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AN INVESTIGATION OF FEATHER KERATIN GENE EXPRESSION

A Thesis submitted to the University of Adelaide, South Australia, for the degree of Doctor of Philosophy.

by

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CONTENTS

SUMMARY

DECLARATION

ACKNOWLEDGEMENTS

CHAPTER 1	INTRODUCTION	PAGE
A.	**Preliminary Comments**	1
1.1	**Feathers and Feather Keratin**	1
1.1.1	Feather Development in the Chick Embryo	2
1.1.2	Feather Keratin Composition, Structure and Time Course of Synthesis	3
1.1.3	Feather Keratin Gene Number and Organization	4
1.1.4	Feather Keratin Gene Structure	5
	(i) 5' Flanking Sequences	5
	(ii) 5' Untranslated Region	6
	(iii) Protein Coding Region	6
	(iv) The Intron	7
	(v) 3' Untranslated Sequences	7
1.1.5	Feather Keratin Gene Transcription, mRNA Processing, Packaging, Transport and Stability **In Vivo**	8
1.1.6	The Nature of Control in Feather Keratinization	9
1.2	**Gene Expression and the Initiation of Transcription**	10
1.2.1	**E. coli** RNA Polymerase	11
1.2.2	Eukaryote RNA Polymerases	12
1.2.3	Promoters and Their Associated Transcription Factors	14

(i) Bacterial Promoters and Transcription Factors 15
(ii) Eukaryote RNA Polymerase I Promoters and Transcription Factors 16
(iii) Eukaryote RNA Polymerase II Promoters and Transcription Factors 17
(a) The TATA Box 18
(b) Immediate Upstream Elements 19
(c) Enhancers 20
(d) Yeast UASs 21
(e) Downstream Elements 22
(iv) Polymerase III Promoters and Transcription Factors 23
1.2.4 The Regulation of Transcription by Specific Factors 24
(i) Sigma Factors of Prokaryotes 25
(ii) The Constitutive and Regulatory Factors of Polymerase II Transcribed Genes 27
(iii) Polymerase III Factors and Regulation of 5S Gene Transcription 32
1.2.5 Effects of Supercoiling and Higher Order Structure 34
1.2.6 Transcriptional Initiation - Concluding Comments 36
1.3 The Xenopus Oocyte Transient Expression System 37
1.4 Thesis Aims 39

CHAPTER 2 MATERIALS AND METHODS

2.1	**Abbreviations**	40
2.2	**Chemicals and Reagents**	40
2.3	**Enzymes**	41
2.4	**General Buffers and Bacterial Media**	42
2.5	**Tissue Culture Media**	42
2.6	**Bacterial Strains**	43
2.7	**Cloned DNA Sequences**	43
2.8	**Cloning Vectors**	43
2.9	**Synthetic Oligonucleotides**	43
2.10	**Recombinant DNA Methods**	44
2.10.1	General Recombinant DNA Methods	44
2.10.2	Transformation Procedure for M13 Recombinants	45
2.10.3	DNA Sequencing by the Dideoxy-Chain Termination Method	46
	(i) Preparation of Single Strand Template for Sequencing	46
	(ii) Sequencing Reactions	46
	(a) Hybridization	47
	(b) Polymerization	47
	(iii) Sequencing Gels	48
2.10.4	Kinasing of Synthetic Oligonucleotides	49
2.11	**Injection of Xenopus Oocytes and Analysis of Oocyte RNA**	49
2.11.1	Animals	49
2.11.2	Oocyte Injection	50
2.11.3	Isolation of RNA from **Xenopus** Oocytes	50
2.11.4	S1 Nuclease Analysis of Oocyte RNA	50
2.11.5	Primer Extension Analysis of RNA	51

2.11.6 Measurement of RNA Accumulation in Oocytes 52
2.11.7 Determination of the Rate of RNA Degradation in Oocytes 53
2.11.8 Quantitation of Transcript Levels from Autoradiographs 53
2.12 Preparation of RNA, Nuclei and Chromatin Salt Wash Fractions from Feather Tissue 54
2.12.1 Feather Tissue 54
2.12.2 Polysomal Feather RNA Preparation and Poly(A)$^+$ RNA Preparation 55
2.12.3 Nuclei Extraction from Feather Tissue 56
2.12.4 Fractionation of Chromatin from Feather Tissue by Sequential Salt Washes 57
2.13 Tissue Culture 57
2.13.1 Culture Conditions 57
2.13.2 Transfection of HeLa, CV1 and Ltk$^-$ Tissue Culture Cells 58
2.13.3 Extraction of RNA from Harvested Tissue Culture Cells 58
2.13.4 Extraction of DNA from Harvested Tissue Culture Cells 59
2.13.5 Cell Staining for Colony Counting 59
2.13.6 Epidermal Feather Cell Culture 59
2.13.7 Chick Embryo Fibroblast Cell Culture 60
2.13.8 Transfection of Chick Embryo Fibroblasts 62
2.14 Virus Associated Protocols 62
2.14.1 Focus Formation Assay 62
2.14.2 Infection of Fibroblasts with Intact RSV 63
2.14.3 Lysis of Virus from Whole Media Extracts 63

2.14.4 Purifying Virus from Culture Medium 63

2.15 Enzyme Assays 63

2.15.1 Reverse Transcriptase Assay 63

2.15.2 Chloramphenicol Acetyl Transferase Assay 64

CHAPTER 3 GENE CONSTRUCTS

A. Introduction and Guide to Reader 66

3.1 97 68

3.2 24 68

3.3 70 68

3.4 1-4E8 68

3.5 SVGene B 69

3.6 BK-i 69

3.7 SVBK-i 70

3.8 BK309HpaIIi 70

3.9 tk (TK/TK) 71

3.10 Eco-tk 71

3.11 SVtk 71

3.12 SVEco-tk 71

3.13 TK/KER 72

3.14 1-4/TK/BglBam 72

3.15 KER/TK 73

3.16 MTK/KER 73

3.17 MTK/KER-BE2 74

3.18 MTK/KER-BE2SMA 74

3.19 MTK/KER-SALSMA 74

3.20 BK-i-3'TK 75

3.21 TK/KER-3'TK 75

3.22 TKKpr 76

3.23	TK/H2B	76
3.24	KER/H2B	76
3.25	KER127TK	77
3.26	KER187TK	77
3.27	KER328TK	78
3.28	KER436TK	78
3.29	BKTK5	79
3.30	BKTK2	79
3.31	KER430TK	79
3.32	KER$\overrightarrow{800}$KER	80
3.33	KER$\overleftarrow{800}$KER	80
3.34	KER$\overrightarrow{617}$KER	80
3.35	KER$\overleftarrow{617}$KER	80
3.36	KER$\overrightarrow{900}$KER	80
3.37	KER$\overleftarrow{900}$KER	80
3.38	KERSp$\overrightarrow{617}$KER	80
3.39	KERSp$\overrightarrow{900}$KER	80
3.40	pJL4B1-4	81

CHAPTER 4 INTRON SEQUENCES MODULATE FEATHER KERATIN GENE TRANSCRIPTION IN XENOPUS OOCYTES

4.1	**Introduction**	82
4.2	**Results**	83
4.2.1	Feather Keratin Gene Transcription Occurs at Low Efficiency in Oocytes and the Detectable Transcripts are Unspliced	83
4.2.2	Transcript Initiation from the TATA Box	86

4.2.3 Removal of the Feather Keratin Intron Increases the Levels of Correctly Initiated Transcript 87
4.2.4 Polyadenylation of Transcripts and 3' End Mapping 88
4.2.5 Test for Enhancer Requirements 89
4.3 Discussion 90
4.3.1 Enhancer Requirements for Feather Keratin Gene Transcription in Oocytes: Clarification of SV40 Enhancer Function 90
4.3.2 Suppressive Effect of the Feather Keratin Intron on Keratin Gene Transcription in **Xenopus** Oocytes 93
4.3.3 Role of Suppressive Effect on Feather Keratin Transcription in Oocytes and its Importance **In Vivo** 95

CHAPTER 5 GENE FUSIONS: KERATIN GENE B AND THE HSV-tk GENE

5.1 Introduction 97
5.2 Experimental Approach and Results 99
5.2.1 Linkage of the Keratin Promoter to the tk Structural Gene Results in a High Level of Transcription in Oocytes 99
5.2.2 Comparisons of Message Stability in Oocytes 101
5.2.3 Investigation of Transcriptional Regulatory Sequences in the Keratin Coding and 3' Untranslated Regions 103

5.2.4 Transcriptional Studies with tk and Keratin Promoters 105

5.2.5 Investigation of the tk Gene Stimulatory Effect: The Importance of Promoter Distance in Relation to the Coding Region 107

5.2.6 Attempts to Characterize the tk Stimulatory Region 108

5.3 Discussion 110

5.3.1 Deductions on the Nature of the tk Stimulatory Region 110

5.3.2 Intragenic Control Sequences 112

5.3.3 The tk Promoter and the tk Intragenic Component are Necessary for Efficient HSV-tk Gene Transcription in Oocytes 115

5.3.4 Feather Keratin Gene Transcription in **Xenopus** Oocytes 116

5.3.5 Warnings in Relation to Promoter Switching Studies 117

CHAPTER 6 ALTERNATIVE EXPRESSION SYSTEMS: THE DEVELOPMENT OF AN HOMOLOGOUS EXPRESSION SYSTEM

6.1 Introduction 118

6.2 Investigation of the Expression of Feather Keratin Genes in Non-Feather Tissue Derived Cell Lines 118

6.2.1 Results and Discussion 118

6.3 **Attempts at Enhancing the Oocyte System by the Co-injection of Chromatin Proteins from Feather Cell Nuclei** 120
6.3.1 Results 120
6.3.2 Discussion 121
6.4 **Epidermal Feather Cell Culture** 123
6.4.1 Results and Discussion 124
6.5 **Proposal for an Homologous Expression System: Transgenesis of Chick Embryos** 126
6.5.1 Experimental Outline of the Transgenic Chick Expression System 127
(i) Fibroblast Cultures 129
(ii) Establishing an Efficient Transfection Procedure 129
(iii) Enzymic Assay for Reverse Transcriptase 131
(iv) Generating Infectious Particles 132
6.5.2 Discussion of the Transgenic Chick Embryo System 133
(i) The Prepared Fibroblast Cultures are Defective in Producing Infectious Virus Particles 133
(ii) Appraisal of the Chick Embryo System for use in Expression Studies 134

CHAPTER 7 CONCLUDING DISCUSSION
7.1 **Feather Keratin Genes and Avian Keratinization** 138
7.2 **Intragenic Control** 140
7.3 **Regulatory Sequences and Specific Factors** 141

BIBLIOGRAPHY

SUMMARY

This thesis presents the results of initial investigations toward understanding the elements regulating the transcription of chicken feather keratin genes. Results were also obtained relating to the transcription of the Herpes Simplex Virus thymidine kinase (HSV-tk) gene.

The **Xenopus** oocyte was chosen as the simplest available expression system. It was observed that the chicken feather keratin genes were transcribed at very low levels in the oocyte and primer extension reactions using selected primers showed that none of the detectable transcripts were spliced. When the single intron, located in the 5' untranslated region of all the known feather keratin genes was removed from feather keratin gene B to produce an intronless gene, a 5-fold increase was observed in transcript initiation from the **in vivo** cap site. This 5-fold increase was retained when the feather keratin intron was replaced with a similar-sized fragment from pBR322, suggesting that the intron sequence was specifically acting to reduce the number of correctly initiated transcripts. That this was so was reinforced by the finding that the low level of keratin transcripts produced from the intact feather keratin gene was not attributable to a degredation of partially-spliced transcripts. The role that this intron mediated repression of transcription may play in regulating feather keratin gene expression **in vivo** is discussed.

Although the level of correctly initiated transcript was increased by intron removal, the level of transcription remained very low when compared with that which occurs **in vivo**. Transcription from the intronless gene was at least an order of magnitude lower than that observed for other genes co-injected into **Xenopus** oocytes. To determine whether the low level of feather keratin gene transcription in oocytes was the result of inefficient functioning of the keratin promoter, gene hybrids were constructed using feather keratin gene B and the HSV-tk gene. The tk gene is expressed very efficiently in oocytes. When the tk promoter was linked to the protein coding region of feather keratin gene B, however, the level of transcript produced was similar to that obtained from the intronless keratin gene. Furthermore, when the keratin promoter was linked to the coding region of the tk gene, the level of transcript produced was 20-fold greater than that from the tk promoter/keratin coding region construct and approached that produced from the unmodified HSV-tk gene. Time-course experiments showed that these results were not due to a decreased stability of transcripts from constructs containing the feather keratin coding region. It was concluded that one of two possible mechanisms was acting, either the keratin coding region was acting to inhibit transcription from the tk promoter, or the tk coding region was acting to stimulate transcription from the keratin promoter.

To test whether the keratin coding region was acting to inhibit transcript initiation from the tk promoter, deletions were made in the keratin coding region of the tk promoter/keratin coding construct to define the location of

any potentially inhibitory regions. None of the gross deletions performed resulted in an increase in transcription from the tk promoter. When the tk promoter was linked to the coding region of the H2B histone gene (which is efficiently expressed in oocytes from its own promoter) a low level of transcription was again observed from the tk promoter. From these results it was concluded that the relatively high level of transcripts initiating from the keratin promoter when linked to the tk coding region was a result of a stimulatory action of the tk coding region on the keratin promoter. This conclusion can also be extended to a possible activation of the tk coding region on its own promoter when the tk gene is transcribed in oocytes.

Further investigations using the keratin promoter/tk gene construct, showed that the tk coding region stimulatory effect was distance dependent. A preliminary investigation to define the region was undertaken by inserting a variety of large fragments from the tk coding region into the coding region of the intronless keratin gene in a position dependent manner. Although the insertion of the entire tk coding region into the coding region of the intronless keratin gene resulted in transcription stimulation from the keratin promoter, inidivdual fragment insertion failed to define the stimulatory region. This was presumably related to disruption of the stimulatory region(s) when the selected fragments were isolated from the tk gene.

The tissue specific expression, and the critically timed activation of keratin gene transcription **in vivo** suggests a possible need for other, tissue specific factors. The lack of such factors in frog oocytes would provide an explanation for the low levels of keratin gene transcripts and the inability to detect spliced transcripts. The hypothetical need for tissue specific factors was further supported by the inability to detect correctly initiated keratin transcripts after the genes were transfected into a variety of non-feather tissue derived cell lines via calcium phosphate mediated DNA transfection.

Co-injection of keratin genes into oocytes with protein fractions purified from the nuclei of transcriptionally active feather cells was considered as a means of confirming the existence of specific activatory factors. Such co-injection experiments were thwarted by the extreme difficulty encountered in obtaining sufficient nuclei from developing feathers from which to extract chromatin proteins. Attention was subsequently focussed on culturing primary chick epidermal cells for DNA transfection purposes. Poor yields of viable cells and their propensity to rapidly keratinize in culture made these cells difficult to maintain for such studies.

The frog oocyte system has provided some interesting results on the expression of feather keratin genes and during the course of this work has revealed some important questions in relation to HSV-tk gene transcription. The need for an homologous expression system is considered paramount for future studies relating to feather keratin gene transcription. A proposal is given for an homologous expression system, which once established, should provide answers to questions pertaining not only to feather keratin gene expression but

also to the expression of the other genes known to be associated with avian keratinization. Preliminary work towards establishing this homologous expression system is presented.

DECLARATION

This thesis contains no material which has been accepted for the award of a degree or diploma in any University and in my belief contains no material which has been published by another person, except where due reference is made in the text.

ANNA MARIA GRAŻYNA KOLTUNOW

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CHAPTER 1.

INTRODUCTION.

A. PRELIMINARY COMMENTS

A considerable effort has been made in this laboratory in order to gain an understanding of chicken feather keratin composition, structure and biogenesis. Detailed information has also been gained regarding the structure of feather keratin genes and their arrangement within the chick genome. Initiation of feather keratin gene transcription is believed to be the primary controlling event in feather keratinization. This thesis presents the results of the first efforts toward understanding the expression of the feather keratin genes at the transcriptional level.

The introduction which follows is intended to familiarize the reader with feather development and with feather keratin. An account of the salient features involved in the initiation of prokaryotic and eukaryotic gene transcription follows, as much of this information was used as a guide whilst investigating the factors involved in feather keratin gene transcription. Finally, aspects of the **Xenopus** oocyte transient expression system are discussed since this system was predominantly used during the course of this work.

1.1 FEATHERS AND FEATHER KERATINS

The feather, a beautifully complex, non-living structure is the culmination of an equally complex series of terminal differentiation events which occur in the avian epidermis. Numerous reviews have dealt extensively with the morphogenesis of feather development (Lucas and Stettenheim, 1972; Matulionis, 1970) and the molecular properties of feather

keratin proteins (Fraser **et al.**, 1972; Rogers, 1984). These aspects will be discussed relatively briefly and as in the case of the most recent review on feather keratins (Gregg and Rogers, 1986) more emphasis will be placed on the recent advances in the area of molecular biology.

1.1.1 FEATHER DEVELOPMENT IN THE CHICK EMBRYO

The work in this thesis is specifically concerned with embryonic chicken feather development. Adult feather development will not be discussed in detail.

Feather development begins at around day 5 of embryonic life in the chicken (Holmes, 1935). An outgrowth of both the epidermis and the dermis occurs to form feather primordia which are essentially a tube of epidermal cells surrounding a core of mesenchyme (Matulionis, 1970). This structure, macroscopically visible at day 9 of development (figure 1-1A), continues to increase in length and diameter by numerous cellular divisions. By day 12, different cell types are apparent; all of them being epidermally derived. The feather at this stage consists of a series of barb ridges in cylindrical arrangement encased externally by sheath cells and supported internally by column cells (figure 1-1B). Keratinization is first observed on day 12 in the cells at the distal tip of the feather. During the next few days, increase in feather length is primarily due to cellular elongation (figure 1-1C) and keratinization continues to proceed rapidly towards the proximal end of the feather. At about 17 to 18 days feather development is essentially complete. When the chick hatches at 21 days the feathers dehydrate, the sheath splits open and the down feathers open up (figures 1-1D and

FIGURE 1-1

FEATHER DEVELOPMENT IN THE

CHICK EMBRYO

A. Scanning electron micrographs of 9 day feathers.

B. Cellular organization in the embryonic feather at 12 days. 1, low magnification. 2 and 3, higher magnification. Arrows indicate magnified areas. P: Peridermal cells, PP: Pulp cells, Pp.C: Pulp cavity, Bl: Basal lamina, BVRC: Barb-vane-ridge cells, Sh: Sheath cells, B.bl: Barbule, B: Barb, C: Column cells (from Matulionis, 1970).

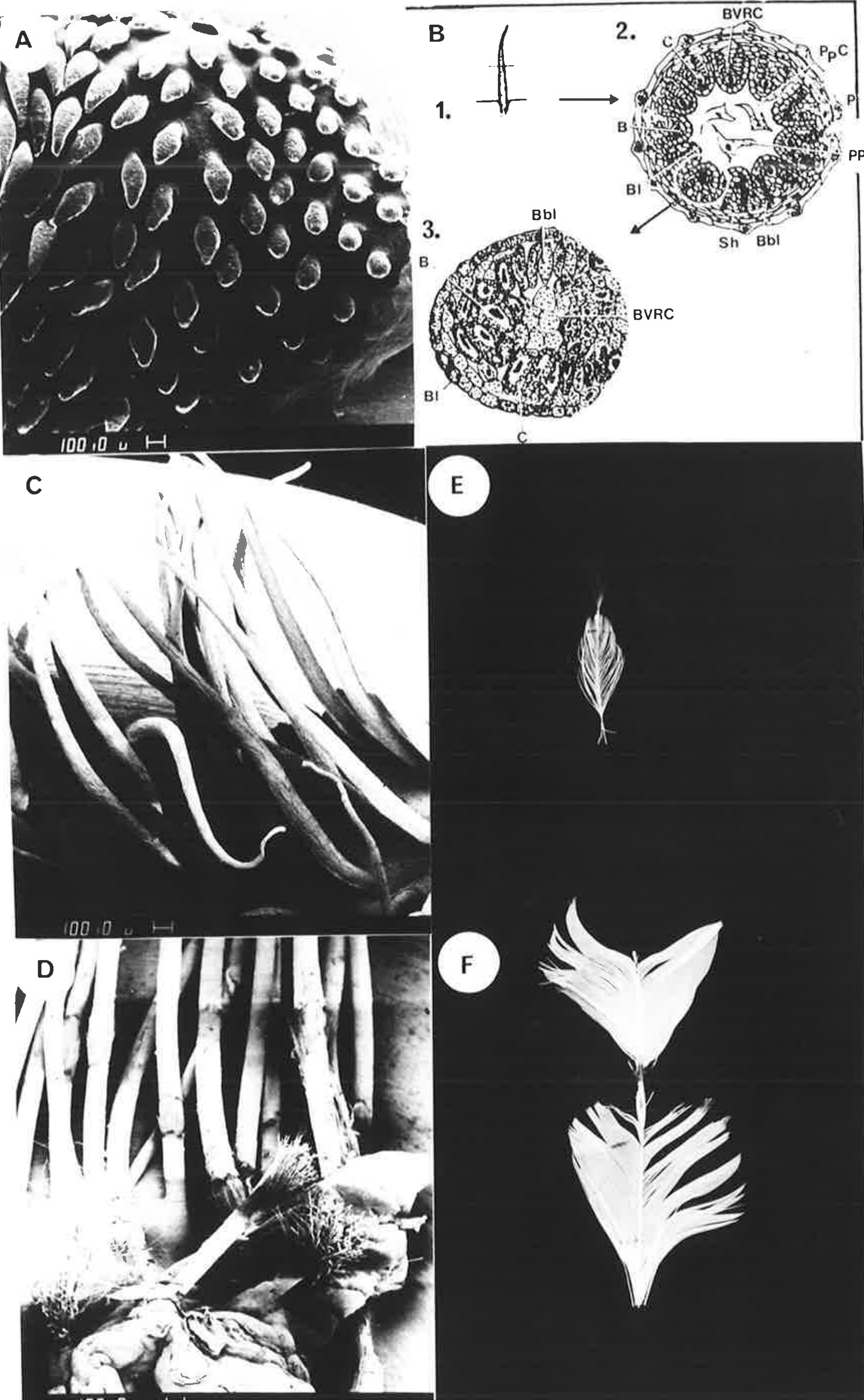
C. Scanning electron micrograph of 14 day feathers.

D. Scanning electron micrograph of 19 day feathers.

E. First moulting showing adult feather with the downy embryonic feather on its tip.

F. A subsequent adult moulting.

(Scanning electron micrographs kindly donated by G.E. Rogers).



A
B
1.
2.
BVRC
C
PpC
P
B
PP
Bl
Sh
Bbl
3.
Bbl
B
BVRC
Bl
C
C
E
D
F

1-1E). Adult feathers which eventually replace the downy embryonic feathers grow from the existing follicles, pushing the embryonic feathers out on their tips as shown in figure 1-1E. Subsequent adult moultings, an example of which is given in figure 1-1F, follow the same process (Lucas and Stettenheim, 1972).

1.1.2 FEATHER KERATIN; COMPOSITION, STRUCTURE AND TIME COURSE OF SYNTHESIS

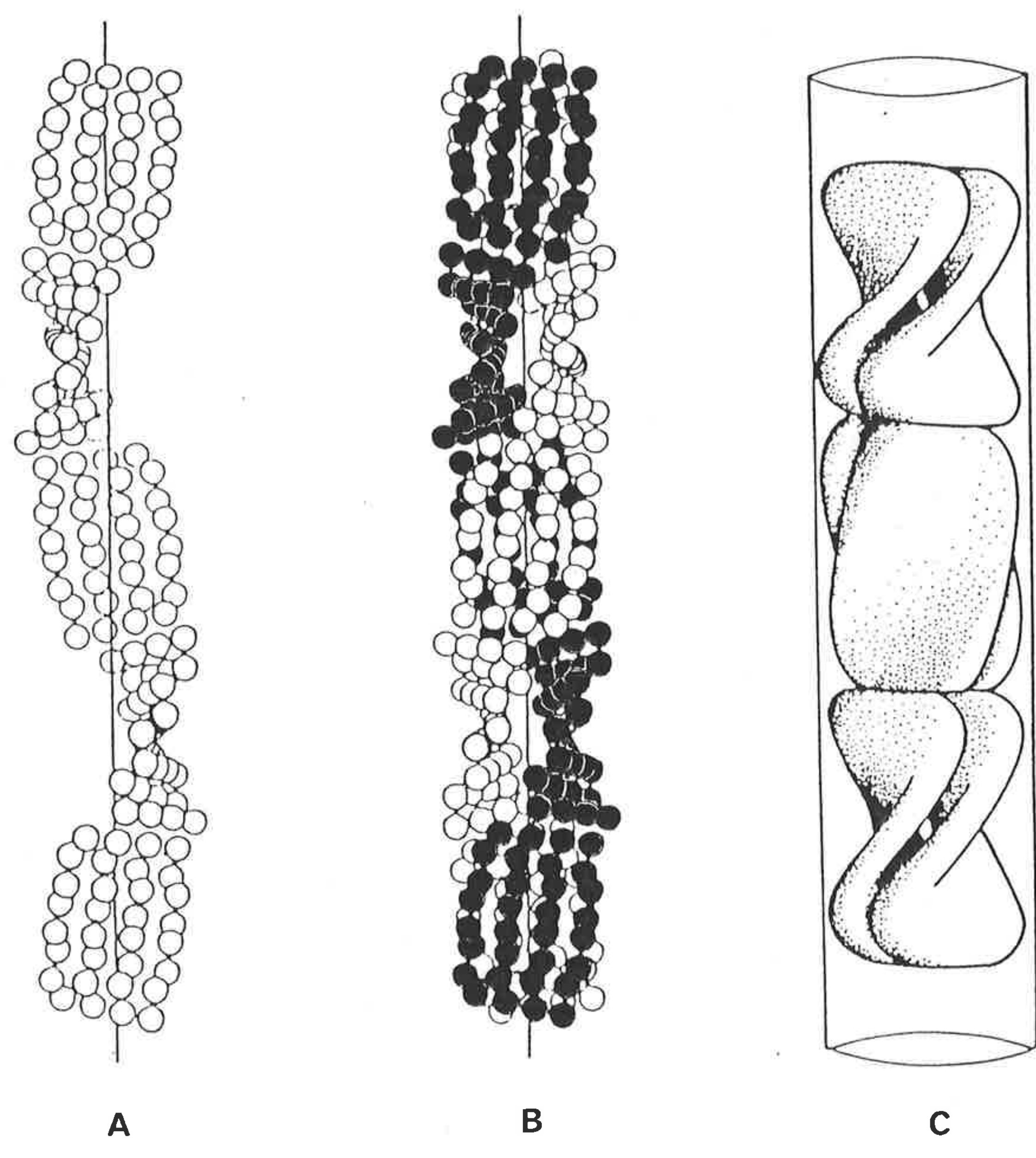
Feather keratin synthesis which is initially observed on day 12 of embryonic life, reaches a maximal rate at around days 14-15 and declines somewhat after day 16 (Powell **et al.**, 1976). During this time a family of closely homologous polypeptide chains are synthesized, members differing only in the substitution of one or two amino acid residues (Walker and Rogers, 1976). Each polypeptide chain is conserved in length to 96-97 amino acids and the monomers have a molecular weight of 10,400 daltons (Harrap and Woods, 1964; Gregg and Rogers, 1986).

The feather keratins are classified as β-keratins because the unit of feather keratin protein structure is a β-pleated sheet. It is believed that each β-pleated unit is derived from one polypeptide chain of 96-97 amino acid residues. The central region of each polypeptide chain is thought to adopt the β-sheet arrangement (Gregg **et al.**, 1984) with the remaining residues on the amino and carboxyl ends, eventually acting to produce covalent/non-covalent interactions between adjacent filaments. The β-sheet units form long linear arrays following a right handed helical path (figure 1-2A; Fraser and MacRae, 1980). Two such twisted sheets, running in opposite

FIGURE 1-2

MODELS FOR FEATHER KERATIN CORE FILAMENTS

The molecular model for the core filament of feather keratin shows the core filament **B** made up of two right handed twisted sheet structures shown in **A** (Fraser and McRae, 1980). **C**, Cartoon representation of the twisted (right handed) β-pleated sheets which make up the core structure of feather keratins (Gregg and Rogers, 1986).


A
B
C

senses make up a core filament which is 3nm in diameter (figures 1-2B and C). This is a process of self assembly and enzymes are not needed to form the long filaments (Fraser **et al.**, 1971). Keratin fibrils, which are bundles of 3nm filaments appear in the feather at day 13 of chick embryogenesis (Filshie and Rogers, 1962).

As the process of keratinization continues, the cells become packed with fibrils and become metabolically inert. Cellular organelles (mitochondria etc.) degenerate into lipid bodies and are reabsorbed (Matulionis, 1970). The cells lose their ability to replicate DNA, cellular levels of DNA polymerase are significantly reduced (Kischer and Furlong, 1967) and there is an active removal of DNA from the keratinizing cells (Kemp **et al.**, 1974). Eventually, the feather keratin fibrils coalesce and are crosslinked into a fibrous lattice. The resultant structure is desiccated, strong and rigid.

1.1.3 FEATHER KERATIN GENE NUMBER AND ORGANIZATION

Restriction enzyme mapping and Southern transfer hybridization using specific gene probes has shown that there are probably no more than 20 feather keratin genes in the chick genome (Presland, 1986). Fifteen of these genes have been isolated and at least partially characterized.

Figure 1-3A shows 11 of the feather keratin genes which are located in a clone designated Cosmid 4 (Presland and Rogers, 1984). The genes are spaced at regular intervals, approximately 3.3 kb from centre to centre. They all appear to be transcribed in the same direction, although the transcriptional orientation of gene 3 is as yet unknown (R.

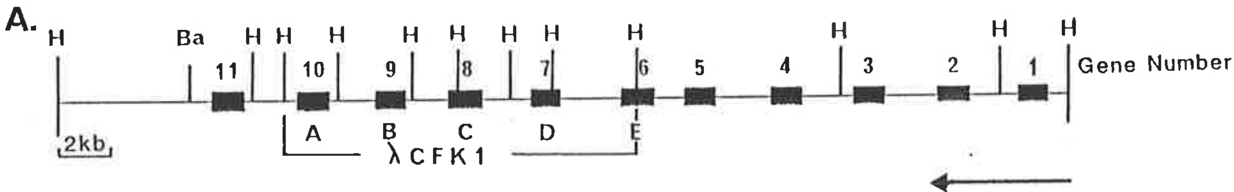
FIGURE 1-3

FEATHER KERATIN GENE ORGANIZATION AND STRUCTURE

A. The spacing of the 11 feather keratin genes located in Cosmid 4. The arrow indicates the direction of transcription. The position of the genes located in the genomic clone λCFK1 is indicated and the diagram is drawn to scale.

B. General structure of embryonic feather keratin genes (Gregg **et al.**, 1983). The marked regions of 3' untranslated sequences represent conserved sequence. Numbers indicate length of gene segments in base pairs.

C. DNA sequence homology among genes A-D in λCFK1 (Gregg **et al.**, 1983).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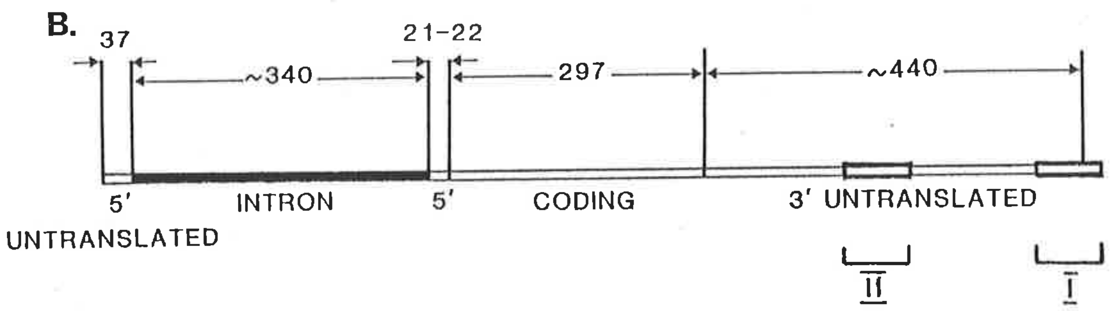
1
Gene Number
2kb
A
B
C
D
E
λCFK1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~440
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INTRON
5'
CODING
3' UNTRANSLATED
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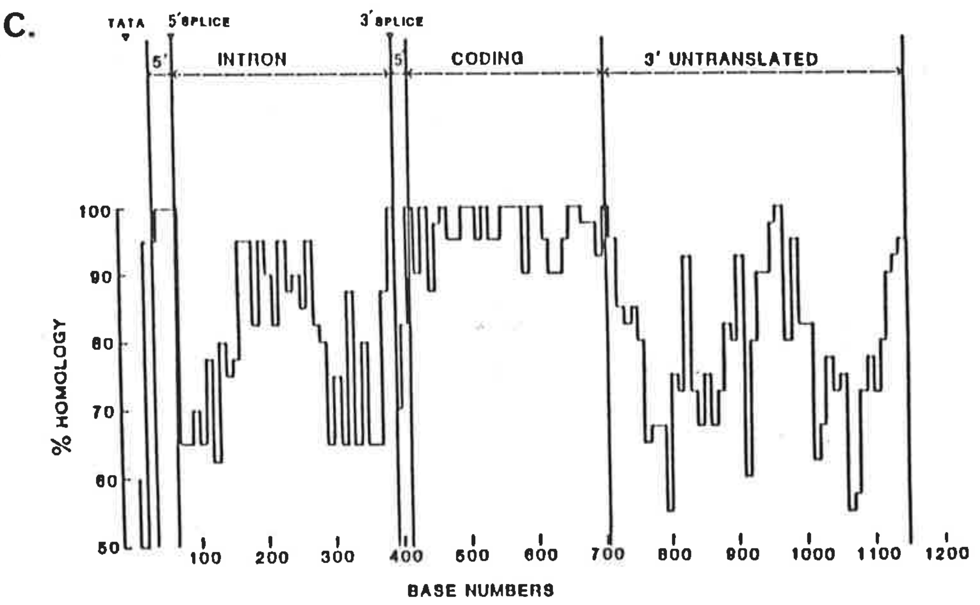

C.
TATA
5'SPLICE
3'SPLICE
5'
INTRON
5
CODING
3' UNTRANSLATED
% HOMOLOGY
100
90
80
70
60
50
100
200
300
400
500
600
700
800
900
1000
1100
1200
BASE NUMBERS

Presland, personal communication). Genes 6-10, originally named A-E, are well characterized and are contained within the charon 4A genomic clone λCFK1 (Molloy **et al.**, 1982).

1.1.4 FEATHER GENE STRUCTURE

All of the feather keratin genes which have been characterized have the same general structure (figure 1-3B; Gregg **et al.**, 1983; Presland, 1986). The following discussion on gene structure is based on comparisons between the four feather keratin genes which have been fully sequenced, A to D (7-10) in λCFK1. The discussion will focus on areas of localized sequence conservation and some speculations will be made on the possible significance of these conserved areas in relation to gene expression. It should be noted that generally the four genes in λCFK1 possess remarkable sequence homology, this is shown diagrammatically in figure 1-3C. The feather gene cluster is believed to have arisen by a process of gene duplication because of the similarities among gene sequences (Gregg **et al.**, 1983).

(i) 5' Flanking Sequences

The TATAAA sequence which is important in the initiation of transcription (see section 1.2.3(iii)a) is present in all four genes of λCFK1 (Gregg **et al.**, 1983) although, in gene 8 the sequence is altered to CATAAA (Molloy **et al.**, 1982). The comparison of the 5' flanking regions of genes 9 and 10, shown in figure 1-4A, indicates that there is a significant level of similarity, supporting the tandem gene duplication hypothesis. However, the sequence conservation in this part of the gene could also be an indicator of keratin-specific promoter

FIGURE 1-4

COMPARISONS OF FEATHER KERATIN GENE SEQUENCES

A. DNA sequence flanking the 5' side of genes 9 and 10. Identical sequence is illustrated by the dark line. Gene numbers refer to those indicated in figure 1-3A.

B. DNA sequence of 5' untranslated regions. Arrow indicates the splice junction. Gene 8-10 sequences are identical to the sequence of gene 7 except where shown.

C. The conserved sequences surrounding the splice junction of the feather keratin gene intron. Conserved regions are identical in the four known sequences (genes 7-10).

D. Gene sequences in the region of the mRNA 3' terminus location I in figure 1-3B. Vertical line indicates the point of polyadenylation of the mRNA. Boxed regions are those consensus sequences which are believed to be associated with the polyadenylation process.

E. The rigidly conserved sequences from location II of the 3' untranslated region (figure 1-3B). The gene listed as pCFK17 is a cDNA clone isolated in this laboratory.

All diagrams were taken from Gregg and Rogers (1986).

A.

GENE

-350
10 AAGCTTCAAGGATTAGGGGAAGAAGAGTCCCAATCATCAGGACAAATATT
9 ———————G —■——————A—G—A—CCT—CGG——GGATGAGT—TTG——GCAA

-300
10 ATTGCTGCCCCAGAGAGTAAATGGCAGACATCCCCTGGAGTGCTGGAACA
9 GGCC—AAAGGT———AG—CTCT—CT—TCCT——GGTTACCCA—TGCTC—T——

-250
10 CTCTTCATCACTGACTGCATCATCCTTGTGATGATGTGTCCTTAACTCTG
9 ——GGA ———————CTG ——TGTG—TGA—G ——C ————■■■—C ———AA■■■■■■■
(Δ TTG)

-200
10 GAAATATTTTGACCTCTAATGCTGGCACTTGAAAGCAGTTGGCACAGTTG
9 ———G ———————G ————————T ———C ————————G ——————A—TGGT—GAA
(Δ C) (Δ AA)

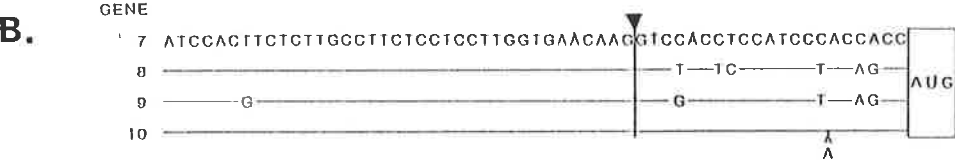
-150
10 AATAGTATGTGCAGAACAGGAAAATAAAAGGAAGCATTACAAGGAAAATA
9 GGGGTAT———CAT ———G——G———G————G————G————G—C—G—

-100
10 ACCAAGACAGTAGTTTTTATAATTGTGATGATTTGCATGGAAGATGAGCT
9 —AA——A—AGAACAC—A——T —————————G——T ————AC——A—C ——————

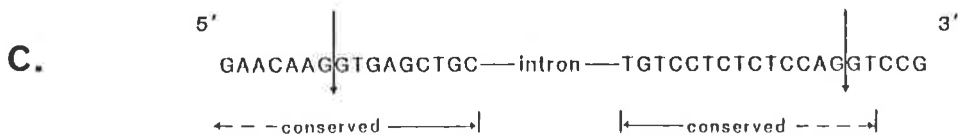
-50
10 TTTGGTAAACACATTACTTTTGCAAAGGGTGCTGCTCTAAGCCCGGGGCA
9 G———A——C—T————A—GTACG—A ——————A———■■————GTA——A—T——GT

mRNA →
10 GGCTAGACCAGTATAAAAGGCCAGTCCAGGACCAGGCTCCCTC|ATCC
9 ——TCCT—A ————————————C ——■————ACT—T ————G ——————|———

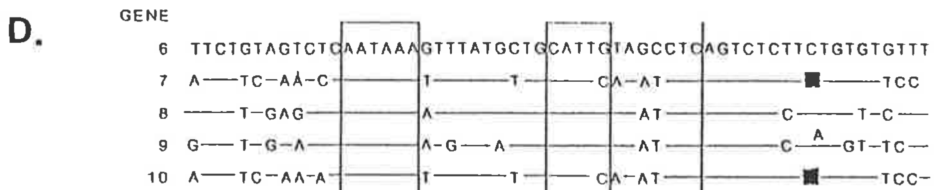
B.



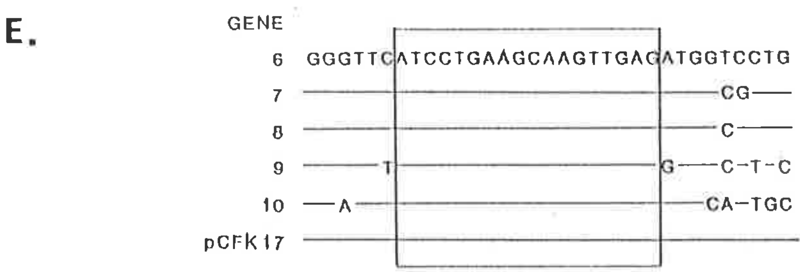
C.



D.



E.



elements. The existence of keratin-specific promoter elements remains to be determined.

(ii) 5' Untranslated Region

Feather keratin messenger RNA (mRNA) has a 5' untranslated region of 59 or 60 bases, depending on the gene from which it was derived. The 5' untranslated region is divided into two by a single intron of about 340-360 nucleotides which lies between bases 37 and 38 of the mature transcript (figure 1-3B). The sequence from base 1 in the mRNA to base 37 is rigidly conserved, with only one base change in the four known sequences (figure 1-4B). This high degree of similarity is greater than that observed in the protein coding sequences (figure 1-3C; Gregg **et al.**, 1983), suggesting that these first 37 bases of untranslated sequence may be of some functional significance, perhaps in regulating gene expression. The remaining 21-22 bases between the 3' splice site and the AUG are less strongly conserved, suggesting that this sequence may not be as important as the 37 base pair leader (Gregg and Rogers, 1986).

(iii) Protein Coding Region

The protein coding regions of the feather keratin genes in λCFK1 are exactly 297 bases long (including "stop" and "start" codons) giving a peptide of 97 amino acids. There are no introns in the protein coding region. The DNA sequence within this region shows greater than 95% similarity amongst the genes (figure 1-3C; Gregg **et al.**, 1983). Such a high degree of sequence conservation is not usually necessary to produce functional proteins given the degeneracy of the amino

acid code and may reflect the recent gene duplication. Alternatively, the feather keratin coding sequences may have a regulatory role as well as being of structural importance. A precedent has already been set for intragenic sequences being important in the regulation of gene expression (refer to section 1.2.3(iii)e).

(iv) The Intron

The position of the feather keratin intron is exactly the same in all cases examined so far. In addition to sequences around the 5' and 3' splice junctions (figure 1-4C), there are other regions of significant conservation in the feather keratin intron (refer to figure 1-3C). Conservation of the feather keratin intron, both in sequence and structural positioning suggests it may have a role in expression. A portion of this project was concerned with testing this hypothesis.

The other known examples of genes with a single intron contained wholly within the 5' non-coding region are also genes associated with avian keratinization, namely the chicken scale keratin, the histidine-rich fast protein genes (Rogers, 1984) and a chicken claw keratin gene (L. Whitbread, personal communication).

(v) 3' Untranslated Region

The 3' untranslated regions are 440-460 bases long and show a considerable degree of sequence divergence except for the two areas indicated in figure 1-3C. One of the regions surrounds the polyadenylation signal (region I in figure 1-3B and shown in figure 1-4D). The sequence motif AAUAAA is

conserved in all of the 3' sequences. Another sequence implicated in the processing of the 3' terminus (consensus CAYUG, Y = pyrimidine; Benoist **et al.,** 1980) is present 8 to 12 bases upstream of the point of polyadenylation (figure 1-4D; Gregg and Rogers, 1986). The second area of conservation, located near the centre of the 3' untranslated region (figure 1-3B, region II), is an 18 base sequence which is perfectly conserved in the 6 known gene sequences (figure 1-4E; Gregg **et al.,** 1983). There is no indication what function this sequence may have. It could exert some controlling effect in transcription, translation or in RNA transport and processing.

1.1.5 FEATHER KERATIN GENE TRANSCRIPTION, mRNA PROCESSING, PACKAGING, TRANSPORT AND STABILITY IN VIVO

The position at which feather keratin gene transcription begins **in vivo** has been identified as an adenine residue 390-420 bases 5' to the protein coding region (Molloy **et al.,** 1982). The primary transcript is "capped" with a 7-methyl guanosine residue (Morris and Rogers, 1979), polyadenylated and is around 1150 bases long. Once the intron is removed the RNA is approximately 800 bases in length, including the length of the poly(A) tail (Gregg **et al.,** 1983).

Numerous attempts have been made in this laboratory to isolate unspliced primary transcripts, none have been successful. It appears that once feather keratin transcription is initiated the intron is rapidly spliced out. There have been no direct observations of RNA packaging or transport of feather keratin transcripts. Pulse labelling studies suggest that translation occurs at the point of

keratin fibril assembly (Kemp **et al.**, 1974).

Studies regarding feather keratin mRNA stability **in vivo** have not been very thorough. Prior to the onset of keratinization, protein synthesis has been shown to be abolished by 24 hours incubation with the RNA synthesis inhibitor actinomycin D. Once keratinization had commenced, similar treatment resulted in only a 40% reduction in protein synthetic activity. The results were interpreted as an indication of increased stability of the mRNA (Bell, 1964). It was not determined, however, whether this was a decrease in degradation or whether the larger amounts of RNA present were taking longer to degrade. Increased mRNA stability would of course be advantageous in allowing the synthesis of the feather keratins to continue during cell senescence.

1.1.6 THE NATURE OF THE CONTROL IN FEATHER KERATINIZATION

Feather keratin messenger RNA is synthesized immediately prior to the onset of keratin synthesis and can form in excess of 50% of the total message content of the cell (Powell **et al.**, 1976). The message is not stored in the cytoplasm in an inactive form before detectable keratin synthesis. Although mechanisms operating at the translational level may be involved in modulating the rate of keratin protein synthesis, the primary controlling event in keratinization is the initiation of transcription of the feather keratin genes (Powell **et al.**, 1976).

1.2 GENE EXPRESSION AND THE INITIATION OF TRANSCRIPTION

Gene expression consists of two major stages, DNA transcription and RNA translation. Modulation of either stage is sufficient to regulate protein production. Control at the translational level is, however, energetically wasteful since it exerts an unnecessary load on the transcription-translation apparatus. Moreover, the transcriptional process supplies an available means for co-ordinate gene regulation. It is not surprising therefore that gene expression in prokaryotes and eukaryotes is, for the most part, regulated at the transcriptional level.

It has become clear that similar molecular principles are used for transcription in both prokaryotes and eukaryotes. Transcription is initiated, nucleotide triphosphates are incorporated resulting in elongation of the RNA and subsequently termination occurs. In eukaryotes, intron-exon gene structure requires processing of the RNA. In addition, the partitioning of eukaryote cells into nucleus and cytoplasm results in a need to transport transcriptional products from one compartment to another. All of these processes are regulated since the purpose of transcription is not just to make RNA but to make the appropriate amounts at the appropriate stage of the cell cycle.

The initiation of transcription in prokaryotes and eukaryotes is itself a major controlling factor in gene expression since initiation specifies what type of RNA is made at a particular developmental stage. Regulation at the elongation and termination stages of the transcription process appears to modulate the amount of the specific RNA made.

The following discussion is concerned with the process of transcriptional initiation and its regulation. The discussion includes prokaryotes as well as eukaryotes to highlight the similarities between these two types of organisms. The different RNA polymerases and their transcription units will be described first, this is followed by a more detailed analysis of promoter structure and the additional effector molecules that interact with promoters. Some models are then presented on how transcription factors may function in the regulation of gene transcription and finally the effects of supercoiling and higher order chromatin structure are discussed.

1.2.1 E. coli RNA POLYMERASE

E. coli RNA polymerase is one of the best studied polymerases. The catalytic core of this enzyme (E) consists of two α subunits (M_r 36,512), a β subunit (M_r 150,619) and a β' subunit (M_r 155,162; Chamberlin, 1982). The core enzyme contains all the necessary catalytic activity and can transcribe any region of DNA at low efficiency. The holoenzyme consists of the core enzyme plus a regulatory subunit, σ (McClure, 1985). Multiple σ factors exist and they are involved in specifying transcriptional initiation (see section 1.2.4(i)).

In prokaryotes, a gene sequence and its respective promoter or control sequences is termed an operon. Polycistronic operons are also common, these contain two or more genes which are transcribed from a common control site. The 5' regions flanking the coding regions are noted for their compactness. Termination sequences are well defined and

numerous mechanisms facilitate termination (Rosenberg and Court, 1979; Greenblatt, 1984).

1.2.2 EUKARYOTE RNA POLYMERASES

There are three RNA polymerases in eukaryotes, I, II and III. Each is involved in the transcription of a particular class of eukaryotic genes. All are multisubunit enzymes and are primarily distinguished by their differential α-amanitin sensitivity. Polymerase II is highly sensitive to α-amanitin, polymerase I highly insensitive and the sensitivity of polymerase III to α-amanitin lies somewhere in between.

Polymerase I transcribes the large ribosomal RNAs (rRNAs); 28S, 18S and 5.8S in a contiguous cistron. The respective RNAs are then cleaved from the primary transcript and incorporated into ribosomes. Little is known about specific termination signals.

Polymerase II is involved in the transcription of mRNA which directs the synthesis of most of the cellular protein. The genes transcribed range from those involved in cellular housekeeping functions such as histones, to tissue specific genes such as ovalbumin. Examples of simple and complex polymerase II transcription units are shown in figure 1-5. The promoter regions of the genes transcribed by this polymerase can extend several hundred base pairs upstream of the transcription initiation site. The transcripts are subject to covalent modifications at both ends (Nevins, 1983). Almost immediately after initiation a 7-methyl guanosine residue or "cap" is added to the first base of the RNA. The region of initiation usually consists of an adenine residue surrounded by pyrimidine residues (Breathnach and Chambon,

FIGURE 1-5

DEFINITION AND EXAMPLES

OF

SIMPLE AND COMPLEX TRANSCRIPTION UNITS

Simple transcription units are depicted in **A** and complex transcription units are depicted in **B**. In each case, DNA is shown at the top and the mRNA(s) underneath. On the former are indicated sites of initiation (**INIT.**) and termination (**TERM.**) of transcription, splicing sites (s) and the position of the sequence which will become the site of poly(A) addition to the processed transcript (●). Figure drawn from Darnell (1982).

			POLY(A)	SPLICE	EXAMPLES
A	SIMPLE Encode 1 protein	1. Init Term	-	-	HISTONES
		2.	+	-	PROT IX ADENOVIRUS α-INTERFERONS, YEAST
		3.	+	+	α-AND β-GLOBINS MANY CELL mRNAs
B	COMPLEX Encode 2 or more proteins	1. 2 poly A sites Init Term	2 OR MORE	+ (all known cases spliced)	ADENOVIRUS LATE IMMUNOGLOBULIN HEAVY CHAINS CALCITONIN
		2. 2 splice sites Init S S S Term	+	2 OR MORE	ONLY VIRAL GENES (can be integrated) ADENO SV40, POLYOMA RETROVIRUSES

1981). The term cap site usually refers to the first base of transcription initiation. Depending on the gene, numerous residues of adenylic acid (poly(A) tail) are also added to the 3' end of the mRNA chain (Nevins, 1983). The position of polyadenylation is designated by a specific signal with consensus AAUAAA, however, additional signals may be required (Benoist **et al.**, 1980; Gil and Proudfoot, 1984). In all cases examined so far, transcription does not terminate at the polyadenylation site, but termination occurs some distance downstream of the polyadenylation signal. In general, transcription termination mechanisms have not been well defined (Nevins, 1983).

Intervening sequences may also be contained within polymerase II transcription units, the function of these for the most part remains obscure. Comparative studies have revealed a consensus sequence of $5'\text{-AG/}\underline{\text{GT}}{}^{\text{A}}_{\text{G}}\text{AGT-3}'$ at the donor or 5' splice site and $5'\text{-}\binom{\text{T}}{\text{C}}_{n}\text{N}^{\text{C}}_{\text{T}}\underline{\text{AG}}\text{/G-3}'$ at the acceptor or 3' splice site. The first two bases (GT) and the last two (AG), underlined in both cases are strictly conserved (Mount, 1982). Small nuclear ribonucleoprotein particles (snurps) are involved in the removal of introns and the production of correct 3' ends from primary gene transcripts (see Turner, 1985 for a recent discussion).

Polymerase III transcribes the transfer RNA (tRNA) genes, 5S ribosomal RNA genes, some small cytoplasmic and nuclear RNAs and some virus associated RNAs. These genes are relatively short when compared with polymerase I and II transcribed genes. A hallmark of polymerase III transcribed genes is that their promoter is always contained within the gene (Korn, 1982; Lassar **et al.**,1983; Brown, 1984).

Transcription termination usually occurs at the first run of four or more thymidine residues following initiation, here too, transcription termination is poorly understood.

In vitro, the three classes of polymerase can only initiate transcription accurately with the assistance of factors from crude cellular extracts (Cizewski and Sollner-Webb, 1983; Davison **et al.**, 1983; Brown,1984).

1.2.3 PROMOTERS AND THEIR ASSOCIATED TRANSCRIPTION FACTORS

The definition of a promoter, as gleaned from both prokaryote and eukaryote definitions, is a segment of DNA that contains signals which direct the proper binding of the RNA polymerase thus enabling transcriptional initiation. The polymerase is not the only protein which recognizes specific sequences in the promoter. Other effector molecules, by binding to specific promoter sequences, can influence the ability of the RNA polymerase to accurately initiate transcription.

The particular nature of the signals recognized by the polymerase and by specific effector molecules have been deduced by analysis of the DNA. Three main methods have been employed. Firstly, DNA sequencing has been used to define regions which occur in high frequency within promoters. Secondly, important regions have been defined using genetic analysis in which bases within the promoter were mutated. The DNA was reintroduced into the cell or an experimental substitute such as an **in vitro** transcription system where increases or decreases in promoter strength could be observed. Thirdly, chemical or enzymic "footprinting" techniques have been used to establish regions which were protected by

polymerase or effector molecule binding. Such techniques involve the use of DNAse I or dimethyl sulphate (Galas and Schmitz, 1978; Siebenlist and Gilbert, 1980).

How do site-specific proteins recognize a specific sequence of base pairs in double stranded DNA? Proteins do not recognize sequences of base pairs **per se**; they recognize specifically placed clusters of DNA hydrogen bond donors and acceptors by complementarity with matrices of hydrogen bond acceptors and donors located in the binding site of the proteins (Takeda **et al.**, 1983). The donors and acceptors of a particular base pair must be located in the major or minor grooves of the DNA double helix since the hydrogen bonds involved in double helix formation are sequestered in the interior of the helix, thus are not available for participating in protein-nucleic acid interaction (Seeman **et al.**, 1976).

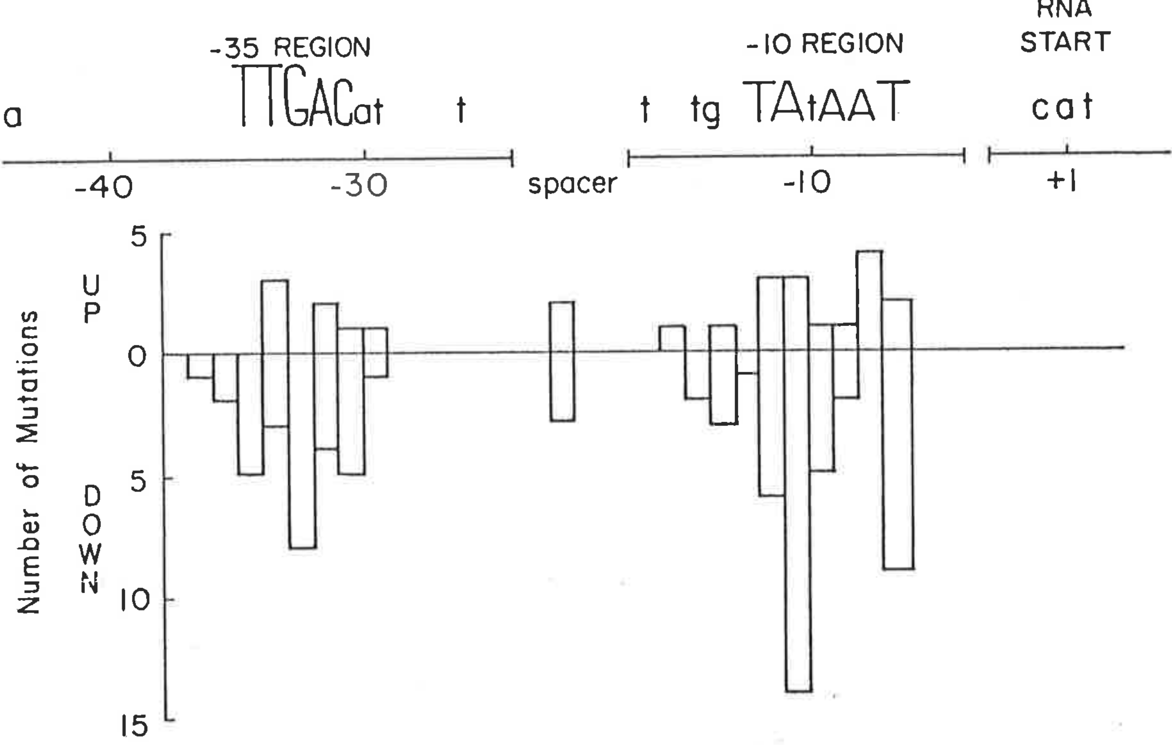
(i) Bacterial Promoters and Transcription Factors

Two regions participate in the RNA initiation reaction in **E. coli**, they are located approximately 35 bp and 10 bp upstream from the RNA start site (McClure, 1985). The current promoter consensus is shown in figure 1-6. In each region, there are three bases that are highly conserved, TTG at the -35 region and TA---T near the -10 region. Conserved and weakly conserved sequence homologies exist around these highly conserved bases, resulting in a sequence consensus of TTGACA for the -35 region and TATAAT for the -10 region. Surprisingly, no single **E. coli** promoter has been found that matches the consensus sequence exactly. This consensus sequence appears to be maximal with respect to promoter

FIGURE 1-6

E. coli PROMOTER CONSENSUS SEQUENCE AND DISTRIBUTION OF MUTATIONS WITHIN KNOWN PROMOTERS

The sequences homologies at positions within the promoter are shown in three classes. The highly conserved bases (>75%; 9 S.D.) are large capital letters. The conserved bases (>50%; 5 S.D.) are small capital letters. The weakly conserved bases (>40%; 3 S.D.) are lower case letters. The distance between the -35 and -10 regions (spacer) is ordinarily 17 ± 1 base pair. Below each position, including the spacer, the number and the nature of known promoter mutations are shown as bars (from McClure, 1985).


-35 REGION
-10 REGION
RNA START
a
TTGACat
t
t
tg
TAtAAT
cat
-40
-30
spacer
-10
+1
Number of Mutations
UP
DOWN
5
0
5
10
15

strength. It appears therefore that promoter function in **E. coli** is optimized for each gene **in vivo** and is not maximized (McClure, 1985).

The activity of **E. coli** RNA polymerase is modulated by activators and repressors (von Hippel **et al.**, 1984; McClure, 1985). All of the known bacterial activator binding sites are located near or upstream of the -35 region. There is considerable diversity in the location of repressors however as they have been found as far upstream as -60 and as far downstream as +12 (McClure, 1985). In contrast to eukaryotic transcriptional effector molecules, bacterial activators and repressors are quite well characterized.

(ii) Eukaryote RNA Polymerase I Promoters and Their Transcription Factors

Sequence homologies around the transcription initiation site of rRNA genes are only really evident when closely related species are compared. For example, figure 1-7 shows the obvious sequence conservation that exists between the upstream regions of **Xenopus** rRNA genes. These regions are not conserved to any major degree in either mouse, rat or human. The only obvious similarity that is evident is that in higher eukaryotes the G at position -16 is conserved, as is the T at position -1 (Sommerville, 1984 and references therein). The significance of these two conserved residues is unknown.

The general lack of sequence conservation amongst the upstream regions of rRNA genes across species is surprising given that rRNA synthesis is a general feature of all cells. There may exist sequences further upstream which are needed for transcription. The -169 to -45 region in mouse rDNA has

FIGURE 1-7

SEQUENCE HOMOLOGIES AROUND THE INITIATION SITES OF rRNA GENES

Major sequence homologies around the transcription initiation site of rRNA genes of mammals, **X. laevis, X. clivii, X. borealis, Drosophila melanogaster, Tetrahymena pyriformis, Dictyostelium discoideum, Physarum polycephalum Sacchromyces calsbergensis** and **Sacchromyces rosei.** The conserved G at position -16 and the conserved T at -1 are indicated (arrow heads) as is the start site and direction of transcription (arrow). The diagram is taken from Sommerville (1984).

	-40	-30	-20	-10	+1	+10
			▽		▽ ⟶	
Mouse	ATCTTT		TATTG		TACTGACACGC	
Rat	ATCTTT		TATTG		TACTGACACGC	
Human	ATCTTT		TTTTGG		TGCTGACACGC	
X. laevis		CTACGCTTTT	GTGCGGGCAGGAAGGTAGGG			
X. clivii		CTACGCGTTT	GTGCCGACAGGAAGGTAGGG			
X. borealis		CTACGCTTTT	GTGCGGACAGGAAGGTAGGG			
D. melanogaster		TTT	G		TAGGT	
T. pyriformis			G		T	
D. discoideum			T	ATACATATA		
P. polycephalum			A	ATACATATA		
S. carlsbergensis			A	AGGTACTTCATGCGAAAGC		
S. rosei			G	AGGAACTTCATGCGAAAGC		

been shown to influence promoter efficiency **in vitro** (Grummt, 1982). In **Xenopus**, duplicated intact promoters and 42 bp repeats extend several thousand base pairs upstream of the promoter closest to the gene (Moss, 1982; Busby and Reeder, 1983). It has been postulated that these 42 bp repeats bind transcription factors. This may explain why in microinjected oocytes, **Xenopus** rRNA genes with multiple repeat units are preferentially transcribed (Reeder **et al.**, 1983).

