DEFINING THE EARLY LYTIC REGION OF COLIPHAGE 186 AND THE CONTROL OF MIDDLE GENE TRANSCRIPTION

A Thesis submitted for
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by

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FOR MY PARENTS

Tony and Miriam Richardson

Murphy`s laws.

If anything can go wrong it will.

Corollaries:

1. Nothing is as easy as it looks.
2. Everything takes longer than you think.
3. If there is a possibility of several things going wrong, the one that will cause the most damage will be the one to go wrong.
4. If you perceive that there are four possible ways in which a procedure can go wrong and circumvent these, then a fifth way will promptly develop.
5. Left to themselves, things tend to go from bad to worse.
6. Whenever you set out to do something, something else must be done first.
7. Every solution breeds new problems.

Extensions of Murphy`s laws:

1. Inside every large problem is a small problem struggling to get out.
2. The solution to a problem changes the nature of the problem.
3. Everything put together falls apart sooner or later.

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SUMMARY

DEFINING THE EARLY LYTIC REGION OF COLIPHAGE 186 AND THE CONTROL OF MIDDLE GENE TRANSCRIPTION.

This thesis describes work carried out to provide an understanding of the expression of the early lytic and middle genes of the temperate coliphage 186. The specific aims of this study were to identify the 186 early lytic genes, and to investigate the mechanism of control of middle gene transcription.

The DNA sequence of the early lytic region was completed. Computer-assisted analysis of the DNA sequence led to the prediction that the early lytic transcript encoded four genes; CP75, CP76, CP77 and CP78. This transcript was predicted to terminate after the CP78 gene at a potential rho-independent terminator structure, tR1. The gene, CP79, following the terminator tR1, was predicted to be the first gene in the middle region. These predicted genes were cloned into a plasmid expression vector and their protein products were identified by SDS-polyacrylamide gel electrophoresis.

The functions of the CP75 and CP76 genes have been determined by other members of the laboratory and are involved in the lysis-lysogeny decision. Thus, the assignment of functions to CP77 and CP78 was required. Two functions have been previously described that are likely to be encoded by CP77 and CP78; Dhr, which results in an inhibition of E. coli DNA replication, and Tom, which was postulated to be an essential function required for 186 middle gene transcription. The investigation of the Dhr function revealed that it was encoded by CP78. CP78 is a non-essential gene but appears to be important in 186 lytic development. It was expected that the CP77 gene would encode the Tom function, however this study also revealed that CP77 is a non-essential gene, the expression of which results in an inhibition of E. coli cell division. CP77 was named the fil gene.

Thus, it appeared that the predicted Tom function was not encoded in the early lytic region.

Previous studies carried out in this laboratory, led to the prediction that middle gene transcription occurs either by antitermination of the early lytic transcript or by promoter activation of a new transcript. As a first step towards understanding the control of middle gene transcription, Northern analysis was used to identify, size and determine the approximate 5'-ends and 3'-ends of the in vivo transcripts from the 186 early lytic and middle regions. The transcription pattern of the early lytic and middle regions was consistent with a mechanism for middle gene transcription involving antitermination and RNaseIII processing.

Studies were carried out to determine whether an antitermination mechanism for middle gene transcription was likely. This study did not provide evidence for the existence of a control mechanism for 186 middle gene transcription, and it is likely that middle gene transcription occurs simply by transcription passing through the relatively weak early terminators. However, these studies revealed that translation was important for transcription of the 186 early lytic and middle regions and it was postulated that an attenuation-type mechanism may be involved in the control of middle gene transcription. The work presented in this thesis provides the basis for further studies concerning 186 middle gene expression.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university unless otherwise stated. To the best of my knowledge and belief, this thesis contains material not previously published or written by any other person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan.

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Helena E. Richardson

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ABBREVIATIONS

min	-	minute
A_{600}	-	Absorbance at 600 nm
DNA	-	deoxyribonucleic acid
RNA	-	ribonucleic acid
kd	-	kilodalton
b	-	base
bp	-	base-pair
kb	-	kilobase (1,000 base-pairs)
kPa	-	kilopascal
Ci	-	Curie
uCi	-	microCurie
mg	-	milligram
ml	-	millilitre
mA	-	milliamp
M	-	Molar
mM	-	millimolar
uM	-	micromolar
mmol	-	millimole
ng	-	nanogram
ug	-	microgram
ul	-	microlitre
um	-	micrometre
u	-	micron
V	-	volts
Kcal	-	Kilocalorie
J	-	Joules
EtBr	-	ethidium bromide
SDS	-	sodium dodecyl sulphate

----- ABBREVIATIONS (contd.) -----

Tris	-	Tris (hydroxymethyl) aminomethane
EDTA	-	ethylenediamine tetra acetate
gm	-	gram
rpm	-	revolutions per minute
g	-	gravitational force
TCA	-	tri-chloro acetic acid
wt	-	wild-type
moa	-	multiplicity of addition
UV	-	ultraviolet
cpm	-	counts per minute
U	-	units
pfu	-	plaque-forming units
cfu	-	colony-forming units
rbs	-	ribosome-binding site
orf	-	open reading-frame
aa	-	amino acids
gp	-	gene-product
rf	-	replicative-form

Other abbreviations are described in Chapter 2.

CHAPTER 1.

INTRODUCTION.

CHAPTER 1. INTRODUCTION.

1.1 COLIPHAGE 186.

Coliphage 186 is a temperate phage of the P2-related group of phage (Bertani and Bertani, 1971). This group includes phages P2, 186, P4, 299, 18 and W and is distinct from the lambdoid group of phages (λ, 434, 424, ϕ 80, ϕ 21, P22) in morphology and genetic organization (Bertani and Bertani, 1971; Hocking and Egan, 1982a; Syzbalsky and Syzbalsky, 1979).

186 has a double-stranded, non-permuted DNA genome of approximately 30 kb in length and a molecular weight of 19.7×10^6 daltons (Wang, 1967; Wang and Schwartz, 1967; Chattoraj *et al.*, 1973; Younghusband *et al.*, 1975). The DNA possesses complementary cohesive ends, 19 bases in length (Baldwin *et al.*, 1966; Wang *et al.*, 1973; Murray and Murray, 1973).

A linear genetic map of 186 has been constructed on the basis of two and three factor crosses (Hocking and Egan, 1982a) and the physical mapping of insertion mutants (Younghusband *et al.*, 1975). A physical map has been constructed (Saint and Egan, 1979) and aligned with the genetic map by analysis of the gene content of cloned DNA restriction fragments (Finnegan and Egan, 1979).

1.2 GENETIC ORGANIZATION OF THE 186 GENOME.

1.2.1 Genes and Predicted Genes.

The genetic map of 186 determined by Hocking and Egan (1982a) and Younghusband *et al.* (1975) is shown in Figure 1.1(a). Twenty two genes essential for lytic development have been identified, and 21 of these genes are located within the 0%-67% region of the 186 genome. These genes include the tail genes (*N* to *D*), head genes (*W* to *Q*) and the lysis gene *P* (Hocking and Egan, 1982c). The function of the *O* gene is not known. The

B gene encodes a function required for late gene transcription (Finnegan and Egan, 1981; Kalionis et al., 1986b).

The region from 67%-100% encodes the essential replication gene A (Hocking and Egan, 1982b) as well as a number of other genes, which include cI, int and cII. The cI, int and cII genes are non-essential genes, involved in the maintenance or establishment of lysogeny. cI encodes a repressor, which is required for the maintenance of lysogeny (Baldwin et al., 1966). The int gene is required for the integration of 186 DNA into the bacterial chromosome during the establishment of lysogeny and also for excision of the 186 prophage from the bacterial chromosome during lysogenic induction (Bradley et al., 1975; J.B. Egan, personal communication). The position of the int and cI genes was indicated by the physical mapping of two insertion mutants ins3 at 70.3% and ins1 at 73.5% (Younghusband et al., 1975), which show the Int^- and cI^- phenotypes, respectively (Bradley et al., 1975). The cII gene encodes a protein required for the establishment of lysogeny (Huddleston, 1970; Carter, 1985). The cII gene has been mapped between the att site and the A gene by Hocking (1977) and was further located to the XhoI-BglII (67.6%-79.6%) region by in vitro recombination (I. Lamont, personal communication; Carter, 1985).

The DNA sequences of the PstI-PstI (65.5%-77.4%) fragment (Kalionis et al., 1986a) and the BglII-BamHI (79.6%-96.0%) fragment (Sivaprasad, 1984) have been determined. The analysis of the PstI (65.5%-77.4%) DNA sequence from the wild-type and mutants enabled the identification of the cI, int, B and D genes (Kalionis et al., 1986a). Computer-assisted analysis of this region led to the prediction of three further genes. These potential genes were named CP69, CP75 and CP76 (Kalionis et al., 1986a; Figure 1.1b), CP standing for computer protein, followed by the chromosomal coordinate approximating the initiation codon of the potential

Figure 1.1 Genetic map of coliphage 186.

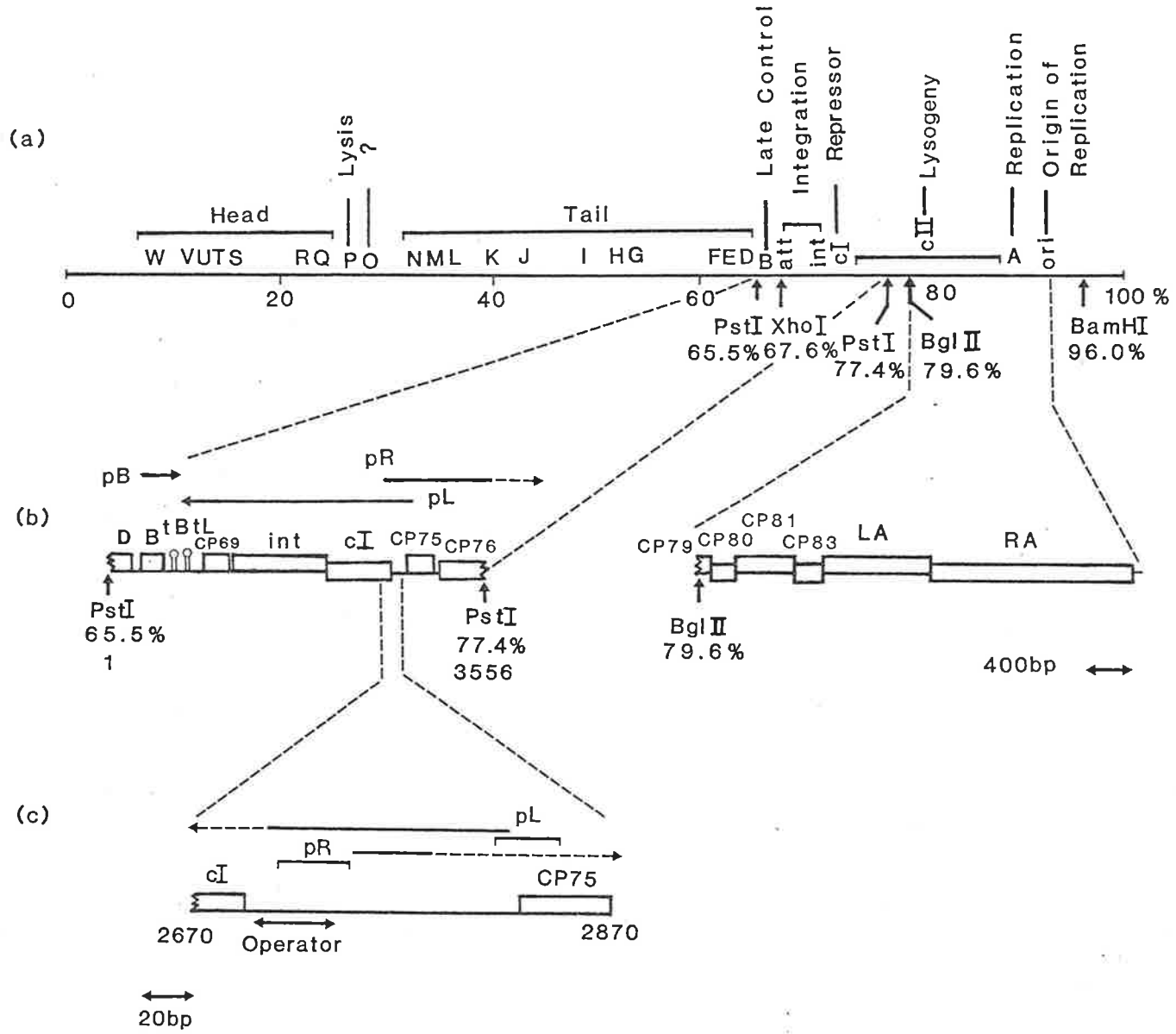
a. A diagrammatic representation of the position of 186 genes on the 186 genome. Map positions of genes are given by Hocking and Egan (1982a). The map position of cII is not known accurately. The functions of the genes are listed above the map.

Relevant restriction sites are shown. The DNA sequence of the PstI (65.5%-77.4%) region and the BglII-BamHl (79.6%-96.0%) region have been determined (Kalionis et al., 1986a; Sivaprasad, 1984).

b. A diagrammatic representation of the genes and predicted genes, which are encoded in the sequenced regions from 65.5% to 93.2%. Genes and potential genes are represented by the boxed regions. D, CP76, and CP79 are only partially represented in the sequenced regions, as indicated by the jagged-edged boxes. Transcription signals and transcripts are indicated by the horizontal arrows.

Sequence numbering is from the PstI (65.5%) site. The sequence coordinates of the restriction sites is of the first base of the site on the l-strand.

c. A diagrammatic representation of the operator region. The position of the operator site is shown relative to the pR and pL promoters and the cI and CP75 genes. (These genes are not fully shown on this diagram.) The convergent overlapping arrangement of transcription from the pL and pR promoters is shown.


(a)
Lysis
?
Late Control
Integration
Repressor
Lysogeny
Replication
Origin of Replication
Head
Tail
W VUTS RQ PO NML K J I HG FED B att int cI cII A ori
0
20
40
60
80
100 %
PstI 65.5%
XhoI 67.6%
PstI 77.4%
BglII 79.6%
BamHI 96.0%
(b)
pB
pR
pL
D B tB tL CP69 int cI CP75 CP76
CP79 CP80 CP81 CP83 LA RA
PstI 65.5% 1
PstI 77.4% 3556
BglII 79.6%
400bp
(c)
pL
pR
cI
CP75
2670
Operator
2870
20bp

gene. Protein products have been identified for the B, CP69, int and cI genes (A. Puspurs, personal communication).

The analysis of the DNA sequence of the BglII-BamHI (79.6%-96.0%) region from the wild-type and mutants has revealed that the replication gene A is actually two genes, LA and RA (Sivaprasad, 1984). Furthermore, this analysis led to the prediction of several other potential genes CP79, CP80, CP81 and CP83 (Figure 1.1b). Protein products of molecular weights commensurate with that predicted from the DNA sequence have been identified for CP80, CP81, CP83, LA and RA (A. Puspurs, personal communication). These genes are arranged such that the initiation codon of each gene overlaps the stop codon of the previous gene, and thus, are likely to be translationally coupled genes (Sivaprasad, 1984; Normark et al., 1983). This arrangement has been shown to be particularly important in the expression of the RA gene, which lacks a ribosome-binding site, and depends upon the translation of LA for expression (Sivaprasad, 1984).

The 67%-100% region of the 186 genome also includes the attachment (att) site and the origin of replication (ori). The att site was located by recombination mapping to between the B and cI genes (Hocking and Egan, 1982a) and by hybridization studies to between the PstI (65.5%) and the XhoI (67.6%) sites (D. Dodd, 1983). The att site is therefore expected to be located after the B gene (67.3%) and before the XhoI (67.6%) site (D. Dodd, 1983; Kalionis et al., 1986a). The 186 ori has been mapped at 92.9% $\pm$ 1.8% by Chattoraj and Inman (1973).

1.2.2 Transcription Promoters.

The computer-assisted analysis of the DNA sequence of the PstI (65.5%-77.4%) region for possible promoters led to the prediction of a leftward promoter pL (at 2817 bp from the PstI site at 65.5%), and two rightward promoters pB and pR (at 263 bp and 2740 bp from the PstI site at 65.5%, respectively) (Kalionis et al., 1986a). Transcriptional studies have

provided evidence that the pL promoter is active in vivo and that it gives rise to a leftward transcript, the size of which is consistent with termination at a Rho-independent terminator (tL) after the CP69 gene (Kalionis, 1985). This transcript is therefore predicted to encode cI, int and CP69 (Kalionis, 1985; Kalionis et al, 1986a; Figure 1.1b). Evidence has been provided that the pB promoter gives rise in vitro and in vivo to a transcript that encodes the B gene and terminates at a Rho-independent terminator (tB) after the B gene (Pritchard and Egan, 1985; Kalionis et al., 1986b; Figure 1.1b). Evidence has also been provided that the pR promoter is active in vitro and in vivo and that transcription initiates from pR at position 2747 (74.7%) from the PstI site at 65.5% (Pritchard and Egan, 1985; Kalionis, 1985; Figure 1.1b). The pR promoter is predicted to be the early lytic promoter (the first promoter to be expressed in 186 lytic development). The pR and pL transcripts initially converge, then overlap and diverge; an arrangement termed convergent overlapping (see Figure 1.1c).

DNA sequencing of 186 virulent mutants (which are resistant to the cI repressor) has located the operator site (the cI repressor binding site) to a region, which overlaps the pR promoter (Kalionis, 1985; I. Lamont, personal communication; Figure 1.1c). The binding of the cI repressor to the operator site is expected to occlude the access of RNA polymerase to the pR promoter and prevent the expression of the 186 lytic genes.

1.3 186 LYTIC GENE TRANSCRIPTION.

As discussed above, the pR promoter is expected to be the first promoter expressed during 186 lytic development. This prediction is supported by the transcription studies of Finnegan and Egan (1981) and Pritchard and Egan (1985). Furthermore, these studies have enabled the

classification of 186 lytic gene expression into three distinct stages;

(1) early

(2) middle

(3) late.

Early lytic transcription occurs immediately after heat-induction of a 186 prophage. Middle transcription includes transcription of the replication genes LA and RA. Late transcription, mediated by the late control gene B (Hocking and Egan, 1982a; Finnegan and Egan, 1981; Kalionis et al., 1986b), includes transcription of the 186 morphological genes and lysis gene in the region 0%-65.5%. The definition of 186 lytic transcription into these three stages has come from in vivo and in vitro transcription studies.

1.3.1 186 Late Gene Transcription.

Finnegan and Egan (1981) hybridized pulse-labelled RNA, prepared at different times after the heat-induction of 186 wild-type or mutant lysogens, to cloned DNA restriction fragments. These results revealed that from 0 to 27 min after heat-induction, transcription from 186 wild-type DNA occurred mainly from the 65.5%-100% region. After this time, transcription of this region decreased slightly. Transcription of the 0%-65.5% region was detected at 25-27 min after heat-induction and increased until cell lysis.

A 186 Bam mutant, which has a mutation in the late control gene B, had two effects on transcription. Firstly, transcription of the 0%-65.5% region did not occur. Secondly, transcription of the 65.5%-100% region was at a higher level at 35-37 min after heat-induction than that of the wild-type. From these results it was concluded that the region from 0%-65.5% represents the late region of 186 (Figure 1.2a) and the B gene is required for the transcription of this region. The higher level of transcription of

Figure 1.2 Definition of the 186 early lytic region.

a. This map shows the 186 early lytic and middle regions as defined by Finnegan and Egan (1981). The location and genetic content of the plasmid-clones used as hybridization probes to detect 186 RNA (Finnegan and Egan, 1981) is shown. The plasmid-clones are derived from pBR322 (Finnegan and Egan, 1979).

pEC35 contains the PstI (65.5%-77.4%) fragment from 186 del1 (5), which contains a deletion (indicated by the shaded box) from 67.9% to 74.0% (Chapter 2.2.1).

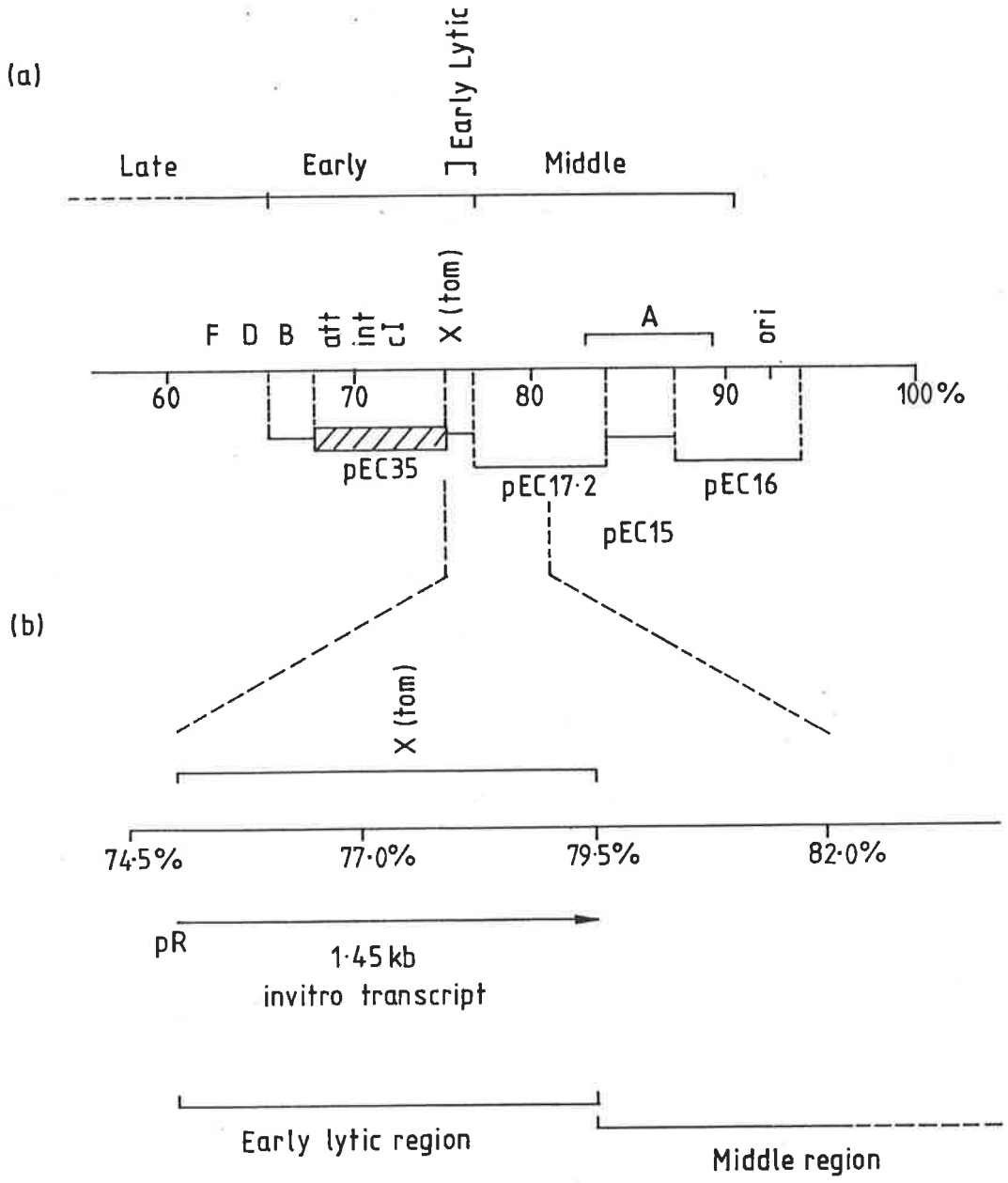
pEC17.2 contains the PstI (77.4%-84.6%) fragment

pEC15 contains the PstI (84.6%-87.5%) fragment

pEC16 contains the PstI (87.5%-94.0%) fragment

The location of the early lytic and middle regions, as determined by the in vivo hybridization studies of Finnegan and Egan (1981) using the probes described above, are indicated. The early lytic region was defined to be from ~74.0% to ~77.4%. From the knowledge of the DNA sequence of the middle region (Sivaprasad, 1984), middle transcription was predicted to terminate soon after the RA gene at 93.2%. The predicted position of the middle control gene tom (X), is also shown.

b. This map shows the 186 early lytic and middle regions as defined by Pritchard and Egan (1985). The location of the 1.45 kb in vitro transcript is shown. The early lytic region is defined by this 1.45 kb in vitro transcript to be from 74.7% to ~79.5%, as indicated on the map. The tom (X) gene is predicted to be encoded by this transcript. The location of the middle region (~79.5%-~93.2%), is also indicated.


(a)
Early Lytic
Late
Early
Middle
F D B att int cI X (tom)
A
ori
60
70
80
90
100%
pEC35
pEC17·2
pEC16
pEC15
(b)
X (tom)
74·5%
77·0%
79·5%
82·0%
pR
1·45 kb
invitro transcript
Early lytic region
Middle region

the 65.5%-100% region from 186 Bam compared with the wild-type led to the prediction that the B gene is directly or indirectly responsible for decreasing transcription of this region.

A 186 Aam mutant, which contains a mutation in the 186 replication gene RA, resulted in several changes in the transcription pattern. Firstly, transcription of the 65.5%-100% region was slightly reduced compared with the wild-type. This was predicted to be due to the decreased template number as a result of the mutation in the replication gene. Furthermore, late gene transcription was absent, and finally, RNA hybridizing to pEC35 (Figure 1.2a), which was considered to be diagnostic for B gene transcription, was dramatically decreased. These results led to the conclusion that the A gene-product is required for B gene transcription, and that activation of the late genes is either directly or indirectly dependent on the A gene-product.

1.3.2 186 Early Lytic and Middle Gene Transcription.

The resolution of the early and the middle stages of 186 lytic development was based on in vivo transcription studies where protein synthesis was inhibited by chloramphenicol (Finnegan and Egan, 1981) and in vitro transcription studies in the absence of any protein synthesis (Pritchard and Egan, 1985).

1.3.2(a) In Vivo Transcription Studies.

When protein synthesis was inhibited by chloramphenicol the only region showing high transcriptional activity was contained within pEC35 (Figure 1.2a), which had a level of transcription similar to that obtained without chloramphenicol. pEC35 contains the PstI (65.5%-77.4%) fragment from 186 del1 (5) (Chapter 2.2.1) and encodes the B gene. Finnegan and Egan (1981) had previously concluded, from transcriptional analysis after induction of an Aam prophage, that B gene transcription was dependent on

the A gene-product. Thus, the B gene was not expected to be transcribed in chloramphenicol-treated cells. It was therefore expected that the transcriptional activity of pEC35 was mainly from the interval 74.0%-77.4% (from the right side of the dell deletion to the PstI site). This result allowed the distinction between the early and middle stages of 186 lytic development. Early lytic transcription was defined as that occurring immediately after the heat-induction of a 186 prophage and requiring no 186 protein products. For the reasons described above, the region from ~74.0% to ~77.4% was assigned as the early lytic region. 186 middle gene transcription was then defined as that occurring in the region from ~77.4% to ~93.2% (the 3'-end of the replication gene RA) and requiring 186 protein synthesis. Finnegan and Egan (1981) postulated that the requirement for 186 protein synthesis for further rightward transcription was due to the need for a specific 186 function, X. The X function was postulated to positively control transcription of the middle region and is now referred to by the more descriptive name Tom (turn-on of middle transcription). This function was predicted to be an essential function and to be located at approximately 76% on the 186 genome (Finnegan and Egan, 1981).

1.3.2(b) In Vitro Transcription Studies.

The in vitro transcription studies of Pritchard and Egan (1985) provided further evidence for the resolution of 186 lytic gene expression into the early and middle stages. In these studies 186 transcripts, which were initiated by unmodified RNA polymerase in the absence of protein synthesis, were identified. Four major transcripts were detected, one of which was a 1.45 kb transcript (band 2), that mapped in the 74.7%-77.4% region (the early lytic region). This transcript was used to more specifically define the early lytic region. The 5'-end of this transcript was located at position 2747 (from the PstI site at 65.5%) and was consistent with initiation of transcription at the pR promoter. This

1.45 kb transcript was shown to terminate after the PstI (77.4%) site since in vitro transcription of PstI-digested 186 DNA resulted in the disappearance of the 1.45 kb transcript and the appearance of a new transcript, sized at 860 b. The size of the 1.45 kb transcript and the position of its 5'-end (at 74.7%) led to the prediction that this transcript terminates at approximately 79.5%, which is to the left of the BglII (79.6%) site. This more specifically defines the early lytic region as being from ~74.7% to ~79.5% (Figure 1.2b). The middle region is therefore defined as the region from ~79.5% to ~93.2% (Figure 1.2). The middle control function Tom, which was postulated to be required for the positive control of middle gene expression (Finnegan and Egan, 1981), was therefore predicted to be encoded in the region ~74.7% to ~79.5% (the early lytic region).

1.4 POSITIVE CONTROL OF GENE TRANSCRIPTION.

Positive control of gene transcription has been well documented (Englesberg and Wilcox, 1974; Raibaud and Schwartz, 1984; Busby, 1986; Galloway and Platt, 1985; Platt, 1986; von Hippel et al., 1984). Mechanisms of positive control fall into two classes :

(1) Positive control exerted at the level of transcription initiation (promoter activation).

(2) Positive control exerted at the level of transcription termination (antitermination).

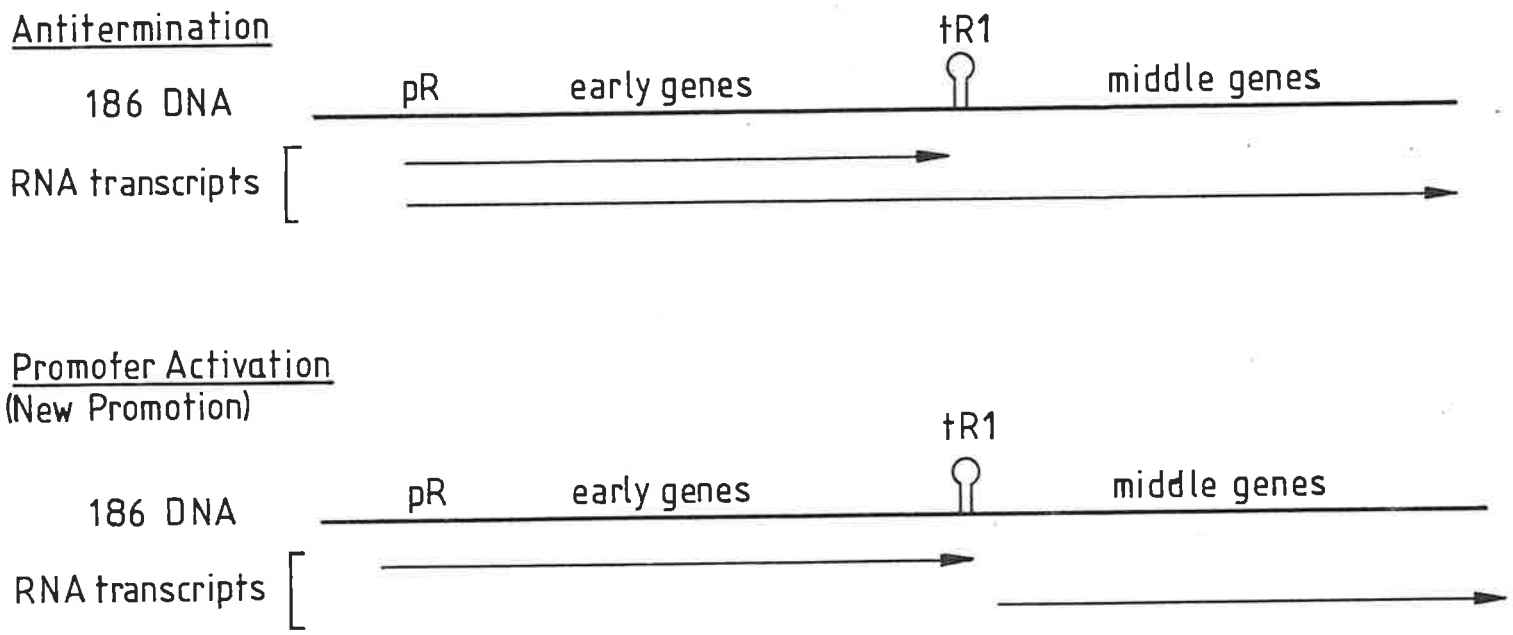
One of the aims of this thesis is to characterize the control of 186 middle gene expression (see Chapter 1.5). 186 middle gene expression has been postulated to be positively regulated by the 186 Tom function. The Tom function was predicted to most likely act by activation of a promoter for middle gene expression or by antitermination of the early lytic transcript (Figure 1.3). For this reason, it is relevant to discuss the

Figure 1.3 The difference between antitermination and promoter activation control mechanisms in 186 middle gene transcription.

The early lytic genes are expressed from a transcript, which initiates at the pR promoter and terminates at the tR1 terminator.

Antitermination : The middle genes are expressed by the extension of the early lytic transcript.

Promoter Activation : The middle genes are expressed by the activation of transcription from a new promoter located before the middle genes.


Antitermination
186 DNA
pR
early genes
tR1
middle genes
RNA transcripts
Promoter Activation
(New Promotion)
186 DNA
pR
early genes
tR1
middle genes
RNA transcripts

respective characteristics of the two known mechanisms of positive control of transcription; promoter activation and antitermination.

1.4.1 Promoter Activation.

Promoter activation is a mechanism used to control gene expression in several bacterial and bacteriophage systems (Raibaud and Schwartz, 1984; Busby, 1986). An activated promoter is defined as a promoter, which requires for its function the participation of a protein factor (the "activator"), that is either not always present or not always active.

1.4.1(a) Activator Proteins.

Activator proteins so far characterized can be catagorized into three distinct classes :

(1) An activator may be an accessory factor, which alters the promoter specificity of RNA polymerase. This group of activators makes up the large majority of the characterized activator proteins. Examples of this group include the E. coli cyclic-AMP receptor protein, Crp, and the arabinose-operon control protein, AraC (de Crombrugghe et al., 1984; Englesberg and Wilcox, 1974), the λ cI and cII proteins (Gussin et al., 1983; Hochschild et al., 1983; Wulff and Rosenberg, 1983; Ho et al., 1983, 1986) and the T4 middle control protein, Mot (Uzan et al., 1983; Rabussay, 1983; Brody et al., 1983; Pulitzer et al., 1985). The late control proteins of 186 (B), P2 (ogr) and P4 (δ) are also likely to be accessory factors (Kalionis et al., 1986b; Sunshine and Sauer, 1975; B. Sauer et al., 1982; Souza et al., 1977).

(2) The activator may be a new sigma factor, which replaces the normal RNA polymerase sigma factor, and thereby changes the promoter specificity. New sigma factors are observed in B. subtilis sporulation, during the E. coli heat shock response (HtpR), nitrogen assimilation (NtrA)

and in bacteriophage T4 (gp55) and Bacillus phage SP01 lytic development (reviewed by Reznikoff et al., 1985; Doi and Wang, 1986).

(3) Alternatively, the activator may be a new RNA polymerase. New RNA polymerases are involved in the expression of bacteriophage T7 (and T3) late genes and in bacteriophage N4 early and middle gene expression (Chamberlin and Ryan, 1982; Haynes and Rothman-Denes, 1985; Zehring et al., 1983; Zehring and Rothman-Denes, 1983).

1.4.1(b) Activated Promoters.

Promoters recognized by E. coli RNA polymerase have a well conserved DNA sequence, in particular at positions -10 and -35 from the transcription start point (Figure 1.4; Rosenberg and Court, 1979; Hawley and McClure, 1983a). Positively controlled promoters are not fully functional or are completely inactive in the presence of E. coli RNA polymerase alone. The DNA sequences of positively controlled promoters have been observed to deviate significantly from the E. coli promoter consensus sequence (Figure 1.4). Activated promoters, which require novel RNA polymerases or a new sigma factor, bear very little resemblance to the E. coli promoter consensus sequence (reviewed by Chamberlin and Ryan, 1982; Reznikoff et al., 1985; Kustu et al., 1986; Grossman and Losick, 1986). Promoters, which require RNA polymerase accessory proteins for their function, have a DNA sequence, which is more similar to the E. coli promoter consensus sequence, nevertheless, they differ markedly in the -35 region from the canonical E. coli promoter sequence (Raibaud and Schwartz, 1984; Busby, 1986). In most cases so far studied, evidence has been obtained that the accessory proteins bind to distinct binding sites on the DNA located close to or overlapping the -35 region of the promoter (Busby, 1986; Raibaud et al., 1985; Gussin et al., 1983; Ho et al., 1986; Brody et al., 1983). These binding sites may show a hyphenated inverted repetitive structure (e.g. the λ cI repressor binding site; Gussin et al., 1983), a

Figure 1.4 Activated promoters.

This Figure presents examples of each of the three types of activated promoters :

a. Activated promoters involving novel RNA polymerases : T7 encodes a novel RNA polymerase, which directs transcription from the T7 late promoters (Chamberlin and Ryan, 1982). The T7 late promoter consensus sequence as given by Oakley and Coleman (1977) and Dunn and Studier (1983) is shown. The point of transcription initiation is indicated by +1.

b. Activated promoter involving novel sigma factors : The T4 gp55 is a sigma factor, which directs transcription from the T4 late promoters (Kassavetis and Geiduschek, 1984; Reznikoff et al., 1985). The T4 late promoter consensus sequence as given by Christensen and Young (1983) and Elliott and Geiduschek (1984), is shown. The point of transcription initiation is indicated by +1.

c. Activated promoters involving RNA polymerase accessory factors : The λ cII protein is a RNA polymerase accessory factor, which activates transcription from the λ pRE, pI and paQ promoters (Wulff and Rosenberg, 1983; Echols and Guarneros, 1983; Ho et al., 1986). The DNA sequence of the λ pRE promoter (Schmeissner et al., 1980) is presented. The -10 and -35 regions are underlined. The point of transcription initiation is indicated by +1. The regions in bold type indicate the direct repeat sequence, which has been shown to be the cII protein binding-site (Ho et al., 1983; 1986).

The E. coli promoter consensus sequence (Rosenberg and Court, 1979; Hawley and McClure, 1983a), is shown for comparison. The -10, -35 and +1 regions, are indicated.

(a) Novel RNA Polymerase

T7 Late Promoter
Consensus Sequence

CGACTCACTATAGGGAGA
+1

(b) Novel Sigma factor

T4 Late Promoter
Consensus Sequence

TATAAATActatt-----
+1

(c) RNA Polymerase
Accessory Factor

λ gpcII Activated
Promoter (pRE)

TTGCGTTTGT**TTGC**ACGAACCATATGTAAGTATTTCCTTAG
-35 -10 +1

E. coli Promoter
Consensus Sequence

TTGACA-----(15-19)-----TATAAT-----cat
-35 -10 +1

direct repeat separated by a few base pairs (e.g. the binding sites for the λ cII and E. coli Pho B activator proteins; Ho et al., 1986; Surin et al., 1984) or be apparently unstructured (e.g. the T4 Mot activator protein binding site; Brody et al., 1983).

1.4.1(c) The Mechanism of Promoter Activation by RNA Polymerase Accessory Factors.

The molecular mechanism of activation of a promoter by an activation protein has only been investigated thoroughly in the cases of λ cI, cII and E. coli Crp. The efficiency of a promoter is dependent upon the recognition of the promoter by RNA polymerase and the efficiency at which bound RNA polymerase achieves a transcriptionally competent state (the open complex) (Rosenberg and Court, 1979; von Hippel et al., 1984; McClure, 1985). Promoters requiring activation by the λ cI, cII or E. coli Crp proteins appear to be defective in the binding of RNA polymerase and/or in the isomerization of RNA polymerase to the open complex (Shih and Gussin, 1984; Hawley and McClure, 1982; Malan et al., 1984). In all three cases, direct activator-RNA polymerase interactions have been shown or are expected to be important in the activation mechanism (Hochschild et al., 1983; Hawley and McClure, 1983b; Ho et al., 1986; Blazy et al., 1980; Spassky et al., 1984; Shanblatt and Revzin, 1983). Furthermore, the binding of the λ cII and E. coli Crp activator proteins to the activator binding sites causes a conformational change in the DNA, which is also expected to be important in activation (Ho et al., 1986; Kolb et al., 1983; Gronenborn et al., 1984; Wu and Crothers, 1984; Weber and Steitz, 1984). However, the exact mechanism by which these interactions and DNA structural changes increase the binding of RNA polymerase and/or open complex formation at the activated promoter, is not known.

1.4.2 Antitermination.

Transcription termination occurs at termination sites, which are generally characterized by a region of hyphenated dyad symmetry in the DNA, that can form a stem-loop structure in the RNA (Adhya and Gottesman, 1978; Rosenberg and Court, 1979; Galloway and Platt, 1985). Termination at these sites requires the release of the RNA transcript and the dissociation of the transcription complex, and may or may not require the termination factor Rho, which aids in the release of the RNA transcript from the DNA (von Hippel et al., 1984; Galloway and Platt, 1985).

Antitermination is a mechanism of positive control whereby a trans-acting factor acts to prevent transcription termination. Antitermination mechanisms are used by bacteriophage λ and other lambdoid phages to control the expression of the delayed-early and late genes (Herskowitz and Hagen, 1980; Friedman and Gottesman, 1983; Rybchin, 1984; Franklin 1985a,b; Tanaka and Matsushiro, 1985). Antitermination similar to λ antitermination has also been observed in the E. coli rRNA operons (reviewed by Morgan, 1986), and the bacteriophage P4 encodes a function, Psu, which antiterminates transcription in P4, P2 and E. coli genes, by a mechanism which is expected to be different to λ antitermination (Sunshine et al., 1976; Sauer et al., 1981; Lagos et al., 1986).

Antitermination may theoretically occur by the modification of the termination signal so that it is no longer recognised as such, or by modification of the transcribing RNA polymerase to a termination-resistant form. In the case of λ, antiterminaton occurs by the modification of RNA polymerase to a termination-resistant form. Since the bacteriophage λ antitermination mechanisms are the best characterized, the essential features of this positive control system will be discussed.

1.4.2(a) The Role of Antitermination in λ Lytic Development.

Immediately following λ infection or prophage induction, transcription by E. coli RNA polymerase occurs from the pL, pR and pR' promoters to give three major immediate-early transcripts (Friedman and Gottesman, 1983; Figure 1.5). Transcription from the pL promoter terminates at several terminators, which are located between ~1.0 kb to 1.7 kb from pL, namely tL1a, tL1b, tL2aI and tL2aIII (Lozeron et al., 1977, 1983; Hyman and Honigman, 1986; Figure 1.5). Transcription from pR terminates at the Rho-dependent terminator tR1 at five positions between ~290 bp to 450 bp from pR (Rosenberg et al., 1978; Lau et al., 1982, 1983; Morgan et al., 1983a,b; Lau and Roberts, 1985). Termination at tR1 is only ~50% efficient and transcription continues rightward and terminates mostly at the Rho-independent terminator tR2 (Court et al., 1980a; Kroger and Hobom, 1982; Couturier et al., 1973; Szybalski et al., 1970, 1983; Figure 1.5). pR' transcripts terminate at tR1' and tR2' (Szybalski et al., 1983; Figure 1.5).

Among the functions encoded by the immediate-early transcripts, is the λ N gene-product (the first gene encoded on the pL transcript), which acts to antiterminate pL and pR transcripts at several terminators (both Rho-dependent and Rho-independent) located downstream of the pR and pL promoters (Roberts, 1969; Lozeron et al., 1977, 1983; Gottesman et al., 1980; Greenblatt and Li, 1982; Szybalski et al., 1983; Figure 1.5). Transcription from λ pL, which has been modified by gpN, continues past the tL2, tL3, tI and tJ' terminators, but finally terminates at gpN-unresponsive terminators located on the nonsense strand in the J to T region (Gottesman et al., 1980; Honigman, 1981; Luk and Szybalski, 1983; Szybalski et al., 1983; Figure 1.5). Transcription from λ pR by λ gpN-modified RNA polymerase allows efficient expression of the λ replication genes O and P, the late control gene Q, and also contributes to λ late gene expression (Dambly and Couturier, 1970). The Q gene-product acts to

tR1 (Court et al., 1980; Rosenberg et al., 1978; Dambly-Chaudiere et al., 1983; Szybalski, 1983).

tR2 (Kroger and Hobom, 1982)

tL1, tL2a, tL2b, tL2c, tL2d, tL3, tJ, t'J1, t'J2, t'J3, t'J4 (Szybalski, 1983).

tL1 has been resolved into two terminators tL1a and tL1b (Hyman and Honigman, 1986).

tL2a has been resolved into two termination sites tL2aI and tL2aII (Hyman and Honigman, 1986).

tI (Schmeissner et al., 1984a, Szybalski, 1983).

Rho-dependent terminators are indicated by the * sign.

λ gpN-modified RNA polymerase is able to transcribe past all of these terminators, with the exception of tJ. tJ appears to be one of the, as yet poorly characterized, gpN-unresponsive terminators in the J-T region.

Figure 1.5 The Genetic and transcriptional map of phage λ.

a. A diagrammatic representation of the λ genome showing the major genes and transcripts. (Not all genes or transcripts are shown.) The map is not drawn to scale. The functions of the genes, are indicated above the map.

λ lytic gene expression initiates from the promoters pL, pR and pR', and terminates (in the absence of λ gpN) at several terminators located downstream from these promoters. The first of these terminators are shown in each case. Transcription, which is partially or completely dependent on the λ gpN antitermination function, is shown by the dashed lines.

Transcription from the pRE and pI promoters requires the λ gpcII activation protein (Wulff et al., 1980; Schmeissner et al., 1980, 1981; Shimatake and Rosenberg, 1981). Transcription from pRM is dependent upon the λ gpcI protein (Gussin et al., 1983).

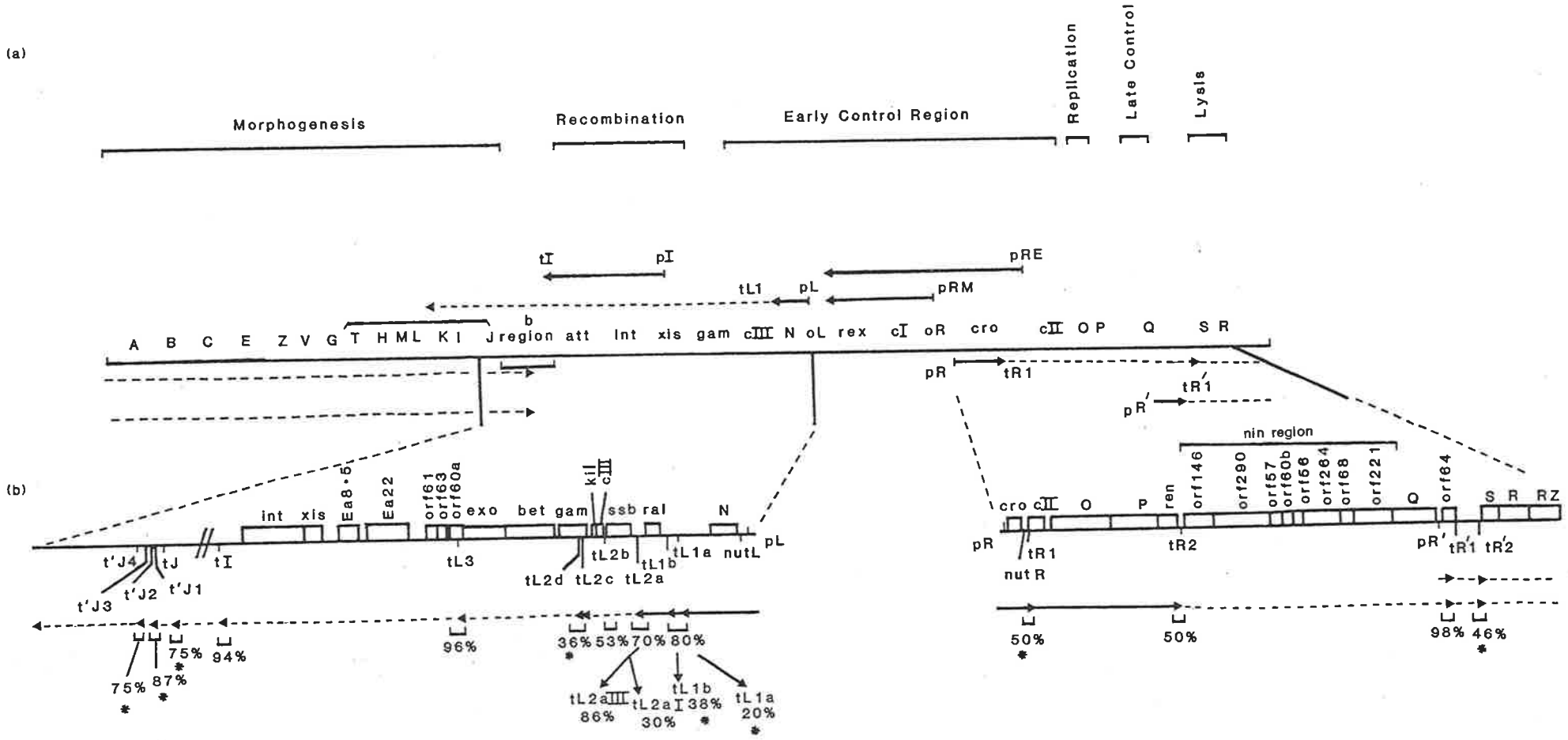
b. An expansion of the λ immediate-early and delayed-early regions showing genes (represented by the boxed regions), promoters, terminators, nut sites and transcripts. The map is drawn approximately to scale. The left region represents ~18 kb and the right region represents ~8.5 kb.

The region to the left of tI is not shown in full. // indicates the region (8.6 kb), which is not shown.

The boxes marked orf, are potential genes (Sanger et al., 1983). Functions or protein products have been identified for the other genes shown on the map (Sanger et al., 1983).

Transcription, which can occur in the absence of λ gpN, is shown by the full arrows, whereas transcription dependent on λ gpN, is shown by the dashed arrows.

The termination efficiency of the termination sites are indicated below the terminators. The efficiency of termination at these sites is from the following sources :


(a)
Morphogenesis
Recombination
Early Control Region
Replication
Late Control
Lysis
tI
pI
pRE
tL1
pL
pRM
b
A B C E Z V G T H ML K I J region att Int xis gam cIII N oL rex cI oR cro cII O P Q S R
pR
tR1
tR'1
pR'
nin region
(b)
int xis Ea8·5 Ea22 orf61 orf63 orf60a exo bet gam kil cIII ssb ral N pL
cro cII O P ren orf146 orf290 orf57 orf60b orf58 orf284 orf68 orf221 Q orf84 S R RZ
t'J4 tJ tI t'J3 t'J2 t'J1
tL3
tL2d tL2c tL2a tL2b tL1b tL1a nutL
pR tR1 nutR tR2 pR' tR'1 tR'2
75% 87% 75% 94%
96%
36% 53% 70% 80%
50% 50%
98% 46%
tL2aIII 86%
tL2aI 30%
tL1b 38%
tL1a 20%

antiterminate transcription initiated at pR' and allow efficient expression of the λ late genes (Roberts, 1975; Forbes and Herskowitz, 1982; Grayhack and Roberts, 1982). gpQ-antiterminated rightward transcription eventually terminates in the b region (Bouvre and Szybalski, 1969; Burt and Brammar, 1982; Figure 1.5).

1.4.2(b) The Requirements of λ gpN and gpQ Antitermination.

The modification of RNA polymerase by λ gpN to a termination-resistant form requires in addition to gpN, several E. coli proteins known as Nus (N utilization substance) and sites of the λ genome known as nut (N utilization) sites.

The role of E. coli proteins (Nus) in λ gpN antitermination was revealed by the isolation of E. coli mutants, which have reduced ability to support λ gpN-mediated antitermination. Five different nus mutants have been isolated, namely nusA, nusB, nusC (rpoB), nusD (rho) and nusE (rplJ) (reviewed by Friedman et al., 1984). As indicated, three of these nus mutants map in previously defined genes; nusC mutations map in the gene encoding the α-subunit of RNA polymerase, nusD mutations map in the gene encoding the termination factor Rho and nusE mutations map in the gene encoding the ribosomal protein S10 (Friedman et al., 1981, 1983, 1985; Friedman and Gottesman, 1983). The isolation of these mutations implicates the involvement of Rho, RNA polymerase and the ribosome in termination and/or gpN-mediated antitermination. nusA and nusB mutants map in previously undefined genes, which appear to play roles in both termination and antitermination in E. coli (Friedman et al., 1984, 1985; Hauser et al., 1985; Plumbridge et al., 1985; Peacock et al., 1985; Garner et al., 1985; Sharrock et al., 1985). Thus, the study of λ gpN has allowed the identification of novel E. coli genes involved in termination, which may further the understanding of the mechanism of transcription termination.

The λ nut sites are located downstream of the pR and pL promoters (nutR is ~250 bp downstream of pR and nutL is ~50 bp downstream from pL), and are essential for the action of gpN (Salstrom and Szybalski, 1978; Rosenberg et al., 1978; de Crombrugghe et al., 1979; Dambly-Chaudiere et al., 1983; Olson et al., 1982). The λ nut sites consist of three conserved sequence elements box A, box B and box C. Box B (AGCCCTGAA RAAGGGCA; R=A or G) is a region of hyphenated dyad symmetry, 17 bp in length that can form a stem-loop structure in the RNA and is likely to be the site of action of gpN (Rosenberg et al., 1978; Friedman and Gottesman, 1983). Box A (CGCTCTTA) is important in the action of the NusA protein (Friedman and Olson, 1983; Friedman et al., 1985). A role for box C (GGTGTRTG; R=A or G) has not yet been found.

λ gpQ-antitermination requires the NusA protein and a DNA sequence termed the qut site located close to the λ pR' promoter (Daniels and Blattner, 1982; Somasekhar and Syzbalski, 1983; Grayhack and Roberts, 1982; Grayhack et al., 1985). The λ qut site contains a region of hyphenated dyad symmetry (which differs from the box B sequence at the nut sites) and a box A sequence (Friedman and Olson, 1983; Daniels and Blattner, 1982).

1.4.2(c) The Mechanism of λ gpN Antitermination.

Genetic and biochemical studies have established that λ gpN antitermination occurs by the modification of RNA polymerase (to a termination-resistant form) at the nut sites by λ gpN in conjunction with Nus factors (Ward and Gottesman, 1982; Friedman and Gottesman, 1983; Friedman et al., 1983, 1984; Greenblatt, 1984). This modification of RNA polymerase occurs, at least partially, at the RNA level since the translation of the nut region prevents gpN action (Olson et al., 1982, 1984; Warren and Das, 1984). The exact mechanism by which RNA polymerase is modified to a termination-resistant form by gpN, is not known, nor is the exact role of the NusA, NusB and NusE proteins in gpN antitermination.

However, recent studies have shown that the NusA, NusB and NusE proteins are directly involved in gpN antitermination (Goda and Greenblatt, 1985; Das et al., 1985; Das and Wolska, 1984; Ghosh and Das, 1984). The NusA protein is known to interact with RNA polymerase and with λ gpN (Greenblatt and Li, 1981a,b; Friedman et al., 1981, 1984, 1985). Since box A has been shown to be important for the action of the NusA protein (Friedman and Olson, 1983; Friedman et al., 1985) it has been proposed that the NusA protein binds to RNA polymerase at box A, enabling the subsequent binding of gpN to the NusA protein at the nut site. The interaction of the NusB and NusE proteins with RNA polymerase, with gpN, or with each other is less well defined. However, recent studies have led to the prediction that the NusE protein (ribosomal protein S10) may function in gpN antitermination while in the ribosome (Das and Wolska, 1984; Das et al., 1985; Friedman et al., 1985). However, translation has been shown not to be involved in λ gpN antitermination, therefore if ribosomes are involved they must function in a way, which does not involve translation (Goda and Greenblatt, 1985; Warren and Das, 1984; Olson et al., 1984).

In summary, RNA polymerase is modified at the λ nut sites by gpN and the Nus factors by a mechanism, which is not yet well defined. RNA polymerase modified by gpN is able to transcribe through both Rho-dependent and Rho-independent termination signals to allow expression of the λ delayed-early genes. The mechanism by which gpN prevents transcription termination at most (but not all terminators), is not known.

1.4.3 Comparison of Promoter Activation and Antitermination Control Mechanisms.

Promoter activation and antitermination mechanisms of control can be distinguished by transcription studies. Positive control mechanisms involving promoter activation should result in the appearance of a new

transcript, whereas those involving antitermination should result in the extension of a smaller transcript (Figure 1.3).

In order to determine whether gene expression is positively controlled and the mechanism by which positive control occurs, it is essential to carry out transcriptional and genetic studies. A knowledge of the DNA sequence, in conjunction with transcription and genetic studies, should allow the detection of transcriptional signals and control sites that are important in the control of gene expression.

1.5 AIMS AND APPROACH.

The aims of this work were as follows :

(1) To identify the functions of 186 genes encoded in the early lytic region (77.4%-79.5%) in order to determine whether the postulated middle control function, Tom, is encoded in this region.

(2) To characterize the transcription pattern of the 186 early lytic and middle regions *in vivo*.

The purpose of this work was to provide a further understanding of 186 early lytic and middle gene expression. In particular, the emphasis was on investigating the postulated requirement of a 186 protein (Tom) for middle gene transcription, and in determining whether middle gene transcription occurs by promoter activation or by antitermination. The broader significance of this study is that it may reveal a new type of promoter activation or antitermination mechanism, and thus, may contribute to the understanding of transcription initiation or termination in *E. coli*.

An essential prerequisite to this work was to complete the DNA sequence of the 186 early lytic region. The DNA sequence is known to the left of the *PstI* (77.4%) site and to the right of the *BglII* (79.6%) site. The approach was then to determine the DNA sequence of the *PstI-BglII* (77.4%-79.6%) region. Potential genes and transcriptional control signals encoded in the DNA sequence of this region can be predicted by analysis of

the sequence with the aid of computer programs (Chapter 2.38). Analysis of the protein-products encoded in the early lytic region can be used to confirm the existence of potential genes.

The function of the early lytic genes can be investigated by the isolation of mutants in these genes, and the characterization of the effect of these mutations on 186. The determination of the functions of these genes is important in understanding 186 phage development, and is expected to reveal the identity of the postulated middle control gene Tom.

The studies of Finnegan and Egan (1981) provided an elementary transcription pattern of the 186 early lytic and middle region. However, the approach used by Finnegan and Egan (1981) did not allow the size, number and position of the RNA transcripts on the DNA sequence to be determined, and was also constrained by the lack of knowledge of the exact gene content of the cloned fragments used as hybridization probes (Chapter 1.3.2a). The characterization of the in vivo RNA transcripts of the 186 early lytic and middle region with respect to their size, number and location on the DNA sequence is important in investigating the mechanism of middle gene transcription. The technique of Northern analysis using specific probes (possible from the knowledge of the DNA sequence of the region of interest) enables the characterization of RNA transcription with respect to the parameters listed above (Thomas, 1980).

The results are presented in three Sections. The first Section is concerned with defining the early lytic region. The second Section investigates the functions of the early lytic genes. The third Section deals with the characterization of the transcription pattern of the early lytic and middle regions, and in investigating the control of middle gene transcription.

CHAPTER 2.

MATERIALS AND METHODS.

CHAPTER 2. MATERIALS AND METHODS.

2.1 BACTERIAL STRAINS.

The bacterial strains used in this study are described in Table 2.1. Bacterial strains constructed in this work were obtained using the methods described in Chapter 2.15.

2.2 BACTERIOPHAGE STRAINS.

2.2.1 186 Strains.

Derivatives of the phage 186 used in this study are described below. Phage 186 strains constructed in this work were obtained using the methods described in Chapter 2.32.

The numbers preceding the phage strains described below are used in the text in association with the phage strain (e.g. 186 del1 (5)) to aid in locating the description of a particular phage.

(1) 186 cItsp : A heat-inducible mutant with a temperature-sensitive repressor (Baldwin et al., 1966; Woods and Egan, 1974).

(2) 186 cI10 : A clear plaque mutant, which has a defective cI gene (Huddleston, 1970).

(3) 186 vir1 : A 186 mutant able to grow on a 186 lysogen, isolated as a spontaneous mutant in a stock of the phage 186 cIam53 (Woods, 1972).

(4) 186 Aam1vir1 : A replication-defective mutant of 186 vir1 (3) (Hocking, 1977) that contains an amber mutation in the LA gene (Sivaprasad, 1984).

(5) 186 del1 : A deletion mutant isolated as a heat-stable phage using the procedure of Parkinson and Huskey (1971) (Dharmarajah, 1975;

Saint, 1979; Finnegan and Egan, 1981; R. O'Connor, unpublished). 186 del1 contains a deletion of 1.84 kb from 67.9%-74.0% (sequence coordinates 716-2551), removing the cI, CP69 and int genes (Kalionis et al., 1986a). This phage gives super-clear plaques (large very clear plaques at 30°C) due to a mutation, which maps outside the 67.6%-79.6% region (data not shown).

(6) 186 del2 : A virulent mutant isolated by the same procedure as described for 186 del1 (5) (Dharamarajah, 1975). 186 del2 contains a deletion of 1.84 kb from 67.9%-74.0% and an insertion of 0.6 kb between the PstI sites at 77.4% and 84.6% (Saint, 1979; R. O'Connor, unpublished). This insertion has been shown to be a tandem duplication of the region 79.0%-81.0% (sequence coordinates 4041-4641), which spans the BglII site at 79.6% so that the phage has two BglII sites (this work). This phage gives super-clear plaques (see 186 del1 (5)) as a result of a mutation, which maps outside the 67.6%-79.6% region (data not shown).

(7) 186 cItspins3 : An insertion mutant, which contains an IS3 element (1.4 kb) at 70.3% in the int gene so that the phage has an Int$^-$ phenotype (Bradley et al., 1975; Younghusband et al., 1975; Saint, 1979; Kalionis et al., 1986a).

(8) 186 cItspAam11 : A replication-defective mutant of 186 cItsp (1) (Hocking, 1977; Hocking et al., 1982b) that contains an amber mutation in the LA gene (Sivaprasad, 1984).

(9) 186 del1Dhr1 : A 186 Dhr$^-$ mutant created by recombination of the 1.78 kb XhoI-BglII (67.6%-79.6%) fragment from the Dhr$^-$

plasmid pEC401 into 186 cItsp (1) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(10) 186 dellXB : A 186 dell phage created by recombination of the 1.78 kb XhoI-BglII (67.6%-79.6%) fragment from the plasmid pEC400 into 186 cItsp (1) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work). This phage does not contain the super-clear mutation in contrast to 186 dell (5).

(11) 186 dellAam11Dhr1 : A 186 Dhr^- mutant created by recombination of the 1.78 kb XhoI-BglII (67.6%-79.6%) fragment from the Dhr^- plasmid pEC401 into 186 cItspAam11 (8) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(12) 186 dellAam11 : A 186 dell mutant created by recombination of the 1.78 kb XhoI-BglII (67.6%-79.6%) fragment from the plasmid pEC400 into 186 cItspAam11 (8) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(13) 186 cItspAam11Dhr1 : A 186 Dhr^- mutant created by recombination of the 2.93 kb XhoI-PstI (67.6%-77.4%) from 186 cItsp (1) and the 0.69 kb PstI-BglII (77.4%-79.6%) fragment from the Dhr^- plasmid pEC401 into 186 cItspAam11 (8) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(14) 186 cItspAam11Dhr2 : A 186 Dhr^- mutant created by recombination of the 2.93 kb XhoI-PstI (67.6%-77.4%) from 186 cItsp and the 0.69 kb PstI-BglII (77.4%-79.6%) fragment from the Dhr^- plasmid pEC402 into 186 cItspAam11 (8) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(15) 186 cItspins3Aam11 : A 186 int^- mutant created by recombination of the 5.02 kb XhoI-BglII (67.6%-79.6%) fragment from

186 cItspins3 (7) into 186 cItspAaml1 (8) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(16) 186 cItspins3Dhrl : A 186 $Dhr^{-}Int^{-}$ mutant created by recombination of the 4.19 kb XhoI-BssHII (67.6%-76.9%) from 186 cItspins3 (7) and the 0.83 kb BssHII-BglII (76.9%-79.6%) fragment from the Dhr^{-} plasmid pEC401 into 186 cItsp (1) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(17) 186 cItspins3Aaml1Dhrl : A 186 $Dhr^{-}Int^{-}$ mutant created by recombination of the 4.19 kb XhoI-BssHII (67.6%-76.9%) from 186 cItspins3 (7) and the 0.83 kb BssHII-BglII (76.9%-79.6%) fragment from 186 cItspAaml1Dhrl (13) into 186 cItsp Aaml1 (8) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(18) 186 cItspdel3 : A 186 mutant containing a deletion of the 0.25 kb HaeIII-HincII (77.8%-78.7%) fragment (sequence coordinates 3703-3950), created by recombination of the 2.93 kb XhoI-PstI (67.6%-77.4%) fragment with the 0.15 kb PstI-HaeIII (77.4%-77.8%) and the 0.3 kb HincII-BglII (78.7%-79.6%) fragment from 186 cItsp (1) into 186 cItsp (1) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work). This deletion removes the 3'-end of the CP77 gene (54 amino acids), and the 5'-end of the CP78 gene (33 amino acids). The 5'-end of the CP77 gene (22 amino acids) is fused to a non-coding frame from the CP78 region (13 amino acids).

(19) 186 cItspdel4 : A 186 mutant containing a deletion of the 0.53 kb SauIIIA-BglII (77.9%-79.6%) fragment (sequence coordinates 3712-4248), created by recombination of the 3.08 kb XhoI-SauIIIA (67.6%-77.9%) fragment from 186 cItsp (1) into

186 cItsp (1) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work). This deletion removes the CP78 gene, the 5'-end of the CP79 gene (43 amino acids) and the 3'-end of the CP77 gene (49 amino acids). The 5'-end of the CP77 gene (26 amino acids) is fused to a non-coding frame from the CP79 region (9 amino acids).

(20) 186 cItspCP77am : An amber mutant in the gene CP77 created by oligonucleotide mutagenesis of the M13mp8-clone mEC401, followed by recombination of the 0.69 kb PstI-BglII (77.4%-79.6%) fragment from the mutated DNA with the 2.93 kb XhoI-PstI (67.6%-77.4%) fragment from 186 cItsp (1) into 186 cItsp (1) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(21) 186 cItspCP78am : An amber mutant in the gene CP78 created by oligonucleotide mutagenesis of the M13mp8-clone mEC401, followed by recombination of the 0.69 kb PstI-BglII (77.4%-79.6%) fragment from the mutated DNA with the 2.93 kb XhoI-PstI (67.6%-77.4%) fragment from 186 cItsp (1) into 186 cItsp (1) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(22) 186 cItspdeltR1 : A 186 mutant, which contains a deletion of 29 bp spanning the potential transcription terminator, tR1, created by oligonucleotide mutagenesis of the M13mp9-clone mEC400, followed by recombination of the 0.66 kb PstI-BglII (77.4%-79.6%) fragment from the mutated DNA with the 2.93 kb XhoI-PstI (67.6%-77.4%) fragment from 186 cItsp (1) into 186 cItsp (1) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

(23) 186 cItspDup : A 186 cItsp (1) recombinant phage containing the duplication from 186 del2 (6) and constructed by recombination of the 0.6 kb BglII-BglII fragment from the phage 186 del2 (6) into the BglII (79.6%) site of 186 cItsp (1), in the r-orientation (this work).

(24) 186 cItspins3Dup : A 186 cItspins3 (7) recombinant phage containing the duplication from 186 del2 (6) and constructed by recombination of the 0.6 kb BglII-BglII fragment from 186 del2 (6) into the BglII (79.6%) site of 186 cItsp ins3 (7), in the r-orientation (this work).

(25) 186 cI10Dup : A 186 cI10 (2) recombinant phage containing the duplication from 186 del2 (6) and constructed by recombination of the 0.6 kb BglII-BglII fragment from 186 del2 (6) into the BglII (79.6%) site of 186 cI10 (2), in the r-orientation (this work).

(26) 186 del1Dup : A 186 del1 (5) recombinant phage containing the duplication from 186 del2 (6) and constructed by recombination of the 0.6 kb BglII-BglII fragment from 186 del2 (6) into the BglII (79.6%) site of 186 del1 (5), in the r-orientation (this work).

(27) 186 del2XB : A recombinant phage containing the 1.78 kb XhoI-BglII (67.6%-79.6%) fragment from 186 del2 (6), which contains the deletion from 67.9% to 74.0%. This fragment was recombined into 186 cItsp (1) DNA at the unique XhoI (67.6%) and BglII (79.6%) sites (this work).

TABLE 2.1

BACTERIAL STRAINS.

stock number	strain	genotype	comments	origin or reference
E251	W3350	F⁻ galK galT strA748	Su⁻	This lab.. Made by transduction of strA allele from CGSC4214 into W3350.
E252	W3350 (186)	E251 (186 cItsp(1))	A Su⁻ 186 lysogen	J. Finnegan (1979).
E298	GC4540	sfiA::Tn5 thr leu hisD strA tif tsl pyrD34	Used to make transducing stocks for transducing sfiA into E536(pKC7) and E1111(pEC400)	From S. Gottesman. D'Ari and Huisman (1983).
E508	C600	tonA supE44 thr leu thi	Su2⁺	Appleyard (1954).
E536	W3350	E594 strA594 F⁻ galK galT	Su⁻	Campbell (1965), Weigle (1966).
E538	S26	h-59 (S26 λ i λ cured of the λ lysogen) F⁻ gal⁺ strˢ	Su⁻	From D. Hogness. Garen et al. (1965).
E539	S26	h-59 (S26 λiλ cured of the λ lysogen) T2ʳ HfrC phoA rel tonA supD	Su1⁺	From D. Hogness.
E540	S26	h-59 (S26 λ i λ cured of the λ lysogen) F⁻ supE gal⁺ strˢ	Su2⁺	From D. Hogness.
E541	S26	h-59 (S26 λ i λ cured of the λ lysogen) F⁻ supF gal⁺ strˢ	Su3⁺	From D. Hogness.
E573	C600 (186)	E508 (186 cI⁺)	A Su⁺ 186 lysogen	This lab..

BACTERIAL STRAINS.

E574	C600 (186)	E508 (186 cItsp(1))	A Su^+ 186 lysogen Source of 186 DNA.	This lab.. Described by Baldwin et al. (1966).
E605	JM101	lac pro supE44 thi F'traD36 proAB $lacI^q$ lacZ delM15	Host for M13 infection	Messing (1979).
E660	CSR603	recA1 uvrA6 phi1 supE44	A Su^+ maxicell strain for the labelling of proteins	Sancar and Rupert, (1978) and Sancar et al. (1979).
E699	W3350 (186)	E536 (186 cI^+)	A Su^- 186 lysogen	J.B. Egan, this lab..
E767	W3350	E251 thyA	Su^-	This work. constructed as described in Chapter 2.15.2.
E777	W3350	E251 thyA thyR	Su^- used in pre-labelling studies	This work. constructed as described in Chapter 2.15.2.
E780	W3350 (186)	E777 (186 cItsp Aam11(8))	A Su^- 186 A^- lysogen used in pre-labelling studies	This work.
E788	W3350 (186)	E251 (186 cItsp Aam11(8))	A Su^- 186 A^- lysogen used in pulse-labelling studies	This work.
E832	M72	lacZam str^R delbio-uvrB deltrpEA2 (λ Nam7Nam53 cI857delH1)	Host for pPLc236 plasmid-clones	From E. Remaut. Castellazzi et al. (1972) and Bernard et al. (1979).
E862	N100	galK sup^+ lac^+ recA pro	Strain used for the analysis of promoters and terminators	From K. McKenney. Shulman et al. (1976).
E863	C600	galK lac thr leu supE44	Strain used for the analysis of promoters and terminators	From K. McKenney. Shulman et al. (1976).

BACTERIAL STRAINS.

E1011	C600 (186)	E508 (186 cItsp Aam11(8))	A Su^+ 186 A^- lysogen Source of 186 A^- DNA	S. Hocking, this lab..
E1111	W3350 (186)	E536 (186 cItsp Aam11(8))	A Su^- 186 A^- lysogen	S. Hocking, this lab..
E2121	N100 (pKC7)	recA gal sup^+ carrying plasmid pKC7	Source of pKC7 DNA	Rao and Rogers (1979).
E2131	N100 (pKO1)	recA gal sup^+ carrying plasmid pKO1	Source of pKO1 DNA	McKenney et al. (1981).
E2161	S37	C600 carrying plasmid pcI857	Source of pcI857 DNA	From E. Remaut Remaut et al. (1983).
E2184	N100 (pKO2)	recA gal sup^+ carrying plasmid pKO2	Source of pKO2 DNA	From K. McKenney de Boer et al. (1984).
E2188	MC1061 (pMC931)	ara galUK strA dellacX74 carrying plasmid pMC931	Source of pMC931 DNA	From P. Reeves. Casadaban et al. (1980).
E2195	N100 (pKL600)	E862 carrying plasmid pKL600	Source of pKL600 DNA	This work.
E2196	M72 (pPLc236)	E832 carrying plasmid pPLc236	Source of pPLc236 DNA	A. Puspurs, this lab..
E2267	pp100	F^+ thi galK carrying plasmid pGP1	Contains the plasmid pGP1, which encodes the Mu kil gene	From N. Goosen. Giphart-Gassler and van de Putte (1979).
E2268	W3350 (186) (pEC400)	E1111 carrying plasmid pEC400	Used for the analysis of 186 early functions	This work.
E2269	W3350 (186) (pKC7)	E1111 carrying plasmid pKC7	Used as a control in the analysis of 186 functions	This work.

BACTERIAL STRAINS.

E2270	W3350 (pKC7)	E536 carrying plasmid pKC7	Used as a control in the analysis of 186 functions	This work.
E2271	W3350 (186) (pEC400)	E2268 sfiA::Tn5	SfiA$^-$	This work. Made by transduction (using P1kc) of sfiA::Tn5 from E298 into E2268.
E2272	W3350 (pKC7)	E2270 sfiA::Tn5	SfiA$^-$	This work. Made by transduction (using P1kc) of sfiA::Tn5 from E298 into E2270.
E2273	W3350 (186) (pEC400)	E2268 recA$^+$ sr1300::Tn10	RecA$^+$	This work. Made by transduction (using P1kc) from the strain JC10241 (recA$^+$ sr1300::Tn10) (Csonka and Clark, 1980).
E2274	W3350 (186) (pEC400)	E2268 recA56 sr1300::Tn10	RecA$^-$	This work. Made by transduction (using P1kc) from the strain JC10240 (recA56sr1300::Tn10)(Csonka and Clark, 1980).
E2275	W3350 (pKC7)	E2270 recA$^+$ sr1300::Tn10	RecA$^+$	This work. See E2273.
E2276	W3350 (pKC7)	E2270 recA56 sr1300::Tn10	RecA$^-$	This work. See E2274.
E4063	pp442	F$^+$thi galK hek	Hek$^-$ (resistant to Mu Kil)	From N. Goosen. Goosen and van de Putte (1984).
E4064	pp442 (186)	E4063 (186 cItsp Aam11(8))	A 186 lysogen of the Hek strain	This work.
E4065	pp442 (186)	E4063 (186 cItsp Aam11Dhr1(13))	A 186 Dhr$^-$ lysogen of the Hek$^-$ strain	This work.
E4066	W3350 (186)	E777 (186 cItsp ins3Aam11(15))	A Su$^-$ 186 Int$^-$A$^-$ lysogen used for pre-labelling studies	This work.

BACTERIAL STRAINS.

E4067	W3350 (186)	E251 (186 cItsp ins3Aam11(15))	A Su^- 186 Int^-A^- lysogen	This work.
E4068	W3350 (186)	E251 (186 cItsp Aam11Dhr1(13))	A Su^- 186 A^-Dhr^- lysogen used for pulse-labelling studies	This work.
E4069	W3350 (186)	E251 (186 cItsp ins3Aam11Dhr1 (17))	A Su^- 186 Int^-A^- Dhr^- lysogen	S. Williams, this lab..
E4070	W3350 (186)	E251 (186 cItsp ins3Dhr1(16))	A Su^- 186 Int^- Dhr^- lysogen	S. Williams, this lab..
E4089	N4903	pgl del8 his ilv str rnc$^+$ gly::Tn5	$RNaseIII^+$	From D. Court. Apirion and Watson (1974).
E4090	N4903	E4089 rnc105	$RNaseIII^-$	From D. Court. Kindler et al. (1973); Apirion and Watson (1974).
E4121	W3350 (186)	E251 (186 cItsp del3(18))	A lysogen of a 186 deletion mutant.	This work.
E4122	W3350 (186)	E251 (186 cItsp del4(19))	A lysogen of a 186 deletion mutant.	This work.
E4123	C600 (186)	E508 (186 cItsp Aam11Dhr1(13))	A Su^+ 186 A^-Dhr^- lysogen. Source of 186 A^-Dhr^- DNA	This work.
E4124	C600 (186)	E508 (186 cItsp Aam11Dhr2(14))	A Su^+ 186 A^-Dhr^- lysogen	This work.
E4125	W3350 (186)	E251 (186 cItsp Aam11Dhr2(14))	A Su^- 186 A^-Dhr^- lysogen	This work.
E4127	N100 (186)	E862 (186 cItsp ins3Dhr1(16))	A $GalK^-$ Su^- 186 Int^-Dhr^- lysogen	This work.
E4128	N100 (186)	E862 (186 cItsp ins3Aam11(15))	A $GalK^-$ Su^- 186 Int^-A^- lysogen	This work.
E4129	N4903 (186)	E4089 (186 cItsp(1))	$RNaseIII^+$	This work.

BACTERIAL STRAINS.

E4130	N4903 (186)	E4090 (186 cItsp(1))	RNaseIII$^-$	This work.
E4134	W3350 (186)	E251 (186 cItsp CP77am(20))	A Su$^-$ 186 CP77$^-$ lysogen	This work.
E4135	W3350 (186)	E251 (186 cItsp deltR1(22))	A Su$^-$ 186 deltR1 lysogen	This work.
E4137	W3350	E536 Dhr1^R	A mutant resistant to the 186 Dhr function	This work.
E4138	W3350 (186)	E251 (186 cItsp CP78am(21))	A Su$^-$ 186 CP78$^-$ lysogen	This work.
E4168	159	F$^-$ uvrA strA Tn10::srl300 gal$^-$ recA56	A Su$^-$ maxicell Strain for protein labelling	A. Puspurs, this lab.. Made by P1kc transduction of recA56 from the strain JC10240 (recA56 srl::Tn10) (Csonka and Clark, 1980) into 159 uvrA (obtained from M. Pearson).
E4170	W3350 (186)	E251 (186 cItsp ins3(7))	A Su$^-$ 186 Int$^-$ lysogen	This work.
E4176	W3350 (186)	E536 Dhr1^R (186 cItsp Aam11(8))	A 186 lysogen of the Dhr1^R mutant	This work.

2.2.2 Other Bacteriophage Strains.

Plkc : This phage was used as a transducing vector for bacterial strain constructions.

2.3 PLASMID-VECTORS AND PLASMID-CLONES.

2.3.1 Plasmid-Vectors.

The plasmids used in this study are described below.

pKC7 : A derivative of the plasmid pBR322, in which the HindIII-BamHI fragment is replaced with the HindIII-BamHI fragment from the transposon Tn5 (Rao and Rogers, 1979; Auerswald et al., 1981; Mazodier et al., 1985). pKC7 is shown diagrammatically in Figure 2.1(a). pKC7 encodes the genes for ampicillin and kanamycin resistance. The plasmid has unique BglII and XhoI sites.

pMC931 : A plasmid derived from pACYC177, which is compatible with pBR322-derived plasmids (Casadaban et al., 1980). pMC931 is shown diagrammatically in Figure 2.1(b). This plasmid encodes the genes for ampicillin and kanamycin resistance.

pPLc236 : A plasmid containing the bacteriophage λ promoter pL for the cloning and controlled expression of genes in hosts encoding the λ cI857 gene (Remaut et al., 1981). pPLc236 is shown diagrammatically in Figure 2.1(c). This plasmid encodes the gene for ampicillin resistance.

pcI857 : A plasmid derived from pMC931, which encodes the lambda cI857 gene (Remaut et al., 1983). This plasmid encodes the gene for kanamycin resistance.

Figure 2.1 Circular maps of cloning and expression vectors.

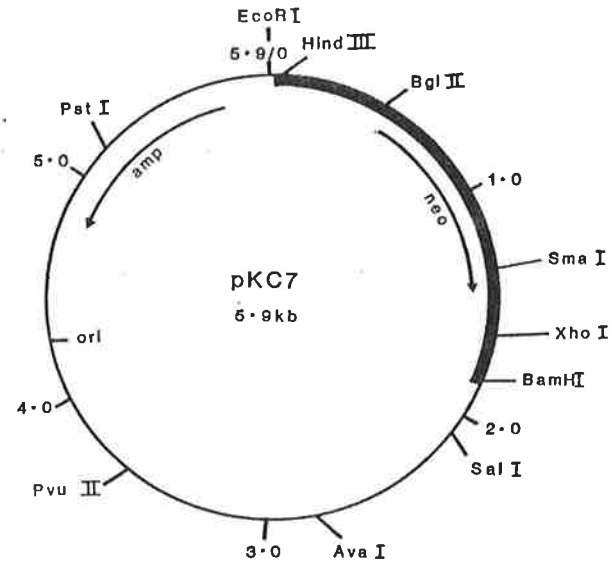
a. pKC7 : pBR322-derived cloning vector. The area shaded represents the region derived from Tn5.

b. pMC931 : pACYC177-derived cloning vector. The area shaded is the region encoding the E. coli lacZYA genes.

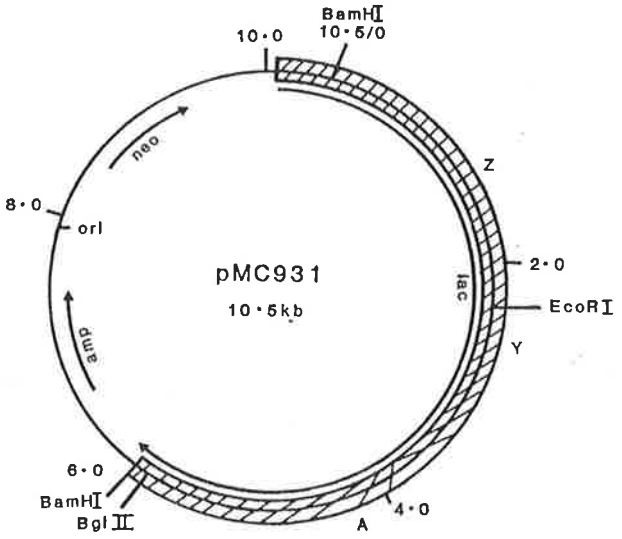
c. pPLc236 : The λ pL-expression vector. The position of the λ pL promoter, is shown.

The positions and direction of transcription of genes for kanamycin resistance (neo) and/or ampicillin resistance (amp) carried by these plasmids, are indicated on the maps.

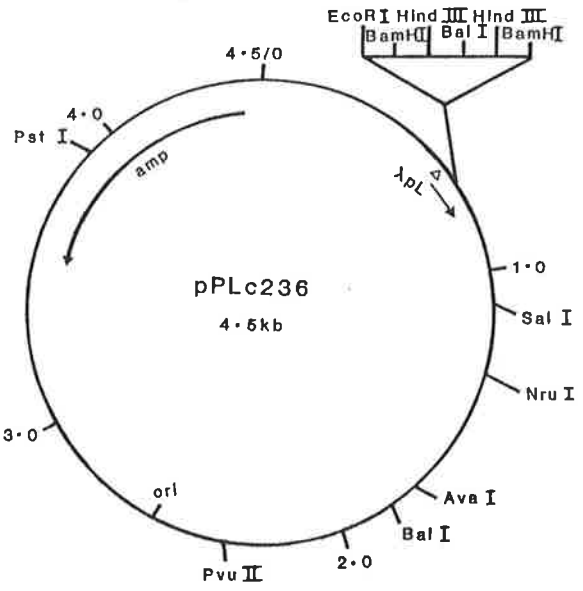
(a)


EcoR I
5·9/0
Hind III
Bgl II
Pst I
5·0
amp
neo
1·0
Sma I
pKC7
5·9kb
ori
Xho I
BamH I
4·0
2·0
Sal I
Pvu II
3·0
Ava I

(b)


BamH I
10·5/0
10·0
neo
Z
8·0
ori
2·0
pMC931
10·5kb
lac
EcoR I
amp
Y
6·0
BamH I
Bgl II
A
4·0

(c)


EcoR I Hind III Hind III
BamH I Bal I BamH I
4·5/0
4·0
Pst I
amp
λpL
1·0
Sal I
pPLc236
4·5kb
Nru I
3·0
ori
Ava I
Bal I
Pvu II
2·0

pKO1 : A plasmid for the cloning and quantitation of promoter activity (McKenney *et al.*, 1981). pKO1 is shown diagrammatically in Figure 2.2(a). This plasmid encodes the gene for ampicillin resistance.

pKO1-T : pKO1 containing the λ *to* terminator inserted after the *galK* gene (M. Rosenberg, personal communication). This plasmid encodes the gene for ampicillin resistance.

pKO2 : A plasmid for the cloning and quantitation of promoter activity (de Boer, 1984). pKO2 is shown diagrammatically in Figure 2.2(b). This plasmid encodes the gene for ampicillin resistance. pKO2 contains a greater number of cloning sites than pKO1.

pKL600 : A plasmid for the cloning and quantitation of terminator activity containing the *pLac* promoter in an orientation such that it allows the expression of *galK*. pKL600 is derived from pKO8 (pKO1 with a *SalI* linker in place of the *EcoRI* site; Figure 2.2a) by insertion of the *PvuII* fragment from M13mp10, which includes the *pLac* promoter and multiple cloning sites. The *EcoRI* site derived from M13mp10 has been end-filled to create stop codons in all three reading-frames. This plasmid was obtained from K. McKenney (personal communication). pKL600 is shown diagrammatically in Figure 2.2(c). This plasmid encodes the gene for ampicillin resistance.

Figure 2.2 Circular maps of McKenney promoter-analysis and terminator-analysis vectors.

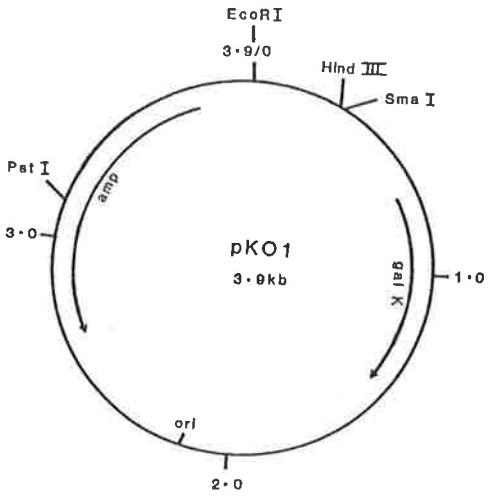
a. pKO1 : Promoter-analysis vector.

b. pKO2 : Promoter-analysis vector.

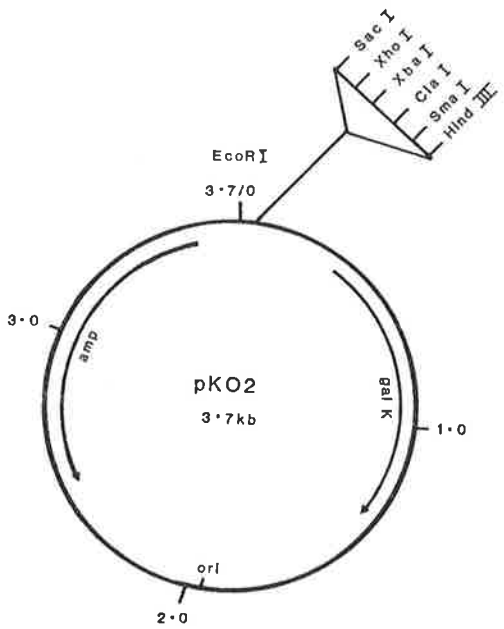
c. pKL600 : Terminator-analysis vector. The position of the pLac promoter, is shown.

The position and direction of transcription of the ampicillin resistance gene (amp) carried by these plasmids, is indicated on the maps.

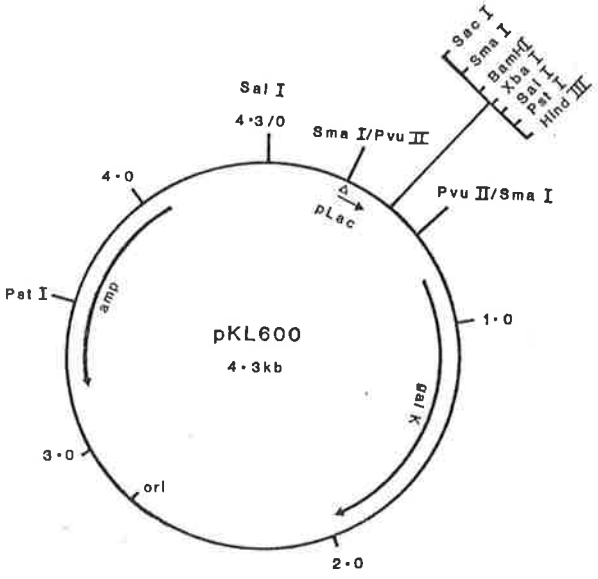
(a)


EcoR I
3·9/0
Hind III
Sma I
Pst I
3·0
amp
pKO1
3·9kb
gal K
1·0
ori
2·0

(b)


Sac I
Xho I
Xba I
Cla I
Sma I
Hind III
EcoR I
3·7/0
3·0
amp
pKO2
3·7kb
gal K
1·0
ori
2·0

(c)


Sac I
Sma I
BamH I
Xba I
Sal I
Pst I
Hind III
Sal I
4·3/0
Sma I/Pvu II
4·0
Pvu II/Sma I
pLac
Pst I
amp
pKL600
4·3kb
1·0
gal K
3·0
ori
2·0

2.3.2 Plasmid-Clones.

Plasmid-clones used in this study are described below. Plasmid-clones constructed in this work were obtained using the methods described in Chapter 2.31.

pEC400 : Contains the 1.78 kb XhoI-BglII (67.6%-79.6%) fragment from 186 del1 (5) cloned into the XhoI and BglII sites of pKC7 (H. Richardson, 1981). This clone encodes the 186 early lytic genes including a lethal function under 186 cI repressor control and is therefore only stable in a 186 lysogen.

pEC401 : pEC400 containing mutations (Dhr1), which allow cell survival in a non-lysogen (H. Richardson, 1981).

pEC402 : pEC400 containing mutations (Dhr2), which allow cell survival in a non-lysogen (H. Richardson, 1981).

pEC403 : pEC400 containing a mutation (Dhr3), which allows cell survival in a non-lysogen (this work).

pEC404 : pPLc236 containing the 0.4 kb PstI-HincII (77.4%-78.7%) fragment from 186 cItsp (1) in the orientation such that rightward genes are expressed from λ pL (r-orientation). The clone was obtained by replacing the 1.58 kb BamHI-PvuII fragment from pPLc236 with the 0.4 kb BamHI (in the cloning site of pPLc236 DNA) to HincII (in the 186 cloned DNA) fragment from pEC503 (this work). This clone encodes the CP77 gene.

pEC405 : pMC931 containing the 2.24 kb NruI-SauIIIA (70.4%-77.6%) fragment from 186 cItsp (1), which encodes the cI, CP75, and CP76 genes. The clone was obtained by subcloning from pEC410

the SauIIIA (in pKC7 DNA, 6 bp from the beginning of the 186 cloned DNA) to SauIIIA (77.6%) fragment into the unique BglII site of pMC931 (this work).

pEC406 : Contains the 0.69 kb PstI-BglII (77.4%-79.6%) fragment from 186 delIDhrI (9) end-filled and cloned into the SmaI site of pKO1 in the orientation such that rightward transcription would read into the galK gene (r-orientation) (this work).

pEC407 : Contains the 0.4 kb PstI-HincII (77.4%-78.7%) fragment from 186 delIDhrI (9). This clone was obtained by subcloning from pEC406 the HindIII (in PKO1 DNA) to HincII 0.41 kb fragment into the HindIII and SmaI sites of pKO1 (this work).

pEC410 : Contains the 2.77 kb NruI-BglII (70.4%-79.6%) fragment from 186 cItsp (1), which encodes the cItsp, CP75, CP76, CP77 and CP78 genes, cloned into pKC7 by replacement of the 1.0 kb BglII-SmaI fragment (this work).

pEC411 : Contains the 0.3 kb HincII-BglII (78.7%-79.6%) fragment from 186 cItsp (1) cloned into the SmaI and BamHI sites of pKL600 such that promotion from pLac results in rightward transcription of 186 DNA (r-orientation) (this work).

pEC412 : Contains the 0.27 kb HincII-BglII (78.7%-79.6%) fragment from deltR1 DNA (isolated from the deltR1 derivative of mEC400) cloned into the SmaI and BamHI sites of pKL600 such that promotion from pLac results in rightward transcription of 186 DNA (r-orientation) (this work).

pEC415 : Contains the 2.71 kb NruI-AhaIII (70.4%-79.4%) fragment encoding the cItsp, CP75, CP76, CP77$^-$ and CP78$^-$ genes from 186 cItspAam11Dhr1 (13) cloned into the SmaI site of pKO2

such that transcription from 186 pR reads into the galK gene (r-orientation) (this work).

pEC417 : Contains the 2.71 kb NruI-AhaIII (70.4%-79.4%) fragment encoding the cItsp, CP75, CP76, CP77 and CP78 genes from 186 cItsp (1) cloned into the SmaI site of pKO2 such that transcription from 186 pR reads into the galK gene (r-orientation) (this work).

pEC420 : pPLc236 containing the 0.53 kb SauIIIA-BglII (77.9%-79.6%) fragment from CP78am DNA in the orientation such that rightward genes are expressed from λ pL (r-orientation). The clone was obtained (as described for pEC421) from the CP78am derivative of mEC401 (this work). This clone encodes the CP78am gene.

pEC421 : pPLc236 containing the 0.53 kb SauIIIA-BglII (77.9%-79.6%) fragment derived from wild-type 186 in the orientation such that rightward genes are expressed from λ pL (r-orientation). The clone was obtained by isolating the SauIIIA-BglII fragment from mEC401 after digestion with SauIIIA, and ligating this fragment into the BamHI site of pPLc236 (this work). This clone encodes the CP78 gene.

pEC422 : pPLc236 containing the 0.4 kb PstI-HincII (77.4%-78.7%) fragment from CP77am DNA in the orientation such that rightward genes are expressed from λ pL (r-orientation). The clone was obtained by replacing the 0.6 kb HindIII-NruI fragment from pPLc236 with the 0.4 kb HindIII-HincII fragment from the CP77am derivative of mEC401 (this work). (The HindIII site is in the cloning site of M13). This clone encodes the CP77am gene.

pEC424 : pPLc236 containing the 0.69 kb PstI-BglII (77.4%-79.6%) fragment derived from wild-type 186 in the orientation such that rightward genes are expressed from λ pL (r-orientation). The clone was obtained by replacing the 0.6 kb HindIII-NruI fragment from pPLc236 with the 0.7 kb HindIII-SmaI fragment from mEC401 (this work). (Both the HindIII and SmaI sites are in the cloning site of M13.) This clone encodes the CP77 and CP78 genes.

pEC426 : pKO2 containing the 4.44 kb NruI-XmnI (70.4%-85.2%) fragment encoding the cItsp, CP75, CP76, CP77, CP78, CP79, CP80, CP81 and CP83 genes from 186 cItsp (1) such that transcription from 186 pR reads into the galK gene (r-orientation). The clone was obtained by ligating the 2.77 kb NruI-BglII (70.4%-79.6%) fragment with the 1.66 kb BglII-XmnI (79.6%-85.2%) fragment, and then recombining the resulting NruI-XmnI fragment into the SmaI site of pKO2 (this work). [The NruI-XmnI (70.4%-85.2%) fragment could not be isolated directly since there is another XmnI site at 74.4%.]

pEC427 : pKL600 containing the 0.23 kb HincII-AhaIII (78.7%-79.4%) fragment from 186 cItsp (1) in an orientation such that rightward 186 genes are expressed from pLac. This clone was obtained by subcloning the HincII-AhaIII fragment from pEC410 into the SmaI site of pKL600.

pEC503 : Contains the 0.69 kb PstI-BglII (77.4%-79.6%) fragment isolated from pEC400, end-filled and cloned into the end-filled HindIII site of pPLc236 in the orientation such that rightward genes are expressed from λ pL (r-orientation) (A. Puspurs, this laboratory). This clone encodes the CP77 and CP78 genes.

pEC601 : Contains the 1.28 kb HaeIII (71.2%-75.5%) fragment from 186 cItsp (1) cloned into the SmaI site of pKO1-T such that transcription from 186 pR reads into galK (r-orientation) (I. Dodd, this laboratory). This clone encodes the cItsp gene and the pR and pL promoters.

pEC602 : Contains the 1.28 kb HaeIII (71.2%-75.5%) fragment from 186 cItsp (1) cloned into the SmaI site of pKO1-T such that transcription from 186 pL reads into galK (l-orientation) (I. Dodd, this laboratory). This clone encodes the cItsp gene and the pR and pL promoters.

pEC604 : Contains the 1.28 kb HaeIII (71.2%-75.5%) fragment from 186 cItsp (1) cloned into the SmaI site of pKO2 such that transcription from 186 pR reads into galK (r-orientation) (I. Dodd, this laboratory). This clone encodes the cItsp gene and the pR and pL promoters.

pEC606 : Contains the 2.0 kb MstI (69.4%-76.1%) fragment from 186 cItsp (1) cloned into the SmaI site of pKO2 such that transcription from 186 pR reads into galK (r-orientation) (I. Dodd, this laboratory). This clone encodes the cItsp and CP75 genes and the pR and pL promoters.

pGP1 : pMB9 containing the Mu c^{ts}, ner, A, B, cim and kil genes (Giphart-Gassler and van de Putte, 1979).

2.4 M13-VECTORS AND M13-CLONES.

2.4.1 M13-Vectors.

The M13-vectors used in this study were M13mp8, mp9 (Messing and Vieira, 1982; Messing, 1983), and mp19 (Norrander et al., 1983).

2.4.2 M13-Clones.

The M13-clones used in this study are described below. M13-clones constructed in this work were obtained using the methods described in Chapter 2.31. The sequence coordinates of the restriction sites refer to the first base of the site on the l-strand.

mEC400 : M13mp9 containing the l-strand of the 0.69 kb PstI-BglII fragment (77.4%-79.6%; sequence coordinates 3556-4244) from 186 cItsp (1) cloned into the PstI-BamHI sites of M13mp9 (this work).

mEC401 : M13mp8 containing the r-strand of the 0.69 kb PstI-BglII fragment (77.4%-79.6%; sequence coordinates 3556-4244) from 186 del1 (5) cloned into the PstI-BamHI sites of M13mp8 (this work).

mEC404 : M13mp9 containing the l-strand of the 142 bp HpaII fragment (76.7%-77.2%; sequence coordinates 3367-3509) from 186 del1Aaml1Dhr1 (11) DNA (this work).

mEC405 : M13mp9 containing the l-strand of the 177 bp HpaII fragment (77.2%-77.8%; sequence coordinates 3509-3687) from 186 cItsp (1) DNA (this work).

mEC406 : M13mp9 containing the l-strand of the 398 bp HpaII fragment (77.8%-79.1%; sequence coordinates 3687-4085) from 186 cItsp (1) DNA (this work).

mEC407 : M13mp9 containing the r-strand of the 398 bp HpaII fragment (77.8%-79.1%; sequence coordinates 3687-4085) from 186 cItsp (1) DNA (this work).

mEC408 : M13mp9 containing the l-strand of the 393 bp HpaII fragment (79.1%-80.4%; sequence coordinates 4085-4478) from 186 cItsp (1) DNA (this work).

mEC500 : M13mp8 containing the l-strand of the 2.18 kb PstI fragment (77.4%-84.6%; sequence coordinates 3556-5733) cloned into the PstI site of M13mp8 (D. Martin, this laboratory). This fragment was obtained from pEC17.2, which contains the PstI (77.4%-84.6%) fragment from 186 cItsp (1) inserted into the PstI site of pBR322. The phenotype of pEC17.2 is consistent with an orientation such that 186 genes are not expressed from the pBR322 pAmp promoter (Finnegan, 1979).

mEC501 : M13mp8 containing the r-strand of the 2.18 kb PstI fragment (77.4%-84.6%; sequence coordinates 3556-5733) and was constructed as described for mEC500 (D. Martin, this laboratory).

mEC700 : M13mp9 containing the l-strand of the HpaII fragment (86.1%-86.9%; sequence coordinates 6174-6417) from 186 cItsp (1) end-filled and cloned into the SmaI site of M13mp9 (Sivaprasad, 1984).

mEC701 : M13mp9 containing the l-strand of the FnuDII fragment (87.3%-88.3%; sequence coordinates 6530-6842) from 186 cItsp (1) cloned into the SmaI site of M13mp9 (Sivaprasad, 1984).

mEC800 : M13mp7 containing the l-strand of the 1.72 kb PstI fragment (65.5%-77.4%; sequence coordinates 1-3556) from 186 del1 (5) cloned into the PstI site of M13mp7. The fragment was obtained from the plasmid-clone pEC35, which contains the PstI (65.5%-77.4%) fragment [from 186 del1 (5)] cloned into

the PstI site of pBR322 (Kalionis, 1985; Finnegan and Egan, 1979).

mEC801 : M13mp7 containing the r-strand of the 1.72 kb PstI fragment (65.5%-77.4%; sequence coordinates 1-3556) from 186 del1 (5) cloned into the PstI site of M13mp7. The fragment was obtained from the plasmid-clone pEC35 (see mEC800; Kalionis, 1985; Finnegan and Egan, 1979).

mEC802 : M13mp9 containing the 1-strand of the 446 bp HpaII fragment (73.8%-75.3%; sequence coordinates 2487-2933) from 186 cItsp (1) cloned into the AccI site of M13mp9 (Kalionis, 1985).

2.5 OLIGONUCLEOTIDES.

The oligonucleotides used during the course of this work for specific mutagenesis and DNA sequencing were constructed by BRESA and are described below.

CP77 amber : (1-strand) 17-mer 5'-CGCCGAAATAGTCAGGT-3' (sequence coordinates 3789-3805). TAG replaces TGG. supD suppressing strains (e.g. E539) replace the amber codon with the correct amino acid, ser.

CP78 amber : (1-strand) 17-mer 5'-GAATTGTTTAGGGTGCC-3' (sequence coordinates 3871-3887). TAG replaces TTG. The amber mutation is not replaced with the correct amino acid, leu, in any of the suppressor strains available.

tR1 terminator deletion (deltR1) : (r-strand) 30-mer (sequence coordinates 4114-4099/4069-4056) 5'-CCTCCTGTTTTTTGGC\TAATTACGTTTAAT-3' (the point of the deletion is indicated by \). The deletion

removes 29 bp spanning the potential terminator tR1 (removing the region of hyphenated dyad symmetry and the following 9 bases containing 6 T-residues).

The positions chosen for the construction of the amber mutations were, where possible, in the middle of the gene to ensure the gene was inactivated and to enable identification of the amber fragment on a protein gel. In addition, the position chosen for the amber mutation was such that the amber codon could be replaced by the correct amino acid in one of the three suppressing strains available (supD, supE and supF). However, it was not possible to simply create an amber mutation in the CP78 gene with the properties described above. The CP78am oligonucleotide that was chosen does not allow the original amino acid to be replaced in either of the three suppressing strains available, and is positioned at the 5'-end of the gene in order to ensure inactivation of the gene (since possible alternative positions were too close to the 3'-end).

2.6 ENZYMES.

Restriction endonucleases : New England Biolabs or Bethesda Research Labs..

E. coli DNA polymerase I (Klenow fragment) : Biotechnology Research Enterprises of South Australia (BRESA).

T4 DNA ligase : Boehringer Mannheim and BRESA.

Calf intestinal phosphatase : BRESA.

Avian myeloblastosis virus (AMV) reverse transcriptase : Life Science Inc., Florida.

E. coli RNA polymerase (holoenzyme) : Boehringer Mannheim.

Lysozyme : Sigma Chemical Co..

Proteinase K : Boehringer Mannheim.

RNAse A : Sigma Chemical Co.. Stock solutions were heated at 80°C for 20 min to inactivate DNAses.

E. coli DNAse I : Boehringer Mannheim.

T4 Polynucleotide Kinase : Boehringer Mannheim.

T4 DNA Polymerase : PL Biochem. Inc..

All enzymes were stored according to the manufacturers' directions.

2.7 RADIOCHEMICALS.

Radiochemicals d[α-^{32}P]CTP and d[α-^{32}P]ATP of specific activity 1700 Ci/mmol, d[γ-^{32}P]ATP of specific activity 2000 Ci/mmol and r[α-^{32}P]GTP of specific activity 1000 Ci/mmol (and all having radioactive concentrations of 5 mCi/ml) were purchased from BRESA. [methyl-^{3}H]-thymine of specific activity 30-60 Ci/mmol (and radioactive concentration of 1 mCi/ml), [methyl-^{3}H] - thymidine of specific activity 22-44 Ci/mmol (and radioactive concentration of 1 mCi/ml), D-[1-^{14}C]-galactose of specific activity of 58 mCi/mmol (and radioactive concentration of 200 uCi/ml) and L-[^{35}S]-methionine of specific activity 1320 Ci/mmol (and radioactive concentration of 14.4 mCi/ml) were obtained from Amersham.

2.8 CHEMICALS.

All chemicals were of analytical grade or of the highest purity available.

Acridine orange : Sigma Chemical Co..

Acrylamide : Sigma Chemical Co..

Agarose : Sigma Chemical Co..

Amine A : Humpko Sheffield, U.S.A..

Ampicillin (Sodium salt) : Sigma Chemical Co..

Ammonium persulphate (APS) : May and Baker. Stock solutions at 25% (w/v) in water, were kept at 4°C for no more than two weeks.

Bacto-tryptone, yeast extract and Bacto-agar : Difco Labs., U.S.A..

Bovine serum albumin (BSA) : Sigma Chemical Co.. Acetylated before use to remove nucleases according to the procedure of Gonzalez et al. (1977) and kept as a 1.5 mg/ml solution in water at -20°C. (Gift from M. Pritchard, this laboratory.)

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIG) : Sigma Chemical Co.. Stock solutions at 20 mg/ml in dimethyl formamide, were kept at -20°C.

Brij 58 (polyoxyethylene 20 cetyl ether) : Sigma Chemical Co..

Calf thymus DNA : Sigma Chemical Co.. Sonicated, heat denatured and stored as a 10 mg/ml solution in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA at -20°C. (Gift from D. Dodd, this laboratory).

Chloramphenicol : Sigma Chemical Co..

Cycloserine : Sigma Chemical Co..

Coumermycin A : Sigma Chemical Co..

CsCl : Bethesda Research Labs..

Deoxyribonucleoside triphosphates (dNTP) : Sigma Chemical Co.. Stock solutions at 20 mM (prepared in 5 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) were kept at -20°C.

Dideoxyribonucleoside triphosphates (ddNTP) : Sigma Chemical Co.. Stock solutions at 20 mM (prepared in 5 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) were kept at -20°C.

Diethylpyrocarbonate : Sigma Chemical Co..

Dithiothreitol : Sigma Chemical Co.. Stored as a 1 M solution in water, in the dark at -20°C.

E. coli tRNA : Sigma Chemical Co.. Extracted three times with phenol/TE before use and kept as a 10 mg/ml solution in TE, at -20°C.

Ethanol : Redistilled before use and stored at -20°C. RNAse-free ethanol was obtained by sterilising the distillation apparatus and collection bottles in a 110°C oven overnight before use.

Ethidium bromide : Sigma Chemical Co.. Stored as a 10 mg/ml solution in water, at 4°C in the dark.

Ethylenediaminetetraacetic acid (EDTA) : Disodium salt. Sigma Chemical Co..

Ficoll 400 : Phamacia Fine Chem..

Formamide : B.D.H. Labs. Aust.. De-ionised (Chapter 2.29.3d) and stored in the dark at -20°C.

Glyoxal : Technical grade glyoxal, was obtained from B.D.H. Labs. Aust.. De-ionised (Chapter 2.29.3d) and stored in the dark at -80°C for long term storage, otherwise stored at -20°C.

Isopropyl-β-D-thiogalactopyranoside (IPTG) : Sigma Chemical Co.. Stock solutions were at 24 mg/ml in water, and kept at -20°C.

Kanamycin (sulphate) : Sigma Chemical Co..

Low-gelling-temperature agarose (LGT) : Bethesda Research Labs..

Mixed bed resin (508-X8(D)) : Bio-Rad Labs..

β-Mercaptoethanol : Sigma Chemical Co..

Methionine assay media (MAM) : Difco Labs., U.S.A..

N-methyl-N'-nitro-N-nitrosoguanidine : Aldrich Chem. Co..

N, N'-methylene-bis-acrylamide (bis) : Sigma Chemical Co..

N, N, N', N'-tetramethylethylenediamine (TEMED) : Eastern Kodak Co..

Nonidet P40 : B.D.H. Labs. Engl..

Phenol : AR grade, was redistilled and stored in the dark at -20°C, B.D.H. Labs. Aust..

Polyethylene glycol (PEG) 8000 : for phage preparations and general use was from Sigma Chemical Co.. M13 phage preparations for sequencing were prepared using PEG 8000 from B.D.H. Labs. Aust..

Polyvinyl pyrrolidone : May and Baker Ltd..

Ribonucleoside triphosphates (NTP) : Sigma Chemical Co.. Stock solutions at 20 mM (prepared in 5 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) were kept at -20°C.

Sequencing primer : (17-mer; 5'-GTAAAACGACGGCCAGT-3') was purchased from New England Biolabs and BRESA.

Sodium azide : Ajax Chemicals Ltd..

Sodium dodecyl sulphate : Sigma Chemical Co..

Tetracycline : Upjohn Pty. Ltd., a kind gift.

Tetramethylammonium chloride : Aldrich Chemical Co..

Toluene Scintillation Fluid contained 0.35% (w/v) PPO (2,5-diphenyl oxazole) and 0.035% (w/v) POPOP (1,4-bis [2-(5-phenyl-oxazolylphenyl)] benzene in toluene. Both PPO and POPOP were obtained from Sigma Chemical Co.. Toluene was obtained from May and Baker Ltd..

Trimethoprim : Burrough Wellcome and Co..

Trizma base and Tris 7-9 : Sigma Chemical Co..

Thymine : Sigma Chemical Co..

Thymidine : Sigma Chemical Co..

Urea : Sigma Chemical Co..

2.9 MEDIA.

2.9.1 Liquid Media.

All media and buffers were prepared in glass distilled water and were sterilised by autoclaving for 25 min at 120° C and 120 kPa.

L (Luria) broth : 1% Bacto-typtone, 0.5% yeast extract, 1% NaCl, pH 7.0.

2x YT broth : 1.6% Bacto-typtone, 1% yeast extract, 0.5% NaCl, pH 7.0.

M13 minimal medium : 1.05% K_2HPO_4, 0.45% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.05% $Na_3citrate.2H_2O$ and water to 1000 ml. This solution was autoclaved, cooled to 45°C and the following added from separately prepared sterile solutions; 10 ml of 20% glucose, 0.8 ml of 1 M $MgSO_4$, 0.5 ml of 1% thiamine-HCl.

TPG-CAA : 0.5% NaCl, 8.0% KCl, 1.1% NH_4Cl, 1% KH_2PO_4, 12.1% Trizma base, 0.8% sodium pyruvate and water added to 90 ml and the pH adjusted to 7.4. After autoclaving and cooling to room temperature the following separately prepared sterile solutions were added to the final concentrations specified; 0.16 mM Na_2SO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 ug/ml $FeCl_3$, 0.2% glucose, and 1% vitamin-free casamino acids.

M63 : 0.1 M KH_2PO_4, 0.015 M $(NH_4)_2SO_4$, 0.002 mM $FeSO_4.7H_2O$ in water adjusted to pH 7.0 with KCl.

M9 : 0.55% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl. After autoclaving sterile solutions of $CaCl_2$ and $MgCl_2$ were added to 0.1 M and 1 mM, respectively.

Additions to growth media were 1.0% vitamin-free casamino acids, 0.2% glucose, 5 ug/ml thiamine-HCl, 2-5 ug/ml thymine, 1 ug/ml biotin, and 50 ug/ml of individual amino acids. Antibiotics were added to rich media at the following concentrations; ampicillin at 50 ug/ml, kanamycin at 50 ug/ml and tetracycline at 20 ug/ml. For minimal media half the above listed concentrations were used.

2.9.2 Solid Media.

Z plates : 1% Amine A, 1.2% Bacto-agar, 0.5% NaCl, pH 7.2.

YGC plates : 1% Amine A, 0.5% Yeast extract, 1% NaCl, 1.5% Bacto-agar then glucose added to 0.1% and $CaCl_2$ added to 2.4 mM.

M9 minimal plates : 1.5% Agar, 0.55% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl and pH adjusted to 7.0. After autoclaving and cooling to 45°C the following sterile solutions were added to the final concentrations indicated; 0.1 mM $CaCl_2$, 1 mM $MgSO_4$,

1 ug/ml thiamine-HCl. If glucose or other sugars and amino acids were required they were added at the concentrations specified above (Chapter 2.9.1, additions to growth media) and the plates poured.

