ON THE EFFECT OF UV-IRRADIATION ON DNA REPLICATION IN Escherichia coli.

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SUMMARY

Hooper *et al.* (1981) showed that UV-irradiated bacteria transiently lost the capacity to host an infection with phage 186, but production of phage λ and P2 was normal. Hooper (1979) proposed that the loss in capacity was due to a *trans*-acting effect on 186 DNA synthesis caused by a depletion of bacterial functions required by the phage.

The aim of this thesis was to identify the cause of the loss in the capacity of irradiated cells to produce 186 and to determine whether or not UV causes a *trans*-acting inhibition of DNA synthesis in *E. coli*.

The work confirmed that 186 DNA synthesis was inhibited in UV-irradiated cells, and it was concluded that the inhibition of 186 DNA synthesis (loss in phage capacity) was due to a *trans*-acting effect induced in UV-irradiated cells. The loss in capacity does not appear to be a *recA*$^+$ *lexA*$^+$- dependent SOS function, nor does it appear to be caused by a depletion of *dnaC* (an *E. coli* initiation function).

It was also found that a UV-irradiated cell was refractory to replication from unirradiated *oriC* (the *E. coli* origin of replication), but not from *ori*λ. Therefore UV induces a *trans*-acting inhibition of

replication from certain replicons in the absence of photoproducts in that replicon. Thus an unusual aspect of the effect of UV on DNA synthesis was uncovered.

STATEMENT

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

I consent to this thesis being made available for photocopying and loan if it is accepted for the award of the degree.

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Meera M. Verma

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ABBREVIATIONS

wt	wild type
moa	multiplicity of addition
R.T.	Room temperature
O.D.	Optical density
UV	Ultraviolet light
cpm	counts per minute
J	Joules
pfu	plaque forming units
cfu	colony forming units

CHAPTER 1

INTRODUCTION

UV-irradiation of *E. coli* cells causes cell-death and increased mutagenesis. Both these effects are the culmination of several intracellular events and are therefore a secondary effect of irradiation. The primary effect of UV on cells appears to be the inhibition of chromosomal DNA synthesis. The inhibition is attributed to a *cis*-acting effect of UV-induced damage, sustained by the DNA. However there exists a situation where the replication of undamaged DNA is inhibited in UV-irradiated cells. When unirradiated 186 phage infect UV-irradiated cells, phage DNA synthesis is transiently inhibited. The current ideas on the effect of UV on DNA synthesis are inadequate at explaining this phenomenon.

1.1 THE TRANSIENT INHIBITION OF PHAGE 186 REPLICATION IN UV-IRRADIATED CELLS

Temperate coliphage 186 has a linear, non-permuted, double-standard genome approximately 30 kb in size (Younghusband *et al.*, 1975). It was originally described as a non-lambdoid, non-inducible phage and classed with P2, the prototype non-inducible phage (Bertani and Bertani, 1971; Jacob and Wollman, 1961).

However, it has been found that the 186 prophage is inducible by UV-irradiation, mitomycin C and naladixic acid (Woods and Egan, 1974) and therefore cannot be classed with P2 in this respect.

While studying 186 induction by UV light, it was noticed that the latent period of phage produced by UV-induction was considerably longer than that seen with heat-induction of a temperature sensitive prophage (Hooper *et al.*, 1981). This was in contrast to UV-induction of λ prophage, which had a latent period only slightly longer than that obtained with heat-induction (Hooper *et al.*, 1981). This was not because the 186 prophage had sustained more damage than λ, since it was found that infection of UV-irradiated cells with unirradiated 186 phage also resulted in an extended latent period, as compared with infection of unirradiated cells. Thus it appeared that prior irradiation of the cells in some way hampered 186 phage production. Neither λ nor P2 phage production was delayed under similar conditions (Fig 1.1)

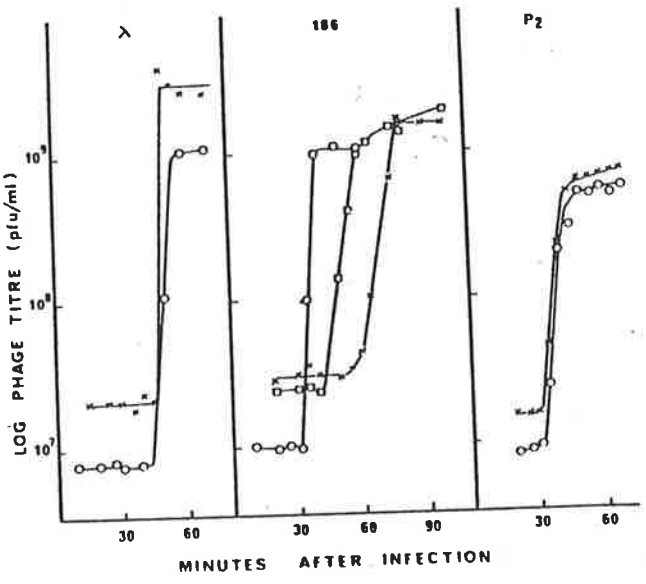


FIG 1.1 Latent period after infection by either λ, 186, or P2 of C600 bacteria either unirradiated (o), or irradiated with 30 J/m², (□) or 45 J/m², (×) before infection (Hooper *et al.*, 1981).

In order to determine which phase of 186 phage infection was affected by irradiation of the host, Hooper *et al.* (1981) studied 186 DNA replication and transcription in irradiated cells.

186 DNA synthesis was studied by pulse-labelling UV-irradiated and unirradiated cells that had been infected with 186. The pulse-labelled samples were hybridised to unlabelled 186 DNA. 186 DNA replication was detected 20 minutes after infection of an unirradiated culture, but in the irradiated (90 J/m² of UV) culture it was not detected until 50 minutes after infection. Therefore 186 DNA replication was transiently inhibited in UV-irradiated cells. In comparison, DNA replication from phage P2 was detected 20 minutes after infection of UV-irradiated cells, consistent with the fact that P2 phage production was not delayed by UV-irradiation (Hooper *et al.*, 1981), and indicating that the delay in the replication of 186 DNA was unlikely to have been an artifact of the labelling procedure in irradiated cells.

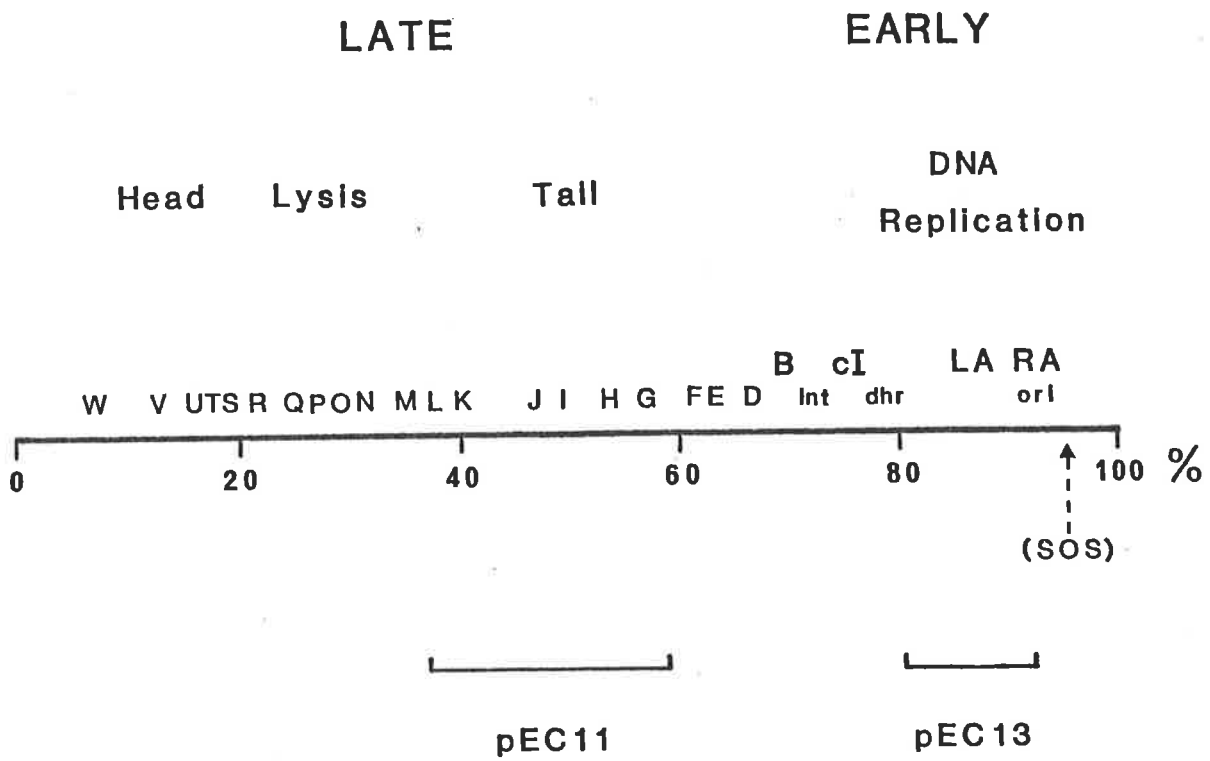
The transient inhibition in 186 DNA synthesis could have been due to either a direct effect of UV on DNA replication, or a UV-induced block in transcription of the 186 replication genes. In order to establish which of these possibilities was the case, 186 transcription of the phage replication genes was studied in UV-irradiated cells.

FIG 1.2 Genetic map of 186 and relevant features

The linear map of coliphage 186 showing the genes involved in phage morphogenesis and replication.

The early (pEC13) and late (pEC11) regions of the genome cloned into pBR322 are shown. The position of the putative SOS box is also shown (↑).

LA, RA - DNA replication
B - late gene turn on
int - phage site-specific integration
cI - phage repressor
dhr - *d*epression of *h*ost *r*eplication
ori - origin of phage DNA replication
W - Q - head
P, - lysis
O - D - tail


LATE
EARLY
Head
Lysis
Tail
DNA
Replication
W V U T S R Q P O N M L K J I H G F E D
B
Int
cI
dhr
LA
RA
ori
0
20
40
60
80
100
%
(SOS)
pEC11
pEC13

The 186 replication genes LA and RA are encoded in the early region of the 186 genome (Hocking and Egan, 1982a,b,c; Sivaprasad, 1984) (Fig 1.2). Transcription of various regions of the 186 genome in UV-irradiated cells was studied by hybridisation of *in vivo* pulse-labelled RNA to pBR322 clones of those regions. RNA hybridisation to a pBR322 clone of the early region of 186, encoding the replication genes (pEC13, Fig 1.2), was similar in irradiated and unirradiated cultures (Hooper *et al.*, 1981; Hooper, 1979). Therefore, UV-irradiation does not inhibit transcription of the replication genes.

In contrast, RNA hybridisation to a 186 DNA fragment from the late region, cloned into pBR322 (pEC11, Fig 1.2) was depressed in UV-irradiated cells (Hooper *et al.*, 1981). Thus, transcription of the late genes was inhibited in irradiated cells, as was total 186 transcription (Hooper *et al.*, 1981). As 186 late gene transcription requires phage replication (Finnegan and Egan, 1981), this inhibition in late gene transcription, is probably a consequence of the inhibition of 186 DNA replication in UV-irradiated cells.

Since transcription of the 186 replication genes is not detectably reduced in UV-irradiated cells, it was concluded that the transient inhibition in phage production was due to a relatively direct block on 186 DNA replication in a UV-irradiated cell (Hooper *et al.*, 1981).

This reveals a unique situation in which the replication of an undamaged replicon is inhibited in a UV-irradiated environment. Hitherto, the inhibition in DNA replication has always been attributed to *cis*-acting photoproducts in the genome (discussed in the following section), whereas here, something other than such photoproducts is acting in *trans* to inhibit DNA replication. As mentioned before, this cannot be explained by the accepted mode of UV-damage on nucleic acid replication.

Study of this phenomenon has the potential to uncover an unsuspected facet of the effect of UV on *E. coli* DNA synthesis.

In order to put the study in perspective I will briefly discuss the known effects of UV on *E. coli* cells and on DNA synthesis in particular. This is not intended to be an indepth review of the field, but rather an examination of certain basic concepts and assumptions, currently held, which are of relevance to this work. This deals with studies conducted in the 1960's and early 1970's, since that was when most of the work on the effect of UV on DNA synthesis was done. In addition, interesting current developments in procaryotic cellular mechanisms of UV repair and increased survival will be briefly discussed.

1.2 EFFECT OF UV ON *E. coli*

UV-irradiation has a complex set of effects on whole cells, two measurable results of which are mutagenesis and cell 'death' i.e. the loss of the cells ability to reproduce itself (Swenson, 1976).

Cell death can be estimated from survival curves which represent the fraction of colony forming individuals as a function of the UV-fluence. Survival curves vary greatly in shape depending on the bacterial strain, conditions of irradiation, pre- and post- irradiation treatments, plating medium and conditions of incubation (Swenson, 1976). However, they are useful in comparing radiation sensitivities of various repair-deficient strains.

The other major effect UV has on the cells is an increase in mutagenesis in the survivors. This is discussed later.

Studies on the effect of UV on *E. coli* cells have dealt mainly with the far UV region of the spectrum (190-300 nm), using lamps which emit predominantly at 254 nm. At this wavelength absorption of energy is primarily by the nucleic acids and, to a lesser extent, by proteins. This is because all nucleotide bases in the nucleic acids absorb at this wavelength while only a few amino acid residues of proteins absorb measurably in this region (Harm, 1980).

Most of the biological effects of UV on the cell are considered to be due to its absorption by DNA. This is based on the fact that the action spectrum for killing of *E. coli* cells is very similar to the absorption spectrum of nucleic acids (Rupert, 1960). In addition chromosomal DNA is present in restricted copies compared with the number of copies of RNA and other macromolecules per cell, again making it a more effective target for the biological killing effect of UV (Cerutti, 1975).

1.2.1 UV inhibits DNA synthesis

The effect of UV on several cellular process, DNA, RNA and protein synthesis and respiration, has been studied. It was found that the primary consequence of UV-irradiation of cells was the transient cessation of DNA synthesis (Kelner, 1953; Hanawalt and Setlow, 1960; Swenson and Setlow, 1966).

Kelner (1953) showed that a UV fluence which caused 90% killing of *E. coli* B/r completely inhibited DNA synthesis for 50 minutes but had only a slight negative effect on RNA synthesis and on growth (measured by an increase in turbidity). Respiration was not affected.

Likewise, the data of Hanawalt and Setlow (1960) and Swenson and Setlow (1966) showed that DNA synthesis was transiently inhibited by UV-fluences which allowed RNA and protein synthesis, albeit at a reduced rate.

Although DNA synthesis appears to be primarily affected, RNA and protein synthesis also suffer a reduction in rate. However, when DNA synthesis resumes, the rate of RNA and protein synthesis also increases (Swenson and Setlow, 1966). Therefore, the effect of UV on these two processes may be a consequence of the inhibition of DNA synthesis.

(a) ***Cause of the UV-induced inhibition of DNA synthesis:*** Absorption of UV by nucleic acids causes photochemical changes in their structure. These are called photoproducts and are mainly pyrimidine derivatives (reviewed by Patrick and Rahn, 1976; and Harm 1980).

Two pyrimidine photoproducts have been strongly implicated in the biological effects of UV, the cyclobutyl dipyrimidine or pyrimidine dimer, and the pyrimidine adduct, 6-4' (pyrimidine-2-one) thymine or pyr- pyr (6-4) photoproduct. (Haseltine, 1983; Walker, 1984). The pyr-pyr (6-4) adduct has recently been implicated as the pre-mutagenic lesion. (Haseltine, 1983).

Pyrimidine dimers in the DNA are thought to be the main cause of cellular lethality. Setlow (1966) summarised the reasons for this conclusion:

(i) The mean lethal dose (i.e. 37% survival) to bacteria and viruses produces a measurable number of dimers in the DNA but makes negligible non-pyrimidine damage.

(ii) Photoreactivation, a light treatment which reactivates many biological systems including cells, transforming DNA and viruses, had only been shown to remove pyrimidine dimers.

(iii) Radiation resistant cells are capable of excising dimers, while sensitive mutants (Uvr^-) do not excise dimers.

Pyrimidine dimers were also thought to cause the UV-induced inhibition of *E. coli* DNA synthesis. This was because reversal of the dimers by photoreactivation caused an early resumption in DNA synthesis (Setlow *et al.*, 1963; Doudney, 1974). In addition, inability to remove pyrimidine dimers (e.g. in an excision repair deficient mutant) resulted in a prolonged inhibition in DNA synthesis (Setlow *et al.*, 1963; Billen and Carreira, 1971). Therefore there is a strong correlation between the presence of dimers in the DNA and the inhibition of DNA synthesis. It has also been shown that pyrimidine dimers block DNA synthesis on single-stranded templates *in vitro* (Moore *et al.*, 1981).

Other forms of damage to DNA, are also induced by UV. Of these protein-DNA crosslinks (Smith, 1962) (induced by UV) may also have a role in cell killing (Smith, 1966; Doudney, 1968). None of the other forms of damage play a predominant role at biologically significant doses (Doudney, 1968).

Pyrimidine dimers do not appear to cause a permanent block in DNA synthesis. (Rupp and Howard-Flanders, 1968; Ganesan and Smith, 1971; Billen and Carreira, 1971; Smith, 1969). Excision deficient mutants of *E. coli* K12 and B (*uvr*A6, *uvr*B, B_{s-1}), which are incapable of excising dimers from the DNA, are capable of synthesising DNA at a reduced rate after low doses of UV. In this case DNA synthesis occurs despite the presence of dimers in the DNA. Based on this and other data Rupp and Howard-Flanders (1968) concluded that the pyrimidine dimers slow the progress of the replication complex rather than stopping it indefinitely. They proposed that DNA replication paused for a short time then reinitiated past the dimer, leaving single stranded gaps in the DNA. The gaps in the daughter strand are repaired using sister strand exchanges (Rupp *et al.*, 1971).

Thus, the extreme sensitivity of excision repair mutants was used as evidence that dimers were involved in the inhibition of DNA synthesis, but since replication can occur in those mutants it was concluded that dimers do not cause a complete block.

(b) ***Post-irradiation replication:*** The classical pattern of pre- and post- irradiation DNA synthesis is as described below. Immediately after irradiation there is a period when DNA synthesis is almost completely inhibited

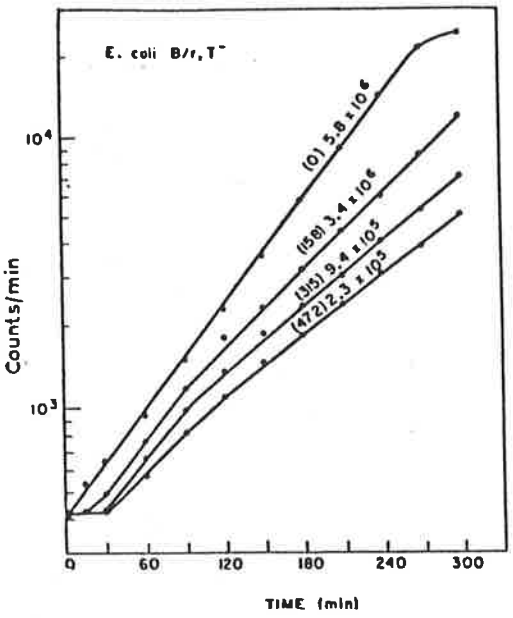


FIG 1.3 The uptake of [^{14}C]-thymine by cultures of *E. coli* B/r, T$^-$ after various doses of UV (in erg/mm^2) shown in parenthesis in the Fig. (Smith and O'Leary, 1968)

(Fig 1.3), the duration of the inhibition depends on the UV-fluence (Doudney, 1974). Subsequently the rate of synthesis is restored to its pre-irradiation rate (Doudney, 1974). This is referred to as the recovery phase. However, after a certain dose, defined as the 'critical dose' (Doudney 1971), the rate of replication during the recovery phase is proportionately decreased.

As mentioned previously, photoreactivation immediately after UV-irradiation shortened the duration of the inhibition and caused an early resumption in recovery DNA synthesis (Setlow *et al.*, 1963; Doudney, 1974). The data of Setlow *et al.* (1963) showed a good correlation between the estimated number of dimers removed from the DNA and the extent of amelioration of the effect of UV on DNA synthesis. Doudney (1974) also showed that at low doses of UV (<20 J/m^2) photoreactivation caused a shortening of the delay. But at doses greater than the 'critical dose'

(40 J/m²) no early resumption in DNA synthesis was obtained instead an increase in the recovery rate was obtained. Therefore the postirradiation inhibition in DNA synthesis appears to be caused by photo-reactivatable and non-photoreactivatable damage. The damage which is non-photoreactivated may be due to dimers which are inaccessible to photoreactivating enzyme. However, that has not been demonstrated.

The recovery of DNA replication requires protein synthesis (Doudney, 1973a) and seems to initiate synchronously at the chromosomal origin (Hewitt and Billen, 1965; Billen, 1969; Doudney, 1973 a; b). The first cycle of recovery DNA synthesis after irradiation appears to be independent of *dna*A but not of *dna*C (Jonczyk and Ciesla, 1979), since replication occurs at the non-permissive temperature after irradiation of a *dnaA* Ts mutant but not in a *dnaC* Ts mutant. The *dnaA* independent cycle of replication can be induced in a *recA441* (tif-1) *dnaA46* double mutant by a temperature shift to 41°C (Jonczyk and Ciesla, 1979). A form of replication called stable DNA replication, which is independent of *dna*A is also induced by UV-irradiation (Kogoma and von Meyenberg, 1983). This is discussed below in more detail.

In summary, UV induces pyrimidine dimers in DNA. Dimers are responsibile for cellular lethality and cause a temporary block in chromosomal DNA synthesis. However, some

non-photoreactivatable damage may also be involved in the inhibition of DNA synthesis.

(c) ***Stable replication:*** Although UV-irradiation transiently inhibits normal DNA replication, low fluences induce a novel form of linear replication called stable DNA replication (SDR) (Kogoma *et al.*, 1979). This replication proceeds in the absence of protein synthesis, whereas normal DNA replication requires *de novo* protein synthesis (Maaloe and Hanawalt, 1961).

Stable DNA replication is induced by several other treatments which inhibit DNA synthesis eg thymine starvation, naladixic acid, and hydroxyurea (Kogoma and Lark, 1975; Kogoma *et al.*, 1979).

A mutant, *dnaT*, abolishes the ability to induce SDR (Lark and Lark, 1979). This mutant maps at 99 minutes on the *E. coli* map. At the same map location, another mutation, *sdr*T renders constitutive the ability to replicate DNA in the absence of protein synthesis (Lark and Lark, 1980). Other mutations (Sdr^c) which allow constitutive stable DNA replication map at the *sdrA* locus near *metD* at 5 minutes on the *E. coli* map (Kogoma, 1978; Kogoma *et al.*, 1981). Thus two separate loci appear to be involved in constitutive stable DNA replication. Constitutive SDR requires the *recA* protein (Lark and Lark, 1980; Lark *et al.*, 1981; Kogoma *et al.*, 1981). However the two loci involved

differ in their mode of dependence on *recA*. The *sdrT* mutations render normal replication (in the presence of protein synthesis) and stable DNA replication dependent on *recA*$^+$ (Lark *et al.*, 1981). But in the *sdrA* mutants only replication in the absence of protein synthesis (stable replication) is *recA*$^+$-dependent (Kogoma *et al.*, 1981). This means that in the *sdrA* mutants, protein synthesis has to be inhibited in order to demonstrate *recA*- dependent replication.

sdrA mutations make the cell independent of both *dnaA* and *oriC* (the *E. coli* origin of replication, Kogoma and von Meyenberg, 1983). (Both the *dnaA* protein and *oriC* are essential for initiation of normal *E. coli* replication, reviewed in Marians, 1984). Therefore *sdrA* mutants seem to confer an alternative mechanism for initiation of chromosomal replication (Kogoma and von Meyenberg, 1983). In fact *sdrA* mutations were found to map at the same locus as *dasF* mutations (extragenic suppressors of *dnaA*), and *rnh* mutations and all the mutants are deficient in RNase H activity (Ogawa *et al.*, 1984). Since *sdrA*, *dasF* and *rnh* appear to be allelic and all confer the phenotype of constitutive SDR (Torrey *et al.*, 1984), it has been proposed that an RNA priming step at initiation of DNA replication may be altered. The proposal suggested that two types of RNA primers for initiation of DNA replication are synthesised in *sdrA*, *dasF* and *rnh* mutants, one in a *dnaA/oriC*-dependent and one in *dnaA/oriC*-independent manner.

and that only the *dnaA/oriC*-dependent primer is involved in the normal DNA replication, with the *dnaA/oriC*-independent primer being selectively degraded by RNase H (Ogawa *et al.*, 1984; Horiuchi *et al.*, 1984). This proposal suggests that the *dnaA* protein protects the *oriC*-primer from RNase H-mediated degradation.

As mentioned earlier a form of stable replication is also induced by UV. Induced-stable replication is also a *recA*$^+$ *lexA*$^+$-dependent SOS function (Lark and Lark, 1979; Kogoma *et al.*, 1979). (The SOS regulatory circuit is discussed in a later section). Induced stable replication also does not need *dnaA* function (Ciesla and Jonczyk, 1980). However, protein synthesis has to be inhibited in order to obtain continued DNA synthesis in the absence of *dnaA* (Ciesla and Jonczyk, 1980). Therefore the absence of protein synthesis seems to be a prerequisite for obtaining SDR, and this form of replication may not occur *in vivo* unless protein synthesis is inhibited (Doudney, 1978).

There have been reports that induced stable replication is UV-resistant (Kogoma *et al.*, 1979). Kogoma *et al.* (1979) showed that induced stable DNA replication could recover after UV-irradiation in the absence of protein synthesis, but normal DNA replication could not. However, it cannot be concluded from this that SDR is UV-resistant since the data only shows that normal synthesis cannot recover in the absence of protein synthesis and that the

replicating sites are permanently inactivated (in the absence of protein synthesis) by UV. In fact, induced SDR in the absence of protein synthesis, experiences a delay and recovers in a manner similar to normal DNA synthesis in the presence of protein synthesis (Sedliakova *et al.*, 1978; Williams, 1982). Therefore since induced stable DNA replication also experiences transient inhibition after UV, it is no more UV-resistant than normal DNA synthesis.

1.2.2 Repair of UV-damage

The lethality caused by UV photoproducts in the DNA can be due to interference with either replication or information transfer (transcription). Repair processes either remove the damage resulting in DNA templates capable of both functions, or they bypass the lesion, redistributing it in the genetic material.

Three mechanisms for dealing with UV damage are:

(i) Photoreactivation
(ii) Excision repair
(iii) Post-replication repair

(a) ***Enzymatic photoreactivation:*** This mode of repair reverses the UV-induced alteration in DNA. The pyrimidine dimers (cis-syn isomers) are monomerised *in situ* by the photoreactivating enzyme in the presence of near UV or short wavelength visible light (310-480 nm) (Sutherland, 1978a).

Photoreactivation reverses several effects of UV-irradiation i.e. lethality (Kelner, 1953), filamentation (Kantor and Deering, 1967) and inactivation of transforming DNA and viruses (Setlow, 1966).

The photoreactivating enzyme or DNA photolyase gene, *phr* has been cloned (Sancar and Rupert, 1978; Rupert and Sancar, 1978) and the enzyme purified (Sutherland, 1978b).

It was recently suggested that photolyase also aids dark repair (Yamamoto *et al.*, 1983a; b; Yamamoto *et al.*, 1984). In fact, Sancar *et al.* (1984) found that photolyase stimulated the removal of pyrimidine dimers by the *uvrABC* excision-nuclease *in vitro* in the dark.

The absence of photoreactivation is desired in most experimental work studying UV-repair mechanisms because photoreversal of the dimers would reduce any other repair mechanism coming into action. Photoreactivation can be prevented by using yellow fluorescent illumination, in the case of *E. coli*, since wavelengths >500 nm are beyond the action spectrum of its photolyase, or the experimental procedures can be carried out in the dark. Alternatively *phr*$^-$ mutant (defective in the photolyase enzyme) can be used.

(b) ***Excision repair***: Excision repair involves enzymatic dimer removal from one strand, with subsequent resynthesis to restore the integrity of that strand (for a review of earlier work see Hanawalt *et al.*, 1979).

Excision repair was discovered in 1964 in *E. coli* K12 (Boyce and Howard-Flanders, 1964) and in *E. coli* B (Setlow and Carrier, 1964). It was found that dimers, identified chromatographically, disappeared from the acid-insoluble fraction of *E. coli* K12 parental DNA and reappeared in the acid-soluble fraction. This did not occur in UV-sensitive *uvrA* mutant cells (Boyce and Howard-Flanders, 1964). Similarly, dimers are excised from the chromosome of *E. coli* B and B/r cells but not from the UV-sensitive B_{s-1} mutant (Setlow and Carrier, 1964). Therefore it was concluded that the cell possessed a mechanism to excise dimers (Pettijohn and Hanawalt, 1964) and that this mechanism was non-functional in the UV sensitive *uvrA* and B_{s-1} mutants.

Subsequently 23 Uvr^- mutants with differing UV-sensitivity were isolated and divided into three complementation groups *uvrA uvrB* and *uvrC* (Howard-Flanders *et al.*, 1966). All three classes of mutant were excision deficient (Howard-Flanders *et al.*, 1966).

Mutations in these *uvr* genes make the cell sensitive to a number of other DNA damaging treatments, such as mitomycin C, psoralin-near-UV and di-chlorodiamine platinum II (Sancar and Rupp, 1983).

The purified products of the *uvrA uvrB* and *uvrC* genes form a complex known as the *uvrABC* excision-nuclease (Sancar and Rupp, 1983). *In vitro*, the *uvrABC* nuclease specifically cleaved plasmid DNA damaged with UV, cis-platinum and psoralen-near-UV, but did not cleave undamaged DNA (Sancar and Rupp, 1983). So the purified proteins recognise the same substrate *in vitro* and *in vivo*.

The *uvrABC* nuclease activity can be resolved in two steps. Firstly *uvrA* and *uvrB* proteins bind to the DNA and translocate to the site of the damage. The translocation occurs *via* the *uvrA* ATPase activity since it is inhibited by ATP(γS). Secondly a UvrA-, B- and C-dependent strand cleavage reaction occurs, probably when the *uvrC* protein binds to the UvrAB/lesion complex (Seeberg and Steinum, 1983).

The mechanism of excision was demonstrated *in vitro* by Sancar and Rupp (1983). They showed that the UvrABC excision nuclease makes two cuts on UV-damaged DNA, one on either side of the lesion. The enzyme hydrolyses the eighth phosphodiester bond on the 5' side of pyrimidine dimers and on the 3' side it cuts the fourth or fifth phosphodiester bond. The 12-13 nucleotide long fragment carrying the lesion is then released from the helix.

In addition to the pyrimidine dimer, the UV-induced pyrimidine-pyrimidone (6-4) photoproduct mentioned earlier, is also excised by the UvrABC nuclease (Sancar and Rupp, 1983; Franklin and Haseltine, 1984).

Another gene *uvrD*, also known as *uvrE*, *recL* or *mutU* has been implicated in excision repair (Ogawa *et al.*, 1968; Nakayama *et al.*, 1983) The *uvrD* gene product is the DNA helicase II and it also has single stranded DNA-dependent ATPase activity (Hickson *et al.*, 1983). Addition of the *uvrD* protein to the UvrABC nuclease greatly stimulated the introduction of strand breaks in irradiated DNA. (Kumura *et al.*, 1985). Therefore the *uvrD* protein stimulates the incision reaction catalysed by the UvrABC enzyme.

In vivo the excision repair process produces patches in DNA which are heterogeneous in size (Cooper and Hanawalt, 1972). The majority of the lesions (99%) are repaired with short patches 20-30 nucleotides in length while 1% are repaired with much longer patches (1,500 nucleotides long). The long patch repair is under the control of the *recA*$^+$ *lexA*$^+$ regulatory circuit (Cooper, 1982). DNA polymease I is suggested to be responsible for both long and short patch synthesis in wild type cells (Cooper, 1982).

Expression of the excision repair genes which occurs constitutively in undamaged cells, is further induced by DNA damage in a *recA*$^+$ *lexA*$^+$ dependent fashion.

Evidence that some excision occurs constitutively comes from the fact that both dimers and TC (6-4) products are removed from DNA in the presence of chloramphenicol (Swenson and Setlow, 1966; Franklin and Haseltine, 1984). Other evidence is that dimers are excised from a *recA*$^-$ *uvr*$^+$ mutant (70% of the dimers are excised compared with 90% in wild type) but are not excised at all from a *uvrB*$^-$ mutant.

In addition to the constitutive repair, expression of the *uvrA*, *uvrB* and *uvrD* genes was found to be induced by DNA damaging treatments (Kenyon and Walker, 1981; Fogliani and Schendel, 1981; Kumura *et al.*, 1983; Nakayama *et al.*, 1983). In the case of the *uvrA* and *uvrB* genes induction is *recA*$^+$ *lexA*$^+$ dependent. Induction of *uvrD* has, so far, only been shown to be *recA*$^+$-dependent (above citations). The *uvrC* gene may also be inducible (Backendorf *et al.*, 1983a).

The *uvrA* and *uvrB* genes have been sequenced and shown to have promotors which are regulated by the *lexA* repressor *in vitro* (Sancar *et al.*, 1982a; Sancar *et al.*, 1982b; van den Berg *et al.*, 1983; Backendorf 1983b). This is consistent with their being damage-inducible and part of the *recA*$^+$ *lexA*$^+$ regulatory circuit. The *uvrB* gene has two promoters P1 and P2 (Sancar *et al.*, 1982b). P1 is proximal to the coding region of the *uvrB* gene, while P2 is distal and carries a *lexA* binding site. *In vivo*, transcription from both P1 and P2 is inducible by UV. However, most of

the transcription is from P1. Transcription, in the uninduced state is entirely from P1 (van den Berg *et al.*, 1983). It was reported recently that the *dnaA* protein may be involved in the regulation of the *uvrB* gene (van den Berg *et al.*, 1985). If this is substantiated it will add another dimension to the control of the excision genes.

(c) ***Post-replication repair:*** As mentioned previously Rupp and Howard-Flanders (1968) found that DNA synthesis continued in an excision-deficient strain after irradiation with 6 J/m² of UV. The newly synthesised DNA existed as short fragments. After incubation for 70 minutes the short fragments were converted to a size which co-sedimented with the full-length control DNA. It was postulated that replication stopped at a dimer lesion then reinitiated past that site resulting in a gap. Post-replication repair, or daughter-strand gap repair, then occurs by sister-strand exchanges, which fill each gap with undamaged DNA from the isopolar parental strand resulting in full length DNA (Rupp *et al.*, 1971). (For a review see Hanawalt *et al.*, 1979).

Post-replication repair requires the *recA* gene (Smith and Meun, 1970) and several other genes, including *lexA*, have also been implicated in the process (Hanawalt *et al.*, 1979). The mechanism appears to involve some form of genetic recombination between the DNA containing single strand gaps (gapped DNA) and the sister-strands. The

possibility that gapped DNA may be a substrate for recombination enzymes has been further studied *in vitro*.

Purified *recA* protein is a single-strand DNA-dependent ATPase and promotes both homologous pairing and strand exchange reactions (McEntee *et al.*, 1979). In the presence of ATP, the *recA* protein leads to efficient pairing of homologous gapped DNA and intact circular duplexes (Cassuto *et al.*, 1980; Cunningham *et al.*, 1980). *recA* protein-mediated reciprocal strand exchange can occur between a gapped circular molecule and another circular duplex nicked at a point opposite the gap. (West *et al.*, 1981). Based on the enzymatic studies and electron microscopic and X-ray crystallographic data on *recA* protein and its complexes with DNA, Howard-Flanders and West (1983) have outlined a model for the mechanism of homologous-pairing and strand-exchange in post-replication repair. The model involves *recA*-mediated pairing of a single DNA strand with the hydrogen bonds in the wide groove of an intact duplex molecule. The protein then condenses on the paired molecules, in a helical fibre, extending along the duplex and surrounding the locally paired DNA. Branch migration and release of *recA* protein subsequently occurs.

This model and several other aspects of post-replication repair remain to be verified *in vivo*.

It has been found *in vivo*, that recombination between λ phages was stimulated by the presence of pyrimidine dimers, in the absence of phage replication. It was concluded that gaps generated by excision of dimers initiated the recombination (Howard-Flanders, 1983).

This form of repair does not remove the UV-induced lesions but redistributes them between the parent and daughter DNA duplexes.

In summary, there are three known DNA repair processes active in UV-irradiated *E. coli* cells, photoreactivation, excision and post-replication repair. As has been mentioned the activity of two of these processes is regulated by the *recA* and *lexA* gene products i.e. the SOS regulatory system. This system is closely involved in cellular repair and enhanced survival.

1.2.3 The SOS regulatory system

'...damage to DNA (or stalled DNA replication) initiates a regulatory signal that causes the simultaneous derepression of various functions, all of which presumably promote the survival of the cell or its phages'. (Witkin, 1976). The group of inducible functions belonging to this regulatory unit are referred to as SOS functions (Witkin, 1976).

The SOS functions are under the co-ordinate control of two genes, *recA* and *lexA* (Radman, 1975). Aspects of this control system have been reviewed by Witkin (1976; 1982), Hanawalt et al. (1979), Gottesman (1981), Echols (1981), Little and Mount (1982), Gottesman and Neidhardt (1983), Walker (1984).

The SOS regulatory system is referred to as a global control system (Gottesman and Neidhardt, 1983) since it is a network of unlinked genes that share a common regulatory signal. This signal controls and changes the physiology of the cell. Global control units or regulons can also be defined as two or more operons that share a common regulator molecule (Gottesman and Neidhardt, 1983).

(a) ***The model of SOS control:*** A model of SOS control has been formulated, several essential features of which have been confirmed and will be discussed here.

The model postulates that a signal (generated by DNA damage) leads to activation of the proteolytic activity of the *recA* protein, which then cleaves cellular and prophage repressors causing the induction of SOS functions which are otherwise repressed. (Gottesman, 1981).

(b) ***The recA protease:*** *recA*$^-$ mutants were found to be extremely UV-sensitive (Clark, 1973). The *recA* protein has an induced proleolytic activity which acts on two classes of repressors, *viz*.

(i) on cellular repressor, of which the *lexA* repressor is the only known example (Little *et al.*, 1980), and

(ii) on phage repressors λ and P22 (Roberts and Roberts, 1975; Roberts *et al.*, 1978; Phyzicky and Roberts, 1980). Proteolytic cleavage occurs at the alanine-glycine bond (Horii *et al.*, 1981) and requires two co-factors, a nucleoside triphosphate and a single-stranded polynucleotide (Craig and Roberts, 1980).

In vitro, under certain conditions, the *lexA* and λcI repressors break down spontaneously in the absence of *recA* protein. This reaction has the same site-specificity to that of the *recA* catalysed reaction (Little 1984). Therefore the protease activity may be inherent to the repressors themselves and the activated *recA* protein may just play a stimulatory role (Little, 1984) in this cleavage.

In vivo however, the *recA* protein plays a critical role in the induction of the SOS regulon. One of the first responses to DNA damage is the induction of *recA* protein production (McEntee, 1977; Gudas and Mount, 1977). This is because the *recA* gene is itself repressed by the *lexA* repressor (Little *et al.*, 1981; Brent and Ptashne 1980). DNA damage activates a basal level of the *recA* protein to

its protease form and cleavage of the *lexA* repressor then leads to induction of the *recA* gene and the full response. This overproduction of the protein is not required for induction of some SOS functions, activation of the basal level is sufficient to cleave the λ repressor (Moreau *et al.*, 1980). In addition, overproduction of the *recA* protein is insufficient to activate the *recA* protease, in the absence of DNA damage (Uhlin and Clark, 1981; Volkert *et al.*, 1981; Quillardet *et al.*, 1982; Ginsberg *et al.*, 1982). Therefore the protease is activated by a damage generated signal. However, there is an exception to this: the *recA441* (formerly tif-1) mutation. SOS functions can be induced in this mutant at 41°C in the absence of DNA damage. The *recA441* protein is a more efficient protease *in vitro* and *in vivo* (Roberts *et al.*, 1978). The mutation alters the polynucleotide requirement of the protease such that shorter oligonucleotides activate it (McEntee and Weinstock, 1981).

Besides being a protease, the *recA* protein plays a role in cellular homologous recombination and has been implicated in the repair of post-replication gaps (Howard-Flanders, 1978; Rupp *et al.*, 1971; Howard-Flanders and West, 1983) discussed in Section 1.2.2.

The recombinational and protease activities of the *recA* protein seem to reside in functionally distinct domains. A mutant *recA430* is competent in recombination but deficient in proteolytic cleavage of the λ repressor, while

another mutant *recA142*, is defective in recombination but capable of limited λ repressor cleavage (Roberts and Roberts, 1981).

(c) ***The lexA repressor:*** As mentioned the *lexA* protein is a substrate for the *recA* protease (Little *et al.*, 1980). Two types of mutations occur at this locus. The first class comprise dominant, UV-sensitive alleles, deficient in the induction of the SOS functions and exemplified by *lexA3* (Mount *et al.*, 1972). The protein from the *lexA3* mutants is not sensitive to *recA* protease (Little *et al.*, 1980). The other class of mutants is UV-resistant compared with *lexA3*, and produces defective or non-functional *lexA* repressor protein. This results in the SOS functions being expressed in the absence of damage, either at high temperatures e.g. *tsl* (Mount *et al.*, 1973) or consitutively e.g. *spr* (Mount, 1977; Pacelli *et al.*, 1979). These mutants made an important contribution to understanding the SOS regulatory circuit.

The current nomenclature of the above mutants is as follows (Walker, 1984):

lexA(Ind$^-$) formerly *lexA*$^-$ e.g. *lexA3*, *lexA1*
lexA(Ts) formerly *tsl* and
lexA(Def) formerly called *spr*

TABLE 1.1

SOS boxes in front of several *lexA* repressed genes

GENE	LexA binding site
recA	TACTGTATGAGCATACAGTA
*lexA*o1	TGCTGTATATACTCACAGCA
*lexA*o2	AACTGTATATACACCCAGGG
uvrA	TACTGTATATTCATTCAGGT
uvrB	AACTGTTTTTTTATCCAGTA
sfiA *	TACTGTACATCCATACAGTA
colE1 1	TGCTGTATATAAAACCAGTG
colE1 2	CAGTGGTTATATGTACAGTA
cloDF13 *	TACTGTGTATATATACAGTA
uvrD	ATCTGTATATATACCCAGCT
umu D/C-1 *	ATCTGCTGGCAAGAACAGAC
umu D/C-2 *	TACTGTATATAAAAACAGTA
consensus	taCTGTaTata-a-aCAGta

* not shown to bind *lexA3* protein

Gottesman and Neidhardt (1983), Brent (1983)

Walker (1984)

Purified *lexA* protein represses both the *recA* and *lexA* genes (Brent and Ptashne, 1981; Little *et al.*, 1981) i.e. it is autoregulatory. Using DNaseI protection studies *lexA* protein binding sites were identified. These binding sites shared considerable homology, and were termed SOS boxes (Little *et al.*, 1981) SOS boxes have been defined in several genes controlled by the SOS regulon (Table 1.1) *uvrA*, *uvrB* (Sancar *et al.*, 1982a; b) *cle1*, *cloDF13* (van den Elzen *et al.*, 1982) *sfiA* (Cole, 1983), *umu D/C* (Elledge *et al.*, 1983) *uvrD* (Walker, 1984). These sites have the consensus sequence taCTGTatata-a-aCAGta (Walker, 1984). Some of the control regions of these genes have two adjacent operator (binding sites) and *lexA* protein binding is then co-operative (Brent, 1983).

(d) ***The SOS functions:*** A brief list of induced *E. coli* SOS functions is given in Table 1.2 (for a more detailed list see Witkin, 1982, and Walker, 1984). Most of these functions, with the notable exception of prophage induction, seem to operate to increase the cells chances of survival after DNA damaging treatments. In the case of the excision repair genes, more efficient removal of damage would ensure faster recovery from damage. In the case of some other induced functions the advantage is not so apparent. For example, cell division may be inhibited to allow multiple genomal copies to accumulate which could increase the chances of post replication recombination repair (Hanawalt, 1982). Mutagenesis and error prone repair

TABLE 1.2

SOS FUNCTIONS

Induced activity	induced gene(s)	reference
Prophage induction	phage genes	Witkin (1976)
Inhibition of cell division (filamentation)	*sfiA (sulA)*	Huisman *et al.* (1980) Cole (1983)
Bacterial mutagenesis and error prone repair	*umuC*	Elledge *et al.* (1983)
Excision repair	*uvrA, uvrB uvrC (?), uvrD*	Kenyon and Walker (1981), Fogliani and Schendel (1981), Backendorf *et al.* (1983a), Kumura *et al.* (1983) Nakayama *et al.* (1983)
Induced radioresistance	*recA*	Witkin (1976)
Induction of *recA* protein	*recA*	McEntee (1977)
Stable DNA replication	-	Kogoma *et al.* (1979)
Alleviation of K-specific restriction of λ	-	Thoms and Wackernagel (1982, 1984)
recF-dependent recombination	-	Armengod (1982)
-	*dinA, B, D, F*	Kenyon and Walker (1981)
Site-specific recombination	*himA*	Miller *et al.* 1981

may be built in adaptive measures (Hanawalt, 1982). Several of the genes shown to be induced by damage (damage inducible or *din*, Kenyon and Walker, 1980) have not yet been assigned functions.