Less is known of the regions that bind transcription factors or even the transcription factors themselves. Accurate initiation of rRNA gene templates **in vitro** is only achieved if all the components in the extract are derived from the same species (Grummt **et al.**, 1982). Hence, in keeping with promoter conservation within a species, it also appears that transcription factors are species specific.

(iii) Eukaryote Polymerase II Promoters and Their Transcription Factors

In polymerase II transcribed genes numerous transcriptional control elements can be defined. Some elements are common to most genes, but are not present in all cases, other elements are specific to individual genes or to groups of genes. Given the functional diversity of polymerase II transcribed genes, this is perhaps not surprising. As a whole the promoter regions can be organized into a common plan. Figure 1-8A shows the arrangement of DNA sequence elements controlling the transcription of a hypothetical higher eukaryote gene. The promoter elements can be divided into three major groups, the TATA box, immediate upstream elements, and far upstream or downstream elements (enhancers).

FIGURE 1-8

ORGANIZATION OF POLYMERASE II

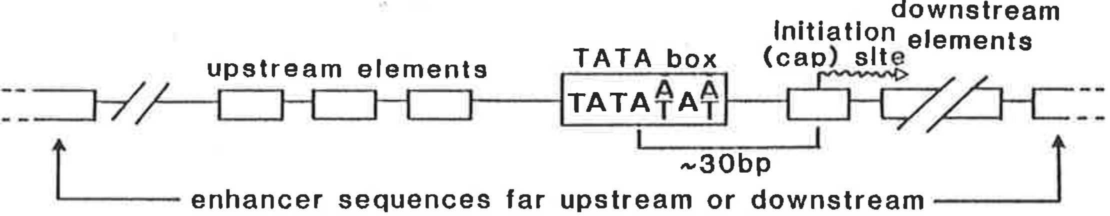
TRANSCRIBED GENES

A. The variety in position of transcriptional control elements with respect to the initiation site in polymerase II transcribed genes. The TATA box (at around -30 bp) determines the cap site, other elements can be located in the immediate upstream region and also far upstream or even downstream of the cap site.

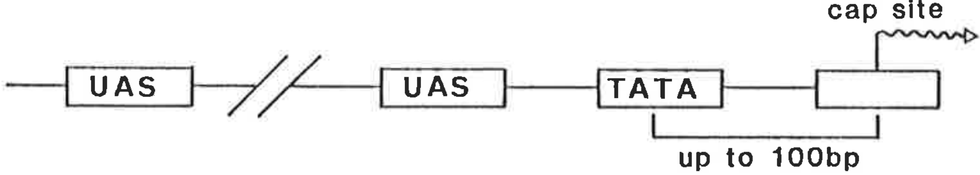
B. Positioning of transcriptional control elements in yeast. Sequences near or at the transcription initiation site determine the cap site. The TATA box appears to be mainly required for efficient transcription and its position varies with respect to the cap site. Upstream activation sites (UASs) bind factors that regulate transcription.

(Adapted from Schaffner, 1985).

A. Higher eukaryotes



B. Yeast



A potential fourth group, slowly gaining members, includes promoter elements downstream of the initiation site but within the gene boundary. These groups will be discussed in the sections below.

(iii) a. The TATA Box

One sequence element is common to nearly all polymerase II transcribed genes. An A-T rich region of DNA (consensus, TATA(A/T)A(A/T); Corden **et al.**, 1980) often referred to as the TATA box is found approximately 25-30 bases upstream from the mRNA start site. The only polymerase II genes shown to lack any semblance of a TATA box are the Simian Virus 40 (SV40) and the polyoma late genes and the E2 and IVa2 genes of adenovirus. Mutations in the TATA box result in a decrease in the amount of correctly initiated transcript, although, the total amount of transcript generated may not be significantly reduced. Thus the TATA box appears to specify the site of transcription initiation, i.e. the cap site (Benoist and Chambon, 1981). The TATA box has been shown to bind specific transcription factors during **in vitro** transcription studies (refer to table 1-1).

Yeast genes are an exception as far as the TATA box is concerned (figure 1-8B). It appears that sequences near or at the transcription start site determine the initiation point whereas the TATA box, located at a variable distance from the initiation site (within 100 bp upstream) is mainly required for efficient transcription (Gaurente, 1984).

(iii) b. Immediate Upstream Elements

Most elements which contribute to the efficiency of mRNA synthesis are located within a region not further than 200 base pairs upstream of the transcription start site, which is termed for the purpose of this discussion, the immediate upstream region. Quite a large number of immediate upstream elements have now been described and footprinting studies have shown that some of these elements directly bind specific factors from cellular extracts **in vitro.** Some of the different factors and in some cases the DNA sequences each factor recognizes are shown in table 1-1.

A number of points can be made in relation to table 1-1:

1. Some factors are gene specific.
2. Multiple factor binding regions can exist within the upstream region of an individual gene.
3. Some sequence elements, notably CTF and Spl binding sites, are present in the immediate upstream region of a wide variety of gene types.
4. The sequence elements listed bind activatory as well as inhibitory factors.
5. Proof of the actual binding of most of these factors **in vivo** awaits verification.
6. In most cases the identification and isolation of the actual protein moiety still needs to be achieved. Spl is the only protein of this type that has been purified (to 95% homogeneity, Kadonaga **et al.**, 1986).

TABLE 1-1

FACTORS WHICH BIND TO PROMOTER ELEMENTS OF POLYMERASE II TRANSCRIBED GENES

The table lists DNA sequences or regions of DNA in a number of different genes which have been shown by DNA footprinting studies to bind specific factors from cellular extracts **in vitro.** A key to the various symbols used is given at the bottom of the table.

FACTOR	ACTIVATORY (A) INHIBITORY (I)	CELL EXTRACT	SEQUENCE RECOGNIZED	GENES WHERE BINDING OBSERVED	REFERENCE
CTF CAAT Binding Transcription Factor	A	HeLa Nuclear Extract	5' Pu Pu C C A A T 3'	HSV-tk Human β-Globin Murine Sarcoma Virus (MSV) Human Heatshock Gene (hsp70)	Kadonaga *et al.*, 1986
TATAA Binding Factors	A	HeLa Nuclear Extract	5' TATA(T/A)A(T/A) 3'	Conalbumin Adenovirus Major Late Promoter	Davison *et al.*, 1983 Sawadogo and Roeder, 1985
		Drosophila SL2 Cell Nuclear Extract	5' TATA(T/A)A(T/A) 3'	*Drosophila* Heat Shock Gene hsp 70, hsp 83	Wu, 1984
Sp1	A	HeLa Nuclear Extract	5' (G/T)GGGCGG(GGC/AAT) 3'	- †HSV-Intermediate Early -3 Gene (all 5 GC boxes ~ -250 to -75) - †Mouse Dihydrofolate Reductase (all 4 GC boxes ~ -190 to -50) - †Human Metallothionein IIA (~ -65) - †SV40 Early Promoter (all 6 boxes ~ -110 to -50) - †Chicken-tk Intron - †HSV-tk (both GC boxes ~ -110 to -50) - †AIDS Virus LTR (all 3 GC boxes ~ -75 to -50)	Kadonaga *et al.*, 1986
Glucocorticoid Receptor	A	Purified Receptor	5' (T/C)GG(T/A)CA(AA/CT)NTGT(T/C)CT 3'	- Mouse Mammary Tumour Virus: MMTV I (-186 to -170) - Mouse Mammary Tumour Virus: MMTV IIA (-129 to -113) - Human Metallothionein IIA Gene (-263 to -243) - Human Growth Hormone Gene (first intron, +109 to +95) - Rabbit Uteroglobin Gene (-2.6 Kb)	Karin *et al.*, 1984 and references therein. Moore *et al.*, 1985 & Slater *et al.*, 1985 Cato *et al.*, 1984
HSTF - Heatshock Transcription Factor	A	*Drosophila* KC Nuclear Extract	3 DOMAINS 1. -62 5' CTNGAATNTTCTAGA 3' -49 2. -189 to -165 3. -258 to -235	- hsp 70 Gene	Topol *et al.*, 1985
Adf-1	A	*Drosophila* KC Nuclear Extract	-85 to - 24	- *Drosophila* Alcohol Dehydrogenase Gene	Heberlein *et al.*, 1985
USF	A	HeLa Nuclear Extract	-63 5' GGCCACGTGACC 3' -52	- Adenovirus Major Late Promoter	Sawadogo & Roeder, 1985
NF-1	A	HeLa Nuclear Extract	5' TGG(A/C)NNNNNGCCAA 3'	- §Immunoglobulin μGene - §Adenovirus-5 - §Human c-myc - §Human Polyoma Virus - §Human/Simian Cytomegalovirus	Hennighansen *et al.*, 1985 and references therein
		Chick Oviduct Nuclei	5' TGG(A/C)NNNNNGCCAA 3'	- §Chick Lysozyme Gene (-11 Kb)	Borgmeyer *et al.*, 1984
Polyoma B Enhancer Factor	A	Mouse 3T6 Nuclei	B element of polyoma enhancer (~ -195)	- Polyoma Virus Early Promoter Region	Piette *et al.*, 1985
Large T Antigen	I	SV40 Infected Cells	Region I SV40 origin - 5' GAGGCTTTTTTGGAGGC 3'	- SV40 Origin Regions I and II	Ryder *et al.*, 1985
α 2	I	Yeast	32 bp sequence (~ -135)	- Yeast *a*-Specific Genes eg. STE6	Johnson & Herskowitz 1985
GAL 4	A	Yeast	5' CGGA(G/C)GAC(A/T)CAG(G/C)AGGC 3'	- UAS of GAL 1 and GAL10 Genes, 4 Positions Beginning -368, -387, -405 and -469	Giniger *et al.*, 1985
H4 Histone S Phase Factor	A	L-Cell Nuclear Extract	-70 to -10	- Mouse H4 Histone Gene Upstream Promoter Region	Capasso & Heintz, 1985
Virion Associated Protein	A		5' TAATGAPuAT 3'	- †Intermediate Early 3 Gene of HSV I (3 boxes - 400 to -250)	Lang *et al.*, 1984 & Preston *et al.*, 1984
Engrailed Homeo Box Binding Factor	A	Synthesized in *E. coli* via Fusion to *lac Z* Gene	3 DOMAINS* 1. -1200 to -200 2. -2100 to -250 3. -2300 to -125	- *Drosophila* Engrailed Gene Upstream Region	Desplan *et al.*, 1985
β-Globin Factors	A	Chicken Erythrocyte Nuclei	2 DOMAINS -196 5' GGGGGGGGGGGGGGGCGGG 3' -177 -159 5' ATCTGGGCACCTTGCCCTGAGCCCCACCCT 3' -35	- Chicken β^{A}-Globin Gene	Emerson *et al.*, 1985

Pu = Purine.

* = Upstream of AUG.

† = Numbers only indicate the stretch of DNA within which the sequence boxes are found.

N = Any nucleotide.

§ = Sequence mentioned is positoned upstream of cap site, for exact position refer to the references listed in Hennighansen *et al.*, 1985.

UNLESS OTHERWISE STATED POSITION OF A SEQUENCE IS GIVEN IN RELATION TO THE CAP SITE.

(iii) c. Enhancers

Enhancers are DNA sequences that are able to stimulate transcription. They normally exist between 100 and 300 base pairs upstream of the transcription initiation site (Khoury and Gruss, 1983), hence in the general scheme of polymerase II promoter structure, enhancers overlap with other stimulatory elements that are immediately upstream. Functionally, they possess unique properties which have been demonstrated **in vitro** and **in vivo.**

In order for a sequence to be classified as an enhancer it must fulfill the following criteria. Firstly, the sequence must stimulate transcription from the correct transcription initiation site of a linked gene (up to two orders of magnitude have been observed). Secondly, the stimulation should be independent of orientation. Thirdly, the enhancement should be evident over long distances (at least 1 kb) irrespective of whether the sequence is positioned 5' or 3' to the transcription initiation site (Schaffner, 1985).

The upstream promoter regions of viruses commonly contain enhancer elements and most viral enhancers have the ability to function in a variety of cell types (Khoury and Gruss, 1983). However, tissue specific enhancers have also been discovered. An example is the mouse immunoglobulin heavy chain enhancer which is found 3' to the cap site in the intron between the J and C segments of the mouse cμ gene (Banerji **et al.,** 1983). This enhancer functions in both orientations, even with heterologous promoters, only if tested in lymphoid cells (Neuberger, 1983). Tissue specific enhancers have also been found upstream of the following genes, rat chymotrypsin

(Walker **et al.**, 1983), albumin (Ott **et al.**, 1984) and the Eß gene of the mouse major histocompatibility gene family (Gillies **et al.**, 1984).

No nucleotide sequence common to all enhancers has been identified. A number of short consensus sequences are shared by different sets of enhancers and enhancers may contain more than one such consensus element. Among the different consensus sequences are the "core" consensus, described as 5'-GTGG(A/T)(A/T)(A/T)G-3' (Laimins **et al.**, 1982; Weiher **et al.**, 1983) and stretches of alternating purines and pyrimidines (Nordheim and Rich, 1983).

The fact that the various viral and cellular enhancers have different species and/or cellular specificities has long suggested that specific factors interact with enhancer elements (Khoury and Gruss, 1983). Recently, specific factors have been shown to interact with the two 72 base pair repeats of the well characterized SV40 enhancer. The level of these factors was observed to vary among cell types, thus affecting the degree of enhancement (Schöler and Gruss, 1984). In addition the B element of the polyoma enhancer has been shown by protection studies to bind specific factors (table 1-1; Piette **et al.**, 1985). Nothing is really known about the mechanism of enhancer action.

(iii) d. Yeast UASs

A yeast promoter may have one or more upstream activatory sequences (UASs) which are recognized by regulatory proteins in response to specific physiological signals. The UAS effect is exerted in an orientation-dependent manner and over a large distance (up to 1500 base pairs). However, the UAS effect is

not a classical enhancer effect since UASs cannot activate transcription from a downstream position (see Gaurente, 1984 for review).

(iii) e. Downstream Elements

In addition to the immunoglobulin enhancer there are elements of other genes downstream of the transcription initiation site which have been shown to be important in transcriptional initiation.

Two elements are located within the sea urchin H2B histone gene. One resides between nucleotides +11 and +76 in the 5' untranslated leader sequence. The other is located near the 3' end of the gene and extends from 90 base pairs upstream of the mRNA 3' terminus to 140 base pairs downstream of the 3' terminus and is unrelated to the termination signal (Mous **et al.**, 1985).

Downstream elements have also been implicated in the tissue specific expression of the chicken thymidine kinase gene (Merrill **et al.**, 1984b) and the human globin genes (Charnay **et al.**, 1984; Wright **et al.**, 1984). A specific binding site for purified glucocorticoid receptor exists in the first intron of the human growth hormone gene, 100 bp downstream from the cap site (Moore **et al.**, 1985).

There are cis-acting control sequences in the yeast mating type (MAT) locus which when linked to heterologous genes have all of the characteristics of enhancers but in reverse, transcription being efficiently repressed. In yeast, these so called silencer sequences usually exert their repressive effect from a downstream position (Brand **et al.**, 1985).

(iv) Polymerase III Promoters and Their Transcription Factors

The region of DNA essential for the promotion of polymerase III transcribed genes is always contained within the structural sequence (Brown, 1984). Polymerase III transcribed genes can be classified into two groups on the basis of their internal promoter structure. Group I includes the tRNA genes and the adenovirus-2 VA1 gene. This group is characterized by an internal split promoter consisting of two components, box A and box B, which are separated by about 30 nucleotides (Ciliberto **et al.**, 1983). Consensus sequences of the two boxes are RRYNNARY-GG for box A and G(A/T)TCRANNC for box B (R = purine, Y = pyrimidine, N = any nucleotide). Initiation of transcription occurs between 10 and 20 nucleotides from box A. Group II contains the 5S RNA genes. Here the internal control region is some 34 base pairs in length, the first 11 bases being structurally (and functionally) homologous to the A box of the tRNA genes. The remaining bases share no homology with the other polymerase III transcribed genes and appear to be specific for the 5S genes. Transcription is initiated approximately 50 nucleotides from the 5' end of the internal promoter region (Sakonju **et al.**, 1980; Ciliberto **et al.**, 1983).

Specific factors in addition to polymerase III are required for the transcription of these genes in cell free extracts. Group I (tRNA) genes require transcription factors B and C (TFIIIB and C; refer to Lassar **et al.**, 1983 for review). Transcription of 5S genes requires, in addition to factors B and C, a 38,000 dalton protein, TFIIIA (Engelke **et**

al., 1980) which binds to the internal promoter of 5S genes (Sakonju **et al.,** 1981). Factors B and C are poorly characterized, TFIIIA however, has been purified (Smith **et al.,** 1984). The need for TFIIIA has also been demonstrated **in vivo** (Brown and Schlissel, 1985).

1.2.4 REGULATION OF TRANSCRIPTION BY SPECIFIC FACTORS

There are two important features in the control of transcriptional initiation. The first is the recognition of the site of transcriptional initiation and the second is the frequency of initiation, which is dependent on promoter strength; the relative affinity of the promoter for the polymerase. Specific factors could conceivably function at both of these control levels by facilitating or inhibiting polymerase binding.

There is, however, another aspect which influences the frequency of initiation and that is the rate-limited assembly of all the components necessary for transcription in the form of a "preinitiation complex". Preinitiation complexes can be viewed as being composed of the polymerase as well as specific effector molecules. Evidence for such complexes has been reported for the genes transcribed by polymerase I (Cizewski and Sollner-Webb, 1983; Wandelt and Grummt, 1983). Little is known about polymerase I preinitiation complexes, however, the complex formed prior to polymerase II transcription is known to require three other enzymic factors in addition to factors such as those listed in table 1-1 (Dynan and Tjian, 1983; Parker and Topol, 1984). Of the eukaryote gene systems, most is known about the transcription complexes which activate the 5S RNA genes of **Xenopus** oocytes (see section 1.2.4(iii)

below). Even in prokaryotes, sigma factors need to associate with the polymerase and activators need to bind to the DNA. Once preinitiation complexes are formed they are quite stable and promoter strength probably becomes the important rate-limiting factor.

The regulation of transcriptional initiation by specific factors is a highly interactive process, thus the transcription factors which will be discussed should always be viewed in the context of their interaction with other components of the transcriptional machinery.

(i) Sigma Factors of Prokaryotes

The core bacterial RNA polymerase can transcribe any region of DNA, at low efficiency. The σ subunit alone has no catalytic activity, however, in combination with the core complex it allows both site and strand selection. (McClure, 1985). Sigma is required only for transcription since it is released some time after transcription has begun and is recycled (Travers and Burgess, 1969).

It has long been hypothesized that each gene or group of genes in **E. coli** could require its own specific σ factor (Burgess **et al.,** 1969). Until recently this has only been demonstrated in **Bacillus subtilis,** where five different σ factors have been identified. The probable occurence of multiple σ factors in **E. coli** is now apparent with the recent discovery of a σ factor which is involved in the transcription of the heat shock genes (Grossman **et al.,** 1984).

Multiple σ factors have also been identified in another prokaryote: **Streptomyces coelicolor** (Westpheling **et al.,** 1985). The potential for other σ factors also exists in the

nitrogen fixation genes of **Klebsiella** and **Rhizobium** (Ausubel, 1984; Beynon **et al.**, 1985).

From the examples listed above it is possible that the use of different σ subunits to specify the transcriptional initiation of different classes of genes could be a wide-spread phenomenon in prokaryotes. However, the transcriptional initiation of a wide variety of genes in **E. coli** by the same σ subunit still leaves an unsolved problem regarding specific gene recognition and initiation.

Repressors of transcription act by binding to specific sites near the promoter and they have a wide spectrum of affinities for the operator region. The tightest binding repressors e.g. **E. coli** lac (10^{-11} M; Barkley and Bourgeois, 1978) can result in an absolute shutdown in transcription initiation. The weaker binding repressors eg cI and cro of λ (10^{-8}-10^{-9} M; Johnson **et al.**, 1979) may simply decrease transcriptional initiation. The mechanism of repression has been attributed to a steric blocking of the RNA polymerase preventing binding to the promoter (von Hippel **et al.**, 1984).

Activator binding can aid in either promoter recognition or increase the frequency of transcription initiation, or both. Like repressors, different activators have different binding affinities for promoter regions (McClure, 1985).

The structures for the cro and cI repressors of λ and for the CAP protein of **E. coli** have been determined by X-ray crystallography (Anderson **et al.**, 1981; McKay and Steitz, 1981; Pabo and Lewis, 1982). It is interesting that despite differences in function, domain organization and tertiary structure, all of these proteins bind to the DNA as a dimer and use α-helices to contact the DNA. One α-helix in each of

these proteins fits into the major groove of the DNA. Contacts are made between exposed functional groups on the bases and amino acid side chains via hydrogen bonds and Van der Waals forces (Pabo and Sauer, 1984). Most of the contacts made by cro and CAP occur on one side of the double helix, however, cI contacts both sides of the helix by a flexible region of protein which wraps around the DNA (Pabo and Sauer, 1984).

Several hypotheses have been proposed for the mode of action of transcriptional activators. Two classes of models exist. The first involves activator-induced changes in the DNA at the promoter site (Dickson **et al.**, 1975; McKay and Steitz, 1981) and the other involves activator-RNA polymerase interactions at the promoter (Ptashne **et al.**, 1980). Evidence tends to support the latter. For example, studies involving cI activation of transcription from the λP_{RM} promoter and cII activation of λP_I indicate that in order for these activators to function they must interact with the RNA polymerase (Hawley and McClure, 1983). How the activation is induced once such protein-protein contacts are made, is unknown.

(ii) The Constitutive and Regulatory Factors of Polymerase II Transcribed Genes

In contrast to prokaryote activators and repressors, little is known about the transcription factors associated with polymerase II transcribed genes. Certainly, no information is available on the regulation of the genes which code for the transcription factors as is the case in prokaryotes.

The best characterized transcription factor in this polymerase class is Spl, a factor isolated from HeLa cells. Spl has been purified to 95% homogeneity although nothing is yet known of its structure. The sequence recognized by Spl is a GC-rich sequence (refer to table 1-2) and promoters that are responsive to transcriptional stimulation by Spl often contain multiple GC boxes. Each binding site can have a different affinity for the Spl protein (Kadonaga **et al.,** 1986; table 1-2). Spl is believed to bind the recognition sequence on only one DNA strand, contact being made in the major groove of the DNA helix (Gidoni **et al.,** 1984). Interestingly, no shared regulatory features have been found among the diverse promoters to which Spl binds.

Inferences have been made about the regulation of transcriptional initiation in polymerase II genes from their general promoter structure. However, there is a distinct lack of information about transcription factors. Polymerase II transcribed genes have promoters in which the regulatory sequences are often spread over long distances from the cap site. This is in contrast to the compact promoter structure of prokaryotes where it is easier to envisage direct effector-polymerase contacts leading to modulation of transcriptional initiation. Any models concerned with the regulation of transcriptional initiation in polymerase II genes also need to accommodate the fact that functionally unrelated genes may contain similar types of promoter elements.

Serfling **et al.** (1985) view promoters as being composed of modular arrangements of small sequence motifs which bind specific factors. Each motif has a specific function conferring for example, tissue specificity, general trans-

TABLE 1-2

Sp1 BINDING AFFINITY

The consensus sequence for Sp1 binding is shown at the top of the table. The sequence was derived from the 19 binding sites listed. The relative affinity of Sp1 for each site was estimated by DNAse I footprinting.

DHFR = Dihydrofolate reductase gene.

CH-TK = chicken tk gene.

MT = metallothionein gene.

HSV IE-3 = HSV intermediate early 3 gene.

The numbers in brackets refer to the position number of the GC box upstream of the initiation site. (From Kadonaga **et al.**, 1986).

CONSENSUS: 5' $^{G}_{T}$GGGCGG$^{GGC}_{AAT}$ 3'

SEQUENCE	RELATIVE AFFINITY	SOURCE
G G G G C G G G G C	High	HSV IE-3 (V); DHFR (I, III) MT IIA; CH-TK INTRON
T G G G C G G G G C	High	HSV IE-3 (III, IV)
T G G G C G G A G T	High	SV40 (III, V)
G G G G C G G A G C	High	DHFR (II, IV)
G G G G C G G G G G	Medium	HSV IE-3 (I)
G G G G C G G G G T	Medium	HSV IE-3 (II)
T G G G C G G G G T	Medium	HSV-TK (II)
T G G G C G G A A C	Medium	SV40 (II)
G G G G C G G G A T	Medium	SV40 (IV)
G G G G C G G G A C	Medium	SV40 (VI)
G G G G C G G A G A	Low	SV40 (I)
G G G G C G G C G C	Low	HSV-TK (I)

criptional enhancement and inducibility. All motifs are grouped into two general functional classes, constitutive and regulatory.

The TATA box whose main function is to specify the site of transcription initiation could be considered a constitutive element. Other candidates for constitutive factors include Sp1 and CTF whose recognition sequences are found in the upstream region of a variety of gene types (table 1-1). A tissue specific factor could also be considered as a constitutive element because without that factor, the gene would not normally be expressed.

Examples of sequence elements which fall into a regulatory category include the steroid hormone receptor binding sites (table 1-1 and references therein) and the metal regulatory region of the metallothionein genes (Karin **et al.**, 1984). Regulatory elements would function to modulate the level of transcription.

The model by Serfling **et al.** (1985) is depicted in figure 1-9 and the explanation of this model is given in the legend on the facing page. The five examples shown in figure 1-9 summarize how different combinations of regulatory and constitutive factors enable regulated gene expression. It is easy to see how different genes with no common regulatory function could share a particular element since the expression of a particular gene containing that element would be dependent on the combination of other modular elements and the availability of other specific factors.

There are some uncertainties with this model in relation to factor interaction. Upstream factors must in some way interact with other components of the transcriptional

FIGURE 1-9

SIMPLIFIED MODEL OF EUKARYOTIC TRANSCRIPTION CONTROL

A. Promoters of weakly expressed genes contain only a few upstream DNA elements for constitutive (c) expression.

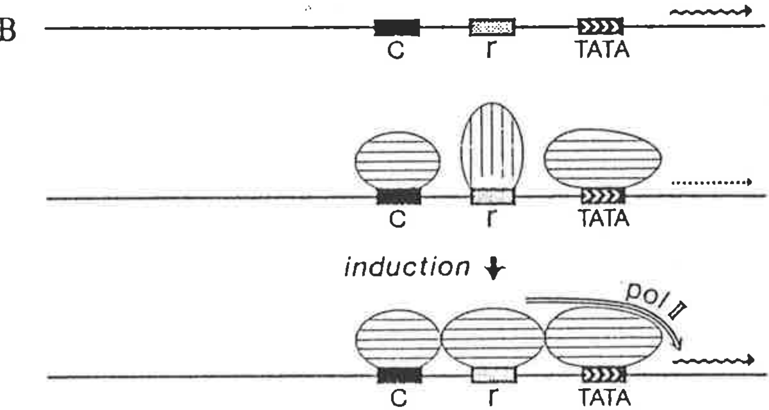
B. Promoters regulated at the level of cell differentiation or by induction with environmental stimuli harbor not only constitutive but also regulatory (r) motifs. A schematic view of regulation of transcription by the interaction of several factors, namely a TATA box binding protein, a constitutive factor (e.g., Spl), and a regulatory factor (e.g., a steroid hormone receptor) is shown underneath. In the uninduced state, the latter factor could be absent, could be present but not bind, or, as shown, could bind in a conformation that does not stimulate but rather represses transcription. Induction presumably involves "bridging" of constitutive factors by a regulatory factor.

C. Promoters of strongly expressed constitutive genes. DNA motifs are arbitrarily numbered to illustrate that several types of elements can be intermingled and repeated. Although certain DNA motifs may occur preferentially in one orientation and close to the TATA box, and others further upstream, the difference between **A** and **C** may well be of a quantitative rather than a qualitative nature (Motifs C_1-C_2-C_1-C_2 would exert an enhancer effect if detached and linked to a test gene).

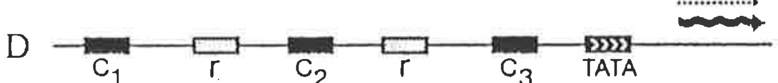
D. Promoters of strongly expressed regulated genes. This example is an extension of situation **B**, with several regulatory motifs interspersed at strategic positions with constitutive elements. The number and spatial arrangement of these motifs probably determines the basal vs. induced level of transcription.

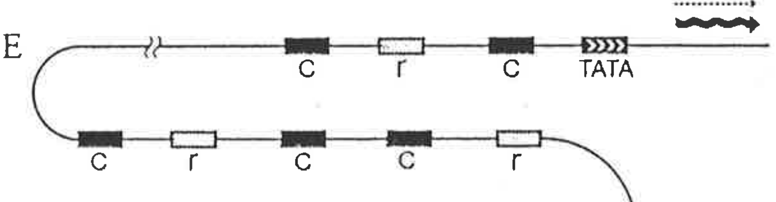
E. Remote control by an enhancer. The sequences just upstream of the TATA box are not sufficient to result in the induction of a high level of transcription by themselves, but they respond to additional elements far away, presumably mediated by protein-protein contacts of the factors involved. (From Serfling **et al.**, 1985).

A
C
TATA


B
C
r
TATA
induction
pol II

C
C1
C2
C1
C2
C3
TATA


D
C1
r
C2
r
C3
TATA


E
C
r
C
TATA
C
r
C
C
r

machinery. An upstream factor, binding near the transcription initiation site, could exert its effect on transcription by inducing some conformational change in the other proteins that make up the initiation complex or alternatively in the DNA structure (refer to figure 1-10A). Direct protein-protein contact mechanisms become more difficult to explain the further the element is from the initiation site. Conceivably, protein interaction with the transcriptional machinery could be maintained if there was a continuous scaffold of protein due to factors binding to numerous elements, thus linking all elements, even the most distal in the promoter. This type of interaction is suggested in the model proposed by Serfling **et al.** (1985).

What of promoters in which a small number of elements may be scattered over a long upstream sequence? Dynan and Tjian (1985) have suggested a number of possible explanations for the ability of upstream factors to act at a distance.

1. Other proteins may bind to fill in the gaps, once again creating a continuous scaffold of protein (figure 1-10B).
2. After an initial binding to a particular site the factor may shift to make interactive contact with other proteins at a nearby site (figure 1-10C). This would require energy to allow the factor to dissociate and then move along the helix.
3. Rather than moving, the factor could induce a conformational change in the helix, producing a signal that can be transmitted over some distance leading to local melting at the TATA box region enabling the polymerase to bind to the DNA.

FIGURE 1-10

MODELS FOR THE ACTION

OF

PROMOTER-SPECIFIC FACTORS

A. Transcription factors (stippled) bound to upstream elements in direct contact with other proteins (shaded) bound at the initiation site.

B-E represents action at a distance.

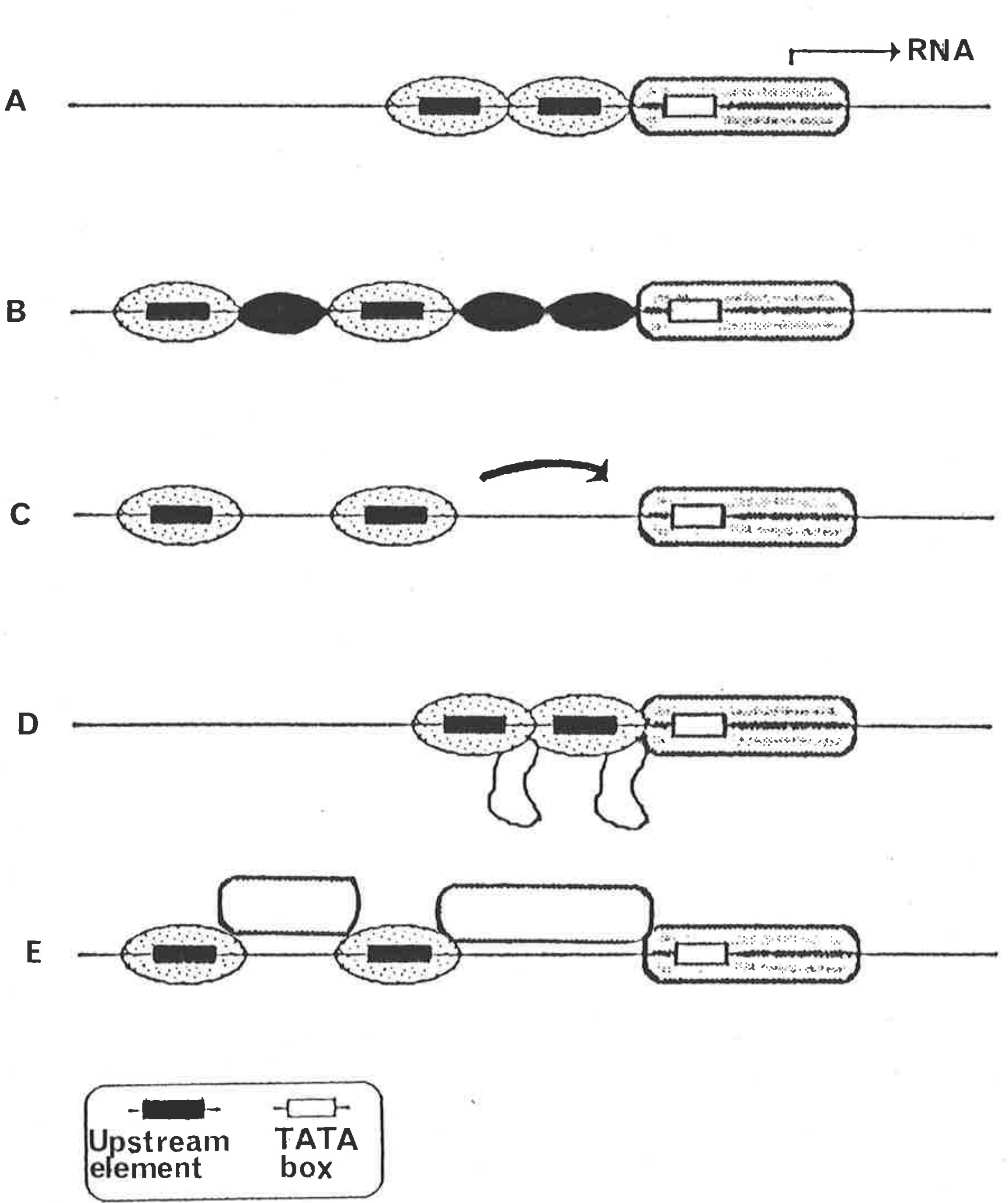
B. Additional proteins bind interstices to create a continuous scaffold of protein in the promoter region.

C. Upstream factor shifts position to contact proteins at initiation site or alternatively, alters conformation of DNA, with effect felt at initiation site.

D. Transcription factors condense into a compact nucleoprotein structure with looping out of the DNA found in between.

E. Rather than additional proteins binding DNA interstices to create a protein scaffold, the additional proteins may form bridges between the promoter-specific proteins, transmitting the effect to the initiation site.

(A-D from Dynan and Tjian, 1985).


RNA
A
B
C
D
E
Upstream element
TATA box

4. Binding of factors could allow the DNA to condense into a nucleoprotein structure (figure 10D; Dynan and Tjian, 1985).

No real evidence exists for any of these interactive models, although a scaffold system could exist in the HSV-tk gene where CTF, the CAAT transcription factor, binds between the two Spl sites (Jones **et al.**, 1985).

Another method of interaction could be provided by the stimulatory transcriptional proteins encoded by viral oncogenes, for example, the Ela protein of adenovirus-2. Ela proteins are known to directly stimulate transcription of a number of genes, both viral and cellular (Kingston **et al.**, 1985, for review). It has been shown that Ela proteins do not bind DNA directly and experiments indicate that Ela does complex with DNA binding proteins in HeLa cell extracts (Ferguson **et al.**, 1985). Perhaps Ela proteins form bridges between different promoter-specific proteins to help facilitate the rate of formation of active transcription complexes (figure 1-10E). Evidence for this hypothesis has not yet been produced.

One major problem with interactive models, such as those described above, is that they tend to view all of the elements necessary for correct transcriptional initiation as existing 5' to the initiation site. It should not be forgotten that a number of transcriptional elements occur 3' to the initiation site, for example, the immunoglobulin enhancer and the elements associated with sea urchin histone gene transcription (refer to sections 1.2.3(iii)c and e). Such elements provide evidence for a type of intragenic control of transcription. Some of the interactions described above could also be used to

explain how these intragenic elements may function in regulating transcriptional initiation.

(iii) Polymerase III Factors: Regulation of 5S Gene Transcription

In eukaryotic genes the interaction between gene promoter elements and transcription factors in order to produce the regulated expression of a specific gene class is best exemplified by the 5S genes of **Xenopus.**

The **Xenopus** genome contains two types of multigene families encoding the 5S RNA genes. Firstly, the oocyte type which comprise greater than 98% of the animal's 5S genes, these are active in growing oocytes but silent in somatic cells. The second type of 5S RNA gene is the somatic type which comprise only 2% of the animal's 5S RNA genes but encode the majority of the 5S RNA synthesized by somatic cells. The differential expression is known to be controlled at the transcriptional level (Brown, 1984).

A minimum of three different components is required to programme both the oocyte and the somatic genes into an active state. These components, TFIIIA, B and C, form a stable complex with the internal control region of the gene. The RNA polymerase III recognizes the transcription complex but is not an integral part of it. The order of factor attachment has been deduced **in vitro** and is TFIIIA followed by TFIIIC then TFIIIB and finally polymerase III (Bieker **et al.,** 1985). Repressed 5S genes do not have these factors bound to them (Ryoji and Worcel, 1984; 1985) as their repressed state is maintained by a nucleosome structure dependent on histone H1 (Brown 1984). The internal control regions of the two types of 5S gene families

have differing affinities for TFIIIA, the somatic type having a 4-fold greater affinity for TFIIIA binding (Wormington **et al.**, 1981). It has been shown by injection of TFIIIA into cleaving embryos that much of the preference for somatic 5S RNA transcription over oocyte 5S RNA transcription in somatic cells is due to the concentration of TFIIIA and to the differences in TFIIIA binding affinity for the two types of genes (Brown and Schlissel, 1985).

All of the above observations provided direct support for a model explaining the asymmetric distribution of active and repressed 5S genes (Brown, 1984). During oogenesis when TFIIIA concentration is in excess, large amounts of both types of 5S RNA are synthesized, the dominance of the oocyte type relating to the higher genomic copy number. The RNA accumulates and TFIIIA concentration decreases as it is recruited into 7S ribonucleoprotein storage particles (Korn, 1982). The 4-fold greater binding affinity of the somatic type genes for TFIIIA results in most of the stable transcription complexes being of the somatic type as the oocyte type 5S genes which have failed to recruit transcription factors become repressed through the binding of H1 histone. The oocyte genes are fully repressed by the end of gastrulation (Brown, 1984).

It was suggested further by Brown (1984) that once the genes are committed for activity by the formation of an active transcription complex binding to the internal control region that the stability of the complex could maintain gene activity.

The structure of TFIIIA itself suggests an explanation of how the 5S transcription complex can remain stable despite the

repeated passage of polymerase III. It has been suggested, from amino acid sequence analysis and footprinting analysis (Smith **et al.**, 1984), that TFIIIA is composed of nine flexible finger-like domains which are the proposed DNA binding regions (see Enver, 1985 for review). It is thought that TFIIIA makes contact with only one strand of the helix (Sakonju and Brown, 1982). Hence, the fingers of TFIIIA, normally interdigitating on one side of the helix could disengage individually to allow polymerase passage whilst the remaining parts of TFIIIA are bound by the finger-like domains. Both the strand specificity and the multiple contact binding of TFIIIA are features in common with Spl which binds to short GC boxes (see section 1.2.4(ii)). Multiple contact binding may be a general feature of eukaryote DNA binding proteins.

1.2.5 EFFECTS OF SUPERCOILING AND HIGHER ORDER STRUCTURE

It is mainly for the purpose of simplicity that DNA has been considered during much of this discussion as though it exists as a simple helix. This is obviously not the case. Cells, prokaryotic and eukaryotic, expend considerable energy to generate negatively supercoiled DNA and to maintain the amount of supercoiling at a rather constant level.

The torsional stress generated within the DNA helix by supercoiling has been shown to have direct effects on certain prokaryote promoters both **in vivo** and **in vitro**. Supercoiling has been shown to decrease as well as increase gene expression in prokaryotes (Gellert, 1981). From **in vitro** studies it appears that a greater diversity in promoter strengths results from supercoiling than from promoter interactions or effector molecules (refer to McClure, 1985 for review).

Supercoiling provides any cell with a powerful mechanism for the regulation of transcription because the overall superhelical stress of closed circular or looped DNA can be altered by a change anywhere in the domain of such a structure. In eukaryotes, for example, this has implications for communication between regulatory elements dispersed over considerable distances and the transcriptional machinery during the process of transcription initiation. It could also be envisaged that DNA supercoiling in both prokaryotes and eukaryotes could affect initiation frequency directly either by favouring or disfavouring the binding of specific protein effectors.

In eukaryotes, the structure of chromatin adds another dimension in complexity to the process of transcriptional initiation. The supercoiled DNA in association with histones is packaged into nucleosome. Strings of nucleosomes wind up into arrays resembling solenoids to form fibres approximately 30 nm in diameter (Igo-Kemenes **et al.**, 1982). One of the major problems with this highly compacted structure is that it potentially prevents the interaction of specific effector molecules with promoters. Thus the structure needs to be unwound to enable transcription to be initiated.

Transcriptionally active genes **in vivo** appear to be free of nucleosomes in the 5' or 3' gene flanking regions and therefore are hypersensitive to digestion by nucleases (Wu, 1980; Elgin, 1984). Studies in the 5' hypersensitive domain of the adult chicken β-globin gene have delineated a hypersensitive sequence extending from about -70 to -270 nucleotides from the cap site (McGhee **et al.**, 1981). Hypersensitivity to nucleases in this domain is only observed

in chicken cells that express this gene. By using footprinting, specific protein factors have been observed to bind to this region **in vitro** (see table 1-1, Emerson **et al.**, 1985).

Recent footprinting experiments on intact chicken erythrocyte nuclei have identified regions within the 5' hypersensitive domain that are protected against nuclease attack (Jackson and Felsenfeld, 1985). The pattern of protection is similar to that observed **in vitro**. It appears, at least in the case of the adult chicken β-globin gene, that nucleosomes are disrupted in the promoter regions making way for the attachment of specific regulatory proteins. This could probably be extended to other eukaryotic genes. Details of the controls that bring about such an exposure are unknown. In general, little is known about the regulation of transcription from the level of higher order chromatin structure.

1.2.6 TRANSCRIPTIONAL INITIATION - CONCLUDING COMMENTS

The promoters of genes can be considered to be the sites of assembly of multiprotein complexes. Promoters contain distinctive control sequences which are required for gene expression. The way that these sequences act to modulate transcription involves specific factors that interact with the DNA and also influence the core transcriptional machinery to initiate RNA synthesis from particular sites. The rate - limited assembly of all the components in the form of a stable transcription complex probably enables careful modulation of transcription initiation.

Once many of the factors that regulate the expression of

genes in eukaryotes have been identified and the way in which they work is understood, attention will then focus on what regulates the regulators; a process already underway in prokaryotes.

1.3 THE XENOPUS OOCYTE TRANSIENT EXPRESSION SYSTEM

The value of oocytes for analysis of the expression of introduced genes has been frequently reviewed. Most of the information on the oocyte system summarized below was obtained, unless otherwise stated, from the following sources, Gurdon and Melton (1981), Wickens and Laskey (1982) and Colman (1984).

Oocytes contain between 10^4 and 10^5 times more of the following constituents than a typical somatic cell: ribosomes; RNA polymerase I, II and III; ribonucleotide triphosphates, and histones H1, H2A, H2B, H3 and H4. The large store of transcriptional machinery in the oocyte has made it an attractive system for use in transcriptional studies.

The 1.2 mm diameter of a fully developed oocyte makes it an amenable subject for microinjection. Although the nucleus is not visible, microinjection can achieve an 80% success rate in depositing DNA. Oocytes are easy to maintain in culture, the equipment for microinjection is relatively simple and the injection procedure is easy to learn.

DNA must be injected into the nucleus, as progressive degradation of introduced DNA occurs in the cytoplasm. The introduced DNA is not integrated into the **Xenopus** genome. Circular DNA molecules are the most useful to inject because they are stable for as long as the oocyte remains viable. The

stability of circular DNA is probably a consequence of its assembly into a chromatin-like structure. Oocytes have the capacity to assemble at least 12 ng of DNA into minichromosome structures which have many properties in common with normal chromatin. Double stranded DNA molecules are not replicated in the oocyte nucleus but single stranded circular DNA molecules are converted into double stranded molecules. The large store of transcriptional machinery means that large amounts of DNA can be injected. For genes transcribed by RNA polymerase II and III maximal RNA synthesis is elicited by an injection of 10 ng of DNA per nucleus.

Genes transcribed by polymerase II have been observed to be transcribed with fidelity when introduced into **Xenopus** oocytes. The transcriptional fidelity of polymerase II transcribed genes appears to depend on whether the oocyte contains the appropriate factors for the transcription of a particular gene. For example, housekeeping genes such as histones (Stunnenberg and Birnstiel, 1982) and HSV-tk (McKnight, 1980) are transcribed efficiently in oocytes and the transcripts are correctly initiated and terminated. An exception is the sea urchin H3 histone gene which terminates inefficiently (Hentschel **et al.**, 1980). Some tissue specific genes such as the immunoglobulin genes are expressed inefficiently (Bentley **et al.**, 1982), others like the rabbit β-globin gene are not expressed at all (de Villiers **et al.**, 1982). In contrast, the tissue specific human zeta-globin genes are transcribed well in oocytes (Proudfoot **et al.**, 1984).

Problems associated with transcriptional initiation and subsequent processing have been overcome by co-injecting

protein components from nuclei of cells from which the gene was derived (Stunnenberg and Birnstiel, 1982; Wigley, 1986). Such an approach was also used in this study and this aspect will be discussed further in chapter 6.

1.4 THESIS AIMS

The aims of this project were as follows:

1. To examine the general control features of feather keratin gene transcription using **Xenopus** oocytes.
2. To use the **Xenopus** oocyte to study gene transcription using the well characterized chicken feather keratin genes in direct comparison with genes not involved in terminal differentiation, specifically, histone genes and HSV-tk.
3. To assess the feasibility of modifying existing expression systems and if necessary to develop a new system in which to study the tissue and stage specific controls involved in feather keratin gene expression.

CHAPTER 2.

MATERIALS AND METHODS.

2.1 ABBREVIATIONS

Abbreviations are as described in "Instructions to authors" (1978). Additional abbreviations are listed below.

BCIG: 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

bis acrylamide: N,N'-methylene-bisacrylamide.

BME: 2-mercaptoethanol.

CAT: chloramphenicol acetyl transferase.

ddNTP: dideoxynucleotide triphosphate.

DMEM: Dulbecco's modified Earle's medium.

DTT: dithiothreitol.

EDTA: ethylenediaminetetra-acetic acid.

EGTA: ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra-acetic acid.

HEPES: N-2-hydroxyethylpiperazine-N'-2ethane-sulphonic acid.

IPTG: isopropyl-β-D-thio-galactopyrosine.

PEG: polyethylene glycol.

PIPES: piperazine-N,N'-bis-(2-ethane-sulphonic acid).

PMSF: phenyl-methyl sulphonyl fluoride.

SDS: sodium dodecyl sulphate.

TEMED: N,N,N',N'-tetramethyl-ethylene-diamine.

2.2 CHEMICALS AND REAGENTS

All chemicals were of analytical reagent grade, or of the highest available purity. Most chemicals and materials were obtained from a range of suppliers, the major source of the more important chemicals and reagents are listed below.

Acrylamide, agarose, ATP, ddNTPs, dNTPs, DTT, bisacrylamide, PMSF and chloramphenicol: Sigma.

Low melting point agarose: B.R.L.

Mixed bed resin AG 501-X8(D): Bio-rad.

TEMED and Xylene cyanol: Tokyo Kasei.

Urea (ultra pure): Merck.

Bromocrescol purple, formamide and PEG 6000: BDH.

M13 universal primer γ-^{32}P-ATP, α-^{32}P-dCTP, α-^{32}P-dATP, α-^{32}P-rGTP (specific activity, >2000 Ci/mmol): Biotechnology Research Enterprises of S.A. (BRESA).

D-threo-(dichloroacetyl-1-^{14}C)-chloramphenicol (54 mCi/mmol): Amersham.

G418 and DMEM: GIBCO.

Tryptose phosphate broth: DIFCO

Fetal calf serum: Commonwealth Serum Laboratories.

Gentamicin and fungizone: Schering Corp., USA.

2.3 ENZYMES

Enzymes were obtained from the following sources:

Acetyl coenzyme A: gift from Dr. J. Wallace, this department.

AMV reverse transcriptase: Molecular Genetic Resources.

Calf intestinal phosphatase and Ribonuclease A: Sigma.

Chloramphenicol acetyl transferase: P.L. Biochemicals.

E. coli DNA-polymerase I, Klenow fragment: Boehringer Mannheim; BRESA.

Proteinase K: Boehringer Mannheim.

Restriction endonucleases: Boehringer Mannheim; New England Biolabs.

S1 nuclease: Boehringer Mannheim.

T4 DNA ligase: New England Biolabs; BRESA.

T4 polynucleotide kinase: Boehringer Mannheim.

Trypsin 1:250: DIFCO.

2.4 GENERAL BUFFERS AND BACTERIAL MEDIA

All bacteria, except JM101, were grown in L-broth or on L-agar plates. JM101 was grown in minimal medium, 2 x YT broth and on minimal plus glucose plates.

L-broth: 1% (w/v) amine A, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0.

L-agar plates contained L-broth with 1.5% (w/v) bacto-agar.

Minimal medium: 2.1% (w/v) K_2HPO_4, 0.9% (w/v) KH_2PO_4, 0.2% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) tri-sodium citrate.

Minimal plus glucose plates contained minimal medium, 0.4% (w/v) glucose, 0.0001% (w/v) thiamine and 1.5% (w/v) bacto-agar.

2 x YT broth: 1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.0.

All buffers and media were prepared with distilled and deionised water and sterilized by autoclaving (20 psi for 25 minutes at 140°C) except heat labile reagents, which were filter sterilized. Glassware and other utensils were rendered RNAse and DNAse free by autoclaving.

2.5 TISSUE CULTURE MEDIA

DMEM, and tryptose phosphate broth were prepared according to the manufacturer, except that gentamicin was added to the DMEM solutions (50 μg/ml). Tissue culture media were prepared using distilled water and were filter sterilized

before use.

2.6 BACTERIAL STRAINS

MC1061 (Casadaban and Cohen, 1980) was a gift from Dr. R. Sturm, this department.

JM101 (Messing, 1979) was a gift from Dr. A. Robins, this department.

2.7 CLONED DNA SEQUENCES

λCFK1 and feather keratin subclones were gifts from Dr. K. Gregg, this department.

Cosmid 4 was a gift from R. Presland, this department.

p7AT, pJL4Bam5 and M13H2BX-X were gifts from Dr. R. Sturm, this department.

H5 was a gift from P. Wigley, this department.

pSV2CAT, pSV2Neo and the SV40 enhancer containing clone were gifts from Dr. M.F. Shannon, this department.

pBR322/SV-0+T/tk was a gift from Dr. M. Capecchi, University of Utah.

1090/-29 was a gift from Dr. J. Sorge, Scripps Clinic, La Jolla, California.

2.8 CLONING VECTORS

pBR322 was a gift from Dr. R. D'Andrea, this department.

M13mp7, M13mp8, M13mp9, M13mp18, M13mp19 were all gifts from Dr. C.P. Morris, this department.

2.9 SYNTHETIC OLIGONUCLEOTIDES

Synthetic DNA primers were synthesized by Dr. D. Skingle and S. Rogers of BRESA. The various primer sequences are

listed below.

H1 26-mer, 5'-dGGCGGGAGCGGTCTCGGACATCGCGG-3'.

H2B 26-mer, 5'-dGGCTCGGGCATAGTGGCACAACGCGC-3'.

H5 26-mer, 5'-dATGGCCGCCGCTTCACCCCACGGCAG-3'.

(H1 and H2B were a gift from Dr. R. Sturm and the H5 primer was a gift from P. Wigley).

Keratin 25-mer, 5'-dTTGGGCCACAGGGACGGCACAGATC-3'.

Keratin 26-mer, 5'-dGAGGGCTCATTGCAGCTGTTGGCCAG-3'.

Keratin 20-mer, 5'-dTGACCTTGTTCACCAACCAG-3'.

Keratin Sp 25-mer, 5'-dTGTTCTGGGGGAAGGAGCTGAGGAT-3'.

HSV-tk 26-mer, 5'-dGCGCTGGCGAAGAGGTGCGGGAGTTT-3'.

The location of the feather keratin primers and the tk primer are shown in figures 3-A and 3-B respectively.

2.10 RECOMBINANT DNA METHODS

2.10.1 GENERAL RECOMBINANT DNA METHODS

The following methods were performed as described in Maniatis **et al.** (1982):

Propagation and maintenance of bacterial and virus strains.

Large scale plasmid preparation by the alkaline lysis procedure.

Purification of closed circular DNA by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients.

Large scale preparation of λ clones.

Quantitation of DNA and RNA.

Manufacture of hybridization probes by random priming.

Nick-translation of DNA.

Kinasing of DNA fragments using T4 polynucleotide kinase.

End labelling of DNA fragments using the Klenow fragment of **E. coli** DNA polymerase I.

Chromatography through Sephadex G-50.

Autoradiography.

Dephosphorylation of DNA.

DNA digestion with restriction endonucleases.

Agarose and polyacrylamide gel electrophoresis.

Recovery of DNA from agarose and acrylamide gels.

Southern transfer, Northern transfer and dot blots.

Subcloning of DNA fragments into plasmid vectors.

Transformation of **E. coli** MC1061 using the calcium chloride procedure.

In situ hybridization of bacterial colonies or bacteriophage plaques.

Rapid small scale isolation of plasmid DNA.

It is important to note that the handling of viable organisms which contained recombinant DNA was carried out under the containment conditions as defined and approved by the Australian Academy of Science Committee on Recombinant DNA and the University of Adelaide.

2.10.2 TRANSFORMATION PROCEDURE FOR M13 RECOMBINANTS

A loopful of **E. coli** strain JM101, from a minimal plus glucose plate, was used to infect 5 ml of minimal medium which was then grown overnight at 37°C with aeration. The overnight culture was diluted 50 fold into 50 ml of 2 x YT and grown with shaking to an A_{600} of 0.4. The cells were pelleted by gentle centrifugation (Sorvall HB-4 rotor, 5000 rpm for 2 minutes), then resuspended in one-twentieth volume of ice cold

50 mM $CaCl_2$ and left on ice for at least 1 hour. 200 µl of this cell suspension was mixed with a sample of the ligation mix and left on ice for 40 minutes. The transformation mix was heated to 42°C for 2 minutes. 3 ml of L-broth containing 0.7% agar, 20 µl of BCIG (20 mg/ml in dimethylformamide), 20 µl of IPTG (24 mg/ml in water) and 0.2 ml of a JM101 overnight culture diluted 1:5 in 2 x YT broth was then added and the mixture plated directly onto a minimal plus glucose plate. Incubation was overnight at 37°C.

2.10.3 DNA SEQUENCING BY THE DIDEOXY-CHAIN TERMINATION METHOD (Sanger et al., 1977)

(i) Preparation of single strand template for sequencing

M13 phage plaques were toothpicked into 1 ml of a fresh JM101 overnight culture (grown in minimal medium) which had been diluted 1:40 in 2 x YT broth. After incubation at 37°C with vigorous shaking for 5 hours, the culture was centrifuged for 5 minutes in an Eppendorf microfuge. The supernatant was poured into an Eppendorf tube containing 200 µl of 2.5 M NaCl, 20% PEG 6000 and left at room temperature for 15 minutes. The single stranded M13 phage particles were collected as a pellet after centrifugation for 5 minutes. The supernatant was aspirated and the pellet resuspended in 100 µl of 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, phenol/chloroform extracted and the DNA recovered by ethanol precipitation. The DNA pellet was resuspended in 25 µl of 10 mM Tris-HCl pH8.0, 0.1 mM EDTA and stored frozen at -20°C.

(ii) Sequencing reactions

Four separate reactions, each specific for one of the

bases in DNA, were used to analyze the sequence of the insert in the M13 single stranded template. In each of the sequencing reactions, the M13 universal primer was extended in the presence of a different ddNTP such that there was a partial incorporation of each, which resulted in termination of synthesis.

The method described below is for the sequencing of one M13 clone but is readily expanded to allow the concurrent sequencing of 8 to 16 clones.

(ii) a. Hybridization

2.5 ng of universal primer (17-mer) was annealed to 5 µl of M13 single-stranded template in a 10 µl volume containing 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$ by heating the solution to 70°C for 3 minutes and then hybridizing at room temperature for 45 minutes.

(ii) b. Polymerization

1 µl of α-^{32}P-dATP (approximately 7 µCi) was lyophilized, the hybridization mix was added, vortexed to dissolve the labelled dATP and then 1 µl of 10 mM DTT added. 1.5 µl of each of the appropriate zero mixes (T^{o} for ddTTP: 10 µM dTTP, 200 µM dCTP, 200 µM dGTP, 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA; C^{o} for ddCTP: 200 µM dTTP, 10 µM dCTP, 200 µM dGTP, 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA; G^{o} for ddGTP: 200 µM dTTP, 200 µM dCTP, 10 µM dGTP, 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA; A^{o} for ddATP: 200 µM of dTTP, dCTP and dGTP, 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and ddNTP solutions (0.1 mM for ddCTP and ddATP, 0.5 mM for ddTTP and ddGTP, each in water) were added together. 2 µl of the zero-ddNTP mixtures were added

separately to four Eppendorf "reaction tubes".

0.5 μl of DNA polymerase I, Klenow fragment (1 unit/μl) was added to the hybridization mixture-label-DTT solution. 2 μl of this was then added to each of the four reaction tubes and the solutions were mixed by centrifugation for 1 minute. After 10 minutes incubation at 37°C, 1 μl of dATP chase (500 μM dATP in 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA) was added to each of the four tubes, mixed by a 1 minute centrifugation and incubated for a further 15 minutes at 37°C.

3 μl of formamide loading buffer (100% formamide, 0.1% (w/v) bromo cresol purple, 0.1% (w/v) xylene cyanol and EDTA to 20 mM was added to stop the reactions and the solutions were mixed by a short centrifugation. Samples were boiled for 3 minutes and then loaded onto a sequencing gel.

(iii) Sequencing gels

Products of the dideoxy-chain terminator sequencing reactions were separated by electrophoresis on polyacrylamide gels which included 7 M urea as a denaturant. The gels used were 40 cm x 40 cm x 0.35 mm. A 6% gel was normally used and it was made in the following way. An 85 ml mixture of acrylamide monomer (20:1, acrylamide to bisacrylamide) in TBE buffer containing 7 M urea, was prepared, 800 μl of 10% (w/v) ammonium persulfate and 65 μl of TEMED added, the mixture poured into a gel mold and allowed to polymerize.

Reservoir tanks contained about 4 litres of TBE buffer and the gels were pre-electrophoresed for 45 minutes at 30 mA. Debris and urea were removed from sample wells prior to loading by flushing with buffer from a syringe. All gels were run at 25-40 mA and kept at high temperature during

electrophoresis to facilitate DNA denaturation.

Gels were fixed with 200 ml of 10% (v/v) acetic acid and washed with 2 litres of 20% (v/v) aqueous ethanol. After drying, the gels were autoradiographed overnight at room temperature.

2.10.4 KINASING OF SYNTHETIC OLIGONUCLEOTIDES

Primers were labelled at their 5' ends with T4 polynucleotide kinase and γ-^{32}P-ATP. Normally 50-100 ng of synthetic primer was kinased in a 10 µl reaction containing 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 5 mM DTT, 10 µl of lyophilized γ-^{32}P-ATP (approximately 70 µCi) and 1 unit of enzyme. The reaction was incubated for 45 minutes at 37°C where upon 5 µl of formamide loading buffer was added (2.10.3(ii)b) and the mixture loaded onto a 20% polyacrylamide gel for purification.

2.11 INJECTION OF XENOPUS OOCYTES AND ANALYSIS OF OOCYTE RNA

2.11.1 ANIMALS

Xenopus laevis females were obtained from Dr. Ray Harris (South Australian Institute of Technology, Pharmacology Department) or Dr. Keith Dixon (Flinders University, School of Biological Sciences). A breeding stock is maintained at Flinders University whereas the Institute colony of 50 females was bought from Ring Shipping Pty. Ltd., Cape Town, South Africa. For best results, wild-type females were maintained under laboratory conditions for at least six months prior to harvesting of oocytes.