M13 minimal plates : 1.5% Bacto-agar was added to M13 minimal media.

MacConkey-galactose plates : 4% MacConkey agar base, autoclaved, then 1% galactose was added and the plates poured.

EMB plates : 1% Bacto-tryptone, 0.1% Yeast extract, 0.5% NaCl, 0.2% K_2HPO_4, 1.2% Agar, autoclaved then 10 ml of EMB dye (4% eosin Y, 0.65% methylene blue) was added and the plates poured.

Antibiotic plates : YGC plates were supplemented with antibiotics at the following concentrations; tetracycline at 20 mg/ml, ampicillin at 50 mg/ml and kanamycin at 50 mg/ml. Plates were poured from 30 ml of the appropriate medium, dried overnight at 37°C and stored at 4°C.

Soft agar overlay : 1% Bacto-tryptone, 0.7% Bacto-agar, 0.5% NaCl, pH 7.0.

YT soft agar overlay : 0.8% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 0.7% Bacto-agar, pH 7.0.

2.10 BUFFERS.

20x SSC : 3.0 M NaCl, 0.3 M Na_3 citrate, pH 7.4.

10x TBE : 0.89 M Tris-HCl, 0.89 M boric acid, 2.7 mM EDTA, pH 8.3.

10x TAE : 0.4 M Tris-acetate, 0.2 M Na acetate, 10 mM EDTA, pH 8.2.

10x TE : 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA.

10x TM : 0.1 M Tris-HCl, pH 8.0, 0.1 M $MgCl_2$.

TM used for preparation and storage of phage (Chapter 2.13, 2.16, 2.26.1) was 10 mM Tris-HCl, pH 7.1, 10 mM $MgCl_2$.

100x Denhardts solution : 2% Ficoll 400, 2% BSA, 2% Polyvinyl pyrrolidone.

6x Glycerol loading buffer : 30% glycerol, 0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 1 mM EDTA.

Formamide loading buffer : 95% Formamide, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol, 10 mM EDTA.

Sodium Phosphate buffer : 1M Na_2HPO_4/NaH_2PO_4, 10 mM EDTA, pH 6.5.

2.11 MOLECULAR WEIGHT MARKERS.

2.11.1 DNA Molecular Weight Markers.

HinfI digest of pBR325 DNA at 100 ng/ul : Made by HinfI digestion of pBR325 DNA followed by phenol extraction and ethanol-precipitation.

HpaII digest of pUC19 DNA at 500 ng/ul : Obtained from BRESA.

HindIII digest of phage lambda DNA at 400 ng/ul : Obtained from Biolabs and from BRESA.

EcoRI digest of phage SPP-1 DNA at 500 ng/ul : Obtained from BRESA.

Sizes were accurately determined from the published sequences and/or restriction maps of pBR325 (Bolivar, 1978; Prentki et al., 1981), pUC19 (Yanisch-Perron et al., 1985), lambda (Sanger et al., 1982; Daniels et al., 1983), SPP-1 (Ratcliff et al., 1979) and are marked on the appropriate Figures.

2.11.2 RNA Molecular Weight Markers.

Cucumber mosaic virus (CMV) RNA was a gift from C. Davies. The sizes as given in Rezaian *et al.* (1985), are as follows : RNA 1, 3387 b; RNA 2, 3035 b; RNA 3, 2193 b; and RNA 4, 1027 b.

Ribosomal RNA markers were : *E. coli* 23S RNA, 2904 b; and *E. coli* 16S RNA, 1541 b (Brosius *et al.*, 1978; Brosius *et al.*, 1980).

2.12 MISCELLANEOUS MATERIALS.

Fuji Rx medical X-ray film : Fuji Photo Film Co..

Positive/negative Land Pack film : Polaroid.

Nitrocellulose : Schleicher and Schuell BA85 (0.45 u).

Ultrafiltration membrane filters : Millipore (0.45 u).

Glass microfilters 2.5 cm : Whatman Ltd..

DE81 2.3 cm filters : Whatman Ltd..

Dialysis membrane (18/32) : Union Carbide.

Centrifuge rotors : Beckman.

2.13 STORAGE OF BACTERIA AND BACTERIOPHAGE.

Bacterial stocks for short term storage were maintained on YGC plates at 4^{o}C, except JM101, which was maintained on M13 minimal plates. Long term storage of bacterial cultures was by freezing at -80^{o}C after addition of an equal volume of 80% glycerol.

Low-titre stocks of M13 recombinant phage were maintained in 2x YT broth at -20^{o}C. Low-titre 186 phage stocks were passed through

0.45 u Millipore membrane filters and stored at 4°C. High-titre 186 phage stocks prepared by CsCl block gradient centrifugation (Chapter 2.26.1) were dialysed three times against one litre of TM and stored at 4°C.

2.14 GROWTH OF BACTERIAL CULTURES.

Stationary phase bacterial cultures were prepared by inoculating broth with a single colony of bacteria from a plate stock (or for JM101, a loopful of cells from the inoculum region) or a loopful of bacteria directly from the glycerol stock, and incubating overnight in capped flasks at the appropriate temperature (usually 30°C or 37°C) in a New Brunswick gyrotary water bath.

Log phase cultures and indicator bacteria were prepared by diluting a fresh stationary culture one hundred-fold into sterile broth and incubating with aeration in a gyrotary water bath at the appropriate temperature, until the required cell density was reached (usually $2x10^8$ cfu/ml, which occurs at A_{600}= 0.8 in L broth or A_{600}= 0.2 in TPG-CAA). Cell density was measured by observing A_{600} using a Gilford 300 T-1 spectrophotometer. Indicator bacteria were chilled and kept on ice until required.

2.15 CONSTRUCTION OF BACTERIAL STRAINS.

2.15.1 186 Lysogens.

The phage were streaked for single plaques on a lawn of the bacterial strain to be lysogenized and the plates were incubated at 30°C overnight. The centre of a turbid plaque was streaked for single colonies on a YGC plate and the plate was incubated overnight at 30°C. Single colonies were tested, along with the appropriate controls of a lysogen and a non-lysogen, for immunity to 186 by cross-streaking against 186 cI10 (2) and 186 vir1 (3). A colony, which was capable of growth over 186 cI^-, but not 186 vir was considered to be lysogenic. This colony was purified two times

by streaking for single colonies on YGC plates at 30°C and testing several of these colonies for immunity to 186 as described above.

To make 186 amber lysogens of Su^- strains, 10^7-10^9 phage were spotted onto an Su^- lawn and the plate was incubated overnight at 30°C. The centre of the spot was then streaked for single colonies on YGC plates and these were tested for lysogeny and purified as described above.

To construct 186 *int*$^-$ lysogens it was necessary to co-infect cells with a helper *int*$^+$ phage that was incapable of lysogenizing by itself. For this purpose 186 *c*I10 (2) was used. Equal numbers (10^7-10^9 pfu) of the 186 *int*$^-$ and 186 *c*I10 phage were spotted onto the lawn of the bacterial strain to be lysogenized and the plate was incubated overnight at 30°C. The centre of the spot was then streaked for single colonies and these were tested for immunity and purified as described above. The lysogens constructed in this way were tested to ascertain that they were pure *int*$^-$ lysogens and were not double lysogens of 186 *c*I*tsp**int*$^-$ and 186 *int*$^+$*c*I$^-$. This was done by testing cultures, which were grown at 30°C or for three hours after heat-induction at 40°C, for the level of background phage. The level of background phage was determined by removing the bacterial cells by centrifugation (9,000 rpm, 5 min, room temperature, JA20 rotor) and spotting dilutions of the supernatant onto a lawn of E508. In fact, many lysogens constructed in this way were found to be double lysogens of these two phage and gave a higher background level of phage at 30°C (due to spontaneous induction) than a single 186 *c*I*tsp* lysogen (10^8 as compared with 10^6 pfu/ml).

2.15.2 Thy- Strains.

Thymine-requiring strains (*thyA*) requiring 50 ug/ml thymine for growth were constructed by trimethoprim selection (of spontaneous mutants) as described by Miller (1972). The strain was then purified two times by streaking on YGC plates containing thymine (50 ug/ml). Low thymine-

requiring strains (thyA thyR; Okada, 1966) requiring 2 ug/ml thymine for growth were selected from the high thymine-requiring strain after spreading the cells on M9 plates containing thymine (2 ug/ml). Such strains were then purified two times through single colony isolations by streaking on YGC plates supplemented with thymine (2 ug/ml).

2.15.3 P1 Transduction.

P1 transducing stocks : The donor strain was grown in L broth containing $CaCl_2$ (5 mM) to A_{600}= 0.8 ($2x10^8$ cfu/ml) and then P1kc phage was added to 0.2 ml of cells in a glass tube at a multiplicity of addition (moa) of 10^7 pfu/ml. The tube was incubated at 37°C for 30 min to allow adsorption of the phage. The mixture was then spread on to YGC plates using 3 ml of layer agar to which $CaCl_2$ (5 mM) had been added. The plates were incubated until lysis was evident, usually for 6-8 hours at 37°C or overnight at 30°C. A control plate was included where cells were not infected with P1kc, to aid in the detection of cell lysis. The phage were harvested by adding 3 ml of L broth containing $CaCl_2$ to the plate and leaving it for 20 min at 4°C. The top layer agar and L broth were scraped off into a 50 ml Oakridge centifuge tube, a few drops of chloroform were added, and the tube was vortexed for 10 min then centrifuged (10,000 rpm, 10 min, 4°C, in a JA20 rotor). The supernatant was filtered through a 0.45 u Millipore membrane, assayed for phage (as described in Chapter 2.16.1, except that YGC plates were used instead of Z plates), and stored at 4°C. This P1kc stock was used to infect the donor strain again as described above and this second stock was used to transduce the recipient strain.

Transduction : The recipient cells were grown in L broth supplemented with $CaCl_2$ (5 mM) until A_{600}= 1.0 and then 1 ml of bacteria was mixed with 0.1 ml of P1kc phage in a glass tube. After incubation at 37°C for 30 min 0.2 ml of 1 M Na_3 citrate (pH 7.0) was added and the cells were collected

by centrifugation (9,000 rpm, 10 min, 4°C, JA20 rotor) and then resuspended in 1 ml of 0.1 M Na citrate (pH 7.0) to prevent further P1kc infection. The cells were then diluted (10^{-1}, 10^{-2} and 10^{-3}), and 0.1 ml of the diluted and undiluted cells were spread onto selective plates supplemented with 0.2 ml of 1 M Na_3citrate (pH 7.0), to prevent infection by P1kc of cells on the plate, and incubated at the appropriate temperature. As a control to assess the background resistant frequency, the bacterial recipient culture not infected with P1kc was plated onto the selective plates. Also, to confirm the absence of donor bacteria in the phage lysate, 0.1 ml of the P1kc stock was spread onto the selective plates. Transductants obtained were subjected to two single colony purifications.

2.15.4 Transformation with Plasmids.

Plasmids were transformed into bacterial strains using the procedure described in Chapter 2.31.3(b) and the strain was purified two times by streaking for single colonies on YGC plates supplemented with the appropriate antibiotic.

2.16 PHAGE AND BACTERIAL ASSAYS.

2.16.1 Phage Assays.

Phage lysates were assayed for phage by mixing 0.1 ml of the phage diluted in TM buffer, 0.2 ml of log phase indicator bacteria (Chapter 2.14) and 3 ml melted (0.7%) soft agar overlay and pouring the mixture onto Z plates. The agar was allowed to solidify and the plates were inverted and incubated overnight at the appropriate temperature (usually 30°C or 37°C). Plaques were counted and scored as plaque-forming units per ml (pfu/ml).

2.16.2 Bacterial Assays.

Bacterial viable counts were measured by diluting a culture of cells in TM and spreading the appropriate dilution onto YGC plates supplemented with antibiotics or thymine when necessary. The plates were incubated at the appropriate temperature overnight, or longer if necessary, and the colonies were counted and scored as colony-forming units per ml (cfu/ml).

2.17 186 BURST ANALYSIS.

186 phage burst analysis was performed either by infection of cells with the phage (for phage strains for which lysogens could not be made, i.e. cI$^-$ strains), or by heat-induction of a cItsp lysogen of the phage. The phage burst size was determined by calculating the difference between the number of infectious centres before the phage burst and the highest number of pfu/ml produced after the phage burst.

2.17.1 Infection.

Cells were grown generally at 37^{o}C or 39^{o}C in L broth to A_{600}= 0.8 or in TPG-CAA to A_{600}= 0.2 ($2x10^8$ cfu/ml). Phage were added at a moa between 1 and 20 and incubation was continued in a gyratory water bath. Infection was allowed to proceed for 5 - 10 min and then unadsorbed phage were inactivated by the addition of 186-specific antisera at K= 1.5. After 10 min of antisera treatment, the infected culture was diluted 1:100 into pre-warmed broth and incubation was continued. Samples were taken at intervals and assayed for phage (Chapter 2.16.1). A viable count was generally taken before infection (Chapter 2.16.2).

2.17.2 Heat-Induction of 186 Lysogens.

The 186 cItsp lysogenic cultures were grown at 30°C in L broth to A_{600}= 0.8, or TPG-CAA to A_{600}= 0.2 ($2x10^8$ cfu/ml) and then the flask was transferred to 40°C or 41.5°C and incubation was continued. Phage production was assayed at appropriate intervals (Chapter 2.16.1). A viable count was generally taken before heat-induction (Chapter 2.16.2).

2.18 DNA LABELLING STUDIES.

To study the effect of 186 on host DNA replication, total cellular DNA was labelled during 186 infection or heat-induction, by pulse-labelling with ^{3}H-thymidine or continuous labelling of thy$^-$ cells with ^{3}H-thymine. The rationales for these procedures are described by O'Donovan (1977).

2.18.1 Pulse-Labelling with ^{3}H-Thymidine.

Cells were grown overnight at the appropriate temperature in TPG-CAA (containing the appropriate antibiotics and/or nutritional requirements) and were then diluted into the same media and grown with aeration to A_{600}= 0.2. The cultures were either infected with 186 (with CsCl-gradient purified phage; Chapter 2.26.1) at 39°C, at a moa of 20, to ensure a high level of infection, or a 186 cItsp lysogen was heat-induced (Chapter 2.17). Samples (200 ul) were removed at intervals and added to pre-warmed 50 ul aliquots of ^{3}H-thymidine in TPG-CAA (final concentration of 20 uCi/ml) in an Eppendorf tube and incubated without aeration for 2 min. The incorporation of labelled thymidine into DNA has been shown to be linear within this pulse-time interval (Hocking, 1977).

Measurement of incorporation of ^{3}H-thymidine into DNA was done essentially as described by Bollum (1966). The pulse was terminated by removing a 100 ul sample, spotting it onto a Whatman GF/A filter, and then immersing the filter into ice-cold 10% trichloroacetic acid (TCA). The filters were batch-washed 4 times in 10% TCA and twice in ethanol and were

then dried overnight at 65°C. The TCA-preciptatable counts were determined, after the addition of toluene scintillation fluid to the samples, in a Packard or Beckman liquid scintillation spectrometer.

2.18.2 Pre-Labelling with ^{3}H-Thymine.

Low-thymine-requiring cells were grown overnight at the appropriate temperature in TPG-CAA containing thymine (2ug/ml) and appropriate nutritional requirements and/or antibiotics. The overnight culture was diluted into the same medium, to which ^{3}H-thymine (final concentration of 4 uCi/ml) had been added, and incubation was continued. (A dilution of 1:200 - 1:500 of the overnight culture was generally carried out to allow three generations of growth in the labelling media. This was to ensure that the DNA was uniformilly labelled before the commencement of the experiment.) When the cultures had reached A_{600}= 0.2 the cells were either infected with phage, or a 186 cItsp lysogen was heat-induced (as described in Chapter 2.18.1).

Samples (100 ul) taken at time intervals before and after infection or induction, were spotted onto a Whatman GF/A filter and the filter was immersed into ice-cold 10% TCA. The filters were batch-washed and TCA-precipitatable counts were determined, as described above (Chapter 2.18.1).

2.19 GALACTOKINASE ASSAYS.

Galactokinase (galK) assays were carried out to quantitate the level of galK expression from clones in the McKenney promoter-analysis or terminator-analysis vectors (pKO2 or pKL600), in galK^{-} E^{+}T^{+} cells (E862 or E863). The level of galK expression from clones in the McKenney promoter-analysis or terminator-analysis vectors correlates with the strength of a promoter or terminator (McKenney et al., 1981; Rosenberg et al., 1983). Crude estimations of the level of galK expression from a particular clone,

were obtained from the colour of colonies containing this clone on MacConkey-galactose plates. $GalK^-$ strains give white colonies and $GalK^+$ strains give red colonies on MacConkey-galactose plates (McKenney et al., 1981).

The galactokinase assay procedure was adapted from the method described by Wilson and Hogness (1966) and Adhya and Miller (1979) with modifications recommended by I. Dodd (this laboratory).

Cultures containing the pKO2 or pKL600-clones of interest (Chapter 2.3) were grown overnight at the appropriate temperature in M63 medium containing 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1% glucose and 25 ug/ml ampicillin (supplemented with the appropriate amino acids and vitamins). The overnight culture was diluted one hundred-fold into the same medium and grown at the appropriate temperature to A_{650}= 0.2 - 0.6. To allow expression of a cloned promoter, which is controlled by a temperature-sensitive repressor, the cultures were grown at 30°C then transferred to 41°C (heat-induction) for the appropriate time (as indicated in the text).

Aliquots (1 ml) were taken from the culture and placed into Eppendorf tubes. (If a weak promoter was to be assayed the cells were concentrated four to five-fold before the lysis procedure.) The cells were lysed by the addition of 40 ul of lysis buffer (100 mM dithiothreitol, 100 mM EDTA, 50 mM Tris-HCl, pH 8.0) and 3 drops of toluene, followed by vortexing for 30 seconds and incubation at room temperature until the toluene had formed a single drop at the surface of the tube. The aqueous phase was removed into another tube and placed on ice. If a strong promoter was to be assayed, a 1/5 to 1/10 dilution of the lysed cells (diluted in the growth media with the appropriate amount of lysis buffer added) was carried out and aliquots were taken from this diluted lysate to be assayed for galactokinase.

To assay for galactokinase activity, 20 ul of the lysate was added to

80 ul of the reaction solution [2 mM rATP, 5 mM $MgCl_2$, 125 mM Tris-HCl, pH 7.9, 1.25 mM dithiothreitol, 4 mM NaF and 200 nM ^{14}C-galactose (8 - 12 uCi)]. The mixture was incubated at 32°C and aliquots (6x 15 ul), were taken at intervals from 0 - 50 min, and spotted onto dry DE81 2.3 cm filters. After all the samples had been taken, filters were batch-washed twice in 1 litre of water, except for 3 - 6 filters, which were not washed so that the total amount of radioactivity added to each filter (average of the 3 - 6 unwashed filters) could be determined. Filters were dried for 2 hours to overnight at 65°C, scintillation fluid was added and radioactivity present on each filter was measured in a Packard or Beckman liquid scintillation spectrometer.

The bound cpm (washed filters) were plotted against time and the slope of the line (Δ cpm/ min) was calculated. Galactokinase enzyme units (nmol galactose phosphorylated/ min/ ml of cells/ A650) were calculated using the following formula :

Units = Δ cpm/min x 1/total cpm x $1/A_{650}$ x 10,400.

The copy number of plasmid-clones in the McKenney vector was determined using the technique of Projan et al. (1983), except for the use of lysozyme instead of lysostaphin to lyse the cells and of RNAse A instead of pancreatic ribonuclease to remove RNA. Any significant differences observed in the copy number of the plasmid-clones, were noted and the galK units were adjusted accordingly.

2.20 MUTAGENESIS OF PLASMIDS AND CELLS.

2.20.1 Nitrosoguanidine Mutagenesis of Cells.

The strain to be mutated was diluted into L broth from an overnight culture and grown to A_{600}= 1.0. Two ml of the culture were removed and placed in a glass tube, to which 50 ul of nitrosoguanidine (2.5 mg/ml, in 95% ethanol) was added, and the tube was incubated at 30°C for 30 min. Cells were collected by centrifugation (9,000 rpm, 10 min, 4°C, JA21 rotor)

and the pellet was resuspended in M63 medium. The cells were again collected by centrifugation (9,000 rpm, 10 min, 4°C, JA21 rotor), resuspended in 10 ml of L broth and grown to stationary phase. Mutants were isolated by spreading the cells onto selective plates.

2.20.2 Nitrosoguanidine Mutagenesis of Plasmid DNA.

Plasmid DNA was mutated with nitrosoguanidine *in vivo*, as described for nitrosoguanidine mutagenesis of cells (Chapter 2.20.1). Plasmid DNA was then prepared from the mutated cells after they had grown to stationary phase (Chapter 2.24.1). Mutated plasmids were selected by transformation of the DNA into a selective strain.

2.21 CURING CELLS OF PLASMIDS.

Cells were cured of pBR322-derived plasmids by several passages of growth in minimal media (M9 + glucose). Cells were then spread onto YGC plates, with or without the selective antibiotic. If more than half of the cell population was sensitive to the antibiotic, the single colonies on the YGC plate were tested by spotting suspensions of the cells (in TM) onto YGC plates, with or without antibiotics. Single colonies, which were not resistant to the antibiotic, were assumed to be cured of the plasmid and purified though single colony isolations.

If the above procedure did not give rise to cells cured of the plasmid, coumermycin A [an inhibitor of DNA gyrase; Danilevskaya and Gragerov (1980)] was added (at 1 - 5 ug/ml) to the M9 + glucose media and cells, which grew under this treatment, were tested for loss of the plasmid, as described above.

2.22 PREPARATION OF PHAGE STOCKS.

2.22.1 Low-Titre Phage Stocks.

Low-titre stocks (10^9 - 10^{10} pfu/ml) of 186 strains were prepared by heat-induction or liquid infection, as described by Hocking and Egan (1982a).

2.22.2 High-Titre Phage Stocks by Heat-Induction.

The 186 lysogenic culture was grown overnight at 30°C and then diluted one hundred-fold into 2x 500 ml L broth and incubated at 30°C with aeration to A_{600}= 0.8. The culture was transferred to a 40°C bath and shaken for three to four hours until lysis was complete.

Chloroform (2 ml) was then added and the culture was left at 4°C for 10 min to lyse any remaining cells. Bacterial debris was removed by centrifugation (9,000 rpm, 4°C, 20 min, JA10 rotor) and the supernatant was decanted. NaCl and PEG 8000 were added to a final concentration of 0.5 M and 10% (w/v), respectively, and precipitation was allowed to proceed overnight at 4°C. The precipitate was collected by centrifugation (9,000 rpm, 4°C, 20 min, JA10 rotor), resuspended in 8 ml of TM and then purified by CsCl block gradient centrifugation (Chapter 2.26.1).

2.22.3 High-Titre Phage Stocks by Liquid Infection.

A fresh overnight culture of E508 was diluted one hundred-fold into 2x 500 ml of L broth (pre-warmed to 37°C) and then incubated with aeration to A_{600}= 0.2. The culture was infected (moa= 0.1) with phage from a low-titre stock (Chapter 2.13). Incubation was continued at 37°C until lysis was complete, or for 4 hours after infection if lysis did not occur. Chloroform was then added, the culture was treated (as described in Chapter 2.22.2) and the phage were purified by CsCl density gradient centrifugation (Chapter 2.26.1).

2.23 PHAGE DNA PREPARATION.

Phage DNA was prepared either by phenol extraction (Chapter 2.27.2) of CsCl-purified high-titre phage stocks (Chapter 2.22.2, 2.22.3, 2.26.1), or by using the method described below.

A 50 ml culture of E508 was infected at A_{600}= 0.2 with phage at a moa of 0.1 and incubated at 37°C until lysis occurred, or for 4 hours if lysis did not occur. Chloroform was then added and the culture was left at 4°C for 10 min. Cell debris was removed by centrifugation (10,000 rpm, 10 min, 4°C, JA20 rotor) and then DNAse I (50 ug) and RNAse A (100 ug) were added and the mixture incubated for 1 hour at 4°C. The lysate was again centrifuged (10,000 rpm, 10 min, 4°C, JA20 rotor) to remove any remaining cell debris. The phage particles were then pelleted by centrifugation (20,000 rpm, 3 hours, 4°C, JA20 rotor) and the pellet was resuspended overnight in 400 ul of TE. Forty ul of 10x proteinase K buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 100 mM EDTA, 5.0% SDS) and a few crystals of proteinase K were then added, and the digestion was allowed to proceed for 1 - 2 hours at 37°C. The phage DNA was then extracted twice with phenol (Chapter 2.27.3) and the DNA ethanol-precipitated using Na acetate (final concentration of 0.3 M) and 2.5 volumes of ethanol, washed with 70% ethanol (v/v in TE), dried and resuspended in 100 ul TE (Chapter 2.27.3).

2.24 PLASMID DNA PREPARATION.

2.24.1 Analytical Preparation.

The following procedure based on the method of Birnboim and Doly (1979) gave sufficient plasmid DNA for several restriction analyses from 5 ml of a stationary phase, plasmid-containing culture.

Bacteria grown in L broth containing the appropriate antibiotic were pelleted by centrifugation (9,000 rpm, 10 min, 4°C) in a 10 ml Oakridge tube and the pellet was resuspended in 250 ul of lysis buffer

(25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose). A volume of 250 ul of freshly prepared lysozyme (8 mg/ml) in lysis buffer was added to the resuspended cells and the solution mixed gently. After 15 min at room temperature, 1 ml of 0.2 N NaOH, 1% SDS was added and the tube left on ice for 10 min. 0.75 ml of 3 M Na acetate (pH 4.6) was then added and the tube was vortexed and placed on ice again for 15 - 30 min. The mixture was centrifuged twice (18,000 rpm, 10 min, 4°C, JA21 rotor) to remove chromosomal DNA and the plasmid DNA in the supernatant was precipitated by addition of 2.5 volumes of ethanol (Chapter 2.27.3). The pellet was washed in 70% ethanol (v/v in TE), dried and redissolved in 20 ul TE (Chapter 2.27.3). RNAse A (final concentration of 50 ug/ul) was added and the solution was incubated for 30 min at 37°C. The DNA was then phenol extracted, and ethanol-precipitated using 0.1 M Na acetate (pH 4.6) and 2.5 volumes of ethanol (Chapter 2.27.3). The pellet was washed with 70% ethanol (v/v in TE), dried and resuspended in 20 ul TE (Chapter 2.27.3) and restriction analysis (Chapter 2.28) was carried out.

2.24.2 Large-Scale Preparation.

Preparative amounts of plasmid DNA were obtained using either of the two procedures described below.

2.24.2(a) Preparative, Modified Birnboim and Doly Method.

A 50 ml overnight culture of the strain carrying the plasmid grown in L broth containing the appropriate antibiotic was used to prepare DNA by this method. The procedure used, was essentally as described in Chapter 2.24.1, except the volumes of solutions added were scaled-up by a factor of 10. The only changes to the procedure were as follows : K acetate was used instead of Na acetate to precipitate chromosomal DNA and protein. After this step the plasmid DNA was precipitated using 0.6 volumes of isopropanol and washed with 70% ethanol (v/v in TE). After RNAse A

digestion, 40 ul of 10x proteinase K buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 100 mM EDTA, 5.0% SDS) and a few crystals of proteinase K were added and the tube was incubated at 37°C for 1 - 2 hours. The DNA was phenol extracted and ethanol-precipitated, as described above (Chapter 2.24.1), and finally resuspended in 200 ul of TE.

2.24.2(b) CsCl Gradient Method.

A plasmid-containing cell culture was grown overnight in L broth containing the appropriate antibiotic and then used to inoculate 2x 500 ml of L broth. The culture was grown with aeration to A_{600}= 1.0 and then 100 mg of chloramphenicol (in 95% ethanol) was added to each flask and amplification of the plasmid was allowed to proceed at 37°C overnight. Cells were removed by centrifugation (9,000 rpm, 10 min, 4°C, JA10 rotor), resuspended in 7.5 ml of 25% sucrose, 50 mM Tris-HCl (pH 8.0) in a 50 ml Oakridge tube, and then 2.0 ml of a freshly prepared 10 mg/ml solution of lysozyme was added. The tube was placed on ice for 30 min and 3 ml of 0.25 M EDTA, pH 8.0, added. After gentle mixing, the tube was again placed on ice for 5 min. Detergent solution (12 ml of 1% Brij 58 (w/v) and 0.4% Na deoxycholate (w/v) in 50 mM Tris-HCl, pH 8.0, 25 mM EDTA) was added to the tube, mixed gently, and the tube was left on ice for a further 10 min. After centrifugation (18,000 rpm, 30 min, 4°C, JA20 rotor), the supernatant was removed and the plasmid DNA was purified by CsCl equilibrium gradient centrifugation (Chapter 2.26.2).

2.25 M13 REPLICATIVE-FORM (RF) PREPARATION.

M13 RF DNA was prepared using either of the two methods described below.

2.25.1 Preparative Modified Birnboim and Doly Method.

An overnight culture of JM101 in M13 minimal medium was used to inoculate 50 ml of 2x YT broth. The culture was grown at 37°C to A_{600}= 0.1 and infected with the required M13 single-stranded DNA phage, either with 50 - 200 ul of single-stranded DNA phage stock (Chapter 2.31.5a) or a single plaque obtained by streaking the phage stock on a lawn of JM101. The culture was grown for six to eight hours and the cells collected by centrifugation (9,000 rpm, 10 min, 4°C, JA20 rotor) and the RF DNA prepared from the cells (as described in Chapter 2.24.2a).

2.25.2 CsCl Gradient Method.

An overnight culture of JM101, grown in M13 minimal medium, was subcultured into 20 ml of 2x YT broth and grown to A_{600}= 0.1, then 50 - 200 ul of the required M13 single-stranded DNA phage stock (Chapter 2.31.5a), or a single plaque derived from this stock, was added. The M13-infected culture was grown at 37°C to A_{600}= 0.5 and was then diluted into 2x 500 ml of 2x YT broth and grown for six to eight hours at 37°C. Cells were collected by centrifugation (9,000 rpm, 10 min, 4°C, JA10 rotor) and the M13 RF DNA isolated by the plasmid preparation procedure described previously (Chapter 2.24.2b), followed by CsCl equilibrium density gradient centrifugation (Chapter 2.26.2) to further purify the RF DNA.

2.26 CsCl DENSITY GRADIENT CENTRIFUGATION.

2.26.1 CsCl Block Density Gradient for Preparation of High-Titre Phage Stocks.

CsCl block density gradient centrifugation was used to prepare high-titre 186 phage stocks for use in infection experiments and to obtain DNA by phenol extraction (Chapter 2.27.2).

Two CsCl solutions of density 1.6 gm/ml and 1.35 gm/ml were prepared in sterile TM and were used to form a block gradient by adding 4 ml of the 1.35 gm/ml solution and underlaying it with 1.0 ml of the 1.6 gm/ml solution in a 10 ml polycarbonate Oakridge tube. The high-titre phage suspension in TM (Chapter 2.22.2, 2.22.3), was carefully layered on top of the gradient and the tube centrifuged for 90 min at 45,000 rpm, 8°C in a Beckman Ti50 rotor.

The opaque phage band was collected by piercing the bottom of the tube and dialysed (using sterile dialysis tubing; Chapter 2.12, 2.30.1b) three times against one litre of TM and stored at 4°C. A titre of 10^{12} - 10^{13} pfu/ml were usually obtained by this method.

2.26.2 CsCl Equilibrium Density Gradient for Plasmid Purification.

CsCl equilibrium density gradient centrifugation was used to prepare plasmid and M13 RF DNA. Plasmid DNA was purified by adding 0.95 gm of CsCl and 200 ul of 10 mg/ml solution of EtBr per ml of plasmid DNA solution (Chapter 2.24.2b, 2.25.2), loading the solution into a 10 ml polycarbonate tube and centrifuging the tubes to equilibrium (42 hours, 45,000 rpm, 20°C, Beckman Ti50 rotor). The bands were visualised under subdued fluorescent light and the lower of the two bands, containing the plasmid DNA, was collected by piercing the tube from the bottom. (The upper band contains chromosomal DNA and nicked plasmid DNA.) EtBr was removed by three extractions with isopropanol equilibrated with 5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. The DNA solution was then diluted by a factor of three with TE and ethanol-precipitated using 2 volumes of ethanol (Chapter 2.27.3). The pellet was washed with 70% ethanol/TE (v/v), dried, resuspended in TE and stored at 4°C (Chapter 2.27.3).

The concentration and purity of the DNA was determined by obtaining the spectra of absorbance over the range 230-340 nm on a Varian Superscan 3 ultra-violet/visible spectrophotometer (A_{260}= 1.0 represents a

concentration of 50 ug/ul). A260/280 and A260/230 ratios were greater than 1.8 for all DNA used, indicating low protein contamination.

2.27 PHENOL EXTRACTION OF DNA.

2.27.1 Phenol Equilibration and Storage.

Buffer equilibration of phenol was carried out by mixing 50 ml redistilled phenol with 50 ml of 1 M Tris-HCl (pH 8.0) and 5 mg of 8-hydroxy-quinoline at room temperature. The phases were allowed to separate and the phenol phase was taken and mixed with 50 ml of TE and allowed to stand until the phases separated. The aqueous phase was again removed and the phenol phase was equilibrated once more with TE. Phenol equilibrated with TE in this manner was stored under TE and kept frozen in 50 ml aliquots at -20^{o}C until required.

2.27.2 Phenol Extraction of Bacteriophage DNA.

A high-titre phage stock (10^{12} pfu/ml; Chapter 2.26.1) was diluted to 0.9 ml in TE, and then 0.1 ml of 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 100 mM EDTA, 5.0% SDS was added, followed by 0.1 mg of proteinase K. After incubation at 37^{o}C for 60 min, the solution was again diluted (to 5 ml) with TE, and an equal volume of TE-equilibrated phenol (Chapter 2.27.1) added. The mixture was gently shaken for 5 min and the phases were separated by centrifugation (7,000 rpm, 5 min, 4^{o}C, JA20 rotor). The aqueous phase was collected and re-extracted at least twice with an equal volume of TE-equilibrated phenol. The phenol phases were washed with an equal volume of TE and Na acetate (pH 4.6) was added to the pooled aqueous phases to a final concentration of 0.3 M, followed by 2.5 volumes of ethanol. DNA was left to precipitate overnight at -20^{o}C and was collected by centrifugation (18,000 rpm, 20 min, 4^{o}C, JA20 rotor). The pellet was washed in 70% ethanol/TE (v/v), dried then finally dissolved in TE and stored at 4^{o}C (as described in Chapter 2.27.3).

The concentration and purity of the DNA was determined, as described in Chapter 2.26.2.

2.27.3 Phenol Extraction and Ethanol-Precipitation of DNA Solutions.

DNA solutions were mixed with one volume of TE-equilibrated phenol in Eppendorf tubes (or in 10 ml or 50 ml Oakridge tubes for larger volumes), vortexed, kept at room temperature for 5 min, vortexed again and centrifuged (10,000 g, 3 min, room temperature). The aqueous phase was removed, and the process repeated until no material was visible at the interface of the aqueous and phenol phases. The phenol phases were washed with an equal volume of TE. The aqueous phases were pooled and Na acetate (pH 4.6) was added to 0.3 M followed by 2.5 volumes of ethanol. DNA was ethanol-precipitated overnight at -20°C, or at -80°C for 30 min and the DNA collected by centrifugation (10,000 g, 20 min, 4°C). The pellet was washed in 70% ethanol/TE (v/v) (by adding 70% ethanol to the pellet followed by centrifugation at 10,000 g, 5 min, 4°C), dried *in vacuo* for 10 min, dissolved in TE and stored at 4°C.

2.28 RESTRICTION ANALYSIS OF DNA.

Analytical digestion of DNA with restriction endonucleases was carried out for two to twelve hours at 37°C (or at 65°C for *TaqI* digestions) with a two to five-fold excess of enzyme (2 to 5 units per ug of DNA) in a volume of 10 - 20 ul. Digestion buffers were those specified by the manufacturers' catalogues and were stored at -15°C as 10x stocks. For double digestions, the enzyme with the lowest NaCl concentration was used first and the concentration of NaCl was then raised to the appropriate amount and the second enzyme was added. Preparative digests of 20 - 50 ug of DNA were in 50 - 200 ul reaction volumes and were incubated overnight at 37°C. Reactions were terminated by heating to 70°C for 10 min and DNA was tested

for complete digestion by agarose gel electrophoresis on minigels (Chapter 2.29.1).

2.29 GEL ELECTROPHORESIS.

2.29.1 Agarose Gel Electrophoresis of DNA.

Agarose gel solution (1% or 2% w/v agarose in TAE) was stored at 65°C. Analytical work was carried out using horizontal minigels prepared by pouring 9 ml of gel solution onto a 7.5 cm x 5.0 cm glass microscope slide or 18 ml of gel solution onto a 7.5 cm x 10.0 cm glass microscope slide, with the appropriate comb set in place. Glycerol loading buffer was added to the samples to a final concentration of 5% glycerol, 0.04% bromophenol blue, 0.02% xylene cyanol, 1.7 mM EDTA (from a 6x stock; Chapter 2.10) and electrophoresis was carried out in TAE buffer at 100 - 200 mA.

DNA was visualised by staining gels with EtBr (0.0004% w/v in TAE). Gels were photographed under short wavelength UV-light. Approximate concentrations of DNA solutions were determined by comparing the intensity of the EtBr-stained bands with that of known concentrations of DNA molecular weight markers (Chapter 2.11.1).

Preparative gels were poured on glass slides, as described above, or in a perspex tray (14 x 11 x 0.3 cm) and run horizontally, submerged in TAE. Low-gelling-temperature (LGT) agarose gel solution (1.0% or 2.0% w/v in TAE) was cooled to 37°C before pouring (Chapter 2.29.1) and electrophoresis was carried out at 100 mA at 4°C. DNA was visualized, as described in Chapter 2.30.1(a).

2.29.2 Agarose Gel Electrophoresis of RNA.

Agarose gels for RNA fractionation were 1.0%, 1.5% or 2.0% agarose (w/v in 10 mM Na phosphate, 0.1 mM EDTA, pH 6.5) and were poured into a perspex tray (14 x 11 x 0.3 cm) and run horizontally, submerged in

10 mM Na phosphate, 0.1 mM EDTA, pH 6.5. The buffer was recirculated every 15 min by hand or continuously by peristaltic pump. Glycerol loading buffer was added to the samples to a final concentration of 5% glycerol, 0.04% bromophenol blue, 0.02% xylene cyanol, 1.7 mM EDTA from a 6x stock (Chapter 2.10) and electrophoresis was carried out at 30 mA - 50 mA. RNA was visualized, as described in Chapter 2.36.3(a).

2.29.3 Polyacrylamide Gel Electrophoresis.

2.29.3(a) Non-Denaturing Gels.

A 30% gel stock (acrylamide:bis, 30:0.8) was prepared by dissolving 146.1 gm acrylamide and 3.9 gm bis-acrylamide in 500 ml of glass distilled water. The solution was de-ionised (Chapter 2.29.3d) and then de-gassed for 30 min using a vacuum pump. A 5% polyacrylamide gel was prepared by mixing 10 ml of the 30% gel stock, 6 ml of 10x TBE, 44 ml water, 300 ul of freshly prepared 25% (w/v) APS and 80 ul TEMED. Twenty percent polyacrylamide gels were prepared by mixing 40 ml of the 30% gel stock, 6 ml 10x TBE, 14 ml of water, 300 ul 25% APS (w/v) and 80 ul TEMED. Gels were poured at room temperature. Polymerization of a sample of the gel solution in a beaker usually occurred within 5 - 10 min. The gel was allowed to sit for 60 min on the bench after polymerization of the sample had occurred to ensure polymerization was complete. Gel dimensions were 20 x 40 x 0.05 cm or 17 x 26 x 0.05 cm. Pre-electrophoresis was at 20 mA for 20 min. Formamide loading buffer (Chapter 2.10) was added to the samples (one volume of formamide loading buffer to one volume of sample) and electrophoresis was at 25 mA unless otherwise stated. End-labelled (Chapter 2.31.2a) pBR325 HinfI or pUC19 HpaII DNA fragments (Chapter 2.11.1) were used as molecular weight markers.

2.29.3(b) Denaturing (Sequencing) Gels.

Stock gel solution (6% polyacrylamide; acrylamide:bis, 19:1; 8 M urea in TBE) was prepared by dissolving 57 gm acrylamide, 3 gm bis-acrylamide and 480.5 gm urea in 400 ml glass-distilled water at room temperature. The solution was made to 900 ml with glass-distilled water and was then de-ionised (Chapter 2.29.3d). One hundred ml of 10x TBE was added and the solution was de-gassed, as described in Chapter 2.29.3(a).

Polymerisation was carried out by adding 400 ul of freshly prepared 25% (w/v) APS and 100 ul of TEMED, to 80 ml of gel stock solution. Gel dimensions were either 20 x 40 x 0.025 cm or 40 x 40 x 0.025 cm. Polymerisation was allowed to occur for 60 min at room temperature.

Pre-electrophoresis was in TBE for 30 min at 800 V with the comb in place to prevent well distortion. The comb was removed immediately prior to loading and the wells were flushed with TBE to remove urea and any unpolymerised material. Electrophoresis was at 1200 V - 1500 V unless otherwise stated.

Band distortion due to localised heating near the centre of the gel was eliminated by sandwiching a plastic bag, of the same dimensions as the gel, between the outside gel plate and another glass plate with the aid of 0.2 cm perspex spacers and clamps. The plastic bag was filled with TBE. The temperature was monitored with the aid of a plate thermometer and was not allowed to exceed $45^{o}C$.

2.29.3(c) Denaturing (Sequencing) Gels to Resolve Band Compressions.

Sequencing gels containing 25% or 40% formamide (v/v) were prepared by including the appropriate amount of de-ionized formamide (Chapter 2.29.3d) in the gel stock solution (Chapter 2.29.3b). Polymerisation required the addition of 600 ul freshly prepared 25% APS (w/v in water) and 140 ul of TEMED to 80 ml of gel stock solution. Pre-electrophoresis was for

2 to 3 hours at 500 V. Electrophoresis conditions were as described in Chapter 2.29.3(b).

2.29.3(d) De-Ionization of Solutions.

Solutions were de-ionized by adding 10% (w/v) of mixed bed resin (Chapter 2.8) and gently stirring the solution at room temperature for 30 min. Mixed bed resin was removed by filtration.

2.29.4 Autoradiography.

Fuji Rx medical X-ray film was used for autoradiography. In general, gels were wrapped in plastic (Vitafilm) and exposed at room temperature for up to 48 hours, or for longer periods at -80°C with Tungstate intensifying screens. All sequencing gels were exposed overnight at room temperature unless otherwise indicated. Specific conditions for autoradiography of gels are given in the Figure legends.

2.30 ISOLATION OF DNA FRAGMENTS FROM GELS.

2.30.1 Extraction of DNA from Agarose Gel Slices.

DNA to be extracted from agarose for the purpose of cloning was detected by staining the gel with acridine orange (0.001% w/v in TAE) for 10 min and then de-staining with TAE for at least 15 min. The bands were visualized under fluorescent light. If the amount of DNA in the band to be isolated was less than 1 ug, the gel was stained with EtBr (0.0004% w/v in TAE) rather than acridine orange, for 5 min and then de-stained for 5 min. The gel was kept in the dark after the addition of EtBr and the bands were visualized by brief exposure to long wavelength UV-light.

Agarose containing the desired DNA fragment was excised from the gel with a sterile scalpel blade and the DNA was removed from the agarose slice by either one of the two methods described below.

2.30.1(a) Extraction of DNA from Low-Gelling-Temperature (LGT) Agarose Gel Slices.

The LGT agarose gel slice containing the desired DNA fragment was melted at 65°C for 30 min in an Eppendorf tube and then one volume of NET (1 M NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA) was added. If the amount of DNA was less than 1 ug, 1 - 2 ul of E. coli carrier tRNA (Chapter 2.8) at 10 ug/ul was added to the tube. The solution was vortex-mixed and kept at 65°C for a further 15 min. The tubes were then transferred to a 37°C heating block for 15 min and the mixture was extracted once with phenol (pre-heated to 65°C in NET), as described in Chapter 2.27.3, except that the tubes were kept at 37°C during the extraction and the phenol phase was washed once with NET. The DNA solution was then diluted to 0.3 M NaCl with TE and the DNA was ethanol-precipitated (as described in Chapter 2.27.3).

A better yield of DNA from LGT agarose was obtained if some modifications were made to the above method. TE was used in place of NET. Ice-cold phenol (equilibrated with TE) was added to the DNA solution at 37°C and the tubes were immediately vortex-mixed and placed on ice for 5 min. The tubes were then placed at room temperature for 1 min before centrifugation (10,000 g, 3 min, room temperature). After phenol extraction Na acetate (pH 4.6) was added to 0.3 M and the DNA was precipitated with ethanol (as described in Chapter 2.27.3).

2.30.1(b) Electro-Elution from Agarose Gel Slices.

Dialysis tubing (Chapter 2.12) was soaked in sterile water for 15 min, and knots were tied in one end. The tubing was boiled for 5 min in the buffer to be used and thoroughly washed with the same buffer at room temperature before use. All dialysis tubing was prepared and used on the same day.

The agarose gel slice containing the desired DNA fragment was placed into the dialysis tubing (tied at one end) with 400 ul of TE, and the tube

was tied at the other end. The dialysis tubing containing the gel slice was placed perpendicularly to the electric field in a horizontal gel apparatus, and electrophoresed in TAE at 100 mA for 10 - 20 min. The current was reversed for one min, the buffer surrounding the slice was removed from the tubing and a portion of this buffer, together with 40 ul of 3 M Na acetate (pH 4.6) was used to rinse out the dialysis bag to remove any DNA sticking to the tubing. The DNA was precipitated with ethanol (Chapter 2.27.3) after addition of 10 - 20 ug of E. coli tRNA (Chapter 2.8) as carrier.

2.30.2 Recovery of DNA from Polyacrylamide Gel Slices.

The radioactive fragments of interest were located after autoradiography (Chapter 2.29.4) by comparison of their sizes to radioactive DNA markers (Chapter 2.11.1). The autoradiograph was aligned with radioactive ink marks, which had been placed on the edges of the gel prior to autoradiography, and was used as a template to locate the bands of interest. The bands were excised from the gel using a sterile scalpel blade. The DNA was eluted from gel slices by adding 500 ul of gel elution buffer (500 mM ammonium acetate, 1 mM EDTA, 0.1% SDS, pH 7.6) to each individual slice in an Eppendorf tube and incubating the tubes overnight at 37°C with constant agitation (Maxam and Gilbert, 1980). The eluate was carefully collected and 2.5 volumes of ethanol added, after which DNA was ethanol-precipitated (as described in Chapter 2.27.3), dissolved in TE and stored at 4°C.

2.31 PLASMID AND M13 CLONING.

2.31.1 Preparation of Vector DNA for Cloning.

Plasmid and M13 cloning vectors were prepared by digesting the RF DNA with the appropriate restriction enzyme(s) (Chapter 2.24.2, 2.25, 2.28). For each 10 ul of DNA, the volume was adjusted to 60 ul and was made to

0.1 M Tris-HCl, pH 8.0, 0.17% SDS. Calf intestinal phosphatase (0.5 - 2.0 U) was added and the mixture was incubated for two hours at 37°C. The phosphatase was inactivated by heating to 70°C for 20 min and the mixture was extracted with phenol and DNA was ethanol-precipitated (Chapter 2.27.3). Vector DNA was purified from any undigested DNA by fractionation on an agarose gel followed by recovery of the vector DNA from a gel slice, as described in Chapter 2.30.1. Vector DNA was finally dissolved in TE at a concentration of 20 ng/ul and stored at 4°C.

2.31.2 End-Labelling and End-Filling.

2.31.2(a) End-Labelling and End-Filling using the Large Fragment of DNA Polymerase I (Klenow).

DNA restriction fragments to be used as radioactive size markers, or for analysis of restriction patterns on polyacramide gels, were 3'-end-labelled with ^{32}P in a reaction mix containing 50 mM NaCl, 6 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 unit of *E. coli* DNA polymerase I large fragment (Klenow) and 2 - 4 uM d[α-^{32}P]CTP or d[α-^{32}P]ATP. After incubation at 37°C for 15 min the reaction was terminated by the addition of formamide loading buffer (Chapter 2.10) and the samples were loaded onto the gel or stored at 4°C until required.

One tenth of any solution containing DNA restriction fragments to be fractionated on polyacrylamide gels (Chapter 2.29.3a) and then cloned (Chapter 2.31.3), was end-labelled to allow detection of the fragments by autoradiography.

End-filling to create blunt-ended DNA restriction fragments for cloning was carried out using the Klenow fragment or by using T4 DNA polymerase (Chapter 2.31.2b). The end-filling reaction using the Klenow fragment was carried out in the same way as end-labelling (as described above) except that the labelled nucleotide was replaced with 0.05 mM of dNTP solution (added from a stock containing 0.25 mM of each of the four

dNTPs in 5 mM Tris-HCl, pH 8.0, 1 mM EDTA). The reaction was terminated by heating at 70°C for 5 min.

2.31.2(b) End-Filling using T4 DNA Polymerase.

T4 DNA polymerase was used for blunt-ending in preference to the Klenow fragment of E. coli DNA polymerase when the restriction site to be end-filled contained a 3'-overhang (e.g. PstI) since T4 DNA polymerase has a more efficient 3'- 5' exo-nuclease activity than the Klenow fragment (Huang and Lehman, 1972; Maniatis et al., 1982). The method used, was essentially as described by Maniatis et al. (1982), and was adapted from the method of O' Farrell (1981). The reaction was carried out at 37°C for 15 min in 0.033 M Tris-acetate, pH 7.9, 0.066 M K acetate, 0.01 M Mg acetate, 0.5 mM dithiothreitol, 0.1mg/ml BSA, 0.1 mM dNTPs and 2 - 4 units of T4 DNA polymerase. The reaction was terminated by heating at 70°C for 5 min.

2.31.3 Ligation and Transformation (Transfection) with Plasmid or M13-Vectors.

2.31.3(a) Ligation with Plasmid-Vectors.

Twenty ng of plasmid-vector, which had been cut with the appropriate restriction enzyme(s), treated with calf intestinal phosphatase and purified (Chapter 2.31.1), was mixed with the DNA fragment to be cloned in a 3:1 molar ratio of insert:vector. The ligation was carried out in a volume of 10 - 20 ul containing 5 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM rATP, and T4 DNA ligase (0.2 units for staggered-end ligations or 1.0 units for blunt-end ligations). The mixture was incubated overnight at 15°C and then transformed into bacterial cells (Chapter 2.31.3b).

2.31.3(b) Transformation with Plasmid-Vectors.

Competent cells were prepared by inoculating L broth with a hundred-fold dilution of a stationary phase culture of the bacterial strain to be transformed and growing the cells with aeration to A_{600}= 0.3. After chilling on ice for 10 min, the cells were placed in a 50 ml Oakridge centrifuge tube and collected by centrifugation (7,000 rpm, 10 min, 4°C, JA20 rotor), resuspended to 1/50 th the original volume, in ice-cold 100 mM $CaCl_2$ and left on ice for at least two hours. Competent cells were always prepared and used on the same day.

An aliquot (1/4 - 1/2 volume) of the ligation mix was added to 0.1 ml competent cells in chilled, sterile glass tubes and kept on ice for 10 min. The cells were then heat-shocked by incubation at 37°C for 5 min and left on ice for a further 10 min. One ml of L broth was then added to each tube and the tubes were incubated in a gyratory water bath at the appropriate temperature for 1 - 2 hours to allow the expression of the antibiotic resistance gene(s) present on the plasmid. An aliquot (0.2 ml) from each tube was plated on YGC or MacConkey-galactose plates supplemented with the appropriate antibiotic and the plates were incubated overnight or longer at the appropriate temperature.

The following controls (which lacked the insert DNA) were included for each bacterial strain to be transformed ; (1) digested and phosphatased vector at 5 ng/plate (to test for uncut vector DNA), (2) digested, phosphatased and religated vector at 5 ng/plate (to test that the phosphatasing reaction had been successful), (3) uncut vector at 1 ng/plate [to test the transformation efficiency of the competent cells (a transformation efficiency of 10^6 - 10^7 transformants/ug was achieved for most strains used)], (4) Untransformed cells (to test for spontaneous antibiotic resistance or contaminants in the competent cells).

2.31.3(c) Ligation with M13-Vectors.

Ligation and transfection using M13-vectors was carried out essentially as described by Messing (1983 and personal communication).

M13-vector DNA, which had been digested with the appropriate restriction enzyme(s), treated with calf intestinal phosphatase and purified (Chapter 2.31.1), was kept at a constant 20 ng per ligation. The DNA fragment to be cloned was mixed with M13-vector in a insert to vector molar ratio of 3:1. The required ratio was achieved by adding 10 ng/kb of insert DNA.

Ligation was carried out in a total volume of 20 ul containing 10 mM $MgCl_2$, 0.1 mM rATP, 2 mM dithiothreitol, 50 mM Tris-HCl, pH 7.5 and T4 DNA ligase (at 0.2 units for staggered-end ligations or 1.0 units for blunt-end ligations). Tubes were incubated overnight at 15°C and the ligation mix was then used to transfect competent cells.

2.31.3(d) Transfection with M13-Vectors.

Competent cells were prepared by inoculating 2x YT broth with a hundred-fold dilution of a stationary phase culture of JM101, which had been grown overnight in M13 minimal medium at 37°C. The cells were grown at 37°C with aeration to A_{600}= 1.0. The culture was left on ice for 10 min, and the cells collected by centrifugation (7,000 rpm, 10 min, 4°C, JA20 rotor). The cells were resuspended in 1/10th the original volume in ice-cold 100 mM $CaCl_2$ and left on ice for at least two hours. Competent cells were always prepared and used on the same day.

An aliquot (1 - 5 ul) of the ligation mix was added to 0.2 ml of competent cells in chilled, sterile glass tubes and the mixture was kept on ice for 40 min. The cells were then heat-shocked by incubation at 45°C for 2 min and 3 ml of molten YT soft agar containing 20 ul of IPTG (24 mg/ml), 20 ul of BCIG (20 mg/ml) and 0.2 ml of log phase (A_{600}= 1.0) JM101 were added. The mixture was poured onto an M13 minimal plate and the plates were

incubated overnight at 37°C unless otherwise stated. M13 recombinants (clones) appeared as white plaques whereas parental M13 phage plaques were blue.

The following controls (which lacked the insert DNA) were included with each transfection ; (1) digested and phosphatased vector at 5 ng/plate (to test for contamination by uncut vector), (2) digested, phosphatased and religated vector at 5 ng/plate (to test that the vector DNA alone was not giving rise to white plaques), (3) uncut vector at 1 ng/plate (to test the transfection efficiency of the competent cells), (4) untransfected cells (to test for M13 contamination).

2.31.4 Identification of Plasmid Recombinants.

Plasmid recombinants were identified by their characteristic phenotype, as described in the relevant Chapters [e.g. the colour of colonies on MacConkey-galactose plates; sensitivity to antibiotics (if the fragment had been cloned into an antibiotic resistance gene); temperature sensitivity or immunity to 186]. Plasmid recombinants, which did not show a characteristic phenotype, were tested for inserted DNA by colony hybridization, as described below. Clones were tested to determine the orientation of the insert by preparing plasmid DNA, as described in Chapter 2.24.1, and then carrying out restriction endonuclease analysis (Chapter 2.28).

Colony hybridization : This procedure was carried out essentially as described by Maniatis et al. (1982) and was based on the procedure of Grunstein and Hogness (1975). Colonies to be tested were spotted onto a YGC plate containing the appropriate antibiotic. Controls containing the plasmid-vector and a clone containing a fragment overlapping the region of interest were spotted onto the same plate. After overnight incubation at the appropriate temperature the plate was then chilled to 4°C for 1 hour, a dry nitrocellulose filter was placed on top of the agar plate and the

filter and underlying agar were marked with a needle (to allow later alignment). When the filter was completely wetted, it was removed and the agar plate was again placed in the oven at the appropriate temperature for overnight incubation, to allow the colony spots to regenerate. The nitrocellulose filter was placed colony-side up, for 5 min, on Whatman 3MM filter papers, which were pre-soaked in each of the following solutions : 5% SDS; Denaturing solution (0.5 M NaOH, 1.5 M NaCl); Neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0); and 2x SSPE (0.36 M NaCl, 20 mM NaH_2PO_4, pH 7.4, 2 mM EDTA). The nitrocellulose filter was then dried colony-side up on Whatman 3MM filter paper for 30 min at room temperature and baked for 2 hours at 80°C in a vacuum oven. The baked filter was floated on the surface of a solution of 6x SSC in a beaker for 1 min and then was submerged for 5 min to thoroughly wet the filter. The filter was then washed in a solution of 50 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1% SDS at 42°C for 2 hours to remove any agar fragments or loose bacterial debris. The filter was placed in a plastic bag and pre-hybridization and hybridization were carried out (as described in Chapter 2.36.3b), using an appropriate radioactive probe, which was prepared as described in Chapter 2.34. After hybridization, the filter was washed 3 - 4 times for 5 - 10 min in 2x SSC, 0.1% SDS at room temperature followed by 2 washes for 1 hour each in 1x SSC, 0.1% SDS at 65°C. The filter was then placed on Whatman 3MM paper, assymetrical marks were made around it with radioactive ink and the filter was then wrapped in plastic (Vitafilm). The filter was autoradiographed, as described in Chapter 2.29.4. The autoradiograph was aligned with the agar plate and colonies, which hybridized with the probe, were tooth-picked from the plate and purified by streaking for single colonies. Plasmid DNA was prepared, as described in Chapter 2.24.1, and tested for insert size and orientation by restriction analysis (Chapter 2.28).

2.31.5 Identification of M13 Recombinants.

To identify M13 recombinants, white plaques were tooth-picked from the appropriate plates (Chapter 2.31.3d) and single-stranded DNA phage stocks were prepared (Chapter 2.31.5a). The phage were analysed for inserted DNA by lysing the phage then sizing the single-stranded DNA by agarose gel electrophoresis (Chapter 2.31.5c). Single-stranded DNA was then prepared (Chapter 2.31.5b) from M13 phage containing inserts identified in this way, and if necessary the DNA was subjected to a complementarity test (Chapter 2.31.5d) to determine the orientation of the insert. The DNA sequences of the clones were then determined (Chapter 2.33).

2.31.5(a) Preparation of M13 Single-Stranded DNA Phage Stocks.

A fresh overnight stationary-phase culture of JM101 grown in M13 minimal medium, was diluted one hundred-fold into 2x YT broth. Two ml aliquots of the diluted culture were dispensed into 10 ml screw-capped polycarbonate tubes. M13 white plaques were tooth-picked into the cultures, which were then incubated at 37°C with constant agitation for 5 - 7 hours. The cultures were then centrifuged (6,000 rpm, 10 min, room temperature, in a bench centrifuge) and the supernatants, containing the M13 single-stranded DNA phage, were carefully transferred into a Eppendorf tube and centrifuged for a further 10 min in an Eppendorf centrifuge. One ml of supernatant was transferred into another Eppendorf tube and 270 ul of PEG solution (20% PEG w/v, 2.5 M NaCl) was added. Phage particles were allowed to precipitate for 15 min at room temperature. The phage pellets were collected by centrifugation (10,000 g, 5 min, room temperature, in an Eppendorf centrifuge) and the supernatants withdrawn by aspiration. Tubes were centrifuged again for 2 seconds and any traces of the supernatant were removed. The phage pellets were resuspended overnight at 4°C in 200 ul

of TE. Phage stocks prepared in this manner were kept at 4°C for up to one week, or for longer periods at -20°C.

2.31.5(b) Preparation of M13 Single-Stranded DNA.

Phage stocks (Chapter 2.31.5a) were phenol extracted with one half volume TE-saturated phenol (Chapter 2.27.1) at room temperature and the phases were separated by centrifugation (10,000 g, 3 min, room temperature). One hundred and fifty ul of the aqueous phase was carefully withdrawn, avoiding the interface of the two phases, and placed into an Eppendorf tube. The DNA was ethanol-precipitated, after the addition of 15 ul of 3 M Na acetate (pH 4.6) and 400 ul of ethanol (as described in Chapter 2.27.3), and then washed with 1 ml of 95% ethanol, dried *in vacuo* and finally dissolved in 24 ul of TE.

2.31.5(c) Sizing of M13 Single-Stranded DNA by Agarose Gel Electrophoresis.

Potential recombinant phage were tested for the presence of the cloned DNA by taking 10 ul of single-stranded DNA phage stock (Chapter 2.31.5a), adding 2 ul of SDS-lysis buffer (0.1% bromophenol blue, 3 mM EDTA, pH 8.0, 300 mM NaCl, 28% glycerol, 2% SDS), heating the mixture at 65°C for one hour and then placing it on ice for 5 min. DNA liberated from the phage in this manner was electrophoresed on minigels (Chapter 2.29.1). Recombinant M13 DNA had a lower mobility on these gels than a control with no inserted DNA.

2.31.5(d) Complementarity Test for M13 Single-Stranded DNA Clones.

To determine the orientation of the cloned DNA, complementation tests were performed, as described below. Two ul of M13 single-stranded DNA template DNA (Chapter 2.31.5b) to be tested was added to 2 ul of reference

DNA (a M13 single-stranded DNA clone of known orientation and spanning the region, from which the test clone was derived) in an Eppendorf tube, which contained 8 ul of 0.25 M NaCl. The mixture was incubated at 60°C for one hour and then placed on ice for 5 min. Glycerol loading buffer was added to the samples and DNA was electrophoresed on minigels (Chapter 2.29.1) at 50 - 100 mA. DNA was visualised by EtBr staining (0.0004% EtBr in TAE). If the test DNA was not complementary to the reference clone a single band was observed, whereas clones containing DNA sequences complementary to the reference clone were able to hybridize in this region, thereby increasing their molecular weight and retarding their mobility on the gel. The following controls were performed : (1) Reference DNA plus M13 single-stranded DNA without an insert was treated, as described above, and was shown not to produce any hybrid bands. (2) As a positive control the reference DNA was hybridized, as described above, to a M13 single-stranded DNA clone of opposite orientation.

2.32 RECONSTRUCTION OF 186 FROM DNA FRAGMENTS BY RECOMBINATION IN VITRO.

This procedure was used to physically map mutations on the 186 genome and to transfer mutations present on plasmid-clones, or M13-clones, into the phage. This procedure relied on the existence of the unique XhoI (67.6%) and BglII (79.6%) sites present on 186 and the existence of unique PstI, SauIIIA, BssHII and SnaBI sites within the small XhoI-BglII (67.6%-79.6%) fragment. Thus, DNA fragments from plasmid-clones, M13-clones, or a different 186 strain, could be recombined with 186 DNA *in vitro* to form complete 186 DNA molecules.

2.32.1 Ligation and Transfection.

The fragments to be ligated into 186 were mixed with the 186 large 26.4 Kb XhoI-BglII fragment in a 1:1 molar ratio, using 40 - 60 ng of the

large 186 fragment. The ligation reaction was performed in 10 - 20 ul of buffer, as described in Chapter 2.31.3(a). Transfection of the ligated DNA into bacteria was performed, as described for transformation of plasmid DNA, except that after the competent cells were heat-shocked and incubated for 15 min on ice, 0.2 ml of log-phase bacteria (A_{600}= 0.8) and 3 ml of 0.7% agar were added to the transformed cells. The mixture was poured onto Z plates and incubated at the appropriate temperature overnight. The strain E508 was used for most transfections except for the construction of 186 amber mutants when the appropriate suppressing strain was used (see Chapter 2.5). The following controls were performed with each transfection ; (1) ligated large 186 fragment, (2) unligated large 186 fragment, (3) uncut 186 DNA at 5 ng/transfection (10^6 - 10^7 transfectants/ug of DNA were obtained for most bacterial strains used), (4) non-transfected competent cells. If three-factor or four-factor ligations (ligations involving the joining of two or three fragments with the large 26.4 kb XhoI-BglII fragment) were performed, additional controls, in which only one of the small fragments was added to the 26.4 kb XhoI-BglII 186 fragment, were carried out. This was to test for any contamination of the small fragments with the uncut XhoI-BglII fragment (67.6%-79.6%) or with other small fragments.

2.32.2 Identification of Recombinants.

186 phage recombinants were distinguished from parental phage by their phenotype (e.g. clear plaques when non-recombinant phage gave turbid plaques, or vice versa). Recombinants were purified and checked for the correct size fragments by restriction analysis. To identify recombinants where the phenotype was unknown, the transfectants were spotted onto a lawn of bacteria with the appropriate controls and grown at the appropriate temperature overnight. Recombinants were then identified by plaque hybridization, as described below.

Plaque hybridization : This procedure was essentially as described by Maniatis et al. (1982), and was based on the method of Benton and Davis (1977). The agar plate, on which the phage to be screened had been spotted, was chilled at 4°C for 1 hour and then the phage spots were transferred to a nitrocellulose filter by placing the filter on the surface of the agar plate for 5 min. The filter and the surface of the agar plate were marked with a needle to (allow later alignment). The filter was removed from the agar plate and immersed, DNA side up, in a shallow tray containing denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 2 min. The filter was then transferred to a tray containing neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for 5 min and rinsed in 2x SSPE (0.36 M NaCl, 20 mM NaH_2PO_4, pH 7.4, 2 mM EDTA) for 5 min. After drying at room temperature for 30 min, the filter was baked at 80°C under vacuum for 2 hours. After baking, the filter was washed in 10 mM Tris-HCl (pH 8.0) by floating the filter, DNA side up, on the surface of the solution for 1 min then submerging it for 5 min. The filter was pre-hybridized and hybridized, as described in Chapter 2.35.4(b), using the appropriate radioactive probe (Chapter 2.34.2), then was washed and autoradiographed (Chapter 2.35.4c). Plaques, which hybridized with the probe under stringent wash conditions, were purified, as described in Chapter 2.35.4(c).

2.33 DNA SEQUENCING.

The dideoxynucleotide chain termination sequencing technique (Sanger et al., 1977a, 1980; Schreier and Cortese, 1979) was used with the modifications recommended by A.V. Sivaprasad (1984).

2.33.1 Annealing.

The DNA to be sequenced was annealed by mixing 1 ul (2.5 ng) of M13 universal primer (17-mer; 5'-GTAAAACGACGGCCAGT-3'), 8 ul of template DNA

(Chapter 2.31.5b) and 1 ul 10x TM in an Eppendorf tube then placing the tube into a 65°C oven for one hour. The tubes were then allowed to cool to room temperature for 30 min.

2.33.2 The Extension Reaction.

The annealed DNA (Chapter 2.33.1) was added to an Eppendorf tube containing 2 ul (10 uCi) of d[α-^{32}P]CTP (2.8 uM), which had been dried *in vacuo* and redissolved in 2 ul of label supplement (16 uM dCTP in 5 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and thoroughly mixed. Reaction mixes were prepared in four Eppendorf tubes by mixing equal volumes of ddNTPs and dNTPs and dispensing 2 ul into each of the four tubes. (ddNTP and dNTP stock solutions were prepared in 5 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The DNA/label solution (2 ul) was added to each tube and mixed with the ddNTPs/NTPs solution. Finally, 2 ul of Klenow enzyme solution (5 units of Klenow enzyme diluted to 0.05 units/ul in TM just before use) was dispensed onto the side wall of the reaction tubes. Final concentrations of ddNTPs and dNTPs were as follows :

A: 4 uM dATP, 35 uM dGTP, 35 uM dTTP, 80 uM ddATP

C: 25 uM dATP, 25 uM dGTP, 25 uM dTTP, 15 uM ddCTP

G: 35 uM dATP, 5 uM dGTP, 35 uM dTTP, 50 uM ddGTP

T: 35 uM dATP, 35 uM dGTP, 5 uM dTTP, 130 uM ddTTP

If the sequencing reaction was required for determining the sequence a long distance from the primer (>250 bases), the amount of label and label supplement was doubled and the final concentration of ddNTPs and dNTPs was altered as follows :

A: 5.3 uM dATP, 46.7 uM dGTP, 46.7 uM dTTP, 53.3 uM ddATP

C: 33.3 uM dATP, 33.3 uM dGTP, 33.3 uM dTTP, 22.5 uM ddCTP

G: 46.7 uM dATP, 6.7 uM dGTP, 46.7 uM dTTP, 33.3 uM ddGTP

T: 46.7 uM dATP, 46.7 uM dGTP, 6.7 uM dTTP, 86.7 uM ddTTP

Sequencing reactions were commenced by a 2 second centrifugation to mix the enzyme solution with the reaction mix and were incubated at 37°C for 15 min. Reactions were "chased" by adding 2 ul of dNTP-enzyme solution (0.25 mM of each dNTP and 0.025 units/ul of Klenow enzyme solution in TE, prepared immediately before use) to each tube and incubating for a further 15 min at 37°C. The reaction was terminated by adding 4 ul of formamide loading buffer (95% de-ionised formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10 mM EDTA, 0.01 M NaOH) and the tubes were heated to 100°C for 3 min. Samples (0.5 ul) were loaded onto 6% denaturing acrylamide gels and electrophoresed (Chapter 2.29.3b,c).

After electrophoresis, one of the glass plates was removed and the gel fixed by washing it with two litres of 10% acetic acid, 20% ethanol (v/v in water). The addition of ethanol prevented the gel from swelling and wrinkling during the fixing process. The gel was dried in a 110°C oven for 15 - 45 min and then autoradiographed (Chapter 2.29.4).

2.34 PREPARATION OF RADIOACTIVE DNA PROBES.

2.34.1 Preparation of Radioactive DNA Probes by Primer Extension on M13 Single-Stranded DNA Clones.

The preparation of ^{32}P-DNA probes from M13 single-stranded DNA clones was adapted from the procedure or Bruening *et al*. (1982). M13 single-stranded DNA clones with inserts of the same polarity as the RNA to be detected, were used to prepare ^{32}P-DNA probes where only the strand complementary to the RNA was made radioactive.

M13 17-mer (2.5 ng) universal primer (5'-GTAAAACGACGGCCAGT-3') was annealed to 8 ul of M13-clone DNA (Chapter 2.31.5b) in 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ at 60°C for one hour and the mixture was allowed to cool to room temperature for 30 min. The extension reaction was performed using 5 U of Klenow in TM, 50 uCi (2.8 uM) each of d[α-^{32}P]ATP, d[α-^{32}P]CTP

and the other two unlabelled dNTPs (each at 36 uM), for 15 min at 37°C. After a 5 min "chase" with 2 ul of all unlabelled dNTPs (each at 0.25 mM), the Klenow enzyme was inactivated by heating at 70°C for 10 min. The extended product was digested with the appropriate restriction enzyme(s), as described in the text and Figure legends, for 4 - 10 hours at 37°C (Chapter 2.28) and the resulting fragments were fractionated by electrophoresis on a 5% polyacrylamide gel (Chapter 2.29.3a).

The radioactive DNA fragment to be used as a probe was located, isolated and extracted from the gel, as described in Chapter 2.30.2, then concentrated by ethanol-precipitation (Chapter 2.27.3).

If a single-stranded DNA probe was required, removal of the non-radioactive strand was achieved as follows : The double-stranded DNA probe was mixed with an excess amount of an M13-clone (10 ug), which contained a sequence complementary to the non-radioactive strand, in 50 ul of TM. The mixture was boiled for 5 min and hybridization was allowed to occur at 65°C for 1 hour, followed by slow cooling to room temperature. The single-stranded radioactive DNA fragment was purified by electrophoresis at 10 mA on a 5% polyacryamide gel (Chapter 2.29.3a). Autoradiography (Chapter 2.29.4) revealed the presence of 2 bands; the lower band corresponding to the double-stranded DNA fragment and the upper band the single-stranded DNA fragment. The upper band was isolated and eluted from the acrylamide gel slice (Chapter 2.30.2) then concentrated by ethanol-precipitation (Chapter 2.27.3).

2.34.2 Preparation of Radioactive DNA Probes by Kinasing Oligonucleotides.

Radioactive DNA probes for use in detecting mutants created by oligonucleotide site-directed mutagenesis (Chapter 2.35), were made by labelling the 5'-end of the oligonucleotide using polynucleotide kinase. Fifty ng of the oligonucleotide in 7 ul H_2O, 1 ul 10x TM (100 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$) and 1 ul 10 mM dithiothrietol was added to 50 uCi of

r[γ-^{32}P]ATP (final concentration 2.5 uM), which had been dried down in vacuo. After thorough mixing, 1 ul of polynucleotide kinase (2 U/ul) was added. Incubation was at 37°C for 1 hour, and then formamide loading buffer (5 ul) was added and the solution was boiled for 5 min. The sample was loaded onto a 20% polyacrylamide gel (Chapter 2.29.3a) and electrophoresis was carried out, as described in Chapter 2.29.3(a). The gel was autoradiographed (Chapter 2.29.4) and the radioactive oligonucleotide was isolated from the gel (Chapter 2.30.2) and eluted from the gel slice by incubation at 65°C for 2 - 10 hours in TE.

2.35 OLIGONUCLEOTIDE SITE-DIRECTED MUTAGENESIS.

The method used for oligonucleotide site-directed mutagenesis of M13-cloned DNA was derived from the procedures of Zoller and Smith (1982, 1984). The oligonucleotides used in this work are described in Chapter 2.5. Before use in the mutagenesis reaction, the oligonucleotides (with the exception of the oligonucleotide prepared to delete the tR1 terminator) were tested to hybridize specifically to the region of interest by using the oligonucleotide as a primer in a sequencing reaction (Chapter 2.35.4d).

2.35.1 Kinasing the Oligonucleotide.

The synthetic oligonucleotides did not contain a 5'-PO_4 so it was necessary to add a phosphate onto the 5'-end using polynucleotide kinase. Fifty ng of the oligonucleotide was mixed with 1 ul of 10 mM rATP, 1 ul of 10 mM dithiothrietol, 1 ul of 10x TM (100 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$) and 1 ul of polynucleotide kinase (2 U/ul) in a final volume of 10 ul. Incubation was at 37°C for 1 hour. The reaction was stopped by the addition of 10 mM EDTA (pH 8.0) and heat inactivation of the enzyme at 70°C for 10 min.

2.35.2 Extension-Ligation Reaction.

M13 single-stranded DNA (200 ng) to be mutagenised was mixed with 10 ng of the oligonucleotide containing the appropriate mutation, and 2 ng of the M13 universal sequencing primer in a volume of 15 ul containing 5 ul of TM (100 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$) and 5 ul 200 mM NaCl. The mixture was heated to 65°C for 5 min then cooled slowly to room temperature. It was found that better annealing occurred if the mixture was placed at 4°C for 15 min, after cooling to room temperature.

After the annealing step, 5 ul of dNTP solution (containing 0.5 mM of each dNTP), 5 ul of rATP (10 mM), 17 ul H_2O, 2 U of Klenow fragment and 1 U of T4 DNA ligase were added to the annealed DNA and the extension/ligation reaction was allowed to proceed at room temperature for 4 hours.

2.35.3 Transfection.

JM101 competent cells were prepared and 2 - 20 ul of the extended-ligated DNA was transfected into cells, as described in Chapter 2.31.3(d), but without the addition of IPTG or BCIG to the agar before plating. The plates were incubated at the appropriate temperature overnight. The following controls were carried out ; (1) 0.2 ul of the untreated single-stranded DNA, (2) un-transfected cells.

2.35.4 Testing Plaques for the Presence of the Mutated DNA.

Plaques obtained after the transfection of the extended-ligated DNA, and non-mutated control plaques, were spotted onto another plate seeded with a lawn of JM101 and the plate was incubated overnight at the appropriate temperature. The phage were then tested for the presence of the mutation, encoded by the oligonucleotide, by plaque hybridization using the relevant oligonucleotide as a probe, as described below.

The solvent TMACl (tetramethylammonium chloride) was used for the stringent washing of filters since TMACl eliminates the preferential

melting of AT versus GC base pairs (Melchior and von Hippel, 1973; Orosz and Wetmur, 1977), and thus, the temperature for stringent washing (in 3M TMACl) is based solely on the length of the probe (Ullrich et al., 1984; Wood et al., 1985).

2.35.4(a) Transfer of Plaques to Nitrocellulose.

A nitrocellulose filter was placed on the agar plate containing the plaques to be tested and left for 5 - 15 min. The filter and the agar plate were marked with assymetric dots using a needle (to aid in the alignment of the filter and the plate for the identification of mutants). The filter was then removed and allowed to dry, DNA-side up, at room temperature for 30 min, after which it was baked at 80°C under vacuum for 2 hours. The filter was then washed in 10 mM Tris-HCl (pH 8.0) by placing the filter on the surface of the solution for 1 min and then submerging it for 5 min. This procedure helped to reduce non-specific (background) hybridization.

2.35.4(b) Hybridization.

Pre-hybridization was performed in plastic bags at 37°C overnight in 4 ml of the following solution : 6x NET (0.9 M NaCl, 90 mM Tris-HCl, pH 7.6, 6 mM EDTA); 5x Denhardts solution; 0.5% (v/v) Nonidet P40; and 100 ug/ml of sonicated and denatured calf thymus DNA. (The calf thymus DNA was boiled for 5 min and then snap-chilled before addition to the rest of the solution.) After pre-hybridization, the solution was removed and 4 ml of the same solution containing the appropriate ^{32}P labelled oligonucleotide (Chapter 2.34.2) was added. Hybridization was at 37°C overnight.

2.35.4(c) Washing.

After hybridization, the filter was removed from the plastic bag and washed twice, non-stringently, in 100 ml of 6x SSC for 10 min at room

temperature. The filters were then rinsed in TMACl wash solution (3M TMACl, 2 mM EDTA, 0.05 M Tris-HCl, pH 8.0, 1% SDS) at room temperature and placed on a piece of Whatman 3MM paper, on which assymetrical marks had been placed using radioactive ink (to allow the orientation of the autoradiograph with the filter). The paper and filter were wrapped in plastic (Vitafilm) and autoradiographed overnight. After autoradiography, all plaques including the unmutated controls showed hybridization to the probe. To identify mutant phage, the filters were washed in TMACl wash solution for 1 hour at the temperature specified by the size of the oligonucleotide, as calculated by Wood et al. (1985). The filter was then autoradiographed with radioactive markers, as described above. After the stringent wash, 1% - 50% of the plaques hybridized with the probe. Plaques, which hybridized with the radioactive oligonucleotide under these stringent wash conditions, were then tooth-picked from the agar plate and purified by streaking for single plaques. To confirm that these purified plaques contained the mutation, the hybridization with the appropriate oligonucleotide, was repeated.

2.35.4(d) Confirmation of the Mutation by DNA Sequencing.

To confirm that the phage identified by plaque hybridization contained the correct mutation, M13 single-stranded DNA was prepared (as described in Chapter 2.31.5a,b), and the DNA sequence was determined (Chapter 2.33) using either the M13 universal sequencing primer or another oligonucleotide, which would anneal to the M13-clone at a position upstream from the mutation. If the primer to be used in the sequencing reaction had a mismatch with the DNA sequence, to which it was to be annealed, the hybridization was carried out at 65°C, cooled slowly to room temperature and was then placed at 4°C for 15 min, to obtain better annealing.

2.36 RNA ANALYSIS.

All procedures for analysis of RNA required care to be taken in avoiding ribonucleases. Gloves were worn at all times and all glassware was sterilised in a 110°C oven overnight. All other equipment was autoclaved or immersed in 1 M KOH for 15 min and rinsed thoroughly with sterile glass-distilled water.

2.36.1 RNA Preparation.

This method was adapted from a procedure by Court et al. (1980) and a protocol supplied by G. Christie (personal communication) with modifications recommended by B. Kalionis (personal communication).

A fresh stationary phase bacterial culture of a 186 cItsp lysogen was diluted one hundred-fold into L broth, incubated with aeration at 30°C to A_{600}= 0.8 ($2x10^8$ cfu/ml) and heat-induced by transfer to a 40°C water bath (Chapter 2.17.2). For infection with 186, cells were grown at 37°C and infected at A_{600}= 0.8 at an moa of 10 (Chapter 2.17.1). Aliquots of 10 ml were taken at the times indicated in the text and Figure legends, placed into 50 ml polypropylene tubes and were transferred immediately into ice. NaN_3 was added to a final concentration of 0.02 M to stop cell metabolism, and the aliquots were kept chilled on ice until all time samples were collected. Cells were collected by centrifugation (9,000 rpm, 10 min, 4°C, JA20 rotor) and resuspended in 2 ml of freshly prepared lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM NaN_3, 4 mg/ml lysozyme). Lysis was accomplished by freezing the samples in a dry ice/ethanol bath and then placing the tubes immediately into a 20°C water bath and allowing the lysate to thaw for 10 min. The freeze/thaw cycle was carried out twice and SDS was then added to a final concentration of 0.2% to ensure complete lysis and to inhibit the action of RNAses. The tubes were incubated at 45°C for 3 min.

Samples were extracted twice with equal volumes of phenol equilibrated with RNA buffer (20 mM Na acetate, pH 5.2, 20 mM KCl, 10 mM $MgCl_2$) and the phenol phases were washed once with RNA buffer. Nucleic acids were precipitated by addition of one-tenth volume of 3 M Na acetate (pH 4.6) and 2.5 volumes RNAse-free ethanol. Tubes were placed at -80°C for 30 min and nucleic acids were collected by centrifugation (18,000 rpm, 20 min, 4°C, JA20 rotor). The pellets were rinsed with RNAse-free ethanol, dried *in vacuo* and finally redissolved in 4.5 ml of 0.1 mM EDTA (pH 8.0).

2.36.2 Removal of DNA from RNA Preparations.

A simple method to remove contaminating DNA (and residual protein) was based on the procedure of Glisin *et al.* (1974) and relies on the high buoyant density of RNA, which allows it to pellet in CsCl solutions whereas both DNA and protein have lower buoyant densities and remain in solution.

The RNA sample (in 4.5 ml of 0.1 mM EDTA, pH 8.0) (Chapter 2.36.1) was mixed with 4.5 ml of 7.5 M CsCl. The solution was carefully overlayed onto a 2.5 ml pad of 5.2 M CsCl in a 10 ml polyallomer tube. After centrifugation (30,000 rpm, 20°C, 16 hours, SW41 rotor), the supernatant (10 ml) was carefully removed by aspiration and the tubes were cut below the level of the CsCl pad with a sterile scalpel blade. The remaining supernatant was removed and the gelatinous pellet was dissolved in 0.1 mM EDTA (pH 8.0). The RNA was ethanol-precipitated twice (with RNAse-free ethanol) and finally resuspended in 0.1 mM EDTA (pH 8.0).

RNA concentrations were determined using the Varian superscan spectrophotometer by measuring the absorbance over the range 190 - 340 nm (A_{260}= 1.0 represents a concentration of 40 ug/ml). RNA was stored at -80°C until required. Yields of DNA-free RNA after centrifugation through the CsCl pad were generally 1 - 6 mg/10 ml aliquot of cell culture.

2.36.3 Northern Transfer and Hybridization.

The methods used for analysis of *in vivo* RNA were adapted from the procedures of Thomas (1980) and McMaster and Carmichael (1977).

2.36.3(a) Glyoxylation and Transfer from Agarose Gels.

Nucleic acid samples were denatured with 1 M de-ionised glyoxal (Chapter 2.29.3d) in 10 - 20 ul of 10 mM Na phosphate, pH 6.5, 0.1 mM EDTA at 50°C for 30 - 45 min. Dimethylsulphoxide (DMSO) was omitted from the glyoxylation procedure of McMaster and Carmichael (1977), because of excessive breakdown of RNA even when redistilled DMSO was used (K. Gordon, personal communication). This did not affect the denaturation process, as judged by the absence of any change in the mobility of molecular weight markers (B. Kalionis, personal communication).

Samples were electrophoresed on 1.8% agarose gels in 10 mM Na phosphate (pH 6.5), at 30 mA (Chapter 2.29.2). Non-radioactive RNA markers (Chapter 2.11.2) were detected by staining with EtBr (0.0004% ug/ml in 10 mM Na phosphate, pH 6.5) and photographed under short wavelength UV-light. Radioactive nucleic acid markers (186 *in vitro* transcripts; Chapter 2.36.5) were either transferred to nitrocellulose, or the track was cut from the agarose gel and immediately autoradiographed. Gel tracks containing RNA to be transferred to nitrocellulose were exposed to long wavelength UV-light for 5 min to fragment the RNA.

RNA was transferred unidirectionally to nitrocellulose (Schleicher and Schuell, BA85, 0.45 u) by blotting, as described by Thomas (1980), using 20x SSC as the transfer buffer. Bidirectional transfers were carried out by the blotting procedure of Smith and Summers (1980), using 20x SSC as the transfer buffer. After transfer, the filters were air-dried for 30 min, RNA side up, baked at 80°C under vacuum for two hours and then placed into 400 ml of 10 mM Tris-HCl, pH 8.0, at 100°C . The filter was agitated slowly until the buffer reached room temperature. This procedure was recommended

for removal of all residual glyoxal, which may interfere with the hybridization reaction (Thomas, 1983).

2.36.3(b) Hybridization and Washing.

Pre-hybridization, and hybridization conditions of ^{32}P-DNA probes to nitrocellulose-bound RNA, were as described by Thomas (1980). Pre-hybridization was at 42°C overnight in plastic bags in 60% formamide, 6x SSC, 6x Denhardts solution, 0.06 M Na phosphate, pH 6.5 and 0.1 mg/ml sonicated and denatured calf thymus DNA. (The calf thymus DNA had been boiled for 5 min then snap-chilled before addition to the pre-chilled pre-hybridization mix.) Hybridization was at 42°C overnight in a solution of 60% formamide, 6x SSC, 1x Denhardts solution, 0.025 M Na phosphate and 0.1 mg/ml sonicated and denatured calf thymus DNA, which contained the radioactive probe ($1x10^6$ - $3x10^7$ cpm). Probes were heat-denatured at 100°C for 5 min, snap-chilled and diluted into pre-chilled hybridization buffer. After hybridization, the hybridization buffer was removed and the filters were washed four times for 5 min at room temperature in 250 ml 2x SSC, 0.1% SDS and then twice at 60°C in 250 ml 0.1x SSC, 0.1% SDS. Filters were covered with plastic film (Vitafilm) and autoradiographed at -80°C (Chapter 2.29.4).

2.36.4 RNA Dot Blots.

RNA dot blots were performed using the procedure of Thomas (1983). RNA was denatured using glyoxal (Chapter 2.36.3a) and then diluted to a volume of 50 ul in 6x SSC (in 10 mM Na phosphate, pH 6.5, 0.1 mM EDTA). RNA was loaded onto nitrocellulose (which had been soaked in 20x SSC for 30 min) by suction using the Schleicher and Schuell Minifold I or II apparatus. Each loading position was pre-washed using 20x SSC and after the RNA solution was loaded, was washed through twice with 20x SSC. The filter was then dried for 30 min at room temperature, RNA-side up, and baked at 80°C under

vacuum for 2 hours. To remove the glyoxal, 10 mM Tris-HCl (pH 8.0) at 100°C was added to the filters and they were agitated slowly until the buffer reached room temperature. To reduce non-specific hybridization, the filters were floated, RNA-side up, in 6x SSC for 1 min and then submerged for 5 min. Pre-hybridization and hybridization were as described in Chapter 2.36.3(b). The filter was autoradiographed, as described in Chapter 2.36.3(b). The intensity of the dots were quantitated using a Zeinch scanning laser densitometer. The intensity of the dots corresponds to the amount of RNA spanning the region of the probe. The specific activity of each probe was normalized by hybridization to known concentrations of DNA (denatured, loaded onto the nitrocellulose filter and probed, as described above), to allow comparison of the amount of RNA hybridizing to one probe relative to another.

2.36.5 In Vitro Transcription of 186 DNA.

Phage 186 *in vitro* RNA transcripts were prepared using *E. coli* RNA polymerase, by the method of Pritchard and Egan (1985). Phage 186 DNA to be used in the *in vitro* transcription reaction, was purified by CsCl equilibrium density gradient centrifugation (Chapter 2.26.1) and phenol extraction (Chapter 2.27.2). 186 restriction fragments to be used in the *in vitro* transcription reaction were isolated from an agarose gel and phenol extracted and ethanol-precipitated several times (Chapter 2.30.1, 2.27.3).

If the 186 *in vitro* transcripts (prepared as described by Pritchard and Egan, 1985) were to be analysed on a denaturing polyacylamide gel, formamide loading buffer (Chapter 2.10) was added to the samples. The samples were then heated at 65°C for 5 min, snap-chilled on ice, and loaded onto a 6% denaturing polyacrylamide gel (Chapter 2.29.3b). After electrophoresis, the gel was fixed, as described in Chapter 2.33.2, and autoradiographed (Chapter 2.29.4). If the 186 *in vitro* RNA transcripts were

to be used for molecular weight markers on 1% - 2% agarose gels, then the samples were glyoxylated, as described in Chapter 2.36.3(a), before electrophoresis (Chapter 2.29.2).

2.36.6 Determination of 5'-Ends of RNA Transcripts by Primer Extension.

This procedure was based on the method described by McKnight et al. (1981) with modifications recommended by R. Sturm (personal communication). Radioactive DNA restriction fragments to be used as primers were prepared, as described in Chapter 2.34.1.

The radioactive DNA primer, and 10 ug of RNA produced in vivo (Chapter 2.36.1) were precipitated with ethanol (Chapter 2.27.3), redissolved in 10 ul of 200 mM NaCl, 10 mM Tris-HCl (pH 8.3), and then heated at 100°C for 3 min. After annealing at 60°C for 3 hours, the tubes were allowed to cool to room temperature and 24 ul of reaction mix was added to give a final concentration of 10 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 10 mM dithiothreitol, 500 uM of each of the four dNTPs, and 60 mM NaCl. One ul (15 units) of AMV reverse transcriptase was added to the reaction mix and the tubes were incubated at 42°C for one hour. Nucleic acids were precipitated with RNAse-free ethanol (as described in Chapter 2.27.3), and redissolved in 2 ul of 0.1 mM EDTA. An equal volume of formamide loading buffer was added to the samples and the samples were heated to 100°C for 3 min, and electrophoresed on a 6% denaturing polyacylamide gel (Chapter 2.29.3b). The gel was fixed, as described in Chapter 2.33.2, and autoradiographed (Chapter 2.29.4).

2.37 PROTEIN ANALYSIS.

The analysis of proteins presented in this work was done in collaboration with A. Puspurs.

To label proteins encoded by a plasmid-clone, the maxicell system of Sancar et al. (1979) was used, and proteins were labelled with

^{35}S-methionine as described by Gilphart-Gassler et al. (1981). Maxicell strains contain two mutations, recA and uvrA, which allows the complete degradation of host DNA and RNA after UV-irradiation, thus, allowing the synthesis of proteins specifically from the plasmid DNA present in these strains. Two maxicell strains were used in this work, an Su^+ strain (E660) and an Su^- strain (E4168). For the analysis of plasmid-clones in the expression vector pPLc236 (which encodes the λ pL promoter) it was necessary transform these clones into derivatives of these maxicell strains, which contained the plasmid, pcI857 (encoding a temperature sensitive λ cI repressor; Chapter 2.3.1). This allowed the genes cloned downstream of the λ pL promoter in pPLc236 to be repressed at 30°C and to be expressed at 42°C.

The maxicell strains containing the plasmid-clones to be analysed were grown overnight at 30°C in CAA media (0.1 M KH_2PO_4 pH 7.0, 0.015 M $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 ug/ml $FeCl_3$, 4% glucose, 1% casamino acids) with the addition of the appropriate antibiotics and 5 ug/ml thiamine for the Su^+ maxicell strain (E660). The overnight cultures were subcultured into the same media and grown at 30°C to A_{600}= 0.4. The cultures were irradiated for 5 sec with UV-light in 12 cm sterile glass petri dishes. [The UV-irradiation was carried out at a distance of 50 cm from the UV-light (a 15 Watt Oliphant Germicidal lamp) and at a fluence rate of 1.5 J/m^2/second.] The cultures were then transferred into foil-wrapped 200 ml flasks and incubated for 2 hours in a gyratory water bath at 30°C. Cycloserine was added to the cultures to prevent growth of cells and incubation was continued overnight. Cells were collected by centrifugation (10 min, 6000 rpm, at 4°C, JA20 rotor), washed 3x in M9 media, resuspended in 0.5 ml of methionine assay buffer [0.1 M KH_2PO_4 pH 7.0, 0.015 M $(NH_4)_2SO_4$, 5 ug/ml $FeCl_3$, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 4% glucose, 1% MAM (methionine assay media), 200 ug/ml cycloserine]. Five mg/ml thiamine, and 50 ug/ml of the amino acids threonine, leucine, proline, and arginine were

added to the resuspended cells of the Su^+ maxicell strain (E660). Cells were left at 4^oC until required.

To label proteins expressed in the maxicell strains, 0.2 ml of the cells, which were prepared as described above, were incubated at the required temperature for 5 min. Ten ul of L-[^{35}S]-methionine at 132 Ci/mmol in MAM was then added and incubation was continued for 1 hour. To stop incorporation of ^{35}S-methionine, unlabelled methionine was added to a final concentration of 6 mM. Cells were collected by centrifugation (10,000 g, 3 min, room temperature, in an Eppendorf centrifuge) and resuspended in 50 ul of sample buffer (0.063 M Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, 0.75 M β-mercaptoethanol, 0.04% bromophenol blue). To lyse the cells, the solution was heated to 100^oC for 3 min and vortexed thoroughly. The incorporation of label was determined by TCA-precipitation (as described in Chapter 2.18.1). Samples were stored at -20^oC until required.

The samples were analysed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) using either 12.5% or 15% polyacrylamide. In order to resolve small proteins, a 6 M urea/15% polyacrylamide-SDS gel was used (Swank and Munkres, 1971; Ley, 1984). As molecular weight markers, methyl-^{14}C labelled proteins (purchased from New England Nuclear, Boston, Mass. U.S.A.) were mixed with lysed maxicells and electrophoresed along with the other samples. The sizes (in daltons) of these proteins are as follows : BSA, 69000; ovalbumen, 46000; carbonic anhydrase, 30000; lactoglobulin A, 18367; cytochrome C, 12300; and insulin, 5766. The gels were fixed and fluorographed as described by Reeve and Shaw (1979) unless stated otherwise.

2.38 COMPUTER-ASSISTED ANALYSIS.

The database management system of Staden (1980) was used for the storage, management and general analysis of DNA sequences. The program HYPLOT, which was used to calculate the number and position of acidic,

basic and hydrophobic amino acids, was written by R. Williams and modified by I. Dodd (personal communication). The program MWCALC (Staden, 1980) was used to determine the molecular weights of proteins.

Predicting protein coding frames (GENE) : The method of assessing DNA sequences for their protein coding potential based on codon usage (Staden and McLachlan, 1982), was used for analysis of DNA sequence data. The program GENE (Kalionis et al., 1986a) was used to analyse the protein coding potential in phage 186 and employed the codon usage of E. coli (Chen et al., 1982) or the bacteriophage λ early and delayed-early genes int, xis, exo, bet, gam, N, cI, cro, cII, O, P and Q (Daniels et al., 1982) as standards. [These particular λ genes were used as they are the best characterized of the early and delayed early λ genes.] Codon usage of all possible reading-frames was compared (in window lengths of 25 - 40 codons) to the codon usage of the standard. Any frame, which had a similar codon usage pattern to that of the standard, was predicted to be a protein coding frame. The program GENEPLOT was used to give a graphic display of the codon usage of a particular sequence (A.V. Sivaprasad, personal communication).

Searching for signals (SCAN) : The program SCAN (Kalionis et al., 1986a) was used to predict the location of potential promoter sequences for transcription, and ribosome-binding sites. This program uses a weight matrix to evaluate each section of the DNA sequence by the same principal used in the program of Staden (1984a).

Promoters were predicted using a weight matrix composed of the frequency of each base at each position of the 112 E. coli promoters compiled by Hawley and McClure (1983a), with variable spacings (15 - 21 bp) between the -10 and -35 positions. The following threshold scores were used for various spacings between the -10 and -35 regions : 66.0 (17 bp); 66.8 (16 and 18 bp); 67.8 (15 and 19 bp); 68.3 (20 bp); and 68.8 (21 bp) (Kalionis et al., 1986a). [A spacing of 17 between the -35 and -10 regions has been shown to be the optimal for promoter activity (Aoyama et al.,

1983).] Using a method similar to the method used in this study Mulligan et al. (1984) showed that the degree of homology of a promoter to known promoters was related to the strength of that promoter in vitro.

Potential ribosome-binding sites were predicted using a weight matrix based on the rules of Stormo et al. (1982). These rules (rule 1 - 7) are based on the degree of homology to the Shine-Dalgarno sequence (Shine and Dalgarno, 1974), rule 7 being the most stringent rule.

Searching for secondary structure : Direct and inverted repeats were searched for using dot matrix analysis (Maizel and Lenk, 1981; Staden 1982). Potential stem-loop structures were searched for using the program COMSTR (A.V. Sivaprasad, personal communication). COMSTR had advantages over dot matrix analysis for the detection of inverted repeat structures (stem-loops) because it displayed the structure in a 2-D form and calculated an approximate Δ G value for the stability of the structure using the rules of Tinoco et al. (1973) as modified by Steger et al. (1984). Dot matrix analysis was also used for the detection of inverted repeat structures since COMSTR does not detect secondary structures, which have assymetrical bulges in the stem (A.V. Sivaprasad, personal communication).

Protein comparison : The comparison of the amino acid sequence of proteins was performed using dot matrix analysis (Maizel and Lenk, 1981; Staden 1982). Matches were analysed for statistical significance using the program ALIGNSIG, which was modified from the program of Doolittle (Jue et al., 1980; Doolittle, 1981) by I. Dodd (this laboratory). The NIH program SEQDP (Needleman and Wunsch, 1970; Dayhoff, 1978; Kanehira, 1982) was used to align two proteins according to the presence of amino acids with similar physical properties (Dayhoff et al., 1978) and to determine the significance of this alignment.

GENE and SCAN programs were written by I. Dodd (this laboratory) and COMSTR was written by A.V. Sivaprasad (this laboratory). All computer analysis was performed on a DEC PDP-11 minicomputer or a VAX 11-785 computer.

RESULTS: SECTION I.

CHAPTER 3.

DNA SEQUENCE ANALYSIS OF THE

186 EARLY LYTIC REGION.

CHAPTER 3. DNA SEQUENCE ANALYSIS OF THE 186 EARLY LYTIC REGION.

3.1 INTRODUCTION.

A detailed knowledge of the gene content of a specific region and the associated transcriptional control signals is an essential prerequisite to understanding the control of gene expression of that region. The most direct approach to obtain this information is to determine the DNA sequence of the region of interest. Computer-assisted analysis of this DNA sequence can be used to predict the gene content and the presence of transcriptional control sites (such as promoters and terminators).

The 186 early lytic region is defined by the 1.45 kb *in vitro* transcript (Pritchard and Egan, 1985) to be from ~74.7% to ~79.5% (Chapter 1.3.2b; Figure 3.1a). The DNA sequence of the 186 early lytic region (Figure 3.1a) is known to the left of the *Pst*I site at 77.4% (Kalionis *et al.*, 1986a). As detailed in Chapter 1.2.2, the analysis of the DNA sequence of the early lytic region 5' to the *Pst*I (77.4%) site, led to the prediction of the presence of a promoter (*p*R) at 74.7% (Kalionis *et al.*, 1986a). The 5'-ends of the *in vivo* and *in vitro* transcripts have been determined and are consistent with initiation at this promoter (Kalionis, 1985; Pritchard and Egan, 1985). Two early lytic genes, *CP75* and *CP76* are predicted to start before the *Pst*I (77.4%) site (Kalionis *et al.*, 1986a).

The 186 middle region is defined as the region from ~79.5% to ~93.2 (Chapter 1.3.2; Figure 3.1a). The DNA sequence of the middle region from the *Bgl*II site at 79.6% to the *Bam*HI site at 96.0% has been determined (Sivaprasad, 1984). Five genes, *CP80*, *CP81*, *CP83*, *LA* and *RA* are encoded between ~80% and 93.2% (Sivaprasad, 1984; Chapter 1.2.1). Preceding this region is the 3'-end of another potential gene, *CP79*, which is expected to start before the *Bgl*II (79.6%) site (Sivaprasad, 1984; Chapter 1.2.1). Computer-assisted analysis failed to reveal the presence of any potential

promoters between 79.6%-93.2% on the 186 genome (Sivaprasad, 1984), therefore, these genes are expected to be transcribed by extension of the early lytic transcript, or by new promotion within the PstI-BglII (77.4%-79.6%) region. The PstI-BglII region is therefore expected to encode transcriptional signals, which will be important in the control of 186 early lytic to middle gene expression.

This Chapter describes the DNA sequencing of the PstI-BglII (77.4%-79.6%) region. The sequence across the PstI (77.4%) and BglII (79.6%) sites was also determined to show that the sequence is contiguous with the neighbouring regions.

3.2 RESULTS AND DISCUSSION.

3.2.1 Sequencing Strategy.

The strategy chosen for sequencing the PstI-BglII (77.4%-79.6%) region was to clone the PstI-BglII fragment from 186 cItsp (1) DNA, and smaller DNA fragments spanning this region, into the vector, M13mp9, followed by determination of the DNA sequence using the modified Sanger dideoxy chain termination method (Chapter 2.4.1, 2.31, 2.33). Figure 3.1 shows the region of 186, which was sequenced and the sequencing strategy.

An M13mp8-clone (mEC501) containing the r-strand of the PstI (77.4%-84.6%) fragment was available (Chapter 2.4.2), and was used to obtain the DNA sequence rightward from the PstI (77.4%) site (Chapter 2.33).

To obtain a clone containing the l-strand of the PstI-BglII (77.4%-79.6%) fragment, so that the DNA sequence could be determined leftward from the BglII site, 186 cItsp DNA was digested with XhoI and BglII (which have unique sites on 186 DNA) and the 3.6 kb XhoI-BglII (67.6%-79.6%) fragment was isolated from an agarose gel (Chapter 2.28, 2.30.1). The XhoI-BglII fragment was then digested with PstI (77.4%), which resulted in two fragments of 2.9 kb (XhoI-PstI) and 0.7 kb (PstI-BglII). The 0.7 kb

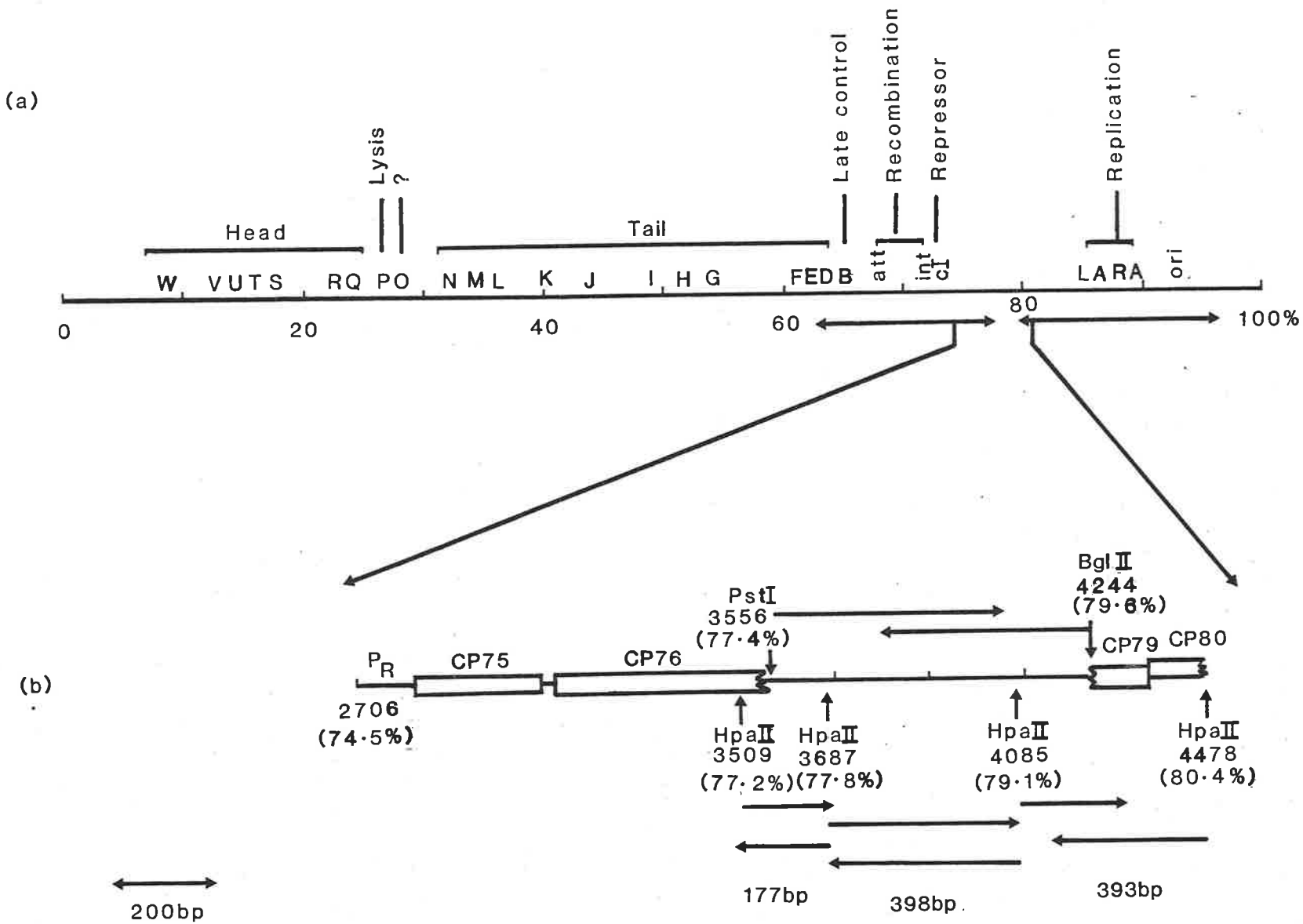
Figure 3.1 Sequencing strategy of the PstI-BglII (77.4%-79.6%) region from 186 cItsp.

a. The genetic map of 186 is shown. The positions of genes are given by Hocking and Egan (1982a). The functions of the genes are listed above the map. The arrows underneath the map represent the regions of the 186 genome, which have been sequenced : PstI (65.5%-77.4%) (Kalionis et al., 1986a) and BglII-BamHI (79.6%-96.0%) (Sivaprasad, 1984).

b. The 74.5%-80.4% region is expanded to show the sequencing strategy of the PstI-BglII (77.4%-79.6%) region. The predicted genes in the adjacent sequenced regions are shown. The 3'-end of CP76 and the 5'-end of CP79 overlap into the PstI-BglII region. The CP80 gene is only partially represented on this diagram, as indicated by the jagged-edged box.

The relevant restriction sites, as determined from the sequencing data of Kalionis et al. (1986a), Sivaprasad (1984) and from this work, are shown. The sequence coordinates of the restriction sites refer to the first base of the site on the l-strand.

The arrows above and below the map represent gel readings used to generate the DNA sequence of the PstI-BglII region and the DNA sequence over the PstI and BglII sites. The arrows above the map represent gel readings from M13-clones of the PstI-BglII fragment, and those below the map represent gel readings from M13-clones of the HpaII fragments. The sizes of these HpaII fragments, are indicated. Rightward arrows represent gel readings used to generate the l-strand sequence, whereas leftward arrows represent gel readings used to generate the r-strand sequence.


(a)
Lysis
?
Late control
Recombination
Repressor
Replication
Head
Tail
W VUTS RQ PO NML K J I H G FEDB
att
int
cI
LARA
ori
0
20
40
60
80
100%
(b)
PR
CP75
CP76
CP79
CP80
2706
(74·5%)
PstI
3556
(77·4%)
BglII
4244
(79·6%)
HpaII
3509
(77·2%)
HpaII
3687
(77·8%)
HpaII
4085
(79·1%)
HpaII
4478
(80·4%)
200bp
177bp
398bp
393bp

fragment was isolated from an agarose gel and was cloned into an M13mp9-vector, which had been digested with PstI and BamHI, to generate the clone mEC400 (Chapter 2.30.1, 2.31). mEC400 was used to determine the sequence of the 1-strand from the BglII (79.6%) site (Chapter 2.33).

DNA sequence data obtained using the clones described above, allowed the sequence of the entire PstI-BglII (77.4%-79.6%) region to be derived. As shown in Figure 3.1(b), the sequence was determined only from one strand except for an overlap of 220 bp in the central region. To determine the sequence of both strands and to obtain sequence data over the PstI (77.4%) and BglII (79.6%) sites, it was necessary to obtain further clones. The preliminary DNA sequence obtained by sequencing the clones containing the PstI-BglII (77.4%-79.6%) region, revealed that HpaII would cut the PstI-BglII region at two sites (77.8% and 79.1%). Furthermore, the knowledge of the DNA sequence of the adjacent regions was used to predict that HpaII would give small fragments, which spanned the PstI and BglII sites (Figure 3.1).

To obtain HpaII clones across the PstI (77.4%) site and subclones of the PstI-BglII (77.4%-79.6%) region, the 3.6 kb XhoI-BglII (67.6%-79.6%) fragment was digested with BssHII (76.9%), which generated two fragments of 2.78 kb (XhoI-BssHII) and 0.84 kb (BssHII-BglII). The 0.84 kb fragment was isolated from an agarose gel and further digested with HpaII (Chapter 2.30.1, 2.28). Restriction fragments obtained from this digest were end-labelled with ^{32}P and analysed by polyacrylamide gel electrophoresis (Chapter 2.31.2, 2.29.3a). The restriction pattern was consistent with that predicted from the preliminary sequence (results not shown). These DNA fragments were "shot-gun" cloned into a M13mp9 AccI vector (Chapter 2.31). To obtain clones spanning the BglII (79.6%) site, 186 cItsp DNA was digested with PstI, and the 2.18 kb PstI (77.4%-84.6%) fragment was isolated after agarose gel electrophoresis (Chapter 2.30.1). The purified 2.18 kb fragment was digested with HpaII and the digestion products were

end-labelled and fractionated by polyacrylamide gel electrophoresis (Chapter 2.31.2, 2.29.3a) (results not shown). A double band containing the 393 bp HpaII (79.1%-80.4%) fragment (which spans the BglII site) and the 398 bp HpaII (77.8%-79.1%) fragment, was isolated from the gel and the fragments were cloned into the AccI site of M13mp9 (Chapter 2.30.2, 2.31). Clones in both orientations, which spanned the BglII (79.6%) site, were identified by DNA sequencing (Chapter 2.33).

The DNA sequences of the HpaII clones described above were determined (Chapter 2.33) as shown in Figure 3.1(b). This allowed the completion of the DNA sequence of the PstI-BglII (77.4%-79.6%) region in both directions and provided sequence data over the PstI (77.4%) and BglII (79.6%) sites. DNA sequence compressions, which occurred in the sequencing gels, were resolved by the addition of 25% or 40% (v/v) de-ionised formamide to the sequencing gel mix (Chapter 2.29.3c,d).

The DNA sequence of the PstI-BglII (77.4%-79.6%) region is presented in Figure 3.2(a), together with the results of the computer-assisted analysis showing potential genes and transcriptional control signals. Also included are the neighbouring sequences extending out to the early lytic promoter, pR (Kalionis et al., 1986a) and to the 3'-end of the potential gene (CP79) overlapping the BglII (79.6%) site. The sequence numbering is from the PstI (65.5%) site (Kalionis et al., 1986a). All relevant restriction sites are also shown.

3.2.2 Analysis of the DNA Sequence.

3.2.2(a) Gene Content.