Mutagenesis is an inducible function and requires the *lexA* repressed *umuC/D* genes, in addition it appears to require activated *recA* protein itself (Blanco *et al.*, 1982; Walker 1984; Ennis *et al.*, 1985). The processes involved in mutagenesis have recently been comprehensively reviewed (Walker, 1984). Most of the UV-induced mutations are targeted (Miller, 1982) ie they occur at the site of, or in the vicinity of lesions in the DNA. The lesion involved appears to be the pyrimidine-pyrimidine (6-4) adduct (Brash and Haseltine, 1982; Haseltine, 1983). Untargeted mutagenesis on the other hand seems to be due to the induction of an SOS-dependent error prone form of replication. Caillet-Fauquet and Maenhaut-Michael (1982) concluded that untargeted mutagenesis probably occurs by an increased mismatch error rate in DNA replication. They also concluded that these mismatches are normally corrected by the mismatch repair system, since an increased frequency of mutations is obtained in cells deficient in mismatch repair.

In addition to mutagenesis another SOS function, stable DNA replication, also requires activated *recA* protein independent of its role in the inactivation of *lexA* repressor (Witkin and Kogoma, 1984).

(e) ***Control of induction of the SOS regulon:*** The SOS system can exist in two extreme states, fully repressed (OFF) or fully expressed (ON). In addition it can exist in an intermediate state where some genes are induced to varying levels (Little, 1983; Walker, 1984).

This can be achieved because the *lexA* repressor has different affinities for its operators - it binds most tightly to the *recA* operator and less tightly (in decreasing order) to the *uvrB*, *sfiA* and *lexA* operators (Brent, 1982, 1983). Since the *lexA* repressor binds its own operator weakly, a significant amount of *lexA* protein is produced in the uninduced cell. If the DNA damage is mild, Brent (1983) proposes that this would lead to only a small drop in the cellular *lexA* protein concentration, causing *uvrB* and *sfiA* genes to be selectively derepressed and thereby allowing the cell to repair the damage. Full induction of the SOS pathway is not always beneficial e.g. when the cell harbours a λ-like prophage, induction causes lysis. Therefore, only relatively severe damage, which results in complete activation of the basal amount of *recA* protease, results in complete inactivation of the *lexA* repressor and consequent induction of the *recA* gene and all other SOS genes.

Inherent to this proposal is the assumption that activation of *recA* protease is proportional to the damage sustained, and therefore, that the amount of *recA* protease controls the extent of turn-on of the SOS regulon.

After induction by UV light, the kinetics of mRNA synthesis from several SOS genes was studied by Markham *et al.* (1984; 1985). They showed that increased rates of mRNA synthesis were detected as early as 5 minutes after induction. After 90 minutes the induced mRNA synthesis declined to the pre-induction level. The authors reasoned that this re-establishment of repression was due to inactivation of the signal which activates the *recA* protease. They also showed that transcription of the *recA* gene is turned off prior to that of the *lexA* gene, consistent with the *lexA* protein re-establishing repression of the other SOS genes before it represses itself.

With respect to the turn-off of the SOS functions Little (1983) has demonstrated that the *recA* protease activity, and by inference the inducing signal, is reduced by a *lexA* repressed function, i.e. the proteolytic activity (signal) does not decay spontaneously. He showed that in a $lex^{+}/lexA3(Ind^{-})$ diploid strain, the $lexA^{+}$ repressor protein was rapidly cleaved for a much longer time, than in a $lexA^{+}$ wild type strain. Therefore the $lexA3(Ind^{-})$ protein was repressing some function which would otherwise have 'inactivated' the *recA* protease. Little (1983) postulated that the *recA* protein itself or the *uvrA* and *uvrB* proteins may be involved in inactivation of the signal, since these proteins play a major role in the repair of DNA damage which is presumably the source of the inducing signal. Therefore

there may be an active mechanism for switching OFF the SOS regulon.

(f) ***The SOS inducing signal:*** As discussed in the preceeding section, the amount of *recA* protease probably controls the state of the SOS regulon (Little, 1983). However, not much is known about the correspondence between DNA damage and activated *recA* protease.

The proteolytic activity of the purified *recA* protein requires two cofactors i.e. nucleotide triphosphate and single-stranded polynucteotide (Craig and Roberts, 1980). Therefore the signal could be something which generates these co-factors, such as DNA degradation products resulting from damage or repair, or single-stranded gaps formed as a replication fork passes a damaged site (Little, 1982). Roberts *et al.* (1982) suggested that an increase in dATP levels obtained on induction, may be an important component of the inducing signal. This could also be true for temperature induction of *recA441*(Ts) (Llagastera *et al.*, 1985).

Pyrimidine dimers are directly or indirectly involved in generating the inducing signal, since to obtain the same level of induction of *recA* protein a 10-fold higher UV dose was necessary in a wild type strain (which excises the dimers) compared with a *uvrA* mutant (Salles and Paoletti, 1983). However the continued presence of dimers in the *uvrA*

mutant did not prevent a decrease in *recA* protein, which decayed to the basal uninduced level in 6-7 hours (Salles and Paoletti, 1983).

In light of the fact that the excision repair system excises pyr-pyr 6-4 adducts as well as dimers, it is probably better to refer to damage induced lesions, rather than pyrimidine dimers, as being responsible for generating the inducing signal.

Some clues about the nature of the inducing signal come from a phenomenon known as indirect induction. Borek and Ryan (1960) showed that introduction of UV-irradiated F^+ factor into unirradiated λ lysogens caused induction of the prophage. Since the cell itself was unirradiated, this induction of the SOS system was referred to as indirect. In addition to prophages, other SOS functions e.g. the *sfiA* gene, are also indirectly induced (D'Ari and Huisman, 1982). The authors showed that UV-irradiated λ, P1 and M13 phage all indirectly induce the expression of an *sfiA*:: lac fusion. P1 and M13 are more efficient inducers than λ. If the phage are incapable of replicating, e.g. in an immune lysogen or if deficient in phage replication genes, a much lower level of induction is seen (D'Ari and Huisman, 1982).

From these findings three things can be concluded about the nature of the SOS inducing signal.

1. Irradiated DNA is sufficient to induce the SOS system.

2. All damaged DNA's do not cause the same level of induction, therefore the lesions themselves are not the only thing involved.

3. Processing of the lesions by attempted replication generates a more efficient inducing signal.

In summary, the SOS response is a global control system which alters the physiology of the cell. The system is inducible by DNA damage and is reversible, since it returns to a normal growth state after recovering from the damage.

1.2.4 Induction of the heat-shock proteins

UV has recently been shown to induce functions from another global control system the heat-shock regulon.

Krueger and Walker (1984) showed that certain heat-shock proteins, *groEL* and *dnaK*, were induced by UV and nalidixic acid. The induction was independent of *recA* and *lexA*, therefore the *groEL* and *dnaK* genes are not part of the SOS regulon. Instead the induction was controlled by the *htpR* gene product (Krueger and Walker, 1984), a positive acting element required for expression of the heat-shock genes (Neidhardt *et al.*, 1983). The heat shock proteins are

induced by a shift to high temperatures and a variety of other treatments.

Thus UV induces both the SOS regulon and genes in the heat shock regulon. However, a higher dose of UV was required to induce the heat shock genes compared with that required to induce the SOS response (50 J/m² *vs* 10 J/m²) (Krueger and Walker, 1984).

1.3 CONSIDERATION OF THE TRANSIENT LOSS IN CELLULAR CAPACITY TO HOST A 186 INFECTION AFTER UV

1.3.1 UV-induced loss in phage capacity

The effect of irradiation on the ability of bacteria to assure the development of an infecting phage ('capacity'), was first studied by Benzer and Jacob (1953), who also coined the term 'capacity' in this connection. It was found that UV-irradiation reduced the 'capacity' of bacteria for several phage, as measured by efficiency of plating (Stent, 1958). The extent of the reduction varied for different phage (Stent, 1958).

The term '186 capacity' is employed in this thesis to describe the irradiated cell's capability to support 186 phage production. As described earlier, Hooper *et al.* (1981) found that cells transiently lost 186 capacity after UV-irradiation. Benzer and Jacob (1953) used the efficiency of plating of phage on UV-irradiated bacteria as a measure

of the loss in capacity. In this work phage burst analysis was used to study the loss in the capacity of irradiated cells to produce 186 phage.

1.3.2 Why study the loss in 186 capacity

The work of Hooper *et al.* (1981) raised the possibility that UV may exert a *trans*-acting inhibition on DNA synthesis, in addition to the *cis*-acting inhibition caused by lesions in the DNA.

The UV-induced inhibition of 186 phage production could be used to distinguish between *cis*- and *trans*-acting factors induced by UV which could not easily be distinguished by studying *E. coli* DNA replication directly.

An added advantage of using 186 phage to investigate the possibility of trans-effects induced by UV, was that the phage requires the host initiation functions *dnaA* and *dnaC* (Hooper and Egan, 1981) and 186 replication may be thus be similar to *E. coli* replication in its requirements. Therefore, identification of UV-induced factors which inhibit 186 replication could uncover a novel effect of UV (*trans*-acting inhibition) on *E. coli* DNA synthesis.

1.3.3 The cause of the UV-induced loss in 186 capacity

The following considerations were entertained as possible causes of the loss in 186 capacity.

(i) 186 replication may require host DNA replication *per se*.

(ii) The loss in capacity may be a UV-induced SOS function.

(iii) UV-irradiation of the cell may result in depletion or inactivation of host functions required by 186 phage.

(ii) and (iii) are not mutually exclusive but were examined to identify possible cellular factors involved in the inhibition.

(a) ***186 dependence on concomitant host replications:*** If 186 replication requires *E. coli* DNA replication *per se*, then UV-inhibition of host synthesis would account for the loss in 186 capacity, making it unnecessary to postulate, a *trans*-acting inhibition specific to UV-irradiated cells. The *cis*-acting inhibition caused by lesions in the host chromosome would, in this case, indirectly be inhibiting 186 replication.

This possibility was entertained because it has been found that every treatment which inhibited host replication also inhibited 186 replication and phage production, e.g. UV, nalidixic acid, mitomycin C, and transfer of *dna*Ts (A,C,B) mutants to the non-permissive temperature.

If 186 has a requirement for host DNA synthesis, it could be a *cis* requirement i.e. the phage DNA has to be integrated in the chromosome to be replicated passively by the host machinery or a *trans* requirement i.e. it just requires simultaneous on going host replication. It is known that 186 does not need to be integrated into the host chromosome in order to replicate since replicating monomeric circles of 186 DNA have been visualised by electron microscopy (Chattoraj and Inman, 1973). In addition *int*$^-$ (defective in site-specific phage integration) mutants of 186 are viable and yield normal phage bursts (Bradley *et al.*, 1975). Therefore 186 does not have a *cis*-requirement for host DNA replication. Instead it replicates as a rolling circle and unidirectionally (Chattoraj and Inman, 1973; 1981).

It is also unlikely that 186 requires concomitant host synthesis since it possesses a *dhr* (formerly known as *dho*) gene (Fig 1.2) (Hocking and Egan, 1982b), which considerably depresses the host DNA replication upon infection of cells with 186.

(b) ***A UV-induced SOS function:*** If the loss in 186 capacity is caused by an induced *trans*-acting function, this function could be part of the SOS regulon since most damage inducible functions seem to be under SOS control. This possibility was supported by the report that the *recA*

protein may be responsible for the transient inhibition in *E. coli* DNA synthesis after UV-irradiation (Trgovcevic *et al.* 1980). A *recA* mutant did not show the characteristic delay in *E. coli* DNA synthesis displayed by its parent strain. Instead it had a reduced rate of DNA synthesis, suggesting that the *recA* protein, in some way, caused the characteristic delay. However, the *recA* mutant and its *recA* parent were irradiated with UV-doses which resulted in the same survival. Consequently, the mutant received a much smaller dose of UV compared with the control. The absence of the delay in replication may have been due to the use of this low dose of UV and not due to the *recA* mutation.

A second unrelated consideration concerns the presence of a possible SOS box on the 186 genome (Sivaprasad, 1984). The consensus sequence for *lexA* protein binding was located on a rightward promotor of 186 located at 95% on the genetic map (Fig 1.2). It could be envisaged that this promotor may control a phage gene involved in the turn-off of phage DNA replication, normally expressed late in infection. In this case the *lexA* repressed gene would be turned on in a UV-irradiated cell by *recA* protease and expression of the replication turn-off gene would inhibit 186 DNA synthesis until the *lexA* repression was re-established. (It must be noted that as yet the putative 'SOS box' in 186 has not been demonstrated to bind *lexA* protein, nor has the promotor been shown to be under *lexA* control).

Thus the SOS regulatory system could cause the transient inhibition in 186 replication, either through a host or phage encoded replication inhibitor. This could be tested using *recA*$^-$ or *lexA*(Ind$^-$) mutants deficient in the expression of the SOS genes.

(c) ***Depletion or inactivation of host replication functions***: In addition to 186 two other phages tested, P1 and Mu, experience a transient inhibition in phage production in UV-irradiated cells (Hooper and Egan, 1981). As mentioned earlier, λ and P2 are not inhibited under the same circumstances.

The block in 186 phage production was shown to be at the level of DNA replication (Hooper *et al.*, 1981). It was considered most unlikely that the block was at the elongation of DNA. Since all the phage tested probably use the *E. coli* elongation functions in fact λ and P2 have been shown to require several *E. coli* elongation genes (Furth and Wickner, 1983; Bowden *et al.*, 1975). It was far more likely that the block acted on the initiation of DNA synthesis, since λ and P2 which are unaffected in UV-irradiated cells do not require the host initiation functions *dnaA* and *dnaC* (Further and Wickner, 1983; Bowden *et al.*, 1975) but 186 requires both functions (Hooper and Egan, 1981). In addition to 186 requiring *dnaC*, P1 and Mu, which were transiently inhibited in UV-irradiated cells, were also

found to require *dnaC* (Hooper and Egan, 1981). Based on this correlation, Hooper (1979) postulated that the *dnaC* gene product is depleted and therefore limiting in UV-irradiated cells so that an incoming *dnaC*-requiring replicon is incapable of replicating until the concentration of the *dnaC* protein is restored.

The mode of *dnaC* utilisation lends theoretical support to the hypothesis. *In vitro*, *dnaC* is required for the initial compilation of the initiation complex of proteins. (Wickner and Hurwitz, 1975; Arai *et al.*, 1981; Fuller *et al.*, 1983) but not for continued fork movement (Nusslein-Crystalla *et al.*, 1982). If, however, a new replisome is created *dnaC* protein is required to insert *dnaB* protein into the complex (Nusslein-Crystalla *et al.*, 1982). Extrapolating this observation to the situation in UV-irradiated cells where *trans*-dimer gap synthesis occurs (Rupp and Howard-Flanders, 1968), if reinitiation past dimers requires *dnaC* protein then multiple initiations could deplete the cells supply of *dnaC* protein.

The above scenario could actually lead to depletion since the *dnaC* protein is present in limited copy number in the cell (Kobori and Kornberg, 1982 ; Lanka and Schuster, 1983).

1.3.4 Is the initiation of *E. coli* DNA synthesis inhibited by UV ?

In this study, 186 was used as a tool (since it is very simple to quantitate phage production) in an effort to identify specific cellular factors which may be involved in a *trans*-acting inhibition of DNA replication. If such factors were found it would have supported the possibility that the UV-induced inhibition affects *E. coli* DNA synthesis, since depletion of host functions or an SOS-controlled inhibitor would presumably act primarily on *E. coli* DNA synthesis and only fortuitously on other replicons. For reasons discussed before it was considered most likely that *E. coli* initiation rather than elongation would be the target for a UV-induced *trans*-acting inhibition. In addition to using 186 it was decided to directly test the premise that UV might inhibit initiation of replication from the *E. coli* origin of replication *oriC*.

oriC is a unique site on the *E. coli* chromosomal region located between *uncB* and *asnA* (von Meyenberg *et al.*, 1977; 1978) where bidirectional replication of the *E. coli* chromosome begins (Kaguni *et al.*, 1982). This region has been cloned into various plasmid and phage vectors and is capable of autonomous replication (von Meyenberg *et al.*, 1978; Kaguni *et al.*, 1982). Bidirectional replication *in vitro* requires the *dnaA* and *dnaC* proteins as well as several other proteins (Fuller *et al.*, 1981; Kaguni *et al.*, 1982; Kaguni and Kornberg, 1984).

A propagating λ clone of the *oriC asnA* region λoriC was constructed by Soll (1980a). Replication of this phage from the *oriC* origin can be studied. Thus introduction of the unirradiated phage into irradiated cells presented a system akin to that described for 186, where the effect of irradiation of the cell on the *oriC*-dependent replication could be studied. The advantage of this system of course, is that no photoproducts are present in the *oriC*-dependent replicon under study so any inhibition of replication has to be due to *trans*-acting factors or refractory conditions in the cell, induced by UV-irradiation.

1.4 AIMS

To determine the reason for the unique sensitivity of 186 phage infection of UV-irradiated host cells and thereby to provide some further insight into the effects of UV on DNA replication.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial Strains

All the bacterial strains used were derivatives of *E. coli* K12 and are listed in Table 2.1

2.1.2 Bacteriophage and Bacteriophage Clones

All the coliphage and coliphage clones are listed in Table 2.2.

2.1.3 Plasmids and Plasmid Clones

These are listed in Table 2.3

2.1.4 Chemicals

The following chemicals were purchased from the companies indicated:

Agarose: Bethesda Research Laboratories (BRL)

NZ-Amine A: Sheffield products of Kraft Inc.

Amino acids (analytical grade): Sigma Chemical Co.

Ampicillin: Sigma Chemical Co.

Bacto-agar, Bacto-peptone and Bacto tryptone: Difco Laboratories

Bovine Serum Albumin: Sigma Chemical Co.

Casamino acids (Vitamin free): Difco Laboratories

Chloramphenical: Sigma Chemical Co.

Cesium chloride (ultra pure): Bethesda Research Laboratories

Ethidium bromide: Sigma Chemical Co.

TABLE 2.1

Bacterial strains

Collection No.	Strain	Relevant genotype	Source/reference
E220	C600 (Mu)	E508 (Mu*cI*ts61)	this laboratory
E237	C600	E508 *thyA thyR*	this laboratory
E251	W3350	*gal strA* sup^+	this laboratory
E252	W3350 (186)	E251 (186*cI*ts*p*)	this laboratory
E264	W3350(186)	E251 (186*cI*ts*p*Bam17)	this laboratory
E508	C600	*thr leu thi tonA supE*	D. Hogness. Appleyard (1954)
E511	C600 (λ)	E508 (λ*c*I^+)	this laboratory
E514	C600 (λ)	E508 (λ*ind*$^-$*c*I857)	D. Hogness
E516	W3350 (λ)	E251 (λ*ind*$^-$*c*I857)	R. Doherty
E528	SY545	F^+ *polA1 endA strA dnaA5 Zi*::Tn10 *tsx*	R Fuller
E573	C600 (186)	E508 (186*c*I^+)	this laboratory
E574	C600 (186)	E508 (186*cI*ts*p*)	this laboratory
E0635	W3350 (186)	E251 (186$^+$)	this laboratory
E0678	W3350 (λ)	E251 (λ*c*I857)	this work
E0679	W3350 (λ)(186)	E251 (λ*c*I857) (186*cI*ts*p*)	this work
E0680	W3350(λ)(186)	E264 (λ*c*I857)	this work
E728	AO992	C00 *recA*	Amos Oppenheim
E746	AB1157	*thr-1 leuB6 proA2 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44*	B. Bachmann. DeWitt and Adelberg (1962)
E749	AB2497	AB1157 *thyA12 deoB16*	B. Bachmann. Howard-Flanders *et al.* (1966)

E750	AB1886	AB1157 *uvrA6*	B. Bachmann. Howard-Flanders *et al.* (1966)
E753	AB2470	AB1157 *recB21*	B. Bachmann. Howard-Flanders and Theriot (1966)
E754	JC5495	AB1157 *recA13 recB21*	B. Bachmann. Willets and Clark (1969)
E761	JC10240	Hfr PO45 *thr-300 ilv-318 rpsE300 recA56 srl-300::Tn10*	A.J. Clark
E764	JC10257	AB1157 *recA441 srl-300*::Tn10	A.J. Clark
E766	JC13067	AB1157 *leu*$^+$ *recB21 recC22 sbcB15 sfiB lexA3 malB*::Tn10	A.J. Clark
E777	W3350	E251 *thyA thyR*	this laboratory
E782	JC11867	AB1157 *recAo281 lexA3 malB*::Tn10 HK19^R	A.J. Clark. Volkert *et al.* (1981)
E783	JC11457	AB1157 *recAo281*	A.J. Clark. Volkert *et al.* (1981)
E793	AB1157 (186)	E746 (186*cItsp*)	this work
E801	AB1157 (λ)	E764 (λ*cI*$^+$)	this work
E808	AB1157	AB1157 (as E746)	D.W. Mount
E818	GW1104	*tif* Δ*lac umuC*::MuD *malE*::Tn5	G. Walker
E832	M12	*lacZam* Sm^R Δ*bio-uvrB* Δ*trpEA2* (λ*Nam7N53cI857*ΔHI)	E. Remaut Castellazi *et al.* (1972)
E849	CM987	F$^-$ *asnA31 asnB32 relA1 spoT1 thi-1 recA*	von Meyenberg *et al.* (1979)
E990	C600	E508 *thyA thyR dnaC2*	this laboratory
E4044	C600	E508 *malB*::Tn10	this work
E4045	C600	E508 *lexA3 malB*::Tn10	this work
E4046	W3350	E777 *Zi*::Tn10	this work

E4047	W3350	E777 *dnaA5 Zi*::Tn10	this work
E4048	C600 (λ)	E237 (λ *ind*$^-$*cI*$^+$)	this work
E4049	C600 (λ)	E990 (λ*ind*$^-$*cI*$^+$)	this work
E4050	C600 (λ)	E990 (λ*ind*$^-$*cI*$^+$) *dnaC*$^+$	this work
E4051	C600	E237 *uvrA6 malE*::Tn5	this work
E4053	C600	E990 *recA56 srl300*::Tn10	this work
E4056	CM987 (λ)	E849 (λ*ind*$^-$*cI*$^+$)	this work
E4057	C600	E237 *malE*::Tn5	this work
E4058	AB1157	E746 *thyA thyR*	this work
E4059	AB1157	E808 (186 *cItsp*)	this work
E4060	AB1157	E746 (186*cItsptum9*)	this work
E4061	AB1157	E808 (186*cItsptum9*)	this work
E4062	AB1157	E746 *recA56 srl-300::Tn10*	this work
E4063	AB1157	E746 *srl-300::Tn10*	this work
E4064	AB1157	E4058 (186*cItsp*)	this work
E4065	AB1157	E749 (186*cItsp*)	this work
E4066	C600	E508 *recA56 srl-300::Tn10*	this work
E4067	AB1157	E508 *srl-300::Tn10*	this work

TABLE 2.2

Bacteriophage and bacteriophage clones

Phage No.	Genotype	Relevant phenotype and comments	source/ reference
399	186*c*I$^+$	wild type immune at 37°C	Jacob and Wollman (1956)
400	186*c*I*ts*p	temperature sensitive repressor, referred to in this thesis as 186*c*I*ts*	Baldwin *et al*. (1966)
505	186*c*I*ts*p*tum*9	prophage non-inducible by mitomycin C and UV	this laboratory
281	186*c*I*am*53*vir*1	insensitive to 186 immunity	this laboratory
410	186*c*I10	non-functional *c*I repressor	this laboratory
70	P1kc	clear plaques on *E. coli* K12	P. Reeves
72	P1*vir*	insensitive to P1 immunity	G. Walker
103	Muc*I*ts61	temperature-sensitive repressor	heat-induced from E220
21	λ*c*I$^+$	wild type	Kaiser, (1957)
67	λ*ind*$^-$*c*I$^+$	non-inducible by UV	Jacob and Campbell (1959)
1	λ*c*I857	temperature-sensitive repressor	Sussman and Jacob (1962)
262	λ*c*I857*ind*$^-$	prophage non-inducible by UV	Sussman and Jacob (1962)
46	λ*c*I72	non-functional *c*I repressor	R. Thomas
12	λ*vir*	insensitive to λ immunity	
52	λ*imm*^{21}*nin*5p*dnaC*	carrying an *E. coli dnaC* gene	Iida (1977)

68	λp*b221c1am14*	*b*221 deletion removes the λ(att-int) region, 19.6-30.0 kb on the λ map, amber mutation in the *c*I repressor	Daniels et al. (1983) L. Soll
λ1020	λ*b221cIam14* p*oriC asnA*	carries the *E. coli oriC asnA region*	Soll (1980a.)
λ1021	λ*b221cI*$^{+}$*Oam29* p*oriC asnA*	amber mutation in the *O* gene and a wild type repressor	Soll (1980a.)
263	λ*b221cII*$^{-}$*Oam29* p*oriC asnA*	clear plaque mutant of λ1021	This work
264	λ*b221cII*$^{-}\Delta$ p(*oriC asnA*)	*oriC asnA deletion of* λ263	*this work*

TABLE 3.0

Plasmids and Plasmid clones

Number	Description	Source and reference
pBR322	amp^R tet^R	Bolivar *et al.* (1977)
pMOB45	cam^R tet^R	Bittner and Vapnek (1981)
pJK169	4.8kb *Bam*H1 *E. coli dnaC* fragment cloned into the *Bam*H1 site of pBR322	Kobori and Kornberg (1982)
pJK129	4.8kb *Bam*H1 *E. coli dnaC* fragment cloned into the *Bam*H1 site of pMOB45	Kobori and Kornberg (1982)

Ficoll 400: Pharmacia Fine Chemicals

Kanamycin: Sigma Chemicals Co.

Mitomycin C: Sigma Chemicals Co.

Nucleotides and nucleoside phosphates: Sigma Chemical Co.

Phenol (redistilled before use): BDH Laboratories

Polyethylene glycol 8000: Sigma Chemical Co.

Polyvinyl pyrrolidone: May and Baker Ltd.

Tetracycline: A gift from Upjohn Pty. Ltd. (Australia)

Trimethoprim: Burroughs Wellcome and Co. (Australia)

Trizma Base and Tris HC1: Sigma Chemical Co.

Yeast Extract: Oxoid Ltd.

Other chemicals were routinely obtained from Sigma Chemical Co., BDH Chemicals Ltd., Ajax Chemicals Ltd. and May and Baker Ltd. and were of either analytical grade or of the highest available purity.

2.1.5 Radionucleotides

[methyl-^{3}H]thymidine (20-40 Ci/mMol) and [methyl-^{3}H]thymine (40-50 Ci/mMol) were purchased from The Radiochemical Centre Amersham, England. α[^{32}P]-dGTP and α[^{32}P]-dCTP were gifts from Dr. R.H. Symons, this department.

2.1.6 Enzymes

Restriction enzymes were purchased either from New England Biolabs or from Bethesda Research Laboratories. DNase I and lysozyme were from Sigma Chemical Co. DNA

polymerase I and Proteinase K were obtained from Boehringer Mannheim, West Germany.

2.1.7 Media

Media were prepared using glass distilled water and sterilized by autoclaving. Aminoacids and antibiotics were added from sterile stock solutions after the media had been autoclaved.

The composition of the media is given per litre.

LB Broth

Sodium chloride	10 g
Bacto Tryptone	10 g
Yeast Extract	5 g
Water	1000 ml

the pH was adjusted to 7.0 before autoclaving.

10 x LB Broth

10 fold the concentration used for LB broth.

LB Broth treated with activated charcoal

The pH of LB broth was lowered to between 4.0 and 5.0 with HC1. Activated charcoal (Ajax Chemicals, Australia), at a concentration of approximately 1 g/100 ml LB broth was addded and the mixture allowed to stand for 5-10 min. The charcoal was removed by filtration. The pH was raised to 7.0 with NaOH and the broth autoclaved. Glucose was added to 0.1% final concentration.

YGC Broth

Sodium chloride	10 g
NZ amine A	10 g
Yeast extract	5 g
Water	1000 ml

the pH was adjusted to 7.0 and the broth was autoclaved then the following solutions were added.

20% sterile glucose	5 ml
0.4 M sterile $CaCl_2$	6 ml

NZCYM

NZ amine A	10 g
NaCl	5 g
Casamino acids	1 g
Yeast extract	5 g
$MgSO_4$	2 g

The pH was adjusted to 7.5 before autoclaving.

TPGCAA

Sodium chloride	0.5 g
Potassium chloride	8.0 g
Ammonium chloride	1.1 g
Trizma Base	12.1 g
Sodium pyruvate	0.8 g
Water	900 ml

The pH was adjusted to 7.4 with HCl. To 90 ml of this medium the following solutions were added:

0.16 M Na_2SO_4	0.1 ml
1 M $MgCl_2$	0.1 ml
0.4 M $CaCl_2$	0.25 ml
0.1 mg/ml $FeCl_3$	0.1 ml
20% Glucose	1.0 ml
10% Casamino acids	2.0 ml

M9CAA

10 x M9 salts*	100 ml
Water	900 ml

Adjusted the pH to 7.0, autoclaved and cooled, then added:

0.4 M $CaCl_2$	0.25 ml
1 M $MgSO_4$	1.0 ml
10 mg/ml thiamine	1.0 ml
20% Maltose	1.0 ml
10% Casamino acids	2.0 ml

*10 x M9 salts:	Na_2HPO_4	60 g
	KH_2PO_4	30 g
	NaCl	5 g
	NH_4Cl	10 g

M9CAA supp

M9CAA

Biotin	1 µg/ml
tryptophan	50 µg/ml

Z Plates

NaCl	5 g
NZ amine A	10 g
Water	1000 ml

The pH was adjusted to 7.2

Agar	12 g

The mixture was autoclaved and 30 ml poured per plate.

YGC Plates

NaCl	10 g
NZ Amine A	10 g
Yeast extract	5 g
Water	1000 ml

The pH was adjusted to 7.0

Agar	15 g

The mixture was autoclaved cooled to 45°C then the following were added:

20% glucose	5 ml
0.4 M $CaCl_2$	2 ml

30 ml was poured into each petri dish.

M9 Plates

10 x M9 salts (See M9CAA)	100 ml
Water	900 ml
Agar	15 g

The pH was adjusted to 7.0, autoclaved cooled to 45°C then the following were added:

0.4 M $CaCl_2$	0.25 ml
1 M $MgSO_4$	1 ml
10 mg/ml thiamine HCl	1 ml

If glucose or other sugars and amino acids were required, they were added and the plates poured.

O.M. Plates

Solution A:	Bacto-Agar	15 g
	Water	500 ml

Solution B:	K_2HPO_4	10.5 g
	KH_2PO_4	9.0 g
	$(NH_4)_2SO_4$	1.0 g
	Sodium Citrate	0.5 g
	Water	500 ml

Solutions A and B were autoclaved separately, cooled and mixed, then the following were added:

1 M $MgSO_4$	0.21 ml
20% maltose	10 ml
10% Casamino acids	20 ml

Top Agar

Bacto-agar	0.7 g
Water	100 ml

Autoclaved

2.1.8 Buffers and Other Solutions

TM: 10 mM Tris HCl
10 mM $MgSO_4$
pH 7.1,

prepared from sterile stock solutions.

TE: 10 mM Tris HCl
0.1 mM EDTA
pH 8.0,

prepared from sterile stock solutions

HI: 0.1 M phosphate buffer pH 7.0
15 mM $(NH_4)_2SO_4$
1 mM $MgSO_4$
1.8 µM $FeSO_4$

prepared from sterile stock solutions

1 M Phosphate Buffer:

KH_2PO_4	136.1 g
Water	1000 ml

adjusted pH to 7.0 with KOH pellets, autoclaved.

1 x SSC: 0.15 M NaCl
0.015 M sodium citrate
pH 7.4.

TAE: (Gel electrophoresis buffer)
40 mM Tris
20 mM sodium acetate (anhydrous)
0.1 mM EDTA
pH 8.2 with acetic acid.

PSB: (Phage storage buffer)

10 mM Tris

100 mM NaCl

10 mM $MgCl_2$

0.05% gelatin

pH 7.4 then autoclaved.

P1 diluent:

20 mM Tris

10 mM $MgSO_4$

1% Ammonium acetate

pH 7.4 with acetic acid then autoclaved.

Scintillation Fluid:

2,5-diphenyloxaxole (PPO)	3.5 g
1,4-bis[2-(5-Phenyloxazolyl)]benzene (POPOP)	0.35 g
toluene	1000 ml

stored in a dark bottle.

1 x Denhardts Solution:

0.02% Ficol

0.02% Bovine serum albumin

0.02% polyvinylpyrrolidone

Stored at -20°C

1 x Glycerol Loading Buffer:

5% glycerol

0.04% Bromophenol blue

25 mM EDTA.

2.2 METHODS

2.2.1 Storage of phage and bacteria

Low titre phage stocks were either millipore filtered or stored over chloroform. Both low and high titre stocks were kept at 4°C. Bacterial strains were kept in 40% glycerol at -80°C for long term storage or kept in nutrient agar slabs and plates at 10°C for short term storage.

2.2.2 Growth of bacterial cultures

Single colonies of bacteria were transferred from nutrient plates into the medium in use and agitated overnight in a New Brunswick gyrotory water bath at 37°C or, if temperature sensitive, at 30°C. The overnight culture was diluted 50-100 fold into fresh medium and agitated as before. Optical density (O.D.) was measured at 600 nm using a Gilford 300T-1 spectrophotometer and the culture was used immediately the required density was reached.

2.2.3 Preparation of phage stocks

Low titre stocks

(a) 186 cIts and λcIts857 by heat induction.

Cells of C600 (186*cIts*), (E574) or C600 (λ*cIts*857), (E514) were cultured in LB broth to 5 x 10^8 cfu/ml at 30°C,

transferred to 39°C and cellular lysis followed by measuring O.D. When lysis was complete, bacterial debris was removed by centrifugation at 10,000 rpm at 5°C for 10 min.

(b) MucIts61 by heat induction.

C600 (MucI*ts*61), (E220) was cultured in LB broth containing glucose (0.2%) and $CaCl_2$ (4 mM) to an O.D. of 0 8 at 30°C, the culture was then transferred to 39°C till lysis, by following O.D., was complete (about 90 mins after transfer). The bacterial debris was removed by centrifugation, 10 min at 10,000 rpm at 5°C. The supernatant was assayed on C600 on YGC plates and stored over $CHCl_3$.

(c) P1kc by liquid infection

E508 or desired bacterial strain was cultured in LB broth containing 10 mM $MgSO_4$ and 4 mM $CACl_2$ to an O.D. of 0.7. Phage were added (moa 0.01-0.1) and the O.D. followed till lysis occurred. Cell debris was sedimented by centrifugation for 10 min at 10,000 rpm. The supernatant was millipore filtered and assayed on YGC plates.

(d) 186cI10 and 186vir1 by liquid infection.

C600 cells (E508) were cultured to an O.D. of 0.7 at 37°C, phage were added (moa 0.1-0.5) and the O.D. observed. If lysis, i.e. decrease in O.D., occurred the debris was removed by centrifugation immediately, if no lysis was evident the cells were incubated overnight at 4°C and

subsequently the culture was centrifuged for 10 min at 10,000 rpm at 4°C. The supernatant in either cases was millipore filtered and assayed on E508 indicator bacteria on Z plates.

(e) P1kc and P1vir by plate stocks.

The desired bacterial cells were cultured in LB broth containing 10 mM $MgSO_4$ and 4 mM $CaCl_2$ to late log phase. 0.1 ml of cells was mixed with 10^5-10^8 pfu and 3 ml of top agar. The mixture was poured onto a YGC plate, wet with 0.2 ml of LB broth containing $CaCl_2$ (4 mM). The plate was incubated at the required temperature till lysis (i.e. clearing of the P1 infected plates compared with an uninfected control bacterial lawn) had occurred. The plate was then flooded with P1 diluent and allowed to stand for 10-15 min, after which the supernatant and top agar were transferred to a centrifuge tube, a few drops of $CHCl_3$ were added and the tube agitated on a vortex mixer. The contents were centrifuged for 10 min at 10,000 rpm, the supernatant millipore filtered and assayed on bacterial indicator cells on a YGC plate for pfu/ml.

(f) λ phage by plate stocks.

Stocks of all the λ phage and λ clones except *λcIts857* were prepared by this method.

The required bacterial cells were cultured in LB broth containing maltose (0.2%) to mid log phage, 0.2 ml of

cells was mixed with either a single plaque or 10^7 pfu and allowed to stand for 5-10 min at room temperature. This was then mixed with 3 ml of top agar and poured onto a Z plate, wet with 0.2-0.5 ml sterile water. The plate was incubated at 37°C for 8-10 hrs, till lysis was apparent compared with a control uninfected bacterial lawn. The plate was flooded with 3 ml of TM buffer or PSB and allowed to stand for 15 min overnight at 10°C. The buffer and top agar were collected and centrifuged to remove the agar and debris. The supernatant was millipore filtered and assayed for pfu/ml.

High titre stocks

(g) 186cIts and λcI857 large volume high titre stocks

8-10 L of LB broth were innoculated with overnight cultures of C600 (*186cIts*) (E574) or C600 (*λcIts857*) (E514) in a 25 L fermentor (New Brunswick Magmaferm Fermentor). Cells were grown up with aeration and agitation (300 rpm) at 30°C to an O.D. of 0.8-1.0. Temperature was shifted up to 39°C and took 10-15 min to stabilise. Cells were harvested when lysis occurred and the O.D. decreased to a minimum level.

Harvesting: NaCl (19 g/L) was added to the lysate and the mixture kept at 10°C for 1 hour. Cells were collected by centrifugation of the lysate at 10,000 rpm, at 5°C for 20 min in 500 ml centrifugation pots in a JA10 rotor (Beckman

Model J21B centrifuge) or in a GS3 rotor (Sorval RC5B centrifuge). The supernatant was collected and pfu/ml were assayed.

PEG precipitation: 100 g/L of polyethylene glycol (P.E.G.) was added to the supernatant and dissolved in it (Yamamoto *et al.*, 1970). The mixture was stored overnight at 10°C. The precipitate was collected by centrifugation at 9,000 rpm at 5°C for 30 min. The pellets were drained of the P.E.G. solution and resuspended in the minimum possible volume of TM buffer. The pellet had to be soaked in TM for a few hours to allow it to be resuspended easily.

CsCl block gradient: The phage were purified from the above TM suspension by centrifugation on a CsCl block gradient as described essentially by Maniatis *et al.* (1982). Either polyallomer or nalgene polycarbonate centrifuge tubes were used. The step gradients were prepared by underlaying 3 ml of CsCl (ρ1.35) with 1 ml of CsCl (ρ1.6). 0.5 g/ml of CsCl was added to the TM suspension and the mixture with about 1.35 was layered over the step gradient and centrifuged at 45,000 rpm at 8°C for 90 min in a Ti50 rotor in a Beckman L8-70 or L5-50 ultracentrifuge.

A whitish phage band formed at the ρ1.35/1.16 interface was removed either by puncturing the side of the polyallomer tube or the base of the polycarbonate tube.

$1-2 \times 10^{13}$ pfu were obtained per litre of culture used.

Reverse CsCl gradient: To purify the phage further they were centrifuged in another step gradient. 3 ml of CsCl ρ 1.6 was layered under 2 ml of CsCl ρ1.35. Then 5-6 ml of the phage suspension plus an equal volume of saturated CsCl (final density about ρ 1.7) was underlayed beneath the CsCl ρ1.6 layer. The gradients were balanced and centrifuged at 45,000 rpm at 8°C for 90 min as described above. A whitish phage band was formed at the interface of ρ1.35/1.6 and collected as above. Recovery of pfu from this gradient was 90-100%.

(h) λ Phage small volume high titre stocks

50 ml of NZCYM broth was innoculated with 0.25 ml of a C600 overnight culture and 10^7 pfu of the desired phage. The cultures were agitated at 37°C. 4 hours later lysis was complete, a few drops of $CHCl_3$ were added and the flasks agitated at 37°C for 10 min. 50 µg DNase and 100 µg RNase were added and the incubation continued for 45 min on ice. The debris was pelleted by a 15 min centrifugation at 10,000 rpm at 4°C. The clarified supernatant was centrifuged at 20,000 rpm for 3 hours at 4°C. The phage pellets were resuspended in 400 µl of Tris (100 mM) pH 8.0/NaCl (300 mM), by standing overnight at 40°C. This method is a modification of that of Kao *et al.*, (1982).

2.2.4 Construction of bacterial strains

(a) ***thy***$^-$: Low thymine requiring strains were constructed by trimethoprim selection as described by Miller (1972). Mutations obtained by this method map mainly in the *thyA* gene and therefore have been designated *thyA*. Low thymine requiring mutations are designed *thyR* (Miller, 1972).

(b) ***lysogens:*** 186*cIts* and λ*cIts*: The phage were streaked for single plaques on a lawn of the bacteria to be lysogenised and the plates were incubated at 30°C overnight. The centre of a turbid plaque was streaked for single colonies on a YGC plate, with nutritional supplements, if any were required and incubated overnight at 30°C. Single colonies were tested for phage immunity by cross-streaking against corresponding *c*I$^-$ and *vir* mutant phage. A colony which was capable of growth through the *c*I mutant streak but not through the *vir* mutant streak was considered to be immune and therefore lysogenic. Appropriate controls ie a non-lysogen and a known lysogen were always tested simultaneously. The lysogenic colony was subjected to two more single colony isolations, each time several colonies were tested for immunity. After the final single colony isolation all the colonies tested were usually immune.

*186c*I$^+$ and λ*c*I$^+$ lysogens were constructed by the same procedure described above, but incubations were at 37°C

rather than 30°C, unless the bacterial cells were temperature sensitive, in which case they were cultured at 30°C.

An Su^- lysogen of a 186*cI*^{ts}*pBam* mutant was constructed by spotting 10^7-10^8 phage on the Su^- lawn. After overnight incubation at 30°C the centre of the spot was streaked for single colonies and these were tested for lysogeny as described above.

(c) **P1 *transduction*:** *Donor lysate:* The donor cells were cultured in LB broth containing $CaCl_2$ (4mM) to late log phase. 0.2 ml of donor cells and 10^6, 10^7, or 10^8 pfu/ml of P1*vir* or P1kc were incubated at 37°C (or 30°C if temperature sensitive) for 30 min, then plated on a YGC plate. Prior to pouring the plate it was wet with 0.5 ml of LB broth plus $CaCl_2$, in addition a drop of 0.1 M $CaCl_2$ was added to 3 ml of top agar.

The plate was incubated, right side up for 8-10 hr at 37°C or overnight at 30°C. The top agar was harvested by flooding the plate with 3 ml of P1 diluent. After 15 min the agar was scrapped into a centrifuge tube. A few drops of chloroform were added and the agar centrifuged for 10 min at 10,000 rpm at 5°C. The supernatant was millipore filtered, assayed and stored at 4°C. The highest titre lysate was subsequently used.

Transduction: The recipient cells were cultured in LB broth containing $CaCl_2$ (4 mM) to late log phase. To 1 ml of the recipient culture, varying volumes (1, 10, 100 µl) of the P1 lysate raised on the donor were added. 2 ml Sarstedt tubes were used. Absorption was allowed at 37°C for 20 mins or at 30°C for 30 mins. Bacteria were sedimented by centrifugation in an Eppendorf centrifuge for 2 mins at RT and resuspended in LB broth containing 0.1 M sodium citrate. If an antiobiotic marker was being transduced, the tubes were incubated at 37°C or 30°C for at least 60 min, except in the case of Kanamycin resistance, where the incubation was overnight, before plating on selective medium. When nutritional markers were being transduced this step was often omitted. 0.1 ml of each transduction tube was spread on the selection plate and incubated at the required temperature. To assess the background reversion/resistance frequency, the bacterial recipient culture treated as above but without the addition of P1 phage was plated on the selective medium. 0.1 ml of the donor P1 lysate was also spread on the selective plate to confirm the absence of donor bacteria.

(d) Transformation with plasmids: Cells were transformed with plasmids according to the calcium chloride method of Maniatis *et al.*, (1982).

2.2.5 Phage and Bacterial Assays

(a) Viable phage: These were assayed by diluting the phage lysate or infected/induced culture in TM buffer and plating 0.1 ml of the dilution with 0.2 ml of a permissive indicator culture (O.D. 0.8-1.0) and 3 ml of melted top agar on an agar plate. 186, λ and Mu were assayed on Z plates while P1 was assayed on YGC plates. The indicator bacteria were normally cultured in LB broth to mid log phase. The plates were incubated overnight at the required temperature and the plaques subsequently counted.

(b) Viable bacteria: Dilutions of the culture to be assayed were made in TM and spread with a sterile spreader on YGC or minimal plates supplemented with nutritional requirements if any. The plates were incubated overnight or, if necessary, longer and the colonies were counted.

(c) Bacterial nutritional requirements: M9 plates were spread with all the required nutrients except the one being tested and the bacteria were spotted on this plate as well as one which was completely supplemented. Appropriate non-requiring and requiring cells were also spotted onto these plates as controls.

2.2.6 UV-irradiation of Bacteria

Cells to be irradiated were either suspended in prewarmed HI buffer or 10 mM $MgSO_4$ or were irradiated in the

minimal medium in which they were cultured (TPGCAA, M9CAA). The suspension was placed in a glass petri dish, to the depth of 1 mm, on an agitating table and irradiated at a distance of 50 cm at a fluence rate of 1.5 J/m²/sec. The source of the UV light was a 15 W Oliphant Germicidal lamp. The control unirradiated culture was treated identically except for the irradiation. The cultures were infected 3-15 min after irradiationn.

When bacterial survival curves were being measured each dose of UV was delivered in a separate petri dish, simultaneously or consecutively. The cultures were then diluted and the viable cells assayed. The time between irradiation and plating never exceeded 20 min.

UV-sensitivity of bacterial strains (transductants) was tested by streaking single colonies across a YGC plate, covering half of the streak with a glass plate and irradiating the other half with 7-30 J/m² of UV, the plates were then incubated overnight and the sensitivity compared to control cultures streaked on the same plate.

2.2.7 Phage Infection Procedures

(a) Removal of unabsorbed phage by millipore filtration: This method of infection was used in Section 6.1 for phages λ, 186, P1 and Mu.