2.11.2 OOCYTE INJECTION

The microinjection technique and the conditions used for oocyte culture have been described (Colman, 1984). In each experiment batches of 30-50 oocytes were injected. Unless otherwise stated each oocyte nucleus received 5 ng of test DNA and also (when necessary) 5 ng of p7AT DNA (section 3.1) in a total injection volume of 50 nl. The oocytes were incubated at 18°C for 22-24 hours in Barths' saline.

2.11.3 ISOLATION OF RNA FROM XENOPUS OOCYTES (Probst et al., 1979)

The oocytes were squashed in a loose-fitting glass homogenizer in a solution containing 10 mM Tris-HCl pH 7.4, 1.5 mM MgCl, 10 mM NaCl, 1% SDS and 0.5 mg/ml proteinase K (0.5 ml of this solution was used per batch of oocytes). After incubation at room temperature for 25 minutes, EDTA was added to 10 mM and the mixture extracted three times with phenol/chloroform. The aqueous phase was made to 0.2 M NaCl and the oocyte RNA precipitated by the addition of 2.5 volumes of ethanol. Each oocyte usually yielded 4-5 μg of total RNA. Oocytes were stored dry at -80°C if the RNA extraction was not carried out immediately after incubation.

2.11.4 S1 NUCLEASE ANALYSIS OF OOCYTE RNA

For 3' end analysis, double stranded DNA restriction fragments were end-filled using DNA polymerase I Klenow fragment in the presence of α-^{32}P-dNTPs. The double stranded restriction enzyme fragments were heat denatured and snap chilled. Fragment hybridization to RNA was carried out in a

volume of 30 µl. The reaction mix contained 80% formamide, 40 mM PIPES, pH 6.4, 1 mM EDTA, 400 mM NaCl and incubations were at 50°C for 12-16 hours. Annealing reactions were diluted with 300 µl of ice-cold S1 buffer (200 mM NaCl, 2 mM $ZnSO_4$, 50 mM sodium acetate, pH 4.6) and 1,000-2,500 units of S1 nuclease were added. Digestions were at 37°C for 1 hour. The mixture was then phenol/chloroform extracted, nucleic acids precipitated with ethanol, and dissolved in formamide loading buffer and electrophoresed on 6% sequencing gels (2.10.3(iii)) prior to exposure.

2.11.5 PRIMER EXTENSION ANALYSIS OF RNA (McKnight et al., 1981).

Typically, 1.5 ng of kinased primer was added to the RNA equivalent of two injected oocytes (8-10 µg of total RNA) and ethanol precipitated. The pellet was resuspended in 10 µl of 200 mM NaCl, 10 mM Tris-HCl pH 8.3. Primers were hybridized to the oocyte RNA for 2 hours at 65°C for double stranded primers (after heat denaturation) and 2 hours at 42°C for single stranded synthetic primers. Following hybridization, the samples were incubated with reverse transcriptase in a volume of 34 µl containing 60 mM NaCl, 10 mM Tris-HCl pH 8.3, 10 mM DTT, 500 µM dNTPs, 10 mM $MgCl_2$ and 8 units of enzyme, for 1 hour at 42°C.

Extension products were ethanol precipitated, washed in 70% aqueous ethanol and dried **in vacuo**. After resuspension in 5 µl of formamide loading buffer, the extended primers were heat denatured at 100°C for 5 minutes and electrophoresed on 6% or 10% sequencing gels and detected by exposure to X-ray film. For the purposes of densitometric scanning, X-ray

films were flashed before exposure (Laskey and Mills, 1975).

2.11.6 MEASUREMENT OF RNA ACCUMULATION IN XENOPUS OOCYTES

Each oocyte was injected with 10 ng of test DNA. Twenty four hours later 1 µCi of α-^{32}P-rGTP was injected into each oocyte. A sample of 10 oocytes was removed at varying times after the injection (2, 5, 10 and 24 hours) with each sample being frozen at -80°C until all samples had been collected. The RNA was extracted as described in section 2.11.3.

The amount of labelled RNA transcribed from the DNA injected was determined by hybridization to nitrocellulose bound DNA. For each time point, a set of 4, 8 x 8 mm nitrocellulose filters was prepared. Two filters were blanks and the other filters contained 0.5 µg of spotted, denatured gene fragment resected from the plasmid stock which was used for the original oocyte injection. Each set of filters was baked at 80°C **in vacuo** for 1.5 hours and then prehybridized for 2 hours at 60°C in sealed plastic bags containing 1 ml of 0.05 x Blotto in 6 x SSC (1 x Blotto = 5% w/v nonfat dry skim milk, 0.01% Antifoam A; Johnson **et al.**, 1984). After prehybridization the solution was removed and replaced with the equivalent of 10 oocytes worth of labelled RNA in 1 ml of 50% formamide, 0.05 x Blotto, 6 x SSC. The sealed bags containing the filters were incubated at 42°C for 16 hours. All filters from all time points were washed twice for 30 minutes with 0.05 x Blotto, 2 x SSC and 1% SDS at room temperature. Final washes were twice for 30 minutes each at 55°C in 0.1 x SSC, 0.1% SDS. The filters were dried and the amount of labelled RNA which hybridized was determined by scintillation spectroscopy.

2.11.7 DETERMINATION OF THE RATE OF RNA DEGRADATION IN OOCYTES

The procedure used was essentially that described by Miller **et al.** (1982). Oocytes were injected with 10 ng of the DNA template and 1 μCi of α-^{32}P-rGTP and incubated for 24 hours at 18°C. Each oocyte was then injected intranuclearly with 10 nl of α-amanitin (100 μg/ml) which has been found to inhibit greater then 95% of RNA polymerase II activity (Miller **et al.,** 1982). Batches of 10 oocytes were collected at varying times after the α-amanitin injection and the amount of labelled transcript remaining at each time was determined by hybridization to nitrocellulose bound DNA as described in section 2.11.6.

2.11.8 QUANTITATION OF TRANSCRIPT LEVELS FROM AUTORADIOGRAPHS

Each track of the autoradiographs obtained was scanned along its centre using either a Joyce Loebel densitometer or an LKB 220Z Ultrascan Laser densitometer with integrator. In experiments involving the SV40 enhancer, the intensities of the bands of interest were determined by cutting out and weighing the respective peaks from the densitometry trace. The intensity of the extension product band representing H2B mRNA was used as an internal standard. The degree of enhancement E was calculated by the equation:

$$E = \frac{W_e}{W_{ht}} \cdot \frac{W_{hc}}{W_o}$$

where W_e and W_o represent primer extension levels for enhancer^{+} and enhancer^{-} constructs respectively. W_{ht} and W_{hc}

represent the primer extension levels for histone gene controls in the test (enhancer$^+$) and control (enhancer$^-$) tracks respectively. The degree of enhancement was calculated from a series of experiments using oocytes from different frogs.

2.11.9 PURIFICATION OF OOCYTE POLY(A)$^+$ RNA

Oligodeoxythymidylic acid cellulose was prepared and used to select poly(A)$^+$ RNA essentially as described by Aviv and Leder (1972).

2.12 PREPARATION OF RNA, NUCLEI AND CHROMATIN WASH FRACTIONS FROM FEATHER TISSUE

2.12.1 FEATHER TISSUE

Fertilized eggs of Leghorn fowls (**Gallus domesticus**) were obtained from the Parafield Poultry Research Station of the Department of Agriculture, Parafield, South Australia. The eggs were stored at 37°C, 54% humidity in a forced draught incubator. Feathers designated as, for example, "14 day feathers" were from embryos incubated for a total of 14 days.

Embryos were removed from the eggs and washed with Hank's balanced salt solution. They were then immersed in the same solution and the body feathers removed by plucking with jewellers' forceps into ice cold KCl buffer (200 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4). The feathers were then washed in ice-cold KCl buffer by repeated slow speed centrifugation performed at 4°C.

2.12.2 POLYSOMAL FEATHER mRNA PREPARATION AND POLY(A)$^+$ RNA PREPARATION

The feathers which were plucked and washed as described in 2.12.1 were pelleted by centrifugation at 3,000 rpm in a bench MSE centrifuge at 4°C. All subsequent procedures were carried out at 4°C. The pelleted feathers were resuspended in two volumes of KCl buffer containing 10 mM DTT and allowed to stand on ice for 10 minutes prior to homogenization by about 10 strokes in a 10 ml glass teflon homogenizer. Cell debris and nuclei were pelleted by centrifugation at 12,000 rpm for 10 minutes at 4°C using a Sorvall HB-4 swing out rotor. The supernatant, termed the feather lysate, was carefully withdrawn by pasteur pipette ensuring that the pellet remained undisturbed.

Feather polysomes were prepared essentially as described by Partington **et al.** (1973). The polysomes were pelleted from the feather lysate by centrifugation in a Beckman Ti50 rotor at 45,000 rpm for 90 minutes at 4°C. Each tube contained 1 ml of 40% w/v sucrose in TK buffer (15 mM KCl, 10 mM Tris-HCl pH 7.4) layered under the lysate, and during centrifugation the sucrose formed a pad which minimized protein contamination of the polysome pellet. The supernatant was decanted and the pellet rinsed with TK buffer. Polysome pellets were resuspended in 2.7 ml of 0.1 M Tris-HCl pH 9, after which 0.3 ml of 1 M Tris-HCl pH 9, 1% SDS was added.

The solution was phenol extracted twice by the addition of an equal volume of water saturated phenol with gentle shaking for 5 minutes and subsequent centrifugation at 3,000 rpm for 10 minutes in an MSE bench centrifuge. The RNA was

ethanol precipitated and poly(A)$^+$ RNA was selected essentially as described by Aviv and Leder (1972).

2.12.3 NUCLEI EXTRACTION FROM FEATHER TISSUE

Feathers were plucked from chick embryos as described in section 2.12.1 except that the feathers were stored and washed in $^1/_6$ x KCl buffer. The procedure used to extract nuclei was modified from that described by Marshall and Burgoyne (1976). Washed feathers were resuspended in two volumes of homogenizing solution (10 mM Tris-HCl pH 8, 1.5 mM $MgCl_2$, 1 mM KCl, 11.6% sucrose (w/v), 0.1 M BME, 19 mM EGTA, 74 mM EDTA) and homogenized by using 6 strokes of a Potter Elvehjem homogenizer per 10 mls of resuspended feathers. The homogenate was filtered through sterile muslin and layered on top of first underlay solution (10 mM Tris-HCl pH 8, 1.5 mM $MgCl_2$, 1 mM KCl, 47% sucrose (w/v), 0.1 M BME, 11 mM EGTA, 37 mM EDTA). The volume of underlay to filtered homogenate was 1:2 per oakridge tube. The solution was centrifuged at 8,000 rpm in a Sorvall HB-4 swing out rotor for 20 minutes at 4°C. All the supernatant was decanted and the slightly bloody pellet was resuspended in second underlay (10 mM Tris-HCl pH 8, 1.5 mM $MgCl_2$, 1 mM KCl, 72% sucrose (w/v), 0.1 M BME, 3.8 mM EGTA, 13.7 mM EDTA). Two volumes of resuspended pellet was layered on top of 1 volume of second underlay solution and the tubes were centrifuged at 21,000 rpm in a Beckman SW41 rotor for 45 minutes at 4°C. Pellets containing nuclei were pooled and resuspended in solution F (10 mM Tris-HCl pH 8, 1.5 mM $MgCl_2$, 1 mM KCl, 11.6% sucrose, 0.1 M BME, 3.8 mM EDTA). Nuclei were washed solution F by centrifugation at 7,000 rpm in a Beckman JA21 rotor for 5 minutes at 4°C and resuspension

in solution F. The nuclei were counted using a haemocytometer.

2.12.4 FRACTIONATION OF CHROMATIN FROM FEATHER NUCLEI BY SEQUENTIAL SALT WASHES

The procedure used to extract proteins from feather cell chromatin was that described by Stunnenberg and Birnstiel (1982). The feather nuclei were lysed in 80 mM NaCl, 1 mM PMSF, 20 mM EDTA, 2 mM EGTA, pH 6.5, by homogenization in a loose fitting glass homogenizer and the chromatin collected by centrifugation at 5,000 x g for 10 minutes at 4°C. The chromatin was sequentially extracted with 150 mM, 300 mM, 450 mM, 600 mM and 2 M NaCl in 20 mM Tris-HCl pH 7.0, 1 mM EDTA, 0.2 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF and centrifuged at 4°C in a Beckman SW41 rotor for 30 minutes at 22,000 rpm through a 10% (w/v) sucrose cushion in the same buffer. The chromatin extracts were concentrated by ammonium sulphate precipitation (0.35 g/ml) dissolved in and extensively dialyzed against protein injection buffer which also served as a storage buffer (5% glycerol, 20 mM Tris-HCl pH 7.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM EGTA, 0.1 mM PMSF, 0.5 mM DTT). Samples were stored at -80°C.

2.13 TISSUE CULTURE

2.13.1 CULTURE CONDITIONS

Unless otherwise stated, cells were grown in DMEM, 5% fetal calf serum, 50 μg/ml gentamicin and incubated at 37°C in a Forma Scientific water-jacketed incubator. Commonly used cell lines were kept continuously growing in T_{75} flasks,

subculturing every 7-10 days.

2.13.2 TRANSFECTION OF HeLa, CV1 AND Ltk$^-$ TISSUE CULTURE CELLS

Cells were seeded approximately 16 hours before transfection at a density of 4-6x10^5 cells/60 mm plate or 8x10^5-1x10^6 cells/10 cm plate. In the case of 60 mm dishes, 2.5 μg of test DNA together with 10 μg of chicken carrier DNA was transfected using the calcium technique described by Graham and van der Eb (1973). The amount of DNA was doubled for 10 cm plates. The calcium precipitate was left on the cells for 24 hours after which the medium was changed and the cells were left incubating for a further 48 hours. Cells were harvested by scraping with a teflon scraper. Residual medium was removed by pelleting the cells (1,000 rpm, bench centrifuge room temperature) and resuspending in PBS.

2.13.3 EXTRACTION OF RNA FROM HARVESTED TISSUE CULTURE CELLS

Total RNA was extracted from cells by pelleting the PBS washed cells and resuspending them in lysis solution containing 0.5% NP40, 10 mM NaCl, 10 mM Tris-HCl pH 8, 10 mM EDTA (500 μl or 1 ml of solution was used for cells harvested from 60 mm or 10 cm plates respectively). The cells were vigorously vortexed and left on ice for 15 minutes with intermittent mixing. After centrifugation in an Eppendorf centrifuge for 15 minutes to pellet nuclei, the supernatant was decanted and subjected to phenol/chloroform extraction and ethanol precipitation. The samples were DNAse treated , phenol chloroform extracted and then precipitated. The RNA pellets were resuspended in water and stored at -80°C.

2.13.4 EXTRACTION OF DNA FROM HARVESTED TISSUE CULTURE CELLS

The pellet of nuclei remaining after cell lysis (2.13.3) was washed with the lysis solution described above (minus the NP40) to remove cytoplasmic residue. After a 5 minute centrifugation in the Eppendorf centrifuge, the remaining solution was aspirated and the nuclei were resuspended in 1% proteinase K, 0.1% SDS in 50 mM Tris-HCl pH 7.5 and left agitating gently overnight at 37°C. After RNAse treatment the DNA was extracted several times with phenol, then chloroform and precipitated with ethanol. The pellet was resuspended in water and stored at -20°C.

2.13.5 CELL STAINING FOR COLONY COUNTING

Colonies were visualized using DIFF-QUIK (AHS, Australia). The method followed was that provided with the solutions.

2.13.6 FEATHER EPIDERMAL CELL CULTURE

Eight day old embryos were removed from their eggs, decapitated, washed in PBS+A solution (PBS, 0.01% fungizone, 50 μg/ml gentamicin) and placed onto a dry sterile petri dish. As many small skin squares as possible were cut from the backs of 5 embryos, the squares being left floating in small culture dishes in PBS until ready for trypsinization. The skin squares were divided into small groups and trypsinized (0.25% trypsin, 0.025% EDTA) at room temperature in a staggered fashion so that the tissue was exposed to the trypsin solution for the minimum amount of time which would enable epidermal-dermal separation (approximately 5 minutes). Signs of

epidermal-dermal separation were monitored by observation under a dissecting microscope. Epidermis was removed from the dermis using watchmakers' forceps and a 21 gauge needle, then the epidermal pieces were rinsed in PBS and minced by cutting with a sterile 21 gauge needle. These manipulations were also carried out under a dissecting microscope. Minced tissue was collected and stored on ice in DMEM, 5% fetal calf serum until all samples had been collected. Collected tissue was pelleted by gentle centrifugation and further trypsinized in 0.1% trypsin, 0.05% EDTA at $37^{o}C$ for 30 minutes with gentle agitation. Dissociation of the tissue at the end of the trypsinization procedure was facilitated by sucking the tissue up and down through a narrow bore pasteur pipette until single cells and small cell clumps could be observed under the microscope. The cells were spun down (1,000 rpm, 5 minutes at room temperature in a bench centrifuge). The trypsin was removed and the cells were resuspended in DMEM, 5% fetal calf serum. Cells were seeded at high density, approximately $2x10^{5}$ cells/35 mm dish, producing 4 dishes. All manipulations were performed under sterile conditions in a lamina flow cabinet.

2.13.7 CHICK EMBRYO FIBROBLAST CELL CULTURE

Chick embryo fibroblast cultures were made from 9 day old chick embryos essentially as described by Rein and Rubin (1968). Modifications were made to the culture medium, tissue dispersion techniques and the method of cell storage.

Four chick embryos were used. Once the embryos were removed from the eggs they were decapitated, rinsed in PBS+A (2.13.6) and placed onto a sterile petri dish. The wings and feet were removed and the embryos were eviscerated. After

rinsing in PBS the embryos were placed onto a fresh petri dish and minced finely with scissors. The tissue was scooped up into a sterile centrifuge tube and 5 mls of 0.25% trypsin, 0.025% EDTA was added. The tube was gently shaken by inversion at room temperature for 5 minutes. Cell clumps were allowed to settle and the supernatant was drawn off with a wide mouthed pasteur pipette and placed into 5 ml of media (DMEM, 10% tryptose phosphate broth, 5% fetal calf serum). This trypsinization procedure was repeated two times, and the final volume of cell-containing supernatant and media totalled approximately 30 ml.

The cells were spun down (1,000 rpm at room temperature, bench centrifuge) and resuspended in 5 ml of media. To dissociate cell clumps further the suspension was drawn up and down 20 times using a wide mouthed pasteur pipette. This was repeated using a narrow bore pasteur. The cells were spun down again and resuspended in 20 ml of medium and counted. All cells were seeded out at a density of $7x10^6$ cells/10 cm plate and cultured at 37^oC for 14 hours. The medium was changed to remove unattached cells and the fibroblasts were selectively removed by washing the plates for 10 minutes in Ca^{2+} and Mg^{2+} free PBS containing 0.02% EDTA (Rhinewald and Green, 1975). The fibroblasts collected were spun down and resuspended in media and counted. The cells were pelleted and resuspended in freezing medium (DMEM, 10% tryptose phosphate broth, 25% fetal calf serum, 5% DMSO) at a density of $5x10^6$ cells/ml and stored in 2 ml aliquots in sealed plastic vials under liquid nitrogen. This procedure yielded a culture which was at least 95% pure in relation to fibroblast content.

2.13.8 TRANSFECTION OF CHICK EMBRYO FIBROBLASTS

The procedure used to transfect chick embryo fibroblasts was identical to that used for other tissue culture cells with respect to cell seeding and the constituents necessary for forming the precipitate (see section 2.13.2, note that rat carrier DNA was used instead of chicken carrier DNA). However, the precipitate was left on the cells for a period of six hours, removed, and the cells were subsequently shocked for 2 minutes with 15% glycerol in DMEM, 10% tryptose phosphate broth, 5% fetal calf serum (2ml/60mm plate, 4 ml/10 cm plate). The glycerol shock medium was then removed, the culture medium was replaced and the cells were incubated as per the usual transfection procedure.

2.14 VIRUS ASSOCIATED PROTOCOLS

2.14.1 FOCUS FORMATION ASSAY (Vogt, 1969)

Fibroblasts were seeded at a density of 4×10^6 cells/60 mm culture dish (5 ml of DMEM, 10% tryptose phosphate broth, 5% fetal calf serum was used per plate). The cultures were infected within 4 hours of seeding with a high and low concentration of Prague A strain RSV (10^4 and 10^2 focus forming units per plate, kindly donated by M. Lineal, Fred Hutchinson Cancer Centre, Seattle, Washington). The inoculum was left on the plate for 16 hours and the medium was replaced with 5 ml of O.L (0.6% agarose in DMEM, 10% tryptose phosphate broth, 5% fetal calf serum) which was left to harden at room temperature for 10 minutes. Two ml of DMEM, 10% tryptose phosphate broth, 5% fetal calf serum was overlayed on top of the plates and they were incubated at 37°C. The plates were

observed regularly for focus formation relative to control plates, the liquid overlay being changed every 4 days.

2.14.2 INFECTION OF FIBROBLASTS WITH INTACT RSV

Fibroblasts were seeded at a density of $4x10^5$ cells/60 mm dish; DMEM, 10% tryptose phosphate broth, 5% fetal calf serum was used as the medium. Within 4 hours of seeding the cells were inoculated with 10^4 focus forming units of donated Prague A RSV (2.14.1). The plates were left for 16 hours after which the infection medium was removed and the normal medium was replaced. The culture was split every 3 days and 20% of the recovered cells were reseeded and left to grow. The remaining unseeded cells and media were stored at -80^oC.

2.14.3 LYSIS OF VIRUS FROM WHOLE MEDIA EXTRACTS

Virus particles from culture media were lysed by the addition of Triton X-100, Tris-HCl pH 8.3 and DTT such that the resultant concentrations of each constituent were 0.2%, 10 mM and 1 mM respectively in 1 ml of culture medium. The mixture was vortexed briefly and kept on ice until ready for use in the reverse transcriptase assay.

2.14.4 PURIFYING VIRUS FROM CULTURE MEDIUM

The method of purification used was that described by Robinson and Lamoreux (1976).

2.15 ENZYME ASSAYS

2.15.1 REVERSE TRANSCRIPTASE ASSAY

The reverse transcriptase assay used was a modification

of that described by Robinson and Lamoreux (1976). Reactions were carried out in the presence of a poly rG.oligo dC template-primer complex and the ability to incorporate α-^{32}P-dCTP was measured. The poly rG.oligo dC template was prepared by mixing poly rG (M_r >150,000) with an oligo dC 12-mer in a ratio of 20:1 by weight and annealing in 10 mM Tris-HCl pH 8.3. Reverse transcriptase reaction mixtures contained 7 µCi of α-^{32}P-dCTP, 10 µg of template and 5.2 µl of sample adjusted to a final volume of 10 µl in 10 mM Tris-HCl pH 8.3, 60 mM KCl, 5 mM DTT and 10 mM $MgCl_2$. Duplicates were set up for each sample dilution. Reactions were incubated at 42°C for 1 hour and the samples were spotted onto DEAE (81) filter discs. After sample drying the discs were washed twice for 5 minutes with constant agitation in 0.5 M NaH_2PO_4 and then water to remove non-specifically bound label. Discs were then rinsed in 95% ethanol to facilitate drying. The discs were counted for Cerenkow radiation after air drying.

2.15.2 CHLORAMPHENICOL ACETYL TRANSFERASE (CAT) ASSAY

The assay was usually performed on cytosol extracts from transfected cells and the method employed was similar to that described by Gorman **et al.** (1982). Cells scraped from tissue culture plates were spun down (1,000 rpm bench centrifuge) and rinsed with PBS. After another spin the PBS was removed and the cells were resuspended in 0.25 M Tris pH 7.6, 10 mM DTT (100 µl/batch of cells harvested from each 10 cm plate). Cells were freeze/thawed rapidly three times (dry ice/ethanol bath/37°C) and then centrifuged for 5 minutes in an Eppendorf centrifuge. The supernatant was retained and the amount of protein present was determined by the absorbance at 280 nm.

The amount of protein extract used per assay ranged between 25 to 100 μg per test extract. Reaction mixtures contained 0.2 μCi of ^{14}C-chloramphenicol, 0.14 M Tris-HCl pH 7.6, 0.5 mM acetyl coenzyme A and the cytosol extract in a total volume of 180 μl. The reactions were incubated at 37°C for 3 hours and then extracted with 1 ml of ethyl acetate. The ethyl acetate phase was removed and lyophilized after which the residue was resuspended in 20 μl of ethyl acetate.

The resuspended ethyl acetate samples were spotted onto a thin layer chromatography (TLC) plate and dried with a hair dryer. The TLC plate was developed in a mixture of chloroform and ethanol (9:1) until the front had moved about 15 cm. The TLC plate was dried and exposed to X-ray film at -80°C with an intensifying screen.

CHAPTER 3.

GENE CONSTRUCTS.

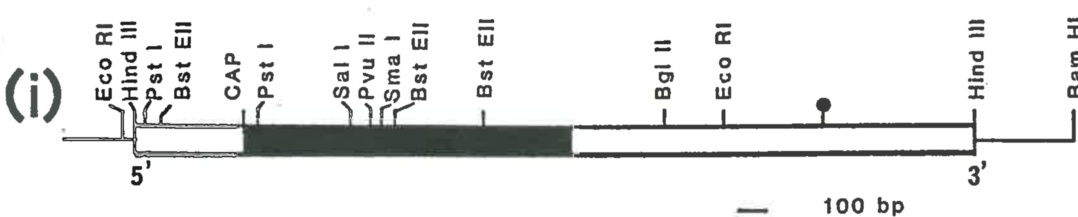
A. INTRODUCTION AND GUIDE TO READER

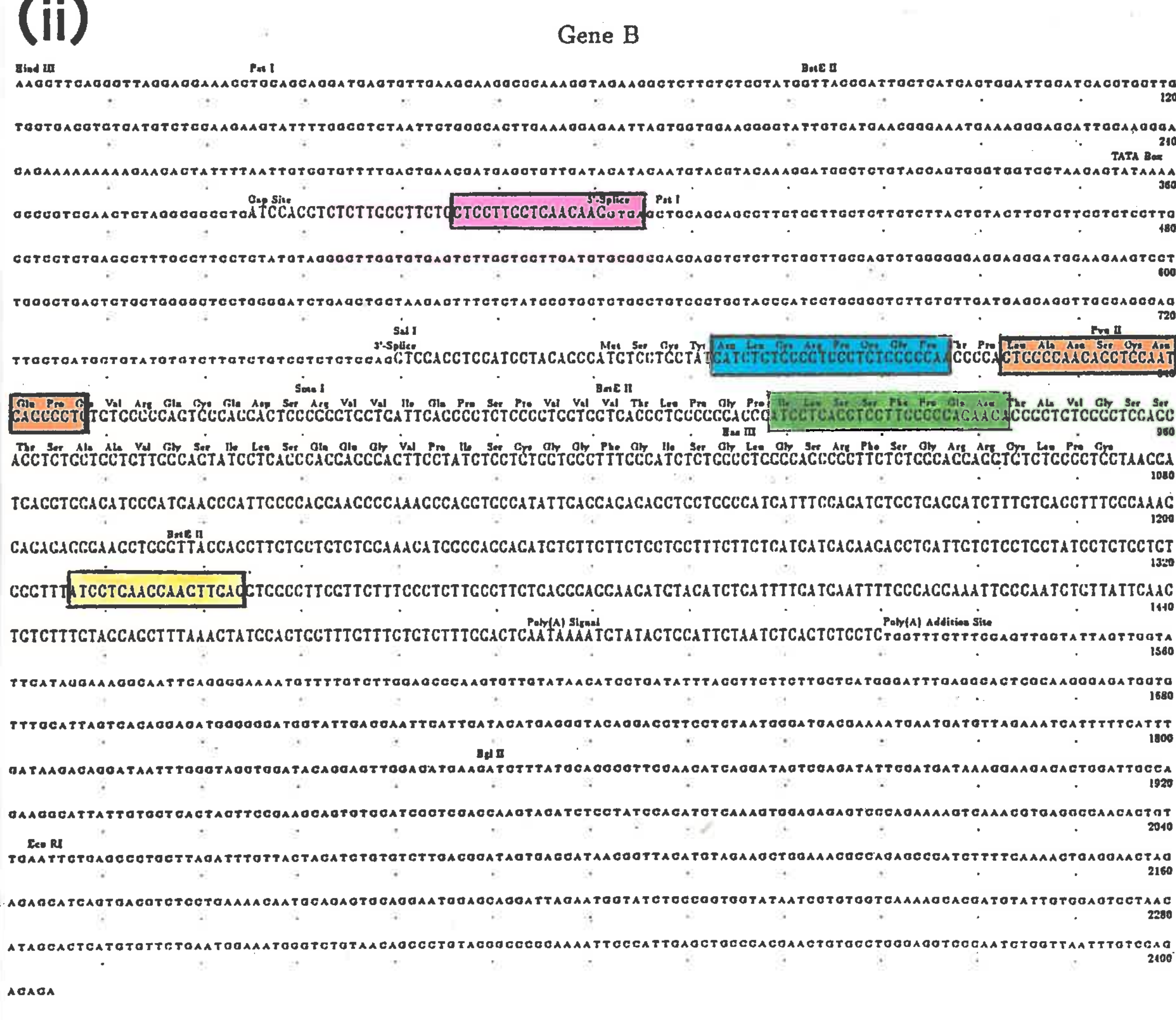
This chapter describes the method of construction of the various recombinant plasmids used in the course of this project. Feather keratin gene B and the HSV-tk gene were the basis for many of the constructs made and their sequences are shown in figures 3-A and 3-B respectively. These figures can be left folded out whilst reading the text of this chapter to facilitate the location of restriction enzyme sites used in the manufacture of a particular construct.

In general, pBR322 was the vector for most of the constructions with MC1061 cells being used in the transformation procedure, however, JM101 cells were transformed with DNA constructs contained in M13 vectors. The various procedures involved in the cloning of DNA fragments were performed as described in Maniatis **et al.** (1982). Where mismatched restriction endonuclease termini were to be ligated, overhangs were treated with **E. coli** DNA polymerase (Klenow fragment) in the presence of deoxynucleotides to produce flush ends. Once made, each construct was checked by restriction enzyme mapping and sequencing. For the sake of brevity, data pertaining to each construction step cannot be presented because of the large number of constructs which need to be described.

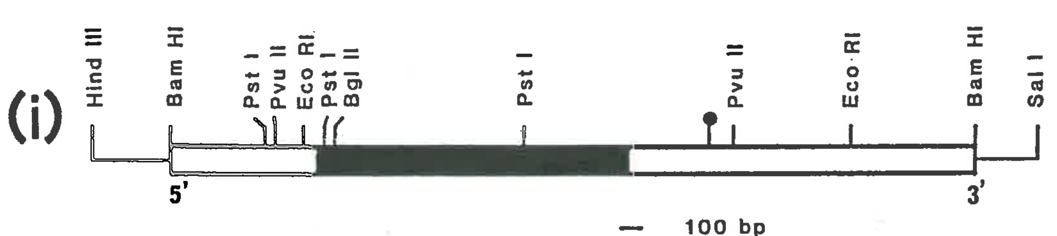
Each construct is assigned a reference number and the method of manufacture acts as a figure legend for the diagram depicted on the opposite page. The diagrams have not been drawn to scale except where indicated. This chapter has also been reproduced in pamphlet form and can be found in a sleeve at the back of this thesis. The pamphlet is intended for use

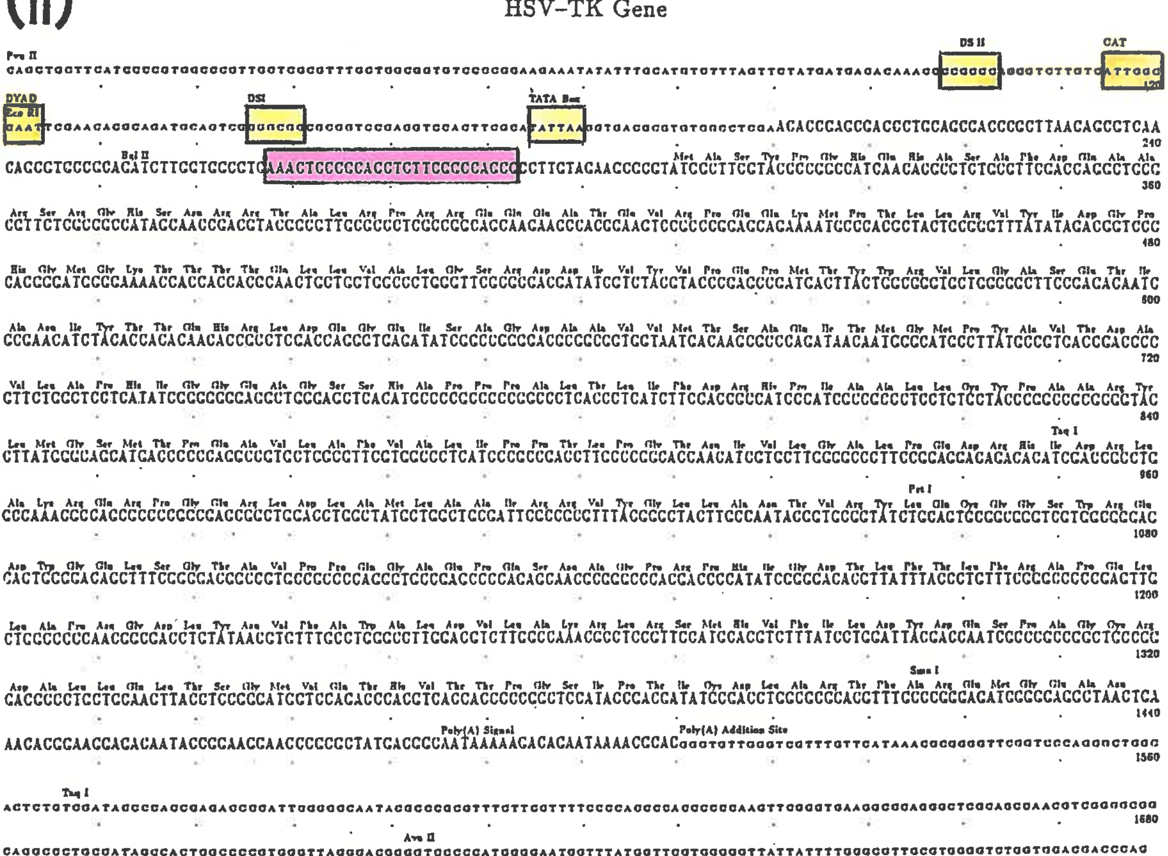
when reading chapters 4, 5 and 6 and will circumvent constant referral to the bound version.





- 20-mer primer
- 25-mer primer
- 26-mer primer
- Sp 25-mer primer
- 18 bp conserved sequence





- promoter related signals
- tk 26-mer

FIGURE 3-A

(i) Feather keratin gene B is contained within a 2.93 kb HindIII fragment which has been inserted into the HindIII site of pBR322. This clone is designated pλCFK1-4. The restriction map of the 2.93 kb fragment shown is drawn to scale and the region that encodes gene B mRNA is shaded.

(ii) The sequence of the 2.93 kb fragment from the 5' HindIII site to the shaded circle in figure 3-A(i). The segment that encodes gene B mRNA begins at nucleotide 385 and ends at nucleotide 1530. The gene has one intron of 337 bases which splits the 5' untranslated region into two sections, 37 and 21 bases respectively. The large capital letters represent the mRNA sequence and the small capital letters the intron sequence and flanking sequences. Other features of interest, for example primer location, are coloured and a key is given at the bottom of the gene sequence. Selected restriction enzyme sites are indicated above the recognized DNA sequence.

FIGURE 3-B

(i) The HSV-1 tk gene is contained within a 3.4 kb BamHl fragment. The fragment was isolated from a clone called pBR322/SV-0+T/tk (Capecchi, 1980) and inserted into the BamHI site of pBR322 (refer to 3.9). Restriction mapping of the fragment showed it was very similar to that described by McKnight (1980) except that the KpnI site was missing. The restriction map of the 3.4 kb fragment shown is drawn to scale. The shaded region represents the segment of DNA which encodes tk mRNA.

(ii) The sequence from the 5' PvuII site to the shaded circle in figure 3-B(i) (taken from McKnight, 1980). The segment that encodes tk mRNA extends from nucleotide 201 to nucleotide 1510. There are no introns in this gene. Large capital letters represent tk mRNA sequence and small capital letters represent flanking sequence. The various features of interest are coloured and a key to the colours is given at the bottom of the sequence.

3.1 97, 3.2 24, 3.3 70

In these constructs, feather keratin gene A was inserted into the chicken histone cluster, p7AT. A more detailed map of p7AT is given on the left hand side of the diagram. The purpose of these clones was to determine whether gene A was being packaged into inactive chromatin in oocytes (section 4.2.1).

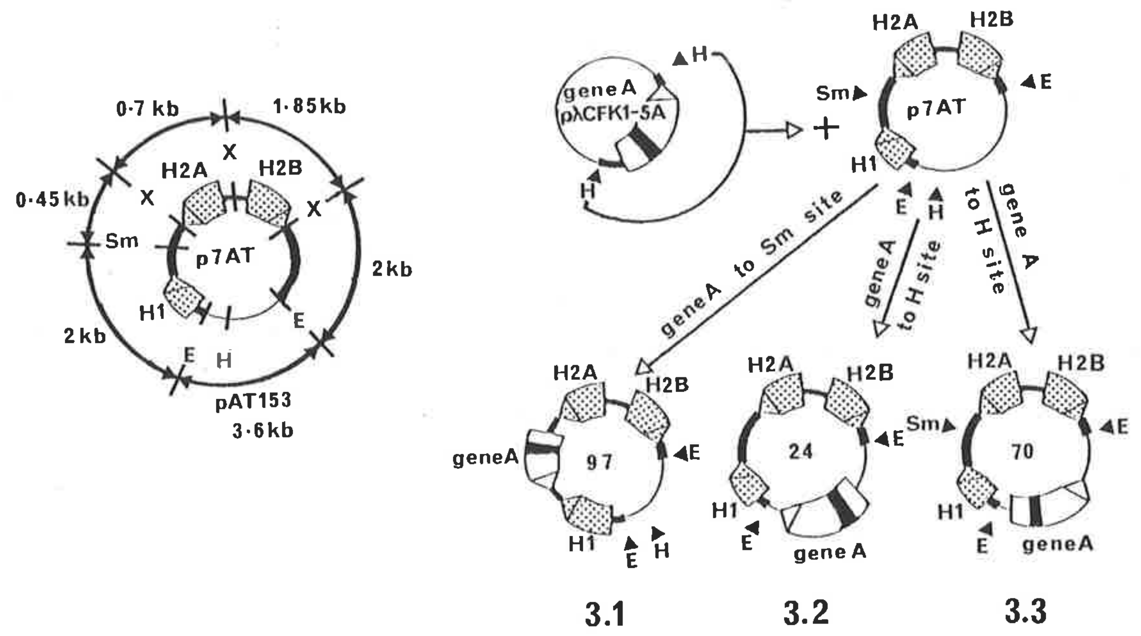
Two vectors were constructed by digesting p7AT with HindIII or SmaI. Gene A was resected from the subclone, pλCFK1-5A by digestion with HindIII (fragment = 2.15 kb). Some of the isolated fragment was inserted into the HindIII cut p7AT vector, generating clones 24 and 70 which contained gene A in two different orientations relative to the direction of transcription of the H1 histone gene. Clone 97 was constructed by inserting the isolated gene A fragment into the SmaI site of p7AT. The size of each of the constructs generated is 12.75 kb.

3.4 1-4E8

1-4E8 is a feather keratin gene B clone which has only one HindIII site; the one 5' to the mRNA start site in pλCFK1-4. This clone was used as the basis for a number of recombinant plasmids.

1-4E8 was constructed by digesting pλCFK1-4 with BglII and BamHI (figure 3-A). After electrophoresis the 5.86 kb fragment, lacking the 1.43 kb bp BglII/BamHI fragment was isolated and the compatible cohesive ends were religated, destroying both the BglII and the BamHI site. The size of the clone is 5.86 kb.

3.1, 3.2 and 3.3



3.4

In all diagrams shown, the following apply, unless otherwise stated: the broad arrows represent the segment of DNA which encodes the mRNA of the gene. The direction of the arrow indicates the direction of transcription. Plain arrows refer to keratin genes, stippled to histone genes and slant-shaded arrows to tk sequences. The shaded block within the arrows representing keratin genes depicts the position of the intron. The thick dark lines represent flanking regions pertaining to each gene and the thin lines pBR322 or M13 derived DNA. A = AluI, Av = AvaII, Ba = BamHI, Bg = BglII, Bs = BstEII, E = EcoRI, EF = end-filled, F = FnuDII, Ha = HaeIII, H = HindIII, Hp = HpaII, P = PstI, Pv = PvuII, S = SalI, Sm = SmaI, Su = SauIIIA. Tq = TaqI, X = XhoI
* = site destroyed.

3.5 SVGene B

This gene B clone contains the SV40 enhancer inserted upstream of the mRNA cap site and was made to test the effect of the enhancer on gene B transcription in oocytes (section 4.2.5).

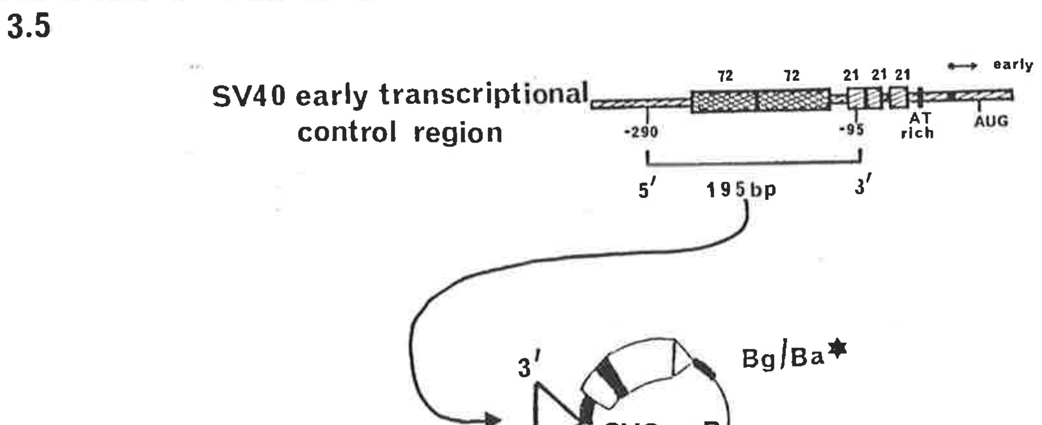
A 195 bp fragment containing the SV40 enhancer was isolated from a donated clone by digestion with EcoRI and HindIII. The enhancer fragment was inserted into the HindIII site of 1-4E8 (section 3.4), 383 bases upstream of the mRNA start site of gene B. The 3' overhangs of the fragments were end-filled before ligation. The size of the clone is 6.05 kb.

3.6 BK-i

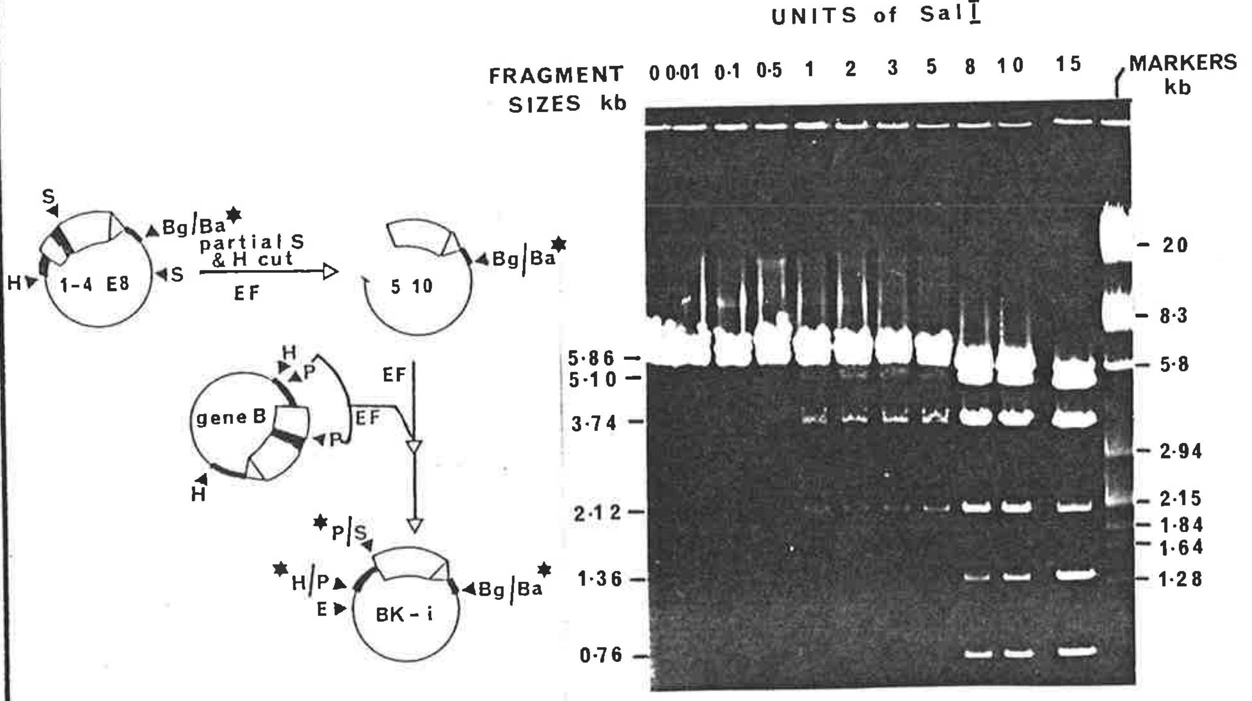
BK-i is a feather keratin gene B-containing clone in which the single intron has been removed from the 5' untranslated region. BK-i was constructed to test the effect of intron removal on transcription in **Xenopus** oocytes (section 4.2.3).

A number of 5 µg aliquots of 1-4E8 DNA (section 3.4) were digested to completion with HindIII and partially with different concentrations of SalI (0.01 to 20 units for 30 minutes at 37°C). The overhangs of the fragments were end-filled and the digests were electrophoresed on a 0.55% low melting point agarose gel in TAE. The 5.10 kb fragment isolated lacked all of the DNA upstream of the 3' splice site of gene B. Sequences missing included the intron, the 37 bp 5' untranslated sequence, cap site and 383 bp of DNA 5' to the cap site. The 5.10 kb fragment acted as the vector for BK-i construction (clone 510). Gene B was digested with PstI in the presence of Klenow fragment and deoxynucleotides and the

3.5



3.6



399 bp fragment containing 356 bases of sequence upstream from the mRNA cap site, the 37 bp 5' untranslated sequence and 6 bases of intron (figure 3-A) was chosen as the keratin promoter. The 399 bp fragment was inserted into the 510 vector to generate BK-i with a size of 5.5 kb.

3.7 SVBK-i

SVBK-i contains the SV40 enhancer inserted upstream of the BK-i cap site. This plasmid was constructed to test the effect of the enhancer on BK-i transcription in oocytes (section 4.2.5).

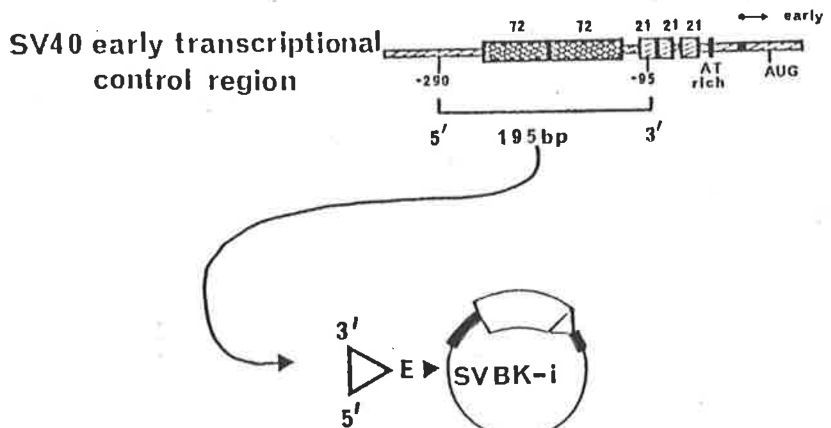
The 195 bp enhancer fragment (section 3.5) was inserted into the EcoRI site of BK-i, 386 bp upstream of the mRNA start site. The orientation of the enhancer fragment is shown in the diagram. The size of the clone is 5.69 kb.

3.8 BK309HpaIIi

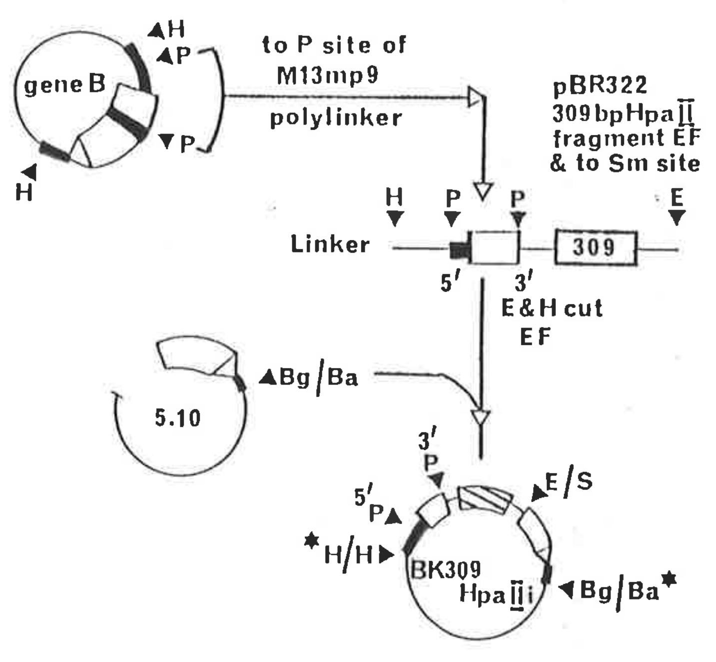
In this clone the intron of feather keratin gene B was replaced with sequences from pBR322. The clone was used to determine whether sequences within the keratin intron were responsible for suppression of transcript initiation (section 4.2.3).

The 399 bp PstI fragment containing the keratin promoter and mRNA start site was inserted into the PstI site of the M13mp9 polylinker (generating M13Pst) and the 309 bp HpaII fragment from pBR322 was inserted into the SmaI site of the same construct. The combined promoter/pseudo-intron was excised with EcoRI and HindIII and inserted into the SalI site of clone 510 (section 3.6). This resulted in a construct with spacing between the mRNA start site and the protein coding initiation point which differs from the unaltered gene by a further 6 bp. The size of the clone is 5.83 kb.

3.7



3.8



3.9 tk (or TK/TK)

The clone containing the HSV-1 tk gene was derived from the plasmid pBR322/SV-0+T/tk (Capecchi, 1980) in which the 3.4 kb fragment containing the entire tk gene was isolated by digestion with BamHI and inserted into the BamHI site of pBR322. The size of this clone is 7.76 kb and it was used in a number of different experiments.

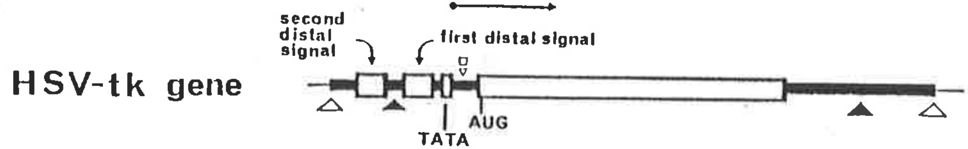
3.10 Eco-tk

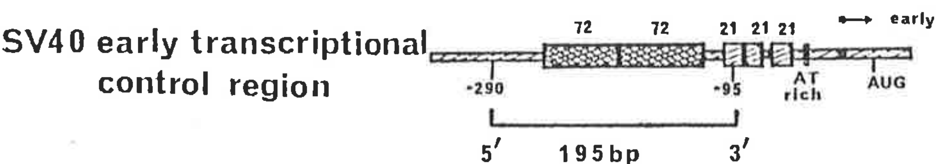
Eco-tk is a derivative of tk in which the second distal transcription signal (McKnight **et al.,** 1984) has been removed. This was achieved by digesting tk with EcoRI and inserting the 2.3 kb fragment containing the tk gene into the EcoRI site of pBR322. The direction of transcription is that shown in the diagram. The size of the resulting clone was 5.46 kb. Eco-tk was used in section 4.2.5.

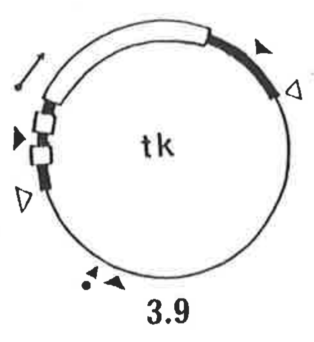
3.11 SVtk and 3.12 SVEco-tk

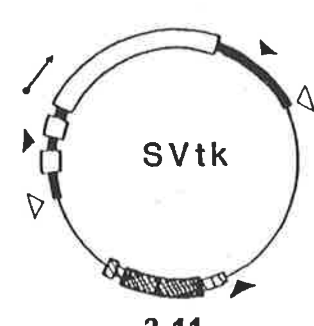
SVtk and SVEco-tk contain the SV40 enhancer and were used to test SV40 enhancer function in **Xenopus** oocytes (section 4.2.5). SVtk and SVEco-tk were constructed by inserting the SV40 enhancer fragment (section 3.5) into the HindIII site of tk (section 3.9) and Eco-tk (section 3.10) respectively. The HindIII site in tk is 976 bases upstream of the mRNA start site whilst in Eco-tk the HindIII site is 111 bp upstream from the mRNA start site. The size of SVtk is 7.95 kb and the size of SVEco-tk is 5.65 kb.

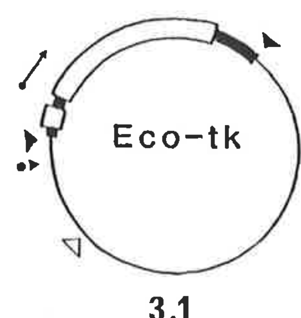
3.9,3.10,3.11 and 3.12

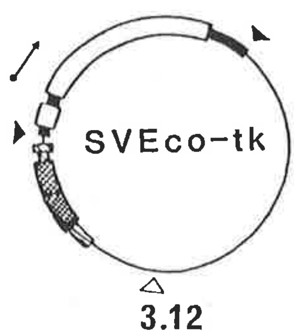












▲ Eco RI site
△ Bam HI site
Hind III site
— pBR322 DNA
Bgl II site

SV40 enhancer
HSV-tk gene
HSV DNA
SV40 DNA
Direction of transcription

3.13 TK/KER

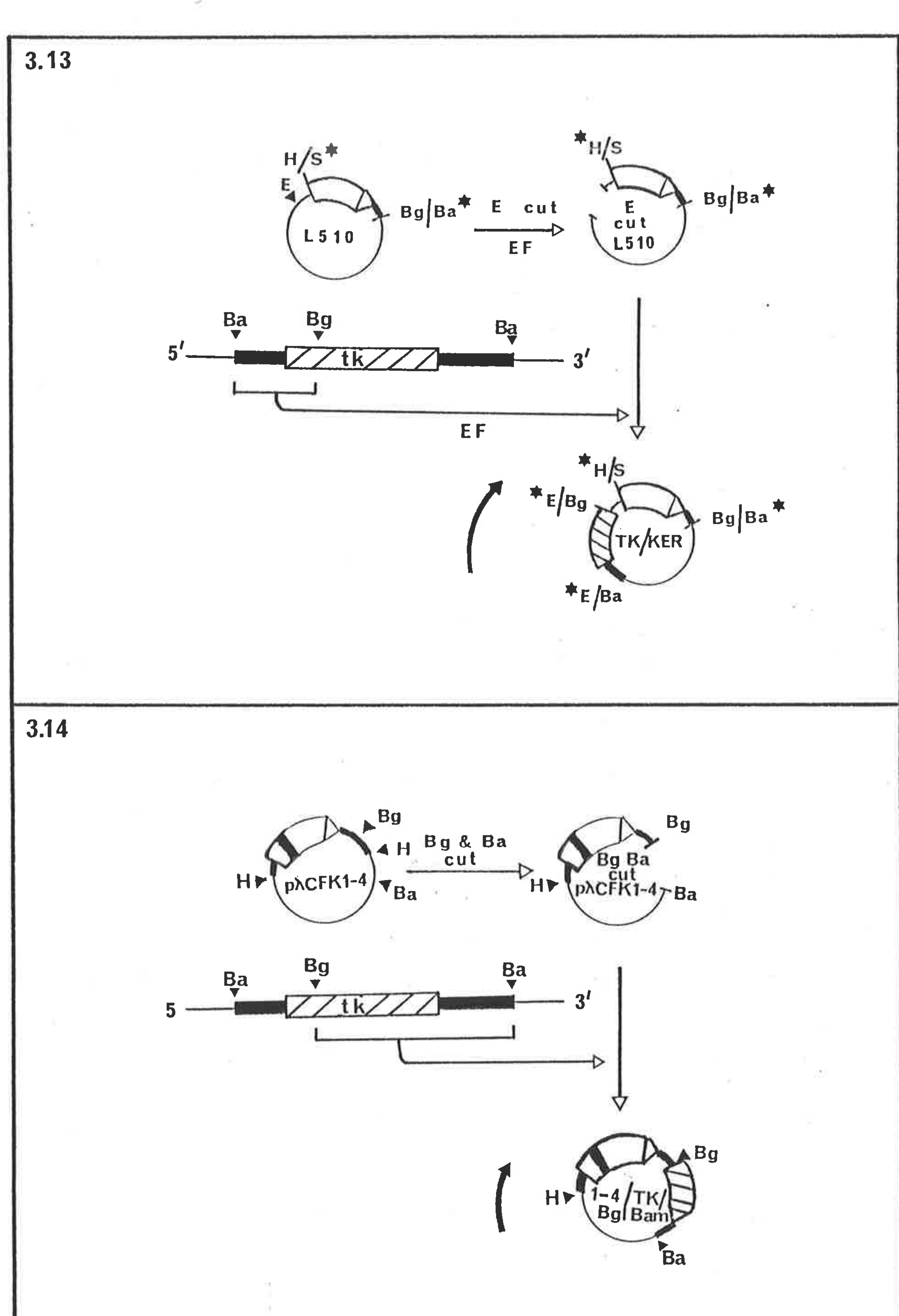
This construct contains the tk promoter fused to keratin gene B sequences found 3' to the coding region SalI site (figure 3-A(ii)). TK/KER was used in section 5.2.1 when testing promoter efficiency in oocytes.

The 5.10 kb fragment described in 3.6 was self-ligated and transformed into MC1061 cells as it was necessary to obtain more of this DNA for further manipulations. This clone was designated L510. Unfortunately, L510 could not be digested with HindIII as this site was destroyed on cloning. L510 was subsequently digested with EcoRI and the 5' overhangs were end-filled; this formed the vector for TK/KER. The tk clone (3.9) was digested with BamHI and BglII and the 680 bp fragment containing the tk promoter was isolated, end-filled and inserted into the L510 EcoRI cut vector as shown. The resulting construct contained 31 bp of pBR322 DNA between the junction of the tk promoter fragment and the keratin gene sequences. The size of the clone is 5.78 kb.

3.14 1-4/TK/BglBam

This clone was an intermediate in the preparation of some of the hybrid gene constructs. It contains all of gene B (including the intron) and the coding region of the tk gene (the 2.7 kb BglII/BamHI fragment, figure 3-B(ii)).

The clone was constructed by digesting pλCFK1-4 with BglII and BamHI. The 5.86 kb fragment isolated was used as a vector for the insertion of the 2.7 kb BglII/BamHI fragment from the tk clone as shown in the figure. Note that the inserted tk fragment is still resectable by digestion with BglII and BamHI. The size of the clone is 8.56 kb.