One approach for predicting the gene content of a region is to locate open reading-frames of at least 40 amino acids, which are associated with a potential initiation codon (ATG or GTG) and a ribosome-binding site (Shine and Dalgarno, 1974; Stormo et al., 1982). This approach is termed "gene search by signal". Another approach is "gene search by content", where

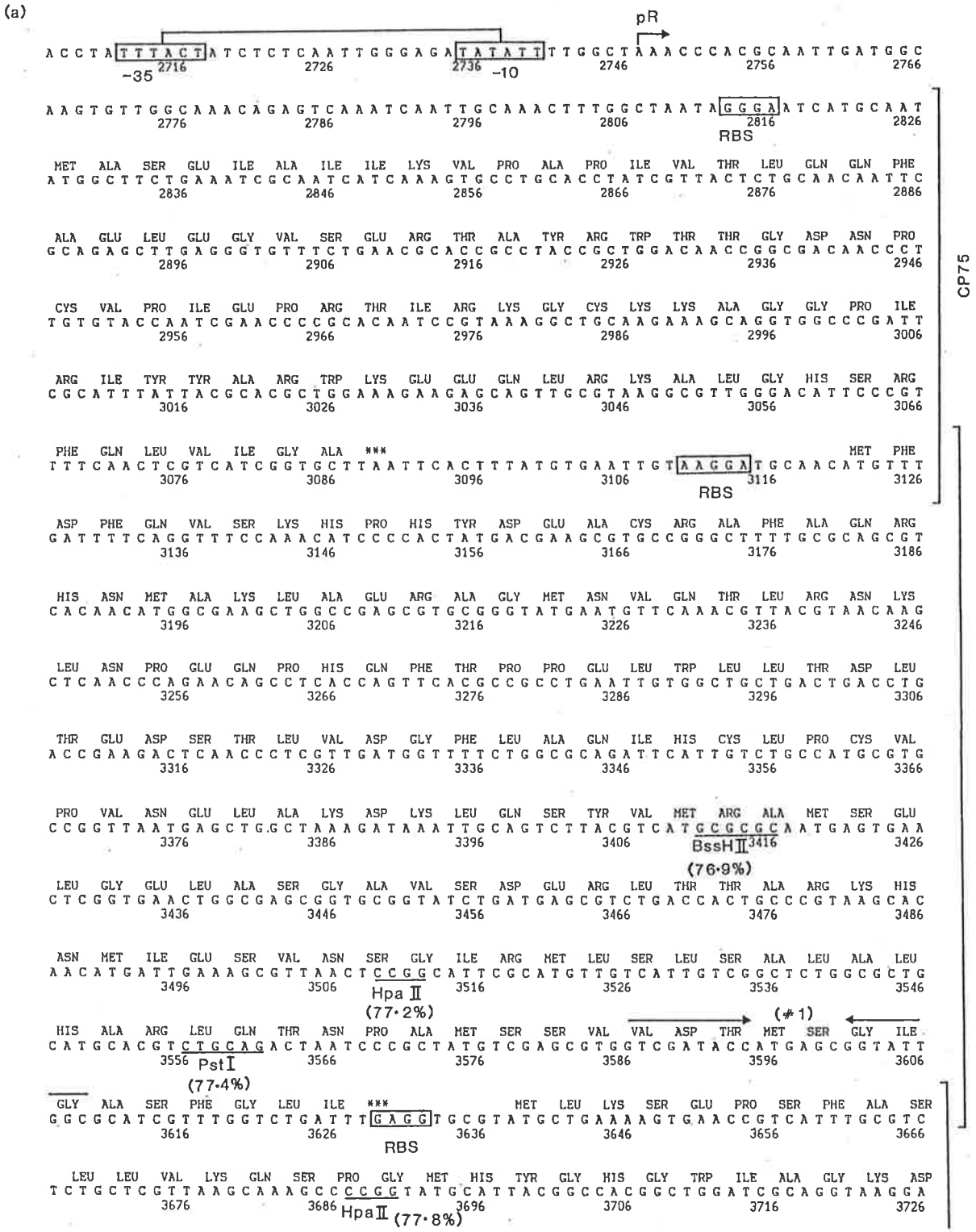
Figure 3.2 DNA sequence of the PstI-BglII (77.4%-79.6%) fragment and adjacent regions from 186 cItsp.

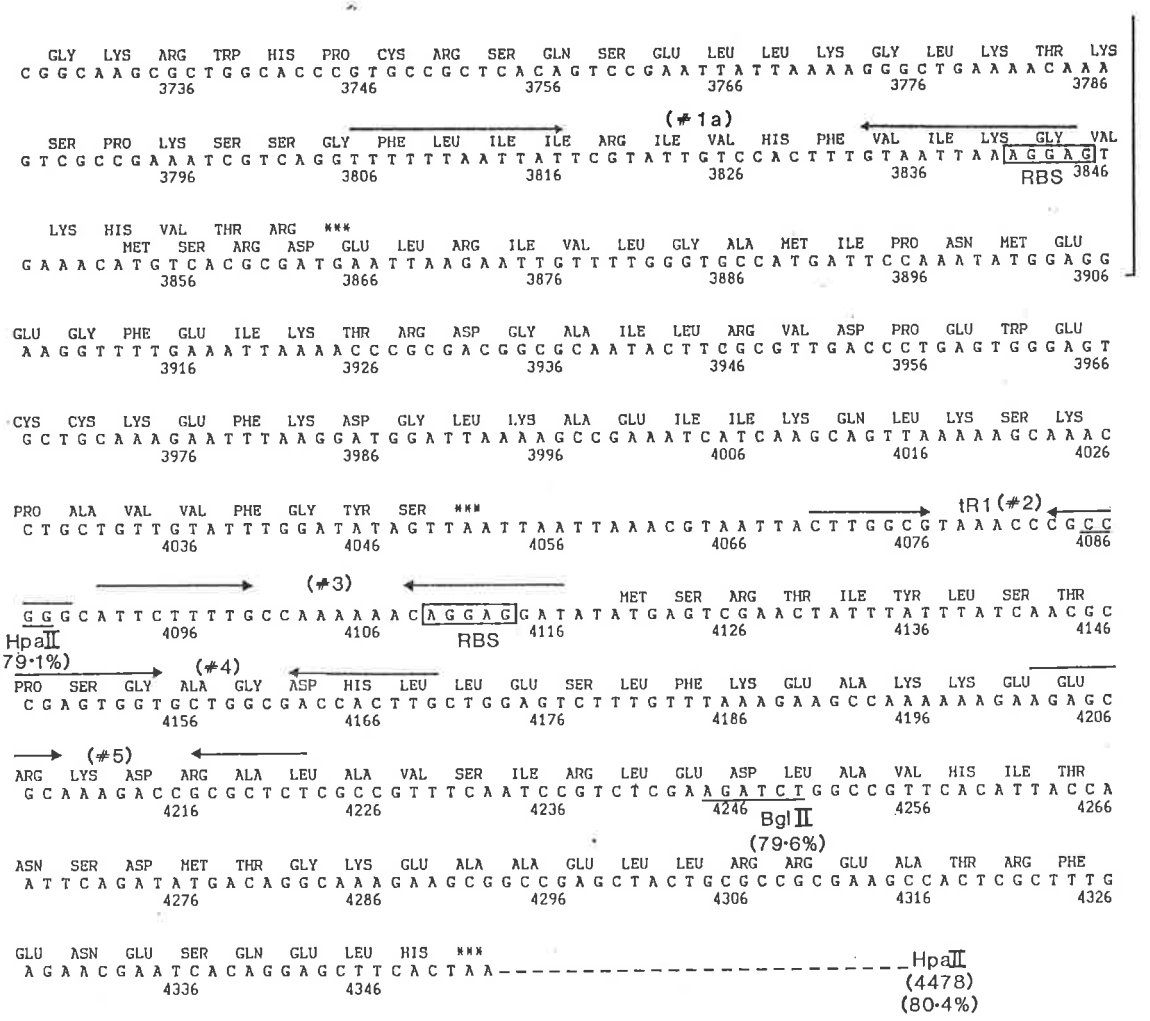
a. This Figure presents the DNA sequence of the l-strand from pR promoter to the 3'-end of CP79, from 186 cItsp. The DNA sequence to the left of the PstI (77.4%) site was determined by Kalionis et al. (1986a) and to the right of the BglII (79.6%) site was determined by Sivaprasad (1984). The numbering of the sequence is from the PstI (65.5%) site.

Transcription and translation is from left to right. Potential genes are indicated on the right of the Figure. The amino acid sequences (in three letter code) of the potential genes, are shown. (*** indicates a termination codon.) All relevant restriction sites are marked beneath the DNA sequence.

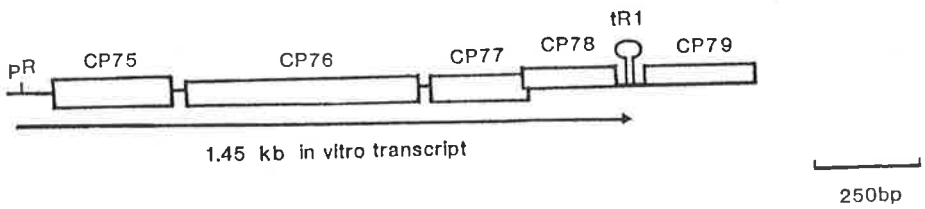
The -35 and -10 regions of the pR promoter are boxed and the startpoint of transcription from the pR promoter is indicated by the horizontal arrow. Ribosome-binding sites (RBS) are boxed. Potential transcription terminators, shown in Figure 3.4, are indicated by the convergent arrows and are numbered #1-#5.

b. A diagrammatic representation of the predicted coding regions in the region pR - CP79. The coding regions are represented by the boxed regions. The 1.45 kb in vitro transcript (which defines the early lytic region) is also shown. This transcript is predicted to terminate at the tR1 terminator (structure #2). The tR1 terminator is represented by the hairpin structure (which is not drawn to scale).


(a)
pR
-35
-10
RBS
CP75
RBS
CP76
BssHII
(76·9%)
Hpa II
(77·2%)
(#1)
PstI
(77·4%)
RBS
HpaII (77·8%)


CP77
CP78
CP79
(#1a)
RBS
tR1 (#2)
(#3)
RBS
HpaII
(79·1%)
(#4)
(#5)
BglII
(79·6%)
HpaII
(4478)
(80·4%)

(b)


tR1
pR
CP75
CP76
CP77
CP78
CP79
1.45 kb in vitro transcript
250bp

differences between genes and "non-genes" can be used to distinguish coding from non-coding regions (Shepherd, 1981; Fickett, 1982; Staden, 1984c; McLachlan et al., 1984). Methods of "gene search by content", which are based on codon usage are particularly useful (Staden and McLachlan, 1982; Gribskov et al., 1984). A combination of the two approaches described above results in a very powerful method for the prediction of genes (Staden, 1984b; Kolaskar & Reddy, 1985).

In this work, possible ribosome-binding sites were searched for using the computer program SCAN (Chapter 2.38), employing the ribosome-binding site rules of Stormo et al. (1982). Coding potential was analysed using the computer program GENE (Chapter 2.38), which determines the coding potential of a region, based on the method of Staden and McLachlan (1982) and by using the codon frequency standards of E. coli genes (Chen et al., 1982) and of λ immediate-early and delayed-early genes (Daniels et al., 1982; Chapter 2.38).

The analysis of the PstI-BglII (77.4%-79.6%) sequence (sequence coordinates 3556-4249) detected four rightward open reading-frames (ORFs) comprising bases 3556-3630, 3638-3865, 3852-4052 and 4119-4249 (Figure 3.2a). No significant leftward ORFs were detected. Three ribosome-binding sites were detected and were associated with three of the four rightward ORFs described above. The site at base 3629 satisfied rule 6 whereas the sites at bases 3841 and 4109 satisfied rule 7 (Stormo et al., 1982). The first and fourth ORFs were not completely contained within the PstI-BglII (77.4%-79.6%) region and represent the 3'-end of CP76 (sequence coordinates 3556-3630; Figure 3.2a) and the 5'-end of CP79 (sequence coordinates 4119-4249; Figure 3.2a). CP76 (sequence coordinates 3121-3630) and CP79 (sequence coordinates 4119-4352) are predicted to encode proteins of 169 amino acids and 77 amino acids, respectively. The two other ORFs have been named CP77 and CP78 (Figure 3.2a) and are predicted to encode proteins of 75 amino acids and 66 amino acids, respectively. The ribosome-binding sites

of the three potential genes initiating within the PstI-BglII (77.4%-79.6%) region, as well as those of the CP75 and CP76 genes (Kalionis et al., 1986a), are shown in Table 3.1.

All four ORFs (CP76, CP77, CP78 and CP79) showed codon usage frequencies similiar to those of both E. coli genes, and λ immediate-early and delayed-early genes (Chapter 2.38). The codon usage plot employing E. coli codon usage frequencies (GENEPLOT; Chapter 2.38) is shown in Figure 3.3, and includes the CP75 gene (Kalionis et al., 1986a) for the sake of comparison. Since CP76, CP77, CP78 and CP79 have similar codon usage to E. coli genes and are preceded by potential ribosome-binding sites, it is likely that they represent genes. The results described in Chapter 4 provide evidence that these ORFs are genes by showing that they encode proteins of sizes, which are consistent with those predicted from the DNA sequence (Table 3.2).

The arrangement of CP77 and CP78 is unusual because an overlap of 14 bp occurs between the 3'-end of CP77 and the 5'-end of CP78. Several examples of genes, which overlap have been reported in E. coli (Platt and Yanofsky, 1975; Nichols and Yanofsky, 1979; Christie and Platt, 1980; McKenney et al., 1981; Barnes and Tuley, 1983; Cole et al., 1983; Yamamoto et al., 1982; A.C. Robinson et al., 1984, 1986), in the single-stranded DNA phages ϕX174 and G4 (Barrel et al., 1976; Sanger et al., 1977b; Godson et al., 1978), in phage T7 (Dunn et al., 1981; Dunn and Studier, 1981, 1983), in phage T4 (Spicer and Konigsberg, 1983; Trojanowska et al., 1984; Macdonald and Mosig, 1984; Gram and Ruger, 1985; Valerie et al., 1986; Hahn et al., 1986), in phage λ (Kroger and Hobom, 1982; Sanger et al., 1982; Daniels et al., 1982) and in phage 186 [the int and cI genes (Kalionis et al., 1986a); and several genes in the middle region (Sivaprasad, 1984)]. Studies with overlapping genes have shown that the 5' gene may have either a positive or negative effect upon the translation of the 3' gene, depending on the degree of overlap. Gene arrangements where the 5' gene

Table 3.1 Ribosome binding sites.

Predicted Protein	Ribosome Binding Site (RBS)(a)	Stormo Rule(b)	Sequence Position(c)
CP75	GGGA- 10 -ATG	- (d)	2827
CP76	AAGGA- 6 -ATG	7	3121
CP77	GAGG- 5 -ATG	6	3638
CP78	AGGAG- 6 -ATG	7	3852
CP79	AGGAG- 5 -ATG	7	4119

Notes to Table 3.1

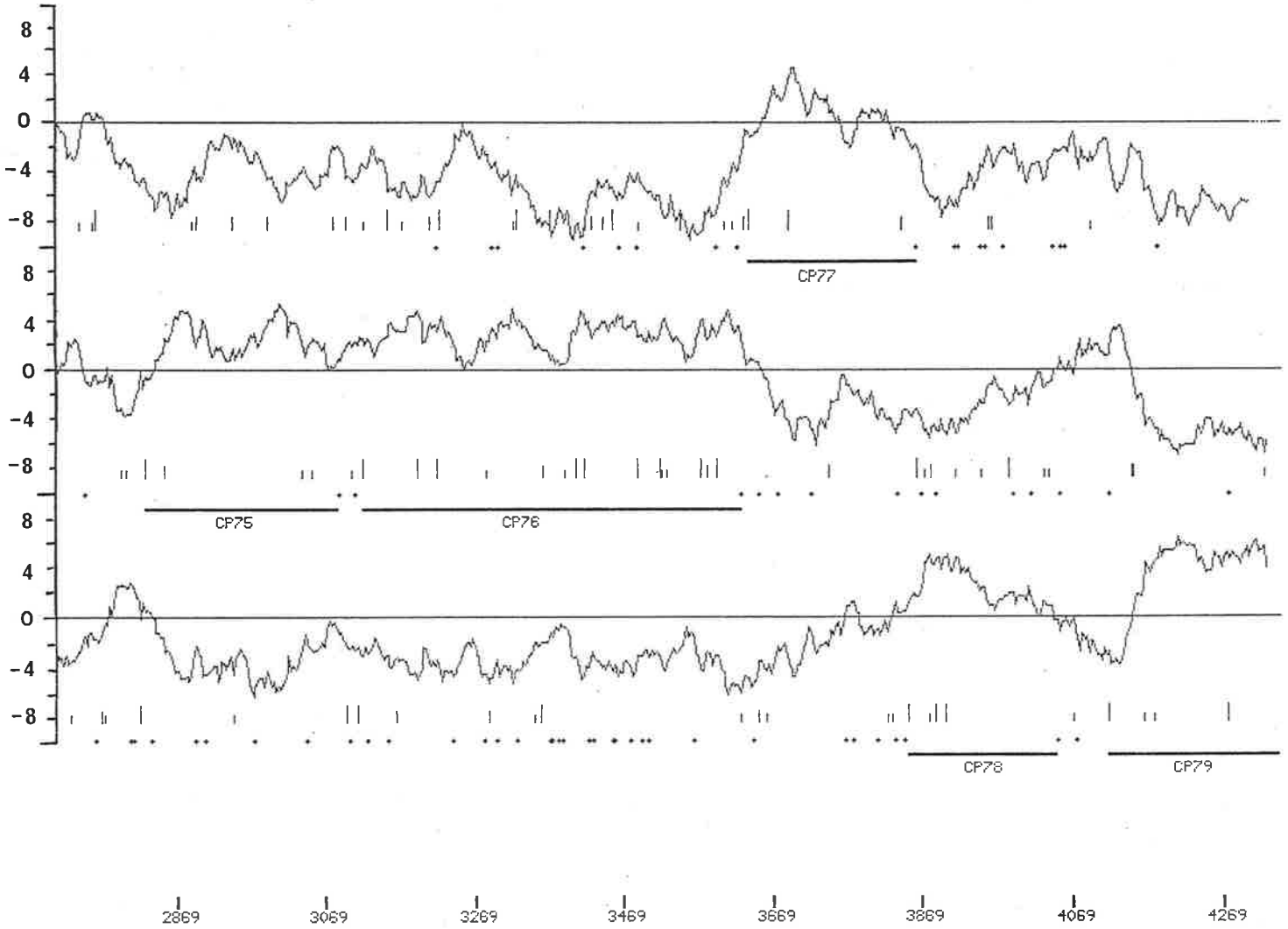
a. The numbers between the proposed ribosome binding site and the initiation codon refer to the number of intervening bases.
b. Stormo et al. (1982)
c. Sequence position corresponding to the A residue of the ATG initiation codon.
d. Sequence was not detected by the Stormo rules but shows homology to the Shine-Dalgarno sequence (Shine and Dalgarno, 1974).

Figure 3.3 Codon usage plots for the DNA sequence of the pR-CP79 region from 186 cItsp.

The codon usage plots for all three reading-frames on the 1-strand of the pR - CP79 region, are shown. The X-axis gives the sequence position and the Y-axis gives the score.

The DNA sequence of the pR-CP79 region was analysed in sections (window lengths) of 25 codons (Chapter 2.38), using the codon usage standards of E. coli genes (Chen et al., 1982). The score [log (P/1-P), with a cut-off range from -10 to +10; Staden and McLaughlan, 1982; Chapter 2.38)] is shown on the Y-axis. The score indicates the degree to which the codon usage of a section of the DNA sequence is related to that of the standard (the codon usage of E. coli genes). A positive score for a region indicates that the region shows similar codon usage to the standard, whereas a negative score indicates that the frame has a poorer fit to the codon usage of the standard.

The positions of termination codons in each of the three reading-frames are indicated by the + signs. Beneath each plot potential start codons are indicated as follows : ATG start codons by the large vertical lines, GTG start codons by the medium-sized vertical lines and TTG start codons by the small vertical lines. The positions of the predicted genes, are indicated beneath the appropriate codon usage plot.


8
4
0
-4
-8
CP77
CP75
CP76
CP78
CP79
2869
3069
3269
3469
3669
3869
4069
4269

overlaps the 3' gene by only a few bases (~4 bp) result in an enhancement of expression (positive coupling) of the distal gene (Oppenheim and Yanofsky, 1980; Yates and Nomura, 1981; Schumperli et al., 1982; Baughman and Nomura, 1983; Das and Yanofsky, 1984; Aksoy et al., 1984; Trojanowska et al., 1984) and this is thought to be important in ensuring correct molar production of the proteins (Normark et al., 1983). Gene arrangements where a greater overlap (~30 bp, or greater) occurs between two genes, result in a decrease in the expression of the distal gene (negative coupling). This occurs with the λ C and Nu3 genes (Shaw and Murialdo, 1980), with bacteriophage MS2 lysis and replicase genes (Berkhout et al., 1985), and with gene overlaps constructed in vitro (Das and Yanofsky, 1984; Schottel et al., 1984). The overlapping arrangement of CP77 and CP78 suggests that the translation of CP77 may have important regulatory consequences on the expression of CP78.

3.2.2(b) Properties of the Predicted Proteins.

Information can be obtained about some of the possible physical properties and functions of a protein as well as its expression, by the analysis of its amino acid sequence and codon usage. Properties such as the degree of basicity, or polarity, give an indication as to whether the protein is likely to interact with DNA or the cell membrane (Daniels et al., 1982; Capaldi and Vanderkooi, 1972). The codon usage of the gene can predict whether the protein will be highly or poorly expressed (Grosjean and Fiers, 1982; McLachlan et al., 1984). The properties of the predicted proteins, together with the amino acid composition of each gene-product are tabulated in Table 3.2. The molecular weight of each predicted protein was determined from the DNA sequence using the MWCALC program (Chapter 2.38). The properties of the CP75 protein (Kalionis et al., 1986a) are included in Table 3.2 to enable the comparison of the characteristics of the predicted genes encoded in the pR-CP79 (74.7%-80.0%) region.

Basic proteins have the potential for interacting with the negatively-charged backbone of DNA and thus, are likely to be control proteins. For instance, the λ DNA-binding control proteins Cro and cII and the λ recombination proteins Int and Xis show a high level of basic residues (16.7%, 17.5%, 16.6% and 25%, respectively) (Roberts et al., 1977; Daniels et al., 1983) compared with the average distribution of basic amino acids (11%) for most proteins (Doolittle, 1981). The percentage of basic (lys, arg), acidic (glu, asp) and hydrophobic (ala, val, leu, ile, phe, trp) amino acid residues in the 186 proteins encoded in the pR-CP79 (74.7%-80.0%) region, were calculated using the program HYPLOT (Chapter 2.38) and are shown in Table 3.2.

The translation products of CP75, CP77, CP78 and CP79 show a high percentage of basic amino acids (16.1%, 18.6%, 16.6% and 16.8%, respectively). The CP76 gene-product is only 8.9% basic, but most of these basic residues are concentrated at the amino-terminal end of the protein (16.7% in the first 42 amino acids of the 169 amino acid protein). This may indicate that all five proteins interact with DNA. Five sequence-specific DNA binding proteins (λ cI, Cro, E. coli CAP, the trp repressor and 434 repressor) have been shown to interact with the DNA by a α helix-turn-α helix structural motif (Pabo and Sauer, 1984; Schevitz et al., 1985; Anderson et al., 1985). The helix-turn-helix regions of these proteins have amino acid sequence homology, and many other known DNA-binding proteins share this homology (Matthews et al., 1982; R.T. Sauer et al., 1982; Pabo and Sauer, 1984). Dodd and Egan (1987) derived a weight matrix for the DNA-binding region of λ Cro-type DNA-binding proteins, which can be used successfully to predict proteins that are likely to be sequence-specific DNA-binding proteins. Using this weight matrix in a modified version of the SCAN program (Chapter 2.38), Dodd and Egan (1987) showed that the CP75 and CP76 gene-products each contain an amino acid sequence, which scores significantly above the threshold value. Thus, the

CP75 and CP76 predicted gene-products are likely to be sequence-specific DNA-binding proteins. The CP77, CP78 and CP79 predicted gene-products did not contain any amino acid sequences, which scored significantly above the threshold value using this analysis. Although this result indicates that the CP77, CP78 and CP79 proteins are unlikely to be sequence-specific DNA-binding proteins of the λ Cro-type, they may still interact with DNA in a non-specific manner, as predicted for the E. coli Hu family of proteins (Geider and Hoffmann-Berling, 1981; Pettijohn, 1982), or by a different binding mechanism. The sequence-specific DNA-binding proteins λ Int and Xis, E. coli IHF (HimA, HimD), phage SPO1 TF1, and phage P22 Arc and Mnt do not show homology to the conserved amino acid sequence of the α helix-turn-α helix motif and thus, probabaly interact with DNA in a different manner to the λ Cro-type DNA-binding proteins (Ross and Landy, 1982, 1983; Better et al., 1983; Yin et al., 1985; Craig and Nash, 1984; Greene and Geiduschek, 1985; Sauer et al., 1983; Dodd and Egan, 1987). A further possiblity is that they may interact with RNA. The phage antitermination functions λ N, λ Q, ϕ80 N, P22 N, and ϕ21 N, the P4 polarity suppressor Psu, the T4 translational repressor RegA and the E. coli termination function Rho, are known or presumed to interact with the RNA (Friedman and Gottesman, 1983; Lagos et al., 1986; Trojanowska et al., 1984; Richardson, 1982) and show a high level of basic amino acids (21.1%, 13.5%, 18.4%, 19.8%, 26.3%, 17.0%, 17.2%, and 13.8%, respectively) (Daniels et al., 1983; Tanaka and Matsushiro, 1985; Franklin, 1985a,b; Dale et al., 1986; Trojanowska et al., 1984; Pinkham and Platt, 1983).

The degree of polarity of a protein (the polarity index) can be calculated by adding together the mole fraction of polar amino acids (asp, asn, glu, gln, lys, ser, arg, thr, his) within a protein (Capaldi and Vanderkooi, 1972). A polarity index of less than 40% indicates that the protein has a low polarity and is likely to interact with the cell membrane. The polarity index of the predicted gene-products of CP75, CP76,

Table 3.2 Properties of proteins predicted from the DNA sequence.

		CP75	CP76	CP77	CP78	CP79
Ala	GCA	5	3	1	1	0
	GCC	1	2	0	2	5
	GCG	1	8	1	0	1
	GCT	2	4	0	1	1
		9	17	2	4	7
Arg	AGA *	0	0	0	1	0
	AGG *	0	0	0	0	0
	CGA *	0	0	1	0	1
	CGC	5	2	2	3	5
	CGG *	0	1	0	0	0
	CGT	3	6	1	0	2
		8	9	4	4	8
Asn	AAC	1	5	0	0	1
	AAT	0	3	0	1	1
		1	8	0	1	2
Asp	GAC	1	3	1	2	2
	GAT	0	5	0	2	2
		1	8	1	4	4
Cys	TGC	1	2	1	2	0
	TGT	1	1	0	0	0
		2	3	1	2	0
Gln	CAA	3	1	1	0	0
	CAG	1	7	1	1	1
		4	8	2	1	1
Glu	GAA	4	7	2	5	6
	GAG	3	3	0	3	5
		7	10	2	8	11
Gly	GGA *	1	0	1	2	0
	GGC	3	2	3	1	2
	GGG *	0	0	1	0	0
	GGT	3	6	3	2	1
		7	8	8	5	3
His	CAC	0	4	3	0	3
	CAT	1	3	2	0	0
		1	7	5	0	3
Ile	ATA *	0	0	0	1	0
	ATC	7	0	1	2	1
	ATT	2	5	4	3	2
		9	5	5	6	3
Leu	CTA *	0	0	0	0	1
	CTC	1	3	1	0	2
	CTG	1	13	3	0	3
	CTT	1	0	0	1	1
	TTA	0	1	3	3	1
	TTG	2	4	0	1	2
		5	21	7	5	10
Lys	AAA	4	3	6	5	5
	AAG	2	3	4	2	0
		6	6	10	7	5
Met	ATG	1	9	2	3	2
		1	9	2	3	2
Phe	TTC	1	1	0	0	0
	TTT	1	5	3	3	2
		2	6	3	3	2
Pro	CCA	1	2	0	1	0
	CCC	1	2	1	0	0
	CCG	1	2	3	0	1
	CCT	3	2	0	2	0
		6	8	4	3	1
Ser	AGC	0	4	1	1	0
	AGT	0	1	1	1	2
	TCA	0	2	3	1	4
	TCC	1	2	1	0	0
	TCG	0	3	2	0	0
	TCT	2	2	1	0	1
		3	14	9	3	7
Ter (Stop)	TAA	1	0	0	1	1
	TAG	0	0	0	0	0
	TGA	0	1	1	0	0
		1	1	1	1	1
Thr	ACA	2	0	1	0	1
	ACC	2	4	0	1	1
	ACG	0	2	1	0	1
	ACT	1	3	0	0	2
		5	9	2	1	5
Trp	TGG	2	1	2	1	0
		2	1	2	1	0
Tyr	TAC	2	1	1	0	0
	TAT	1	1	0	1	1
		3	2	1	1	1
Val	GTA	1	1	1	1	0
	GTC	1	2	2	0	0
	GTG	1	2	1	0	0
	GTT	2	5	1	3	2
		5	10	5	4	2
TOTAL AMINO ACIDS		87	169	75	66	77
BASIC [a]		14	15	14	11	13
BASIC (%)		16.1	8.9	18.6	16.6	16.8
ACIDIC [a]		8	18	3	12	15
ACIDIC (%)		9.2	10.6	4.0	18.2	19.5
HYDROPHOBIC [a]		46	85	38	34	30
HYDROPHOBIC(%)		52.9	50.3	50.6	51.0	39.0
POLARITY INDEX, % [b]		41	47	47	42	60
MODULATING CODONS * [c]		1	1	3	4	2
MODULATING CODONS * (%)		1.1	0.6	4.0	6.0	2.6
MOL. WT.		9770	18671	8399	7522	8817

Notes to Table 3.2

a. Basic (Lys+Arg), acidic (Glu+Asp) and hydrophobic (Ala+Val+Leu+Ile+Phe+Trp)
b. Proteins below a polarity index of 40% are considered likely candidates for membrane-associated proteins (Capaldi and Vanderkooi, 1972).
c. Proteins that are strongly expressed have a low percentage (0.6%) and weakly expressed proteins have a higher percentage (>5.2%) of modulating codons (ATA,AGA,AGG,CGA,CGG,GGA,GGG,CTA) as described by Grosjean and Fiers (1982).

CP77, CP78 and CP79 are shown in Table 3.2. None of these predicted proteins show a polarity index of less than 40% and they are therefore unlikely to represent cell membrane-associated proteins.

The codon usage of a gene can give an indication as to whether the protein will be expressed at low or high levels. The percentage of modulating codons (rare codons, corresponding to minor tRNAs) in genes have been shown to correlate with how well a gene is expressed (Ikemura, 1981a,b; Grosjean and Fiers, 1982). Strongly expressed genes have a low percentage (0.6%) of modulating codons, whereas weakly expressed genes have a higher percentage (5.2%) of modulating codons. Several workers have postulated (Grosjean and Fiers, 1982; Gouy and Gautier, 1982; Konigsberg and Godson, 1983) that rare codons might be translated more slowly. Indeed evidence has been presented that rare codons slow the translation rate of genes both in vivo and in vitro (Pedersen 1984a,b; M. Robinson et al., 1984; Bonekamp et al., 1985), and may even cause translational pausing (Varenne et al., 1984). The percentage of modulating codons (ATA, AGA, AGG, CGA, CGG, GGA, GGG, CTA) in CP75, CP76, CP77, CP78 and CP79 are shown in Table 3.2.

CP77, CP78 and CP79 have a high level of modulating codons (4%, 6% and 2.6%), whereas CP76 (0.6%) and CP75 (1.1%) have a low level of modulating codons. Therefore, it is expected that CP77, CP78 and CP79 will be poorly expressed while CP76, CP75 should be highly expressed genes.

3.2.2(c) Transcriptional Control Signals.

Gene transcription is controlled by specific signals encoded in the DNA (reviewed by Pribnow, 1979; Rosenberg and Court, 1979). Initiation sites for RNA synthesis are associated with a well conserved DNA sequence, the promoter, which contains highly conserved sequences in the -35 and -10 positions (Siebenlist et al., 1980; Hawley and McClure, 1983a; Galas et al., 1985; McClure et al., 1985). The degree of homology of a promoter

to known promoters has been shown to be related to the strength of that promoter *in vitro* (Mulligan *et al.*, 1984).

In this study, a weight matrix, which was derived from the occurrence of each base at each position of the promoter using the 112 promoters compiled by Hawley and McClure (1983a) (Kalionis *et al.*, 1986a; Chapter 2.38), was used to scan the PstI-BglII (77.4%-79.6%) sequence and adjacent regions for possible promoters and to predict their strength, using the computer program SCAN (Chapter 2.38).

This analysis led to the prediction of two low scoring (compared with λ pL; Hawley and McClure, 1983a) rightward promoters at positions 3873 and 3911, and two relatively high scoring leftward promoters at positions 3821 and 3830. [The position refers to the first base of the -10 region (Rosenberg and Court, 1979).] These promoters were named pR784, pR785, pL782 and pL783 for the sake of reference. (R and L, signify transcription direction while the numbers approximate the chromosomal location to the first decimal place.) The sequences of the four presumptive promoters are recorded in Table 3.3. Although the homology scores of the pL783 and pL782 potential leftward promoters were relatively high, it is pertinent to note that pL783 lacks the highly conserved T-residue at the first position of the -10 region and pL782 shows poor homology to the -10 region. Since the -10 region is important for promoter function (Hawley and McClure, 1983a), the homology scores obtained for these promoters may not reflect the strength of these promoters (i.e. they may be relatively weak promoters).

The termination of RNA synthesis occurs at specific termination sites, which belong to two major classes, the Rho-independent and the Rho-dependent terminators (Adhya and Gottesman, 1978; Galloway and Platt, 1985; Platt, 1986). Rho-independent terminators are capable of functioning *in vitro* without the need for any additional proteins other than RNA polymerase, and are characterized by a region of GC-rich hyphenated dyad symmetry (that can form a stem-loop structure in the RNA) followed by a

TABLE 3.3 Possible promoters encoded in the PstI-BglII sequence.

PROMOTER [a] (POSITION)	SEQUENCE	SCORE [b]	OCCURENCE BY CHANCE [c]
pR784 (3873)	GTGAAACATGTCACGCGATGAATTAAGAA	66.1	1/3 KB
pR785 (3911)	GTGCCATGATTCCAAATATGGAGGAAGGT	66.4	1/6 KB
pL782 (3821)	TTCACTCCTTTAATTACAAAGTGGACAAT	66.6	1/10 KB
pL783 (3830)	GTGACATGTTTCACTCCTTTAATTACAAA	67.3	1/36 KB
E. coli CONSENSUS	TTGACA TATAAT		
	-35 -10		

Notes to Table 3.3

a. The position refers to the sequence position of the first base in the -10 region on the l-strand.
b. The score was obtained using the promoter matrix of Kalionis et al. (1986a). This matrix is derived from the occurence of each base at each position of the promoter, using the 112 promoters compiled by Hawley and McClure (1983a). The score was calculated as described in Chapter 2.38.
c. Shows the frequency of this sequence occuring by chance in a randomised sequence.

Using the promoter matrix of Kalionis et al. (1986a), other promoters scored as follows: 186 pR 67.2, 186 pL 66.7 (Kalionis et al., 1986a), λ pR 67.6, λ pL 67.2, and λ pRM 65.4 (Hawley and McClure, 1983a).

string of consecutive T-residues (Holmes et al., 1983; Galloway and Platt, 1985; Platt, 1986). Termination of transcription generally occurs at one of these T-residues. Rho-dependent terminators need the transcription factor Rho (Roberts, 1969) for termination to occur and are less well defined than Rho-independent terminators. Transcription stop points, which are Rho-dependent are generally, but not always, characterized by a region of GC-rich hyphenated dyad symmetry and associated with a region of >70 bases of untranslated, unstructured RNA, promoter-proximal to the termination site (Lowery and Richardson, 1977; Adhya et al., 1979; Morgan et al., 1983a,b, 1985; von Hippel et al., 1984; Lau and Roberts, 1985; Galloway and Platt, 1985). The region of untranslated, unstructured RNA has been shown to be required for the interaction of Rho with the RNA (Richardson and Macy, 1981; Richardson, 1982; Sharp and Platt, 1984; Ceruzzi et al., 1985; Chen et al., 1986). Analysis of the region 5' to Rho-dependent termination sites has not revealed any striking sequence homologies (Morgan et al., 1985), but did detect an abundance of C-residues (Richardson and Macy, 1981) and a low G-residue content (Ceruzzi et al., 1985).

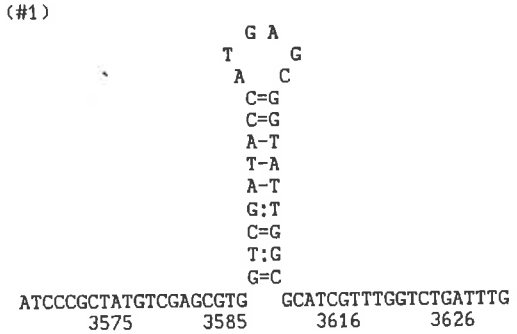
In this study, potential transcription terminators (stem-loop structures) were searched for using dot matrix analysis and the computer program COMSTR (Chapter 2.38). COMSTR searches for stem-loop structures and calculates the ΔG values of these structures using the rules of Tinoco et al. (1973) as modified by Steger et al. (1984).

The analysis of the PstI-BglII (77.4%-79.6%) region for possible terminator structures revealed several stem-loop structures, the most stable of which are shown in Figure 3.4. Four of the structures [#1 (3586-3609), #1a (3805-3844), #4 (4147-4169) and #5 (4202-4222)] are located within potential genes, while the other two [#2 (4070-4089) and #3 (4091-4116)] are located between CP78 and CP79 (Figure 3.2). Structure #2 is consistent with a Rho-independent terminator, with a GC-rich stem immediately followed by a T-rich (6/9) tail (Figure 3.4). Structures #1,

Figure 3.4 Potential terminator structures in the PstI-BglII (77.4%-79.6%) region from 186 cItsp.

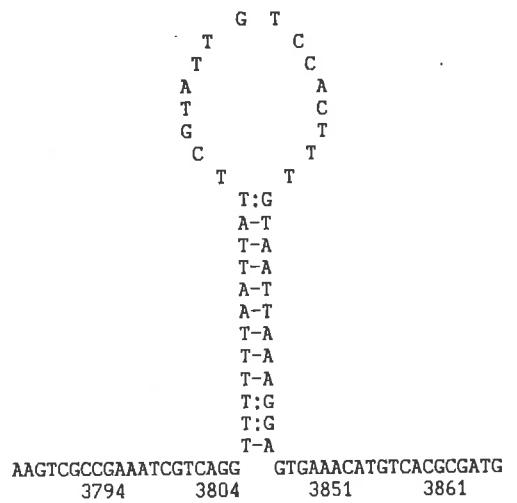
This Figure shows the most stable stem-loop structures encoded within the PstI-BglII (77.4%-79.6%) region. A threshold value of $\Delta G = -8.0$ was arbitrarily chosen. These stem-loop structures were predicted using the computer program COMSTR and by dot matrix analysis (Chapter 2.38). The free energy (ΔG) values were calculated using the rules of Steger et al. (1984), and are listed beneath each structure. The DNA sequence coordinates are listed beneath each structure.

(#1)



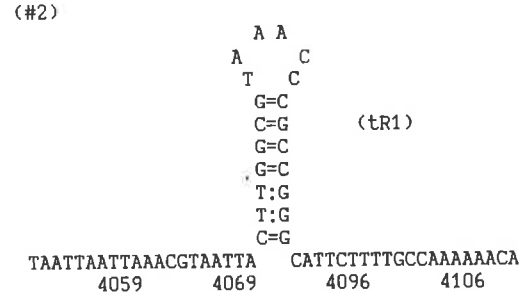
ΔG = -10.5 Kcal/mol

(#1a)



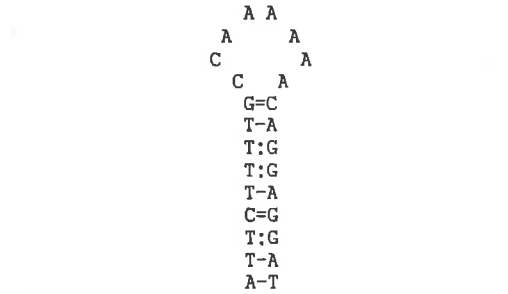
ΔG = -10.8 Kcal/mol

(#2)



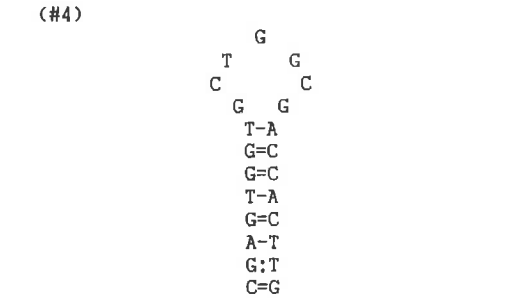
ΔG = -10.2 Kcal/mol

(#3)



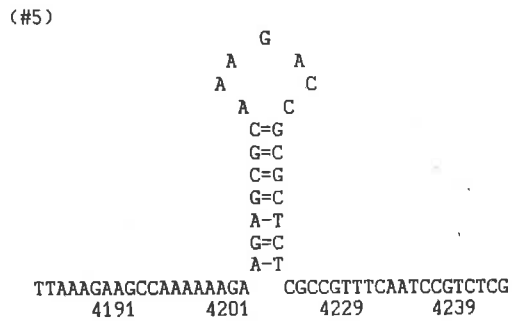
ΔG = -8.4 Kcal/mol

(#4)



ΔG = -9.0 Kcal/mol

(#5)



ΔG = -10.2 Kcal/mol

#1a, #3, #4, and #5 are not immediately followed by a T-rich region and are therefore unlikely to be Rho-independent terminators. These structures also do not fall into the general description of Rho-dependent terminators since they are located in potentially translated regions or they are not preceded by >70 bases of untranslated RNA. However, Rho-dependent terminators have been observed in translated regions of the λ genome (e.g. tLIIc and tLIId; Figure 1.5) (Szybalski et al., 1983; Chapter 1.4.2a). For this reason structures #1, #1a, #3, #4 and #5 were considered to be potential Rho-dependent terminators.

It is pertinent to note that structures #1a and #3 overlap the presumptive ribosome-binding sites of CP78 and CP79, respectively (Figure 3.2). Local secondary structure in the RNA, spanning the ribosome-binding site of a gene, has been reported to decrease the efficiency of translation initiation (Hall et al., 1982; Kastelein et al., 1983; Woods et al., 1984; Schottel et al., 1984; Buell et al., 1985; Stanssens et al., 1985; Coleman et al., 1985; Looman et al., 1986). Thus, structures #1a and #3 may result in a decrease in the expression of CP78 and CP79.

As discussed in Chapters 1.3.2(b) and 3.1, the early lytic transcript (the 1.45 kb in vitro transcript) was expected to terminate close to the BglII (79.6%) site (Pritchard and Egan, 1985). Thus, the stem-loop structures #2, #3, #4 and #5, which are encoded close to the BglII site (sequence coordinate 4244), are likely candidates for the transcription termination signal of the early lytic transcript. Since structure #2 was the only potential Rho-independent terminator detected and therefore, the only terminator expected to function in vitro (without the addition of Rho), this structure was considered to be the most likely transcription termination signal for the early lytic transcript. This potential terminator (structure #2) was named tR1. Termination of the early lytic pR transcript at the presumptive tR1 terminator would result in a transcript of approximately 1360 bases in length, which is consistent (within 7%) with

the size estimated for the in vitro pR transcript, band 2 (1450 bases; Pritchard and Egan, 1985). Thus, tR1 was considered to be the signal that terminates the early lytic transcript. CP75, CP76, CP77 and CP78 are therefore predicted to be early lytic genes (genes encoded on the early lytic transcript) and CP79 is predicted to be the first middle gene.

The analysis of the DNA sequence of the early lytic region for potential promoters and terminators has led to two important observations. Firstly, the position of the predicted rightward promoters pR784 and pR785, is such that these two promoters are located approximately 200 bases to the left of the potential terminator tR1 (Figure 3.2a). Should these promoters be functional and be of importance in the transcription of the middle region, a control mechanism must exist to enable efficient transcription to proceed past tR1. Secondly, the two leftward promoters pL782 and pL783, may play a role in the establishment phase of cI transcription in the lysis-lysogeny decision. For instance, they may act in an analagous way to λ pRE (Wulff and Rosenberg, 1983). Therefore, it is important to determine whether or not these promoters are functional in vivo. This will be investigated in Chapters 7 and 8.

3.3 SUMMARY.

The sequence of the PstI-BglII (77.4%-79.6%) fragment has completed the sequence of the 186 early lytic region and the analysis of the sequence (summarized in Figure 3.2b) has revealed two important findings that may have relevance to the control of lytic gene expression.

(1) Transcription initiating from the early lytic promoter pR (Kalionis, 1985; Kalionis et al., 1986a; Pritchard and Egan, 1985) is likely to encode four genes, CP75, CP76, CP77 and CP78 and to terminate at a putative Rho-independent terminator tR1 located in the CP78-CP79 intergenic region. CP75, CP76, CP77 and CP78 are defined as early lytic genes and CP79 as the first gene in the middle region.

(2) The CP75 and CP76 gene-products are likely to be control proteins since they contain an amino acid sequence, which has significant homology to the DNA-binding region of λ Cro-type DNA-binding proteins (Dodd and Egan, 1987; Kalionis et al., 1986a), and they may be candidates for functions involved in the lysis/lysogeny decision and/or candidates for the postulated middle control gene (tom), which was discussed in Chapter 1.3.2.

CHAPTER 4.

GEL ANALYSIS OF THE PROTEIN PRODUCTS ENCODED IN THE 186 EARLY LYTIC REGION.

CHAPTER 4. GEL ANALYSIS OF THE PROTEIN PRODUCTS ENCODED IN THE 186 EARLY LYTIC REGION.

4.1 INTRODUCTION.

As detailed in Chapter 3, the sequencing and analysis of the PstI-BglII (77.4%-79.6%) region, together with the adjacent regions (Kalionis et al., 1986a; Sivaprasad, 1984) has led to the prediction that four genes CP75, CP76, CP77 and CP78 are encoded on the early lytic transcript, with CP79 the first gene of the middle region. This Chapter deals with the gel analysis of the proteins produced from the early lytic region.

Evidence can be obtained for the existence of genes by determining whether the protein products expressed from a plasmid-clone of this region are of a molecular weight approximating that predicted from the translated sequence. Alternatively, fusion-proteins produced from the fusion of potential genes with an ORF on the plasmid, or truncated proteins produced from amber mutations, can be used to obtain evidence that genes encode proteins and that the reading-frame is correct.

In this work, early lytic proteins were expressed from plasmid-clones in maxicells and analysed by SDS-polyacrylamide gel electrophoresis (Sancar et al., 1979; Chapter 2.37). All clones used were constructed during the course of this work (Chapter 2.3.2), unless stated otherwise. The analysis of proteins in all the experiments described below was carried out in collaboration with A. Puspurs. Specific reference to the work carried out by A. Puspurs is detailed in the Figure legends.

4.2 RESULTS AND DISCUSSION.

4.2.1 The 186 Early Lytic Gene-Products.

The XhoI-BglII (67.6%-79.6%) DNA fragment from 186 del1 (5) (containing a deletion, which removes the 3'-end of cI gene, and the complete int and CP69 genes) includes the early lytic region of 186, but does not contain the irrelevant lysogenic genes (Figure 4.1a). This fragment was cloned into the plasmid pKC7 (Chapter 2.3.1) to give the clone, pEC400 (Chapter 2.3.2). pEC400 encodes a lethal gene (Chapter 5.2.2) that is not expressed in a strain, which is lysogenic for 186. The Su^+ maxicell strain (E660) is infected poorly by 186 and is therefore difficult to lysogenize. For this reason a mutated derivative of pEC400, namely pEC401 (that contains point mutations in the CP77, CP78 and CP79 genes), which is capable of survival in a non-lysogenic host (Chapter 5.2.2; Figure 4.1a) was used to transform the Su^+ maxicell strain (E660). Proteins produced from pEC401 and labelled with ^{35}S-methionine in the maxicell strain were analysed, as described in Chapter 2.37. The fluorograph is shown in Figure 4.2. Candidate proteins were observed, which were consistent with the predicted molecular weights (see Table 3.2, Figure 4.1) for CP76, and the fusion-proteins of CP79 and cI, but there were only poorly defined bands in the regions expected for CP75, CP77, and CP78.

The appearance of a protein product from pEC401, consistent in size with that expected for the CP76 protein, increases the likelihood that the computer-predicted gene CP76 is a real gene. Furthermore, the appearance of a protein product from pEC401, consistent with the size expected for the CP79-fusion-protein (protein expected from the fusion of CP79 with a plasmid ORF), confirms the reading-frame for CP79 and provides supporting evidence that CP79 is a real gene. The poor representation of the CP75, CP77 and CP78 gene-products may be explained by the expected poor

Figure 4.1 Gene content of the plasmid-clones used to analyse the protein products of the 186 early lytic region.

a. A diagrammatic representation of the gene content of the XhoI-BglII (67.6%-79.6%) region from 186. Genes are represented by the boxed regions and promoters are represented by the arrows. Relevant restriction sites are shown. The sequence coordinates of the restriction sites refer to the first base of the site on the l-strand.

Plasmid-clones used in this study for the analysis of protein products of the early lytic genes are shown. The construction of these plasmid-clones is described in Chapter 2.3.2. The restriction sites used to construct these clones are indicated (Chapter 2.3.2).

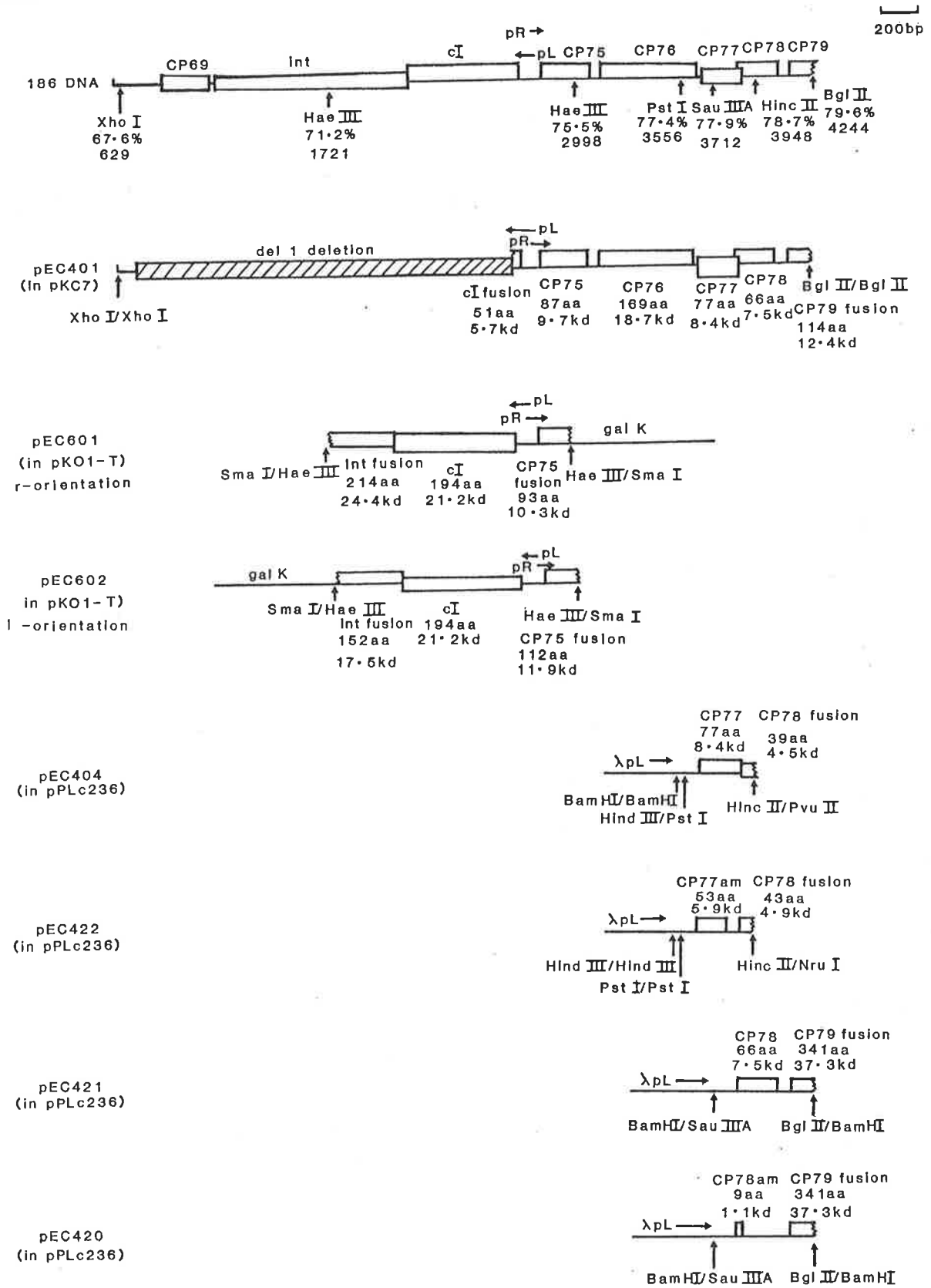
The genes present on these clones are shown. The sizes of the expected protein products in the number of amino acids (aa) and by molecular weight (kd), are shown. For plasmid-clones in the expression vector pPLc236 the λ pL promoter-proximal fusion-protein is not expected to be produced, and therefore is not indicated on the diagram. The position of the galK gene carried by clones in the vector, pKO1-T, is indicated.

Genes that are only partially represented on the plasmid-clones are indicated by the jagged-edged boxes. The sizes of the fusion-proteins expected for these genes are indicated.

The del1 deletion (67.9%-74.0%) carried by pEC401, is represented by the shaded box.

b. The DNA sequence and amino acid sequence (in one letter code) of all three reading-frames of the fusion genes expected from these plasmid-clones. (* indicates a termination codon.) The sizes of the predicted fusion-products are shown in the number of amino acids (aa) and by molecular weight (kd). The restriction site-fusions are also indicated.

(a)


200bp
pR→
186 DNA
CP69
Int
cI
←pL CP75
CP76
CP77
CP78
CP79
Xho I 67·6% 629
Hae III 71·2% 1721
Hae III 75·5% 2998
Pst I 77·4% 3556
Sau IIIA 77·9% 3712
Hinc II 78·7% 3948
Bgl II 79·6% 4244
pEC401 (In pKC7)
del 1 deletion
←pL
pR→
Xho I/Xho I
cI fusion 51aa 5·7kd
CP75 87aa 9·7kd
CP76 169aa 18·7kd
CP77 77aa 8·4kd
CP78 66aa 7·5kd
Bgl II/Bgl II
CP79 fusion 114aa 12·4kd
pEC601 (in pKO1-T) r-orientation
gal K
Sma I/Hae III
Int fusion 214aa 24·4kd
cI 194aa 21·2kd
CP75 fusion 93aa 10·3kd
Hae III/Sma I
pEC602 (in pKO1-T) l-orientation
Sma I/Hae III
Int fusion 152aa 17·5kd
cI 194aa 21·2kd
Hae III/Sma I
CP75 fusion 112aa 11·9kd
pEC404 (in pPLc236)
CP77 77aa 8·4kd
CP78 fusion 39aa 4·5kd
λpL→
BamHI/BamHI
Hind III/Pst I
Hinc II/Pvu II
pEC422 (in pPLc236)
CP77am 53aa 5·9kd
CP78 fusion 43aa 4·9kd
λpL→
Hind III/Hind III
Pst I/Pst I
Hinc II/Nru I
pEC421 (in pPLc236)
CP78 66aa 7·5kd
CP79 fusion 341aa 37·3kd
λpL→
BamHI/Sau IIIA
Bgl II/BamHI
pEC420 (in pPLc236)
CP78am 9aa 1·1kd
CP79 fusion 341aa 37·3kd
λpL→
BamHI/Sau IIIA
Bgl II/BamHI

b.

(a) CP79-fusion with plasmid DNA in pEC401

```
 M S R T I Y L S T P S G A G D H L L E S L F K E A K K E E R K D R A L A V S I R
  * V E L F I Y Q R R V V L A T T C W S L C L K K P K K K S A K T A L S P F Q S
   E S N Y L F I N A E W C W R P L A G V F V * R S Q K R R A Q R P R S R R F N P
ATGAGTCGAACTATTTATTTATCAACGCCGAGTGGTGCTGGCGACCACTTGCTGGAGTCTTTGTTTAAAGAAGCCAAAAAAGAAGAGCGCAAAGACCGCGCTCTCGCCGTTTCAATCCGT
      7180       7190       7200       7210       7220       7230       7240       7250       7260       7270       7280       7290

 L E D L D P L R H Q I L G G K K A I Q F T L Q G F P T L P E G A P A G N S G S L
V S K I L I P C A I R-S L A A R K P S S L L C R A S Q P Y Q R A P Q L A I P V R
 S R R S * S P A P S D P W R Q E S H P V Y F A G L P N L T R G R P S W Q F R F A
CTCGAAGATCTTGATCCCCTGCGCCATCAGATCCTTGGCGGCAAGAAAGCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTTACCAGAGGGCGCCCCAGCTGGCAATTCCGGTTCGCTT
        7300 BglⅡ/BglⅡ

 A V H K T A Q S S Y R H V S P L Q A T C F L F A L A F S L V Q I A Q *   114 aa
L L S I K P P S L A I A M * A H C K L P A F S L R L R F P L S R * P S
 C C P * N R P V * L S P C K P T A S Y L L S L C A C V F P C P D S P V
GCTGTCCATAAAACCGCCCAGTCTAGCTATCGCCATGTAAGCCCACTGCAAGCTACCTGCTTTCTCTTTGCGCTTGCGTTTTCCCTTGTCCAGATAGCCCAGTAG  12·4 kd
```

(b) CP78-fusion with plasmid DNA in pEC404

```
 M S R D E L R I V L G A M I P N M E E G F E I K T R D G A I L R V L P R A F R *
  C H A M N * E L F W V P * F Q I W R K V L K L K P A T A Q Y F A F C L A R F G D
   V T R * I K N C F G C H D S K Y G G R F * N * N P R R R N T S R S A S R V S V
ATGTCACGCGATGAATTAAGAATTGTTTTGGGTGCCATGATTCCAAATATGGAGGAAGGTTTTGAAATTAAAACCCGCGACGGCGCAATACTTCGCGTTCTGCCTCGCGCGTTTCGGTGA
      3861       3871       3881       3891       3901       3911       3921       3931       3941
                                                                                  HincⅡ/PvuⅡ

 * R * K P L T H A A P G D G H S L S V S G C R E Q T S P S G R V S G C W R V S G
  D G E N L * H M Q L P E T V T A C L * A D A G S R Q A R Q G A S A G V G G C R G
M T V K T S D T C S S R R R S Q L V C K R M P G A D K P V R A R Q R V L A G V G
TGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGG

R S H        39 aa
 A A M
A Q P *
CGCAGCCATGA  4·5 kd
```

(c) CP78-fusion with plasmid DNA in pEC422

```
 M S R D E L R I V L G A M I P N M E E G F E I K T R D G A I L R V R R E A G W P
  C H A M N * E L F W V P * F Q I W R K V L K L K P A T A Q Y F A F D A R L D G L
   V T R * I K N C F G C H D S K Y G G R F * N * N P R R R N T S R S T R G W M A
ATGTCACGCGATGAATTAAGAATTGTTTTGGGTGCCATGATTCCAAATATGGAGGAAGGTTTTGAAATTAAAACCCGCGACGGCGCAATACTTCGCGTTCGACGCGAGGCTGGATGGCCT
      3861       3871       3881       3891       3901       3911       3921       3931       3941
                                                                                  HincⅡ/NruⅠ

 S P L * F F S L P A A S G C P R C R P C C P G R * M T T I R D S F K D R S R L L
  P H Y D S S R F R R H R D A R V A G H A V Q A G R * R P S G T A S R I A R G S Y
F P I M I L L A S G G I G M P A L Q A M L S R Q V D D D H Q G Q L Q G S L A A L
TCCCCATTATGATTCTTCTCGCTTCCGGCGGCATCGGGATGCCCGCGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCGCTCGCGGCTCTTA

 P A * L R S L D R * S S R R F M P P R R A H G T G W H G L * A P P Y T L S A S P
  Q P N F D H W T A D R H G D L C R L G E H M E R V G M D C R R R P I P C L P P R
T S L T S I T G P L I V T A I Y A A S A S T W N G L A W I V G A A L Y L V C L P
CCAGCCTAACTTCGATCACTGGACCGCTGATCGTCACGGCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACCTTGTCTGCCTCCCCG

 R C V A V H G A G P P R R P   43 aa
  V A S R C M E P G H L D L
A L R R G A W S R A T S T *
CGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGA  4·9 kd
```

(d) CP79-fusion with plasmid DNA in pEC420 and pEC421

```
M S R T I Y L S T P S G A G D H L L E S L F K E A K K E E R K D R A L A V S I R
 * V E L F I Y Q R R V V L A T T C W S L C L K K P K K K S A K T A L S P F Q S V
  E S N Y L F I N A E W C W R P L A G V F V * R S Q K R R A Q R P R S R R F N P
ATGAGTCGAACTATTTATTTATCAACGCCGAGTGGTGCTGGCGACCACTTGCTGGAGTCTTTGTTTAAAGAAGCCAAAAAAGAAGAGCGCAAAGACCGCGCTCTCGCCGTTTCAATCCGT
      4128       4138       4148       4158       4168       4178       4188       4198       4208       4218       4228       4238

L E D P L R R T H R G R H H R R H R C G C W R L Y R R H H R W G R S G S P L R A
 S K I L Y A G R I V A G I T G A T G A V A G A Y I A D I T D G E D R A R H F G L
S R R S S T P D A S W P A S P A P Q V R L L A P I S P T S P M G K I G L A T S G
CTCGAAGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCT
      4248 BglⅡ/Bam HⅠ

H E R L F R R G Y G G R P V A G G L L G A I S L H A P F L A A A V L N G L N L L
 M S A C F G V G M V A G P W P G D C W A P S P C M H H S L R R R C S T A S T Y Y
S * A L V S A W V W W Q A R G R G T V G R H L L A C T I P C G G G A Q R P Q P T
CATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCGTGGCCGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTA

L G C F L M Q E S H K G E R R P M P L R A F N P V S S F R W A R G M T I V A A L
 W A A S * C R S R I R E S V D R C P * E P S T Q S A P S G G R G A * L S S P H L
T G L L P N A G V A * G R A S T D A L E S L Q P S Q L L P V G A G H D Y R R R T
CTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATGACTATCGTCGCCGCACTT

M T V F F I M Q L V G Q V P A A L W V I F G E D R F R W S A T M I G L S L A V F
 * L S S L S C N S * D R C R Q R S G S F S A R T A F A G A R R * S A C R L R Y S
Y D C L L Y H A T R R T G A G S A L G H F R R G P L S L E R D D D R P V A C G I
ATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCATTTTCGGCGAGGACCGCTTTCGCTGGAGCGCGACGATGATCGGCCTGTCGCTTGCGGTATTC

G I L H A L A Q A F V T G P A T K R F G E K Q A I I A G M A A D A L G Y V L L A
 E S C T P S L K P S S L V P P P N V S A R S R P L S P A W R P T R W A T S C W R
R N L A R P R S S L R H W S R H Q T F R R E A G H Y R R H G G R R A G L R L A G
GGAATCTTGCACGCCCTCGCTCAAGCCTTCGTCACTGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGCTGGGCTACGTCTTGCTGGCG

F A T R G W M A F P I M I L L A S G G I G M P A L Q A M L S R Q V D D D H Q G Q
 S R R E A G W P S P L * F F S L P A A S G C P R C R P C C P G R * M T T I R D S
V R D A R L D G L P H Y D S S R F R R H R D A R V A G H A V Q A G R * R P S G T
TTCGCGACGCGAGGCTGGATGGCCTTCCCCATTATGATTCTTCTCGCTTCCGGCGGCATCGGGATGCCCGCGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAG

L Q G S L A A L T S L T S I T G P L I V T A I Y A A S A S T W N G L A W I V G A
 F K D R S R L L P A * L R S L D R * S S R R F M P P R R A H G T G W H G L * A P
A S R I A R G S Y Q P N F D H W T A D R H G D L C R L G E H M E R V G M D C R R
CTTCAAGGATCGCTCGCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGCTGATCGTCACGGCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCC

A L Y L V C L P A L R R G A W S R A T S T *  341 aa
  P Y T L S A S P R C V A V H G A G P P R P
R P I P C L P P R V A S R C M E P G H L D L
GCCCTATACCTTGTCTGCCTCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGA 37·3 kd
```

(e) CP75-fusion with plasmid DNA in pEC601

```
M A S E I A I I K V P A P I V T L Q Q F A E L E G V S E R T A Y R W T T G D N P
 W L L K S Q S S K C L H L S L L C N N S Q S L R V F L N A P P T A G Q P A T T
  G F * N R N H Q S A C T Y R Y S A T I R R A * G C F * T H R L P L D N R R Q P
ATGGCTTCTGAAATCGCAATCATCAAAGTGCCTGCACCTATCGTTACTCTGCAACAATTCGCAGAGCTTGAGGGTGTTTCTGAACGCACCGCCTACCGGCTGGACAACCGGCGACAACCCT
      2836       2846       2856       2866       2876       2886       2896       2906       2916       2926       2936       2946

C V P I E P R T I R K G C K K A G G G N K G C T R T F I R L C C A P P P Y V N L
L V Y Q S N P A Q S V K A A R K Q V G A I R A A R A L L S A S A A L R H R T * I
 L C T N R T P H N P * R L Q E S R W G Q * G L H A H F Y P P L L R S A T V R K F
TGTGTACCAATCGAACCCCGCACAATCCGTAAAGGCTGCAAGAAAGCAGGTGGGGGCAATAAGGGCTGCACGCGCACTTTTATCCGCCTCTGCTGCGCTCCGCCACCGTACGTAAATTTA
      2956       2966       2976       2986       2996
                                                  Hae Ⅲ/Sma Ⅰ

W L V M K C W Q R P S E T *    93 aa
Y G W L * N A G R D P A R P
 M V G Y E M L A E T Q R D L
TGGTTGGTTATGAAATGCTGGCAGAGACCCAGCGAGACCTGA   10·3 kd
```

(f) CP75-fusion with plasmid DNA in pEC602

```
M A S E I A I I K V P A P I V T L Q Q F A E L E G V S E R T A Y R W T T G D N P
 W L L K S Q S S K C L H L S L L C N N S Q S L R V F L N A P P T A G Q P A T T
  G F * N R N H Q S A C T Y R Y S A T I R R A * G C F * T H R L P L D N R R Q P
ATGGCTTCTGAAATCGCAATCATCAAAGTGCCTGCACCTATCGTTACTCTGCAACAATTCGCAGAGCTTGAGGGTGTTTCTGAACGCACCGCCTACCGCTGGACAACCGGCGACAACCCT
      2836       2846       2856       2866       2876       2886       2896       2906       2916       2926       2936       2946

C V P I E P R T I R K G C K K A G G G M G S K L G R A Q H V T G P E V V L A C P
L V Y Q S N P A Q S V K A A R K Q V G G W G V S L A G L S T L P D Q K L S W H A
 L C T N R T P H N P * R L Q E S R W G D G E * A W P G S A R Y R T R S C P G M P
TGTGTACCAATCGAACCCCGCACAATCCGTAAAGGCTGCAAGAAAGCAGGTGGGGGGATGGGGAGTAAGCTTGGCCGGGCTCAGCACGTTACCGGACCAGAAGTTGTCCTGGCATGCCCA
      2956       2966       2976       2986       2996  Hae Ⅲ/Sma Ⅰ

A E Q H T H V A V V T S V T F T H Q A D I V S P P S K I R F S G * W C D S L H H
Q R N S T H M S R W L R P S R S R I R R I S L A H P A K F G F L A D G A I V F T
 S G T A H T C R G G Y V R H V H A S G G Y R * P T Q Q N S V F W L M V R * S S P
GCGGAACAGCACACACATGTCGCGGTGGTTACGTCCGTCACGTTCACGCATCAGGCGGATATCGTTAGCCCACCCAGCAAAATTCGGTTTTCTGGCTGATGGTGCGATAGTCTTCACCAT

V K H P L C G G Q V F C C P P L A G A L   112 aa
M S N I H S A A V R S S A V P H L L A L *
C Q T S T L R R S G L L L S P T C W R S
GTCAAACATCCACTCTGCGGCGGTCAGGTCTTCTGCTGTCCCCCACTTGCTGGCGCTCTGA 11·9 kd
```

(g) Int-fusion with plasmid DNA in pEC601

```
M T V R K N P A G G W I C E L Y P N G A K G K R I R K K F A T K G E A L A F E Q
 * P S V K I R L A V G F V N S T Q T V Q K A N V S E R N S L L K A R L W R L S
  D R P * K S G W R L D L * T L P K R C K R Q T Y Q K E I R Y * R R G S G V * A
ATGACCGTCCGTAAAAATCCGGCTGGCGGTTGGATTTGTGAACTCTACCCAAACGGTGCAAAAGGCAAACGTATCAGAAAGAAATTCGCTACTAAAGGCGAGGCTCTGGCGTTTGAGCAG
      6993      7003      7013      7023      7033      7043      7053      7063      7073      7083      7093      7103

Y T V Q N P W Q E E K E D R R T L K E L V D S W Y S A H G I T L K D G L K R Q L
S T P F K T R G R K K R K T G A R * K S W L I H G I A L H A L H * K M V * N A S
V H R S K P V A G R K G R Q A H V K R A G * F M V * R S W H Y T E R W F E T P V
TACACCGTTCAAAACCCGTGGCAGGAAGAAAAGGAAGACAGGCGCACGTTAAAAGAGCTGGTTGATTCATGGTATAGCGCTCATGGCATTACACTGAAAGATGGTTTGAAACGCCAGTTA
      7113      7123      7133      7143      7153      7163      7173      7183      7193      7203      7213      7223

A M H H A F E C M G E P L A R D F D A Q M F S R Y R E K R L K G E Y A R S N R V
* P C T M L L S V W A N H S H A I S M R R C F P A T E K N G * K V S M P V Q T E
S H A P C F * V Y G R T T R T R F R C A D V F P L P R K T V K R * V C P F K Q S
GCCATGCACCATGCTTTTGAGTGTATGGGCGAACCACTCGCACGCGATTTCGATGCGCAGATGTTTTCCCGCTACCGAGAAAAACGGTTAAAAGGTGAGTATGCCCGTTCAAACAGAGTG
      7233      7243      7253      7263      7273      7283      7293      7303      7313      7323      7333      7343

K E V S P R T L N L E L G G W G V S L A G L S T L P D Q K L S W H A Q R N S T H
* K R Y R L A R L I L S W G D G E * A W P G S A R Y R T R S C P G M P S G T A H
E R G I A S H A * S * A G G H G S K L G R A Q H V T G P E V V L A C P A E Q H T
AAAGAGGTATCGCCTCGCACGCTTAATCTTGAGCTGGGGGGATGGGGAGTAAGCTTGGCCGGGCTCAGCACGTTACCGGACCAGAAGTTGTCCTGGCATGCCCAGCGGAACAGCACACAC
      7353      7363      7373
                    Hae III/Sma I

M S R W L R P S R S R I R R I S L A H P A K F G F L A D G A I V F T M S N I H S
T C R G G Y V R H V H A S G G Y R * P T Q Q N S V F W L H V R * S S P C Q T S T
H V A V V T S V T F T H Q A D I V S P P S K I R F S G * W C D S L H H V K H P L
ATGTCGCGGTGGTTACGTCCGTCACGTTCACGCATCAGGCGGATATCGTTAGCCCACCCAGCAAAATTCGGTTTTCTGGCTGATGGTGCGATAGTCTTCACCATGTCAAACATCCACTCT

A A V R S S A V P H L L A L *   214 aa
L R R S G L L L S P T C W R S
C G G Q V F C C P P L A G A L
GCGGCGGTCAGGTCTTCTGCTGTCCCCCACTTGCTGGCGCTCTGA   24·4 kd
```

(h) Int-fusion with plasmid DNA in pEC602

```
M T V R K N P A G G W I C E L Y P N G A K G K R I R K K F A T K G E A L A F E Q
 * P S V K I R L A V G F V N S T Q T V Q K A N V S E R N S L L K A R L W R L S
  D R P * K S G W R L D L * T L P K R C K R Q T Y Q K E I R Y * R R G S G V * A
ATGACCGTCCGTAAAAATCCGGCTGGCGGTTGGATTTGTGAACTCTACCCAAACGGTGCAAAAGGCAAACGTATCAGAAAGAAATTCGCTACTAAAGGCGAGGCTCTGGCGTTTGAGCAG
      6993      7003      7013      7023      7033      7043      7053      7063      7073      7083      7093      7103

Y T V Q N P W Q E E K E D R R T L K E L V D S W Y S A H G I T L K D G L K R Q L
S T P F K T R G R K K R K T G A R * K S W L I H G I A L H A L H * K M V * N A S
V H R S K P V A G R K G R Q A H V K R A G * F M V * R S W H Y T E R W F E T P V
TACACCGTTCAAAACCCGTGGCAGGAAGAAAAGGAAGACAGGCGCACGTTAAAAGAGCTGGTTGATTCATGGTATAGCGCTCATGGCATTACACTGAAAGATGGTTTGAAACGCCAGTTA
      7113      7123      7133      7143      7153      7163      7173      7183      7193      7203      7213      7223

A M H H A F E C M G E P L A R D F D A Q M F S R Y R E K R L K G E Y A R S N R V
* P C T M L L S V W A N H S H A I S M R R C F P A T E K N G * K V S M P V Q T E
S H A P C F * V Y G R T T R T R F R C A D V F P L P R K T V K R * V C P F K Q S
GCCATGCACCATGCTTTTGAGTGTATGGGCGAACCACTCGCACGCGATTTCGATGCGCAGATGTTTTCCCGCTACCGAGAAAAACGGTTAAAAGGTGAGTATGCCCGTTCAAACAGAGTG
      7233      7243      7253      7263      7273      7283      7293      7303      7313      7323      7333      7343

K E V S P R T L N L E L G A I R A A R A L L S A S A A L R H R T * I Y G W L * N
* K R Y R L A R L I L S W G Q * G L H A H F Y P P L L R S A T V R K F M V G Y E
E R G I A S H A * S * A G G N K G C T R T F I R L C C A P P P Y V N L W L V M K
AAAGAGGTATCGCCTCGCACGCTTAATCTTGAGCTGGGGGCAATAAGGGCTGCACGCGGCACTTTTATCCGCCTCTGCTGCGCTCCGCCACCGTACGTAAATTTATGGTTGGTTATGAAAT
      7353      7363      7373
                    Hae III/Sma I

A G R D P A R P   152 aa
M L A E T Q R D L
C W Q R P S E T *
GCTGGCAGAGACCCAGCGAGACCTGA   17·6 kd
```

expression of these genes, predicted on the basis of the number of modulating codons present in these genes (Grosjean and Fiers, 1982; Chapter 3.2.2b) and/or they may be unstable proteins.

To further examine the existence of the presumptive CP75, CP77 and CP78 genes, the strategy used was to express, in the maxicell system, plasmid-clones encoding individual genes from the wild-type, and amber mutants or fusions of these genes, and to analyse the proteins produced by SDS-polyacrylamide gel electrophoresis. The appearance of protein bands of the expected size for the fusion-product (protein expected for the fusion of genes with plasmid ORFs) or the disappearance of a specific protein band (and the appearance of a truncated protein) when the gene contains an amber mutation will provide evidence that the predicted reading-frame is correct and that the computer-predicted gene encodes a protein.

4.2.2 Evidence that CP75 Encodes a Protein.

To confirm that CP75 encodes a protein, clones that produced CP75-fusion-proteins were analysed after labelling the proteins with ^{35}S-methionine in maxicells (Strain E660), followed by electrophoresis on an SDS-polyacrylamide gel (Chapter 2.37). The clones used were pEC601 and pEC602, which contain the HaeIII (71.2%-75.5%) fragment from 186 cItsp (1) cloned into the SmaI site of pKO1-T in either orientation (I. Dodd, personal communication; Chapter 2.3.2; Figure 4.1a). This fragment encodes the cItsp gene and creates fusion-proteins for CP75 and Int (see Figure 4.1a,b). The labelling was done at either 30°C (cI is expected to repress expression of the CP75-fusion gene from pR), or at 37°C (when transcription from pR can occur and the CP75-fusion gene expressed). Figure 4.3 shows that protein bands were obtained at 37°C of molecular weights (11.4 kd for pEC601 and 12.4 kd for pEC602), which were consistent with the predicted sizes of the CP75-fusion-proteins (10.3 kd for pEC601 and 11.9 kd for pEC602; Figure 4.1b). Proteins of a size consistent with the cI protein

Figure 4.2 The protein products of the 186 early lytic region.

This Figure shows protein products prepared from the plasmid-clone containing the 186 early lytic region (pEC401; Chapter 2.3.2). The gene content of pEC401 is shown in Figure 4.1.

Maxicells were prepared from E660 (pEC401) or E660 (pKC7) and proteins were labelled with ^{35}S-methionine at 37°C (Chapter 2.37). Samples (at approximately 30,000 cpm) were fractionated on a 15% polyacrylamide-SDS gel overnight at 95 V. Fluorography was as described by Reeve and Shaw (1979). The fluorograph was developed after 2 days exposure at -80°C.

Gel Tracks 1. Protein products in maxicells carrying the plasmid-vector pKC7.

2. Protein products in maxicells carrying pEC401.

3. Molecular weight markers (Chapter 2.37).

The sizes of the protein molecular weight markers are indicated on the right of the Figure. The sizes of the protein products from pEC401, as determined by their migration relative to the molecular weight markers, are indicated on the left of the Figure. The likely identity of these protein bands is indicated. The molecular weights in brackets are the sizes of the proteins determined from the translated DNA sequence.

E660 (pKC7) gave rise to several protein products that were not present in the sample prepared from E660 (pEC401). This is presumably due to the replacement in pEC401, of the XhoI-BglII fragment from the vector (containing the kanamycin gene) with the 1.78 kb XhoI-BglII fragment from 186 (Chapter 2.3.2). The intense band beneath the ampicillin protein is most likely the kanamycin gene-product (29.0 kd). The identity of the other protein bands observed in sample from E660 (pKC7) is not known. ^{35}S-methionine labelled-samples from E660 (not carrying any plasmids) did not give rise to any protein bands (data not shown).

The preparation of maxicells, labelling of proteins, gel electrophoresis and the fixing of the gel were carried out by A. Puspurs.

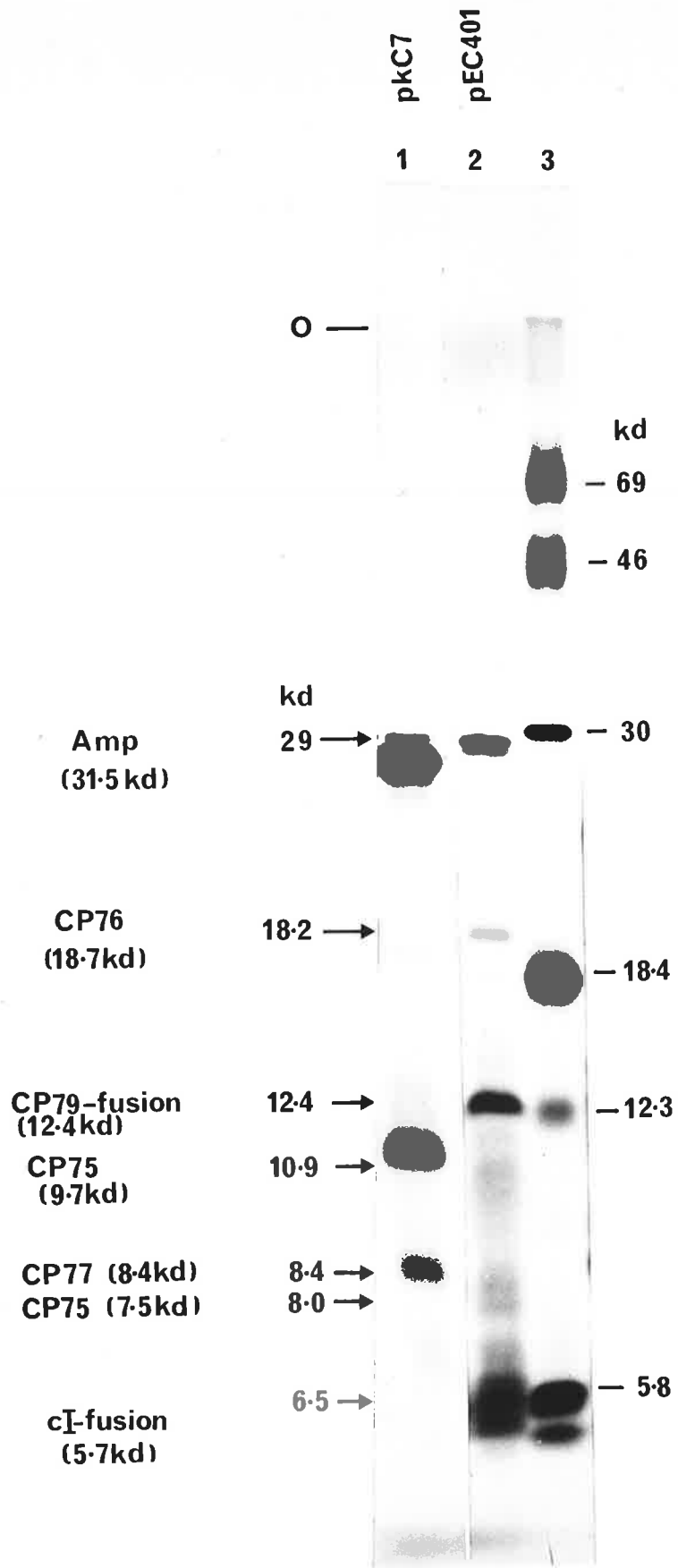

pkC7
pEC401
1
2
3
O
kd
69
46
kd
Amp
(31·5 kd)
29
30
CP76
(18·7kd)
18·2
18·4
CP79–fusion
(12·4kd)
12·4
12·3
CP75
(9·7kd)
10·9
CP77 (8·4kd)
8·4
CP75 (7·5kd)
8·0
6·5
5·8
cI-fusion
(5·7kd)

Figure 4.3 The CP75-fusion protein.

Maxicells were prepared from E660 carrying either pEC601, pEC602 or the parent vector, pKO1-T (Chapter 2.37). Proteins were labelled with ^{35}S-methionine at 30°C or 37°C. Samples (at approximately 10,000 cpm) were fractionated on a 12.5% polyacrylamide-SDS gel, overnight at 100 V. The gel was fixed and fluorographed, as described by Reeve and Shaw (1979). The gel was fluorographed for 1 week at -80°C.

Gel Tracks 1. Molecular weight markers (Chapter 2.37).

2 and 3. Protein products in maxicells carrying pKO1-T at 30°C and 37°C, respectively.

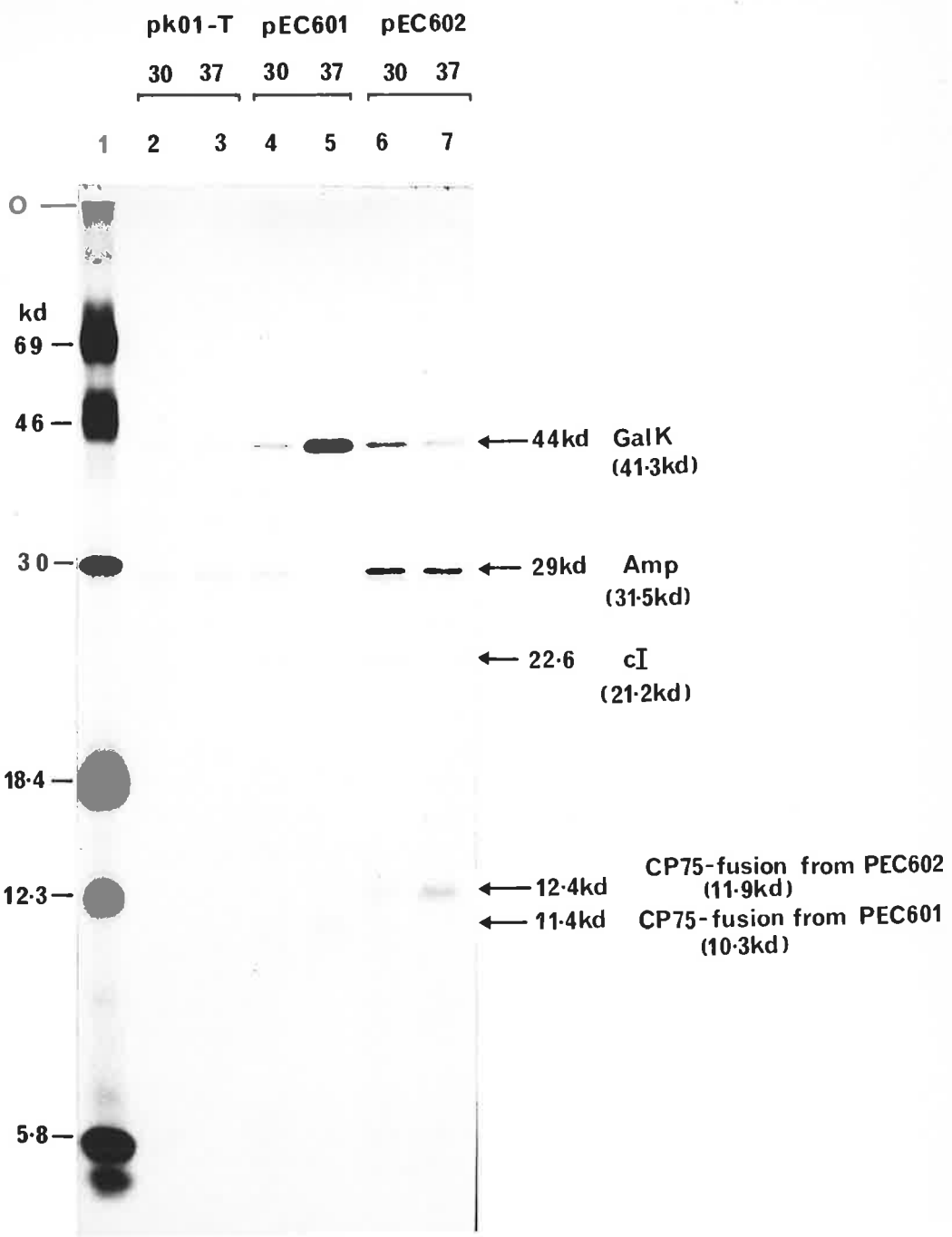
4 and 5. Protein products in maxicells carrying pEC601 at 30°C and 37°C, respectively.

6 and 7. Protein products in maxicells carrying pEC602 at 30°C and 37°C, respectively.

The gene content of pEC601 and pEC602 is shown in Figure 4.1. The clones were constructed by I. Dodd, as described in Chapter 2.3.2.

The sizes of the molecular weight markers are indicated on the left of the Figure. The sizes of the major protein products from pEC601 and pEC602, as determined by their migration relative to the molecular weight markers, are indicated to the right of the Figure. The likely identity of these protein bands is indicated. The molecular weights in brackets are the sizes of the proteins determined from the translated DNA sequence (Figure 4.1).

The preparation of maxicells, protein labelling, gel electrophoresis and fixing of the gel were carried out by A. Puspurs.


pk01-T
pEC601
pEC602
30
37
30
37
30
37
1
2
3
4
5
6
7
O
kd
69
46
30
18·4
12·3
5·8
44kd GalK
(41·3kd)
29kd Amp
(31·5kd)
22·6 cI
(21·2kd)
12·4kd CP75-fusion from PEC602
(11·9kd)
11·4kd CP75-fusion from PEC601
(10·3kd)

(21.2 kd) were faintly detected, however, candidates for the Int-fusion-proteins were not visible (Figure 4.1, 4.3). The inability to detect the Int-fusion-proteins may reflect poor expression due to the large number of modulating codons (Grosjean and Fiers, 1982; Chapter 3.2.2b) present in these fusion genes (7% and 5.3% for the Int-fusion from pEC601 and pEC602, respectively) and/or instability of these proteins.

These results provide evidence that the predicted reading-frame for CP75 is correct and that CP75 encodes a protein.

4.2.3 Evidence that CP77 and CP78 Encode Proteins.

The strategy used to obtain evidence for the existence of CP77 and CP78 was to clone the genes individually into an expression vector (pPLc236) and to analyze the proteins produced. Furthermore, clones of CP77am and CP78am mutants in pPLc236 were constructed in order to compare the proteins obtained from these mutants with those obtained from clones of CP77 and CP78 from the wild-type. The CP77am and CP78am mutations were created by oligonucleotide site-directed mutagenesis (Chapter 2.35) of the M13-clone, mEC401, which encodes the PstI-BglII (77.4%-79.6%) fragment (Chapter 2.4.2). The oligonucleotides used to create the CP77am and CP78am mutations are shown in Chapter 2.5 and their positions on the DNA sequence are shown in Figure 4.4. The mutations were confirmed by determining the DNA sequence of the CP77 and CP78 region, using as primers, the M13 universal primer, the CP77am and CP78am oligonucleotides (Chapter 2.33, 2.35.4d, 2.5; data not shown).

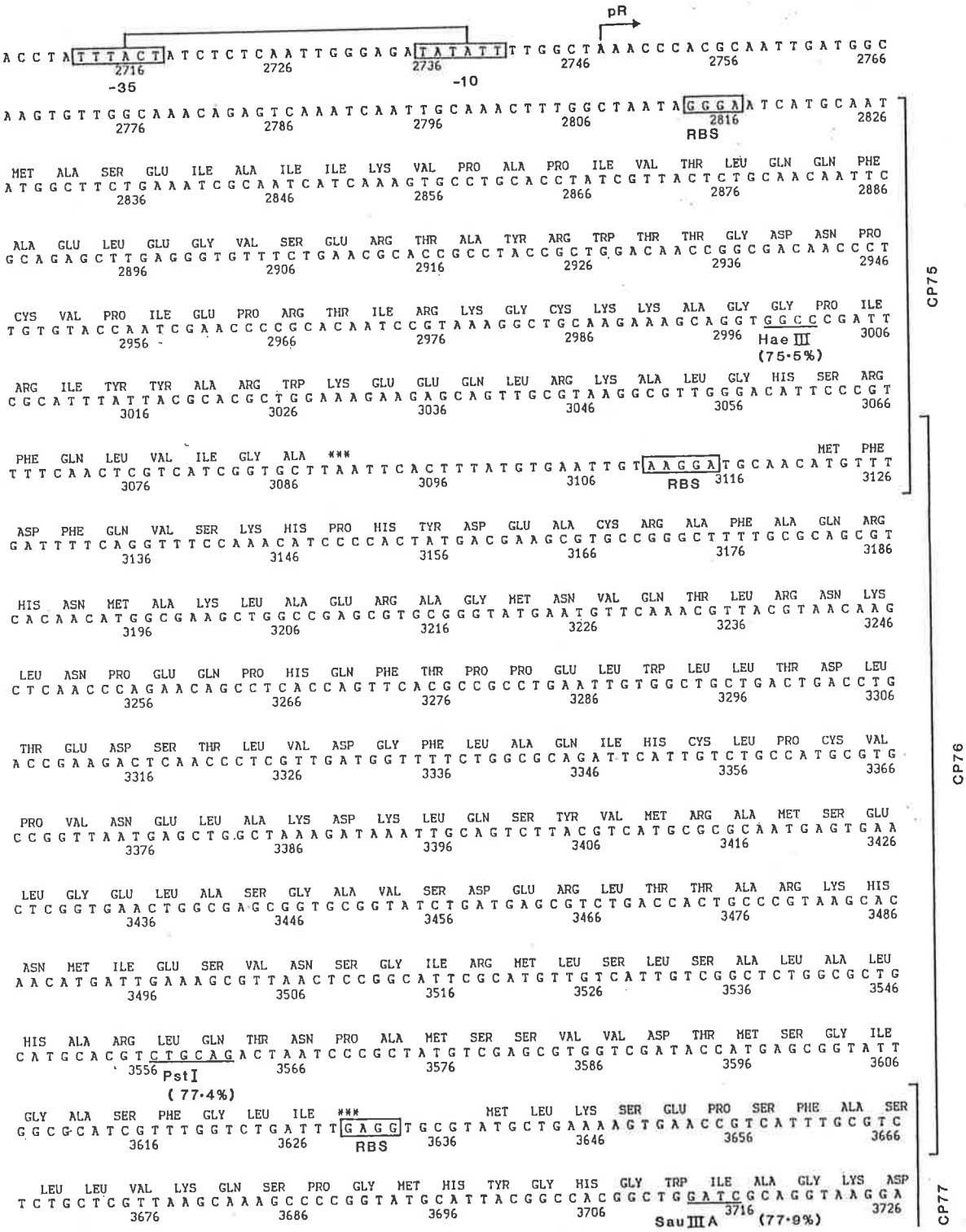
Clones of DNA fragments from the CP77am and CP78am mutants and of the corresponding DNA fragments from the wild-type were obtained in the expression vector, pPLc236 (Chapter 2.3; 2.31). pPLc236 contains the λ pL promoter and thus, the expression of genes cloned 3' to this promoter can be controlled by the presence of the λ cI857 repressor, which is expressed from a compatible plasmid-clone (pcI857) (Chapter 2.3.1). At 28°C the

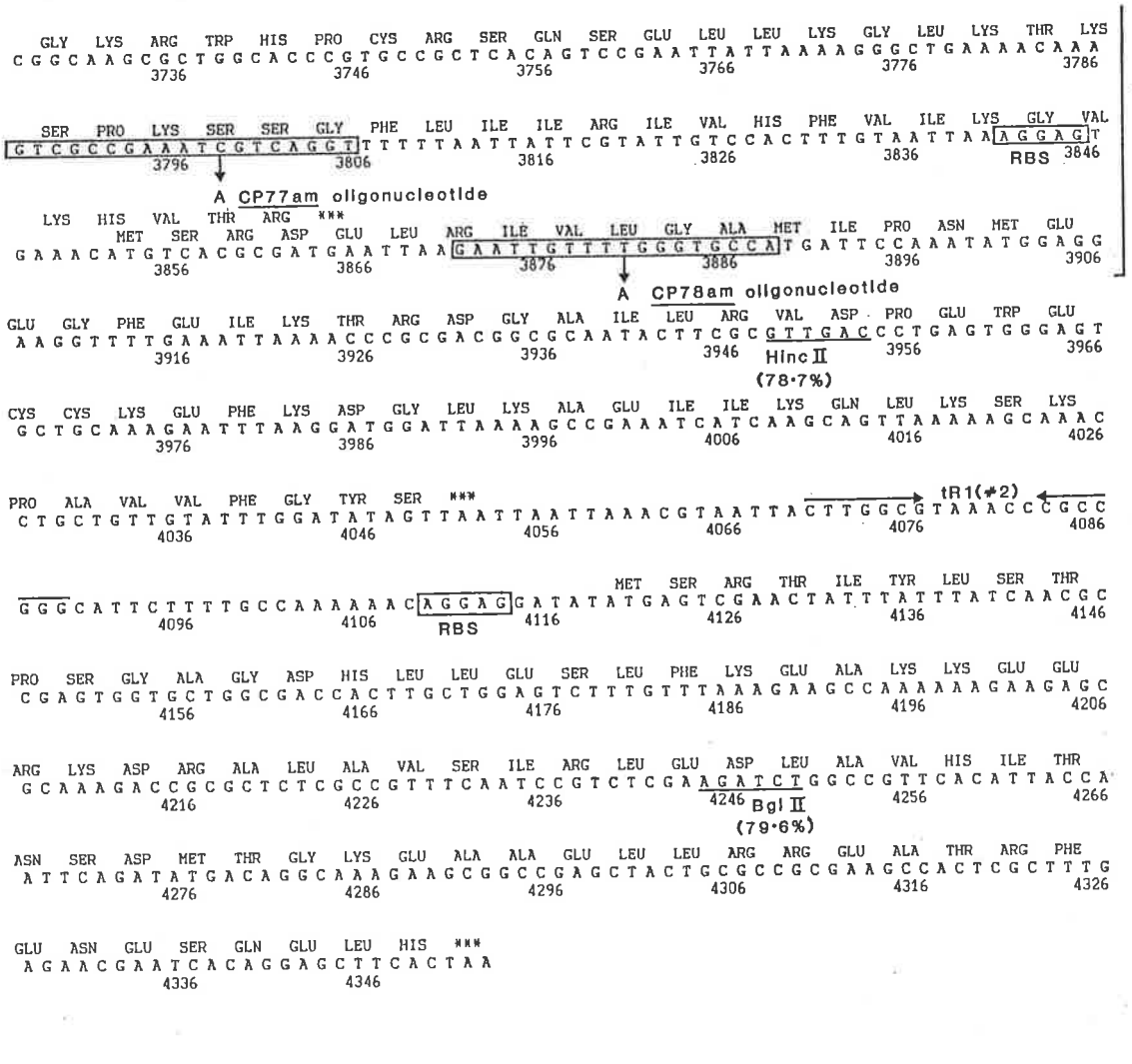
Figure 4.4 Sequence positions of the CP77am and CP78am oligonucleotides.

This Figure shows the DNA sequence of the l-strand from the pR promoter to the 3'-end of CP79 from 186 cItsp (see Figure 3.2). Potential genes are indicated on the right of the Figure. Ribosome-binding sites (Chapter 3.2.2a) are boxed. The pR promoter is indicated. The tR1 terminator (structure #2) is indicated by the convergent arrows.

The positions on the DNA sequence of the CP77am and CP78am oligonucleotides, which were used to create amber mutants in CP77 and CP78, (Chapter 2.5), are shown. The base changes created by these oligonucleotides (which give rise to amber stop codons, TAG) are indicated.

Relevant restriction sites used in the construction of plasmid-clones are also indicated.


pR
-35
-10
RBS
Hae III
(75·5%)
RBS
PstI
(77·4%)
RBS
SauIIIA
(77·9%)
CP75
CP76
CP77


GLY LYS ARG TRP HIS PRO CYS ARG SER GLN SER GLU LEU LEU LYS GLY LEU LYS THR LYS
C G G C A A G C G C T G G C A C C C G T G C C G C T C A C A G T C C G A A T T A T T A A A A G G G C T G A A A A C A A A
3736 3746 3756 3766 3776 3786
SER PRO LYS SER SER GLY PHE LEU ILE ILE ARG ILE VAL HIS PHE VAL ILE LYS GLY VAL
G T C G C C G A A A T C G T C A G G T T T T T A A T T A T T C G T A T T G T C C A C T T T G T A A T T A A A G G A G T
3796 3806 3816 3826 3836 RBS 3846
A CP77am oligonucleotide
LYS HIS VAL THR ARG ***
MET SER ARG ASP GLU LEU ARG ILE VAL LEU GLY ALA MET ILE PRO ASN MET GLU
G A A A C A T G T C A C G C G A T G A A T T A A G A A T T G T T T T G G G T G C C A T G A T T C C A A A T A T G G A G G
3856 3866 3876 3886 3896 3906
A CP78am oligonucleotide
GLU GLY PHE GLU ILE LYS THR ARG ASP GLY ALA ILE LEU ARG VAL ASP PRO GLU TRP GLU
A A G G T T T T G A A A T T A A A A C C C G C G A C G G C G C A A T A C T T C G C G T T G A C C C T G A G T G G G A G T
3916 3926 3936 3946 HincII 3956 3966
(78·7%)
CYS CYS LYS GLU PHE LYS ASP GLY LEU LYS ALA GLU ILE ILE LYS GLN LEU LYS SER LYS
G C T G C A A A G A A T T T A A G G A T G G A T T A A A A G C C G A A A T C A T C A A G C A G T T A A A A A A G C A A A C
3976 3986 3996 4006 4016 4026
PRO ALA VAL VAL PHE GLY TYR SER ***
C T G C T G T T G T A T T T G G A T A T A G T T A A T T A A T T A A A A C G T A A T T A C T T G G C G T A A A C C C G C C
4036 4046 4056 4066 4076 4086
tR1(#2)
M E T SER ARG THR ILE TYR LEU SER THR
G G G C A T T C T T T T G C C A A A A A A C A G G A G G A T A T A T G A G T C G A A C T A T T T A T T T A T C A A C G C
4096 4106 RBS 4116 4126 4136 4146
PRO SER GLY ALA GLY ASP HIS LEU LEU GLU SER LEU PHE LYS GLU ALA LYS LYS GLU GLU
C G A G T G G T G C T G G C G A C C A C T T G C T G G A G T C T T T G T T T A A A G A A G C C A A A A A A A G A A G A G C
4156 4166 4176 4186 4196 4206
ARG LYS ASP ARG ALA LEU ALA VAL SER ILE ARG LEU GLU ASP LEU ALA VAL HIS ILE THR
G C A A A G A C C G C G C T C T C G C C G T T T C A A T C C G T C T C G A A G A T C T G G C C G T T C A C A T T A C C A
4216 4226 4236 4246 BglII 4256 4266
(79·6%)
ASN SER ASP MET THR GLY LYS GLU ALA ALA GLU LEU LEU ARG ARG GLU ALA THR ARG PHE
A T T C A G A T A T G A C A G G C A A A G A A G C G G C C G A G C T A C T G C G C C G C G A A G C C A C T C G C T T T G
4276 4286 4296 4306 4316 4326
GLU ASN GLU SER GLN GLU LEU HIS ***
A G A A C G A A T C A C A G G A G C T T C A C T A A
4336 4346
CP78
CP79

cI857 repressor is active and transcription from λ pL is repressed, whereas at 42°C, the cI857 repressor is inactive and transcription of the cloned genes in pPLc236 occurs from λ pL. pEC422 contains the PstI-HincII (77.4%-78.9%) fragment from CP77am, and pEC404 contains the corresponding fragment from $CP77^+$ (Chapter 2.3.2). pEC420 contains the SauIIIA-BglII (77.9%-79.6%) fragment from CP78am and pEC421 contains the corresponding fragment from $CP78^+$ (Chapter 2.3.2). These clones and the sizes of the protein products expected to be expressed from them are shown in Figure 4.1.

The cloned genes were expressed in the Su^- maxicell strain (E4168) carrying the plasmid pcI857 (which encodes the λ cI857 repressor; Chapter 2.2.1), at 42°C and proteins were labelled with ^{35}S-methionine. Labelled proteins were analysed by urea/SDS-polyacrylamide gel electrophoresis in order to resolve the low molecular weight proteins (Chapter 2.37; Figure 4.1). Protein samples labelled at 28°C (the temperature at which the cloned genes are not expressed) were also analysed. The fluorograph is presented in Figure 4.5.

The $CP77^+$ clone (pEC404) at 42°C resulted in an intense, but broad, band sized at approximately 8.9 kd (Figure 4.5, lane 2). The $CP78^+$ clone (pEC421) at 42°C also gave rise to an intense band sized at 8.9 kd (Figure 4.5, lane 6). The sizes of these protein bands (8.9 kd) are greater than the sizes expected for the CP77 (8.4 kd) and CP78 (7.5 kd) proteins (Figure 4.1). This is probably due to the small size and highly basic nature expected for the CP77 and CP78 proteins (Chapter 3.2.2b). Small basic proteins are often difficult to size accurately because their gel migration is retarded and they are resolved poorly on SDS-polyacrylamide gels (Birkeland and Lindqvist, 1986; Christie et al., 1986). Since CP77 and CP78 proteins are expected to be small basic proteins and thus, to migrate abberantly on SDS-polyacrylamide gels it is therefore likely that the 8.9 kd protein band observed for the $CP77^+$ clone and the $CP78^+$ clone are the respective protein products of the CP77 and CP78 genes.

The analysis of the protein products obtained from the clones of the amber mutants provided some evidence to support the assignment of the 8.9 kd protein bands as the products of the CP77 and CP78 genes. The CP77am and CP78am mutants were expected to lead to the disappearance of the 8.9 kd bands. Furthermore, the CP77am mutant was predicted to give rise to an amber protein-fragment of 5.9 kd. A CP78 amber protein-fragment was not expected to be visualized due to its small size (1.1 kd).

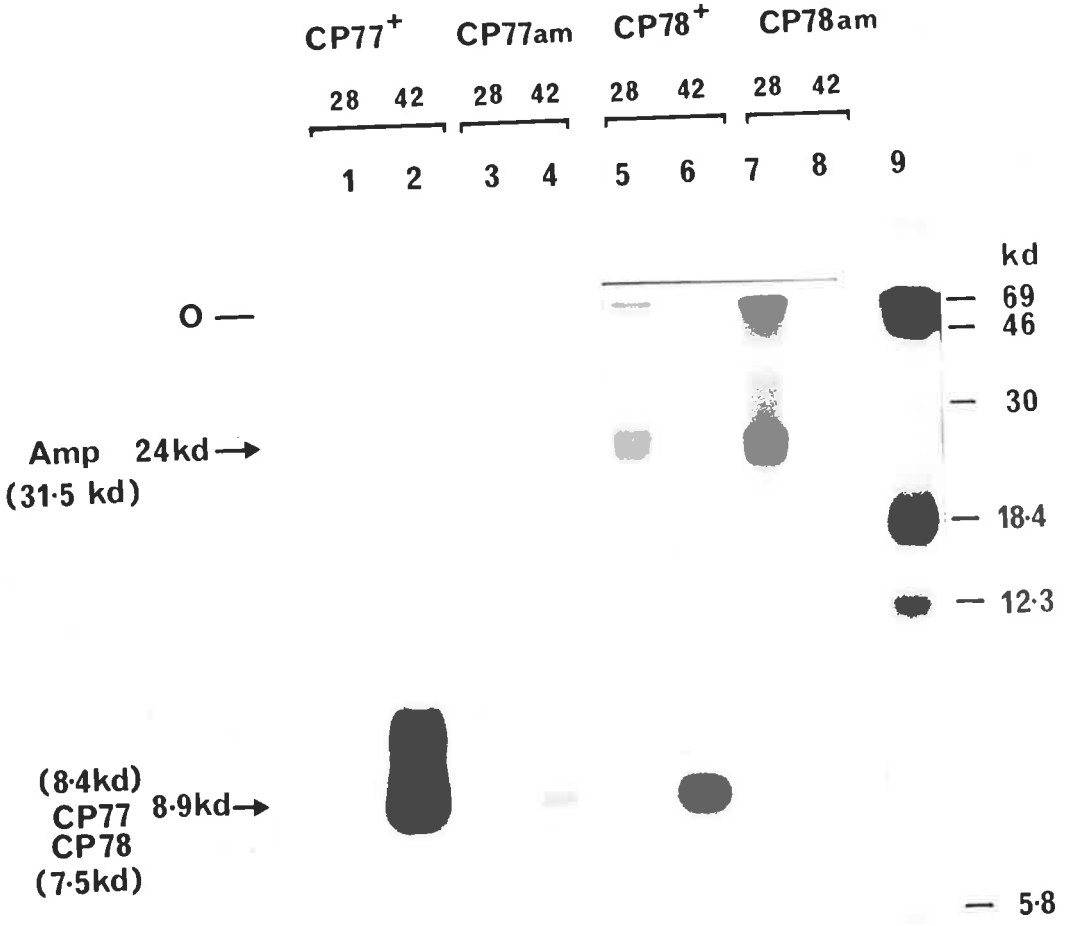
The CP77am clone (pEC422) and the CP78am clone (pEC420) at 42°C (Figure 4.5, lane 4 and 8) resulted in 8.9 kd bands of a significantly reduced intensity compared with the 8.9 kd bands obtained for the clones of the wild-type genes (Figure 4.5, lane 2 and 6).

These results provide some support for the assignment of the 8.9 kd proteins from the CP77 clone (pEC404) and the CP78 clone (pEC421) as the respective CP77 and CP78 gene-products. However, this must be stated with reservation, as there were some apparent inconsistencies. Firstly, the 8.9 kd bands were not completely absent in the protein samples prepared from the CP77am and CP78am clones. This suggests that the amber mutants are leaky and that translation is not terminated at the amber stop codons with 100% efficiency. Several cases of mis-reading of translational stop codons have been reported (Ryoji et al., 1983; Yates et al., 1977; Engleberg-Kulka et al., 1979; Miller and Albertini, 1982; Bossi, 1983; Weiner and Weber, 1971). Secondly, candidates for fusion-proteins or for the CP77 amber protein-fragment were not observed. This may be due to degradation of these abnormal proteins. There are many examples where abnormal proteins, such as fusion-proteins, proteins containing missense mutations or those due to premature termination at nonsense codons, show poor stability (Goldschmidt, 1970; Platt et al., 1970; Lin and Zabin, 1972; Bukhari and Zipser, 1973; Gottesman and Zipser, 1978; Kowit and Goldberg, 1977; Pakula et al., 1986).

the sizes of the proteins determined from the translated DNA sequence (Figure 4.1).

^{35}S-methionine labelled-samples from E4168 (not carrying any plasmids) did not give rise to any protein bands (data not shown).

The gel electrophoresis and fixing of the gel was carried out by A. Puspurs.


CP77+
CP77am
CP78+
CP78am
28 42 28 42 28 42 28 42
1 2 3 4 5 6 7 8 9
kd
O —
69
46
30
Amp 24kd →
(31·5 kd)
18·4
12·3
(8·4kd)
CP77
CP78
(7·5kd)
8·9kd →
5·8

4.3 SUMMARY

The results described above have provided evidence that CP76, CP77 and CP78 encode proteins and CP75 and CP79 encode fusion-proteins of sizes approximating the predicted sizes (Figure 4.1). Although there are some reservations, these results provide support that these computer-predicted genes are real genes and that the reading-frames are correct. Thus, CP75, CP76, CP77, CP78 and CP79 will henceforth be referred to as real genes. Definite confirmation that the reading-frames of these genes are correct can be achieved by determining the amino acid sequence of the proteins encoded by these genes using standard techniques (Walsh et al., 1981).

RESULTS: SECTION II.

CHAPTER 5.

THE 186 EARLY LYTIC GENES dhr AND fil.

CHAPTER 5. THE 186 EARLY LYTIC GENES dhr AND fil.

5.1 INTRODUCTION.

The analysis of the DNA sequence of the early lytic region (Chapter 3) and the identification of the proteins encoded in this region (Chapter 4) led to the conclusion that there are four early lytic genes; CP75, CP76, CP77 and CP78. The next step was to characterize the functions of these genes.

Recent studies carried out in this laboratory have enabled the assignment of functions to the CP75 and CP76 genes. I. Dodd has described a function Cpl, that controls the expression of the lysogenic genes from the 186 pL promoter (comparable to the λ Cro function; Gussin et al., 1983), and has shown that this function is encoded by CP75. The cII function, which is necessary for the establishment of lysogeny (Chapter 1.2.1), is encoded by CP76 (Carter, 1985). Both cpl and cII are unlikely to be essential for 186 lytic development since deletion and/or amber mutations have been obtained in these genes (H. Richardson, results not shown; I. Dodd, personal communication).

Thus, two early lytic genes CP77 and CP78 have yet to be assigned functions. There are two functions, which have been previously described that could be encoded in the early lytic region. The first of these functions is Dho, that acts to inhibit E. coli DNA replication, early after 186 prophage induction (Hocking and Egan, 1982b). This function has been renamed Dhr (for depression of host replication) and the effect it has on E. coli DNA replication has been termed the Dhr Effect. The second of these functions is the Tom function (previously X), which was postulated by Finnegan and Egan (1981) to be required for 186 middle gene transcription (Chapter 1.3.2).

This Chapter concerns the characterization of the Dhr function and the identification of the *dhr* gene. The results obtained, allow the identification of *CP78* as the *dhr* gene. The results presented in this Chapter also reveal that *CP77* encodes a gene (*fil*), which causes cell filamentation.

5.2 RESULTS AND DISCUSSION.

5.2.1 The Dhr Effect.

The experiment of Hocking and Egan (1982b), which demonstrated the *Dhr Effect*, was repeated and the results are shown in Figure 5.1. After heat-induction of a 186 *cItsp* (1) lysogen, the rate of DNA replication, as monitored by pulse-labelling with ^{3}H-thymidine (Chapter 2.18.1) was reduced. The depression of DNA replication began at approximately 5 min after the temperature shift and the rate continued to fall to a level of about 60% of the initial value at 25 min, before rising again to a maximum at 35 min due to 186 DNA synthesis (Hooper and Egan, 1981; Hocking and Egan, 1982b). The same depression in the rate of DNA synthesis was seen after the heat-induction of a culture lysogenic for the replication mutant 186 *cItspAam11* (8), and the rate remained depressed until 45 min when it gradually increased (Figure 5.1). This reduction in the rate of host DNA replication is the *Dhr Effect*. The increase in the rate of DNA synthesis, which occurred 45 min after heat-induction of the A^{-} mutant corresponded to the appearance in the culture of non-lysogenic cells (data not shown) that presumably arose by segregation after excision of a prophage that could not replicate.

These results confirm those of Hocking and Egan (1982b), which suggest that Dhr acts early in lytic development and therefore is likely to be an early lytic function. The next stage was to obtain evidence that the Dhr function is an early lytic gene, by isolating Dhr^{-} mutants and mapping the position of the mutations on the 186 genome.

Figure 5.1 The rate of DNA replication after heat-induction of 186 cItsp lysogens : The Dhr Effect.

Cultures of E252 [Su^- (186 cItsp (1))], E788 [Su^- (186 cItsp Aam11 (8))] and E251 [Su^-], which were grown overnight at 30°C in TPG-CAA medium, were diluted into the same medium and incubated with aeration at 30°C to A_{600}= 0.2 ($2x10^8$ cfu/ml). Cultures were transferred to 40°C at 0 min and incubation with aeration was continued. Samples (200 ul) were taken every 5 min and the rate of DNA replication was determined by pulse-labelling with ^{3}H-thymidine, as described in Chapter 2.18.1.