C600 (E508) cells were cultured in LB broth to an O.D. of 1.0, sedimented by centrifugation for 2 min in an Eppendorf centrifuge at room temperature (R.T.), resuspended in 37°C H.I. buffer and UV-irradiated at R.T. The cells were again sedimented and resuspended in an equal volume of LB broth (prewarmed to 37°C) and transferred to a flask covered in aluminium foil. 1.0 ml of the culture was infected with the appropriate phage at an moa (multiplicity of addition) of 1.0 and incubated at 37°C in a gyrotary shaker for 10 min. The cells were collected on a millipore HA 0.45 nm filter while the unabsorbed phage remained in the filtrate. The infected cells were resuspended from the filter by agitation in 2 ml of prewarmed LB broth, then diluted 10^{-4} into LB broth (prewarmed to 37°C) and phage production was followed by assaying aliquots for pfu/ml. 0 min was the time of addition of phage.

(b) Inactivation of unabsorbed phage by specific antiserum: This was used for both λ and 186 infection of cells cultured in either minimal of rich medium.

In minimal medium: Cells were cultured, in the medium and at the temperature required to an O.D. of 0.3-0.4. If irradiation was required, the culture was exposed to UV for the desired fluence, then phage were added to an moa (multiplicity of addition) of 1.0-20 as required. Cultures were incubated, in flasks wrapped in aluminium foil to prevent photo-reactivation, at the experimental

temperature and 3-5 mins later specific antiserum was added. 5 mins later the culture was diluted either 10^{-4} or 10^{-5} into prewarmed medium and the incubation continued at the desired temperature. Phage production was measured by assaying for pfu/ml at intervals.

In rich medium: The cells were cultured in the medium and at the temperature required to an O.D. of 0.7-0.9, sedimented (2 min in an Eppendorf centrifuge at R.T.), resuspended in either HI buffer or 10 mM $MgSO_4$, and UV-irradiated. If the cells were suspended in H.I. they were sedimented by centrifugation (2 min at R.T. in an eppendorf centrifuge) and resuspended in fresh prewarmed broth. If the cells were in 10 mM $MgSO_4$, 10 x LB broth was added to 1 x after the UV treatment. Cultures were transferred to flasks wrapped in aluminium foil. 0.5 ml of the cells in broth were infected with phage for 3-10 min then specific antiserum was added to a final concentration of 1-3 K, and incubation was continued at the required temperature. 5 mins later the culture was diluted (10^{-4} or 10^{-5}) away from the antiserum into fresh broth (prewarmed) and phage production was followed by assaying for pfu/ml.

0 mins was the time of addition of phage and occurred 3-15 min after UV-irradiation.

2.2.8 Heat induction of the prophage from 186*cIts* and λ*cIts*857 lysogens.

The lysogens were cultured in LB broth at 30°C to an O.D. of 0.8-1.0, the cells were sedimented by centrifugation and were resuspended in HI buffer (prewarmed to 30°C), UV irradiated and then diluted into fresh LB broth (39°C), transferred to a flask covered with aluminium foil and phage production followed by assaying for pfu/ml with time. 0 min was the time of transfer of the cultures to 39°C.

2.2.9 Measurement of radioactive nucleotide incorporation into DNA by TCA-precipitation

This was done essentially as described by Bollum (1966). 50-100 µl of the labelled culture was spotted onto a Whatman GF/A filter and dropped into an ice cold 10% TCA solution. The filters were batch washed with 3 changes of TCA (trichloro acetic acid) followed by two washes in ethanol. After drying at 65°C for at least 40 min, the filters were counted in scintillation fluid in a Packard tricarb or Beckman LS 7500 liquid scintillation spectrometer.

2.2.10 Radioactive labelling of bacterial and phage DNA *in vivo*

Continuous labelling: *thy*$^-$ bacteria were cultured in the required medium overnight then diluted to an O.D. of 0.05 into fresh medium containing thymine (1-2 µg) and [methyl-^{3}H]thymine (2-60 µCi/ml). Incorporation of

thymine was followed by measuring the acid insoluble radioactivity as TCA-precipitable cpm (Section 2.2.9). About 3 generations later (at an O.D. of 0.4 for minimal medium and 0.8 for LB broth), cultures were UV-irradiated and infected with phage as described previously (Section 2.2.7b), except that after irradiation the cultures were transferred to 37°C and all manipulations carried out under a yellow fluourescent National 40 W G.E.C. lamp. After phage infection the cultures were not diluted. Incorporation of radioactivity into total DNA was measured by TCA-precipitation and 200 µl samples were removed for estimation of the labelled phage DNA by hybridization. Incorporation of [^{3}H]thymine was stopped by dilution of the precursor pool with unlabelled thymine (200 µg/ml, final concentration).

2.2.11 Phage DNA preparation

Proteinase K treatment, phenol extraction

Purified DNA from both 186 and λ phage, for hybridization and *in vitro* labelling, was prepared by this method.

High titre stocks of phage (approximately 10^{13} pfu/ml were diluted to 10^{12} pfu/ml in TE buffer. 5-10 ml of the diluted stock were treated with proteinase K as follows:

NaCl and SDS were added to a final concentration of 10 mM and 0.5% respectively. Proteinase K enzyme (1 mg/5ml) was added and the solution incubated at 37°C for 30 min.

The proteinase K treated stock was then phenol extracted and ethanol precipitated as follows:

Redistilled phenol was washed with 1 M Tris buffer pH 9.0. The aqueous phase was removed and the phenol phase was washed 2-3 times with TE buffer. For the final wash the phenol was heated and cooled to R.T. to allow it to equilibrate with the TE buffer.

10 ml of equilibrated phenol were added to the proteinase K treated stock and gently mixed for 5 mins, then centrifuged for 5 min at 10,000 rpm at 25°C. The aqueous phase was removed with a J shaped pasteur pipette.

10 ml of TE was added to the phenol phase and 10 ml of equilibrated phenol was added to the aqueous phase and the above procedure carried out. If no white precipitate was formed at the interface then the two aqueous phases were pooled. If a precipitate was formed, then the aqueous phase was re-extracted with phenol until the interface was clear.

The phage DNA in the aqueous phases was concentrated and separated from residual traces of phenol by ethanol precipitation.

NaCl, to a final concentration of 0.2 M and 2 volumes of redistilled ethanol were added. The solution was thoroughly mixed and allowed to stand in a dry ice/ethanol bath for 5 min, or at -80°C for 30 min, then centrifuged at 10,000 rpm for 15 min at 5°C. The pellet was drained, rinsed with 95% ethanol (-20°C), dried in a dessicator and resuspended in TE buffer (1-2 ml).

Purity of the DNA was checked by performing a spectrophotometric scan on a dilution of the sample, in a Varian Superscan 3 spectrophotometer. If A_{260}/A_{280} and A_{260}/A_{240} were >2.0 the DNA was considered to be free from protein contamination.

200-500 µg of phage DNA were routinely obtained from 5-10 ml of phage suspension (10^{12} pfu/ml).

2.2.12 Radioactive labelling of phage DNA *in vitro* by nick-translation

Solution A:

phage DNA	1 µg/20 µl
DNA polymerase 1	1 µl (1 unit)
dATP (0.25 mM)	1 µl
dTTP (0.25 mM)	1 µl
DNase 1 (10 ng/ml)	1 µl
Cocktail*	6 µl
Water	20 µl

*Cocktail:	1 M $MgCl_2$	2.5 µl
	1 M Tris pH 7.8	25 µl
	1 M β mercaptoethanol	5 µl
	1 mg/ml B.S.A.	27 µl

α^{32}P-dGTP and α^{32}P-dCTP were added to Solution A at a final concentration of 5 µM each.

The mixture was incubated at 14°C for 2 hr and the reaction stopped by adding EDTA to a final concentration of 10 mM. The mixture was extracted with equilibrated phenol and the labelled DNA separated from unincorporated nucleotides on a G-50 Sephadex mini column. The radioactive fractions were counted and those from the labelled DNA peak were pooled and ethanol precipitated.

2.2.13 DNA-DNA hybridization

Preparation of nitrocellulose filters with bound phage DNA: 2.5 cm diameter nitrocellulose filters, Schleicher and Schuell BA 85, 0.45 µm, either precut or punched out before use, were washed in distilled water for 10 min.

Phage DNA was bound to these filters by a modification of the method described by Kafatos (1979).

The DNA isolated from CsCl purified phage was diluted to a concentration of 5 µg/400 µl in 0.4 N NaOH. The

solution was heated at 75°C for 10 min and subsequently chilled on ice. An equal volume of chilled 2 N ammonium acetate was added and the mixture agitated on a vortex mixer and stored on ice.

A stack of dry clean, Kleenex paper towels was covered with a sheet of wet 3 mm filter paper. A washed, wet sheet of nitrocellulose was layered over this. Each of the washed, wet, 2.5 cm diameter filters was placed on this sheet and the air bubbles removed by rolling a pipette gently across the surface.

400 µl of the denatured DNA solution, prepared above, was loaded onto each filter by pouring it through a clean glass tube 12 mm in diameter pressed against the filter. The solution was allowed to drain slowly through the filter, then each filter was rinsed with 200 µl of 1 M ammonium acetate by the method described above.

The filters were air dried and baked at 80°C *in vacuo* for 2 hours. Filters were always used within 24 hrs of preparation.

Prehybridization of the filters: The filters were prehybridized in batches of 10 in glass petri dishes, essentially by the method of Denhardt (1966). 10-20 ml of 10 x Denhardt's solution/4x SSC was added per petri dish and the filters incubated at 65°C for 2-3 hours.

Preparation of hybridisation mixtures: 200 µl aliquots of labelled cells were lysed by a modification of the method of Kuempel (1972).

The aliquots were centrifuged for 3 min in an eppendorf centrifuge at R.T. and the supernatant carefully removed and discarded, the pellet was washed in 50 µl TE and resuspended in 100 µl of 10 mM Tris, 1.0 mM EDTA, 3% sucrose and 1 mg/ml lysozyme and allowed to stand at R.T. for 10 min. The cells were subjected to 1 cycle of freezing in a dry ice/ethanol bath and thawing at R.T.. 350 µl of sterile water and 50 µl of 5 N NaOH were added, the solution was heated at 94°C for 8 mins and chilled, followed by the addition of 50 µl of 5 N HCl and 0.65 ml of 10x Denhardt's solution/4x SSC. The pH of the solution was adjusted to 7 by further additions of acid or alkali if required.

The whole procedure was carried out in 1.5 ml screw capped tubes and the prehybridized filters carrying the phage DNA were added to the hybridisation mixture in these tubes, one filter/tube. The mixture was incubated at 65°C for 15-20 hr.

Washing of nitrocellulose filters: Filters were batch washed by one of the two methods described below.

(a) After the hybridisation at 65°C the filters were removed and batch washed in 3 changes of 3 mM Tris pH 9.0 (Warnaar and Cohen, 1966) at R.T. for 30 min each. The filters were dried *in vacuo* for 60 mins and counted in scintillation fluid.

(b) Filters were washed in two changes of 2xSSC/0.1% SDS at R.T. for 15 min each, followed by two washes in 0.1xSSC/0.1% SDS at 41°C for 15 min each. They were then dried and counted as above.

2.2.14 Extraction of Plasmid DNA

Three methods were used to extract plasmid DNA.

(a) Modified Birnboim and Doly (1979) method I:

2 ml of cells were centrifuged in 2 ml low temperature SARSTEDT tubes for 3 min in an eppendorf centrifuge at 37°C and the supernatant carefully decanted off. The pellets were gently resuspended in 90 µl of TE containing sucrose (15%), 10 µl of lysozyme (1 mg/100 µl) was added (Solution I), gently mixed and held for 30 mins on ice. 200 µl of freshly prepared 0.2 N NaOH/0.1% SDS solution (Solution II) was added, the tube was gently inverted to mix the solutions and held on ice for 5-7 mins. 150 µl of 3 M sodium acetate (chilled) (Solution III) was added, again the tube gently inverted to facilitate mixing and after 60 min on ice, the mixture was centrifuged at R.T. for 3 min. The supernatant was recentrifuged in an

Eppendorf tube for 4 min at R.T. to remove all traces of the white precipitate.

Two volumes of ethanol were added and the tubes held at -20°C for 30 mins, followed by centrifugation for 4 min at R.T. The pellet was drained, rinsed with 70% ethanol, dried in a Savant speed vac concentrate or vacuum desiccator and resuspended in 10 µl of TE.

(b) Modified Bimboim and Doly (1979) method II:

Cells were suspended in solution I and held on ice for 4 min. Solution II was added and the mixture was held on ice for 5-7 min. Solution III was added and the incubation on ice continued for 10 min. The ethanol precipitation was carried out at R.T. for 2 min. The rest of the procedure was the same as described in 2.2.14(a).

(c) The rapid or Grosveld et al. (1981) method:

1-2 ml of the culture was centrifuged for 1 min at R.T. in an Eppendorf centrifuge. The pellet was resuspended in 100 µl of glucose (50 mM), Tris HC1 pH 8.0 (25 mM), EDTA (10 mM) and held at RT for 5 mins. 200 µl of NaOH (0.2 N)/SDS (1%), made fresh, was added and the mixture kept on ice for 5 mins followed by the addition of 3 M sodium or potassium acetate and incubation on ice for 5 min. The mixture was centrifuged for 2 min at R.T. and the supernatant was recentrifuged if necessary. 2 volumes of ethanol were added and the mixture held for 2 min at R.T.

The pellet was sedimented by a 2-4 min centrifugation at R.T. in an Eppendorf centrifuge, washed with 70% ethanol, dried *in vacuo* and resuspended in TE buffer.

2.2.15 Restriction analysis of DNA

This was performed by the methods recommended in the New England Biolabs catalogue 1983-84.

2.2.16 Agarose Gel Electrophoresis

1% agarose was prepared in TAE buffer and dissolved by heating. When the solution had cooled to 65°C, 10-25 ml were poured onto horizontal glass slides (5 x 7 or 8 x 8 cm) using the required comb. The gel was immersed in TAE buffer in a horizontal apparatus. The samples were loaded in glycerol loading buffer and electrophoresed at R.T. at a constant current of 150-200 mA. Within 40-60 min the bromo-phenol-blue dye had run off the bottom of the gel. The gel was then removed, stained with ethidium bromide (0.0004% w/v in 1 x TAE) and photographed using short wavelength UV from a transilluminator model TS-36, UV products Inc., with a Polaroid camera using land pack positive/negative film.

2.2.17 Fluorography of ^{3}H-labelled DNA on agarose

The gel was prepared for fluorography by a modification of the method of Laskey and Mills (1975).

After the gel was photographed it was soaked in two, 30 min changes of methanol (about 50 ml each). The gel was

then soaked, agitating in a solution of 8% naphthalene and 1% PPO (Gill *et al.*, 1981) for 60 min at R.T.

The gel was then washed in two changes of distilled water (10 min each) and dried on 2 sheets of Whatman 3 mm paper on a slab gel drier model SE 1140 from Hoefer Scientific Instruments at about 40°C for 40-60 min. The gel shrank slightly during this treatment.

The dried gel was placed in contact with Fuji X-ray film (RX) and the film was exposed at -80°C for various times (6-48 hr).

2.2.18 Statistical methods

(i) the mean and standard deviation of samples were calculated using the formulae:

$$\text{mean} \quad \bar{x} = \frac{1}{n} \sum x$$

where the sample had n number of observations of x_1, $x_2 \ldots\ldots, x_n$

and sample standard deviation $S_x = \sqrt{Sx^2}$

where $Sx^2 = \frac{1}{n-1} \sum (x - \bar{x})^2$

(Bailey, 1968, Wilks, 1961)

(ii) When the mean of one sample, $\text{Sample}_{(1)}$ was substracted from the mean of another ($\text{Sample}_{(2)}$) the standard deviation of the difference was given by the formula:

$$= \sqrt{Sx_1^2 + Sx_2^2}$$

where Sx^2 refers to the variance of each sample.

(iii) Confidence limits for the unknown population mean μ were calculated using the 'Student's' t-distribution.

$$\mu = \bar{x} \pm t \frac{s}{n}$$

where t is a value dependent on the degrees of freedom (n-1) and the probability (α or P). The appropriate value of t was obtained from Wilks (1961).

(iv) A one-tailed test was performed by using the t values for twice the desired probability e.g. the t value for 0.9 (or 0.1) was used to obtain 95% confidence limits (Bailey, 1968).

(v) The Poisson distribution was used as described by Ellis and Delbruck (1939), to predict the number of samples containing 1 or more cells.

The fraction p_r of samples containing r particles (cells in this case) is given by:

$$p_r = \frac{n^r e^{-n}}{r!}$$

where n is the average number of particles in the sample. If n is unknown it can be calculated from the experimental value of p_r e.g. from p_0 (i.e. the fraction of samples not containing any particles) $n = -\ln p_0$

The fraction p_r was then multiplied by the total number of samples to obtain the number of samples predicted to contain r particles (cells).

CHAPTER 3

UV-INDUCED INHIBITION OF 186 DNA SYNTHESIS

The loss in 186 capacity in UV-irradiated cells is due to the inability of the phage to replicate its DNA in these cells (Hooper *et al.*, 1981). As discussed in Chapter 1 the inability to replicate 186 DNA is not due to a block in transcription of the phage replication genes, since early RNA synthesis is normal in UV-irradiated cells (Hooper *et al.*, 1981).

This chapter re-examines the inhibition of 186 DNA replication which occurs in UV-irradiated cells.

3.1 METHODOLOGY FOR STUDYING 186 DNA SYNTHESIS

Hooper *et al.* (1981) studied replication of 186 DNA in UV-irradiated cells, by pulse-labelling thy^+ cells with [^{3}H]thymidine. However, there are reservations about the validity of studying DNA replication in UV-irradiated cells by this method (Smith and O'Leary, 1968). Degradation of DNA after UV-irradiation can increase the level of free unlabelled thymine, thus diluting the endogenous pool of radio-labelled nucleotides. This can result in a reduction in the amount of [^{3}H]thymine incorporated into replicating DNA, and lead to an artificial depression in DNA synthesis. Smith and O'Leary (1968) recommended a different protocol for studying the effect of radiation on DNA synthesis kinetics. The protocol involved labelling bacterial DNA with a labelled precursor (e.g. [^{3}H]thymine), for several generations both before and after UV-irradiation, preferably using mutant cells deficient in the synthesis of the

precursor used (e.g. *thy*-cells). They also recommended that the data be plotted as log cpm incorporated versus time. In support of this protocol, Doudney (1971) showed that in a *thy*-mutant prelabelled with [^{3}H]thymine, incorporation of the label into DNA was directly related to the quantity of DNA synthesised in the cells.

It was decided to confirm the transient UV-induced inhibition in 186 DNA synthesis observed by Hooper *et al.* (1981), by examining 186 DNA replication in irradiated and unirradiated cells in which the DNA had been prelabelled with [^{3}H]thymine. Synthesis of 186 DNA was measured by DNA-DNA hybridisation.

3.2 STANDARDISATION OF HYBRIDISATION CONDITIONS

3.2.1 Attachment of unlabelled 186 DNA to nitrocellulose filters

The DNA filters were prepared by a modification of the method described by Kafatos (1979). The efficiency of binding of DNA to the nitrocellulose was measured using 186 DNA labelled, by nick-translation, with ^{32}P (see Section 2.2.12). Using the method described in Section 2.2.13, approximately 85% of the DNA applied to the nitrocellulose filters was retained. (If the DNA was applied in a more concentrated solution 5 µg/200 µl instead of 5 µg/400 µl, only 60% of the DNA was retained, so 5 µg/400 µl was routinely used.) The filters were then baked *in vacuo* and incubated overnight in the prehybridisation solution. After

this treatment approximately 98% of the DNA originally bound to the filter was still retained.

3.2.2 Efficiency of hybridisation

The efficiency of hybridisation was measured and optimised by determining the extent of hybridisation of purified ^{32}P-labelled 186 DNA to unlabelled 186 DNA attached to nitrocellulose filters. The extent of hybridisation is expressed as the radioactivity (as cpm) bound as a percentage of the input cpm.

Initially, the effect of altering the amount of unlabelled DNA attached to the filters, on the percentage hybridisation was examined. This was to determine the amount of DNA that was non-limiting, to be used per filter in subsequent experiments. The amount of labelled DNA added in these tests was 200 ng per filter since this is in excess of the maximum amount of 186 DNA expected in an aliquot of 200µl of 186-infected cells.#

#Maximum amount of 186 DNA/200 µl aliquot of infected cells:

If the culture has 2 x 10^8 cfu/ml
the aliquot (200 µl) has 4 x 10^7 cfu
given a burst of 100 pfu/infected cell,
the number of phage/aliquot (200µl) = 4 x 10^9pfu.

The molecular weight of 186 phage DNA is about 2 x 10^7

Using Avogadro's number
6 x 10^{23} molecules of 186 = 2 x 10^7g = 2 x 10^{13} µg

Therefore the mass of 4 x 10^9 molecules of 186 = 0.133 µg
= 133 ng

This is the upper limit since the aliquots are removed prior to lysis, when the number of pfu/cell is lower than the final burst size.

Table 3.1a shows that 200 ng of labelled 186 DNA hybridised to 2 and 4 μg of unlabelled DNA (attached to filters), with an efficiency of approximately 40% (calculated as a percentage of the input cpm binding to the filters). In the absence of DNA on the filter, the efficiency was 0.3%.

Although 2 μg of DNA on the filter was not limiting for hybridsation of 200 ng of labelled 186 DNA, it was decided to use an excess (5 μg/filter) to ensure that the DNA on the filter was not a limiting factor in subsequent experiments.

The ultimate intention was to study replication of 186 DNA by hybridisation, using lysates of 186-infected cells and not purified DNA. Therefore, to test whether the presence of the bacterial debris had any effect on the efficiency of hybridisation, ^{32}P-labelled 186 DNA was added to aliquots of bacterial lysates (E251 cells) (prepared as in section 2.2.13) and the mixtures were denatured and incubated with 186 DNA (5 μg attached per filter). Table 3.1b shows that hybridisation of 186 DNA was not affected by the presence of the lysate and remained about 40% efficient.

Wahl *et al.* (1979) reported that sodium dextran sulphate improved the efficiency and shortened the time required for DNA-DNA hybridisation in a two-phase system. However, addition of sodium dextran sulphate 10% (w/v) did

TABLE 3.1

EFFICIENCY OF 186 DNA-DNA HYBRIDISATION

Expt. No.	µg DNA/ filter	addition to hyb^{n} mix	ng of ^{32}[P-] -186 DNA added	input 186 cpm	cpm bound to filters*	Percentage 186 hybridisation
a.	4	none	200	1.48×10^5	65835 ± 1.6%	44.4
	2	none	200	1.48×10^5	62520 ± 20%	42.0
	0	none	100	1.45×10^5	570	0.3
b.	5	Bacterial lysate	100	5.8×10^4	24043 ± 13%	43
	0	Bacterial lysate	100	5.8×10^4	60	0.08

* mean of duplicate ($\bar{x}$) + % standard deviation (Sn-1)

Purified 186 DNA was labelled with ^{32}P by nick-translation as described in Section 2.2.12. Unlabelled 186 DNA was added to obtain the required concentration (see table). Samples were denatured as described in Section 2.2.13 and added to nitrocellulose filters carrying the indicated amount of unlabelled 186 DNA. Hybridisations were carried out as described (Section 2.2.13)

a. Purified ^{32}P-labelled 186 DNA added to different amounts of unlabelled 186 DNA attached to nitrocellulose filters.

b. Bacterial lysates were prepared as in Section 2.2.13. ^{32}P-labelled 186 DNA was added to the lysates. The mixture was denatured and hybridised to 186 DNA attached to filters, as described in Section 2.2.13.

not significantly increase the percentage hybidisation of 186 DNA after incubation at 65°C for 15 hours (data not shown). Hybridisation was complete within 15 hours whether sodium dextran sulphate was present or not.

On the basis of the data in this section, hybridisations were performed for 18-20 hours at 65°C, using 5 µg of unlabelled 186 DNA bound to nitrocellulose filters.

3.2.3 Linearity of hybridisation

While optimising the conditions for hybridisation, the extent of 186 DNA-DNA binding was measured using *in vitro*-labelled (nick-translated) 186 DNA. The planned experimental procedures however, involved 186 DNA labelled *in vivo*. Therefore it was necessary to determine whether the amount of DNA hybridised, varied linearly with the amount added. This included testing whether or not 5µg of 186 DNA attached to the filters was limiting over the same range.

Cells of a 186*cI*^{ts} lysogen were pre-labelled with [^{3}H]thymine for several generations. The prophage was heat-induced and after 40 minutes the cells were lysed. DNA in the lysate was denatured and varying volumes were added to unlabelled 186 DNA on filters (see Section 2.2.13). After hybridisation the filters were washed and the amount of radioactivity (as cpm) retained was measured.

In addition, the total phage produced in the experiment and the burst size per cell were measured 90 minutes after heat-induction. 4 x 10^9 pfu/ml were produced in total with an average of 75 pfu/cell.

In these experiments, the efficiency of 186 hybridisation could not be measured directly by the percentage of input cpm, bound to the filter, since the aliquot contained ^{3}H-labelled *E. coli* DNA as well as ^{3}H-labelled 186 DNA. Instead the 186-specific cpm were calculated as the hybridised cpm derived from the 186 infected culture minus those derived from the uninfected culture. The values are plotted in Fig 3.1. 186 specific cpm hybridised linearly for cellular aliquots from 100 - 500µl in volume. Since it was intended to measure hybridisations using 200 µl aliquots of 186 infected cells, it was concluded that 186 hybridisation was linear within a range from 0.5 to 2.5 times the amount of 186 DNA to be used.

3.2.4 Media for studying phage infection and DNA replication

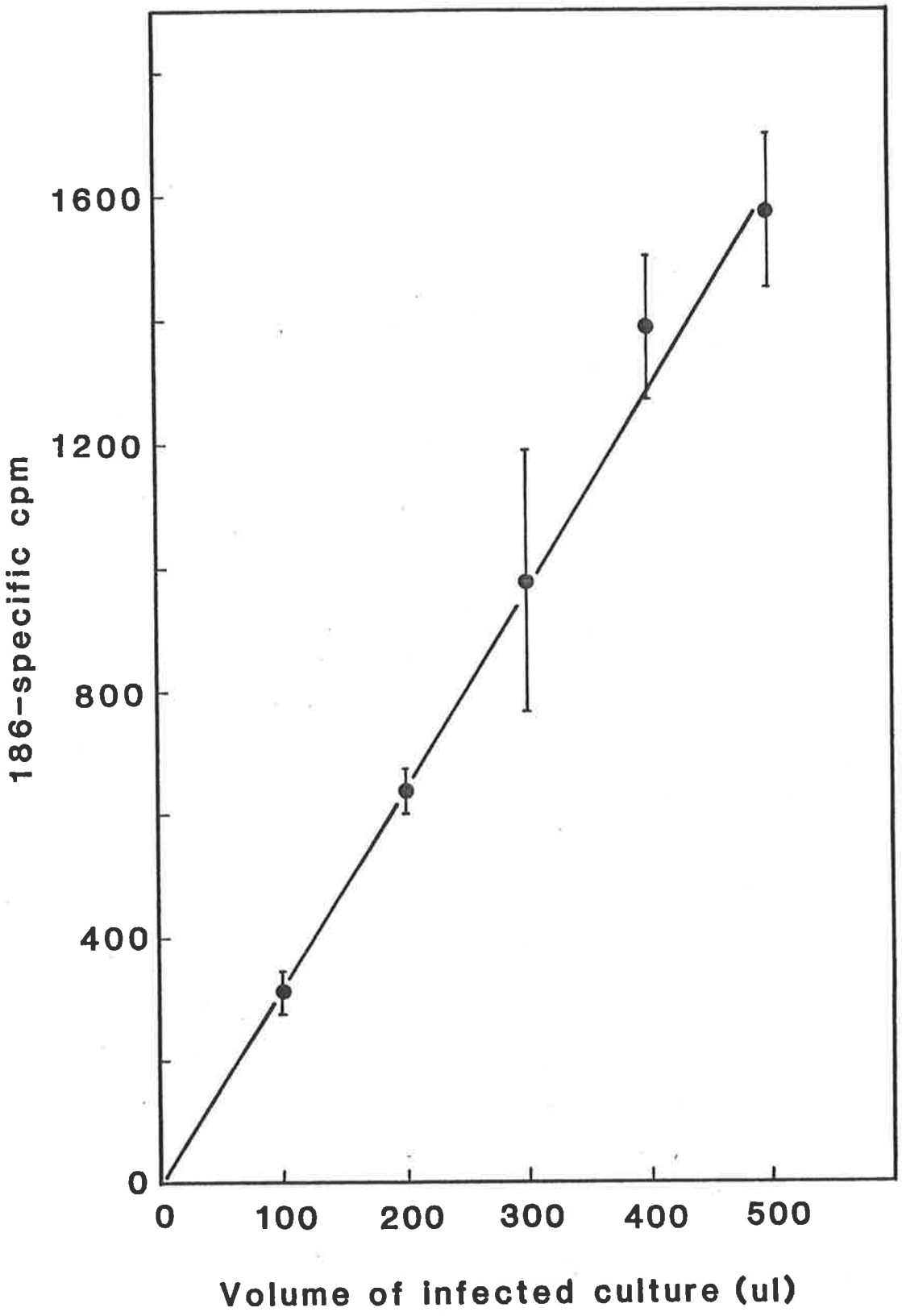
The latent period of 186 phage production was usually determined in bacteria cultured in LB broth. In experiments in which the cultures were UV-irradiated, the cells were collected by centrifugation, resuspended in HI buffer or 10 mM $MgSO_4$, and irradiated. The cells were then restored to LB broth and infected with the phage. (It is necessary to

FIG 3.1 Linearity of 186 DNA-DNA hybridisation using in vivo labelled DNA

thy-cells lysogenic for 186*cIts* phage(E4064) and control non-lysogenic *thy*-cells (E4058) were grown in LB broth containing [^{3}H]thymine (16 µCi/µg/ml) to pre-label the DNA. The cultures were heat-induced by incubation at 39°C for 40 minutes. Aliquots 100-500 µl were removed and lysates were lysed by the method of Kuempel (1972) (Section 2.2.13). The DNA was denatured and hybridised (see Section 2.2.13) to filters carrying unlabelled 186 DNA (5 µg/ml).

The filters were washed and the radioactivity bound to the filters (as cpm) was counted. 186-specific cpm were calculated by subtracting the bound cpm derived from the non-lysogenic culture (background), from those derived from the 186-lysogenic culture.

The mean of duplicate values (given by the vertical bars) is plotted.


1600
1200
800
400
0
186-specific cpm
0
100
200
300
400
500
Volume of infected culture (ul)

irradiate in buffer as LB broth absorbs the UV light and protects the cells from irradiation).

For experiments where the cells were continuously labelled with [^{3}H]thymine, this method was inconvenient because the label had to be maintained in the buffer and through all the centrifugation steps. Therefore, a different method was devised. Cultures were grown in minimal medium (TPGCAA) containing thymine and [^{3}H]thymine at the desired concentrations. Cells were irradiated in this medium and were subsequently infected with 186. Hooper (1979) showed that TPGCAA does not significantly reduce the dose of UV-radiation received by the cells relative to the dose received by cells irradiated in HI buffer. The absorption of TPGCAA at 260 nm is 1-2 OD units, whereas that of LB broth is 45 OD units. Also the survival of *uvrA* mutant bacteria was not significantly different when UV-irradiated in either TPGCAA or HI buffer (Hooper, 1979).

One possible disadvantage of the above method was irradiation of thymine in the medium, which could damage the precursor. However, UV-irradiation of the medium does not reduce the amount of thymine to limiting levels (Williams, 1982). Williams (1982) found that prior irradiation (45.6 J/m^2 of UV) of minimal medium containing thymine (1-2 µg/ml) did not reduce the rate of DNA replication of a *thy*-mutant. In addition, when *thy*-cells, irradiated in TPGCAA and thymine recover the ability to synthesis DNA, they do so at

a rate similar to the preirradiation rate. Therefore, cells were cultured and irradiated in TPGCAA and thymine. To study DNA synthesis in the culture, [^{3}H]thymine was added several generations prior to irradiation and incubation was continued without any further additions of the radioactive precursor.

3.3 186 PHAGE INFECTION AND BURST IN IRRADIATED AND UNIRRADIATED CELLS

186 infection of cells cultured in TPGCAA was examined as a prelude to studying the replication of 186 DNA in cells cultured in this medium.

C600 *thy*$^-$ cells (E237) were cultured in TPGCAA containing thiamine (5 µg/ml) and thymine (2 µg/ml) to an OD of 0.2-0.4 186 *cIts* phage were added at a mutiplicity (moa) of 20 and incubation was continued for 5 minutes at 37°C. Unabsorbed phage were inactivated using 186 specific antiserum. After 5 minutes the culture was diluted away from the antiserum and assayed for infectious centres and later for free phage. Comparing the number of infectious centres with the viable cell count at the time of infection shows the percentage of cells which were infected. Under the conditions described above, close to 100% cell infection was achieved. Free phage produced during the course of the experiment were assayed on C600 indicator bacteria.

In the unirradiated culture, there were about 5 x 10^7 infectious centres. 40 minutes after infection the cells started to lyse and release free phage. The phage burst was about 50 pfu/ml (Fig 3.2). Cells irradiated with 45 J/m^2 of UV prior to infection had a reduced capacity to produce 186 phage. The burst size was delayed and smaller than that obtained with unirradiated cells (Fig 3.2).

The loss in 186 capacity was thus demonstrable in TPGCAA, so this medium was used in experiments involving labelling of cells with [^{3}H]thymine to study 186 DNA replication.

The linearity of hybridisation studied previously (Section 3.2.3), was measured with cells grown in LB broth instead of the TPGCAA medium used here. However, the total number of phage produced (4 x 10^9) and the burst size obtained (75 pfu/ml) in LB broth were similar to those obtained in TPGCAA. Therefore 186 hybridisation should also be linear for 186-infected cells cultured in TPGCAA medium.

3.4 REPLICATION OF 186 DNA IN PRELABELLED UV-IRRADIATED C600 *thy*- CELLS

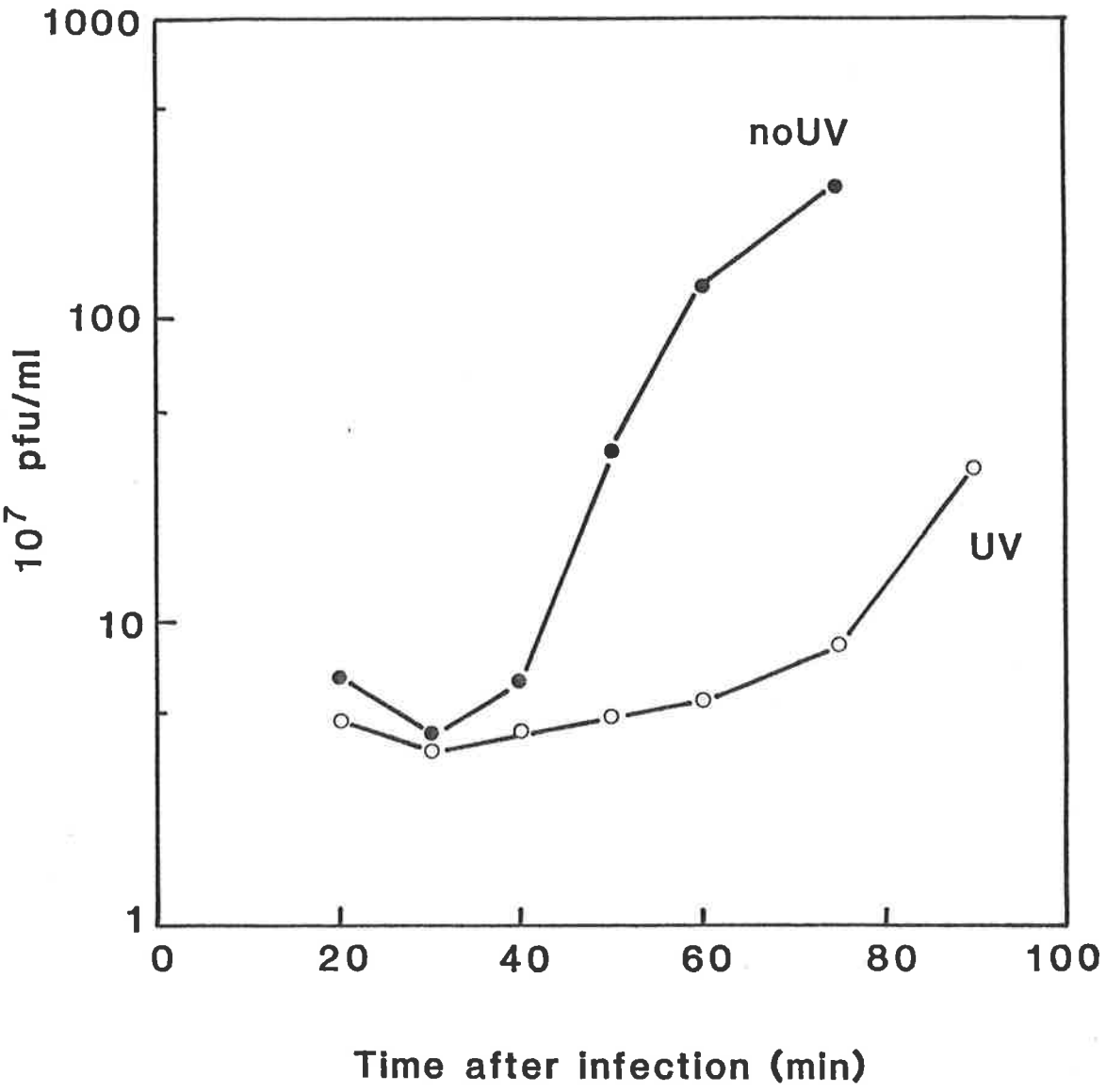
Cells of strain C600 *thy*- (E237) were cultured in TPGCAA containing thiamine and [^{3}H]thymine. At the required density the culture was irradiated with 0 and 45J|m^2 of UV.

FIG 3.2 Effect of UV-irradiation of cells on production of 186 phage in TPGCAA medium

Cells of strain C600 thy- (E237) were cultured in TPGCAA containing thymine (2 µg/ml) and thiamine (5 µg/ml) at 37°C to an OD 0.2. The cells were irradiated with 0 or 45 J/m² of UV. 186 *cIts* phage were added at 0 minutes at a multiplicity (moa) of 20 and infection was continued at 37°C. After 5 minutes unabsorbed phage were inactivated with 186 specific antiserum. Incubation was continued at 37°C and aliquots were removed at the times indicated and assayed for pfu on C600 indicator bacteria.

(● - ●) unirradiated cells

(○ - ○) 45 J/m²-irradiated cells.


1000
100
10
1
10^7 pfu/ml
noUV
UV
0
20
40
60
80
100
Time after infection (min)

The unirradiated and irradiated cultures were infected with 186 phage or left uninfected. Unabsorbed phage were inactivated using antiserum. Aliquots were removed 0, 15 and 35 minutes ater infection, to determine the 186 DNA replication. The DNA in the aliquots was denatured and hybridised to unlabelled 186 as described (Section 2.2.13). *E. coli* DNA synthesis was followed in uninfected control cultures, by measuring the acid-insoluble radioactivity. *E. coli* DNA synthesis proceeded linearly in the unirradiated culture (Fig 3.3a), but in the irradiated culture it was inhibited for a time after irradiation, after which it recovered with a rate similar to that in the unirradiated control (Fig 3.3a).

As described in the protocol above, unlabelled phage were used to infect prelabelled cells, so any radioactivity (cpm) bound to the 186 DNA filters, above background is an indication of 186 replication.

The 186-specific cpm were measured as the radioactivity (cpm) bound with 200 µl of 186 infected cells, minus that bound with 200 µl of uninfected cells (background), and were treated as a measure of 186 DNA replication.

The data are shown in Table 3.2. 186-specific cpm (Table 3.2b) were absent 0-1 minutes after infection of both unirradiated and UV-irradiated cells. In the unirradiated

cells replication commenced sometime between 1 and 15 minutes. From 15 minutes to 30 minutes 186-specific cpm increased 10 fold. Therefore, 186 replication was detectable in unirradiated, 186-infected cells.

Replication of 186 DNA did not occur up to 15 minutes after infection, in cells which had been UV-irradiated (45 J/m^2) prior to infection. By 30 minutes some 186-specific cpm were detectable but these were 10-fold less than those obtained with the unirradiated cells.

Fig 3.3b is a diagrammatic representation of the data in Table 3.2. For the purposes of plotting the data on a log scale, the negative values (0 minutes and 15 minutes in the irradiated sample) have been treated as 1 cpm. It is clear from Fig 3.3b and Table 3.2 that the replication of 186 DNA is delayed in UV-irradiated cells. The small number of samples means that the length of the delay cannot be accurately measured but it must be between 15 and 30 minutes under the conditions used in this experiment.

Although unlabelled phage DNA was used to infect labelled cells, this was not a point of concern for two reasons:

(i) The infecting phage were circular, undamaged molecules which should not be susceptible to UV-induced exonuclease attack.

TABLE 3.2

EFFECT OF UV ON SYNTHESIS OF 186 DNA

	cpm bound*					
UV dose	UNIRRADIATED			45 J/m² IRRADIATED		
TIME (min after infection)	0	15	35	0	15	35
a.						
186 infected	122 ± 17	317 ± 30	1767 ± 10	106 ± 2	75 ± 22	272 ± 20
uninfected	129 ± 10	168 ± 63	93 ± 1	120 ± 16	96#	111 ± 4
b.						
186-specific cpm	-7 ± 20	149 ± 69	1674 ± 10	-14 ± 16	-21 ± 22	161 ± 20

* mean of duplicates ± standard deviation

\# the duplicate sample was lost.

Cells of strain C600 thy- (E237) were cultured at 37°C in TPGCAA containing thiamine (5 µg/ml) and [³H]thymine (20 µci/2 µg/ml) for 3 generations to an OD 0.4 cells were irradiated with 0 or 45 J/m² and were infected with 186 (moa 20) or left uninfected. After 5 minutes, unabsorbed phage were inactivated with 186-specific antiserum. Incubation was continued at 37°C. Samples were removed from the cultures . The cells were lysed and the DNA hybridised to 186 (5 µg attached to nitrocellulose filters) as described in Section 2.2.13. The filters were washed and the radioactivity (as cpm) was counted

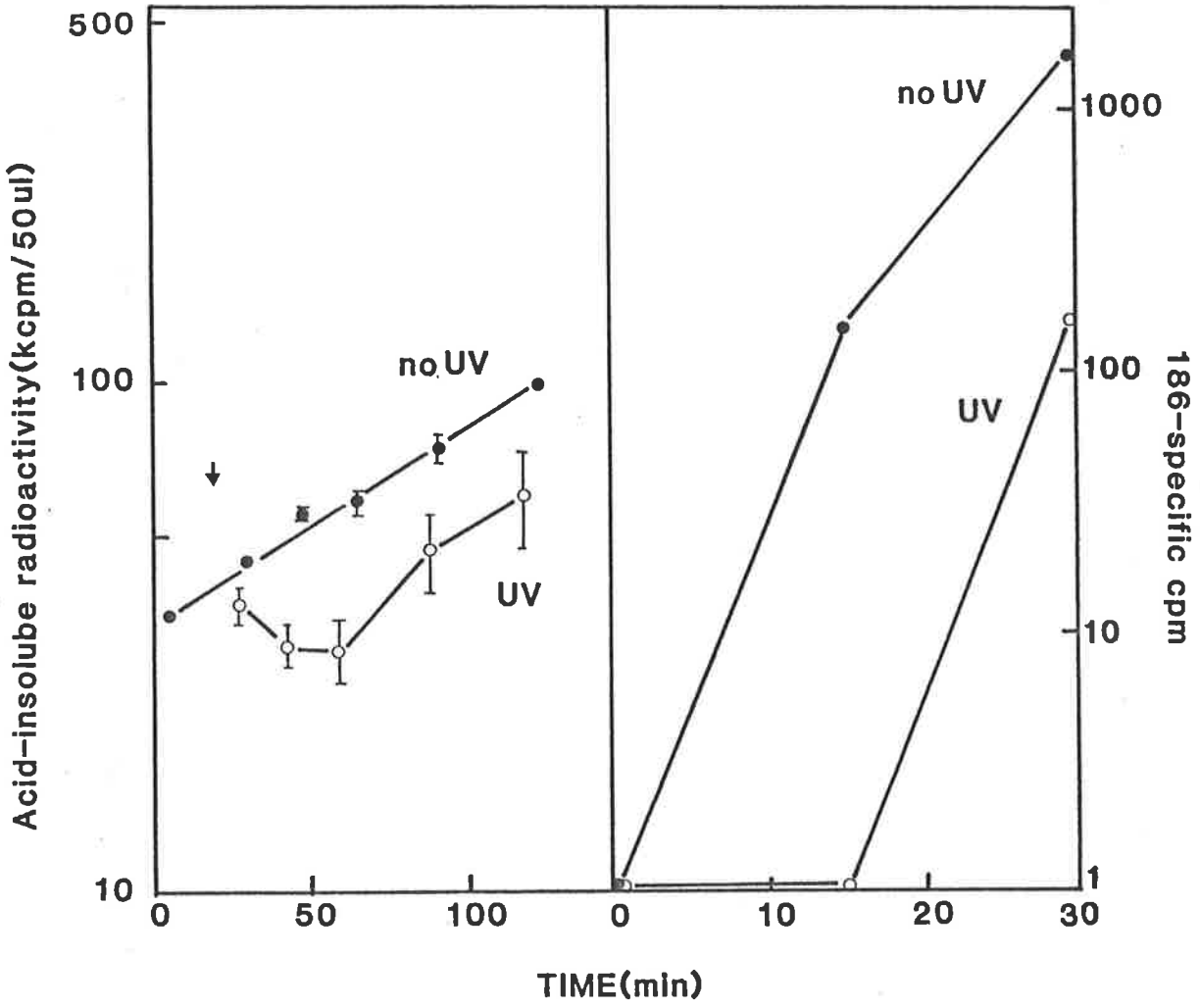
a. The cpm bound from uninfected and 186-infected cultures ± standard deviation (Sn-1).

b. 186 specific cpm = (mean cpm bound with 186 infected cells$_{(1)}$) - (mean cpm bound with uninfected cells$_{(2)}$) ± $\sqrt{S.D.^2_1 + S.D.^2_2}$

FIG 3.3 Effect of UV on the synthesis of *E. coli* DNA (a.) and 186 DNA (b.)

a. C600 *thy*-cells (E237) were cultured in TPGCAA containing thiamine (5 µg/ml) and [^{3}H]thymine (20 µCi/2 µg/ml) for 3 generations at 37°C and were irradiated with 0 or 45 J/m^2 of UV. Samples (50 µl) were removed at intervals after irradiation and tested for the amount of acid-insoluble cpm incorporated into chromosomal DNA (see Section 2.2.9). Cultures were irradiated at the time indicated (↓).

b. Cells treated as above were infected with (moa 20) (0 minutes in the figure) after the irradiation. Aliquots were removed from 186-infected and control uninfected cultures at various times and hybridised to unlabelled 186 DNA (5 µg) as described in Section 2.2.13. The values were taken from Table 3.2.