3.13
H/S
E
L510
Bg/Ba
E cut
EF
E cut L510
Bg/Ba
Ba
Bg
Ba
5′
tk
3′
EF
H/S
E/Bg
Bg/Ba
TK/KER
E/Ba
3.14
Bg
H
H
pλCFK1-4
Ba
Bg & Ba cut
Bg
Bg Ba cut
H
pλCFK1-4
Ba
Ba
Bg
Ba
5
tk
3′
Bg
H
1-4/TK/
Bg/Bam
Ba

3.15 KER/TK

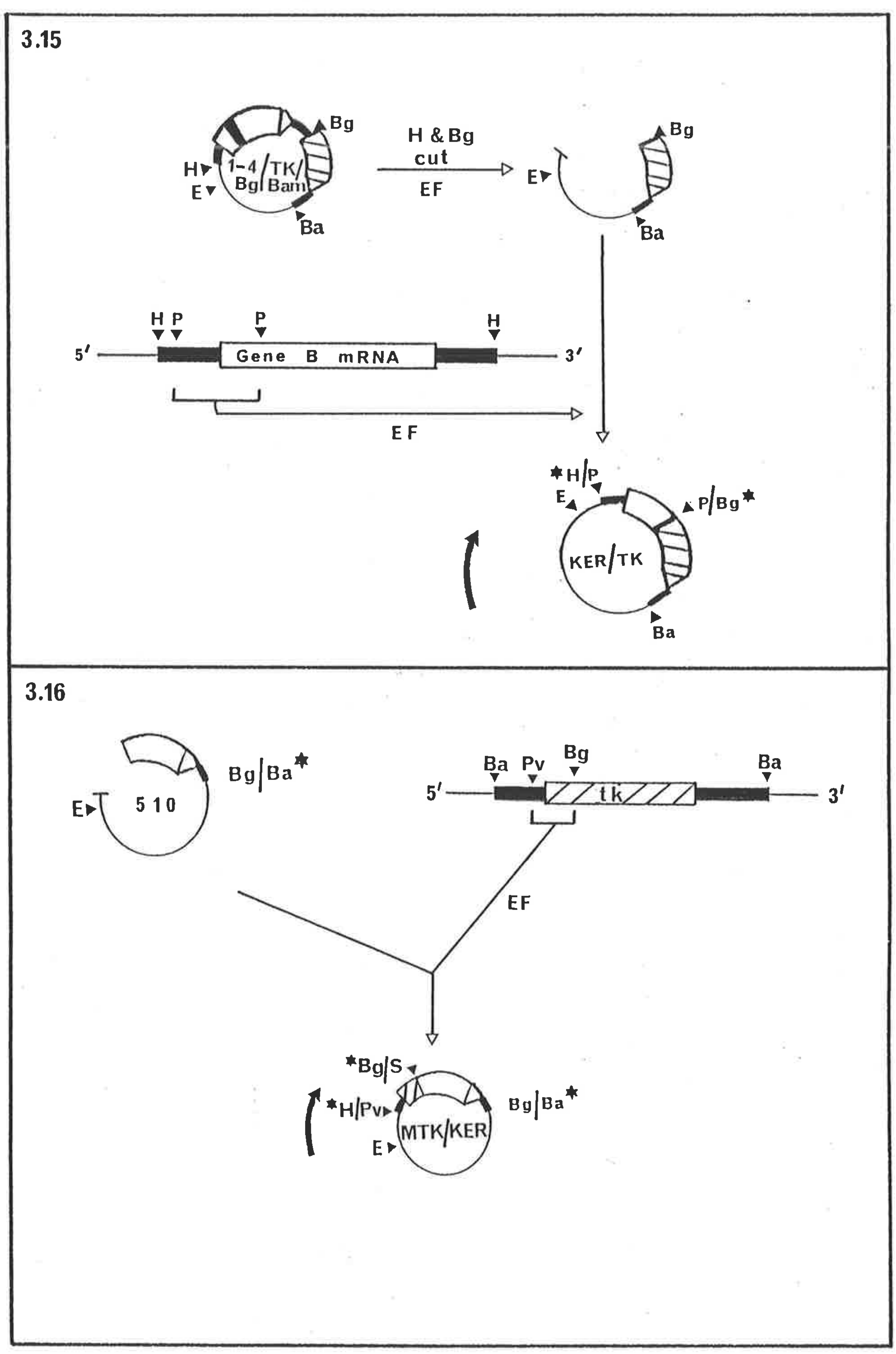
In KER/TK the keratin promoter is fused to the 2.7 kb BglII/BamHI fragment of the HSV-tk gene. This construct was used in section 5.2.1 to test keratin promoter efficiency.

KER/TK was constructed by digesting 1-4/TK/BglBam (section 3.14) with HindIII and BglII to remove all keratin sequences (1.84 kb). A 399 bp Pstl fragment from the 5' end of keratin gene B (see section 3.6) was inserted into the HindIII/BglII cut 1-4/TK/BglBam clone as shown in the diagram. The size of the clone is 7.12 kb.

3.16 MTK/KER

In this construct the tk promoter was fused to keratin gene sequences, however, MTK/KER lacks the pBR322 DNA found between the tk promoter and keratin gene sequences in TK/KER (section 3.13). MTK/KER was used as the basis for a number of deletion constructs and reference is made to this clone in sections 5.2.1 and 5.2.3 of the text.

MTK/KER was constructed by isolating a 250 bp PvuII/BglII fragment, containing the promoter sequences, from the tk clone (figures 3-B(i) and (ii)). The 250 bp fragment was inserted into the 510 vector used in the construction of BK-i (section 3.6). The size of the clone is 5.35 kb. The arrow indicates the direction of transcription.



3.15
Bg
H & Bg
cut
EF
H
E
1-4/TK/
Bg/Bam
Ba
E
Bg
Ba
H P
P
H
5′
Gene B mRNA
3′
EF
*H/P
E
P/Bg*
KER/TK
Ba
3.16
Bg/Ba*
E
5 1 0
Ba
Pv
Bg
Ba
5′
tk
3′
EF
*Bg/S
*H/Pv
MTK/KER
Bg/Ba*
E

3.17 MTK/KER-BE2

MTK/KER-BE2 is a derivative of MTK/KER and lacks the 316 bp fragment from the keratin coding region. This clone was used in section 5.2.3 to test the effect of keratin sequences on transcript initiation.

MTK/KER-BE2 was constructed by digesting MTK/KER with BstEII. (A detailed diagram of the MTK/KER clone is provided to facilitate the location of the sites). The 5.03 kb fragment, missing the 316 bp BstEII fragment, was isolated and the ends of the fragment were religated.

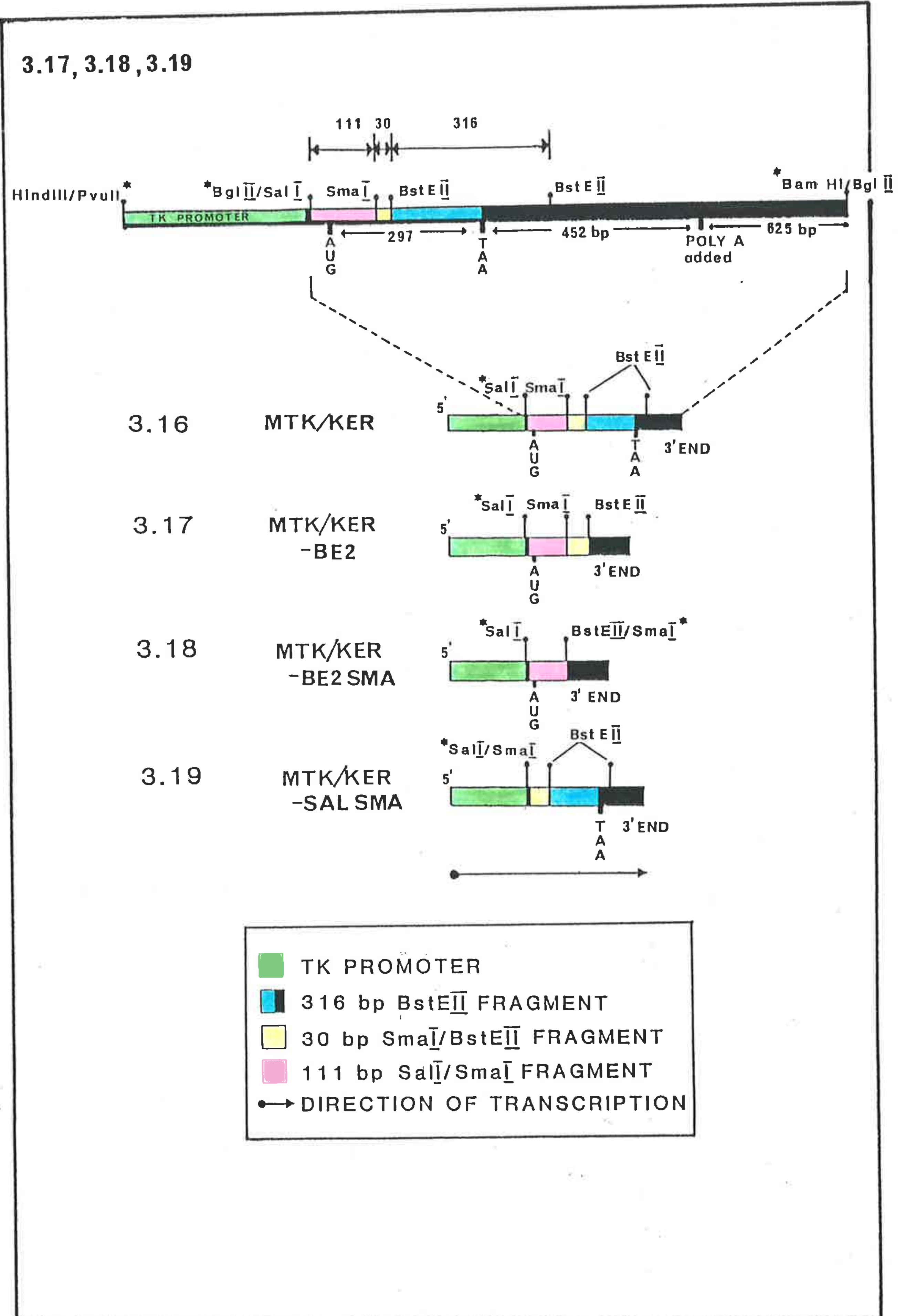
3.18 MTK/KER-BE2SMA

This clone is also derived from MTK/KER and has 346 bp of keratin coding region missing. This was achieved by digesting MTK/KER with BstEII and SmaI, end-filling the BstEII overhang, isolating the 5 kb fragment (lacking the 346 bp BstEII/SmaI fragment) and religating the DNA. The use of this construct is discussed in section 5.2.3.

3.19 MTK/KER-SALSMA

This clone was constructed to test the effect of keratin sequences on transcription from the tk promoter in oocytes (section 5.2.3).

Clone 1-4E8 (section 3.6) was digested with HindIII and SmaI. This removed all of the keratin sequences 5' to the SmaI site in the coding region of gene B (870 bp) resulting in a 5 kb fragment. The 5 kb fragment was isolated to act as a vector and the ends of the fragment were filled. A 250 bp PvuII/BglII fragment, containing the tk promoter (section 3.16), was inserted into the 5 kb vector generating MTK/KER-SALSMA (size = 5.25 kb).


3.17, 3.18, 3.19
111
30
316
HindIII/PvuII*
*BglII/SalI
SmaI
BstEII
BstEII
*Bam HI/BglII
TK PROMOTER
AUG
297
TAA
452 bp
POLY A added
625 bp
3.16
MTK/KER
*SalI
SmaI
BstEII
5'
AUG
TAA
3'END
3.17
MTK/KER -BE2
*SalI
SmaI
BstEII
5'
AUG
3'END
3.18
MTK/KER -BE2 SMA
*SalI
BstEII/SmaI*
5'
AUG
3' END
3.19
MTK/KER -SAL SMA
*SalI/SmaI
BstEII
5'
TAA
3'END
TK PROMOTER
316 bp BstEII FRAGMENT
30 bp SmaI/BstEII FRAGMENT
111 bp SalI/SmaI FRAGMENT
DIRECTION OF TRANSCRIPTION

3.20 BK-i-3'TK

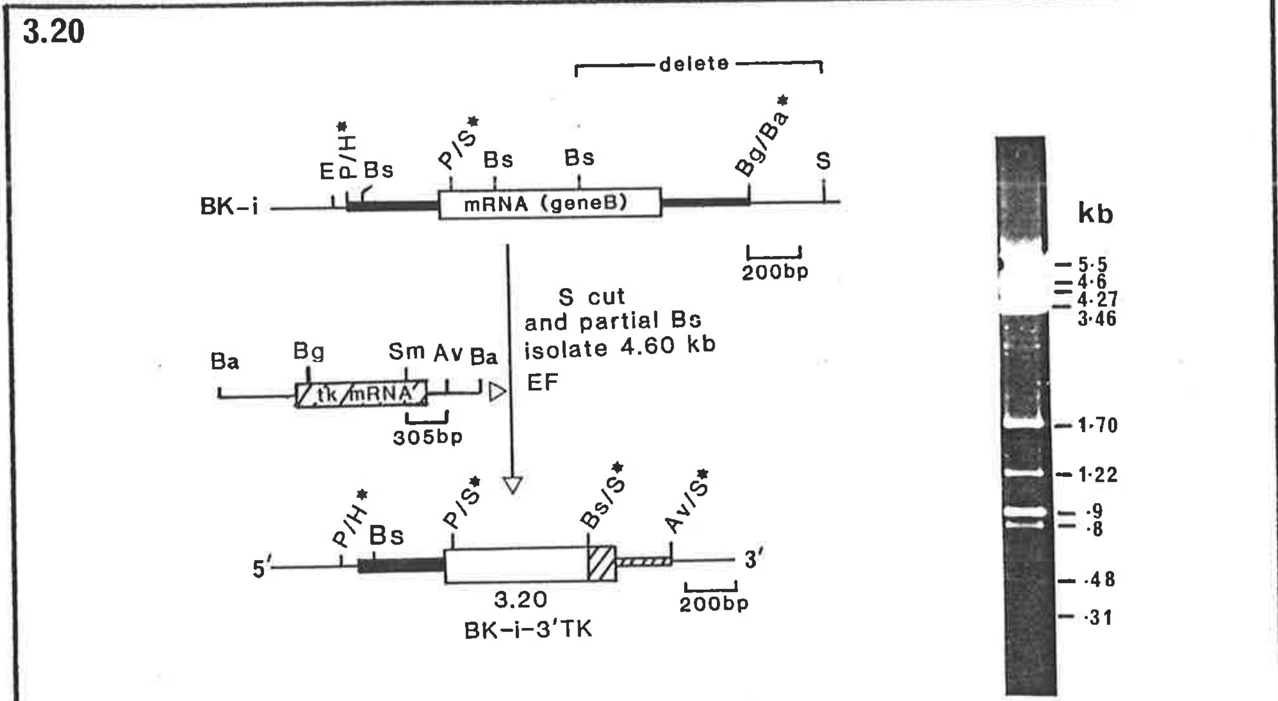
This clone was made to test whether the 18 bp sequence in the 3' untranslated region of the keratin gene had any effect on transcription from the keratin promoter in oocytes (section 5.2.3). The clone is similar to BK-i except that the 3' end of the gene containing the polyadenylation signal and associated 3' flanking DNA has been removed and replaced with the tk polyadenylation signal and 3' sequences.

BK-i-3'TK was constructed by digesting 5 µg of BK-i with SalI and then partially digesting with BstEII (2 units, 60°C for 30 minutes). The ends of the fragments were filled and the DNA was electrophoresed on a 0.55% agarose gel in TAE. The 4.60 kb fragment, missing 900 bp of sequence from the SalI site in pBR322 up to the third BstEII site in keratin gene B (figure 3-A) was isolated. The 4.60 kb fragment served as a vector for the insertion of a 305 bp SmaI/AvaII fragment isolated from the 3' end of the tk gene (3-B(ii)). The resultant clone was 4.9 kb in size.

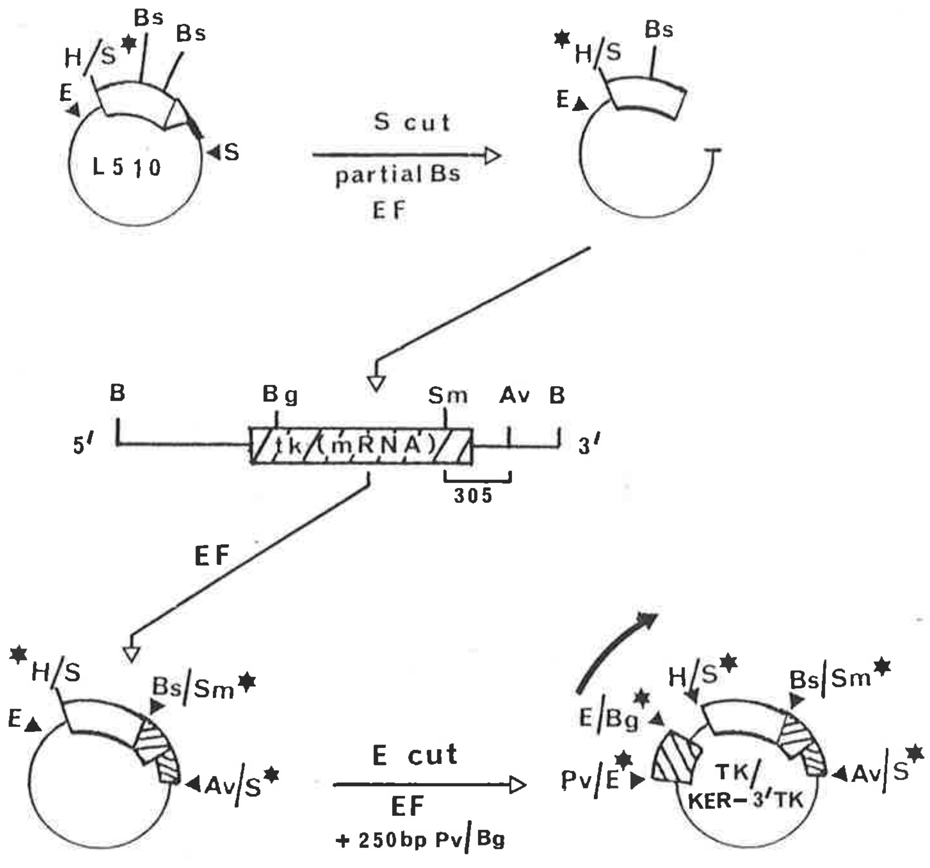
3.21 TK/KER-3'TK

This construct is similar to BK-i-3'TK (3.20) except that the keratin promoter has been replaced with the tk promoter. This clone was used in section 5.2.3 and was constructed by partially digesting L510 (section 3.13) with BstEII. The digestion removed the same 900 bp fragment which had been deleted from the 3' end of BK-i in the construction of BK-i-3'TK. A 305 bp SmaI/AvaII fragment from the 3' end of the tk gene (3.20) was inserted in place of the deleted 900 bp fragment. The resulting construct was digested with EcoRI and the 250 bp PvuII/BglII fragment, containing the tk promoter

3.20



3.21



(see 3.16) was inserted to generate TK/KER-3'TK (4.75 kb). Note that 31 bp of pBR322 DNA is present at the tk-keratin junction.

3.22 TKKpr

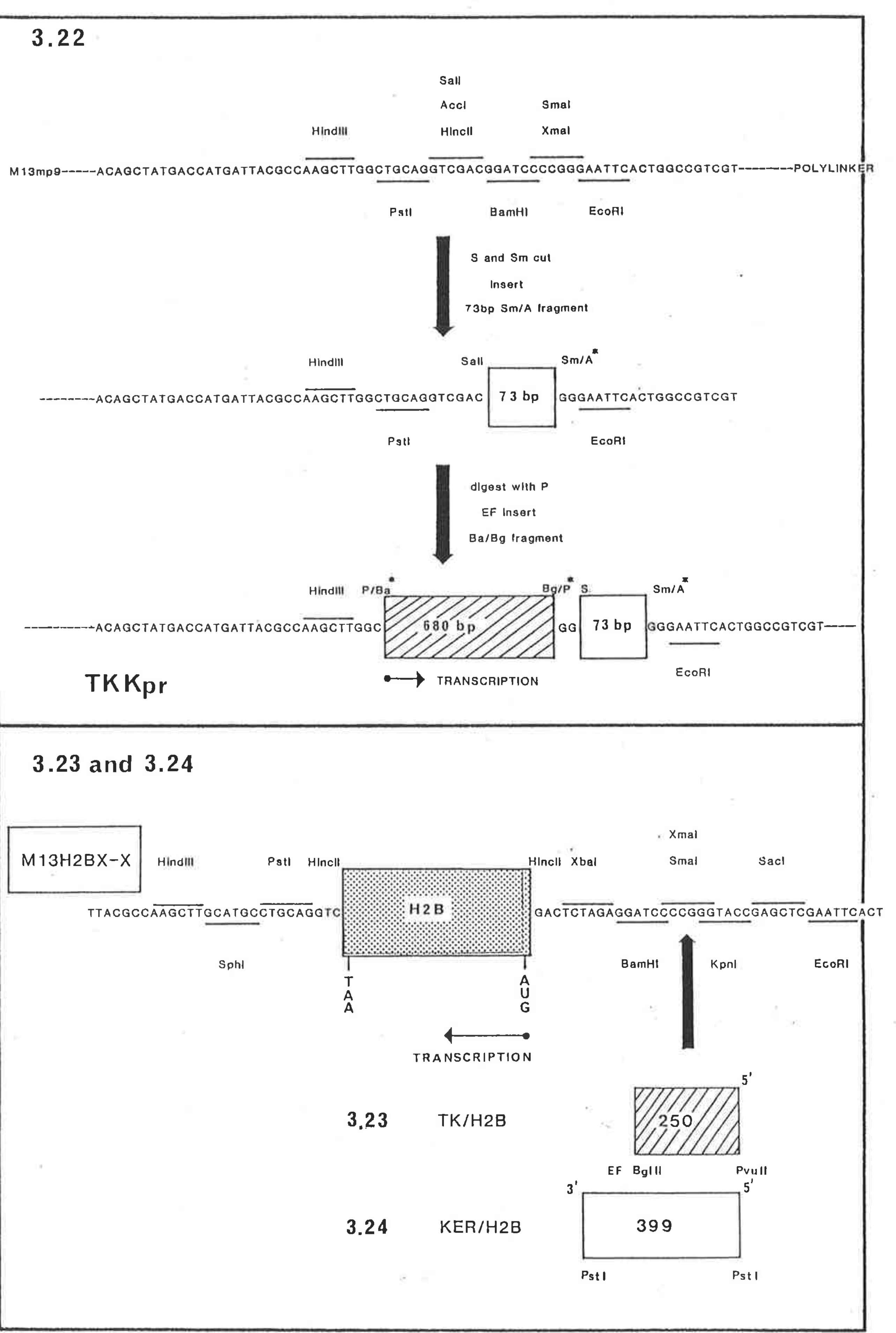
This construct was used in section 5.2.3 to test the efficiency of the tk promoter in the presence of 73 bp of keratin DNA.

A 73 bp SalI/AluI fragment from the coding region of keratin gene B (figure 3-A(ii)) was inserted directionally into M13mp9 which had been digested with SmaI and SalI. The clone generated, M1373, was digested with PstI. A 680 bp BamHI/BglII fragment (figure 3-B(i)) containing the tk promoter was inserted into the PstI site of M1373 to generate TKKpr. The 73 bp keratin DNA fragment contained the keratin 25-mer primer sequence. The size of the clone is 7.98 kb.

3.23 TK/H2B and 3.24 KER/H2B

TK/H2B and KER/H2B were constructed to test the efficiency of the tk and keratin promoters when linked to the coding region of the chicken H2B histone gene (section 5.2.3).

An M13 clone, M13H2BX-X (containing the coding region of the H2B gene (1.85 kb) inserted in the HincII site of M13mp19) was used as a vector to insert the tk and keratin promoter sequences. M13H2BX-X was digested with SmaI. To construct TK/H2B, the 250 PvuII/BglII fragment containing the tk promoter (section 3.16) was inserted into the SmaI site of M13H2BX-X as shown in the diagram. To construct KER/H2B the 399 bp PstI fragment from feather keratin gene B containing the keratin promoter was inserted into the SmaI site as shown. The size of TK/H2B is 9.35 kb and the size of KER/H2B is 9.5 kb.



3.22
SalI
AccI
HincII
SmaI
XmaI
HindIII
M13mp9-----ACAGCTATGACCATGATTACGCCAAGCTTGGCTGCAGGTCGACGGATCCCCGGGAATTCACTGGCCGTCGT--------POLYLINKER
PstI
BamHI
EcoRI
S and Sm cut
Insert
73bp Sm/A fragment
HindIII
SalI
Sm/A*
--------ACAGCTATGACCATGATTACGCCAAGCTTGGCTGCAGGTCGAC
73 bp
GGGAATTCACTGGCCGTCGT
PstI
EcoRI
digest with P
EF insert
Ba/Bg fragment
HindIII
P/Ba*
Bg/P*
S
Sm/A*
---------ACAGCTATGACCATGATTACGCCAAGCTTGGC
680 bp
GG
73 bp
GGGAATTCACTGGCCGTCGT---
EcoRI
TKKpr
TRANSCRIPTION
3.23 and 3.24
M13H2BX-X
HindIII
PstI
HincII
HincII
XbaI
XmaI
SmaI
SacI
TTACGCCAAGCTTGCATGCCTGCAGGTC
H2B
GACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACT
SphI
BamHI
KpnI
EcoRI
TAA
AUG
TRANSCRIPTION
3.23
TK/H2B
5'
250
EF BglII
PvuII
3.24
KER/H2B
3'
5'
399
PstI
PstI

3.25 KER127TK

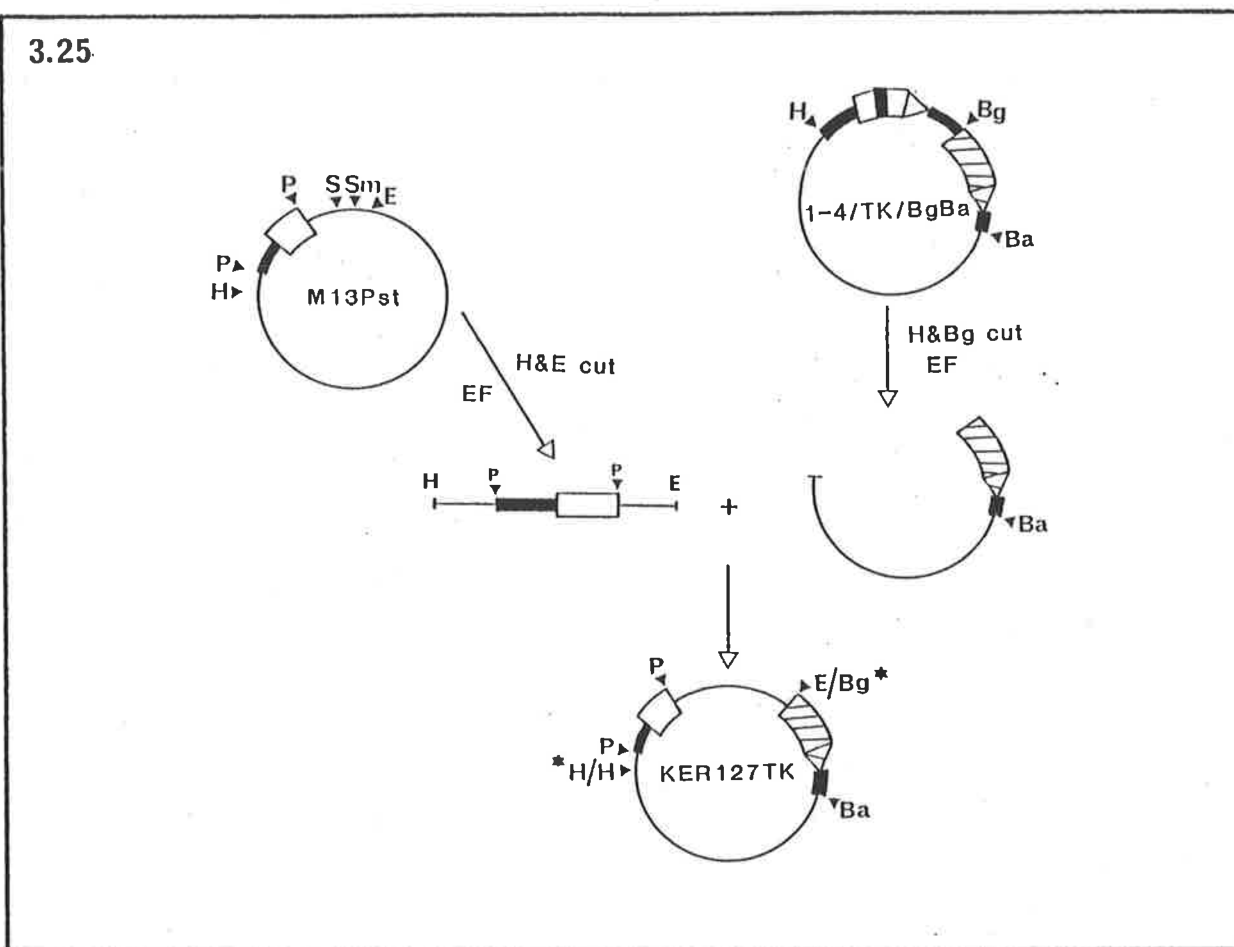
This construct, together with 3.26, 3.27 and 3.28, were used in section 5.2.5 to test the effect of displacing the keratin promoter from tk coding sequences.

KER127TK was constructed by digesting M13Pst (section 3.8) with EcoRI and HindIII to remove the keratin promoter. The excised, end-filled fragment was 27 bases longer on the 3' end of the keratin promoter because of the associated M13 DNA. The fragment was inserted into 1-4/TK/BglBam (3.14) which had been digested with HindIII and BglII to remove all of the keratin sequences (1.84 kb). In the resultant construct the distance from the cap site in the keratin promoter to the AUG of the tk gene was 127 bases (size = 7.15 kb).

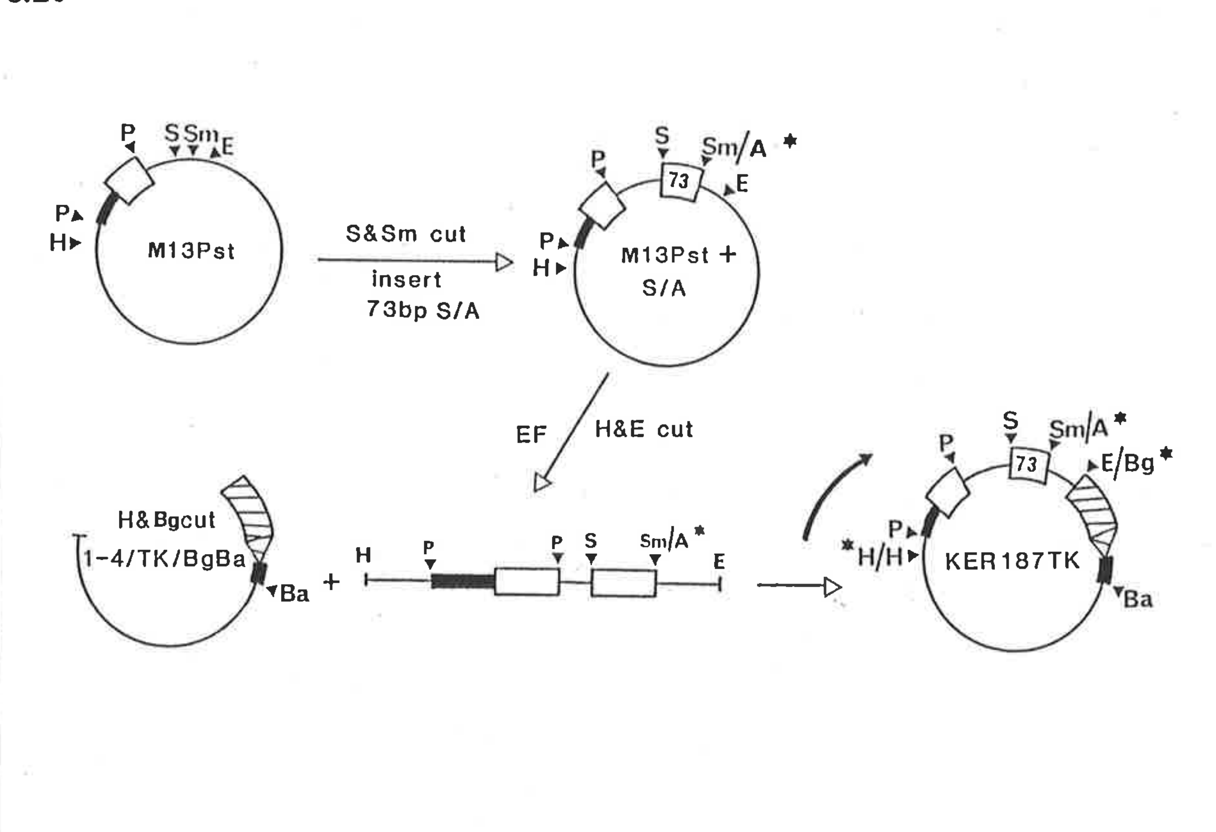
3.26 KER187TK

This construct was made by digesting M13Pst (section 3.8) with SmaI and SalI and inserting a 73 bp SalI/AluI fragment from the coding region of keratin gene B (section 3.22). The combined fragments were excised with EcoRI and HindIII and inserted into 1-4/TK/BglBam (section 3.14) which had been digested with HindIII and BglII to remove keratin sequences (1.84 kb). The distance from the cap site in the keratin promoter to the AUG of the tk gene was 187 bases (size = 7.20 kb).

3.25



3.26



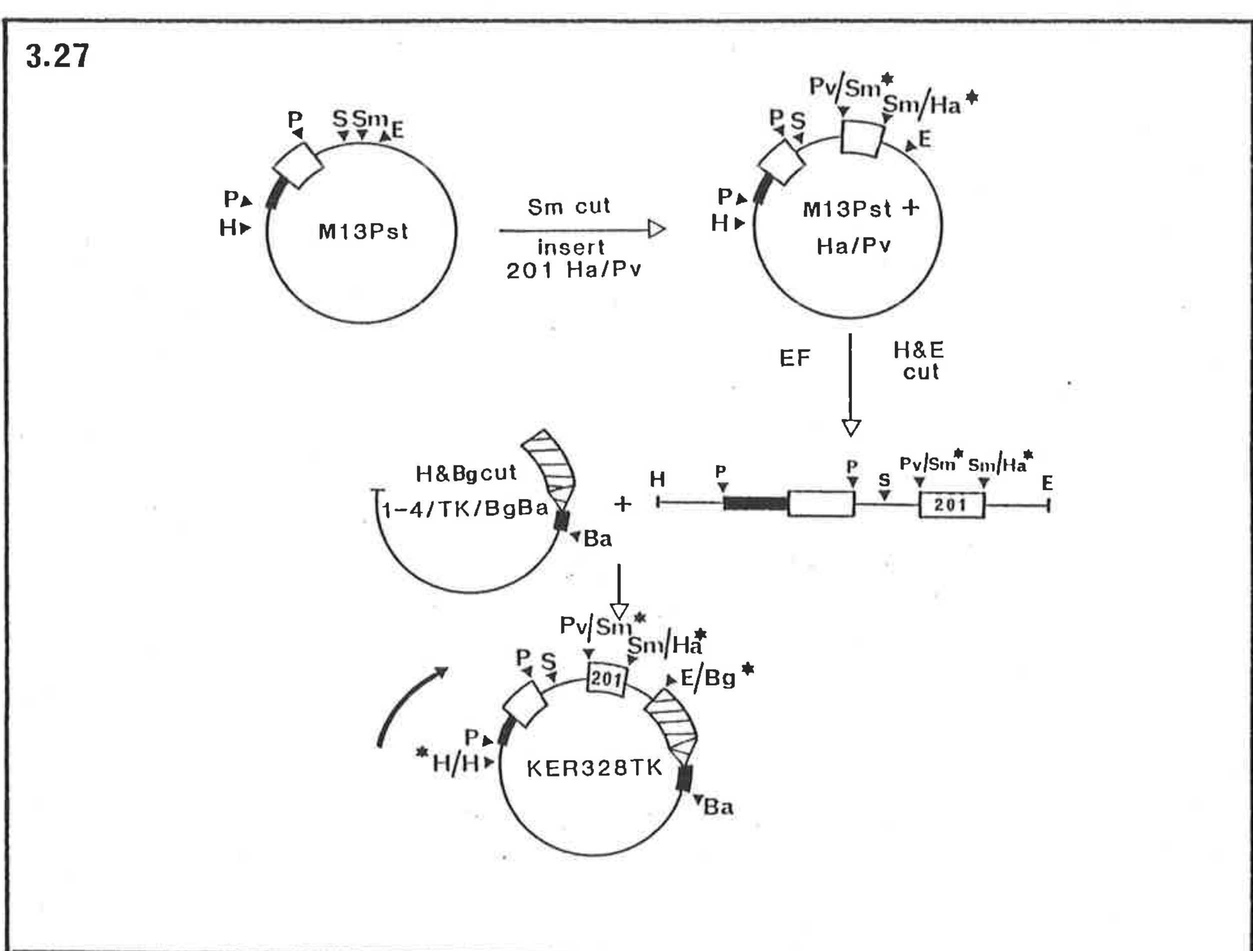
3.27 KER328TK

To make KER328TK, a 201 bp PvuII/HaeIII fragment from the coding region of gene B (figure 3-A(ii)) was inserted into M13Pst (3.8) which had been digested with SmaI. The combined fragments were excised by digesting with EcoRI and HindIII. The EcoRI/HindIII fragment was inserted into 1-4/TK/BglBam (section 3.16) which had been digested with HindIII and BglII to remove the keratin sequences.

The distance from the cap site in the keratin promoter to the AUG of the tk gene was 328 bases (size = 7.33 kb).

3.28 KER436TK

The construction of KER436TK involved inserting a 309 bp HpaII fragment from pBR322 into M13Pst (section 3.18) which had been digested with SmaI. The keratin promoter, linked to the 309 bp fragment was excised by digestion with EcoRI and HindIII and inserted into 1-4/TK/BglBam (section 3.14) which had been digested with HindIII and BglII to remove keratin DNA. The distance from the cap site in the keratin promoter to the AUG of the tk gene was 436 bases (size = 7.45 kb).



3.28

Sm/Hp*
P S
Hp/Sm*
E
309
P
H
M13Pst +
309Hp
H&E cut
EF
H
P
P
S
Sm/Hp*
Hp/Sm*
E
309
H&Bgcut
1-4/TK/BgBa
Ba
Sm/Hp*
P S
Hp/Sm*
E/Bg*
P
*H/H
KER436TK
Ba

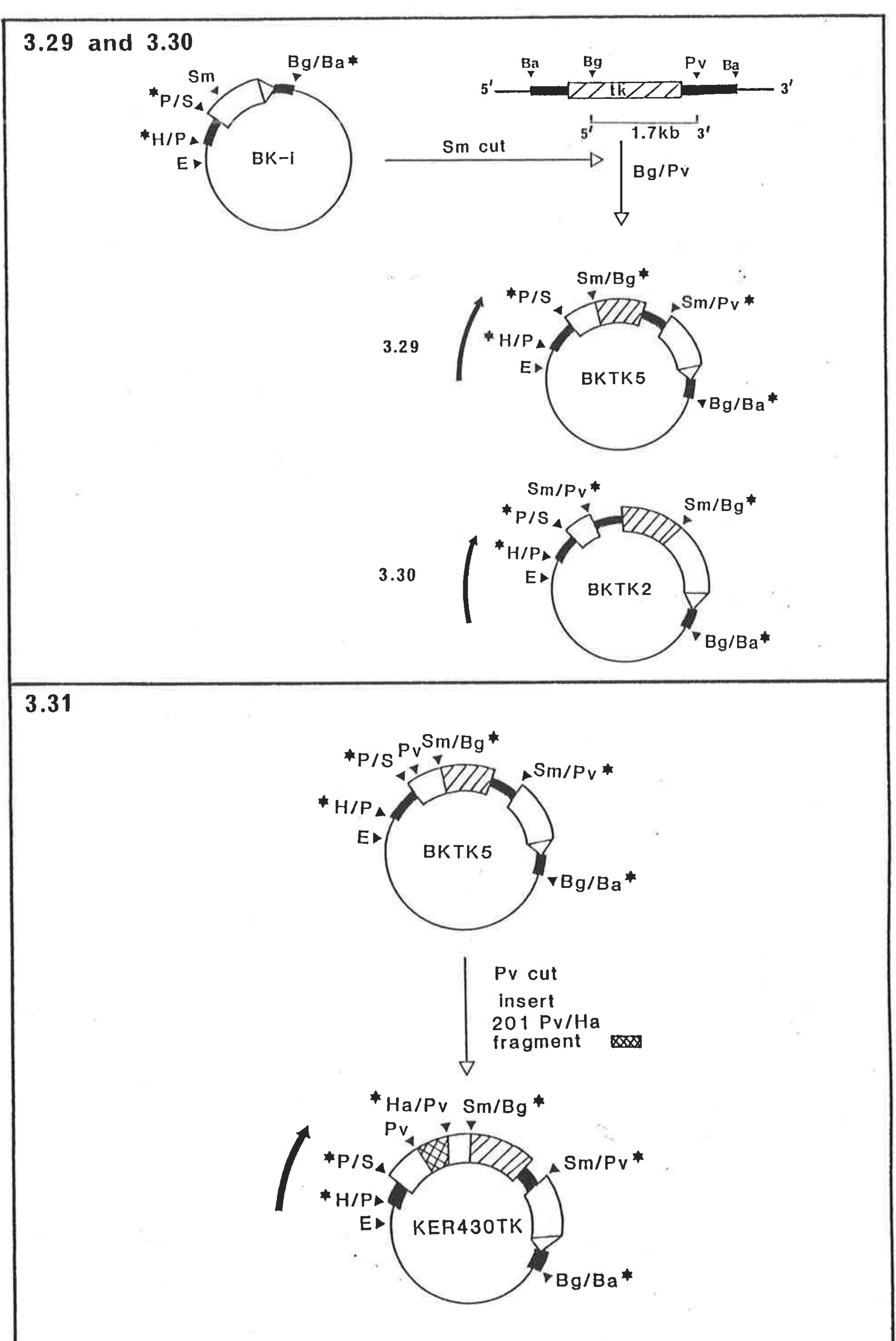
3.29 BKTK5 and 3.30 BKTK2

These constructs which contain a 1.7 kb BglII/PvuII fragment from the tk gene (figure 3-B(i)) inserted into the SmaI site of BK-i, were used in experiments described in section 5.2.6. The diagram shows the method of construction. In BKTK5, the orientation of the tk fragment, relative to the keratin promoter is the same as that in the intact tk gene. The tk fragment in BKTK2 is inserted in the opposite direction to that in BKTK5. The size of both constructs is 7.2 kb.

3.31 KER430TK

This construct is a derivative of BKTK5 (section 3.29) and the cap site of the keratin promoter has been displaced from the AUG of the tk gene by 430 bases of keratin DNA, rather than the pBR322 sequences that were inserted in construct KER436TK (section 3.28).

BKTK5 was digested partially with PvuII and a 201 bp PvuII/HaeIII fragment of keratin coding DNA (section 3.27) was inserted into the PvuII site, 38 bp 5' to the SmaI site where the 1.7 kb BglII/PvuII tk fragment had been originally inserted in the construction of BKTK5. KER430TK was used in experiments described in section 5.2.5. The size of the construct is 7.40 kb.



3.29 and 3.30
Sm
Bg/Ba
P/S
H/P
E
BK-i
Ba
Bg
Pv
Ba
5'
tk
3'
1.7kb
Sm cut
Bg/Pv
Sm/Bg
P/S
Sm/Pv
H/P
3.29
E
BKTK5
Bg/Ba
Sm/Pv
Sm/Bg
P/S
H/P
3.30
E
BKTK2
Bg/Ba
3.31
P/S
Pv
Sm/Bg
Sm/Pv
H/P
E
BKTK5
Bg/Ba
Pv cut
insert
201 Pv/Ha
fragment
Ha/Pv
Sm/Bg
Pv
P/S
Sm/Pv
H/P
E
KER430TK
Bg/Ba

3.32 KER▶800KER, 3.33 KER◀800KER, 3.34 KER▶617KER

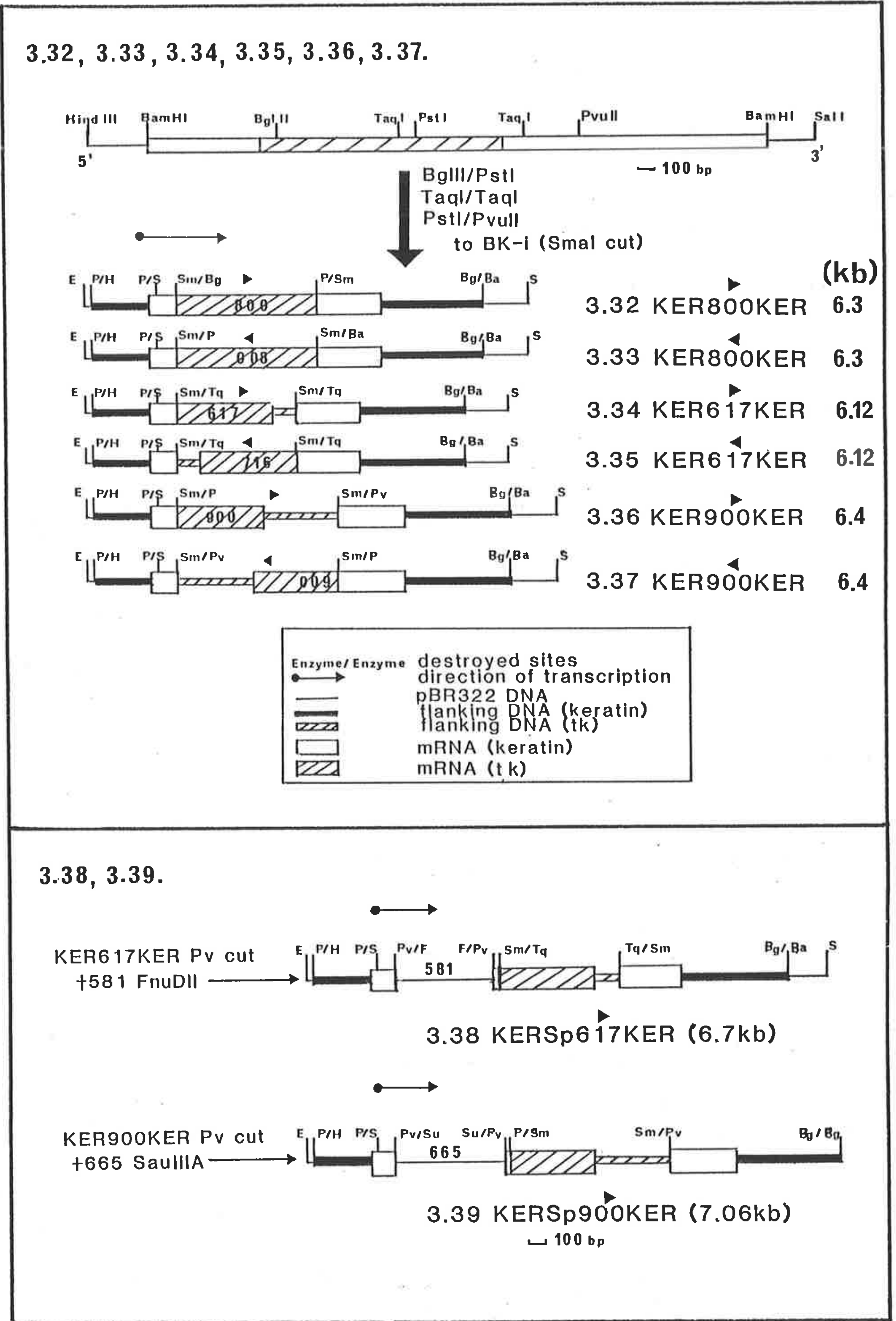
3.35 KER◀617KER, 3.36 KER▶900KER, 3.37 KER◀900KER

In all of these clones different sized fragments of DNA from the 1.7 kb BglII/PvuII fragment of the tk gene were inserted, in both orientations, into the SmaI site of BK-i. An 800 bp BglII/PstI fragment, a 900 bp PstI/PvuII fragment and a 617 TaqI fragment were inserted, after the overhangs were end-filled, into the SmaI site of BK-i as shown in the diagram. In each case, the arrow above the fragment size (▶) indicates the orientation of the fragment. These constructs were used in the experiments described in section 5.2.6. The size of each clone is given in brackets.

3.38 KERSp617KER and **3.39 KERSp900KER**

These constructs are derivatives of constructs 3.34 and 3.36 respectively. Sequences from pBR322 were inserted to move the 617 bp and 900 bp fragments in a 3' direction away from the keratin promoter so that they would occupy a position similar to their normal position in the intact tk gene (w.r.t distance from the cap site).

To generate KERSp▶617KER, KER▶617KER was digested partially with PvuII and a 581 bp FnuDII fragment from pBR322 was inserted into the PvuII site, 38 bp 5' to the SmaI site where the 617 bp fragment had been originally inserted. Similarly, a 665 bp Sau3A fragment, isolated from pBR322 was inserted into the PvuII site of KER▶900KER to produce KERSp▶900KER. The sizes of the clones are given in brackets. These constructs were used in the experiments described in section 5.2.6.

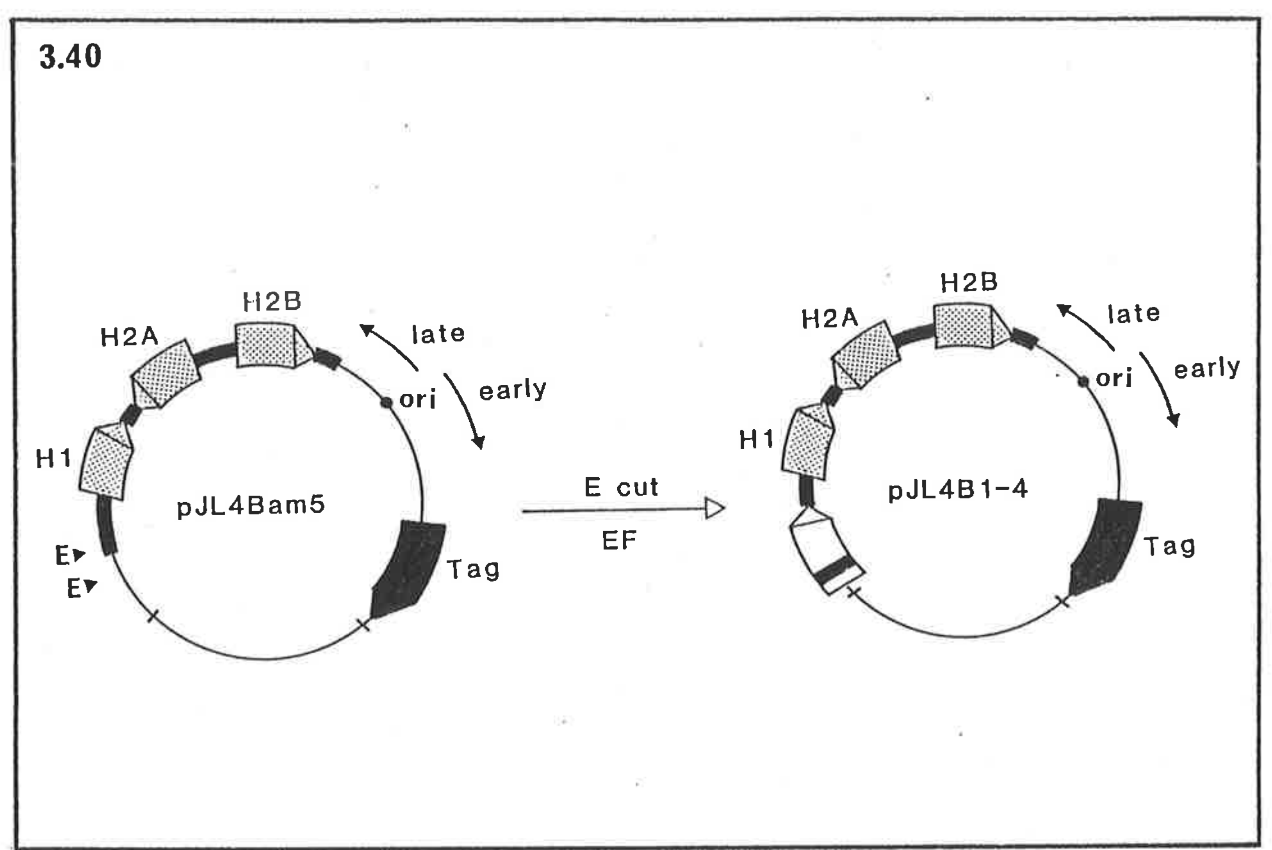


3.32, 3.33, 3.34, 3.35, 3.36, 3.37.
Hind III
BamHI
Bgl II
Taq I
Pst I
Taq I
PvuII
BamHI
Sal I
5'
3'
100 bp
BglII/PstI
TaqI/TaqI
PstI/PvuII
to BK-I (SmaI cut)
(kb)
3.32 KER800KER 6.3
3.33 KER800KER 6.3
3.34 KER617KER 6.12
3.35 KER617KER 6.12
3.36 KER900KER 6.4
3.37 KER900KER 6.4
Enzyme/Enzyme destroyed sites
direction of transcription
pBR322 DNA
flanking DNA (keratin)
flanking DNA (tk)
mRNA (keratin)
mRNA (tk)
3.38, 3.39.
KER617KER Pv cut
+581 FnuDII
581
3.38 KERSp617KER (6.7kb)
KER900KER Pv cut
+665 SauIIIA
665
3.39 KERSp900KER (7.06kb)
100 bp

3.40 pJL4B1-4

This clone contained feather keratin gene B inserted into a eukaryotic, self-replicating vector, pJL4Bam5. The clone pJL4Bam5 contains the SV40 ori, early and late promoter, T antigen coding region and 5 kb of chicken histone DNA containing the same histone genes found in p7AT (H1, H2A and H2B). These features are shown in the diagram.

pJL4Bam5 was digested with EcoRI and the 5' overhangs were end-filled. A 2.93 kb HindIII fragment containing gene B was isolated from pλCFK1-4 and inserted into the EcoRI-digested pJL4Bam5. The orientation of gene B is shown in the diagram. The size of the construct is 13.96 kb.


3.40
H2B
H2A
late
ori
early
H1
pJL4Bam5
E
E
Tag
E cut
EF
H2B
H2A
late
ori
early
H1
pJL4B1-4
Tag

CHAPTER 4.

INTRON SEQUENCES MODULATE

FEATHER KERATIN GENE TRANSCRIPTION

IN XENOPUS OOCYTES.

4.1 INTRODUCTION

The expression of cloned genes can be investigated in a number of systems including cell free extracts, tissue culture cells, oocytes and whole animals. At the commencement of this project the **Xenopus** oocyte transient expression system had recently been established in this department, whereas tissue culture facilities were at a minimum. Already, considerable insight into the control sequences involved in the transcription of histone genes (Probst **et al.**, 1979; Grosschedl and Birnstiel, 1980) and the HSV-tk gene (McKnight and Kingsbury, 1982) had been obtained using oocytes. Therefore, the **Xenopus** oocyte was chosen as the system in which to examine general transcriptional control features of the tandem feather keratin genes recently sequenced in this laboratory.

The conserved structure of feather keratin genes A-D in the genomic clone λCFK1 has been described in section 1.1.4 and recent work has shown this general structure to be maintained throughout the 15 known genes which code for feather keratin proteins (Presland, 1986). In all cases, an intron divides the 5' untranslated region of the gene into two segments and there is significant cross-hybridization of intron sequences throughout the gene cluster (Presland, 1986). It is interesting to note that other genes known to be associated with avian keratinization, such as the scale gene family (Wilton, 1984) a claw keratin gene (L. Whitbread, unpublished observations) and the histidine-rich fast protein genes (Morris, 1985), also possess a single intron in the 5' untranslated region, suggesting that this feature may be of some significance to gene expression during keratinization.

The functional significance of the feather keratin intron was tested in the work described below.

4.2 RESULTS

4.2.1 FEATHER KERATIN GENE TRANSCRIPTION OCCURS AT LOW EFFICIENCY IN OOCYTES AND THE DETECTABLE TRANSCRIPTS ARE UNSPLICED

A primer extension assay was used to detect transcripts from feather keratin genes injected into oocytes (see section 2.11.5). Early attempts to detect transcripts used kinased restriction fragments isolated from the coding regions of the genes of interest. These experiments used a variety of templates in which the number of feather keratin genes and associated flanking regions were progressively increased. For example, the feather keratin genes were injected singly, as plasmid sub-clones or circularized, resected inserts, or as a cluster of genes in a charon 4A clone (λCFK1, containing 4 genes) or a cosmid clone (Cosmid 4, containing 11 genes). Irrespective of the type of clone injected, no feather keratin gene transcripts could be detected despite the ease with which transcripts from the control chicken histone cluster (p7AT; see section 3.1) were observed (data not shown).

To test the possibility that the keratin gene plasmids and larger clones were being packaged into inactive chromatin whereas the histone genes were packaged into active chromatin, three combined plasmids were constructed by inserting keratin gene A into the histone cluster p7AT (constructs 97, 24 and 70, see sections 3.1, 3.2 and 3.3). In all cases the histone genes were transcribed but transcripts could not be detected

FIGURE 4-1

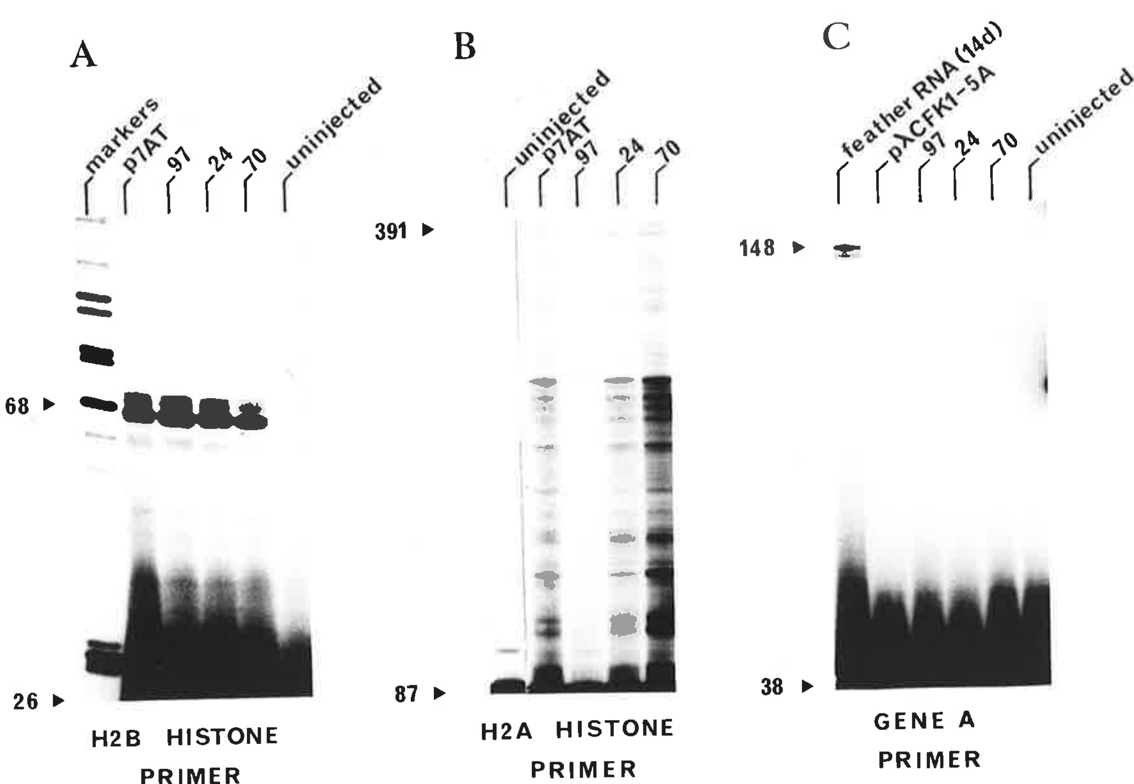
PRIMER EXTENSIONS ON RNA ISOLATED FROM OOCYTES INJECTED WITH CONSTRUCTS 97, 24 AND 70

Oocytes from the same frog were injected with constructs 97, 24 and 70 which contained feather keratin gene A inserted into the histone cluster p7AT (sections 3.1, 3.2 and 3.3). Two oocytes worth of RNA was used in the primer extension analysis and reaction products were electrophoresed on a 6% denaturing polyacrylamide gel.

A. Primer extensions performed using a kinased synthetic 26-mer, specific for the detection of H2B transcripts. The H2B primer extension product is 68 bases long.

B. Primer extensions performed using a kinased 87 bp restriction fragment specific for the detection of H2A gene transcripts giving an extension product of 391 bases.

C. Primer extensions using a kinased 38 bp restriction fragment primer specific for feather keratin gene A detection. The primer was from the region in gene A similar to that spanning the PvuII/SmaI sites in gene B (figure 3-A(ii)). The expected extension product band is at 148 bases as shown by the feather RNA track where the primer extension reaction was performed on 10 μg of polysomal RNA isolated from 14 day embryonic chick feather tissue.


A
markers
p7AT
97
24
70
uninjected
68
26
H2B HISTONE
PRIMER
B
uninjected
p7AT
97
24
70
391
87
H2A HISTONE
PRIMER
C
feather RNA (14d)
pλCFK1-5A
97
24
70
uninjected
148
38
GENE A
PRIMER

from the feather keratin gene (figure 4-1).

The differential level of transcripts observed from the H2B gene compared with those from the H2A and feather keratin genes in figure 4-1 was probably related to the fact that a single stranded primer was used to detect H2B transcripts. The relatively inefficient H2A extension for example, could have been attributed to a competition between the primer hybridizing to the H2A mRNA and hybridizing to itself to reform the double stranded restriction fragment. Even the signal intensity of the primer extension products derived from polysomal feather keratin message was quite low using a double stranded primer given the relatively large amount of RNA used for extension (10 µg; figure 4-1C).

In order to increase the sensitivity of transcript detection a single stranded synthetic oligonucleotide was utilized (25-mer, see figure 4-2C and figure 3-A(ii)). The 25-mer was designed to hybridize with perfect match to the coding region of at least 4 feather keratin mRNAs (encoded by genes A-D in λCFK1). The signal intensity increased markedly using the 25-mer as 100 ng of polysomal mRNA used in the extension reaction resulted in an intense product band (figure 4-2A).

The primer extension products obtained when RNA from oocytes injected with gene B (pλCFK1-4) was analyzed using the coding region 25-mer are shown in figure 4-2A. Only after a considerable exposure time were the reaction products observed. However, they did not have the predicted length of 95 bases expected from correctly spliced transcripts, but were instead, about 330 bases larger.