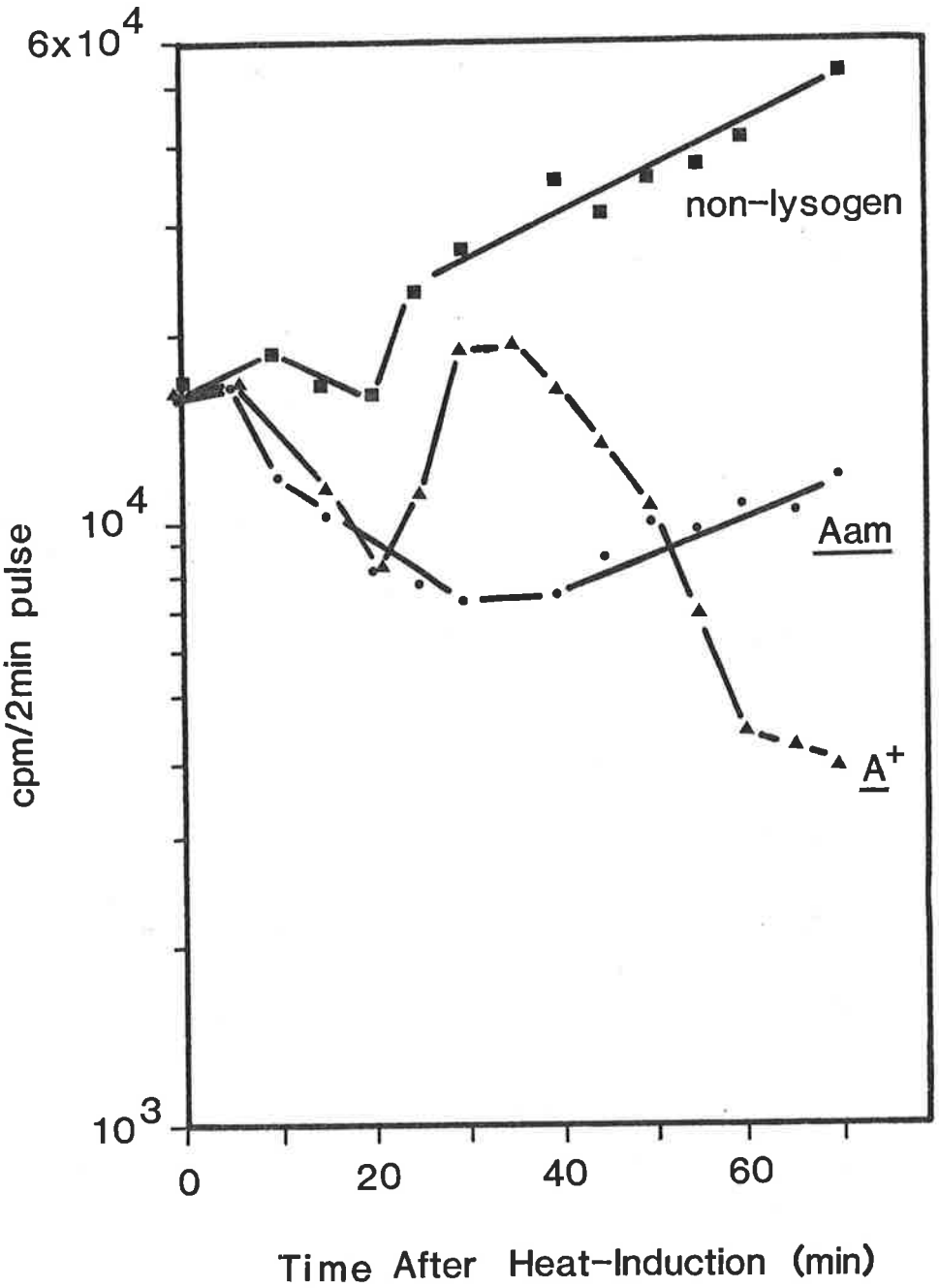
Symbols : ▲ = E252 [E251 (186 cItspA$^+$ (1))]

● = E788 [E251 (186 cItspAam11 (8))]

■ = E251 (non-lysogen)

Bacterial and bacteriophage strains are described in Chapters 2.1 and 2.2.1.

The Dhr Effect is the depression in DNA replication observed when 186 lysogens are heat-induced.


6x10^4
10^4
10^3
cpm/2min pulse
non-lysogen
Aam
A+
0
20
40
60
Time After Heat-Induction (min)

5.2.2 Isolation of Dhr⁻ Mutants.

Some of the results presented in this section (specifically, the isolation of pEC400, pEC401 and pEC402) have been described previously (H. Richardson, 1981). These results have relevance to subsequent studies and thus, it is important to describe them again here.

A possible approach for the isolation of a Dhr⁻ mutant came from the cloning studies of Finnegan (1979). It was found that a plasmid-clone pEC32 (Figure 5.2) containing the HindIII-BglII (61.3%-79.6%) fragment from 186 del1 (5) (which is cI⁻), could only be obtained in lysogenic strains and was lethal when used to transform a non-lysogen. These results suggested that pEC32 contained a lethal gene under cI repressor control. Further cloning studies carried out by Finnegan (1979) suggested that this gene was encoded within the PstI-BglII (77.4%-79.6%) region (the early lytic region). Since the Dhr function, as an inhibitor of E. coli DNA replication, may be expected to be lethal when overexpressed from a plasmid-clone and since the timing of the Dhr Effect suggested that the gene involved was an early lytic gene, it was predicted that the lethal gene described by Finnegan (1979) was the dhr gene. Therefore, the approach chosen to isolate a dhr mutant, was to isolate a non-lethal mutant of a clone expressing the early lytic genes.

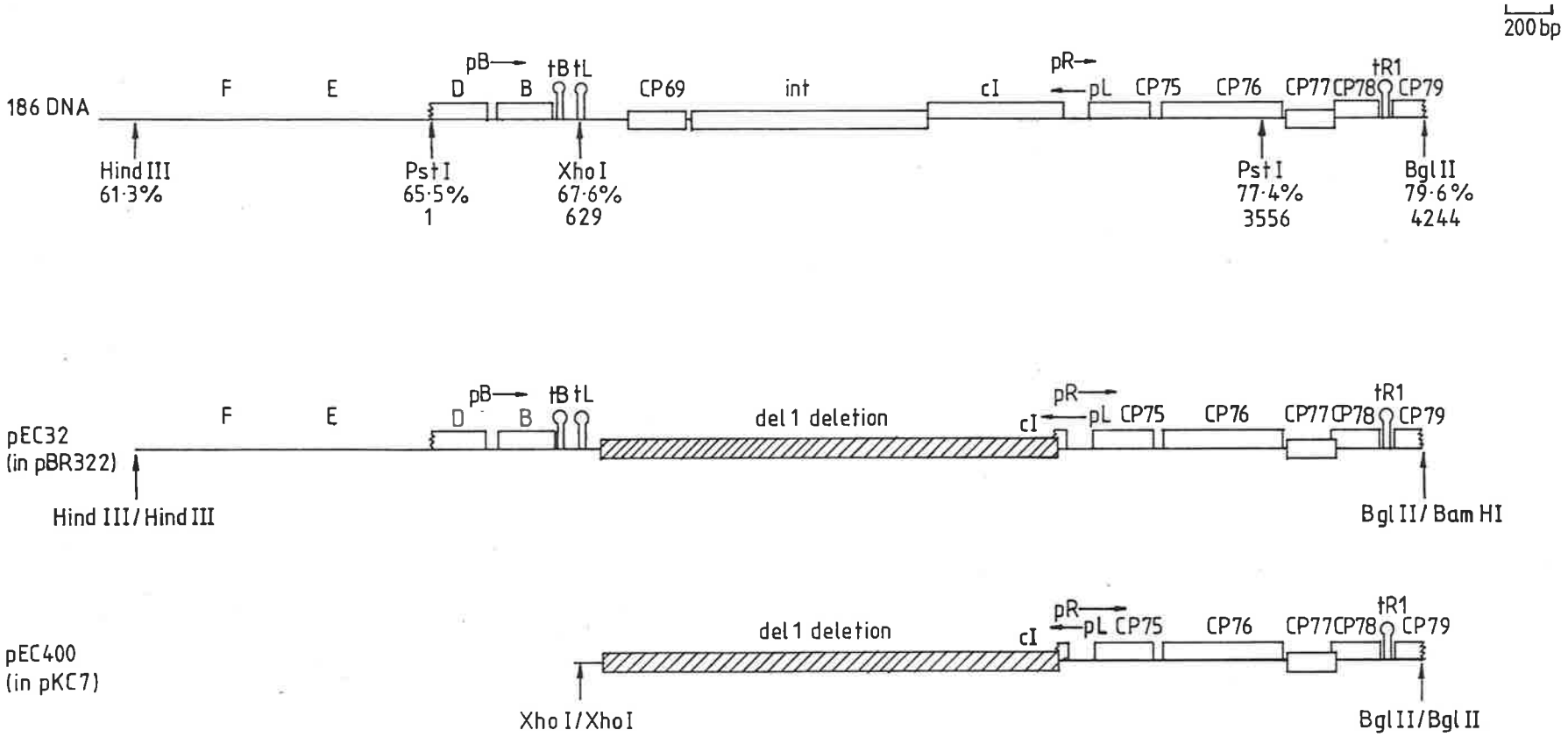
To obtain a clone of the early lytic region, the XhoI-BglII (67.6%-79.6%) fragment from the phage 186 del1 (5) (which contains a deletion removing int and part of cI; Figure 5.2) was ligated into the plasmid-vector, pKC7, at the unique XhoI and BglII sites. It was necessary to obtain this clone from 186 del1, since clones in pBR322-derived vectors containing the 186 immunity region are highly unstable (even in a lysogen) presumably as a result of the expression of the 186 int gene (Finnegan, 1979). To obtain this clone, the ligated DNA was transformed into a 186 cI⁺ lysogenic strain (E699) to prevent the expression of the lethal

Figure 5.2 Gene content of pEC32 and pEC400.

A diagrammatic representation of the gene content of the HindIII-BglII (61.3%-79.6%) region from 186. Genes, which have been sequenced, are represented by the boxed regions and promoters are represented by the arrows. Relevant restriction sites are shown. The sequence coordinates of the restriction sites refer to the first base of the site on the l-strand.

The gene content of pEC32 and pEC400 are shown. The construction of pEC32 is described by Finnegan (1979). The construction of pEC400 is described in Chapter 2.3.2. The restriction sites used to construct these plasmid-clones are indicated. Genes that are only partially represented on the plasmid-clones are indicated by the jagged-edged boxes.

These plasmid-clones contain the del1 deletion (67.9%-74.0%), which is represented by the shaded boxes.


200 bp
186 DNA
F
E
pB
D
B
tB tL
CP69
int
cI
pR
pL
CP75
CP76
CP77 CP78
tR1
CP79
Hind III
61·3%
Pst I
65·5%
1
Xho I
67·6%
629
Pst I
77·4%
3556
Bgl II
79·6%
4244
pEC32
(in pBR322)
del 1 deletion
Hind III / Hind III
Bgl II / Bam HI
pEC400
(in pKC7)
Xho I / Xho I
Bgl II / Bgl II

gene. The resulting clone was named pEC400 (Chapter 2.3.2). The location of the fragment used to construct pEC400 is shown in Figure 5.2.

Consistent with the prediction of Finnegan (1979), pEC400 was shown to contain a lethal gene, as it was unable to transform a non-lysogenic recipient [<10 transformants/ug DNA for the non-lysogenic recipient (E536) compared with $7x10^5$ transformants/ug for the lysogenic recipient (E699)].

To provide evidence that the early lytic region encodes the dhr gene, the following experiment was carried out. pEC400 was used to transform a Su^- 186 cItspAam11 (8) lysogen at 30°C (a temperature at which the expression of dhr is repressed by the cI repressor). This strain was then heat-induced and the effect of the expression of the cloned genes on E. coli DNA replication was determined by using pulse-labelling (Chapter 2.18.1). As shown in Figure 5.3, the expression of early lytic genes from pEC400 results in a more enhanced and prolonged Dhr Effect (Chapter 5.2.1) than observed in the same strain carrying the parent plasmid pKC7 instead of pEC400. This is presumably due to a gene dosage effect and suggests that the dhr gene is encoded in pEC400.

To isolate pEC400 Let^- (non-lethal) mutants, nitrosoguanidine mutagenesis (Chapter 2.20.2) was used. Mutagenized pEC400 DNA was used to transform a non-lysogen (E536), which gave rise to rare transformants among the recipient cells, indicative of potential mutations in the lethal gene. Two of these transformants, Let1 and Let2, were purified through two single colony isolations and were shown to carry plasmids (pEC401 and pEC402, respectively). These plasmids were confirmed to contain the XhoI-BglII fragment by restriction analysis (Chapter 2.28), and to display a non-lethal phenotype as judged by their ability to transform both non-lysogenic and lysogenic cells at equal efficiency ($7x10^5$ transformants/ug DNA).

The next step was to test the Let^- mutants for the Dhr^- phenotype. The Dhr phenotype of the Let1 mutant was initially investigated. The XhoI-BglII (1.78 kb) fragment was recovered from pEC401 (Let1) and ligated into

Figure 5.3 The rate of DNA replication after heat-induction of a 186 lysogen carrying pEC400.

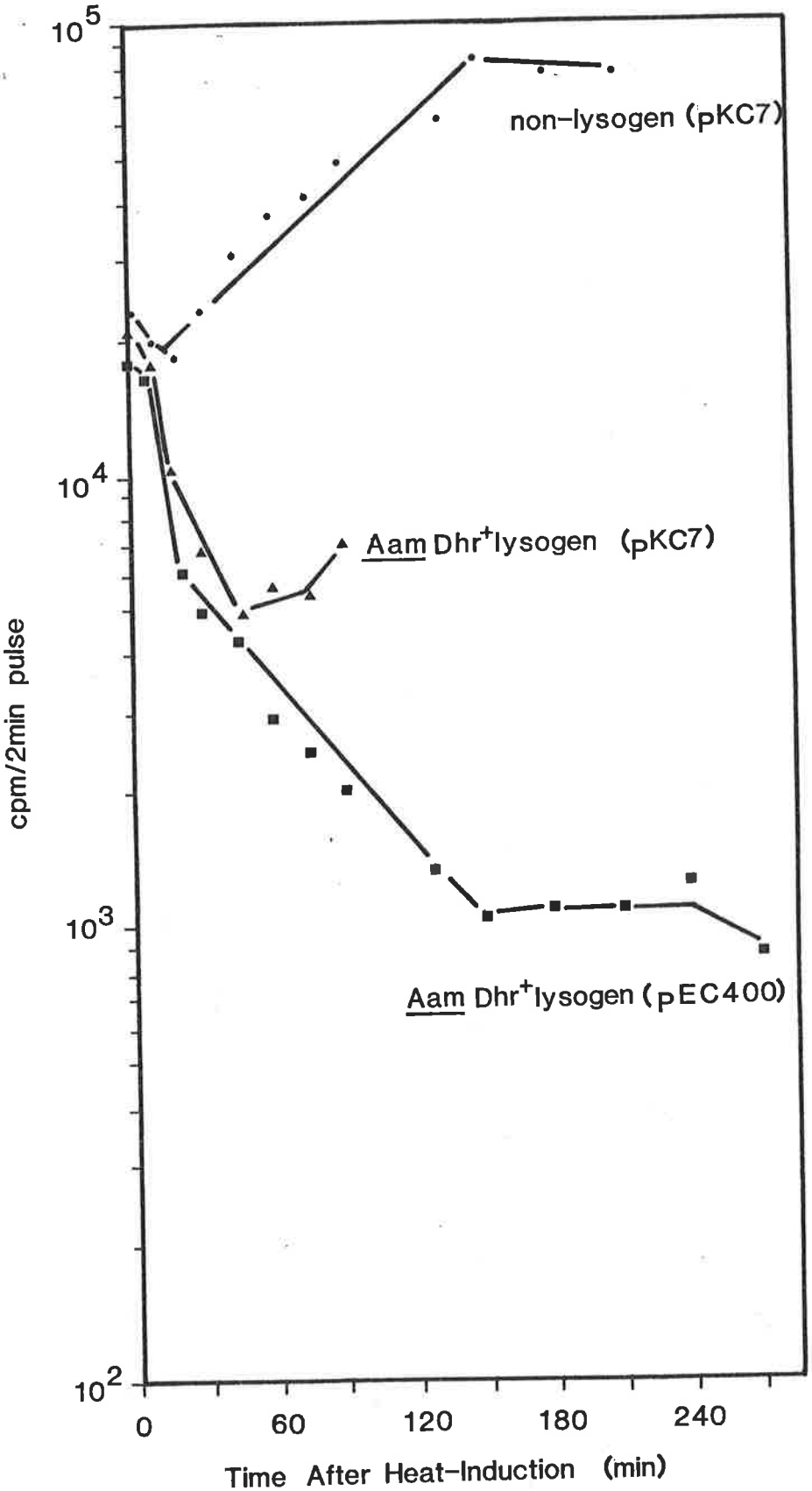
Cultures of E2268 [Su^- (186 cItspAam11 (8)) (pEC400)], E2269 [Su^- (186 cItspAam11 (8)) (pKC7)] and E2270 (Su^- (pKC7)], grown overnight at 30°C in TPG-CAA medium (containing the appropriate antibiotics), were diluted into the same broth and incubated with aeration at 30°C to A_{600}= 0.2 ($2x10^8$ cfu/ml). Cultures were transferred to 40°C at 0 min and incubation with aeration continued. Samples (200 ul) were taken at the indicated times and the rate of DNA replication determined by pulse-labelling with ^{3}H-thymidine, as described in Chapter 2.18.1.

Symbols : ■ = E2268 [E536 (186 cItspAam11 (8)) (pEC400)]

▲ = E2269 [E536 (186 cItspAam11 (8)) (pKC7)]

● = E2270 [E536 (pKC7)]

Bacterial and bacteriophage strains are described in Chapters 2.1 and 2.2.1. Plasmid and plasmid-clones are described in Chapter 2.3.


10^5
10^4
10^3
10^2
cpm/2min pulse
non-lysogen (pKC7)
Aam Dhr+lysogen (pKC7)
Aam Dhr+lysogen (pEC400)
0
60
120
180
240
Time After Heat-Induction (min)

186 cItspAam11 (8) DNA at the unique XhoI and BglII sites to yield the non-lethal (Let$^-$) recombinant phage, 186 del1Aam11Let1 (11) (Chapter 2.32). As a control, the same restriction fragment from pEC400 was ligated into XhoI/BglII digested 186 cItspAam11 (8) DNA to give the appropriate Let$^+$ phage (186 del1Aam11Let$^+$ (12)). These phage were tested for their ability to display the Dhr Effect (Chapter 2.18.1, 5.2.1). The results shown in Figure 5.4, reveal that the rate of DNA synthesis in the Let$^+$ infected culture, compared with that of the uninfected culture, dropped soon after infection and remained constant until 45 min (the Dhr Effect), after which it increased at a rate that paralleled that of the uninfected culture. In contrast, the rate of DNA synthesis after infection by the Let1 phage increased at a rate that essentially followed that of the uninfected culture (i.e. showed no Dhr Effect). It was therefore concluded that the Let1 mutant was indeed a Dhr$^-$ mutant. This result supports the prediction of Finnegan (1979) that the let gene is the dhr gene, although, further genetic analysis is necessary to confirm this assignment. For convenience the Let$^-$ mutants will henceforth be referred to as Dhr$^-$ mutants.

Having determined that pEC401 contains a mutation in the dhr gene it was then important to determine the approximate location of this gene. To determine whether the dhr gene mapped to the left or the right of the PstI (77.4%) site, the PstI-BglII (77.4%-79.6%) fragment was recovered from pEC401 (Dhr1) and pEC402 (Dhr2), recombined in vitro with the XhoI-PstI (67.6%-77.4%) fragment from a Dhr$^+$ phage (186 cItsp (1)), and the XhoI-BglII (67.6%-79.6%) fragment formed was ligated into 186 Aam11cItsp (8) DNA by these unique restriction sites (Chapter 2.32) to give the phage 186 cItspAam11Dhr1 (13) and 186 cItspAam11Dhr2 (14), respectively. The resulting recombinant phage did not display the Dhr Effect as shown by pulse-labelling studies after heat-induction of the corresponding lysogens (Figure 5.5). These results show that the dhr gene maps between the PstI (77.4%) and BglII (79.6%) sites on the 186 genome, and suggest that

Figure 5.4 The rate of DNA replication after infection of E251 with 186 AamLet$^+$ or 186AamLet1.

A culture of E251 (Su^-), which was grown overnight at 39°C in TPG-CAA medium, was diluted into the same broth and incubated with aeration at 39°C to A_{600}= 0.2 ($2x10^8$ cfu/ml). Cultures were infected with phage [186 del1 Aam11Let$^+$ (12) or 186 del1Aam11Let1 (11)] at a moa of 20 (as described in Chapter 2.18.1) at 0 min. Samples (200 ul) were taken at the indicated times and the rate of DNA replication was determined by pulse-labelling with ^{3}H-thymidine, as described in Chapter 2.18.1.

Symbols : ■ = E251 infected with 186 del1Aam11Let$^+$ (12)

△ = E251 infected with 186 del1Aam11Let1 (11)

• = E251 uninfected.

Bacterial and bacteriophage strains are described in Chapters 2.1 and 2.2.1.

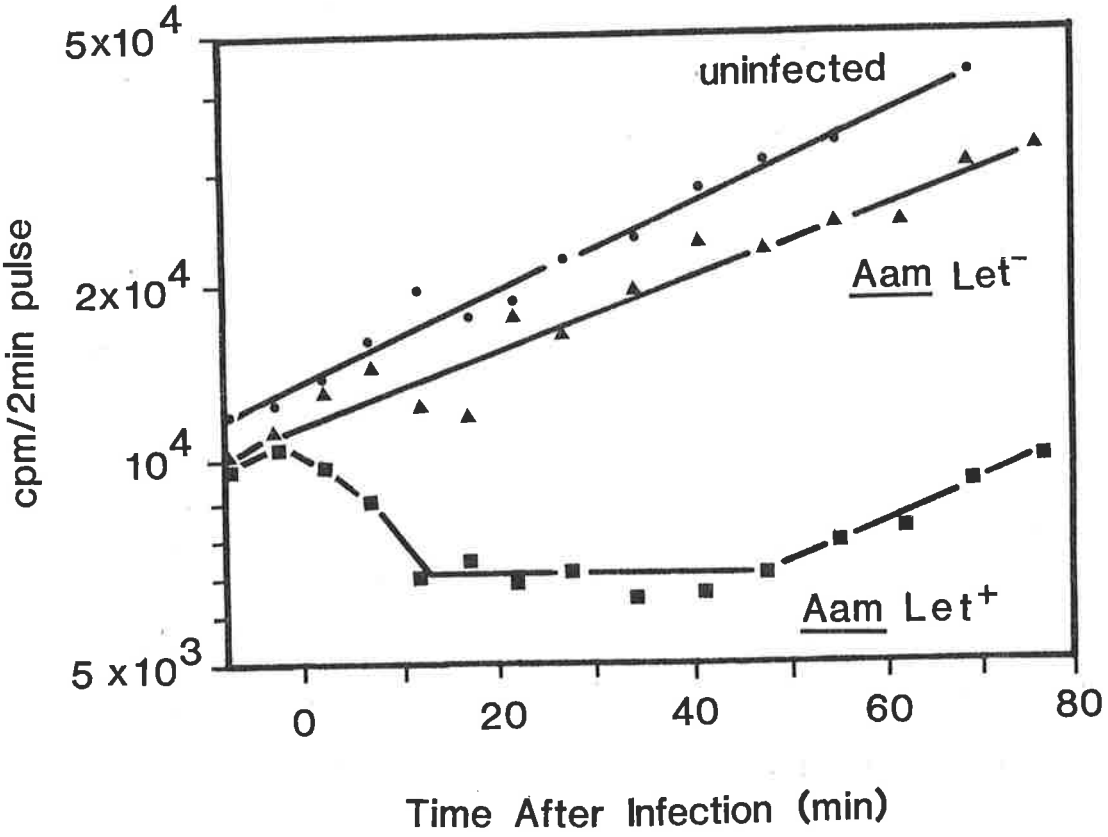

5x10^4
2x10^4
10^4
5 x10^3
cpm/2min pulse
uninfected
Aam Let⁻
Aam Let⁺
0
20
40
60
80
Time After Infection (min)

Figure 5.5 The rate of DNA replication after the heat-induction of 186 cItspDhr1 and 186 cItspDhr2 lysogens.

Cultures of E4068 [Su^- (186 cItspAam11Dhr1 (13))], E4125 [Su^- (186 cItspAam11Dhr2 (14))], E788 [Su^- (186 cItspAam11 (8))], and E251 [Su^-], which were grown overnight at 30°C in TPG-CAA medium, were diluted into the same broth and incubated with aeration at 30°C to A_{600}= 0.2 ($2x10^8$ cfu/ml). Cultures were transferred to 40°C at 0 min and incubation with aeration was continued. Samples (200 ul) were taken at the indicated times and the rate of DNA replication was determined by pulse-labelling with ^{3}H-thymidine, as described in Chapter 2.18.1.

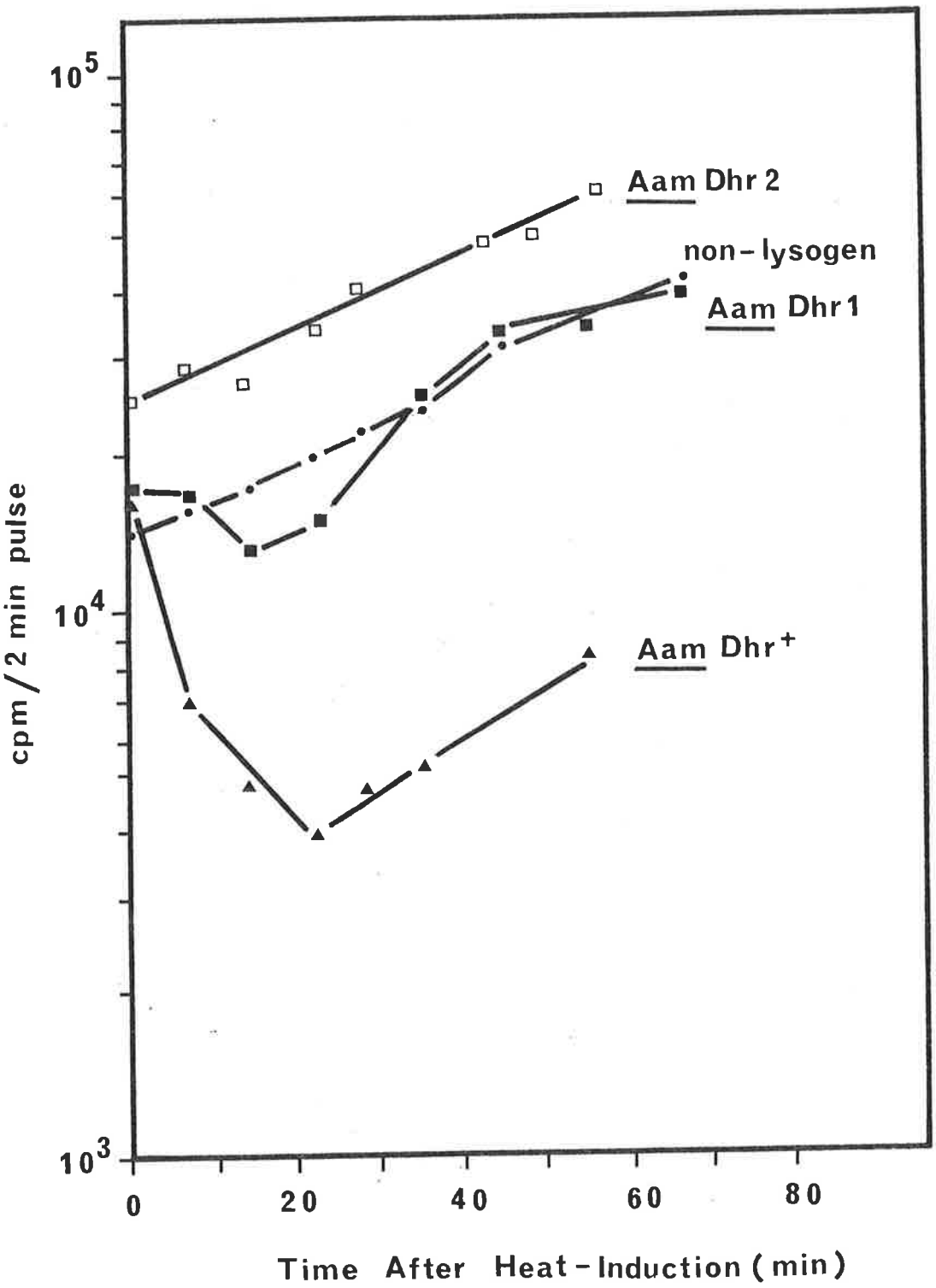
Symbols : ■ = E4068 [E251 (186 cItspAam11 Dhr1 (13))]

□ = E4125 [E251 (186 cItspAam11 Dhr2 (14))]

▲ = E788 [E251 (186 cItspAam11 (8))]

• = E251 (non-lysogen)

Bacterial and bacteriophage strains are described in Chapters 2.1. and 2.2.1.


10^5
10^4
10^3
cpm / 2 min pulse
Aam Dhr 2
non-lysogen
Aam Dhr 1
Aam Dhr$^+$
0
20
40
60
80
Time After Heat-Induction (min)

dhr is either an early lytic gene (CP77 or CP78) or the first middle gene (CP79).

5.2.3 The Effect of the Dhr⁻ Mutations on the Phage.

The isolation of viable 186 Dhr⁻ phage suggested that the dhr gene is unlikely to be an essential gene. However, it was noticed that both Dhr⁻ phage (Dhr1 and Dhr2) gave rise to smaller plaques than the Dhr⁺ control, particularly at higher temperatures (> 37°C). Infection parameters (in the strain E508) were determined for a Dhr1 phage, (186 delldhr1 (9); which was constructed, as described in Chapter 2.2). This phage showed a reduced burst size (70%), and an extended latent period and rise time (both by 5 min) compared with the Dhr⁺ control [186 dellXB (10), constructed as described in Chapter 2.2.1] (data not shown). Furthermore, the burst size of the Dhr2 phage was determined by heat-induction of a Su⁺ 186 cItspAam11Dhr2 (14) lysogen (E4124). The Dhr2 phage also showed a reduced burst size (51%) compared with the control 186 cItspAam11 (8) (strain E1011) (data not shown).

These results suggest that the Dhr function, although not essential to 186, is advantageous to the phage. For this reason it was of interest to further characterize the effect of Dhr on the host, with the purpose of gaining a better understanding of the function of Dhr in 186 development.

5.2.4 The Effect of Dhr on the Host.

5.2.4(a) The Effect of Dhr on E. coli DNA Replication.

Pulse-labelling studies have shown that Dhr results in a depression of E. coli DNA replication (Chapter 5.2.1, 5.2.2). However, the Dhr Effect (as revealed by pulse-labelling studies) may not be a true depression of host DNA replication, but rather reflect some restriction in entry of the labelled thymidine into the intracellular pool. Such a restriction in entry

of labelled thymidine into the cell has been reported to occur upon the expression of another phage function, the λ Hin^+ function (Court et al., 1980b). To test this possibility, pre-labelling studies (Chapter 2.18.2) were pursued. With pre-labelling, the radioactive thymine is added several generations before infection so that the host DNA and the precursor pool will effectively maintain a constant specific activity regardless of perturbations to the cell. After pre-labelling thy cells with ^{3}H-thymine for three generations, the cultures were infected with the Dhr^+ phage (186 delAam11 (12)) or the Dhr^- phage (186 delAam11Dhr1 (11)) and DNA synthesis was monitored (Chapter 2.18.2). Figure 5.6(a) shows that the rate of DNA synthesis in Dhr^- infected cells was approximately the same as the uninfected control whereas the rate in Dhr^+ infected cells was reduced. This result is consistent with the suggestion that Dhr interferes with E. coli DNA synthesis.

In the experiment described above, the effect of Dhr on host DNA replication could only be examined for 45 min after infection since after this time DNA replication in non-infected cells [which presumably resulted from segregation away from the replication-defective 186 template (Chapter 5.2.1)], began to contribute to TCA-precipitable counts (Chapter 2.18.2; data not shown). For this reason an Int^- phage (186 cItspins3Aam11 (15)) that could not excise from the bacterial chromosome after heat-induction, was constructed (as described in Chapter 2.2.1). A Thy^- lysogen of this phage was constructed, as described in Chapter 2.15.1, and the effect of the expression of 186 genes on host DNA replication was analysed by pre-labelling studies (Chapter 2.18.2).

The results presented in Figure 5.6(b), show that the rate of E. coli DNA replication in the presence of Dhr was substantially depressed compared with the non-lysogenic control and that this depression was maintained for the duration of the experiment (300 min). These results can not be

Figure 5.6 DNA replication, measured by pre-labelling studies, after infection with 186 or heat-induction of a 186 cItspInt$^-$ Aam11Dhr$^+$ lysogen.

Figure 5.6(a) Infection of cells with 186 Dhr$^+$ or 186 Dhr$^-$.

A culture of E777 [Su$^-$ thyA thyR], which was grown overnight at 39^{o}C in TPG-CAA + thymine (2 ug/ml), was diluted (400-fold) into the same medium containing ^{3}H-thymine (4 uCi/ml). The cultures were grown at 39^{o}C to A_{600}= 0.2 (2x10^8 cfu/ml), and then (at 0 min) infected with phage [186 dellAam11Dhr$^+$ (12) or 186 dellAam11Dhr1 (11)] at a moa of 20 (as described in Chapter 2.18.1). Samples (100 ul) were taken at the indicated times and the incorporation of ^{3}H-thymine into DNA was determined, as described in Chapter 2.18.2.

Symbols : ▲ = E777 infected with 186 dellAam11Dhr$^+$ (12)

■ = E777 infected with 186 dellAam11Dhr1 (11)

● = E777 uninfected.

Bacterial and bacteriophage strains are described in Chapters 2.1. and 2.2.1.

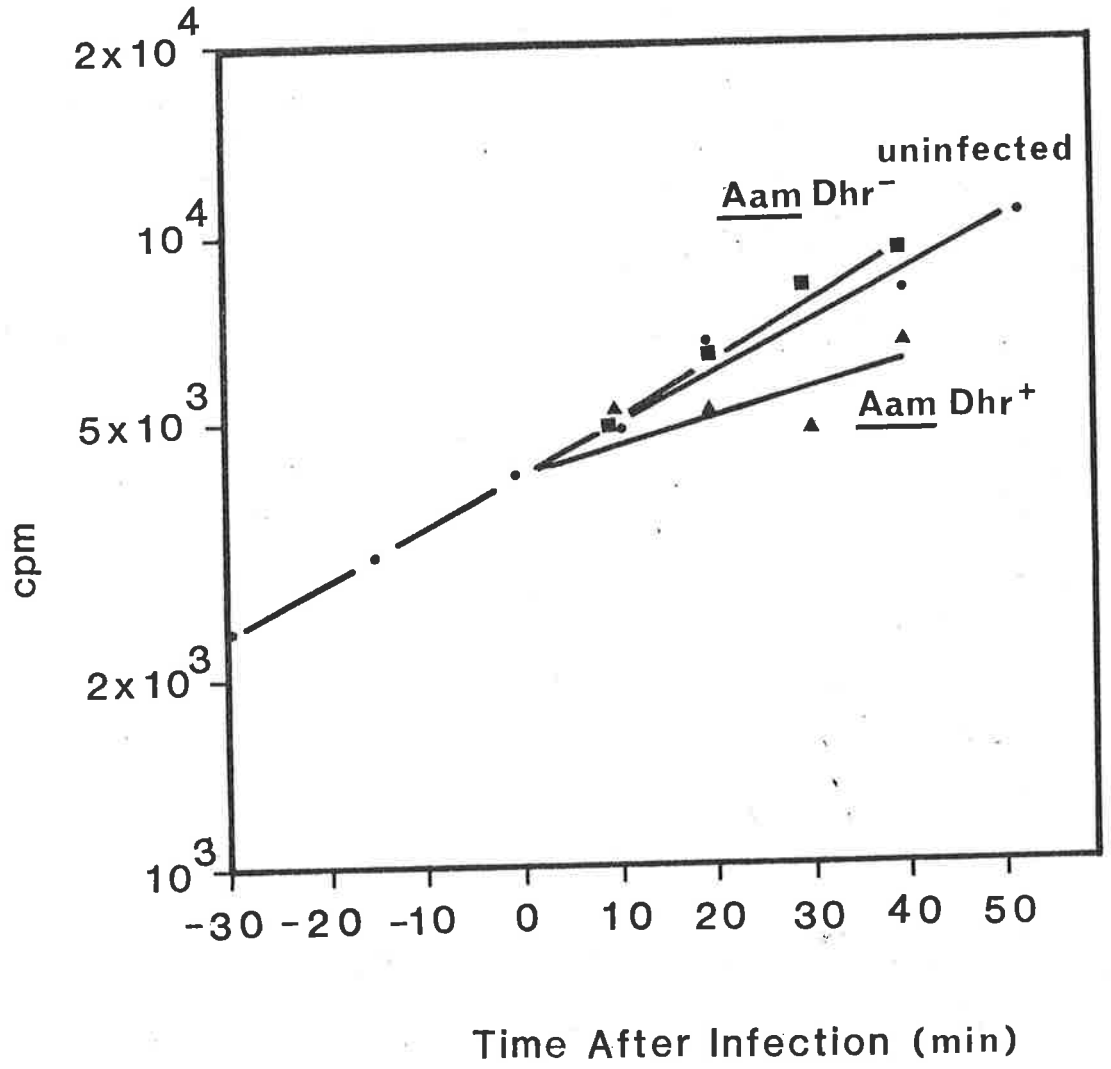

2x10^4
10^4
5x10^3
2x10^3
10^3
cpm
uninfected
Aam Dhr⁻
Aam Dhr⁺
-30 -20 -10 0 10 20 30 40 50
Time After Infection (min)

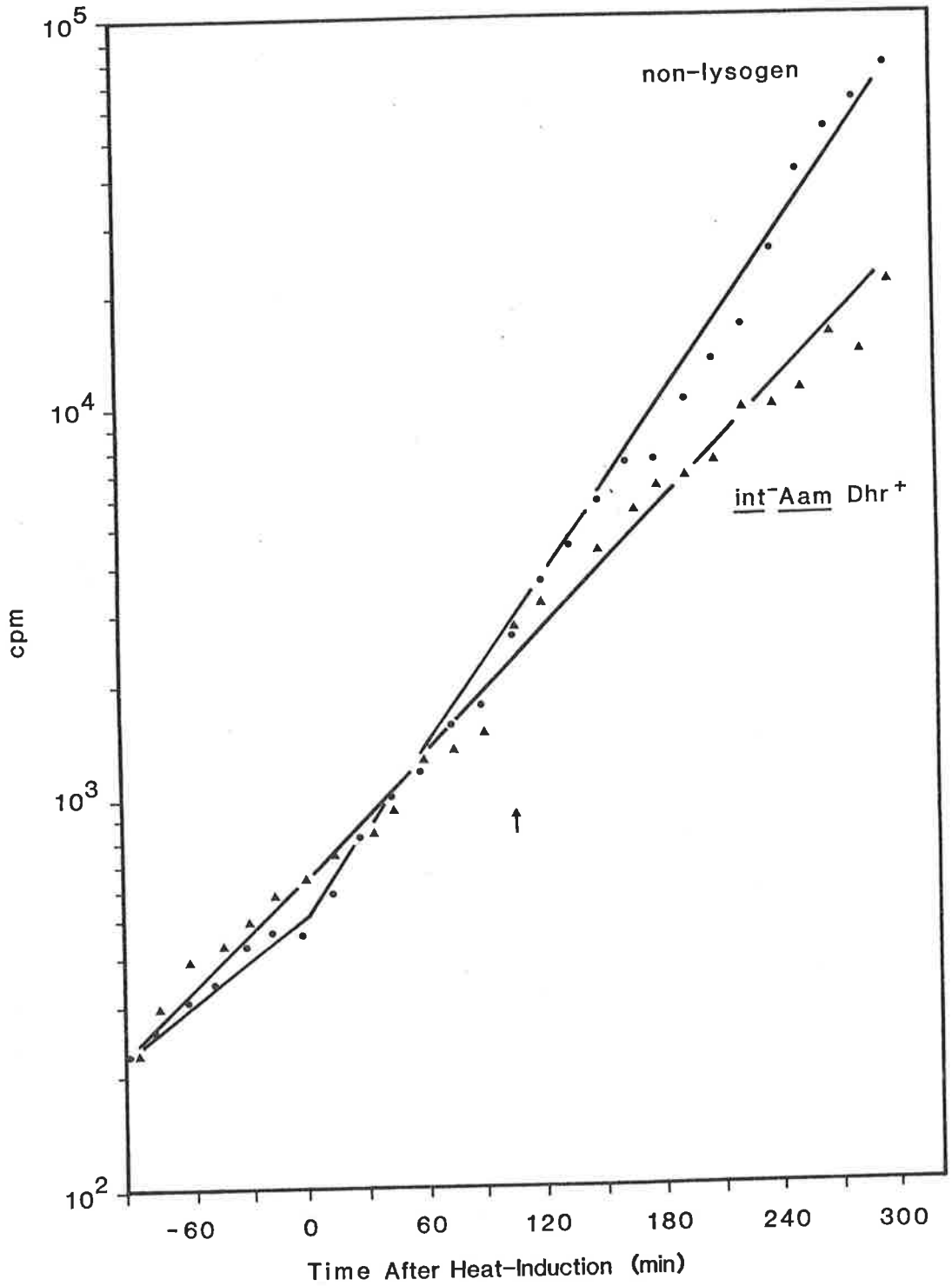
Figure 5.6(b) Heat-induction of a 186 cItspAam11Int$^-$Dhr$^+$ lysogen.

Cultures of E4066 [Su$^-$ thyA thyR (186 cItspAam11ins3 (15))] and E777 [Su$^-$ thyA thyR], which were grown overnight at 30°C in TPG-CAA + thymine (2 ug/ml), were diluted into the same medium containing ^{3}H-thymine (4 uCi/ml) and incubated with aeration at 30°C to A_{600}= 0.2 (2x10^8 cfu/ml). Cultures were transferred to 40°C at 0 min and incubation with aeration was continued. Samples (100 ul) were taken at the indicated times and the incorporation of ^{3}H-thymine into DNA was determined, as described in Chapter 2.18.2. At 105 min (indicated by the arrow) after heat-induction the cultures were diluted ten-fold into pre-warmed medium and incubation continued. Samples were taken from this diluted culture from 105 min to 300 min after heat-induction.

Symbols : ▲= E4066 [E251 thyA thyR (186 cItspAam11ins3 (15))]

● = E777 [E251 thyA thyR]

Bacterial and bacteriophage strains are described in Chapters 2.1 and 2.2.1.


10^5
10^4
10^3
10^2
cpm
non-lysogen
int⁻Aam Dhr⁺
-60
0
60
120
180
240
300
Time After Heat-Induction (min)

explained by variations in the precursor pool composition and lead to the conclusion that Dhr acts to depress host DNA replication.

5.2.4(b) The Effect of Dhr on Cell Viability.

The effect of Dhr expression on cell viability was initially investigated using a heat-induced 186 cItspInt$^-$A$^-$Dhr$^+$ (15) lysogen and determining the number of viable cells with time. Figure 5.7(a) shows that for at least 180 min after heat-induction the optical density of the culture increased with time at only a slightly reduced rate compared with the non-lysogenic control. However, the colony forming ability showed an immediate reduction in the rate of increase, but without evidence of cell death (Figure 5.7b). However, cell death was apparent after overnight incubation of the 186 cItspInt$^-$A$^-$Dhr$^+$ lysogen on YGC plates at 41.5°C. This lysogen gave a survival of 1% (compared with the viable count at 30°C), whereas the control (a 186 cItspInt$^-$A$^-$Dhr1 (17) lysogen; constructed as described in Chapter 2.2.1, 2.1, 2.15.1) showed 100% survival (Table 5.1).

The effect of excess Dhr on cell viability was investigated by heat-inducing a Su$^-$ lysogen of 186 cItspAam11Dhr$^+$ (8) carrying the Dhr$^+$ plasmid pEC400, and determining the number of viable cells with time. The viable count initially increased with time, but at a reduced rate compared with the controls (the Su$^-$ lysogen carrying pKC7, and the Su$^-$ non-lysogen carrying pKC7) (Figure 5.8a). However, after 30 min the viable count decreased rapidly and by 240 min was reduced to 0.03% of the number of viable cells present at time zero. In contrast, the optical density of the culture continued to increase at the same rate as the control cultures, at least until 180 min after heat-induction when it decreased slightly, suggesting the possibility of cell lysis (Figure 5.8b).

These results have demonstrated that the onset of death from the expression of dhr from pEC400 is at ~30 min after heat-induction. Since

Figure 5.7 Relative cell mass and viable count after heat-induction of a 186 cItspAam11Int$^-$Dhr$^+$ lysogen.

Figure 5.7(a) Relative cell mass after heat-induction of a 186 cItsp Aam11Int$^-$Dhr$^+$ lysogen.

Cultures of E4067 [Su$^-$ (186 cItspAam11ins3 (15))] and E251 [Su$^-$], which were grown overnight at 30°C in L broth, were diluted into the same medium and grown with aeration at 30°C to A_{600}= 0.8 ($2x10^8$ cfu/ml). Cultures were transferred to 41.5°C at 0 min and incubation was continued. At 60 min after heat-induction the cultures were diluted one hundred-fold into pre-warmed L broth and incubation was continued. Samples were taken from the diluted culture from the time indicated by the arrow.

The optical density (at 600 nm) was followed for 180 min before heat-induction and for 360 min afterwards at the times indicated.

Symbols : ▲= E4067 [E251 (186 cItspAam11ins3 (15))]

● = E251 (non-lysogen).

Bacterial and bacteriophage strains are described in Chapters 2.1 and 2.2.1.

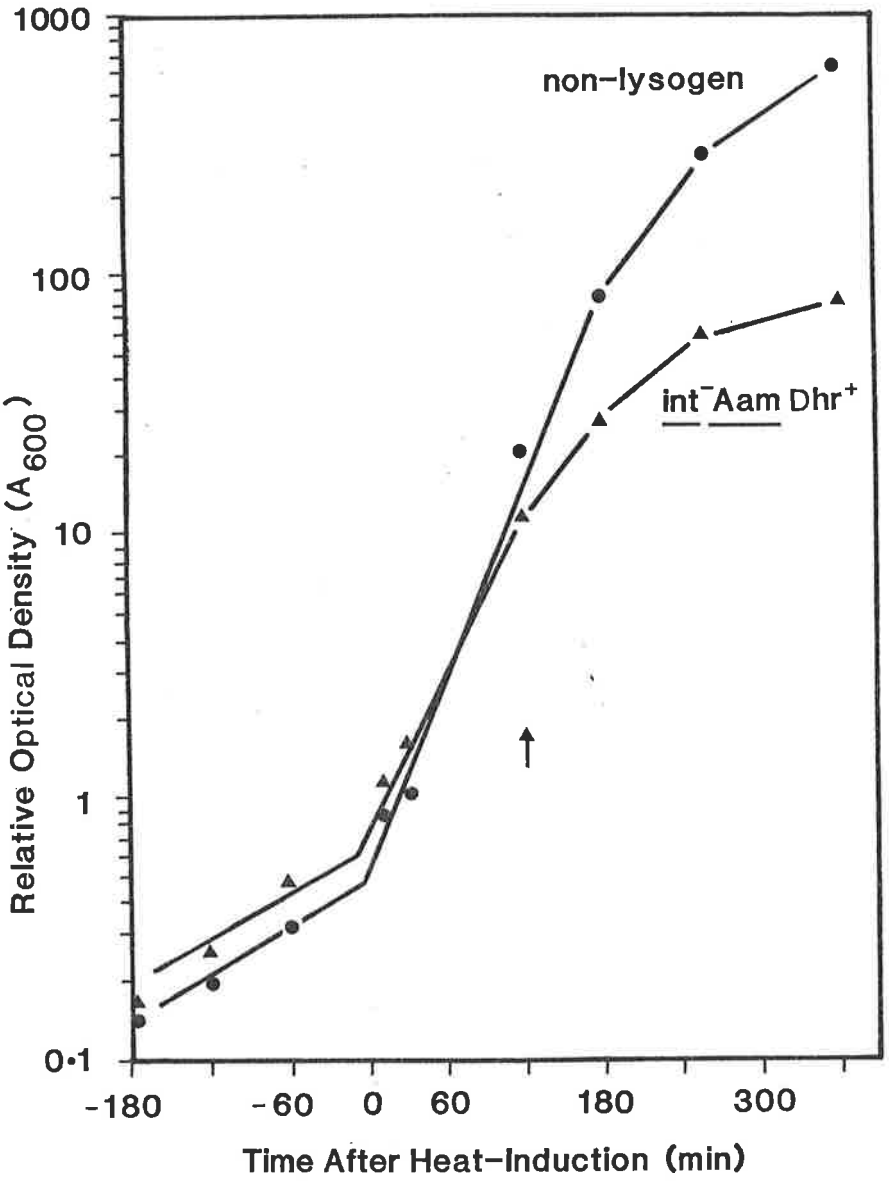

1000
100
10
1
0·1
Relative Optical Density (A_{600})
non-lysogen
int⁻Aam Dhr⁺
-180
-60
0
60
180
300
Time After Heat-Induction (min)

Figure 5.7(b) Relative viable count after heat-induction of a 186 cItsp Aam11Int^{-} Dhr^{+} lysogen.

Growth conditions were as described in Figure 5.7(a). The viable counts (as cfu/ml) of the cultures were determined at the times indicated (as described in Chapter 2.16.2).

Symbols : ▲= E4067 [E251 (186 cItspAam11ins3 (15))]

● = E251 (non-lysogen).

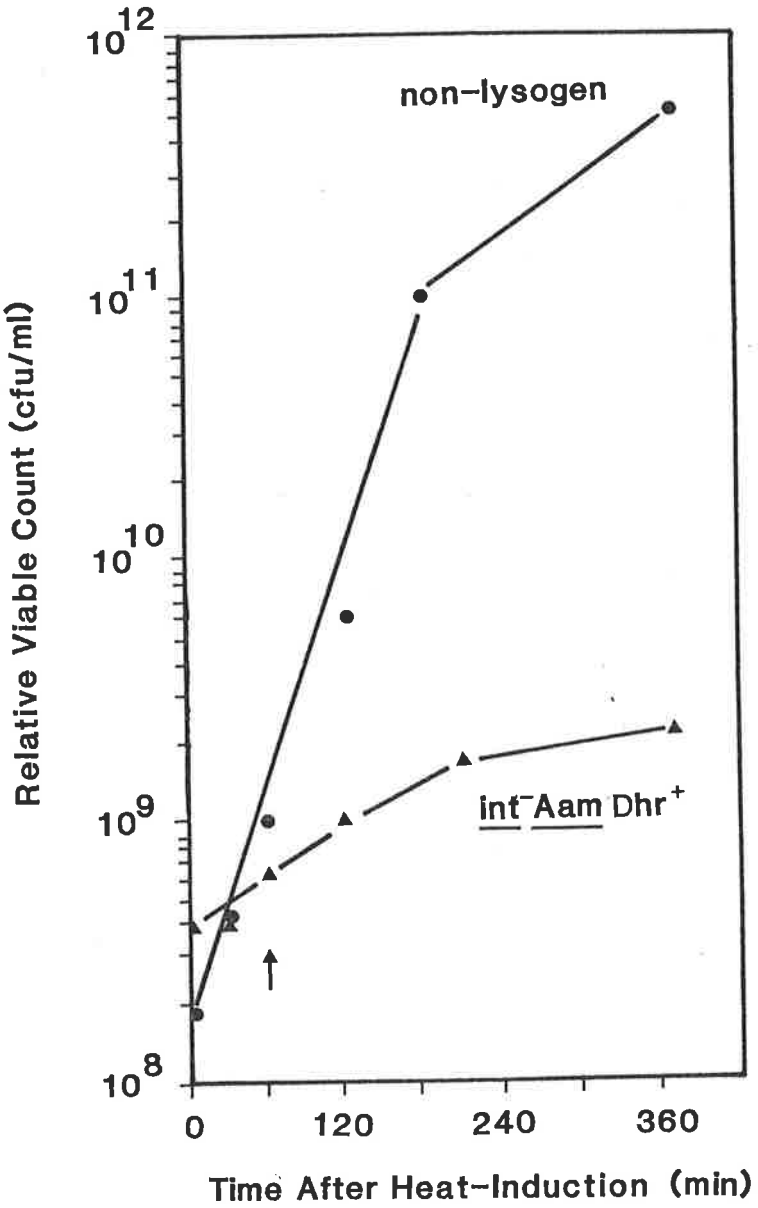

Relative Viable Count (cfu/ml)
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
non-lysogen
int⁻ Aam Dhr⁺
0
120
240
360
Time After Heat-Induction (min)

Figure 5.8 Relative viable count and cell mass after heat-induction of a 186 lysogen carrying pEC400.

Figure 5.8(a) Relative viable count after heat-induction of a 186 lysogen carrying pEC400.

Cultures of E2268 [Su^- (186 cItspAam11 (8)) (pEC400)], E2269 [Su^- (186 cItspAam11 (8)) (pKC7)] and E2270 [Su^- (pKC7)], which were grown overnight at 30°C in L broth (containing the appropriate antibiotics), were diluted into the same medium and grown with aeration at 30°C to A_{600}= 0.8 ($2x10^8$ cfu/ml). Cultures were transferred to 41.5°C at 0 min and incubation was continued. At 60 min after heat-induction the cultures were diluted one hundred-fold into pre-warmed L broth and incubation was continued. Samples were taken from the diluted culture from the time indicated by the arrow.

The viable count (as cfu/ml) of the cultures were determined at the times indicated (as described in Chapter 2.16.2).

Symbols : ■ = E2268 [E536 (186 cItspAam11 (8)) (pEC400)]

• = E2269 [E536 (186 cItspAam11 (8)) (pKC7)]

▲ = E2270 [E536 (pKC7)]

Bacterial and bacteriophage strains are described in Chapters 2.1 and 2.2.1. Plasmids and plasmid-clones are described in Chapter 2.3.

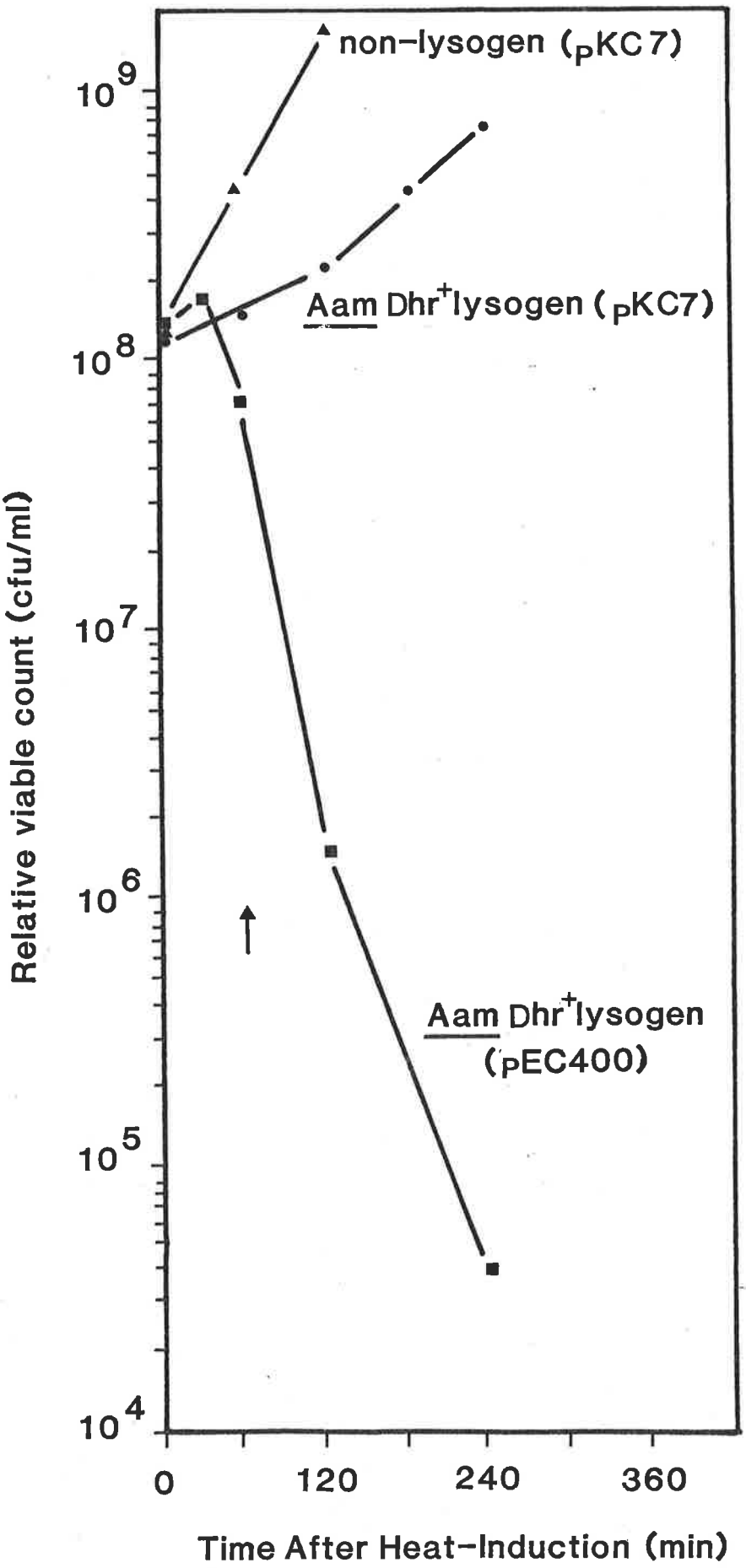

non-lysogen (pKC7)
Aam Dhr+lysogen (pKC7)
Aam Dhr+lysogen
(pEC400)
Relative viable count (cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
0
120
240
360
Time After Heat-Induction (min)

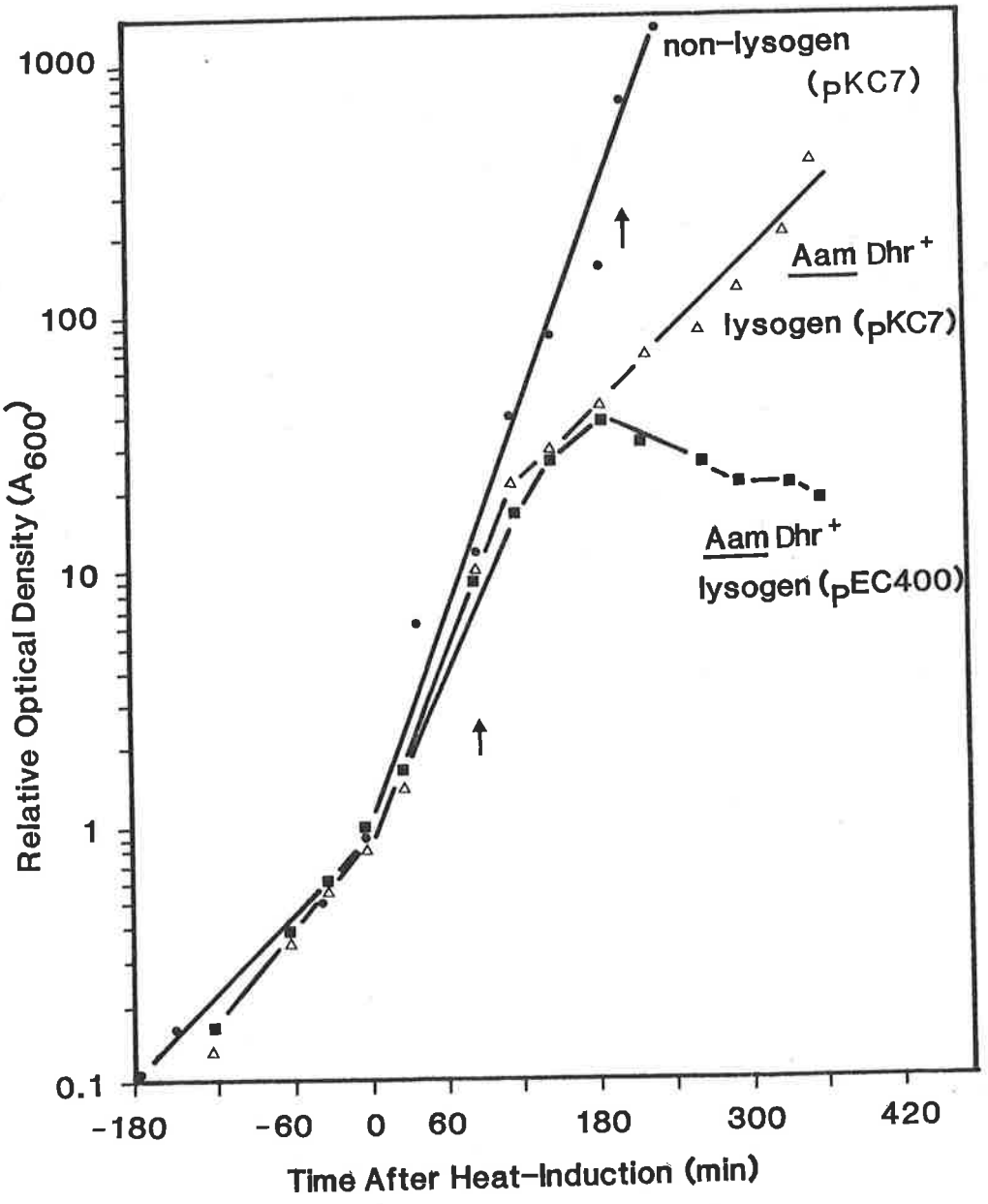
Figure 5.8(b) Relative cell mass after heat-induction of a 186 lysogen carrying pEC400.

Growth conditions were as described in Figure 5.8(a). The optical density (at 600 nm) was followed for 180 min before heat-induction and for 360 min afterwards, at the times indicated. Cultures were diluted one hundred-fold at 60 min after heat-induction and samples were taken from the diluted cultures from the time indicated by the first arrow. E2268 was again diluted one hundred-fold at 180 min after heat-induction and samples were taken from this diluted culture from the time indicated by the second arrow.

Symbols : ■ = E2268 [E536 (186 cItspAam11 (8)) (pEC400)]

△ = E2269 [E536 (186 cItspAam11 (8)) (pKC7)]

• = E2270 [E536 (pKC7)]


non-lysogen
(pKC7)
Aam Dhr+
lysogen (pKC7)
Aam Dhr+
lysogen (pEC400)
1000
100
10
1
0.1
-180
-60
0
60
180
300
420
Relative Optical Density (A600)
Time After Heat-Induction (min)

the Dhr Effect begins at ~5 min after heat-induction of pEC400 (Figure 5.3) it is possible that the inhibition of E. coli DNA replication by Dhr ultimately results in the observed lethality.

Excess Dhr also appeared to be the main cause of lethality when Dhr was expressed from an Int^- lysogen that could replicate. The 186 cItsp $Int^-A^+Dhr^+$ (7) lysogen (constructed as described in Chapter 2.15.1) showed a survival of 0.0001% after overnight incubation at 41.5°C (compared with the viable count of 30°C) while the 186 cItspInt^-A^+Dhr1 (16) lysogen (constructed as described in Chapter 2.2.1, 2.1, 2.15.1) gave a survival of 100% (Table 5.1).

5.2.4(c) The Effect of Dhr on Cell Division.

The results shown in Figures 5.7 and 5.8 revealed that the viable count of the culture was more sensitive to Dhr than was the optical density (i.e. the optical density increased with time while the viable count remained the same for the Int^- Dhr^+ lysogen, or decreased for cells expressing Dhr from pEC400). This suggests that cell division is inhibited, but cell growth is continuing. To determine the effect of Dhr on cell morphology the lysogen carrying pEC400 was heat-induced for 4 hours at 42°C, and the cells were viewed under a microscope using phase contrast optics. The control culture (the induced non-lysogen carrying pKC7) showed only normal cells (Figure 5.9a) and the uninduced lysogen carrying pEC400 also gave mostly normal cells with a few elongated cells (Figure 5.9b). In contrast, the induced lysogen carrying pEC400 resulted in elongated cells (filamentous cells) of 20-50x normal cell length (Figure 5.9c). As an additional control a mock heat-induction was carried out on non-lysogenic cells carrying the Dhr1 plasmid pEC401 and this gave a similar result to the uninduced strain carrying pEC400 (data not shown).

Cell filamentation was also apparent after the heat-induction of a 186 cItsp$Int^-A^-Dhr^+$ (15) lysogen (strain E4067; Chapter 2.1) for 4 hours at

TABLE 5.1

Survival of 186 Int$^-$ lysogens at 41.5°C.[a]

Strain[b]	Phenotype	Survival %[c]
E4067 [E251(186cItspins3Aam11(15))]	Int$^-$A$^-$Dhr$^+$	1%
E4069 [E251(186cItspins3Aam11Dhr1(17))]	Int$^-$A$^-$Dhr$^-$	100%
E4170 [E251(186cItspins3(7))]	Int$^-$A$^+$Dhr$^+$	0.0001%
E4070 [E251(186cItspins3Dhr1(16))]	Int$^-$A$^+$Dhr$^-$	100%

Notes to Table 5.1

a. Cultures were grown in L broth at 30°C to A_{600} = 0.8. Samples were taken and dilutions of the culture were spread on YGC plates. Plates were incubated at 30°C or 41.5°C overnight and the viable counts were determined (Chapter 2.16.2).

b. The construction of the 186 strains and the bacterial strains are described in Chapters 2.2.1 and 2.1.

c. The survival percentage refers to the viable count at 41.5°C relative to that obtained at 30°C.

42°C. This lysogen gave filaments of 2-10x greater length than normal cells (data not shown).

These results have shown that the expression of Dhr from a plasmid-clone, or from a prophage, also results in the inhibition of E. coli cell division and result in the appearance of long filamentous cells. It is pertinent to note that there were no obvious septa observed in the filamentous cells (Figure 5.9c). This suggests that the stage of cell division, which is inhibited is an early event in the cell division cycle involving the formation of septa (Slater and Schaechter, 1974; Mendelson, 1982; Donachie et al., 1984).

5.2.5 Investigation of the Mechanism of Action of Dhr.

5.2.5(a) The Role of the SOS Response in the Dhr Effect.

The results presented above provide evidence to suggest that Dhr acts to inhibit E. coli DNA replication and cell division. It was considered possible that the inhibition of cell division could be due to the induction of the SOS response (Witkin, 1976; Walker, 1984, 1985). The SOS pathway is controlled by the RecA protease and can be induced by perturbations to DNA replication (e.g. thymine starvation, UV-irradiation or growth of temperature-sensitive mutants in dnaE, dnaB or polA at the non-permissive temperature) (Witkin, 1976; Blanco and Pomes, 1977). One of the effects of the SOS response is an inhibition of cell division, which is mediated by the sfiA gene-product (Huisman and D'Ari, 1981; Mizusawa et al., 1983; Huisman et al., 1983, 1984).

To investigate the possibility that the inhibition of cell division by Dhr may be due to the expression of the sfiA gene, a sfiA::Tn5 lysogen carrying pEC400 (strain E2271) was constructed (Chapter 2.1, 2.15.3). As a control a sfiA$^-$ non-lysogen carrying pKC7 (strain E2272) was also constructed (Chapter 2.1, 2.15.3). These two strains and their sfiA$^+$

Figure 5.9 Cell morphology of the 186 lysogen carrying the Dhr$^+$ plasmid-clone, pEC400 at 42^{o}C.

Cultures of E536 carrying pKC7 (strain E2270), or the E536 (186 cItspAam11Dhr$^+$ (8)) lysogen carrying pEC400 (strain E2268), were grown in L broth at 30^{o}C to A_{600}= 0.2. The cultures were diluted 1/10 into fresh media and were grown for four hours at either 30^{o}C or 42^{o}C. Cells were photographed at 400x magnification under the microscope using phase contrast optics.

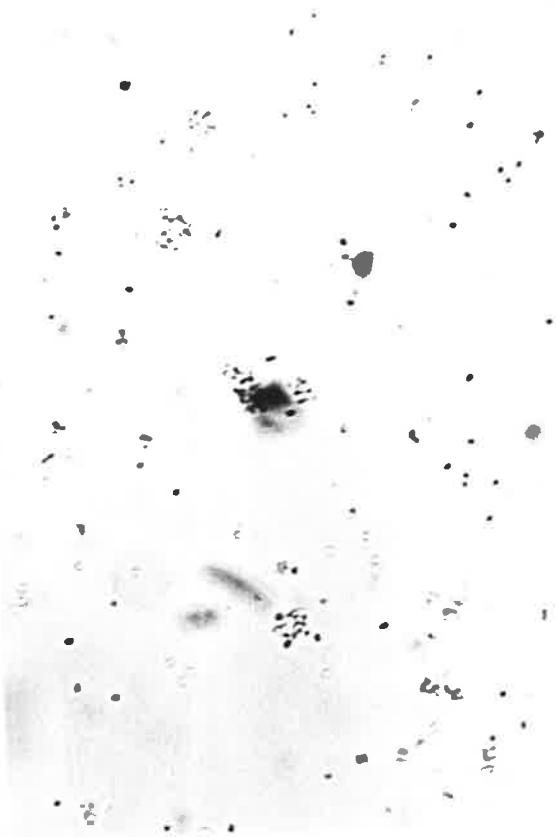
(a) E536 (pKC7) at 42^{o}C.

(b) E536 (186 cItspAam11Dhr$^+$ (8)) (pEC400) at 30^{o}C.

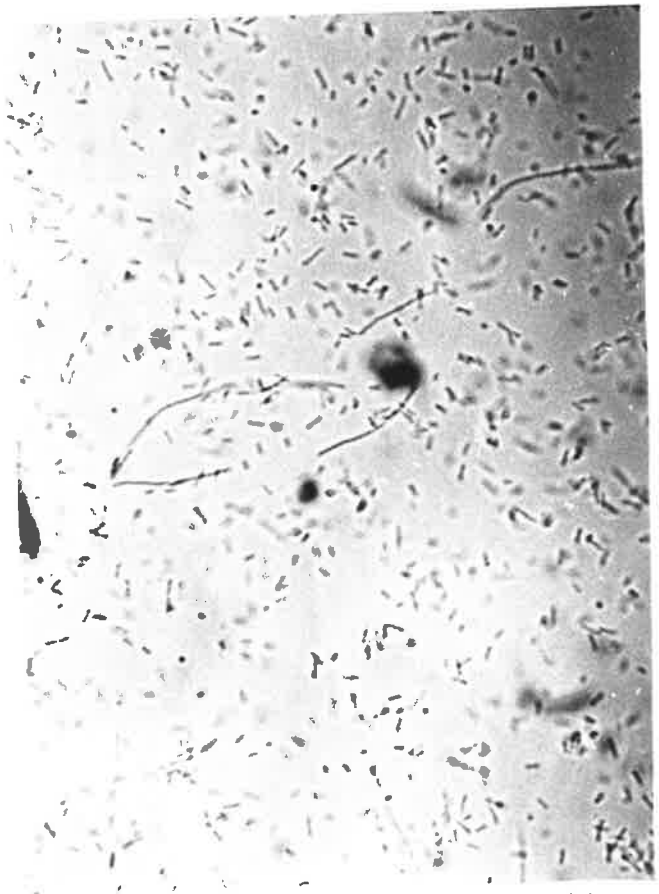
(c) E536 (186 cItspAam11Dhr$^+$ (8)) (pEC400) at 42^{o}C.

E536 (pKC7) grown for four hours at 30^{o}C gave normal cells (data not shown).

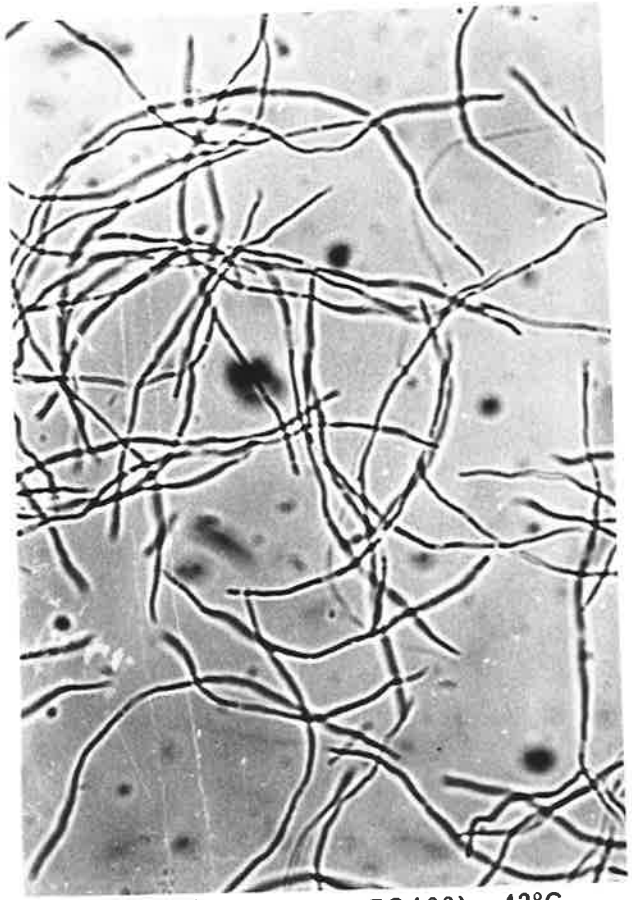
The gene content of pEC400 is shown in Figure 5.2.



(a) non-lysogen (pKC7), 42°C



(b) A^- Dhr^+ lysogen (pEC400), 30°C



(c) A^- Dhr^+ lysogen (pEC400), 42°C

equivalents (strains E2268 and E2270; Chapter 2.1) were incubated at 42°C for 4 hours and the effect of the sfiA mutation on Dhr-induced filamentation was then examined by viewing the cells under a microscope using phase contrast optics. This investigation revealed that the sfiA mutation did not noticeably reduce the filamentation caused by the expression of genes from pEC400 (data not shown).

These results suggest that the sfiA gene-product was not involved in the filamentation caused by the expression of the early lytic genes from pEC400. However, it was possible that another SOS gene was involved. sfiA-independent recA-dependent filamentation has been reported (Huisman et al., 1980; Burton and Holland, 1983; D'Ari and Huisman, 1983; Maguin et al., 1986). Therefore, to test this possibility, a recA$^-$ (recA56) lysogen carrying pEC400 (strain E2274) and as a control a recA$^-$ non-lysogen carrying pKC7 (strain E2276) were constructed (Chapter 2.1, 2.15.3). As controls, the equivalent recA$^+$ lysogen carrying pEC400 (strain E2273) and the recA$^+$ non-lysogen carrying pKC7 (strain E2275) were constructed (Chapter 2.1, 2.15.3). These strains were tested for filamentation, as described above. The presence of the recA$^-$ mutation did not reduce the filamentation caused by the expression of early lytic genes from pEC400 (data not shown).

These results have shown that filamentation caused by the expression of dhr from the plasmid-clone pEC400 is not due to the induction of the SOS response.

5.2.5(b) Isolation of Dhr-Resistant Host Mutants.

As an initial step towards understanding the mechanism by which Dhr depresses E. coli DNA replication, host mutants resistant to the lethal effect of Dhr were isolated and characterized. Such mutants were expected among the surviving colonies when the dhr gene was over-expressed from the clone, pEC400 (Figure 5.8a). When cells of the 186 cIts pAam11 (8) lysogen

carrying pEC400 (strain E2268) was spread onto YGC plates containing ampicillin, and incubated at 41.5°C overnight, surviving colonies appeared at a frequency of $3x10^{-5}$, although only 1/100 of these was truly $temp^R amp^R$. The remaining colonies would not grow when restreaked on ampicillin plates at 41.5°C. Surprisingly, none of the surviving colonies tested were found to be host mutants. These surviving colonies instead were found to be due to either of the following mutations; Let^+ to Let^- mutations on the plasmid (as indicated by the fact that the plasmid isolated from these colonies was able to transform non-lysogenic cells at a high frequency), or cItsp to cI^+ reversions on the prophage (as revealed by the fact that the surviving colonies were immune to 186 cI10 (2) infection at 41.5°C). One of the Let^- plasmid mutants (pEC403 or Dhr3) was retained for later investigation.

To increase the likelihood of isolating host mutants resistant to Dhr, nitrosoguanidine mutagenesis was used (Chapter 2.20.1). After nitrosoguanidine mutagenesis, the cell survival frequency increased to $1x10^{-4}$ and lethal-resistant (Let^R) host mutants were obtained, as indicated by the finding that the plasmids isolated from these mutants were unable to transform non-lysogenic cells (suggesting that the plasmid was still Let^+), and these mutants were sensitive to 186 cI10 (2) infection at 41.5°C (suggesting that a cItsp to cI^+ mutation on the prophage had not occurred). Some of these Let^R host mutants showed cold-sensitivity as their growth at 30°C was markedly reduced compared with that of the parent strain (E2268). One of these Let^R mutants (Let^R1) was then cured of the plasmid pEC400 (Chapter 2.21) and characterization of this mutant was carried out. This mutant was found to be sensitive to 186 cI10 at 30°C, indicating that it no longer carries the 186 cItspAam11 prophage. However, the 186 plaques formed on Let^R1 were smaller than normal. This is consistent with the smaller 186 plaques obtained with the 186 Dhr^- mutants (Chapter 5.2.3). It was also noted that the cold-sensitivity of this mutant (cured of pEC400) was reduced considerably compared with that obtained in the presence of

pEC400 (Table 5.2). This result indicates that it is the presence of pEC400 that is largely responsible for the cold-sensitivity and suggests that this mutant is only resistant to the lethal effect of pEC400 at high temperatures (i.e. is a temperature-sensitive mutation).

The Let^R1 mutant was confirmed to be truly Let-resistant by its ability to be retransformed with the plasmid pEC400 at the same efficiency as the parent plasmid ($1x10^6$ transformants/ug of DNA). The Let^R1 mutant was then tested for Dhr-resistance. A 186 cItspAam11 lysogen of this strain was heat-induced at 40^oC and the rate of E. coli DNA replication monitored by pulse-labelling (Chapter 2.18.1). The results presented in Figure 5.10 shows that the Let^R1 lysogen did not show the Dhr Effect. This confirms that the Let^R1 mutant is Dhr-resistant. The Let^R1 mutant will henceforth be referred to as the Dhr^R1 mutant.

The Dhr^R1 mutant was tested to determine whether the mutation prevented the filamentation, which occurs when early lytic genes are expressed from pEC400. Dhr^R1 carrying pEC400 or cured of the plasmid was grown at either 30^oC or 41.5^oC for 4 hours and cells were examined under the microscope using phase contrast optics (Table 5.2). At 41.5^oC, the Dhr^R1 strain carrying pEC400 and Dhr^R1 cured of the plasmid gave normal cells, whereas the control of the parent strain carrying pEC400 (Strain E2268) gave filamentous cells (data not shown). This result indicates that the Dhr^R1 mutant is resistant to pEC400-induced filamentation at 41.5^oC. At 30^oC the control strain (E2268) and Dhr^R1 gave rise to mostly normal cells, however, Dhr^R1 carrying pEC400 gave rise to long filamentous cells (10-50x greater in length than normal cells) (data not shown; Table 5.2). This result suggests that the Dhr^R1 mutant is only resistant to pEC400-induced filamentation at high temperatures. This is consistent with the

TABLE 5.2

Characterization of the Let1^R (Dbr1^R) mutant.

(a) GROWTH

Strain	Temperature 30°C	41.5°C
E2268 [E536(186 cItspAam11(8))(pEC400)]	+++	-
E4137(pEC400) [E536 Let1^R(pEC400)]	-	+++
E4137 [E536 Let1^R]	+	+++

(b) CELL MORPHOLOGY

Strain	Temperature 30°C	41.5°C
E2268 [E536(186cItspAam11(8))(pEC400)]	normal (few small filaments)	filamented (10-50x)
E4137(pEC400) [E536 Let1^R(pEC400)]	filamented (10-50x)	normal (few small filaments)
E4137 [E536 Let1^R]	normal	normal

Notes to Table 5.2

(a) E2268 was grown at 30°C, whereas E4137 and E4137(pEC400) were grown at 41.5°C in L broth (containing ampicillin if required) to an A_{600} = 0.8. A loopful of cells from each culture were streaked for single colonies on YGC plates [or YGC plates supplemented with ampicillin for E4137(pEC400)]. The plates were incubated overnight at 30°C or 41.5°C and the growth of the bacterial colonies was compared. +++ indicates good growth, + indicates some growth, and - indicates no growth or poor growth.

(b) E2268 was grown at 30°C, whereas E4137 and E4137(pEC400) were grown at 41.5°C in L broth (containing ampicillin if required) to A_{600} = 0.8. Cultures were then halved and grown for four hours at either 30°C or 41.5°C. Samples from the cultures were removed and viewed under the microscope using phase contrast optics (at 400x magnification). The morphology of the cells was recorded.

The bacterial and bacteriophage strains are described in Chapters 2.1 and 2.2.1. pEC400 is described in Chapter 2.3.2 and Figure 5.2.

Figure 5.10 The rate of DNA replication after the heat-induction of a 186 cItspDhr$^+$ lysogen of the Let1^R strain.

Cultures of E4176 [Su$^-$ Let1^R (186 cItspAam11 (8))], E4137 [Su$^-$ Let1^R], E1111 [Su$^-$ (186 cItspAam11 (8))] and E536 [Su$^-$], which were grown overnight at 30^{o}C in TPG-CAA medium, were diluted into the same broth and incubated with aeration at 30^{o}C to A_{600}= 0.2 (2x10^8 cfu/ml). Cultures were transferred to 40^{o}C at 0 min and incubation with aeration was continued. Samples (200 ul) were taken at the indicated times and the rate of DNA replication was determined by pulse-labelling with ^{3}H-thymidine, as described in Chapter 2.18.1.

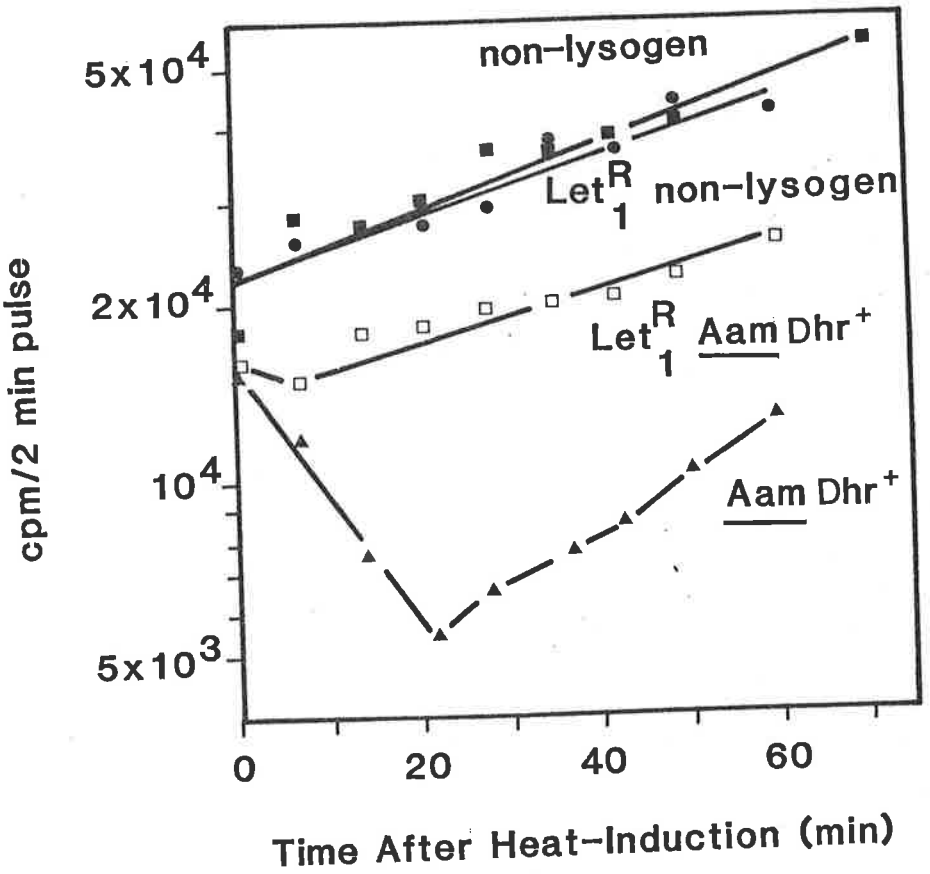
Symbols : □ = E4176 [E536 Let1^R (186 cItspAam11 (8))]

● = E4137 [E536 Let1^R] (non-lysogen)

▲ = E1111 [E536 (186 cItspAam11 (8))]

■ = E536 (non-lysogen)

Bacterial and bacteriophage strains are described in Chapters 2.1 and 2.2.1.


non-lysogen
Let$^{R}_{1}$ non-lysogen
Let$^{R}_{1}$ Aam Dhr^{+}
Aam Dhr^{+}
5x10^{4}
2x10^{4}
10^{4}
5x10^{3}
cpm/2 min pulse
0
20
40
60
Time After Heat-Induction (min)

previous results, which suggested that Dhr^R1 contains a temperature-sensitive mutation, preventing the action of Dhr at temperatures above 40°C, but not at 30°C.

The results presented above have established that Dhr^R1 is a host mutant, which is resistant to Dhr at high temperatures. Studies are currently underway by S. Williams of this laboratory to determine the precise location of the Dhr^R1 mutation on the E. coli chromosome. The map position of the Dhr^R1 locus on the bacterial chromosome may enable the identification of the host function involved in the inhibition of E. coli DNA replication and cell division by Dhr and may provide insights into the function of Dhr in 186 lytic development.

5.2.6 The Identification of the dhr Gene.

The results presented in Chapters 5.2.1 to 5.2.5, have established that dhr is a 186 gene encoded within the PstI-BglII (77.4%-79.6%) region, and have characterized the effect of Dhr on 186 and on the host. The next step was to identify which of the genes encoded within the PstI-BglII (77.4%-79.6%) region, is the dhr gene. The first approach used to identify the dhr gene was to determine the DNA sequences of the two characterized Dhr^- mutants (Dhr1 and Dhr2).

5.2.6(a) DNA Sequencing of Dhr^- Mutants.

The strategy to identify the dhr gene was to determine the DNA sequence of the PstI-BglII (77.4%-79.6%) region from the Dhr1 (pEC401) and Dhr2 (pEC402) mutants and compare this sequence with the DNA sequence obtained from the PstI-BglII region from the parent clone, pEC400 (Chapter 2.3.2, 5.2.2).

The PstI-BglII (77.4%-79.6%) fragments were isolated from pEC401, pEC402 and pEC400 and cloned into the PstI and BamHI sites of M13mp8 or mp9 to create clones of the r-strand and l-strand (Chapter 2.30.1, 2.31). In

addition, the PstI-BglII fragment from these plasmids were digested with HpaII or TaqI and the resulting fragments were "shot-gun" cloned into the AccI site of M13mp9 (Chapter 2.30.2, 2.31).

The sequencing strategy used to determine the sequence of the PstI-BglII (77.4%-79.6%) region of pEC400, pEC401 and pEC402 is shown in Figure 5.11(a),(b),(c). The DNA sequence was determined using the modified Sanger dideoxy chain termination method (Chapter 2.33). The DNA sequence of the PstI-BglII (77.4%-79.6%) region of pEC400 was the same as that determined for 186 cItsp (1) (Chapter 3.2.1). The PstI-BglII region of the Dhr1 mutant (pEC401) was found to differ from the wild-type sequence at three positions, each involving a C to T base change, namely at base 3671 in CP77 to give a missense mutation of leucine to phenylalanine, at base 3954 in CP78 to create another missense mutation of proline to serine and at base 4225 in CP79 to give a third missense mutation of alanine to valine. The PstI-BglII (77.4%-79.6%) region of the Dhr2 mutant (pEC402) contained mutations in CP77 (a G to A base change at base 3739 resulting in a tryptophan to an opal nonsense mutation) and in CP78 (a G to A base change at position 3910 resulting in a glycine to asparagine amino acid change). Figures 5.12 and 5.13 present photographs of the autoradiographs of sequencing gels showing these mutations and Figure 5.15 shows the position of these mutations in the DNA sequence of the PstI-BglII (77.4%-79.6%) region. The results obtained from the DNA sequencing of the PstI-BglII region of the Dhr1 and Dhr2 mutant has shown that CP76 and CP79 are not the dhr gene, however, they did not allow the identification of the dhr gene since both CP77 and CP78 contained mutations.

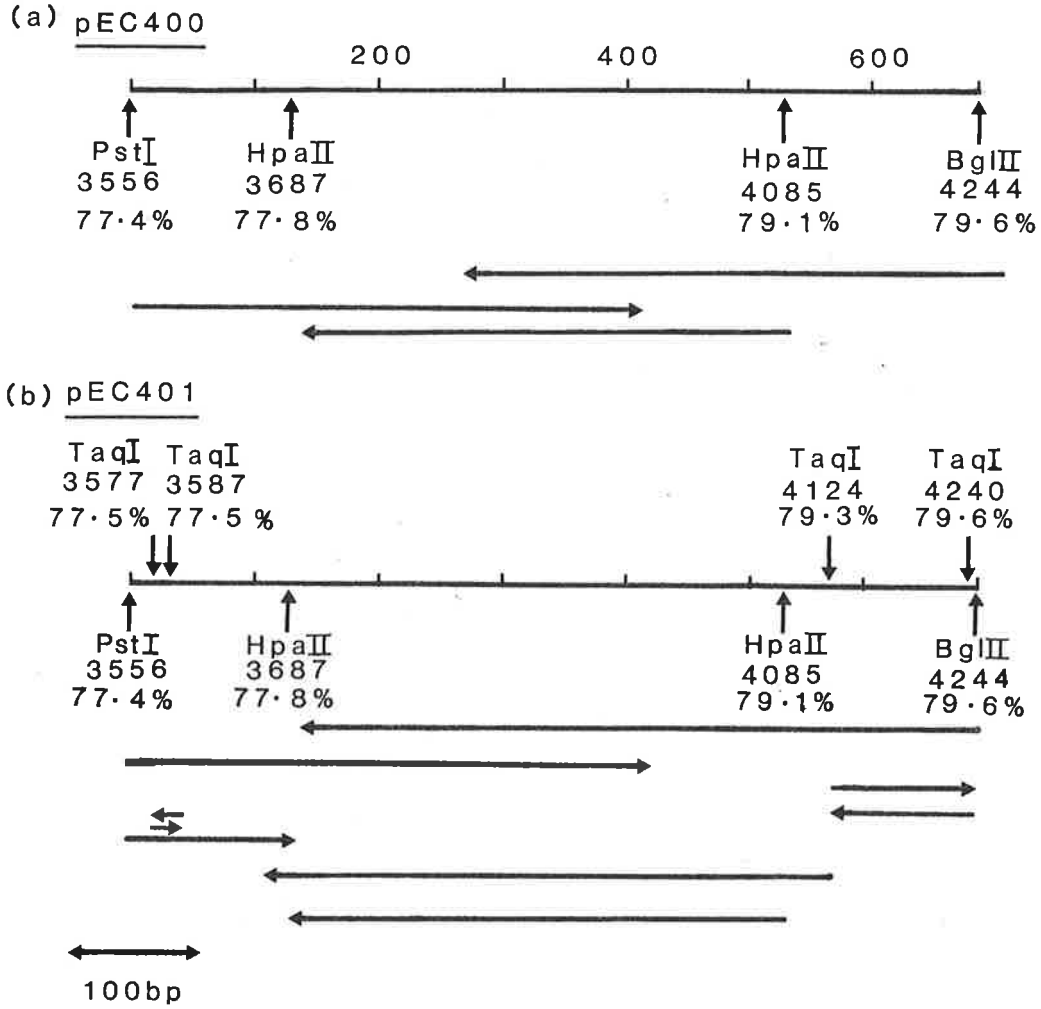
These results have revealed that either CP77 or CP78 may be the dhr gene. The multiple mutations possessed by the Dhr1 and Dhr2 mutants may be a consequence of using nitrosoguanidine as the mutagen, which has been reported in some cases to cause multiple mutations (Guerola et al., 1971).

Figure 5.11 The sequencing strategy of the PstI-BglII (77.4%-79.6%) regions from the wild-type (pEC400), Dhr1 (pEC401), Dhr2 (pEC402) and Dhr3 (pEC403).

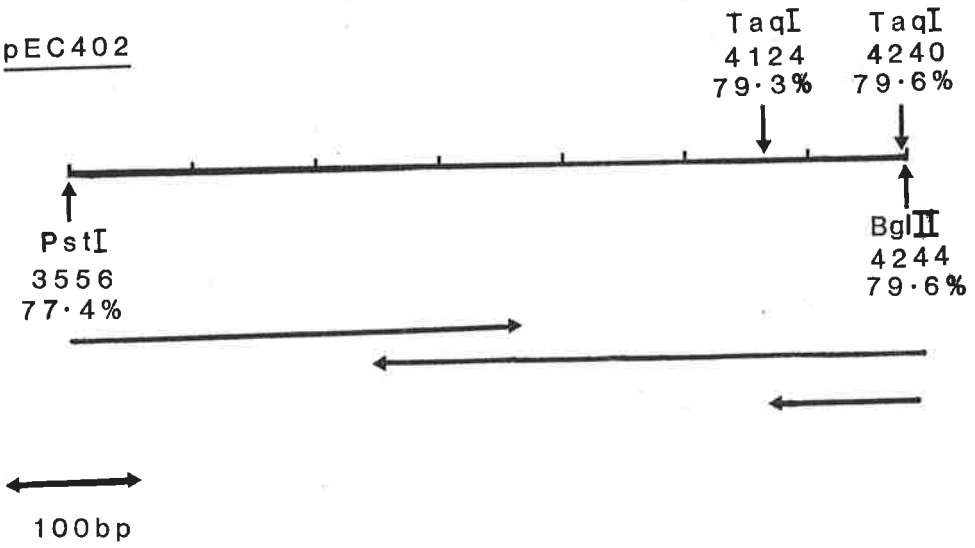
A restriction map of the PstI-BglII (77.4%-79.6%) region is shown, for each plasmid-clone. The marks on the maps of pEC400, pEC401 and pEC402 represent 100 bp, and on the map of pEC403 represent 200 bp. Relevant restriction sites are indicated. The sequence coordinates of the restriction sites refer to the first base of the site on the l-strand.

The arrows below the map represent gel readings used to generate the DNA sequence. Rightward arrows represent gel readings of the l-strand, whereas leftward arrows represent gel readings of the r-strand sequence.

The position of the IS1 element in pEC403, is indicated.


(a) pEC400
200
400
600
PstI
3556
77·4%
HpaII
3687
77·8%
HpaII
4085
79·1%
BglII
4244
79·6%
(b) pEC401
TaqI
3577
77·5%
TaqI
3587
77·5%
TaqI
4124
79·3%
TaqI
4240
79·6%
PstI
3556
77·4%
HpaII
3687
77·8%
HpaII
4085
79·1%
BglII
4244
79·6%
100bp

(c) pEC402



(d) pEC403

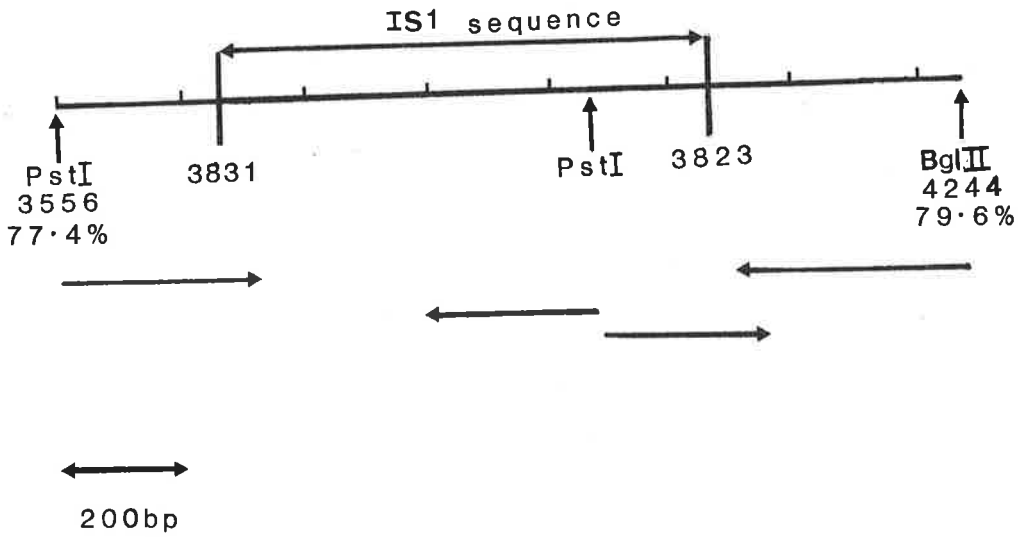
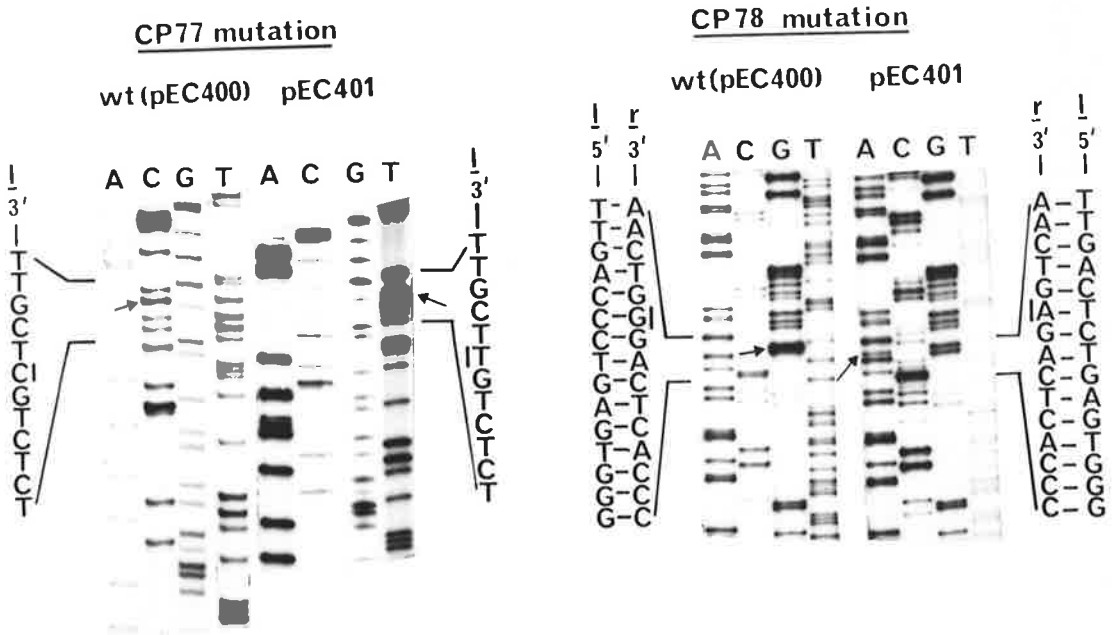


Figure 5.12 DNA sequence of the PstI-BglII (77.4%-79.6%) region from the 186 Dhr1 mutant.

This Figure shows the DNA sequence of the mutations in Dhr1 (pEC401) compared with the DNA sequence of the wild-type (pEC400). The DNA sequence of the CP77 mutation is from the l-strand, whereas the DNA sequence of the CP78 and CP79 mutations are from the r-strand. The positions of the base pair changes, are indicated on the autoradiograph by the arrows, and are summarized in Figure 5.15.

The DNA sequencing was carried out as described in Chapter 2.33. Electrophoresis and autoradiography conditions were as described in Chapter 2.29.3(c) and 2.29.4.

Dhr 1


CP77 mutation
wt(pEC400) pEC401
A C G T A C G T
CP78 mutation
wt(pEC400) pEC401
A C G T A C G T

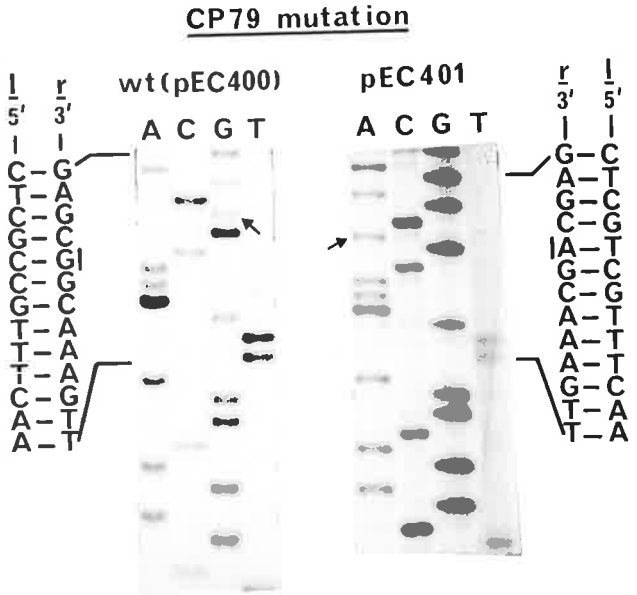

CP79 mutation
wt(pEC400)
pEC401
A C G T
A C G T

Figure 5.13 DNA sequence of the PstI-BglII (77.4%-79.6%) region from the 186 Dhr2 mutant.

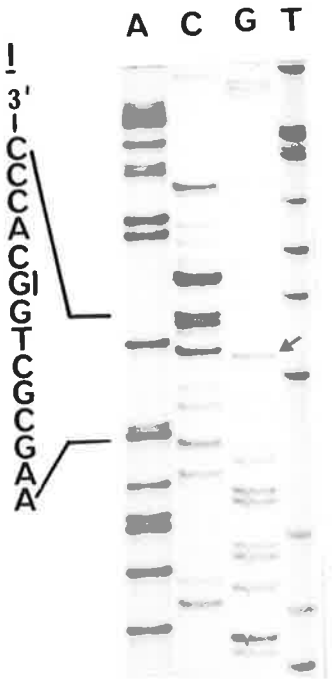
This Figure shows the DNA sequence of the mutations in Dhr2 (pEC402) compared with the DNA sequence of the wild-type (pEC400). The DNA sequence of the CP77 mutation is from the l-strand, whereas the DNA sequence of the CP78 mutation is from the r-strand. The positions of the base pair changes, are indicated on the autoradiograph by the arrows, and are summarized in Figure 5.15.

The DNA sequencing was carried out as described in Chapter 2.33. Electrophoresis and autoradiography conditions were as described in Chapter 2.29.3(c) and 2.29.4.

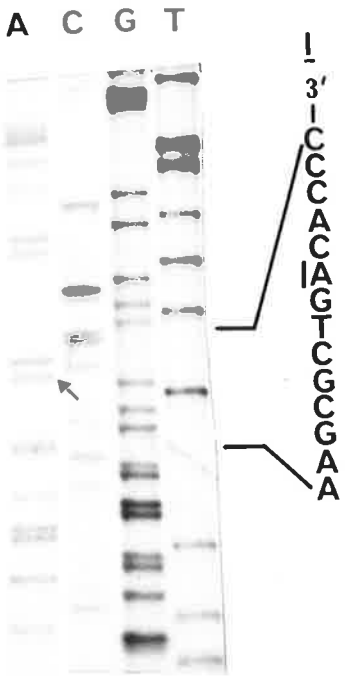
Dhr 2

CP77 mutation

wt (pEC400)

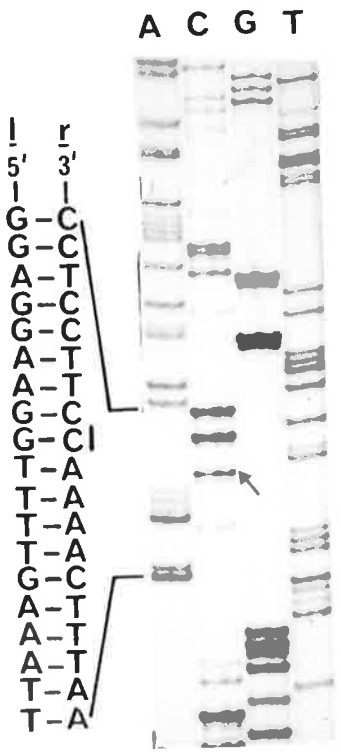


pEC402

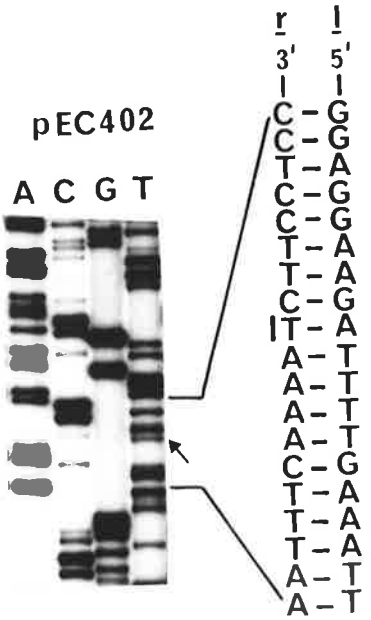


CP78 mutation

wt (pEC400)



pEC402



Since the DNA sequencing of the Dhr1 and Dhr2 mutants did not allow the identification of the *dhr* gene because of the presence of multiple mutations, it was decided to determine the DNA sequence of the PstI-BglII region of the Dhr$^-$ mutant, Dhr3, which had been isolated as a spontaneous mutant (Chapter 5.2.5b).

The plasmid DNA (pEC403) from the Dhr3 mutant was isolated and analysed by restriction analysis (Chapter 2.24.2, 2.28). The plasmid-clone pEC403, was found to have an insertion of ~800 bp within the PstI-BglII (77.4%-79.6%) region (data not shown). This insertion contained a PstI site, and PstI digestion of the XhoI-BglII (67.6%-79.6%) fragment from pEC403 gave three fragments, namely 1.2 kb (XhoI-PstI), 0.86 kb (PstI-PstI) and 0.6 kb (PstI-BglII) (data not shown). The 0.86 kb and 0.6 kb fragments were cloned into M13mp8 and mp9 to give clones in both orientations and these clones were sequenced using the modified Sanger dideoxy method (Chapter 2.33). The sequencing strategy is shown in Figure 5.11(d). The analysis of the sequencing data showed that Dhr3 contained an IS1 insertion element (Johnsrud, 1979) at position 3831 in *CP77*, which resulted in the duplication of 9 bp of 186 DNA at the position of the insertion (i.e. this 9 bp sequence was found at the 5'-end and 3'-end of the IS1 element). Figure 5.14 presents a photograph of the autoradiographs of sequencing gels revealing the junctions of the IS1 insertion element with 186 DNA. Figure 5.15 shows the position of this insertion element in the DNA sequence of the PstI-BglII (77.4%-79.6%) region.

Since IS1 in either orientation has polar effects on the expression of genes, which are located promoter-distal to the position of the insertion (Besemer, 1977; Das *et al.*, 1977), the dilemma remained as to whether *CP77* or *CP78* was the *dhr* gene. This result with Dhr3 and the double mutations in *CP77* and *CP78* obtained for the Dhr1 and Dhr2 mutants, raised the possibility that mutations in both *CP77* and *CP78* may be required for the Let$^-$ (lethal$^-$) phenotype [the phenotype used to select these clones

Figure 5.14 DNA sequence of the PstI-BglII (77.4%-79.6%) region from the 186 Dhr3 mutant.

This Figure shows the DNA sequence of the 186 DNA-IS1 insertion junctions in Dhr3 (pEC403) compared with the DNA sequence of the wild-type (pEC400). The DNA sequence is from the 1-strand. The first and last positions of the IS1 sequence, are indicated by arrows. The 9 bp region, which is duplicated in Dhr3, is indicated. The results are summarized in Figure 5.15.

The DNA sequencing was carried out as described in Chapter 2.33. Electrophoresis and autoradiography conditions were as described in Chapter 2.29.3(c) and 2.29.4.

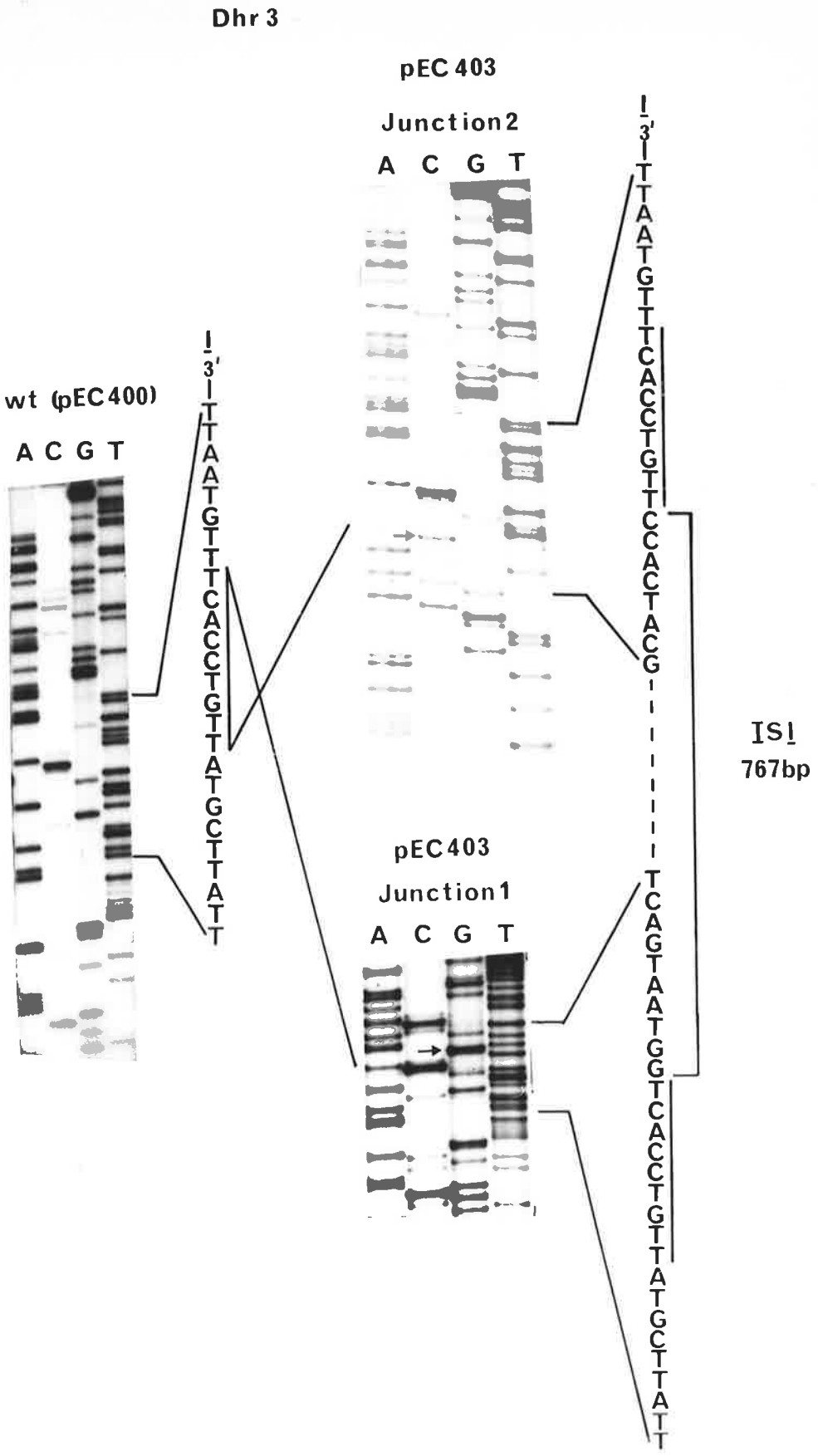
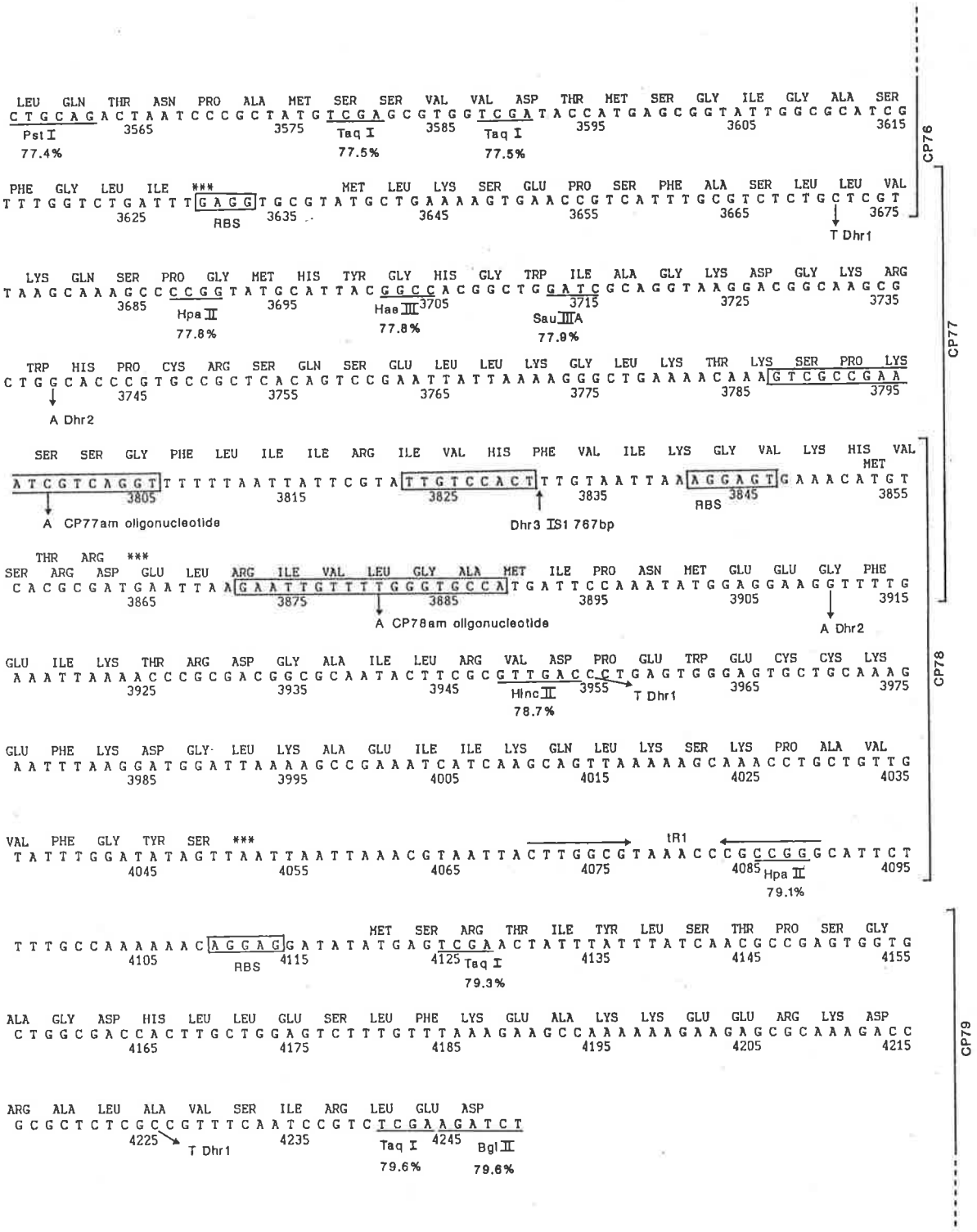

Dhr 3
pEC 403
Junction2
A C G T
wt (pEC400)
A C G T
5' TTAATGTTTCACCTGTTATGCTTATT
5' TTAATGTTTCACCTGTTCCACTACG - - - - - - - - - - TCAGTAATGGTCACCTGTTATGCTTATT
IS1
767bp
pEC403
Junction1
A C G T

Figure 5.15 Location of the Dhr1, Dhr2, and Dhr3 mutations.