500
100
10
Acid-insolube radioactivity(kcpm/50ul)
no UV
UV
0
50
100
0
10
20
30
TIME(min)
no UV
UV
1000
100
10
1
186-specific cpm

(ii) At 30 kb the 186 genome is a little less than 1% the size of the *E. coli* genome. Assuming 20 phage (an moa of 20 was used for the experiment) infect each cell, breakdown, if it occurs, would contribute at most 20% non-radioactive precursors to the total breakdown pool so that this dilution effect could not account for the 90% depression in 186-specific cpm seen in the experiment.

3.5 CONCLUSION

The results described in this chapter show that replication of 186 DNA is inhibited in UV-irradiated, continuously-labelled cells of *E. coli*, confirming the data of Hooper et al. (1981). Therefore the loss in 186 capacity was concluded as being due to the transient inhibition of 186 DNA replication in irradiated cells. Since the loss in 186 capacity was an easier parameter to study than 186 DNA synthesis, it was used in the following experiments to determine the cause of the inhibition.

CHAPTER 4

NON-DEPENDENCE OF 186 DNA REPLICATION ON *E. coli* CHROMOSOMAL REPLICATION

One explanation for the UV-induced loss in 186 capacity is that 186 DNA replication requires host DNA synthesis *per se* i.e. replication of 186 DNA can take place only in actively replicating host cells. If this is so, the delay in 186 phage production after UV-irradiation of the host would be explained by the transient inhibition in host DNA replication. This possibility is discussed in Chapter 1.

The possibility would be disproved if a situation existed where *E. coli* DNA-synthesis was inhibited, but 186 phage were produced. But, all the physical and chemical inhibitors of DNA synthesis which have been tested (UV, mitomycin C, nalidixic acid), as well as all the *dna* mutants tested (*dnaA*, *dnaC*, *dnaB*) inhibited the replication of 186 as well as *E. coli*, so that such a situation has not yet been discovered.

The UV-irradiated *uvrA* cell offered another system for testing non-dependence of 186 on *E. coli* DNA synthesis. Irradiation of an excision-repair deficient mutant of *E. coli*B with 20 J/m² of UV, completely inhibits host chromosomal replication for at least 80 minutes. (Billen and Carriera, 1971). This may also be true of an excision repair deficient mutant of the *E. coli* K-12 strain, which is the strain used extensively for the study of 186 replication and phage production. If UV-irradiation does inhibit host DNA replication for long periods of time, it

would be informative to see whether 186 phage can be produced while host replication is inhibited.

To test this isogenic *thy*$^-$ *uvrA*$^+$ and *thy*$^-$ *uvrA*$^-$ strains were constructed. They were then tested to find whether a moderate fluence of UV inhibited *E. coli* DNA synthesis for an extended period of time.

4.1 CONSTRUCTION AND UV-SENSITIVITY OF *uvrA* DERIVATIVES

Isogenic *uvrA*$^+$ and *uvrA6* derivatives of strain C600 *thy*$^-$ were constructed by P1 transduction of the *uvrA* locus with *malE*::Tn5. The *uvrA* and *malE* loci map at 92 min on the *E. coli* K-12 linkage map (Bachmann, 1983). Firstly, Kanamycin resistance (Tn5) was transduced from a *uvrA*$^+$ *malE*::Tn5 mutant (E818) into AB1157 *uvrA6* (E750) to give AB1157 *uvrA6 malE*::Tn5. From this strain kanamycin resistance was transduced into C600 *thy*$^-$ (E237) cells. UV-sensitive (*uvrA6*) and UV-resistant (*uvrA*$^+$) derivatives were isolated (See section 2.2.4C) and purified to give the required strains.

E4051 - C600 *thy*$^-$ *uvrA6 malE*::Tn5 and

E4057 - C600 *thy*$^-$ *uvrA*$^+$ *malE*::Tn5

The survival of strain C600 *thy*$^-$ *uvrA*$^+$ and its *uvrA6* derivative was assayed after irradiation with varying doses of UV. The results are recorded in Fig 4.1. Survival of the *uvrA6* mutant was slightly better on minimal (M13) than on rich (YGC) medium. As expected, the *uvrA6* mutant was

much more sensitive to UV-irradiation than the *uvrA*$^+$ excision proficient strain.

4.2 DNA REPLICATION IN UV-IRRADIATED *uvrA*$^+$ AND *uvrA6* CULTURES

C600 *thy*$^-$ *uvrA*$^+$ (E4057) and C600 *thy*$^-$ *uvrA6* (E4051) were cultured in TPGCAA containing thymine and prelabelled with [^{3}H]thymine for three generations. DNA replication was followed by measuring the acid-insoluble radioactivity (as cpm) before and after irradiation.

Unirradiated cultures of the *uvrA6* mutant replicated DNA at approximately the same rate before and after the time of irradiation (Fig 4.2). The slight depression in rate immediately after irradiation was probably due to the cells being transferred to a petri dish for the time that the test samples were being irradiated. An unirradiated culture of the *uvrA*$^+$ cells gave similar results (data not shown).

After irradiation with 45 J/m^2 of UV. the control *uvrA*$^+$ culture showed a 20-30 minute delay in DNA replication after which it recovered (Fig 4.2), with a rate similar to its unirradiated control. In contrast, the DNA synthesis in the *uvrA6* culture irradiated with 45 J/m^2 was inhibited and did not recover before the end of the experiment i.e. 70 minutes after irradiation. Therefore a dose of 45 J/m^2 of UV inhibits DNA replication of the *uvrA6* mutant for at

FIG 4.1 Survival curves of *uvrA*$^+$ and *uvrA6* cultures

C600 *thy*$^-$ *uvrA*$^+$ (E4057) and C600 *thy*$^-$ *uvrA6* (E4051) cells were cultured in TPGCAA medium containing thymine (2µg/ml) to an OD of 0.2. Samples were UV-irradiated with the desired fluences. Each irradiation was carried out in a separate dish. The cells were diluted in TM buffer and plated on minimal plates (M13 containing thymine 50 µg/ml, leucine 20 µg/ml and threonine 20 µg/ml) and YGC plates (containing thymine 20 µg/ml, which were incubated at 37°C for survivors. Colonies on YGC plates were counted after 24 hours incubation. Minimal plates were incubated for a further 24 hours to allow the colonies to reach a countable size.

(▲ - ▲) *uvrA*$^+$ cells on YGC plates

(△ - △) *uvrA*$^+$ cells on minimal plates

(▼ - ▼) *uvrA6* cells on YGC plates

(▽ - ▽) *uvrA6* cells on minimal plates

Duplicate samples were tested at the relevant doses in the *uvrA6* culture.

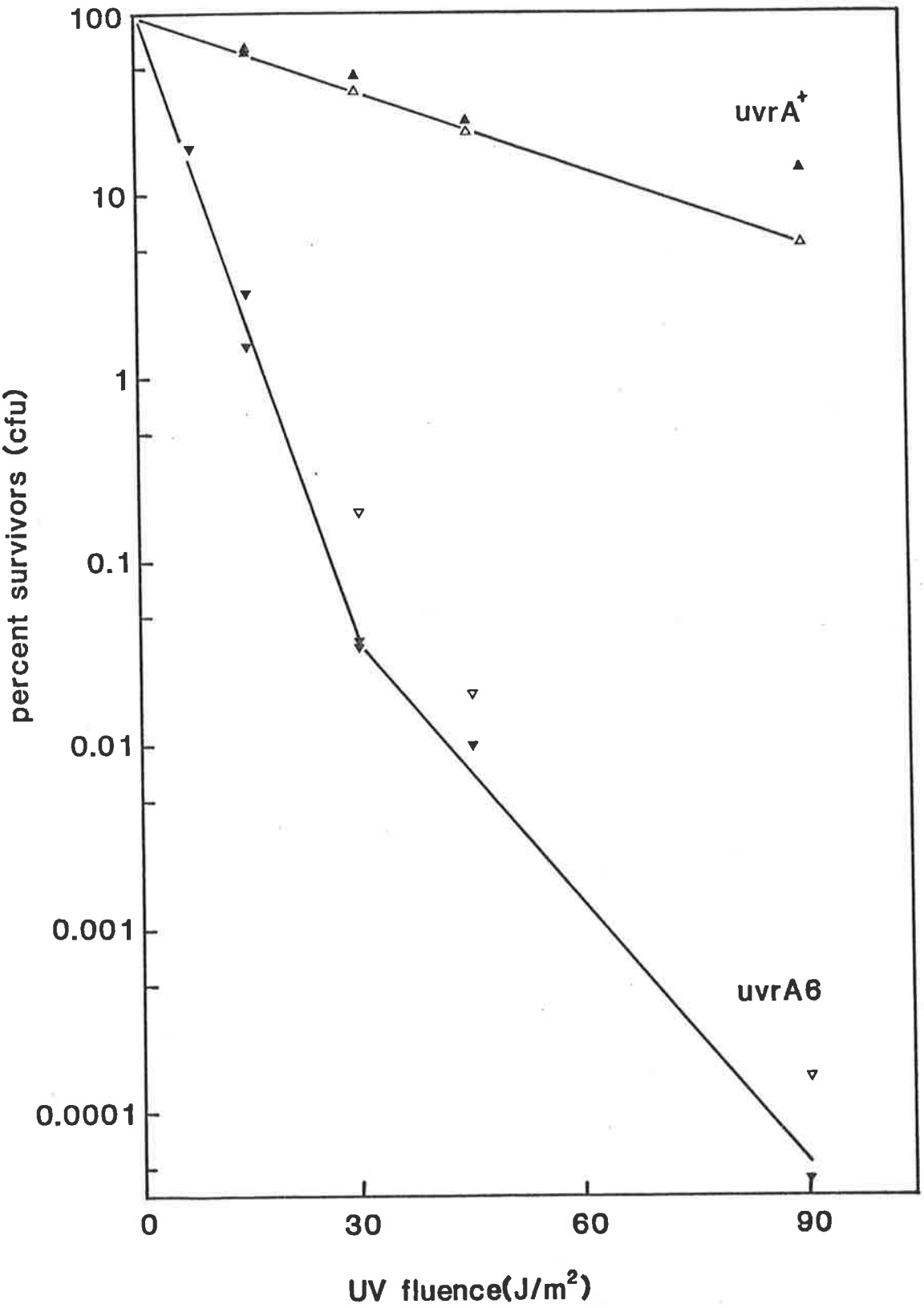

100
10
1
0.1
0.01
0.001
0.0001
percent survivors (cfu)
uvrA+
uvrA6
0
30
60
90
UV fluence(J/m2)

FIG 4.2

Effect of UV on synthesis of *E. coli* DNA in the *uvrA*$^+$ and *uvrA6* cultures

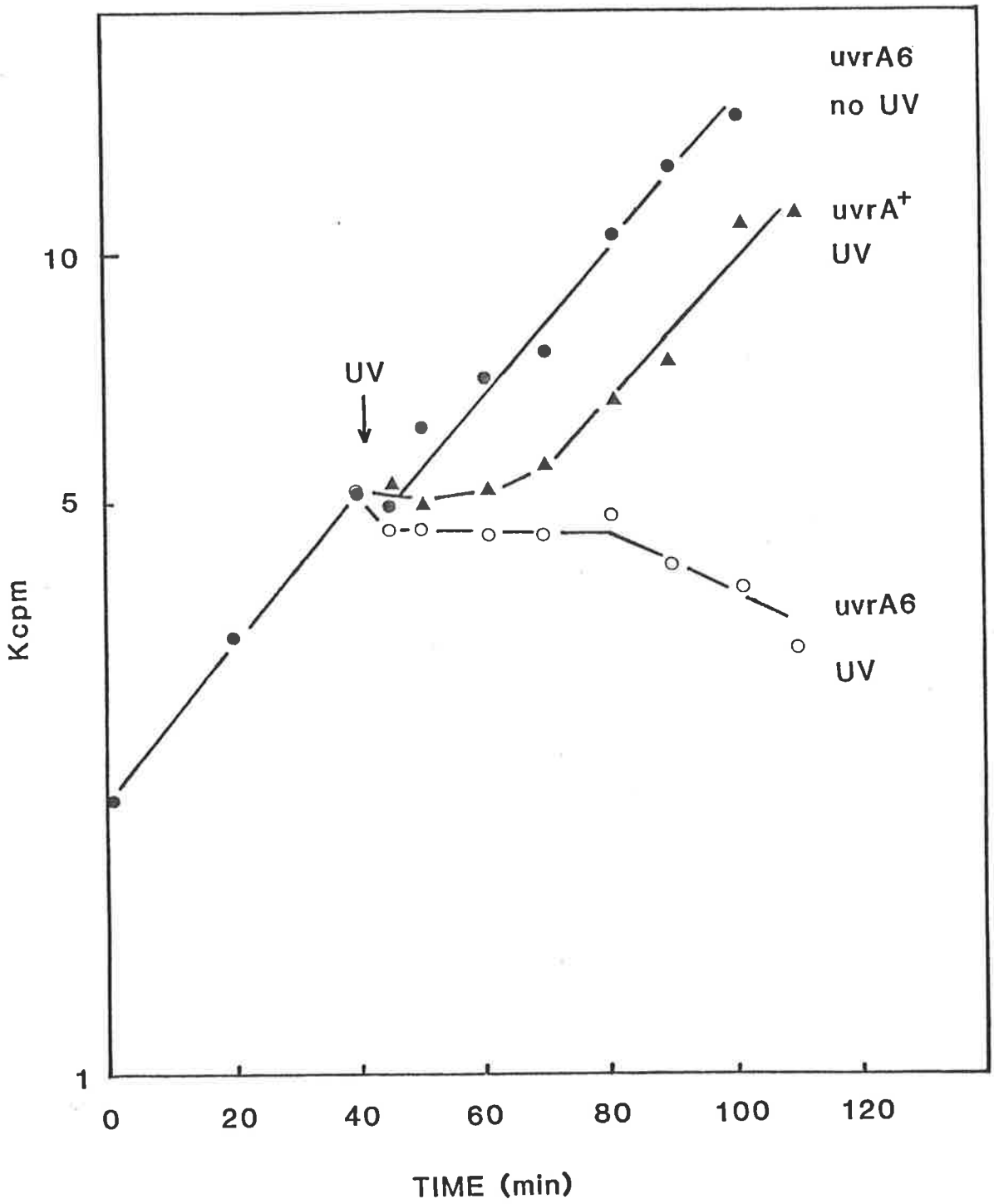
C600 *thy*$^-$ *uvrA*$^+$ (E4057) and C600 *thy*$^-$ *uvrA6* cells (E4051) were cultured in TPGCAA containing [^{3}H]thymine (6µci/2µg/ml) for 3 generations. Portions of the cultures were irradiated with 0 or 45 J/m^2 of UV at the time indicated (↓). Acid-insoluble cpm in 50 µl aliquots were determined by TCA-precipitation (Section 2.2.9) at intervals after the irradiation.

(● - ●) *uvrA6* unirradiated

(o - o) *uvrA6* 45 J/m^2 UV-irradiated

(▲ - ▲) *uvrA*$^+$ 45 J/m^2 UV-irradiated

Replication in the unirradiated *uvrA*$^+$ culture was similar to that in the unirradiated *uvrA6* culture.


uvrA6
no UV
uvrA+
UV
UV
uvrA6
UV
10
5
1
Kcpm
0
20
40
60
80
100
120
TIME (min)

least 70 minutes. This dose of UV causes a transient loss in the capacity of *uvr*$^+$ cells to produce 186 phage (Fig 3.2), however, these cells are capable of producing some 186 phage. I wished to test whether the *uvrA6* mutant could yield 186 phage under the same conditions.

4.3 186 PHAGE PRODUCTION IN UV-IRRADIATED AND UNIRRADIATED *uvrA*$^+$ AND *uvrA6* CULTURES

C600 *thy*$^-$ *uvrA*$^+$ (E4057) and C600 *thy*$^-$ *uvrA6* (E4051) cells were cultured, irradiated and infected with 186 phage as described in the legend to Fig 4.3. Phage production was studied by assaying phage on C600 indicator bacteria at intervals after infection.

The unirradiated *uvrA*$^+$ culture had a burst size of approximately 30, after irradiation the burst size was 6 and phage production was delayed. The unirradiated *uvrA6* culture also had a burst size of about 30. The irradiated *uvrA6* mutant was capable of producing 186 phage, with a burst size of about 7.

Therefore, although the UV-irradiated *uvrA6* mutant cells are incapable of replicating chromosomal DNA, they can produce 186 phage to the same extent as UV-irradiated wild type cells. This shows that 186 can replicate in the absence of detectable concomitant host chromosomal DNA replication.

FIG 4.3 Phage production by 186 in irradiated and unirradiated *uvrA*$^+$ and *uvrA6* cultures

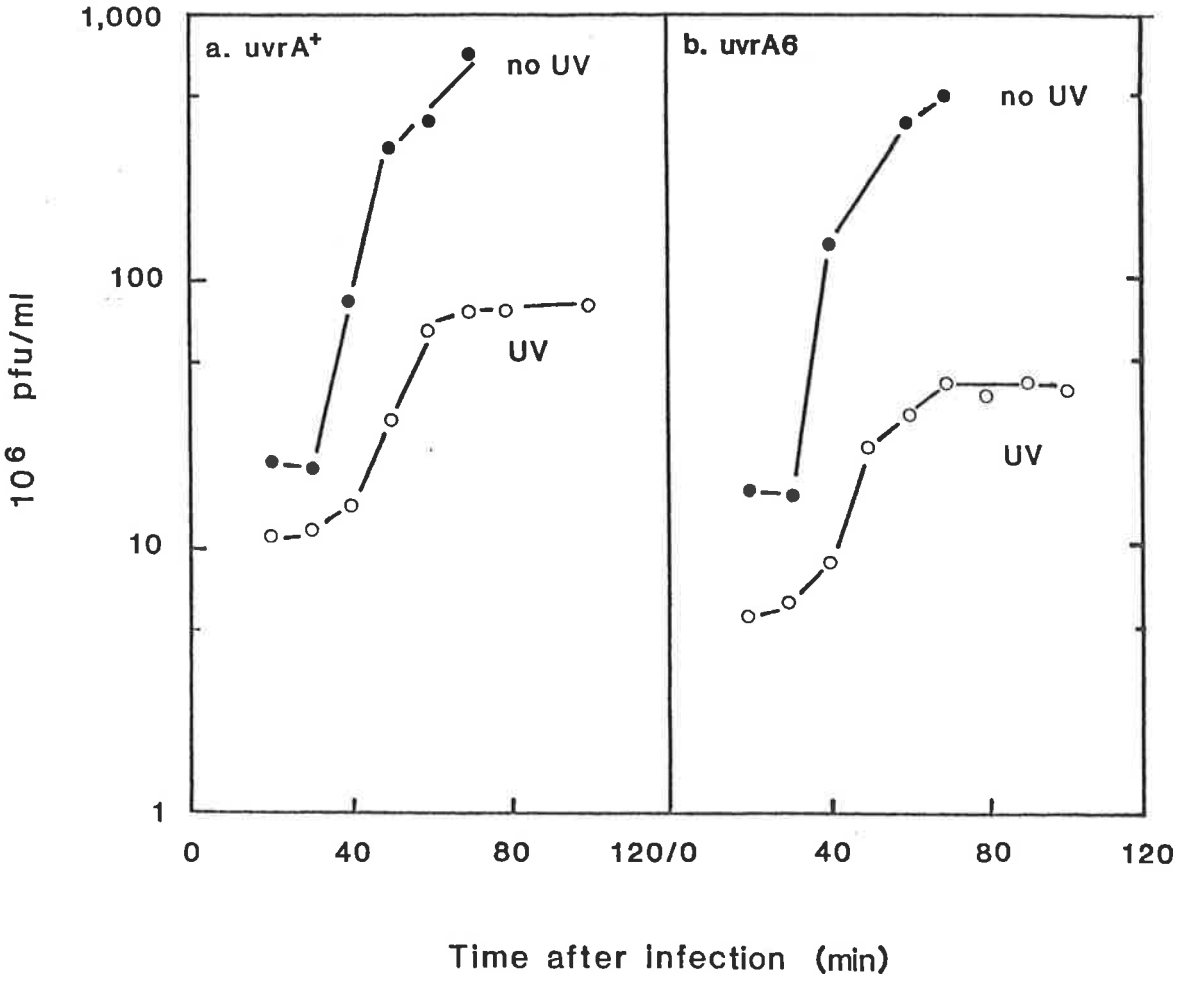
C600 *thy*$^-$ *uvrA*$^+$ cells (E4057) and C600 *thy*$^-$ *uvrA6* cells (E4051) were cultured in TPGCAA containing thymine (2μg/ml) to an OD of 0.25. A portion of each culture was irradiated with 0 or 45 J/m². 186 phage were added at an moa of 10. Incubation was continued for 2.5 minutes at 37°C, then unabsorbed phage were removed by antiserum treatment. Samples were assayed for pfu/ml on C600 indicator bacteria.

a. E4057

b. E4051

(● - ●) unirradiated cells

(o - o) 45 J/m² irradiated cells.


1,000
100
10
1
10^6 pfu/ml
a. uvrA+
no UV
UV
b. uvrA6
no UV
UV
0
40
80
120/0
40
80
120
Time after infection (min)

4.5 CONCLUSION

The data clearly show that 186 phage production can occur in the absence of *E. coli* DNA synthesis. Therefore, the transient inhibition in chromosomal DNA synthesis obtained in UV-irradiated cells is not the cause of the cells' loss in 186 capacity.

CHAPTER 5

THE LOSS OF 186 CAPACITY : AN SOS FUNCTION?

It was of interest to determine whether the UV-induced loss of cellular capacity to host a normal 186 infection was a *recA*$^+$ *lexA*$^+$-dependent function (for reasons described in Chapter 1). Practically, the UV fluence employed to examine the premise is important. It appears to be the general practice, when comparing UV^S mutants with their UV^r counterparts, to irradiate both strains with fluences which yield the same survival (Trogovcevic *et al.*, 1980; Quillardet *et al.*, 1982). In doing so, the sensitive strain (UV^S) is invariably irradiated with a lower fluence of UV than the resistant (UV^r) strain. Therefore, the cells do not sustain the same amount of initial damage.

So far, 186 phage production has been studied in 45 J/m^2-irradiated cells. If, however, the UV^S (i.e. *recA*, *lexA* mutant) and control cells were irradiated, to obtain the same survival and 186 phage production was studied in the cultures and no loss in 186 capacity occurred in the *recA*, *lexA* mutant cells irradiated with the lower dose, it would have been concluded that the loss in capacity was dependent on *recA*$^+$ *lexA*$^+$. In fact, the same fluence would not alter 186 capacity in wild type cells either. In addition it was known from the data in Chapter 4, that UV-irradiated *uvrA6* and *uvr*$^+$ cells produce 186 to the same extent, although the *uvrA6* culture had an extremely low viable count. Thus, in the above case the loss in 186 capacity was independent of cellular survival. Therefore irradiating the *recA* and *lexA* mutants and control cells to

the same survival could lead to a false conclusion about the effect of these mutations.

Thus it was decided to irradiate the mutants and their control cultures with the same fluence of UV.

Most of the mutations required to test the proposition were available in an isogenic AB1157 background. Therefore it was decided to do the study in this strain. Stocks of the various mutant cultures were obtained, mainly from the Centre for Genetic Stock Cultures (CGSC) in Yale. Sources are detailed in Table 2.1

5.1 THE EFFECT OF A *recA* MUTATION ON 186 PHAGE PRODUCTION AFTER UV-IRRADIATION

If the loss in 186 capacity in UV-irradiated cells is caused by activated *recA* protein, then in a *recA* mutant, 186 phage production should be returned to the normal level obtained in unirradiated cells.

In anticipation of a need to study the effect of the mutation on DNA replication it was considered prudent to do the experiment in a *recB* background, so as to minimise the 'reckless' DNA degradation seen in UV-irradiated *recA* bacteria (Howard-Flanders and Boyce, 1966, Willets and Clark, 1969).

AB1157 *recB21* (E753) and AB1157*recA13 recB21* (E754) were cultured in LB broth, UV-irradiated in 10 mM $MgSO_4$, 10x LB broth was added to a final concentration of 1x and the cells were infected with 186.

The *recB21* cells were irradiated with a range of fluences to find the minimum amount of UV for which 186 experienced difficulties. When the cells were irradiated with 15 J/m^2 of UV, one-third the fluence used to study the 186 delay in C600 cells, 186 phage production was inordinately sensitive (Fig 5.1a).

This was unusual but could be of advantage since the *recA recB* mutant could also be irradiated with this reduced UV fluence (15 J/m^2) resulting in higher survival than that obtained with 45 J/m^2, which is the fluence normally required to demonstrate UV-sensitivity of 186 phage production.

The presence of the *recA13* mutation in the *recB21* strain restored, almost completely the irradiated-cells capacity to host a 186 phage infection (Fig 5.1b). Therefore it was concluded that the 186 sensitivity in a UV-irradiated cell was a $recA^+$-dependent function.

In order to determine whether the increased 186 sensitivity seen in the *recB21* mutant (Fig 5.1a) was due to the *recB* mutation or characteristic of the AB1157 strain,

FIG 5.1 The effect of a *recA* mutation on the loss of 186 capacity

AB2470 (*recB21*, E753), JC5495 (*recA13 recB21*, E754) and AB1157 (wt, E746) were cultured in LB broth at 37°C to an OD 1.0 (approximately 4 x 10^8 cfu/ml) sedimented and resuspended in 10 mM $MgSO_4$ (37°C) and UV-irradiated with 0 or 15 J/m². 10 x LB broth was added to a final concentration of 1x and infected with 186 *cIts* (moa = 2). After 2.5 minutes unabsorbed phage were removed with antiserum and phage production was assayed at intervals. Cellular survival after 15 J/m² was: *recB* - 1.5%, *recA recB* - 0.01%, w.t. - 89%

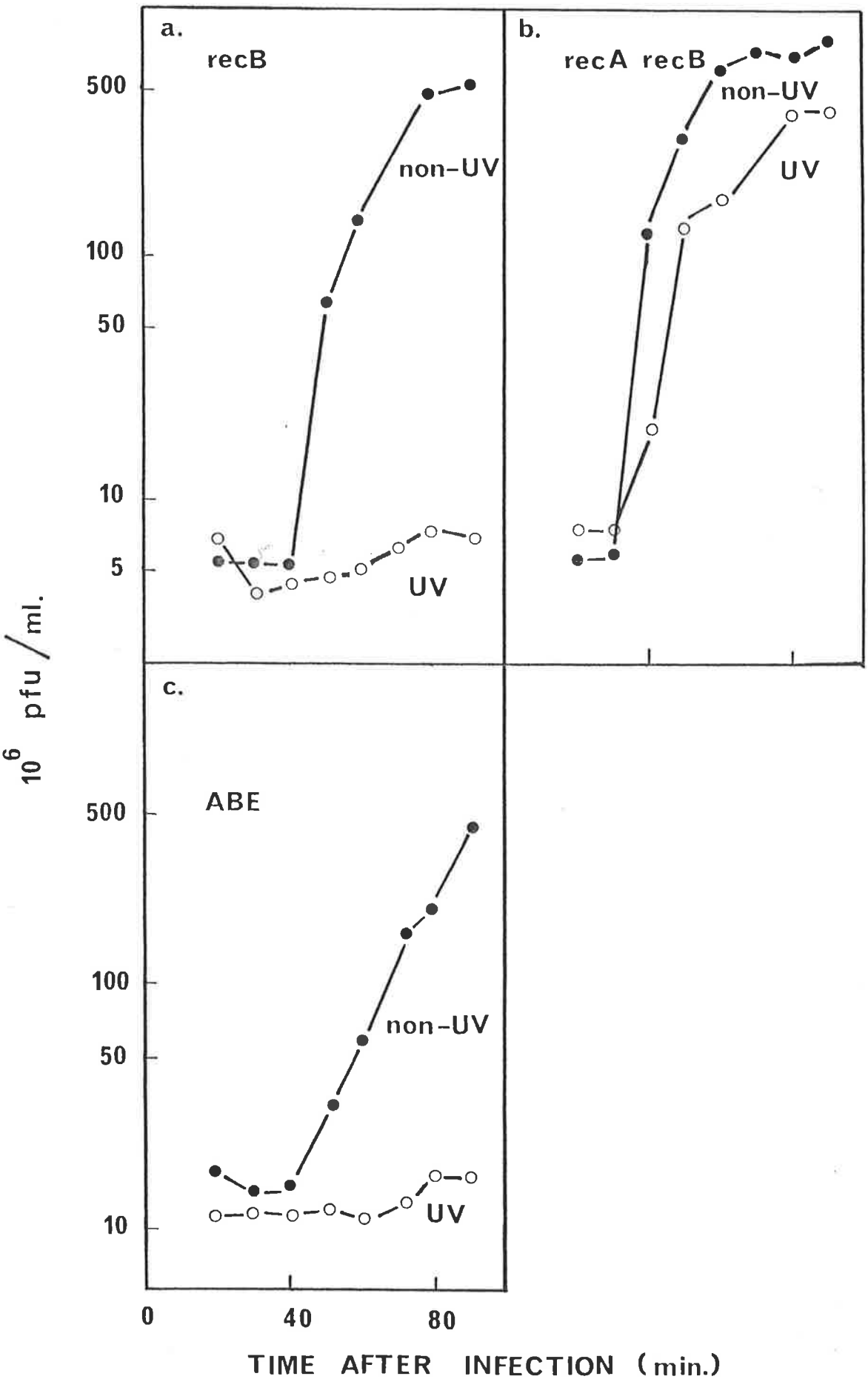
a. AB1157 *recB*21

b. AB1157 *recA*13 *recB*21

c. AB1157

(● - ●) - unirradiated cells

(o - o) - 15 J/m² irradiated cells


a.
recB
non-UV
UV
b.
recA recB
non-UV
UV
c.
ABE
non-UV
UV
500
100
50
10
5
0
40
80
10^6 pfu / ml.
TIME AFTER INFECTION (min.)

186 phage infection was examined in the UV irradiated (15 J/m²) and unirradiated AB1157 cells (E746). 186 infection of the 15 J/m²-irradiated AB1157 culture is as sensitive as that of the *recB21* mutant (Fig 5.1c). Therefore the increased sensitivity appears to be characteristic of the AB1157 strain.

5.2 186 PHAGE PRODUCTION IN A *recA441* (tif-1) MUTANT AT ELEVATED TEMPERATURES

The *recA441* or (tif 1) mutation causes the induction of SOS functions at elevated temperatures (40-42°C) in the absence of UV. This is due to activation of the mutant *recA* protease, as discussed in Chapter 1. Since the loss in 186 capacity is dependent on $recA^+$ (above) it should be induced, in the absence of UV, at 40°C in the *recA441* mutant.

To determine the conditions for activation of *recA441* at 40°C, λ prophage induction from a *recA441* (λcI^+) lysogen was studied under conditions which are known to activate the *recA* protease (minimal medium and adenine) and those which depress it (LB broth).

An AB1157 *recA441* (λcI^+) lysogen (E801) was cultured overnight in LB broth at 30°C and diluted into either TPGCAA or LB broth. At an OD of 0.4 the cells were diluted into TPGCAA, TPGCAA containing adenine (100 μg/ml), and LB broth respectively. Incubation was continued at 40°C for 120 minutes. Phage were assayed at intervals on C600 indicator

bacteria on Z plates. The data obtained is shown in table 5.1).

The 0 min sample gives the free phage present in the culture. $<10^4$ pfu/ml were detectable in the cells cultured in LB broth throughout, while ~10^6 pfu/ml were present in the cells cultured in minimal medium (Table 5.1). By 100 minutes, no increase in phage is seen in LB, but the cells cultured in TPGCAA show an increase, of three orders of magnitude. If adenine was added to the medium the increase was 20 fold greater than in its absence. Therefore *recA441* protease was activated in TPGCAA containing adenine (100 µg/ml) at 40°C.

186 infection of the *recA441* mutant was tested under these conditions with AB1157 as a control.

AB1157 and AB1157 *recA441* cells were cultured overnight in LB broth at 30° and diluted into TPGCAA to an OD of 0.4. Adenine (100 µg/ml) was added to both cultures and they were transferred to 40°C for 20 minutes, after which they were infected at 40°C with 186. Unabsorbed phage were inactivated with antiserum after 2.5 minutes. Incubation was continued at 40°C and 186 phage production was studied.

186 did not suffer any increase in the latent period nor was its phage production significantly impaired in the

TABLE 5.1

Induction of λcI$^+$ prophage from a *recA441* (λcI$^+$) lysogen

	pfu/ml			
Time after transfer to 40°C	0′	50′	100′	120′
Medium				
TPGCAA	9.4×10^5	9.5×10^7	1×10^9	6.2×10^9
TPGCAA + adenine	1.0×10^6	–	2×10^{10}	4×10^{10}
LB broth	$<10^4$	$<10^4$	$<10^4$	$<10^5$

recA441 (λ*c*I$^+$ lysogen (E801), cultured overnight in LB broth at 30°C, was diluted into TPGCAA and LB broth and cultured at 30° to an OD of 0.4. Adenine (100 μg/ml) was added to a portion of TPGCAA and all three cultures were transferred to 40°C. Phage production was assayed on C600 bacteria.

heat-activated *recA441* mutant compared with *recA* control (Fig 5.2). Therefore the loss in 186 capacity was not induced by heat-activation of the *recA441* protease.

5.3 THE EFFECT OF *lexA3*(Ind$^-$) ON 186 PHAGE PRODUCTION IN UV-IRRADIATED CELLS

In order to resolve the contradiction that the loss in 186 capacity, was dependent on *recA*$^+$ but not temperature-inducible in a *recA441* (tif-1) mutant, the next step was to determine whether it was under *lexA* repressor control or directly dependent on *recA*.

If the sensitivity is caused by a *lexA* repressed function, normal phage production should be obtained in a *lexA3*(Ind$^-$) mutant since the mutant repressor cannot be cleaved by the *recA* protease and is non-inducible by UV. However, *lexA* protein also represses the *recA* gene and in the above mutant *recA* protein production cannot be induced so one would be unable to distinguish between *recA* and *lexA* dependency.

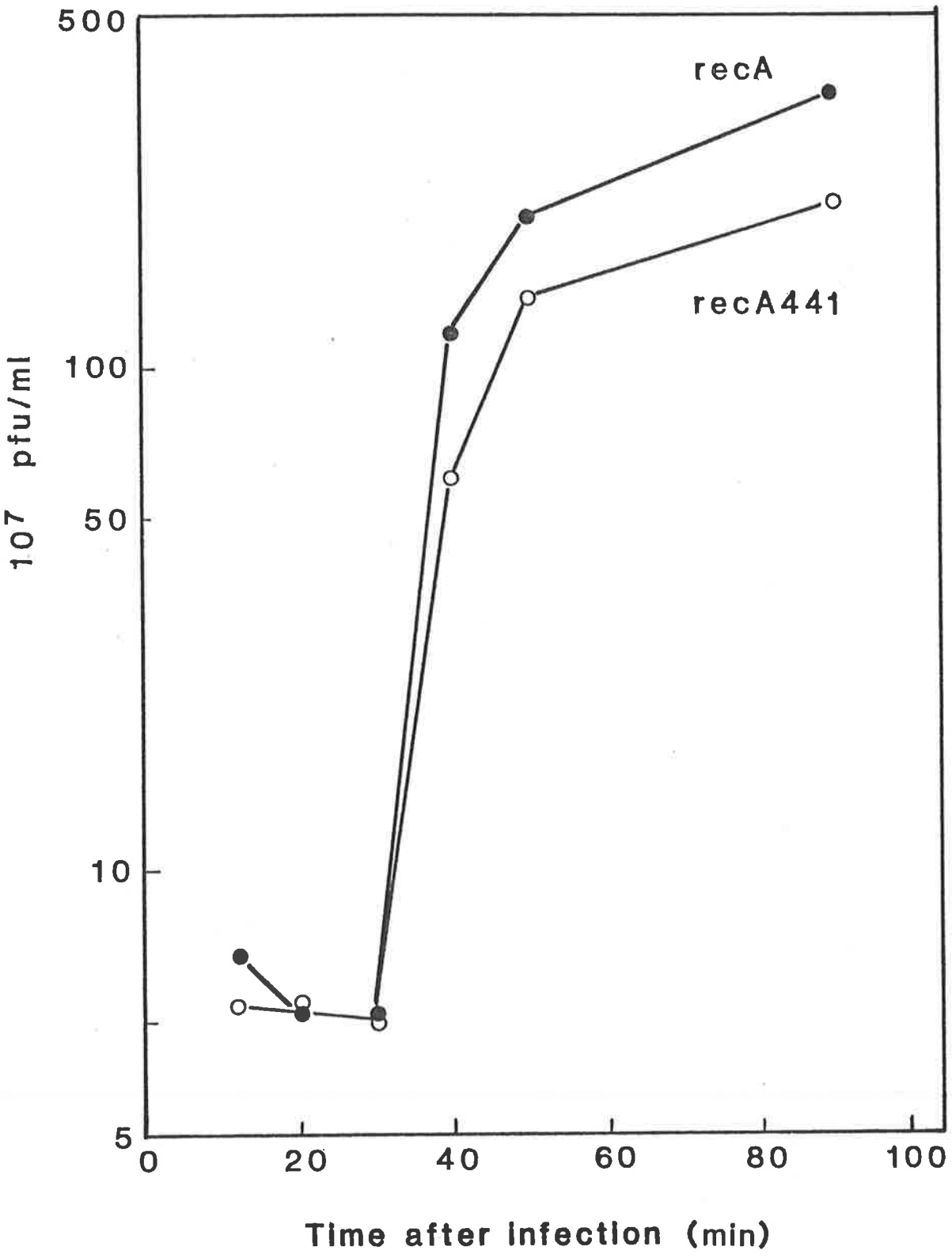
A mutation, in the *recA* operation, *recAo*, allows constitutive high levels of *recA* protein production. In a *recAo lexA3* double mutant, the production of *recA* protein is independent of the *lexA* repressor and the protease can be overproduced and is activated by UV. If the loss in 186 capacity is caused by the *recA* protein alone, it will still be obtained in the *recAo lexA3* mutant. But if it is a *lexA*

FIG 5.2 Effect of heat-activation of *recA441* on 186 phage production

AB1157 *recA441* (E764) and AB1157 (E808) were cultured overnight in LB broth at 30°C diluted in TPGCAA and cultured at 30°C to an OD of 0.4. Adenine (100 µg/ml) was added to both cultures, which were then transferred to 40°C and after 20 minutes were infected with 186*c*Its. Unabsorbed phage were inactivated 2.5 minutes later with antiserum. Phage were assayed on C600 indicator bacteria.

(● - ●) *recA*$^{+}$

(o - o) *recA441*


500
100
50
10
5
10^7 pfu/ml
recA
recA441
0
20
40
60
80
100
Time after infection (min)

repressed function, phage production in the strain, after UV-irradiation, should be normal.

The control strain carried the *recAo* mutation but was *lexA*$^{+}$.

AB1157 *recAo* (E783) and AB1157 *recAo lexA3* (E782) cells were cultured, irradiated with 0 or 15 J/m^{2} and infected with 186 as described for the *recA recB* and *recB* cultures.

Contrary to expectation, the *recAo* mutant did not display the extreme loss in 186 capacity seen in AB1157 and *recB* cells irradiated with 15 J/m^{2} (Fig 5.3a). Nor did 186 infection of UV-irradiated *recA lexA3* experience any undue sensitivity (Fig 5.3b). In fact 186 phage production was impaired to the same extent in the UV-irradiated *recAo* and *recAo lexA3* cultures. Therefore, the presence of the *lexA3* mutation did not restore to normal the cells' capacity to produce 186 phage. (Fig 5.3b).

Two major conclusions can be made from the above data. Firstly, the 186 sensitivity does not appear to be a *lexA*-dependent function since 186 phage production is as impaired in the *lexA3*(Ind^{-}) mutant as in the control *lexA*$^{+}$ strain. Secondly, 186 infection of the irradiated control *recAo* strain is not as sensitive as it is in the AB1157 parent strain. This was something of a puzzle till it was realised

FIG 5.3 Effect of the *lexA3* mutation on the cells' loss in 186 capacity

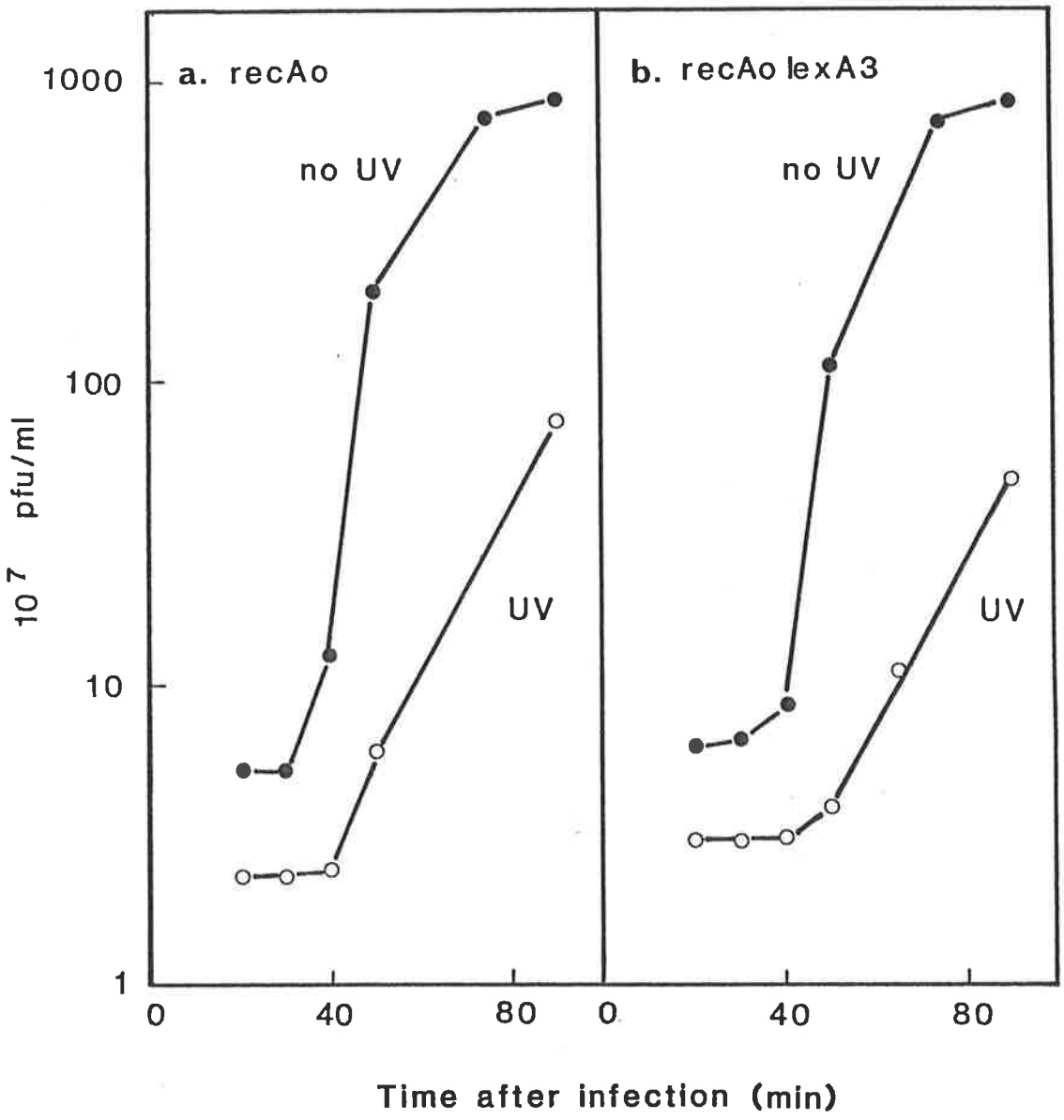
JC11457 (*recAo*, E783) and JC11867 (*recAo lexA3*, E782) cells were cultured in LB broth at 37° to an OD of 1.0 (approximately 2 x 10^8 cfu/ml) sedimented resuspended in 10 mM $MgSO_4$ (37°C) and UV-irradiated with 0 or 15 J/m² of UV. 10 x LB broth was added to a final concentration of 1x and cells were infected with 186*cIts* (moa 2). After 2.5 minutes at 37°C, unabsorbed phage were removed with antiserum and phage production was assayed at intervals. Cellular survival after irradiation with 15 J/m² was: *recAo* - 99%, *recAo lexA3* - 22%

a. *recAo*

b. *recAo lexA3*

● - ● unirradiated cells

o - o 15 J/m² UV-irradiated cells


a. recAo
b. recAo lexA3
no UV
UV
1000
100
10
1
10^7 pfu/ml
0
40
80
Time after infection (min)

that the extent of the loss in capacity seen in the *recAo* strains was similar to that expected from studies with C600 cells, while the high sensitivity with the parent AB1157 was unusual.