Although the length of the extension made it difficult to

FIGURE 4-2

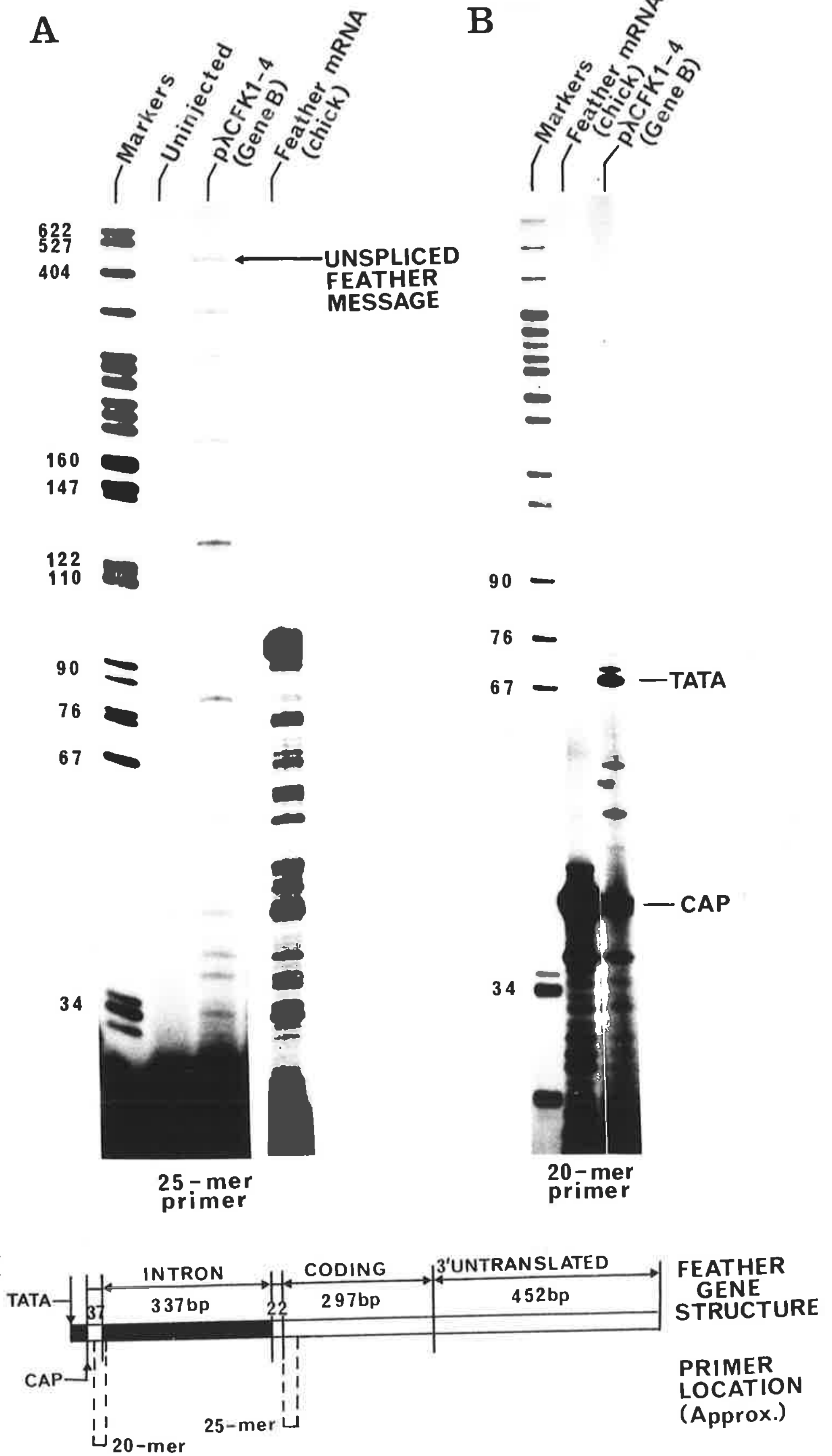
PRIMER EXTENSION ANALYSIS ON RNA ISOLATED FROM OOCYTES INJECTED WITH GENE B (pλCFK1-4)

Oocytes were injected with feather keratin gene B and two oocytes worth of RNA was used in the primer extension analysis.

A. A long exposure of the primer extension products produced when a coding region primer (25-mer) was used in the reaction mix. The extension reaction terminated at approximately 430 bases indicating the existence of unspliced transcripts. Spliced transcripts should migrate at 95 bases in a manner identical to primer extension products from the **in vivo** transcripts (100 ng of polysomal RNA from 14 day feather tissue was used). Reaction products were electrophoresed on a 6% denaturing polyacrylamide gel.

B. A long exposure of the results obtained when primer extensions were performed on the same samples used in **A** except that the 20-mer primer was used instead of the 25-mer. Primer extension products specific for keratin gene transcripts electrophorese at 41 bases and also 39-40 bases with products generated by transcripts initiating from the TATA box migrating at around 68-72 bases. 20 ng of polysomal feather RNA was used in the extension reaction represented by the track marked feather mRNA chick. Reaction products were electrophoresed on a 10% denaturing acrylamide gel.

C. A diagrammatic representation of feather keratin gene structure and the relative positions of the synthetic primers.



A
Markers
Uninjected
pλCFK1-4 (Gene B)
Feather mRNA (chick)
622
527
404
160
147
122
110
90
76
67
34
UNSPLICED FEATHER MESSAGE
25-mer primer
B
Markers
Feather mRNA (chick)
pλCFK1-4 (Gene B)
90
76
67
34
TATA
CAP
20-mer primer
C
TATA
CAP
37
INTRON
337bp
22
CODING
297bp
3'UNTRANSLATED
452bp
FEATHER GENE STRUCTURE
PRIMER LOCATION (Approx.)
20-mer
25-mer

define the exact termination point under the electrophoresis conditions used, the reaction corresponded in size to unspliced feather keratin transcripts. It was also conceivable that non-specific transcript initiation was occurring at a site 330 bases away from the expected feather keratin initiation site. In order to discriminate between these alternatives a different oligonucleotide primer (20-mer) was designed to hybridize to the conserved sequences in the 5' untranslated region of the feather keratin genes A-E (see section 1.1.4(ii) and figure 4-2C). Re-examination of the RNA using the 20-mer showed that the oocyte transcripts from feather keratin gene B were initiating from the same position as that observed **in vivo** (figure 4-2B).

Similar results were obtained when the RNA from oocytes injected with λCFK1, Cosmid 4 and construct 97 was analyzed using the two different synthetic primers (figure 4-3). The shorter (and fainter) extension products observed in figures 4-2 and 4-3 probably resulted from either partially degraded RNA or pause sites encountered during reverse transcription (McKnight **et al.,** 1981).

Figure 4-3 shows that feather keratin gene A was expressed at levels similar to feather keratin gene B in oocytes. The amount of transcript derived from oocytes originally injected with λCFK1 and Cosmid 4 was not significantly greater than that observed from gene B even though those two clones contained 4 and 11 genes respectively. In the case of λCFK1 this was probably due to the fact that

FIGURE 4-3

ANALYSIS OF TRANSCRIPTS FROM OOCYTES INJECTED WITH 97, GENE B, λCFK1, AND COSMID 4

Oocytes from the same frog were injected with either construct 97 (containing feather keratin gene A inserted into the histone cluster p7AT), feather keratin gene B, λCFK1 (containing 4 intact feather keratin genes, A-D, size = 38.1 kb with 15 kb being keratin DNA) or Cosmid 4 (containing 11 intact feather keratin genes, 47 kb in size, 41 kb being keratin DNA; figure 1-3A). Primer extension analysis was performed using the keratin 20-mer primer on two oocytes worth of RNA. The reaction products were electrophoresed on a 10% denaturing polyacrylamide gel. The positions of the cap site and bands indicative of transcripts initiating from the TATA box are shown.

Gene A (97)
Gene B (pλCFK1-4)
λCFK1
Cosmid 4
Uninjected

◁ TATA

◁ CAP

all clones were injected on a per weight basis and in the case of Cosmid 4 it was possible that some of the genes were not being expressed.

It appears that feather keratin transcripts observed at steady state levels in the oocyte are only present at very low levels and are not spliced. Gene B was chosen (arbitrarily) for further expression studies investigating the parameters responsible for these results.

4.2.2 TRANSCRIPT INITIATION FROM THE TATA BOX

With all keratin constructs tested, a low level of transcription was also initiated from the TATA box (e.g. figures 4-2B, 4-3, 4-4A). This phenomenon has previously been reported to occur in transfected cells (Proudfoot **et al.**, 1984) and in microinjected oocytes (R. Palmiter and G. Partington, personal communication). Transcript initiation from the TATA box was also observed in a number of other microinjected genes during the course of this work, e.g. tk and H2B histone (figures 5-1, 5-2, 5-4A and 5-7A). Transcript initiation from the TATA box was quite low in frequency, but within the one batch of oocytes the frequency of initiation was constant irrespective of the particular construct type (figures 4-3, 4-4A). Interestingly, the frequency of initiation from the TATA box was observed to vary from one batch of oocytes to the next, as exemplified by the intensity of the extension product observed in figure 4-2B compared with that in figure 4-4A.

4.2.3 REMOVAL OF THE FEATHER KERATIN INTRON INCREASES THE LEVELS OF CORRECTLY INITIATED TRANSCRIPT

There is an intron present in the 5' untranslated region of all the isolated keratin and keratin-related genes associated with avian keratinization. To test the effect of the intron on feather keratin gene transcription in the oocyte, the intron was removed from feather keratin gene B. This produced the construct BK-i (see section 3.6).

Removal of the keratin intron increased the level of correctly initiated transcript 5-fold (figure 4-4A) as determined by densitometric scanning of primer extension autoradiographs. As an internal control, histone DNA (p7AT) was co-injected with each keratin gene construct. Figure 4-4B shows that the level of H1 transcript was the same in all cases. Concurrent primer extension analysis on mRNA isolated from developing feather tissue confirmed that a majority of oocyte transcripts from the intronless keratin gene were initiated from the same position as the **in vivo** transcripts (figure 4-4A).

Transcript initiation also occurred from the keratin gene TATA box but the presence or absence of the feather keratin intron had no effect on the level of these transcripts (figure 4-4A). The constant level of TATA box initiation, irrespective of the keratin construct type, suggested that the lower level of accurately initiated transcript observed in the presence of the feather keratin intron was unlikely to be caused by instability of the unspliced transcript or by losses due to partial splicing.

It is important to note that, although the level of

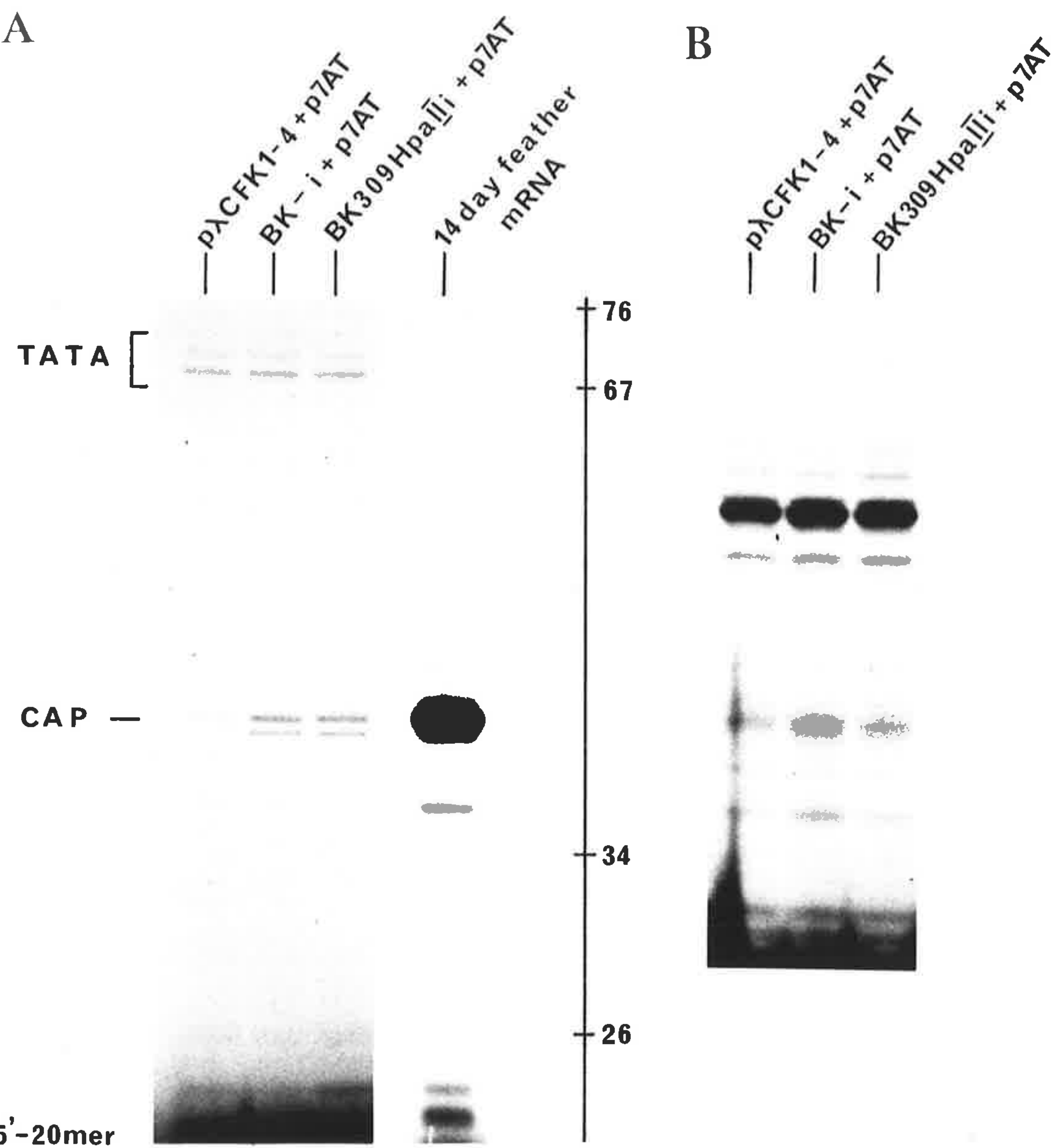
FIGURE 4-4

EXTENSION ANALYSIS ON RNA FROM OOCYTES INJECTED WITH GENE B, BK-i AND BK309HpaIIi

Oocytes were injected with either gene B, BK-i (gene B minus the intron) or BK309HpaIIi (gene B, but intron replaced with pBR322 fragment). An equal amount of p7AT histone DNA was co-injected with each construct to act as an internal control.

A. A kinased synthetic 20-mer was used to detect keratin transcripts and the reaction products were electrophoresed on a 10% denaturing polyacrylamide gel. The primer extension products for each construct electrophoresed at 41 bases with products generated by transcripts initiating from the TATA box migrating at around 68-72 bases.

B. Primer extensions performed on the same RNA as in **A**, using the H1 histone primer instead of the keratin 20-mer. A 6% polyacrylamide denaturing gel was used and the H1 histone extension products electrophoresed at 58 bases.


A
pλCFK1-4 + p7AT
BK-i + p7AT
BK309HpaIIi + p7AT
14 day feather mRNA
TATA
CAP
5'-20mer
76
67
34
26
B
pλCFK1-4 + p7AT
BK-i + p7AT
BK309HpaIIi + p7AT

correctly initiated transcript was increased by removal of the feather keratin intron, it was still at least an order of magnitude lower than that of the histone genes, the herpes simplex virus thymidine kinase gene and even the tissue specific H5 histone gene (figure 4-5).

To determine whether the DNA sequence within the intron was causing a specific reduction in the detectable level of transcripts, the keratin intron was replaced with the 309 bp HpaII fragment from pBR322 (construct BK309HpaIIi, see section 3.8). This manipulation left the 5' splice sequence intact but eliminated the 3' splice junction. The resultant spacing between the mRNA start site and the protein coding initiation point differed from the unaltered gene by 6 bp. After injection of BK309HpaIIi, transcripts were detected at levels essentially identical to those from BK-i (figure 4-4A). Thus, the presence of an intron-sized interruption is not, in itself, sufficient to suppress transcription from the feather keratin promoter. The suppressive effect must therefore be related to the nature of sequences within the keratin intron.

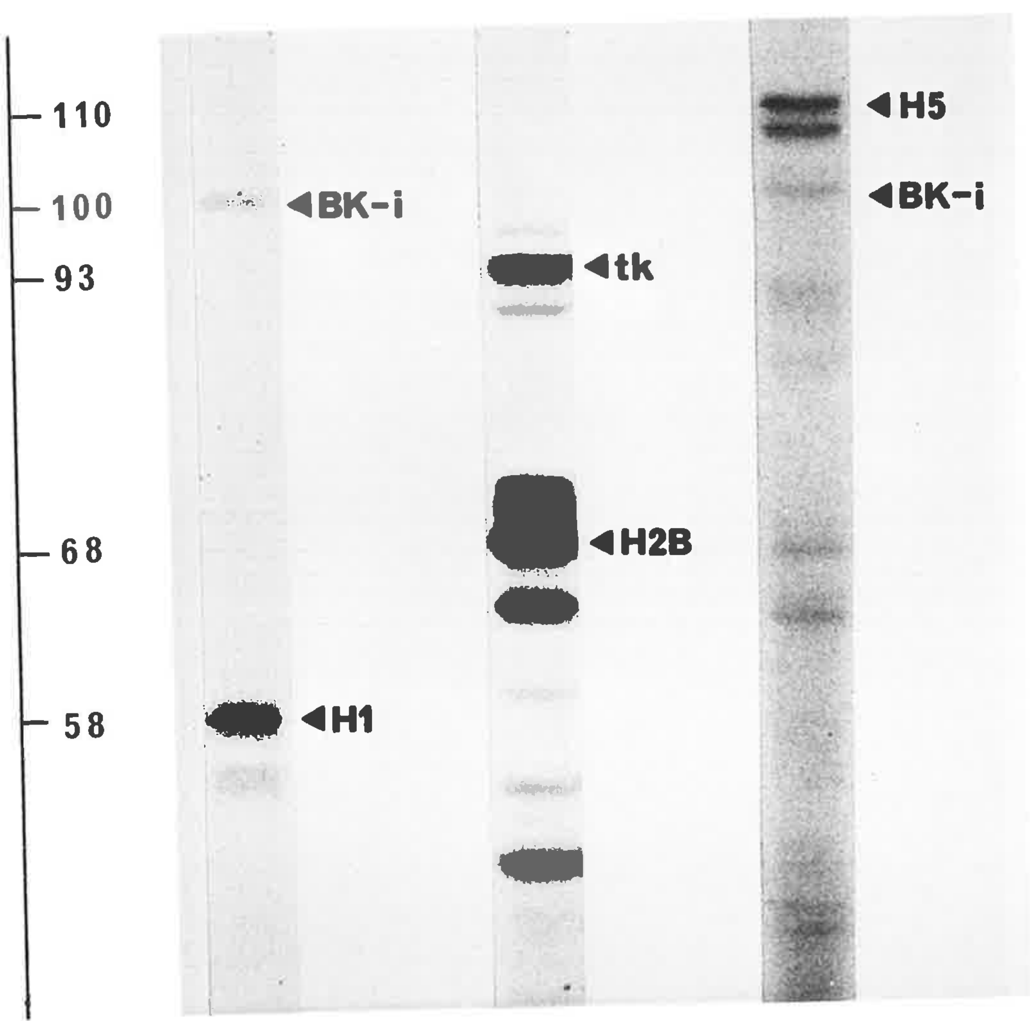
4.2.4 POLYADENYLATION OF TRANSCRIPTS AND 3' END MAPPING

Feather keratin transcripts are polyadenylated **in vivo** (Powell **et al.**, 1976). BK-i was used to test for polyadenylation of keratin mRNA in the oocyte because it produced the most readily detectable product. Binding of the RNA to oligo (dT)-cellulose resulted in the retention of approximately 20% of the BK-i transcripts (figure 4-6A) as determined by densitometric scanning. This corresponds to a similar efficiency of polyadenylation to that reported for mRNA from other introduced genes (Wickens and Gurdon, 1983).

FIGURE 4-5

EXTENSIONS ON RNA FROM OOCYTES INJECTED WITH A VARIETY OF DNA TEMPLATES

Each oocyte was injected with 12 ng of a DNA mixture containing 3 ng quantities of each of the following clones; BK-i (gene B minus intron), p7AT, tk (tk gene) and a pBR322 subclone containing the H5 histone gene. The **left** track shows primer extension products generated using the keratin 25-mer and the H1 histone primer. The **central** track shows primer extension products generated when the tk and H2B primers were used in the same reaction mix. The **right** track shows extension products using the H5 histone gene primer and the keratin 25-mer. All of the primers used were of similar specific activity and the reaction products were electrophoresed on a 6% denaturing polyacrylamide gel. The size of each extension product is indicated by the numbers on the left. The intensity of the bands indicates the relative amount of transcript produced from each gene after a 24 hour incubation period.



110
100
93
68
58
BK-i
H1
tk
H2B
H5
BK-i

FIGURE 4-6 A

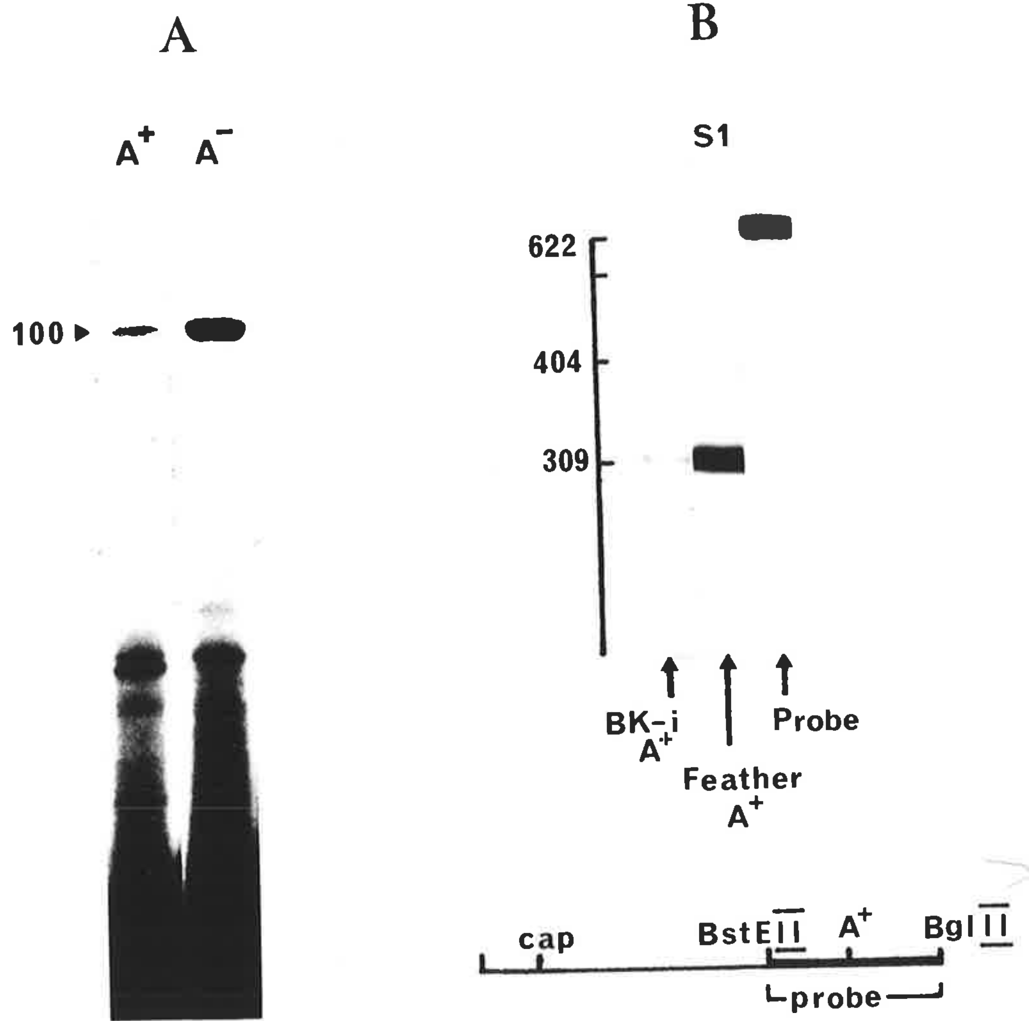
PRIMER EXTENSION ANALYSIS OF POLY(A)$^+$ AND (A)$^-$ TRANSCRIPTS

Poly(A)$^+$ and poly(A)$^-$ RNA, isolated from oocytes injected with BK-i, was assayed by primer extension. The keratin 25-mer and the RNA equivalent of 5 oocytes was used in the reaction. The reaction products were electrophoresed on a 6% denaturing polyacrylamide gel.

FIGURE 4-6 B

3' S1 ANALYSIS OF BK-i TRANSCRIPTS

The equivalent of 10 oocytes worth of poly(A)$^+$ RNA was used in the reaction. The S1 probe was an end-labelled 632 bp BstEII/BglII fragment from the 3' end of gene B (as indicated in the diagram). The length of the probe after S1 digestion **(left)** was the same as that obtained when 500 ng of polysomal feather mRNA from 14 feathers was used in the reaction (approximately 314 bp, **centre**). A small sample of undigested probe was loaded in the **third** track.


A
A+
A−
100
B
S1
622
404
309
BK-i
A+
Feather
A+
Probe
cap
BstEII
A+
BglII
probe

At this stage it is not known whether the unbound mRNA completely lacked a poly(A) tail or whether tailing was simply too short to allow binding to the column.

A 632 bp BglII/BstEII fragment was used to map the 3' terminus of polyadenylated BK-i message by S1 protection analysis. The point of addition of the poly(A) tail was observed to be approximately the same as that observed **in vivo** (figure 4-6B).

4.2.5 TEST FOR ENHANCER REQUIREMENTS

The enormous burst of feather keratin gene transcription during feather development may be facilitated by an enhancer-like stimulation. It is possible that enhancer sequences may occur in the DNA already injected but may not be recognized in the oocyte. Alternatively, such sequences may be present in the more distal parts of the feather keratin cluster beyond the termini of Cosmid 4. To test these possibilities gene B and the intronless keratin gene (BK-i) were modified to include the SV40 enhancer producing SVGene B and SVBK-i (sections 3.5 and 3.7). No increase in transcription was observed from either gene in the presence of the enhancer (figure 4-7). This was not due to an inability of the SV40 enhancer to function in the oocyte because the attachment of the enhancer to the thymidine kinase gene clone tk (SVtk, see section 3.11) resulted in a low but reproducible 4-fold increase in tk gene transcription (figure 4-8).

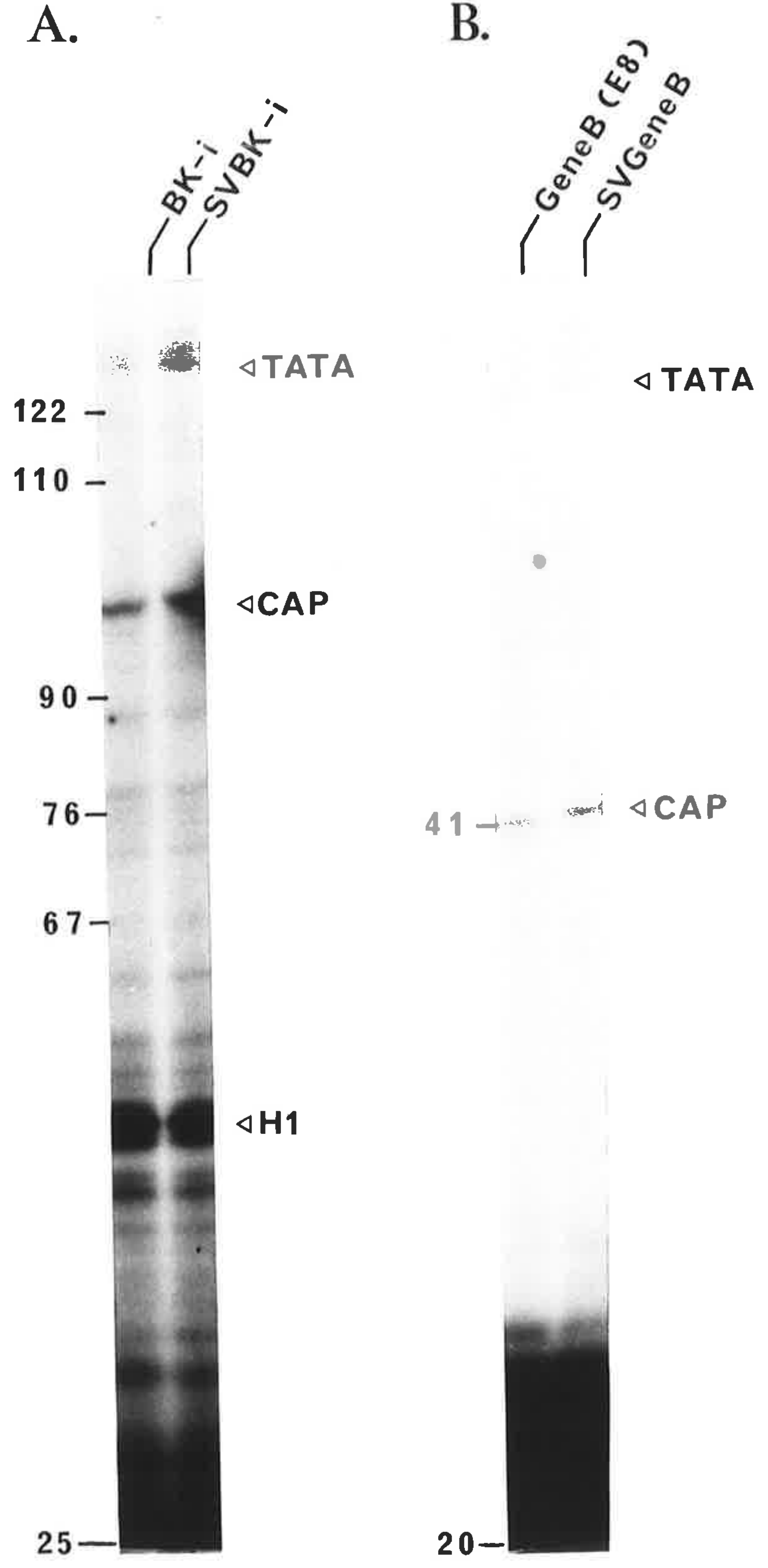
It was possible that the low level of tk gene enhancement was related to an already maximal transcriptional efficiency of this gene in the oocyte. To test this possibility the second distal transcription signal was removed

FIGURE 4-7

PRIMER EXTENSIONS ON FEATHER KERATIN GENE B CONSTRUCTS CONTAINING THE SV40 ENHANCER

A. Oocytes were injected with BK-i (gene B minus intron; section 3.6) and SVBK-i (gene B minus intron and SV40 enhancer; section 3.7). An equal amount of p7AT containing the chicken H1 histone gene was included in each injection mix to act as an internal control. Two oocytes worth of RNA, the H1 primer and the keratin 25-mer were used in the same extension mix. The H1 extension product at 58 bp is indicated in the diagram. BK-i and SVBK-i extension products electrophoresed at 100 bp. Bands representing initiations from the keratin TATA box are also indicated. The reaction products were electrophoresed on a 6% denaturing polyacrylamide gel.

B. Oocytes were injected with gene B (clone 1-4E8; section 3.4) and SVgene B (clone 1-4E8 plus SV40 enhancer; section 3.5). The extension reaction was performed on the RNA equivalent of two oocytes and the keratin 20-mer was used in the reaction. The products were electrophoresed on a 10% denaturing polyacrylamide gel and migrated at 41 bases. Bands representing initiations from the TATA box are also indicated.



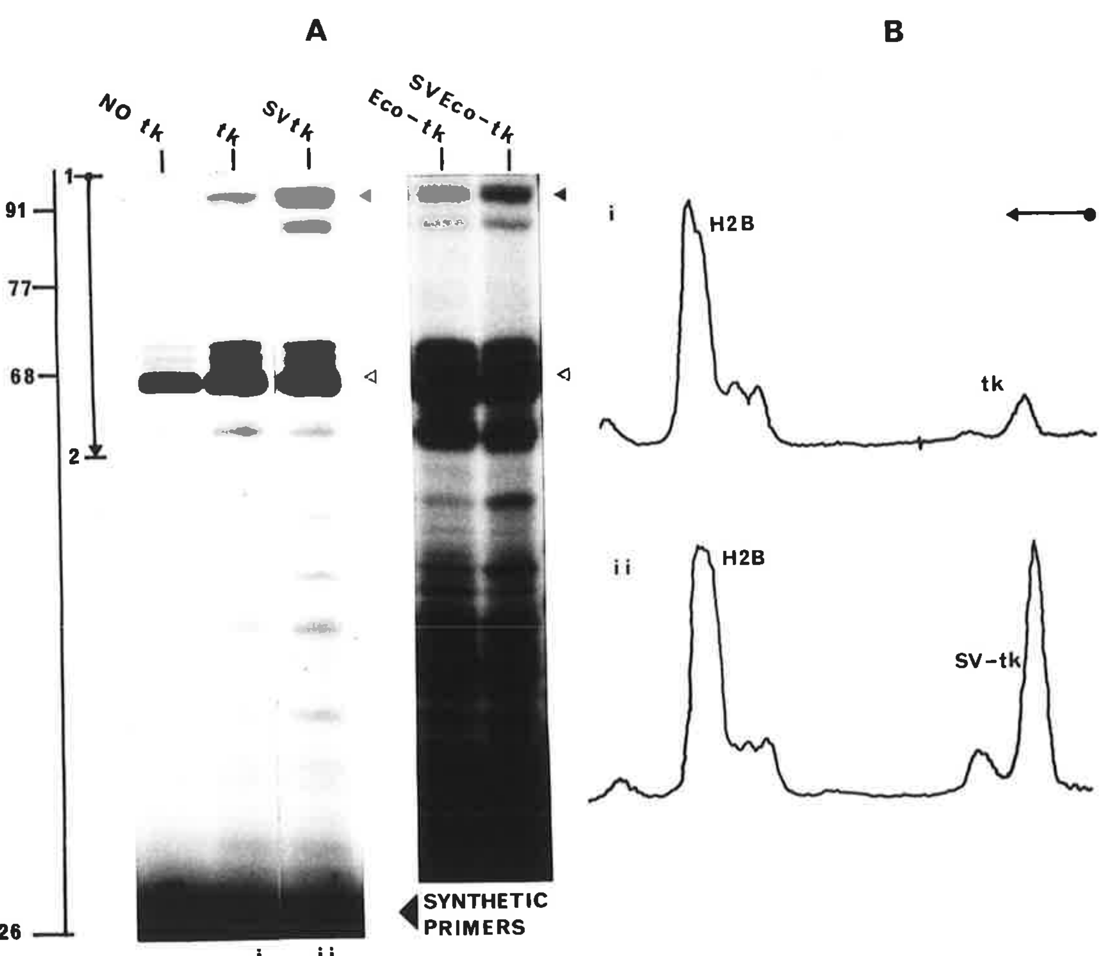
A.
B.
BK-i
SVBK-i
GeneB (E8)
SVGeneB
TATA
122
110
CAP
90
76
67
H1
25
TATA
41
CAP
20

FIGURE 4-8

EFFECT OF THE SV40 ENHANCER ON HSV-tk GENE TRANSCRIPTION IN XENOPUS OOCYTES

A. Oocytes were injected with the following plasmids, tk (tk gene; section 3.9), SVtk (tk gene plus SV40 enhancer; section 3.11), Eco-tk (tk gene minus second distal promoter; section 3.10) and SVEco-tk (tk gene minus second distal promoter plus SV40 enhancer; section 3.12). An equal amount of p7AT DNA containing the H2B histone gene was co-injected with each tk construct to act as an internal control. The shaded arrowheads show the position of nucleotide extension products representing tk mRNA at 87 and 93 bp. The open arrowheads show the position of nucleotide extension products indicative of H2B mRNA at 68 bp. Symbols **(i)** and **(ii)** refer to the tracks whose representative densitometric scans are depicted in **B.**

B. Direction and length of scan from points **1** to **2** shown on the left of **A.**


A
B
NO tk
tk
SVtk
Eco-tk
SVEco-tk
91
77
68
26
1
2
SYNTHETIC
PRIMERS
i
ii
H2B
tk
SV-tk

from the tk gene promoter, producing clone Eco-tk (see section 3.10). Removal of the second distal signal reduces transcriptional efficiency to 4% of normal (McKnight **et al.**, 1984). When reduced to such low efficiencies of transcription the extent of enhancement by the SV40 enhancer (SVEco-tk, see section 3.12) was similar to that of the unmodified gene, about 4-fold (figure 4-8).

4.3 DISCUSSION

4.3.1 ENHANCER REQUIREMENTS FOR FEATHER KERATIN GENE TRANSCRIPTION IN XENOPUS OOCYTES: CLARIFICATION OF SV40 ENHANCER FUNCTION

An enormous burst of feather keratin gene transcription occurs in the epidermal cells of the developing feather around day 13 of embryonic life in the chicken. During this time feather keratin message derived from up to 20 genes forms in excess of 50% of the total message content of the cell (Powell **et al.**, 1976). In the transcriptional studies described here more than 10^6 copies of feather keratin gene B were injected per oocyte, and at no time was there observed an efficiency of transcription comparable to that which occurs **in vivo**. The possibility that the keratin DNA was being selectively degraded was ruled out in experiments where a feather keratin gene was inserted into the histone cluster and it was observed that histone genes continued to be expressed efficiently while transcription from the feather keratin gene remained low.

Enhancer-type sequences may be necessary to produce the required level of transcription since it is known that feather keratin gene amplification does not occur (Gibbs, 1977). Two

possibilities were evident. Firstly, enhancer sequences might occur within the DNA already injected, but may not be recognized by the oocyte transcriptional complex. Alternatively, transcriptionally important sequences may exist in the more distal parts of the keratin gene cluster, beyond the termini of the cosmid clone (Cosmid 4) used in this study. Attaching the SV40 enhancer to a keratin subclone containing gene B and also to BK-i had no effect on the levels of transcripts produced from either construct.

At this time there were discrepancies in the literature relating to the functional capability of the SV40 enhancer in **Xenopus** oocytes. In one study the rabbit β-globin gene was found to be only weakly expressed in early **Xenopus** embryos despite the presence of the SV40 enhancer (Rusconi and Schaffner, 1981). However, when rabbit β-globin gene recombinants were transfected into HeLa cells, transcriptional efficiency of this gene was increased 200-fold when compared to the enhancerless gene (Banerji **et al.**, 1981). From these results it was inferred that the SV40 enhancer was not functional in **Xenopus** oocytes (de Villiers **et al.**, 1982). This conclusion is erroneous because of the biological differences which exist between oocytes and the eggs or early embryos of **Xenopus**. For example, **Xenopus** oocytes are intensively active in RNA synthesis whereas eggs and embryos up to the mid-blastula stage synthesize very little RNA and are transcriptionally relatively quiescent (Gurdon and Melton, 1981).

To try and resolve whether the SV40 enhancer was functional in oocytes the effect of the SV40 enhancer on HSV-tk gene transcription was tested. The HSV-tk gene was

specifically chosen because it is efficiently expressed in **Xenopus** oocytes (McKnight and Kingsbury, 1982) and the SV40 enhancer is known to increase its expression in cell culture (Luciw **et al.**, 1983).

The 4-fold increase in HSV-tk gene transcription observed here is lower than the 10-20 fold increase in tk enzyme activity seen in Hela cells in the presence of the enhancer (Fromm, 1983). It has been shown that specific factors interact with the SV40 enhancer and that the level of these factors varies among cell types (Schöler and Gruss, 1984; Sassone-Corsi **et al.**, 1985). This could be part of the reason for the lower level of stimulation observed in oocytes. A recent publication by Spinelli and Ciliberto (1985) has also verified that the SV40 enhancer is functional in oocytes. Spinelli and Ciliberto (1985), however, observed a 10-fold increase in chloramphenicol acetyl transferase (CAT) activity in the presence of the SV40 enhancer. It is interesting that transcription from the tk gene and the CAT gene is stimulated to differing degrees in oocytes by the SV40 enhancer while feather keratin gene B appears to be enhancer independent.

The difference in the degree of transcriptional enhancement in the oocyte may be a reflection on the type of gene to which the SV40 enhancer is linked. It is possible that the complement of promoter elements possessed by a gene may have some influence on the ability of the SV40 enhancer to stimulate transcription. For example, other factors which bind to specific promoter elements may not interact favourably with enhancer factors making it difficult to form the initiation complex necessary for transcription (see section 1.2.4). The availability of all the necessary factors in a

particular expression system would also have a direct bearing on the degree of transcriptional stimulation.

4.3.2 SUPPRESSIVE EFFECT OF THE FEATHER KERATIN INTRON ON KERATIN GENE TRANSCRIPTION IN XENOPUS OOCYTES

Feather keratin transcripts, derived from keratin genes microinjected into **Xenopus** oocytes, were present at very low levels and, surprisingly, all of the transcripts detected at steady state levels were observed to be unspliced. The absence of any spliced feather transcripts in injected oocytes is unusual because the excision of introns by oocytes is well documented. For example, oocytes injected with SV40 DNA synthesize virion protein VPI (De Robertis and Mertz, 1977) and the large tumour antigen (Rungger and Turler, 1979) the production of functional mRNA for both of these proteins requires the excision of a single intron. SP6-derived globin message is efficiently spliced when injected into oocytes (Green **et al.**, 1983). All six introns must be excised from the ovalbumin gene when it is injected into oocytes to produce the detected protein product (Wickens **et al.**, 1980). In addition, the chicken ALA-synthetase gene, which contains two introns, is effeciently expressed in oocytes with correct splicing (J. Loveridge, personal communication).

It is possible that oocytes lack molecules which are specifically necessary for the processing of feather keratin transcripts, for example, small nuclear ribonucleoprotein particles (snurps). It has already been reported that the oocyte lacks a specific snurp which is necessary for the correct termination of sea urchin H3 histone transcripts (Galli **et al.**, 1983).

Two observations suggest that intron sequences in the intact feather keratin gene may be inhibiting transcriptional initiation in the oocyte. Firstly, there was a 5-fold increase in the level of correctly initiated gene B transcripts when its intron was removed. Secondly, the 5-fold increase was retained when the keratin intron was replaced with a similar-sized fragment from pBR322.

Another explanation for these results is that the low level of correctly initiated transcripts produced from the intact feather keratin gene B were caused by degradative losses due to partial splicing and injection of a gene lacking the intron circumvented this, thus resulting in a greater number of observable transcripts. However, replacement of the keratin intron with a pBR322 fragment resulted in a construct which lacked a functional 3' splice site, yet transcript levels were identical to those obtained from the intronless keratin gene, BK-i.

Furthermore, a low level of transcription was initiated at the keratin gene TATA box in the microinjected oocytes. Impaired transcript stability due to inefficient splicing should reduce the yield of transcripts from the TATA box by the same factor as it reduced the yield from the **in vivo** initiation site. This was clearly not the case, as the amount of transcript initiated from the TATA box was constant, irrespective of the presence or absence of the intron from the injected construct. The only change observed with the clone containing the keratin gene intron was a decrease in the number of transcripts initiated at the cap site.

The fact that removal of the intron did not result in the elevation of the keratin gene transcripts to levels which were

at least comparable to other genes injected into oocytes suggests that there may be sequences within the keratin gene, other than the intron, which could prevent feather keratin transcription. This aspect is investigated further in chapter 5.

4.3.3 ROLE OF THE SUPPRESSIVE EFFECT ON FEATHER KERATIN GENE TRANSCRIPTION IN OOCYTES AND ITS IMPORTANCE IN VIVO

In the chicken, the production of feather keratin protein in large amounts occurs during the terminal differentiation of the feather keratinocytes and no doubt contributes to the death of those cells. This feature suggests that the unscheduled expression of these genes would be potentially lethal to a cell and implies a need for strict controls on their expression.

It is unlikely that oocytes possess specific factors which could function to repress feather keratin transcription because the β-keratins of avian tissues have no direct equivalent in **Xenopus** (Fraser **et al.**, 1972). Instead, a specific factor(s) may be required to bind to the feather keratin intron in order to facilitate transcriptional activation. However, removal of the feather keratin intron did not increase transcription in oocytes to levels comparable with those observed **in vivo**, hence, additional factors may be necessary for maximum transcriptional activity.

The fact that no spliced transcripts can be detected in oocytes provides another possible control against the unscheduled expression at the level of RNA maturation. It seems unlikely that any unspliced messages would be translated efficiently because of the presence of numerous AUG codons in

the intron, which according to the scanning hypothesis proposed by Kozak (1983) may significantly reduce the likelihood of correct translation. Thus, controls on both the initiation of feather keratin gene transcription and RNA processing could reduce the probability of cell mortality from any unscheduled expression.

The role of the intron **in vivo** may in fact be to keep the feather keratin genes transcriptionally inactive until the correct elements combine to stimulate transcription, at the appropriate stage in development. Similar considerations may be relevant to the expression of the scale keratin and histidine-rich fast protein genes because these genes possess a similarly located intron in the 5' untranslated region and are also involved in avian keratinization.

It is not known however whether the observed suppressive effect of the feather keratin intron on transcriptional initiation actually occurs **in vivo.** At this stage in the project, a decision was made to postpone characterization of the intron sequences which may be responsible for the suppression until the effect could be verified in an homologous expression system, such as that described in chapter 6.

CHAPTER 5.

GENE FUSIONS: KERATIN GENE B

AND THE HSV-tk GENE.

5.1 INTRODUCTION

The low level of transcripts observed in oocytes even when the intron was removed from feather keratin gene B may have been related to an inefficient functioning of the keratin promoter. In order to test this possibility, hybrid recombinant gene constructs were made using feather keratin gene B and the HSV-tk gene. Before describing the experiments performed, recent information pertaining to the expression of the HSV-tk gene and the definition of its promoter elements will be presented. For details relating to tk gene structure refer to figure 3-B.

The expression of the Herpes simplex type 1 viral genes in infected cells is regulated in a cascade fashion. Immediately after infection, viral mRNAs are translated into gene products (α-polypeptides) that activate the transcription of the next major group of genes, the β-polypeptides, of which the tk gene is a member. Subsequent transcriptional activation of the third and final group of viral genes (γ) requires both the presence of α and β polypeptides (Honess and Roizman, 1974; 1975).

The tk gene has been shown to be efficiently expressed in an "unregulated" manner in **Xenopus** oocytes (McKnight and Gavis, 1980; McKnight and Kingsbury, 1982) and in numerous cell lines where it has been used as a selectable marker. The cis-acting signals in the promoter, required for the efficient transcription of the tk gene in **Xenopus** oocytes have been determined (McKnight **et al.**, 1984; see also discussion, this chapter). The signals consist of the TATA sequence; the first distal signal (DSI), a GC-rich hexanucleotide located 50 bp

upstream from the mRNA cap site; the CAT dyad, located 80 bp upstream from the cap site and the second distal signal (DSII) containing another GC-rich hexanucleotide sequence which is found 100 bp upstream of the cap site. The region extending from 50 to 100 bases upstream from the cap site has also been shown to be required for maximal promoter activity in virally induced HeLa cells (El Kareh **et al.**, 1985).

DNAse I footprinting experiments using HeLa cell extracts have shown that the Spl transcription factor binds to the two distal transcription signals and that the CAT transcription factor (CTF) binds to the CAT dyad (Jones **et al.**, 1985). The importance of each of these protein binding sequences for transcriptional efficiency, as judged by mutational analysis, varies from system to system. For example, in HeLa cell extracts, mutations which alter the binding of Spl to DSI and DSII reduce transcription to a greater extent than mutations which alter the binding of CTF (Jones **et al.**, 1985). In oocytes, the two different factor binding sites appear to be more comparable in importance (McKnight and Kingsbury, 1982). In contrast, when the tk gene is transfected into an expression system which is more like the **in vivo** situation (HeLa cells previously transfected with a tk^- mutant of HSV) the CTF binding sequences appear to be more important for tk promoter function than the Spl binding sites (El Kareh **et al.**, 1985). Thus the relative efficiencies of transcription factors appear to vary in the different systems. The exact mechanism of action of the two factors in the activation of tk gene transcription is still unknown.

5.2 EXPERIMENTAL APPROACH AND RESULTS

The results from each experiment dictated the design of subsequent experiments, thus in some sections below, approaches, results and some conclusions are treated as integrated units.

5.2.1 LINKAGE OF THE KERATIN PROMOTER TO THE tk STRUCTURAL GENE RESULTS IN A HIGH LEVEL OF TRANSCRIPTION IN OOCYTES

To test whether the low transcriptional efficiency of the feather keratin gene was caused by promoter deficiencies, hybrid genes were constructed. The keratin promoter from gene B was fused to the tk protein coding region and associated 3' flanking DNA to produce the construct designated KER/TK. Conversely, the tk promoter was fused to the protein coding region and associated 3' flanking DNA of feather keratin gene B to yield the construct TK/KER (refer to sections 3.13 and 3.15 for the construction details). The keratin intron was omitted from the constructs made to prevent possible suppressive effects on transcript initiation.

If inefficient keratin promoter function was responsible for the low level of transcripts observed from the intronless keratin gene, the following results would be expected:

1. Transcripts would be detected in low levels from KER/TK (the construct which contains the keratin promoter) possibly in similar amounts to that observed from the intronless keratin gene, BK-i.

2. A higher level of transcription would be expected from the TK/KER construct since the tk gene is known to function efficiently in oocytes.

Figure 5-1 shows the results obtained from this experiment. TK/KER was expressed at very low levels, comparable to the intronless keratin gene, and KER/TK was expressed extremely well in oocytes, at a level approaching that of the intact tk gene (TK/TK). Densitometric scanning showed that the number of transcripts produced from TK/TK (track 3) and KER/TK (track 4) were 30-fold greater and 20-fold greater respectively than those produced from BK-i (track 6). Interestingly, use of the tk cap site at position 207 was favoured over that at position 201 (figure 3-B(iii)) when the tk promoter was linked to the keratin sequences. This is obvious in later figures and the reason for this is not known.

The amount of transcript produced from the control H2B histone gene, which was co-injected with each test construct, was identical in all cases (figure 5-1B). This demonstrated that the test results were not due to variations in the experimental procedure. To ensure that all elements necessary for the correct functioning of the tk promoter were intact, the tk promoter was resected from the poorly expressed TK/KER construct using PvuII, cloned into M13mp18 and sequenced. The sequence obtained was identical to that published previously (McKnight, 1980) except for one base change, C to G, 41 bases upstream from the mRNA cap site. This base change was also observed on sequencing a tk promoter fragment which had been obtained from the intact tk gene (data not shown). The base change at this position is not critical for efficient tk promoter function (McKnight and Kingsbury, 1982).

FIGURE 5-1

LINKAGE OF THE KERATIN PROMOTER TO THE tk STRUCTURAL GENE RESULTS IN A HIGH LEVEL OF TRANSCRIPTS

Oocytes from the same frog were injected with either TK/TK (intact tk gene), KER/TK (keratin promoter fused to the tk gene), TK/KER (tk promoter fused to the keratin gene) or BK-i. An equal amount of p7AT DNA containing the chicken histone H1 gene was included as an internal control. Primer extensions were performed using the RNA equivalent of two oocytes. The primers were of the same specific activity and the extension products were electrophoresed on a 6% denaturing polyacrylamide gel.

TRACK 1 HpaII cut pBR322 markers; the sizes are given to the left of the track.

TRACK 2 Extension reactions performed on uninjected oocyte RNA using the tk 26-mer, keratin 26-mer and H1 26-mer.

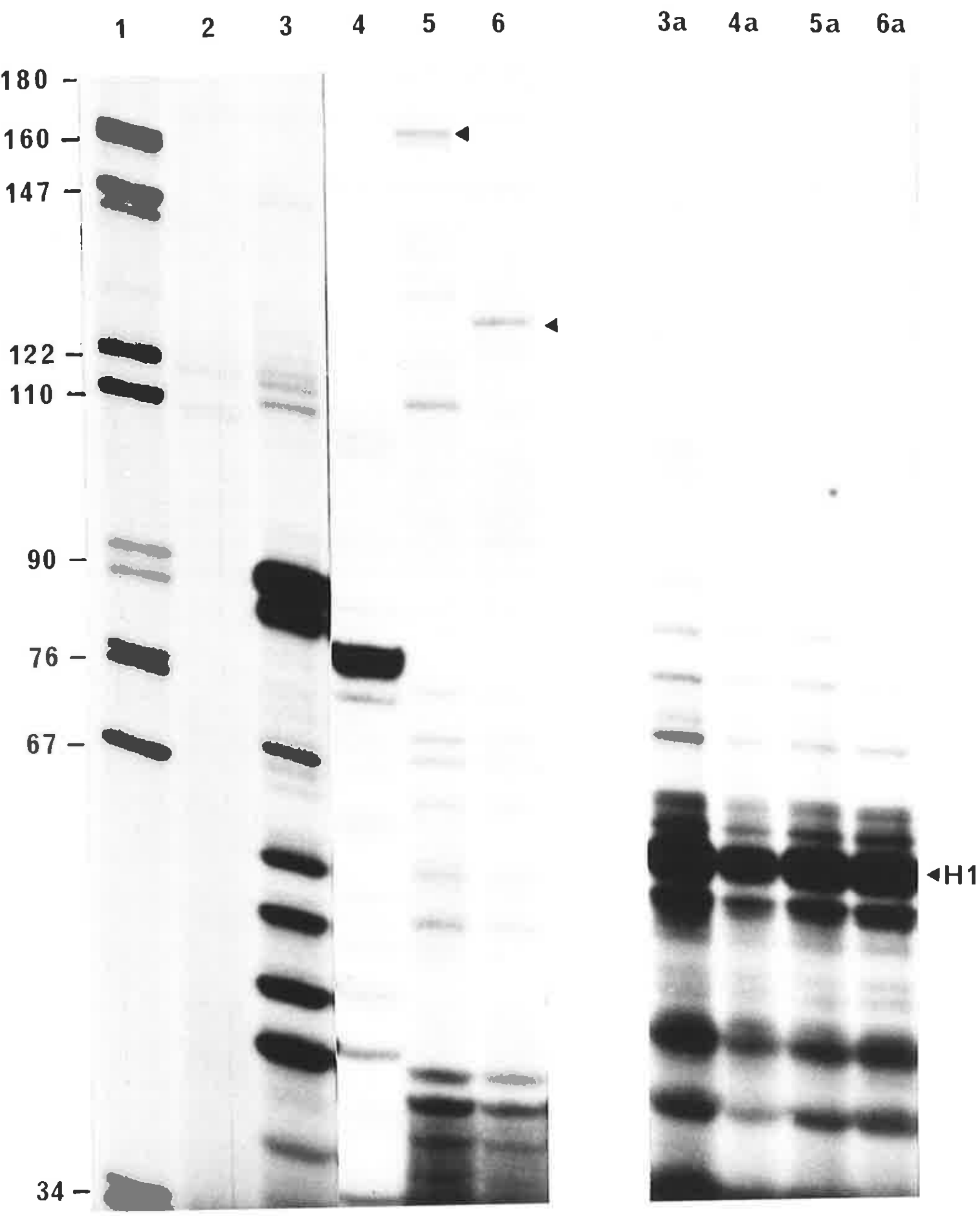
TRACK 3 Extension products from tk (section 3.9) injected oocyte RNA using the tk 26-mer. The products migrated at 87 and 93 bases.

TRACK 4 Extension products from KER/TK (section 3.15) injected oocyte RNA using the tk 26-mer. The products migrated at 84 bases.

TRACK 5 Extension products from TK/KER (section 3.13) injected oocyte RNA using the keratin 26-mer. The products electrophoresed at 173 bases and are indicated by the arrow. (The cap site at position 207 (figure 3-B(ii)) was used in favour of the cap site at position 201).

TRACK 6 Extension products from BK-i injected oocyte RNA using the keratin 26-mer. The products migrated at 131 bases.

TRACKS 3a to 6a Primer extensions were performed on the same RNA as that used in the tracks **3** to **6** except the H1 primer was used in the extension reactions. The products electrophoresed at 58 bases.


1
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5
6
3a
4a
5a
6a
180
160
147
122
110
90
76
67
34
H1

It was concluded that the keratin promoter was able to function very efficiently in the oocyte when linked to the tk gene coding and 3' untranslated regions. Furthermore, the efficiency of the tk promoter appeared to be reduced when linked to the feather keratin protein coding and associated 3' untranslated region. Four possibilities were considered likely explanations for these results.

1. The original TK/KER construct possessed 31 bp of pBR322 DNA between the tk promoter and the keratin gene and this may have affected tk promoter efficiency. To eliminate this possibility, a modified TK/KER construct was made (MTK/KER; section 3.16) which did not contain the pBR322 DNA. Figure 5-2 shows that the presence of the pBR322 DNA had no effect on transcript levels (compare tracks 4 and 5).
2. Constructs containing the feather keratin protein coding and associated 3' untranslated region are degraded very rapidly in oocytes and are thus present in low amounts at steady state levels.
3. Sequences in the keratin gene may be able to inhibit transcription from the tk promoter.
4. The tk protein coding and associated 3' flanking DNA could act to stimulate transcription from the keratin promoter.

Experiments to test possibilities 2 to 4 are described in the following sections.

5.2.2 COMPARISONS OF MESSAGE STABILITY IN OOCYTES

To establish whether there was a difference in message stability, the rate of message degradation was determined for

FIGURE 5-2

DELETIONS IN THE KERATIN SEQUENCES OF TK/KER

Oocytes were injected with constructs derived from TK/KER. Each construct had a deletion in the keratin sequences. An equal amount of p7AT DNA, containing the H1 histone gene, was co-injected with each construct and primer extensions using the H1 26-mer acted as an internal control (indicated). The RNA equivalent of 2 oocytes was used. Primers were of the same specific activity and reaction products were electrophoresed on a 6% denaturing polyacrylamide gel. Information for each track (except number 1) is presented in the following order: sample injected, (construct reference) primer used and the length of the extension product. Where RNA from constructs containing the tk promoter were assayed, the last number indicates the location of the cap site used (see figure 3-B(ii)). The symbol (◀) denotes transcripts initiating from the cap site and (◁) indicates transcripts initiating from the TATA box.

TRACK 1 HpaII cut pBR322 markers (end-labelled).

TRACK 2 TK/TK (3.9), tk 26-mer, 93 and 87 bases, 201 and 207.

TRACK 3 KER/TK (3.15), tk 26-mer, 84 bases.

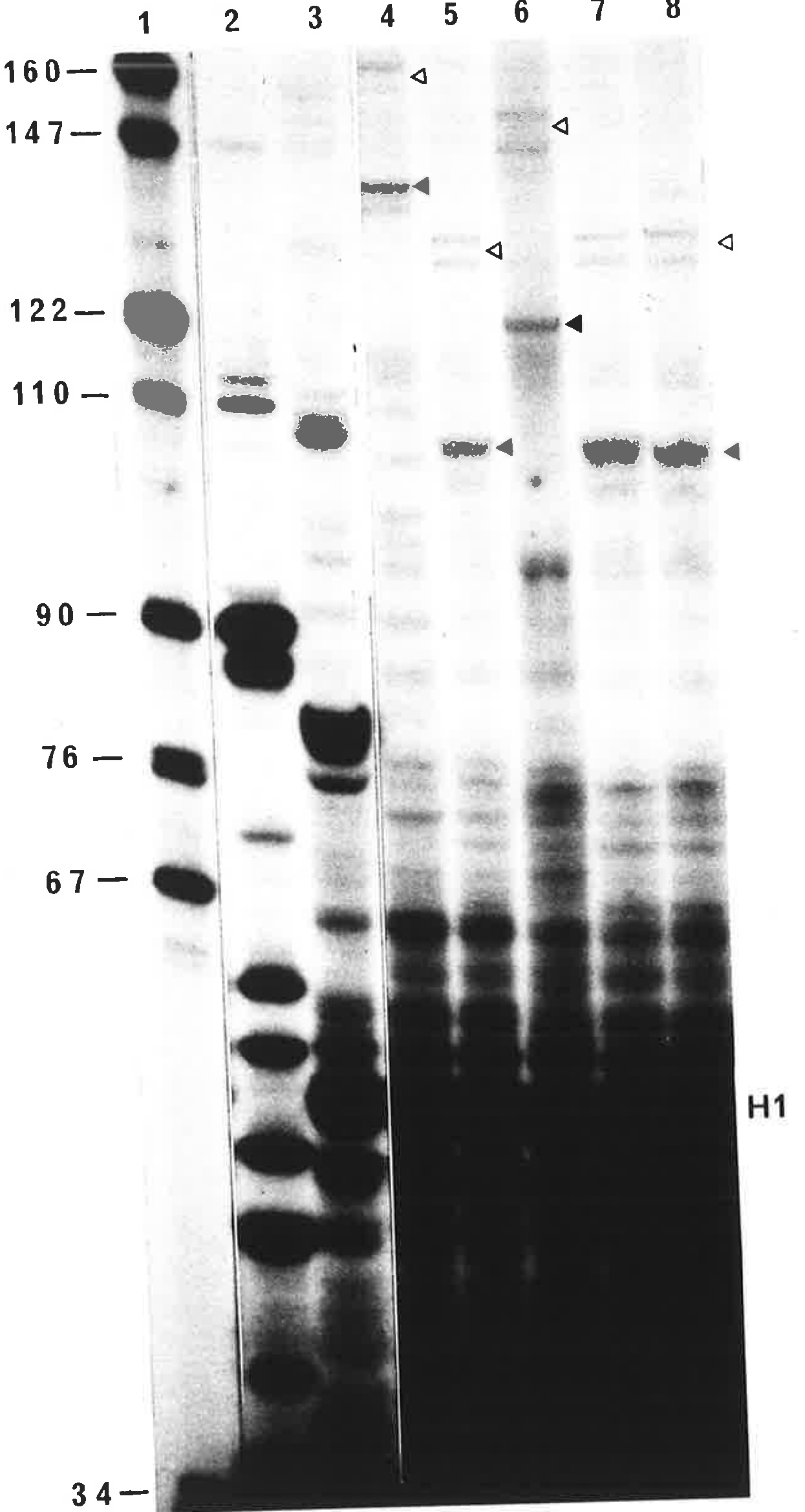
TRACK 4 TK/KER (3.13), keratin 25-mer, 142 bases, 207.