The DNA sequence of the 1-strand of the PstI-BglII (77.4%-79.6%) region is presented, and the mutations detected in the Dhr1, Dhr2 and Dhr3 mutants are indicated. The position of the IS1 insertion in Dhr3, is indicated by the arrow and the 9 bp region, which is duplicated in Dhr3, is boxed.

The positions of the genes, are indicated on the right of the Figure. Ribosome-binding sites are boxed. The tR1 terminator is indicated by the convergent arrows. All relevant restriction sites are shown.

The positions of the CP77am and CP78am oligonucleotides on the DNA sequence, are also shown.


CP76
CP77
CP78
CP79
Pst I
77.4%
Taq I
77.5%
Taq I
77.5%
RBS
T Dhr1
Hpa II
77.8%
Hae III
77.8%
Sau IIIA
77.8%
A Dhr2
A CP77am oligonucleotide
Dhr3 IS1 767bp
RBS
A CP78am oligonucleotide
A Dhr2
HincII
78.7%
T Dhr1
tR1
Hpa II
79.1%
RBS
Taq I
79.3%
T Dhr1
Taq I
79.6%
Bgl II
79.6%

(Chapter 5.2.5b)]. Should mutations in both CP77 and CP78 be necessary for the Let$^-$ phenotype then the sequencing of further Dhr$^-$ mutants isolated by the selection for the Let$^-$ phenotype would not allow the identification of the dhr gene. For this reason another approach was used to determine whether CP77 or CP78 encoded the dhr gene. The approach used was to analyse the effect on the host of the expression of CP77 or CP78 from plasmid-clones.

5.2.6(b) Analysis of Clones of CP77 and CP78.

Plasmid-clones in the expression vector, pPLc236 (which contains the λ pL promoter upstream of the cloning site), of the CP77 gene (pEC404) and the CP78 gene (pEC421) were obtained, as described in Chapters 4.2.3 and 2.3.2. These clones are shown diagrammatically in Figure 5.16. The expression of CP77 or CP78 from these clones was controlled in a strain (E832), which carries a defective λ prophage encoding the cI repressor gene containing a temperature-sensitive mutation (cI857). The clones were tested for their lethality to the host cells, and their ability to demonstrate the Dhr Effect upon heat-induction at 41.5°C (the expression temperature).

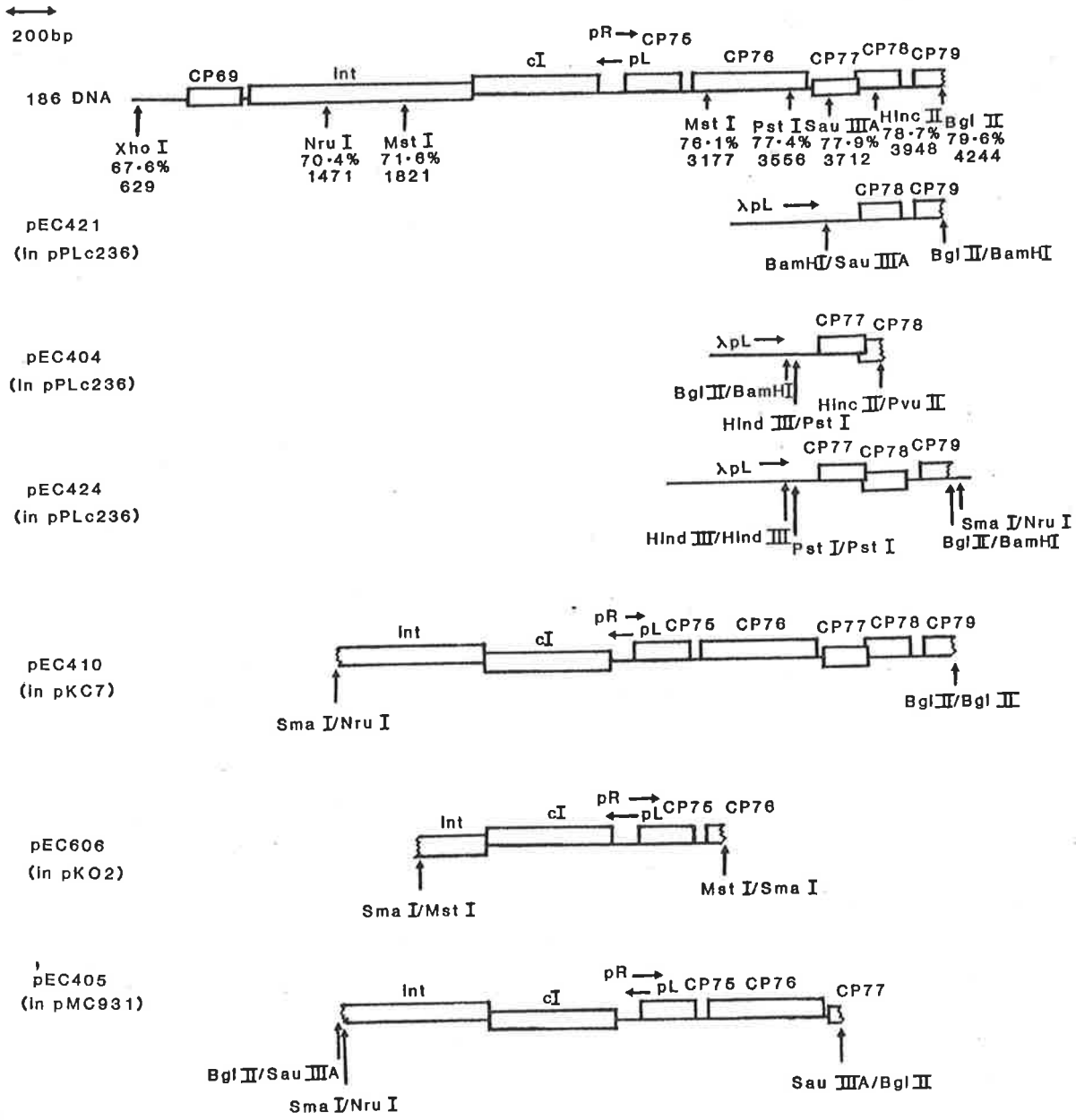
Cells were grown overnight at 41.5°C on YGC plates containing ampicillin, and the viable counts were determined. The strain carrying the pPLc236 vector, showed 100% survival at 41.5°C compared with that obtained at 30°C. The clone of the CP77 gene (pEC404) showed a slight reduction in viable count (~50-70% survival), whereas the CP78 clone (pEC421) showed a significant reduction in viable count (0.13%-3.3% survival) compared with that obtained at 30°C. This result suggests that CP78 encodes the lethal gene described by Finnegan (1979). However, CP77 also results in a small, but significant, decrease in cell survival at 41.5°C. The observation that the expression of both CP77 and CP78 decreases the survival of the host may

Figure 5.16 Gene content of pEC421, pEC404, pEC424, pEC410, pEC606 and pEC405.

A diagrammatic representation of the gene content of the XhoI-BglII (67.6%-79.6%) region from 186. Genes are represented by the boxed regions, and promoters are represented by the horizontal arrows. Relevant restriction sites are shown. The sequence coordinates of the restriction sites refer to the first base of the site on the l-strand.

The gene content of pEC421, pEC404, pEC424, pEC410, pEC606 and pEC405 are shown. The construction of these plasmid-clones is described in Chapter 2.3.2. The restriction sites used to construct these clones are indicated.

The gene content of these clones is indicated. For plasmid-clones in the vector, pPLc236, the λ pL promoter-proximal fusion-gene is not expected to be expressed, and therefore is not shown on the diagram. Genes that are only partially represented on the plasmid-clones are indicated by the jagged-edged boxes.


200bp
186 DNA
CP69
Int
cI
pR
CP75
pL
CP76
CP77
CP78
CP79
Xho I
67·6%
629
Nru I
70·4%
1471
Mst I
71·6%
1821
Mst I
76·1%
3177
Pst I
77·4%
3556
Sau IIIA
77·9%
3712
Hinc II
78·7%
3948
Bgl II
79·6%
4244
pEC421
(in pPLc236)
λpL
BamHI/Sau IIIA
Bgl II/BamHI
pEC404
(in pPLc236)
Bgl II/BamHI
Hinc II/Pvu II
Hind III/Pst I
pEC424
(in pPLc236)
Hind III/Hind III
Pst I/Pst I
Sma I/Nru I
Bgl II/BamHI
pEC410
(in pKC7)
Sma I/Nru I
Bgl II/Bgl II
pEC606
(in pKO2)
Sma I/Mst I
Mst I/Sma I
pEC405
(in pMC931)
Bgl II/Sau IIIA
Sma I/Nru I
Sau IIIA/Bgl II

explain the presence of mutations affecting both CP78 and CP77 in the Dhr1, Dhr2 and Dhr3 mutants, which were isolated as non-lethal mutants.

The survival of cells carrying the clone of CP78 (pEC421) was similar to that obtained for a clone encoding both the CP77 and CP78 genes (pEC424) and a clone encoding the entire early lytic region (pEC410). [pEC424 and pEC410 were constructed, as described in Chapter 2.3.2 (Figure 5.16; Table 5.3).] This result indicates that CP78 is the gene largely responsible for the lethality, which occurs when the early lytic genes are expressed from a plasmid-clone. In further support of this conclusion, the expression of genes from clones of CP75 (pEC606), and CP75 and CP76 (pEC405), constructed as described in Chapter 2.3.2 (Figure 5.16), did not show a reduction in cell survival (Table 5.3).

To determine whether CP77 or CP78 encoded the dhr gene, pEC421 (CP78), pEC404 (CP77) and pEC424 (CP78, CP77) and the parent vector, pPLc236, were tested for their ability to show the Dhr Effect, after heat-induction at 41.5°C (Figure 5.17). The expression of genes from pEC424 resulted in the depression of E. coli DNA replication (the Dhr Effect) as expected. The expression of CP78 (from pEC421) also resulted in the Dhr Effect, whereas pEC404 and pPLc236 did not cause the Dhr Effect. This result shows that CP78 is the gene responsible for the Dhr Effect and thus, encodes the dhr gene.

As discussed in Chapter 5.2.4(c), cell division inhibition was also associated with the Dhr Effect. Therefore, it was of interest to observe the effect of the expression of CP78 (the dhr gene) on cell morphology. Cells carrying the CP78 plasmid-clone (pEC421) or the parent vector, pPLc236, were grown at 42°C for 4 hours, then viewed under the microscope using phase contrast optics. The cell morphology of cells carrying pPLc236 were normal at 42°C (Figure 5.18a). Surprisingly, the expression of CP78 from pEC421 also gave normal cells (Figure 5.18b). This result suggests that the CP78 (dhr) gene is not responsible for the cell filamentation,

TABLE 5.3

Survival at 41.5°C of E832 carrying clones of the 186 early lytic genes.[a]

Plasmid-clones (Plasmids)	186 genes encoded	Survival %[d]
pPLc236	-	100%
pEC421[b]	CP78	0.13%-3.3%[e]
pEC404[b]	CP77	50%-70%
pEC424[b]	CP77,CP78	1.8%-3.6%[e]
pKC7	-	100%
pEC410[c]	cItsp,CP75,CP76,CP77,CP78	2%-4%[e]
pKO2	-	100%
pEC606[c]	cItsp,CP75	100%
pMC931	-	100%
pEC405[c]	cItsp,CP75,CP76	100%

Notes to Table 5.3

a. Cultures of E832 carrying plasmid-clones (or plasmids) were grown in L broth (containing the appropriate antibiotic) at 30°C to A_{600} = 0.8. Samples were taken and dilutions of the culture were spread onto YGC plates containing the appropriate antibiotic. Plates were incubated at 30°C or 41.5°C overnight and the viable counts were determined (Chapter 2.16.2). The results presented here were obtained from several experiments.

b. The plasmid-clones pEC421, pEC404 and pEC424 have the 186 genes cloned downstream from the λ pL promoter (Chapter 2.3.2). The expression of the cloned genes from the λ pL promoter is controlled by the λ cI857 repressor, which is expressed from the defective λ prophage present in E832. Thus, at 30°C the cloned genes are not expressed, but they are expressed at 41.5°C.

c. The expression of 186 early lytic genes from pEC410, pEC606 and pEC405 is controlled by the 186 cItsp repressor, which is encoded on the plasmid-clone (Chapter 2.3.2). Thus, at 30°C 186 early lytic genes are not expressed whereas at 41.5°C these genes are expressed.

d. The survival percentage refers to the viable count at 41.5°C relative to that obtained at 30°C. E832 containing pEC421, pEC404, pEC424 and pEC410 gave variable levels of survival, as indicated.

e. It is pertinent to note that there is a strain difference in the survival frequency obtained for the clones encoding CP78, between the strains E832 and E536 (Chapter 5.2.2; data not shown). For example, when cells carrying pEC410 are grown overnight on YGC plates containing ampicillin at 41.5°C (the expression temperature) the survival percentage of E832 is 100-1000-fold greater than that obtained for E536 (data not shown). This result suggests that E832 contains mutations that allow partial resistance to the lethal effect of the expression of early lytic genes from a plasmid-clone.

which is observed when early lytic genes are expressed from pEC400 (Chapter 5.2.4c). However, the conclusion that the dhr gene resulted in filamentation was based on the assumption that the Dhr1 and Dhr2 mutants contained single mutations (Chapter 5.2.2) and the results presented here have shown that these mutants contain mutations in CP77 as well as CP78. It was therefore possible that CP77 was responsible for the filamentation observed when early lytic genes are expressed from pEC400 (Chapter 5.2.4c).

To determine whether the expression of CP77 resulted in an inhibition of E. coli cell division, cells carrying pEC404 were grown for 4 hours at 42°C and then viewed under the microscope using phase contrast optics. The heat-induction of pEC404 resulted in filamentous cells (~10-20x larger than normal cells at 42°C; Figure 5.18c). This result shows that the expression of CP77 leads to an inhibition in cell division. However, this inhibition in cell division caused by CP77 can not be complete and most cells must eventually divide since the CP77 clone (pEC404) only results in a small decrease in viable count.

The analysis of the clones encoding CP77 and CP78 has allowed the identification of CP78 as the dhr gene, a gene responsible for the Dhr Effect and largely responsible for the lethality observed when 186 early lytic genes are expressed from a plasmid-clone. CP77 was identified as being responsible for cell filamentation, and thus, was named the fil gene. Having assigned functions to CP77 and CP78 the next step was to determine whether these genes are important to 186 lytic development.

Figure 5.17 The rate of DNA replication after the heat-induction of strains carrying pEC421, pEC404 or pEC424.

Cultures of E832 (pcI857) carrying either pEC421, pEC404, pEC424 or the parent vector pPLc236, which were grown overnight at 30°C in TPG-CAA medium (containing the appropriate growth supplements and antibiotics), were diluted into the same broth and incubated with aeration at 30°C to A_{600}= 0.2 ($2x10^8$ cfu/ml). Cultures were transferred to 41.5°C at 0 min and incubation with aeration was continued. Samples (200 ul) were taken at the indicated times and the rate of DNA replication was determined by pulse-labelling with ^{3}H-thymidine, as described in Chapter 2.18.1.

Symbols : ○ = pEC421 (CP78)

■ = pEC404 (CP77)

• = pEC424 (CP77, CP78)

▲ = pPLc236

Bacterial and bacteriophage strains are described in Chapters 2.1 and 2.2.1. Plasmids and plasmid-clones are described in Chapter 2.3 and Figure 5.16.

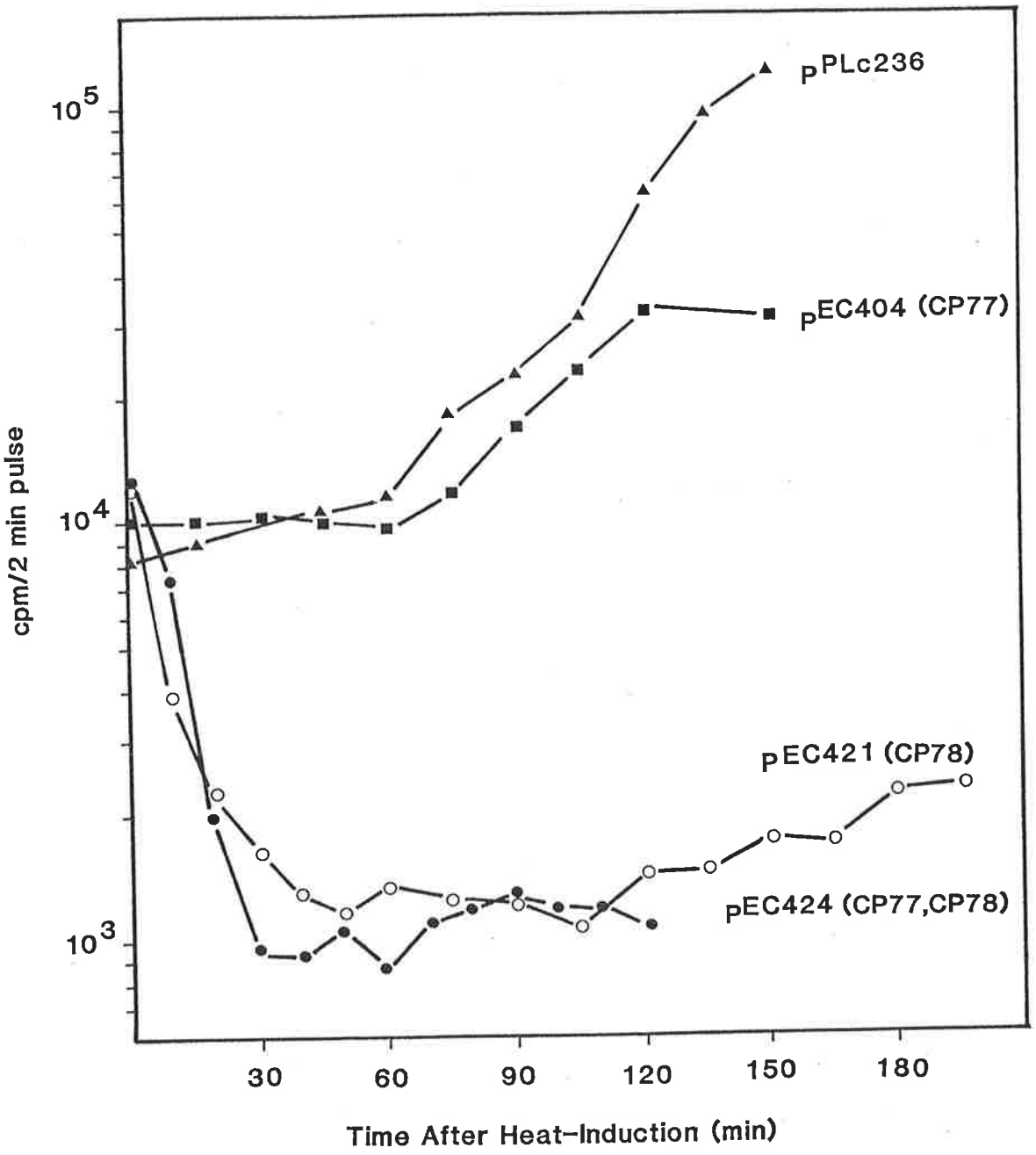

pPLc236
pEC404 (CP77)
pEC421 (CP78)
pEC424 (CP77,CP78)
10^5
10^4
10^3
cpm/2 min pulse
30
60
90
120
150
180
Time After Heat-Induction (min)

Figure 5.18 Cell morphology of E832 carrying clones of CP78 (pEC421) or of CP77 (pEC404) at 42°C.

Cultures of E832 carrying pPLc236, pEC421 or pEC404 were grown in L broth at 30°C to A_{600}= 0.2. The cultures were diluted 1/10 into fresh media and were grown for four hours at either 30°C or 42°C. Cells were photographed at 400x magnification under the microscope using phase contrast optics.

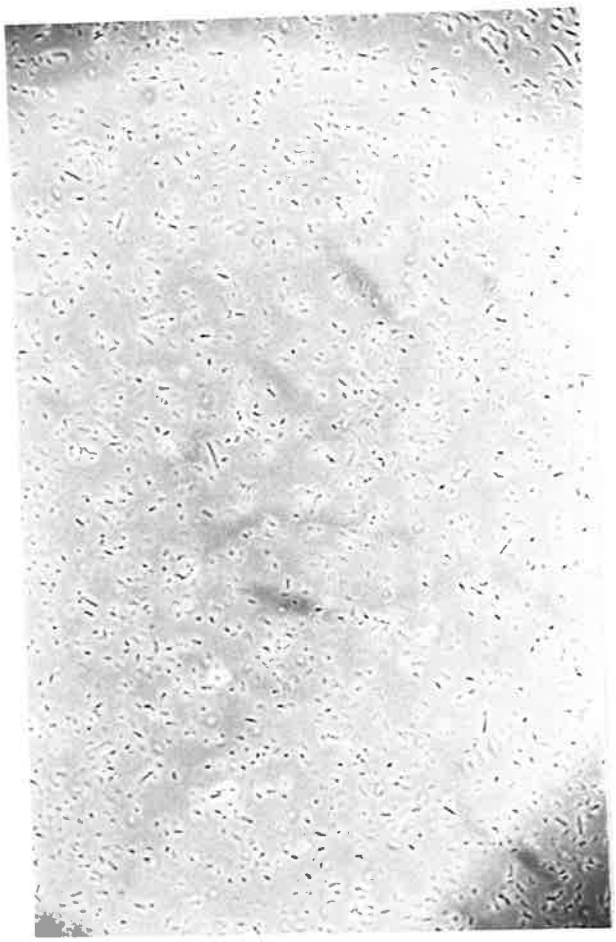
(a) E832 carrying pPLc236 at 42°C.

(b) E832 carrying the CP78 clone (pEC421) at 42°C.

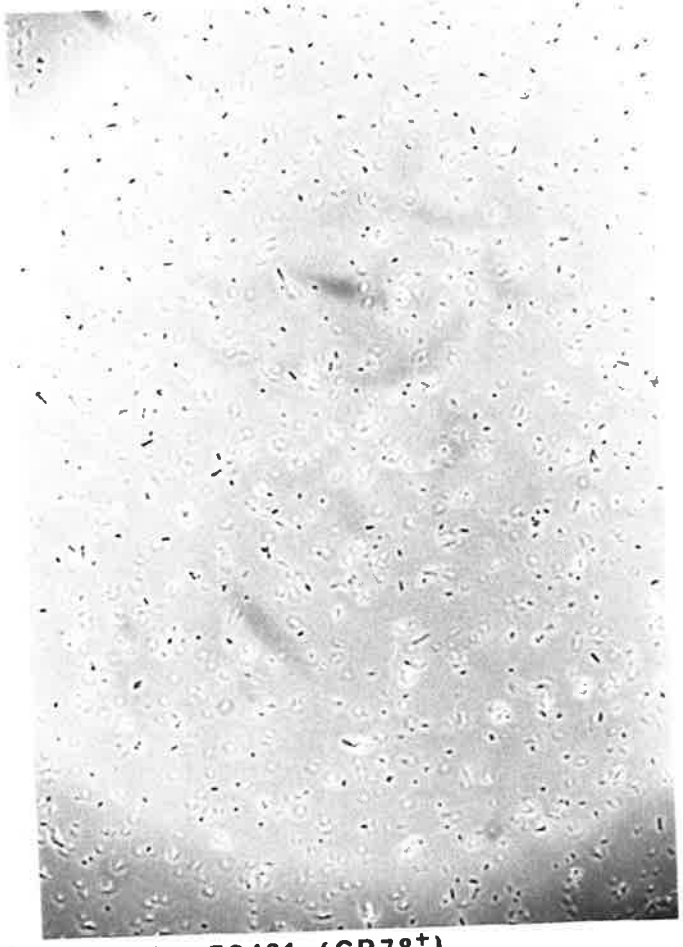
(c) E832 carrying the CP77 clone (pEC404) at 42°C.

These strains gave normal cells when grown for four hours at 30°C (data not shown).

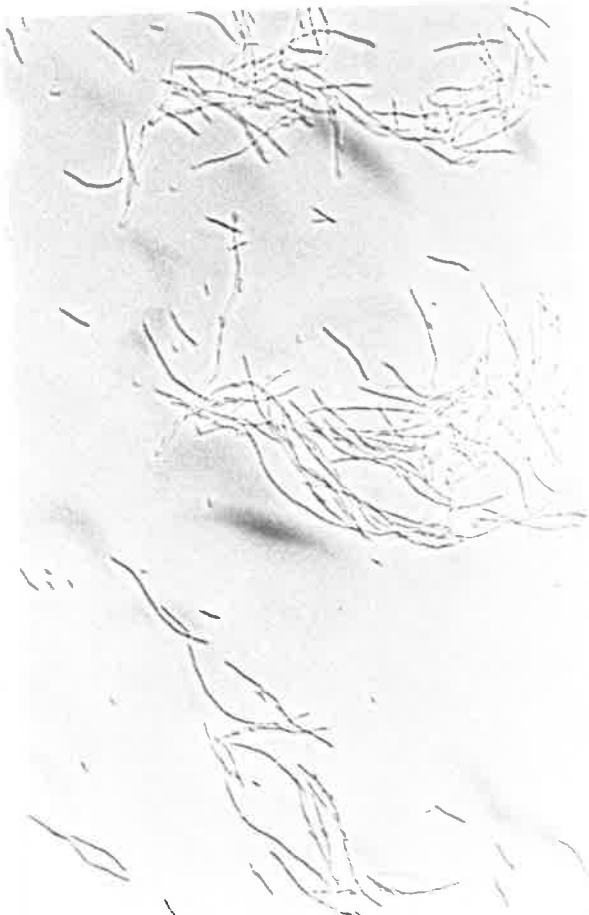
The gene content of pEC421 and pEC404 is shown in Figure 5.16.



(a) pPLc236



(b) pEC421 (CP78^{+})



(c) pEC404 (CP77^{+})

5.2.7 Investigation as to whether fil (CP77) and dhr (CP78) are Essential to 186.

5.2.7(a) Amber Mutants in CP77 and CP78.

The 186 Dhr1 and Dhr2 mutants have mutations in both fil (CP77) and dhr (CP78). These mutants give a reduced burst size indicating that either CP77 or CP78 are important to 186 lytic development (Chapter 5.2.3). However, it was possible that these mutants are not completely Dhr^- and Fil^-, so that the possibility remained that dhr and/or fil may be essential for 186.

To assess the importance of fil (CP77) and dhr (CP78) to 186, amber mutants in these genes were created. As discussed in Chapter 4.2.3, CP77am and CP78am mutants were obtained in the M13-clone, mEC401 (Chapter 2.4.2) using oligonucleotide site-directed mutagenesis and were confirmed by DNA sequencing (Chapter 2.35). To create 186 CP77am and 186 CP78am mutants, the PstI-BglII (77.4%-79.6%) fragments from the CP77am and CP78am derivatives of mEC401 were recombined with the XhoI-PstI (67.6%-77.4%) fragment from 186 cItsp (1), and each resulting XhoI-BglII fragment was ligated into 186 cItsp using these unique sites (Chapter 2.30.1, 2.32) to form 186 cItspCP77am (20) and 186 cItspCP78am (21). These phage were shown to carry the CP77am and CP78am mutations, respectively, by plaque hybridization with the oligonucleotides used for their creation, as probes (Chapter 2.5, 2.32.2; Figure 5.15; data not shown). On non-suppressing strains (E538, E251) the 186 CP77am mutant gave plaques indistinguishable from wild-type plaques in their size and appearance. The CP78am mutant gave very small plaques, particularly at high temperatures (37^oC-41.5^oC) (data not shown). These results suggest that it is the CP78 (dhr) mutation in the 186 Dhr1 and Dhr2 mutants that is responsible for the decreased burst size of these mutants. In fact, the plaque size of the CP78am mutant was even smaller than that of the plaques obtained for the 186 Dhr1 and Dhr2

mutants (data not shown). This result indicates that Dhr1 and Dhr2 may not result in a completely defective dhr gene, or that the fil (CP77) mutations present in these mutants may partially suppress the effects of the mutations in the dhr gene.

The mutant phenotype of the CP78am mutant was not suppressable in any of the three available suppressing strains Su1 (E539), Su2 (E540) or Su3 (E541). This may be explained by the fact that these suppressing strains do not replace the amber stop signal with the correct amino acid, leucine (Chapter 2.5; Figure 5.15), and indicates the importance of the leucine residue at this position. The degree to which the 186 CP78am and CP77am mutations affect 186 development was investigated, by determining the burst size after the heat-induction of the corresponding lysogens (E4138, E4134). 186 CP77am showed a slightly reduced burst size (80%-90%) whereas 186 CP78am gave a significantly reduced burst size (25%) compared with the wild-type lysogen (E252) (data not shown).

These results suggest that dhr (CP78) is important to the phage while fil (CP77) is not important. However, although these results suggest that both fil and dhr are not essential to 186, this cannot be stated with conviction since it is possible that these amber mutants may be slightly leaky, as was observed when these mutant genes were expressed from a plasmid-clone (Chapter 4.2.3), and thus, the phages may not be completely defective in these genes.

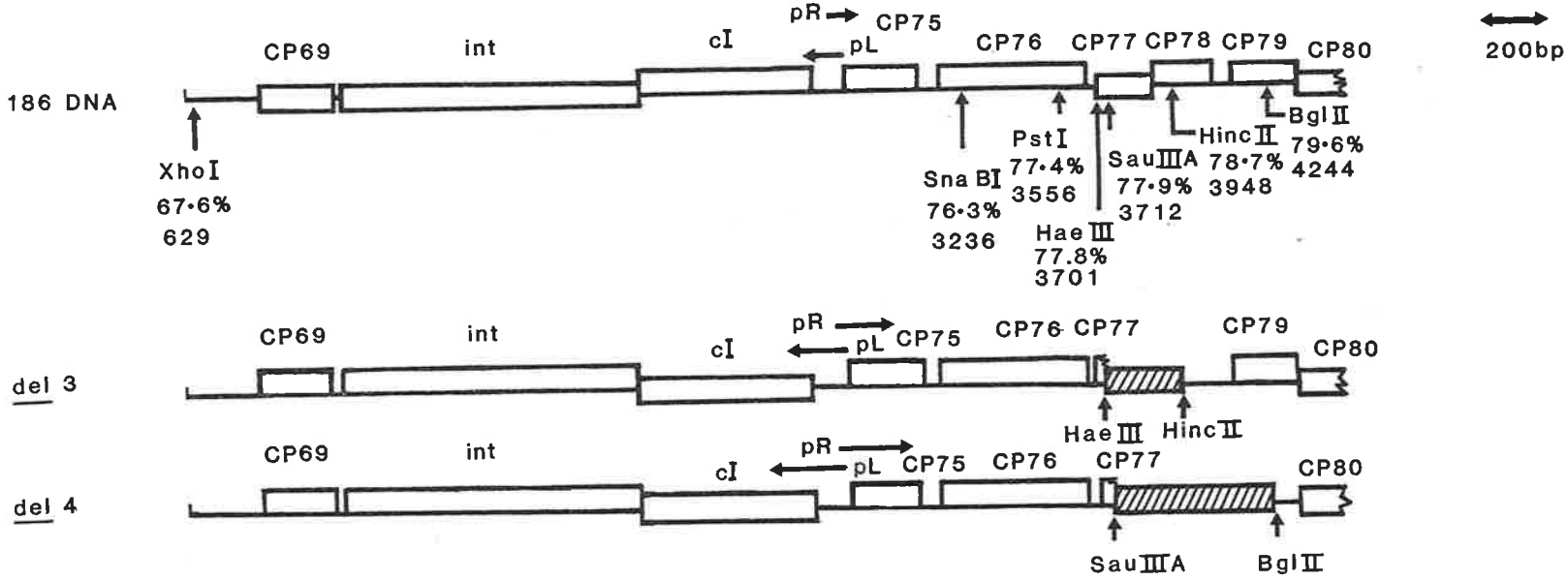
5.2.7(b) Deletions of CP77 and CP78.

To determine whether CP77 and CP78 were essential to 186 an attempt was made to isolate two deletion mutants within the CP77-CP79 region, by exploiting suitable restriction sites within the PstI-BglII (77.4%-79.6%) region. The two deletions that were attempted were from HaeIII-HincII (77.9%-78.9%), sequence coordinates 3703-3950 (del3) and from SauIIIA-BglII (77.9%-79.6%), sequence coordinates 3712-4258 (del4) (Figure 5.19,

Figure 5.19 Location of the del3 and del4 deletions.

A diagrammatic representation of the gene content of the XhoI-CP80 (67.6%-80.3%) region from 186. Genes are represented by the boxed regions and promoters are represented by the arrows. Relevant restriction sites are shown. The sequence coordinates of the restriction sites refer to the first base of the site on the 1-strand.

The gene content of this region from 186 del3 (18) and 186 del4 (19), is shown. These 186 deletion mutants were constructed as described in Chapter 2.2.1. The deletions are represented by the shaded boxes. The restriction sites used to construct these deletions are indicated. Genes, which are not expected to be expressed (due to the removal of their ribosome-binding sites by the deletions), are not shown on these diagrams.


186 DNA
CP69
int
cI
pR
pL
CP75
CP76
CP77
CP78
CP79
CP80
200bp
XhoI
67·6%
629
Sna BI
76·3%
3236
PstI
77·4%
3556
Hae III
77.8%
3701
SauIIIA
77·9%
3712
HincII
78·7%
3948
BglII
79·6%
4244
del 3
del 4

5.15). Both of these phage deletions (186 cItspdel3 (18) and 186 cItspdel4 (19)) were isolated. The construction of these deletions is described in Chapter 2.2.1. Both deletion phage gave small plaques compared with the wild-type. The del3 deletion removes the 3'-end of the fil (CP77) gene (54/75 amino acids) and the 5'-end of the dhr (CP78) gene (33/66 amino acids), whereas the del4 deletion removes the 3'-end of the fil (CP77) gene (49/75 amino acids), the complete dhr (CP78) gene and the 5'-end of the CP79 gene (26/77 amino acids). The viability of these phages provides evidence to suggest that the fil (CP77), and the dhr (CP78) gene are not essential to 186. Furthermore, these results show that the first middle gene, CP79, is probably also a non-essential gene.

The burst sizes of the 186 del3 and del4 phage were determined by heat-induction of the corresponding lysogens (E4121 and E4122; Chapter 2.1) and both phage gave a burst size of ~30% of the wild-type (data not shown). This burst size is similar to that obtained for the 186 Dhr^- phage, 186 cItspCP78am (21). The similarity in burst size of the two deletion phage (which are expected to be Dhr^- and Fil^-) to that of the Dhr^- phage (186 cItspCP78am) provides further evidence to suggest that fil (CP77) is not important to 186 lytic development.

5.3 SUMMARY.

The results presented in this Chapter have shown that CP78 encodes the dhr gene, a gene responsible for the depression of host DNA replication (the Dhr Effect) and that CP77 encodes the fil gene, which results in the inhibition of E. coli cell division. The dhr (CP78) gene was also shown to be largely responsible for the lethality observed when 186 early lytic genes are expressed from a plasmid-clone, although fil (CP77) also resulted in a small decrease in cell survival. The fil and dhr genes are not essential to 186, although dhr appears to be important to 186 lytic

development. The possible function of Dhr and Fil in 186 development will be considered in Chapter 10.2.

The Tom function (postulated to be required for 186 middle gene transcription) was expected to be an early lytic gene (Finnegan and Egan, 1981; Chapter 1.3, 5.1). However, all early lytic genes (CP75, CP76, CP77 and CP78) have now been assigned functions. To accommodate the tom gene in the early lytic region one of the early lytic genes must encode more than one function. Since CP75 (cpl) and CP76 (cII) have defined roles in 186 development, it was considered unlikely that either of these genes encodes the Tom function. It is therefore possible that tom is encoded by dhr (CP78) or fil (CP77), which have as yet undefined roles in 186 development. However, Tom was postulated to be an essential function and therefore mutants in this gene should be lethal to the phage. Since dhr or fil are not essential to 186, this raises the possibility that Tom may be encoded in another region (for example the 186 middle region) or that if Tom is encoded in the early lytic region (by dhr or fil) it is not an essential gene. Whether dhr or fil encode the Tom function is a subject investigated later in this study (Chapter 8).

The results presented in Chapters 3, 4 and 5 were concerned with the definition of the early lytic region and determining the functions encoded by the early lytic genes. The next Chapters are concerned with the transcription of the 186 early lytic and middle regions and the control of middle gene expression.

RESULTS SECTION III.

CHAPTER 6.

THE ANALYSIS OF THE IN VIVO RIGHTWARD RNA TRANSCRIPTS OF THE 186 EARLY LYTIC AND MIDDLE REGIONS.

CHAPTER 6. THE ANALYSIS OF THE IN VIVO RIGHTWARD RNA TRANSCRIPTS OF THE 186 EARLY LYTIC AND MIDDLE REGIONS.

6.1 INTRODUCTION.

A knowledge of the in vivo transcription pattern of a specific region of DNA is important in understanding the control of gene expression of that region. As discussed in Chapter 1.3, in vivo and in vitro transcription studies have established that rightward transcription occurs from the pR promoter and results in a 1.45 kb transcript in vitro (Finnegan and Egan, 1981; Kalionis, 1985; Pritchard and Egan, 1985). This Chapter is concerned with determining the sizes and approximate 5'-ends and 3'-ends of the in vivo RNA transcripts from the 186 early lytic and middle regions.

To determine the transcription pattern of the early lytic and middle regions of 186, Northern analysis (Chapter 2.36.3) was used. In Northern analysis, RNA fractionated on an agarose gel is transferred to a nitrocellulose filter and RNA species are then detected by hybridization with radioactive probes. The technique of Northern analysis enables the detection and sizing of RNA transcripts, which are encoded in a specific region. The availability of a library of M13-clones spanning the 186 early lytic and middle regions (sequencing clones: this work; Chapter 2.4.2; Kalionis et al., 1986a; Sivaprasad, 1984) allowed the construction of specific hybridization probes for the detection of rightward RNA (Chapter 2.34.1). These probes were used to identify and map the approximate 5'-ends and 3'-ends of the early lytic and middle transcripts.

Northern analysis has two major disadvantages. Firstly, this technique detects accumulated RNA species and thus, the products of RNA degradation are also detected. This problem can be minimized by taking RNA samples as soon as practicable after the heat-induction of a 186 lysogen.

Secondly, Northern analysis has the disadvantage of not being quantitative due to the poor transfer of small and large RNAs from the agarose gel to the nitrocellulose filter (Thomas, 1983). Thus, to quantitate the amount of transcription occuring across a certain region, RNA dot blot analysis was used (Thomas, 1983; Chapter 2.36.4).

6.2 RESULTS AND DISCUSSION.

6.2.1 The Quantitation of RNA Produced from the Early Lytic and Middle Regions during 186 Lytic Development.

As an initial step in characterizing the transcription of the early lytic and middle regions, it was important to determine the levels of RNA produced from the early lytic and middle regions during 186 lytic development. This would provide confirmation of the time course studies of Finnegan and Egan (1981) (Chapter 1.3) and enable the selection of an appropriate time, at which to take samples for Northern analysis.

RNA was prepared at different times after the heat-induction of a 186 cItsp lysogen (Chapter 2.36.1, 2.36.2) and the amount of RNA produced from the early lytic and middle regions was quantitated using RNA dot blot analysis (Chapter 2.36.4), using probes specific to the early lytic region or the middle region (constructed as described in Chapter 2.34.1). Table 6.1 shows the results obtained. At 5 min after heat-induction, transcription of the early lytic and middle regions was barely detectable, however, this transcription had increased dramatically by 15 min and continued to increase to reach a plateau at 35 min. Comparing the amounts of early lytic RNA relative to middle RNA, revealed that early lytic transcription was ~10-fold greater than middle transcription at 5 min after heat-induction, but by 15 min middle transcription was ~1.7-fold greater than early lytic transcription. This apparent delay in the onset of middle

TABLE 6.1

Quantitation of RNA from the 186 early lytic and middle regions with time after heat induction of a 186 cItsp lysogen.[a]

Source of RNA	Level of hybridization			
	Early Probe[b]		Middle probe[c]	
		-zero[d]		-zero[d]
non-lysogen	1175	-	407	-
186 cItsp lysogen				
0 min	1764	0	444	0
5 min	5465	3701	825	381
15 min	122856	121092	215002	214558
25 min	191510	189746	230214	229770
35 min	179703	177939	298672	298228

Notes to Table 6.1

a. RNA hybridizing to the 186 early and the middle regions was quantitated by RNA dot blot analysis (Chapter 2.36.4). Samples were taken with time (0 min to 35 min, as indicated in the Table) after the heat-induction of the 186 cItsp (1) lysogen (E252) and RNA was prepared (Chapter 2.36.1, 2.36.2). RNA from the non-lysogen (E251) was prepared 35 min after a mock heat-induction. Two ug of each sample of RNA was loaded onto the nitrocellulose filters as described in Chapter 2.36.4. Radioactive probes were prepared as described in Chapter 2.34.1 and Figure 6.1. Hybridization and autoradiography were as described in Chapter 2.36.3(b). The level of hybridization of the probe to RNA was quantitated by scanning the autoradiograph using a Zeinch scanning laser densitometer. The specific activity of the probes was normalized by the quantitation of the amount of probe hybridizing to known concentrations of DNA. The RNA dot blot intensities presented here are normalized.

b. The Early probe (1) was the HinfI-HpaII (2819-2935) DNA fragment prepared from mEC802 (Figure 6.1).

c. The Middle probe was the BglII-HpaII (4249-4480) DNA fragment prepared from mEC408 (Figure 6.1).

d. The amount of RNA hybridizing with the probes at zero minutes after heat-induction was deducted from the dot blot intensities obtained at later times, for the purpose of comparison.

transcription is consistent with the requirement of a 186-encoded protein for middle gene expression.

These results were in general agreement with the results of Finnegan and Egan (1981), which were obtained by the hybridization of labelled RNA to plasmid DNA probes. However, the difference obtained in the ratio of early lytic to middle RNA at 5 min compared with 15 min was not obtained by Finnegan and Egan (1981). This difference may be explained by the poor specificity of the probe (pEC35; Chapter 1.3.2) used by Finnegan and Egan (1981) to detect early lytic RNA (see Chapter 10.4.1a).

The quantitation of RNA from the early lytic and middle regions has shown that the level of RNA increases drammatically from 0 min to 15 min after heat-induction, but does not show a marked increase from 15 min to 35 min. Therefore, the sampling time of 20 min after heat-induction was chosen to prepare RNA for all analysis described in Chapters 6, 7 and 8, unless otherwise stated.

6.2.2 Detection of the 186 Early lytic and Middle Transcripts.

RNA prepared 20 min after the heat-induction of a 186 cItsp lysogen was glyoxylated, fractioned on a 2% agarose gel and transferred bidirectionally to nitrocellulose filters (Chapter 2.36.1, 2.36.2, 2.29.2, 2.36.3). Radioactive probes (prepared as described in Chapter 2.34.1), specific for the early lytic region (HinfI-HpaII, sequence coordinates 2819-2935) or the middle region (BglII-HinfI, sequence coordinates 4249-4335) (Figure 6.1), were hybridized to the filters. The results obtained are presented in Figure 6.2.

The early probe detected a transcript (E2) corresponding in size to the 1.45 kb *in vitro* transcript, which initiates from pR (Figure 6.2, lane 1). Since the *in vitro* 1.45 kb transcript most likely terminates at tR1 (Chapter 3.2.2c; Figure 6.2), this result suggests that tR1 is functional *in vivo*. However, this 1.45 kb transcript was not the only

HhaI (3734-3934) probe : obtained from mEC406 by digestion of the DNA with HhaI.

FnuDII-HhaI (3860-3934) probe : obtained from mEC406 by digestion of the DNA with FnuDII/HhaI.

HinfI-HincII (3894-3950) probe : obtained from mEC406 by digestion of the DNA with HinfI/HincII.

HincII-HpaII (3951-4087) probe : obtained from mEC406 by digestion of the DNA with HincII and BamHI (in the M13 cloning site).

HhaI-HpaII (3935-4087) probe : obtained from mEC406 by digestion of the DNA with HhaI and BamHI (in the M13 cloning site).

HincII-HpaII (3951-4087) probe : obtained from mEC400 by digestion of the DNA with HincII/HpaII.

HpaII-BglII (4088-4248) probe : obtained from mEC400 by digestion of the DNA with HpaII. (There is a HpaII site in the cloning site of M13.)

HinfI (4125-4176) probe : obtained from mEC400 by digestion of the DNA with HinfI.

BglII-HinfI (4249-4335) probe : obtained from mEC408 by digestion of the DNA with BglII/HinfI.

BglII-HpaII (4249-4480) probe : obtained from mEC408 by digestion of the DNA with BglII and BamHI (in the M13 cloning site).

HinfI (5513-5606) probe : obtained from mEC500 by digestion of the DNA with HinfI.

SacII-HpaII (6268-6419) probe : obtained from mEC706 by digestion of the DNA with SacII and EcoRI (in the M13 cloning site).

FnuDII-PstI (6533-6605) probe : obtained from mEC701 by digestion with PstI. (There is a PstI site in the cloning site of M13.)

PstI-FnuDII (6606-6840) probe : obtained from mEC701 by digestion with PstI and EcoRI (in the M13 cloning site).

Figure 6.1 Location of probes used for Northern analysis and RNA dot blot analysis.

The DNA sequence of the l-strand from 2706-7005 from 186 cItsp showing restriction sites used for the construction of probes. The DNA sequence from 4626-5401 is not included in this Figure (indicated by --//--). The pR promoter and the tR1 terminator are shown. All relevant restriction sites are shown. The arrows above the sequence represent the site of cleavage on the r-strand.

The probes used in this study for Northern analysis and RNA dot blot analysis were constructed as described in Chapter 2.34.1 and were purified by electrophoresis on a polyacrylamide gel (Chapter 2.29.3a, 2.30.2). The M13-clones and restriction sites used to construct these probes are described below. The M13-clones are described in Chapter 2.4.2. These probes are specific for rightward RNA. The sequence coordinates listed below indicate the region of 186 DNA contained in the probe.

HinfI-HpaII (2819-2935) probe : obtained from mEC802 by digestion of the DNA with HinfI and BamHI (in the M13 cloning site).

HpaII (3370-3511) probe : obtained from mEC404 by digestion of the DNA with PstI/BamHI (in the M13 cloning site).

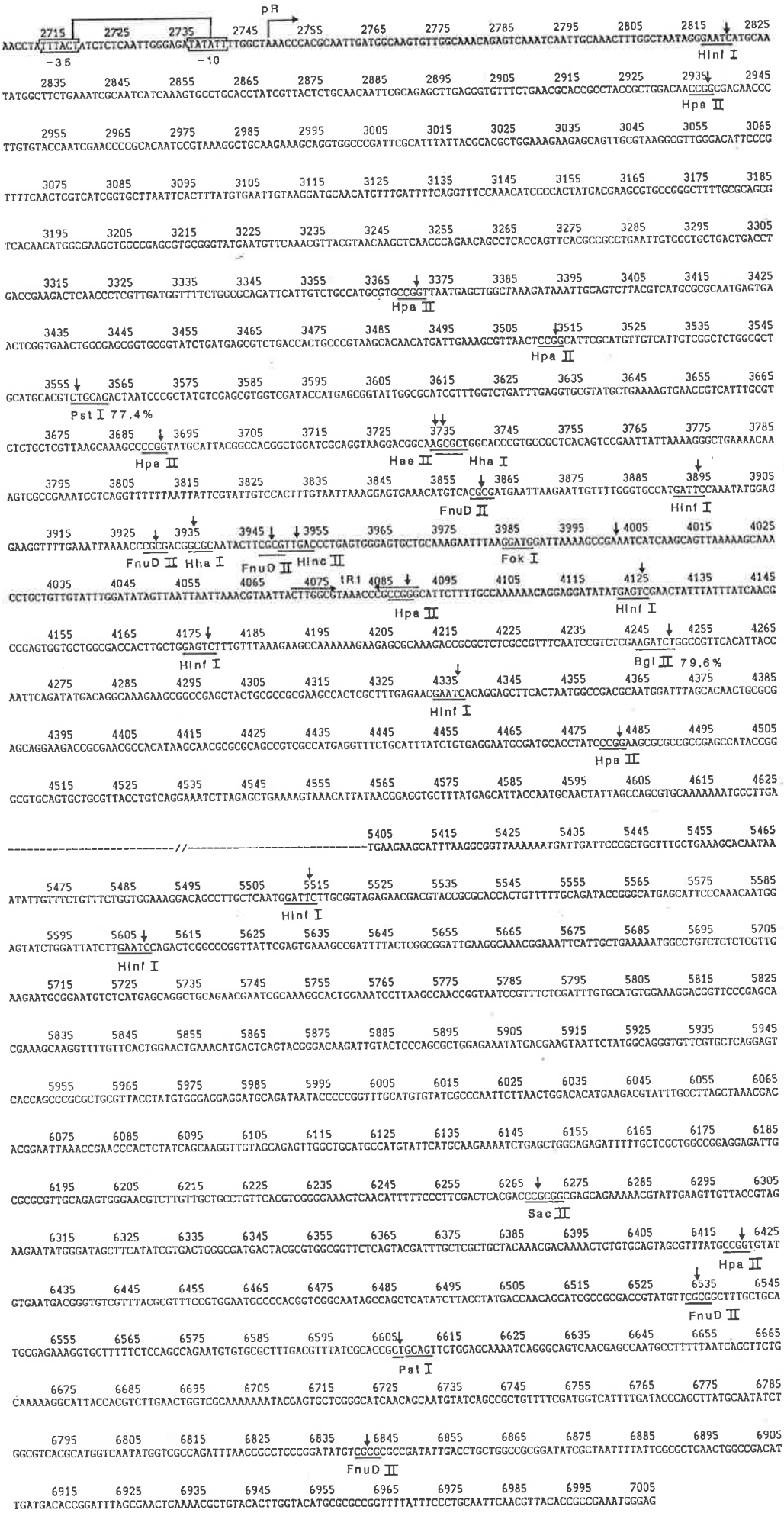
PstI-HpaII (3557-3689) probe : obtained from mEC405 by digestion of the DNA with PstI and BamHI (in the M13 cloning site).

HpaII (3512-3689) probe : obtained from mEC405 by digestion of the DNA with PstI/BamHI (in the M13 cloning site).

HpaII-HaeII (3690-3732) probe : obtained from mEC406 by digestion of the DNA with PstI (in the M13 cloning site) and HaeII.

HpaII-HhaI (3690-3733) probe : obtained from mEC406 by digestion of the DNA with PstI (in the M13 cloning site) and HhaI.

HpaII-FnuDII (3690-3859) probe : obtained from mEC406 by digestion of the DNA with PstI (in the M13 cloning site) and FnuDII.


pR
-35
-10
HInf I
Hpa II
Hpa II
Hpa II
Pst I 77.4%
Hpa II
Hae II
Hha I
FnuD II
HInf I
FnuD II
Hha I
HInc II
Fok I
tR1
Hpa II
HInf I
HInf I
Bgl II 79.6%
HInf I
Hpa II
HInf I
HInf I
Sac II
Hpa II
FnuD II
Pst I
FnuD II

The major RNA transcripts are indicated by the arrows. Non-radioactive CMV RNA and rRNA molecular weight markers (Chapter 2.11.2) are indicated on the Figure.

RNA prepared from the non-lysogen (E251) after a mock heat-induction showed no hybridization with either the early or middle probes (data not shown).

Figure 6.2 186 early lytic and middle transcripts after fractionation of RNA on a 2% agarose gel.

This Figure shows the transcription pattern of the 186 early lytic and middle regions after fractionation of RNA on a 2% agarose gel. The positions on the DNA sequence of the probes used to detect 186 early lytic and middle RNA, are shown diagrammatically. Genes are represented by the boxed regions. The pR promoter, tR1 terminator and the 1.45 kb in vitro transcript are also shown. The tR1 terminator, represented by the hairpin structure, is not drawn to scale. The probes used are as follows :

Early probe (E) : HinfI-HpaII (2819-2935)

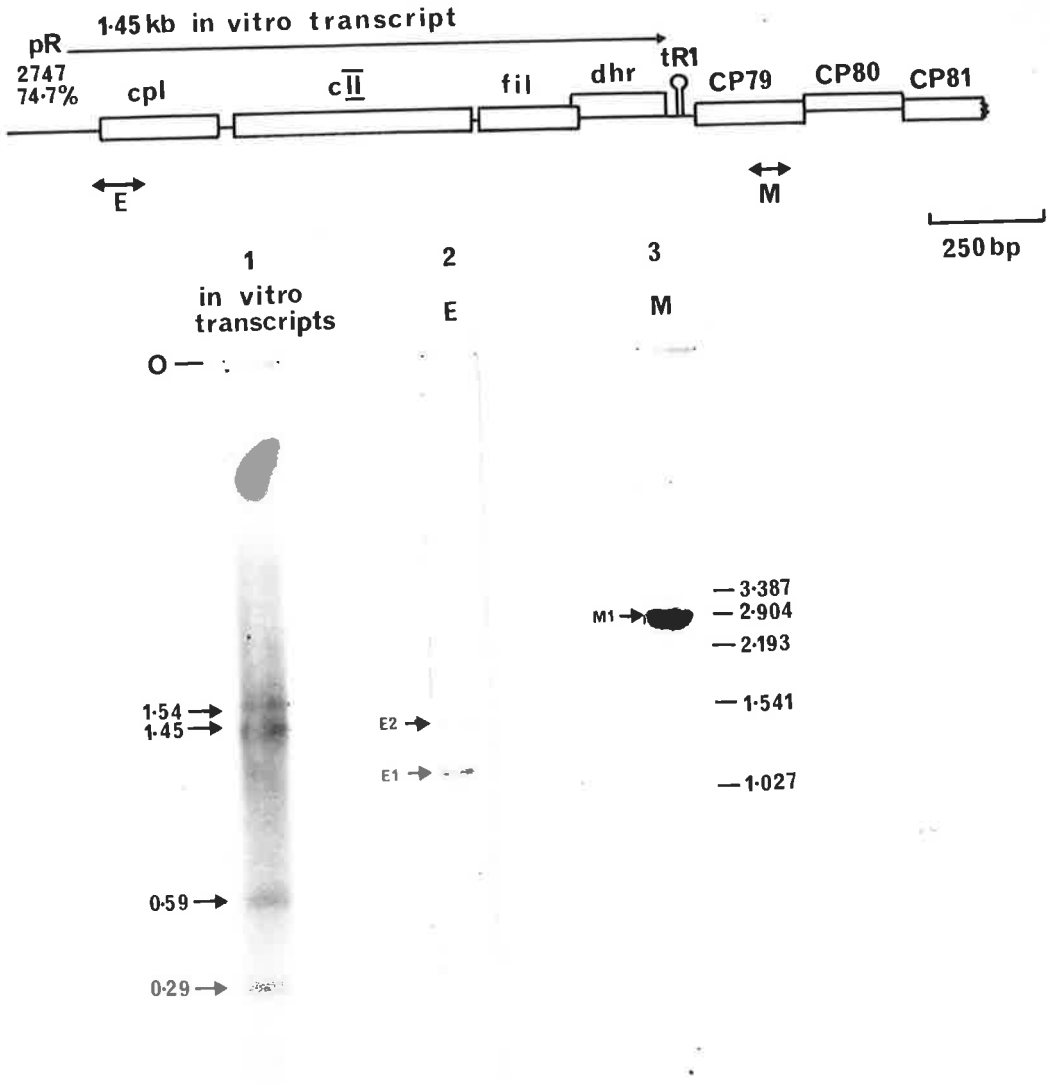
Middle probe (M) : BglII-HinfI (4249-4335)

The construction of these probes is described in detail in the legend to Figure 6.1.

RNA was prepared 20 min after the heat-induction of E252 [E251 (186 cItsp (1))], as described in Chapter 2.36.1 and 2.36.2. Twenty ug of RNA was denatured with glyoxal and fractionated on a 2% agarose gel (Chapter 2.36.3a, 2.29.2). 186 in vitro transcripts (prepared as described in Chapter 2.36.5) and RNA molecular weight standards (Chapter 2.11.2) were also denatured with glyoxal and fractionated on the 2% agarose gel. RNA was then transferred bi-directionally to nitrocellulose and hybridized with the probe specific to the early lytic region or to the middle region (Chapter 2.36.3). The autoradiographs were exposed for 2 days at $-80^{o}C$ with an intensifying screen.

Gel Tracks :

1. 186 in vitro transcripts.
2. E252 RNA hybridized with the early probe.
3. E252 RNA hybridized with the middle probe.


1·45 kb in vitro transcript
pR
2747
74·7%
cpl
cII
fil
dhr
tR1
CP79
CP80
CP81
E
M
250 bp
1
in vitro
transcripts
2
E
3
M
O
1·54
1·45
0·59
0·29
E2
E1
M1
3·387
2·904
2·193
1·541
1·027

transcript present *in vivo*, nor was it the major transcript present. A lower molecular weight band (E1) was present at a much greater intensity than E2 (Figure 6.2, lane 2). The middle probe detected a very intense band (M1), of a high molecular weight (Figure 6.2, lane 3). Since the early probe did not detect this transcript, the 5'-end of M1 RNA is at least 178 bases to the right of *p*R. This simple transcription pattern is consistent with that expected if middle transcription is due to new promotion (Chapter 1.4).

To determine the molecular weight of early lytic and middle RNA transcripts, RNA from the derepressed 186 lysogen and RNA molecular weight standards (Chapter 2.11.2) were fractionated on a lower percentage agarose gel (1%) to obtain better resolution of the bands. Northern analysis, using probes specific to the early lytic or middle region, was used to detect early lytic and middle RNA. The transcription pattern obtained in Figure 6.3, was more complex than the transcription pattern shown in Figure 6.2. M1 RNA was resolved into two transcripts, which were sized at 2.8 kb and 3.1 kb (Figure 6.3, lane 3). E2 RNA was also resolved into two transcripts, which were sized at 1.4 kb and 1.5 kb (Figure 6.3, lane 2). E1 RNA was sized at 1.1 kb. These transcripts will henceforth be referred to by their determined sizes. To further complicate the transcription pattern, a higher molecular weight RNA transcript sized at 4.0 kb, was detected with the early probe (Figure 6.3). This RNA transcript was also visualized after longer exposure of filters hybridized with the middle probe in Figure 6.3, or the early probe and middle probe in Figure 6.2 (data not shown). This 4.0 kb RNA transcript is therefore probably due to initiation at *p*R and readthrough of the *t*R1 terminator (Chapter 3.2.2c; Figure 6.2, 6.3).

Having detected the transcripts encoded in the early lytic and middle regions, it was important to determine the approximate positions of the 5'-ends and 3'-ends of these transcripts. The 5'-end of all transcripts

The major RNA transcripts are indicated by the arrows. The sizes of these transcripts were determined using RNA molecular weight standards (Chapter 2.11.2)

RNA prepared from the non-lysogen (E251) after a mock heat-induction showed no hybridization with either the early or middle probes (data not shown).

Figure 6.3 186 early lytic and middle transcripts after fractionation of RNA on a 1% agarose gel.

This Figure shows the transcription pattern of the 186 early lytic and middle region after fractionation of RNA on a 1% agarose gel. The positions on the DNA sequence of the probes used to detect 186 early lytic and middle RNA, are shown diagrammatically. Genes are represented by the boxed regions. The pR promoter, tR1 terminator and the early lytic transcript are shown. The tR1 terminator, represented by the hairpin structure, is not drawn to scale. The probes used are as follows :

Early probe (E) : HpaII (3512-3689)

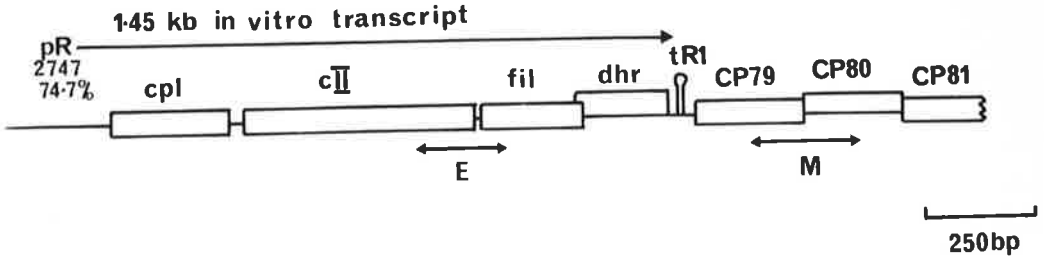
Middle probe (M) : BglII-HpaII (4249-4480).

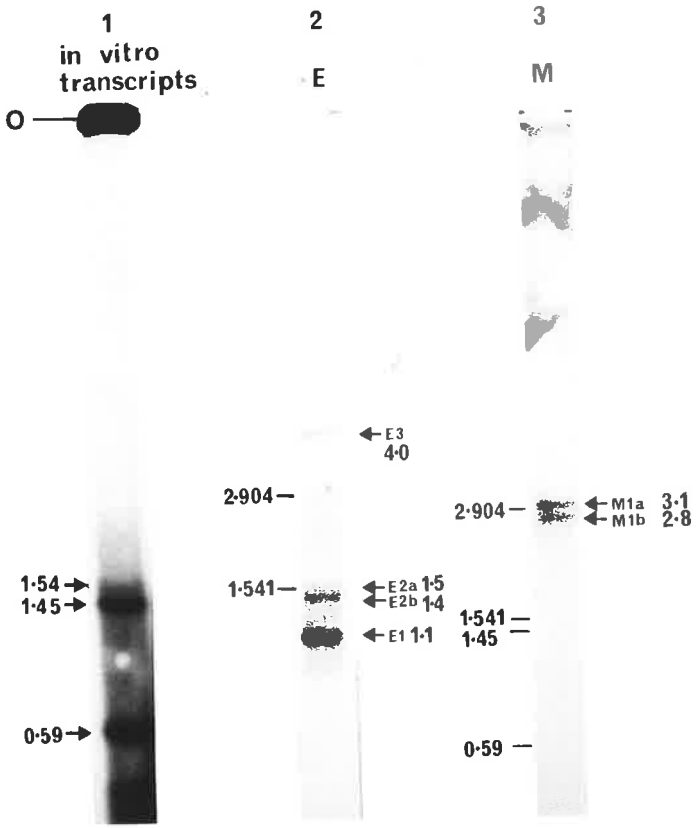
The construction of these probes is described in detail in the legend to Figure 6.1.

Twenty ug of RNA, which was prepared 20 min after the heat-induction of E252 [E251 (186 cItsp (1))] (Chapter 2.36.1, 2.36.2), was denatured with glyoxal and fractionated on a 1% agarose gel (Chapter 2.36.3a, 2.29.2). 186 in vitro transcripts (prepared as described in Chapter 2.36.5) and RNA molecular weight standards (Chapter 2.11.2) were also denatured with glyoxal and fractionated on the 1% agarose gel. RNA was transferred bi-directionally to nitrocellulose and hybridized with the probes specific for early lytic RNA or middle RNA (Chapter 2.36.3). The autoradiographs were exposed for 1 week at -80^{o}C with an intensifying screen.

Gel Tracks :

1. 186 in vitro transcripts
2. E252 RNA probed with the early probe
3. E252 RNA probed with the middle probe


1·45 kb in vitro transcript
pR
2747
74·7%
cpI
cII
fil
dhr
tR1
CP79
CP80
CP81
E
M
250bp


1
in vitro
transcripts
2
E
3
M
O
E3
4·0
2·904
1·541
E2a 1·5
E2b 1·4
E1 1·1
1·54
1·45
0·59
2·904
M1a 3·1
M1b 2·8
1·541
1·45
0·59

hybridizing with an early probe (HinfI-HpaII, sequence coordinates 2819-2935; the same probe as used in this work to detect the early lytic transcripts) were determined by Kalionis (1985) to be located at the pR promoter (position 2747). The work described below is concerned with determining the approximate 3'-ends of the 1.1 kb, 1.4 kb and 1.5 kb early lytic transcripts and the 5'-ends and 3'-ends of the 3.1 kb and 2.8 kb middle transcripts.

6.2.3 Mapping the 3'-Ends of the 1.5 kb, 1.4 kb and 1.1 kb Early Lytic Transcripts.

The approximate 3'-ends of the 1.5 kb, 1.4 kb and 1.1 kb early lytic transcripts were determined by Northern analysis using the probes shown in Figure 6.4. Four hybridization patterns were detected (Figure 6.4): pattern #1, where the 1.1 kb, 1.4 kb and 1.5 kb transcripts were detected; pattern #2, where the 1.4 kb and 1.5 kb transcripts were detected; pattern #3, where only the 1.5 kb transcript was detected; and pattern #4, where none of the early lytic transcripts were detected. The transcription patterns obtained using the probes shown in Figure 6.4, positioned the 3'-ends of the early lytic transcripts, as described below. However, these positions are only approximate, since a partial overlap of the 3'-end of a transcript with the probe may not be detected due to poor hybridization. The 3'-end of the 1.1 kb transcript was shown to be within the HpaII-HaeII (3690-3732) region, since this transcript was detected using the HpaII-HaeII (3690-3732) probe, but not with the HhaI (3734-3934) probe (Figure 6.4). The 3'-end of the 1.4 kb transcript was positioned within the HincII-HinfI (3951-4124) region since the HincII-HpaII (3951-4087) probe hybridized to the 1.4 kb transcript, but the HinfI (4125-4176) probe did not detect this transcript (Figure 6.4). The 1.5 kb transcript was detected with the HpaII-BglII (4088-4248) and with the HinfI (4125-4176) probes, but not with the BglII-HinfI (4249-4335) probe (Figure 6.4),

indicating that the 3'-end of this transcript was between the HinfI-BglII (4125-4248) region.

The position obtained for the 3'-end of the 1.4 kb and 1.5 kb transcripts are consistent with termination at the potential terminator structures discussed previously in Chapter 3.2.2(c) (Figure 3.2a, 3.4). Structure #2 (tR1), (4070-4089) and structure #3 (4091-4116) are within the region (3951-4124) where the 3'-end of the 1.4 kb transcript maps (Figure 6.11). Therefore, it is possible that the 1.4 kb transcript terminates at one of these two structures. Since tR1 is a potential Rho-independent terminator and is the more stable of these two structures (Chapter 3.2.2c), it is more likely that the 1.4 kb transcript terminates at this terminator structure. The 3'-end of the 1.5 kb transcript also maps in a region (4125-4248) where two potential terminators are located, namely structures #4 (4147-4169) and structure #5 (4202-4222), (Chapter 3.2.2c; Figure 6.11), and this transcript may be due to termination at either or both of these structures. Structures #4 and #5 are potential Rho-dependent terminator structures since the stem-loops are not followed by consecutive T-residues (Chapter 3.2.2c). Structures #4 and #5 will henceforth be referred to as tR2 and tR3, respectively. The 3'-end of the 1.1 kb transcript is located in a region (3690-3732) where no potential terminator structures were predicted. This transcript may result from termination at a transcription terminator that was not detected in the sequence analysis (Chapter 3.2.2c), or from RNA processing or degradation.

To investigate whether the 1.4 kb transcript was due to termination at tR1, a deletion of this stem-loop structure and the following consecutive T-residues was constructed by oligonucleotide site-directed mutagenesis using the deltR1 oligonucleotide shown in Chapter 2.5. The deltR1 oligonucleotide was used to create a deletion of this region (sequence coordinates 4070-4098) in the M13-clone, mEC400, using the method described in Chapter 2.35 (Figure 6.11). The deletion mutant created by this method

Probe A gave pattern #1; showing all 3 early lytic transcripts. Probe B and C gave pattern #2; showing the 1.4 kb and the 1.5 kb transcripts. Probe D and E gave pattern #3; showing the 1.5 kb transcript. Probe F gave pattern #4; showing none of the early lytic transcripts.

The 1.1 kb, 1.4 kb and 1.5 kb early lytic transcripts are indicated by the arrows. RNA molecular weight markers (Chapter 2.11.2) are indicated next to each track.

RNA prepared from the non-lysogen (E251) after a mock heat-induction showed no hybridization with any of these probes (data not shown).

Figure 6.4 Mapping the 3'-ends of the 186 early lytic transcripts.

A diagrammatic representation of the probes used to establish the 3'-ends of the 1.1 kb, 1.4 kb and 1.5 kb early lytic transcripts. The positions of relevant restriction sites, are shown. The sequence coordinates of the restriction sites refers to the position on the r-strand to the right of the restriction cut (Figure 6.1). The positions of the probes, are shown underneath the restriction map. The construction of these probes is described in the legend to Figure 6.1. The probes are given numbers according to the transcription pattern they give, as described below and in the text.

RNA was prepared 20 min after the heat-induction of E252 [E251 (186 cItsp (1))] (Chapter 2.36.1, 2.36.2). Twenty ug of RNA was denatured with glyoxal and fractionated on a 1.0% or 1.5% agarose gel (Chapter 2.36.3a, 2.29.2). RNA was transferred bi-directionally to nitrocellulose and hybridized with the relevent probes (Chapter 2.36.3). The autoradiographs were exposed for 2 days to 1 week at -80^{o}C with an intensifying screen.

The gel tracks represent E252 RNA hybridized with the probes labelled A-F (shown beneath the restriction map). These probes contain the following regions from 186 :

A. HpaII-HaeII (3690-3732)

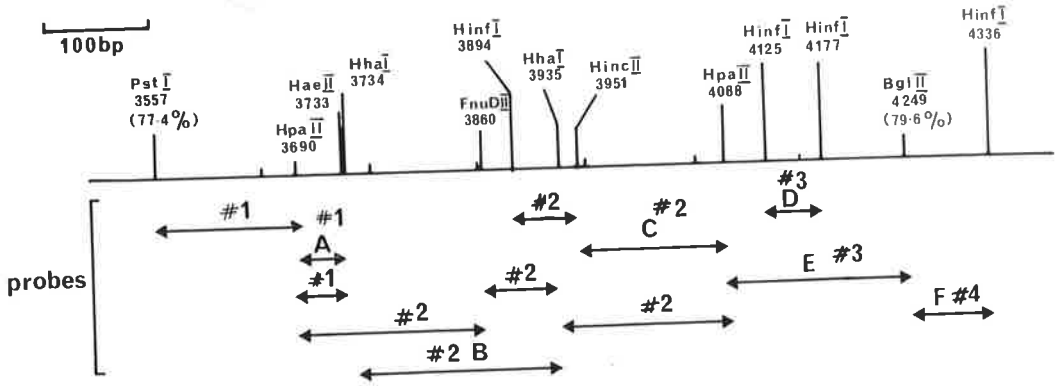
B. HhaI (3734-3934)

C. HincII-HpaII (3951-4087)

D. HinfI (4125-4176)

E. HpaII-BglII (4088-4248)

F. BglII-HinfI (4249-4335)


100bp
Pst I 3557 (77·4%)
Hae II 3733
Hpa II 3690
Hha I 3734
Hinf I 3894
FnuD II 3860
Hha I 3935
Hinc II 3951
Hpa II 4088
Hinf I 4125
Hinf I 4177
Bgl II 4249 (79·6%)
Hinf I 4336
probes
#1
A
#2
B
C
D
E
F #4
#3

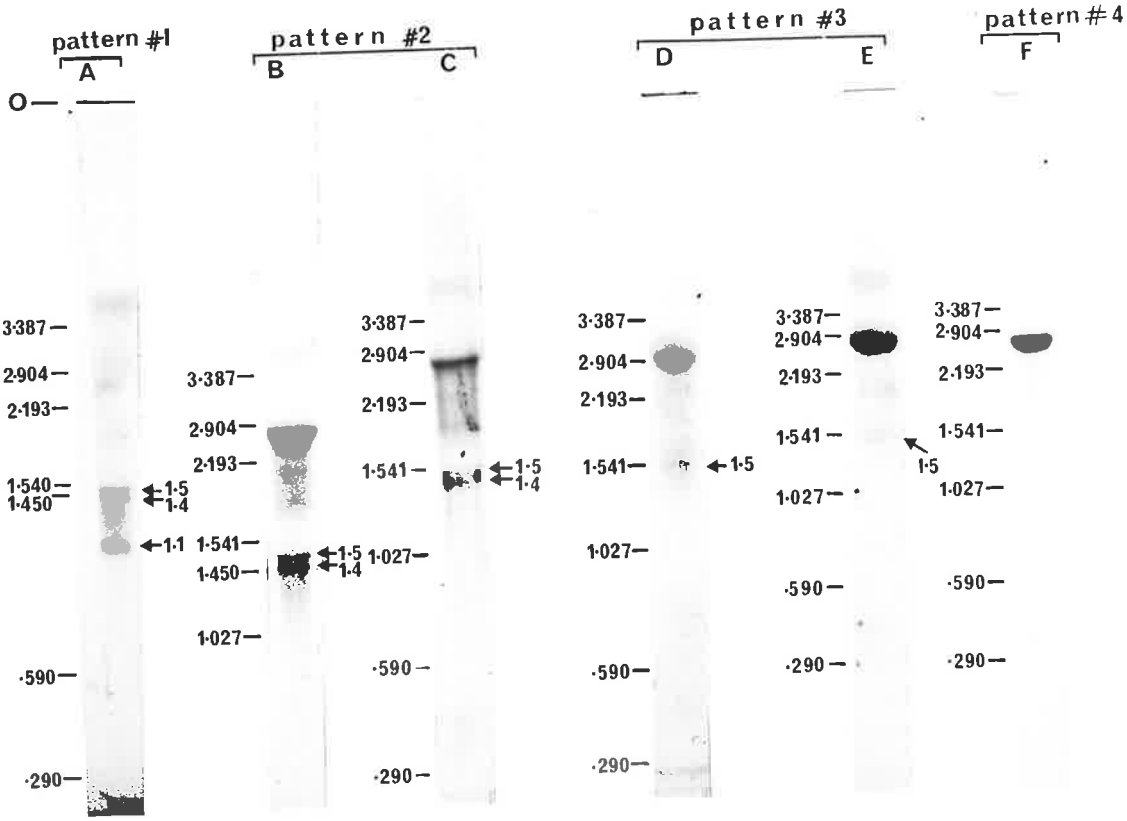

pattern #1
A
pattern #2
B
C
pattern #3
D
E
pattern #4
F
O
3·387
2·904
2·193
1·540
1·450
1·541
1·027
·590
·290
1·5
1·4
1·1

Figure 6.5 The early lytic transcription pattern of 186 cItspdeltR1 (22).

This Figure shows the transcription pattern of 186 cItspdeltR1 compared with the wild-type (186 cItsp) obtained using a probe specific for early lytic RNA [the HinfI-HpaII (2819-2935) probe]. The construction of this probe is described in the legend to Figure 6.1. The position on the DNA sequence of the probe specific for 186 early lytic RNA, is shown diagrammatically. Genes are represented by the boxed regions. The pR promoter and the tR1 terminator are shown. The position of the deltR1 deletion, is shown. The arrow next to the pR promoter represents the direction of transcription. The tR1 terminator, represented by the hairpin structure, is not drawn to scale.

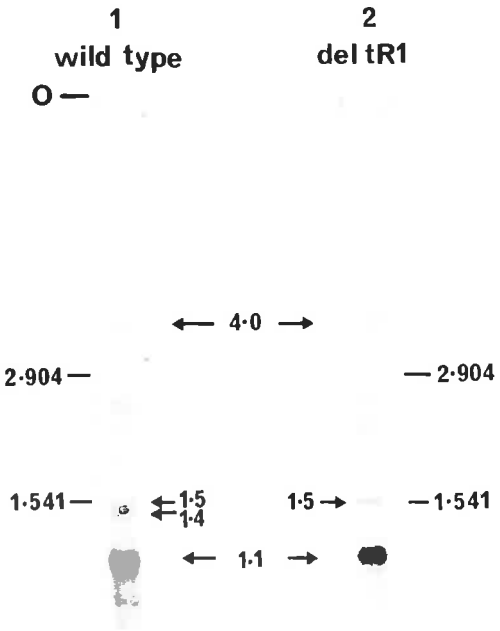
RNA was prepared 20 min after the heat-induction of E4135 [E251 (186 cItspdeltR1 (22))] or E252 [E251 (186 cItsp (1))] (Chapter 2.36.1, 2.36.2). Twenty ug of RNA was denatured with glyoxal and fractionated on a 1.5% agarose gel (Chapter 2.36.3a, 2.29.2). RNA was transferred bi-directionally to nitrocellulose and one filter was hybridized with the probe specific to the early lytic region (Chapter 2.36.3). The autoradiograph was exposed for 7 hours at -80°C with an intensitying screen.

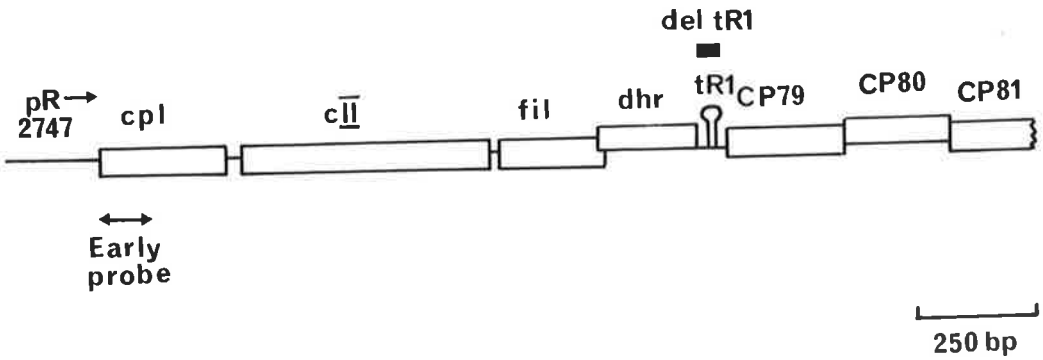
The gel tracks represent RNA from the following lysogens hybridized with the early probe.

1. wild-type : RNA from E252 [E251 (186 cItsp (1))]

2. deltR1 : RNA from E4135 [E251 (186 cItspdeltR1 (22))]

The major RNA transcripts are indicated by the arrows. RNA molecular weight markers (Chapter 2.11.2) are indicated on the Figure.


1
wild type
2
del tR1
O
4·0
2·904
2·904
1·541
1·5
1·4
1·5
1·541
1·1


del tR1
pR
2747
cpI
cII
fil
dhr
tR1
CP79
CP80
CP81
Early
probe
250 bp

was confirmed by DNA sequencing using the M13 universal primer, and then recombined *in vitro* into 186 to create the phage 186 cItspdeltR1 (22), as described in Chapter 2.2.1. (186 deltR1 gave a burst size similar to the wild-type; data not shown.) A lysogen of 186 cItspdeltR1 was heat-induced for 20 min and RNA was prepared, glyoxylated, fractionated on a 1.5% agarose gel and transferred to a nitrocellulose filter (Chapter 2.36.1, 2.36.2, 2.36.3). This filter was hybridized with the HinfI-HpaII (2819-2935) early probe, described in Figure 6.1, and the results obtained are presented in Figure 6.5. RNA prepared from the 186 cItspdeltR1 lysogen did not give the 1.4 kb transcript. This result is consistent with the prediction that the 1.4 kb transcript terminates at the tR1 terminator.

In summary, these results have shown that the 3'-end of the 1.1 kb transcript maps within the region 3690-3732, and that the 3'-end of the 1.5 kb transcript maps within the 4125-4248 region. The 1.4 kb transcript most likely terminates within the region 4070-4098, which encodes the tR1 terminator, suggesting that this potential terminator is functional *in vivo*. Termination at these positions would result in transcripts of 0.94-0.99 kb, 1.32-1.35 kb and 1.38-1.5 kb, all of which are consistent (within 10%) with the sizes determined for the three major early lytic transcripts (1.1 kb, 1.4 kb and 1.5 kb) by Northern analysis (Chapter 6.2.2).

6.2.4 Mapping the 5'-Ends of the 2.8 kb and 3.1 kb Middle Transcripts.

To determine the approximate 5'-ends of the 2.8 kb and 3.1 kb transcripts, the Northern analysis mapping procedure described in Chapter 6.2.3 was carried out, using the probes shown in Figure 6.6. These probes gave three different hybridization patterns: pattern #1, showing the early lytic transcripts, but no middle transcripts; pattern #2, showing the 3.1 kb middle transcript; and pattern #3, showing both the 2.8 kb and 3.1 kb middle transcripts (Figure 6.6). The transcription patterns obtained

using the probes shown in Figure 6.6, positioned the 5'-ends of the middle transcripts, as described below. The 5'-end of the 3.1 kb transcript was located within the HhaI-FnuDII (3734-3859) region, since the 3.1 kb transcript was detected using the HhaI (3734-3934) probe and the HpaII-FnuDII (3690-3859) probe, but not with the HpaII-HhaI (3690-3733) probe (Figure 6.1, 6.6). The 5'-end of the 2.8 kb transcript was located within the HpaII-BglII (4088-4248) region, since the HpaII-BglII (4088-4248) probe detected the 2.8 kb transcript, whereas the HhaI-HpaII (3934-4087) probe did not detect this transcript (Figure 6.1, 6.6). Furthermore, the 5'-end of the 2.8 kb transcript is predicted to lie in the 4070-4098 region, since it was noted that the deletion mutant 186 deltR1 (which contains a deletion of the region 4070-4098; Chapter 6.2.3) did not give rise to the 2.8 kb transcript (Figure 6.6, lane 7 compared with lane 6). This result indicates that the 4070-4098 region is important for the production of the 2.8 kb transcript and it is possible that the 5'-end of this transcript is located in this region.

Having mapped the 5'-end of the 3.1 kb transcript to a small region by the Northern mapping procedure, it was possible to further define the 5'-end by using the technique of primer extension (Chapter 2.36.6). In this method, a radioactive primer is annealed to total cellular RNA and then extended with AMV reverse transcriptase in the presence of all four unlabelled dNTPs. The primer will specifically hybridize to 186 RNA transcripts that contain the complementary sequence and will be extended to the 5'-end of the RNA. This generates a specific extension product(s) that can be accurately sized by comparing its mobility to a DNA sequencing ladder on a 5% denaturing polyacrylamide gel (Chapter 2.29.3b).

The 41 b HinfI-HhaI (3894-3934) fragment (which is located 35 b-160 b from the predicted 5'-end of the 3.1 kb transcript) was used as a primer in an extension reaction on RNA isolated 20 min or 35 min after heat-induction of a 186 cItsp lysogen, or 35 min after the mock heat-induction of a non-

Probe A gave pattern #1; showing none of the middle transcripts. Probe B, C and D gave pattern #2; showing the 3.1 kb middle transcript. Probe E gave pattern #3; showing both the 3.1 kb and 2.8 kb middle transcripts.

Probe F was hybridized to RNA from the wild-type lysogen (E252) and from the 186 deltR1 lysogen (E4135), as indicated on the Figure.

The 2.8 kb and 3.1 kb middle transcripts are indicated by the arrows. RNA molecular weight markers (Chapter 2.11.2) are indicated next to each track.

RNA prepared from the non-lysogen (E252) after a mock heat-induction showed no hybridization with any of these probes (data not shown).

Figure 6.6 Mapping the 5'-end of the 186 middle transcripts.

A diagrammatic representation of the probes used to establish the 5'-ends of the 2.8 kb and 3.1 kb middle transcripts. The positions of relevant restriction sites, are shown. The sequence coordinates of the restriction sites refers to the position on the r-strand to the right of the restriction cut (Figure 6.1). The positions of the probes are shown underneath the restriction map. The construction of these probes is described in the legend to Figure 6.1 The probes are given numbers according to the transcription pattern they give, as described below and in the text.

RNA was prepared 20 min after the heat-induction of E252 [E251 (186 cItsp (1))] or E4135 [E251 (186 cItspdeltR1 (22))] (Chapter 2.36.1, 2.36.2). Twenty ug of RNA was denatured with glyoxal and fractionated on a 1.0% or 1.5% agarose gel (Chapter 2.36.3a, 2.29.2). RNA was transferred bi-directionally to nitrocellulose and hybridized with the relevant probes (Chapter 2.36.3). The autoradiographs were exposed for 4 hours to 1 week at -80^{o}C with an intensifying screen.

The gel tracks represent RNA hybridized with the probes labelled A-F (shown beneath the restriction map). These probes contain the following regions from 186 :

A. HpaII-HaeII (3690-3732)

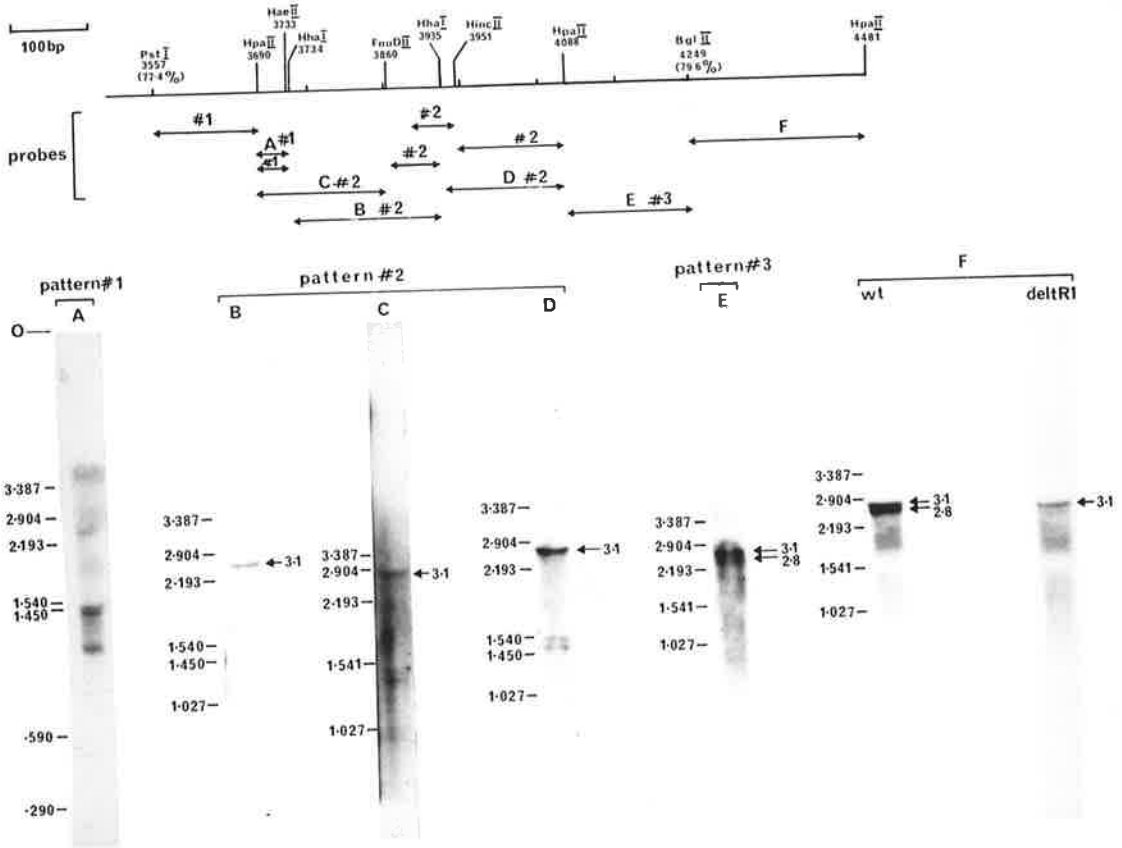
B. HhaI (3734-3934)

C. HpaII-FnuDII (3690-3859)

D. HhaI-HpaII (3935-4087)

E. HpaII-BglII (4088-4248)

F. BglII-HpaII (4249-4480)


100bp
Pst I 3557 (77·4%)
Hae III 3233
Hpa II 3690
Hha I 3734
Fnu DII 3860
Hha I 3935
Hinc II 3951
Hpa II 4088
Bgl II 4249 (79·6%)
Hpa II 4481
probes
#1
A #1
#1
#2
#2
#2
F
C #2
D #2
B #2
E #3
pattern #1
A
pattern #2
B
C
D
pattern #3
E
F
wt
deltR1
O
3·387
2·904
2·193
1·540
1·450
·590
·290
1·027
1·541
3·1
2·8

lysogen. Six major extension products were obtained for RNA isolated from the heat-induced lysogen, but not from the non-lysogen (Figure 6.7). These major extension products are marked with arrows and their positions are shown on the DNA sequence in Figure 6.8. The largest extension product was a double-band, the 5'-ends of which corresponded to the positions 3768 and 3770 ± 2 bases. The same position was obtained for the 5'-ends of the largest extension products of other primers (shown in Figure 6.8) used (data not shown). The smaller extension products may represent real 5'-ends or may result from pausing of AMV reverse transcriptase at secondary structures in the RNA (McKnight et al., 1981). However, the first possibility was considered unlikely since the smaller extension products obtained using other primers did not give the same 5'-ends as obtained for the 41 b primer (data not shown).

In summary, these results have shown that the 5'-ends of the 3.1 kb transcript are located at positions 3768 and 3770 ± 2 b and that the 5'-end of the 2.8 kb transcript is located in the region 4088-4248. Furthermore, the region 4070-4098 appears to be important for the production of the 2.8 kb transcript, since the 2.8 kb transcript is not detected when this region is deleted (in 186 deltR1). Since neither of these transcripts begin immediately 3' to the potential rightward promoters (pR784 at 3873 and pR785 at 3911) predicted in Chapter 3.2.2(c), it is unlikely that these promoters are functional.

6.2.5 Mapping the 3'-Ends of the 2.8 kb and 3.1 kb Middle Transcripts.

The 3'-ends of the 2.8 kb and 3.1 kb transcripts are predicted, on the basis of their size and the position of their 5'-ends, to be located in the region ~6600-6900 (allowing a 10% error in the size determination results obtained from an agarose gel; Chapter 6.2.2). To map the position of the 3'-ends of these transcripts, Northern analysis was carried out using the probes SacII-HpaII (6268-6419) and PstI-FnuDII (6606-6840) (Figure 6.1).

Figure 6.7 Primer extension of the 41 b HinfI-HhaI (3894-3934) primer on RNA from the heat-induced 186 lysogen : The 5'-end of the 3.1 kb middle transcript.

RNA was prepared 20 min or 35 min after the heat-induction of E252 [E251 (186 cItsp (1))] or 35 min after the mock heat-induction of the non-lysogen E251 (Chapter 2.36.1, 2.36.2). The 41 b HinfI-HhaI (3894-3934) primer was constructed as described in the legend to Figure 6.8. This primer was denatured, annealed to 10 ug of RNA and extended with AMV reverse transcriptase in the presence of all four NTPs, as described in Chapter 2.36.6. Electrophoresis was as described in Chapter 2.29.3(b). The autoradiograph was exposed for 24 hours at -80°C with an intensifying screen.

Gel Tracks 1. Primer extension of RNA prepared 20 min after the heat-induction of the 186 cItsp lysogen.

2. Primer extension of RNA prepared 35 min after the heat-induction of the 186 cItsp lysogen.
3. Primer extension of RNA prepared 35 min after the mock heat-induction of the non-lysogen.
4. Identical to track 1 except that AMV reverse transcriptase was omitted from the reaction mix.
5. Identical to track 2 except that AMV reverse transcriptase was omitted from the reaction mix.
6. Identical to track 3 except that AMV reverse transcriptase was omitted from the reaction mix.

A sequencing ladder was included to provide size markers. The size from the 5'-end of the M13 universal primer is given on the left. The major extension products and the sizes of these bands are marked on the right.

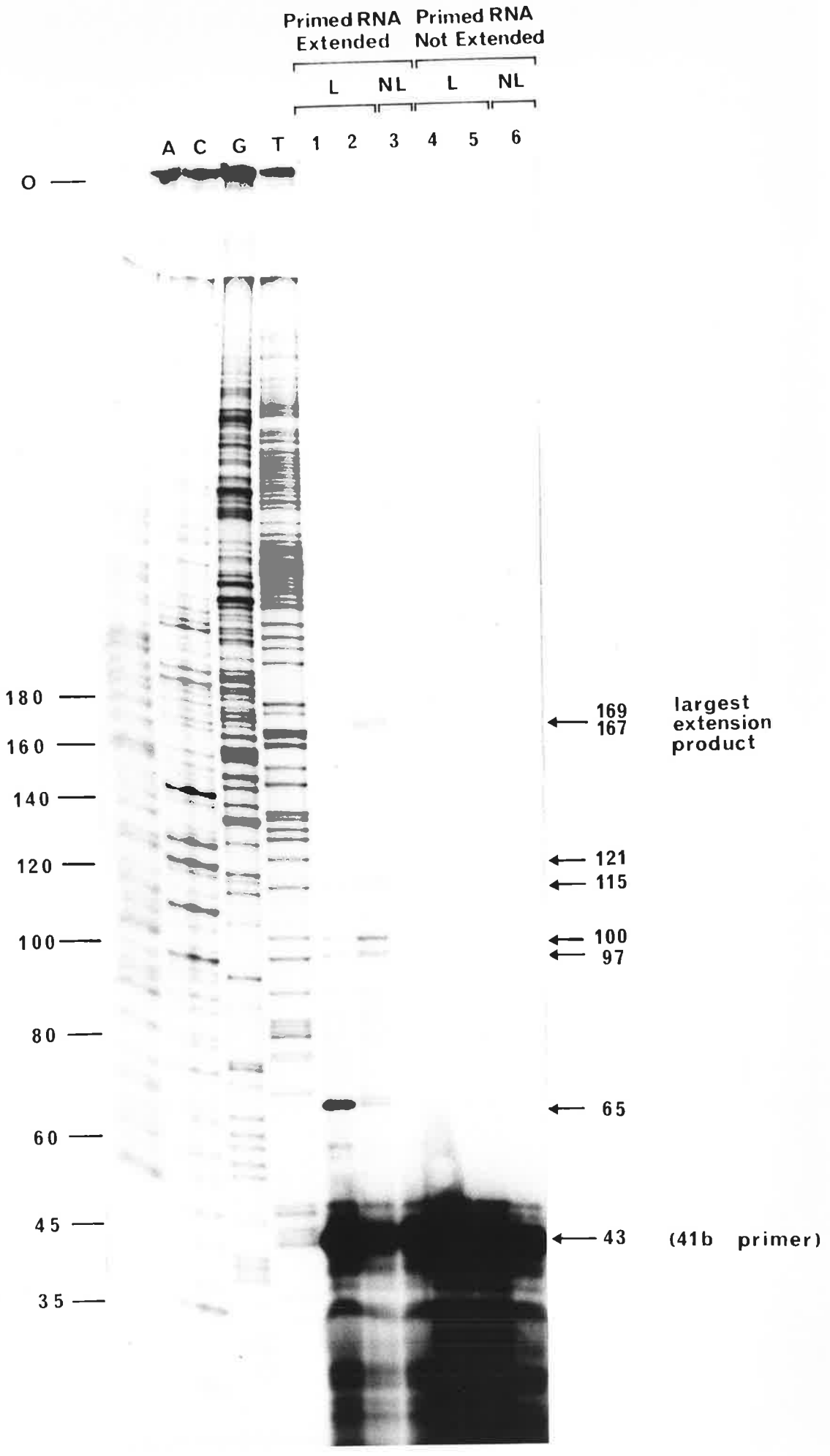

Primed RNA Extended
Primed RNA Not Extended
L
NL
L
NL
A C G T 1 2 3 4 5 6
O
180
160
140
120
100
80
60
45
35
169
167
largest extension product
121
115
100
97
65
43
(41b primer)

Figure 6.8 Positions of primer extension products on the DNA sequence.

The DNA sequence of the PstI-Bg1II (3556-4249) region from 186 cItsp is shown. The upper strand is the l-strand. Relevant restriction sites are marked. The arrows above and below the restriction sites refer to the positions of cleavage by the restriction enzymes. The region HhaI-FnuDII (3734-3859), which was determined to contain the 5'-end of the 3.1 kb transcript by Northern analysis (Figure 6.6), is shaded. Primers used to determine the location of the 5'-end of the 3.1 kb transcript are shown beneath the sequence and are identified by their size. These primers are as follows :

34 b FnuDII-HinfI (3860-3893)

35 b HinfI-FnuDII (3894-3928)

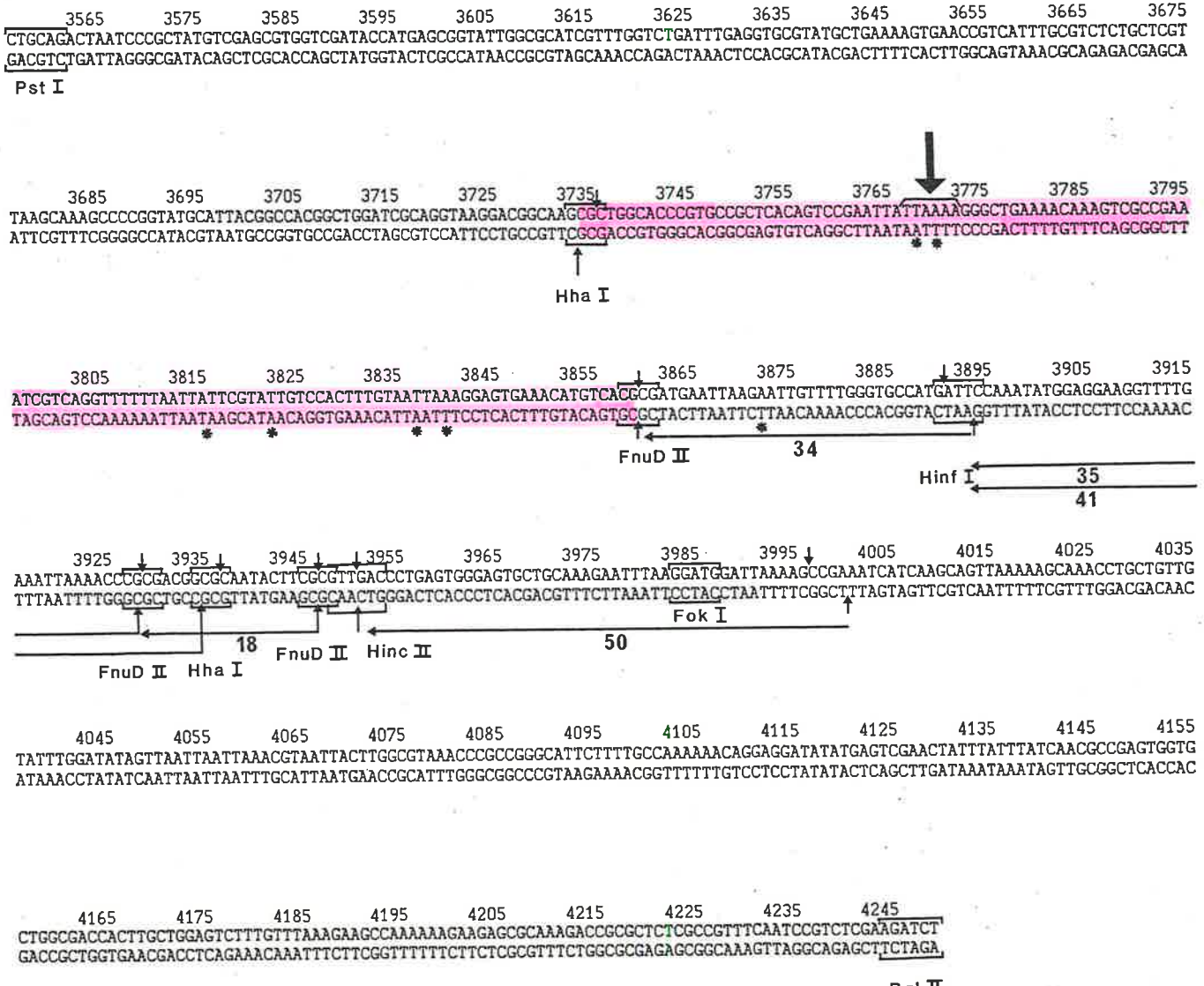
41 b HinfI-HhaI (3894-3934)

18 b FnuDII (3929-3946)

50 b HincII-FokI (3951-4000)

These primers were constructed from mEC406 by digestion with the appropriate restriction enzymes (Chapter 2.34.1).

The positions on the DNA sequence, of the extension products obtained for the 41 b HinfI-HhaI primer, are indicated by *. The position of the largest extension product obtained with all 5 primers, is indicated by the large vertical arrow.


3565 3575 3585 3595 3605 3615 3625 3635 3645 3655 3665 3675
CTGCAGACTAATCCCGCTATGTCGAGCGTGGTCGATACCATGAGCGGTATTGGCGCATCGTTTGGTCTGATTTGAGGTGCGTATGCTGAAAAGTGAACCGTCATTTGCGTCTCTGCTCGT
GACGTCTGATTAGGGCGATACAGCTCGCACCAGCTATGGTACTCGCCATAACCGCGTAGCAAACCAGACTAAACTCCACGCATACGACTTTTCACTTGGCAGTAAACGCAGAGACGAGCA
Pst I
3685 3695 3705 3715 3725 3735 3745 3755 3765 3775 3785 3795
TAAGCAAAGCCCCGGTATGCATTACGGCCACGGCTGGATCGCAGGTAAGGACGGCAAGCGCTGGCACCCGTGCCGCTCACAGTCCGAATTATTAAAAGGGCTGAAAACAAAGTCGCCGAA
ATTCGTTTCGGGGCCATACGTAATGCCGGTGCCGACCTAGCGTCCATTCCTGCCGTTCGCGACCGTGGGCACGGCGAGTGTCAGGCTTAATAATTTTCCCGACTTTTGTTTCAGCGGCTT
Hha I
3805 3815 3825 3835 3845 3855 3865 3875 3885 3895 3905 3915
ATCGTCAGGTTTTTTAATTATTCGTATTGTCCACTTTGTAATTAAAGGAGTGAAACATGTCACGCGATGAATTAAGAATTGTTTTGGGTGCCATGATTCCAAATATGGAGGAAGGTTTTG
TAGCAGTCCAAAAAATTAATAAGCATAACAGGTGAAACATTAATTTCCTCACTTTGTACAGTGCGCTACTTAATTCTTAACAAAACCCACGGTACTAAGGTTTATACCTCCTTCCAAAAC
FnuD II
34
Hinf I
35
41
3925 3935 3945 3955 3965 3975 3985 3995 4005 4015 4025 4035
AAATTAAAACCCGCGACGGCGCAATACTTCGCGTTGACCCTGAGTGGGAGTGCTGCAAAGAATTTAAGGATGGATTAAAAGCCGAAATCATCAAGCAGTTAAAAAGCAAACCTGCTGTTG
TTTAATTTTGGGCGCTGCCGCGTTATGAAGCGCAACTGGGACTCACCCTCACGACGTTTCTTAAATTCCTACCTAATTTTCGGCTTTAGTAGTTCGTCAATTTTTCGTTTGGACGACAAC
Fok I
18
FnuD II
Hha I
FnuD II
Hinc II
50
4045 4055 4065 4075 4085 4095 4105 4115 4125 4135 4145 4155
TATTTGGATATAGTTAATTAATTAAACGTAATTACTTGGCGTAAACCCGCCGGGCATTCTTTTGCCAAAAAACAGGAGGATATATGAGTCGAACTATTTATTTATCAACGCCGAGTGGTG
ATAAACCTATATCAATTAATTAATTTGCATTAATGAACCGCATTTGGGCGGCCCGTAAGAAAACGGTTTTTTGTCCTCCTATATACTCAGCTTGATAAATAAATAGTTGCGGCTCACCAC
4165 4175 4185 4195 4205 4215 4225 4235 4245
CTGGCGACCACTTGCTGGAGTCTTTGTTTAAAGAAGCCAAAAAAGAAGAGCGCAAAGACCGCGCTCTCGCCGTTTCAATCCGTCTCGAAGATCT
GACCGCTGGTGAACGACCTCAGAAACAAATTTCTTCGGTTTTTTCTTCTCGCGTTTCTGGCGCGAGAGCGGCAAAGTTAGGCAGAGCTTCTAGA
Bgl II

The results presented in Figure 6.9, reveal that the SacII-HpaII (6268-6419) probe detected the 2.8 kb and 3.1 kb middle transcripts and the 4.0 kb transcript (which begins at pR), whereas the PstI-FnuDII (6606-6840) probe did not detect any of these transcripts. This result indicates that the 2.8 kb and 3.1 kb middle transcripts and the 4.0 kb transcript terminate within the region 6268-6605.

Although the PstI-FnuDII (6606-6840) probe did not detect the 2.8 kb, 3.1 kb or 4.0 kb transcripts it did weakly hybridize to two transcripts sized at 2.1 kb and 1.8 kb. The 2.1 kb transcript was also detected with the SacII-HpaII (6268-6419) probe, but was not detected with the HinfI (5513-5606) probe (Figure 6.1, 6.9). These results indicate that the 5'-end of the 1.8 kb transcript is located within the 6420-6840 region and the 5'-end of the 2.1 kb transcript is located within the 5607-6419 region. It was not within the scope of this work to further investigate these transcripts.

RNA dot blot analysis (Chapter 2.36.4) was used to quantitate the amount of RNA hybridizing to the SacII-HpaII (6268-6419) and the PstI-FnuDII (6606-6840) probes and to another probe FnuDII-PstI (6533-6605) (Figure 6.1). These results showed that the transcription of the PstI-FnuDII (6606-6840) and the FnuDII-PstI (6533-6605) region is approximately 10% of the level of transcription of the SacII-HpaII (6268-6419) region (data not shown). These results indicate that most RNA transcribing the middle region terminates in the region 6268-6532. The RNA detected in the region promoter-distal to the 6268-6532 region, by RNA dot blot analysis, is probably largely represented by the 1.8 kb and 2.1 kb transcripts, which were detected with the PstI-FnuDII (6606-6840) probe.

Analysis of the DNA sequence of this region (6268-6605) revealed several potential transcription termination structures (Chapter 2.38, 3.2.2c). These structures are shown in Figure 6.10. The most stable of these structures, tM1, was also noted by Sivaprasad (1984), and contains a

Figure 6.9 Mapping the 3'-ends of the 186 middle transcripts.

This Figure shows the transcription pattern obtained using probes located in the region predicted to encode the 3'-ends of the 186 middle transcripts. The positions on the DNA sequence of the probes used in this study, are shown diagrammatically. The appropriate restriction sites are shown. The sequence coordinates of the restriction sites refer to the position on the r-strand to the right of the restriction cut (Figure 6.1). Genes are represented by the boxed regions. The position of the tM1 terminator (described in Figure 6.10), is also shown. The tM1 terminator, represented by the hairpin structure, is not drawn to scale.

The probes used are as follows :

A. HinfI (5513-5606)

B. SacII-HpaII (6268-6419)

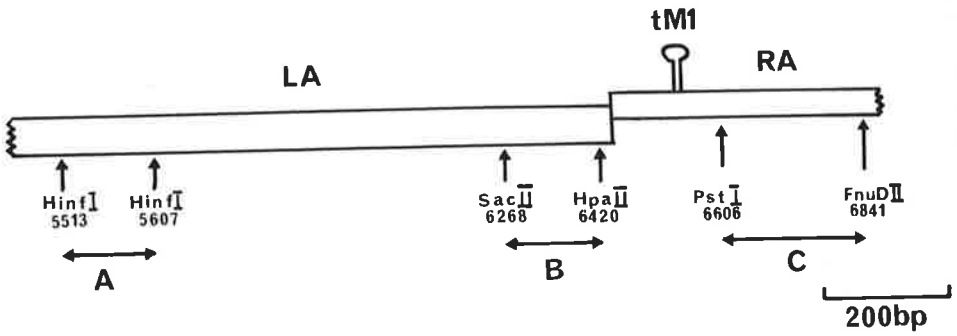
C. PstI-FnuDII (6606-6840)

The construction of these probes is described in the legend to Figure 6.1.

RNA was prepared 20 min after the heat-induction of E252 [E251 (186 cItsp (1))] (Chapter 2.36.1, 2.36.2). Twenty ug of RNA was denatured with glyoxal and fractionated on a 1.5% agarose gel (Chapter 2.36.3a, 2.29.2). RNA was transferred bi-directionally to nitrocellulose and filters were hybridized with either of the three probes described above (Chapter 2.36.3). The autoradiographs were exposed for 1 day to 1 week (1 day for probes A and B, 1 week for probe C) at $-80^{o}C$ with an intensifying screen.

The gel tracks are labelled A, B and C and correspond to RNA, which has been hybridized with each of the probes described above. The major RNA transcripts are indicated by the arrows. RNA molecular weight markers (Chapter 2.11.2) are indicated on the Figure.

RNA prepared from the non-lysogen (E251) after a mock heat-induction did not hybridize with any of these probes (data not shown).


tM1
LA
RA
HinfI 5513
HinfI 5607
SacII 6268
HpaII 6420
PstI 6606
FnuDII 6841
A
B
C
200bp

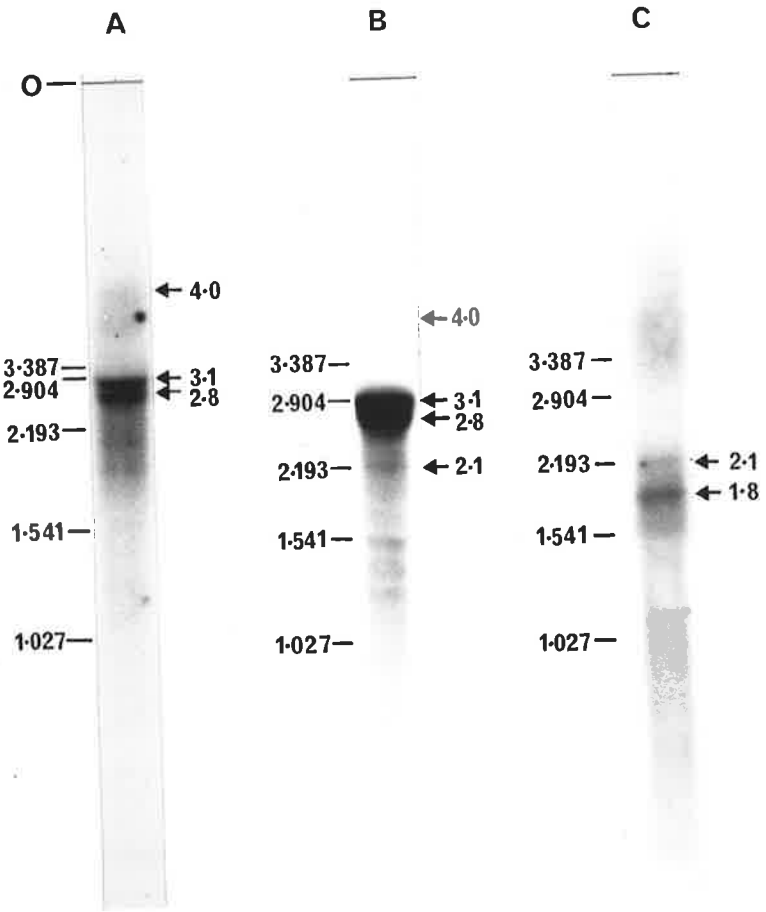
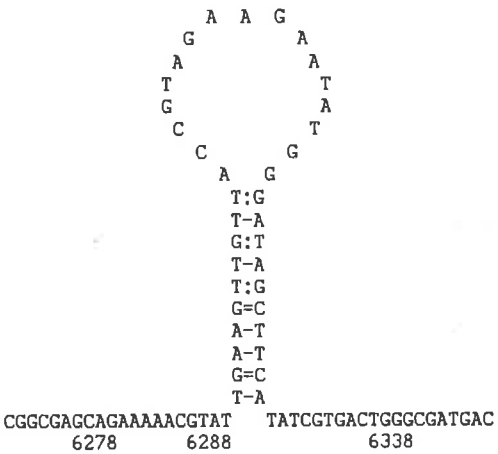

A
B
C
O
4·0
3·387
2·904
3·1
2·8
2·193
2·1
1·8
1·541
1·027

Figure 6.10 Potential terminator structures in the region 6268-6605.