It was also noticed, that there was a correlation between strains which did not show the extreme loss in 186 capacity and the laboratory of their origin. The *recA recB*, *recAo* and *recAo lexA3* mutants strains all originated in J. Clark's laboratory in Berkeley on the West Coast of the USA while the strains which experienced the unusual loss in 186 capacity originated in P. Howard-Flanders laboratory in New Haven on the East Coast of the USA. This raised the suspicion that there may be two cultivars of the AB1157 parent in use, the East Coast strains, which lose the 186 capacity after low fluences of UV and the West Coast strains which only lose capacity after irradiation with larger fluences of UV.

If this was true, any effect that individual mutations might have could be masked by the variation in the parental strains.

In accordance with this idea an isolate of the AB1157 parent was obtained from the West Coast (D. Mount) and was called ABW for AB-West (E808). The AB1157 obtained from B. Bachmann was named ABE for the AB-East (E746).

5.4 TWO CULTIVARS OF AB1157

186 phage production was studied by infecting UV-irradiated (15 J/m²) and unirradiated cultures of ABE and ABW as described previously (Fig 5.1).

ABE and ABW behave completely differently as far as the loss in 186 capacity is concerned (Fig 5.4). The ABE culture reflected the extreme loss in capacity (Fig 5.4a) observed previously in *recB* cells (Fig 5.1a) but ABW did not. Rather it showed a slight loss in capacity (Fig 5.4b) similar to the *recA recB* cells (Fig 5.1b).

Therefore a variation exists in the AB1157 parent strains. In light of this observation, the previous conclusion, that the loss in 186 capacity was *recA*-dependent, could not be made since the results could be an artifact of the strains used.

Various properties of the ABE and ABW strains were studied in order to get a slightly better understanding of the difference between the two strains.

(i) ***Genotype***: To confirm that the two strains being used were indeed AB1157, several nutritional requirements were tested. The genotypes of ABE and ABW were found to be identical in the markers tested - *his pro leu thr thi ara lac gal xyl* str^R.

FIG 5.4 Two cultivars of strain AB1157

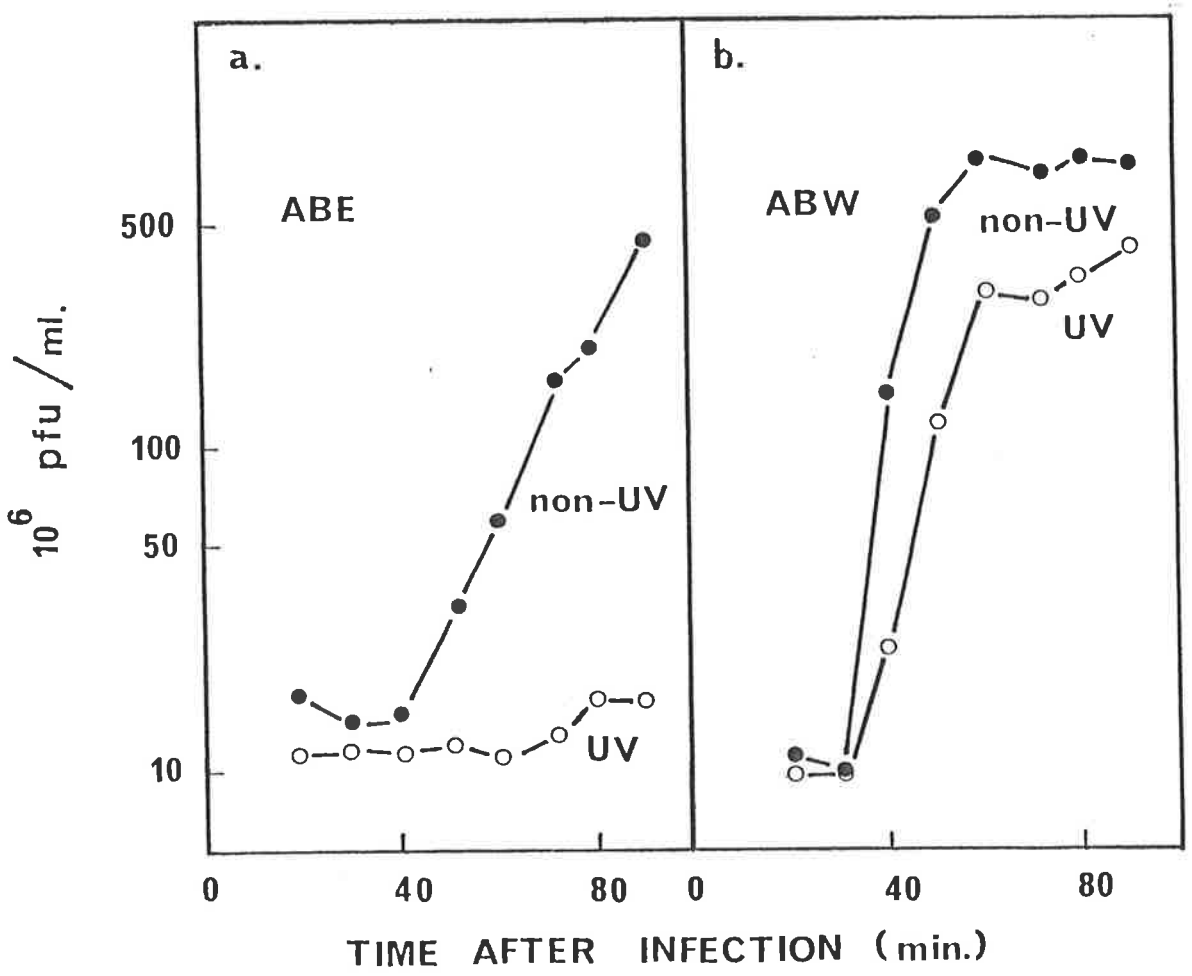
AB1157-EAST (ABE, E746) and Ab1157-WEST (ABW, E808) cells were cultured in LB broth at 37°C to an OD of 1.5 (3 x 10^8 cfu/ml), sedimented resuspended in 10 mM $MgSO_4$ (37°C) and irradiated with O or 15 J/m^2 of UV 10 x LB broth was added to a final concentration of 1x, cultures were infected with 186*c*Its (moa = 2). After 2.5 minutes at 37°C antiserum was added to inactivate unabsorbed phage. Phage production was assayed at intervals. Cellular survival was 90% for both cultures.

a. ABE

b. ABW

(● - ●) unirradiated cells

(o - o) 15 J/m^2 UV-irradiated cells


a.
b.
ABE
ABW
non-UV
UV
non-UV
UV
500
100
50
10
10^6 pfu / ml.
0
40
80
0
40
80
TIME AFTER INFECTION (min.)

(ii) ***UV-sensitivity***: Cellular survival and DNA replication were studied after UV-irradiation. Survival curves for the ABE and ABW strains were very similar. (Fig 5.5a).

To test DNA replication, a *thy*$^-$ derivative of ABE (E4058) was constructed by trimethoprim selection (Section 2.2.4a). AB2497 cells (E749) were considered to be ABW *thy*$^-$ on the basis of 186 phage production after UV.

The generation times of ABE *thy*$^-$ (E4058) and ABW *thy*$^-$ (E749) cultures in LB broth and thymine (1 µg/ml) were significantly different (50 minutes *vs* 30 minutes). This was not caused by limiting levels of thymine since the rates of DNA replication and generation time were the same in LB broth supplemented with either 1 or 2 µg/ml of thymine.

DNA synthesis was followd by TCA precipitation in ABE*thy*$^-$ and ABW*thy*$^-$ cells cultured in LB broth containing [^{3}H]thymine (4 µCi/µg/ml). The cells were sedimented resuspended in prewarmed 10 mM $MgSO_4$ containing [^{3}H]thymine (4 µCi/µg/ml) and UV-irradiated with 0 or 15 J/m^2. 10 x LB broth was added to a final concentration of 1x. (If thymine was omitted from the irradiation buffer, a long delay and drop in total cpm was observed even in the absence of UV-irradiation. Despite all the precautions taken in handling the cultures i.e. pre-warmed petri dishes kept at 37°C as much as possible, a slight drop in total cpm was always observed).

FIG 5.5 Cellular survival and DNA synthesis in unirradiated and UV-irradiated ABE and ABW cells

a. AB1157-EAST (ABE, E746) and AB1157-WEST (ABW, E808) cells were cultured in LB broth, sedimented and resuspended in 10 mM $MgSO_4$ (37°C) aliquots were irradiated with increasing doses of UV. Surviving cfu/ml were assayed on YGC plates at 37°C.

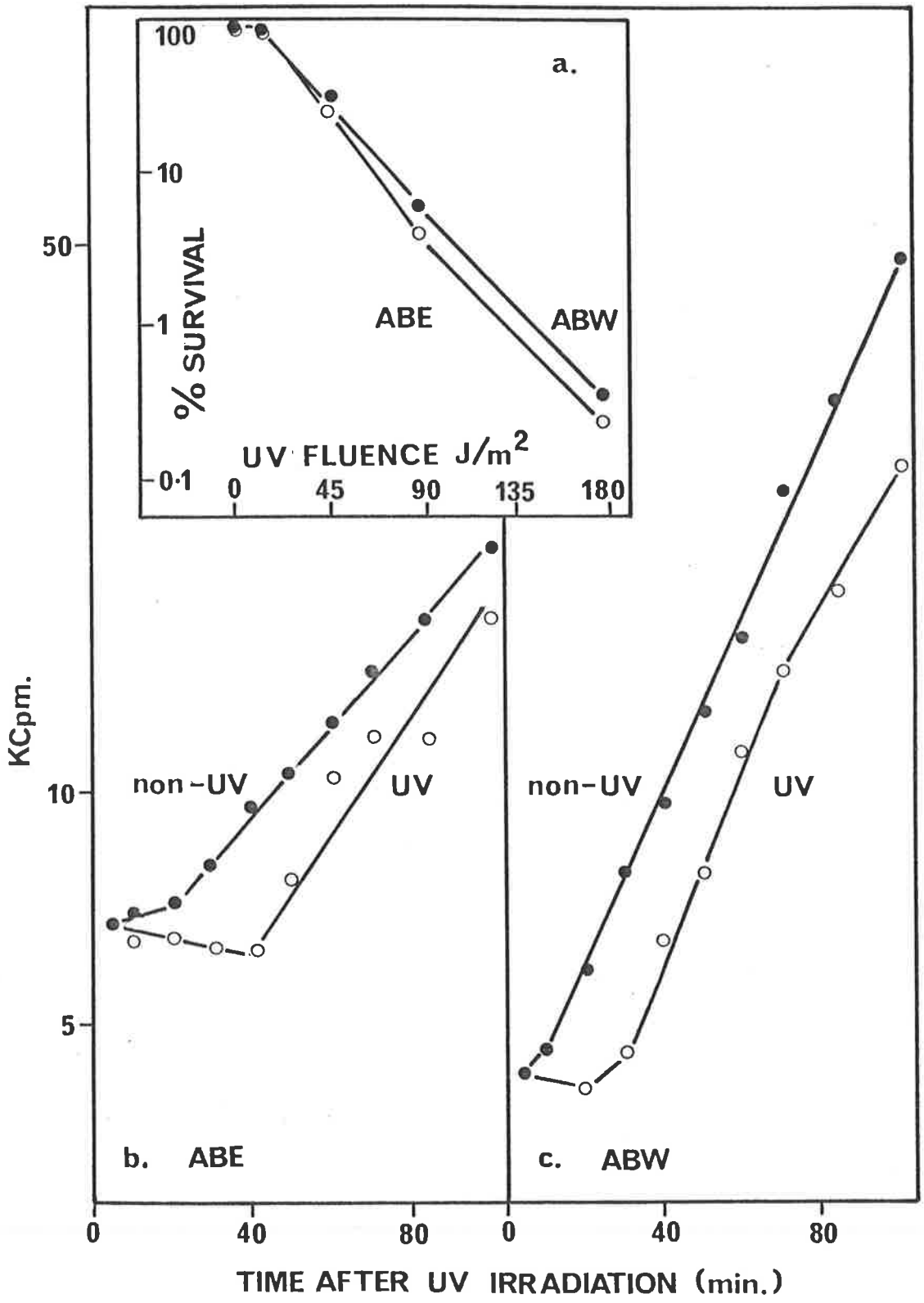
o - o ABE

● - ● ABW

b,c. DNA synthesis was measured in cells of ABE *thy*⁻ (E4058) and ABW *thy*⁻ (E740) cultured in LB broth containing [^{3}H]thymine (4 µCi/µg/ml). At an OD of 0.4, the cells were sedimented, resuspended in MgSO4 (10 mM containing [^{3}H]thymine (4 µCi/µg/ml), irradiated with 0 and 15 J/m² of UV. 10 x LB was added to each culture and the incubation continued at 37°C. Acid-insoluble cpm incorporated were measured at intervals after irradiation.

(● - ●) unirradiated cells

(o - o) 15 J/m² UV-irradiated cells


a.
100
10
1
0·1
% SURVIVAL
ABE
ABW
UV FLUENCE J/m²
0
45
90
135
180
KCpm.
50
10
5
non-UV
UV
non-UV
UV
b. ABE
c. ABW
0
40
80
0
40
80
TIME AFTER UV IRRADIATION (min.)

Both the ABE *thy*⁻ and ABW *thy*⁻ experienced a transient inhibition in DNA replication in the irradiated cultures compared with the unirradiated control cultures. The duration of the inhibition was approximately the same in both strains. (Fig 5.5b and c).

Therefore the ABE strain is not significantly more UV-sensitive than the ABW strain.

(iii) ***186 phage production***: By chance, two conditions other than UV-irradiation, were found under which the ABE and ABW strains behaved differently.

To ^{3}H-label the *thy*⁻ cultures in LB broth for the previous experiment, initially, activated charcoal treated-LB broth was used as a medium. This was to remove the endogenous thymine, prior to the addition of [^{3}H]thymine. When 186 phage production was studied in this medium, by heat-induction of a temperature sensitive prophage in ABW and ABE lysogens, phage were produced normally from the ABW lysogen but no phage burst was seen from the ABE lysogen (Fig 5.6a). Instead the level of infectious centres remained constant, in the heat-induced ABE lysogen, for the duration of the experiment. The fact that the ABE strain can produce an infectious center means that it is capable of producing 186 phage but not in the same manner as ABW.

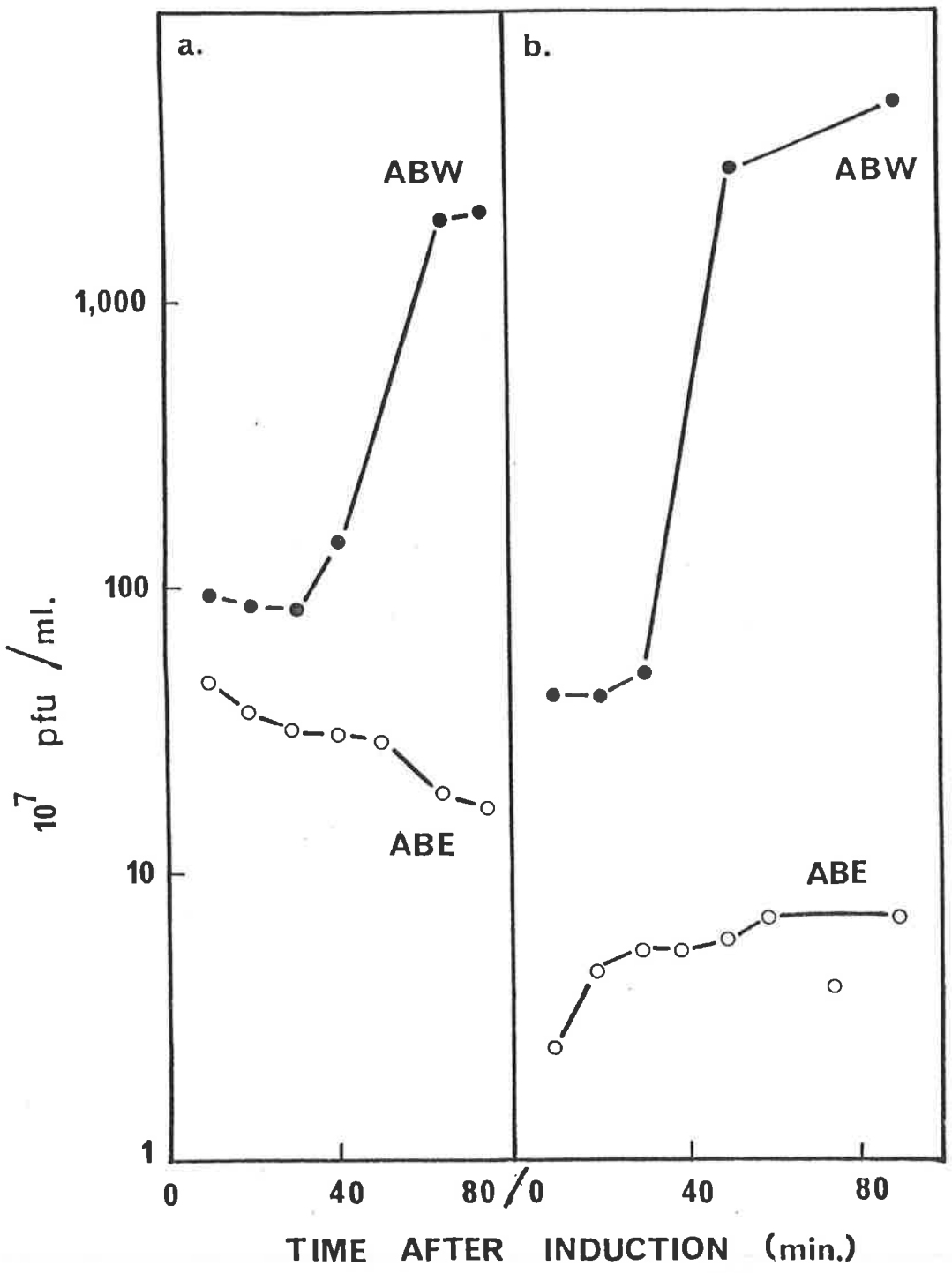
FIG 5.6 Heat-induction of ABE and ABW 186 lysogens in activated charcoal treated LB broth and 186*tum9* lysogens in LB broth

a. ABE (186*cIts*) (E793) and ABW (186*cIts*) (E4059) lysogens were cultured at 30° in LB broth pretreated with activated charcoal, to an OD of 1.0, then shifted to 39°C and phage production was assayed at intervals.

b. ABE (186*cItstum9*) (E4060) and ABW (186*cItstum9*) (E4061) lysogens were cultured at 30°C in LB broth to an OD 0.8 and the prophage was heat-induced by transferring the cultures to 39°C. Phage production was assayed at intervals.

(● - ●) ABW

(o - o) ABE


a.
b.
ABW
ABW
ABE
ABE
1,000
100
10
1
10^7 pfu /ml.
0
40
80
0
40
80
TIME AFTER INDUCTION (min.)

The other phenomenological observation was that a 186 *tum9* mutant, which forms a non-inducible prophage (Woods and Egan 1974) cannot produce a productive infection in the ABE strain but it does in ABW, again in the absence of any irradiation.

Fig 5.6b shows the data from the heat-induction of an ABE (186*cItstum9*) lysogen and an ABW(186*cItstum9*) lysogen. The ABW strain produces a burst but the ABE strain does not.

Thus the ABE and ABW cultivars of AB1157 do not differ in genotype or UV-sensitivity but their *thy*$^-$ derivatives have differing generation times and they differ in their ability to host a 186 infection under certain conditions *viz*.

(i) after UV-irradiation

(ii) in LB treated with activated charcoal

(iii) in LB broth with a 186*cItstum9* mutant.

5.5 THE INDEPENDENCE OF THE LOSS IN 186 CAPACITY AND *recA* IN THE ABE STRAIN

With the realisation that there were two parental strains of AB1157 it became apparent that in order to determine the *recA* dependence of the 186 sensitivity one would have to construct isogenic *recA*$^+$ and *recA*$^-$ cells. The most logical thing was to transfer the required mutations into the C600 background since the 186 phenomenon has been studied predominantly in this strain. I decided initially, however, to see if I could use ABE in this instance. At the time this seemed to have an advantage. The loss in 186

capacity was apparent at a much lower dose of UV than that needed for C600 cells. Consequently 186 infection could be studied in the *recA*$^-$ cells after irradiation with a lower UV fluence than that normally required.

*recA56 srl::*Tn10 was P1 transduced into ABE cells and UV^S (E4062) and UV^R (E4063) *ter*^{R} transductants were purified. (The *recA* gene cotransduces with the *srl* locus). 186 phage production was studied in these strains with and without UV-irradiation.

The *recA*$^+$ showed the characteristic extreme loss in 186 capacity after irradiation with 15 J/m². (Fig 5.7a). The presence of the *recA* mutation did not restore the irradiated cells capacity to produce 186 (Fig 5.7b). In fact it reduced the number of cells capable of producing 186 phage i.e. the number of infectious centres.

Therefore, absence of *recA* function does not reverse the effects of irradiation and one can tentatively conclude that the loss of 186 capacity is not a *recA*-dependent function.

So far it has been established that there are two cultivars of AB1157 and 186 phage production in the two cultivars irradiated with UV, is affected to a greater or lesser extent. This difference makes it difficult to interpret whether certain UV sensitive mutations restore the

loss in 186 capacity, since there is uncertainty about the control strain for the mutant. Despite this difficulty it was evident that both *recA56* and *lexA3* mutants also lost the capacity to produce 186 phage, suggesting that the loss in capacity is not a *recA*$^{+}$ *lexA*$^{+}$-dependent function. In addition the loss of capacity was not induced in a *recA441* (tif-1) culture when the *recA* protease was activated by temperature. This further suggested that the loss in capacity was not *recA*-induced.

In order to confirm whether or not the 186 sensitivity is a *recA*$^{+}$ *lexA*$^{+}$ dependent SOS function. These mutations were introduced into C600 cells to construct isogenic parent and test strains.

5.6 EFFECT OF *recA* AND *lexA3* MUTATIONS, IN C600 CELLS, ON THE LOSS OF 186 CAPACITY

recA56 srl300::Tn10 as transduced (Section 2.2.4c) from E761 into E508 (C600) cells and tetracycline resistant transductants were selected. UV^{S} (*recA56*) and UV^{R} (*recA*$^{+}$) colonies were purified.

lexA3 malB::Tn10 was transduced (Section 2.2.4c) from E766 into E508 (C600) and tetracycline resistant transductants were selected (both *lexA* and *malB* map at 92 min, Bachmann, 1983). UV^{S} (*lexA3*) and UV^{R} (*lexA*$^{+}$) colonies were purified and shown to be *mal*$^{-}$, i.e. tetracycline resistance was shown to co-transduce with loss in the ability to utilise maltose.

FIG 5.7 Effect of *recA56* on the capacity of UV-irradiated ABE cells to product 186 phage

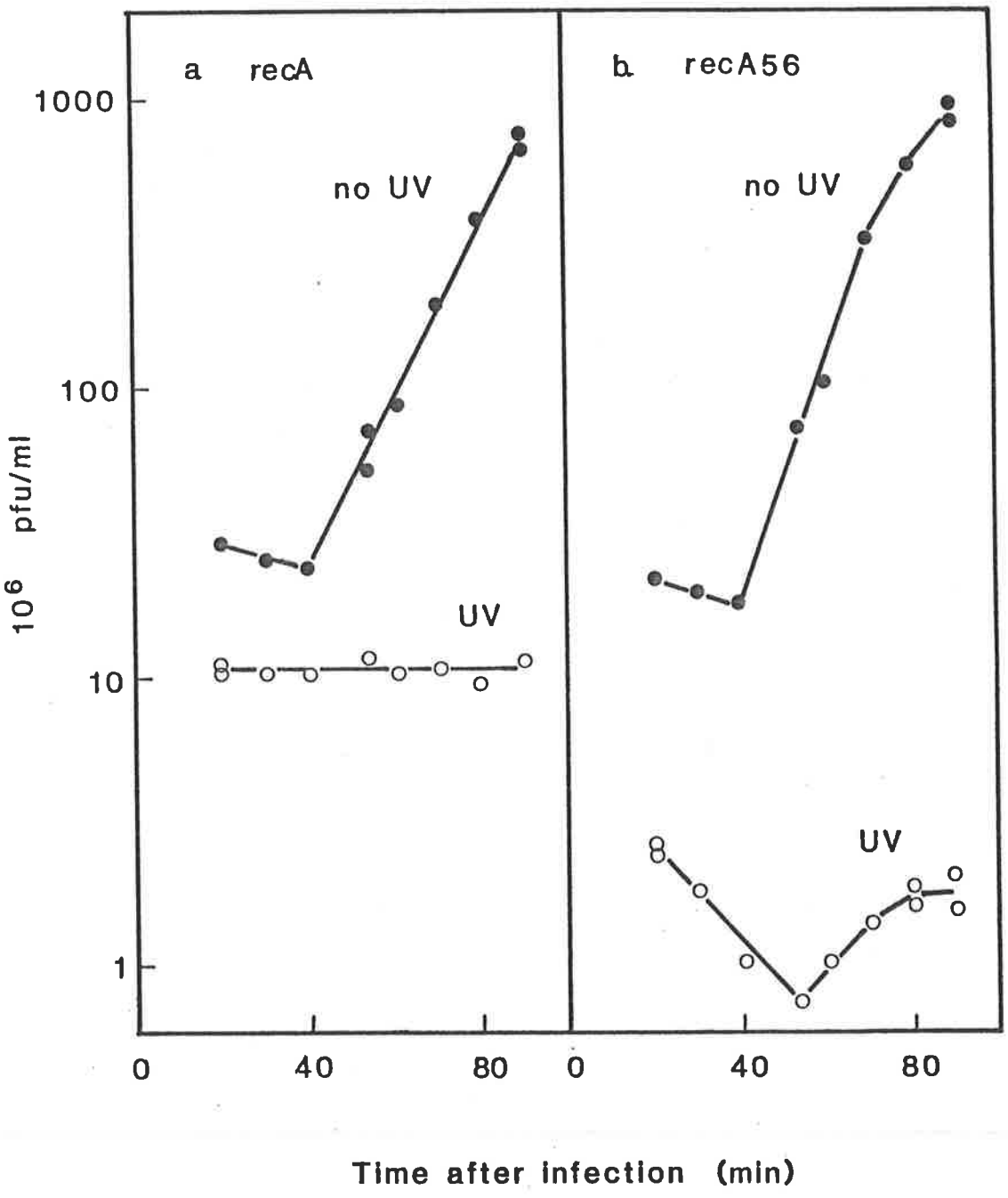
Cells of ABE *srl300*::Tn10 (E4063) and ABE *recA56* *srl300*::Tn10 (E4062) strains were cultured in LB broth at 37°C to an OD of 1.0 (5 x 10^8 cfu/ml). The cells were collected by centrifugation and resuspended in prewarmed (37°) $MgSO_4$ (10 mM). They were then irradiated with 0 or 15 J/m² of UV.LB broth was added (10x to a final of 1x) and the cells were infected with 186*cIts* phage (moa 0.2). After 2.5 minutes at 37°C, the unabsorbed phage were inactivated with 186 specific antiserum. After 5 minutes the culture was diluted away from antiserum, into fresh prewarmed LB broth and the incubation was continued at 37°C. Aliquots were removed at intervals and assayed for pfu on C600 indicator bacteria.

a. E4063

b. E4062

(● - ●) unirradiated cells

(o - o) 15 J/m² UV irradiated cells


a recA
b recA56
no UV
UV
1000
100
10
1
10^6 pfu/ml
0
40
80
Time after infection (min)

Cells were cultured in either LB broth (*lexA3* and *lexA*$^+$) or TPGCAA (*recA56* and *recA*$^+$) and irradiated in 10 mM $Mgso_4$ or TPGCAA respectively. They were then infected with 186 and phage production was assayed at intervals.

A UV-fluence of 45 J/m^2 was used in the following experiments, similar to that used previously with C600 cells.

The loss in 186 capacity was observed in the UV-irradiated control *recA*$^+$ and *lexA*$^+$ cultures (Fig 5.8a, c). The presence of the *lexA3* or *recA56* mutations did not restore to normal the cells capacity to produce 186 phage (Fig 5.8b, d).

5.7 186 DNA REPLICATION IN IRRADIATED ABE CULTURES

The difference between the ABE and ABW cultivars could be exploited to try and gain insight into the UV-induced loss in cellular capacity. This was assuming that the loss in capacity in the ABE strain was an exaggerated or extreme form of the normal UV-induced loss in 186 capacity i.e. it was caused by an extreme form of the same function, and that the extreme loss in capacity was due to inhibition of 186 DNA replication. Identification of the function responsible was, potentially, a direct lead into the cause of the loss in capacity.

FIG 5.8 Effect of *recA56* and *lexA3* on the capacity of UV irradiated C600 cells to produce 186 phage

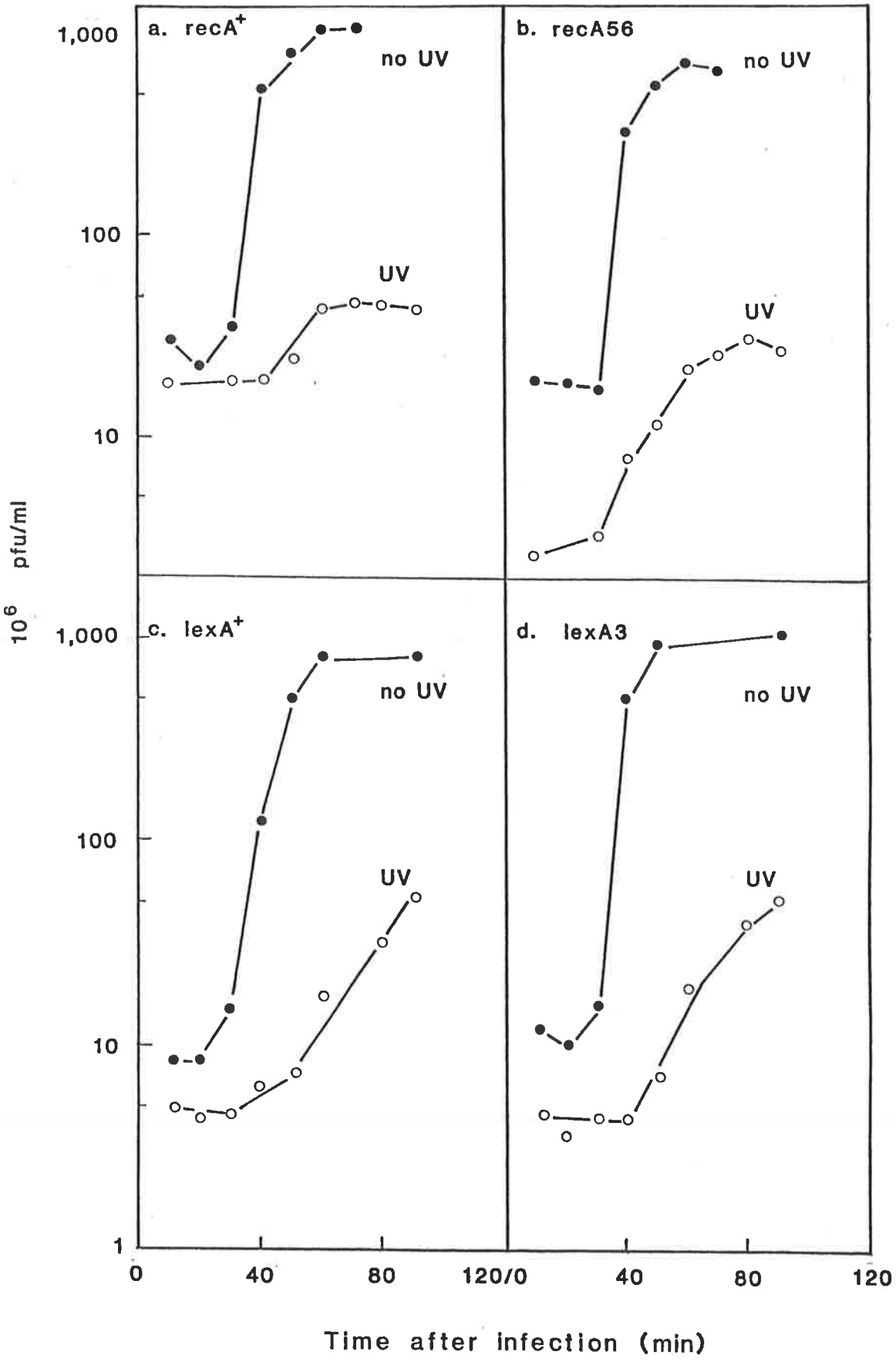
a.b. C600 *srl300*::Tn10 (E4067) and C600 *recA56 srl300*::Tn10 (E4066) cells were cultured in TPGCAA at 37°C to an OD of 0.4 (2 x 10^8 cfu/ml) and were irradiated with O or 45 J/m^2 of UV.

c.d. C600 *malB*::Tn10 (E4044) and C600 *lexA3 malB*::Tn10 (E4045) cells were cultured in LB broth at 37°C to an OD of 1.0 (2 x 10^8 cfu/ml). The cells were collected by centrifugation and resuspended in 37°C prewarmed $MgSO_4$ (10 mM). They were then irradiated with 0 or 45 J/m^2 of UV and LB was added to the suspensions (10x to a final concentration of 1x).

The unirradiated and UV-irradiated cultures were infected with 186*cIts* phage (moa 10), 2.5 minutes later the unabsorbed phage were inactivated with 186 specific antiserum. Incubation was continued at 37°C and aliquots were assayed at intervals for pfu on C600 indicator bacteria.

(● - ●) unirradiated

(o - o) 45 J/m^2 UV-irradiated.


a. recA+
no UV
UV
b. recA56
no UV
UV
c. lexA+
no UV
UV
d. lexA3
no UV
UV
1,000
100
10
1
10^6 pfu/ml
0
40
80
120/0
40
80
120
Time after infection (min)

The second assumption ie that 186 DNA replication would be inhibited by low UV-fluences in ABE cells, was tested. 186 DNA replication was studied by hybridisation in continuously labelled *thy*⁻ derivatives of the two strains.

186 *cIts* lysogens of the ABE *thy*⁻ (E4058) and ABW *thy*⁻ (E749) cells were constructed. Phage production from these lysogens was studied in unirradiated and 15 J/m²-irradiated cultures, by heat-induction of the prophage.

As expected the ABE *thy*⁻ lysogen experienced a greater loss in 186 capacity than did the ABW *thy*⁻ lysogen (Fig 5.9). So the two strains reflect the ABE and ABW difference.

To study the 186 DNA synthesis, the cultures were prelabelled in LB broth with [^{3}H]thymine (20 µCi/µg/ml). At an OD of 0.6, cells were sedimented, resuspended in 10 mM $MgSO_4$ containing [^{3}H]thymine (20 µCi/µg/ml) and were irradiated with 0 or 15 J/m² of UV. 10 x LB broth was added to a final volume of 1x and the cultures were shifted to 39°C to induce the prophage. Samples were removed and hybridised to 186 DNA attached to nitro-cellulose filters to estimate the 186 DNA-DNA hybridisation.

In this experiment the prophage DNA is labelled before phage replication begins. Therefore, the increase in cpm bound to 186 DNA indicates 186 replication. No attempt

was made to subtract the cpm derived from non-specific *E. coli* DNA binding to the filters. This was because previous experiments had shown that the *E. coli* background was <0.1% of the input cpm. In addition, no significant increase in non-specific *E. coli* DNA binding occurred within the 60 minutes of the experiment (186 has a *dhr* function mentioned in Chapter 1 which depresses host DNA synthesis). Instead the cpm obtained due to binding of labelled DNA to blank filters (filters with no DNA attached) was subtracted as background.

186 DNA replication in the ABW *thy*$^-$ lysogen was only silghtly, if at all, delayed in the 15 J/m^2-irradiated culture compared with replication in the unirradiated culture. (Fig 5.10a.). This was as expected from the phage production in this strain (Fig 5.9a). In the ABE *thy*$^-$ strain also, after a transient inhibition, significant 186 DNA synthesis was detected (Fig 5.10b). This was not as expected since phage production in this strain was completely depressed (Fig 5.9b).

Therefore, it was concluded that although 186 DNA replication in the ABE strain was more sensitive than that in the ABW strain it was not as sensitive as phage production. Thus the inability of 186 to form pfu in the irradiated ABE strain may be due to an adverse effect on phage transcription or morphogenesis and not due entirely to an inhibition in phage DNA synthesis. Therefore, it was of

FIG 5.9 Heat induction of 186*cIts* from ABW*thy*$^-$ and ABE*thy*$^-$ lysogen

ABE*thy*$^-$186*cIts*, (E4064) and ABW*thy*$^-$186*cIts*, (E4065) cells were cultured in LB and thymine (2 µg/ml) to about 2 x 10^8 cfu/ml and irradiated in 10 mM $MgSO_4$ containing thymine (2 µg/ml) with 0 and 15 J/m² of UV. Cells were diluted into LB containing thymine (2µg/ml) (prewarmed) and transferred to 39°C. Phage production was assayed at intervals on C600 indicator bacteria.

a. ABW thy$^-$ (186*cIts*)

b. ABE thy$^-$ (186*cIts*)

(● - ●) unirradiated cells

(o - o) 15 J/m² UV-irradiated cells

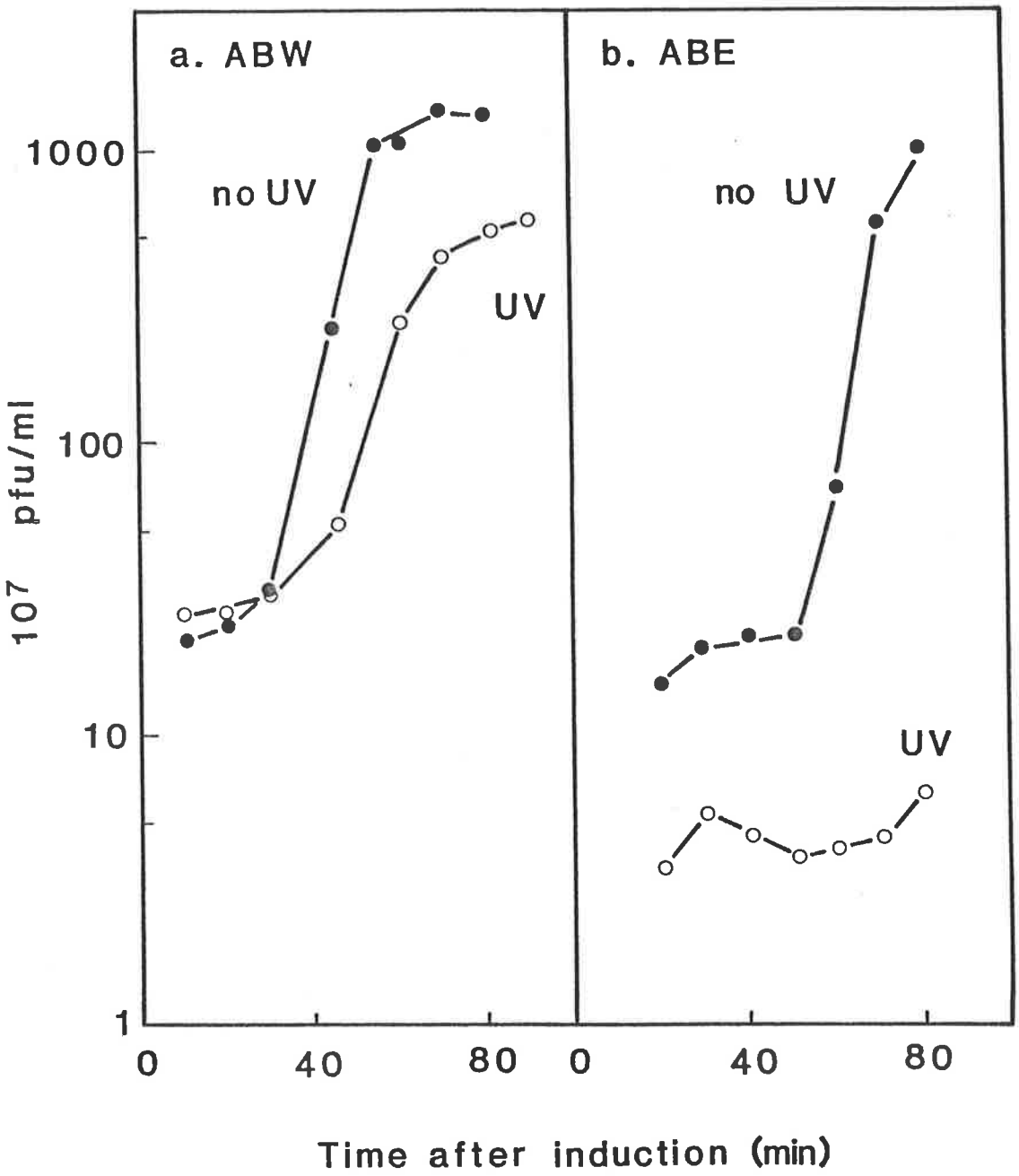

a. ABW
b. ABE
1000
100
10
1
no UV
UV
no UV
UV
10^7 pfu/ml
0
40
80
0
40
80
Time after induction (min)

FIG 5.10 186 DNA replication in 186*cIts* lysogens of ABE and ABW

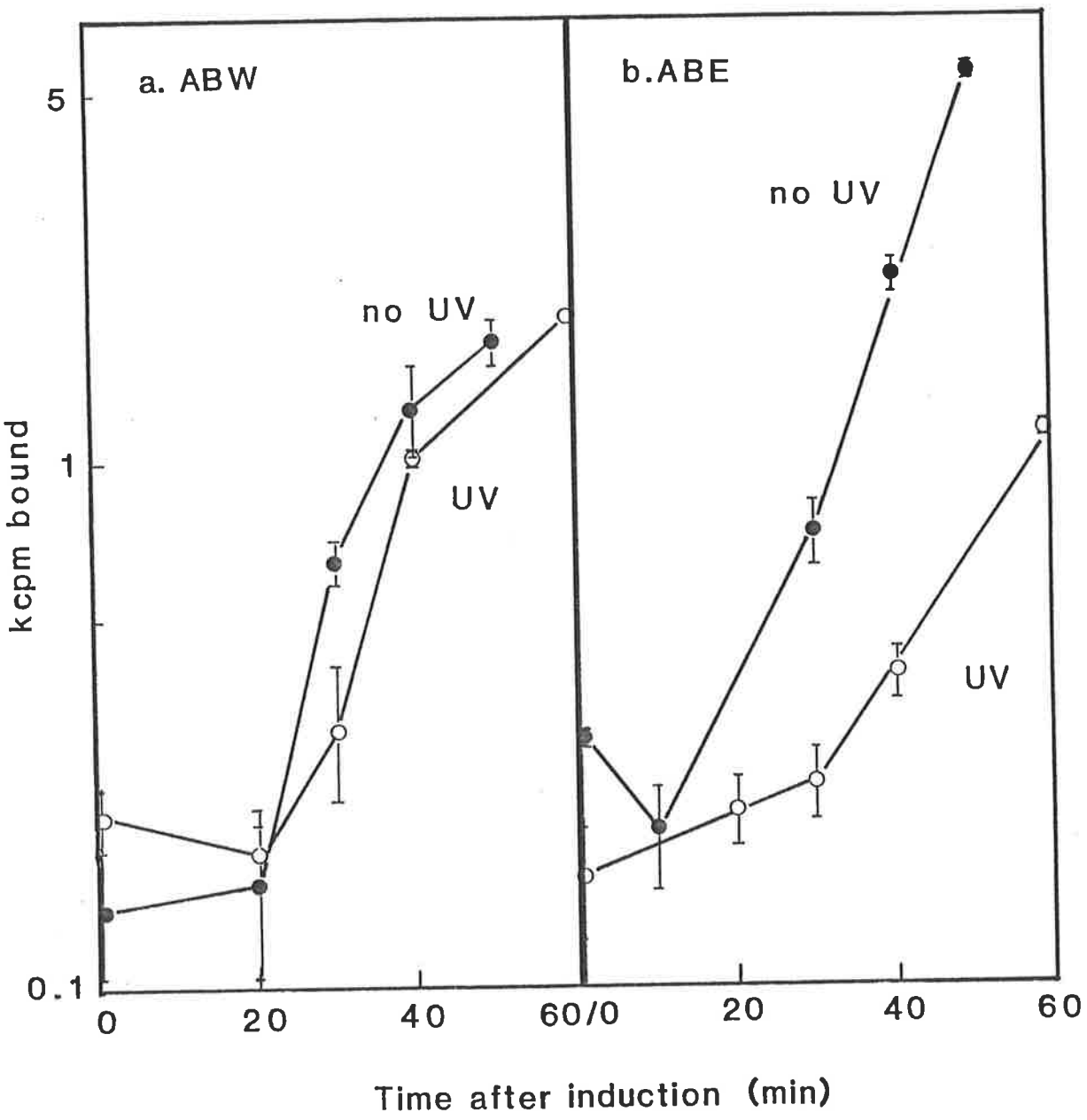
ABEthy$^-$ (186*cIts*), (E4064) and ABWthy$^-$ (186*cIts*), (E4065) lysogens were prelabelled for 3 generation with [^{3}H]thymine (20 µCi/µg/ml) in LB broth. Cells were irradiated in $MgSO_4$ (10mM) containing [^{3}H]thymine (20 µCi/µg/ml) with 0 and 15 J/m^2 of UV. 10 x LB was added to a final concentration of 1x and the prophage heat-induced by transferring the cells to 39°C. Samples were removed and hybridised to 186 DNA on nitrocellulose filters. Mean cpm bound (minus background, see text) from duplicate samples (shown as bars) have been plotted versus time after induction.

a. - ABW thy$^-$

b. - ABE thy$^-$

(● - ●) unirradiated

(o - o) irradiated with 15 J/m^2 of UV


a. ABW
b.ABE
no UV
UV
no UV
UV
5
1
0.1
kcpm bound
0
20
40
60/0
20
40
60
Time after induction (min)

no immediate value to study or identify the difference between the ABE and ABW cultivars.