TRACK 5 MTK/KER (3.16), keratin 25-mer, 107 bases, 207.

TRACK 6 MTK/KER-SALSMA (3.19), keratin Sp 25-mer, 122 bases, 207.

TRACK 7 MTK/KER-BE2 (3.17), keratin 25-mer, 107 bases, 207.

TRACK 8 MTK/KER-BE2SMA (3.18), keratin 25-mer, 107 bases.



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8
160
147
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110
90
76
67
34
H1

messages derived from each of the following constructs, BK-i, TK/KER, KER/TK and TK/TK.

The method used to measure message degradation was that described by Miller **et al.** (1982) and involved co-injecting oocyte nuclei with the construct to be tested and with α-^{32}P-rGTP. The oocytes were then incubated for 24 hours to synthesize and accumulate labelled transcript. α-amanitin was injected into the oocyte at a concentration known to inhibit more than 95% of polymerase II activity (final concentration of 1 μg/ml; Miller **et al.**, 1982). The rate of degradation of the labelled message was subsequently followed by measuring the amount of transcript remaining in oocytes at a series of times after the α-amanitin treatment. In each case the amount of labelled RNA remaining was measured by hybridization to nitrocellulose bound DNA (refer to section 2.11.6 and 2.11.7).

Figure 5-3A shows the amount of labelled transcript synthesized during a 24 hour incubation period. The amount of transcript produced from each of the constructs injected confirmed the results obtained previously by primer extension (section 5.2.1). The rate of message degradation after α-amanitin treatment is shown in figure 5-3B, with the results being expressed as a percentage of the amount of specific transcript present in oocytes immediately before the α-amanitin injection. The degree of scatter amongst the points could be explained by a difference in response to α-amanitin between the batches of injected oocytes because of slight variations in the amount of α-amanitin delivered per oocyte.

The biphasic nature of the curve has been observed previously and it was shown from similar studies with SV40 transcripts that a large majority of the RNA synthesized in

FIGURE 5-3 A

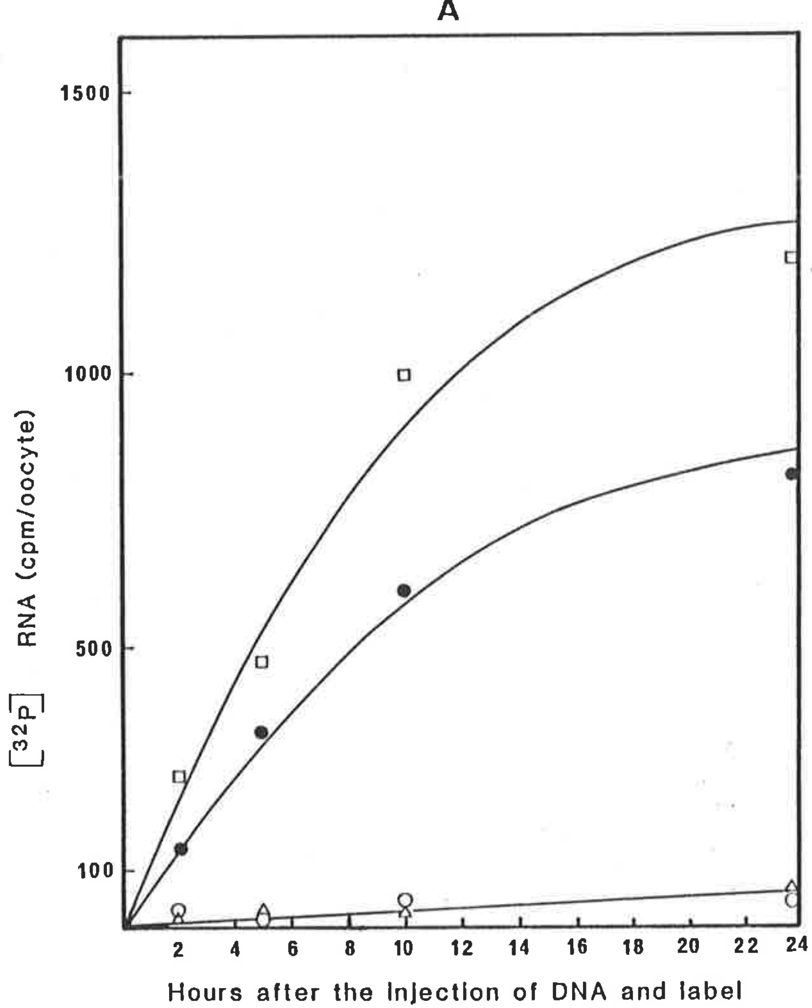
SYNTHESIS OF LABELLED RNA IN OOCYTES

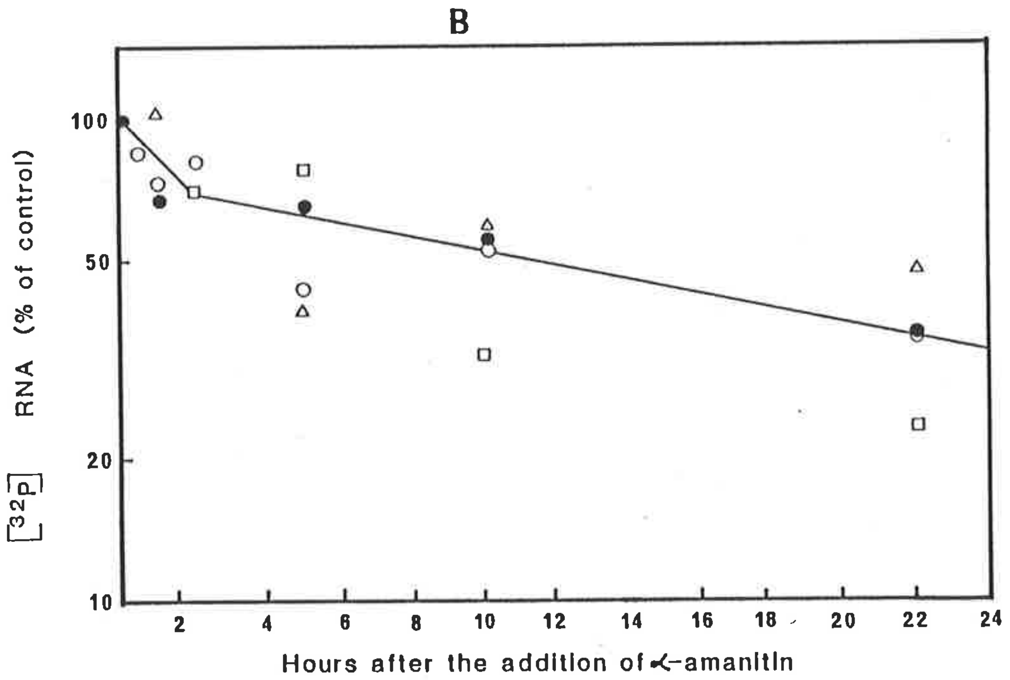
Oocytes were injected with 10 ng of one of four constructs; BK-i (Δ), TK/KER (○), KER/TK (●) or TK/TK (□). Twenty four hours later 1 μCi of α-^{32}P-rGTP was injected into each oocyte. A sample of 10 oocytes was removed at varying times after the injection of label and the amount of labelled RNA produced from each construct was determined by hybridization to nitrocellulose bound DNA (2.11.6). The points represent an average of two experiments.

FIGURE 5-3 B

DISAPPEARANCE OF LABELLED RNA UNDER α-AMANITIN CHASE CONDITIONS

Oocytes were co-injected with 10 ng of the DNA template (symbols as above) and 1 μCi of α-^{32}P-rGTP. The oocytes were then incubated for 24 hours. Each oocyte was injected with 10 nl of α-amanitin (100 μg/ml). Batches of 10 oocytes were removed at varying times after the α-amanitin injection and the amount of labelled transcript remaining was determined by hybridization to nitrocellulose bound DNA (2.11.6). The points represent an average of two experiments and the data was normalized to the amount of radioactivity present at the time of addition of α-amanitin.


A
1500
1000
500
100
[32P] RNA (cpm/oocyte)
2
4
6
8
10
12
14
16
18
20
22
24
Hours after the injection of DNA and label


B
100
50
20
10
[32P] RNA (% of control)
2
4
6
8
10
12
14
16
18
20
22
24
Hours after the addition of α-amanitin

oocytes is degraded within the first few hours (Miller **et al.**, 1982). Message half-life, in the experiments shown here was calculated from the portion of the curve representing the longer-lived transcripts.

The most important constructs in which to compare message stability are TK/KER and KER/TK. The percentage of labelled transcript remaining from both of these constructs is essentially identical at each time point. In general, messages from all of the injected constructs had similar rates of degradation in the oocyte with half-lives of approximately 12-14 hours. Interestingly, similar half-lives have also been reported for sea urchin H2B transcripts (Georgiev and Birnstiel, 1985) and SV40 transcripts in oocytes (Miller **et al.**, 1982). The similarity in message degradation among the four constructs tested here confirms that the measured differences in transcript levels were a function of transcription frequency and not message stability.

5.2.3 INVESTIGATION OF TRANSCRIPTIONAL REGULATORY SEQUENCES IN THE KERATIN CODING AND 3' UNTRANSLATED REGIONS

With the possibility of differential message stability eliminated, the existence of regulatory regions within the structural portion of feather keratin gene B was investigated. The construct MTK/KER was chosen for this purpose on the basis that the tk promoter has been extensively characterized (McKnight **et al.**, 1984). The experimental strategy involved deletion of DNA sequences within the keratin component of the construct and subsequent determination of whether any of the gross deletions performed would alter transcriptional efficiency.

Three different deletions were made within the keratin portion of the MTK/KER construct, producing constructs designated MTK/KER-BE2, MTK/KER-BE2SMA and MTK/KER-SALSMA (for construction details see sections 3.17, 3.18 and 3.19). These constructs were injected into oocytes with an equal amount of p7AT DNA as an internal control. None of the deletions, however, increased transcriptional efficiency from the tk promoter (figure 5.2, cf tracks 5, 6, 7 and 8 with track 3).

In the production of all of the above constructs an effort was made to preserve the polyadenylation signal as it was not certain at the time what effect removal of the polyadenylation signal would have on message stability in oocytes. Subsequent work by Green **et al.** (1983) has shown that the only prerequisite for continued stability of SP6-derived transcripts in oocytes is that the message needs to be capped.

Preservation of the normal keratin polyadenylation signals in the above deletion constructs also resulted in the retention of the 18 bp perfectly conserved sequence (see section 1.1.4(v)) which is present in all of the feather keratin genes sequenced to date. The 18 bp sequence was removed from TK/KER and the deletion of this sequence also eliminated all of the 3' untranslated region keratin sequences which had not been deleted previously. Unfortunately, the keratin polyadenylation signal was also removed so it was replaced by the polyadenylation signal belonging to the tk gene and the construct generated was designated TK/KER-3'TK (refer to section 3.21). No effect on the levels of transcript were observed with this construct and transcript levels remained low in keeping with those from the parental

FIGURE 5-4

EFFECT OF THE REMOVAL OF THE 18 bp 3' SEQUENCE ON TRANSCRIPTION IN OOCYTES

A. Oocytes were injected with either TK/KER-3'TK (section 3.21) or TK/KER (section 3.13). An equal amount of p7AT DNA, containing the H2B chicken histone gene was co-injected with each construct. The RNA equivalent of two oocytes was used in the primer extension reaction with the keratin 26-mer and the reaction products were electrophoresed on a 6% denaturing acrylamide gel.

TRACK 1 HpaII cut pBR322 markers (end-labelled).

TRACK 2 TK/KER; extension product at 173 bases.

TRACK 3 TK/KER-3'TK; extension product at 173 bases.

TRACKS 2a & 3a Extensions on the same RNA used in **2** and **3** except the H2B primer was used. H2B extension products migrated at 68 bases.

B. Oocytes were injected with either BK-i (section 3.6) or BK-i-3'TK (section 3.20). Histone DNA (p7AT) was co-injected with each construct. The RNA equivalent of two oocytes was used in the primer extension with the keratin 25-mer and the H1 primer. The reaction products were electrophoresed on a 6% denaturing polyacrylamide gel.

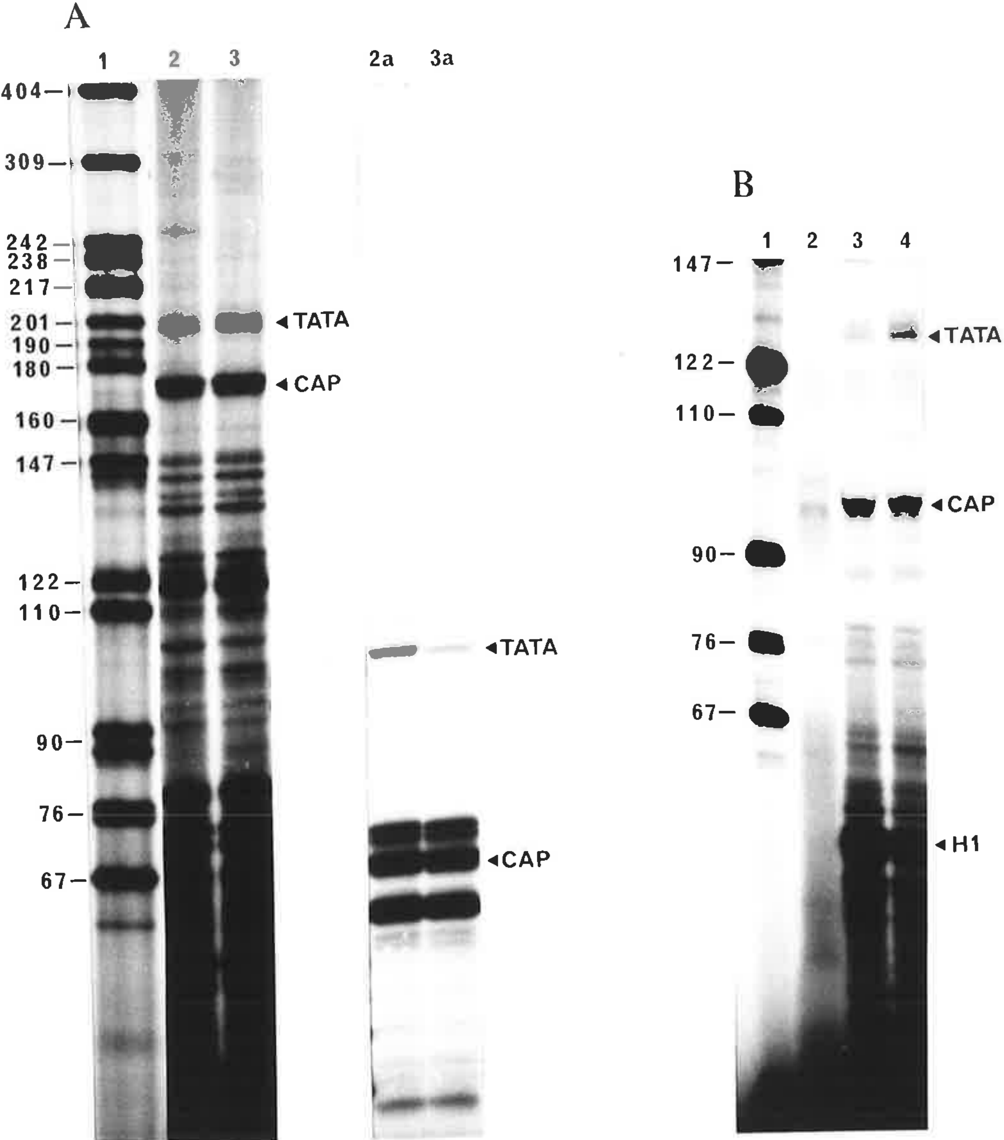
TRACK 1 HpaII cut pBR322 markers (end-labelled).

TRACK 2 Extensions on uninjected oocytes.

TRACK 3 BK-i extension product (**CAP**) at 100 bases.

TRACK 4 BK-i-3'TK extension product at 100 bases.

The position of the H1 extension product (58 bases) is indicated.



A
1
2
3
2a
3a
404
309
242
238
217
201
190
180
160
147
122
110
90
76
67
TATA
CAP
TATA
CAP
B
1
2
3
4
147
122
110
90
76
67
TATA
CAP
H1

construct TK/KER (figure 5-4A).

In order to verify that the 18 bp sequence was not critical for feather keratin gene transcription in oocytes, the sequence was removed from the intronless keratin gene BK-i and the missing polyadenylation signal was again replaced with that belonging to the tk gene, producing BK-i-3'TK (see section 3.20). Transcripts obtained from BK-i-3'TK were identical in amount to those obtained from the intronless keratin gene (figure 5-4B). It appears that in oocytes at least the 18 bp sequence is not important in transcription.

One further deletion construct was made, TKKpr, which was similar to MTK/KER-BE2SMA, except that a further 38 bp of keratin sequence was removed and the construct lacked a polyadenylation signal (see section 3.22). The amount of transcript produced from TKKpr was low and identical to that obtained from the intronless keratin gene (figure 5-5) and hence comparable to the amount from the original TK/KER construct.

To summarize the results from this section, none of the deletions performed in the keratin portion of the MTK/KER construct had any effect on transcription from the tk promoter.

5.2.4 TRANSCRIPTIONAL STUDIES WITH tk AND KERATIN PROMOTERS

The results of the deletions made in the keratin DNA sequences of MTK/KER suggested two possibilities:

1. The presence of any portion of keratin sequence in a construct was sufficient to exert an inhibitory effect on the tk promoter as none of the deletions described were devoid of keratin DNA.

FIGURE 5-5

PRIMER EXTENSIONS

ON

TKKpr AND BK-i

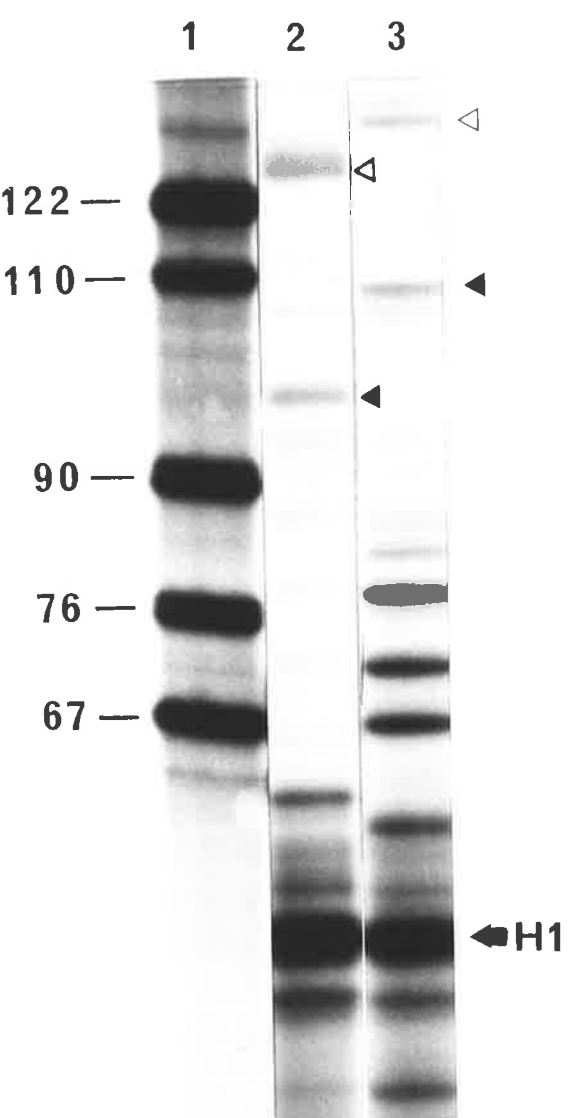
Oocytes were co-injected with either TKKpr (section 3.22) or BK-i (section 3.6). An equal amount of p7AT DNA, containing the H1 gene was injected with each construct. Two oocytes worth of RNA was used in the extension reaction with the H1 primer and the keratin 25-mer. The reaction products were electrophoresed on a 6% denaturing polyacrylamide gel.

TRACK 1 HpaII cut pBR322 markers (end-labelled).

TRACK 2 BK-i extension products (◀) at 100 bases.

TRACK 3 TKKpr extension products (◀) at 109 bases. The cap site at position 207 in figure 3-B(ii) was used in favour of the cap site at position 201.

Transcripts which initiated from the TATA box are indicated by the symbol (◁) and the position of H1 primer extension products is also shown.



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67
H1

2. The promoter of the tk gene may be weak when separated from its structural gene sequences.

To try and discriminate between these two possibilities, the tk promoter was fused to the coding region of the H2B histone gene (construct TK/H2B, see section 3.23). For comparative purposes the keratin promoter was also fused to the H2B protein coding region (construct KER/H2B, section 3.24).

The H2B gene is efficiently expressed in oocytes, hence linkage of the tk and keratin promoters with this heterologous gene should give some indication of their relative strength, independent of keratin and tk gene sequences. Both constructs, TK/H2B and KER/H2B, were co-injected with an equal amount of intact tk gene to act as an internal control. The results of the primer extensions obtained are shown in figure 5-6. Very low levels of transcripts were obtained from both TK/H2B and KER/H2B when compared to the co-injected, intact tk gene. The level of transcript from TK/H2B was also low when compared with transcripts from the intact H2B gene (data not shown).

A number of conclusions can be drawn in the light of these results. Firstly, the keratin gene promoter functions inefficiently in oocytes when it is linked to either its own protein coding region or to that of the H2B gene. Secondly, the tk promoter only functions efficiently when linked to the protein coding and 3' untranslated region of the tk gene. Thus, the tk gene sequences must somehow stimulate transcription from both the tk and keratin promoters. The stimulation is cis-acting since co-injection of the intact tk gene with TK/H2B did not result in an increase in trans-

FIGURE 5-6

LEVEL OF TRANSCRIPTION FROM tk AND KERATIN PROMOTERS WHEN LINKED TO THE H2B GENE

TK/H2B (section 3.23) and KER/H2B (section 3.24) were injected into oocytes and an equal amount of intact tk gene was co-injected with each construct. The KER/TK construct was also injected into one batch of oocytes. The RNA was extracted after 22 hours and primer extensions were performed using the RNA equivalent of 2 oocytes. All primers were of the same specific activity and reaction products were electrophoresed on a 6% denaturing polyacrylamide gel. Information relating to each track, except tracks **1** and **2** is given in the following manner: construct injected, primer used, extension product size, gene co-injected, primer used, extension product size.

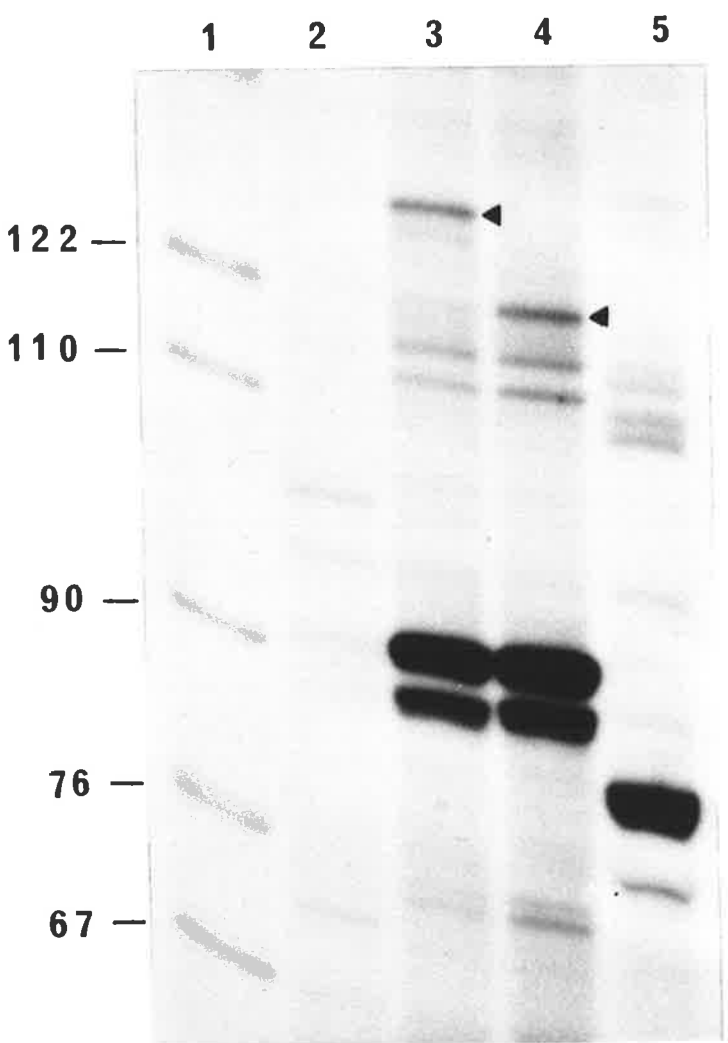
TRACK 1 HpaII cut pBR322 markers.

TRACK 2 Extensions performed on uninjected oocytes using the tk 26-mer and H2B 26-mer.

TRACK 3 TK/H2B, H2B 26-mer, 127 bases (◄), TK/TK, tk 26-mer, 93 and 87 bases.

TRACK 4 KER/H2B, H2B 26-mer, 120 bases (◄), TK/TK, tk 26-mer, 93 and 87 bases.

TRACK 5 KER/TK, tk 26-mer, 84 bases.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cription from the TK/H2B construct.

The possibility remained that the presence of any keratin coding and 3' untranslated sequence could depress transcription from any linked promoter down to a minimum level such as that observed from TKKpr.

5.2.5 INVESTIGATION OF THE tk GENE STIMULATORY EFFECT: THE IMPORTANCE OF PROMOTER DISTANCE IN RELATION TO THE CODING REGION

The first step in the characterization of the tk stimulatory effect was an investigation of whether the activation was distance dependent. Fragments of different sizes from the coding region of feather keratin gene B were placed between the keratin promoter and tk gene junction (refer to sections 3.25, 3.26 and 3.27). This resulted in the production of KER127TK, KER187TK and KER328TK where the central number represents the distance from the keratin cap site to the AUG of the tk gene. Keratin DNA was chosen on the proviso that if transcription from all the spacer constructs was low, other constructs containing different spacer DNA would be made in order to discount the possibility that the keratin DNA spacer was inhibiting transcription.

Injection of the three spacer constructs into oocytes resulted in the same level of transcription as was observed from the parental KER/TK construct (figure 5-7A). It was not until the distance between the keratin cap site and the AUG of the tk gene was increased to 436 bases by inserting pBR322 (construct KER436TK) that the amount of correctly initiated transcript was observed to decrease to a level which was quantitatively comparable to that observed from the intronless

FIGURE 5-7

THE EFFECT OF SEPARATING THE KERATIN PROMOTER FROM tk GENE SEQUENCES

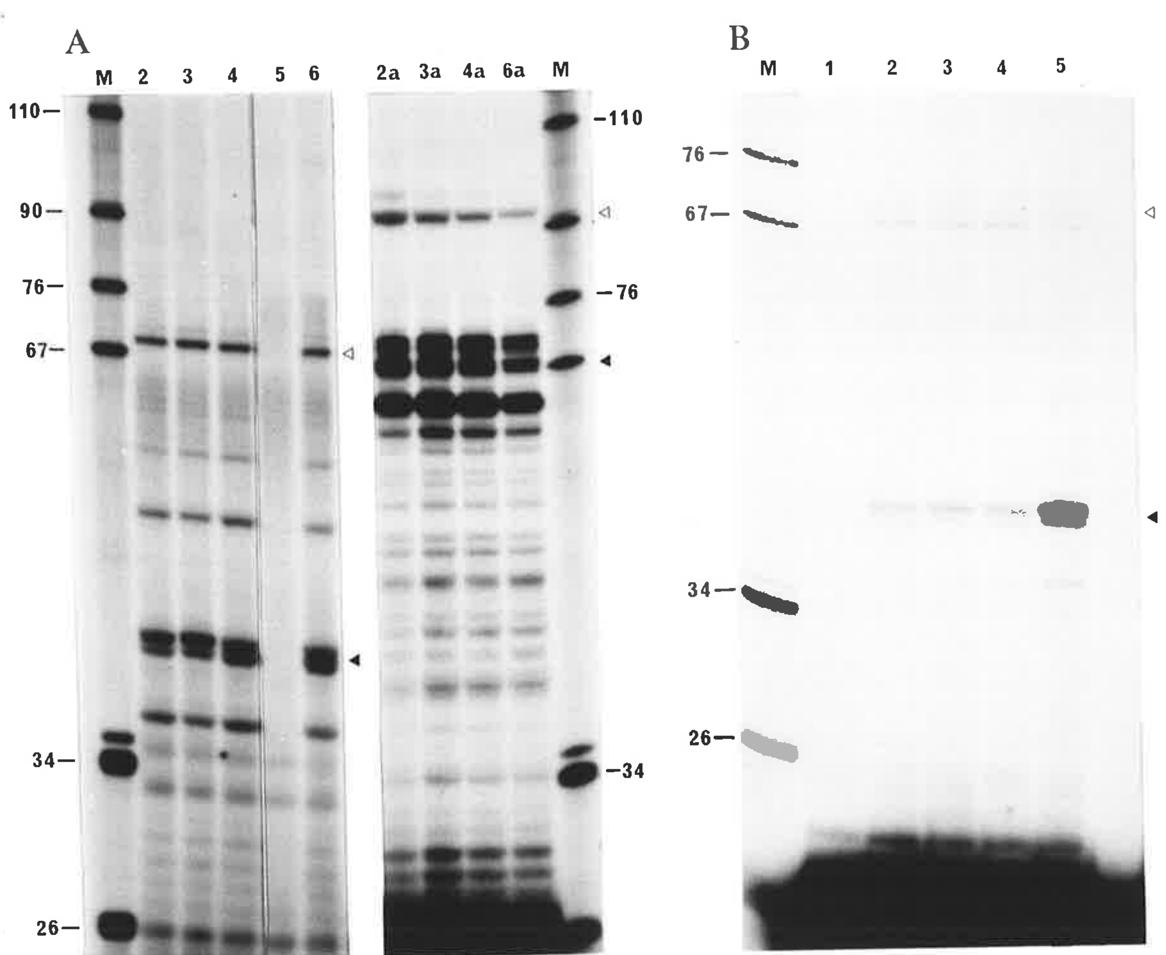
Oocytes were injected with a number of constructs in which the spacing between the keratin promoter and the tk coding region was progressively increased. The central number of the construct name denotes the distance from the cap site of the keratin promoter to the AUG of the tk gene. All constructs in panel **A** were co-injected with p7AT DNA. Primer extensions were performed on 2 oocytes worth of RNA. The keratin 20-mer primer was used in all cases to detect the spacer transcripts and the H2B primer was used to detect histone transcripts. Reactions were electrophoresed on 10% denaturing polyacrylamide gels. It should be noted that the oocytes injected in **A** were from a different frog to those injected in **B**. **M** denotes HpaII cut pBR322 markers, (▶) represents transcript initiation from the cap site and (▷) represents transcripts initiating from the TATA box.

A.

TRACK 2	KER127TK (section 3.25)	Keratin 20-mer used, extension products migrated at 41 bases.
TRACK 3	KER187TK (section 3.26)	
TRACK 4	KER328TK (section 3.27)	
TRACK 5	Uninjected Oocyte RNA	
TRACK 6	KER/TK (section 3.15)	
TRACKS 2a to 6a	Same as **2-6** above, except the H2B primer was used in the extension reaction. The products migrated at 68 bases.	

B.

TRACK 1	Uninjected Oocyte RNA	
TRACK 2	BK-i (section 3.6)	Keratin 20-mer used, extension products migrated at 41 bases.
TRACK 3	KER436TK (section 3.28)	
TRACK 4	KER430TK (section 3.31)	
TRACK 5	KER/TK (section 3.15)	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6a
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110
76
34
B
M
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3
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67
34
26

keratin gene BK-i (figure 5-7B).

The possibility that the decrease in transcript initiation in the KER436TK construct was related to sequences within the introduced pBR322 fragment was tested by the construction of another recombinant plasmid, KER430TK (section 3.31). In KER430TK the space between the keratin cap site and the AUG of the tk gene was increased to 430 bp by inserting the same keratin DNA which was used in the previous spacer constructs. The injection of KER430TK resulted in the production of the same low level of transcript as was observed for KER436TK (figure 5-7B).

In summary, it appears that the activatory effect of the tk gene on the keratin promoter can still be observed if the distance from the keratin cap site to the AUG of the tk gene is increased from the original 101 bp to 328 bp. The effect is not observed if the distance from the keratin cap site to the tk AUG is increased to 430 bp.

5.2.6 ATTEMPTS TO CHARACTERIZE THE tk STIMULATORY REGION

To try and define the sequences within the tk gene responsible for activation, the strategy used was to place tk gene fragments into the coding region of the intronless keratin gene, BK-i. This strategy was facilitated by the presence of a number of convenient restriction sites into which selected tk fragments could be inserted. Transcriptional stimulation was directly compared to the KER/TK construct which was used as an experimental control.

The first step in this strategy was to ensure that surrounding keratin sequences would not inhibit the tk stimulatory effect. A fragment containing the entire tk gene

FIGURE 5-8

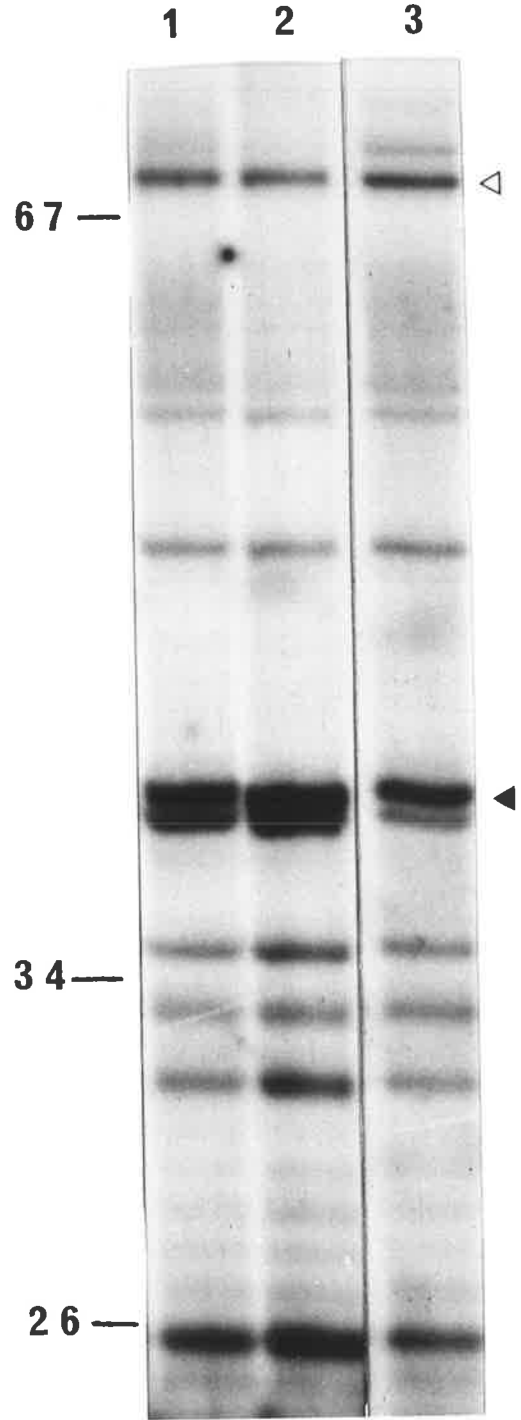
EFFECT OF INSERTING THE LARGE
1.7 kb tk GENE FRAGMENT INTO BK-i

BKTK5 (section 3.29) and BKTK2 (section 3.30), containing the 1.7 kb BglII/PvuII tk gene fragment inserted into the SmaI site of the BK-i were injected into oocytes. KER/TK was also injected for comparison. Primer extension analysis was performed on the RNA equivalent of two oocytes and the keratin 20-mer primer was used. The reaction products were electrophoresed on a 10% denaturing polyacrylamide gel. The position of the extension products representing transcripts initiating from the keratin cap site is indicated by the symbol (◄) at 41 bases. The position of extension products representing transcripts initiating from the TATA box is shown by the symbol (◁).

TRACK 1 KER/TK

TRACK 2 BKTK5

TRACK 3 BKTK2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26

(minus promoter) was inserted into the coding region of BK-i, producing BKTK5 (section 3.29). The orientation of the tk gene relative to the keratin promoter was the same as that in the KER/TK construct. The amount of correctly initiated transcript produced from this construct after injection into oocytes was identical to that observed from KER/TK (figure 5-8). A second construct was made, BKTK2 (section 3.30), with the orientation of the tk fragment inverted relative to the keratin promoter. Surprisingly, the amount of transcript produced from BKTK2 was also identical to that produced from KER/TK.

For the experiments described in this section, transcript initiation from the TATA box of each injected construct was used as the internal control. Initiations from the TATA box appear to remain constant within the one batch of oocytes irrespective of the construct injected. This circumvented the need to repeat primer extensions on transcripts obtained from co-injected histone genes.

To determine the location of sequences which may be responsible for the stimulatory effect, fragments were taken from the tk gene and reinserted into the intronless keratin gene. Three sub-genic fragments were chosen: an 800 bp fragment, a 900 bp fragment contiguous with the 800 bp fragment and a 617 bp fragment which overlapped the junction between the 800 and 900 bp fragments (refer to the figure in section 3.32 for more detailed information).

Initially, the three fragments were individually inserted into the same SmaI site within the intronless keratin gene producing 6 constructs (refer to sections 3.32, 3.33, 3.34, 3.35, 3.36, 3.37 and to the legend of figure 5-9A). A repro-

FIGURE 5-9

THE EFFECT OF INSERTING DIFFERENT FRAGMENTS FROM THE tk GENE INTO BK-i

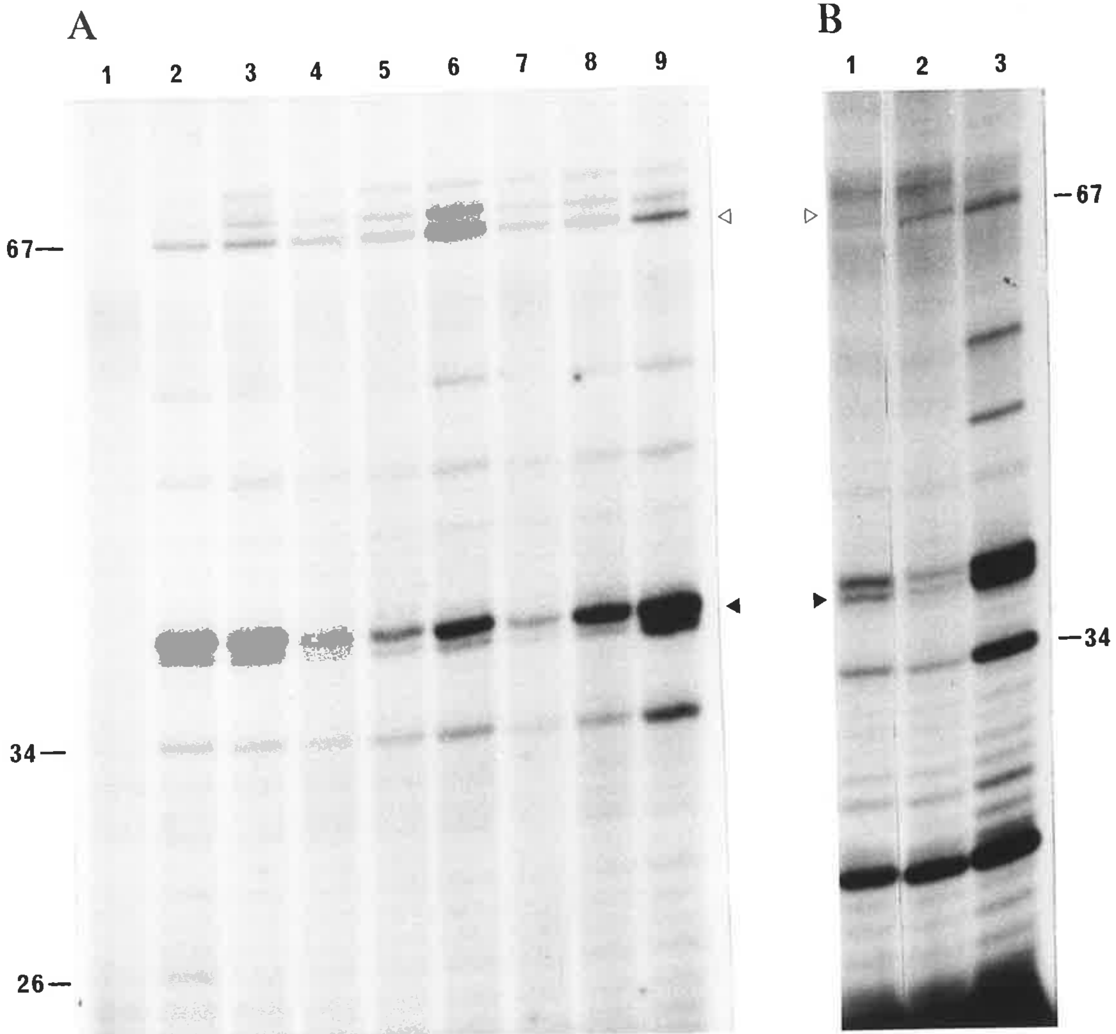
In **A** and **B**, different constructs (listed below) containing tk gene fragments inserted into BK-i were injected into oocytes. Primer extension analysis was performed using the equivalent of two oocytes worth of RNA and the keratin 20-mer primer. Reaction products were electrophoresed on a 10% denaturing polyacrylamide gel. The symbol (▶) denotes the extension product at 41 bases representing initiation from the keratin cap site. The symbol (▷) denotes initiations from the TATA box. Note that the oocytes used in **A** were obtained from a different frog to those used in **B.** The key below lists the constructs with which the oocytes were originally injected.

A.

TRACK 1	Uninjected	
TRACK 2	BK-i	(section 3.6)
TRACK 3	KER8▶00KER	(section 3.32)
TRACK 4	KER8◀00KER	(section 3.33)
TRACK 5	KER6▶17KER	(section 3.34)
TRACK 6	KER6◀17KER	(section 3.35)
TRACK 7	KER9▶00KER	(section 3.36)
TRACK 8	KER9◀00KER	(section 3.37)
TRACK 9	KER/TK	(section 3.15)

B.

TRACK 1	KERSp6▶17KER	(section 3.38)
TRACK 2	KERSp9▶00KER	(section 3.39)
TRACK 3	KER/TK	(section 3.15)


A
1
2
3
4
5
6
7
8
9
67
34
26
B
1
2
3
67
34

ducible increase in transcription was not observed from the keratin promoter regardless of the orientation of the inserted fragments (figure 5-9A, tracks 2 to 9). In most cases a decrease in transcription was observed relative to BK-i.

Insertion of the 617 bp fragment and the 900 bp fragment into the SmaI site of the intronless keratin gene had displaced the normal positioning of these fragments such that they were respectively 497 and 691 bp closer than usual to the keratin mRNA cap site. The results described in section 5.2.5 demonstrated that there was a defined maximum distance that the keratin promoter could be moved away from the tk gene in order to retain the stimulatory effect. It was possible that moving the promoter closer to the coding sequences may have had adverse effects on transcriptional stimulation. DNA from pBR322 was inserted to position the 617 and the 900 bp fragments in the coding region of the intronless keratin gene such that the position of each tk fragment relative to the keratin promoter was similar to that in the parental KER/TK construct (see sections 3.38 and 3.39). Nevertheless, the correctly located 617 bp fragment (KERSp6▶17KER) and 900 bp fragment (KERSp9▶00KER) did not stimulate transcription from the keratin promoter to the level of KER/TK (figure 5-9B).

5.3 DISCUSSION

5.3.1 DEDUCTIONS ON THE NATURE OF THE tk STIMULATORY REGION

The tk gene is expressed very efficiently in **Xenopus** oocytes, yet when the tk promoter is linked to heterologous genes the amount of transcript obtained is very low. The

presence of the tk coding region was observed to stimulate transcription from the keratin promoter. The observation that the stimulation of transcription occurs when the tk gene is inserted, in either orientation into the intronless keratin gene is interesting as it may provide clues for the location of the region. This result suggests that the region(s) may be centrally located, or that the sequences could be palindromic in nature.

The lack of success in defining the stimulatory region could result from the disruption of the region(s) when the selected fragments were isolated from the tk gene. If, for example, the components spanned the 5' end of the 617 fragment and proceeded in a 3' direction crossing the junction of the 900 and 800 bp fragments (a total distance of approximately 100 bp) each of the fragments chosen would have disrupted the stimulatory region (see figure 3-B and section 3.32). This problem could be solved by choosing other restriction fragments. Alternatively, the components necessary to elicit stimulation may be present in dispersed regions throughout the tk gene and the entire complement may be needed to produce the high level of transcription. If this is the case, a better approach to define the dispersed regions would be to make discreet mutations along the coding region of the tk gene. The KER/TK construct could be used for this purpose.

However, the work was terminated at this point because of problems relating to transcriptional efficiency in the oocytes being used. Levels of transcript obtained from all injected constructs including KER/TK and the intact tk gene were extremely low. This was also observed in other trans- criptional studies involving this particular stock of **Xenopus**

frogs (P. Wigley and J. Loveridge, personal communication). The problem was attributed to a change in the condition of the animals from which the oocytes were being obtained, possibly because of seasonal variation. These phenomena have been reported previously (Miller **et al.**, 1982; Jones **et al.**, 1983; G. Partington personal communication) and restocking of the non-breeding colony is currently in progress.

5.3.2 INTRAGENIC CONTROL SEQUENCES

The precedent has already been set for intragenic sequences influencing the transcriptional efficiency of viral genes. A mutation has been observed within the protein coding region of the adenovirus Ela gene which reduces transcription to 2% of wild type. The wild type sequence in this region has striking homology to the SV40 and polyoma enhancers, although the sequence has not yet been subjected to the usual enhancer tests (Osborne **et al.**, 1984). The transcriptional stimulation exerted by the tk coding region on its own promoter may reflect the presence of an enhancer but sequences homologous to the core enhancer sequence are not obvious in the coding region of the tk gene.

There are other polymerase II transcribed genes where intragenic control has been implicated in transcriptional expression. These include the sea urchin H2B histone gene, human growth hormone gene, mouse immunoglobulin gene, the human globin genes and the chicken thymidine kinase gene (Mous **et al.**, 1985; Moore **et al.**, 1985; Banerji **et al.**, 1983; Wright **et al.**, 1984; Charnay **et al.**, 1984; Merrill **et al.**, 1984b). Specific sequences have not been defined for the latter two examples. Intragenic controls in all of the examples

described above, excepting the chicken tk gene, are stimulatory in nature.

Much work has been done on the transcriptional regulation of tk gene expression and it is somewhat surprising that such an intragenic stimulatory effect has not already been noted. Two possible explanations for this are:

1. The response observed here may be totally artefactual, with the high levels of transcription from the KER/TK construct arising fortuitously by some unknown mechanism in oocytes. This could be further tested by linking the promoter of another gene which is weakly expressed in oocytes (e.g. the H5 histone gene promoter) to the tk structural gene and measuring transcriptional efficiency.
2. The work done to date on the transcriptional regulation of this gene may have been non-conducive to the discovery of the stimulatory region.

Most of the work carried out on tk expression has retained an intact structural gene and investigations have concentrated on defining the 5' promoter elements. Early work delineated the functional boundaries corresponding to the 5' and 3' termini of the gene within the 3.4 kb BamHI fragment containing the intact gene (McKnight and Gavis, 1980). McKnight **et al.** (1981) subsequently located the position of a transcriptional control region using 5' and 3' deletion mutants in the oocyte system. Sequences located between 40 and 100 nucleotides upstream of the start site were observed to promote transcription in the absence of the coding component of the gene. The efficiency of transcription from these deletion mutants which lacked the coding region is not

certain as the level of transcription was not directly compared to that of the intact tk gene. Sequences between 16 and 32 nucleotides upstream of the cap site (containing the TATA box) were observed to specify the exact start site of transcription (McKnight **et al.**, 1981). Subsequently, linker scanning mutants were made in the DNA region 120 bases upstream to 20 bases downstream of the cap site. Injection of these mutants into oocytes and analysis of transcript levels defined the promoter elements as being two GC-rich boxes surrounding a CAT dyad (McKnight and Kingsbury, 1982; McKnight **et al.**, 1984).

Merrill **et al.** (1984b) have reported the existence of intragenic elements responsible for the regulated expression of the chicken tk gene. When tk-deficient mouse myoblasts were transformed to the tk positive phenotype using both the cellular chicken tk gene and the HSV-tk gene, chicken tk activity decreased upon terminal differentiation, while in HSV-tk transformed cells, tk activity remained high during differentiation. The chicken tk and HSV-tk gene sequences are not very similar. The chicken tk gene possesses a number of introns whilst the HSV-tk gene does not contain any. In addition, the coding region of the HSV-tk gene is nearly twice the size of the coding region of the chicken tk gene (Merrill **et al.**, 1984a).

Hybrid genes were constructed using the chicken and viral tk genes, producing two constructs, HSV_p/CH_{st} (HSV-tk promoter linked to the chicken tk structural gene) and CH_p/HSV_{st} (chicken tk promoter linked to the HSV-tk structural gene). A regulated pattern of expression was observed only after transfection with HSV_p/CH_{st}. The CH_p/HSV_{st} construct showed

the constitutive pattern of tk expression characteristic of cells transformed with the intact HSV-tk gene. Removal of the 3' end (containing the polyadenylation signal) from the HSV_p/CH_{st} construct and its replacement with the viral tk gene 3' terminus still resulted in regulated expression. It was concluded that the element of the chicken tk gene necessary to specify regulated expression was entirely intragenic.

There is another explanation for these results if they are considered in the light of the results obtained here. The decrease in chicken tk expression on terminal differentiation could be due to a lack of the appropriate activatory information within the structural portion of the chicken tk gene. Many genes become non-functional in terminally differentiated cells and perhaps continued expression of a particular gene in such cells requires active stimulation. Thus, the expression of the HSV-tk gene during muscle differentiation may be a function of the intragenic activatory region.

5.3.3 THE tk PROMOTER AND THE tk INTRAGENIC COMPONENT ARE NECESSARY FOR EFFICIENT HSV-tk GENE TRANSCRIPTION IN OOCYTES

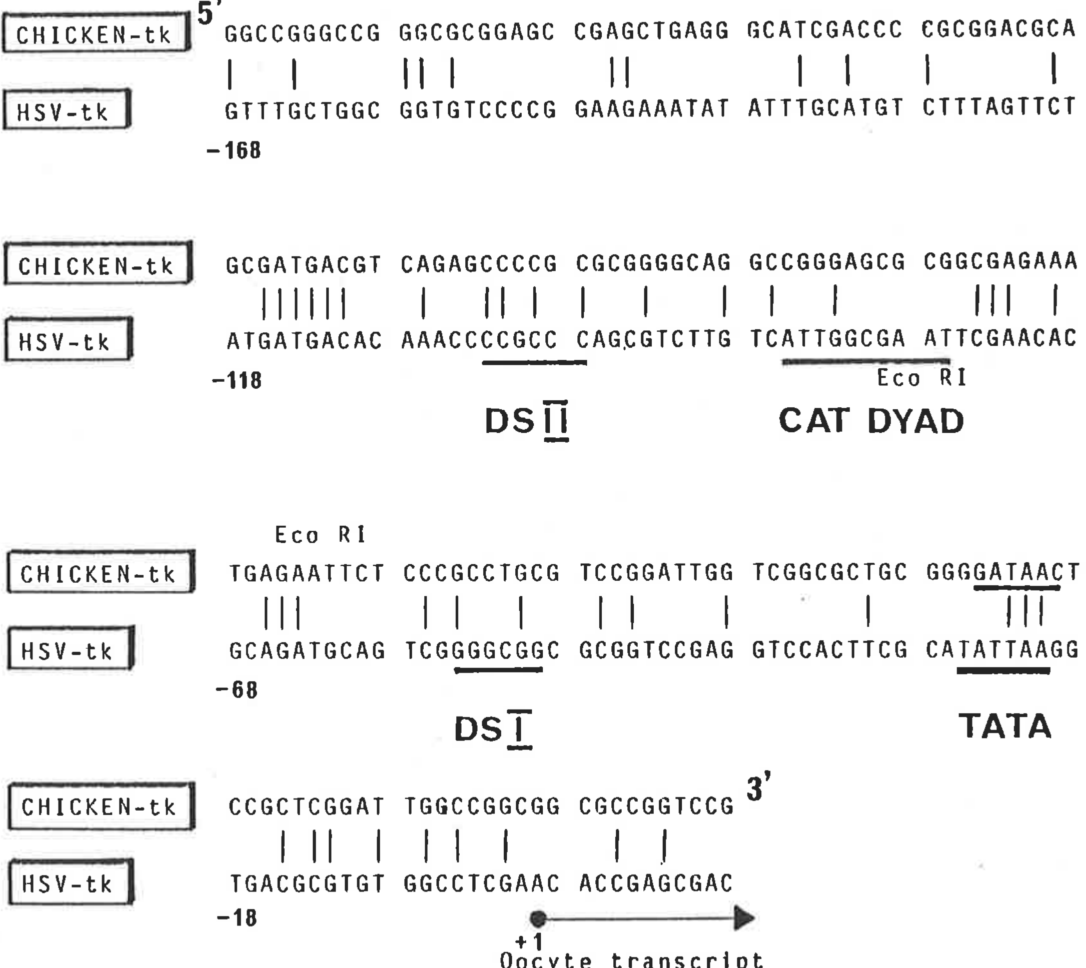
Apart from the TATA box, the chicken tk promoter and the feather keratin promoters lack any strong similarities when compared to the HSV-tk promoter (figure 5-10). The chicken tk and the feather keratin promoters do not possess the distinct GC boxes 5' to the cap site (even though the chicken tk promoter region is GC-rich) or the CAT dyad of the HSV-tk gene (feather keratin gene B does possess a CCAAT box, see figure 3-A(ii)). Data is not available for the transcriptional

FIGURE 5-10

COMPARISON OF CHICKEN-tk

AND HSV-tk PROMOTERS

The sequences of the chicken and HSV-tk promoters have been aligned so that the point of transcript initiation in the oocyte is the same in both cases. The distinctive promoter elements of the HSV-tk gene and the position of the chicken-tk TATA box are marked. Vertical lines indicate sequence similarities that exist under the alignment conditions. The sequence for the chicken-tk promoter was obtained from Merrill **et al.** (1984a) and the HSV-tk promoter sequence is from McKnight (1980).


CHICKEN-tk
5'
GGCCGGGCCG GGCGCGGAGC CGAGCTGAGG GCATCGACCC CGCGGACGCA
HSV-tk
GTTTGCTGGC GGTGTCCCCG GAAGAAATAT ATTTGCATGT CTTTAGTTCT
-168
CHICKEN-tk
GCGATGACGT CAGAGCCCCG CGCGGGGCAG GCCGGGAGCG CGGCGAGAAA
HSV-tk
ATGATGACAC AAACCCCGCC CAGCGTCTTG TCATTGGCGA ATTCGAACAC
-118
Eco RI
DS II
CAT DYAD
Eco RI
CHICKEN-tk
TGAGAATTCT CCCGCCTGCG TCCGGATTGG TCGGCGCTGC GGGGATAACT
HSV-tk
GCAGATGCAG TCGGGGCGGC GCGGTCCGAG GTCCACTTCG CATATTAAGG
-68
DS I
TATA
CHICKEN-tk
CCGCTCGGAT TGGCCGGCGG CGCCGGTCCG
3'
HSV-tk
TGACGCGTGT GGCCTCGAAC ACCGAGCGAC
-18
+1
Oocyte transcript

efficiency of the chicken tk/HSV-tk gene hybrids in **Xenopus** oocytes (Merrill **et al.,** 1984b). This study, however, showed that the intact tk gene produces approximately 30-fold more transcripts than the intronless keratin gene (BK-i) while KER/TK produces approximately 20-fold more transcripts than BK-i. From these values the stimulatory tk intragenic component appears responsible for as much as 66% of the transcriptional efficiency with the classical tk promoter elements contributing only 33%. Thus, it appears that maximal transcriptional efficiency of the HSV-tk gene in oocytes relies on both the distinctive promoter elements and the as yet undefined intragenic component. Conceivably, mutations in either region would affect transcriptional efficiency of the tk gene.

There are at least two speculations which can be made about the nature of the tk stimulatory effect. Firstly, the effect could be enhancer-like with the stimulation extending only over a defined range. Secondly, stimulation may somehow be mediated by the binding of a protein factor which is present in the oocyte.

5.3.4 FEATHER KERATIN GENE TRANSCRIPTION IN XENOPUS OOCYTES

One possibility which was not clearly resolved in the work described above relates to the deletions performed in the TK/KER construct. The possibility remains that any portion of keratin DNA is able to suppress transcription from the tk promoter (refer to section 5.2.4). An approach to resolve this problem would be to delete the remaining piece of keratin DNA in TKKpr, attach the tk polyadenylation signal and compare the level of transcription from this construct to that from

the other deletion constructs and also to TK/H2B. However, this was not considered a critical step in determining the cause of the high level of transcription from KER/TK once it was discovered that transcription from the tk promoter was low in the absence of the tk coding region.

In all cases (except when the keratin promoter was linked to the tk gene) the level of feather keratin gene transcription was low in oocytes. The most likely explanation is that the oocyte lacks the factors necessary for the efficient transcription of feather keratin genes. A more homologous expression system is required in which to study the expression of these genes. From the results described here the preferred approach to elucidate sequences important in feather keratin gene transcription would be to make relatively discreet deletions or mutations in the area of interest, with minimal introduction of sequences from other genes.

5.3.5 WARNINGS IN RELATION TO PROMOTER SWITCHING STUDIES

In general, the results of this work extend a note of caution to studies where test promoters are linked to marker genes as a means of identifying promoter elements. The results of such studies may be misleading in that they could be biased by the actual gene to which the test promoter is linked. This may in some cases provide an inaccurate assessment of possible control elements and promoter efficiency.

CHAPTER 6.

ALTERNATIVE EXPRESSION SYSTEMS.

THE DEVELOPMENT OF

AN HOMOLOGOUS EXPRESSION SYSTEM.

6.1 INTRODUCTION

Although the injection of keratin, histone and thymidine kinase gene constructs into **Xenopus** oocytes has yielded some interesting results (chapters 4 and 5) it has become obvious that the oocyte system is not the most suitable system for the study of specific controls involved in the expression of feather keratin genes. Feather keratin genes were shown to be expressed at low levels in the oocyte and the transcripts were observed to be unspliced. The inefficient expression of these genes in oocytes may relate to the lack of specific transcription and processing factors in this system or to a lack of more general factors.

The aim of the work presented in this chapter was to try to adapt other, existing expression systems, or if necessary to develop a new system in which to study the factors involved in the tissue and stage specific control of feather keratin gene expression.

A number of different approaches were used before arriving at a suitable expression system. In the following sections each approach will be treated individually, the results obtained will be presented and subsequently discussed.

6.2 INVESTIGATION OF THE EXPRESSION OF FEATHER KERATIN GENES IN NON-FEATHER TISSUE DERIVED CELL LINES

6.2.1 RESULTS AND DISCUSSION

It was necessary to test whether the low level of feather keratin gene transcription in oocytes was due to the lack of keratin specific factors or the lack of an appropriate

combination of general transcription factors. Thus, commonly used cell lines, HeLa, CV1 and Ltk$^-$, were tested for their ability to express feather keratin gene B. Gene B was inserted into a vector, pJL4Bam5, which contained the three chicken histone genes present in p7AT to act as internal controls (see section 3.40). The construct produced (designated pJL4B1-4, refer to section 3.40) was transfected into HeLa, CV1 and mouse Ltk$^-$ cells using calcium phosphate precipitation (section 2.13.2). The total RNA produced within the next 48 hours was isolated and assayed by primer extension for the presence of H2B histone gene and gene B transcripts.

Correctly initiated gene B transcripts, either spliced or unspliced, were not detected in any of these cell lines even though correctly initiated H2B transcripts were obtained. Figure 6-1 shows an example of the results obtained with CV1 cells. Histone genes were expressed more efficiently in CV1 cells than in the other two cell lines tested. Presumably this is because the monkey CV1 cell is the natural host for SV40, thus enabling replication of the SV40 ori-containing plasmid to high copy number which is beneficial for transient expression assays.

The inability to detect correctly initiated gene B transcripts indicated that the cells lines tested were less viable alternatives for use as expression systems than the **Xenopus** oocyte. It also indicates that general cellular factors are not the only deficiency in **Xenopus** oocytes.

FIGURE 6-1

TRANSFECTION OF pJL4B1-4

INTO

CV1 CELLS

pJL4B5 DNA (containing a chicken histone gene cluster in a SV40 vector; section 3.40) and pJL4B1-4 DNA (containing feather keratin gene B in pJL45B; section 3.40) were transfected into CV1 cells exactly as described in section 2.13.2. Primer extension analysis was performed on 15 μg of extracted RNA using the keratin 25-mer and the H2B 26-mer in all cases. Reaction products were electrophoresed on a 6% denaturing polyacrylamide gel.