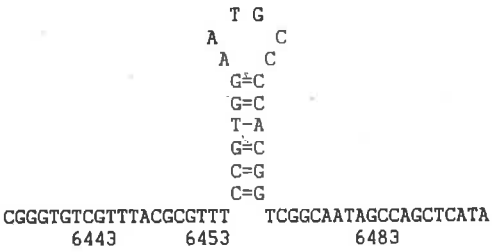
This Figure shows the most stable stem-loop structures encoded in the 6268-6605 region (the 3'-end of the 2.8 kb, 3.1 kb and 4.0 kb transcripts). A threshold value of $\Delta G = -8.0$ was arbitrarily chosen. The stem-loop structures were predicted using the computer program COMSTR and by dot matrix analysis (Chapter 2.38). The free energy (ΔG) values were calculated using the rules of Steger et al. (1984), and are listed beneath each structure. The DNA sequence coordinates of each structure, are indicated. The most stable structure was named tM1 and was considered most likely to represent the terminator for the 2.8 kb, 3.1 kb and 4.0 kb transcripts.

6289-6325



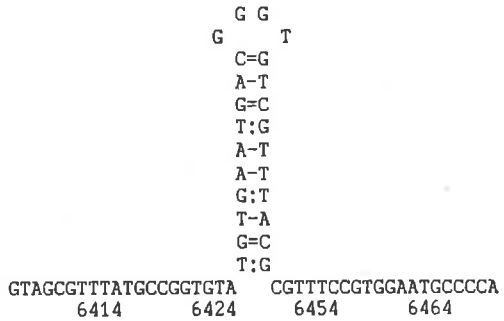
ΔG = -8.7 Kcal/mol

6454-6471



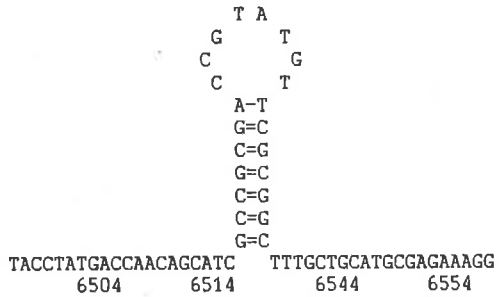
ΔG = -8.7 Kcal/mol

6425-6448



ΔG = -9.1 Kcal/mol

6515-6536 (tM1)



ΔG = -12.8 Kcal/mol

GC-rich stem of 7 bp and a loop of 7 b involving bases 6515-6536. This structure is followed by 3 consecutive T-residues, and thus, is a potential Rho-independent terminator (Chapter 3.2.2c). Transcription termination of this structure would result in transcripts of 2.8 kb and 2.5 kb, which compare favourably in size (within 10%) with the sizes of the middle transcripts (3.1 kb and 2.8 kb) determined by agarose gel fractionation (Chapter 6.2.2).

6.3 SUMMARY OF THE TRANSCRIPTION PATTERN OF THE 186 EARLY LYTIC AND MIDDLE REGIONS AND THE RELEVANCE TO THE CONTROL OF MIDDLE TRANSCRIPTION.

The results obtained for the positions of the 5'-ends and 3'-ends of the early lytic and middle transcripts are summarized in Figure 6.11. Transcription from pR gives rise to four major transcripts; the 1.1 kb, 1.4 kb, 1.5 kb and the 4.0 kb transcript. The 3'-end of the 1.5 kb transcript maps within the CP79 gene and may result from termination at two potential Rho-dependent terminators, tR2 (#4) and tR3 (#5) (Figure 6.11). The 1.4 kb transcript probably results from termination at the tR1 terminator, located in the intergenic region between dhr and CP79 (Figure 6.11). The 3'-end of the 1.1 kb transcript maps within the fil gene at a position where no stable terminator structures were predicted (Chapter 3.2.2c; Figure 6.11). The 1.1 kb transcript may therefore result from termination at a terminator that was not detected in the analysis (Chapter 3.2.2c), or from processing or degradation of RNA.

Two transcripts start within the early-middle region; the 3.1 kb and the 2.8 kb transcript. The 5'-end of the 3.1 kb transcript maps within the fil gene and the 5'-end of the 2.8 kb transcript is most likely located in the intergenic region between dhr and CP79 (Figure 6.11). The 3'-ends of the 2.8 kb and 3.1 kb middle transcripts and the 4.0 kb transcript (which initiates at pR) map to a region (at least between 6268-6605 and probably

between 6268-6532; Figure 6.11) containing a potential Rho-independent terminator, tM1, at the beginning of the replication gene RA.

Weakly hybridizing transcripts were detected using a probe after the tM1 terminator, and were sized at 1.8 kb and 2.1 kb. These transcripts start between 6420-6840 and 5607-6419, respectively (Figure 6.11b). It is possible from the sizes of the 1.8 kb and 2.1 kb transcripts (allowing a 10% sizing error on agarose gels) and the approximate positions of their 5'-ends, that these transcripts could encode the RA gene (Figure 6.11b). The level of transcription 3' to tM1 is only ~10% of the level of transcription before tM1. This result is consistent with the results of Finnegan and Egan (1981), where poor hybridization of RNA was obtained to a plasmid-clone of this region (PstI-PstI, 87.5%-94.0%; Chapter 1.3.2a; Figure 1.2a). Since RA is transcribed at a relatively low level, it is predicted that RA will be a poorly expressed gene. This prediction is consistent with the results obtained from protein gels, which have shown that the RA protein is present at low levels compared with other proteins encoded in the 186 middle region (Sivaprasad, 1984).

The experiments described in this Chapter were carried out in order to gain insight into the mechanism of middle gene expression. As discussed in Chapter 1.4, it was expected that middle genes may be expressed either by the activation of a promoter or by antitermination of the early lytic pR transcript (Figure 1.3). Promoter activation was expected to result in a transcription pattern where a transcript encoding the middle genes, but not the early lytic genes, was detected. Whereas antitermination would be expected to result in a transcription pattern where a transcript encoding both the early lytic and middle regions was detected. The transcription pattern obtained here for the 186 early lytic and middle regions, has features of both promoter activation and antitermination mechanisms. Two new transcripts arise within the PstI-BglI (77.4%-79.6%) region that transcribe the middle genes, but a transcript of sufficient size to encode

The positions of the 1.1 kb, 1.4 kb, 1.5 kb 2.8 kb, 3.1 kb and 4.0 kb transcripts, are indicated by the arrows. The approximate relative abundance of the transcripts are indicated by the thickness of the arrows. The 1.1 kb, 1.4 kb, 1.5 kb and 4.0 kb transcripts most likely initiate from pR (Kalionis, 1985; Chapter 6.2.2). The bracketed regions at the 3'-ends and 5'-ends of the transcripts indicate the regions to which these ends have been mapped.

The positions of the 5'-ends of the 2.1 kb and 1.8 kb transcripts (Chapter 6.2.5), are also indicated. The 3'-ends of these transcripts have not been mapped, as indicated by ?.

Figure 6.11 Summary of the DNA sequence positions of the early lytic and middle transcripts.

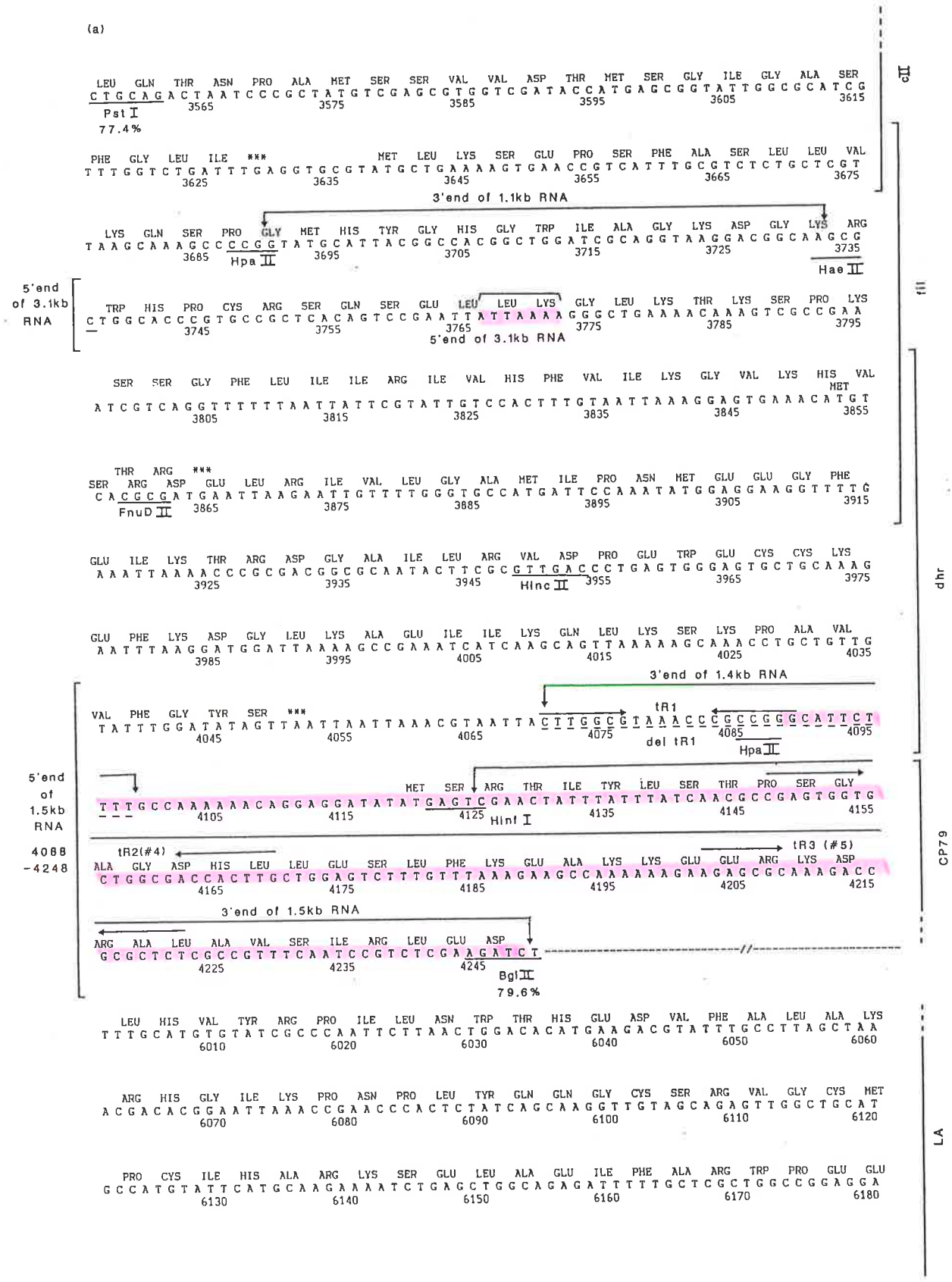
(a) The DNA sequence of the l-strand from the PstI site (at 3556) to 6960, is shown. Not all the sequence is shown (--//-- indicates that the sequence is not contiguous in this region). Genes are indicated on the right of the Figure. All relevant restriction sites are shown and the arrow above the restriction site represents the site of cleavage on the r-strand. The tR1, tR2, tR3 and tM1 potential terminator structures are indicated by the convergent arrows. The deltR1 deletion (4070-4098) is indicated by the dashed line beneath the sequence.

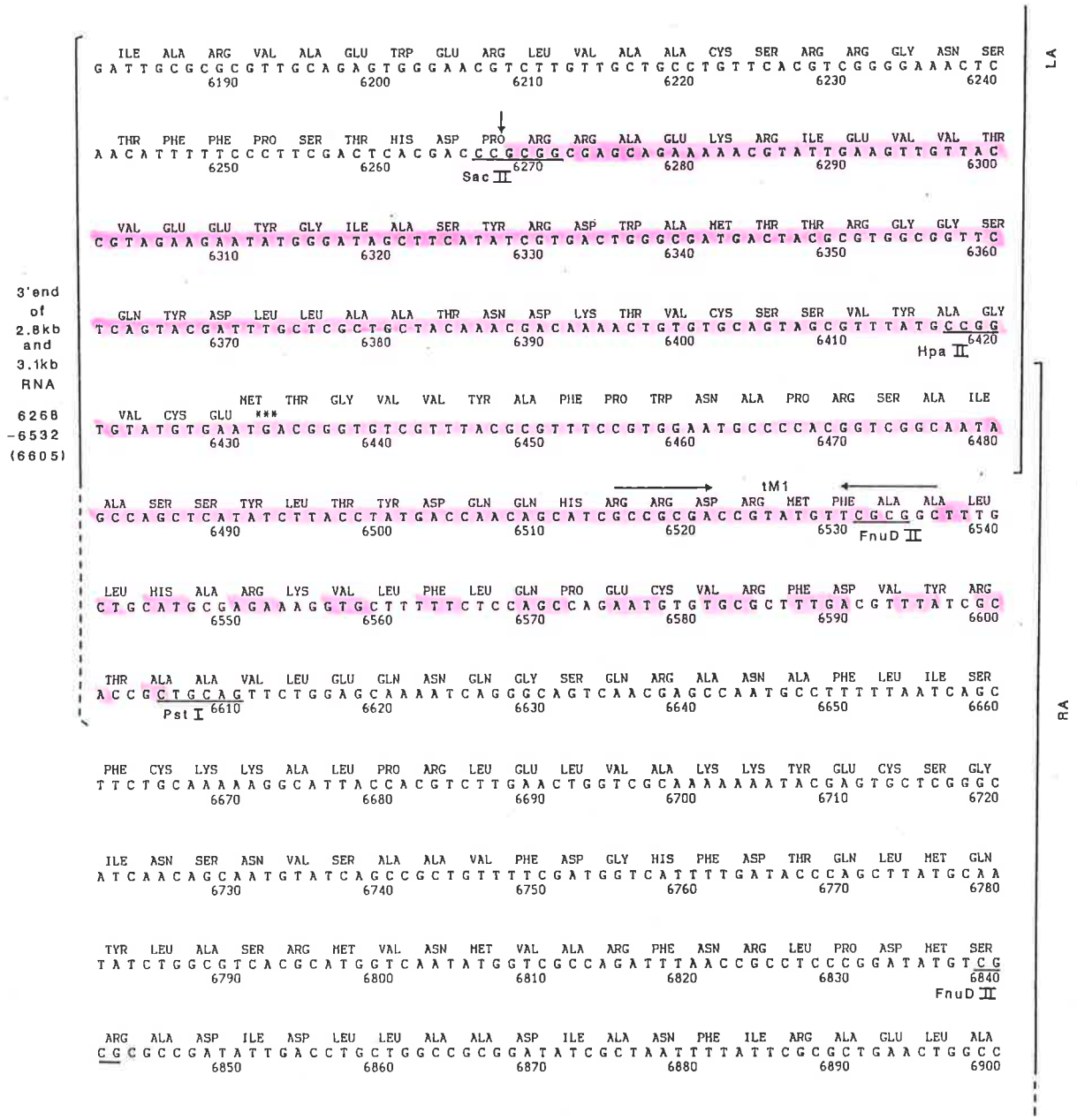
The 3'-ends of the 1.1 kb, 1.4 kb and 1.5 kb transcripts are indicated by the lines above the DNA sequence.

The regions encoding 5'-ends of the 2.8 kb and 3.1 kb transcripts are indicated by the bracketed regions on the left of the Figure and by the shaded region on the DNA sequence.

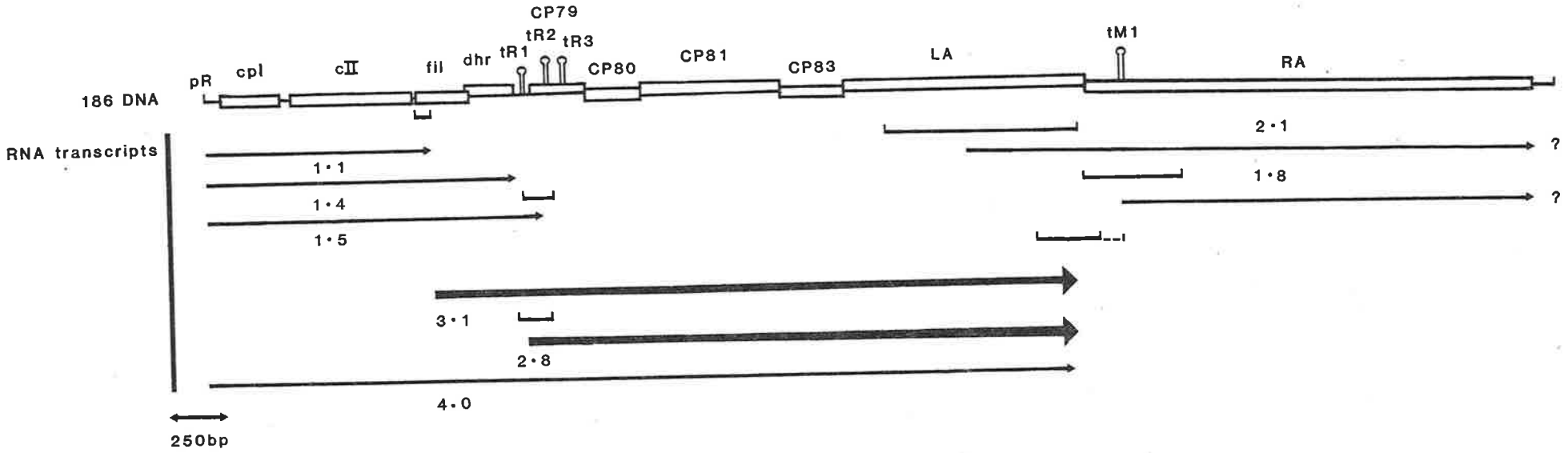
The regions encoding 3'-ends of the 2.8 kb, 3.1 kb and 4.0 kb transcripts are indicated by the bracketed region to the left of the Figure and by the shaded region on the DNA sequence. The sequence coordinates of these regions are listed. The dashed region refers to the fact that the 3'-ends of these transcripts were mapped to the 6268-6605 region by Northern analysis mapping, but from the results of RNA dot blot analysis (Chapter 6.2.5), these transcripts most likely terminate in the vicinity of the FnuDII (6532) site or 5' to this region.

(b) A diagrammatic representation of the gene content of the 186 early lytic and middle regions. Genes are represented by the boxed regions. The potential terminators tR1, tR2, tR3 and tM1 are also indicated. (The stem-loop structures are not drawn to scale.)


(a)
LEU GLN THR ASN PRO ALA MET SER SER VAL VAL ASP THR MET SER GLY ILE GLY ALA SER
CTGCAGACTAATCCCGCTATGTCGAGCGTGGTCGATACCATGAGCGGTATTGGCGCATCG
Pst I
77.4%
3565 3575 3585 3595 3605 3615
PHE GLY LEU ILE *** MET LEU LYS SER GLU PRO SER PHE ALA SER LEU LEU VAL
TTTGGTCTGATTTGAGGTGCGTATGCTGAAAAGTGAACCGTCATTTGCGTCTCTGCTCGT
3625 3635 3645 3655 3665 3675
3'end of 1.1kb RNA
LYS GLN SER PRO GLY MET HIS TYR GLY HIS GLY TRP ILE ALA GLY LYS ASP GLY LYS ARG
TAAGCAAAGCCCCGGTATGCATTACGGCCACGGCTGGATCGCAGGTAAGGACGGCAAGCG
3685 Hpa II 3695 3705 3715 3725 3735
Hae III
5'end of 3.1kb RNA
TRP HIS PRO CYS ARG SER GLN SER GLU LEU LEU LYS GLY LEU LYS THR LYS SER PRO LYS
CTGGCACCCGTGCCGCTCACAGTCCGAATTATTAAAAGGGCTGAAAACAAAGTCGCCGAA
3745 3755 3765 3775 3785 3795
5'end of 3.1kb RNA
SER SER GLY PHE LEU ILE ILE ARG ILE VAL HIS PHE VAL ILE LYS GLY VAL LYS HIS VAL
MET
ATCGTCAGGTTTTTTAATTATTCGTATTGTCCACTTTGTAATTAAAGGAGTGAAACATGT
3805 3815 3825 3835 3845 3855
THR ARG ***
SER ARG ASP GLU LEU ARG ILE VAL LEU GLY ALA MET ILE PRO ASN MET GLU GLU GLY PHE
CACGCGATGAATTAAGAATTGTTTTGGGTGCCATGATTCCAAATATGGAGGAAGGTTTTG
FnuD II 3865 3875 3885 3895 3905 3915
GLU ILE LYS THR ARG ASP GLY ALA ILE LEU ARG VAL ASP PRO GLU TRP GLU CYS CYS LYS
AAATTAAAACCCGCGACGGCGCAATACTTCGCGTTGACCCTGAGTGGGAGTGCTGCAAAG
3925 3935 3945 HInc II 3955 3965 3975
GLU PHE LYS ASP GLY LEU LYS ALA GLU ILE ILE LYS GLN LEU LYS SER LYS PRO ALA VAL
AATTTAAGGATGGATTAAAAGCCGAAATCATCAAGCAGTTAAAAAGCAAACCTGCTGTTG
3985 3995 4005 4015 4025 4035
3'end of 1.4kb RNA
VAL PHE GLY TYR SER ***
TATTTGGATATAGTTAATTAATTAAACGTAATTACTTGGCGTAAACCCGCCGGGCATTCT
4045 4055 4065 4075 tR1 4085 4095
del tR1
Hpa II
5'end of 1.5kb RNA
MET SER ARG THR ILE TYR LEU SER THR PRO SER GLY
TTTGCCAAAAAAACAGGAGGATATATGAGTCGAACTATTTATTTATCAACGCCGAGTGGTG
4105 4115 4125 HInf I 4135 4145 4155
4088 -4248
tR2(#4)
tR3 (#5)
ALA GLY ASP HIS LEU LEU GLU SER LEU PHE LYS GLU ALA LYS LYS GLU GLU ARG LYS ASP
CTGGCGACCACTTGCTGGAGTCTTTGTTTAAAGAAGCCAAAAAAAGAAGAGCGCAAAGACC
4165 4175 4185 4195 4205 4215
3'end of 1.5kb RNA
ARG ALA LEU ALA VAL SER ILE ARG LEU GLU ASP
GCGCTCTCGCCGTTTCAATCCGTCTCGAAGATCT
4225 4235 4245
Bgl II
79.6%
LEU HIS VAL TYR ARG PRO ILE LEU ASN TRP THR HIS GLU ASP VAL PHE ALA LEU ALA LYS
TTTGCATGTGTATCGCCCAATTCTTAACTGGACACATGAAGACGTATTTGCCTTAGCTAA
6010 6020 6030 6040 6050 6060
ARG HIS GLY ILE LYS PRO ASN PRO LEU TYR GLN GLN GLY CYS SER ARG VAL GLY CYS MET
ACGACACGGAATTAAACCGAACCCACTCTATCAGCAAGGTTGTAGCAGAGTTGGCTGCAT
6070 6080 6090 6100 6110 6120
PRO CYS ILE HIS ALA ARG LYS SER GLU LEU ALA GLU ILE PHE ALA ARG TRP PRO GLU GLU
GCCATGTATTCATGCAAGAAAATCTGAGCTGGCAGAGATTTTTGCTCGCTGGCCGGAGGA
6130 6140 6150 6160 6170 6180
dp
fil
dhr
CP79
LA


LA
RA
3'end of 2.8kb and 3.1kb RNA
6268
-6532
(6605)
Sac II
Hpa II
tM1
FnuD II
Pst I
FnuD II

b.


CP79
tR2
tR3
tM1
pR
cpI
cII
fiI
dhr
tR1
CP80
CP81
CP83
LA
RA
186 DNA
RNA transcripts
2·1
?
1·1
1·8
1·4
?
1·5
3·1
2·8
4·0
250bp

the early lytic and middle genes (the 4.0 kb transcript) was detected using an early or a middle probe. Considering these results, two mechanisms were envisaged for the expression of middle genes (Figure 6.12).

The first mechanism, involves promoter activation (giving rise to the 2.8 kb and 3.1 kb transcripts) as the major way in which middle genes are expressed, although some expression also occurs from a low level of readthrough past the early terminators (to give the apparently low abundance 4.0 kb transcript) (Figure 6.12). The second mechanism involves antitermination and processing. The early lytic transcripts may be antiterminated to give rise to the 4.0 kb transcript, which is partially processed to give rise to the 3.1 kb and 2.8 kb middle transcripts with some of the 4.0 kb transcript remaining unprocessed (Figure 6.12). The apparently greater abundance of the 2.8 kb and 3.1 kb middle transcripts relative to the early lytic transcripts may be due to degradation of the early lytic RNAs.

Of the two mechanisms discussed above, the second mechanism was considered to be the most likely for the following reasons. Firstly, the proximity of the 3'-end of the 1.1 kb transcript to the 5'-end of the 3.1 kb transcript and the 3'-end of the 1.4 kb transcript to the 5'-end of the 2.8 kb transcript (Figure 6.11) is suggestive of processing of a larger transcript at two alternative positions (Figure 6.12). Secondly, should the 3.1 kb and 2.8 kb transcripts be due to the activation of promoters (presumably by the same activator protein) it is expected that the DNA sequence spanning the 5'-ends of these transcripts (where the activated promoters should be located) should share some homology (Chapter 1.4.1). The analysis of the DNA sequence spanning the 5'-end of the 3.1 kb transcript with the DNA sequencing spanning the 5'-end of the 2.8 kb transcript did not reveal any significant homology (data not shown). Thirdly, if the 3.1 kb and 2.8 kb transcripts are due to promoter activation, the position of their 5'-ends relative to the early terminators

Figure 6.12 The mechanism of 186 middle gene transcription : New promotion, or antitermination with RNA processing.

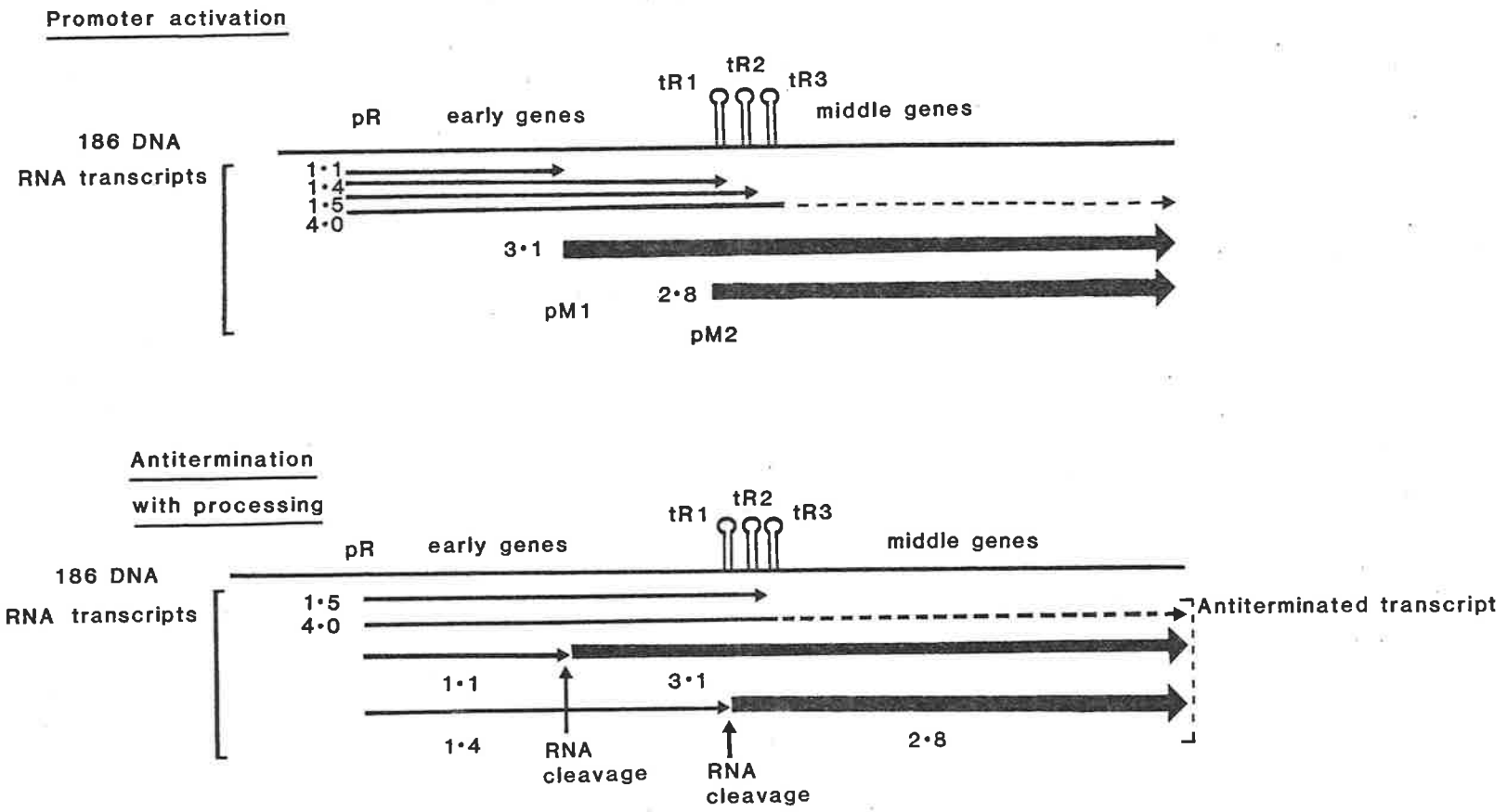
A diagrammatic representation of the two predicted mechanisms for the control of transcription of the 186 middle genes, that are consistent with the transcription pattern (Figure 6.11). Transcripts are represented by the horizontal arrows. The approximate relative abundance of the transcripts are indicated by the thickness of the arrows. The early terminators are represented by the hairpin structures.

New Promotion.

Transcription from the pR promoter gives rise to four transcripts; the 1.1 kb, 1.4 kb, 1.5 kb and 4.0 kb transcripts. The 4.0 kb transcript is due to a small degree of transcription proceeding past the early terminators into the middle region. The 3.1 kb and 2.8 kb transcripts initiate within the early lytic region from the pM1 and pM2 promoters, respectively. Transcription from these middle promoters is able to proceed past the early terminators and express the middle genes.

Antitermination with processing.

Transcription from the pR promoter gives rise to the 1.5 kb transcript, which terminates at the tR2 or tR3 terminators. The 1.5 kb transcript is antiterminated to give rise to the 4.0 kb transcript. The 4.0 kb transcript is processed at two sites to give rise to the 1.1 kb and 3.1 kb transcripts, and the 1.4 kb and 2.8 kb transcripts. The 1.1 kb and 1.4 kb transcripts are then degraded, which leads to their lower abundance relative to the 3.1 kb and 2.8 kb transcripts. A small amount of the 4.0 kb transcript remains unprocessed.


Promoter activation
tR1
tR2
tR3
pR
early genes
middle genes
186 DNA
RNA transcripts
1·1
1·4
1·5
4·0
3·1
2·8
pM1
pM2
Antitermination
with processing
tR1
tR2
tR3
pR
early genes
middle genes
186 DNA
RNA transcripts
1·5
4·0
Antiterminated transcript
1·1
3·1
1·4
RNA
cleavage
RNA
cleavage
2·8

(the 5'-ends of these transcripts are located to the left of the early terminators; Figure 6.11), suggests that a significant percentage of transcripts initiating at the presumptive promoters may terminate at the early terminators. No such transcripts were detected (Figure 6.4, 6.6). Thus, for a promoter activation mechanisms to be viable it is necessary to also propose an antitermination mechanism to allow the middle transcripts to read through the early terminators. Lastly, 186 deletion mutants have been isolated (Chapter 5.2.7b, 2.2.1) that contain deletions spanning the 5'-end of the 3.1 kb transcript (186 del3 (18)) or the 5'-ends of both the 2.8 kb and 3.1 kb transcripts (186 del4 (19)). The viability of these mutants suggests that it is unlikely that the middle transcripts are due to promoter activation, although it is possible that these promoters are not essential for 186 lytic development or the need for these promoters is by-passed in these mutants by the deletion of other control signals.

Experiments to determine whether the 2.8 kb and 3.1 kb middle transcripts are due to promoter activation or arise by antitermination of early lytic transcripts followed by RNA processing are described in the next Chapter.

CHAPTER 7.

INVESTIGATION OF THE MECHANISM OF PRODUCTION OF THE 186 EARLY LYTIC AND MIDDLE TRANSCRIPTS.

CHAPTER 7. INVESTIGATION OF THE MECHANISM OF PRODUCTION OF THE 186 EARLY LYTIC AND MIDDLE TRANSCRIPTS.

7.1 INTRODUCTION.

The results presented in Chapter 6, revealed the transcription pattern of the 186 early lytic and middle regions (Figure 7.1). This transcription pattern can be accounted for either by a mechanism involving the activation of promoters, or by antitermination with RNA processing (Chapter 6.3). The aim of this Chapter, is to determine which of these mechanisms is involved in the production of the early lytic and middle transcripts.

Two approaches were used to investigate whether the middle transcripts arise by transcriptional initiation from an activated promoter, mediated by a 186-encoded activation protein. The first approach is based on the fact that transcripts, which arise by transcriptional initiation contain three phosphates on their 5'-nucleotide. Such transcripts can be distinguished from processed transcripts, which contain one or no phosphates on the 5'-nucleotide, by attempting to label the transcripts at the 5'-ends with [α-^{32}P]-rGTP using the enzyme guanylyl transferase (Furiuchi *et al.*, 1975). Guanylyl transferase only labels transcripts, which contain a 5'-nucleotide which has two or three phosphates (i.e. transcripts, which arise by transcriptional initiation). Thus, if the middle transcripts are due to transcription initiation, then it should be possible to label these transcripts using guanylyl transferase.

Another approach to test whether the middle transcripts arise by promoter activation, is to attempt to activate transcription from a plasmid-clone of the possible promoters by supplying 186 functions in *trans*. 186 functions can be supplied by the heat-induction of a 186 prophage. A plasmid-clone of the promoters can be obtained in the McKenney

promoter-analysis vector, pKO1. pKO1 encodes the galK gene, but lacks a promoter for this gene. A galE$^+$T$^+$K$^-$ strain carrying pKO1 normally forms white colonies on MacConkey-galactose indicator plates, as it is unable to ferment galactose. Clones in pKO1, which contain promoters in the correct orientation, result in the expression of the galK gene and give rise to red colonies on MacConkey-galactose plates (McKenney et al., 1981). Thus, activated promoters can be detected by determining whether there is an increase in galK expression from a plasmid-clone containing these promoters when 186 functions are supplied from a 186 prophage.

Alternatively, if the middle transcripts are due to antitermination and RNA processing of the 4.0 kb antiterminated transcript, then this mechanism may be revealed by transcription studies in E. coli mutants defective in RNA processing. E. coli encodes four known endoribonucleolytic processing enzymes : RNaseIII, RNaseE, RNaseP and RNaseF (Robertson et al., 1968; Dunn, 1976; Kole and Altman, 1981; Misra and Apirion, 1979; Gurevitz et al., 1982; Watson and Apirion, 1981). These enzymes are involved in the processing of E. coli rRNA and tRNA, and in the processing of the tRNA of the T4 and related phage (Abelson, 1979; Gegenheimer and Apirion, 1981; Apirion, 1983). In addition, RNaseIII has been shown to process mRNAs of E. coli and of bacteriophage T7 and λ (Gegenheimer and Apirion, 1981; Dunn and Studier, 1983; Lozeron et al., 1983; Hyman and Honigman, 1986), and to play a role in the control of gene expression; for example, in the retroregulation of λ int gene expression (Gottesman et al., 1982; Court et al., 1983a,b; Echols and Guarneros, 1983; Schmeissner et al., 1984b).

Since the E. coli endoribonuclease RNaseIII cleaves bacteriophage mRNA, it was considered possible that RNaseIII may be involved in the production of the 186 early lytic and middle transcripts. A RNaseIII$^-$ mutant (rnc105) has been isolated, which shows less than 1% RNaseIII activity compared with the wild-type (Kindler et al., 1973; Apirion and

Watson, 1974). This mutation is not lethal to E. coli (although it increases the generation time). Transcription studies in the rnc105 strain will determine whether RNaseIII is involved in the production of 186 early lytic and middle transcripts.

7.2 RESULTS AND DISCUSSION.

7.2.1 Investigation as to whether the 3.1 kb and 2.8 kb Middle Transcripts are due to Initiation from Activated Promoters.

To determine whether the middle transcripts arise by activation of promoters, guanylyl transferase 5'-end-labelling of these transcripts was attempted using a technique based on that described by Haynes and Rothman-Denes (1985). However, this technique did not allow sufficient labelling with (α-^{32}P]-rGTP of either 186 or E. coli RNAs to provide any conclusive results (data not shown). Since the efficiency of 5'-end-labelling with guanylyl transferase is often poor (J.B. Egan, personal communication) this approach was not persisted with.

The next approach was to test whether 186 functions could activate transcription from a plasmid-clone of the region spanning the 5'-ends of the middle transcripts (and thus, the possible activated promoters). The 5'-end of the 3.1 kb transcript is located approximately at position 3768 and the 5'-end of the 2.8 kb transcript is located in the region 4088-4248 (Chapter 6.2.4; Figure 6.11, 7.1).

Activated promoters so far characterized have not included sequences more than 100-200 bases upstream from the transcription initiation point that are required for activation (Chapter 1.4.1; Raibaud and Schwartz, 1984; Busby, 1986). The PstI-BglII (77.4-79.6%) region (sequence coordinates 3556-4249) would therefore be expected to encode the presumptive promoters for the middle transcripts. It is important to note that the PstI-BglII region also contains the early terminators

Chapter 2.34.1. The probe was excised from the M13-vector by digestion with PstI and BamHI (in the M13 cloning site), and purified by electrophoresis on a polyacrylamide gel (Chapter 2.29.3a, 2.30.2).

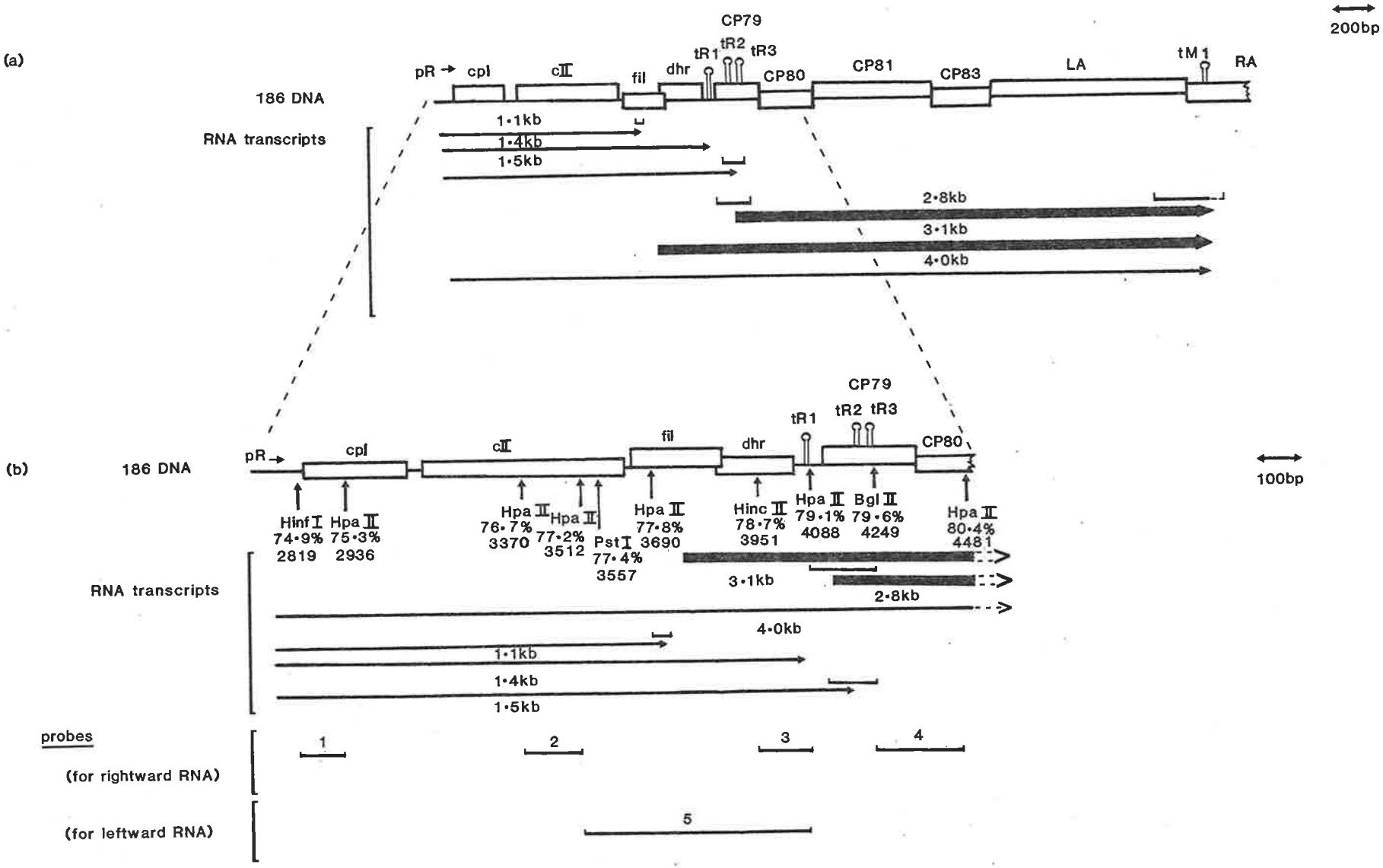
Figure 7.1 Summary of the transcription pattern of the 186 early lytic and middle regions and the positions of probes used in this Chapter.

(a) A diagrammatic representation of the gene content of the 186 early lytic and middle regions. Genes are represented by the boxed regions. The RA gene is only partially represented on the diagram, as indicated by the jagged-edged box. The position of the pR promoter is indicated by the short horizontal arrow. The potential terminators tR1, tR2, tR3 and tM1 are also indicated. (The stem-loop structures are not drawn to scale.)

The positions of the RNA transcripts are indicated by the arrows beneath the genetic map. The approximate relative abundance of the transcripts are indicated by the thickness of the arrows. The bracketed regions at the 3'-ends and 5'-ends of the transcripts indicate the regions to which these ends have been mapped (Figure 6.11).

(b) The region from pR to the middle of CP80 is expanded to show the positions of the probes used for RNA analysis in this Chapter. The positions of relevant restriction sites are shown. The sequence coordinates of the restriction sites refer to the position on the r-strand to the right of the restriction cut (Figure 6.1).

The transcription map of this region is shown beneath the map. The positions of the probes, used in this Chapter, relative to these transcripts are shown. Probes 1-4 are specific for rightward RNA and have been described previously in the legend to Figure 6.1. Probe 5 is specific for leftward RNA. This probe was constructed from the M13-clone, mEC407 (Chapter 2.4.2), using the method described in


(a)
200bp
CP79
tR1
tR2
tR3
pR
cpI
cII
fil
dhr
CP80
CP81
CP83
LA
tM1
RA
186 DNA
RNA transcripts
1·1kb
1·4kb
1·5kb
2·8kb
3·1kb
4·0kb
(b)
186 DNA
100bp
pR
cpI
cII
fil
dhr
CP79
tR1
tR2 tR3
CP80
HinfI 74·9% 2819
HpaII 75·3% 2936
HpaII 76·7% 3370
HpaII 77·2% 3512
PstI 77·4% 3557
HpaII 77·8% 3690
HincII 78·7% 3951
HpaII 79·1% 4088
BglII 79·6% 4249
HpaII 80·4% 4481
RNA transcripts
3·1kb
2·8kb
4·0kb
1·1kb
1·4kb
1·5kb
probes
(for rightward RNA)
(for leftward RNA)
1
2
3
4
5

(Chapter 6.3), which may mask promoters. For this reason, it was important to also examine a region, which does not contain the early terminators. The PstI-HincII (77.4%-78.7%) region (sequence coordinates 3556-3948) does not contain the early terminators, but contains the region upstream of the 5'-end of the 3.1 kb transcript. The strategy was to determine whether 186 functions, supplied by heat-induction of a 186 cItspInt$^-$ prophage (which can not excise from the host chromosome), could activate transcription from a clone of the PstI-BglII fragment or the PstI-HincII fragment, in the McKenney promoter-analysis vector, pKO1 (Chapter 2.3, 2.19). The validity of the conclusions derived from the results of this experiment are based on the assumption that the proposed activator function is produced from the Int$^-$ prophage in sufficient amounts to activate the presumptive activated promoters and that the proposed activator can act in trans.

Plasmid-clones of the PstI-BglII (77.4%-79.6%) fragment and the PstI-HincII (77.4%-78.7%) fragment (derived from the Dhr1 phage) were obtained in the McKenney promoter-analysis vector, pKO1, (pEC406 and pEC407, respectively; Chapter 2.3.2). [It was necessary to clone the PstI-BglII and PstI-HincII fragments from the Dhr1 phage, since the expression of genes from these fragments from the wild-type phage is lethal to the host and the Dhr1 phage contains mutations (in the dhr and fil genes), which prevent this lethality (Chapter 5.2.2, 5.2.6a).]

The galK$^-$ strain (E862) carrying pEC406 or pEC407 gave white colonies on MacConkey-galactose plates. This indicates that there are no rightward promoters encoded within the PstI-BglII region that are recognised by unmodified E. coli RNA polymerase. The next step was to test the effect of the expression of 186 genes from a galK$^-$ 186 cItspInt$^-$A$^-$Dhr$^+$ (15) lysogen (strain E4128) and a galK$^-$ 186 cItspInt$^-$A$^+$Dhr$^-$ (16) lysogen (strain E4127) on the expression of galK from pEC406 or pEC407. [These lysogens were used, since the Int$^-$A$^+$Dhr$^+$ lysogen does not grow at 41^{o}C (the expression temperature), whereas these lysogens show either some growth (A$^-$Dhr$^+$) or

good growth (A^+Dhr^-) at 41°C (Chapter 5.2.4b). Also, these two lysogens were used rather than a E862 186 $Int^-A^-Dhr^-$ (17) lysogen since it is possible that Dhr, Fil, CP79 (which is also mutated in the Dhr1 mutant; Chapter 5.2.6a) or LA are involved in promoter activation.]

The expression of 186 genes from these lysogens did not effect the expression of galK from pEC406 or pEC407 (as judged by colony colour on MacConkey-galactose plates). These results suggest that the PstI-BglII or PstI-HincII regions do not encode activated promoters. However, as previously stated, it is possible that not enough of the activation protein is supplied from the Int^- prophage to activate the presumptive promoters, or that the presumptive activator can only function when supplied in cis. Although promoter activation has not been completely ruled out by this experiment, it was considered more likely that the 3.1 kb and 2.8 kb middle transcripts are due to antitermination of the early lytic transcripts followed by RNA processing of the 4.0 kb antiterminated transcript. The results described below are consistent with this model.

7.2.2 186 Early Lytic and Middle Transcription in an $RNaseIII^-$ Strain.

In order to test the possibility that 186 early lytic and middle transcripts arise by RNaseIII processing, 186 cItsp lysogens of a strain carrying the $RNaseIII^-$ mutation, rnc105, and the isogenic rnc^+ strain were heat-induced for 20 min and RNA was prepared from the cells (Chapter 2.36.1, 2.36.2). Northern analysis was carried out using probes specific to the 186 early lytic or middle regions (Chapter 2.36.3; Figure 7.1b), and the results are shown in Figure 7.2.

RNA from the rnc^+ strain that was hybridized with the early probe showed the 1.1 kb, 1.4 kb, 1.5 kb and 4.0 kb transcripts, as expected (Chapter 6.2.2; Figure 7.2, lane 2). However, when RNA from the rnc105 strain was hybridized with the early probe the 1.1 kb transcript was only faintly detected, whereas the intensity of the 4.0 kb transcript increased

markedly (Figure 7.2, lane 3). This result is consistent with the suggestion that the 1.1 kb transcript results from RNaseIII processing of the 4.0 kb transcript (Chapter 6.3). The 1.4 kb and 1.5 kb transcripts did not show any noticable reduction (or increase) in intensity in the RNaseIII$^-$ strain compared with the RNaseIII$^+$ strain, suggesting that processing was not involved in their formation and therefore, they are most likely due to transcription termination.

The comparison of RNA from the *rnc*$^+$ and *rnc*$^-$ strain that was hybridized with the middle probe (Figure 7.2, lanes 4 and 5), showed that in the *rnc*$^-$ strain the 2.8 kb and 3.1 kb transcripts were reduced markedly in intensity, while the 4.0 kb transcript was increased in intensity. This result establishes that the 2.8 kb and 3.1 kb transcripts result from RNAseIII processing of the 4.0 kb transcript.

The results obtained in this study are summarized in Figure 7.3. These results are consistent with the notion that the middle region is transcribed from the *pR* promoter (presumably by antitermination of the 1.4 kb and 1.5 kb early lytic transcripts) to give a transcript of 4.0 kb. This 4.0 kb transcript is processed by RNaseIII at two sites, the first located within the *CP77* gene and the second within the *CP78*-*CP79* region (Chapter 6.3; Figure 6.11), to generate the 3.1 kb and the 2.8 kb middle transcripts, respectively. Cleavage of the 4.0 kb transcript at the first site to give rise to the 3.1 kb transcript presumably also gives rise to the 1.1 kb early lytic transcript. Similarly, cleavage of the 4.0 kb transcript at the second site to give rise to the 2.8 kb transcript was expected to also give rise to the 1.4 kb transcript. However, if the 1.4 kb transcript is the product of RNaseIII cleavage it would be expected that this transcript should show a significant reduction in intensity in the RNaseIII$^-$ strain. This expected reduction was not obtained, which suggested that the observed 1.4 kb RNA band is not due to RNaseIII processing, but to termination. However, it is pertinent to note that the

Gel Tracks 1. 186 *in vitro* transcripts

2. RNA from the RNaseIII$^+$ 186 *cItsp* lysogen hybridized with the early probe

3. RNA from the RNaseIII$^-$ 186 *cItsp* lysogen hybridized with the early probe

4. RNA from the RNaseIII$^+$ 186 *cItsp* lysogen hybridized with the middle probe

5. RNA from the RNaseIII$^-$ 186 *cItsp* lysogen hybridized with the middle probe

The major RNA transcripts are indicated by the arrows. RNA molecular weight markers (Chapter 2.11.2) are indicated on the Figure.

Figure 7.2 186 early lytic and middle transcription in an RNaseIII$^-$ strain.

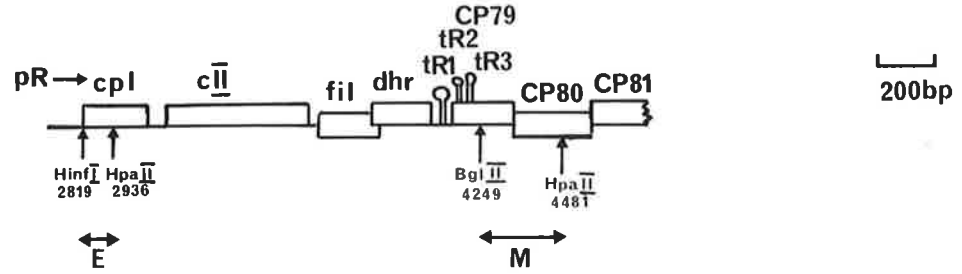
This Figure shows the transcription pattern of the 186 early lytic and middle regions obtained for RNA from a RNaseIII$^-$ 186 cItsp lysogen compared with an RNaseIII$^+$ 186 cItsp lysogen. The positions on the DNA sequence of the probes used to detect 186 early lytic and middle RNA, are shown diagrammatically. The appropriate restriction sites are shown. The sequence coordinates of the restriction sites refers to the position on the r-strand to the right of the restriction cut (Figure 6.1). Genes are represented by the boxed regions. The positions of the pR promoters and the tR1, tR2 and tR3 terminators, are also shown. The arrow next to pR represents the direction of transcription. The terminators, represented by the hairpin structures, are not drawn to scale. The probes used are as follows :

Early probe (E) : HinfI-HpaII (2819-2935)

Middle probe (M) : BglII-HpaII (4249-4480)

The construction of these probes is described in the legend to Figure 6.1.

RNA was prepared 20 min after the heat-induction of the RNaseIII$^-$ 186 cItsp (1) lysogen (E4130) and the RNaseIII$^+$ 186 cItsp (1) lysogen (E4129) (Chapter 2.36.1, 2.36.2). Twenty ug of RNA was denatured with glyoxal, fractionated on a 1.5% agarose gel and transferred unidirectionally to nitrocellulose (Chapter 2.36.3a, 2.29.2). Nitrocellulose-bound RNA was hybridized with the probe specific to the early lytic region or to the middle region (Chapter 2.36.3). The autoradiographs were exposed for 1 week at -80^{o}C with an intensifying screen.


CP79
tR2
pR
cpI
cII
fil
dhr
tR1
tR3
CP80
CP81
200bp
HinfI 2819
HpaII 2936
BglII 4249
HpaII 4481
E
M

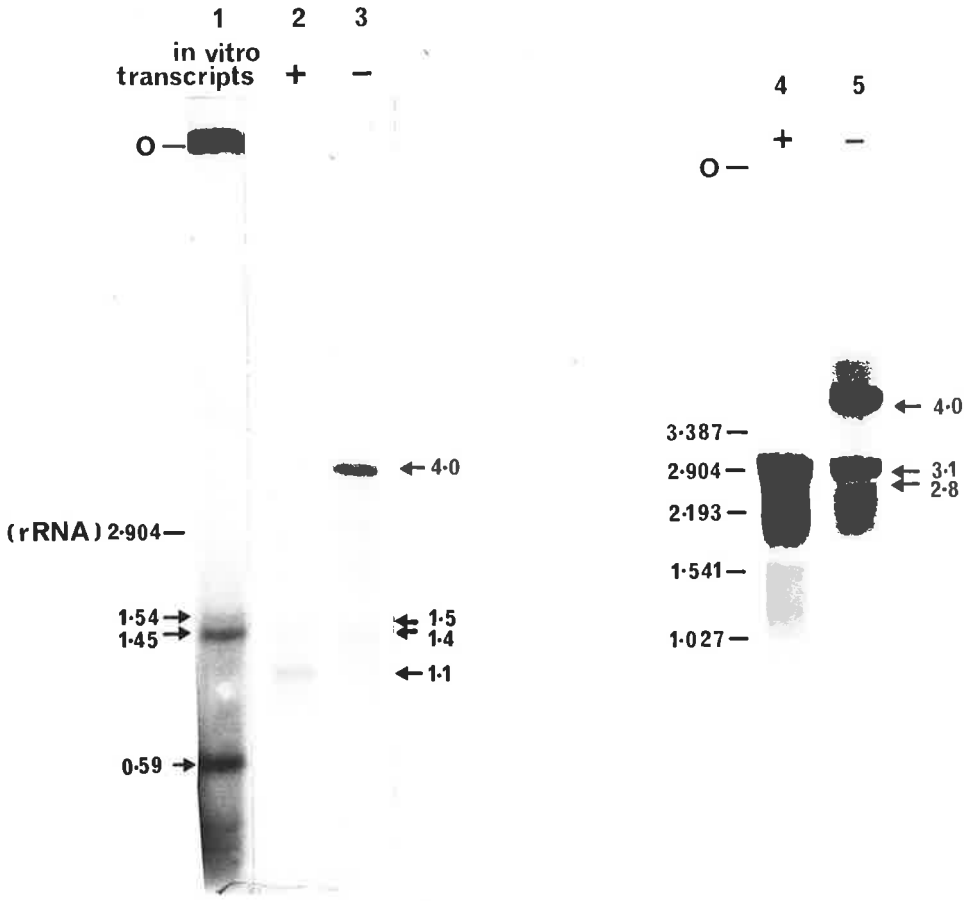
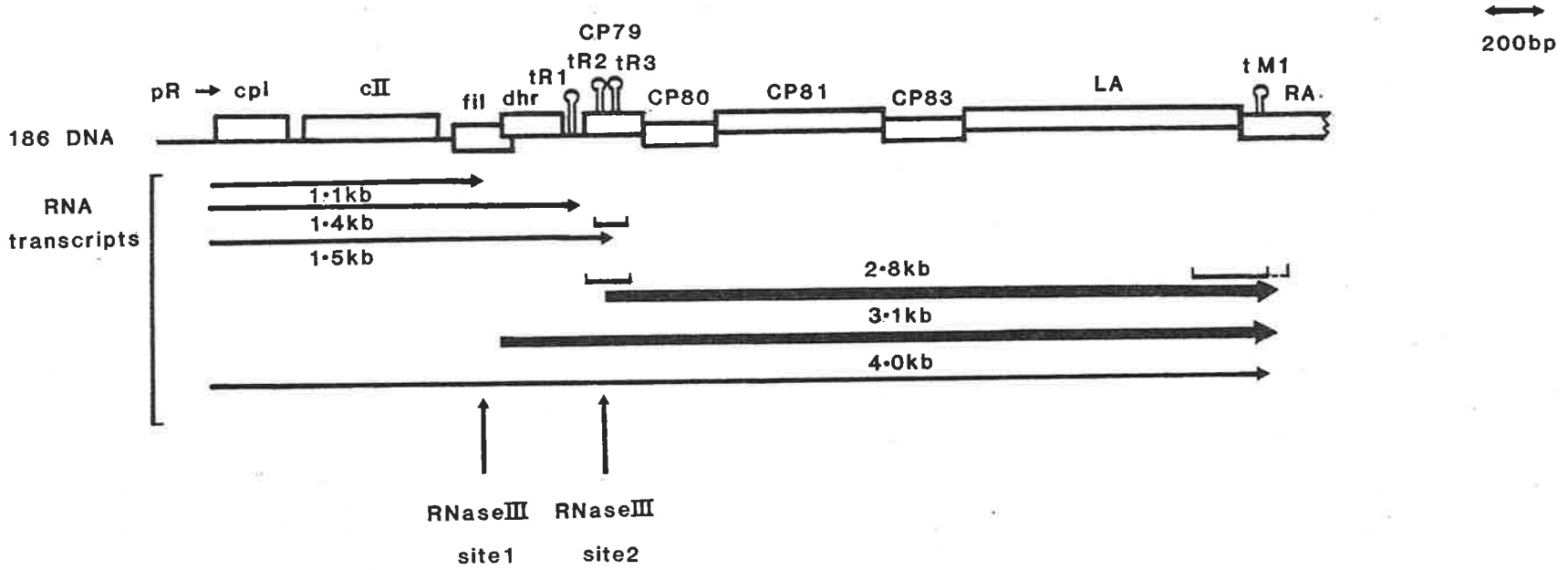

1
2
3
in vitro transcripts
+
–
O
4·0
(rRNA) 2·904
1·54
1·45
0·59
1·5
1·4
1·1
4
5
+
–
O
4·0
3·387
2·904
2·193
1·541
1·027
3·1
2·8

Figure 7.3 The positions of RNaseIII cleavage in the 186 early lytic-middle region.

A diagrammatic representation of the gene content of the 186 early lytic and middle regions. Genes are represented by the boxed regions. The RA gene is only partially represented on the diagram, as indicated by the jagged-edged box. The position of the pR promoter is indicated by the short horizontal arrow. The potential terminators tR1, tR2, tR3 and tM1 are also indicated. (The stem-loop structures are not drawn to scale.)

The positions of the RNA transcripts are indicated by the arrows beneath the genetic map. The approximate relative abundance of the transcripts are indicated by the thickness of the arrows. The bracketed regions of the 3'-ends and 5'-ends of the transcripts, indicate the regions to which these ends have been mapped (Figure 6.11).

The positions of the RNaseIII cleavage sites are shown. Cleavage at site 1 gives rise to the 3.1 kb middle transcript and the 1.1 kb early transcript, whereas cleavage at site 2 gives rise to the 2.8 kb middle transcript. The 1.4 kb and the 1.5 kb transcripts most likely arise by transcription termination.


200bp
CP79
tR2 tR3
tR1
t M1
pR → cpI
cII
fil dhr
CP80
CP81
CP83
LA
RA
186 DNA
RNA
transcripts
1·1kb
1·4kb
1·5kb
2·8kb
3·1kb
4·0kb
RNaseIII
site1
RNaseIII
site2

RNA species detected by Northern analysis represent accumulated RNAs and therefore the relative stabilities of each RNA transcript becomes important. Two possible explanations of the apparent absence of the 1.4 kb RNaseIII cleavage product are as follows (Figure 7.4). The following explanations assume that there are two initial sources for a 1.4 kb transcript; one due to termination (1.4^{t}) and the other due to processing (1.4^{p}).

In the first possibility, the 1.4^{p} kb transcript is generated by cleavage of the 4.0 kb transcript, but is then either rapidly degraded or further processed so that it is not detected, whereas the 1.4^{t} kb transcript is stable (Figure 7.4a). This is possible if the 1.4^{t} kb transcript has a different 3'-end to that of the 1.4^{p} kb transcript. The 3'-end of transcripts have been shown to be important in the stability of the RNA (Higgins and Smith, 1985; Panayotatos and Truong, 1985; Plamann and Stauffer, 1985; Hayashi and Hayashi, 1985; Wong and Chang, 1986; Zwieb et al., 1986). Thus, if the 1.4^{t} kb transcript has a different 3'-end to the 1.4^{p} kb transcript, it may be less susceptible to degradation. In addition, a different 3'-end may affect the secondary structure of the RNA molecule such that it is no longer susceptible to endonucleolytic cleavage. In these circumstances, where the 1.4^{t} kb transcript is stable and the 1.4^{p} kb transcript unstable, the 1.4^{t} kb transcript would be largely responsible for the observed 1.4 kb RNA band. Thus, in the RNaseIII^{-} strain, where the production of the 1.4^{p} kb transcript is prevented, there would be no change in the intensity of the observed 1.4 kb RNA band.

Secondly, the 1.4^{t} kb and 1.4^{p} kb transcripts may both be processed at the first RNaseIII site to give rise to the 1.1 kb transcript and a 0.3 kb transcript (which is either further processed or rapidly degraded, since it is not detected by Northern analysis; Chapter 6.2.3, 6.2.4; Figure 6.4, 6.6). Thus, the observed 1.4 kb RNA band may consist of both the 1.4^{t} kb and the 1.4^{p} kb transcripts. In a RNaseIII^{-} strain the 1.4^{p} kb transcript

would not be produced. However, the effective decrease in the observed 1.4 kb RNA band from the absence of the 1.4^{p} kb band would be compensated for by an increase in the 1.4^{t} kb transcript, which is no longer processed at the first RNaseIII site. Thus, overall the intensity of the observed 1.4 kb RNA band would not change significantly in the RNaseIII^{-} strain compared with the RNaseIII^{+} strain. Further studies (described in Chapter 10.3.4) are required to distinguish between these two possibilities for the apparent absence of the 1.4 kb RNaseIII cleavage product.

In summary, these results have shown that the 3.1 kb and 2.8 kb middle transcripts are generated by RNaseIII processing of the 4.0 kb transcript. Thus, it appears that the middle genes are expressed by antitermination of the early lytic transcripts to produce the 4.0 kb RNA, which is processed by RNaseIII. The possible mechanism of the RNaseIII cleavage of 186 RNA and its role in 186 lytic development will now be considered.

7.2.3 Investigation of the Mechanism of RNaseIII Cleavage of the 4.0 kb Transcript.

RNaseIII cleaves at regions of double-stranded RNA (Robertson *et al.*, 1968; Robertson, 1982). It is possible that an RNaseIII-sensitive RNA structure may be formed by a leftward transcript hybridizing with the rightward 4.0 kb transcript. Two leftward promoters (*p*L782 at 3821 and *p*L783 at 3830) were predicted to be encoded in the *Pst*I-*Bgl*II region (Chapter 3.2.2c). Thus, it is possible that RNaseIII cleavage of the 4.0 kb transcript is a consequence of the formation of double-stranded RNA by hybridization of leftward RNA (transcribed from these leftward promoters) with the rightward 4.0 kb transcript.

To determine whether 186 encodes a leftward RNA in the region encoding the 5'-ends of the 3.1 kb and 2.8 kb transcripts, RNA dot blot analysis was performed using a single-stranded probe, which was specific for leftward RNA [the *Hpa*II (3700-4087) fragment, constructed as described in the legend

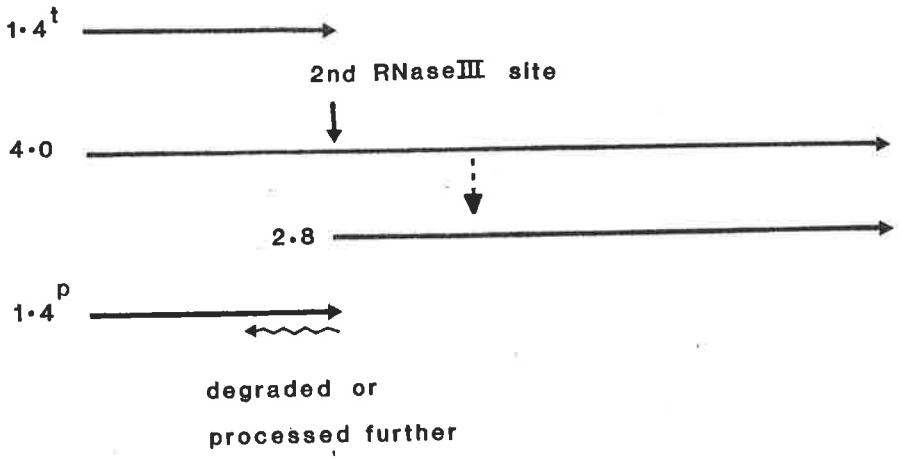
Figure 7.4 Explanation for the apparent absence of the 1.4 kb RNaseIII cleavage product.

A diagrammatic representation of the two proposed mechanisms for the production of the 1.4 kb RNA. Both of these mechanisms are based on the assumption that there are two sources for the 1.4 kb RNA band; one due to termination (1.4^t) and the other due to processing (1.4^p).

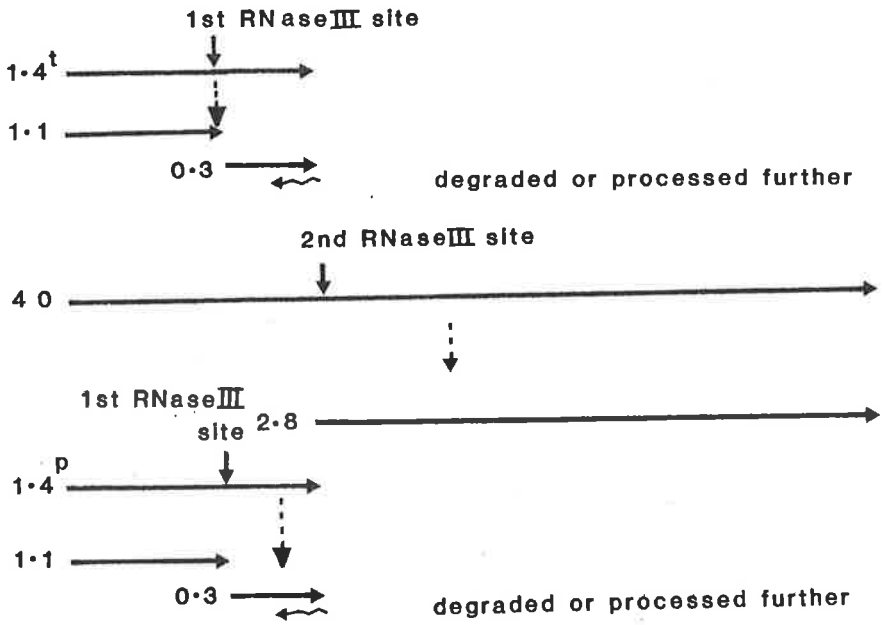
(a) Transcription from the pR promoter gives rise to the 1.4^t kb transcript, which is stable, and the 4.0 kb transcript. The 4.0 kb transcript is processed by RNaseIII at the second RNaseIII site to generate the 2.8 kb middle transcript and the 1.4^p kb transcript. The 1.4^p kb transcript is then degraded or processed further and therefore does not significantly contribute to the observed 1.4 kb RNA band.

(b) Transcription from the pR promoter gives rise to the 1.4^t kb and the 4.0 kb transcripts. The 1.4^t kb transcript is processed by RNaseIII at the first RNaseIII site to generate the 1.1 kb and the 0.3 kb transcripts. The 0.3 kb transcript is degraded or processed further and therefore is not detected. The 4.0 kb transcript is processed by RNaseIII at the second RNaseIII site to generate the 2.8 kb middle transcript and the 1.4^p kb transcript. The 1.4^p kb transcript is then processed by RNaseIII at the first RNaseIII site to generate the 1.1 kb and the 0.3 kb transcript. The 0.3 kb transcript is degraded or processed further and therefore is not detected.

(a)


1·4t
2nd RNaseIII site
4·0
2·8
1·4p
degraded or
processed further

(b)


1st RNaseIII site
1·4t
1·1
0·3
degraded or processed further
2nd RNaseIII site
4 0
1st RNaseIII
site
2·8
1·4p
1·1
0·3
degraded or processed further

to Figure 7.1b]. This probe, did not show any significant hybridization to RNA prepared 20 min after heat-induction of a 186 lysogen (either a RNaseIII$^+$ or RNaseIII$^-$ lysogen), yet was able to detect 0.025 fmoles of 186 DNA (data not shown). In contrast, a probe covering part of the same region, but specific for rightward RNA [the HincII-HpaII (3950-4085) fragment, constructed as described in the legend to Figure 7.1b], that was capable of detecting 0.1 fmoles of 186 DNA, showed strong hybridization (data not shown). These results suggest that it is unlikely that leftward transcription occurs in this region at a very high level and so indicates that the predicted leftward promoters (pL782 and pL783) are not functional, at least not 20 min after prophage induction. Thus, it is unlikely that hybridization between a leftward RNA and the 4.0 kb rightward transcript is responsible for the generation of the RNaseIII cleavage sites. Therefore, it is more likely that local secondary structure in the RNA results in the formation of the two detected RNaseIII cleavage sites located within the PstI-BglII (77.4%-79.6%) region. This will be considered further in the concluding discussion (Chapter 10.3.2).

7.2.4 Investigation of the Role of RNaseIII Processing in 186 Lytic Development.

RNaseIII processing is not essential to 186, since 186 forms plaques on the rnc105 strain. Furthermore, the 186 deletion mutants, described in Chapter 5.2.7(b), remove either one processing site (del3) or both processing sites (del4), yet are viable.

A possible effect of RNaseIII processing in 186 lytic development was revealed by the significantly lower intensities (observed by Northern analysis) of the 1.1 kb and 1.4 kb early lytic transcripts compared with the 2.8 kb and 3.1 kb middle transcripts (Chapter 6.2.3, 6.2.4; Figure 6.4, 6.6, 6.11). This result suggests that the early lytic transcripts may be rapidly degraded. To investigate this possibility, RNA dot blot analysis

(Chapter 2.36.4) was used to quantitate the amount of RNA transcribed from the early lytic region compared with that transcribed from the middle region. Two early lytic region-specific probes were used (one close to the pR promoter and the other 435 b downstream), and one middle region-specific probe [located after the BglII (79.6%) site] was used. The amount of 186 early lytic and middle RNA obtained in the RNaseIII$^+$ strain was compared with that obtained in the RNaseIII$^-$ strain. In addition, early lytic and middle RNA was also quantitated from a lysogen of the 186 del4 deletion mutant, which contains a deletion removing both processing sites (186 cItspdel4 (19); Chapter 5.2.7b), and compared with that obtained from a lysogen of 186 cItsp (1). The results are presented in Table 7.1.

The early/middle RNA ratio obtained from the RNaseIII$^+$ strain was approximately 0.5 - 0.8, and this ratio increased by a factor of 1.5 - 3.0 for RNA prepared from the RNaseIII$^-$ strain, or from the lysogen of the 186 del4 deletion mutant in the RNaseIII$^+$ strain (Table 7.1). These results suggest that RNA 5' to the processing sites is degraded, resulting in a reduced level of 186 early lytic RNA compared with middle RNA. Degradation of the RNaseIII cleaved-early lytic RNA most likely occurs from the 3'-end by RNaseII or polynucleotide phosphorylase (Singer and Tolbert, 1965; Gupta et al., 1977; Portier, 1975; Kimhi and Littauer, 1968; Donovan and Kushner, 1986), since there does not appear to be a 5'-exoribonuclease present in E. coli (Apirion, 1973; Datta and Niyogi, 1976; Deutscher, 1985). It is possible that RNaseIII processing and the subsequent degradation of RNA 5' to the processing sites may act to decrease the level of expression of the early lytic genes (cpl, cII, fil and dhr) relative to middle genes, which otherwise may be inhibitory to a productive lytic infection.

To test the proposal that a high level of early lytic genes may be inhibitory to 186 development and that RNaseIII processing and degradation of early lytic RNA may be important in decreasing expression of early lytic

TABLE 7.1

Quantitation of RNA from the 186 early lytic and middle regions.[a]

Source of RNA	Level of hybridization						
	Early[b] probe 1	Early[c] probe 2	Middle[d] probe	E(1)[e] / M		E(2)[e] / M	
E4129 (rnc+, 186cItsp(1))	111025	90215	145054	0.8	1.4x	0.6	2.9x
E4130 (rnc105, 186cItsp(1))	147414	232708	131201	1.1		1.8	
E252 (E251 186cItsp(1))	129234	77541	166933	0.8	2.3x	0.5	3.1x
E4122 (E251 186cItspdel4(19))	156112	133146	92528	1.7		1.4	

Notes to Table 7.1

a. RNA hybridizing to the 186 early and the middle regions was quantitated by RNA dot blot analysis (Chapter 2.36.4). RNA was prepared 20 min after the heat-induction of the lysogens (Chapter 2.36.1, 2.36.2). Two ug of each sample of RNA was loaded onto the nitrocellulose filters as described in Chapter 2.36.4. Radioactive probes were prepared as described in Chapter 2.34.1 and Figure 6.1. Hybridization and autoradiography were as described in Chapter 2.36.3(b). The level of hybridization of the probe to RNA was quantitated by scanning the autoradiograph using a Zeinch scanning laser densitometer. The specific activity of the probes was normalized by the quantitation of the amount of probe hybridizing to known concentrations of DNA. The RNA dot blot intensities presented here are normalized.

b. The Early probe (1) was the HinfI-HpaII (2819-2935) DNA fragment prepared from mEC802 (Figure 7.1b).

c. The Early probe (2) was the HpaII (3370-3511) DNA fragment prepared from mEC404 (Figure 7.1b).

d. The Middle probe was the BglII-HpaII (4249-4480) DNA fragment prepared from mEC408 (Figure 7.1b).

e. The ratio of RNA hybridizing to the early probes to that hybridizing to the middle probe.

genes, the following experiment was performed. Cells carrying a plasmid-clone encoding the 186 cItsp and cpl genes (pEC606; Chapter 2.3.2; Figure 7.5) were grown at 39°C (a temperature at which the cpl gene is expressed) and the plating efficiency (eop) of 186 virl (3) on this strain was determined and compared with the eop obtained on the control strains, which carried either the parent plasmid (pKO1) or a plasmid-clone encoding only the 186 cItsp gene (pEC604; Figure 7.5). As shown in Table 7.2, 186 virl gave an eop of 51% on the strain carrying the plasmid (pEC606) expressing cpl, and furthermore, resulted in very small plaques. This result shows that high expression of cpl is inhibitory to 186 virl infection. To test whether the high expression of cII further inhibited 186 virl infection, 186 virl was plated on a strain expressing both cpl and cII from a plasmid-clone (pEC405; Chapter 2.3.2; Figure 7.5). Contrary to expectations, 186 virl gave an eop of 95% on this strain compared with the control carrying the parent plasmid (pMC931). This result suggests that cII may antagonize to some extent the inhibition of 186 virl infection by Cpl. It was noted however, that 186 virl plaques remained small on the strain expressing cpl and cII, indicating that 186 virl infection was still inhibited. To examine the effect of RNaseIII processing at RNaseIII sites 1 and 2 on cpl (and cII) gene expression, 186 virl was plated at 39°C on a strain carrying the plasmid-clone pEC415 [containing the NruI-AhaIII (70.2%-79.4%) fragment from 186 cItspDhrl (13); Chapter 2.3.2; Figure 7.5], which in addition to encoding cpl and cII, encodes the region involved in RNaseIII processing. 186 virl gave an eop of ~100% on this strain compared with the control (cells carrying pKO2 or pEC604) and gave large plaques (Table 7.2). This suggests that RNaseIII processing followed by degradation of early lytic RNA, may play a role in decreasing the level of cII and cpl expression (and presumably also fil and dhr expression), which may otherwise inhibit 186 lytic development.

Figure 7.5 The gene content of pEC604, pEC606, pEC405 and pEC415.

A diagrammatic representation of the gene content of the region XhoI-BglII (67.6%-79.6%). Genes are represented by the boxed regions. The positions of the pR and pL promoters are indicated by the short horizontal arrows. (The arrows represent the direction of transcription.) The position of the tR1 terminator is indicated by the stem-loop structure. (The stem-loop structure is not drawn to scale.) Relevant restriction sites are shown. The sequence coordinates of the restriction sites refer to the first base of the site on the l-strand.

The gene content of pEC604, pEC606, pEC405 and pEC415 is shown. The construction of these plasmid-clones is described in Chapter 2.3.2. The restriction sites used to construct these clones are indicated. Genes that are only partially represented on the clones are indicated by the jagged-edged boxes. Promoters and terminators are indicated, as described above. The positions of the RNaseIII sites are also shown. The position of the galK gene carried by clones in the vector, pKO2, is indicated.

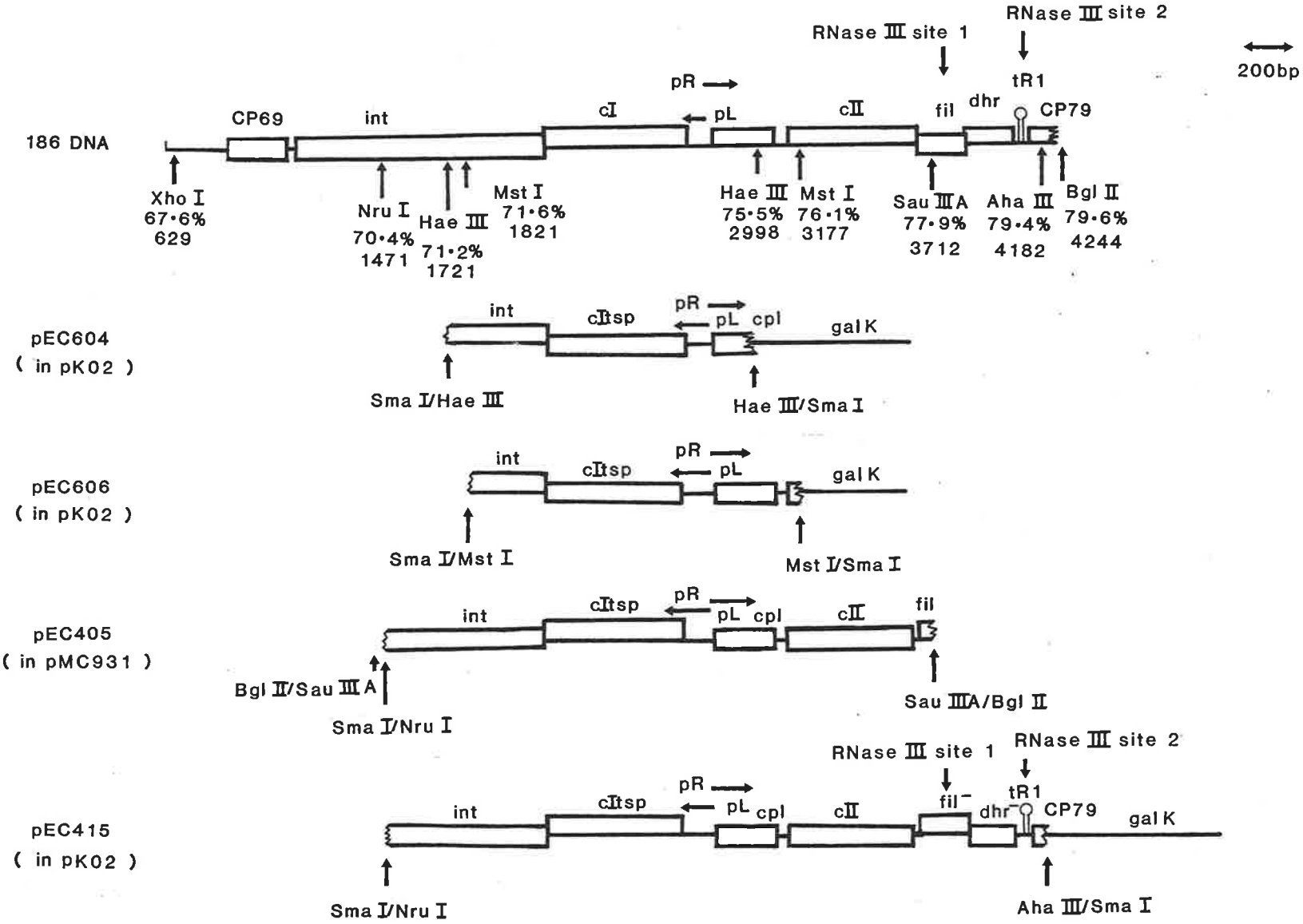

RNase III site 1
RNase III site 2
200bp
pR
tR1
186 DNA
CP69
int
cI
pL
cII
fil
dhr
CP79
Xho I
67·6%
629
Nru I
70·4%
1471
Hae III
71·2%
1721
Mst I
71·6%
1821
Hae III
75·5%
2998
Mst I
76·1%
3177
Sau IIIA
77·9%
3712
Aha III
79·4%
4182
Bgl II
79·6%
4244
pEC604
(in pK02)
cItsp
pL
cpI
galK
Sma I/Hae III
Hae III/Sma I
pEC606
(in pK02)
Sma I/Mst I
Mst I/Sma I
pEC405
(in pMC931)
Bgl II/Sau IIIA
Sma I/Nru I
Sau IIIA/Bgl II
pEC415
(in pK02)

TABLE 7.2

Plating efficiencies of 186 vir on E863 carrying plasmid-clones.[a]

Clones[b] (Plasmids)	186 genes encoded	Efficiency of[c] plating (eop)	Plaque size
pKO2	-	100%	large
pEC604	cItsp	128%	large
pEC606	cItsp, cpl	51%	very small
pMC931	-	100%	large
pEC405	cItsp, cpl, cII	95%	small
pEC415	cItsp, cpl, cII (contains the region encoding RNaseIII processing sites)	119%	large

Notes to Table 7.2

a. Cultures of E863 carrying plasmid-clones (or plasmids) were grown at 30°C in L broth containing the appropriate antibiotic to A_{600} = 0.8. Dilutions of 186 virl (3) were plated with cells (0.2 ml) from each strain (Chapter 2.16.1). Plates were incubated at 39°C overnight and the number of plaques were counted.

b. Plasmids and plasmid-clones are described in Chapter 2.3 and Figure 7.5. pEC604, pEC606 and pEC415 are clones in the vector pKO2, and pEC405 is a clone in the vector pMC931.

c. The efficiency of plating (eop) is expressed as a percentage of the number of 186 virl (3) plaques obtained on strains carrying the plasmid-clones compared to that obtained on the strains carrying the parent vector.

7.3 SUMMARY.

The results presented in this Chapter did not provide evidence for the presence of activated promoters in the 186 early lytic-middle region, but did show that the 3.1 kb and 2.8 kb middle transcripts and the 1.1 kb early lytic transcript arise from RNaseIII processing of the 4.0 kb transcript derived from pR (summarized in Figure 7.3). The 1.4 kb and 1.5 kb transcripts are most likely due to transcription termination, although the 1.4 kb transcript may in part be due to RNA processing. The processing of the 4.0 kb transcript possibly plays a role in decreasing the level of early lytic RNA by the subsequent RNA degradation of the 1.1 kb (and 1.4 kb) processed transcripts from the 3'-end. This would result in a reduction in the expression of the early lytic genes, which may otherwise be inhibitory to 186 lytic development.

The aim of this Chapter was to determine whether middle transcription results from promoter activation or antitermination with RNA processing. These results have shown that the transcription pattern of the 186 early lytic and middle regions is consistent with a mechanism of middle gene transcription involving antitermination and RNaseIII processing. The 4.0 kb transcript, which spans the early lytic and middle regions, is expected to arise by antitermination of the 1.4 kb and 1.5 kb early lytic transcripts, presumably mediated by a 186-encoded antitermination function. Chapter 8 is concerned with determining whether middle gene expression involves an antitermination mechanism, and with identifying the proposed antitermination function.

CHAPTER 8.

INVESTIGATION OF THE CONTROL

OF 186 MIDDLE GENE TRANSCRIPTION.

CHAPTER 8. INVESTIGATION OF THE CONTROL OF 186 MIDDLE GENE TRANSCRIPTION.

8.1 INTRODUCTION.

The results obtained in Chapters 6 and 7 (summarized in Figure 8.1a), have shown that the transcription pattern of the 186 early lytic and middle region is consistent with an antitermination mechanism of control of 186 middle gene expression. Such a mechanism would be expected to act at the terminators for the 1.4 kb transcript (tR1) and the 1.5 kb transcript (tR2 or tR3) to allow transcription to proceed through these terminators and to give rise to the 4.0 kb transcript.

As discussed in Chapter 1.3.2, the involvement of a middle control mechanism was predicted from the in vivo transcription studies of Finnegan and Egan (1981), which were carried out when protein synthesis was inhibited by chloramphenicol, and the in vitro transcription studies of Pritchard and Egan (1985). However, the studies of Finnegan and Egan (1981) were limited by the use of probes of poorly defined genetic content (Chapter 1.3.2). Thus, it was important to reinvestigate the results of Finnegan and Egan (1981) using specific probes of known gene content.

The studies of Pritchard and Egan (1985) revealed a 1.45 kb transcript in vitro, which was consistent with initiation of transcription at pR and termination at the Rho-independent terminator tR1 (Chapter 3.2.2c). However, although transcription has been shown to terminate efficiently at tR1 in vitro (see Chapter 9.2.4), this terminator may be inefficient in vivo. It was therefore important to determine the strength in vivo of the tR1 terminator, and also of the tR2 and tR3 terminators.

The aim of this Chapter is to investigate whether an antitermination mechanism acting at the early terminators is likely to be involved in the control of 186 middle gene expression. Three approaches were used.

Firstly, the effect of inhibiting protein synthesis on 186 early lytic and middle transcription was investigated, using the techniques of RNA dot blot analysis and Northern analysis. This approach should reveal whether there is a specific transcription block at the early terminators, which would be expected if an antitermination mechanism is involved in 186 middle gene expression. Secondly, the intrinsic strengths of the early terminators were investigated *in vivo*. Should antitermination be involved in the control of the expression of the 186 middle genes then it would be expected that the early terminators would be intrinsically efficient terminators *in vivo*. Thirdly, 186 functions were tested to determine whether they increased the expression of the middle genes by using plasmid-clones in the McKenney promoter-analysis vector.

8.2 RESULTS AND DISCUSSION.

8.2.1 Investigation of 186 Early Lytic and Middle Transcription after the Inhibition of Protein Synthesis.

To assess the effect of inhibiting protein synthesis on 186 early lytic and middle transcription, the RNaseIII$^-$ strain (E4090) was used to decrease the complications associated with RNaseIII cleavage and subsequent degradation of early lytic RNA (see Chapter 7.2.4). RNA was prepared as follows. The RNaseIII$^-$ strain was grown to A_{600}= 0.6 in L broth, and protein synthesis was inhibited by the addition of chloramphenicol (as described by Finnegan and Egan, 1981). Chloramphenicol was added at 400 ug/ml, a level which was shown to inhibit *E. coli* protein synthesis (determined by measuring the optical density of the culture; data not shown). The concentration of chloramphenicol used was twice that used by Finnegan and Egan (1981), in order to compensate for the use of the richer growth medium (L broth compared with TPGCAA). The chloramphenicol-treated culture was infected with a 186 A$^-$ phage (186 *Aam1lvir1* (4)) 10 min after

the addition of chloramphenicol. A control culture (not treated with chloramphenicol) was also infected with the 186 A^- phage. [Infection with a 186 A^- strain was carried out to minimize the difference between the chloramphenicol-treated culture and the untreated culture. Infection was used as opposed to heat-induction since chloramphenicol may inhibit excision of the prophage from the host chromosome, and the 186 A^- (replication defective) phage was used since chloramphenicol was expected to prevent 186 replication (due to the absence of protein synthesis), both of which may effect the level of early lytic and middle transcription. Thus, the only difference between the control and the chloramphenicol-treated culture is the absence of 186 protein synthesis in the latter.]

RNA prepared 20 min after infection of the cultures (Chapter 2.36.1, 2.36.2), was analysed by RNA dot blot analysis (Chapter 2.36.4) using two probes specific for early lytic transcription [E(1) located near the pR promoter and E(2) located 435 b downstream] and a probe specific for middle transcription [located after the BglII (79.6%) site] (Figure 8.1b,c). Should a 186 protein be required for middle gene expression, it would be expected that the amount of RNA detected with the early probe will be the same with or without chloramphenicol, whereas middle gene transcription will be markedly reduced by chloramphenicol.

As shown in Table 8.1, transcription detected by the probe specific for middle transcription was markedly reduced by chloramphenicol. Transcription detected by the two early probes [E(1) and E(2); Table 8.1] also showed a dramatic decrease with chloramphenicol.

These results show that when protein synthesis is inhibited by chloramphenicol, 186 early lytic and middle transcription are both significantly decreased. This result is contrary to the results obtained by Finnegan and Egan (1981), who found no significant decrease in early lytic transcription with chloramphenicol. The difference in these results is probably due to the inability of the probe used by Finnegan and

arrows. The position of the deltR1 deletion (sequence coordinates 4070-4098) is indicated above the sequence. All relevant restriction sites are shown. The position of cleavage on the r-strand (indicated by the vertical arrows) is shown for restriction sites used for the construction of probes.

Figure 8.1 Summary of the transcription pattern of the 186 early lytic and middle regions, and the positions of probes and restriction sites used in this Chapter.

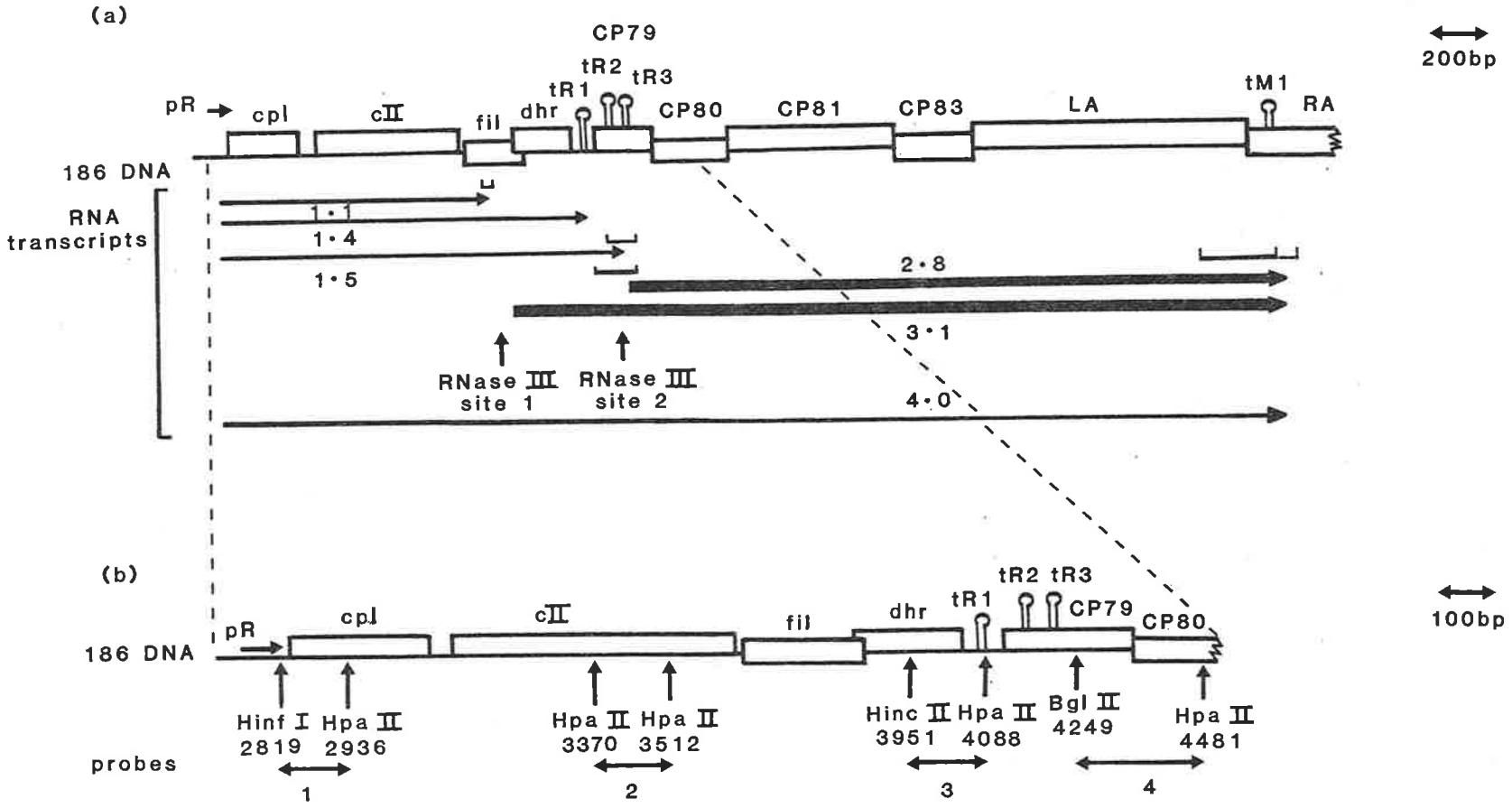
(a) A diagrammatic representation of the gene content of the 186 early lytic and middle regions. Genes are represented by the boxed regions. The RA gene is only partially represented on the diagram, as indicated by the jagged-edged box. The position of the pR promoter is indicated by the short horizontal arrow. The potential terminators tR1, tR2, tR3 and tM1 are indicated by the stem-loop structures. (The stem-loop structures are not drawn to scale.)

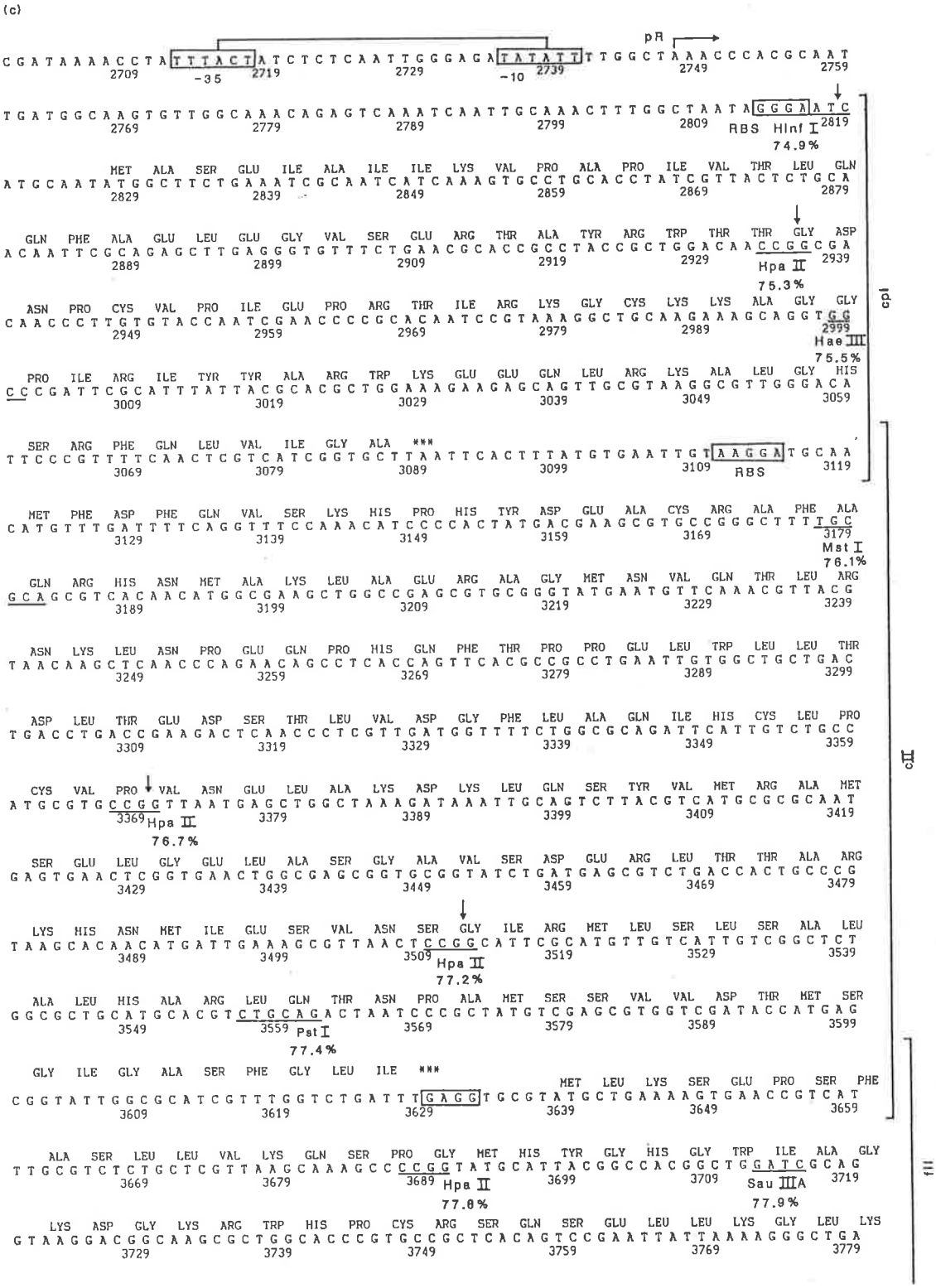
The positions of the RNA transcripts are indicated by the arrows beneath the genetic map. The approximate relative abundance of the transcripts are indicated by the thickness of the arrows. The bracketed regions at the 3'-ends and 5'-ends of the transcripts indicate the regions to which these ends have been mapped (Figure 6.11). The RNaseIII cleavage positions are also shown.

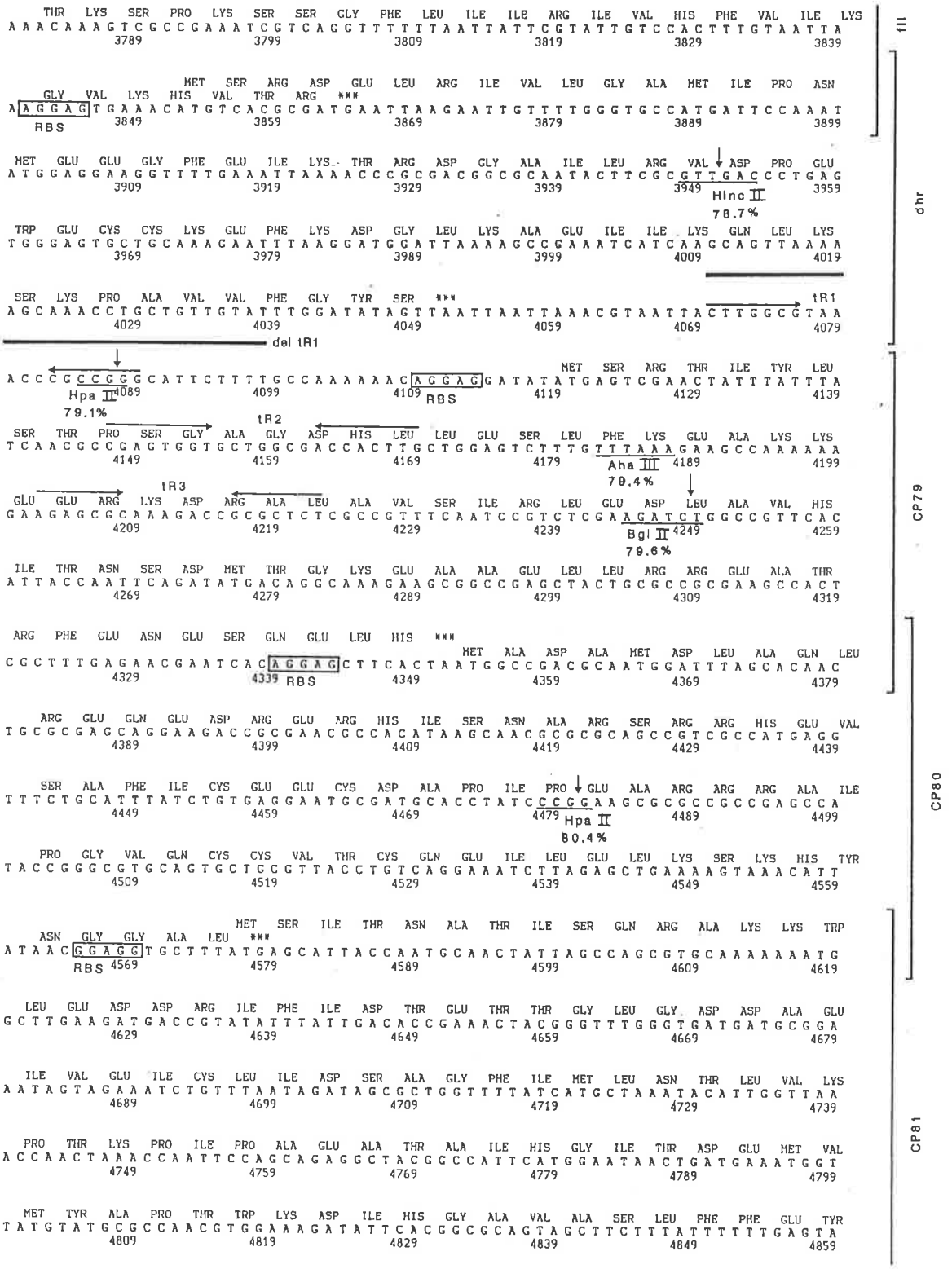
(b) The region from pR to the middle of CP80 is expanded to show the positions of the probes used for RNA analysis in this Chapter. The positions of relevant restriction sites are shown. The sequence coordinates of the restriction sites refer to the positions on the r-strand to the right of the restriction cut (Figure 8.1c).

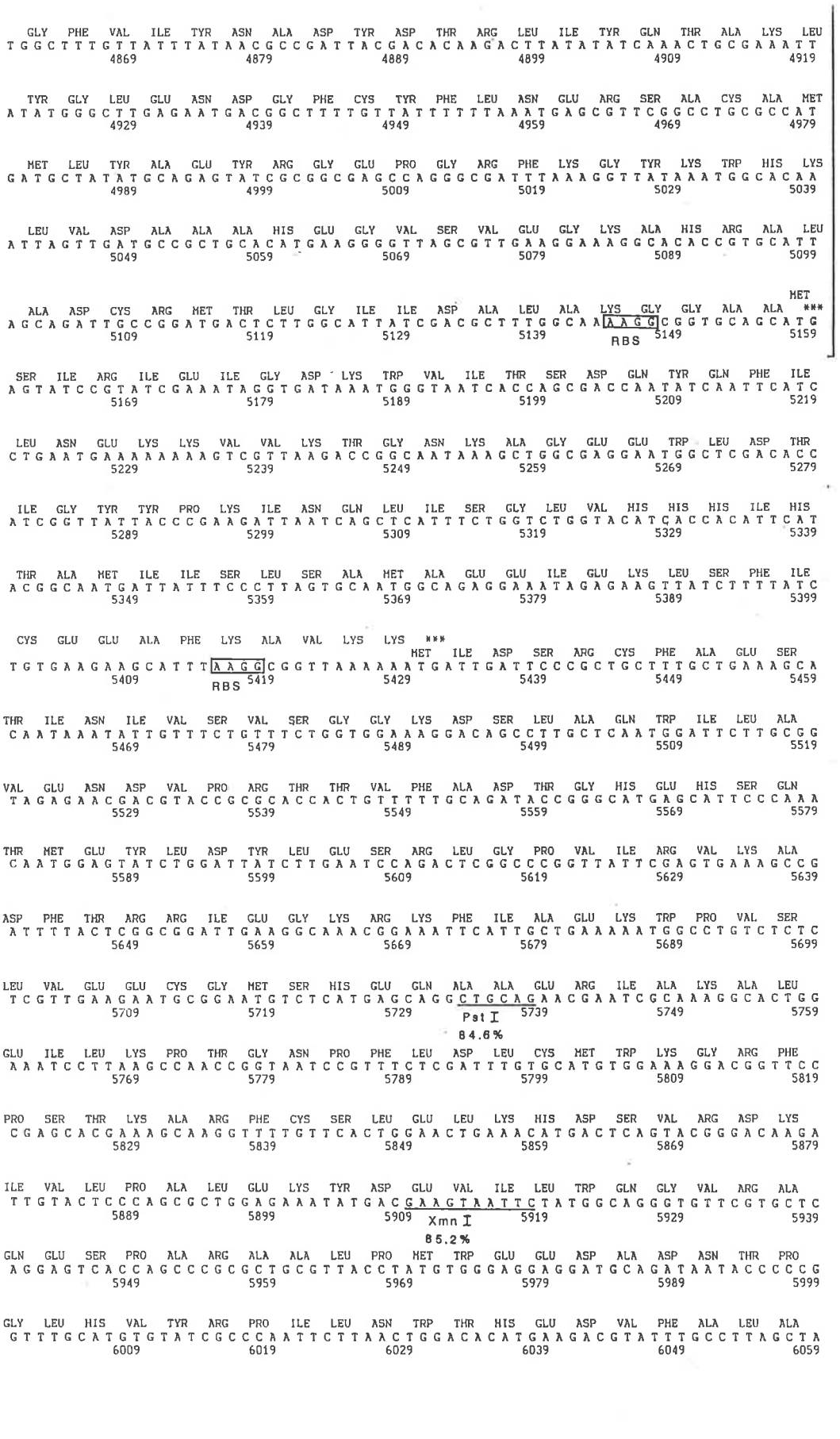
The probes were constructed as described in the legend to Figure 6.1.

(c) The DNA sequence of the l-strand from pR promoter to the middle of the LA gene (sequence coordinates 2700-6059), is shown. Genes are indicated to the right of the Figure. The -35 and -10 regions of the pR promoter are boxed and the startpoint of transcription from the pR promoter is indicated by the horizontal arrow. The potential terminator structures tR1, tR2 and tR3 are indicated by the convergent


(a)
CP79
tR2
tR3
tR1
200bp
pR
cpI
cII
fil
dhr
CP80
CP81
CP83
LA
tM1
RA
186 DNA
RNA transcripts
1·1
1·4
1·5
2·8
3·1
4·0
RNase III site 1
RNase III site 2
(b)
tR2 tR3
100bp
tR1
CP79
dhr
CP80
pR
cpI
cII
fil
186 DNA
Hinf I 2819
Hpa II 2936
Hpa II 3370
Hpa II 3512
Hinc II 3951
Hpa II 4088
Bgl II 4249
Hpa II 4481
probes
1
2
3
4


(c)
pR
-35
-10
RBS
Hinf I
74.9%
Hpa II
75.3%
Hae III
75.5%
RBS
Mst I
76.1%
Hpa II
76.7%
Hpa II
77.2%
Pst I
77.4%
Hpa II
77.8%
Sau IIIA
77.9%
cpI
cII
cIII
fII


fII
dhr
CP79
CP80
CP81
RBS
Hinc II
78.7%
tR1
del tR1
Hpa II
79.1%
tR2
tR3
RBS
Aha III
79.4%
Bgl II
79.6%
RBS
Hpa II
80.4%
RBS


GLY PHE VAL ILE TYR ASN ALA ASP TYR ASP THR ARG LEU ILE TYR GLN THR ALA LYS LEU
TGGCTTTGTTATTTATAACGCCGATTACGACACAAGACTTATATATCAAACTGCGAAATT
4869 4879 4889 4899 4909 4919
TYR GLY LEU GLU ASN ASP GLY PHE CYS TYR PHE LEU ASN GLU ARG SER ALA CYS ALA MET
ATATGGGCTTGAGAATGACGGCTTTTGTTATTTTTTAAATGAGCGTTCGGCCTGCGCCAT
4929 4939 4949 4959 4969 4979
MET LEU TYR ALA GLU TYR ARG GLY GLU PRO GLY ARG PHE LYS GLY TYR LYS TRP HIS LYS
GATGCTATATGCAGAGTATCGCGGCGAGCCAGGGCGATTTAAAGGTTATAAATGGCACAA
4989 4999 5009 5019 5029 5039
LEU VAL ASP ALA ALA ALA HIS GLU GLY VAL SER VAL GLU GLY LYS ALA HIS ARG ALA LEU
ATTAGTTGATGCCGCTGCACATGAAGGGGTTAGCGTTGAAGGAAAGGCACACCGTGCATT
5049 5059 5069 5079 5089 5099
MET
ALA ASP CYS ARG MET THR LEU GLY ILE ILE ASP ALA LEU ALA LYS GLY GLY ALA ALA ***
AGCAGATTGCCGGATGACTCTTGGCATTATCGACGCTTTGGCAAAAGGCGGTGCAGCATG
5109 5119 5129 5139 5149 5159
RBS
SER ILE ARG ILE GLU ILE GLY ASP LYS TRP VAL ILE THR SER ASP GLN TYR GLN PHE ILE
AGTATCCGTATCGAAATAGGTGATAAATGGGTAATCACCAGCGACCAATATCAATTCATC
5169 5179 5189 5199 5209 5219
LEU ASN GLU LYS LYS VAL VAL LYS THR GLY ASN LYS ALA GLY GLU GLU TRP LEU ASP THR
CTGAATGAAAAAAAAAGTCGTTAAGACCGGCAATAAAGCTGGCGAGGAATGGCTCGACACC
5229 5239 5249 5259 5269 5279
ILE GLY TYR TYR PRO LYS ILE ASN GLN LEU ILE SER GLY LEU VAL HIS HIS HIS ILE HIS
ATCGGTTATTACCCGAAGATTAATCAGCTCATTTCTGGTCTGGTACATCACCACATTCAT
5289 5299 5309 5319 5329 5339
THR ALA MET ILE ILE SER LEU SER ALA MET ALA GLU GLU ILE GLU LYS LEU SER PHE ILE
ACGGCAATGATTATTTCCCTTAGTGCAATGGCAGAGGAAATAGAGAAGTTATCTTTTATC
5349 5359 5369 5379 5389 5399
CYS GLU GLU ALA PHE LYS ALA VAL LYS LYS ***
MET ILE ASP SER ARG CYS PHE ALA GLU SER
TGTGAAGAAGCATTTAAAGGCGGTTAAAAAATGATTGATTCCCGCTGCTTTGCTGAAAGCA
5409 5419 5429 5439 5449 5459
RBS
THR ILE ASN ILE VAL SER VAL SER GLY GLY LYS ASP SER LEU ALA GLN TRP ILE LEU ALA
CAATAAATATTGTTTCTGTTTCTGGTGGAAAGGACAGCCTTGCTCAATGGATTCTTGCGG
5469 5479 5489 5499 5509 5519
VAL GLU ASN ASP VAL PRO ARG THR THR VAL PHE ALA ASP THR GLY HIS GLU HIS SER GLN
TAGAGAACGACGTACCGCGCACCACTGTTTTTGCAGATACCGGGCATGAGCATTCCCAAA
5529 5539 5549 5559 5569 5579
THR MET GLU TYR LEU ASP TYR LEU GLU SER ARG LEU GLY PRO VAL ILE ARG VAL LYS ALA
CAATGGAGTATCTGGATTATCTTGAATCCAGACTCGGCCCGGTTATTCGAGTGAAAGCCG
5589 5599 5609 5619 5629 5639
ASP PHE THR ARG ARG ILE GLU GLY LYS ARG LYS PHE ILE ALA GLU LYS TRP PRO VAL SER
ATTTTACTCGGCGGATTGAAGGCAAACGGAAATTCATTGCTGAAAAATGGCCTGTCTCTC
5649 5659 5669 5679 5689 5699
LEU VAL GLU GLU CYS GLY MET SER HIS GLU GLN ALA ALA GLU ARG ILE ALA LYS ALA LEU
TCGTTGAAGAATGCGGAATGTCTCATGAGCAGGCTGCAGAACGAATCGCAAAGGCACTGG
5709 5719 5729 5739 5749 5759
PstI
84.6%
GLU ILE LEU LYS PRO THR GLY ASN PRO PHE LEU ASP LEU CYS MET TRP LYS GLY ARG PHE
AAATCCTTAAGCCAACCGGTAATCCGTTTCTCGATTTGTGCATGTGGAAAGGACGGTTCC
5769 5779 5789 5799 5809 5819
PRO SER THR LYS ALA ARG PHE CYS SER LEU GLU LEU LYS HIS ASP SER VAL ARG ASP LYS
CGAGCACGAAAGCAAGGTTTTGTTCACTGGAACTGAAACATGACTCAGTACGGGACAAGA
5829 5839 5849 5859 5869 5879
ILE VAL LEU PRO ALA LEU GLU LYS TYR ASP GLU VAL ILE LEU TRP GLN GLY VAL ARG ALA
TTGTACTCCCAGCGCTGGAGAAATATGACGAAGTAATTCTATGGCAGGGTGTTCGTGCTC
5889 5899 5909 5919 5929 5939
XmnI
85.2%
GLN GLU SER PRO ALA ARG ALA ALA LEU PRO MET TRP GLU GLU ASP ALA ASP ASN THR PRO
AGGAGTCACCAGCCCGCGCTGCGTTACCTATGTGGGAGGAGGATGCAGATAATACCCCCG
5949 5959 5969 5979 5989 5999
GLY LEU HIS VAL TYR ARG PRO ILE LEU ASN TRP THR HIS GLU ASP VAL PHE ALA LEU ALA
GTTTGCATGTGTATCGCCCAATTCTTAACTGGACACATGAAGACGTATTTGCCTTAGCTA
6009 6019 6029 6039 6049 6059
CP81
CP83
LA

Cf Table 7.1 production

TABLE 8.1

Quantitation of RNA from the 186 early lytic and middle regions after chloramphenicol treatment.[a]

Source of RNA	Level of hybridization		
	Early[b] probe 1	Early[c] probe 2	Middle[d] probe
E4090[e] infected with 186vir1(3)	475120	432487	251265
E4090[e] infected with 186vir1(3) and treated with chloramphenicol	70777	26636	7149
-cam/+cam[f]	7	16	35

Notes to Table 8.1

a. RNA hybridizing to the 186 early and the middle regions was quantitated by RNA dot blot analysis (Chapter 2.36.4). RNA was prepared 20 min after infection of the cultures with 186 vir1 (3) (Chapter 2.36.1, 2.36.2). The cultures were either untreated or treated with chloramphenicol (400 ug/ml) 10 min prior to infection. Two ug of each sample of RNA was loaded onto the nitrocellulose filters as described in Chapter 2.36.4. Radioactive probes were prepared as described in Chapter 2.34.1 and Figure 6.1. Hybridization and autoradiography were as described in Chapter 2.36.3(b). The level of hybridization of the probe to RNA was quantitated by scanning the autoradiograph using a Zeinch scanning laser densitometer. The specific activity of the probes was normalized by the quantitation of the amount of probe hybridizing to known concentrations of DNA. The RNA dot blot intensities presented here are normalized.

b. The early probe (1) was the HinfI-HpaII (2819-2935) DNA fragment prepared from mEC802 (Figure 8.1b).

c. The early probe (2) was the HpaII (3370-3511) DNA fragment prepared from mEC404 (Figure 8.1b).

d. The middle probe was the BglII-HpaII (4249-4480) DNA fragment prepared from mEC408 (Figure 8.1b).

e. E4090 is an RNaseIII$^-$ strain and was used in order to decrease RNA degradation (see Table 7.1).

f. The ratio of RNA hybridizing to each probe from the untreated culture compared with that from the chloramphenicol-treated culture.