5.8 CONCLUSION

The UV-induced loss in 186 capacity is not simply a *recA*$^+$ *lexA*$^+$ dependent function.

CHAPTER 6

TESTING THE DnaC-DEPLETION HYPOTHESIS

The coliphage which were tested for phage production in UV-irradiated cells can be divided into two groups. Phage 186, P1 and Mu all have a delay and require *dnaC*, whereas λ and P2 are not delayed and do not require *dnaC* (Hooper and Egan, 1981). As discussed in Chapter 1, this gave rise to the idea that the pool of *dnaC*-protein may be depleted in UV-irradiated cells, i.e. the DnaC-depletion hypothesis.

If the depletion of DnaC caused the loss in 186 capacity of irradiated cells, increasing the amount of cellular DnaC should restore the cells' capacity to produce 186. To test this, the number of *dnaC* gene copies per cell was increased and the effect of this on the cells' capacity to produce 186 was studied. The results are described in this Chapter.

Before this could be done the effect of irradiation with increasing UV-fluence on the capacity of the cells to produce either 186, P1, Mu or λ was further examined.

6.1 Influence of UV-dose on cellular capacity to produce phage

Cells of strain C600 (E508) were UV-irradiated and infected with either λ, 186, P1 or Mu phage as described in section 2.2.7a. Aliquots were assayed for pfu at intervals. Ca^{++} (4mM) was added to the medium for P1 infections, this had no significant effect on cellular survival.

The phage produced are plotted versus time in Fig 6.1. UV fluences up to 90 J/m², delivered to the cells prior to infection with λ, did not alter the capacity of the cells to produce λ phage whereas irradiation with 180 J/m² of UV, caused some loss in capacity to produce λ. (Fig 6.1a). However the capacity of the cells to produce 186, P1 and Mu phage decreased steadily with increasing UV-fluence at lower doses than for λ (Fig 6.1b, c and d).

This data showed that phage production of P1 and Mu, as well as that of 186, was transiently inhibited in UV-irradiated cultures, confirming the findings of Hooper *et al* (1981). It also extended their observations to include the effect of irradiation of the cells with UV fluences >45 J/m².

The results show that the loss in the capacity of the cells to replicate *dnaC* - requiring replicons becomes more severe with increasing UV-fluence. This is consistent with the proposed DnaC-depletion hypothesis, on the basis that the greater the UV-fluence the more severe would be the depletion of *dnaC* protein.

6.2 Effect of increased *dnaC* gene dosage on 186 infection of a UV-irradiated cell

The copy number of a cellular protein can be increased by introducing a multicopy clone, producing the required

FIG 6.1 Infection of unirradiated and UV-irradiated cells with λ, 186, P1 or Mu phage

Cells of strain C600 (E508) were cultured in LB broth to an OD of 1.0 at 37°C. The cells were collected by centrifugation and UV-irradiated in HI buffer (37°C). The cells were sedimented by centrifugation, resuspended in LB broth (prewarmed to 37°C) and infected with the required phage (moa < 1.0) at 0 minutes. After 10 minutes incubation at 37°C, unabsorbed phage were removed by millipore filtration (Section 2.2.7a). The infected culture was diluted into prewarmed (37°C) LB broth and incubation was continued at 37°C. Aliquots were assayed for pfu on C600 indicator bacteria, at various intervals.

a. λ *cIts857*

b. 186*cIts*

c. P1kc

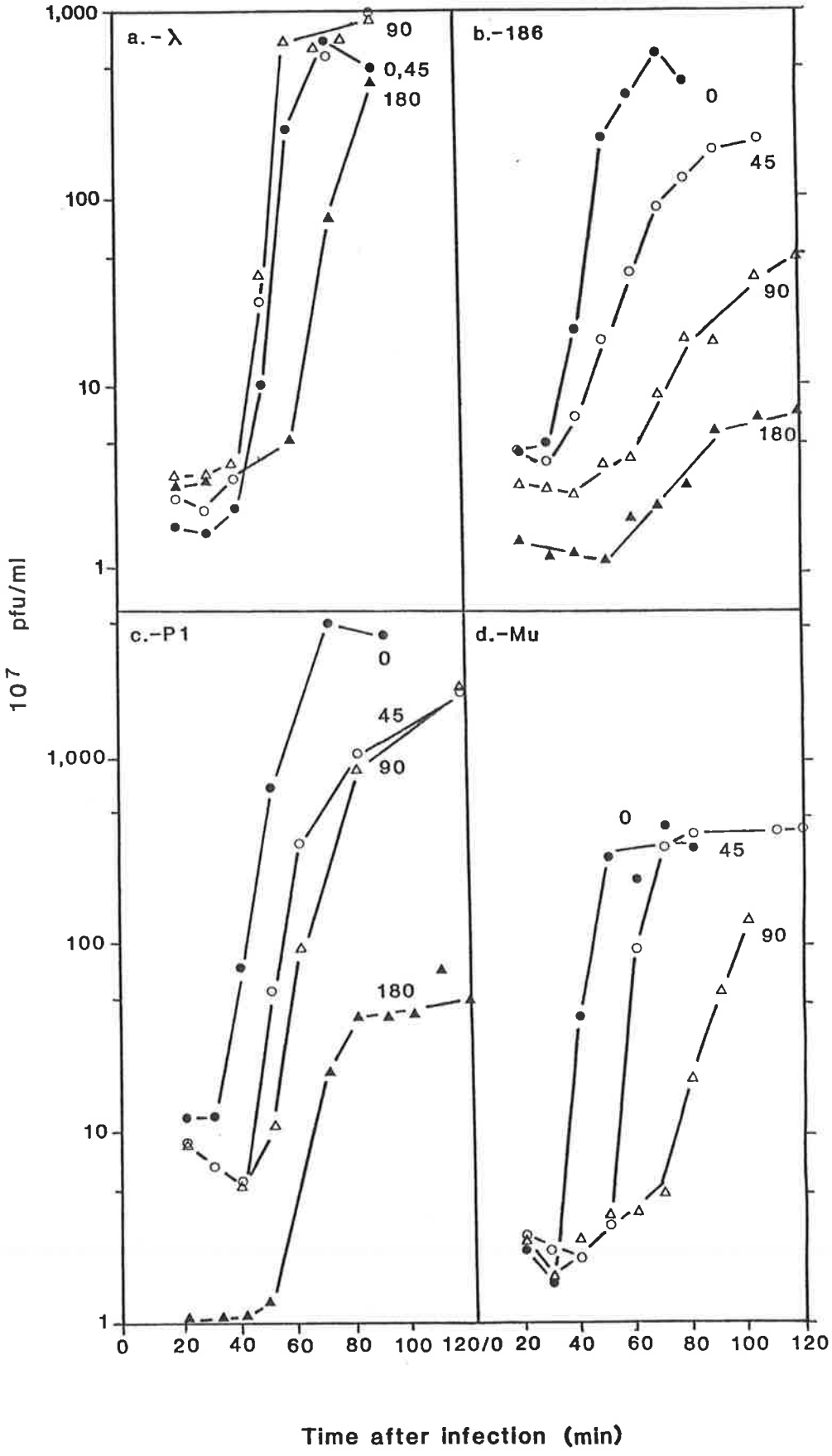
d. Mu*cIts61*

(● - ●) unirradiated cells

(o - o) 45 J/m² - irradiated cells

(Δ - Δ) 90 J/m² - irradiated cells

(▲ - ▲) 180 J/m² - irradiated cells


a.- λ
b.-186
c.-P1
d.-Mu
0
45
90
180
0,45
1,000
100
10
1
0
20
40
60
80
100
120
10⁷ pfu/ml
Time after infection (min)

protein, into a cell. To further investigate the hypothesis, this was done using *dnaC* cloned into phage and plasmid vectors.

It was decided to use a phage vector, rather than plasmid vector, to introduce and amplify the *dnaC* gene in the cell. This was because a phage clone could be introduced into the cells after the culture was irradiated and so circumvent damage to the clone. A plasmid clone would be present at the time of irradiation and the element would probably sustain some UV damage.

Initially, therefore, a λp*dnaC* clone (Iida, 1977) was used to increase the number of copies of the *dnaC* gene. However, it was found that λ and 186 were incompatible. Subsequently, multicopy plasmid clones of *dnaC* (Kobori and Kornberg, 1982) were used.

6.2.1 Use of λp*dnaC*: The *dnaC* gene was cloned into propagating λ by Iida (1977) and was obtained from him. The intention was to co-infect UV-irradiated cells with 186 and λp*dnaC* so that replication of the λ phage would increase the copy number of the *dnaC* gene and presumably result in increased amounts of the gene product.

It was found however that co-infection of unirradiated cells with λ and 186 resulted in reduced burst sizes for both phage as compared with single infections. This led to

the possibility that λ and 186 were detrimental to each other and could not replicate together in the same cell. If this were the case, obviously co-infection with λp*dnaC* could not be used to increase cellular levels of *dnaC* in the presence of 186.

In order to test whether λ and 186 interfered with each other, a double lysogen of the two phage was constructed, with each prophage carrying its mutant temperature sensitive repressor. Heat-induction of the double lysogen should therefore result in a mixed burst from each cell.

Single lysogens of λ*cIts* (E0678) and 186*cIts* (E252) burst at approximately the same time when heat-induced (See section 2.2.8) (Fig 6.2a.).

186 phage bursts from both the single and double lysogens were assayed on a λ*c*I$^+$ lysogen (E511), while λ phage bursts were assayed on a 186 *c*I$^+$ lysogen (E573). The efficiency of plating of the phage on these lysogens compared with non-lysogens is 90-100%.

Heat-induction of the double lysogen (E0679) gave approximately the same burst-size for 186 phage as that obtained from the single lysogen (Fig 6.2b). However, the number of λ phage produced by 90 minutes was only about 10% that obtained from the single lysogen (Fig 6.2b). The level

FIG 6.2 Heat-induction of 186 and λ temperature sensitive prophages from single and double lysogens

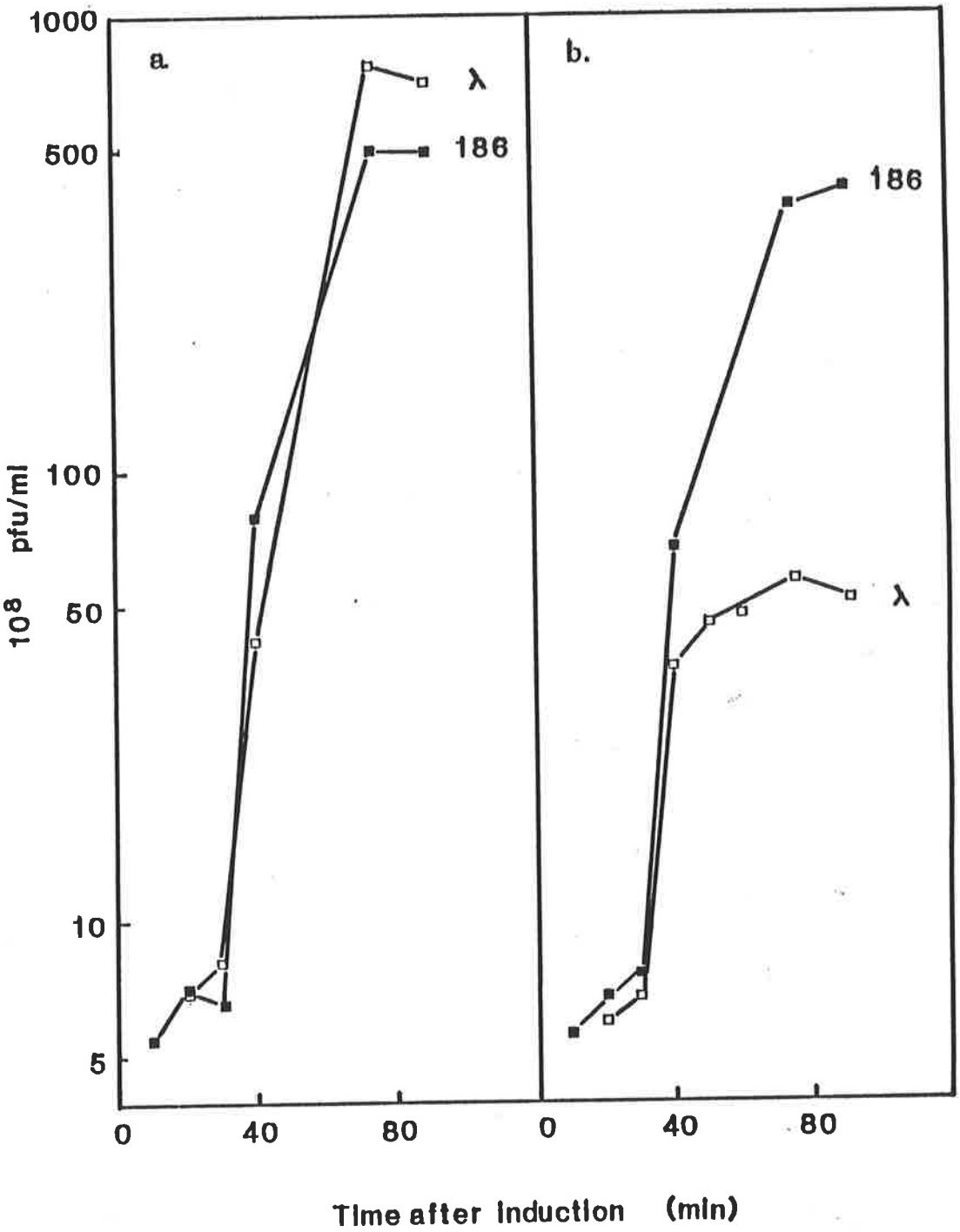
Cells lysogenic for λ*c*I*ts*(E0678), 186*cIts* (E252) or for both phages (E0679) were grown in LB broth at 30° to 5 x 10^8 cfu/ml and transferred to 39°C. The phage produced were assayed at intervals. λ phage were assayed on a 186*c*I^+ lysogen (E573) and 186 phage on a λ*c*I^+ lysogen (E511).

a. single lysogens E0678 and E252

b. double lysogen E0679

(■ — ■) 186 phage

(□ — □) λ phage


1000
500
100
50
10
5
10^8 pfu/ml
a.
b.
λ
186
186
λ
0
40
80
0
40
80
Time after Induction (min)

of infectious centres 20 minutes after induction of the double lysogen was similar for λ and 186 (Fig 6.2b). Showing that both prophage were derepressed and both phage were produced from each cell, although the number of λ phage produced was reduced.

This result means that λ phage production is impaired in the presence of 186 phage production. There was however an earlier report (Baldwin *et al*, 1966) that λ and 186 did not interfere with each other. So single cell analysis was undertaken to study whether both phage were indeed produced from each cell in reduced numbers or whether different sub-populations of the culture produced one phage type and not the other.

Cultures of the required 186*cIts* or λ*cIts* lysogen were cultured in LB broth to an OD of 1.0. The number of cells in the culture was more accurately determined using a haemocytometer. The culture was then diluted to a concentration of 1 cell/ml. The diluted culture (5 ml) was divided into fifty 0.1 ml aliquots, so that only one in ten aliquots should contain a cell. Each aliquot was incubated at the inducing temperature (39°C) for 75 minutes to allow prophage induction and phage production, followed by cell lysis. The contents of the aliquots were then assayed for phage on the appropriate indicator bacteria. In experiments using the double lysogen 10 ml of the diluted culture was divided into fifty 0.2 ml aliquots. After heat-induction

and lysis each aliquot was divided into two portions, one was assayed for λ and the other for 186. This modification allowed both phage to be assayed from each aliquot.

The fraction of aliquots which resulted in an event (ie phage production), was used to predict the number of events which arose from 1 or more cells using the Poisson distribution (See Section 2.2.18).

Since for single lysogens the entire contents of each aliquot were assayed, the number of plaques on each assay plate (pfu/sample tube) was an estimate of the phage burst size per cell, assuming that the aliquot originally contained a single cell. For the double lysogen, since the aliquot was divided in half, the number of plaques/plate was multiplied by 2 to give the burst size per cell.

Table 6.1 is a compilation of the data obtained with single and double lysogens of 186 and λ. The first column shows the burst obtained with 186 phage on heat-induction of a single lysogen. 4/48 aliquots resulted in phage production. Of these all 4 were predicted to have come from single cells. An average of these bursts therefore is the average 186 burst from a single cell, approximately 100 pfu/cell. Similarly for λ, the second column in Table 6.1 shows that 14/47 aliquots yielded phage and of these 12 were predicted to arise from single cells and 2 from 2 cells

TABLE 6.1

Lysogens of 186*cIts* (E252) and λ*cIts* (E0678) and the double lysogen of 186 and λ (E0679) were cultured in LB broth at 30° to an OD of approximately 1.0 (the cell count using the haemocytometer was about 4 x 10^8 cfu/ml) and diluted to about 1 cell/ml. 5 ml of the single lysogens were divided into aliquots (0.1 ml) and 10 ml of the double lysogen were divided into aliquots (0.2 ml). The aliquots were incubated at 39°C for 75 min and then assayed for phage using the indicator strains E573 for λ and E511 for 186 phage. The entire contents of the 0.1 ml aliquots were assayed. The 0.2 ml aliquots were divided into two equal portions of which one was assayed for λ and the other for 186 phage.

a. The total number of samples assayed in each experiment and the number of samples (plates) which contained pfu.

b. The number of samples, predicted by the Poisson distribution, to have originally contained 1 or more cells. (See section 2.2.18).

c. The number of pfu/sample assayed as described above.

TABLE 6.1

Analysis of single cell bursts of phages λ and 186 in single and double lysogens

Lysogen used	186*cIts* (E252)	(λ*cIts*) (E0678)	186*cIts*/λ*cIts* (E0679)
a.			
Total no. of samples	48	47	50
number of samples with no phage	44	33	35
number of samples with phage	4	14	15
b. predicted number of aliquots which contained:			
1 cell	3.82	11.60	12.45
2 cell	0.16	2.05	2.21
3 cells	0.04	0.24	0.26

c.	Number of pfu/sample tube					
Phage assayed for	186	λ		186 #		λ #	
	1	466*	520*	26	42	0	0
	53	160	91	14	46	0	0
	83	193	278	16	88	2	2
	164	211	381	142*	60	0	0
		602*	224	128*	72	6	0
		225	238	80	32	4	0
		260	184	58	34	0	0
				20		0	0
Average burst size/cell	100	222		40		2.6	

* events thought to arise from 2 or more cells

\# plaques/plate x 2 gives the pfu/sample

(Table 6.1b). However, 3 values of pfu/sample tube appeared to be significantly larger than the rest and may arise from more than 1 cell (Table 6.1c). These three values were omitted and the average λ burst size cell was calculated to be approximately 200 pfu.

Columns three and four (Table 6.1c) are derived from the 186/λ double lysogen. They show that the λ phage production/sample was greatly reduced, and most cells that hosted 186 phage production did not produce any λ. From the data, 2 samples were predicted to contain 2 cells so excluding the 2 with a larger number of plaques, the average 186 burst size/cell was about 40% that from the single lysogen. The λ burst size was reduced to 1.0% that obtained with a single lysogen.

The results show that λ phage production is severely depressed in the presence of 186 phage production, and depresses the latter to some extent. However, there is a discrepancy between the extent of depression between these results and the data plotted in Fig 6.2b. Firstly, from Fig 6.2b it can be seen that λ and 186 had approximately the same number of infectious centres, i.e. each cell that produced a 186 phage also produced a λ phage but Table 6.1 shows a greatly reduced number of cells capable of producing both phage. In view of the small numbers of λ phage produced per cell in the latter experiment this difference could to some extent, be due to technical reasons. So that,

dividing each aliquot for the assay of λ and 186 in the single cell analysis may have led to an underestimate of the number of aliquots which contained λ phage. Thus, more cells may have produced 1-2 λ than were detected. Secondly, the burst sizes for both λ and 186 were lower in the double lysogen in Table 6.1 than in Fig 6.2b. This could be due to experimental variation since a repeat of single burst analysis (data not shown) gave a burst of 100 for 186 and 7.5 for λ.

Despite qualitative differences both results show that λ phage production is greatly reduced in a 186/λ double lysogen. Although the data plotted in Fig 6.2a suggested that 186 and λ phage burst the cell at about the same time, it was possible that 186 may have lysed the cell prior to λ packaging. In which case λ replication would be normal but no phage would be obtained. To avoid 186-directed lysis a 186*Bam* mutant was utilised. This mutant continues to replicate phage DNA, but cannot activate the late functions of the phage necessary for lysis (Hocking and Egan 1982b).

A non-amber suppressing su⁻ double lysogen of 186 *Bam*c*Its* and λ*c*I*ts* was constructed (E0680) and single cell bursts were studied in this strain. The average λ burst/cell was only 7% that obtained from a single lysogen. No 186 phage were recovered in this experiment as expected, since the 186*Bam* mutant cannot form progeny phage in an Su⁻ cell.

Thus it can be concluded that λ phage production is impaired in a 186-infected cell and this is due to an early function expressed by 186, since the interference is also observed with a 186*Bam* mutant.

It was not demonstrated whether the interference acted on λ DNA replication or on phage morphogenesis. However, if λ DNA replication was inhibited by replicating 186 a λp*dnaC* clone could not be used to increase cellular DnaC, since no amplification of the *dnaC* gene would be obtained. Also the interference of λ with 186 could affect the interpretation of results. Therefore, it was preferable not to use λp*dnaC* in an attempt to increase cellular *dnaC*, at least until the interference phenomenon was better understood.

6.2.2 Use of *dnaC* cloned into multicopy plasmids:

Kobori and Kornberg (1982) cloned the *dnaC* gene of *E. coli* on a 4.8kb fragment into the *Bam*HI sites of pBR322 (Bolivar, 1977) and pMOB45 (Bittner and Vapnek, 1981).

pMOB45 is derived from R1*drd*19, a runaway replication mutant of plasmid R1. (Uhlin *et al.*, 1979; Bittner and Vapnek, 1981). The runaway replication is temperature-inducible and results in the high copy number of the plasmid.

It was reported by Kobori and Kornberg (1982) that the pBR322-*dnaC* clone (pJK169) grown in LB at 37°C, produced 3.3 fold more *dnaC* protein than the control strain, while heat-induction of the pMBO45-*dnaC* clone (pJK129) overproduced *dnaC* protein 45 fold. In fact up to 150 fold overproduction was reported with this plasmid under certain conditions in a 300L fermenter (Kobori and Kornberg, 1982).

The above clones were obtained and used to study the DnaC-depletion hypothesis in the presence of low and high amounts of the *dnaC* protein.

It was desirable to study the effect of these plasmids in the C600 background, since the loss in 186 capacity had been best characterised in this strain. Although Kobori and Kornberg (1982) utilised a *dnaC*$^-$ *recA*$^-$ (SY562) background to study *dnaC* protein overproduction, overproduction also occurs in a *dnaC*$^+$ cell (J Kobori, personal communication). I tested these plasmids for expression of the *dnaC* protein in C600 cells. Both the pBR322 and pMOB45 clones were able to complement the temperature sensitivity of a C600 *dnaC2*(Ts) *recA56* mutant (E4053, this strain was constructed by P1 transduction of *recA56srl*::Tn10 from E761 into E990). Thus showing that the dnaC protein is produced in this strain.

Kobori and Kornberg (1982) assayed the extent of *dnaC* overproduction in an *in vitro* reconstitution assay, using conversion of ϕX174 single stranded DNA to the duplex RF form. I lacked the purified proteins and enzyme fractions to carry out this assay and so could not measure overproduction under the conditions used. Polyacrylamide gel electrophoresis was not a good method to confirm overproduction since at least 100 fold increase in the protein was required to visualise it (Kobori and Kornberg, 1982). Since the conditions used here, were expected to result in only 3-40 fold overproduction of the protein it was not surprising that the protein was not detectable on an acrylamide gel (data not shown).

Since there was no obvious reason why these multicopy plasmids should not overproduce the protein in C600 cells it was assumed that increased amounts of *dnaC* protein were present under the conditions described and 186 phage bursts were studied in irradiated and unirradiated cells.

Initially, the effect of the pBR322-*dnaC* clone on the loss in 186 capacity was tested. C600 cells with and without the pBR322 clone were UV-irradiated and infected with 186 (moa of 5.0), and the number of phage produced was measured (Fig 6.3).

Irradiation of the control C600 culture with 45 J/m² of UV resulted in the expected loss in 186 capacity (Fig.

FIG 6.3 The effect of *dnaC* cloned into pBR322 on the UV-induced loss in 186 capacity

Cells of strain C600 (E508) and C600/pBR322-dnaC (pJK169) were cultured in LB broth at 37°C to a density of 2 x 10^8 cfu/ml, UV-irradiated (45 J/m^2) in HI (37°C) and infected with 186*c*Its (moa of 5.0). After 5 minutes at 37°C, unabsorbed phage were inactivated by antiserum. Aliquots were assayed for pfu on C600 indicator bacteria at intervals after infection.

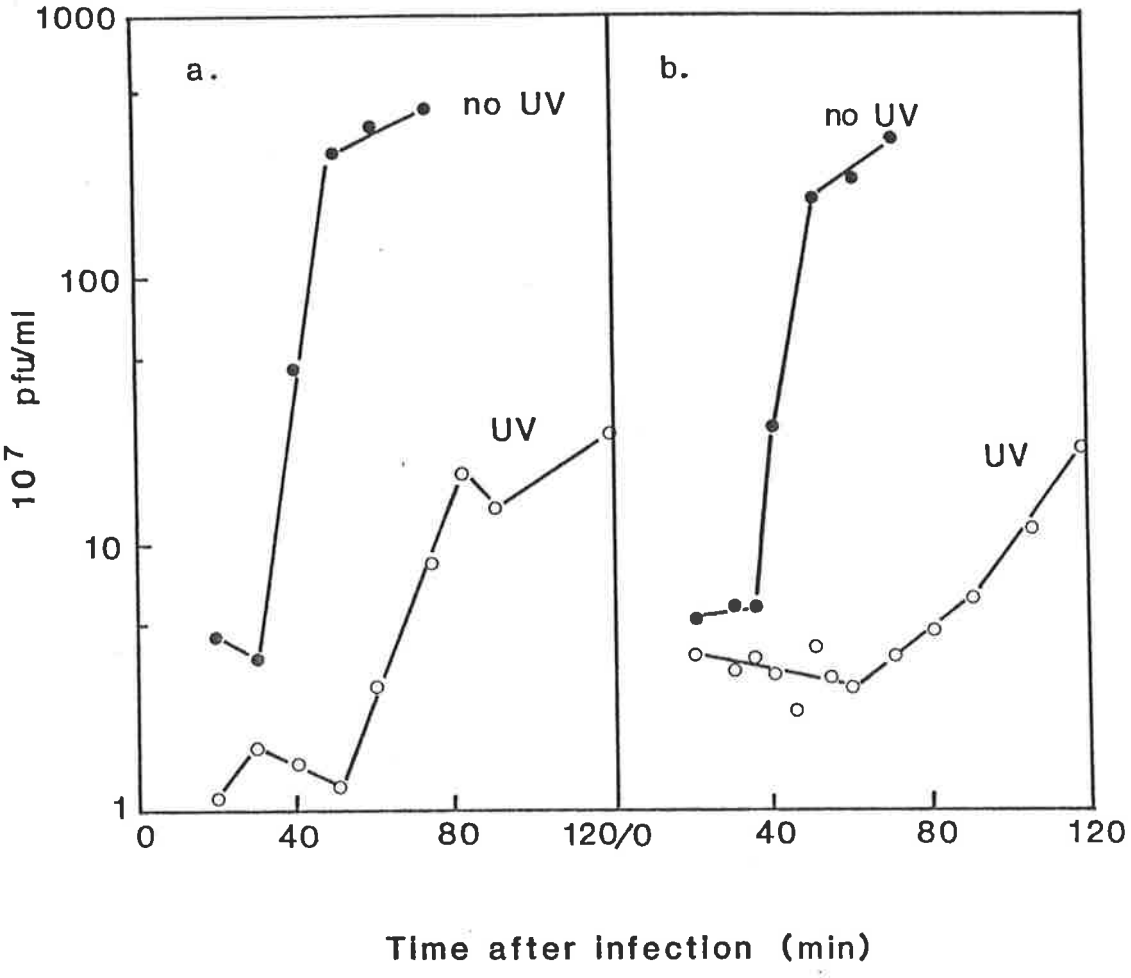
Cellular survival after irradiation with 45 J/m^2 was 45-50% in both cultures.

a. C600

b. C600/pBR322-*dnaC*

(● - ●) unirradiated cells

(o - o) 45 J/m^2 UV-irradiated cells


1000
100
10
1
10^7 pfu/ml
a.
b.
no UV
no UV
UV
UV
0
40
80
120/0
40
80
120
Time after infection (min)

6.3a). The presence of the pBR322-*dnaC* clone did not restore the capacity of the irradiated cells to produce a normal 186 phage infection (Fig 6.3b).

Since the pBR322-*dnaC* clone results in only a 3 fold increase in *dnaC* protein (Kobori and Kornberg, 1982) the pMOB45-*dnaC* clone was tested for its effect on 186 phage production in UV-irradiated cultures, using the conditions of Kobori and Kornberg (1982) which resulted in 40-50 fold overproduction of *dnaC*. The conditions they used to obtain 100-150 fold overproduction were impractical for these experiments as they employed a 300L fermentor.

C600, C600/pMOB45 and C600/pMOB45-*dnaC* cells were cultured, UV-irradiated and infected with 186 phage as described in the legend to Fig 6.4. Phage production was measured, at intervals. The presence of *dnaC* on the pMOB45 vector did not significantly improve 186 phage production in the irradiated cultures (Fig 6.4a and b) compared with the vector alone. But the pMOB45 vector alone exacerbated the effect of UV-irradiation on 186 phage production (Fig 6.4a and c.).

The pMOB45 vector and clone also affected the cell density in an unirradiated culture and the cellular survival after irradiation. At an OD of 1.0 an unirradiated C600/pMOB45 culture had only 60% of the viable count of a C600 control culture, but the percentage cellular survival

FIG 6.4 Effect of pMOB45 and pMOB45-*dnaC* on the UV-induced loss of 186 capacity

Cells of strain C600, C600/pMOB45 and C600/pMOB45-*dnaC* (pJK129) were cultured in LB broth at 30°C to an OD of 0.1, then incubated at 37°C to an OD of 1.0 and irradiated in $MgSO_4$ (10mM, 37°C) with either 0 or 45 J/m² of UV. Cells were sedimented and resuspended in LB broth and infected with 186 phage (moa 10) at 0 minutes. After 5 minutes at 37°C, unabsorbed phage were inactivated with antiserum, the infected culture was diluted into fresh prewarmed LB broth and incubation continued at 37°C. Aliquots were assayed at intervals for pfu on C600 indicator bacteria.

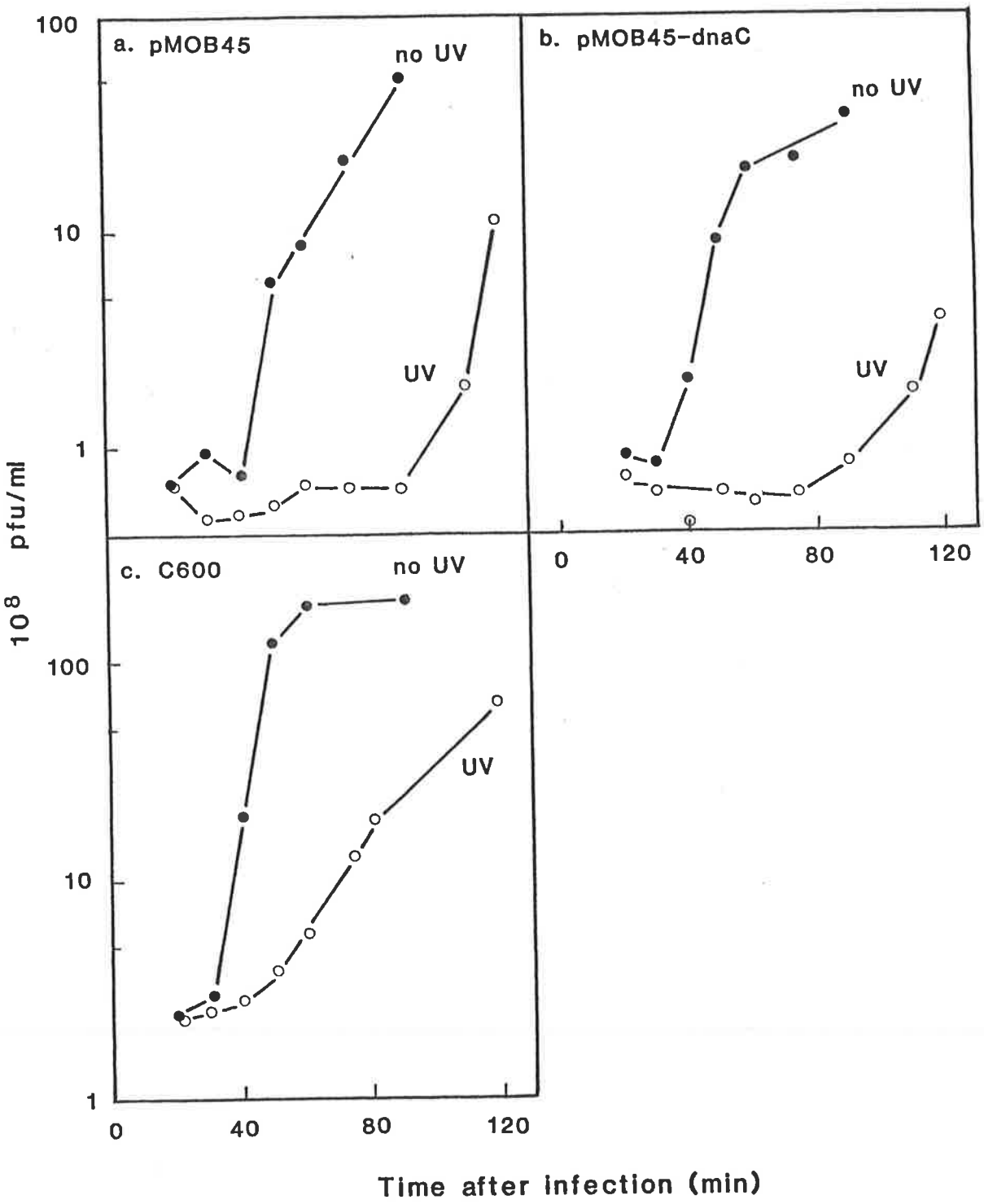
a. C600/pMOB45

b. C600/pMOB45-*dnaC*

c. C600

● - ● unirradiated cells

o - o 45 J/m²-irradiated cells


a. pMOB45
no UV
UV
b. pMOB45-dnaC
no UV
UV
c. C600
no UV
UV
100
10
1
100
10
1
0
40
80
120
0
40
80
120
10^8 pfu/ml
Time after infection (min)

(45-50%) after 45 J/m² of UV was the same as the control C600 cells. So pMOB45 does not affect UV-survival of the cells. Whereas a C600/pMOB45-*dnaC* culture, at an OD of 1.0, had only 20% of the viable count of the C600 control culture, and cellular survival after 45 J/m² of UV was reduced to 20-25% of the unirradiated culture. Therefore the pMOB45-dnaC clone affects the viability and UV-survival of the C600 cells.

6.3 Conclusion

The decease in cellular capacity to produce 186, P1 and Mu phages becomes more marked with increasing UV-fluence. Attempts to use λp*dnaC* to increase the *dnaC* gene copy number did not succeed, since 186 and λ interfered with each other. Using plasmid clones to increase the *dnaC* gene copy number, and therefore to amplify the amount of *dnaC* protein per cell, did not ameliorate the effect of UV-irradiation of cells on 186 phage production.

It was tentatively concluded that depletion of *dnaC* protein was not responsible for the loss in capacity of UV-irradiated to produce 186 phage. The ramifications and qualifications of this conclusion are discussed elsewhere (Chapter 8).

CHAPTER 7

UV-INDUCED INHIBITION OF *oriC*-DEPENDENT REPLICATION

UV-irradiation of cells exerts a trans-acting inhibition on 186 DNA synthesis. Study of this effect led to the possibility that such a UV-induced inhibition may act primarily against *E. coli* chromosomal replication and fortuitously against other replicons like 186, which shared a requirement for certain functions with the host. Presumably this inhibition, either due to an inhibitor or due to the induction of refractory conditions, would disallow the initiation of replication from *oriC* (the origin of *E. coli* replication). It was unlikely that such an inhibition would act against the elongation of DNA synthesis since some replicons λ and P2, which require several elongation functions (Furth and Wickner, 1983; Bowden *et al.*, 1975), are unaffected in irradiated cells. To test whether UV does induce such *trans*-acting effects, *oriC*-dependent replication was studied in UV-irradiated cells.

Studying the effect of UV on initiation of replication from the resident chromosomal origin would be complicated by the presence of photoproducts in the chromosome. These photoproducts are known to interfere with elongation, making it difficult to differentiate between the *cis*-acting inhibition induced by the photoproducts and the proposed *trans*-acting inhibition of initiation. Therefore a means of introducing an undamaged replicon, dependent on *oriC*, into UV-irradiated cells was sought.

Soll (1980a) constructed a specialised transducing λ phage, λ*oriC*, carrying the *oriC asnA* region of the *E. coli* chromosome. This phage retains all its lytic functions including *ori*λ and is therefore capable of forming plaques. In addition, initiation from the cloned *oriC* allows the circularised phage DNA to replicate extrachromosomally as a plasmid. The phage can thus replicate from both origins, *oriC* and *ori*λ. If initiation from *ori*λ is inhibited, phage DNA synthesis becomes dependent on initiation from *oriC*. Under these conditions the phage can be used to transport an undamaged *oriC*-dependent replicon into an irradiated cell. Replication of the phage then becomes an assay for *oriC* activity. λ*oriC* was therefore used to investigate whether or not UV-irradiation induces a *trans*-acting 'inhibition' of *oriC*-dependent DNA synthesis.

The λ*oriC* phage (λ1020) was obtained from L. Soll and tested for its expected phenotype.

7.1 TESTING THE PHENOTYPE OF λ*oriC* PHAGE

Phage λ*b221cIamporiCasnA* (λ1020) and λ*b221cIam14* (λ68) will be referred to as λ*oriC* and λ, respectively, in this section.

The *b221* deletion removes the *att* site and *int* gene and thus prevents λ site-specific recombination, so the only way the λ*oriC* phage can stably transduce *recA*-Asn-(*asnA*-*asnB*-cells) to Asn$^+$ (*asn*A$^+$) is if replication occurs from

oriC. At the same time *ori*λ must be repressed to prevent lysis of the cells.

To test for the presence of *asnA*, the phage were plated on a lawn of C600 bacterial cells for single plaques. The plate was treated with $CHCl_3$ vapour to kill the bacterial lawn. Single plaques were toothpick-transferred onto a lawn of Asn-*recA*-(λ*c*I$^+$) cells (E4056). The cells were plated at a density of 5 x 10^8 cfu per plate on an M9 plate unsupplemented with asparagine. The λ*c*I$^+$ repressor was used to repress *ori*λ. Stabs from control λ plaques did not result in visible bacterial growth while stabs from λ*oriC* plaques did. The growth was not due to transfer of Asn$^+$ cells from the original C600 lawn, since there was no growth in control stabs taken from the $CHCl_3$-treated lawn. Therefore λ*oriC* can transduce Asn$^+$ and it can be concluded that the phage carries the *asnA* gene as expected.

In order to decide whether the phage still carried functional *oriC*, it had to be tested whether the Asn$^+$ transductants obtained with λ*oriC* were stable. This was because *oriC*-*asnA* phage are capable of giving rise to unstable Asn$^+$ transductants. (Bradley, 1983).

Therefore to test if the phage carried *oriC* as well as *asnA*, 100-200 phage were plated directly onto a bacterial lawn of E4056 cells (5 x 10^8/plate) on an unsupplemented M9 plate . Thus individual phage infected cells in the lawn.

Asn^+ transductant would arise as colonies only if these phage were stably maintained from *oriC*. Large colonies grew on Asn^- lawns transduced with λ*oriC* but not λ. A background of minute colonies was present even on the uninfected control lawn and could be due to reversion of the *asnA*$^-$ or *asnB*$^-$ allele. Therefore λ*oriC* gives rise to stable Asn^+ transductants.

To ensure that the Asn^+ transductants were not the result of a spurious integration event and that the phage was maintained as an extrachromosomal plasmid, some of the large colony-forming transductants and minute background colonies were tested for the presence of the λ*oriC* plasmid.

Plasmid DNA was extracted from the above colonies by the method of Grosveld *et al.* (1981) (see Section 2.2.14c) and electrophoresed on an agarose gel. The minute colony from the uninfected and λ infected controls did not contain a large plasmid (Fig. 7.1, track 1 and 2) but all 5 colonies of λ*oriC* derived transductant had a large band (>23.1 kb, M track) (Fig. 7.1, tracks 3-7). This confirmed that λ*oriC* is maintained autonomously. Therefore, since individual λ*oriC* phage are capable of stably transducing Asn^- cells to Asn^+ even though the phage DNA is incapable of either integrating into the chromosome or replicating from *ori*λ, it must carry the alternative origin *oriC*.

FIG 7.1 Plasmid preparations from Asn$^+$ transductants derived from infections with λ and λ*oriC* phage.

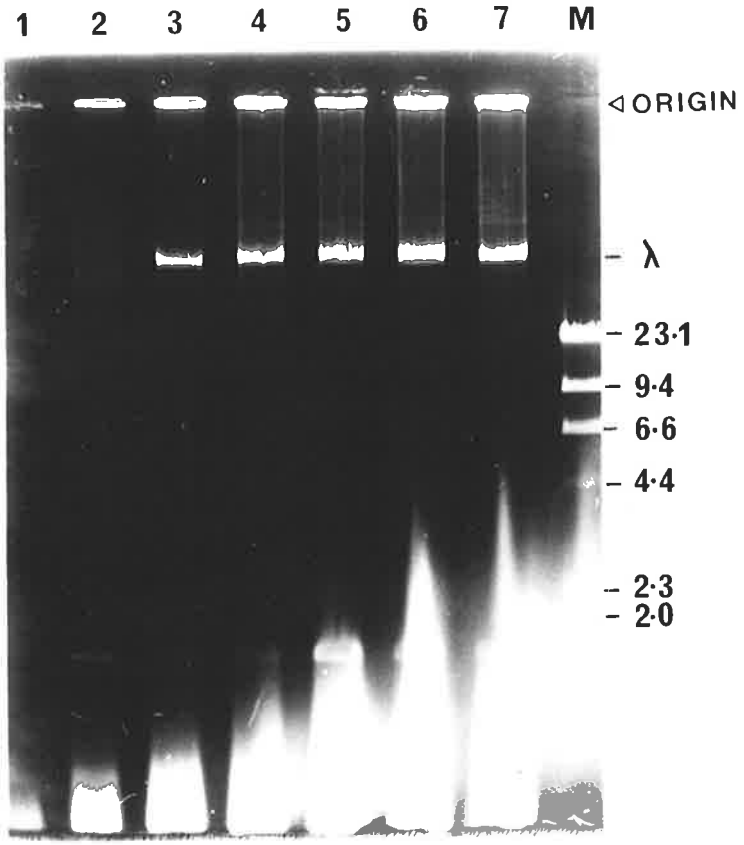
λ*oriC*- and λ- derived Asn$^+$ transductant colonies of *asnA asnB recA*$^-$ (λ*c*I$^+$) were cultured in M9CAA medium, cells were lysed and plasmid extracted by the method described in section 2.2.14c and aliquots were electrophoresed on 1% agarose gels. The gel was stained with ethidium bromide and photographed. The top of the gel is labelled 'ORIGIN' in the figure, and the plasmid band is labelled 'λ'.

track 1 - minute revertant colony

track 2 - minute colony off plate transduced with λ

track 3 - 7 large colonies of λ*oriC* transduced *Asn*$^+$ cells

track M - λ *Hin*dIII restriction fragments used, as size markers, approximately 23.1 kb, 9.4kb, 6.6kb, 4.4kb, 2.3kb and 2.0kb in size.


1
2
3
4
5
6
7
M
ORIGIN
λ
23·1
9·4
6·6
4·4
2·3
2·0

Thus the presence of the *oriC asnA* region on the λ*oriC* phage obtained from L. Soll, was confirmed and it was used to study replication from *oriC*.

In principle, replication from *oriC* could be examined by infecting cells under conditions in which replication from *ori*λ is inhibited, so that the phage burst would represent the amount of replication from *oriC*. However replication from *oriC* is subject to copy number control (von Meyenberg *et al.*, 1978; Ogura *et al.*, 1980) and the λ*oriC* plasmid replicating from *oriC*, has an apparent copy number with a lower limit of 2-3 copies per host chromosome. The actual copy number may be somewhat higher (Soll, 1980a), but apparently this is not high enough to give a measurable burst. Therefore 3 other methods were developed to allow detection and/or quantitation of the low level of *oriC* dependent λ replication in both irradiated and unirradiated cells.

These were:

(i) studying phage bursts from λ*oriC*, using conditions which made phage production dependent on initial rounds of replication from *oriC*, with subsequent replication from *ori*λ resulting in a measurable burst (Section 7.2).

(ii) Following incorporation of radioactive precursor into λ*oriC* replicating extrachromosomally as a plasmid from *oriC* (Section 7.3).

(iii) Estimating the extent of *oriC*-dependent λ replication, by DNA-DNA hybridisation (Section 7.4).

7.2 THE EFFECT OF UV-IRRADIATION ON *oriC*-DEPENDENT PHAGE PRODUCTION FROM λ*oriC*

Conditions were used where derepression of *ori*λ was contingent on initial replication from *oriC*. The phage burst resulting from replication from *ori*λ was therefore dependent on *oriC*.