TRACK 1 Extensions on RNA from pJL4B1-4 transfected cells.

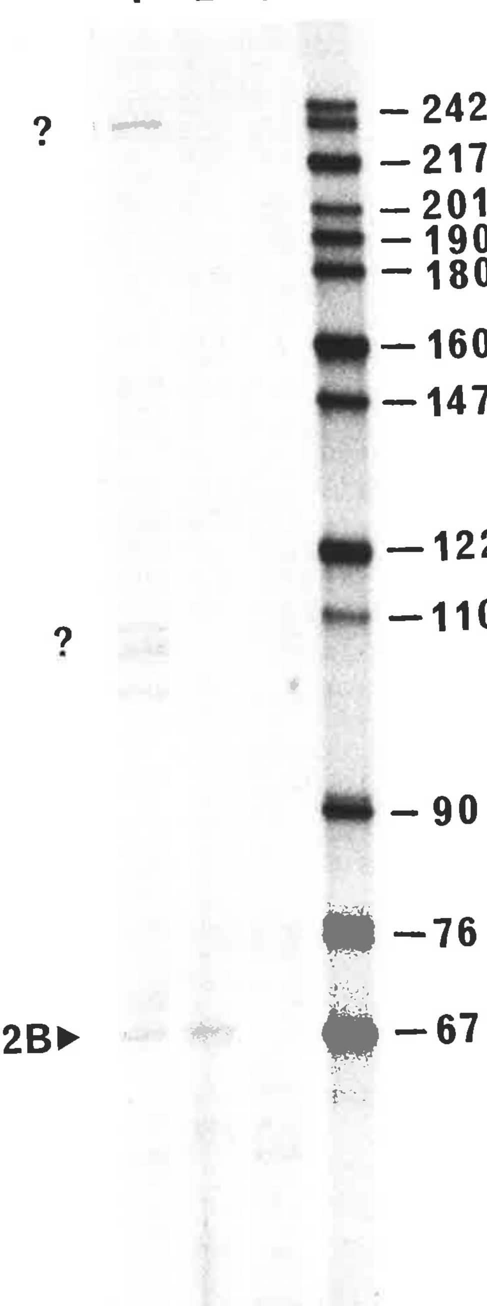
TRACK 2 Extensions on RNA from pJL4B5 transfected cells.

TRACK 3 Extensions on RNA from untreated cells.

TRACK 4 HpaII cut pBR322 end-labelled markers.

The H2B extension products at 68 bases are indicated. The (**?**) symbols denote keratin transcripts which initiate at non-specific sites.

1 2 3 4


?
?
H2B
242
217
201
190
180
160
147
122
110
90
76
67

6.3 ATTEMPTS AT ENHANCING THE OOCYTE SYSTEM BY THE CO-INJECTION OF CHROMATIN PROTEINS FROM FEATHER CELL NUCLEI

In some studies, the oocyte transient expression system has been adapted to overcome tissue specificity problems associated with transcript initiation and processing. Improvements have been obtained by co-injecting protein components from the nuclei of cells from which the particular genes were derived. This complementation procedure has resulted in the successful isolation of a small nuclear ribonucleoprotein particle which is involved in the processing of the 3' terminus of sea urchin H3 histone mRNA (Stunnenberg and Birnstiel, 1982; Galli **et al.**, 1983) and also a nuclear fraction which stimulates transcription of the tissue specific H5 histone gene (Wigley, 1986). Attempts were made to use this complementation assay in order to study the expression of feather keratin genes.

6.3.1 RESULTS

No chicken epidermal feather derived cell line exists from which nuclei could readily be obtained in order to purify non-histone nuclear proteins. Thus, nuclei were extracted from 14 day old chick embryo feathers on the assumption that all the relevant transcription factors should be present as these are involved in the active transcription of feather keratin genes.

Chromatin was prepared from the isolated nuclei and chromatin proteins were subsequently fractionated by extraction with different concentrations of salt (150 mM to

2 M NaCl). The fractions were concentrated, dialyzed and prepared for injection (see section 2.12.4). A total of only 80 μg of protein was obtained after fractionating chromatin from $2.5x10^8$ nuclei. Recoveries per salt fraction were as follows: 150 mM NaCl, 5 μg; 300 mM NaCl, 5 μg; 450 mM NaCl, 7 μg; 600 mM NaCl, 5 μg; 2 M NaCl, 58 μg. The greater protein yield in the 2 M fraction probably represents histone proteins.

In the two successful studies where the oocyte complementation system was employed, approximately 200 ng of chromatin protein was injected into the cytoplasm of each oocyte after the initial nuclear injection of the test DNA (Stunnenberg and Birnstiel, 1982; Wigley, 1986). Except for the 2 M fraction containing the histone proteins, the yield of feather cell chromatin proteins obtained from the other fractions was sufficient to inject only 25 oocytes with each fraction. Feather keratin gene B was used as the test DNA template. However, no increase in transcriptional efficiency or transcript processing was observed (data not shown).

6.3.2 DISCUSSION

The major problem encountered with this approach was obtaining sufficient nuclei from the developing feathers from which to extract chromatin proteins. Processing of feathers plucked from a total of 200, fourteen day old chick embryos yielded only $2.5x10^8$ nuclei. The low yield may result from the fact that there is a gradient of keratinization in the feather, with the degree of keratin filament crosslinking increasing towards the feather tip, where the cells are dead. Partially keratinized cells require a greater degree of force

to disrupt in order to release nuclei and the degree of force permissible is limited by the amount of damage which would be caused to the free nuclei. In addition, the keratin filaments may inflict damage on the nuclei during the homogenization procedure.

Large losses of protein must have occurred during the salt wash fractionation and dialysis procedure as approximately 250 µg of histone protein should have been obtained from $2.5x10^8$ feather cell nuclei in the 2 M salt wash fraction alone (calculated assuming that there are 2.6 pg of DNA/feather cell (Gibbs, 1977) and assuming that histones are present in amounts equimolar to DNA). Wigley (1986) also reported relatively large losses of chromatin protein when using this extraction procedure on nuclei from cultured TS34 chicken cells. However, final protein yields were greater than those observed here because the availability of the cell line yielded $2x10^9$ nuclei as starting material.

The inability to observe an increase in transcriptional efficiency or transcript processing from the injected gene B subclone with any of the salt wash fractions could be a function of a number of possibilities:

1. The factors could be labile.
2. Factor degradation could have occurred during the isolation procedure.
3. The low amounts of protein isolated may not have been representative of the specific factors necessary to stimulate transcript initiation and facilitate processing.

4. The fractionation procedure used to separate the histone component from the other chromatin proteins may have divided up an interactive group of transcription factors into separate components.
5. Sequences flanking feather keratin gene B or even the entire feather keratin gene cluster may be needed in order to observe the effects of factor interaction.

On the basis of the results obtained here, in order to simply test possibility number 5, using for example, 10 different DNA templates with each salt wash fraction would require at least 10^9 feather cell nuclei as starting material for fractionation. At least 800 embryos would need to be plucked. Given the labour intensiveness of this task and the number of animals required for subsequent experiments, this approach was not continued.

6.4 EPIDERMAL FEATHER CELL CULTURE

The synthesis of feather keratin occurs in the specialized epidermal cells of the developing feather. Thus, the transfection of primary cultured feather epidermal cells with feather keratin genes was considered as the next possibility for use as an expression system, not ignoring the fact that the underlying dermis is known to play a role in the differentiation of feather tissue (discussed in 6.4.1). Any introduced genes would of course need to be distinctively tagged because of the high degree of similarity amongst the feather keratin genes. However, primary cultures of feather epidermal cells had to be established first.

6.4.1 RESULTS AND DISCUSSION

Tissue from 7, 8 and 9 day old chick embryos was chosen as the source of epidermal cells because at these ages feather primordia are small and it was thought that this would facilitate the isolation of the epidermal cell layer. Squares of dermal-epidermal tissue, cut from the backs of 7, 8 and 9 day old chicks were trypsinized to separate the two tissue layers. It was difficult to peel the epidermis cleanly away from the dermis when 9 day old chick skin was used. Seven day skin produced the best results with respect to dermal-epidermal separation although the process was tedious as it was difficult to cut large sections of skin for trypsinization because of tissue fragility. Thus, 8 day skin was chosen as the source of epidermal cells.

Epidermal fragments were dissociated as described in the methods (section 2.13.6). The cell population consisted of single cells and varying sized clumps of cells. The cell clumps settled rapidly after plating and cells from some of the smaller clumps (10 cells or less) began to divide rapidly. After 24 hours most of the single cells still remained in suspension and the larger clumps of cells had begun to detach. Most of the cells in the larger clumps appeared dead. Contaminating fibroblasts formed approximately 15% of the cell population and proliferated steadily.

A change of medium at this time removed detached cells and only a few epidermal cells remained. After a further 48 hours the small groups of epidermal cells appeared more compact and formed small sheets. Epidermal cell survival diminished rapidly after this point.

The procedure obviously needed careful refining. Rapid division of the small clumps of settled cells and their arrangement into sheets was indicative of the cells beginning to terminally differentiate. The propensity of the cells to do this may have been caused by the concentration of $CaCl_2$ (1.2 mM) in the medium. Hennings **et al.** (1980) have reported that media calcium concentrations such as those used here cause mouse epidermal cells to stratify in culture by 1-2 days and terminally differentiate, with cells sloughing by 3-4 days. Lowering of calcium in the medium to 0.5-0.1 mM and keeping all other solutions in the preparative stages calcium-free enabled their cells to grow as a monolayer for several months and permitted subculturing.

There are two reasons why further efforts were not made to establish primary cultures of chick feather cells. Firstly, it was obvious that an intensive effort would be required to establish such cultures. Secondly, the absence of the dermis in the epidermal cultures raises some doubts about the usefulness of this system with respect to the tissue and stage specific expression of the feather keratin genes.

Transplantation experiments have shown that the underlying dermis has a dramatic effect on keratinizing tissue. If the epidermis from a potentially feathered region is transposed to the dermis of the lower leg of the chicken the differentiated epidermis produced is typical of the scale tissue of the leg (Sengel, 1958; Rawles, 1963). The interaction between the dermis and the epidermis is a complex one as the chick dermis can only exert its influence on the epidermis at a specific time in development. In addition, the epidermis appears to pass through a distinct phase where it is

receptive to dermal influence (Gregg and Rogers, 1986). Nothing is known of the factors which are involved in this process.

To enable a dermal-epidermal interaction in culture, plugs of dermal-epidermal tissue from chick embryos could be cultured but transfection of altered feather keratin genes into such multicellular masses would be highly inefficient.

6.5 PROPOSAL FOR AN HOMOLOGOUS EXPRESSION SYSTEM: TRANSGENESIS OF CHICK EMBRYOS

The best system for the study of feather keratin gene expression is the chicken itself. The introduction of feather keratin genes into a developing chick embryo, via a retroviral vector, would provide a uniquely homologous system. One vector suitable for such studies is a Rous sarcoma virus (RSV) vector (1090/-29) which contains all the genetic components necessary for the formation of infectious particles, **in vivo** and **in vitro,** but lacks the v-src gene involved in malignant transformation (Sorge and Hughes, 1982; refer to figure 6-2 for the retroviral life cycle and genomic structure). The deleted v-src gene in 1090/-29 allows the insertion of approximately 1.5 kb of DNA without affecting viral functioning. Feather keratin genes, tagged internally with an easily identifiable marker would be inserted into this site. Transfection of the keratin gene-virus DNA hybrids into chick fibroblasts should result in the packaging of the DNA into infectious virus particles which could subsequently be used to infect 2-3 day old chick embryos or even adult oviducts. The introduction of the virus into fertilized eggs would either be by direct injection into the yolk sac or intravenously.

FIGURE 6-2

A. Retroviruses are a group of single stranded RNA tumour viruses. In the virus, the RNA is packaged as a dimer of two identical, capped and polyadenylated RNAs which are hydrogen bonded together. These are enclosed together with a reverse transcriptase molecule in an icosahedral shell composed of core proteins, which in turn is surrounded by an outer envelope obtained from the membrane of infected cells during the budding process. Normally associated with the RNA is either a tRNA molecule or a tRNA binding site which is important for replication. The associated tRNA or host tRNA acts as a primer during replication.

Post penetration, reverse transcriptase converts the (+)RNA into a linear, duplex DNA molecule in the cytoplasm of the infected cell. During this process, long terminal repeats (**LTRs**) are generated at each end of the molecule. The linear DNA is circularized and transported to the host nucleus where it is integrated at ramdomly selected sites. The integrated viral DNA is flanked by the LTRs forming the provirus. Once incorporated, the provirus is transcribed by host machinery. Synthesis of the primary transcript is initiated from the promoter in the 5' LTR. The primary transcript is capped at the 5' end and polyadenylated at the 3' end. Some of this message is spliced in various ways to produce subgenomic mRNAs which are representative of the main coding regions. A typical example of a retroviral genome contains three coding regions, **gag, pol** and **env.** The gag region gives rise to protein units forming the nucleoprotein core of the virion, env codes for components of the envelope and pol is responsible for reverse transcriptase production. Thus, the transcription of proviral DNA results in the production of RNA for virion genomes and RNA for the structural and enzymic proteins. Virus particles are assembled on the cell membrane and bud off into the surrounding environment. Virus particles continue to be released throughout the life cycle of the cell.

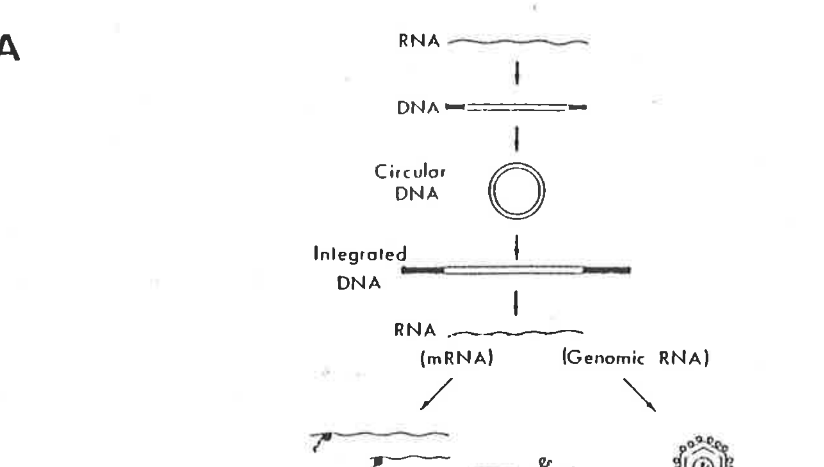
(Summarized from Varmus, 1982; diagram from Hayward and Neel, 1981.)

B. Representation of the wild type genome of Rous sarcoma virus, Schmidt-Ruppin strain A showing the position of the **v-src** gene, involved in malignant transformation. The size of the genome is approximately 10 kb (Hayward and Neel, 1981).

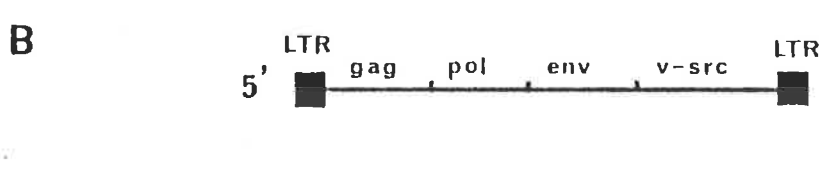
C. A map of the v-src and surrounding envelope (env) gene region (from Sorge **et al.**, 1983).

D. The vector 1090/-29 was constructed by deleting the v-src gene from position 1090 to -29 shown in **C** and inserting the altered proviral clone into the ampicillin half of pBR322 from EcoRI to PvuII. (For further information on construction see Sorge and Hughes, 1982). The restriction map is redrawn from one kindly provided by J. Sorge. (✱) represents sites destroyed, and numbers outside the circle represent sizes of the EcoRI fragments. Foreign DNA is inserted into the ClaI site.

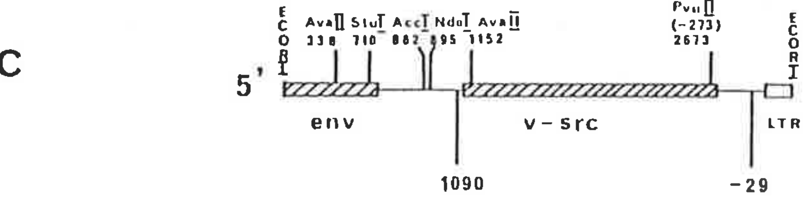
A



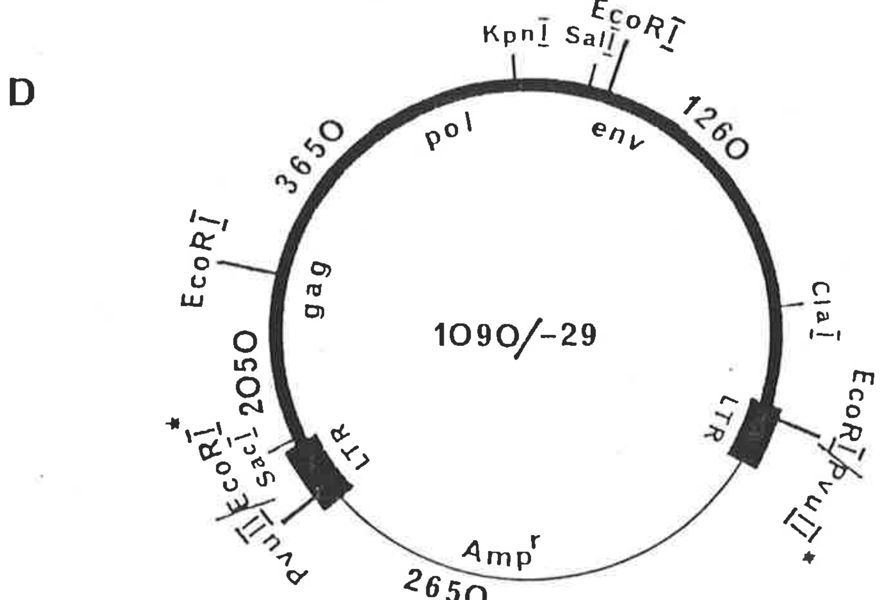
B



C



D



Feather tissue would be harvested at 14 days of embryonic development, the time of active feather keratin synthesis. The DNA isolated would be assayed for the presence of the integrated, tagged, feather keratin gene and the RNA assayed for the presence of specific keratin transcripts.

The feasibility of this system has been demonstrated by experiments in which the RSV vector, 1090/-29, has been used to insert a chicken growth hormone cDNA clone into 9 day old Leghorn chick embryos. Fifty per cent of infected embryos hatched and the concentration of growth hormone in the sera of 50% of the newly hatched chicks was 3 to 10-fold higher than control animals. However, there was no statistically significant difference in the weight of the infected chickens compared to control birds at 20 weeks of age (Souza **et al.**, 1984). Unfortunately, no blotting data was provided to show that the cDNA clone had successfully been integrated into the chick genome.

6.5.1 EXPERIMENTAL OUTLINE OF THE TRANSGENIC CHICK EXPRESSION SYSTEM

Limitations on the time available for this project prevented full development of the transgenic chick embryo system. However, the following is a proposed outline of the components needed for the successful establishment of this system.

1. The virus-keratin gene hybrids must be packaged into infectious particles and a suitable packaging system needs to be established. The system employed by Souza **et al.** (1984) involved the transfection of chick embryo fibroblasts.

2. Once the DNA has been transfected into fibroblast cells, an enzymic assay for reverse transcriptase is necessary to detect the presence of virus particles in the culture medium.
3. The virus particles produced must be characterized to ensure that the keratin sequences are present in their entirety. This involves purifying the virus from collected media by centrifugation (Robinson and Lamoreux, 1976), isolation of viral RNA (Sorge and Hughes, 1982) and subsequent analysis of the RNA by S1 or primer extension (see sections 2.11.4 and 2.11.5).
4. The best procedure for delivery of the purified virus particles into the chick embryos needs to be established. If direct injection into the yolk sac is unsuccessful, a procedure for intravenous injection has been described by Barnes and Julian (1958). The possibility of directly infecting the parental, chicken oviduct could also be investigated.
5. Finally, analysis of the expression of the introduced keratin genes would proceed by established methods, for example, primer extension and S1 analysis on the RNA from the feather tissue of the transgenized chicks.

Those parts of the foregoing proposal that have been completed, or at least commenced, will be discussed in the sections below.

(i) Fibroblast Cultures

As a stock cell line for the preparation of packaged recombinant virus, chick embryo fibroblast cultures were made. Unfortunately, methodological aspects pertaining to fibroblast cell culture were lacking in the paper by Souza **et al.** (1984). Fibroblast cultures were thus made from 9 day old chick embryos essentially as described by Rein and Rubin (1968) except that modifications were made with respect to the type of medium used, tissue dispersion and cell storage (refer to section 2.13.7).

All of the cells obtained from 4 embryos were plated out and cultured for 14 hours, after which fibroblasts were selectively removed (by exposure to Ca^{2+}-Mg^{2+} free PBS), resuspended in a freezing medium and stored in liquid nitrogen (refer to section 2.13.7). The procedure resulted in a culture which contained more than 95% fibroblasts. All experiments were thus performed using secondary cultures.

Growth of chick embryo fibroblasts in culture is density dependent (Rhein and Rubin, 1968). It was found that rapid growth of the fibroblasts prepared here could be maintained for at least one month if the cells were passaged frequently and at high densities. In practice, T_{75} flasks were seeded with 2.5 to $3x10^6$ cells/flask and passaged every 2 to 4 days.

(ii) Establishing an Efficient Transfection Procedure

To test methods for the introduction of viral DNA into chick embryo fibroblasts a model test system was necessary. A plasmid containing the neomycin gene (pSV2Neo) was used for this purpose. Transfer of the neomycin gene into eukaryote

cells confers resistance to normally toxic levels of the neomycin analogue, G418 (Colbere-Garapin **et al.**, 1981).

The procedure described previously for calcium phosphate-mediated DNA transfection (section 2.13.2) involved exposure of the cells to the calcium precipitate overnight but similar treatment of the chick embryo fibroblasts resulted in high cell mortality. The cells survived if exposed to the calcium precipitate for only 6 hours, however, the maximum time of exposure that allowed survival was not determined. When the cells were transfected with pSV2Neo using the 6 hour exposure procedure, very few colonies were observed relative to the amount of transfected DNA (table 6-1).

There have been reports in the literature that transfection efficiency could be increased if cells are treated with DMSO or glycerol after exposure to the calcium-DNA precipitate (Lopata **et al.**, 1984). To test these methods, cells which had been exposed to the pSV2Neo-calcium precipitate for 6 hours were "shocked" with glycerol or DMSO or rinsed with PBS before replacing the culture medium. After a 24 hour incubation period, selection for G418 resistance began. The optimal concentration of G418 for selection in these cells was determined in a previous experiment and found to be 200 μg/ml. The cells were exposed to that concentration of G418 and within 8 days the majority of cells had ceased dividing and had begun to send out long processes. After a further 8 days small colonies of cells were evident and selection continued for a further 5 days after which colonies of cells were counted (see 2.13.5).

The results of these experiments are shown in table 6-1. The DMSO shock was toxic to the cells, few survived to be

TABLE 6-1

ESTABLISHING THE MOST EFFICIENT METHOD FOR TRANSFECTING CHICK EMBRYO FIBROBLASTS

Chick embryo fibroblasts were transfected with 2.5 µg of pSV2Neo DNA/60 mm plate as described in 2.13.2. The precipitate was left on the cells for 6 hours after which the medium was removed. The plates (in triplicate) were either washed in PBS for 2 minutes, shocked with 2 ml of DMSO solution (25% DMSO in DMEM, 10% tryptose phosphate broth, 5% fetal calf serum) or shocked for 2 minutes with 2 ml of glycerol solution (15% glycerol in DMEM, 10% tryptose phosphate broth, 5% fetal calf serum). The number of colonies obtained after selection in medium containing 200 µg/ml G418 are shown for each treatment (average of two experiments).

Constitutent or Treatment	Dish Number			
	1	2	3	4
Carrier DNA	+	+	+	+
pSV2Neo DNA	-	+	+	+
6 h exposure to precipitate	+	+	+	+
PBS wash	+	+	-	-
DMSO shock	-	-	+	-
Glycerol shock	-	-	-	+
Average colony number obtained	0	12	0	94

treated with G418 and the plates were discarded. The glycerol shock treatment increased the number of colonies almost 8-fold and this procedure was chosen for further calcium phosphate-mediated DNA transfection of chick embryo fibroblasts.

(iii) Enzymic Assay For Reverse Transcriptase

Souza **et al.** (1984) gave no reference for an assay for reverse transcriptase activity. Thus, the method of Robinson and Lamoreux (1976) was chosen but it was extensively modified in the following ways: a poly rG.oligo dC 12-mer template was used to measure the incorporation of α-^{32}P-dCTP in the presence of reverse transcriptase, the total reaction volume was reduced to 10 µl and some of the reaction constituents were also altered (section 2.15.1). Incubation of the reaction was carried out at 42°C and the resultant mixture was spotted onto DEAE (81) filter discs, which were then washed to remove non-specifically bound label. After drying, the discs were measured for Cerenkow radiation.

This procedure was more rapid than that described by Robinson and Lamoreux (1976) and was proven to give a quantitative measure of reverse transcriptase activity since the amount of radioactivity incorporated using purified enzyme was linear with sample dilution (figure 6-3A). A reverse transcriptase concentration of 0.1 units resulted in a readily detectable 2.4x10^{5} counts per minute (Cerenkow) of incorporated α-^{32}P-dCTP, above control values. The sensitivity of the assay was tested using culture media from a donated chicken fibroblast cell line transformed with the Prague A strain of RSV. As little as 3x10^{-3} units of reverse transcriptase activity could be detected in a 1.2 µl sample of

FIGURE 6-3

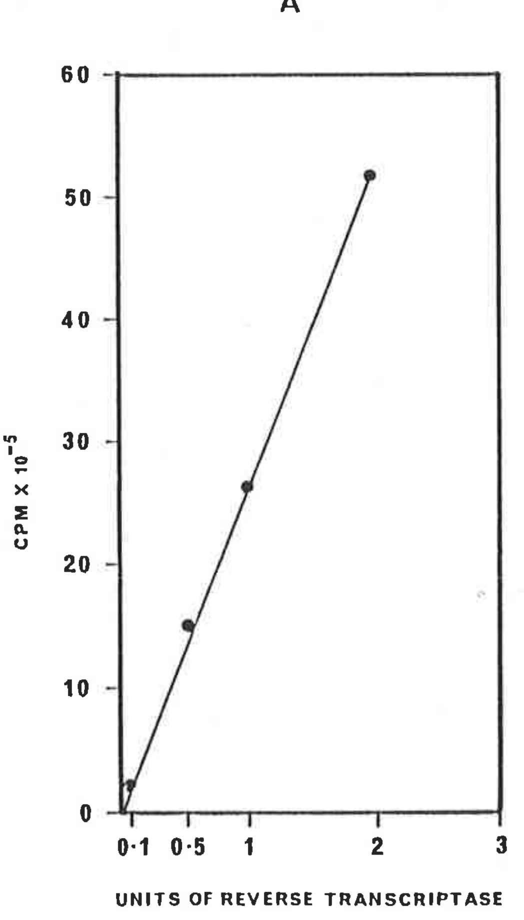
TESTS TO DETERMINE THE SENSITIVITY OF THE MODIFIED REVERSE TRANSCRIPTASE ASSAY

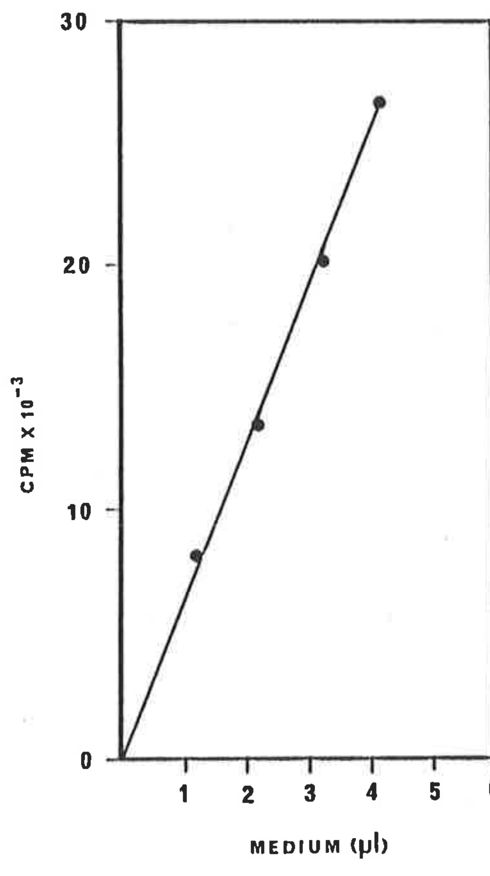
The assay was performed as described in section 2.15.1.

A. The graph shows the counts per minute incorporated when purified enzyme was used in the assay.

B. Representation of the counts per minute incorporated when culture medium from a chicken fibroblast cell line, transformed with Prague A RSV was tested in the assay. Virus particles in the culture medium were lysed as described in 2.14.3.

Each point in **A** and **B** represents the average of two experiments.


A
CPM X 10^{-5}
60
50
40
30
20
10
0
0·1
0·5
1
2
3
UNITS OF REVERSE TRANSCRIPTASE


B
CPM X 10^{-3}
30
20
10
0
1
2
3
4
5
6
MEDIUM (µl)

medium (figure 6-3B).

(iv) Generating Infectious Particles

The vector 1090/-29 was used to transfect cultures of chick embryo fibroblasts. Post-transfection, culture medium was sampled daily for the presence of reverse transcriptase activity, however none could be detected even after 14 days. During the 14 day sampling period, the cells were split every 3 days and an attempt was made to purify virus from the pooled media samples (Robinson and Lameroux, 1976). Reverse transcriptase activity could not be detected from the purified "virus" extracts.

DNA and RNA was purified from fibroblasts on the 14th day post-transfection (see sections 2.13.3 and 2.13.4). Dot blot analysis showed that 1090/-29 DNA had not integrated into the fibroblast genome (figure 6-4A) and no viral RNA (figure 6-4B) could be detected.

In a second series of experiments, 1090/-29 DNA was co-transfected with a vector containing the chloramphenicol acetyl transferase gene (pSV2CAT) to ensure that the transfection procedure was successful. Cultures were split 3 days post-transfection and the fibroblasts were harvested on the 5th day. The cells were then lysed and the cytosol fractions were assayed for the presence of both CAT and reverse transcriptase activity. CAT activity was high (figure 6-5) but no reverse transcriptase activity could be detected in the same cytosol extracts (data not shown). Dot blot analysis on the nuclear fraction from the lysed fibroblasts showed that the transfected DNA had not been integrated into genomic DNA (data not shown).

FIGURE 6-4

DOT BLOT ANALYSIS OF DNA AND RNA ISOLATED FROM FIBROBLASTS TRANSFECTED WITH 1090/-29 DNA

Cultured chick embryo fibroblasts were transfected with 5 µg of 1090/-29 DNA/10 cm plate (section 2.13.8). Fourteen days later, DNA and RNA was isolated from the harvested cells (sections 2.13.3 and 2.13.4 respectively) and spotted onto nitrocellulose filters. The filters were probed with a 3.65 kb nick-translated EcoRI fragment from 1090/-29 (figure 6-2). The hybridization conditions for the RNA and DNA dot blots were as described in Maniatis **et al.** (1982). After hybridization the filters were washed, the final stringency being 0.1 x SSC at 65°C.

A. DNA DOT BLOTS

1 and 2 10 µg of untransfected fibroblast DNA.

3 and 4 10 µg of DNA from fibroblasts transfected with 1090/-29 DNA.

5 and 6 150 ng of 1090/-29 DNA.

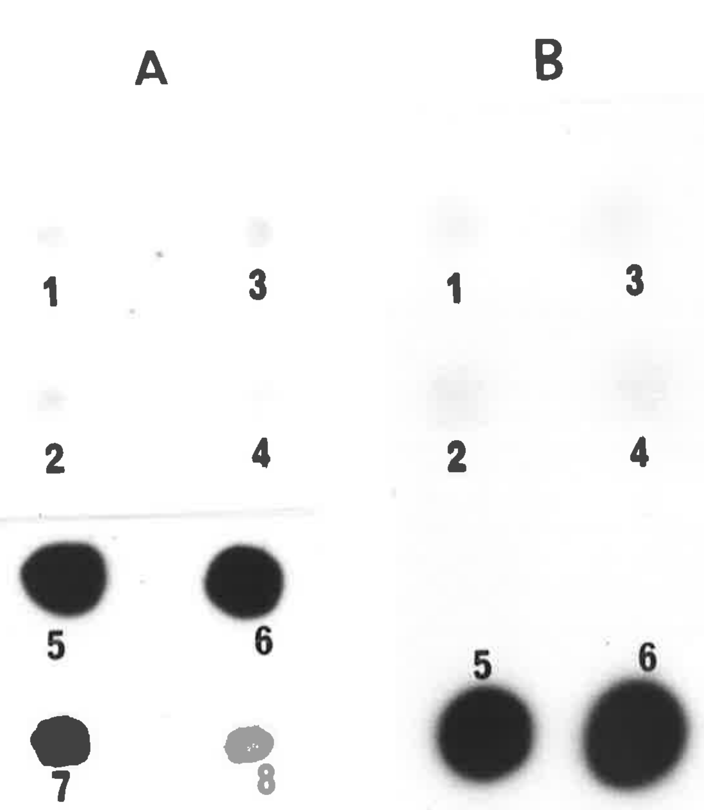
7 and 8 150 ng of pBR322 DNA.

B. RNA DOT BLOTS

1 and 2 20 µg of RNA from untransfected fibroblasts.

3 and 4 20 µg of RNA from fibroblasts transfected with 1090/-29 DNA.

5 and 6 250 ng of 1090/-29 DNA.



A
B
1
3
1
3
2
4
2
4
5
6
5
6
7
8

FIGURE 6-5

CO-TRANSFECTION OF FIBROBLASTS WITH
1090/-29 DNA AND pSV2CAT DNA:
ANALYSIS OF THE CYTOSOL EXTRACTS

Fibroblasts were transfected with 5 µg of pSV2CAT DNA and 5 µg of 1090/-29 DNA/10 cm plate. 15 µg of rat carrier DNA was also used in the transfection. The cultures were split 3 days post-transfection and the cells were harvested on the 5th day. After cell lysis, protein extracts were assayed for the presence of reverse transcriptase and CAT activity. Reverse transcriptase activity was negative in all cases (not shown). The diagram shows the results obtained when 25 µg (**A**), 50 µg (**B**) and 100 µg (**C**) of protein extract was assayed for CAT activity. In all cases (**i**) represents protein from fibroblasts transfected with rat carrier DNA only, (**ii**) represents protein from fibroblasts transfected with the pSV2CAT, 1090/-29 DNA mixture.

A B C

i ii i ii i ii

In an attempt to discover at which point the transfection process was blocked, viable Rous sarcoma virus particles (Prague A strain) were used to infect cultured fibroblasts. After 14 days, foci of cells were not observed, reverse transcriptase activity could not be detected in the medium and dot blot analysis of the DNA failed to detect RSV DNA (data not shown). The interpretation of these results is discussed below.

6.5.2 DISCUSSION OF THE TRANSGENIC CHICK EMBRYO SYSTEM

(i) The Prepared Fibroblast Cultures are Defective in Producing Infectious Virus Particles

The results of this study suggest that the fibroblast cultures utilized were resistant to Prague A strain RSV infection and were also defective in the ability to integrate transfected 1090/-29 DNA (1090/-29 is derived from the Schmidt-Ruppin A strain of RSV). Numerous studies have reported the existence of non-permissive fibroblast cultures and it has been determined that cellular resistance against infection of a given RSV strain can result from the genetic composition of the cells or from congenital infection with an avian leukosis virus (Tooze, 1973 for review; Ignjatovic and Bagust, 1985). Perhaps the observed inability of transfected 1090/-29 DNA to integrate into the fibroblast DNA is another aspect of such non-permissive cells.

The chicken stocks from which embryos were obtained to make fibroblast cultures are probably resistant to the A type strain of RSV, indicating a need for a chicken type with a guaranteed susceptibility. Apparently the most commonly

encountered phenotypes among chicken lines in Australia are those susceptible to infection with RSV viruses of subgroup C and resistant to subgroups A, B, D and E (Ignjatovic and Bagust, 1985). Fortunately, a recent study has established the susceptibility of a number of Australian chicken lines to different strains of RSV (Ignjatovic and Bagust, 1985) and a supplier of chick embryos with guaranteed susceptibility to all RSV subgroups has been secured. For the continuation of this work it is now possible to obtain fibroblast cultures that would serve as the cell source for packaging the transfected DNA into infectious particles.

(ii) Appraisal of the Chick Embryo System for Use in Expression Studies

The success of transcription studies using this system will depend upon the ability of the RSV vector, with the inserted keratin gene, to integrate into the DNA of feather epidermal cells such that the feather gene constructs are expressed in them. Infection of the embryos at an extremely early age will enhance this possibility. In principle there is no reason why integration into feather DNA should not be successful as transforming RSV particles have been detected in the feather follicles of adult chickens (Robinson and Lamoreux, 1976). If successful integration cannot be achieved using this vector, another retroviral system is available which is based on the Moloney murine leukaemia virus. This system has a wide range of cell and species infectivity but the generation of infectious particles using this system is much more complex (Miller **et al.**, 1985).

In the study by Souza **et al.** (1984) expression of the

chicken growth hormone cDNA was presumably under the control of the promoter in the LTR because the inserted gene lacked promoter signals. When an exogenous gene is inserted into a retroviral vector, the initiation and termination signals for mRNA synthesis can be provided by the exogenous gene or by the sequences that the virus itself utilizes for expression and replication. Problems occur if the exogenous gene promoter is inserted in the same orientation as the promoter in the 5' LTR. In a study where the chicken tk gene was inserted into a retroviral vector in the same orientation as the 5' LTR, deletions had occurred in both the virus and in the tk gene when the virus particles were recovered from transfected fibroblasts. Splicing of the chicken tk gene sequences was also observed (Bandyopadhyay and Temin, 1984). When the tk gene was in the opposite orientation to the promoter in the LTR these phenomena were not observed, although viral titre was decreased, presumably because of interference of RNA synthesis from the converging promoters. In some cases if an exogenous gene is inserted in the same orientation as the LTR, the polyadenylation signal may cause transcript pretermination which results in extremely low levels of virus (Shimotohno and Temin, 1981).

For the results of transcription studies using this system to be meaningful, transcription must be under the control of the feather keratin promoter and the keratin gene must be integrated containing its intron. Insertion of the feather keratin gene in the opposite orientation to the viral promoter should allow expression from the keratin promoter once the gene is integrated into the chick genome. In addition, the insertion of the gene in that orientation should

prevent phenomena such as intron excision and transcript pretermination when the recombinant viral-keratin RNA is being packaged into infectious particles in the fibroblasts. The problems related to low viral titre can be overcome by pooling and concentrating virus samples.

One fundamental problem with this type of transgenic expression system is that the integration of the DNA into the genome is random. Whether the introduced gene will be expressed from any integrated position is not known. The fact that the feather keratin genes are clustered in the chick genome may indicate a need for the introduced gene to integrate close to the cluster for efficient expression. Infection of a number of embryos would increase the probability of the introduced gene being integrated into a transcriptionally suitable position within the genome. Furthermore, infecting the embryos at an early age would ensure a more homogenous distribution of the gene throughout all cell types, maximizing the number of feather cells which would contain the test gene.

The obvious advantage of the system is that it is self-contained with respect to all the necessary transcription factors, and cell layer interactions. The introduced feather keratin gene would also be subjected to the variety of controlling events which occur during keratinization. Another attractive feature is the relatively high level of embryo survival as reported by Souza **et al.** (1984).

Initially, the system could be used to define the keratin promoter elements and to determine whether the intron does play a role in transcript initiation **in vivo**. If these studies prove successful the system could be used to study the

transcriptional control features of other genes involved in avian keratinization, for example the claw, scale keratin and histidine-rich fast protein genes.

Thus, the transgenic chick embryo system provides a new avenue to study avian keratin gene expression and the most exciting work is still to come.

CHAPTER 7.

CONCLUDING DISCUSSION.

7.1 FEATHER KERATIN GENES AND AVIAN KERATINIZATION

The most significant aspect of keratin gene expression lies in the fact that keratinization is a major form of terminal differentiation. During keratinization, the synthesis of large amounts of protein coincides with the degeneration of subcellular organelles and cell death. The mechanisms by which such processes are prevented in actively growing tissues and how they are triggered at the appropriate developmental stage are questions of importance in generally understanding somatic development.

The amount of information gained here regarding the specific controls in feather keratinization has admittedly been limited. However, all the initial aims of this preliminary study have been achieved. In the process of examining the general control features of feather keratin gene transcription using the **Xenopus** oocyte it was discovered that DNA sequences within the positionally conserved feather keratin intron may be involved in the suppression of unscheduled feather keratin gene transcription.

Hybrid genes constructed using feather keratin gene B and the HSV-tk gene have shown that the keratin gene promoter is not dependent upon highly specific activators in order to function in oocytes. Furthermore, this work led to the discovery of stimulatory sequences within the transcribed region of the HSV-tk gene which were capable of increasing the functional efficiency of the keratin promoter. This discovery acts as an important caution against the potential anomalies which may arise using a gene fusion approach to study promoter

action. It is becoming increasingly clear that isolated parts of a gene cannot be expected to yield reliable information about their **in vivo** function. A holistic approach appears to be required.

The inefficient level of feather keratin gene transcription and transcript processing in oocytes, when compared with other genes not involved in terminal differentiation, made it obvious that factors pertaining to the expression of keratin genes were lacking in this system. Investigations subsequently focused on testing other existing systems for their suitability in studying feather keratin gene expression. All proved unsuitable and a genuinely homologous system was proposed which involves the use of retroviral vectors to transfer feather keratin genes into chick embryos. The introduction of genes that had been mutagenized in selected regions would allow studies of transcriptionally important structural features. The work undertaken towards establishing this system using a Rous sarcoma virus vector has indicated that development of the system is indeed feasible.

If the retroviral-chick embryo system proves useful for studying the structural features important in efficient feather keratin gene transcription, the work could be extended, with little extra development, to similar studies on other avian genes such as the scale and claw keratin genes and the histidine-rich fast protein genes. The results of these studies should determine whether the retroviral system has the potential for uncovering the mechanisms involved in regulating the co-ordinate expression of each of the avian keratin gene families during development. At this time however, there is no doubt that the retroviral chick embryo system is the most

promising available system for future work on the tissue and stage specific expression of the avian keratin genes.

7.2 INTRAGENIC CONTROL

The most interesting aspect of the work presented in this thesis has been the involvement of intragenic sequences in controlling the efficiency of transcription. This study has discovered, albeit in a surrogate system, the existence of two influential gene segments: a suppressive region within the intron of feather keratin gene B and a stimulatory effect exerted by sequences 3' to the initiation site of the HSV-tk gene. These two examples can now be added to the small but growing list of elements, found 3' to gene cap sites, whose influence on transcriptional efficiency has only recently been realized (Banerji **et al.**, 1983; Charnay **et al.**, 1984; Merrill **et al.**, 1984b; Wright **et al.**, 1984; Moore **et al.**, 1985; Mous **et al.**, 1985).

The dispersal of sequences controlling transcription upstream and downstream of the cap site adds to the complexity of understanding the transcriptional regulation of a particular gene. The presence of transcriptionally important elements 3' to the mRNA cap site suggests that coding regions of a gene may have a dual role in expression, possessing an involvement in the regulation of transcript initiation as well as the production of a specific protein. Such a system of regulation is self-sustaining as it has an innate specificity with regard to controlling gene expression.

7.3 REGULATORY SEQUENCES AND SPECIFIC FACTORS

Is there really a plethora of specific and different factors which recognize all of the controlling sequences in polymerase II transcribed genes? Perhaps not, as the economics of such a system appear unsound. Consider the fact that many (perhaps most) genes may have two or three elements in their promoters necessary for transcription. When multiplied by the number of different genes coding for the various structural proteins of a cell, this hypothesis would require the existence of an enormous number of factors. Then the genes that code for these factors need to be considered, and also the factors that regulate them. The requirement for individually specific factors would result in a vastly greater number of control genes than there are structural genes. It should be noted that a highly specific cascade systems does not provide any explanation for the nature of gene control but merely adds extra steps to the mechanistic view of the process.

The observation that similar factors appear to be involved in the regulation of functionally unrelated genes (Kadonaga **et al.**, 1986) suggests that there may be a more limited contingent of factors. In addition, the ability of certain genes to be expressed in non-homologous cell types suggests that unrelated cells do possess similar types of factors. Different combinations of promoter sequences, each recognized by a particular member of the factor repertoire, could accommodate the specific regulation of a large number of the less stringently regulated housekeeping genes.

The production of tissue specific factors, however, may

be required at a particular point in development to allow a cell to proceed along a particular differentiation pathway and to guard against the possibility of unscheduled differentiation. An alternative to producing a new subset of factors for differentiated tissues would be to covalently modify existing members of the factor repertoire by, for example, phosphorylation, acetylation, ribosylation etc. Such covalent modifications could alter the binding affinity of a protein for a sequence or alter protein function at a specific stage in development without requiring large scale reorganization of cellular pools of protein factors.

Much of this is of course, speculation. Most of the protein factors involved in the transcription of polymerase II genes still need to be purified, characterized and in turn the genes that code for them need to be isolated and studied. Only when such information is obtained on a variety of factors will it be possible to gain some insight into the specific mechanisms involved in activating and regulating gene transcription.

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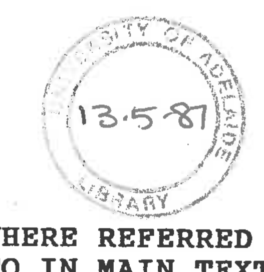
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CONSTRUCT INDEX

NUMBER	CONSTRUCT NAME	PAMPHLET PAGE	WHERE REFERRED TO IN MAIN TEXT (SECTION)
3.1	97	3	4.2.1
3.2	24	3	4.2.1
3.3	70	3	4.2.1
3.4	1-4E8	3	-
3.5	SVGene B	4	4.2.5
3.6	BK-i	4	4.2.3
3.7	SVBK-i	5	4.2.5
3.8	BK309HpaIIi	5	4.2.3
3.9	tk (TK/TK)	6	Numerous
3.10	Eco-tk	6	4.2.5
3.11	SVtk	6	4.2.5
3.12	SVEco-tk	6	4.2.5
3.13	TK/KER	7	5.2.1
3.14	1-4/TK/BglBam	7	-
3.15	KER/TK	8	5.2.1
3.16	MTK/KER	8	5.2.1 and 5.2.73
3.17	MTK/KER-BE2	9	5.2.3
3.18	MTK/KER-BE2SMA	9	5.2.3
3.19	MTK/KER-SALSMA	9	5.2.3
3.20	BK-i-3'TK	10	5.2.3
3.21	TK/KER-3'TK	10	5.2.3
3.22	TKKpr	11	5.2.3
3.23	TK/H2B	11	5.2.4
3.24	KER/H2B	11	5.2.4
3.25	KER127TK	12	5.2.5
3.26	KER187TK	12	5.2.5

3.27	KER328TK	13	5.2.5
3.28	KER436TK	13	5.2.5
3.29	BKTK5	14	5.2.6
3.30	BKTK2	14	5.2.6
3.31	KER430TK	14	5.2.5
3.32	▶ KER800KER	15	5.2.6
3.33	◀ KER800KER	15	5.2.6
3.34	▶ KER617KER	15	5.2.6
3.35	◀ KER617KER	15	5.2.6
3.36	▶ KER900KER	15	5.2.6
3.37	◀ KER900KER	15	5.2.6
3.38	▶ KERSp617KER	15	5.2.6
3.39	▶ KERSp900KER	15	5.2.6
3.40	pJL4B1-4	16	6.2.1

3.1 97, 3.2 24, 3.3 70

In these constructs, feather keratin gene A was inserted into the chicken histone cluster, p7AT. A more detailed map of p7AT is given on the left hand side of the diagram. The purpose of these clones was to determine whether gene A was being packaged into inactive chromatin in oocytes (section 4.2.1).

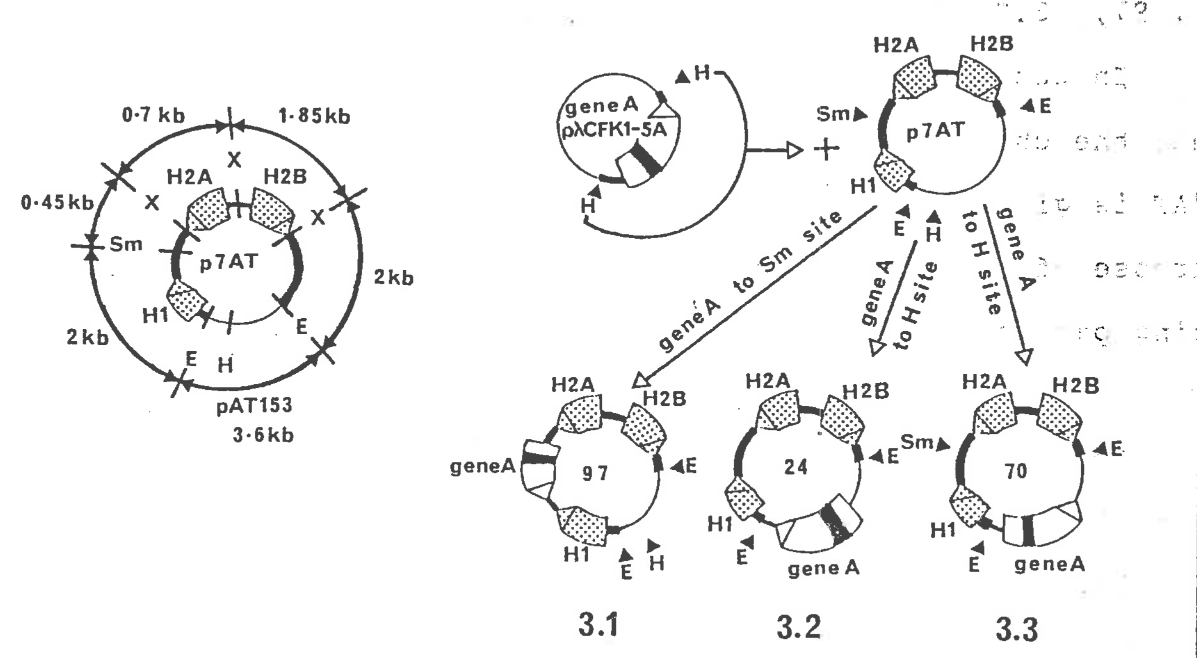
Two vectors were constructed by digesting p7AT with HindIII or SmaI. Gene A was resected from the subclone, pλCFK1-5A by digestion with HindIII (fragment = 2.15 kb). Some of the isolated fragment was inserted into the HindIII cut p7AT vector, generating clones 24 and 70 which contained gene A in two different orientations relative to the direction of transcription of the H1 histone gene. Clone 97 was constructed by inserting the isolated gene A fragment into the SmaI site of p7AT. The size of each of the constructs generated is 12.75 kb.

3.4 1-4E8

1-4E8 is a feather keratin gene B clone which has only one HindIII site; the one 5' to the mRNA start site in pλCFK1-4. This clone was used as the basis for a number of recombinant plasmids.

1-4E8 was constructed by digesting pλCFK1-4 with BglII and BamHI (figure 3-A). After electrophoresis the 5.86 kb fragment, lacking the 1.43 kb bp BglII/BamHI fragment was isolated and the compatible cohesive ends were religated, destroying both the BglII and the BamHI site. The size of the clone is 5.86 kb.

3.1, 3.2 and 3.3



3.4

H
gene B
pλCFK1-4
Bg
H
Ba
Bg & Ba
cut
religate
H
1-4 E8
Bg/Ba*

In all diagrams shown, the following apply, unless otherwise stated: the broad arrows represent the segment of DNA which encodes the mRNA of the gene. The direction of the arrow indicates the direction of transcription. Plain arrows refer to keratin genes, stippled to histone genes and slant-shaded arrows to tk sequences. The shaded block within the arrows representing keratin genes depicts the position of the intron. The thick dark lines represent flanking regions pertaining to each gene and the thin lines pBR322 or M13 derived DNA. A = AluI, Av = AvaII, Ba = BamHI, Bg = BglII, Bs = BstEII, E = EcoRI, EF = end-filled, F = FnuDII, Ha = HaeIII, H = HindIII, Hp = HpaII, P = PstI, Pv = PvuII, S = SalI, Sm = SmaI, Su = SauIIIA. Tq = TaqI, X = XhoI
* = site destroyed.

3.5 SVGene B

This gene B clone contains the SV40 enhancer inserted upstream of the mRNA cap site and was made to test the effect of the enhancer on gene B transcription in oocytes (section 4.2.5).

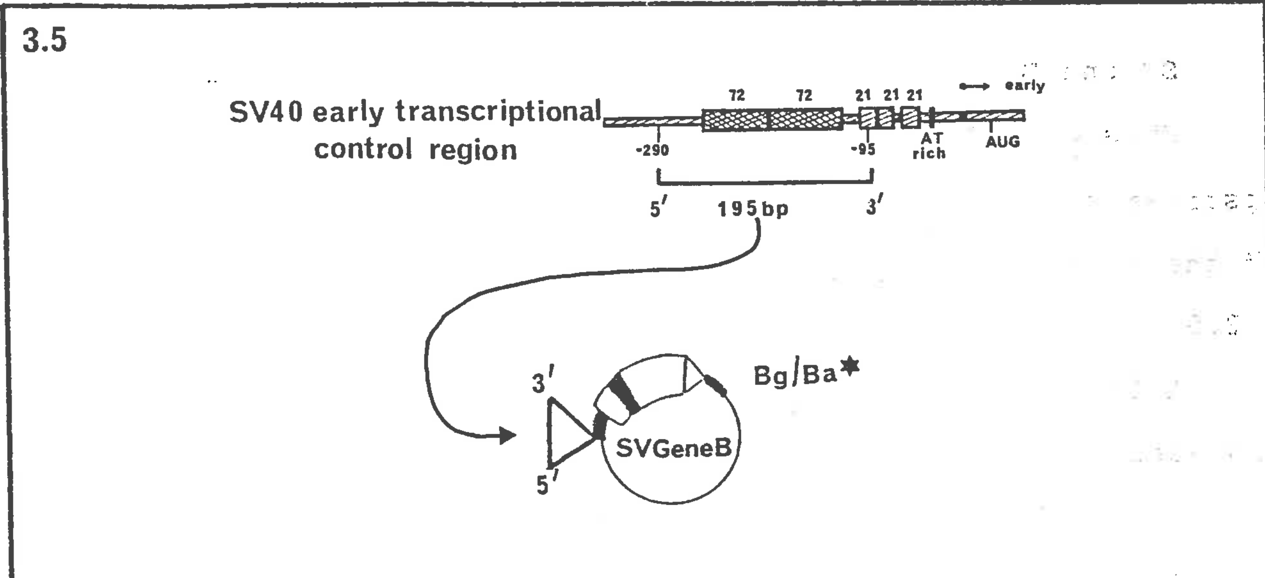
A 195 bp fragment containing the SV40 enhancer was isolated from a donated clone by digestion with EcoRI and HindIII. The enhancer fragment was inserted into the HindIII site of 1-4E8 (section 3.4), 383 bases upstream of the mRNA start site of gene B. The 3' overhangs of the fragments were end-filled before ligation. The size of the clone is 6.05 kb.

3.6 BK-i

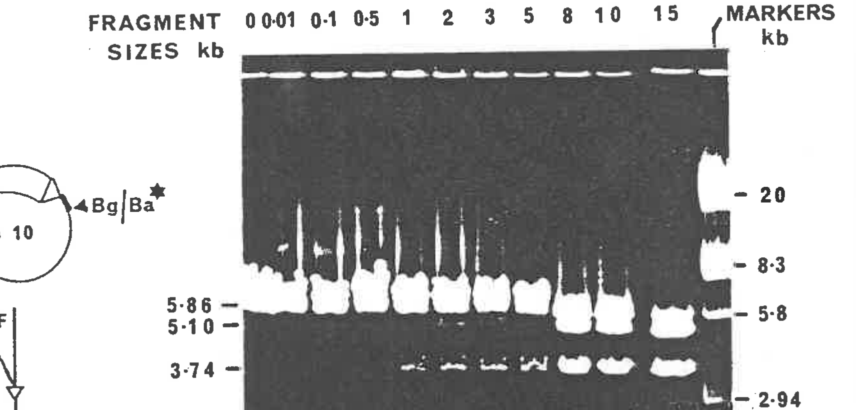
BK-i is a feather keratin gene B-containing clone in which the single intron has been removed from the 5' untranslated region. BK-i was constructed to test the effect of intron removal on transcription in **Xenopus** oocytes (section 4.2.3).

A number of 5 μg aliquots of 1-4E8 DNA (section 3.4) were digested to completion with HindIII and partially with different concentrations of SalI (0.01 to 20 units for 30 minutes at 37°C). The overhangs of the fragments were end-filled and the digests were electrophoresed on a 0.55% low melting point agarose gel in TAE. The 5.10 kb fragment isolated lacked all of the DNA upstream of the 3' splice site of gene B. Sequences missing included the intron, the 37 bp 5' untranslated sequence, cap site and 383 bp of DNA 5' to the cap site. The 5.10 kb fragment acted as the vector for BK-i construction (clone 510). Gene B was digested with PstI in the presence of Klenow fragment and deoxynucleotides and the

3.5



3.6



399 bp fragment containing 356 bases of sequence upstream from the mRNA cap site, the 37 bp 5' untranslated sequence and 6 bases of intron (figure 3-A) was chosen as the keratin promoter. The 399 bp fragment was inserted into the 510 vector to generate BK-i with a size of 5.5 kb.

3.7 SVBK-i

SVBK-i contains the SV40 enhancer inserted upstream of the BK-i cap site. This plasmid was constructed to test the effect of the enhancer on BK-i transcription in oocytes (section 4.2.5).

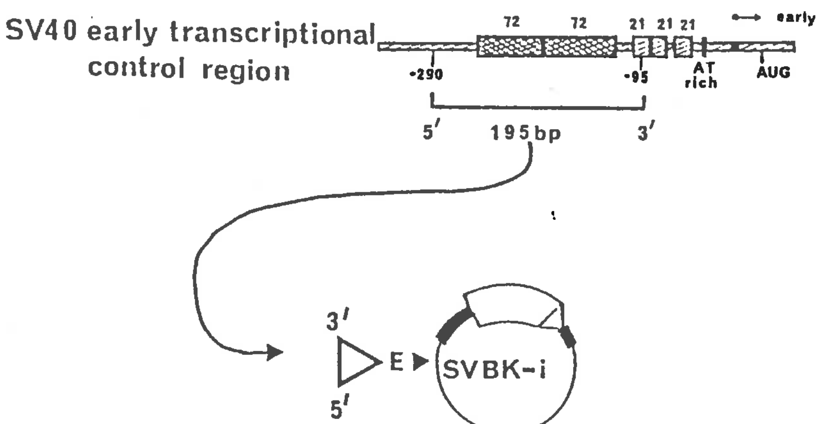
The 195 bp enhancer fragment (section 3.5) was inserted into the EcoRI site of BK-i, 386 bp upstream of the mRNA start site. The orientation of the enhancer fragment is shown in the diagram. The size of the clone is 5.69 kb.

3.8 BK309HpaIIi

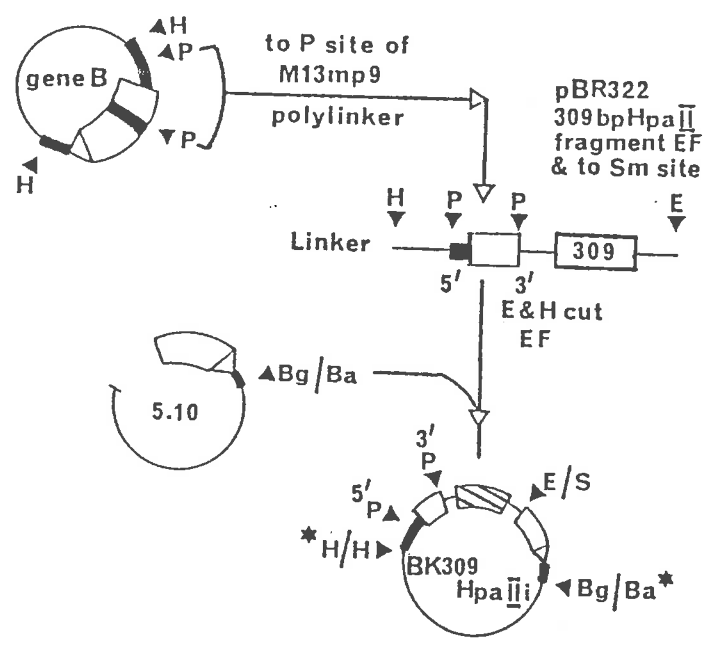
In this clone the intron of feather keratin gene B was replaced with sequences from pBR322. The clone was used to determine whether sequences within the keratin intron were responsible for suppression of transcript initiation (section 4.2.3).

The 399 bp PstI fragment containing the keratin promoter and mRNA start site was inserted into the PstI site of the M13mp9 polylinker (generating M13Pst) and the 309 bp HpaII fragment from pBR322 was inserted into the SmaI site of the same construct. The combined promoter/pseudo-intron was excised with EcoRI and HindIII and inserted into the SalI site of clone 510 (section 3.6). This resulted in a construct with spacing between the mRNA start site and the protein coding initiation point which differs from the unaltered gene by a further 6 bp. The size of the clone is 5.83 kb.