Egan (1981) to detect only 186 early lytic transcription (Chapter 1.3.2). However, consitent with the requirement of an antitermination mechanism, which acts at the early terminators, the results obtained in this Chapter, revealed that although early lytic transcription was decreased by chloramphenicol, middle transcription was decreased to a greater extent (a 16-fold decrease compared with a 35-fold decrease; Table 8.1). Furthermore, chloramphenicol resulted in a greater decrease in RNA detected by the promoter-distal early probe E(2) than by the promoter-proximal early probe E(1) (a 16-fold decrease compared with a 7-fold decrease; Table 8.1). This result indicates that the presence of chloramphenicol results in transcription termination at a significant level in the region 2936-3511, which is 5' to the tR1, tR2 and tR3 termination signals.

In order to determine the number and sizes of transcripts, which are produced when 186 protein synthesis is prevented by chloramphenicol, Northern analysis of RNA prepared from the chloramphenicol-treated or untreated cultures was performed using probes specific to the early lytic or middle region (Figure 8.1). Should an antitermination control mechanism be required for middle transcription, it would be expected that chloramphenicol should result in a transcription block at the early terminators and lead to an increase in the 1.4 kb and 1.5 kb transcripts relative to the 4.0 kb transcript. The results presented in Figure 8.2 reveal that the 1.4 kb and 1.5 kb early lytic transcripts and the 4.0 kb transcript (and also the 1.1 kb, 2.8 kb and 3.1 kb transcripts, present due to residual RNaseIII processing in the RNaseIII$^-$ strain) were present in RNA prepared from the chloramphenicol-treated culture, but only in very low amounts. The detection of the 4.0 kb transcript (although only faintly visible on a long exposure) shows that transcription occurs past the early terminators. These results also show that the 1.4 kb and 1.5 kb transcripts are present at a greater level than the 4.0 kb transcript;

1. RNA hybridized with the early probe.
2. RNA from the chloramphenicol-treated culture hybridized with the early probe
3. RNA hybridized with the middle probe.
4. RNA from the chloramphenicol-treated culture hybridized with the middle probe.

The 1.1 kb, 1.4 kb, 1.5 kb, 2.8 kb, 3.1 kb and 4.0 kb RNA transcripts are indicated by the arrows. RNA molecular weight markers (Chapter 2.11.2) are indicated on the Figure.

RNA prepared from the uninfected cells (E4090) showed no hybridization with either of these probes (data not shown).

Figure 8.2 186 early lytic and middle transcription when translation is inhibited by chloramphenicol.

This Figure shows the transcription pattern of the 186 early lytic and middle regions after the inhibition of protein synthesis by chloramphenicol. The positions on the DNA sequence of probes used to detect 186 early lytic or middle RNA, are shown diagrammatically. The appropriate restriction sites are shown. The sequence coordinates of the restriction sites refer to the position on the r-strand to the right of the restriction cut (Figure 6.1). Genes are represented by the boxed regions. The positions of the pR promoter and tR1, tR2 and tR3 terminators, are shown. The arrow next to the pR promoter represents the direction of transcription. The terminators, represented by the hairpin strctures, are not drawn to scale. The probes used are as follows :

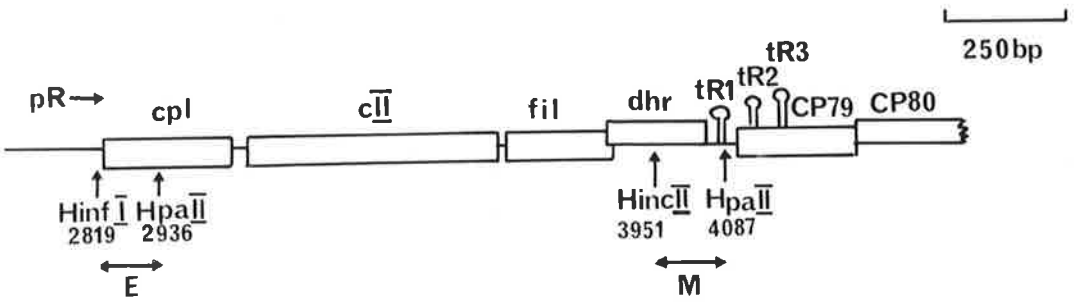
Early probe (E) : HinfI-HpaII (2819-2935)

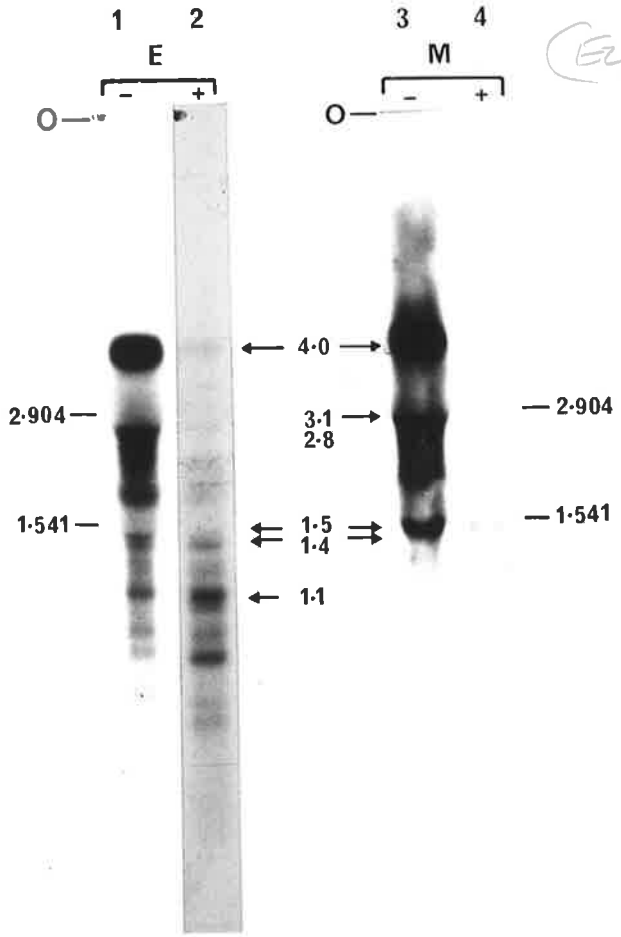
Middle probe (M) : HincII-HpaII (3951-4087)

The construction of these probes is described in the legend to Figure 6.1.

The RNaseIII$^-$ strain (E4090) was treated with chloramphenicol (400 ug/ml) for 10 min and then infected with 186 Aam11vir1 (4). Cells untreated with chloramphenicol were also infected with 186 Aam11vir1 (4). RNA was prepared 20 min after infection, as described in Chapter 2.36.1 and 2.36.2. Twenty ug of RNA was denatured with glyoxal and fractionated on a 1.5% agarose gel (Chapter 2.36.3). RNA was transferred bi-directionally to nitrocellulose and filters were hybridized with the probe specific to the early lytic region or to the middle region (Chapter 2.36.3a, 2.29.2). The autoradiographs were exposed for 2 days at -80^{o}C with an intensifying screen.

The gel tracks represent RNA prepared from the infected culture either treated (+) or untreated (-) with chloramphenicol. The tracks are as follows :


250bp
pR
cpI
cII
fil
dhr
tR1
tR2
tR3
CP79
CP80
Hinf I
2819
HpaII
2936
E
HincII
3951
HpaII
4087
M


1
2
E
3
4
M
O
O
4·0
2·904
3·1
2·8
2·904
1·541
1·5
1·4
1·541
1·1

however, there is not a specific accumulation of the 1.4 kb and 1.5 kb transcripts. The most abundant early lytic transcript appears to be a transcript sized at 0.7 kb (which would have been detected by both early probes used in the RNA dot blot analysis).

In summary, these results have shown that the inhibition of 186 protein synthesis by chloramphenicol does not result in a specific transcription block at the early terminators, but in a general decrease in transcription in both the early lytic and middle regions. This decrease in transcription may reflect the requirement of an antitermination mechanism at termination sites, which are promoter-proximal to the early terminators tR1 and tR2 or tR3, but was considered to be more likely a result of transcriptional polarity.

Transcriptional polarity can occur when translation is prevented either by a mutation in a gene (Adhya and Gottesman, 1978), by a translational repressor (Singer and Nomura, 1985; Cole and Nomura, 1986) or by inhibitors of protein synthesis, such as chloramphenicol (Imamoto, 1973; Mackie and Wilson, 1972; Graham et al., 1982; Nakamura et al., 1979; Schlessinger et al., 1983) and is due to the premature termination of transcription (Franklin and Yanofsky, 1976; Adhya and Gottesman, 1978). This premature termination is thought to be caused by the binding of the Rho termination factor to sites on the RNA that would normally be inaccessable due to translation (Adhya and Gottesman, 1978; Galloway and Platt, 1985).

From the results obtained with these chloramphenicol studies, it was concluded that since transcription proceeds past the early terminators in the absence of protein synthesis, there is no absolute requirement for an antitermination mechanism for middle gene expression. However, no conclusions could be made concerning the existence of an antitermination mechanism that acts to increase the transcription of the middle genes,

because of the possibility that transcriptional polarity was occurring in the absence of translation.

Since transcription proceeds past the early terminators in the absence of translation, the next step was to determine the intrinsic strengths of the early terminators.

8.2.2 The Strength of the 186 Early Terminators In Vivo.

To determine the strength of the early terminators the McKenney terminator-analysis vector, pKL600, was used. pKL600 encodes the galK gene downstream from the pLac promoter, and it was planned to induce expression from pLac with IPTG. However, the pLac promoter was found to be active in the absence of IPTG, as indicated by the finding that cells containing pKL600 form red colonies on MacConkey-galactose plates (data not shown), possibly due to titration of the lac repressor by the multiple copies of the lac operator encoded on the high copy number plasmid. Also, IPTG was found to give only a small enhancement in galK expression from pKL600 as determined by assaying for the galK gene-product, galactokinase (Chapter 2.19; data not shown), and therefore was not used. Cloning of a terminator between pLac and galK in pKL600 results in a reduction in the expression of galK, which corresponds to the strength of the terminator (McKenney et al., 1981).

A clone containing the terminators for the 1.4 kb transcript (tR1) and the 1.5 kb transcript (tR2 or tR3) in pKL600, was constructed (Chapter 2.31). This clone, pEC411 (Chapter 2.3.2), contains the 0.3 kb HincII-BglII (78.9%-79.6%) fragment from 186 (Figure 8.3a, 8.1c). It is pertinent to note that this fragment encodes the 3'-end of the dhr gene (99 bp), which is not translated (since translation from the plasmid-encoded lac gene is prevented from entering into the cloned region by the presence of stop codons in all three reading-frames; Chapter 2.3.1). Since premature termination may occur in this region due to transcriptional polarity, the

Figure 8.3 The gene content of plasmid-clones in the McKenney terminator-analysis and promoter-analysis vectors.

(a) Plasmid-clones in the McKenney terminator-analysis vector, pKL600.

A diagrammatic representation of the gene content of the region from pR to the middle of CP80 (See Figure 8.1c). Genes are represented by the boxed regions. The position of the pR promoter is indicated by the horizontal arrow. The positions of the tR1, tR2 and tR3 terminators are indicated by the stem-loop structure. (The stem-loop structures are not drawn to scale.) The position of the deltR1 deletion is indicated. Relevant restriction sites are shown. The sequence coordinates of the restriction sites refer to the first base of the site on the l-strand.

The gene content of pEC411, pEC412 and pEC427 is shown. The construction of these plasmid-clones is described in Chapter 2.3.2. The restriction sites used to constuct these clones are indicated. The 3'-end of the dhr gene is not shown since it is not expected to be expressed. Genes that are only partially represented on the plasmid-clones are indicated by the jagged-edged boxes. The terminators that are present on these clones are indicated. The genes are expressed from the pLac promoter present on the vector. The position of the galK gene encoded by the vector is also shown.

(a) Clones in Mckenney terminator–analysis vector,pKL600

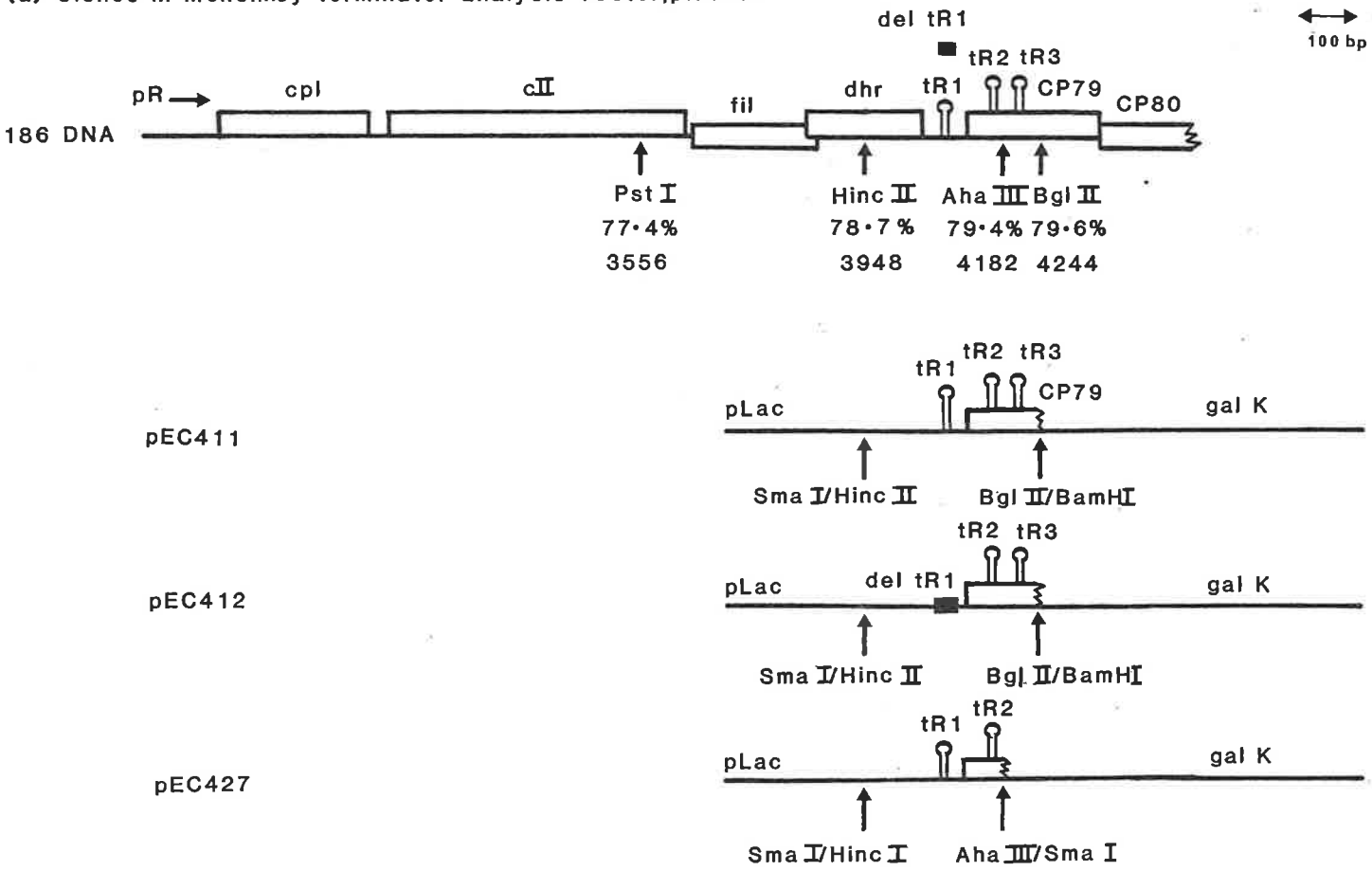
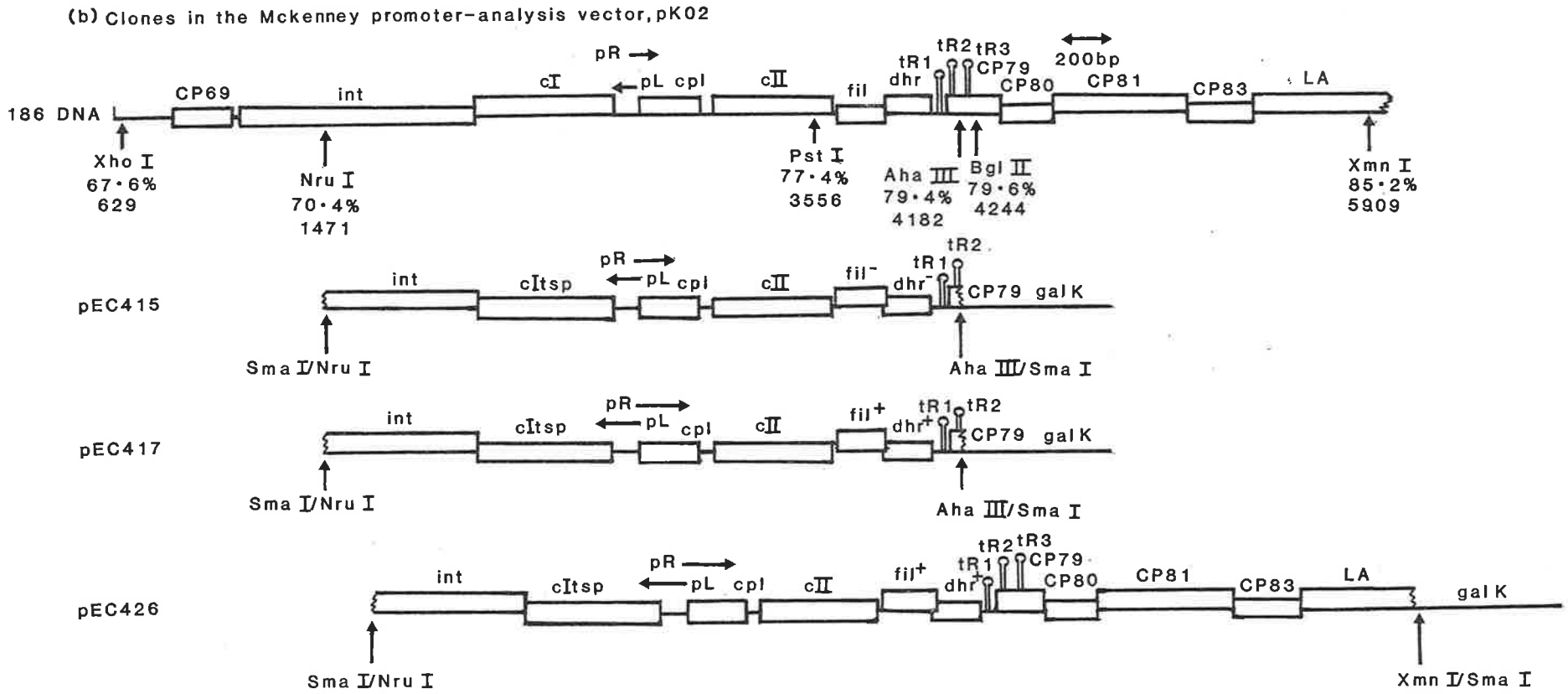


Figure 8.3(b) Plasmid-clones in the McKenney promoter-analysis vector, pKO2.

A diagrammatic representation of the gene content from the XhoI site to the middle of the LA gene. Genes are represented by the boxed regions. The positions of the pR and pL promoters are shown and the direction of transcription is indicated by the horizontal arrows. The positions of the tR1, tR2 and tR3 terminators are indicated by the stem-loop structures. (The stem-loop structures are not drawn to scale.) Relevant restriction sites are shown. The sequence coordinates of the restriction sites refer to the first base of the site on the l-strand.

The gene content of pEC415, pEC417 and pEC426 is shown. The construction of these plasmid-clones is described in Chapter 2.3.2. The restriction sites used to construct these clones are indicated. Genes that are only partially represented on the plasmid-clones are indicated by the jagged-edged boxes. The promoters and terminators are also indicated. The position of the galK gene carried by the vector is also shown.


(b) Clones in the Mckenney promoter-analysis vector, pK02
186 DNA
CP69
int
cI
pR
pL cpl
cII
fil
tR1
dhr
tR2
tR3
CP79
200bp
CP80
CP81
CP83
LA
Xho I
67·6%
629
Nru I
70·4%
1471
Pst I
77·4%
3556
Aha III
79·4%
4182
Bgl II
79·6%
4244
Xmn I
85·2%
5909
pEC415
cItsp
fil⁻
dhr⁻
CP79 galK
Sma I/Nru I
Aha III/Sma I
pEC417
fil⁺
dhr⁺
pEC426
galK
Xmn I/Sma I

efficiency of termination obtained with this clone may be greater than that obtained in the phage.

The level of galK expression from pEC411 was quantitated by assaying for galactokinase (Chapter 2.19) and was compared with that obtained from pKL600. The results presented in Table 8.2(a), show that galK expression is reduced by 56% by the presence of the fragment encoding tR1, tR2 and tR3. This confirms that this region contains transcription terminators.

To determine whether this termination was due mostly to tR1, which is known to be active in vitro (Chapter 8.1, 9.2.4), the HincII-BglII (78.7%-79.6%) fragment was cloned from deltR1 into pKL600 to give pEC412 (Chapter 2.3.2, 2.31, 6.2.3; Figure 8.3a, 8.1c). This clone showed a 48% reduction in the level of galK expression compared with pKL600 (Table 8.1a). As shown in Figure 8.4, this result suggests that tR1 by itself results only in a 15% reducton in galK expression and is therefore a relatively weak terminator in vivo. Therefore, most of the termination, which occurs within the HincII-BglII region, probably occurs at tR2 or tR3.

To determine whether the tR3 terminator was likely to be responsible for the 48% termination observed with pEC412, the HincII-AhaIII (78.7%-79.4%) fragment containing tR1 and tR2 (but not tR3) was cloned into pKL600 to form pEC427 (Chapter 2.3.2, 2.31; Figure 8.3a, 8.1c). The level of galK expression in this clone was quantitated and the results are presented in Table 8.2(b). The results show that the presence of the HincII-AhaIII (78.7%-79.4%) fragment reduced galK expression by 53%. This is approximately the same level of expression as obtained with the clone encoding tR1, tR2 and tR3 (pEC411). Thus, it appears that tR3 does not function as a termination signal. From the knowledge of the strength of the tR1 terminator (15%), the apparent termination efficiency at tR2 is 45% (Figure 8.4). These results indicate that the 1.5 kb transcript (which was predicted in Chapter 6.2.3 to terminate at tR2 or tR3) mostly likely terminates at tR2.

TABLE 8.2

The expression of galK from clones in the McKenney terminator-analysis vector (pKL600) : The efficiency of termination at the 186 early terminators.[a]

Plasmid-clone (Plasmid)[b]	GalK units	% expression[c]	% termination[d]
(A)			
pKL600	88±0	100%	0%
pEC411 [HincII-BglII (78.7%-79.6%)]	39±3	44%	56%
pEC412 [HincII-BglII (78.7%-79.6%) from 186deltR1(22)]	47±3	52%	48%
(B)			
pKL600	111±1	100%	0%
pEC411 [HincII-BglII (78.7%-79.6%)]	48±4	43%	57%
pEC427 [HincII-AhaIII (78.7%-79.4%)]	52±1	47%	53%

Notes to Table 8.2

a. Cultures of E863 carrying plasmid-clones (or plasmids) were grown at 37°C in M63 supplemented media (containing ampicillin) to A_{600} = 0.2 - 0.6. Galactokinase units were determined as described in Chapter 2.19. The galactokinase units presented here are an average of two measurements. (The standard deviation is shown.)

b. Plasmids and plasmid-clones are described in detail in Chapter 2.3 and in Figure 8.3(a). The region from 186 contained in the clones is listed.

c. The expression of galK from these plasmid-clones relative to that obtained for pKL600, is shown.

d. % termination = 100 - % expression.

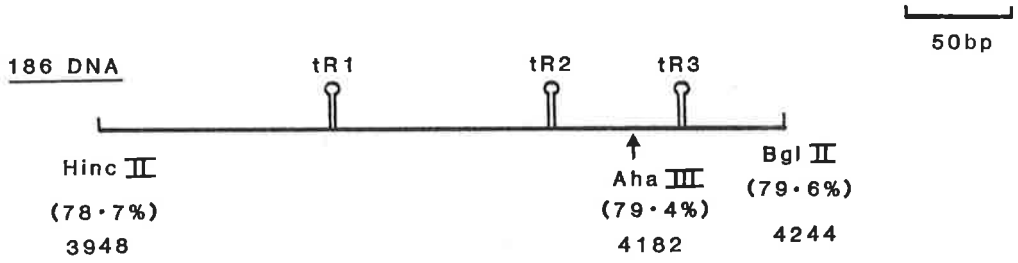
Figure 8.4 The termination efficiency of the early terminators.

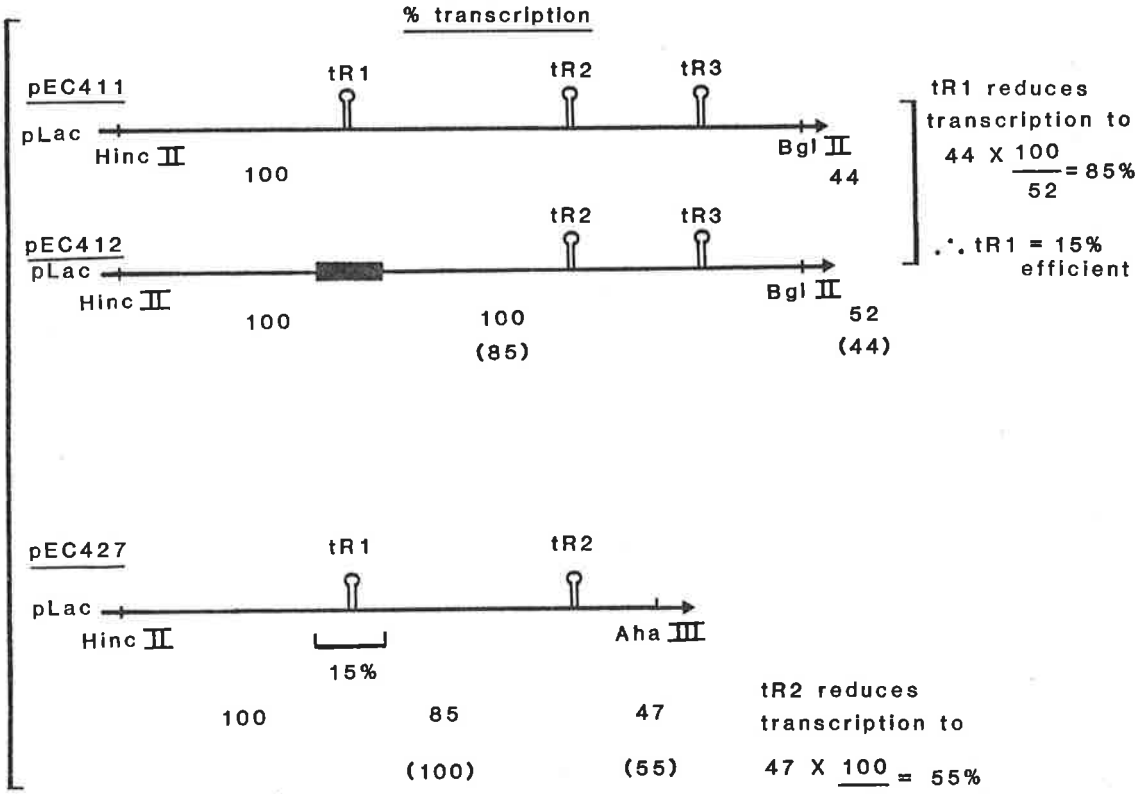
The HincII-BglII (78.7%-79.6%) region is shown diagrammatically. The positions of the tR1, tR2 and tR3 terminators are indicated by the stem-loop structures. The position of the AhaIII site is shown. The sequence coordinates refer to the first base of the site on the l-strand (see Figure 8.1c).

Transcription from the pLac promoter of the regions from 186 encoded in pEC411, pEC412 and pEC427 is indicated by the horizontal arrows. Terminators present on these transcripts are shown and the position of the deltR1 deletion is indicated by the shaded box. The percentage of transcription obtained for these clones relative to that obtained for the plasmid-vector, pKL600 (Table 8.1), is shown to the right of the diagram.

The termination efficiency at tR1 was deduced from the percentage of transcription of pEC411 compared with pEC412 (which contains a deletion of tR1), as shown to the right of the Figure.

The termination efficiency of tR2 was deduced from the percentage of transcription of pEC427 and the knowledge of the termination efficiency of tR1, as shown at the bottom of the Figure.


50bp
186 DNA
tR1
tR2
tR3
Hinc II
(78·7%)
3948
Aha III
(79·4%)
4182
Bgl II
(79·6%)
4244


% transcription
pEC411
pLac
Hinc II
tR1
tR2
tR3
Bgl II
100
44
tR1 reduces transcription to
44 X 100/52 = 85%
∴ tR1 = 15% efficient
pEC412
pLac
Hinc II
tR2
tR3
Bgl II
100
100
(85)
52
(44)
pEC427
pLac
Hinc II
tR1
tR2
Aha III
15%
100
85
(100)
47
(55)
tR2 reduces transcription to
47 X 100/85 = 55%
∴ tR2 = 45% efficient

In summary, these results have shown that tR1 is 15% efficient, in vivo. This result was unexpected since tR1 appears to be an efficient terminator in vitro (Chapter 9.2.4). This difference will be discussed in Chapter 10.4.1(b). Secondly, the results have shown that the removal of the AhaIII-BglII region (containing tR3) does not significantly effect the level of termination, suggesting that most termination occurs at tR2. However, as discussed previously, the clones used to determine this termination efficiency do not necessarily reflect the normal situation since the 3'-end of the dhr gene is not translated, and it is therefore possible that termination is occurring within the 3'-end of the dhr gene. Thirdly, these results revealed that total intrinsic strength of the early terminators is only 56%, at the most (since the clones used to determine this termination efficiency do not necessarily reflect the normal situation and it is possible that this level of termination is an over-estimation). Thus, at least 44% of the transcription passing through this region is able to proceed into the middle region without the need for an antitermination mechanism. Although this high level of readthrough may argue against the need for an antitermination mechanism for middle gene transcription, it does not rule out the involvement of such a mechanism, which may increase the level of readthrough into the middle region from 44% to nearly 100%.

8.2.3 Investigation of the Involvement of 186 Early Lytic and Middle Functions in Middle Gene Transcription.

The result obtained in Chapter 8.2.2, revealed that at least 44% of transcription of the early lytic region passes into the middle region. Since a significant level of middle gene expression occurs in the absence of 186 proteins, three points can be made concerning the putative middle control (antitermination) protein, Tom (Chapter 1.3.2). Firstly, the proposed Tom function may not exist and middle genes may be expressed due to the inefficiency of termination at the early terminators. Secondly, the

proposed Tom function may not be essential to 186, but merely required for efficient 186 lytic development. Thirdly, the proposed Tom function may itself be a middle function.

The early lytic genes have been studied (Chapter 5; I. Dodd and D. Carter, personal communication) and of these genes cpl and cII have defined functions in the 186 life cycle and are therefore unlikely to also be involved in antitermination. The fil and dhr genes both affect the host cell, but play an undefined role in 186 development (Chapter 5), and thus, are candidates for the proposed Tom function. The middle genes CP79, CP80, CP81 and CP83 are nonessential genes (J. Dibbens, personal communication), the functions of which are not known, whereas the middle genes LA and RA are essential genes involved in 186 replication (Hocking and Egan, 1982b; Sivaprasad, 1984). Since definite functions have not been assigned to CP79, CP80, CP81 and CP83, these genes are also candidates for the presumptive Tom function.

To determine if an antitermination mechanism was likely to be involved in 186 middle gene expression, the strategy was to investigate whether the dhr, fil, CP79, CP80, CP81 or CP83 gene-products increased readthrough past the early terminators.

At this point it is pertinent to consider the possible mechanism of action of the postulated antitermination function. Antitermination mechanisms in phage λ involve the binding of the antitermination protein (gpN or gpQ) to RNA polymerase at specific sites in the RNA (nut or qut sites) (Chapter 1.4.2b). This binding converts the RNA polymerase into a termination-resistant form such that transcription can proceed past termination signals. The presumptive 186 antitermination mechanism may also share features of the λ antitermination mechanism (Chapter 1.4.2b). It is possible that the 186 antiterminator (Tom) functions by binding to RNA polymerase and that this binding occurs at specific sites in the RNA. These specific sites may be located at the early terminators, 5' to the

early terminators (as occurs in λ antitermination mechanisms; Chapter 1.4.2b) or may even be located at the 186 pR promoter. It is therefore important to examine the effect of 186 early lytic and middle functions on termination at the early terminators, using the entire pR-early terminator region.

8.2.3(a) The Effect of Fil and Dhr on Termination at the 186 Early Terminators.

To assess the effect of Fil and Dhr on termination at the early terminators (tR1 and tR2), two clones were obtained in the McKenney promoter-analysis vector, pKO2. The NruI-AhaIII (70.4%-79.4%) fragment, containing the pR promoter, the cItsp gene, all four early lytic genes and the early terminators, was isolated from the wild-type phage (186 cItsp (1)) and cloned into pKO2 in an orientation such that transcription from pR reads into the galK gene (Chapter 2.31, 2.3.2; Figure 8.3b, 8.1c). A similar clone was obtained from a fil$^-$ dhr$^-$ phage (186 cItspAam11Dhr1 (13)). [Dhr1 contains point mutations in the fil and dhr genes, which eliminate the phenotypes associated with these genes (Chapter 5.2.6a). This mutant was chosen for use in this study rather than fil amber (CP77am) or dhr amber (CP78am) mutants, since these amber mutants appear to be slightly leaky when present on a plasmid-clone (Chapter 4.2.3).]

These clones, pEC415 (from the fil$^-$ dhr$^-$ phage) and pEC417 (from wild-type 186) (Figure 8.3b), encode the cItsp gene, which represses pR at 30°C and allows the expression of early lytic genes (and then galK) from pR at 41°C. The level of galK expression from pEC415 can be compared with that obtained from pEC417 at 41°C (Chapter 2.19), in order to determine if Fil or Dhr have an affect on termination at the early terminators.

To determine the time of heat-induction at 41°C needed to reach a steady state level of galK expression from 186 pR, a time course experiment

was carried out after heat-induction of galK$^-$ cells carrying pEC417. Samples taken at different times after heat-induction were assayed for the amounts of galactokinase present (Chapter 2.19). The results presented in Figure 8.5, show that after heat-induction there is a very rapid increase in galK expression from pEC417 to reach a steady state level of 286 units at 60 min. Thus, for all clones encoding pR and the cItsp gene, samples were taken 60 min after heat-induction at 41^{o}C.

The level of galK expression from pEC415 and pEC417 after 60 min at 41^{o}C is shown in Table 8.3. pEC415 and pEC417 show very similar levels of galK expression. Should dhr or fil encode an antitermination protein, it would be expected that the level of galK expression from the dhr$^+$ fil$^+$ clone (pEC417) would be greater than that obtained for the dhr$^-$ fil$^-$ clone (pEC415). On the basis of the strength of the early terminators (which are 44% efficient; Chapter 8.2.2) a 2.2-fold (100/44) difference may have been expected if fil or dhr encoded the putative antitermination function and gave complete antitermination. Since the dhr$^-$ fil$^-$ clone (pEC415) did not show a lower level of galK expression than the dhr$^+$ fil$^+$ clone (pEC417), it can be concluded that fil and dhr are not likely to encode an antitermination function.

8.2.3(b) The Effect of CP79, CP80, CP81, and CP83 on Termination at the 186 Early Terminators.

A clone encoding the cItsp gene, the pR promoter, the four early lytic genes, the early terminators and CP79, CP80, CP81 and CP83 was obtained in the McKenney promoter-analysis vector, pKO2, in an orientation such that transcription from pR reads into galK. The construction of this clone, pEC426, which contains the NruI-XmnI (70.4%-85.2%) fragment is described in Chapter 2.3.2 (Figure 8.3b, 8.1c).

The level of galK expression from pEC426 was determined after heat-induction of a galK$^-$ strain carrying this clone at 41^{o}C for 60 min

Figure 8.5 Expression of galK from pEC417 with time after heat-induction.

E863 carrying pEC417 was grown in M63-supplemented media (described in Chapter 2.19) at 30°C to A_{650}= 0.2. The zero time sample was then taken and concentrated 5-fold. The culture was heat-induced at 41°C and samples were taken at the indicated times. Lysates were prepared from all samples and assayed for galactokinase, as described in Chapter 2.19. Lysates of the samples taken from 20 min to 70 min were diluted 5-fold (as described in Chapter 2.19) before assaying for galactokinase.

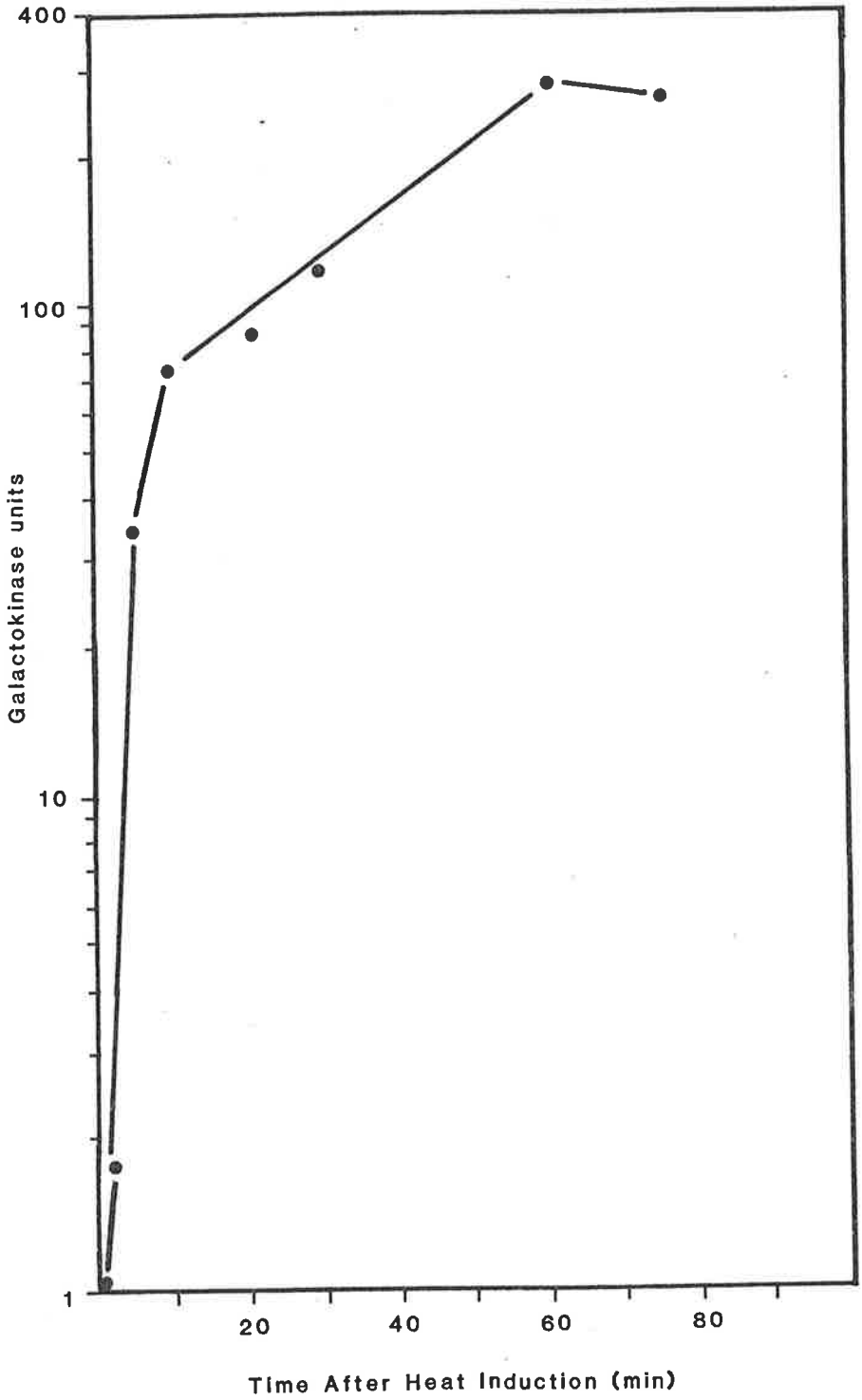

400
100
10
1
Galactokinase units
20
40
60
80
Time After Heat Induction (min)

TABLE 8.3

The expression of *galK* from clones in the McKenney promoter-analysis vector (pKO2) : Test for the effect of Fil and Dhr on termination at the 186 early terminators.[a]

Plasmid-clone (plasmid)[b]	Temperature	GalK units
pKO2	30°C	2.1
	41°C	7.2
pEC417 (*cItsp*, *cpl*, *cII*, *fil*$^+$,*dhr*$^+$)	30°C	0.2
	41°C	150.0±12
pEC415 (*cItsp*, *cpl*, *cII*, *fil*$^-$,*dhr*$^-$)	30°C	0.4
	41°C	166.0±7

Notes to Table 8.3

a. Cultures of E863 carrying plasmid-clones (or plasmids) were grown at 30°C in M63 supplemented media (containing ampicillin) to A_{600} = 0.2 - 0.4. The cultures were then heat-induced for 60 min at 41°C. As controls duplicate cultures were grown at 30°C for 60 min. Galactokinase units were determined as described in Chapter 2.19. The galactokinase units presented for pEC417 and pEC415 at 41°C are an average of three measurements. (The standard deviation is shown.)

b. Plasmids and plasmid-clones are described in detail in Chapter 2.3 and in Figure 8.3(b). The 186 genes carried by these plasmid-clones are listed.

(Chapter 2.19). As a control, the level of galK expression from pEC417 (encoding cItsp, pR, the early lytic genes and the early terminators; Chapter 2.3.2; Figure 8.3b), was determined and compared with that obtained for pEC426. It was expected, that if CP79, CP80, CP81 or CP83 encode the Tom function, then the level of galK expression from pEC426 would be greater (theoretically 2.2x) than that obtained for pEC417. pEC426 did not result in a greater level of galK expression than pEC417 (Table 8.4). This suggests that CP79, CP80, CP81 and CP83 do not encode the Tom function. pEC426 in fact gave a lower level of galK expression than pEC417 (54% lower). This result suggests that in pEC426, additional termination is occurring in the AhaIII-XmnI (79.4%-85.2%) region, and most likely in the BglII-XmnI (79.6%-85.2%) region since the AhaIII-BglII (79.4%-79.6%) region does not appear to result in significant transcription termination (Chapter 8.2.2).

8.3 SUMMARY.

This Chapter was concerned with determining whether an antitermination mechanism was likely to be involved in the control of 186 middle gene expression. Three approaches were used to investigate this, and all three failed to provide evidence for the existence of such a mechanism.

Transcription studies using chloramphenicol to inhibit protein synthesis were carried out to determine whether there was a specific transcription block at the early terminators. These studies showed that early lytic and middle transcription were both significantly reduced by chloramphenicol. Middle transcription was reduced more than early lytic transcription (approximately two-fold); however, these results could not be used to support the existence of an antitermination mechanism because of the probability that transcriptional polarity was resulting in this decrease in transcription.

TABLE 8.4

The expression of galK from clones in the McKenney promoter-analysis vector (pKO2) : Test for the effect of middle functions on termination at the 186 early terminators.[a]

Plasmid-clone (plasmid)[b]	Temperature	GalK units
pKO2	30°C	2.5±0.2
	41°C	8.0±0.3
pEC417 (cItsp, cp1, cII, fil, dhr)	30°C	0.3±0.2
	41°C	186.0±7.0
pEC426 (cItsp, cp1, cII, fil, dhr, CP79, CP80, CP81, CP83)	30°C	3.0±0.1
	41°C	103.0±7.0

Notes to Table 8.4

a. Cultures of E863 carrying plasmid-clones (or plasmids) were grown at 30°C in M63 supplemented media (containing ampicillin) to A_{600} = 0.2 - 0.4. The cultures were then heat-induced for 60 min at 41°C. As controls duplicate cultures were grown at 30°C for 60 min. Galactokinase units were determined as described in Chapter 2.19. The galactokinase units presented here are an average of two measurements. (The standard deviation is shown.)

b. Plasmids and plasmid-clones are described in detail in Chapter 2.3 and in Figure 8.3(b). The 186 genes carried by these plasmid-clones are listed.

Plasmid-clones in the McKenney terminator-analysis vector were used to determine the intrinsic strength of the early terminators *in vivo*. The early terminators together were only 56% efficient. However, this level of termination may be an over-estimate since the clones used in this study contain the 3'-end of the *dhr* gene, which is not translated. As discussed previously (Chapter 8.2.1), untranslated regions may serve as binding sites for the Rho termination factor and lead to transcriptional polarity effects. Thus, these clones may not necessarily reflect the normal situation. These studies also revealed that the *t*R1 terminator (the terminator *in vivo* for the 1.4 kb early lytic transcript) was only 15% efficient in termination and the potential termination structure, *t*R3, was unlikely to play a role in termination. Thus, regions other than *t*R1 and the *Aha*III-*Bgl*II (79.4%-79.6%) region (encoding *t*R3) are involved in the termination occurring within the *Hinc*II-*Bgl*II (78.7%-79.6%) region. It is possible that *t*R2 may be responsible for this termination, although the involvement of other sequences (e.g. the untranslated 3'-end of the *dhr* gene) can not be ruled out.

Since the early terminators are at most only 56% efficient, it was concluded that an antitermination mechanism may not be involved in the control of 186 middle gene transcription, or if an antitermination mechanism does exist then it is probably not essential. Furthermore, if the putative antitermination protein, Tom, exists then it may be encoded by a middle gene.

The 186 early lytic and middle functions; Fil, Dhr, CP79, CP80, CP81 and CP83, were tested for their effect on increasing transcription past the early terminators using clones in the McKenney promoter-analysis vector. These results showed that it is unlikely that *fil*, *dhr*, *CP79*, *CP80*, *CP81* or *CP83* encode the postulated middle control (antitermination) function, Tom.

In conclusion, these studies have not provided evidence for an antitermination mechanism or for the involvement of an antitermination

function in 186 middle gene control. However, the possibility that antitermination may play some non-essential role in 186 lytic development has not been excluded. The question of whether a control mechanism for middle gene expression exists is discussed in Chapter 10.4.1.

CHAPTER 9.

CHARACTERIZATION OF A VIRULENT MUTANT WITH AN INSERTION IN THE 186 EARLY LYTIC REGION AND IMPLICATIONS FOR MIDDLE GENE TRANSCRIPTION.

CHAPTER 9. CHARACTERIZATION OF A VIRULENT MUTANT WITH AN INSERTION IN THE 186 EARLY LYTIC REGION AND IMPLICATIONS FOR MIDDLE GENE TRANSCRIPTION.

9.1 INTRODUCTION.

The results presented in Chapter 8, have not provided evidence for the existence of a control mechanism for middle gene transcription, but nor have they completely ruled out the existence of such a mechanism. Another approach for the study of the presumptive middle control mechanism would be to investigate 186 mutants in which transcription of the middle region is altered (for example, reduced relative to early lytic transcription or expressed constitutively). Such mutants may reveal control sites or genes, which are important in middle gene transcription.

There exists a 186 mutant, 186 del2 (6), which is potentially of the class of mutants, in which middle genes are expressed constitutively. 186 del2 was isolated from the same stock of phage as 186 del1 (5), as a surviving phage after a series of heat challenges followed by infectious cycles (Dharmarajah, 1975). However, unlike 186 del1, 186 del2 is a virulent phage (capable of growth in the presence of the 186 cI repressor). 186 del1 contains a deletion from 67.9% to 74.0%, which removes the int, cI and CP69 genes (Saint, 1979; R. O'Connor, unpublished; Kalionis et al., 1986a). 186 del2 also contains a deletion in the int-cI region and this deletion was expected to extend into the operator region (Chapter 1.2.2) thereby preventing repression of the pR promoter by the cI repressor. However, restriction mapping (Saint, 1979) and heteroduplex mapping under the electron microscope (R. O'Connor, unpublished results) suggested that the 186 del2 deletion was no larger than the deletion in 186 del1. Furthermore, these studies showed that 186 del2 carried an insertion to the

right of the PstI (77.4%) site (Figure 9.1). As the deletion of 186 del2 would need to be much larger (~160 bp) than that of 186 del1 to include the operator site (Kalionis et al., 1986a; Chapter 1.2.2) it was likely that the insertion was bestowing the virulent phenotype. Thus, it appeared that the essential replication functions encoded in the middle region were being expressed when 186 del2 infected a lysogen even though transcription from pR would be blocked.

Since 186 del2 appeared to be a 186 mutant with altered middle gene transcription, an investigation of 186 del2 was commenced with the expectation that it might provide some novel approach to understanding the putative control of middle gene transcription.

The approach initially used to characterize 186 del2, was to determine the DNA sequence of the region of the insertion. The DNA sequence of the 186 del2 deletion and operator-promoter region (Chapter 1.2.2) was also determined to confirm that there were no mutations in this region, which were likely to confer virulence.

The positions of all restriction sites referred to in this Chapter are that of wild-type 186. DNA fragment sizes are as deduced from the DNA sequence of wild-type 186 or 186 del1 (5), (Chapter 3; Kalionis et al., 1986a; Sivaprasad, 1984), or sized from an agarose gel.

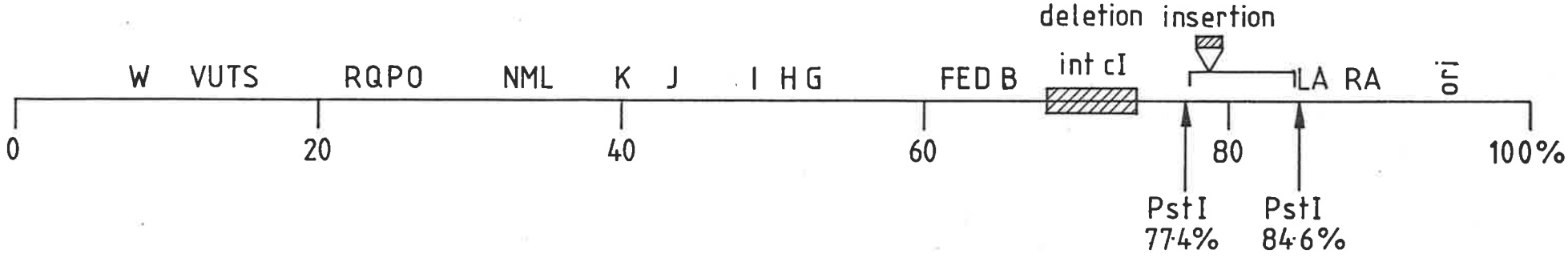
9.2 RESULTS AND DISCUSSION.

9.2.1 Restriction Analysis of 186 del2.

Restriction analysis was used to further investigate the nature of the 186 del2 insertion. The digestion of 186 del2 DNA with BglII resulted in the production of a 0.6 kb fragment (data not shown), indicating that the insertion contained a BglII site. This result, and the fact that the insertion was close to the BglII (79.6%) site, suggested that this insertion may be a duplication of the region spanning the BglII site. Indeed restriction analysis of the 2.9 kb PstI (77.4%-84.6%) fragment from

Figure 9.1 Genetic map of 186 del2.

This map shows the positions of the deletion and insertion in 186 del2 (6) relative to the 186 genes. The deletion and insertion are indicated by the shaded boxes. The deletion removes the int gene and CP69 gene (not shown on the diagram), and part of the cI gene (Chapter 2.2.1). The insertion occurs within the PstI (77.4%-84.6%) region.


deletion
insertion
int cI
W VUTS RQPO NML K J I H G FED B LA RA ori
0
20
40
60
80
100%
PstI
77·4%
PstI
84·6%

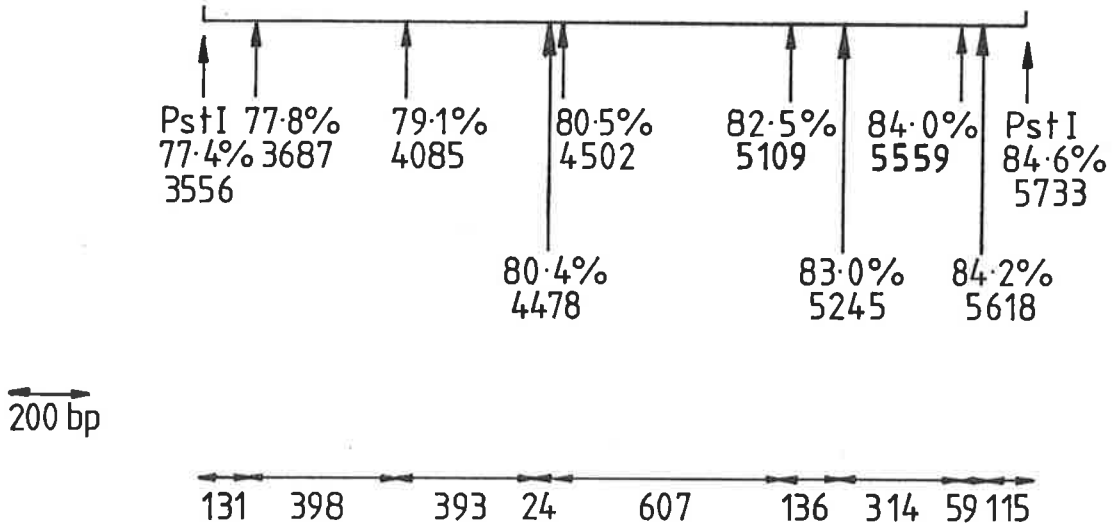
186 del2, using 4 bp restriction endonucleases, revealed that this was the case. Figure 9.2 shows the HpaII restriction pattern obtained for the 2.9 kb PstI (77.4%-84.6%) fragment from 186 del2 compared with that obtained for the 2.1 kb PstI (77.4%-84.6%) fragment from 186 cItsp. The results obtained show that the HpaII restriction pattern for 186 del2 is the same as that obtained for 186 cItsp except for the appearance of one novel fragment (sized at 190 bp). Similar results were obtained for other 4 bp restriction endonucleases used (data not shown). These results have revealed that 186 del2 contains a duplication within the PstI (77.4%-84.6%) region. Furthermore, the appearance of only one novel fragment shows that this duplication is a direct duplication, as opposed to an inverted duplication (Figure 9.3). [An inverted duplication is expected to result in two novel fragments (Figure 9.3).]

The nature of the deletion in 186 del2 was also further investigated using restriction analysis. The 1.8 kb XhoI-BglII (67.6%-79.6%) fragment (sized from an agarose gel) from 186 del2 was digested with TaqI and the restriction pattern obtained was compared with that obtained from the digestion of the 1.78 kb XhoI-BglII (67.6%-79.6%) fragment from 186 del1 (5). Since 186 del2 was isolated from the same stock of phage as 186 del1 (Dharmarajah, 1975; Chapter 9.1), it is likely that they contain the same deletion (from 715 to 2551; Kalionis et al., 1986a). Indeed, the TaqI restriction pattern obtained for the XhoI-BglII (67.6%-79.6%) fragment from 186 del2 was the same as that obtained for the XhoI-BglII fragment from 186 del1 (data not shown) indicating that both phage almost certainly contain the same deletion.

9.2.2 DNA Sequence Analysis of the 186 del2 Deletion and the pR Promoter Region.

To determine whether the 186 del2 deletion was exactly the same as the 186 del1 deletion (Kalionis et al., 1986a) and to ensure that 186 del2 did

The HpaII restriction pattern from 186 cItsp is shown below.



fragment sizes in descending order :

607, 398, 393, 314, 136, 131, 115, 59, 24

These DNA fragments are indicated on the left of the Figure. 186 del2 gave rise to a novel fragment, which was sized at 190 b, as indicated.

Figure 9.2 Restriction analysis of the PstI (77.4%-84.6%) fragment from 186 del2.

186 del2 (6) and 186 cItsp DNA were digested with PstI (Chapter 2.28). DNA was fractionated on a 1% agarose gel. The 2.7 kb PstI (77.4%-84.6%) fragment from 186 del2 PstI-digested DNA (containing the insertion), and the 2.1 kb PstI (77.4%-84.6%) fragment from 186 cItsp PstI-digested DNA were isolated (Chapter 2.29.1, 2.30.1b).

The PstI (77.4%-84.6%) fragments from 186 del2 and 186 cItsp were digested with HpaII (Chapter 2.28). The DNA was end-labelled (Chapter 2.31.2a) and fractionated in a non-denaturing 5% polyacrylamide gel in TBE buffer (Chapter 2.29.3a).

pBR325 HinfI DNA fragments were also end-labelled and fractionated on the gel to serve as size markers (Chapter 2.11.1).

Electrophoresis and autoradiography were as described in Chapters 2.29.3(a) and 2.29.4. The gel was autoradiographed overnight at room temperature.

Gel Tracks :

1. 186 cItsp 2.1 kb PstI (77.4%-84.6%), digested with HpaII
2. 186 del2 2.7 kb PstI (77.4%-84.6%), digested with HpaII
3. pBR325 HinfI molecular weight markers (Chapter 2.11.1)

The sizes of the pBR325 HinfI fragments are indicated on the right of the Figure.

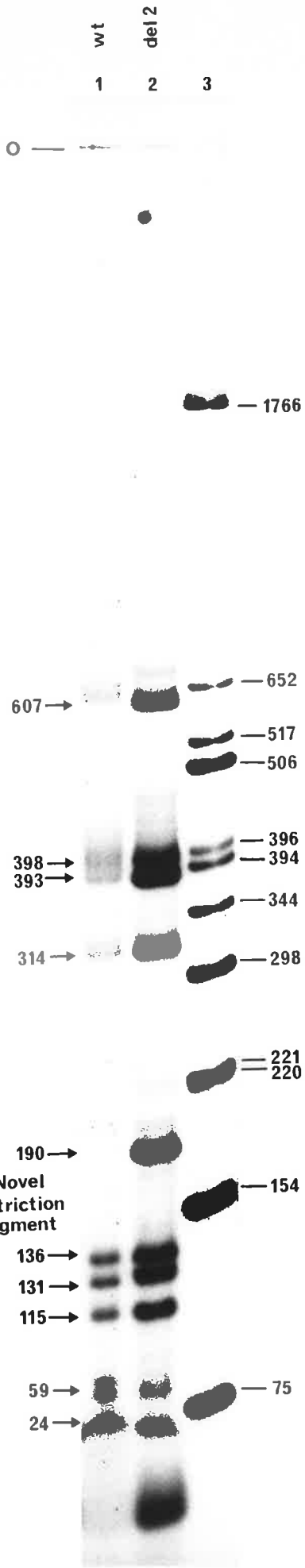
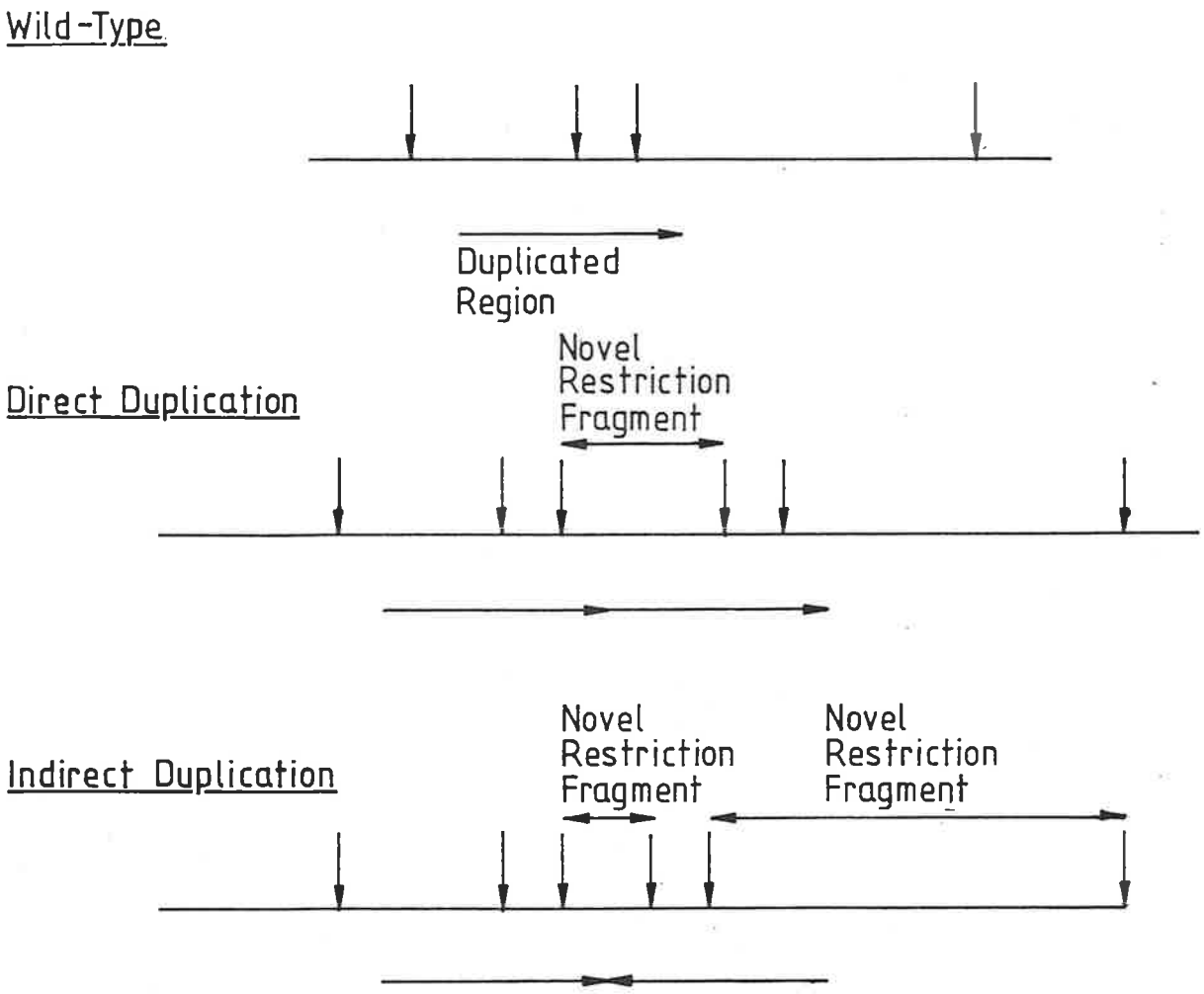

wt
del 2
1
2
3
O
1766
652
607
517
506
396
398
394
393
344
314
298
221
220
190
Novel
Restriction
Fragment
154
136
131
115
59
75
24

Figure 9.3 The difference in restriction patterns between a direct and indirect duplication.

A diagrammatic representation of the restriction maps expected for a direct duplication compared with an indirect duplication.

The restriction sites are indicated by the vertical arrows. The duplicated region is indicated by the horizontal arrow.

A direct duplication results in one novel restriction fragment, whereas an indirect duplication results in two novel restriction fragments, as indicated on the diagram.


Wild-Type
Duplicated Region
Direct Duplication
Novel Restriction Fragment
Indirect Duplication
Novel Restriction Fragment
Novel Restriction Fragment

not contain a mutation in the early lytic promoter pR or operator sites, the DNA sequence of these regions was obtained. M13-clones spanning these regions were obtained by digestion of the 3.7 kb HindIII-BglII (61.3%-79.6%) fragment with TaqI, and cloning the 132 bp, 102 bp and 288 bp TaqI fragments (isolated after fractionation on a 5% polyacrylamide gel) into the AccI site of M13mp19 (Chapter 2.29.3b, 2.30.2, 2.31; Figure 9.4). As a control, the same fragments were isolated from 186 del1 and cloned into the AccI site of M13mp19. The DNA sequence of these fragments was obtained as shown in Figure 9.4 (Chapter 2.33).

The results obtained from the analysis of the DNA sequence revealed that the 186 del2 deletion is exactly the same as the 186 del1 deletion (Figure 9.5), namely a deletion of the region between the short direct-repeats GAG at 713-715 and 2549-2551 (Kalionis et al., 1986a). In addition, the 186 del2 DNA sequence spanning the pR promoter was shown to be the same as that of wild-type 186, as was the 186 del1 DNA sequence in this region (data not shown). Thus, the virulent phenotype of 186 del2 is not due to the 1.83 kb deletion (from 715 to 2551), or to a pR promoter mutation or an operator mutation, and is most likely due to the presence of the duplication spanning the BglII (79.6%) site.

9.2.3 DNA Sequence Analysis of the 186 del2 Duplication.

M13-clones of the region spanning the duplication were obtained as follows. The 2.9 kb PstI (77.4%-84.6%) fragment from 186 del2 was digested with BglII and the 0.69 kb PstI-BglII (77.4%-79.6%) fragment was cloned into the PstI and BamHI sites of M13mp8 and mp9, while the 0.6 kb BglII fragment (the fragment expected to span the novel junction of the duplication; Chapter 9.2.1) was cloned into the BamHI site of M13mp8. In addition, the 0.6 kb BglII fragment was digested with HpaII and the resulting fragments were "shot-gun" cloned into the AccI site of M13mp9. To obtain the DNA sequence across the BglII sites, the 398 bp HpaII

Figure 9.4 The Sequencing Strategy of the 186 del2 deletion, pR promoter region and duplication.

(a) The genetic map of 186 del2 showing the positions of the deletion and duplication. The deletion and duplication are indicated by the shaded boxes (see Figure 9.1). Relevant restriction sites are shown. The sequence coordinates refer to the first base of the restriction site on the l-strand.

(b) A restriction map of the region spanning the deletion and duplication from 186 del2. Relevant restriction sites are indicated. The sequence coordinates of the restriction sites refer to the first base of the site on the l-strand. The sequence coordinates of the region deleted and the region duplicated are indicated. The horizontal arrows below the map represent gel readings used to generate the sequence. Rightward arrows represent gel readings of the l-strand sequence, whereas leftward arrows represent gel readings of the r-strand sequence. The sizes of the fragments are indicated. ? indicates that it was not known which of these regions was sequenced, because of the fact that the fragment lies in the duplication region and was isolated from the 2.7 kb PstI (77.4%-84.6%) fragment. Several clones of both the l-strand and the r-strands were sequenced so it is likely that both regions were sequenced.

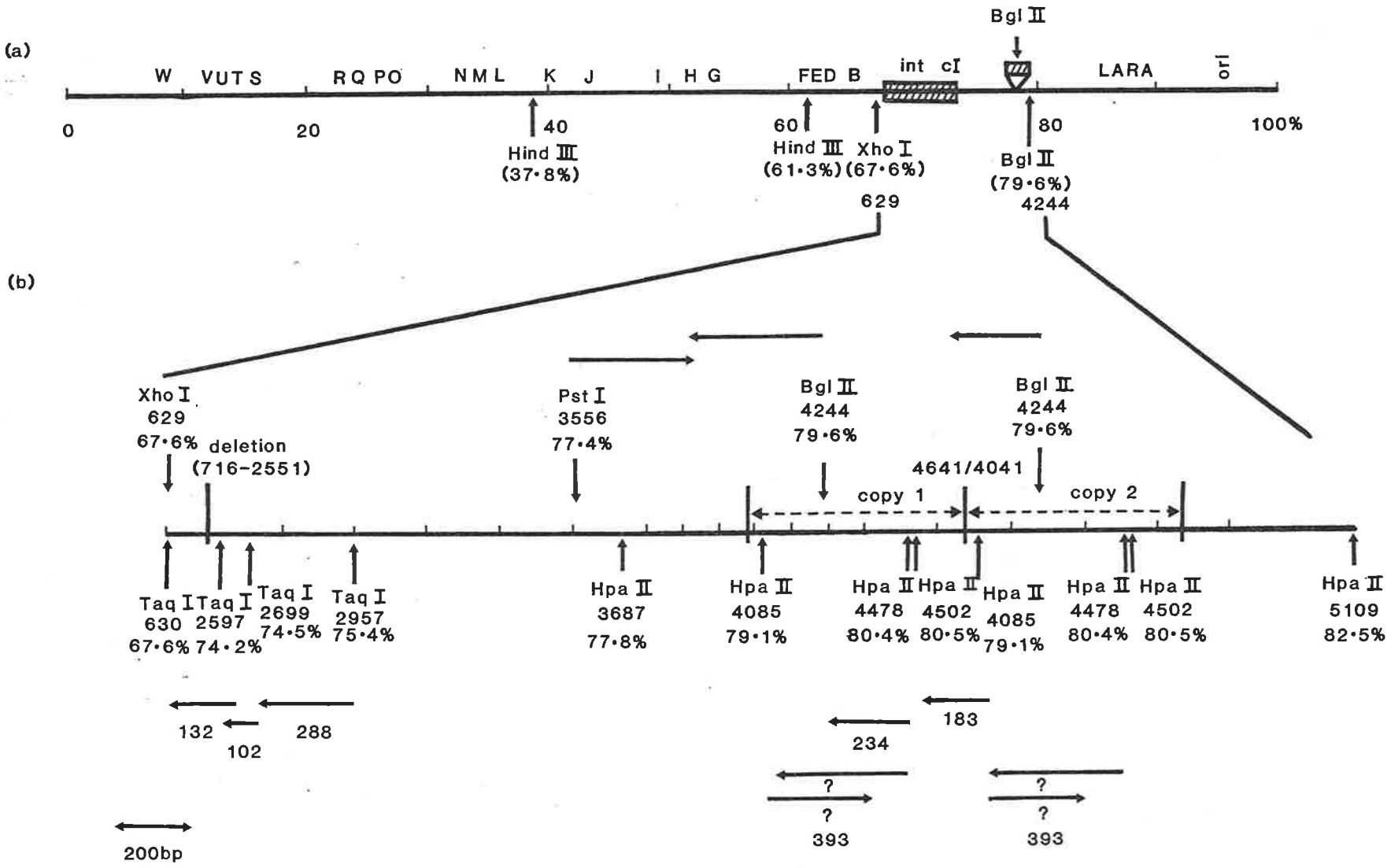
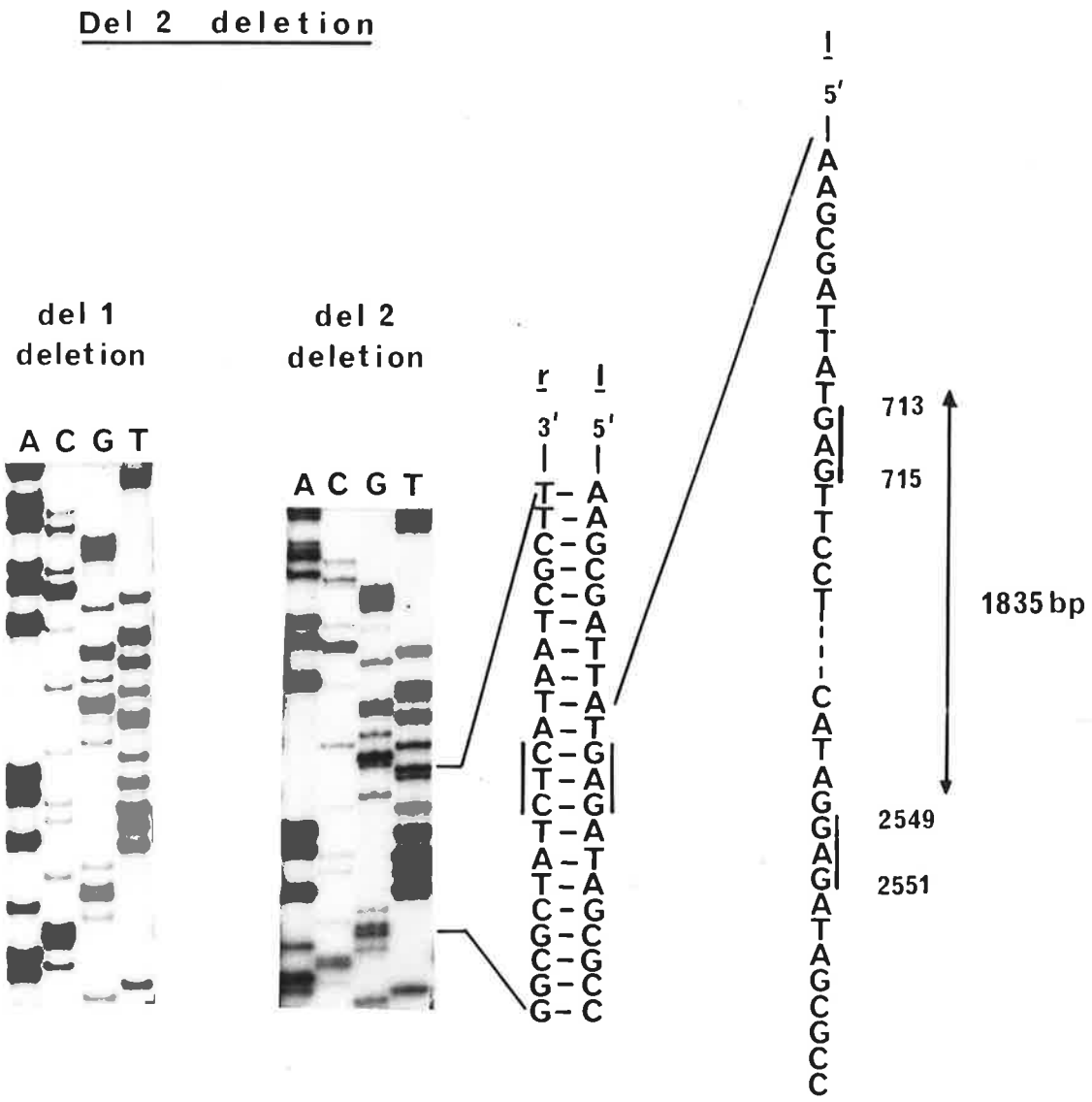

(a)
W VUT S RQ PO NML K J I H G FED B int cI LARA ori
Bgl II
0
20
40
60
80
100%
Hind III (37·8%)
Hind III (61·3%)
Xho I (67·6%)
Bgl II (79·6%)
629
4244
(b)
Xho I 629 67·6%
deletion (716–2551)
Pst I 3556 77·4%
Bgl II 4244 79·6%
Bgl II 4244 79·6%
4641/4041
copy 1
copy 2
Taq I 630 67·6%
Taq I 2597 74·2%
Taq I 2699 74·5%
Taq I 2957 75·4%
Hpa II 3687 77·8%
Hpa II 4085 79·1%
Hpa II 4478 80·4%
Hpa II 4502 80·5%
Hpa II 4085 79·1%
Hpa II 4478 80·4%
Hpa II 4502 80·5%
Hpa II 5109 82·5%
132
102
288
183
234
?
?
393
?
?
393
200bp

Figure 9.5 The DNA sequence spanning the 186 del2 deletion.

This Figure shows the DNA sequence of the region spanning the 186 del2 deletion. The DNA sequence of the equivalent region from 186 del1 is also shown. The DNA sequence is that of the r-strand. The 186 del2 deletion is exactly the same as the 186 del1 deletion and occurs between two short direct repeats, GAG, at postions 713-715 and 2549-2551, as indicated on the right of this Figure.

The DNA sequencing was carried out as described in Chapter 2.33. Electrophoresis and autoradiography conditions were as described in Chapter 2.29.3(c) and 2.29.4.


Del 2 deletion
del 1 deletion
A C G T
del 2 deletion
A C G T
r
l
3'
5'
l
5'
713
715
2549
2551
1835 bp

fragment (which spans the BglII sites; Figure 9.4) was obtained from the 2.9 kb PstI (77.4%-84.6%) fragment of 186 del2 and cloned into the AccI site of M13mp9 (Chapter 2.30.2, 2.31). The DNA sequencing strategy is shown in Figure 9.4 (Chapter 2.33). The DNA sequence of the novel junction is shown in Figure 9.6, and the position of the duplication in the DNA sequence of the PstI (77.4%-84.6%) region is presented in Figure 9.7(a).

The analysis of the DNA sequence obtained, showed that 186 del2 contains a duplication of the region 4041-4641. This region is bounded by short direct-repeats, TATTT. The novel junction created by the 186 del2 duplication occurs in the 5'-end of the CP81 gene (at the sequence coordinate 4641) and the 3'-end of dhr (at the sequence coordinate 4041). This junction results in an in-frame fusion of the CP81 and dhr genes and results in a fusion gene encoding 25 amino acids (Figure 9.7a). Computer-assisted analysis of the DNA sequence of the duplicated region enabled the prediction that a very strong promoter (compared to λ pL; Hawley and McClure, 1983a) was encoded at the novel junction (Figure 9.6, 9.7a). This predicted promoter gave a homology score of 69.0 (which occurs by chance only once in every 450 kb of random sequence), using the promoter matrix of Kalionis et al. (1986a) (Chapter 2.38), and thus, is expected to be stronger than λ pL (which has a homology score of 67.2; Kalionis et al., 1986a). The duplicaton of the 4041-4641 region creates this potential promoter by linkage of a potential -10 region at the 3'-end of the dhr gene to a potential -35 region at the 5'-end of CP81 with an optimal spacing of 17 bp between these regions (Figure 9.6, 9.7a; summarized in Figure 9.7b). This predicted promoter created at the novel junction was named pDup. Should pDup be functional in 186 del2, it provides a means by which this phage can by-pass the need for transcription from the early lytic promoter pR, which is under cI repressor control, to give transcription of the middle genes (CP79, CP80, CP81, CP83 and the essential replication

Figure 9.6 The DNA sequence spanning the novel junction of the 186 del2 duplication.

This Figure shows the DNA sequence of the r-strand of the region spanning the novel junction of the 186 del2 duplication. The DNA sequence of the r-strand from the wild-type (186 cItsp) is also shown. The duplication occurs at two short direct repeats, TATTT, at sequence coordinates 4637-4641 and 4036-4040, as indicated on the right of the Figure. The duplication of the 4041-4641 region creates a potential promoter by the fusion of a potential -10 region (4044-4049) to a potential -35 region (4622-4627) with an optimal spacing of 17 bp between these regions, as indicated on the Figure.

DNA sequencing was carried out as described in Chapter 2.33. Electrophoresis and autoradiography conditions were as described in Chapter 2.29.3(c) and 2.29.4.

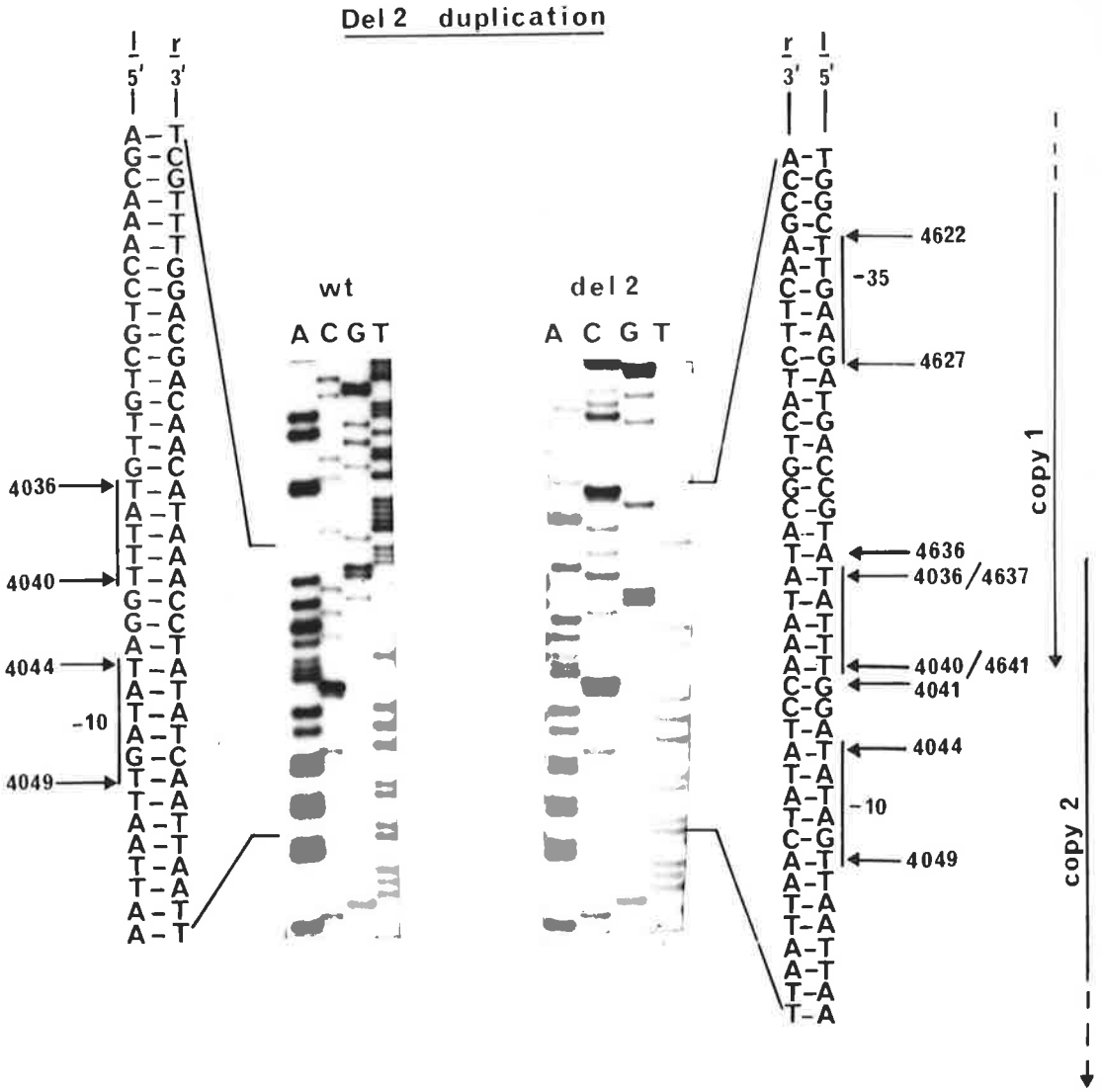
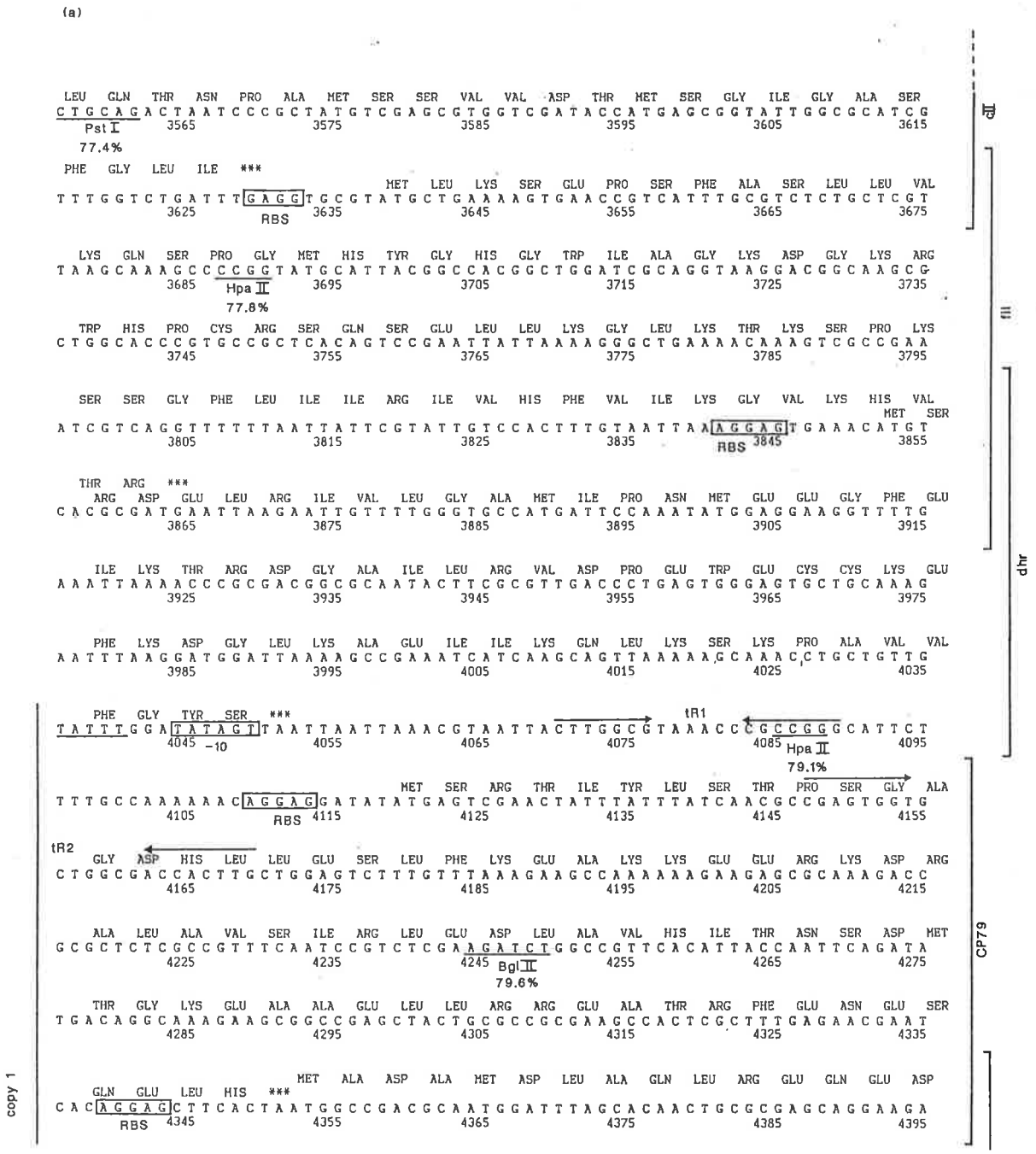

Del 2 duplication
l
5'
r
3'
wt
A C G T
del 2
A C G T
4036
4040
4044
-10
4049
r
3'
l
5'
4622
-35
4627
4636
4036/4637
4040/4641
4041
4044
-10
4049
copy 1
copy 2

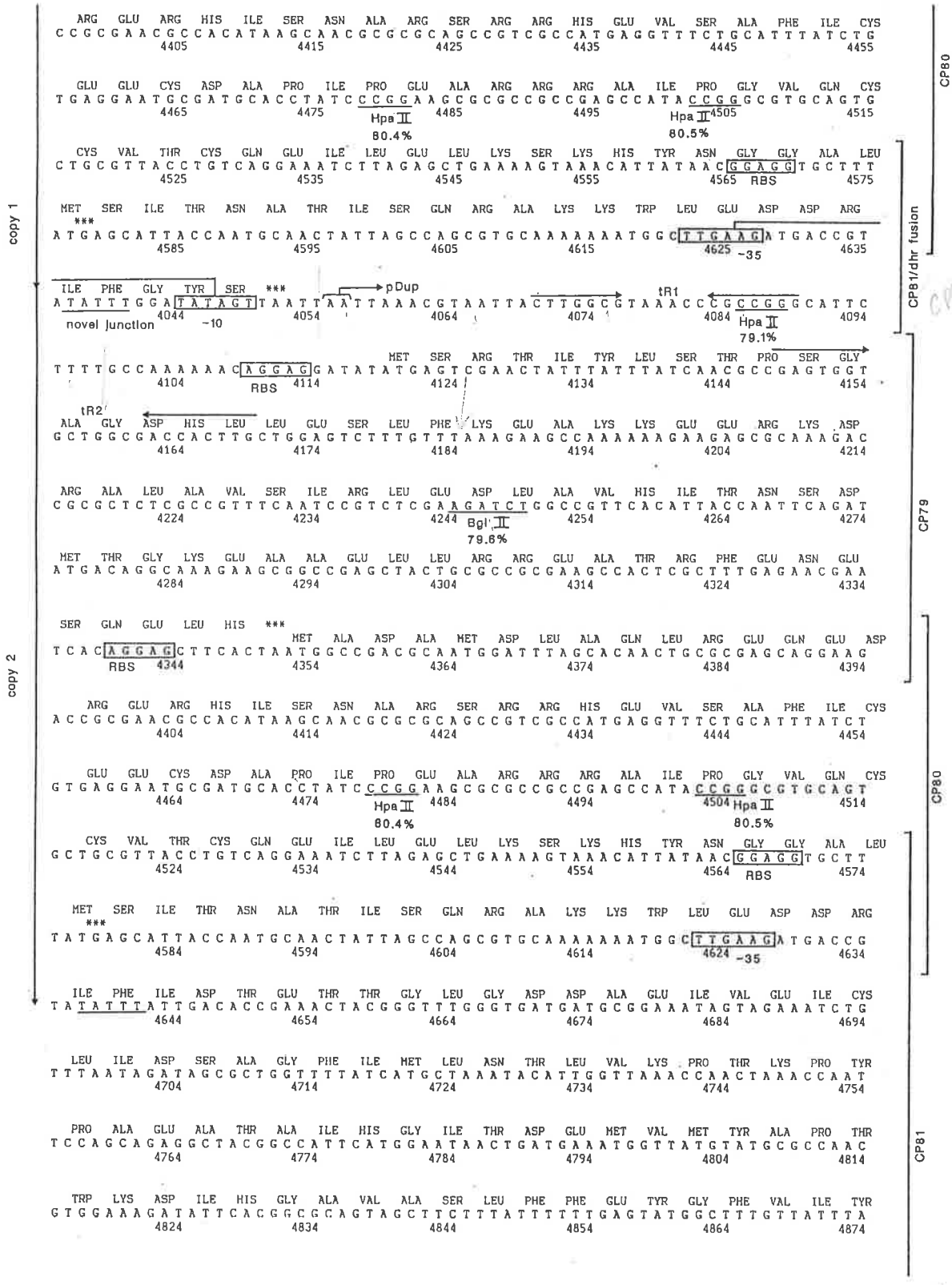
Figure 9.7 The DNA sequence of the 186 del2 duplication.

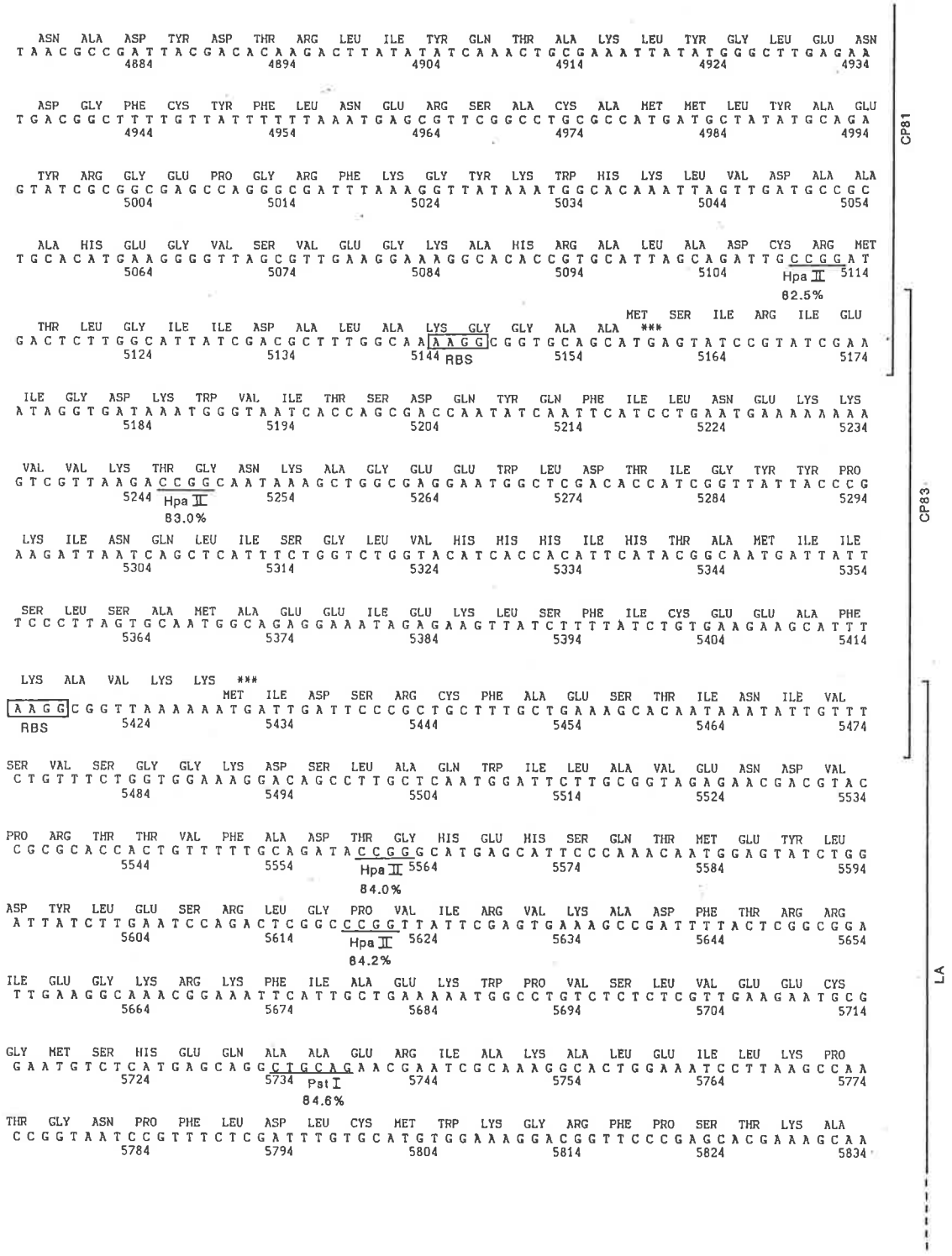
(a) The DNA sequence of the l-strand from the PstI (77.4%) site to 85% (sequence coordinates 3556-5834) from 186 del2 (6), is presented. Genes and potential genes are indicated. Ribosome-binding sites are boxed. The tR1 and tR2 terminators are indicated by the convergent arrows. All relevant restriction sites are shown.

The region duplicated in 186 del2 is indicated on the left of the Figure. Copy 1 and copy 2 refer to the two copies of the duplicated region. The duplication occurs at the sequence TATTT, which is underlined, and results in the formation of a promoter, pDup, by joining together a -10 region (at the 3'-end of the dhr gene) with a -35 region (at the 5'-end of CP81). These -10 and -35 regions are boxed. Transcription from pDup occurs at one of the two A residues at position 4055 (Figure 9.6), as indicated in the Figure.

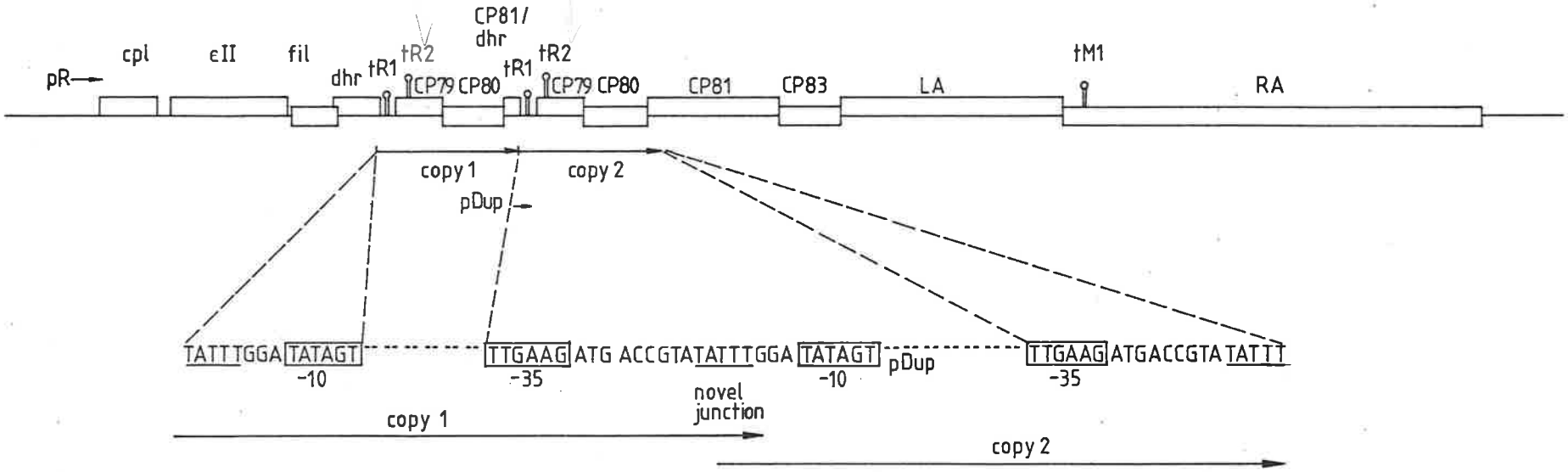
(b) A diagrammatic representation of the position of the duplication in the pR to RA region from 186 del2. The genes are represented by the boxed regions. Promoters are represented by the arrows. The tR1, tR2 and tM1 terminators (represented by the hairpin structure) are not drawn to scale. The region duplicated is indicated beneath the map. The sequence of the novel junction, and at either end of the two copies of the duplicated region is shown. The TATTT sequence, at which the duplication occurred, is underlined and the -10 and -35 regions are boxed.


(a)
LEU GLN THR ASN PRO ALA MET SER SER VAL VAL ASP THR MET SER GLY ILE GLY ALA SER
CTGCAGACTAATCCCGCTATGTCGAGCGTGGTCGATACCATGAGCGGTATTGGCGCATCG
Pst I
77.4%
PHE GLY LEU ILE ***
MET LEU LYS SER GLU PRO SER PHE ALA SER LEU LEU VAL
TTTGGTCTGATTTGAGGTGCGTATGCTGAAAAGTGAACCGTCATTTGCGTCTCTGCTCGT
RBS
LYS GLN SER PRO GLY MET HIS TYR GLY HIS GLY TRP ILE ALA GLY LYS ASP GLY LYS ARG
TAAGCAAAGCCCCGGGTATGCATTACGGCCACGGCTGGATCGCAGGTAAGGACGGCAAGCG
Hpa II
77.8%
TRP HIS PRO CYS ARG SER GLN SER GLU LEU LEU LYS GLY LEU LYS THR LYS SER PRO LYS
CTGGCACCCCGTGCCGCTCACAGTCCGAATTATTAAAAGGGCTGAAAACAAAGTCGCCGAA
SER SER GLY PHE LEU ILE ILE ARG ILE VAL HIS PHE VAL ILE LYS GLY VAL LYS HIS VAL
MET SER
ATCGTCAGGTTTTTTAATTATTCGTATTGTCCACTTTGTAATTAAAGGAGTGAAACATGT
RBS
THR ARG ***
ARG ASP GLU LEU ARG ILE VAL LEU GLY ALA MET ILE PRO ASN MET GLU GLU GLY PHE GLU
CACGCGATGAATTAAGAATTGTTTTGGGTGCCATGATTCCAAATATGGAGGAAGGTTTTG
ILE LYS THR ARG ASP GLY ALA ILE LEU ARG VAL ASP PRO GLU TRP GLU CYS CYS LYS GLU
AAATTAAAACCCGCGACGGCGCAATACTTCGCGTTGACCCTGAGTGGGAGTGCTGCAAAG
PHE LYS ASP GLY LEU LYS ALA GLU ILE ILE LYS GLN LEU LYS SER LYS PRO ALA VAL VAL
AATTTAAGGATGGATTAAAAGCCGAAATCATCAAGCAGTTAAAAAAGCAAACCTGCTGTTG
PHE GLY TYR SER ***
tR1
TATTTGGATATAGTTAATTAATTAAACGTAATTACTTGGCGTAAACCCGCCGGGCATTCT
-10
Hpa II
79.1%
MET SER ARG THR ILE TYR LEU SER THR PRO SER GLY ALA
TTTGCCAAAAAACAGGAGGATATATGAGTCGAACTATTTATTTATCAACGCCGAGTGGTG
RBS
tR2
GLY ASP HIS LEU LEU GLU SER LEU PHE LYS GLU ALA LYS LYS GLU GLU ARG LYS ASP ARG
CTGGCGACCACTTGCTGGAGTCTTTGTTTAAAGAAGCCAAAAAAAGAAGAGCGCAAAGACC
ALA LEU ALA VAL SER ILE ARG LEU GLU ASP LEU ALA VAL HIS ILE THR ASN SER ASP MET
GCGCTCTCGCCGTTTCAATCCGTCTCGAAGATCTGGCCGTTCACATTACCAATTCAGATA
Bgl II
79.6%
THR GLY LYS GLU ALA ALA GLU LEU LEU ARG ARG GLU ALA THR ARG PHE GLU ASN GLU SER
TGACAGGCAAAGAAGCGGCCGAGCTACTGCGCCGCGAAGCCACTCGCTTTGAGAACGAAT
MET ALA ASP ALA MET ASP LEU ALA GLN LEU ARG GLU GLN GLU ASP
GLN GLU LEU HIS ***
CACAGGAGCTTCACTAATGGCCGACGCAATGGATTTAGCACAACTGCGCGAGCAGGAAGA
RBS
copy 1
dhr
II
CP79


copy 1
copy 2
CP80
CP81/dhr fusion
CP79
CP80
CP81
Hpa II
80.4%
Hpa II
80.5%
RBS
-35
pDup
tR1
novel junction
-10
Hpa II
79.1%
tR2
RBS
Bgl II
79.8%
RBS
Hpa II
80.4%
Hpa II
80.5%
RBS
-35


ASN ALA ASP TYR ASP THR ARG LEU ILE TYR GLN THR ALA LYS LEU TYR GLY LEU GLU ASN
TAACGCCGATTACGACACAAGACTTATATATCAAACTGCGAAATTATATGGGCTTGAGAA
4884 4894 4904 4914 4924 4934
ASP GLY PHE CYS TYR PHE LEU ASN GLU ARG SER ALA CYS ALA MET MET LEU TYR ALA GLU
TGACGGCTTTTGTTATTTTTTAAATGAGCGTTCGGCCTGCGCCATGATGCTATATGCAGA
4944 4954 4964 4974 4984 4994
TYR ARG GLY GLU PRO GLY ARG PHE LYS GLY TYR LYS TRP HIS LYS LEU VAL ASP ALA ALA
GTATCGCGGCGAGCCAGGGCGATTTAAAGGTTATAAATGGCACAAATTAGTTGATGCCGC
5004 5014 5024 5034 5044 5054
ALA HIS GLU GLY VAL SER VAL GLU GLY LYS ALA HIS ARG ALA LEU ALA ASP CYS ARG MET
TGCACATGAAGGGGTTAGCGTTGAAGGAAAGGCACACCGTGCATTAGCAGATTGCCGGAT
5064 5074 5084 5094 5104 Hpa II 5114
82.5%
MET SER ILE ARG ILE GLU
THR LEU GLY ILE ILE ASP ALA LEU ALA LYS GLY GLY ALA ALA ***
GACTCTTGGCATTATCGACGCTTTGGCAAAAGGCGGTGCAGCATGAGTATCCGTATCGAA
5124 5134 5144 RBS 5154 5164 5174
ILE GLY ASP LYS TRP VAL ILE THR SER ASP GLN TYR GLN PHE ILE LEU ASN GLU LYS LYS
ATAGGTGATAAATGGGTAATCACCAGCGACCAATATCAATTCATCCTGAATGAAAAAAAA
5184 5194 5204 5214 5224 5234
VAL VAL LYS THR GLY ASN LYS ALA GLY GLU GLU TRP LEU ASP THR ILE GLY TYR TYR PRO
GTCGTTAAGACCGGCAATAAAGCTGGCGAGGAATGGCTCGACACCATCGGTTATTACCCG
5244 Hpa II 5254 5264 5274 5284 5294
83.0%
LYS ILE ASN GLN LEU ILE SER GLY LEU VAL HIS HIS HIS ILE HIS THR ALA MET ILE ILE
AAGATTAATCAGCTCATTTCTGGTCTGGTACATCACCACATTCATACGGCAATGATTATT
5304 5314 5324 5334 5344 5354
SER LEU SER ALA MET ALA GLU GLU ILE GLU LYS LEU SER PHE ILE CYS GLU GLU ALA PHE
TCCCTTAGTGCAATGGCAGAGGAAATAGAGAAGTTATCTTTTATCTGTGAAGAAGCATTT
5364 5374 5384 5394 5404 5414
LYS ALA VAL LYS LYS ***
MET ILE ASP SER ARG CYS PHE ALA GLU SER THR ILE ASN ILE VAL
AAGGCGGTTAAAAAATGATTGATTCCCGCTGCTTTGCTGAAAGCACAATAAATATTGTTT
RBS 5424 5434 5444 5454 5464 5474
SER VAL SER GLY GLY LYS ASP SER LEU ALA GLN TRP ILE LEU ALA VAL GLU ASN ASP VAL
CTGTTTCTGGTGGAAAGGACAGCCTTGCTCAATGGATTCTTGCGGTAGAGAACGACGTAC
5484 5494 5504 5514 5524 5534
PRO ARG THR THR VAL PHE ALA ASP THR GLY HIS GLU HIS SER GLN THR MET GLU TYR LEU
CGCGCACCACTGTTTTTTGCAGATACCGGGCATGAGCATTCCCAAACAATGGAGTATCTGG
5544 5554 Hpa II 5564 5574 5584 5594
84.0%
ASP TYR LEU GLU SER ARG LEU GLY PRO VAL ILE ARG VAL LYS ALA ASP PHE THR ARG ARG
ATTATCTTGAATCCAGACTCGGCCCCGGTTATTCGAGTGAAAGCCGATTTTACTCGGCGGA
5604 5614 Hpa II 5624 5634 5644 5654
84.2%
ILE GLU GLY LYS ARG LYS PHE ILE ALA GLU LYS TRP PRO VAL SER LEU VAL GLU GLU CYS
TTGAAGGCAAACGGAAATTCATTGCTGAAAAATGGCCTGTCTCTCTCGTTGAAGAATGCG
5664 5674 5684 5694 5704 5714
GLY MET SER HIS GLU GLN ALA ALA GLU ARG ILE ALA LYS ALA LEU GLU ILE LEU LYS PRO
GAATGTCTCATGAGCAGGCTGCAGAACGAATCGCAAAGGCACTGGAAATCCTTAAGCCAA
5724 5734 Pst I 5744 5754 5764 5774
84.6%
THR GLY ASN PRO PHE LEU ASP LEU CYS MET TRP LYS GLY ARG PHE PRO SER THR LYS ALA
CCGGTAATCCGTTTCTCGATTTGTGCATGTGGAAAGGACGGTTCCCGAGCACGAAAGCAA
5784 5794 5804 5814 5824 5834
CP81
CP83
LA

(b)


CP81/
dhr
pR
cpl
eII
fil
dhr
tR1
tR2
CP79
CP80
tR1
tR2
CP79
CP80
CP81
CP83
LA
tM1
RA
copy 1
copy 2
pDup
TATTTGGA TATAGT
-10
TTGAAG ATG ACCGTATATTTGGA TATAGT pDup
-35
-10
TTGAAG ATGACCGTA TATTT
-35
novel
junction
copy 1
copy 2

genes LA and RA), and thus, allowing the growth of 186 del2 in a 186 lysogen.

9.2.4 In Vitro Transcription Studies with pDup.

To determine whether the predicted pDup promoter was functional, the 0.6 kb BglII fragment containing pDup was used in an in vitro transcription reaction (Chapter 2.36.5). As shown in Figure 9.8, this fragment gave rise to an intense transcript, which was determined to be 193 b when sized against a sequencing ladder. This transcript was most likely to be a run-off transcript, since undigested 186 del2 DNA did not give rise to a band of this size (Figure 9.8, lane 3). Since computer-assisted analysis did not predict the presence of any leftward promoters in this region it was assumed that this transcript was a rightward transcript. From the size of this run-off transcript, the position of the 5'-end of this RNA corresponds to one of the two A residues at position 4055, 6 or 7 b from the pDup promoter (Figure 9.7a). This result suggests that pDup is functional in vitro.

The in vitro transcription pattern of the 0.6 kb BglII fragment and of undigested 186 del2 DNA also resulted in four bands of lesser intensity and of lower molecular weight than the 193 b transcript (Figure 9.8, lanes 1 and 3). These bands were sized at 44 b, 45 b, 48 b and 49 b, and from the knowledge of the position of the 5'-end of the 193 b transcript were estimated to be due to termination of positions between 4198-4204 (Figure 9.9, 9.7a), which is immediately after the run of T-residues at the Rho-independent tR1 terminator (Chapter 3.2.2c, 6.2.3). The level of transcription termination at the tR1 terminator was calculated to be 70% (see the legend to Figure 9.9), by determining the level of radioactivity in the 44 b, 45 b, 48 b and 49 b transcripts compared with that of the 193 b run-off transcript (by scanning the autoradiograph using a scanning laser densitometer; Chapter 2.36.4) and taking into account the relative

number of G-residues in these transcripts (since [α-^{32}P]-rGTP was used to label the transcripts).

9.2.5 Investigation of 186 del2 Virulence.

The evidence presented so far is consistent with the idea that the duplication in 186 del2 generates a promoter pDup. This promoter is expected to allow the transcription of 186 middle genes independent of transcription from the pR promoter and thus, give 186 del2 a virulent phenotype (the ability to grow on a 186 lysogen in the presence of the cI repressor). To confirm this, the 0.6 kb BglII fragment encoding pDup was recombined into the BglII (79.6%) site of 186 cItsp (1) in the r- orientation to form 186 cItspDup (23) (Chapter 2.2.1, 2.32), and this recombinant phage, which formed clear-plaques, was tested for its ability to form plaques on a 186 cI$^+$ lysogen (E573) (Table 9.1). Surprisingly, this phage did not give plaques on the 186 lysogen at 30^{o}C (cItsp active) or at 38.5^{o}C (cItsp partially inactive). (Higher temperatures, where cItsp is even less active, were not tested since 186 del2 does not form plaques at temperatures greater than 40^{o}C.) Since the 0.6 kb BglII fragment from 186 del2 did not confer virulence in conjunction with the cItsp mutation, it was possible that the XhoI-BglII (67.6%-79.6%) fragment from 186 del2 contained the virulent mutation in a region, which had not been sequenced (Figure 9.4). To test this possibility, this fragment was recombined into 186 cItsp DNA to form 186 del2XB (27) (as described in Chapter 2.2.1). This recombinant phage was tested for virulence and shown not to be virulent (Table 9.1). This result shows that the XhoI-BglII (67.6%-79.6%) region from 186 del2, alone was not sufficient to cause virulence.