Soll (1980b) reported that λ*oriC*, but not λ, was capable of forming minute plaques on a λ*cIts*857 lysogen at 35°C but not at 30°C. His interpretation of this observation was that the intermediate temperature (35°C) caused partial inactivation of the temperature-sensitive repressor, although the reduced concentration of the active repressor was still sufficient to repress the resident prophage and an incoming λ phage. If the incoming phage was λ*oriC*, replication would initiate from *oriC*, producing a few cycles of λ*oriC* replication. The resultant limited increase in copy number of the infecting phage may be sufficient to titrate out the remaining active λ repressor, derepressing *ori*λ and giving rise to a phage burst.

The ability of λ*oriC* to give rise to a phage burst in a λ*cIts857* host at 34-36°C was tested, first in unirradiated and then in UV-irradiated cells.

7.2.1 *oriC* dependent phage production in unirradiated cells

Initially, an Su-(λ*cIts857*) lysogen (E516) was selected as the bacterial host for these experiments. The sup° allelle ensured that the *cIam* gene on λ*oriC* and λ would not contribute to the cellular repressor pool.

The λ*cIts857* lysogen (E516) was grown at 30°C in M9CAA supp medium to an OD of 0.2 and infected with λ*oriC* or λ at low moa (between 1-5 for different experiments). The cultures were transferred to temperatures ranging from 34-36°C. After 4 min, unabsorbed phage were inactivated with λ-specific antiserum and the culture was diluted away from the antiserum into fresh prewarmed medium. Incubation was continued at the desired higher temperature (34-36°C). Samples were removed at intervals and were assayed for pfu on C600 indicator bacteria.

Control lysogenic cells which had been treated as above, except that they were not infected with either λ or λ*oriC*, gave rise to minute plaques on the lawn of indicator bacteria, indicating that the prophage in some cells was being derepressed. The derepression could occur at two stages; during incubation of the culture at 34-36°C, or

during incubation of the plates at 37°C overnight. If it was due to the latter incubation, then treating each aliquot with $CHCl_3$ should lyse the lysogenic infectious centres and prevent derepression and plaque formation by the prophage. $CHCl_3$ treatment, however, did not prevent plaque formation from the uninfected lysogenic cells. Therefore, the minute plaques must have resulted from derepression of λ prophage during the incubation at 34-36°C. Strain E516 was, therefore, an unsuitable host to study phage production from λ*oriC*.

To overcome the problem of infecting a lysogen a ΔHI λ*cIts857* defective lysogen (E832) was used. The ΔHI deletion removes all the rightward lytic functions of λ resulting in a defective prophage, it also deletes some *E. coli* genes (*chlA bio uvrB*). However it retains the λ*c*I857 gene (Castellazi *et al.*, 1972). Since the defective prophage lacks the *cro* function (Castellazi *et al*, 1972), there was a good chance that the drop in active *c*I repressor at 34-36°C which was sufficient to derepress the intact λ*cIts857* prophage, would not result in the *cro* dependent turn-off of *c*I production and so the level of active repressor would be maintained.

λ*oriC* and λ phage production was tested in E832 cells at temperatures varying from 34-36°C by the procedure described earlier. The culture had a viable count of 2×10^8 cfu/ml and the phage were added to a multiplicity of

1.0. (The moa of phage was kept at approximately 1.0 in these experiments to avoid derepression of *ori*λ with large numbers of incoming phage). Phage production from the two phage was assayed at intervals. At 34.5°C production of λ*oriC* phage was consistently better than that of λ, both in the total number of phage produced and in the number of infectious centres obtained. The total number of phage was 20- to 50- fold greater, the burst size was 5- to 10- fold higher and 2- to 5- fold more infectious centres were obtained.

Fig. 7.2 records the data from a typical experiment. In this experiment, a λ*oriC*-infected culture contained 20 times as many phage as a λ-infected culture 135 min after infection. λ*oriC* had a burst approximately 10 times greater than λ, and infection with λ*oriC* resulted in about 3 times as many infectious centres as infection with λ.

A noticeable qualitative difference between the two infections was the size of the infectious centres. λ*oriC*-infected cells gave normal-sized infectious centres, whereas λ-infected cells resulted in mostly minute infectious centres.

Although λ was capable of producing some phage in the presence of the *cIts* repressor at 34.5°C, the presence of *oriC* greatly improved its ability to do so. The difference in phage production, burst size, number and size of

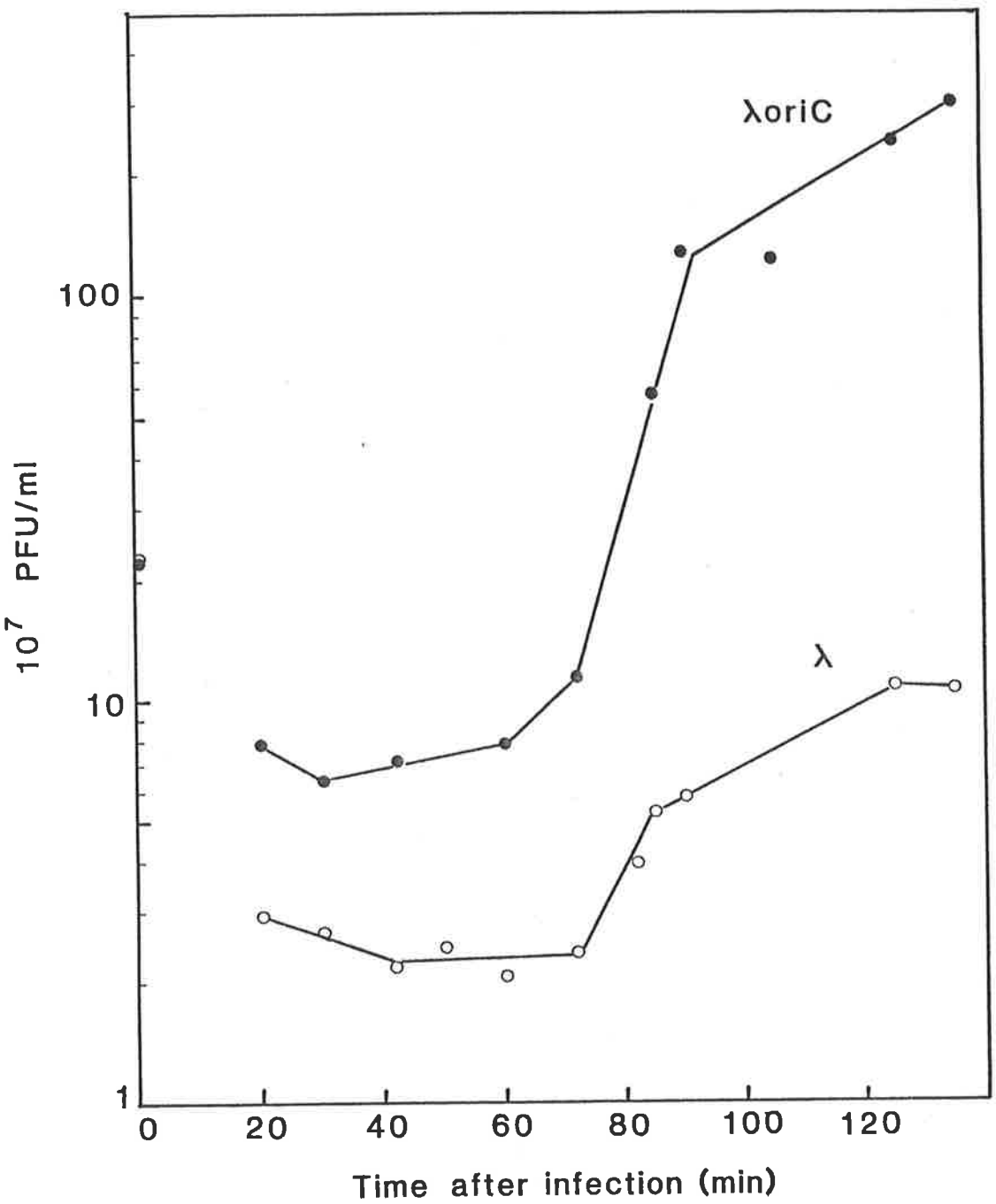
FIG 7.2 *oriC*- dependent λ phage production in ΔHI (λ*c*I857) cells at 34.5°C

ΔHI (λ*c*I857) (E832)cells were cultured at 30°C in M9 supp medium to an O.D. of 0.2 (2 x 10^8 cfu/ml), infected with λ*oriC* (λ1020) and λ (λ68) (moa of 1.0) and incubated at 34.5°C. After 4 minutes unabsorbed free phage were inactivated with λ-specific antiserum and incubation continued at 34.5°C.

The number of phage was assayed on C600 indicator bacteria at intervals after infection.

(● - ●) λ*oriC* (λ1020)

(o - o) λ (λ68)


λoriC
λ
100
10
1
10^7 PFU/ml
0
20
40
60
80
100
120
Time after infection (min)

infectious centres between λ and λ*oriC* was therefore, attributed to replication from *oriC*.

At lower temperatures (34°) the production of both phage was reduced and at temperatures greater than 35°C the phage production from λ (background) was quite high, nearly the same as that from λ*oriC*, so 34.5°C was selected as the optimum temperature to study *oriC*-dependent phage production. It should be noted that the λ control was capable of producing a normal burst size and latent period in non-lysogenic cells at 37°C (Fig. 7.17), showing that it was not a defective phage.

7.2.2 *oriC*-dependent phage production in UV-irradiated cells

UV-irradiation of a λ lysogen causes induction of the prophage due to *recA*-dependent cleavage of the λ*c*I repressor, as discussed previously. Cleavage and inactivation does not occur if the repressor carries an *ind*$^-$ mutation. The prophage present in strain E832, ΔHI, was derived from a λ*c*I*ts*857. Since the *c*I857 mutation was originally isolated in an *ind*$^-$ phage (Sussman and Jacob, 1962), it was expected that the ΔHI prophage would be *ind*$^-$. If this were not the case, strain E832 would not be suitable for use in this study, as inactivation of the *c*I repressor by UV would allow incoming phage to replicate from *ori*λ.

To confirm that the *c*I repressor of the prophage was an *ind*- mutant, the ability of the UV-irradiated ΔHI strain to produce phage from an incoming λ phage at 34.5°C, was studied. The ΔHI cells (E832) were cultured in M9CAA supp medium at 30°C and were then irradiated with 90 J/m² of UV and infected with λ as described in the previous section. The number of phage produced was assayed at intervals.

Irradiation of the cells with 90 J/m² prior to the infection with λ did not significantly change either the amount of phage produced or the burst size (Fig. 7.3a) although the latent period and rise time were a little shorter. However, the UV treatment did not increase or decrease the background phage production obtained with λ phage.

Therefore, under the conditions used, UV does not significantly inactivate the λ repressor at 34.5°C and the system is suitable for study of the effect of UV on *oriC*-dependent phage production.

A dose of 45 J/m² of UV was decided upon for this experiment since both Uvr^+ and Uvr^- cells irradiated with this fluence have a decreased capacity to host a 186 infection (see Chapter 4) and the ΔHI strain is a *uvrB* mutant.

FIG 7.3 The effect of UV on *oriC*-dependent λ phage production

Cells of the ΔHI(λ*c*I857) strain (E832) were cultured at 30°C in M9 supp medium to an O.D. of 0.2 (2 x 10^8 cfu/ml). They were irradiated with either 0, 45 or 90 J/m² of UV and were then infected with λ*oriC* or λ. The cells were then incubated at 34.5°C for 3 minutes, after which time unabsorbed phage were inactivated using λ-specific antiserum. Phage production was assayed at intervals after infection, using C600 indicator bacteria.

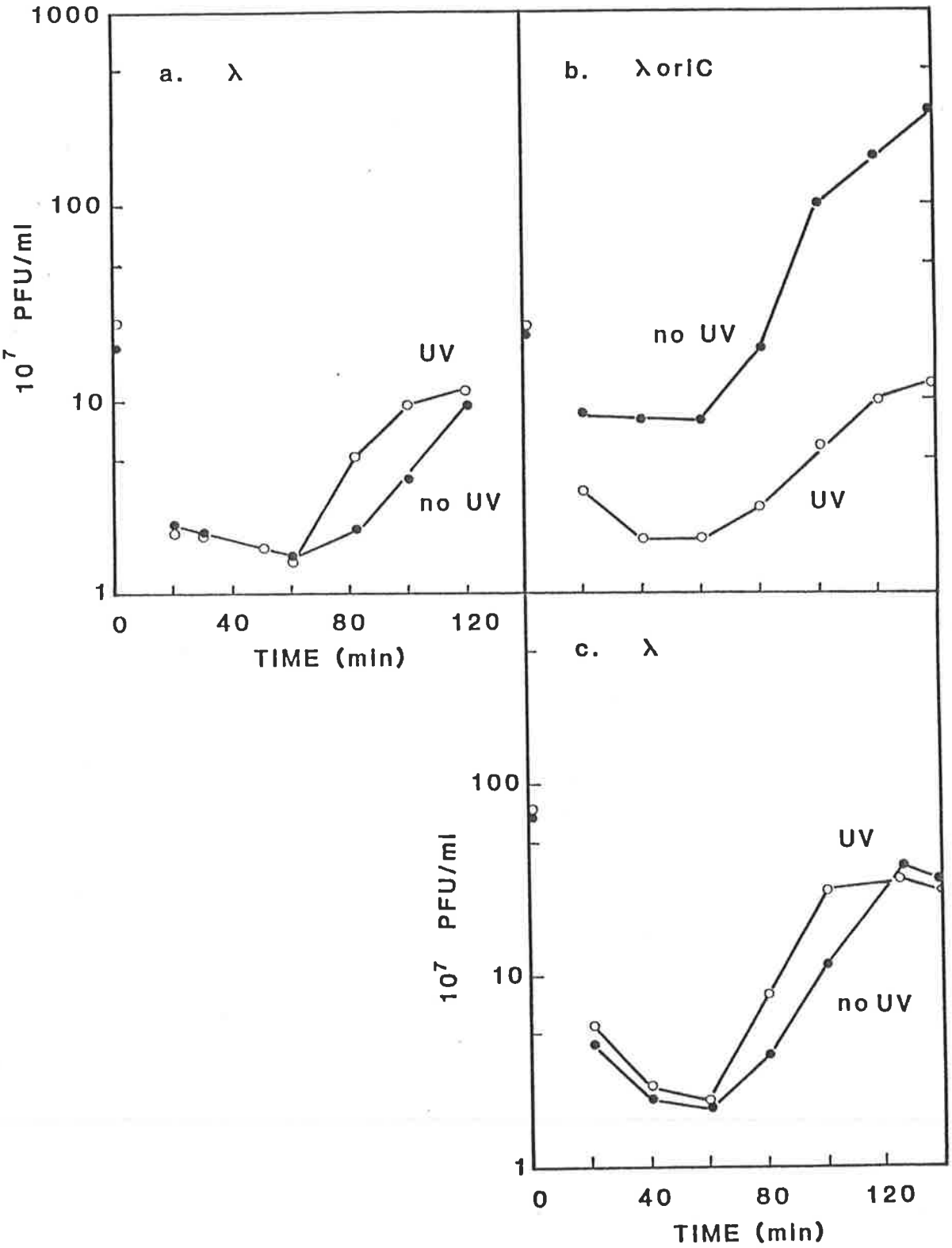
a. λ (moa 1.0), 0 and 90 J/m² of UV

b. λ*oriC* (moa 1.0), 0 and 45 J/m² of UV

c. λ (moa 3.0), 0 and 45 J/m² of UV

(● - ●) unirradiated cells

(o - o) UV-irradiated cells


a. λ
b. λoriC
c. λ
1000
100
10
1
10^7 PFU/ml
UV
no UV
0
40
80
120
TIME (min)

ΔHI cells (E832) cultured in M9CAA supp medium at 30°C were irradiated with 0 and 45 J/m² of UV and infected with λ*oriC* and λ as described in the previous section. The moa of λ*oriC* was 1.0 and that for λ was 3. Phage production was measured at intervals after infection.

λ*oriC* phage production in the unirradiated cells was the same as described earlier (Figs. 7.3b and 7.2). In contrast, λ*oriC* phage production in 45 J/m²-irradiated cells was completely depressed. Total phage production was 20-fold lower than in the unirradiated control. The burst size was 5 times less and the number of infectious centres was 4 times lower than in the unirradiated cells. In addition the size of the infectious centres was much smaller than the control culture.

The pattern of λ*oriC* infection of UV-irradiated cells closely resembles, the pattern seen with λ infection of unirradiated cells (Fig. 7.2 and 7.3a). That is, only the background of phage production from *ori*λ is obtained in the irradiated cells. Therefore *oriC*-dependent replication does not appear to occur in UV-irradiated cells.

Background λ phage production under the same conditions in 45 J/m²-irradiated cells is not significantly affected (Fig. 7.3c). The slightly better phage production in this experiment in the unirradiated cells, compared with the experiments described in Figs. 7.2 and 7.3a is probably

due to the higher moa (3 vs 1). Despite this it is clear that the background is not depressed by 45 J/m² of UV (Fig. 7.3c). In fact, even irradiation of the cells with twice the fluence (90 J/m²) does not depress the background phage production from *ori*λ (Fig. 7.3a).

Therefore the drop in phage production obtained with λ*oriC* in the UV-irradiated cells is not due to a decrease in the level of background replication.

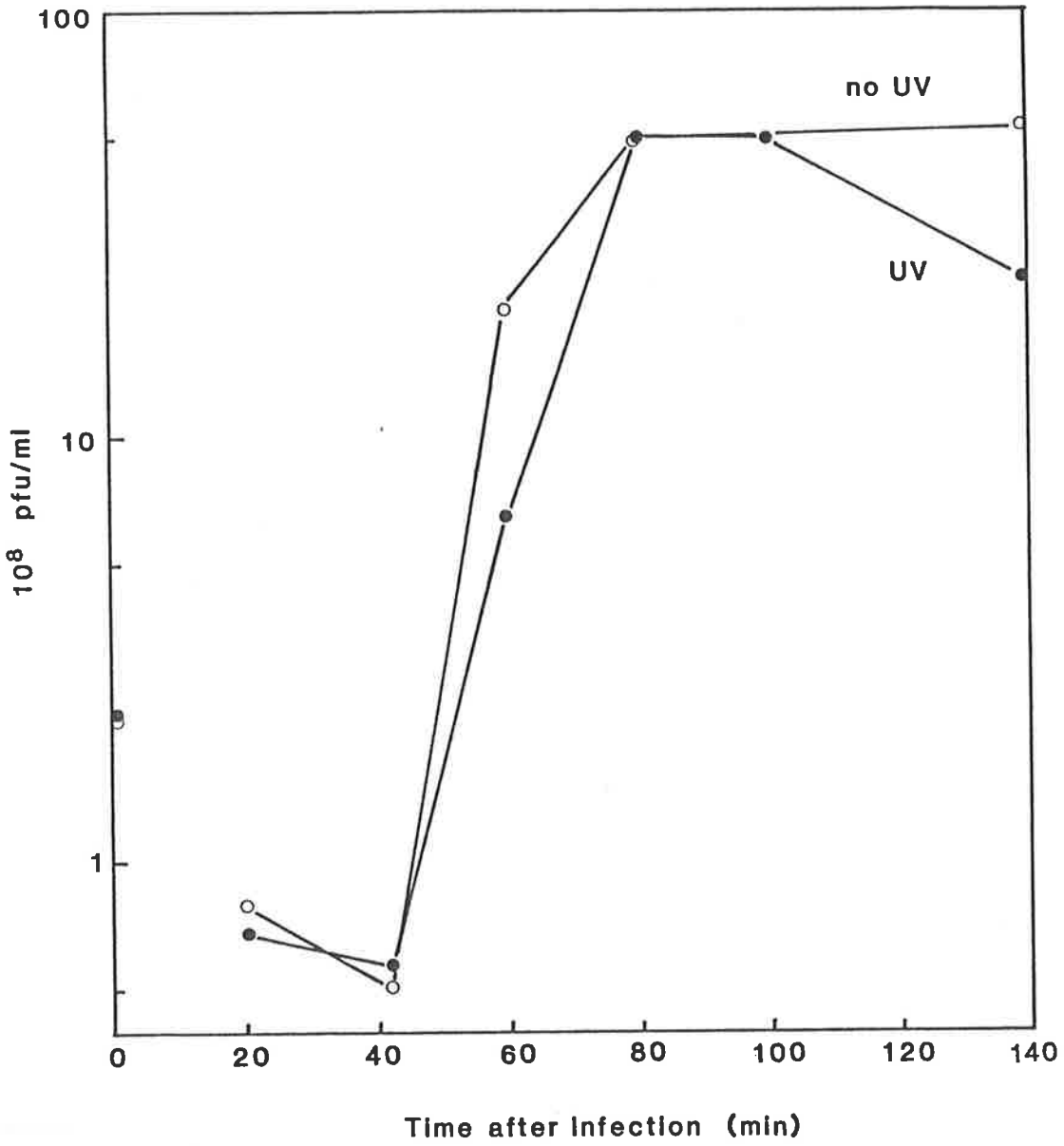
These experiments were performed in an excision repair deficient *uvrB* mutant. To test whether the high UV-sensitivity of these cells irradiated with the doses used, allowed replication from *ori*λ, the irradiated culture at 34.5°C was infected with λ*vir* and phage production was assayed at intervals. λ*vir* is insensitive to repression by the *c*I protein so that phage production should be unaffected by the presence of the λ prophage. The results showed that this was so (Fig. 7.4). After a latent period of 40 minutes a burst size of 100 pfu was obtained. Infection of the 45 J/m²-irradiated culture was very similar, so at the dose of UV used the *uvrB* mutant cells were not significantly altered in their capacity to support a λ infection. It was therefore, concluded that UV-irradiation of cells of strain E832 (ΔHI *uvrB*), does not have a general effect on production of λ phage, but specifically inhibits *oriC*-dependent phage production.

FIG 7.4 Effect of UV on *ori*λ-dependent phage production from λ*vir* in ΔHI(λ*c*I857) cells at 34.5°C

ΔHI(λ*c*I857) cells (E832) were cultured in M9 supp medium at 30°C to an O.D of 0.2 (2 x 10^8 cfu/ml), irradiated with 0 or 45 J/m^2 of UV, infected with λ *vir* (moa 1.0) and incubated at 34.5°C. Unabsorbed phage were inactivated with λ antiserum. 5 minutes later the culture was diluted away from the antiserum into fresh prewarmed medium and the incubation was continued at 34.5°C. Aliquots were assayed for pfu at intervals, on C600 indicator bacteria.

(o - o) unirradiated cells

(● - ●) 45 J/m^2 UV-irradiated cells.


100
10
1
10^8 pfu/ml
no UV
UV
0
20
40
60
80
100
120
140
Time after infection (min)

In order to make the more general conclusion that UV irradiation of cells inhibits replication from *oriC*, two points need to be addressed. Firstly the data were collected in an excision repair-deficient strain and need to be repeated in an excision proficient wild type cell.

Secondly, estimation of replication from *oriC* in the experiment described above is dependent on two additional events: replication from *oriλ*, and phage production. It was therefore desirable to have a more direct assay of *oriC*-replication in a strain which was proficient in excision repair.

7.3 The effect of UV on replication from *oriC* as studied by labelling the *λoriC* plasmid

To study replication from *oriC* directly it was decided to follow incorporation of radioactive thymine into the *λoriC* DNA replicating as a plasmid and visualise this on an agarose gel.

A *thy*-(λ *cI*$^+$) lysogen was prelabelled with [^{3}H]thymine and infected with *λoriC* or λ. Replication was allowed in the continued presence of the label. The presence of the *cI*$^+$ protein represses replication from *oriλ*. Under these conditions *λoriC* circularises and replicates extrachromosomally, initiating from *oriC*. The incorporation of label into this plasmid is an indication that replication has occurred. Plasmid separation techniques were used to distinguish the *λoriC* plasmid from chromosomal DNA.

7.3.1 Optimisation of the technique in unirradiated cells

In order to determine whether *oriC*-dependent replication could be detected in a λ*c*I$^+$ lysogen, C600 *thy*-(λ*c*I$^+$*ind*-) cells (E4048) were cultured in M9CAA containing thymine (2 μg/ml) and prelabelled with [^{3}H]thymine (30-60 μCi/ml for different experiments) for one generation. The cultures were then infected with either λ*oriC* or λ. Unabsorbed phage were not removed, as they were not expected to affect the results, as the phage were not labelled and would not contribute any background radioactivity. Incorporation of label was halted at various times after infection by addition of 100-fold excess of unlabelled thymine. DNA was extracted by the method of Birnboim and Doly (1979), except that sucrose was used in the first solution, instead of glucose (see Section 2.2.14a). The extracted DNA was electrophoresed on a 1% agarose gel which was then stained with ethidium bromide and photographed. The gel was then fluorographed (see Section 2.2.17) for 12-36 hours. Only DNA which had replicated and incorporated [^{3}H]thymine was visible on the fluorograph of the gel. An advantage of this method is that a photograph of the ethidium bromide stained gel allows the amount of phage DNA present as a plasmid to be estimated. Even in samples where no phage DNA replication had occurred (e.g. λ-infected cells), sufficient phage DNA was usually present to be visualised.

Thus in the absence of an internal control, the efficiency of separate extractions could be compared qualitatively using the photographs of the stained gel.

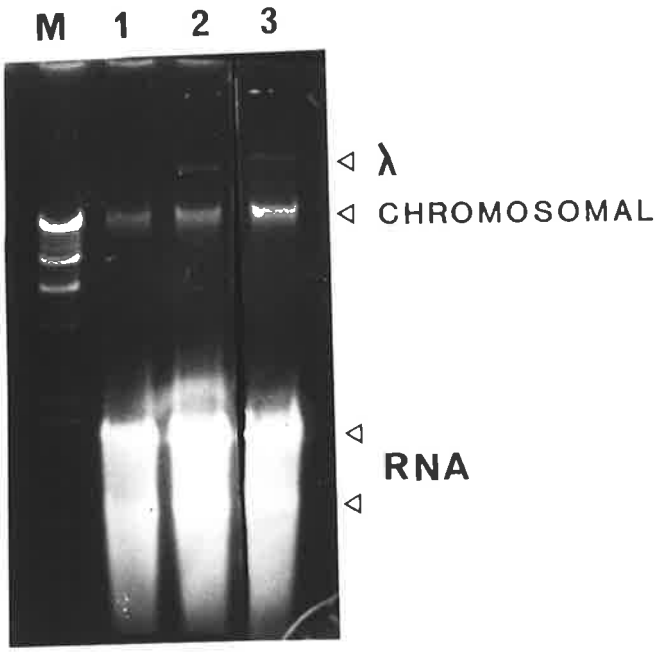
Detection of replication from λoriC: C600 *thy*$^-$ (λ*cI*$^+$*ind*$^-$) cells (E4048) were cultured and labelled as described above and were divided into 3 portions. One was not infected with phage, one was infected with λ and one was infected with λ*oriC*. The samples were incubated at 37°C for 60 min. Plasmid DNA from 2 ml aliquots was extracted and electrophoresed on agarose. The gel was stained and photographed (Fig. 7.5a). All three samples gave a band of chromosomal DNA which co-migrated with the largest (23.1 kb) λ *Hin*dIII restriction fragment. Also, all the samples contained RNA which migrated ahead of the 2 kb λ *Hin*dIII restriction fragment in the marker track (the RNA bands were absent if the sample was treated with RNase). Tracks derived from the λ- and λ*oriC*- infected cells had another band above the chromosomal band (Fig. 7.5a, track 2 and 3 respectively), which co-migrated approximately with 50 kb circular cosmid clones (data not shown). Since the 50 kb band was about the expected size of the λ and λ*oriC* phage DNA and was absent from uninfected cells, it was attributed to the infecting phage. That it was λ DNA was confirmed by hybridisation of nick-translated λ DNA to the phage DNA in the gel *in situ*, (data not shown). Therefore this band will be referred to as the λ plasmid band.

FIG 7.5 Replication of the λ plasmid from *oriC*

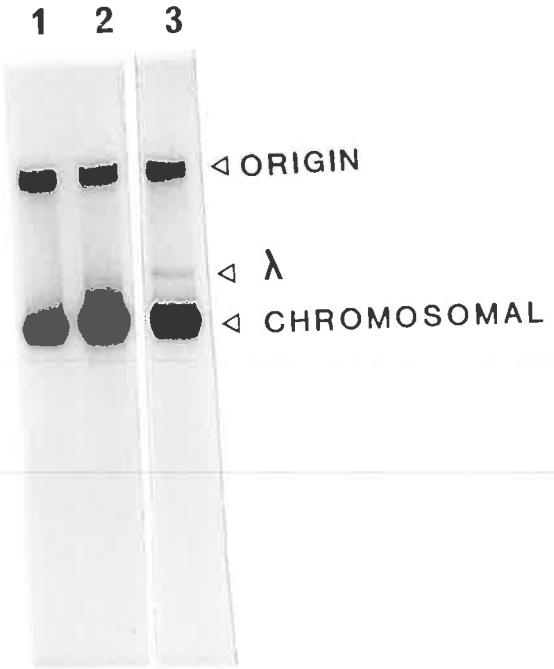
C600 *thy*$^-$ (λ*c*I$^+$ind$^-$) cells (E4048) cultured in M9CAA containing thymine (2μg/ml) at 37°C were prelabelled with [^{3}H]thymine (60 μci/2μg/ml) for one generation. Aliquots were left uninfected or were infected with λ*oriC* (moa 2) or λ (moa 2). After 60 minutes at 37°C, [^{3}H]thymine incorporation was stopped by addition of 200 μg/ml non-radioactive thymine. Plasmid extractions were carried out as described in Section 2.2.14a, the DNA was electrophoresed on 1% agarose and the gel was stained and photographed (a.) and then fluorographed (b.). The λ plasmid DNA is labelled 'λ' and the *E. coli* DNA is labelled 'chromosomal. The top of the gel is labelled 'origin'.

track number 1 - uninfected cells
track number 2 - λ-infected cells
track number 3 - λ*oriC*-infected cells
track number M - λ *Hin*dIII fragments used as size markers, 23.1kb, 9.4kb, 6.6kb and 4.4kb in size

a.



b.



Fluorography of the gel shown in Fig. 7.5a showed that [^{3}H]thymine had been incorporated into the chromosomal band in all three tracks (Fig. 7.5b). The plasmid band had incorporated a [^{3}H]thymine in only the λ*oriC* infected cells. (Fig. 7.5b, track 3).

Therefore, replication of the λ*oriC* plasmid had occurred. Since no replication of the λ plasmid was seen, it was concluded that replication from *oriC* but not *ori*λ was occurring under these conditions.

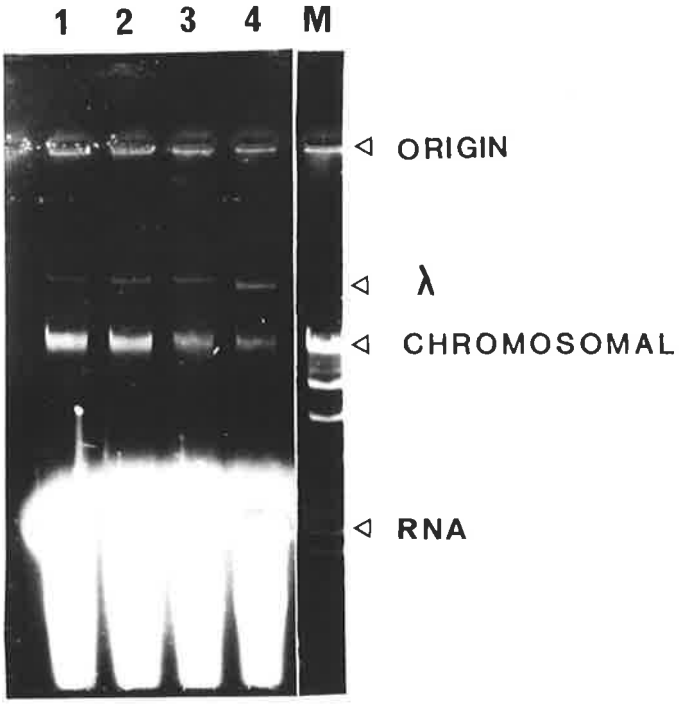
Earlier detection of oriC-dependent replication after infection: C600 *thy*-(λ*cI*$^+$*ind*-) cells (E4048) prelabelled with [^{3}H]thymine (30 μCi/2 μg/ml) were infected with λ*oriC* and λ. Samples were removed from the λ*oriC*- infected culture 10, 30 and 60 min after infection and from the λ-infected culture 60 min after infection. Plasmid DNA was extracted and electrophoresed on agarose. A photograph of the gel (Fig. 7.6a) shows that the λ plasmid DNA was visible even 10 min after infection. However, incorporation of [^{3}H]thymine into λ*oriC* was not detected until 30 min after infection (Fig. 7.6b, tracks 1, 2 and 3). As expected, no incorporation occurred in the λ plasmid band in λ-infected cells (Fig. 7.6b, track 4).

FIG 7.6 Detection of *oriC*-dependent replication at earlier times after infection

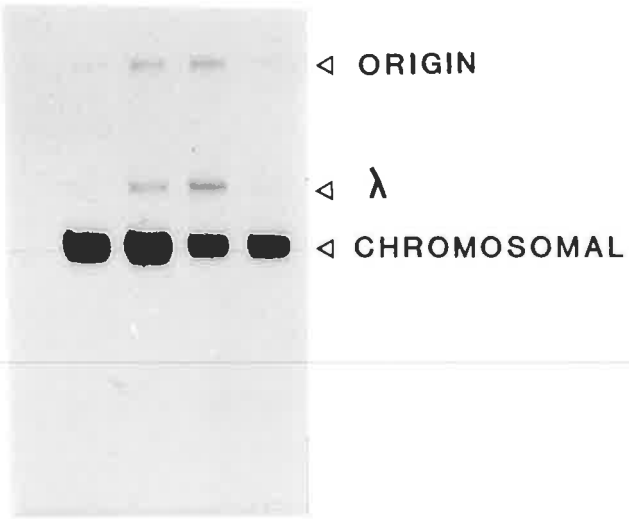
C600 *thy*$^-$ ($\lambda cI^+ ind^-$) cells (E4048) were cultured in M9CAA (37°C) and prelabelled with [^{3}H]thymine (30μci/2μg/ml) for one generation. Cells were infected with either λ or λ*oriC* and incubated at 37°C. Aliquots were removed from the λ*oriC* culture 10 minutes and 30 minutes and from both cultures 60 minutes after infection. Plasmid DNA was extracted (Section 2.2.14a) and electrophoresed on agarose. The gel was photographed (a.), then fluorographed (b.). The λ plasmid DNA is labelled 'λ' and the *E. coli* DNA is labelled 'chromosomal'.

track 1 - λ*oriC*-infected cells, 10 min
track 2 - λ*oriC*-infected cells, 30 minutes
track 3 - λ*oriC*-infected cells, 60 minutes
track 4 - λ-infected cells, 60 minutes
track M - size markers - λ*Hin*dIII restriction fragments, 23.1kb, 9.4kb, 6.6kb and 4.4kb in size

a.



b.



Minimisation of chromosomal contamination:

ColE1-derived plasmid DNA extracted by the Birnboim and Doly (1979) method is not usually contaminated by chromosomal DNA. In the experiments described above the chromosomal contamination was attributed to the relatively large amount of the preparation loaded on the gel, the entire contents extracted from 2 ml aliquots of cells. Usually only 1/10 to 1/50th as much DNA would have been loaded per track. If 1/10th of a λ*oriC* plasmid preparation was used no chromosomal DNA was visible, but neither was the λ plasmid DNA.

To minimise the chromosomal DNA in the preparations, the Grosveld *et al*. (1981) modification of the Birnboim and Doly (1979) method was used. This method omits lysozyme from the lysis solution and uses shorter (5 min) incubations (see Section 2.2.14c). Use of this method completely removed the chromsomal DNA in a λ*oriC* infected culture (Fig. 7.7a, track 2), compared with DNA extracted by the Birnboim and Doly (1979) method (Fig. 7.7a, track 1). However, the ^{3}H-labelled λ plasmid DNA extracted by the Grosveld *et al*. (1981) method was consistently obscured by a smear of radioactive material (Fig. 7.7b, track 2).

Several minor variations of these methods were undertaken to try to remove either the smear over the plasmid band or the chromosomal contamination. These are detailed in the legend to Fig. 7.8. These tests showed that

FIG 7.7 Minimisation of chromosomal DNA in the plasmid extract

C600 *thy*$^-$ (λc*I*$_{ind}^-$) cells (E4048) were cultured in M9CAA medium and prelabelled with [^{3}H]thymine (50μci/μg/ml) for one generation, then infected with λ*oriC*. Aliquots were removed at 20 min later and plasmid DNA was extracted either by the method of Grosveld *et al*. 1981 (Section 2.2.14c) or by the modified Birnboim and Doly (1979) method (Section 2.2.14a.), and electrophoresed on 1% agarose. The gel was stained and photographed (a.) and then fluorographed (b.). The λ plasmid DNA is labelled 'λ' and the *E. coli* DNA is labelled 'chromosomal'.

track 1 - λ*oriC*, Birnboim and Doly 1979)

track 2 - λ*oriC*, Grosveld *et al*. (1981)

track M - size markers - λ *Hin*dIII restriction fragments, 23.1kb, 9.4kb, 6.6kb, 4.4kb, 2.3kb and 2.0kb in size.

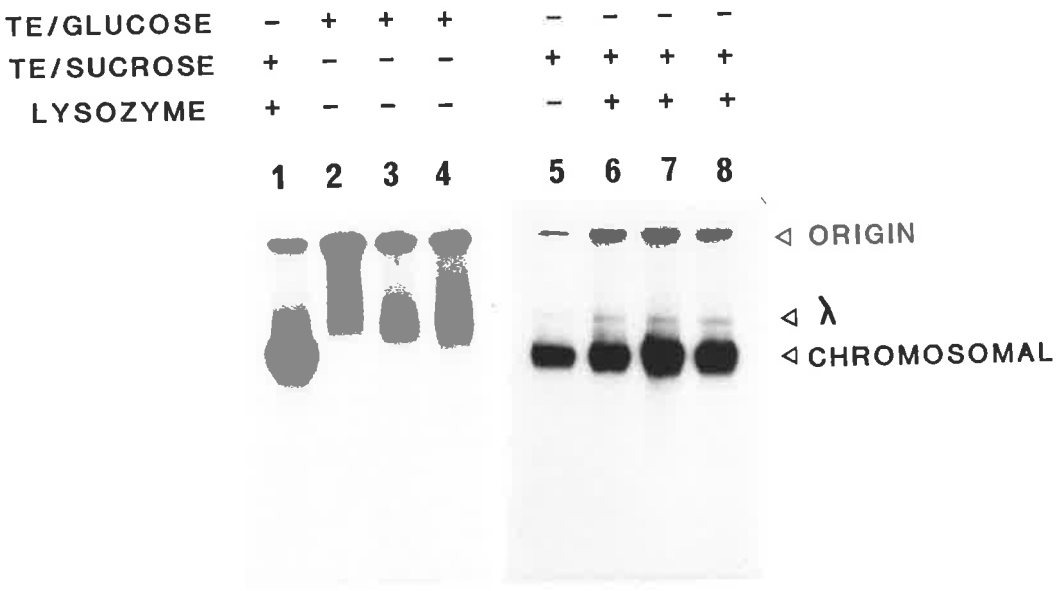
a.

b.

FIG 7.8 The effect of altered methods of plasmid extraction on the detection of labelled λ*oriC* plasmid

C600 *thy*$^-$ (λ*c*I$^+$*ind*$^-$) cells (E4048) were cultured prelabelled and infected with λ*oriC* as described in Fig 7.7. DNA was extracted as detailed below and in Section 2.2.14.

tracks	1	2	3	4	5	6	7	8
resuspension buffer	TE Sucrose lysozyme 20 min ice	TE Glucose 5 min, RT			TE Sucrose 5 min, RT	TE Sucrose/ lysozyme ice 5 min	10 min	20 min
NaOH/SDS	5 min on ice							
NaOAc sol.	5 min on ice							
centrifugation temp	2 min RT	2 min RT	5 min RT	2 min 4°C	4°C	2 min	RT	
EtOH. precipitation	2 min RT	2 min RT	2 min 4°C	2 min dry ice		2 min	RT	
centrifugation	3 min RT	3 min RT	3 min RT	4 min 4°C		3 min	RT	


TE/GLUCOSE
TE/SUCROSE
LYSOZYME
1 2 3 4 5 6 7 8
ORIGIN
λ
CHROMOSOMAL

when glucose was used in the first buffer no chromosomal DNA was obtained but there was always a smear, if sucrose was used the smear disappeared but the chromosomal DNA was present (Fig. 7.8).

It was therefore, decided to continue to use the Birnboim and Doly (1979) method using sucrose buffer, with shortened incubations i.e. 4 min with the lysozyme solution instead of 40 min and 10 min at 0°C with the NaOAc instead of 60 min at 0°C. All the subsequent centrifugation steps were carried out at room temperature (see Section 2.2.14b). Thus the smearing around the plasmid band was avoided but some chromsomal contamination was inevitable.

7.3.2 *oriC*-dependent replication of λ*oriC* plasmid in UV-irradiated cells

The effect of irradiating cells with UV, prior to infection, on *oriC*-dependent replication of phage was studied.

C600 *thy*-(λ*c*I^{+}*ind*-) cells were labelled with [^{3}H]thymine (either 30 or 60 μCi/2 μg/ml), and irradiated with either 0 or 67.5 or 90 J/m^{2} of UV. These were then infected with λ*oriC* or λ phage. Samples were removed at the required times after infection and DNA was extracted as described in the previous section.

The effect of irradiation of the cells with the lower dose (67.5 J/m²) of UV on *oriC*-dependent replication is shown in Fig. 7.9. This fluence was sufficient to prevent incorporation of label into the λ*oriC* plasmid (Fig. 7.9b, track 2), although incorporation occurred in unirradiated cells (Fig. 7.9b, track 1), as found previously. λ plasmid did not show any incorporation of label (Fig. 7.9b, tracks 3 and 4) showing that *ori*λ was repressed under these conditions. Irradiation with a higher dose of UV (90 J/m²) had a similar effect (data not shown).

From this data, it appeared that UV-irradiation of the cells inhibited replication from *oriC*. However a closer look at the photographs in these experiments (Fig. 7.9a) revealed an anomaly. The amount of λ DNA present in the samples derived from UV-irradiated cultures (Fig 7.9, tracks 2 and 4), appeared to be less than that in the corresponding unirradiated samples. Often the chromosomal contamination was also reduced in samples taken from UV-irradiated cells. This was corroborated by the observation that precipitates (obtained following addition of sodium acetate to the DNA), from irradiated samples were more compact and easier to remove from the centrifuge tubes than were those from unirradiated samples.

This raised the possibility that the infecting phage DNA was not extracted as efficiently from UV-irradiated cells as it was from unirradiated cells. If this was the

FIG 7.9 The effect of reduced UV-irradiation on replication of *λoriC*

C600 thy$^-$ (*λcI*$^+$*ind*$^-$) cells (E4048) were treated, infected and processed as in the legend for Fig 7.6 except that 60 μCi/2 μg/ml [^{3}H]thymine was used to prelabel the cells and the cultures were irradiated with 0 or 67.5 J/m^2 of UV and samples were removed 15 minutes after infection. (a.) shows a photograph of the gel, and (b.) a fluorograph of the gel. The λ plasmid DNA is labelled 'λ' and the *E. coli* DNA is labelled 'chromosomal'. The top of the gel is labelled 'origin'.

track 1 - *λoriC* in unirradiated cells

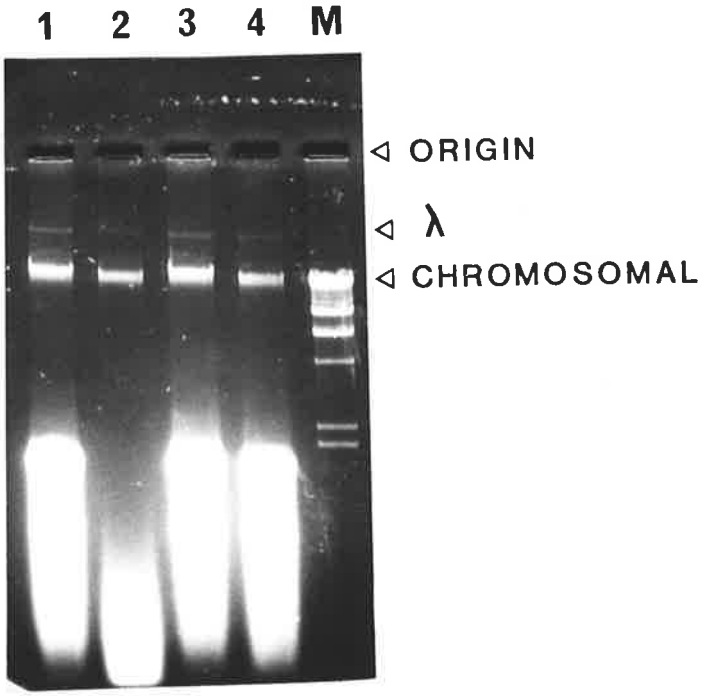
track 2 - *λoriC* in 67.5 J/m^2-irradiated cells

track 3 - λ in unirradiated cells

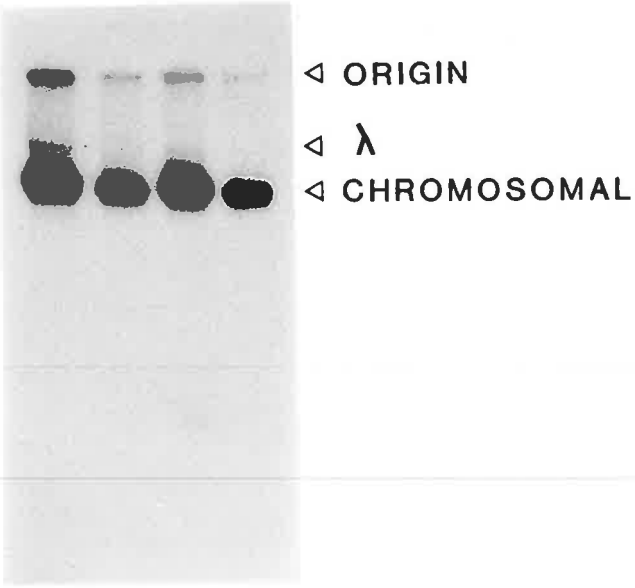
track 4 - λ in 67.5 J/m^2 irradiated cells

track M - λ*Hin*dIII restriction fragments, 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0kb in size

a.



b.



case, the absence of labelled λ*oriC* plasmid in the irradiated cells (Fig. 7.9b, track 2 vs 1) would not reflect the absence of *oriC*-dependent replication but instead would be an artifact of the plasmid extraction procedure. To test this possibility, phage λ replication in a nonlysogen and λ*vir* replication in the λ*cI*$^{+}$*ind*$^{-}$ lysogen was examined in unirradiated and UV-irradiated cells. As mentioned before λ phage production in a non-lysogen is not affected if the cells are irradiated with doses of UV up to 90 J/m². To test whether this was also true of λ*vir*-infection of irradiated λ lysogenic cells, phage production for λ*vir* was studied in unirradiated and 67.5 J/m²-irradiated C600 thy-(λ*cI*$^{+}$*ind*$^{-}$) cells. Phage production of λ*vir* occurred with a latent period of 30 min and a burst size of 40 and 70 phage respectively, in the unirradiated and UV-irradiated cultures (Fig. 7.10), showing that pre-irradiation of the bacteria by UV did not affect replication of λ*vir*. It was therefore expected that incorporation of [^{3}H]thymine into λ*vir* DNA would be the same in unirradiated and pre-irradiated cells.