3.7



3.8



3.9 tk (or TK/TK)

The clone containing the HSV-1 tk gene was derived from the plasmid pBR322/SV-0+T/tk (Capecchi, 1980) in which the 3.4 kb fragment containing the entire tk gene was isolated by digestion with BamHI and inserted into the BamHI site of pBR322. The size of this clone is 7.76 kb and it was used in a number of different experiments.

3.10 Eco-tk

Eco-tk is a derivative of tk in which the second distal transcription signal (McKnight **et al.**, 1984) has been removed. This was achieved by digesting tk with EcoRI and inserting the 2.3 kb fragment containing the tk gene into the EcoRI site of pBR322. The direction of transcription is that shown in the diagram. The size of the resulting clone was 5.46 kb. Eco-tk was used in section 4.2.5.

3.11 SVtk and 3.12 SVEco-tk

SVtk and SVEco-tk contain the SV40 enhancer and were used to test SV40 enhancer function in **Xenopus** oocytes (section 4.2.5). SVtk and SVEco-tk were constructed by inserting the SV40 enhancer fragment (section 3.5) into the HindIII site of tk (section 3.9) and Eco-tk (section 3.10) respectively. The HindIII site in tk is 976 bases upstream of the mRNA start site whilst in Eco-tk the HindIII site is 111 bp upstream from the mRNA start site. The size of SVtk is 7.95 kb and the size of SVEco-tk is 5.65 kb.

3.9,3.10,3.11 and 3.12

3.13 TK/KER

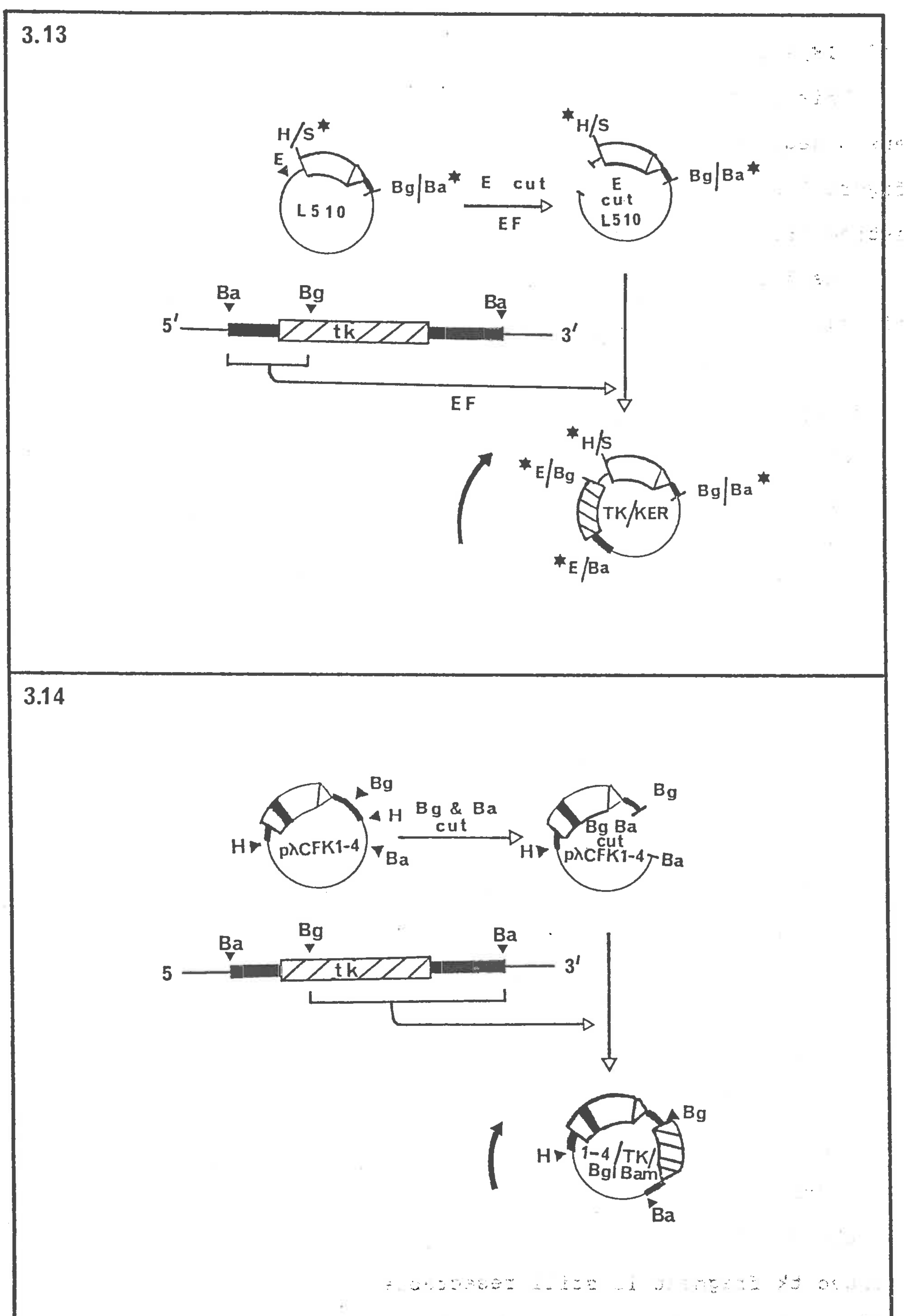
This construct contains the tk promoter fused to keratin gene B sequences found 3' to the coding region SalI site (figure 3-A(ii)). TK/KER was used in section 5.2.1 when testing promoter efficiency in oocytes.

The 5.10 kb fragment described in 3.6 was self-ligated and transformed into MC1061 cells as it was necessary to obtain more of this DNA for further manipulations. This clone was designated L510. Unfortunately, L510 could not be digested with HindIII as this site was destroyed on cloning. L510 was subsequently digested with EcoRI and the 5' overhangs were end-filled; this formed the vector for TK/KER. The tk clone (3.9) was digested with BamHI and BglII and the 680 bp fragment containing the tk promoter was isolated, end-filled and inserted into the L510 EcoRI cut vector as shown. The resulting construct contained 31 bp of pBR322 DNA between the junction of the tk promoter fragment and the keratin gene sequences. The size of the clone is 5.78 kb.

3.14 1-4/TK/BglBam

This clone was an intermediate in the preparation of some of the hybrid gene constructs. It contains all of gene B (including the intron) and the coding region of the tk gene (the 2.7 kb BglII/BamHI fragment, figure 3-B(ii)).

The clone was constructed by digesting pλCFK1-4 with BglII and BamHI. The 5.86 kb fragment isolated was used as a vector for the insertion of the 2.7 kb BglII/BamHI fragment from the tk clone as shown in the figure. Note that the inserted tk fragment is still resectable by digestion with BglII and BamHI. The size of the clone is 8.56 kb.


3.13
H/S
E
Bg|Ba
L510
E cut
EF
E
cut
L510
Ba
Bg
Ba
5'
tk
3'
EF
H/S
E/Bg
Bg|Ba
TK/KER
E/Ba
3.14
Bg
H
H
pλCFK1-4
Ba
Bg & Ba
cut
Bg
Bg Ba
cut
pλCFK1-4
Ba
Ba
Bg
Ba
5
tk
3'
Bg
H
1-4/TK/
Bg/Bam
Ba

3.15 KER/TK

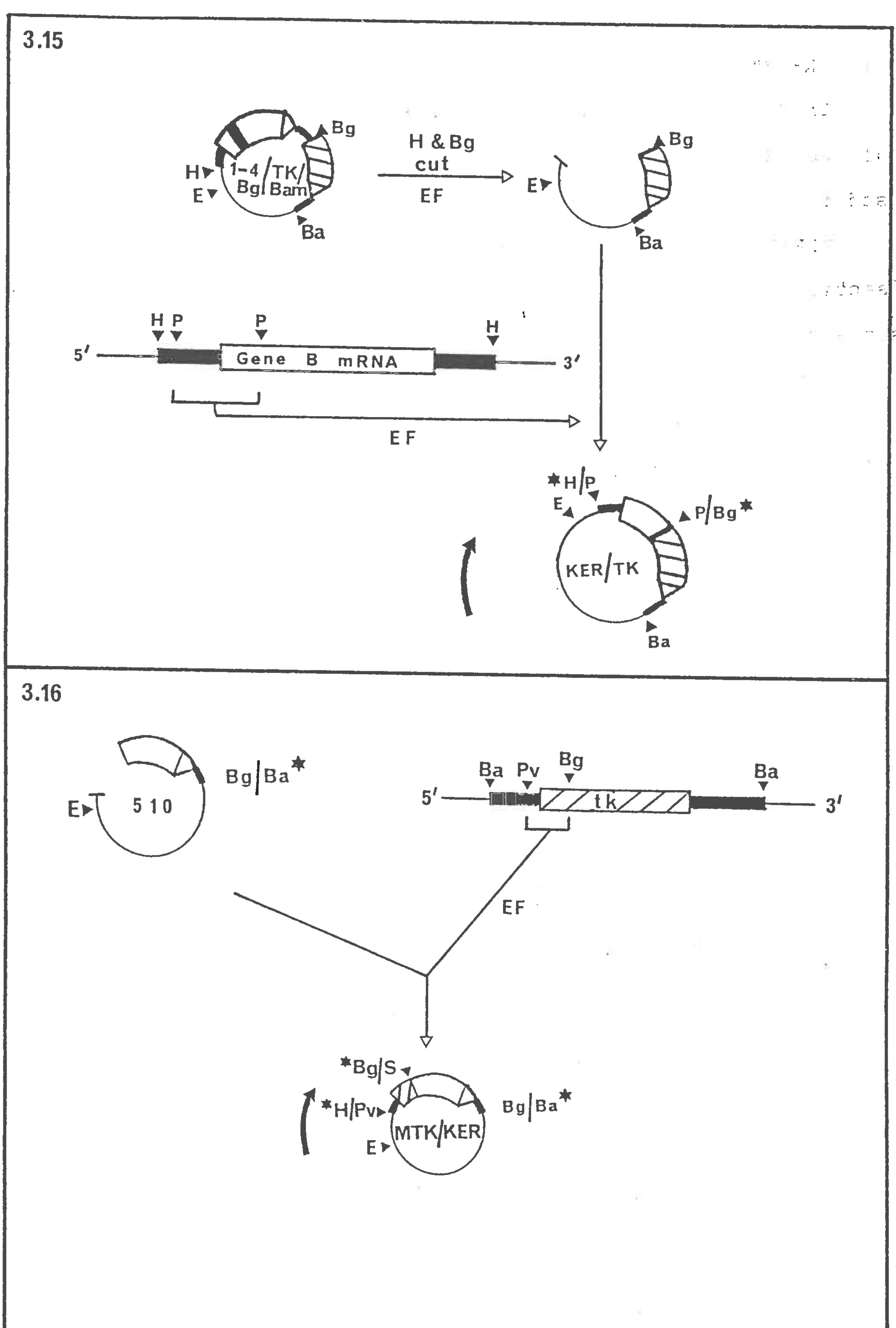
In KER/TK the keratin promoter is fused to the 2.7 kb BglII/BamHI fragment of the HSV-tk gene. This construct was used in section 5.2.1 to test keratin promoter efficiency.

KER/TK was constructed by digesting 1-4/TK/BglBam (section 3.14) with HindIII and BglII to remove all keratin sequences (1.84 kb). A 399 bp Pstl fragment from the 5' end of keratin gene B (see section 3.6) was inserted into the HindIII/BglII cut 1-4/TK/BglBam clone as shown in the diagram. The size of the clone is 7.12 kb.

3.16 MTK/KER

In this construct the tk promoter was fused to keratin gene sequences, however, MTK/KER lacks the pBR322 DNA found between the tk promoter and keratin gene sequences in TK/KER (section 3.13). MTK/KER was used as the basis for a number of deletion constructs and reference is made to this clone in sections 5.2.1 and 5.2.3 of the text.

MTK/KER was constructed by isolating a 250 bp PvuII/BglII fragment, containing the promoter sequences, from the tk clone (figures 3-B(i) and (ii)). The 250 bp fragment was inserted into the 510 vector used in the construction of BK-i (section 3.6). The size of the clone is 5.35 kb. The arrow indicates the direction of transcription.


3.15
Bg
H
E
1-4/TK/Bgl Bam
Ba
H & Bg
cut
EF
E
Bg
Ba
H P
P
H
5′
Gene B mRNA
3′
EF
*H/P
E
P/Bg*
KER/TK
Ba
3.16
Bg|Ba*
E
510
Ba
Pv
Bg
Ba
5′
tk
3′
EF
*Bg/S
*H/Pv
Bg|Ba*
MTK/KER
E

3.17 MTK/KER-BE2

MTK/KER-BE2 is a derivative of MTK/KER and lacks the 316 bp fragment from the keratin coding region. This clone was used in section 5.2.3 to test the effect of keratin sequences on transcript initiation.

MTK/KER-BE2 was constructed by digesting MTK/KER with BstEII. (A detailed diagram of the MTK/KER clone is provided to facilitate the location of the sites). The 5.03 kb fragment, missing the 316 bp BstEII fragment, was isolated and the ends of the fragment were religated.

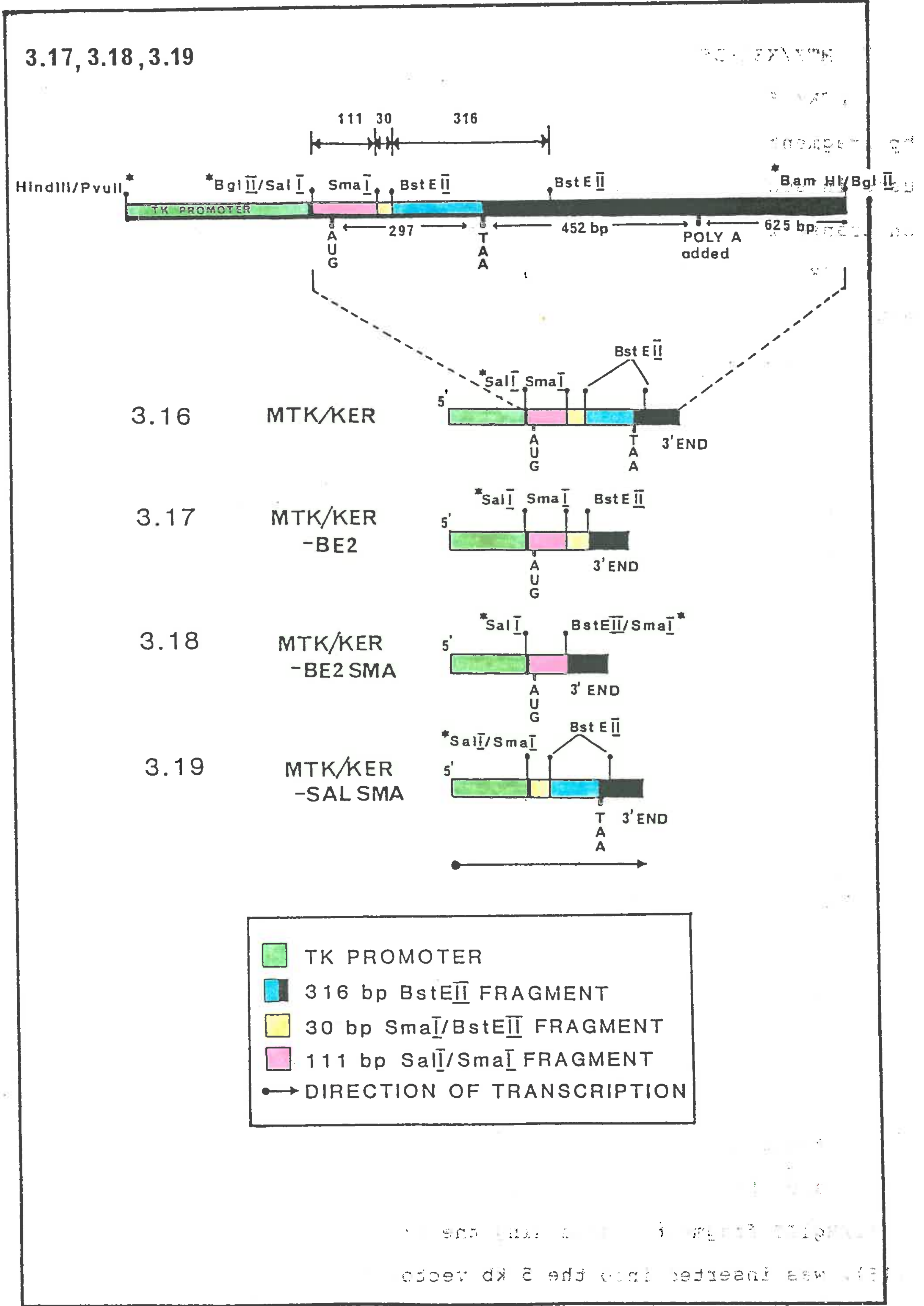
3.18 MTK/KER-BE2SMA

This clone is also derived from MTK/KER and has 346 bp of keratin coding region missing. This was achieved by digesting MTK/KER with BstEII and SmaI, end-filling the BstEII overhang, isolating the 5 kb fragment (lacking the 346 bp BstEII/SmaI fragment) and religating the DNA. The use of this construct is discussed in section 5.2.3.

3.19 MTK/KER-SALSMA

This clone was constructed to test the effect of keratin sequences on transcription from the tk promoter in oocytes (section 5.2.3).

Clone 1-4E8 (section 3.6) was digested with HindIII and SmaI. This removed all of the keratin sequences 5' to the SmaI site in the coding region of gene B (870 bp) resulting in a 5 kb fragment. The 5 kb fragment was isolated to act as a vector and the ends of the fragment were filled. A 250 bp PvuII/BglII fragment, containing the tk promoter (section 3.16), was inserted into the 5 kb vector generating MTK/KER-SALSMA (size = 5.25 kb).


3.17, 3.18, 3.19
111
30
316
HindIII/PvuII
*BglII/SalI
SmaI
BstEII
BstEII
*Bam HI/BglII
TK PROMOTER
AUG
297
TAA
452 bp
POLY A added
625 bp
3.16
MTK/KER
3.17
MTK/KER -BE2
3.18
MTK/KER -BE2 SMA
3.19
MTK/KER -SAL SMA
*SalI
SmaI
BstEII
BstEII/SmaI*
*SalI/SmaI
5'
3' END
TK PROMOTER
316 bp BstEII FRAGMENT
30 bp SmaI/BstEII FRAGMENT
111 bp SalI/SmaI FRAGMENT
DIRECTION OF TRANSCRIPTION

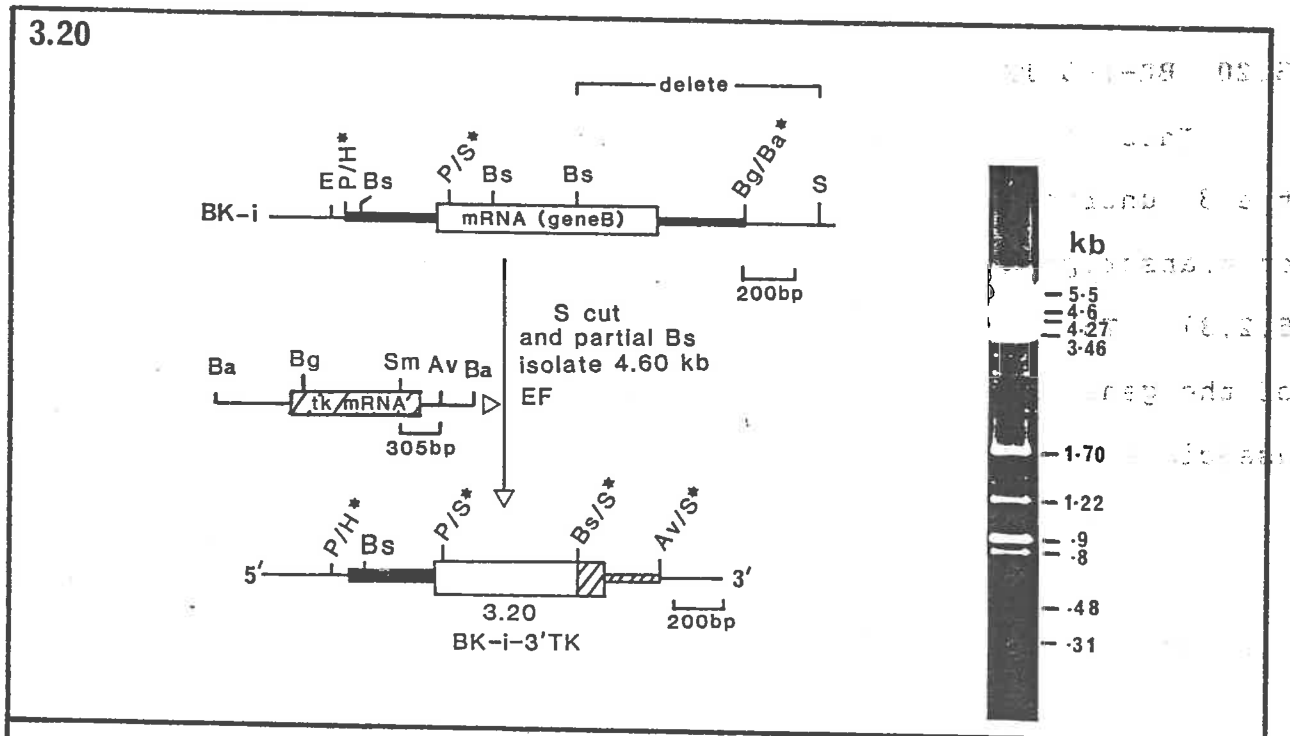
3.20 BK-i-3'TK

This clone was made to test whether the 18 bp sequence in the 3' untranslated region of the keratin gene had any effect on transcription from the keratin promoter in oocytes (section 5.2.3). The clone is similar to BK-i except that the 3' end of the gene containing the polyadenylation signal and associated 3' flanking DNA has been removed and replaced with the tk polyadenylation signal and 3' sequences.

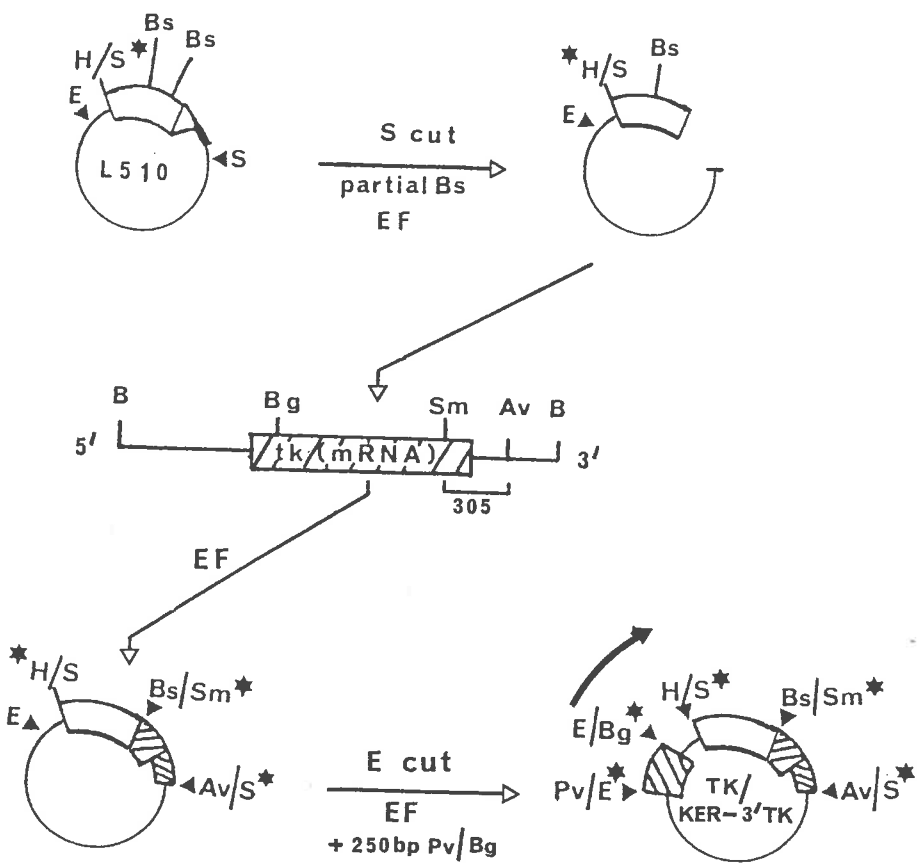
BK-i-3'TK was constructed by digesting 5 μg of BK-i with SalI and then partially digesting with BstEII (2 units, 60°C for 30 minutes). The ends of the fragments were filled and the DNA was electrophoresed on a 0.55% agarose gel in TAE. The 4.60 kb fragment, missing 900 bp of sequence from the SalI site in pBR322 up to the third BstEII site in keratin gene B (figure 3-A) was isolated. The 4.60 kb fragment served as a vector for the insertion of a 305 bp SmaI/AvaII fragment isolated from the 3' end of the tk gene (3-B(ii)). The resultant clone was 4.9 kb in size.

3.21 TK/KER-3'TK

This construct is similar to BK-i-3'TK (3.20) except that the keratin promoter has been replaced with the tk promoter. This clone was used in section 5.2.3 and was constructed by partially digesting L510 (section 3.13) with BstEII. The digestion removed the same 900 bp fragment which had been deleted from the 3' end of BK-i in the construction of BK-i-3'TK. A 305 bp SmaI/AvaII fragment from the 3' end of the tk gene (3.20) was inserted in place of the deleted 900 bp fragment. The resulting construct was digested with EcoRI and the 250 bp PvuII/BglII fragment, containing the tk promoter



3.21



(see 3.16) was inserted to generate TK/KER-3'TK (4.75 kb). Note that 31 bp of pBR322 DNA is present at the tk-keratin junction.

3.22 TKKpr

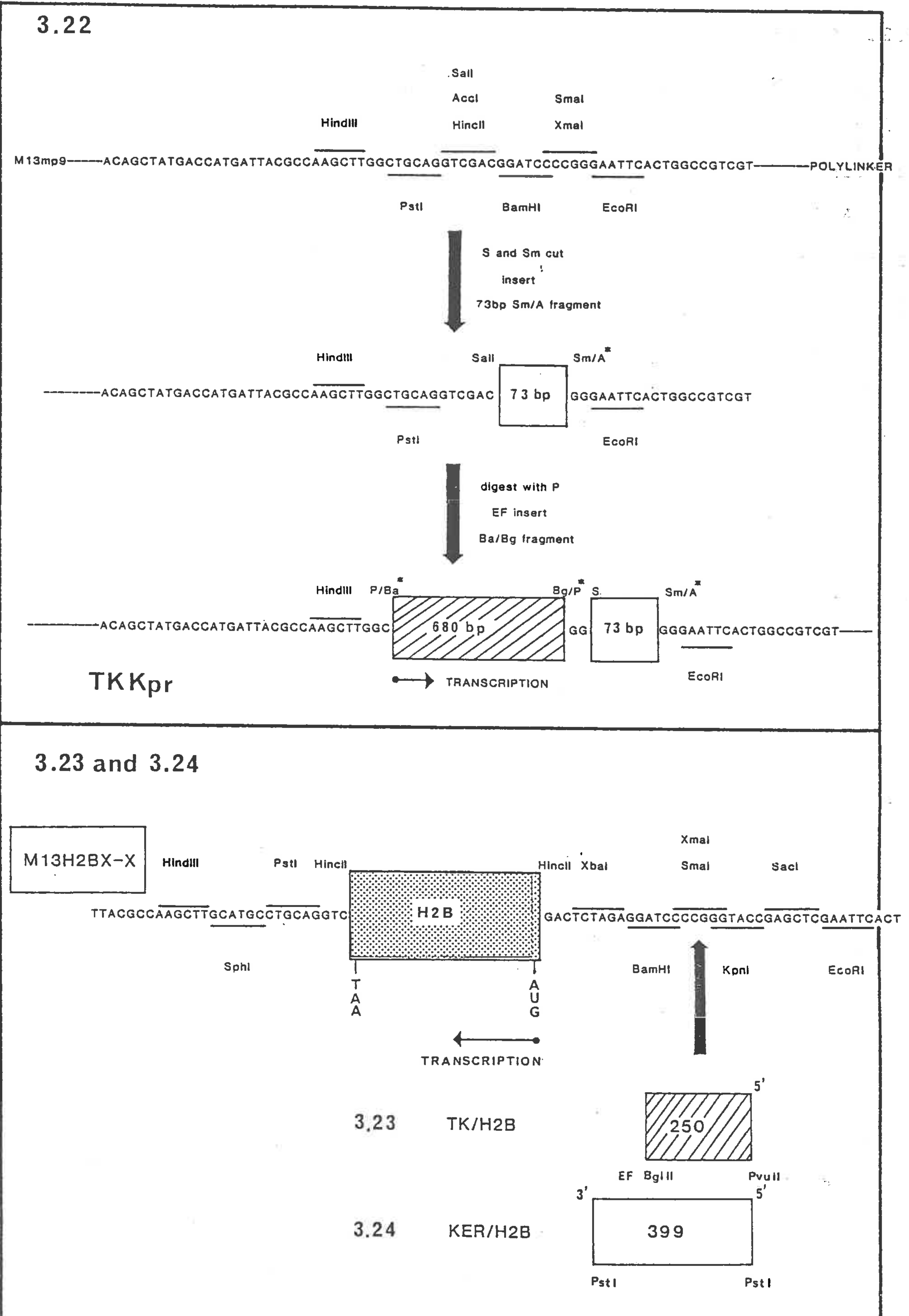
This construct was used in section 5.2.3 to test the efficiency of the tk promoter in the presence of 73 bp of keratin DNA.

A 73 bp SalI/AluI fragment from the coding region of keratin gene B (figure 3-A(ii)) was inserted directionally into M13mp9 which had been digested with SmaI and SalI. The clone generated, M1373, was digested with PstI. A 680 bp BamHI/BglII fragment (figure 3-B(i)) containing the tk promoter was inserted into the PstI site of M1373 to generate TKKpr. The 73 bp keratin DNA fragment contained the keratin 25-mer primer sequence. The size of the clone is 7.98 kb.

3.23 TK/H2B and 3.24 KER/H2B

TK/H2B and KER/H2B were constructed to test the efficiency of the tk and keratin promoters when linked to the coding region of the chicken H2B histone gene (section 5.2.3).

An M13 clone, M13H2BX-X (containing the coding region of the H2B gene (1.85 kb) inserted in the HincII site of M13mp19) was used as a vector to insert the tk and keratin promoter sequences. M13H2BX-X was digested with SmaI. To construct TK/H2B, the 250 PvuII/BglII fragment containing the tk promoter (section 3.16) was inserted into the SmaI site of M13H2BX-X as shown in the diagram. To construct KER/H2B the 399 bp PstI fragment from feather keratin gene B containing the keratin promoter was inserted into the SmaI site as shown. The size of TK/H2B is 9.35 kb and the size of KER/H2B is 9.5 kb.


3.22
SalI
AccI
HincII
SmaI
XmaI
HindIII
M13mp9——ACAGCTATGACCATGATTACGCCAAGCTTGGCTGCAGGTCGACGGATCCCCGGGAATTCACTGGCCGTCGT————POLYLINKER
PstI
BamHI
EcoRI
S and Sm cut
insert
73bp Sm/A fragment
HindIII
SalI
Sm/A*
————ACAGCTATGACCATGATTACGCCAAGCTTGGCTGCAGGTCGAC
73 bp
GGGAATTCACTGGCCGTCGT
PstI
EcoRI
digest with P
EF insert
Ba/Bg fragment
HindIII
P/Ba*
Bg/P*
S.
Sm/A*
————ACAGCTATGACCATGATTACGCCAAGCTTGGC
680 bp
GG
73 bp
GGGAATTCACTGGCCGTCGT——
EcoRI
TKKpr
TRANSCRIPTION
3.23 and 3.24
M13H2BX-X
HindIII
PstI
HincII
HincII
XbaI
XmaI
SmaI
SacI
TTACGCCAAGCTTGCATGCCTGCAGGTC
H2B
GACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACT
SphI
BamHI
KpnI
EcoRI
TAA
AUG
TRANSCRIPTION
3.23
TK/H2B
5'
250
EF BglII
PvuII
3'
5'
3.24
KER/H2B
399
PstI
PstI

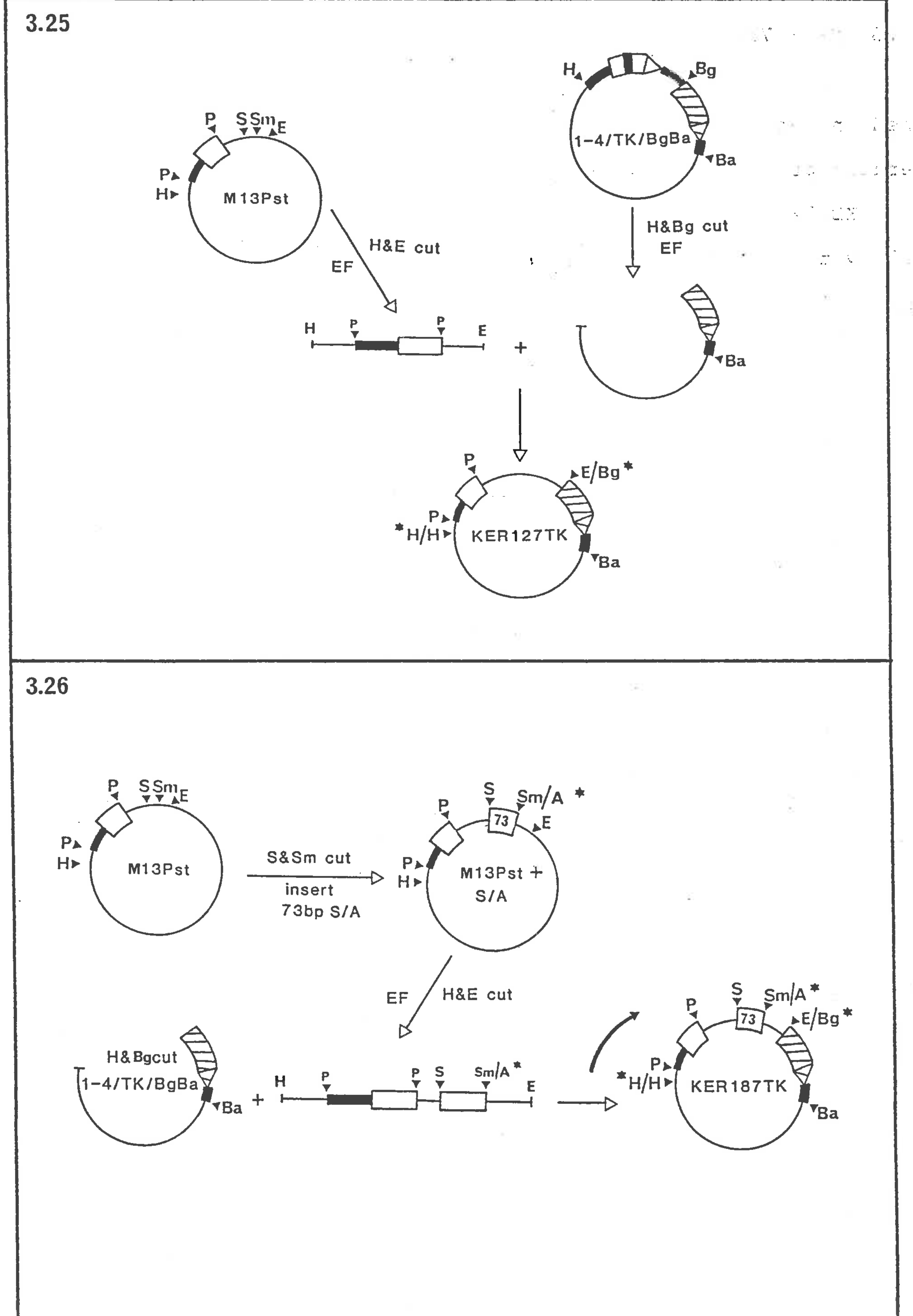
3.25 KER127TK

This construct, together with 3.26, 3.27 and 3.28, were used in section 5.2.5 to test the effect of displacing the keratin promoter from tk coding sequences.

KER127TK was constructed by digesting M13Pst (section 3.8) with EcoRI and HindIII to remove the keratin promoter. The excised, end-filled fragment was 27 bases longer on the 3' end of the keratin promoter because of the associated M13 DNA. The fragment was inserted into 1-4/TK/BglBam (3.14) which had been digested with HindIII and BglII to remove all of the keratin sequences (1.84 kb). In the resultant construct the distance from the cap site in the keratin promoter to the AUG of the tk gene was 127 bases (size = 7.15 kb).

3.26 KER187TK

This construct was made by digesting M13Pst (section 3.8) with SmaI and SalI and inserting a 73 bp SalI/AluI fragment from the coding region of keratin gene B (section 3.22). The combined fragments were excised with EcoRI and HindIII and inserted into 1-4/TK/BglBam (section 3.14) which had been digested with HindIII and BglII to remove keratin sequences (1.84 kb). The distance from the cap site in the keratin promoter to the AUG of the tk gene was 187 bases (size = 7.20 kb).


3.25
P
S Sm E
P
H
M13Pst
H
Bg
1-4/TK/BgBa
Ba
H&E cut
EF
H&Bg cut
EF
H
P
P
E
+
Ba
P
E/Bg *
P
*H/H
KER127TK
Ba
3.26
P
S Sm E
P
H
M13Pst
S&Sm cut
insert
73bp S/A
S
Sm/A *
P
73
E
P
H
M13Pst +
S/A
EF
H&E cut
H&Bgcut
1-4/TK/BgBa
Ba
+
H
P
P
S
Sm/A *
E
S
Sm/A *
P
73
E/Bg *
P
*H/H
KER187TK
Ba

3.27 KER328TK

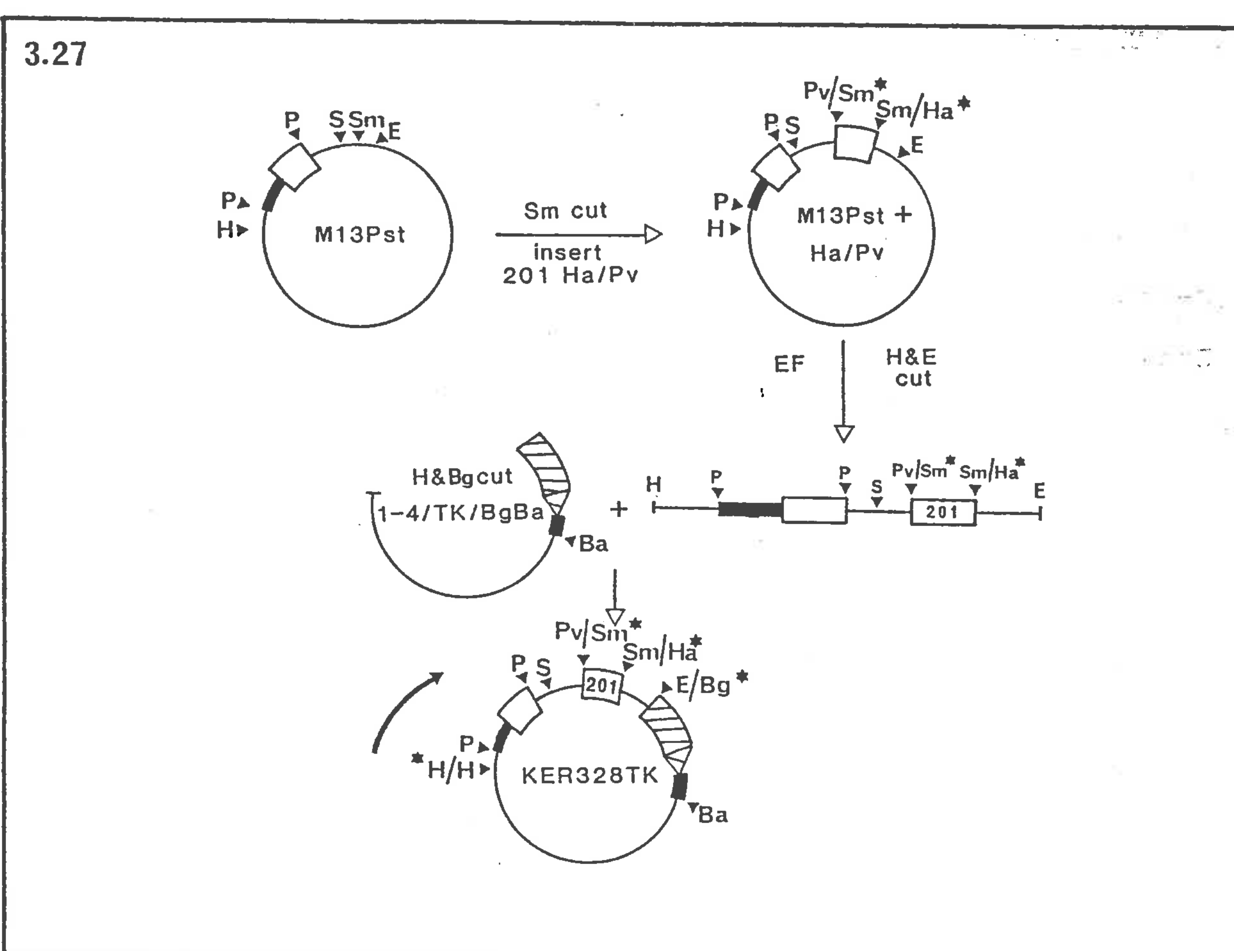
To make KER328TK, a 201 bp PvuII/HaeIII fragment from the coding region of gene B (figure 3-A(ii)) was inserted into M13Pst (3.8) which had been digested with SmaI. The combined fragments were excised by digesting with EcoRI and HindIII. The EcoRI/HindIII fragment was inserted into 1-4/TK/BglBam (section 3.16) which had been digested with HindIII and BglII to remove the keratin sequences.

The distance from the cap site in the keratin promoter to the AUG of the tk gene was 328 bases (size = 7.33 kb).

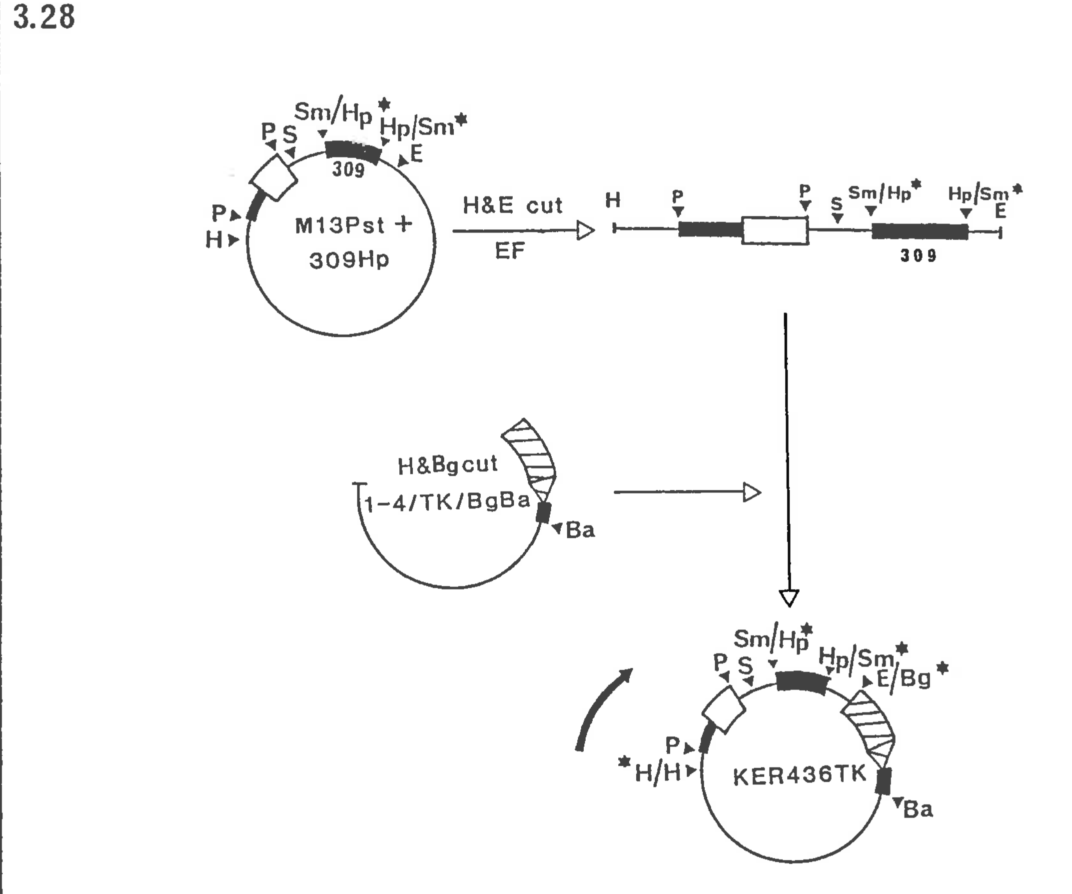
3.28 KER436TK

The construction of KER436TK involved inserting a 309 bp HpaII fragment from pBR322 into M13Pst (section 3.18) which had been digested with SmaI. The keratin promoter, linked to the 309 bp fragment was excised by digestion with EcoRI and HindIII and inserted into 1-4/TK/BglBam (section 3.14) which had been digested with HindIII and BglII to remove keratin DNA. The distance from the cap site in the keratin promoter to the AUG of the tk gene was 436 bases (size = 7.45 kb).

3.27



3.28



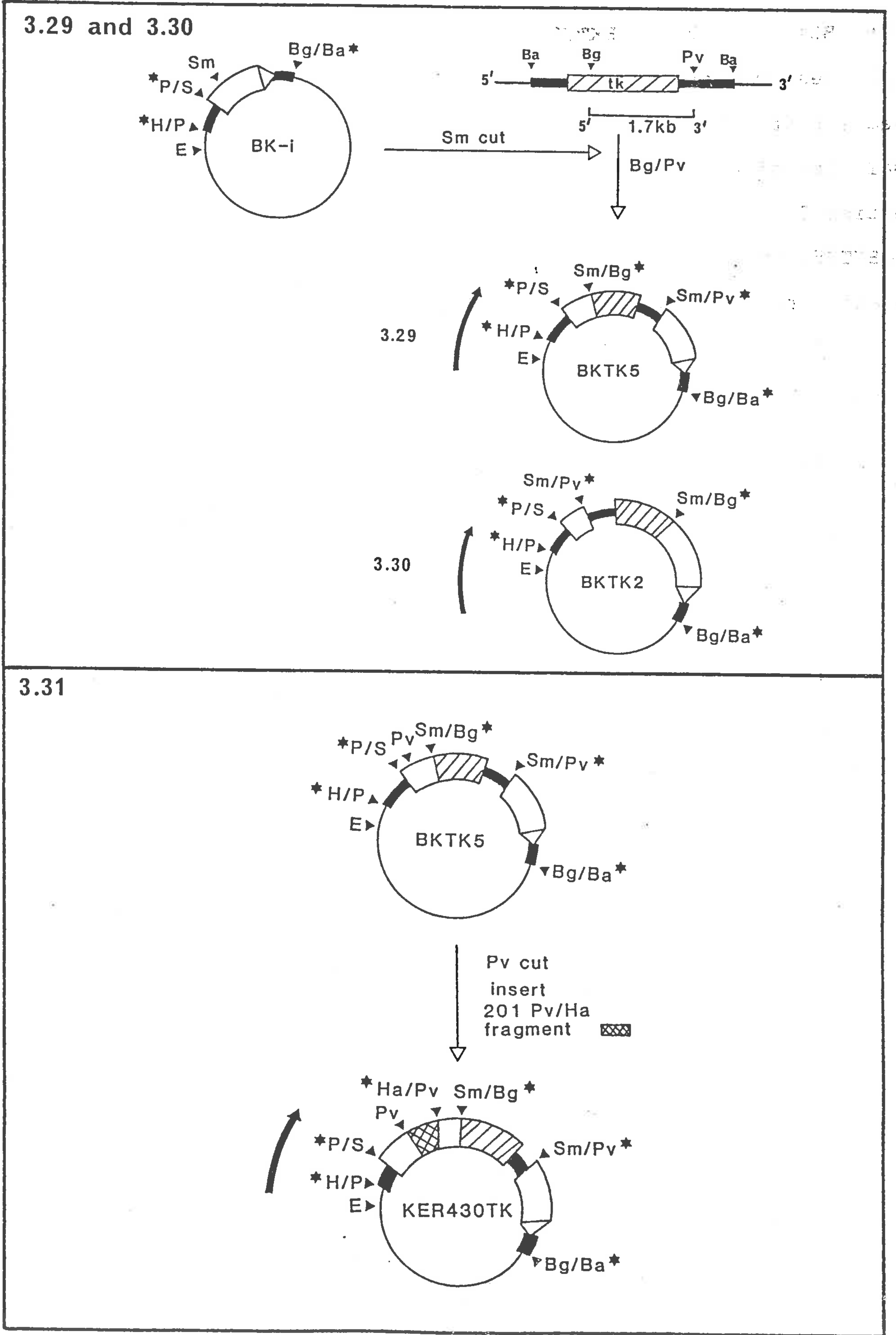
3.29 **BKTK5 and 3.30 BKTK2**

These constructs which contain a 1.7 kb BglII/PvuII fragment from the tk gene (figure 3-B(i)) inserted into the SmaI site of BK-i, were used in experiments described in section 5.2.6. The diagram shows the method of construction. In BKTK5, the orientation of the tk fragment, relative to the keratin promoter is the same as that in the intact tk gene. The tk fragment in BKTK2 is inserted in the opposite direction to that in BKTK5. The size of both constructs is 7.2 kb.

3.31 KER430TK

This construct is a derivative of BKTK5 (section 3.29) and the cap site of the keratin promoter has been displaced from the AUG of the tk gene by 430 bases of keratin DNA, rather than the pBR322 sequences that were inserted in construct KER436TK (section 3.28).

BKTK5 was digested partially with PvuII and a 201 bp PvuII/HaeIII fragment of keratin coding DNA (section 3.27) was inserted into the PvuII site, 38 bp 5' to the SmaI site where the 1.7 kb BglII/PvuII tk fragment had been originally inserted in the construction of BKTK5. KER430TK was used in experiments described in section 5.2.5. The size of the construct is 7.40 kb.



3.29 and 3.30
Sm
Bg/Ba
*P/S
*H/P
E
BK-i
Sm cut
Ba
Bg
Pv
Ba
5'
tk
3'
5'
1.7kb
3'
Bg/Pv
Sm/Bg*
*P/S
Sm/Pv*
3.29
*H/P
E
BKTK5
Bg/Ba*
Sm/Pv*
*P/S
Sm/Bg*
*H/P
3.30
E
BKTK2
Bg/Ba*
3.31
*P/S
Pv
Sm/Bg*
Sm/Pv*
*H/P
E
BKTK5
Bg/Ba*
Pv cut
insert
201 Pv/Ha
fragment
*Ha/Pv
Sm/Bg*
Pv
*P/S
Sm/Pv*
*H/P
E
KER430TK
Bg/Ba*

3.32 KER▶800KER, 3.33 KER◀800KER, 3.34 KER▶617KER

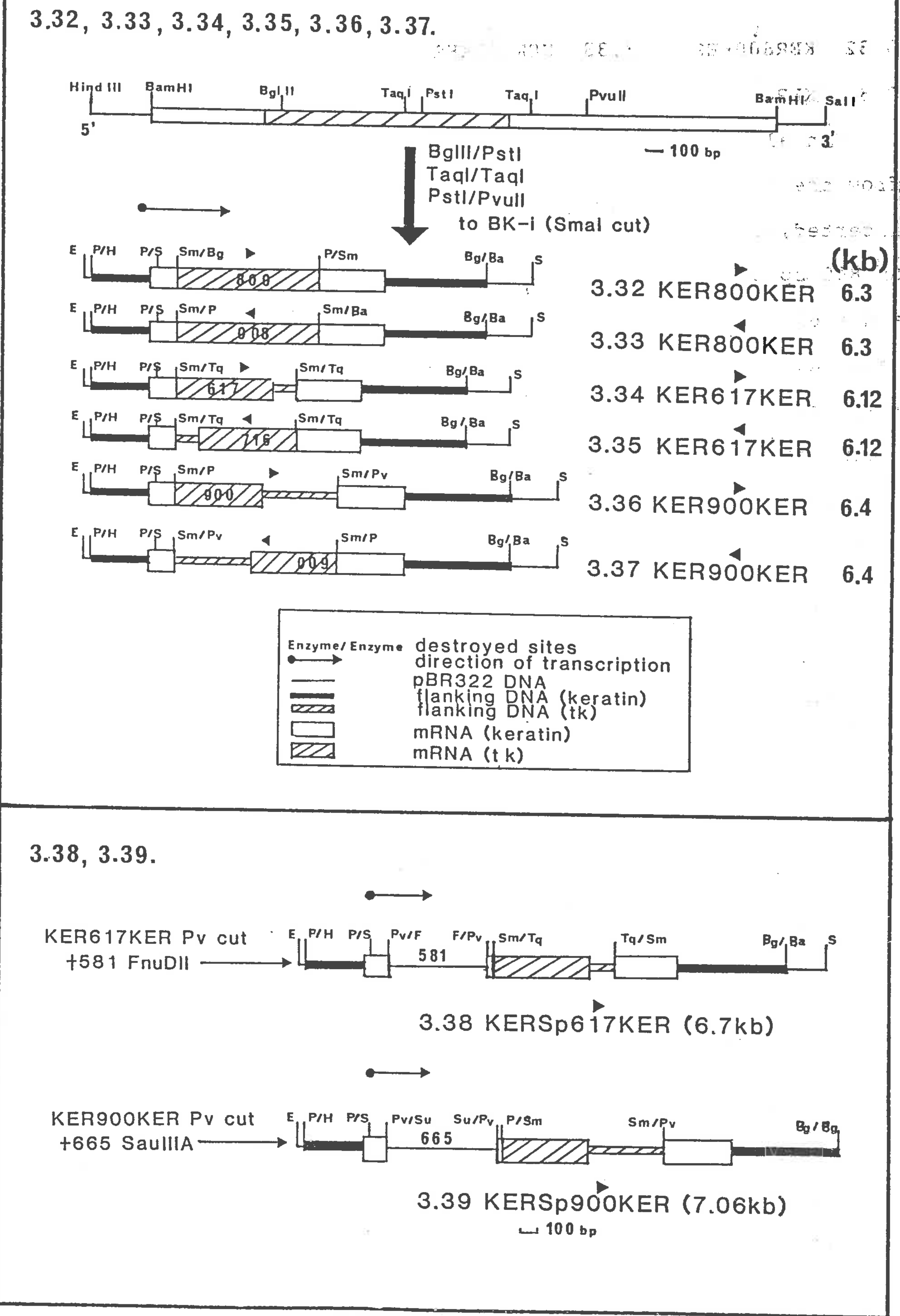
3.35 KER◀617KER, 3.36 KER▶900KER, 3.37 KER◀900KER

In all of these clones different sized fragments of DNA from the 1.7 kb BglII/PvuII fragment of the tk gene were inserted, in both orientations, into the SmaI site of BK-i. An 800 bp BglII/PstI fragment, a 900 bp PstI/PvuII fragment and a 617 TaqI fragment were inserted, after the overhangs were end-filled, into the SmaI site of BK-i as shown in the diagram. In each case, the arrow above the fragment size (▶) indicates the orientation of the fragment. These constructs were used in the experiments described in section 5.2.6. The size of each clone is given in brackets.

3.38 KERSp617KER and 3.39 KERSp900KER

These constructs are derivatives of constructs 3.34 and 3.36 respectively. Sequences from pBR322 were inserted to move the 617 bp and 900 bp fragments in a 3' direction away from the keratin promoter so that they would occupy a position similar to their normal position in the intact tk gene (w.r.t distance from the cap site).

To generate KERSp▶617KER, KER▶617KER was digested partially with PvuII and a 581 bp FnuDII fragment from pBR322 was inserted into the PvuII site, 38 bp 5' to the SmaI site where the 617 bp fragment had been originally inserted. Similarly, a 665 bp Sau3A fragment, isolated from pBR322 was inserted into the PvuII site of KER▶900KER to produce KERSp▶900KER. The sizes of the clones are given in brackets. These constructs were used in the experiments described in section 5.2.6.

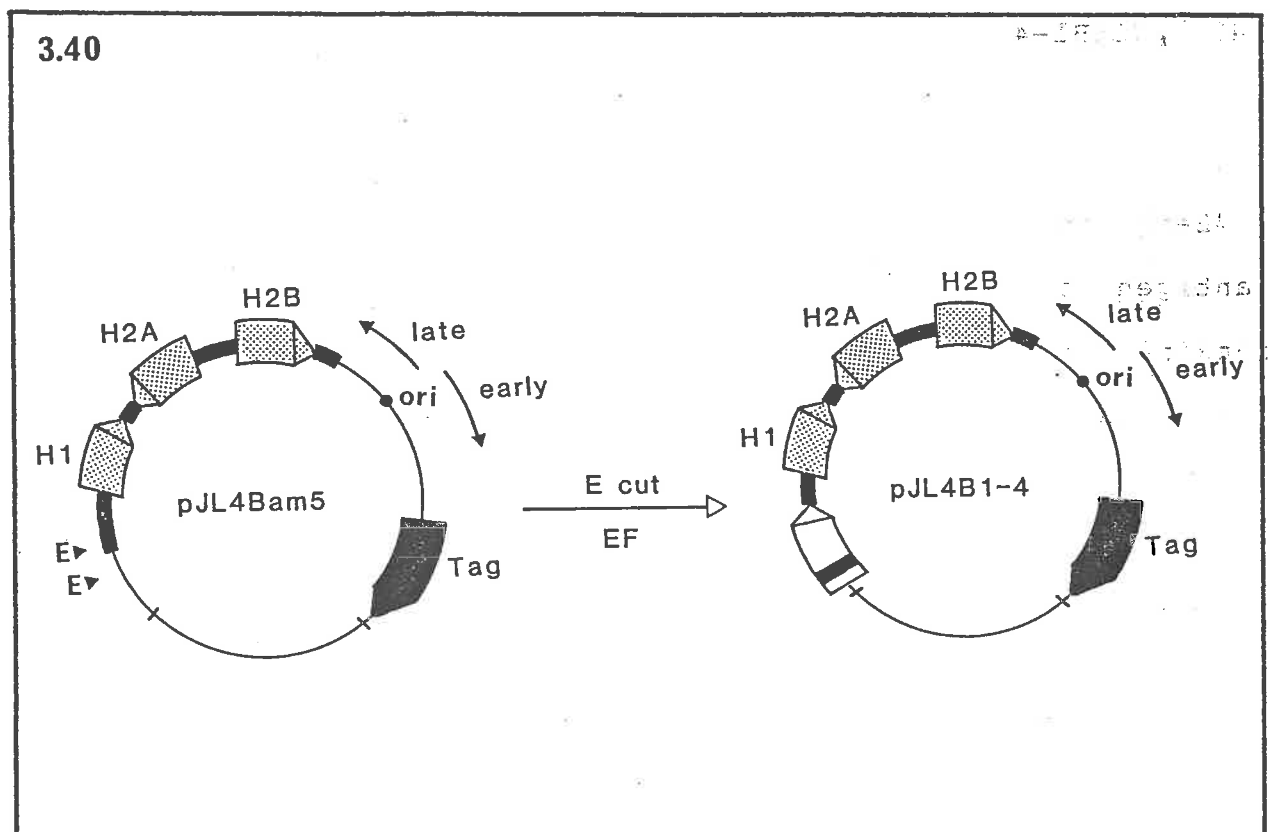


3.32, 3.33, 3.34, 3.35, 3.36, 3.37.
Hind III
BamHI
Bgl II
Taq I
Pst I
Taq I
Pvu II
BamHI
Sal I
5'
3'
100 bp
BglII/PstI
TaqI/TaqI
PstI/PvuII
to BK-i (SmaI cut)
(kb)
3.32 KER800KER 6.3
3.33 KER800KER 6.3
3.34 KER617KER 6.12
3.35 KER617KER 6.12
3.36 KER900KER 6.4
3.37 KER900KER 6.4
Enzyme/Enzyme destroyed sites
direction of transcription
pBR322 DNA
flanking DNA (keratin)
flanking DNA (tk)
mRNA (keratin)
mRNA (t k)
3.38, 3.39.
KER617KER Pv cut
+581 FnuDII
581
3.38 KERSp617KER (6.7kb)
KER900KER Pv cut
+665 SauIIIA
665
3.39 KERSp900KER (7.06kb)
100 bp

3.40 pJL4B1-4

This clone contained feather keratin gene B inserted into a eukaryotic, self-replicating vector, pJL4Bam5. The clone pJL4Bam5 contains the SV40 ori, early and late promoter, T antigen coding region and 5 kb of chicken histone DNA containing the same histone genes found in p7AT (H1, H2A and H2B). These features are shown in the diagram.

pJL4Bam5 was digested with EcoRI and the 5' overhangs were end-filled. A 2.93 kb HindIII fragment containing gene B was isolated from pλCFK1-4 and inserted into the EcoRI-digested pJL4Bam5. The orientation of gene B is shown in the diagram. The size of the construct is 13.96 kb.


3.40
H2B
H2A
late
early
ori
H1
pJL4Bam5
E
E
Tag
E cut
EF
H2B
H2A
late
ori
early
H1
pJL4B1-4
Tag