Since the 0.6 kb BglII fragment alone was not sufficient for virulence, it was possible that the 1.83 kb deletion was required in combination with the 0.6 kb BglII fragment for virulence. To test this possibility the 0.6 kb BglII fragment was recombined into the BglII (79.6%)

Figure 9.8 In vitro transcription of the 0.6 kb BglII fragment from 186 del2.

186 del2 (6) DNA was digested with BglII and the DNA was fractionated on a 1% agarose gel (Chapter 2.28, 2.29.1). The 0.6 kb BglII fragment, containing pDup, was electroeluted from the agarose (Chapter 2.30.1b) and purified, as described in Chapter 2.36.5.

The 0.6 kb BglII fragment (2.5 ug) was used in an in vitro transcription reaction (Chapter 2.36.5). Uncut 186 del2 (6) DNA and 186 cItsp DNA (2.5 ug of each) were also used in an in vitro transcription reaction.

The transcripts were separated on a sequencing gel (Chapter 2.29.3b). The bromophenol blue was run to the bottom of the gel. The gel was fixed and autoradiographed, as described in Chapters 2.29.3(b) and 2.29.4. The autoradiograph was exposed for 24 hours at room temperature.

Gel Tracks 1. 186 del2 (6) 0.6 kb BglII fragment in vitro transcripts.

2. 186 cItsp DNA in vitro transcripts.

3. 186 del2 (6) DNA in vitro transcripts.

A DNA sequencing ladder was included to provide size markers. The sizes given on the left of the Figure are from the 5'-end of the M13 universal primer. The in vitro transcripts from the 186 del2 BglII fragment are indicated by the arrows. The sizes were determined by comparison with the DNA sequencing ladder. The 193 b transcript is a run-off transcript.

The high molecular weight bands seen in tracks 2 and 3 (not marked on the Figure) correspond to the 1540 b, 1450 b, 590 b and 290 b 186 in vitro transcripts that were described by Pritchard and Egan (1985).

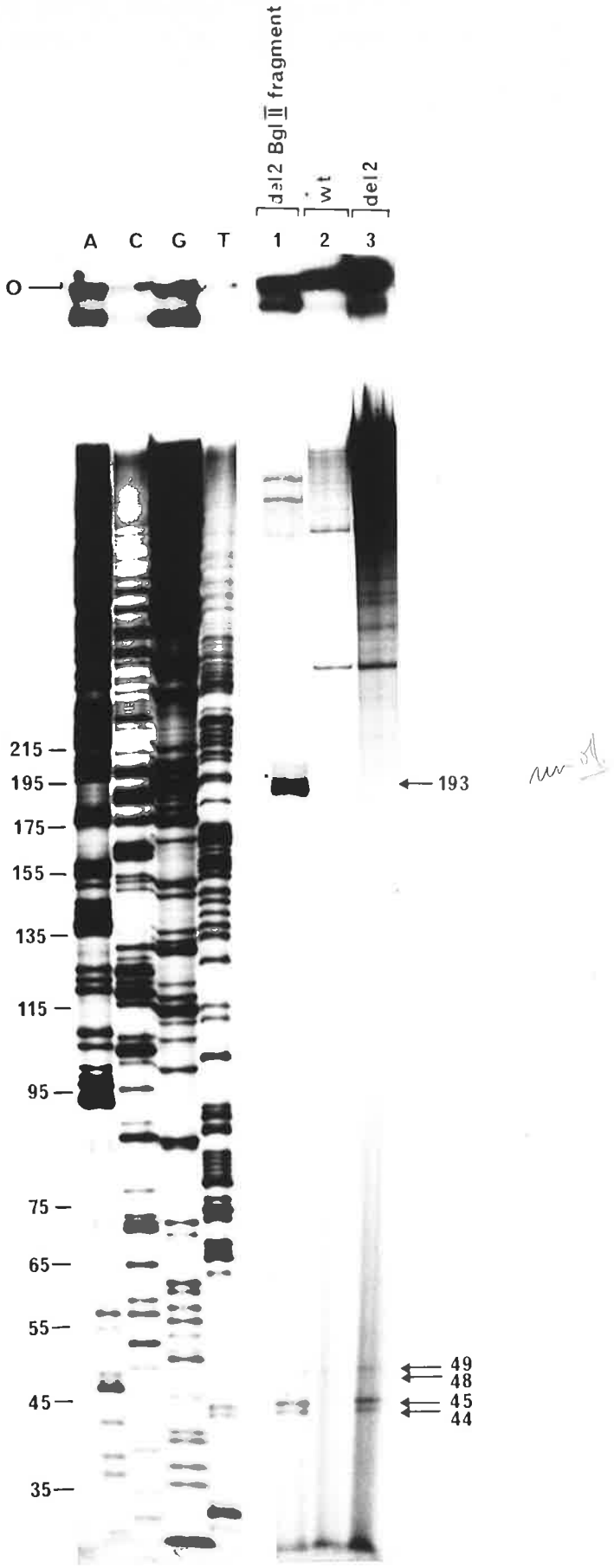
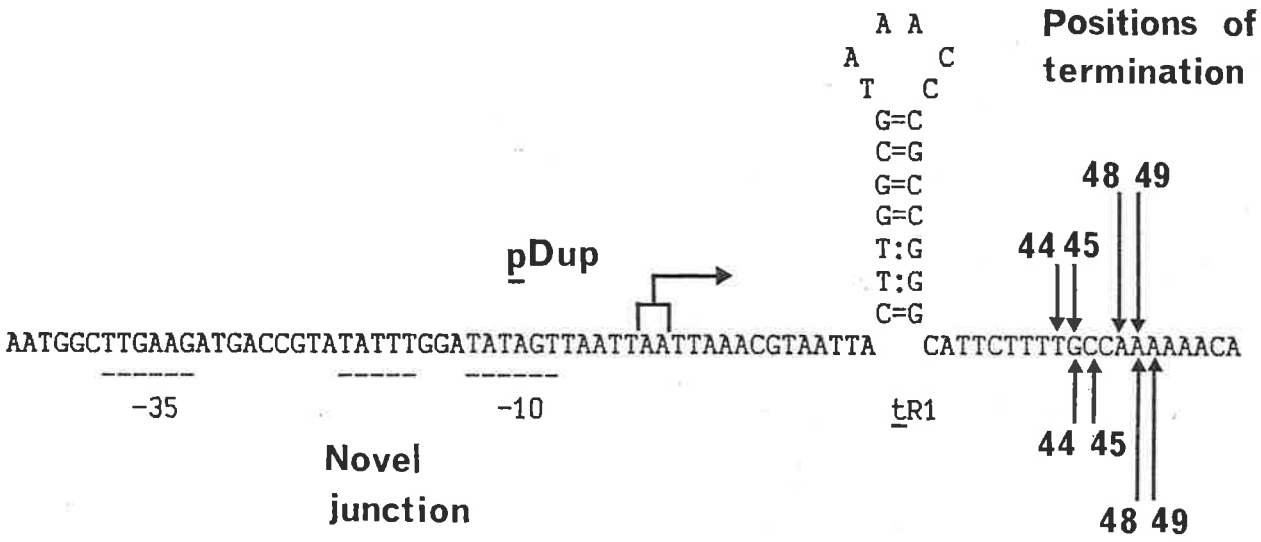

del2 Bgl II fragment
wt
del2
A
C
G
T
1
2
3
O
215
195
175
155
135
115
95
75
65
55
45
35
193
49
48
45
44

Figure 9.9 Positions of termination at tR1.

The DNA sequence of the pDup promoter and tR1 terminator is shown. The positions of the -35, -10 regions and the novel junction, are shown. The transcription start point (determined from the run-off in vitro transcription studies; Figure 9.8) is indicated by the horizontal arrow. The positions of the 3'-ends of the 44 b, 45 b, 48 b and 49 b transcripts on the DNA sequence, are shown by the vertical arrows. The vertical arrows at the top of the sequence indicate the positions of termination if the pDup transcript starts at the first A, whereas the vertical arrows at the bottom of the sequence indicate the positions of termination if the pDup transcripts starts at the second A.

The amount of termination at tR1 was determined as follows. The intensity of the 193 b run-off transcript, and the 44 b, 45 b, 48 b and 49 b transcripts were quantitated by scanning the autoradiograph with a scanning laser densitometer (Chapter 2.36.4). The transcripts were labelled with α^{32}P-rGTP, and thus, the relative G-residue content of the transcripts must be taken into account. The 193 b run-off transcript contains 44 G-residues, whereas the 44 b, 45 b, 48 b and 49 b transcripts contain 9 G-residues

Transcript	Intensity		relative level	
193 b	830406		830406	30%
49 b	90045			readthrough
48 b	105072	555291	2929867	70%
45 b	223752	x 44/9		termination
44 b	180422			


A A
A C
T C
G=C
C=G
G=C
G=C
T:G
T:G
C=G
Positions of termination
48 49
44 45
pDup
AATGGCTTGAAGATGACCGTATATTTGGATATAGTTAATTAATTAAACGTAATTA CATTCTTTTGCCAAAAAACA
-35
-10
tR1
44 45
48 49
Novel junction

site of 186 del1 (5) to form 186 del1Dup (26) (Chapter 2.2.1, 2.32). This phage (a reconstuction of 186 del2) was virulent (Table 9.1). The del1 deletion removes the cI, Int and CP69 genes (Figure 9.1) and it is therefore likely that the expression of one of these genes from the 186 cItspDup recombinant phage was preventing this phage from growing on a 186 cI^+ lysogen. To test if the expression of the cI or int genes from the recombinant phage was responsible for preventing virulence, pDup recombinants of a cI^- phage (186 cI10Dup (25)) and an Int^- phage (186 cItspins3Dup (24)) were constructed (Chapter 2.2.1, 2.32), and tested for virulence. The cI^- Dup recombinant phage was virulent, whereas the Int^- Dup recombinant phage was not virulent (Table 9.1). This result suggests that it is the production of the cI repressor from the cItsp Dup recombinant phage that is preventing the growth of this phage on a 186 cI^+ lysogen.

To determine if the cI-sensitivity of the pDup recombinant phage was a cis-effect due to the presence of this gene on the phage, or if it was a trans-effect possibly due to higher levels of cI repressor in the cell, 186 del2 was used to infect a strain carrying a plasmid-clone of the cItsp gene (pEC602) (Chapter 2.3.2; Figure 4.1a). Since this plasmid-clone is present in the cell at a high copy number (McKenney et al., 1981) the level of the cI protein produced from this plasmid-clone is presumably greater than that obtained from the expression from a 186 prophage (in which there is only one copy of the cI gene). Table 9.2 records the plating efficiency of 186 del2 on this strain at 30^oC compared with that at 30^oC of the virulent mutant 186 vir1 (3), which is virulent because of mutations in the operator (the binding site of the cI repressor; Kalionis, 1985; Kalionis et al., 1986a; Chapter 1.2.2). The plating efficiency of these phage on a non-lysogen and on a 186 cI^+ lysogen are also shown in Table 9.2. 186 del2 could not form plaques at 30^oC on cells carrying the cItsp plasmid-clone, whereas it could do so, at a relatively low efficiency, on the 186 cI^+

TABLE 9.1

The virulence of 186 del2 recombinant phage.[a]

Phage Strain[b]	Phenotype	Virulence[c]
186del1(5)	$cI^{-}Int^{-}$	-
186del2(6)	$cI^{-}Int^{-}Dup$	+
186cItspDup(23)	$cI^{-}Int^{-}Dup$	-
186del2XB(27)	$cI^{-}Int^{-}$	-
186del1Dup(26)	$cI^{-}Int^{-}Dup$	+
186cItspins3(7)	$cI^{+}Int^{-}$	-
186cItspins3Dup(24)	$cI^{+}Int^{-}Dup$	-
186cI10(2)	$cI^{-}Int^{+}$	-
186cI10Dup(25)	$cI^{-}Int^{+}Dup$	+

Notes to Table 9.1

a. Cultures of E573 [E508(186 cI^{+})] and E508 (non-lysogen) were grown in L broth at 30°C to A_{600}= 0.8. Dilutions of 186 del2 (7) or 186 vir1 (3) were plated with the cells (0.2 ml) from each strain (Chapter 2.16.1). Plates were incubated at 30°C overnight and the ability of phage to form plaques on E573 noted. E508 served as a control.

b. Bacteriophage strains were constructed as described in Chapter 2.2.1.

c. +, refers to the ability of the phage to form plaques on E573 (the 186 cI^{+} lysogen). -, indicates that the phage could not form plaques on E573. All phage strains formed plaques on E508 (data not shown).

TABLE 9.2

The plating efficiency of 186 del2 compared with 186 vir1 at 30°C.[a]

Bacterial Strain[b]	The efficiency of plating (eop)[c]	
	186del2	186vir1
non-lysogen (E508)	100%	100%
186cI$^+$ lysogen (E573)	12%	82%
non-lysogen (E863) carrying pEC602[d]	<0.025%	83%

Notes to Table 9.2

a. Cultures were grown in L broth (containing ampicillin if required) at 30°C to A_{600} = 0.8. Dilutions of 186 del2 (7) or 186 vir1 (3) were plated with the cells (0.2 ml) from each strain (Chapter 2.16.1). Plates were incubated at 30°C overnight and the number of plaques were counted.

b. All bacterial strains used were derivatives of C600 (see Chapter 2.1).

c. The efficiency of plating (eop) is expressed as a percentage of the number of plaques obtained on the non-lysogen (E508).

d. pEC602 contains the HaeIII-HaeIII (71.2%-75.5%) fragment from 186 cItsp (1) and carries the 186 cItsp gene (Chapter 2.3.2; Figure 4.1a). E863 carrying the parent vector (pKO1) gave a plating efficiency of 100% for both 186 del2 and 186 vir1 (data not shown).

lysogen. 186 vir1 was able to plate at a much greater efficiency on both the 186 cI$^+$ lysogen and the strain carrying the cItsp plasmid-clone at 30^{o}C.

The observation that 186 del2 could not form plaques on the strain carrying the cItsp plasmid-clone at 30^{o}C (which is expected to have a higher level of the cI repressor than the 186 cI$^+$ lysogen), whereas it could form plaques on the 186 cI$^+$ lysogen at 30^{o}C, suggests that 186 del2 is sensitive to the level of cI repressor in the cell. These results also show that 186 del2 is sensitive to the cI repressor when it is supplied in trans (from the cItsp plasmid-clone). Therefore, it can be concluded that 186 del2 is sensitive to the level of cI repressor in the cell, when cI is supplied in cis or in trans. Furthermore, since the expression of the cI gene alone prevented 186 del2 from forming plaques, it is likely that the inability of 186 del2 to grow on a lysogen is solely caused by the cI repressor without influence from other lysogen-encoded products (i.e. Int and CP69).

9.3 SUMMARY AND RELEVANCE TO THE REQUIREMENTS OF 186 LYTIC DEVELOPMENT.

The DNA sequence analysis of 186 del2 has shown that this phage contains the same deletion as 186 del1 (i.e from 716-2551; Figure 9.5), but unlike 186 del1 it also contains a tandem duplication of the region 4041-4641 (Figure 9.6, 9.7). Both of these recombinational events occurred at small direct repeats, namely 5'-GAG for the deletion and 5'-TATTT for the duplication. Many examples of deletions and duplications generated at small direct-repeats are known (e.g. Albertini et al., 1982; McCorkle and Altman, 1982; Jones et al., 1982; Edlund and Normark, 1981; Nakano et al., 1984; Charlier et al., 1979, 1983).

The analysis of the DNA sequence of the region spanning the 186 del2 duplication predicted that the duplication results in the formation of a strong promoter (pDup). Indeed, transcription studies in vitro provided

evidence that the pDup promoter is functional. Although interesting, the formation of pDup by a duplication is by no means a novel event, since there have been several examples reported where DNA rearrangements result in the formation of new promoters (Sibold and Elmerich, 1982; Bedouelle, 1983; Bitoun et al., 1983; Charlier et al., 1983; Gragerov et al., 1984). Interestingly, there is a virulent mutant (vir37) of phage P2 (of the same group of phage as 186), which has a tandem duplication in the early lytic region of the P2 genome (Bertani and Bertani, 1974; Chattoraj and Inman, 1974).

pDup is positioned on the 186 genome so that it will allow expression of the middle genes. This provides a means by which 186 del2 can by-pass the need for transcription from the pR promoter (which is under cI repressor control), and grow on a 186 cI^+ lysogen. However, biological studies have shown that pDup by itself is not sufficient to confer virulence on a phage and that a cI^- mutation is also needed for the phage to be virulent. These studies have also shown that 186 del2 is affected by the level of cI repressor in the cell.

A possible explanation for the apparent cI-sensitivity of 186 del2 is that 186 del2 requires a function, which is under the control of the cI repressor. The pR promoter is the only known promoter in 186, which is under cI control (Finnegan and Egan, 1981; Kalionis, 1985; I. Lamont, personal communication), and therefore it is likely that this function is under the control of the pR promoter. The pDup promoter is positioned so that all middle genes (CP79, CP80, CP81, CP83 and the essential replication genes LA and RA) are transcribed from the pDup promoter. Therefore, it is expected that when a pDup phage infects a strain where the level of cI repressor is high, all middle genes are likely to be expressed. However, the high level of cI repressor in the cell will be expected to prevent transcription of the early lytic genes from the pR promoter. In situations where the level of cI repressor in the cell is

lower, a small degree of transcription from pR of the early lytic region may be possible. This transcription may be necessary to allow a phage carrying pDup to form plaques on a 186 cI^+ lysogen.

This requirement for transcription of the early lytic region may be to allow the expression of the four early lytic genes (cpl, cII, fil and dhr), that are not expressed from pDup, one or more of which may be needed to allow productive lytic development of this phage. However, the results obtained in Chapter 5, suggested that none of the early lytic functions are essential for 186 lytic development. Further studies are needed to resolve this apparent paradox (see Chapter 10.4.1d).

In summary, although the study of 186 del2 has not provided evidence for a control mechanism for middle gene transcription or increased our knowledge of middle gene expression, it has revealed a possible essential role for a 186 early lytic function in 186 lytic development. This will be discussed further in Chapter 10.4.1(d).

CHAPTER 10.

CONCLUDING DISCUSSION.

10. CONCLUDING DISCUSSION.

10.1 INTRODUCTION.

As discussed in Chapter 1, the major aims of this thesis were to identify the functions encoded in the 186 early lytic region and to characterize the *in vivo* transcription pattern of the 186 early lytic and middle regions. The purpose of these studies was to identify the proposed *tom* gene and to investigate the control of middle gene transcription.

The study of the early lytic functions did not identify the proposed Tom function, but did reveal the existence of the early lytic functions, Dhr and Fil. The possible roles of these genes in 186 lytic development will be discussed and the genes will be compared with similar genes in other phage. The characterization of the *in vivo* transcripts of the early lytic and middle regions revealed a transcription pattern that was consistent with an antitermination mechanism of control of middle gene transcription with RNaseIII processing. Further studies however, failed to provide evidence for the involvement of an antitermination mechanism in the transcription of the middle region. RNaseIII processing and the question of the control of middle transcription will be discussed.

10.2 THE 186 EARLY LYTIC FUNCTIONS DHR AND FIL.

10.2.1 The Mechanism of Action of Dhr and Fil and their Role in 186 Lytic Development.

The existence of the *dhr* gene was revealed by the observation that *E. coli* DNA replication was inhibited upon the induction of a 186 prophage (Hocking and Egan, 1982b), a phenomenon known as the *Dhr Effect*. The investigation of the *Dhr Effect*, presented in this thesis, suggested that lethality and an inhibition of *E. coli* cell division were also associated

with the Dhr Effect (Chapter 5.2.4). However, further studies revealed that these effects were due to the expression of not one, but two, 186 early lytic genes; dhr and fil (Chapter 5.2.6). The expression of dhr results in the inhibition of E. coli DNA replication and causes cell death, whereas the expression of fil results in an inhibition of E. coli cell division and also causes a reduction in cell survival.

The revelation that the expression of fil also results in a reduction in cell survival suggests that the host mutant Dhr^R1 (which at high temperatures is resistant to the lethality that occurs upon expression of the early lytic genes from pEC400, and does not show the Dhr Effect or show cell filamentation; Chapter 5.2.5b), may in fact contain mutations in two E. coli genes; one involved in the action of Dhr and the other involved in the action of Fil. The mapping studies presently being carried out (by S. Williams of this laboratory) should reveal whether one or two E. coli genes are mutated in the Dhr^R1 mutant.

dhr is not an essential gene, but it appears to be important in 186 lytic development as 186 Dhr^- mutants show a significant reduction in burst size (Chapter 5.2.3, 5.2.7). The role of Dhr in 186 lytic development is not known, however, it is possible it may play a role in 186 replication, as detailed below. The immediacy of onset of the inhibition of host DNA replication by Dhr (5 min after heat-induction of a 186 prophage or of a plasmid-clone containing the dhr gene) suggests that Dhr may act to inhibit the elongation step of E. coli DNA replication. Since 186 requires every host replication function that has been tested (Hooper, 1979; Hooper and Egan, 1981), the Dhr function (by inhibiting E. coli DNA replication) may lessen the competition from the host for some limiting components needed for 186 replication.

The fil gene is also not essential and does not appear to be important in 186 lytic development. The mechanism of Fil-induced cell filamentation does not appear to involve the induction of the SOS system (Chapter

5.2.5a), therefore it is possible that this filamentation (caused by Fil) is the result of a direct interference with the process of E. coli cell division. Several E. coli cell division genes have been described (Mendelson, 1982; Donachie et al., 1984), and it is possible that Fil may inhibit the action or the expression of one of these genes. The expression of Fil results in filamentous cells, which show no obvious septa (Chapter 5.2.4c), suggesting that the stage of cell division inhibited is an early event in the E. coli cell cycle (Slater and Schaechter, 1974; Mendelson, 1982; Donachie et al., 1984). The role that Fil-induced filamentation plays in 186 lytic development, may be to prevent segregation of an uninfected daughter cell from the 186-infected cell. Such uninfected cells may compete with the 186-infected or lysogenised cells for nutrients or could be infected by competing 186 phage. Furthermore, a greater 186 burst may be obtained from a large undivided cell than from a smaller newly-divided cell. In this regard, it is pertinent to note that the 186 filam mutant gave a slightly reduced burst compared with that obtained for the wild-type (80%-90%; Chapter 5.2.7a).

10.2.2 Comparison of dhr and fil to Similar Genes Encoded by other Phage.

Host DNA replication and cell division is inhibited during infection by a variety of coliphage. The phage λ, ϕX174 and T4 encode genes, which act to inhibit host DNA replication, and λ, ϕX174 and P4 encode genes, which act to cause filamentation. Genes, which are lethal to the host, that act by an undefined mechanism, have also been reported in several phage; in P2, P4, λ and Mu. These genes encoded by other phage will be compared and contrasted to the 186 dhr and fil genes.

10.2.2(a) P2 Functions.

Whether the 186-related phage, P2, encodes gene(s), which inhibit host DNA replication or result in filamentation of cells is not known. However,

when a P2 cIts prophage (which is unable to excise) is heat-induced, cell death results (Bertani, 1968; Nilsson and Bertani, 1977). This is similar to the lethality observed when a 186 Int$^-$ lysogen is heat-induced and suggests that P2 may encode a gene, which is similar to dhr. In addition, a P2 mutant, sig5, has been obtained, which no longer displays this lethality (Lindahl et al., 1971), and it has been shown that the sig5 mutation is due to the presence of an insertion element in the P2 early lytic region before the B gene (Chattoraj et al., 1975). Furthermore, plasmid-clones encoding this region have been reported to be lethal to the host, unless the expression of these genes is repressed (Saha et al., 1987).

The DNA sequence of the P2 early lytic region has been determined (Haggard-Ljungquist et al., 1987), and a comparison of the translated sequence of the genes encoded in this region with the amino acid sequence of the Fil and Dhr proteins (using the computer programs described in Chapter 2.38) did not reveal any significant homology.

10.2.2(b) P4 Functions.

Phage P4 is a satellite phage of the P2 family (Calendar et al., 1977). P4 has been reported to encode a gene, kil, which results in cell death after infection of cells that do not contain a helper P2 or 186 phage (Calendar et al., 1981; Alano et al., 1986). The map position of this gene is not known. P4 has also been reported to cause cell filamentation in its lysogenic plasmid mode of propagation (Deho et al., 1984). Furthermore, a mutant in the P4 β gene has been reported to inhibit host macromolecular synthesis and eventually kill the host (Gibbs et al., 1973). The β gene is non-essential and has not been sequenced. Further work is necessary to elucidate these effects of P4 on the host and to determine the P4 genes responsible for these effects.

10.2.2(c) The ϕX174 A* Function.

The virulent single-stranded DNA phage ϕX174 inhibits host DNA replication 10-15 min after infection (Linqvist and Sinshiemer, 1967). The ϕX174 A* protein, which binds to ϕX174 RF and single-stranded DNA and has a single-stranded DNA-specific endonucleolytic activity (Eisenberg and Ascarelli, 1981; Langeveld et al., 1979, 1981; van der Ende et al., 1981, 1982; Dubeau and Denhardt, 1981), has been implicated to be involved in this inhibition (Martin and Godson, 1975; Funk and Snover, 1976; Eisenberg and Ascarelli, 1981). It is not known if ϕX174 A* is an essential gene since A* is encoded within the essential A gene (Linney and Hayashi, 1974). However, it has been suggested that the A* protein may be important in the ϕX174 life cycle, since it may play a role in the transition from the semi-conservative RF DNA replication to viral single-stranded DNA synthesis (Eisenberg and Ascarelli, 1981). Infection with ϕX174 also results in the inhibition of cell division (Stone, 1970), which probably results from the inhibition of host DNA replication by gpA* since it has recently been shown that the expression of gpA* from a plasmid-clone not only inhibited host DNA replication, but also caused cell division inhibition and cell death (Colasanti and Denhardt, 1985).

The comparison of the amino acid sequence from the A* gene, which encodes a protein of 37 kd (Sanger et al., 1977b), with the Dhr and Fil amino acid sequences (Chapter 2.38) did not reveal any significant homology.

10.2.2(d) The T4 Ndd Function.

The virulent phage T4 inhibits host replication 4 min after infection, due to the activity of the ndd gene, which causes nuclear disruption by moving the host chromosome to a position closely associated with the cell membrane (Snustad and Conroy, 1974; Snustad et al., 1974, 1976, 1983). Host DNA is then degraded 10 min after infection to supply precursors for

phage DNA synthesis (Koerner and Snustad, 1979). The ndd gene maps near the rIIB gene on the T4 genome and the Ndd protein has been tentatively identified as a basic protein of a molecular weight of ~16 kd (Snustad et al., 1983). The DNA sequence of the ndd gene has not been determined so a comparison of the amino acid sequences derived from the ndd and dhr genes could not be carried out.

10.2.2(e) λ Functions.

Whether the temperate coliphage λ acts to inhibit host replication is still in doubt. Cohen and Chang (1970) concluded, from pulse-labelling and DNA-DNA hybridization studies, that λ depressed host DNA replication (as well as RNA and protein synthesis), but indicated in their discussion the divergent views on this point in the literature. Cohen and Chang (1970) mapped the function(s) involved in this inhibition to two regions, namely a region encoding the exo gene and a region from gam to ral (see Figure 1.5). Court et al. (1980b) suggested that this inhibition of host macromolecular synthesis may be more apparent than real, due to the effect in the pulse-labelling experiments of λ Hin (host inhibition) function, encoded on the pL transcript between 62.5% to 66.3% (encoding the exo gene region), that could restrict entry of exogenous precursors into the intracellular pool. The Hin function maps to the same region as one of the functions involved in the host inhibition observed by Cohen and Chang (1970), however, in contrast to the results of Cohen and Chang (1970), Court et al. (1980b) did not obtain evidence for a function encoded in the gam to ral region. Court et al. (1980b) point out however, that their system involves induction of an extensively deleted prophage, while that of Cohen and Chang (1970) involves infection with conditional lethal phage mutants. As prophage induction shows increased pL transcription compared with infection (Volpi et al., 1983), which would be further enhanced by the absence of the Cro function due to the ΔH1 deletion, then the influence of

Hin would be maximal in the system used by Court *et al.* (1980b). Therefore, it was possible that the inhibition in host macromolecular synthesis observed by Cohen and Chang (1970) is due to the expression of function(s) other than Hin. Evidence to support this comes from the work of Georgiou *et al.* (1979). These authors were studying the Tro phenotype, which is due to the over expression of λ *p*L functions after infection or heat-induction with the Cro$^-$ phage, λ *c*I857*cro*22*Sam*17. The Tro effect results in the inhibition of host macromolecular synthesis (leading to cell death) and the inhibition of heteroimmune phage growth (Eisen *et al.*, 1975; Folkmanis *et al.*, 1977). They showed that the inhibition of host macromolecular synthesis, as observed by pulse-labelling studies, represented a real inhibition and was not due to a decrease in the radioactivity of the intracellular pool or the inhibition in the uptake of precursors. Georgiou *et al.* (1979) showed that the Tro phenotype was due to two regions, that of the *Eal0* (*ssb*) gene, which encodes the single-stranded DNA binding protein (Hendrix, 1971; Szybalski and Szybalski, 1979; Court and Oppenheim, 1983), and the *N* gene, which is involved in antitermination of the immediate-early transcripts to allow delayed-early gene expression (Chapter 1.4.2a; Roberts, 1969; Friedman and Gottesman, 1983).

The system used by Georgiou *et al.* (1979) to examine the Tro phenotype is similar to that used by Court *et al.* (1980b), it was therefore surprising that Court *et al.* (1980b) did not observe the Tro phenotype. This may be explained by the fact that the Δ*H*1 lysogen used by Court *et al.* (1980b) may have contained mutations in the *ssb* gene due to the method used to isolate this deleted prophage (Castellazzi *et al.*, 1972). The Δ*H*1 deletion was isolated as a thermo-resistant (42^{o}C) survivor of a λ *c*I857*Nam*7*Nam*57 prophage (Castellazzi *et al.*, 1972) and, since the Tro phenotype (due to *ssb*) is still partially observed with this *N*$^-$ mutant (Georgiou *et al.*, 1979), it is likely that the Δ*H*1 prophage may also have

acquired a mutation in the ssb gene to allow survival of the lysogen at 42°C. Alternatively, as suggested by Georgiou et al. (1979), ssb and N may not be expressed at high enough levels from the λ ΔH1 lysogen to cause the Tro phenotype.

Comparison of the amino acid sequence of ssb and N (which encode proteins of 122 and 133 amino acids, respectively; Sanger et al., 1982), with 186 Dhr and Fil did not reveal any significant homology (Chapter 2.38; data not shown). Neither did Dhr and Fil show significant homology with the amino acid sequence of the λ orf61, Ea22 or Ea8.5 genes (Sanger et al., 1982), which are candidates for the hin genes (Court and Oppenheim, 1983) (Chapter 2.38; data not shown).

Coliphage λ also encodes a gene, kil, on the pL transcript (Greer, 1975a), the expression of which leads to cell death, apparently due to damage of the host cell membrane (Greer, 1975b; Volpi et al., 1983), with inhibition of E. coli DNA replication and filamentation as delayed secondary effects. The system used by Greer (1975a) to study the kil gene, involved the heat-induction of ΔH1 lysogens. Thus, as discussed previously, the Tro effect was not expected to occur.

E. coli kil-resistant mutants have been isolated, and it is interesting to note that some of these mutants are cold-sensitive for growth (Greer, 1975b; Court and Oppenheim, 1983) as are some Dhr^R mutants. However, these kil-resistant mutants have been mapped in the nusB gene (a gene required for λ gpN-mediated antitermination; Court and Oppenheim, 1983; Chapter 1.4.2) and thus, probably reflect lack of kil gene expression rather than tolerance to the Kil protein.

The λ kil gene is most likely encoded to the immediate left of the cIII gene (Greer, 1975a; Sanger et al., 1982; Knight and Echols, 1983). The comparison of the amino acid sequence derived from λ kil (which encodes a protein of 47 amino acids) with that of 186 Fil and Dhr (Chapter 2.38) did not reveal any significant homology (data not shown).

10.2.2(f) Mu Functions.

The temperate phage Mu also encodes a gene, kil, which causes cell death (van de Putte et al., 1977). When Mu kil is expressed from a multicopy plasmid-clone (pGP1) it results in cell death (Schumann et al., 1978; Gilphart-Gassler et al., 1979; Gilphart-Gassler and van de Putte, 1979) with survival kinetics similar to that obtained upon the expression of 186 dhr from the plasmid-clone, pEC400 (Chapter 5.2.2). However, Mu kil appears to cause cell death by a different mechanism to 186 dhr, since when Mu genes are expressed from pGP1 (in the strain E2267; Chapter 2.1, 2.3.2) there is no effect on E. coli DNA replication as recorded by pulse-labelling studies (data not shown). Furthermore, a Mu kil-resistant E. coli mutant, hek (Goosen and van de Putte, 1984; strain E4063; Chapter 2.1), was made lysogenic for 186 Dhr^+ (strain E4064) or 186 Dhr^- (strain E4065) and it was shown that the Dhr^+ lysogen gave a normal Dhr Effect while the Dhr^- lysogen did not give the Dhr Effect, as expected (data not shown).

The expression of Mu early genes has also been reported to cause cell filamentation (Boeckh et al., 1986). Filamentation was observed upon the expression of Mu early genes from a λ-clone containing the same fragment from Mu as the plasmid-clone, pGP1, used by Giphart-Gassler and van de Putte (1979), and encoding the Mu cI^{ts}, ner, A, B, cim, kil, and gam genes. Filamentation was attributed to the Mu B gene or a gene downstream from it (Boeckh et al., 1986). However, filamentation does not appear to occur when the Mu early genes were expressed from pGP1 (in the strain E2267; Chapter 2.1) (data not shown). This difference may be explained by the possibility that the filamentation observed with the λ-clone of this region (Boeckh et al., 1986) may be dependent on a λ gene.

The Mu kil gene maps between the cim and gam genes and encodes a protein of ~8 kd (Gilphart-Gassler et al., 1981), which is similar in size to 186 Dhr. However, comparison of the amino acid sequence of Mu Kil

derived from the DNA sequence (D. Kamp, personal communication) with the amino acid sequence of Dhr and Fil (Chapter 2.38) did not reveal any significant homology (data not shown). The Mu cim and gam genes (D. Kamp, personal communication) also showed no significant homology with 186 dhr or fil at the amino acid level (Chapter 2.38; data not shown).

10.2.2(g) General Comparisons.

This comparison of functions that act to inhibit host DNA replication, cause filamentation or cell death from various phage with the 186 Dhr and Fil functions, has shown that 186 Dhr and Fil have many similarities to the functions encoded by other phage.

Of the phage functions which have been well characterized, the ϕX174 A^*, λ N and Ssb functions show the greatest similarity to 186 Dhr, in that they are both lethal to the host and inhibit E. coli DNA replication. However, unlike the ϕX174 A^* function, 186 Dhr does not cause cell filamentation (Chapter 5.2.6b), and unlike λ gpN and gpSsb, 186 Dhr does not appear to inhibit E. coli RNA synthesis (Finnegan, 1979) or protein synthesis (since the optical density of plasmid-clones expressing 186 dhr continues to increase although E. coli DNA replication is inhibited; Chapter 5.2.4a,b).

The 186 Fil function shows similarities to the two characterized phage functions, which inhibit E. coli cell division, ϕX174 A^* and λ Kil. However, unlike these functions, 186 Fil does not appear to affect E. coli DNA replication (Chapter 5.2.6b).

The precise mechanism by which the phage functions described above act to inhibit E. coli DNA replication, cell division or cause cell death is not known. Also not known is the exact requirement of these functions in phage development (except for λ N; Friedman and Gottesman, 1983; Chapter 1.4.2). Many of these functions, including 186 Dhr and Fil, appear to be non-essential for phage growth (Snustad and Conroy, 1974; Court and

Oppenheim, 1983; Goosen et al., 1982; Chapter 5.2.7), the exception being λ N (Friedman and Gottesman, 1983).

In summary, although 186 Dhr and Fil show similarities to functions encoded by other phage, they appear to be unique in their effects on the host. Furthermore, 186 Dhr and Fil show no significant amino acid sequence homology to that of any similar functions that have been sequenced. Future studies on the effect of 186 Dhr and Fil on the host should contribute to the understanding of the interaction of 186 with E. coli during 186 lytic development, and may provide insights into the mechanisms by which other phage functions interact with the host and the role they play in phage development.

10.2.3 Future Studies.

As previously discussed (Chapter 5.2.5b), the map position of the Dhr^R1 mutation(s) on the E. coli chromosome and the characterization of this gene(s) will provide a first step to determining the mechanism of action of Dhr and Fil. To further elucidate the E. coli genes that are required for the action of Dhr or Fil, more host mutants that are resistant to the lethal effects of Dhr or Fil can be isolated, by using plasmid-clones containing either the dhr gene (pEC421; Chapter 2.3.2) or the fil gene (pEC404; Chapter 2.3.2), and the chromosomal map position of these mutations can be determined, using standard techniques (Miller, 1972). This approach may not only provide insights into the mechanism of action of Dhr and Fil, but may reveal novel E. coli genes involved in DNA replication or cell division.

The possible role of Dhr in 186 replication can be investigated by determining the amount of 186 DNA produced with time after the heat-induction of a 186 Dhr^- mutant compared with a Dhr^+ phage, using DNA-DNA hybridization studies (Kafatos et al., 1979).

10.3 RNaseIII CLEAVAGE OF 186 EARLY LYTIC AND MIDDLE TRANSCRIPTS.

10.3.1 RNaseIII Cleavage Sites.

RNaseIII cleaves at double-stranded RNA (Robertson et al., 1968). RNaseIII sites previously characterized in natural systems have been shown to occur at regions of secondary structure in the RNA, which can be classified into two major types (Robertson, 1982); those which show imperfect double-stranded RNA stem-loop structures, and those which show perfect double-stranded regions. RNaseIII sites, which show imperfect double-stranded RNA stem-loop structures (i.e. contain two small stems of 7-10 bp separated by a "bubble" of unpaired bases), result in a single RNaseIII cleavage in the "bubble" region. Whereas, RNaseIII sites, which show a more perfect double-stranded region of 20-25 bp in length, result in two RNaseIII cleavages, on opposite sides of the stem and staggered by 2 bp. Sequence homology between RNaseIII sites is very limited, except for the presence of the sequence 5'-AAG/3'-TTC, which occurs near most RNaseIII cleavage sites (Gegenheimer and Apirion, 1981; Szeberenyi et al., 1984; Gurevitz and Apirion, 1985).

10.3.2 RNaseIII Cleavage Sites in the 186 Early Lytic-Middle Region.

As detailed in Chapter 7, two RNaseIII cleavages occur in the 186 early lytic-middle region to give rise to the 3.1 kb and 2.8 kb middle transcripts and the 1.1 kb early lytic transcript. The 5'-end of the 3.1 kb transcript has been determined to be approximately at the sequence coordinate 3768, whereas the 5'-end of the 2.8 kb transcript is located in the region 4088-4248 (Chapter 6.2.4). Furthermore, the region 4070-4098 (deleted in 186 deltR1 (22); Chapter 2.2.1, 6.2.4), appears to be important in the production of the 2.8 kb transcript. The 3'-end of the 1.1 kb transcript was mapped to the region 3690-3732 (Chapter 6.2.3).

Since it is possible that these RNaseIII cleavages occur as a result of the hybridization of two RNA molecules (Robertson et al., 1968) and because two potential leftward promoters are encoded within the PstI-BglII (77.4%-79.6%) region (Chapter 3.2.2c), RNA dot blot analysis was carried out to determine whether there are leftward transcripts in this region (Chapter 7.2.3). This study failed to provide evidence for the existence of a leftward transcript, suggesting that these potential leftward promoters are not active. Thus, RNaseIII cleavage within the early lytic-middle region is likely to be a result of the formation of secondary structures in the RNA.

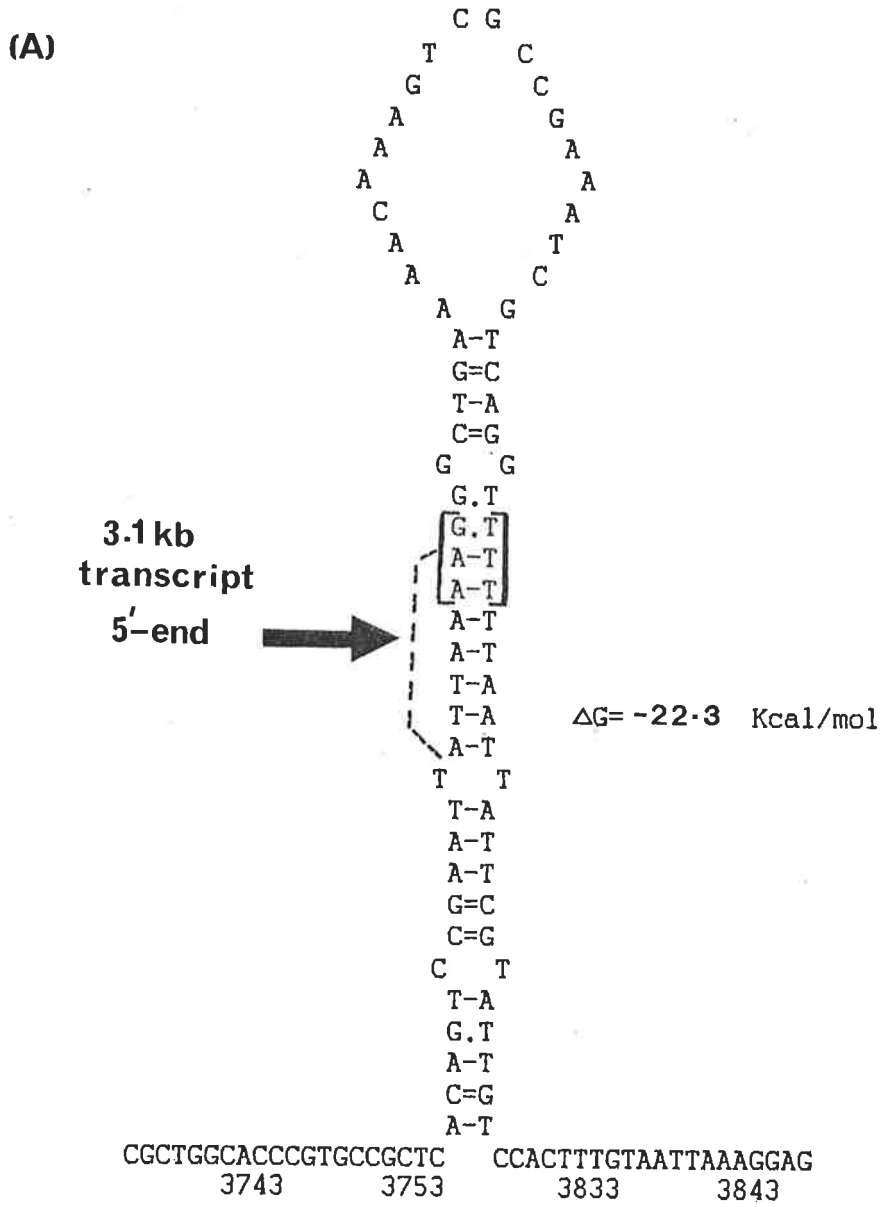
The DNA sequences of the region spanning the 5'-ends of the 3.1 kb and 2.8 kb transcripts were analysed for possible secondary structures using dot matrix analysis (Chapter 2.38). Several secondary structures, that resembled the RNaseIII structures described by Robertson (1982) (Chapter 10.3.1) were predicted. The most stable of these structures are shown in Figures 10.1, 10.2, 10.3 and 10.4.

The structures shown in Figure 10.1 span the 5'-end of the 3.1 kb transcript. In addition, the structures shown in Figures 10.1(b) and 10.1(c) also span the region containing the 3'-end of the 1.1 kb transcript. RNaseIII cleavage of these structures (shown in Figure 10.1b,c) may occur at two positions resulting in the generation of the 1.1 kb and the 3.1 kb transcripts. Should the structure shown in Figure 10.1(a) be cleaved by RNaseIII to generate the 3.1 kb transcript, a different secondary structure must be cleaved by RNaseIII to generate the 1.1 kb transcript, since the region encoding the 3'-end of the 1.1 kb transcript (3690-3732) is not included in this structure.

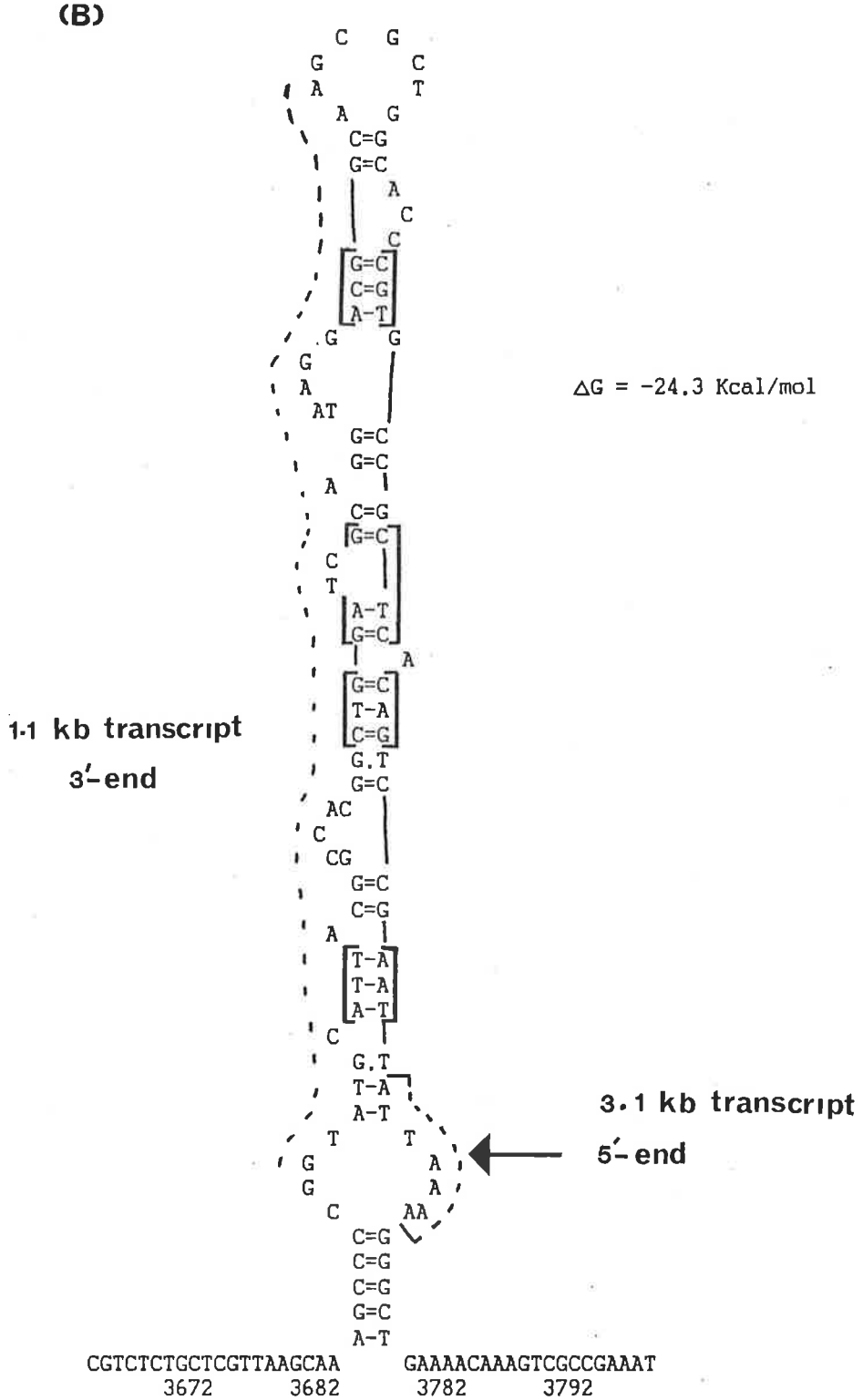
The structures shown in Figure 10.2 span the region 4070-4098, which was shown to be important in the generation of the 2.8 kb transcript (Chapter 6.2.4). Removal of the region 4070-4098 would be expected to

Figure 10.1 Potential RNaseIII-cleavage structures located in the region encoding the 5'-end of the 3.1 kb transcript.

Structures were predicted using dot matrix analysis (Chapter 2.38). The free energy (ΔG) values were calculated using the rules of Steger et al. (1984), and are listed next to the structures. The DNA sequence coordinates are indicated. The position of the 5'-end of the 3.1 kb transcript, is shown by the arrow. Sequences resembling the conserved sequence (5'-AAG/3'-TTC) are boxed. Structures 10.1(b) and 10.1(c) also contain the region encoding the 3'-end of the 1.1 kb transcript, which is indicated on the structures by the dashed line.


(A)
3.1 kb
transcript
5′-end
ΔG= -22·3 Kcal/mol
CGCTGGCACCCGTGCCGCTC CCACTTTGTAATTAAAGGAG
3743 3753 3833 3843

(B)


ΔG = -24.3 Kcal/mol
1.1 kb transcript
3′-end
3.1 kb transcript
5′-end
CGTCTCTGCTCGTTAAGCAA
GAAAACAAAGTCGCCGAAAT
3672
3682
3782
3792

(C)

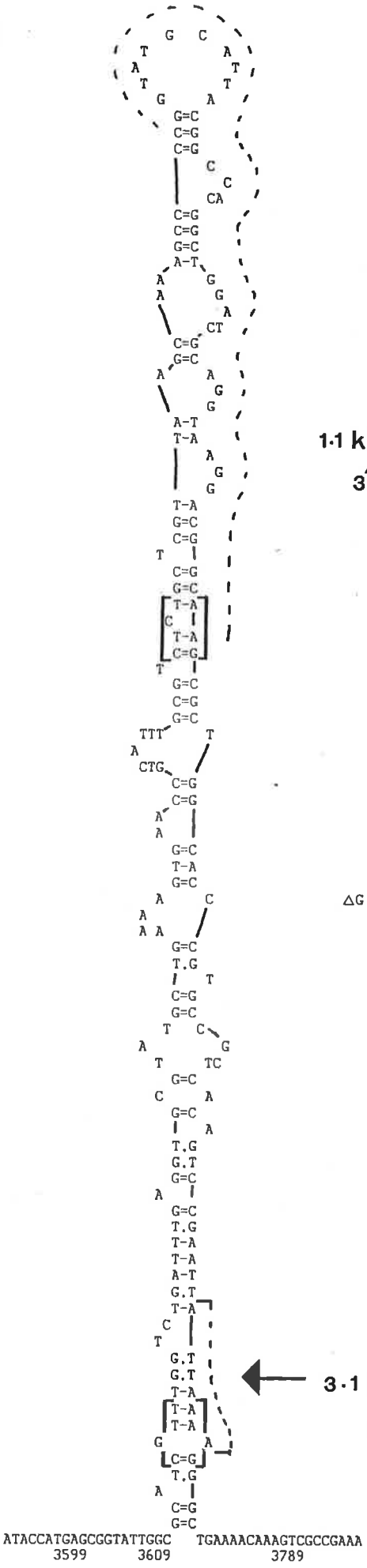
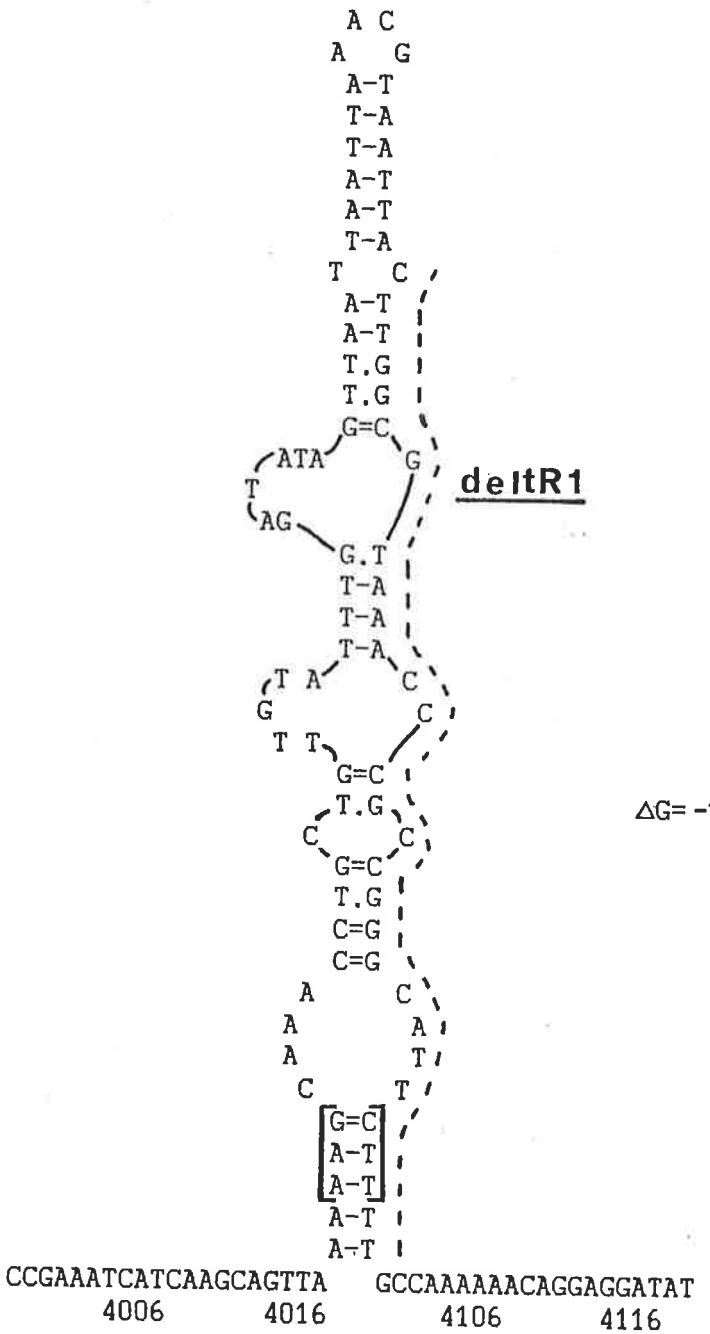

1·1 kb transcript
3′-end
ΔG = -40.5 Kcal/mol
3·1 kb transcript
5′-end
ATACCATGAGCGGTATTGGC TGAAAACAAAGTCGCCGAAA
3599 3609 3789

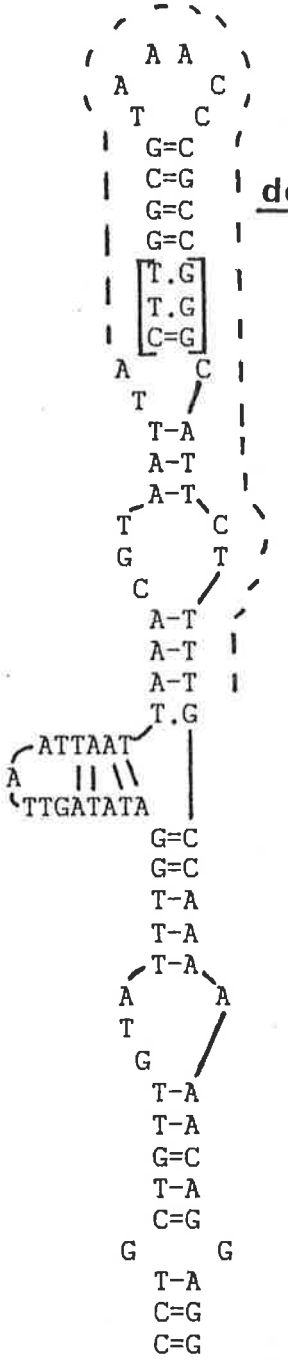
Figure 10.2 Potential RNaseIII-cleavage structures at the 5'-end of the 2.8 kb transcript.

The structures were predicted using dot matrix analysis (Chapter 2.38). The free energy (ΔG) values were calculated using the rules of Steger et al. (1984), and are listed next to the structures. The DNA sequence coordinates are indicated. The position of the deltR1 deletion, is indicated on each structure by the dashed line. Sequences resembling the conserved sequence (5'-AAG/3'-TTC) are boxed.

(A)


delta R1
ΔG= -14·8 Kcal/mol
CCGAAATCATCAAGCAGTTA GCCAAAAAACAGGAGGATAT
4006 4016 4106 4116

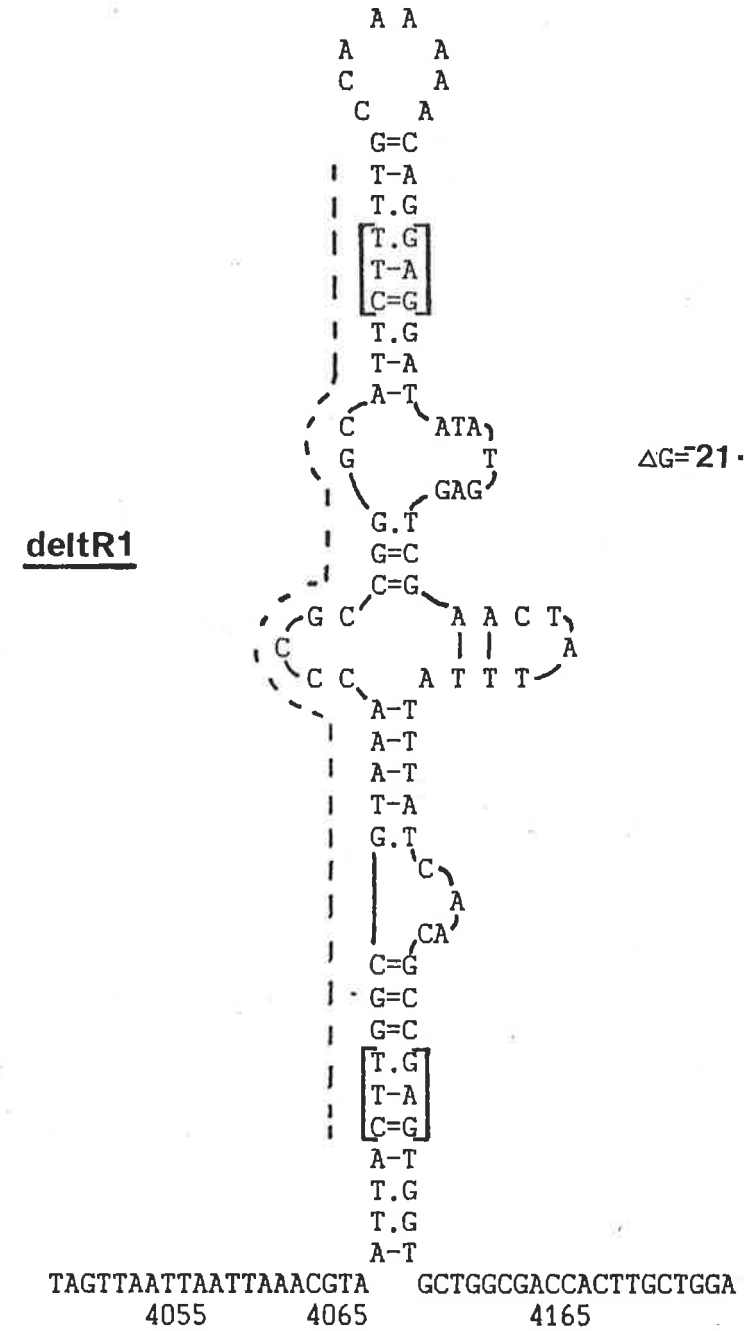
(B)



ΔG=-26·9 Kcal/mol

TCAAGCAGTTAAAAAGCAAA ATATATGAGTCGAACTATTT
4015 4025 4125

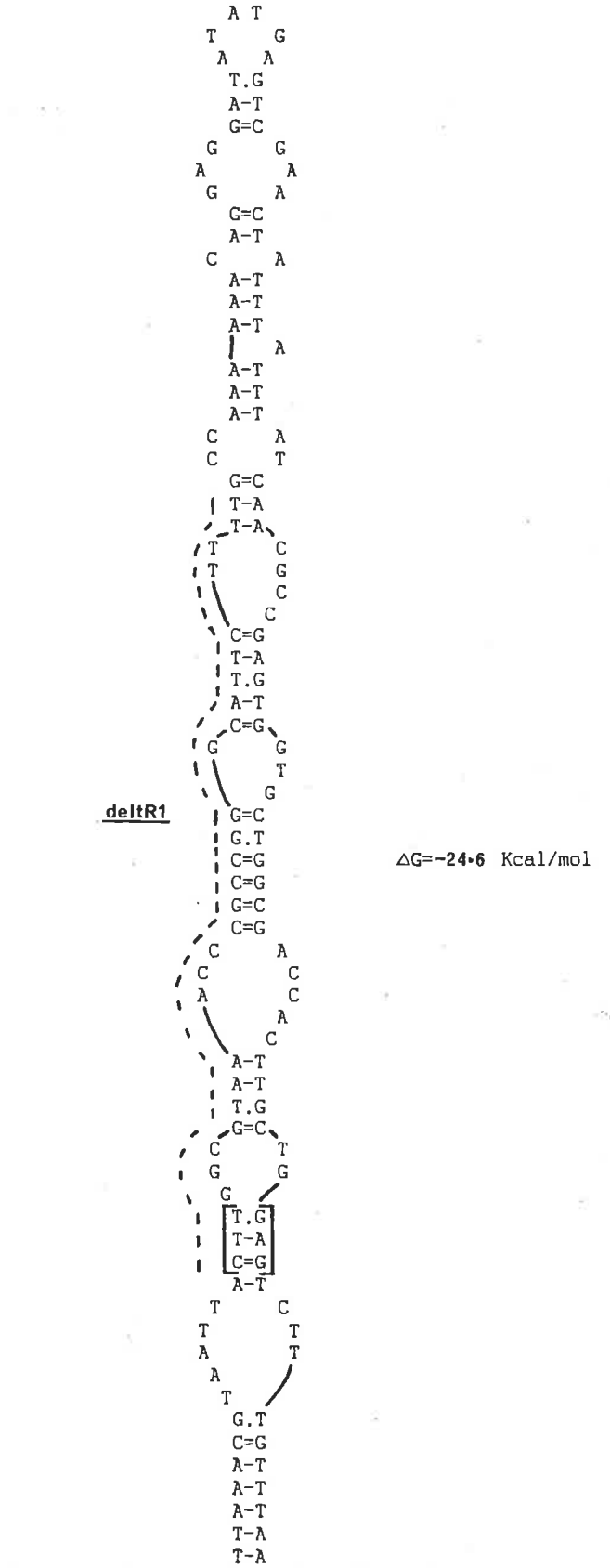
(C)



deltR1

ΔG=-21·3 Kcal/mol

(D)


delltR1
ΔG=-24.6 Kcal/mol
ATTTGGATATAGTTAATTAA AGAAGCCAAAAAAGAAGAGC
4046 4056 4196 4206

disrupt these potential RNaseIII structures and to prevent the production of the 2.8 kb transcript.

The structures, presented in Figures 10.1 and 10.2, show only partial double-strandedness and it is therefore expected that RNaseIII would cleave at these potential sites on only one side of the structure, and that this cleavage would occur in a "bubble" region (see Chapter 10.3.1). However, contrary to expectations, the position determined for the 5'-end of the 3.1 kb transcript occurs in a region of double-strandedness in the structures shown in Figures 10.1(a) and 10.1(c). The structure shown in Figure 10.1(b) however, contains the region spanning the 5'-end of the 3.1 kb transcript in a "bubble" region, and is thus more consistent with previously characterized RNaseIII cleavage sites (Chapter 10.3.1).

As detailed in Chapter 10.3.1, the conserved sequence 5'-AAG/3'-TTC has been observed in most RNaseIII structures. Of the potential RNaseIII structures shown in Figures 10.1 and 10.2, only Figure 10.2(a) contains the conserved sequence. However, the structure shown in Figure 10.1(a) contains the sequence 5'-AAG/3'-TTT, which is similar to the conserved sequence, and is located 2 bp 5' to the position of the 5'-end of the 3.1 kb transcript. This sequence (5'-AAG/3'-TTT) has also been observed in the T4 tRNA RNaseIII processing site (Gurevitz and Apirion, 1985). The potential RNaseIII structures shown in Figures 10.1(b), 10.1(c), 10.2(b), 10.2(c) and 10.2(d) also contain sequences, which are similar to the conserved sequence (as indicated on the structures).

Two other potential RNaseIII structures located within the PstI-BglII (77.4%-79.6%) region are shown in Figures 10.3 and 10.4. The structure shown in Figure 10.3 is encoded between the first and second RNaseIII sites (Chapter 7.2.2). The cleavage of this potential structure by RNaseIII may possibly explain why the ~330 b RNA (expected from the cleavage of RNA at the first and second RNaseIII sites) was not visualized when RNA was

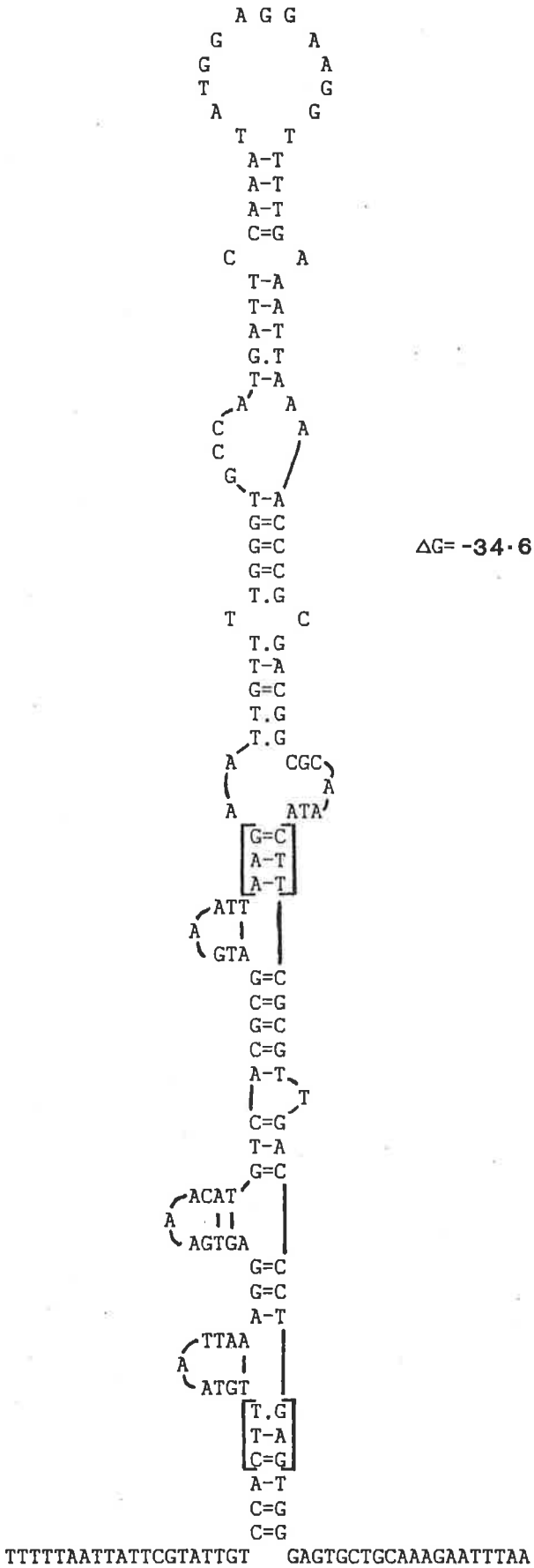
hybridized with specific probes to this region (Chapter 7.2.2). This structure contains the 5'-AAG/3'-TTC conserved sequence.

The structure shown in Figure 10.4 is a very large secondary structure, which includes the regions important in the first and second RNaseIII cleavages (Chapter 7.2.2). This structure places the first RNaseIII cleavage site in a "bubble" region, which is more consistent with the RNaseIII cleavage sites reported in the literature. This structure also includes part of the region in which the 3'-end of the 1.1 kb transcript is located. Sequences resembling the conserved sequence are marked on the structure. Should this structure form, it may be expected that removal of the region 4070-4098 in the deletion mutant 186 deltR1 would result in the destabilization of the complete structure, preventing the generation of the 3.1 kb and 1.1 kb transcripts as well as the 2.8 kb transcript. However, 186 deltR1 only prevents the production of the 2.8 kb transcript (i.e. prevents the second RNaseIII cleavage). Further analysis of the sequence of 186 deltR1 revealed that an alternative local secondary structure could form in the region of the structure disrupted by the deltR1 deletion (Figure 10.4). Therefore, although removal of the region 4070-4098 may prevent the second RNaseIII cleavage, perhaps because the 5'-AAG/3'-TTC sequence is removed, the first RNaseIII cleavage (which results in the generation of the 3.1 kb and the 1.1 kb transcripts) should not be prevented.

This analysis of the DNA sequence spanning the 5'-ends of the 3.1 kb and 2.8 kb transcripts has revealed several possible secondary structures that may function as RNaseIII sites. However, although these structures can be drawn it does not indicate that they will form in 186 RNA or act as RNaseIII sites. Further studies are needed to determine the sequences required for RNaseIII cleavage in the early lytic-middle region.

Figure 10.3 A potential RNaseIII-cleavage structure located in the region between the 5'-ends of the 3.1 kb and 2.8 kb transcripts.

The structure was predicted using dot matrix analysis (Chapter 2.38). The free energy (ΔG) value was calculated using the rules of Steger et al. (1984), and is listed next to the structure. The DNA sequence coordinates, are indicated. Sequences resembling the conserved sequence (5'-AAG/3'-TTC) are boxed.

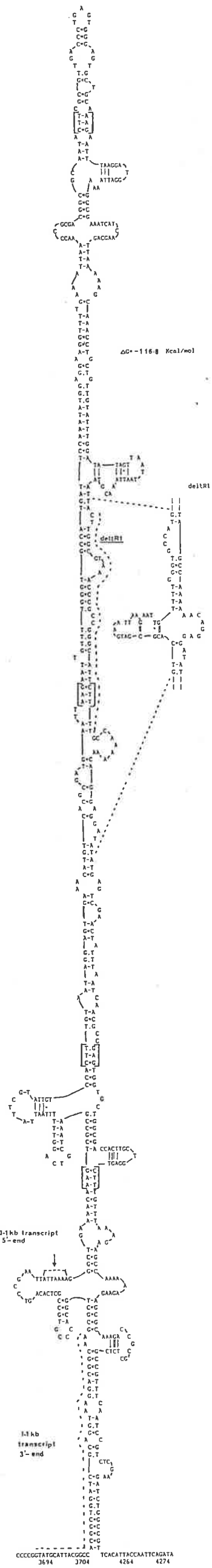


ΔG= -34·6 Kcal/mol

Figure 10.4 A potential RNaseIII-cleavage structure that includes the regions encoding the 5'-ends of the 2.8 kb and 3.1 kb middle transcripts and the 3'-end of the 1.1 kb transcript.

The structure was predicted using dot matrix analysis (Chapter 2.38). The free energy (ΔG) value was calculated using the rules of Steger *et al.* (1984), and is listed next to the structure. The DNA sequence coordinates, are indicated. Sequences resembling the conserved sequence (5'-AAG/3'-TTC) are boxed. The position of the 5'-end of the 3.1 kb transcript, is indicated by the arrow. The positions of the *delt*R1 deletion and the region containing the 3'-end of the 1.1 kb transcript, are indicated on the structure by the dashed lines.

An alternative secondary structure spanning the region removed by the *delt*R1 deletion is shown.


ΔG= -116.8 Kcal/mol
delt81
(ΔG= -92.2 Kcal/mol)
3.1kb transcript
5'- end
1.1kb
transcript
3'- end
CCCCGGTATGCATTACGGCC
3694
3704
TCACATTACCAATTCAGATA
4264
4274

10.3.3 Possible Involvement of RNaseIII Cleavage in the Generation of the 1.8 kb and the 2.1 kb Transcripts that were Detected after tM1.

As discussed in Chapter 6.2.5, two transcripts sized at 1.8 kb and 2.1 kb were detected in the region after tM1. The 5'-end of the 1.8 kb transcript is located in the region 6420-6840, whereas the 5'-end of the 2.1 kb transcript is located in the region 5607-6419 (Figure 6.1, 6.11b). Computer-assisted analysis of this region did not predict any transcription promoters (data not shown). Thus, it is possible that these transcripts are generated by RNaseIII cleavage. These regions were analysed using dot matrix analysis for possible RNaseIII structures. The most stable of these structures are shown in Figure 10.5. Sequences resembling the conserved sequence (5'-AAG/3'-TTC) were found in all of these structures. Transcription studies in a RNaseIII$^-$ strain using probes to the region promoter-distal to tM1 are necessary to determine whether the 1.8 kb and 2.1 kb transcripts arise by RNaseIII cleavage. In addition, further studies are needed to establish whether these putative RNaseIII structures are functional.

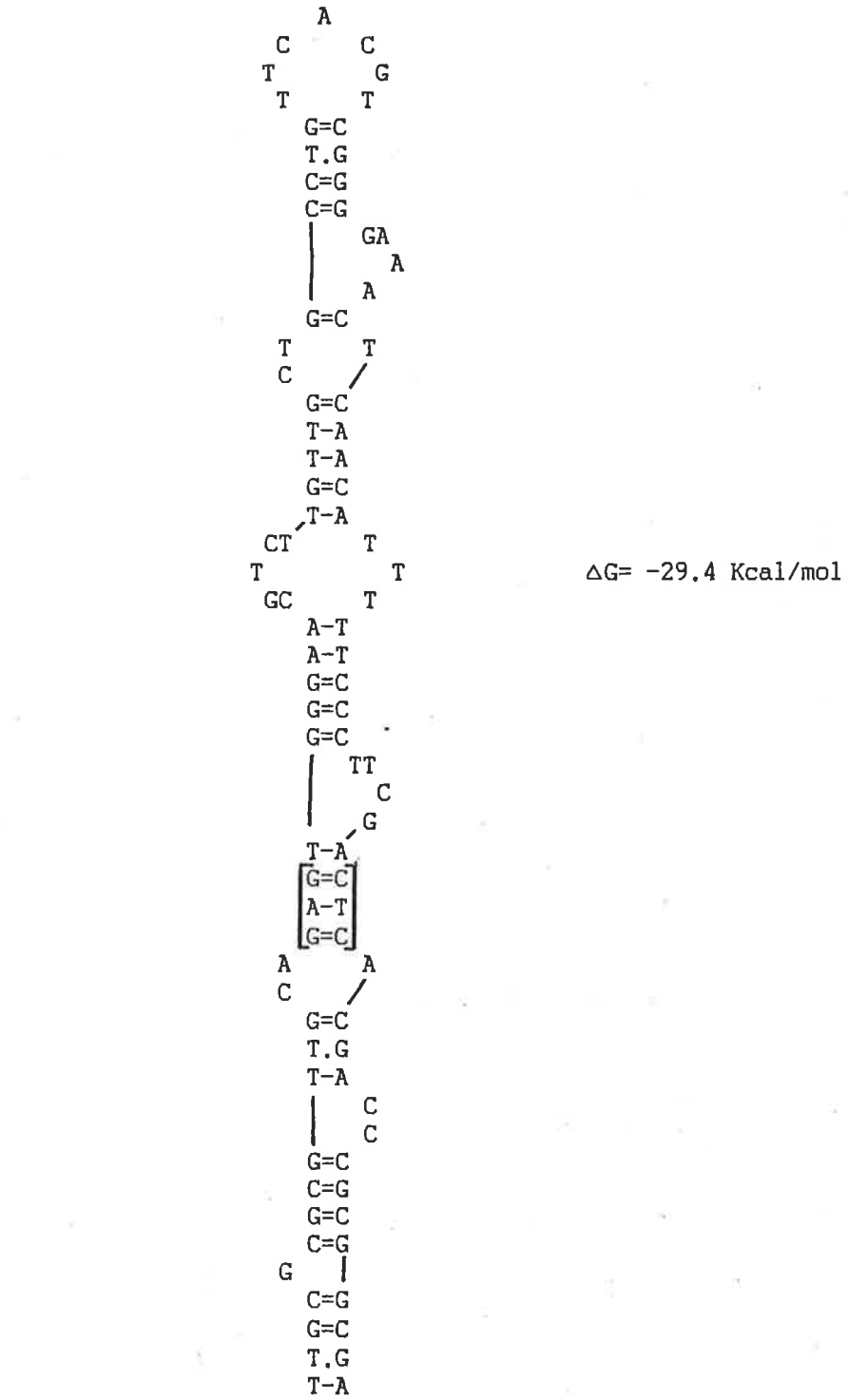
10.3.4 Future Studies.

Further studies are needed to establish the regions, which are required for RNaseIII cleavage within the early lytic-middle region, and within the middle region (should RNaseIII cleavage be involved in the generation of the 1.8 kb and 2.1 kb transcripts). This requires more specific location of the 5'-ends of the 2.8 kb, 1.8 kb and 2.1 kb transcripts and the 3'-end of the 1.1 kb transcript using the techniques of primer extension (McKnight et al., 1981) or S1 mapping (Berk and Sharp, 1977; Burke, 1984). Mutation studies (the creation of small deletions and point mutations) can then be carried out to determine the regions, which are important in the formation of the RNaseIII structures.

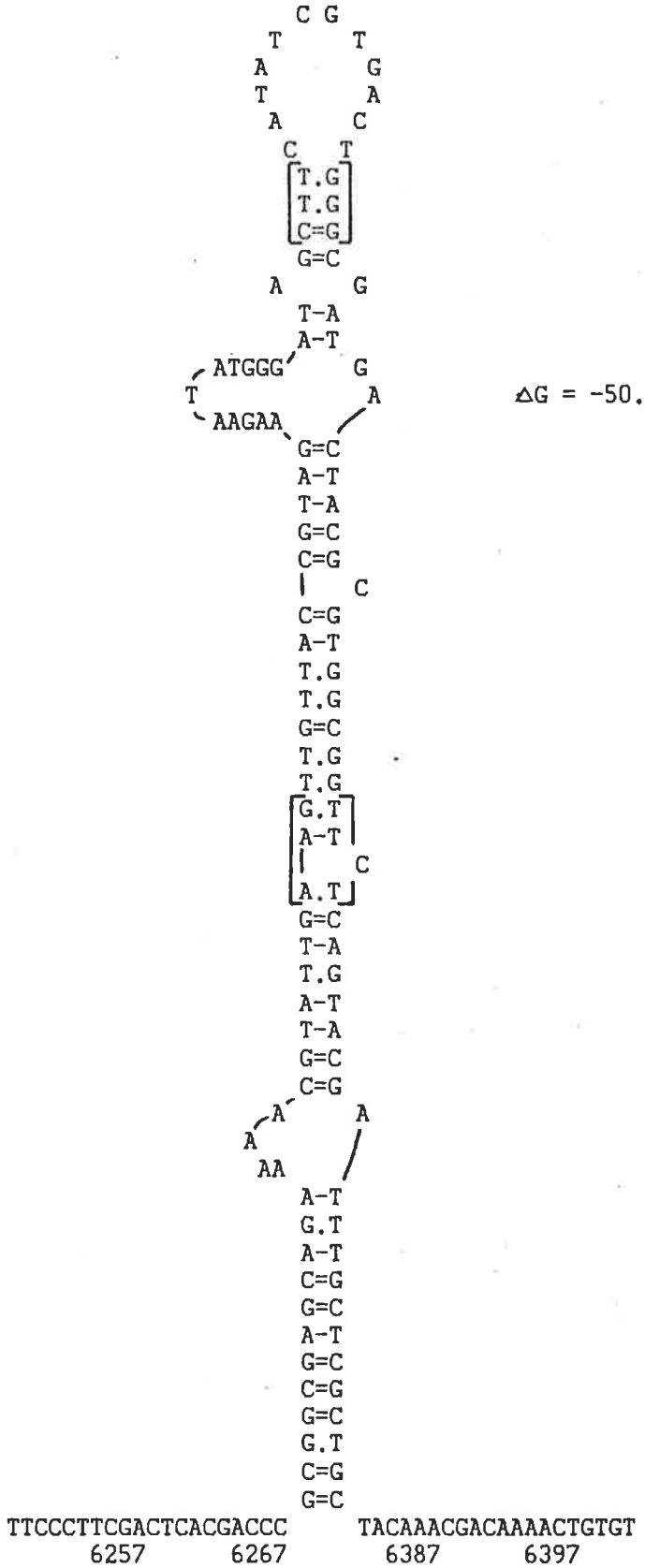
Figure 10.5 Potential RNaseIII-cleavage structures in the region encoding the 5'-end of the 2.1 kb transcript (5607-6419) and the 5'-end of the 1.8 kb transcript (6420-6840).

The structures were predicted using dot matrix analysis (Chapter 2.38). The free energy (ΔG) value was calculated using the rules of Steger et al. (1984) and are listed next to the structures. The DNA sequence coordinates, are indicated. Sequences resembling the conserved sequence (5'-AAG/3'-TTC) are boxed.

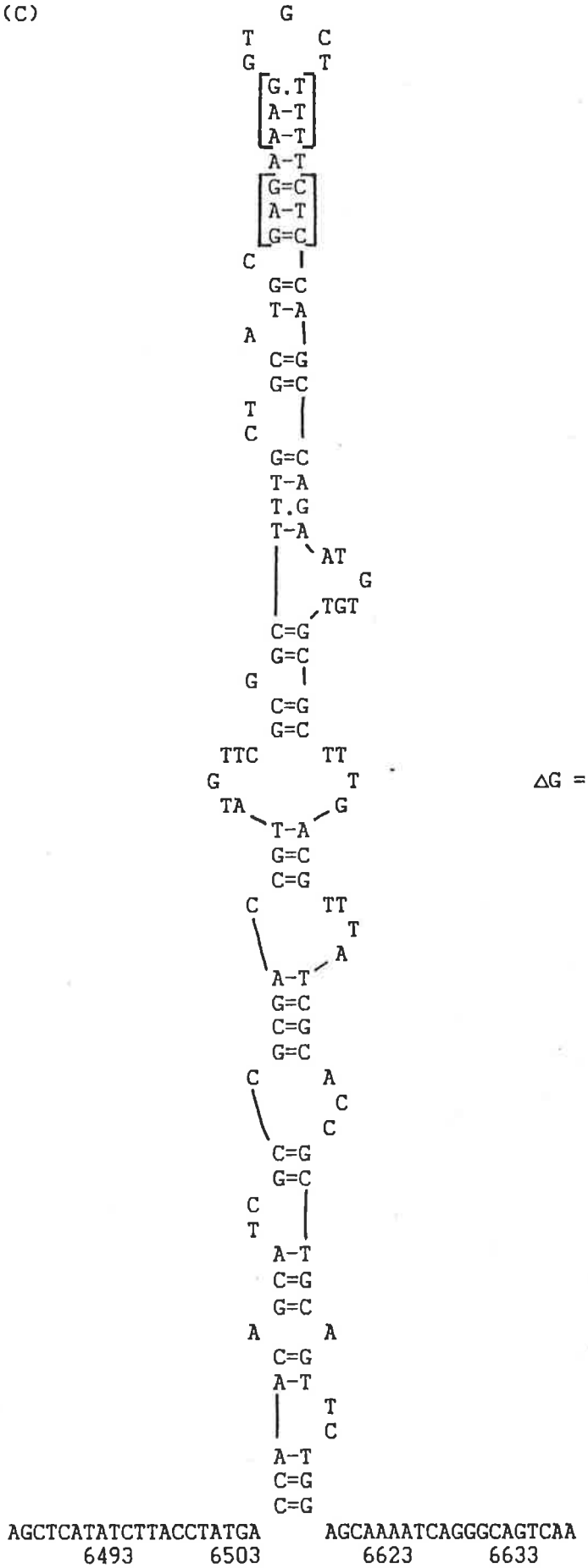
(A)


ΔG= -29.4 Kcal/mol
TTGCTCGCTGGCCGGAGGAGA GCAGAAAAACGTATTGAAGT
6172 6182 6284 6294

(B)

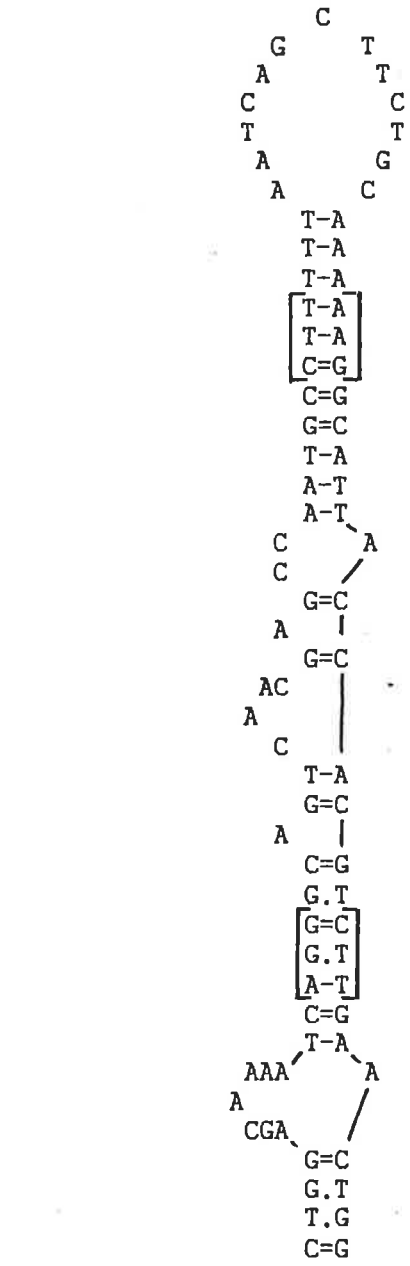

ATGGG
AAGAA
ΔG = -50.3 Kcal/mol
TTCCCTTCGACTCACGACCC
TACAAACGACAAAACTGTGT
6257
6267
6387
6397

(C)



ΔG = -31.1 Kcal/mol

(D)

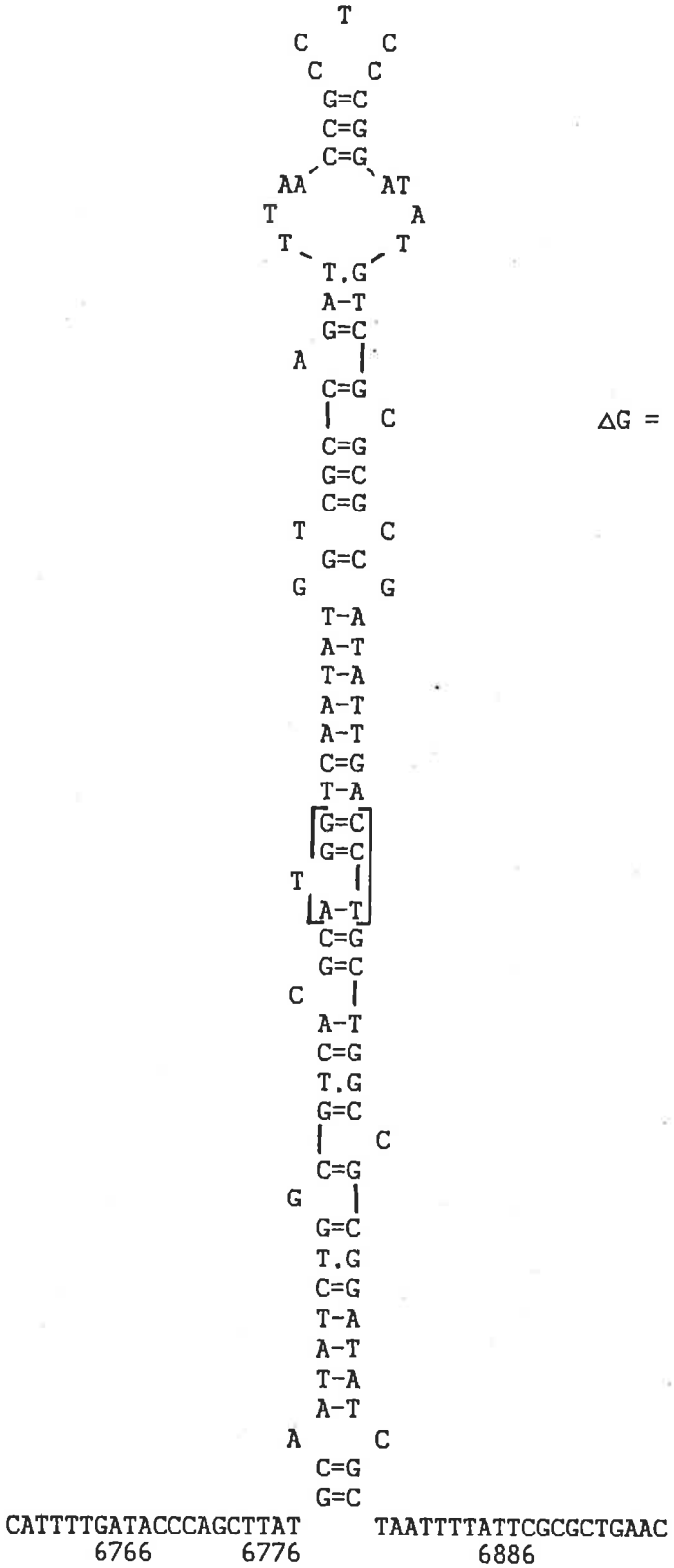


ΔG = -21.9 Kcal/mol

TTTATCGCACCGCTGCAGTT TCGCAAAAAAAATACGAGTGC

6602 6612 6702 6712

(E)


ΔG = -42.7 Kcal/mol
CATTTTGATACCCAGCTTAT
TAATTTTATTCGCGCTGAAC
6766
6776
6886

As discussed in Chapter 7.2.2, it is not known whether the 4.0 kb transcript is the only transcript that is processed by RNaseIII or if the 1.5 kb and 1.4 kb transcripts are also processed. To examine RNaseIII cleavage of the 4.0 kb, 1.5 kb and 1.4 kb RNAs, it is necessary to isolate these RNAs. This can be achieved by $^{32}PO_4$ labelling of *in vivo* 186 RNA in a RNaseIII$^-$ strain, followed by enrichment of these RNAs using the technique of hybridization-elution with specific probes (Salditt-Georgieff and Darnell, 1983; Bouvre and Szybalski, 1971). RNaseIII cleavage of these transcripts can then be examined *in vitro* using conditions, which favour RNaseIII cleavage of double-stranded RNA (Robertson *et al.*, 1968; Sten *et al.*, 1982), and the cleavage products can be analysed by agarose gel electrophoresis. This experiment should also elucidate whether the 1.4 kb transcript is generated (in part) by RNaseIII cleavage at the second RNaseIII site (as described in Chapter 7.2.2).

RNaseIII cleavage within the early lytic-middle region is likely to lead to the degradation of early lytic RNA and result in decreased expression of the early lytic genes (Chapter 7.2.4). To quantitate the degree to which RNaseIII processing and subsequent degradation of early lytic RNA decreases the expression of early lytic genes, the following experiment can be carried out. A clone of the region encoding the RNaseIII processing sites can be obtained in the McKenney terminator-analysis vector (pKL600), such that this region is correctly inserted 3' to the *galK* gene. The effect of this region on *galK* expression from the plasmid-clone can then be investigated in a *galK*$^-$ *rnc*$^+$ strain and compared with that obtained in a *galK*$^-$ *rnc*$^-$ strain. A similar experiment was used to investigate the control of λ *int* gene expression by retroregulation (Rosenberg and Schmeissner, 1982). This system can also be used to investigate the regions, which are important in RNaseIII cleavage.

10.4 THE CONTROL OF 186 MIDDLE GENE TRANSCRIPTION.

10.4.1 Does a Control Mechanism Exist for the Transcription of 186 Middle Genes?

The transcription studies of Finnegan and Egan (1981) and Pritchard and Egan (1985) led to the prediction that a control mechanism exists for the transcription of the 186 middle genes (Chapter 1.3.2). The aim of the *in vivo* transcription studies carried out in this thesis was to further investigate the putative control mechanism for 186 middle gene transcription. The transcription pattern determined for the 186 early lytic and middle regions was consistent with a mechanism of control for middle gene transcription involving antitermination at the early terminators. As discussed in Chapters 8 and 9, four approaches were used to determine whether antitermination was involved in the expression of 186 middle genes. These studies did not provide evidence for a control mechanism for middle gene transcription suggesting that such a control mechanism may not exist. This conclusion is contrary to the conclusions of Finnegan and Egan (1981) and Pritchard and Egan (1985). The results and conclusions obtained from the studies presented in this thesis will now be discussed and compared with the results of Finnegan and Egan (1981) and Pritchard and Egan (1985).

10.4.1(a) The In Vivo Transcription Pattern of the 186 Early Lytic and Middle Regions in the Absence of Translation.

As discussed in Chapter 8.2.1, the *in vivo* transcription pattern obtained in the absence of protein synthesis did not reveal a specific block at the early terminators, but rather a general decrease in 186 early lytic and middle transcription. Although a greater decrease in transcription was obtained at pR promoter-distal regions compared with promoter-proximal regions, this was considered to result from

transcriptional polarity (which occurs when translation is inhibited; Galloway and Platt, 1985) rather than reflecting a need for a 186-encoded protein for middle gene transcription.

The results obtained in this study differ to those obtained by Finnegan and Egan (1981). Finnegan and Egan (1981) deduced from their in vivo transcription results that, when 186 protein synthesis is inhibited by chloramphenicol, middle transcription was markedly reduced, whereas early rightward transcription was not significantly reduced. This led to their proposal that a 186 protein was required for 186 middle gene transcription. Contrary to the results of Finnegan and Egan (1981), the results obtained in this thesis (Chapter 8.2.1) showed that both 186 early lytic and middle transcription was significantly reduced when protein synthesis was inhibited by chloramphenicol. The difference between these results can be explained by the inability of the hybridization probe for early lytic transcription, used by Finnegan and Egan (1981), to specifically detect early lytic transcription. This probe, pEC35 [containing the PstI (65.5%-77.4%) fragment from 186 del1 (5); Chapter 1.3.2], also encodes the B gene, a gene which has since been shown to be autoregulated (i.e. B transcription is negatively controlled either directly or indirectly by the B protein) (Kalionis et al., 1986b). Therefore, in the presence of an inhibitor of protein synthesis (such as chloramphenicol), B gene transcription will be expected to increase and may significantly contribute to the labelled-RNA hybridizing to pEC35. Thus, the results obtained by Finnegan and Egan (1981) can not be used as evidence for the existence of a control mechanism for 186 middle gene expression.

10.4.1(b) The Strength of the Early Terminators In Vivo.

The results presented in Chapter 8.2.2, revealed that the Rho-independent terminator, tR1, is only 15% efficient in vivo. This result is

contrary to the results of Pritchard and Egan (1985), which suggested that transcription terminates in this region efficiently in vitro (Chapter 1.3.2b). Furthermore, the studies presented in Chapter 9.2.4, revealed that tR1 was 70% efficient in vitro. This difference between the termination efficiency at tR1, in vivo and in vitro, demonstrates that the in vitro transcription studies of Pritchard and Egan (1985) can not be used to provide evidence for a control mechanism for middle gene transcription, and illustrates the importance of in vivo transcription studies.

These results also revealed that the total level of termination at the early terminators in vivo, was only at the most 56%. Thus, the early terminators are inefficient terminators. This result, and the results obtained from the in vivo transcriptional studies carried out in the absence of translation (Chapter 8.2.2, 10.4.1a), suggest that there is no absolute requirement for an antitermination mechanism for 186 middle gene expression. However, it does not rule out the possibility that antitermination plays a non-essential, but presumably important, role in 186 middle gene transcription, by increasing readthrough past the early terminators from 44% to nearly 100%.

10.4.1(c) The Involvement of 186 Early Lytic and Middle Functions in Antitermination at the Early Terminators.

In order to investigate whether an antitermination mechanism was involved in 186 middle gene transcription, 186 functions were tested to determine if they increased transcription readthrough past the early terminators (Chapter 8.2.3). The early lytic functions Fil and Dhr were considered the most likely candidates for the postulated Tom (Turn on middle) function (Chapter 1.3.2), since although these fuctions effect the host, they have undefined roles in 186 lytic development. The early lytic functions Cpl and cII have defined roles in the 186 life cycle (Carter, 1985; I. Dodd, personal communication) and were therefore considered

unlikely to encode the Tom function. Fil and Dhr were tested for their effect on termination at the early terminators. These studies showed that Fil or Dhr are unlikely to be the postulated Tom function (Chapter 8.2.3a).

Since at least 44% of transcription reaching the early terminators is able to proceed into the middle region (Chapter 8.2.2), it was considered possible that a middle gene may encode the Tom function. The middle functions, CP79, CP80, CP81 and CP83, were tested for their ability to increase middle gene transcription. These studies revealed that it is unlikely that these middle functions encode the Tom function (Chapter 8.2.3b).

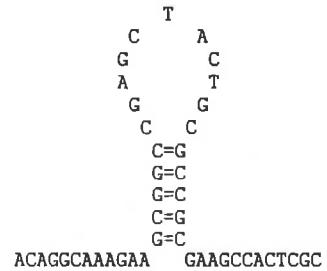
The middle functions, LA and RA, were not tested for their involvement in middle gene expression since the work of Sivaprasad (1984) showed that these functions were involved in 186 replication, and thus, were considered unlikely to also encode the Tom function. However, recent studies (Jarvinen, 1986) suggest that LA may not be directly involved in replication. Thus, in order to rule out the involvement of a 186 middle function in 186 middle gene transcription, LA should be tested for its effect on transcription termination at the early terminators. This is a subject for future work.

Transcription studies revealed that termination was occurring within the AhaIII-XmnI (79.4%-85.2%) region. Since transcription termination was shown not to occur in the AhaIII-BglII (79.4%-79.6%) region (Chapter 9.2.2), this result suggests that there are additional transcription terminators located in the BglII-XmnI region (sequence coordinates 4244-5909). This region (Figure 8.1c) was analysed for potential terminators (stem-loop structures) using the computer program COMSTR and dot matrix analysis (Chapter 2.38). Several potential terminators were predicted, and the most stable of these are shown in Figure 10.6. Whether these potential terminators are functional is a subject for future studies.

Figure 10.6 Potential terminator structures in the region BglII-XmnI (4244-5909).

This Figure shows the most stable terminator structures in the region 4244-5909. [50 bp to the left of the BglII site (at sequence coordinate 4244) was also included in the analysis.] A threshold value of $\Delta G = -6.0$ was arbitrarily chosen. The stem-loop structures were predicted using the computer program COMSTR and by dot matrix analysis (Chapter 2.38). The free energy (ΔG) values were calculated using the rules of Steger et al. (1984), and are listed below each structure. The DNA sequence coordinates of each structure, are indicated.

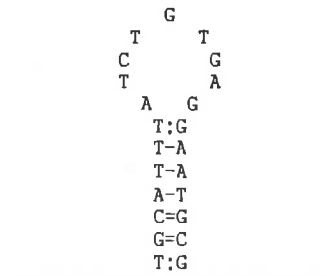
4290-4310



ΔG = -6.5 Kcal/mol

(in CP79)

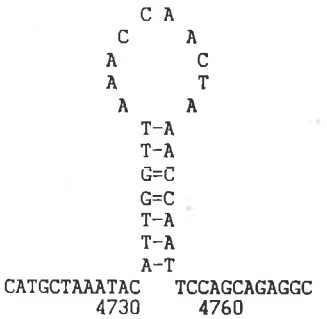
4444-4466



ΔG = -6.5 Kcal/mol

(in CP80)

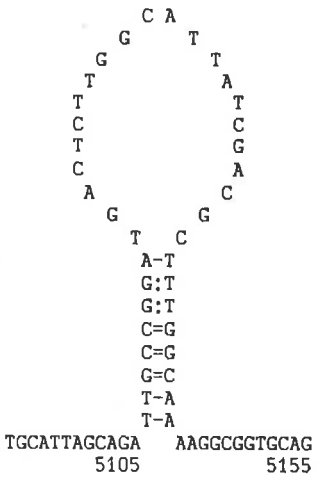
4731-4754



ΔG = -6.4 Kcal/mol

(in CP81)

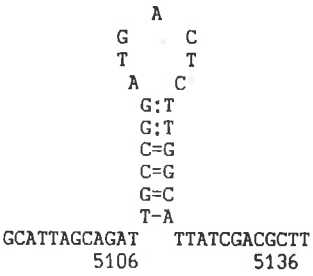
5106-5143



ΔG = -8.4 Kcal/mol

(3'-end of CP81)

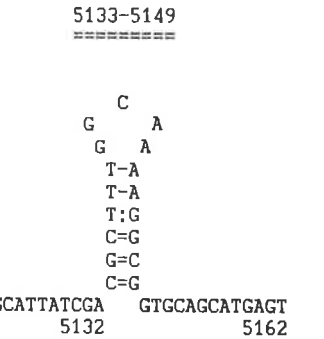
5107-5125



ΔG = -6.9 Kcal/mol

(3'-end of CP81)

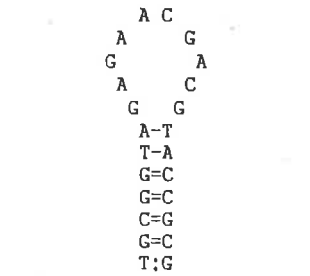
5133-5149



ΔG = -7.0 Kcal/mol

(3'-end of CP81,
overlaps the CP83 rbs)

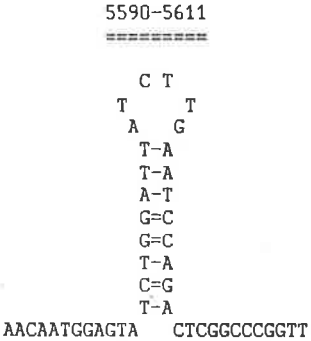
5515-5538



ΔG = -9.6 Kcal/mol

(5'-end of LA)

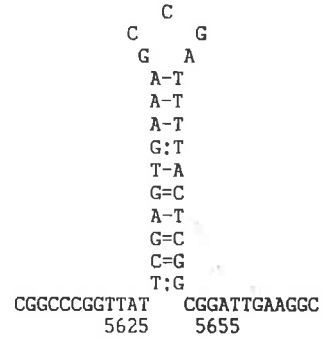
5590-5611



ΔG = -9.0 Kcal/mol

(5'-end of LA)

5626-5650



ΔG = -12.2 Kcal/mol

(5'-end of LA)

One of these structures sequesters the predicted ribosome binding site of the CP83 gene (Figure 8.1c), and if this structure forms, it would be expected to decrease translation of this gene (Chapter 3.2.2c). Furthermore, a decrease in the level of translation of CP83 may also decrease the translation of the downstream genes, LA and RA since the 186 middle genes are expected to be translationally-coupled genes (Sivaprasad, 1984; Chapter 1.2.1, 3.2.2a). Since the 3'-end of CP81 overlaps the 5'-end of CP83 (Figure 8.1c), it may be expected that translation of CP81 may be required to destabilize this potential structure and allow translation initiation at the CP83 rbs. This may provide a means to ensure that CP83 is not expressed unless CP81 has been translated. Further studies are required to determine whether this structure forms and whether it is important in controlling translation initiation at the CP83 rbs.

10.4.1(d) The Analysis of the Virulent Phage 186 del2.

As discussed in Chapter 9.1, the virulent phage 186 del2 potentially belonged to a class of mutants, which had constitutive expression of the middle genes, but not of the early lytic genes. The analysis of this mutant was expected to reveal genes or sites important in the control of transcription of the middle region. However, the analysis of this mutant revealed that this phage was virulent as a result of the generation (by a DNA duplication) of a promoter for the middle genes (Chapter 9.2.3, 9.2.4). Although the study of this phage did not provide evidence for a control mechanism for 186 middle gene transcription, it did reveal a possible essential role for a 186 early lytic function, as discussed below.

The analysis of 186 del2, revealed that this phage was sensitive to the level of cI repressor (Chapter 9.2.5). This result was explained by suggesting that high levels of cI repressor blocks transcription of an essential function from pR. The studies carried out in Chapter 5, and by Carter (1985) and I. Dodd (personal communication), have suggested that the

early lytic functions are not essential to 186. However, although these functions are not essential to 186 during infection of a non-lysogen, one or more of them may be essential during infection in the presence of high levels of the cI repressor. In this regard it is pertinent to note that the early lytic function Cpl acts to decrease the expression of the cI gene by blocking transcription from pL (I. Dodd, personal communication; Chapter 1.2.2). High levels of cI repressor may inhibit 186 lytic development (e.g. cI repressor may bind to low affinity operator sites positioned at promoters for essential genes in the late region of 186). Should this occur, the requirement of Cpl would simply be to decrease the expression of cI, and thereby allow 186 late gene expression.

Alternative explanations for the cI-sensitivity of 186 del2 infection are possible. For example, although mutations in individual early lytic genes are not lethal to 186 (Chapter 5), preventing the expression of all four early lytic genes may be lethal to the phage. Alternatively, the pDup promoter may not be strong enough in vivo to allow sufficient expression of the 186 middle genes, and some transcription from the pR promoter may be necessary to allow a productive lytic infection. An extension from this is that transcription from pDup may terminate at a high efficiency at the tR1 terminator, which is located 15 b downstream from pDup and results in effective termination in vitro (Chapter 9.2.4; Figure 9.9). Transcription initiation from pR would allow the region 5' to tR1 (the CP81/dhr fusion-gene) to be translated, and this may be required (perhaps the presence of ribosomes at the 3'-end of the CP81/dhr fusion-gene may prevent the formation of the tR1 terminator structure) to allow efficient transcription to read past tR1 into the middle region. Thus, a productive infection by 186 del2 may require some transcription from pR, in addition to transcription from pDup.

Further studies are necessary to determine the reason for the cI-sensitivity of 186 del2. To determine whether Cpl or another early lytic

function is essential for the lytic development of 186 del2, 186 early lytic genes can be supplied in trans from a plasmid-clone and tested for their ability to allow 186 del2 to grow in the presence of high levels of the cI repressor. This experiment should also reveal whether the prevention of the expression of all early lytic genes is the reason for the cI-sensitivity of 186 del2. In vivo transcriptional studies are necessary to investigate the possibility that pDup may be a poor promoter in vivo or that transcription from pDup terminates at tR1 at a high efficiency in vivo.

10.4.1(e) Concluding Comment on the Control of 186 Middle Gene Transcription.

The results discussed above, suggest that there is possibly no specific middle control function encoded by 186, and that middle transcription results simply from the inefficiency of the termination signals in the early lytic region. Further indication that an antitermination mechanism of the λ-type does not exist in 186 comes from the observation that 186 can form plaques on Nus mutants (J.B. Egan, personal communication), which are defective in λ gpN-mediated antitermination (Chapter 1.4.2). However, the dramatic decrease in transcription, which occurs when translation is inhibited by chloramphenicol, suggests that the coupling of transcription with translation is very important in the transcription of the middle genes. Support for this coupling of transcription and translation comes from a phenomenon known as the Strep Effect.

Under certain conditions 186 is unable to infect strains carrying the streptomycin-resistant str594 allele (N. Gough, unpublished data). This is known as the Strep Effect. The block in 186 infection in the Str594 strain occurs at the level of transcription and it has been shown that rightward transcription from the early lytic promoter (pR) of 186 is severely

depressed (N. Gough, unpublished data). Streptomycin-resistant ribosomes are known to have slow translation rates (Zengel et al., 1977; Galas and Branscomb, 1976), therefore it is possible that during the Strep Effect, transcription and translation of the early lytic genes is uncoupled and this leads to premature transcription termination.

This apparent requirement for the coupling of transcription and translation may simply be due to transcriptional polarity. However, it may reflect the involvement in 186 early lytic and middle transcription, of an attenuation-type mechanism of control (Yanofsky, 1981; Kolter and Yanofsky, 1982; Bauer et al., 1983). Thus, unless translation occurs past specific sites in the early lytic region, transcription may mostly terminate at terminators located promoter-distal to this region. This mechanism may differ from the classic type of attenuation of biosynthetic operons, in that the region required to be translated may encode a gene (e.g. CP75, CP76, CP77 or CP78).

Potential terminator structures encoded in the PstI-BglII (77.4%-79.6%) region are described in Chapter 3.2.2(c) (Figure 3.4, 3.2). To complete the analysis of the early lytic region for possible terminator structures, which may act to terminate transcription should translation be inhibited, the pR-PstI (74.7%-77.4%) region was analysed using the computer program COMSTR and by dot matrix analysis (Chapter 2.38). The most stable of the potential terminator structures encoded in this region are shown in Figure 10.7. The activity of these structures in transcription termination when protein synthesis is inhibited, is a subject for further studies.

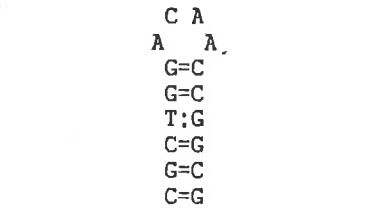
10.4.2 The Control of RA Gene Expression.

The expression of the essential replication gene RA was not considered in detail in this study. Nevertheless, from the results obtained in Chapter 6.2.5, some comment can be made concerning the expression of RA.

Figure 10.7 Potential terminator structures in the region from pR to PstI (2747-3556).

This Figure shows the most stable terminator structures in the region 2747-3556. [50 bp to the right of the PstI site (sequence coordinate 3556) was also included in the analysis.] A threshold value of $\Delta G = -6.0$ was arbitrarily chosen. The stem-loop structures were predicted using the computer programme COMSTR and by dot matrix analysis (Chapter 2.38). The free energy (ΔG) values were calculated using the rules of Steger et al. (1984), and are listed below each structure. The DNA sequence coordinates of each structure are indicated.

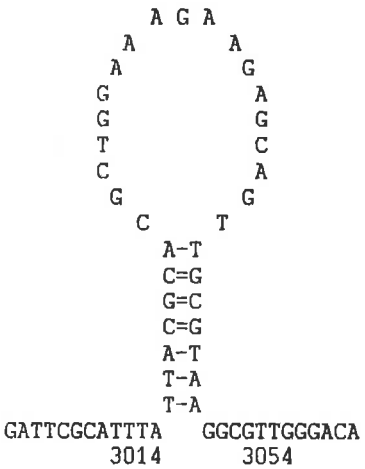
2923-2938
=========



ΔG = -7.1 Kcal/mol

(middle of CP75)

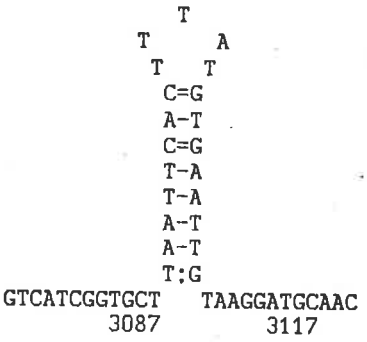
3015-3047
=========



ΔG = -6.8 Kcal/mol

(3'-end of CP75)

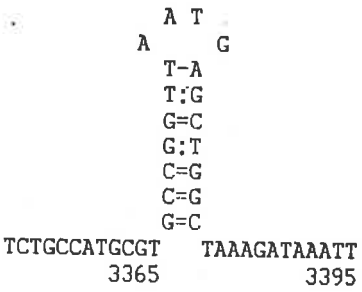
3088-3108
=========



ΔG = -7.4 Kcal/mol

(CP75-CP76 intergenic region)

3366-3383
=========



ΔG = -7.3 Kcal/mol

(middle of CP76)

The results presented in Chapter 6.2.5, revealed that most transcription terminated within the region encoding the tM1 terminator at the beginning of the RA gene. Two weakly hybridizing transcripts sized at 1.8 kb and 2.1 kb were detected promoter-distal to tM1. The 5'-end of the 1.8 kb transcript was mapped to the region 6420-6532, whereas the 5'-end of the 2.1 kb transcript was located in the region 5605-6419 (Chapter 6.2.5). As discussed in Chapter 6.3, it is likely that these transcripts encode the RA gene; however, they do not encode the complete LA gene (which begins at position 5429; Figure 6.1, 8.1c).

The study of Sivaprasad (1984) showed that the translation of the RA gene, which has no recognisable ribosome-binding site, is dependent upon the prior translation of the LA gene. Since the 2.1 kb and 1.8 kb transcripts do not include the 5'-end of the LA gene (and thus, the LA gene is not expected to be translated), it is unlikely that the RA gene encoded by these transcripts is expressed. The question then arises as to how the RA gene is expressed. This is a subject for future studies.

10.4.3 Future Studies.

Future studies on 186 early lytic and middle gene transcription will be directed towards understanding the Strep Effect and the expression of the RA gene. In vivo transcription studies can be used to investigate the transcription pattern obtained during the Strep Effect, in order to determine whether most transcription terminates at a specific region. Northern analysis using RNA probes of greater sensitivity, which can be obtained by using either the SP6 or T7 polymerase system (Melton et al., 1984; Tabor and Richardson, 1985), can be used to detect and map the presumptive low abundance transcripts that express the RA gene.

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