Infection of prelabelled, irradiated (67.5 J/m²) and unirradiated C600 *thy*$^{-}$(λ*cI*$^{+}$*ind*$^{-}$) cells (E4048) by λ*vir* or λ*oriC* and of prelabelled, irradiated (67.5 J/m²) and unirradiated C600 *thy*$^{-}$ cells (E237) by λ was carried out for 15 minutes. Samples were removed, the DNA was extracted and was electrophoresed on a gel and the gel was fluorographed as described. A marked decrease in incorporation into the plasmid band was seen in both the λ*vir*- and λ- infected UV-

FIG 7.10 Effect of UV on phage production from λ *vir* in lysogenic cells

C600 *thy*$^-$ (λ*cI*$^+$*ind*$^-$) cells (E4048) cultured in M9CAA containing thymine (2μg/ml) at 37°C to 2 x 10^8 cfu/ml, were irradiated with 0 or 67.5 J/m² of UV. The cultures were infected with λ*vir* (moa 0.05) and 6 minutes later they were diluted into prewarmed fresh medium. Since <10^5 pfu/ml were left unabsorbed, no antiserum treatment was carried out. Phage were assayed at intervals on C600 indicator bacteria.

(● - ●) unirradiated cells

(o - o) 67.5 J/m²-irradiated cells

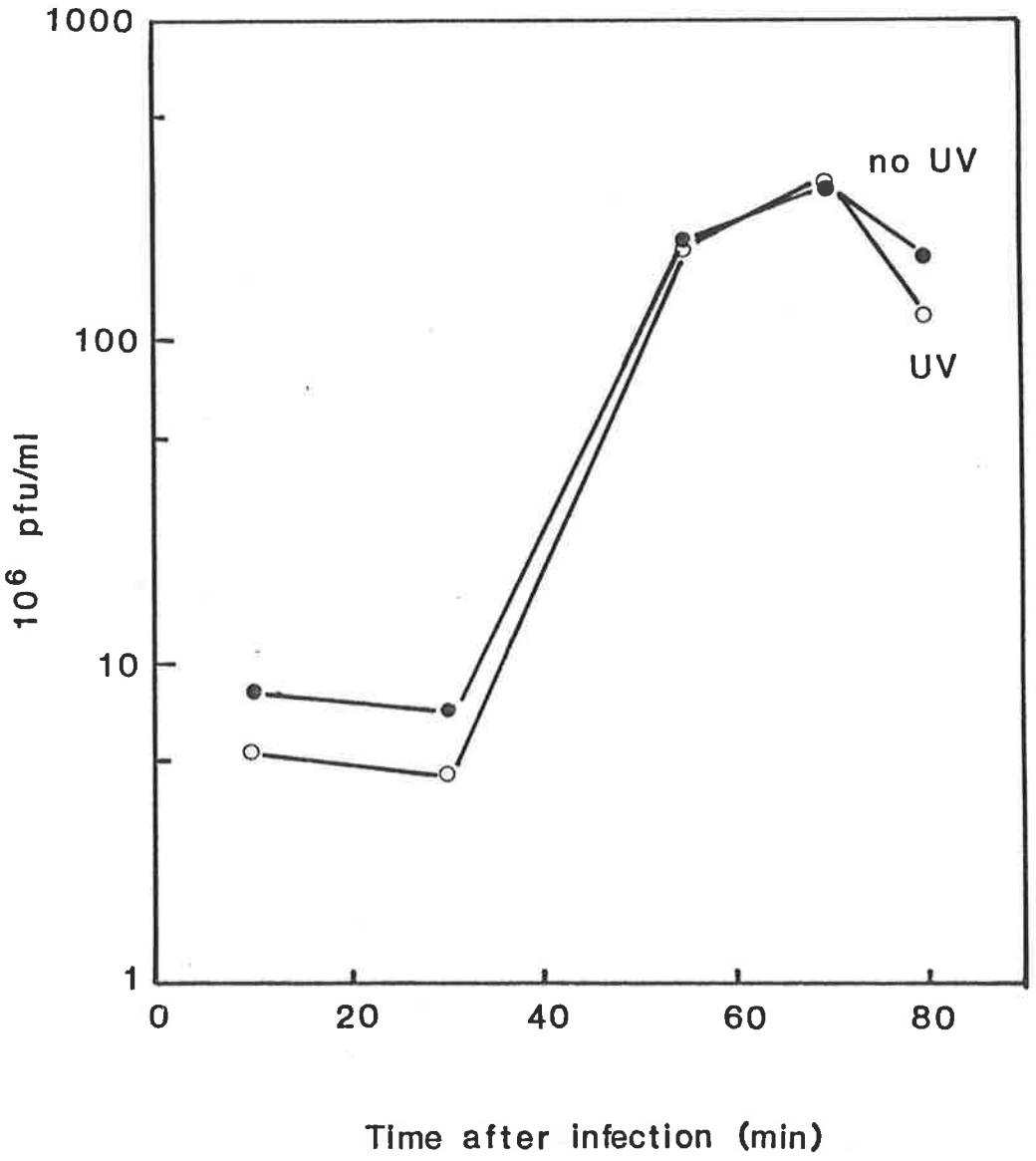

1000
100
10
1
0
20
40
60
80
no UV
UV
10^6 pfu/ml
Time after infection (min)

FIG 7.11 Effect of UV on *ori*λ- dependent replication, using λ*vir* and λ phages

Cells of strains C600 *thy*⁻ (λ*cI ind*⁻) (E4048) and C600 *thy*⁻ (E237) were cultured in M9CAA to an OD of 0.2 and then prelabelled for one generation with [³H]thymine (60 µci/2 µg/ml). A portion of each culture was irradiated with either 0 or 67.5 J/m² of UV. Cells of strain E4048 were infected with λ*oriC* and λ*vir* (moa 2.5) and the cells of strain E237 were infected with λ. After 15 minutes, aliquots were removed from each culture and incorporation of [³H]thymine was stopped by the addition of non-radioactive thymine (200 µg/ml). Plasmid DNA was extracted as described in Section 2.2.14b and electrophoresed on 1% agarose. The gel was fluorographed. The λ DNA is labelled 'λ' while the *E. coli* DNA is labelled 'chromosomal'. The top of the gel is labelled 'origin'.

track 1 - λ*vir* in unirradiated cells of strain E4048

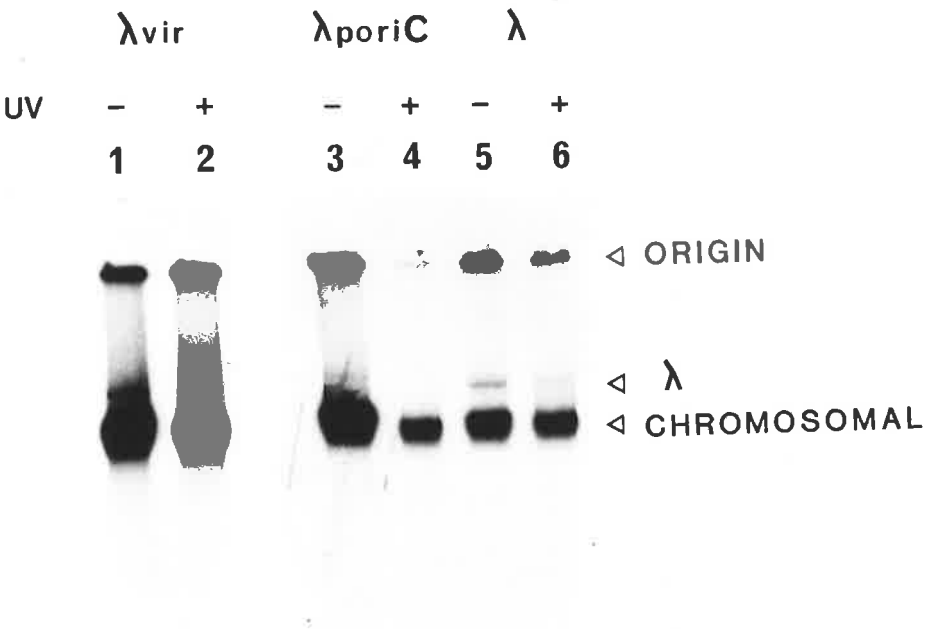
track 2 - λ*vir* in 67.5 J/m² - irradiated cells of strain E4048

track 3 - λ*oriC* in unirradiated cells of strain E4048

track 4 - λ*oriC* in 67.5 J/m²-irradiated cells of strain E4048

track 5 - λ in unirradiated cells of strain E237

track 6 - λ in 67.5 J/m²-irradiated cells of strain E237.


λvir
λporiC
λ
UV
–
+
–
+
–
+
1
2
3
4
5
6
ORIGIN
λ
CHROMOSOMAL

irradiated cultures (Fig. 7.11). (Photographs of these gels have not been shown). The intensity of the band resulting from λ*vir* was less in the irradiated cells (Fig. 7.11, track 2) than in unirradiated cells (Fig. 7.11, track 1). Similarly for λ (Fig 7.11, tracks 5 and 6) and for λ*oriC* (Fig. 7.11, tracks 3 and 4). Therefore UV-irradiation of the bacteria had reduced either the amount of incorporation into the λ*vir* and λ DNA or the amount of λ DNA extracted from the cells.

Since λ- and λ*vir*- phage production in these cells is unaffected by UV, it was concluded that UV was artificially depressing the level of plasmid DNA isolated in these experiments. Therefore the technique could not be used to study replication in irradiated cultures, unless an internal control could be devised to normalise the extraction procedure. Since this was not possible, λ DNA replication was studied by DNA-DNA hybridisation.

7.4 *oriC*-DEPENDENT REPLICATION STUDIED BY DNA-DNA HYBRIDISATION

For reasons given above, visualisation of DNA on a gel was not adequate as a means of investigating replication from *oriC* in UV-irradiated cells. Therefore a different method involving DNA-DNA hybridisation, was devised. This involved infecting prelabelled *E. coli* cells with unlabelled λ*oriC*, allowing replication to occur in the presence of [^{3}H]thymine, lysing the cells and then testing for the presence of [^{3}H]labelled DNA which would hybridise to λ DNA.

Two potential problems were anticipated. The first was that the small number of λ*oriC* genome copies due to copy number control may be difficult to quantitate by hybridisation. The second was that the use of a λ*c*I$^+$ lysogen to repress *ori*λ could lead to unacceptably high backgrounds, since the experimental plan used prelabelling of the cells with the radioactive precursor in preference to pulse labelling (for reasons discussed in Chapter 3). A high background generated by labelling of the prophage would compound the effect of the low copy number, complicating the interpretation of the data.

It was decided therefore to use a λ*oriC Oam* mutant in a non-lysogenic Su$^-$ background to inhibit replication from *ori*λ. The gene O protein is phage encoded and is required for λ replication *in vivo* (Ogawa and Tomizawa, 1968) and *in vitro* (Anderl and Klein, 1982; Tsurimoto and Matsubara, 1982; Wold *et al.*, 1982). It interacts with the phage origin and another phage encoded replication protein *P*. (Furth, 1978; 1979). The *O* protein is required for initiation of λ replication but not for elongation of the leading strand *in vitro* and is probably not required for lagging strand synthesis either (Erdile and Inman, 1984). Therefore in an Su$^-$ (non-suppressing) strain replication from *ori*λ would be inhibited due to the amber mutation in the *O* gene and replication of λ*oriC* would be dependent on initiation from *oriC*.

λ*oriC Oam* (λ1021), obtained from L. Soll, carries a *c*I$^+$ allele and gives low titre phage lysates. It was therefore desirable to have a *c*$^-$ derivative of this phage to try and improve the stock titre. A control λ*Oam* Δ(*oriC*) phage was also required, to estimate the amount of residual replication from *ori*λ. So two phage mutants were isolated before the hybridisations were undertaken.

A spontaneous clear plaque mutant of the λ*oriC Oam* phage was obtained and from this a spontaneous Δ(*oriC asnA*) deletion mutant was obtained as described below.

7.4.1 Construction of λc$^-$*oriC Oam* and λc$^-$Δ(*oriC asnA*)*Oam* phage

λ*c*II$^-$*Oampori*C: λ*b*221*c*I$^+$*Oampori*C*asnA* phage (λ1021) was plated onto a lawn of cells of strain C600 *recA*$^-$ (E728), to give semi-confluent plaques. Spontaneous clear plaques were isolated and then tested for *asnA* transducing ability (Section 7.1). One of the clear plaques capable of transducing Asn$^-$*recA*$^-$(λ*c*I$^+$) cells to Asn$^+$, was purified by single plaque isolations and studied further. It was shown to have a mutation in the *c*II gene by complementation. This phage (λ260 - collection number) will be referred to as λ*oriC Oam* in the following sections.

λ*c*II$^-$*Oam* Δ(*oriCasnA*): The construction of the λ*oriC* phage by Soll (1980a) resulted in the *oriC asnA* insert being

flanked by IS10 elements. Due to the presence of the elements, spontaneous deletions of the *oriC asnA* region occur at a high rate (Soll, 1980a).

Spontaneous deletion mutants were selected by plating the phage constructed above onto a lawn of C600 *recA*$^-$ (E728) cells on O.M. plates using top agar. O.M. plates contain citrate which chelates Mg^{++} ions. Phage heads containing full length genomes lyse under these conditions rendering the phage particles inactive. However, deletion mutants, with shorter genomes are stable and can form plaques. Minute plaques were obtained on the O.M. plate and were toothpick transferred onto a lawn of C600 cells on a Z plate. These plaques were tested for the ability to transduce Asn$^-$ cells to Asn$^+$ (see Section 7.1). A non-transducing phage identified by this method was purified and a small volume high titre stock was prepared by the method described in Section 2.2.3h. The phage DNA was phenol extracted and digested with *Eco*RI, *Bgl*II and *Hin*dIII restriction endonucleases and then electrophoresed on 1% agarose. The bands associated with the *oriC asnA* insert (Soll, 1980a) were absent (data not shown), indicating that the deletion which had rendered the phage non-transducing for *asnA* had removed the entire *oriC asnA* insert.

To confirm the loss of *oriC* the phage was tested, by the plasmid method described in the previous section, for the ability to replicate in Su$^-$ *thy*$^-$ cells. The presence of

the *Oam* mutation prevented initiation of replication from *oriλ*.

Briefly, the experiment involved prelabelling Su$^-$ *thy*$^-$ cells (E777) with [^{3}H]thymine (60 μCi/2 μg/ml) and infecting samples with either λΔ(*oriC*)*Oam* (the deletion carrying test phage) or λ*oriC Oam* (the parent phage). The DNA was extracted by the modified Birnboim and Doly procedure (Section 2.2.14b) electrophoresed on an agarose gel and the gel was stained, photographed and fluorographed. A band of *E. coli* chromosomal DNA was observed in both the uninfected and λ-infected tracks in the photograph (Fig. 7.12a, tracks 1 and 2) comigrating with the 23.1 kb band in the marker track (Fig. 7.12a, track M). The larger λ plasmid band was present in both the λ *oriC Oam* and λΔ(*oriC*)*Oam* samples (Fig. 7.12a, tracks 1 and 2). The plasmid from the deletion mutant migrates ahead of the parent plasmid, probably reflecting the size difference between the two phage genomes. The chromosomal band was labelled in both tracks showing that cellular DNA was replicated in both cases (Fig. 7.12b). The plasmid band had incorporated label (i.e. replicated) only in the λ*oriC Oam*-infected cells (Fig. 7.12b, track 1). The λΔ(*oriC*)*Oam* plasmid was not labelled, i.e. no incorporation had occurred into this DNA. Therefore it was concluded that the λΔ(*oriC Oam*) DNA had not replicated.

FIG 7.12 *λoriC Oam* and *λΔ(oriC) Oam* replication in Su⁻ *thy*⁻ cells

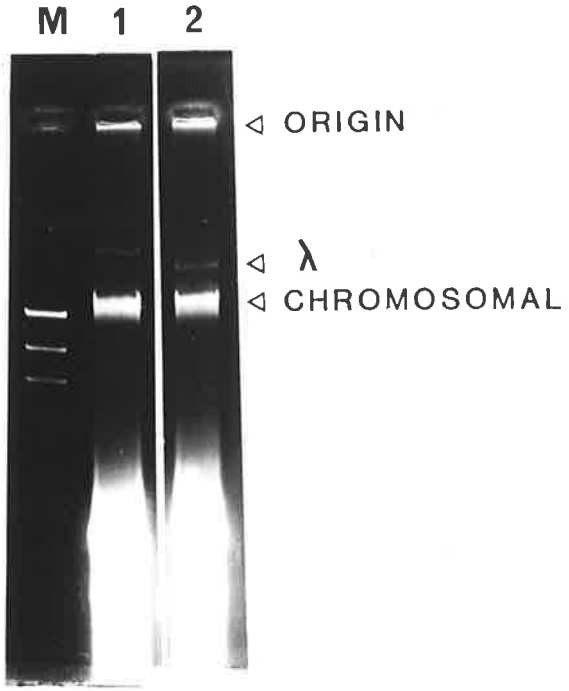
Su⁻ *thy*⁻ cells (E777), were cultured in M9CAA containing thymine (2μg/ml) at 37°C and prelabelled for one generation with [³H]thymine (60μci/μg/ml), then infected with *λoriCOam* and *λΔ(oriC) Oam* (moa 3.o) and incubation continued at 37°C, aliquots were removed and plasmid DNA extracted (see Section 2.2.14b.) and electrophoresed on 1% agarose. The gel was stained and photographed (a.) and then fluorographed (b.). The λ DNA is labelled 'λ' and the *E. coli* DNA 'chromosomal'. The top of the gel is labelled 'origin'

track 1. *λoriC Oam*

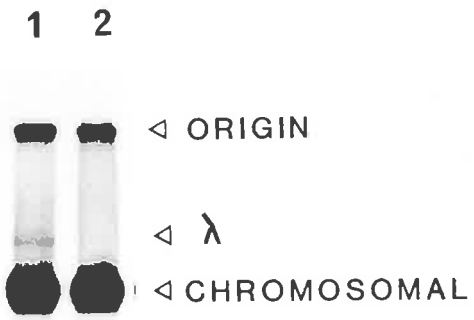
track 2. *λΔ(oriC) Oam*

track M. size markers, λ *Hin*dIII restriction fragments, 23.1, 9.4, 6.6 and 4.4kb in size.

a.



b.



This showed that the test phage did not carry *oriC* so that the deletion mutant had lost *oriC* as well as *asnA*.

7.4.2 Quantitative assay for *oriC*-carrying phage

The λ*oriC Oam* and λΔ(*oriC*)*Oam* phage stocks were raised on *recA*$^-$Su$^+$ bacteria to minimize recombination between the bacterial insert carried by the phage and the bacterial chromosome. However the titre of these stocks was low. Whereas the titres obtained on Su$^+$*recA*$^+$ bacteria were higher, but there was the danger that the *oriC* insert could be rearranged through *recA*-mediated recombination, so that the stock raised on *recA*$^+$ cells may have had a large proportion of phage which had lost functional *oriC*. To test if a significant proportion of the stock raised on *recA*$^+$ cells had lost the *oriC asnA* region a quantitative assay had to be developed.

Soll (1980a) assayed for *oriC* activity by plating λ*c*I$^+$*oriC asnA* phage on a lawn of cells of an Asn$^-$ strain on medium containing a limiting concentration of asparagine. This allowed faint plaques to be seen. If the phage carried *asnA* (with or without *oriC*) the cells in the centre of the plaque were lysogenised and transduced to Asn$^+$ giving rise to visible bacterial growth in the centres of the plaques. However this assay could not be used since the phage used here are *c*II mutants and will not efficiently lysogenise cells in the plaques.

Instead the test described in Section 7.1 was used quantitatively by plating the same volume of the test phage on a lawn of Su^+ bacteria to find the number of pfu and on a lawn of Asn^- *recA*$^-$ (λcI^+) bacteria to find the number of *asnA* transducing phage. A comparison of the numbers of Asn^+ colonies with the numbers of plaques gave the proportion of the phage which carried *asnA*. The Asn^+ transductants were stable (capable of forming single colonies), showing that the phage carried *oriC*, so that this test also gave the proportion of phage carrying *oriC*.

Based on this test, at least 99% of the phage in the *λoriC Oam* stocks carried the *oriC asnA* insert, regardless of whether the stock was raised on a *recA*$^+$ or a *recA*$^-$ host. As expected ,the stock of *λΔ(oriC)Oam* did not give Asn^+ transductants.

7.4.3 The dependence of replication from *oriC* on *dnaC*+ and *dnaA*+

oriC-dependent replication of *λoriC* DNA should have a requirement for functional *dnaA* and *dnaC* proteins, if it initiates in the same manner as the chromosomal *oriC*. This was tested using the plasmid method described in Section 7.3. *oriC*-dependent incorporation of [^{3}H]thymine into the *λoriC Oam* plasmid was studied in temperature-sensitive *dnaC* and *dnaA* mutants at the permissive and non-permissive temperatures. Chromosomal replication ceased in both the *dnaC2* and *dnaA5* mutants about 40 min after shift up to the non-permissive temperature (Fig. 7.13a and b).

FIG 7.13 ***E. coli DNA*** **replication in** ***dnaC2 and dnaA5*** **mutants**

a. C600 *thy*$^-$ (λcI$^+$*ind*$^-$) *dnaC2* (E4049) and *dnaC*$^+$ (E4050) cells cultured in NZCYM medium containing [^{3}H]thymine (5µci/2µg/ml).

b. Su$^-$*thy*$^-$*dnaA5* *Zi*::Tn10 (E4047) and Su$^-$*thy*$^-$ *dnaA*$^+$ (E4046) *Zi*::Tn10 cells cultured in M9CAA containing thymine (20 µCi/2µg/ml).

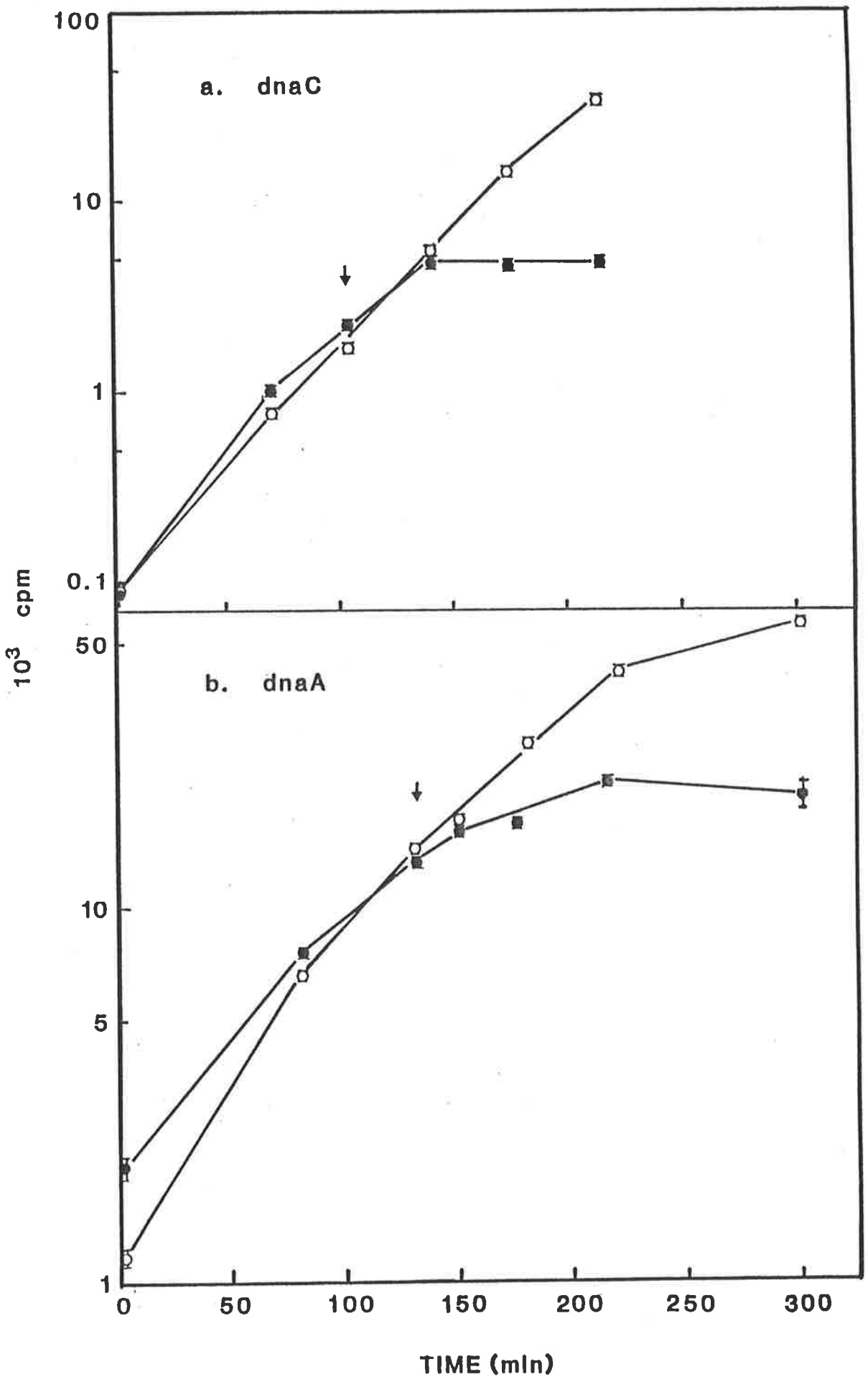
The DNA was continuously labelled and acid-insoluble radioactivity incorporated (as cpm) was measured at intervals by TCA precipitation of 50 µl aliquots.

The cultures were transfered to the non-permissive temperature (40.5°C for *dnaC* and 41 °C for *dnaA*) at the time indicated (↓).

(o - o) wild-type cultures

(● - ●) temperature-sensitive mutants.

The mean of duplicate values (given as bars) was plotted.


a. dnaC
b. dnaA
100
10
1
0.1
50
10
5
1
0
50
100
150
200
250
300
10^3 cpm
TIME (min)

dnaC requirement : C600 *thy*$^-$ *dnaC*2Ts(λ*c*I$^+$) (E4049) and C600 *thy*$^-$*dnaC*$^+$(λ*c*I$^+$) (E4050) cells were cultured in NZCYM medium containing thymine (2 μg/ml) at 30°C and were prelabelled for one generation. The cells were incubated at either the permissive temperature (30°C) or the restrictive temperature (40.5°C) for 30 min. The cultures were then infected with either λ*oriC Oam* or λΔ(*oriC*)*Oam* and incubation continued at the same temperature for 30 min. Plasmid was extracted (see Section 2.2.14b) and electrophoresed on a 1% agarose gel, which was then stained, photographed (Fig. 7.14a) and fluorographed (Fig. 7.14b). As expected the plasmid band was present in all the samples (Fig. 7.14a). Infection with λΔ(*oriC*)*Oam* did not give incorporation of [^{3}H]thymine into the plasmid band, in any of the cultures (Fig. 7.14b tracks 1,3 and 5), since the λ*c*I$^+$ prophage represses replication from *ori*λ. (C600 cells are Su$^+$ and would have suppressed the phage Oam mutation). Therefore no replication occurred from *ori*λ. Replication from *oriC* was detected in the *dnaC*2 mutant infected with λ*oriC Oam* at 30°C (Fig. 7.14b, track 2) and also in the *dnaC*$^+$ culture at 40.5°C (Fig. 7.14b, track 4) but not in the *dnaC*2 mutant at 40.5°C (Fig 7.14b, track 6). Therefore replication from *oriC* was inhibited in the *dnaC*Ts mutant at the non-permissive temperature.

The increased temperature does not directly interfere with replication from *oriC* since replication occurs in the

FIG 7.14 Effect of *dnaC2* on replication from *oriC*

Cells of strain C600 *thy*$^-$*dnaC2* (λcI$^+$), (E4049) and C600 *thy*$^-$*dnaC*$^+$ (λcI$^+$) (E4050) were cultured in NZCYM medium containing thymine (2μg/ml) at 30°C and were prelabelled for one generation with [^{3}H]thymine (60 μCi/2μg/ml).

A portion of both cultures was incubated at 40.5°C. After 30 minutes the cultures were infected with either λ*oriC Oam* or λΔ(*oriC*) *Oam* (moa 3) and incubation was continued at the same temperatures for 30 minutes. Incorporation of label was stopped by the addition of non-radioactive thymine (200 μg/ml), DNA was extracted from each culture and electrophoresed on 1% agarose. The gel was then stained, photographed (a.) and fluorographed (b.) The λ DNA is labelled, λ, and the *E. coli* DNA is labelled, chromosomal.

Track 1 λΔ(*oriC*)*Oam* in *dnaC2* cells at 30°C

Track 2 λ*oriCOam* in *dnaC2* cells at 30°C

Track 3 λΔ(*oriC*)*Oam* in *dnaC*$^+$ cells at 40.5°C

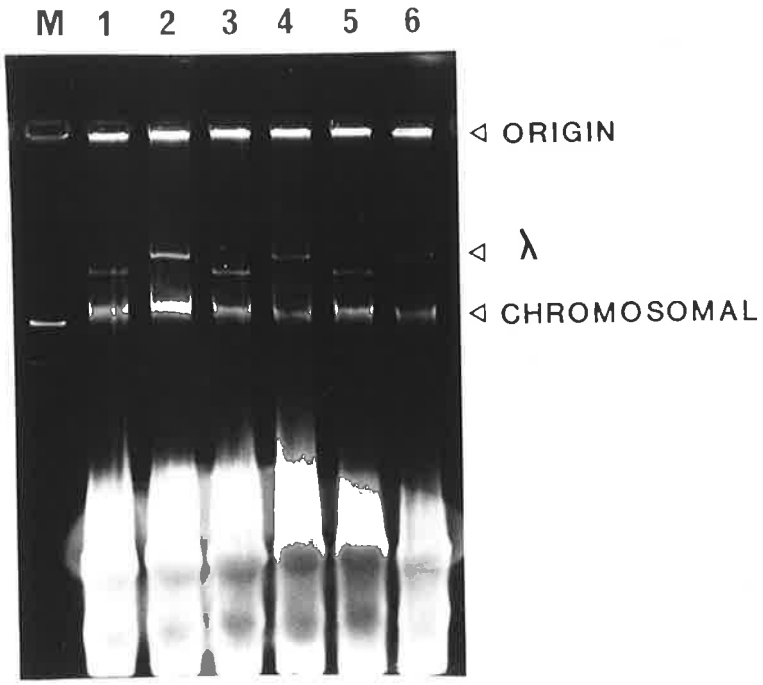
Track 4 λ*oriCOam* in *dnaC*$^+$ cells at 40.5°C

Track 5 λΔ(*oriC*)*Oam* in *dnaC2* cells at 40.5°C

Track 6 λ*oriCOam* in *dnaC2* cells at 40.5°C

Track M marker track, λ*Hin*dIII fragments, 23.1, and 9.4 kb in size

a.



b.

dnaC$^+$ strain at 40.5°C (Fig. 7.14b, track 4). The *dnaC*Ts strain itself is not refractory to *oriC* since replication occurs in it at 30°C. Therefore replication from *oriC* requires *dnaC*.

dnaA requirement : Su$^-$*thy*$^-$*dnaA5 Zi*::Tn10 (E4047) and Su$^-$*thy*$^-$*dnaA*$^+$ *Zi*::Tn10 (E4046) strains were constructed by transducing *dnaA5 Zi*::Tn10 from E528 into Su$^-$ *thy*$^-$ (E777) cells. Since the cells were Su$^-$ they did not suppress the λ*O am* mutation, making it unnecessary to use a λ*c*I$^+$ lysogen.

To test the effect of the *dnaA*Ts mutation on replication from *oriC*, prelabelled *dnaA*Ts cells were infected with λ*oriCOam* and λ*O*$^+$ at either the permissive (30°) or non-permissive (41°C) temperature. After 30 min samples were removed and tested for incorporation of [^{3}H]thymine into the λ plasmid as described in the legend to Fig. 7.15. As expected, uninfected cells did not have any plasmid band (Fig. 7.15a, track 1). λ*oriC O*$^+$ - infected cells incorporated [^{3}H]thymine into the λ-plasmid at 41°C (Fig. 7.15b, track 2) even though no λ band was visible in the photograph (Fig. 7.15a, track 2). λ*oriC Oam* did not incorporate label into the plasmid band at 41°C in the *dnaA5* mutant, but it did so at 30°C (Fig 7.15b, tracks 3 and 4). In addition λ*oriCOam* replicated in the *dnaA*$^+$ strain at 41° and 30°C (data not shown). Therefore replication from *oriC* is also dependent on *dnaA*$^+$.

FIG 7.15 Effect of *dnaA5* on *oriC*-dependent replication of *λoriC*

Su⁻*thy*⁻ *dnaA5 Zi*::Tn10 cells were cultured in M9CAA and thymine (2μg/ml) and prelabelled for one generation with [³H]thymine (60 μCi/ml). At an OD of 0.3 half of the culture asa transferred to 41°C for 30 minutes. The cultures were infected with *λoriCOam* (moa 2), and with *λoriCO*⁺ (moa 2.0). Incubation was continued for 40 minutes at the same temperatures. Incorporation of [³H]thymine was stopped by the addition of non-radioactive thymine (200 μg/ml). DNA was extracted from the samples (see Section 2.2.14b) and electrophoresed on 1% agarose. The gel was photographed (a.) and fluorographed (b.). The λ DNA is labelled 'λ' *E. coli* DNA is labelled 'chromosomal'. The top of the gel is labelled 'origin'.

Track 1. uninfected, 41°C culture

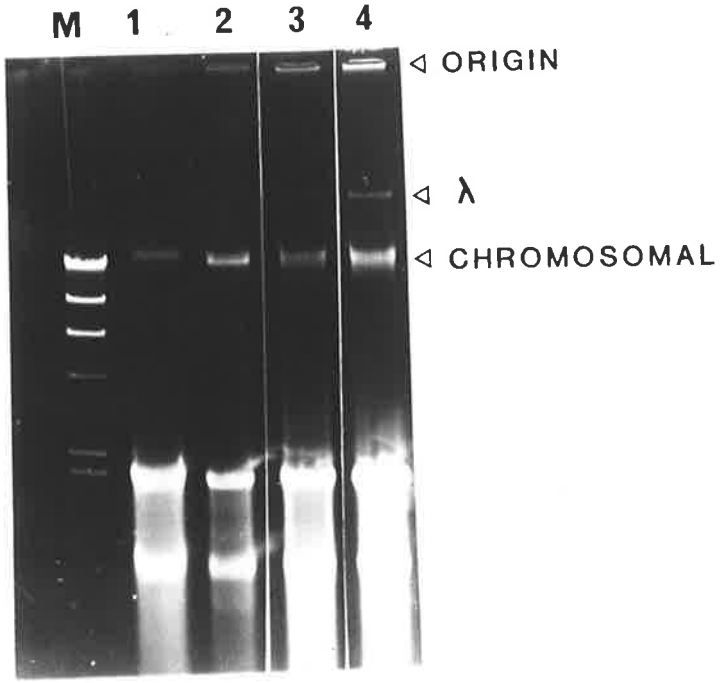
Track 2. *λoriC O*⁺ infected, 41°C

Track 3. *λoriC Oam* infected, 41°C culture

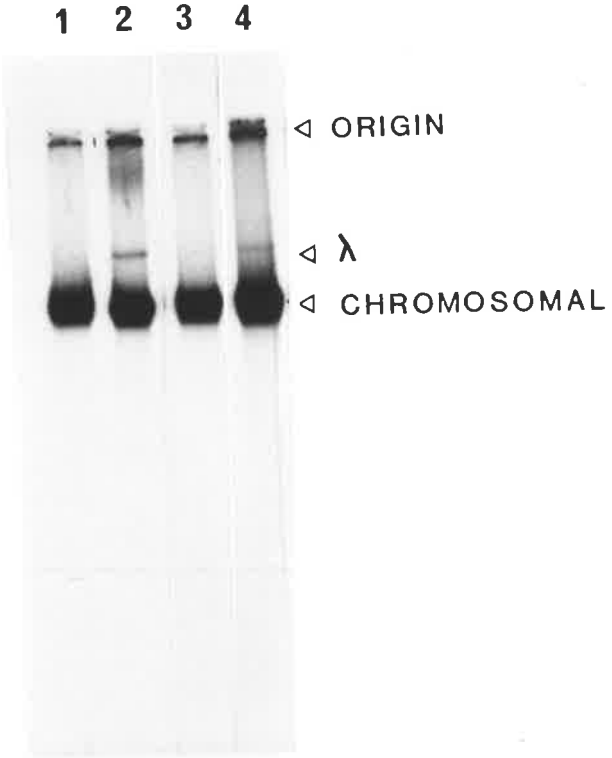
Track 4. *λoriC Oam* infected, 30°C culture

Track M. λ*Hin*dIII restriction fragments, 23.1, 9.3, 6.6, 4.4, 2.3 and 2 kb in size

a.



b.



These experiments showed that, like the chromosomal *oriC*, the *oriC* carried by λ*oriC0am* requires both *dnaA* and *dnaC*.

7.4.4 Standardisation of the hybridisation procedure

Su⁻*thy*⁻ cells (E777) were cultured in M9CAA medium and prelabelled with [³H]thymine for 3 generations. Portions were infected with λ*oriC 0am* or λΔ(*oriC*)*0am*, or were left uninfected, and the labelling was continued. At the required times aliquots (200 µl) were removed and the cells were lysed (Section 2.2.13). The DNA was denatured and hybridised to filters carrying λ DNA (unlabelled). After 15-20 h the filters were washed and the radioactivity (as cpm) on the filters was counted.

The following aspects of the technique were considered in order to optimise the method involving the hybridisation of λ DNA.

(i) Conditions of hybridisation and washing

(ii) Linearity of hybridisation

(iii) Reduction of non-specific background hybridisation.

(i) ***Conditions for hybridisation and washing of filters:***

In order to optimise conditions for maximum λ DNA-DNA hybridisation with minimum background binding of *E. coli* DNA, purified λ DNA ³²P- labelled by nick translation was added to [³H]labelled Su⁻ *thy*⁻ cellular lysates and

hybridised to unlabelled λ DNA. This allowed the percentage of the λ-specific binding and the percentage of the non-specific chromosomal binding to be measured.

The prehybridisation and hybridisation buffers and the washing conditions were varied, and the effect of these changes on the efficiency of hybridisation (i.e. the percentage of λ-specific binding) were studied. The details of the combinations used and the results obtained are listed in Table 7.1.

Four of the combinations (numbers 3, 4, 5 and 6) were essentially equivalent, giving reasonably efficient hybridisation of λ DNA and low background binding of *E. coli* DNA. The third procedure was chosen for use in all further experiments. The detailed procedure was :- prehybridisation of filters using a mixture of 10x Denhardts solution 4x SSC at 65°C for 2-3 hours, followed by hybridisation in 6x Denhardts solution and 2.5x SSC at 65°C for 18-20 hours. The filters were washed twice, for 15 min each time, with 2x SSC containing 0.1% SDS at room temperature (R.T.) and then twice for 15 min each time, with 0.1 x SSC containing 0.1% SDS, at 41°C.

(ii) ***Linearity of Hybridisation*** : This was assessed by two methods.

TABLE 7.1

The effect of varying the prehybridisation, hybridisation and washing conditions on the binding of labelled λ and *E. coli* DNA to λ DNA

No.	INPUT cpm Prehybridisation	Hybridisation	Washing	2.6×10^5 ^{3}H-coli DNA Percentage	3.3×10^4 ^{32}PλDNA Hybrid^n#
1.	10 x Denhardts 4 x SSC 65°C	6 x Denhardts 2.5 x SSC 65°C	3x30min 3mM Tris pH9.0 R.T.	0.31 ±.02	39.39 ± 3.42
2.	10 x Denhardts 4 x SSC 65°C	6 x Denhardts 2.5 x SSC 65°C	3x30min 3mM Tris pH9.0 65°C	0.07	1.31 ± 0.192
3.	10 x Denhardts 4 x SSC 65°C	6 x Denhardts 2.5 x SSC 65°C	2x15 min (2xSSC/ 0.1%SDS) R.T. 2x15 min (0.1xSSC/ 0.1% SDS) 41°C	0.27 ±.02	43.93 ± 2.14
4.	10 x Denhardts 4 x SSC	6 x Denhardts 2.5 x SSC	2x15 min (2xSSC/ 0.1% SDS) 41°C 2x15 min (0.1xSSC/ 0.1%SDS) 65°C	0.26 ±.05	40.75 ± 6.21
5.	BLOTTO 6 x SSC (Johnson *et al.* 1874)	0.6 x BLOTTO 3.5xSSC	2x30 min BLOTTO R.T. 2x15 (0.1xSSC/ 0.1%SDS) 65°C	0.24 ±.05	45.45
6.	10 x Denhardts 4 x SSC 50% formamide	6 x Denhardts 2.5 x SSC 30% formamide 41°C	2x15 min (2xSSC/ 0.1%SDS) 41°C 2x15 min (0.1xSSC/ 0.1%SDS) 65°C	0.26 ±.01	43.18 ±2.35
B.	NO DNA ON THE FILTER 10 x Denhardt 4 x SSC 65°C	6 x Denhardt 2.5 x SSC	3x30min Tris pH9.0 R.T.	0.039 ±.01	0.089 ±.02

70 ng of ^{32}P-labelled nick translated λ DNA was hybridised to non-radioactive λ DNA (5 μgm) attached to nitrocellulose filters, in the presence of ^{3}H-labelled *E. coli* (E777) cellular lysates from 200 μl aliquots (for method see Section 2.2.13). The mean cpm bound ± standard deviation are shown.

The first method involved the addition of varying amounts of ^{32}P-labelled purified λ DNA to *E. coli* cell lysates (see Section 2.2.13) followed by hybridisation of the denatured mixture to λ DNA (5 μg) bound to nitrocellulose filters. The maximum amount of λ DNA anticipated to be present in an experimental sample was 10 ng#. Therefore linearity was tested for hybridisation of λ DNA with a range of concentrations from 1-100 ng.

The extent of hybridisation was linear within a range of 1 to 50 ng of added ^{32}P-labelled λ DNA (Fig. 7.16a). This method measures the efficiency of hybridisation of *in vitro*-labelled λ DNA. In order to assess the linearity of *in vivo*-labelled λ*oriC Oam* DNA, hybridisation mixtures were prepared which contained varying proportions of ^{3}H-labelled λ*oriC Oam* DNA to ^{3}H-labelled *E. coli* chromsomal DNA. The extent of binding to λ DNA was measured using these mixtures. The varying proportions of λ DNA in the mixture were obtained by mixing ^{3}H-labelled λ*oriC Oam*-infected cell

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If 2 x 10^8 cells are infected with λ*oriC Oam* and a 200 μl aliquot removed i.e. 4 x 10^7 λ infected cells and if each λ*oriC Oam* molecule replicates 2 times i.e. 4 copies/cell there is a total of 1.6 x 10^8 molecules of λ DNA/aliquot.

The approximate mol. wt of λ*oriC* = 3.3 x 10^7 D.

Using Avogadro's number
6 x 10^{23} molecules of λ*oriC* = 3.3 x 10^7g = 3.3 x 10^{16}ng
therefore 1.6 x 10^8 molecules = 8.8ng

therefore the 200 μl aliquot of infected cells contains 8.8ng of λ DNA.

lysates with ^{3}H-labelled uninfected cell lysates in 100 µl aliquots such that the total amount of radioactivity in all the samples was the same but the proportion of labelled λ DNA to *E. coli* DNA varied. If λ DNA hybridisation in these samples is linear, the radioactivity binding to the filter should increase linearly above a background of *E. coli* DNA binding.

Details of the experiment are given in Fig. 7.16b. Duplicates of each sample were used. Fig. 7.16b is a plot of the cpm bound vs the volume of λ*oriC Oam*-infected lysate present in each aliquot. The background level of *E. coli* DNA binding was measured from the aliquot with no λ sequences present. Above the background, cpm binding to λ DNA increased linearly although the total input cpm were constant. Therefore, *in vivo*-labelled λ*oriC Oam* DNA hybridises linearly within the range tested. But the background cpm bound were fairly high compared to the λ*oriC Oam* cpm.

(iii) ***Reduction of non-specific background hybridisation:***

The non-specific background binding was measured as the cpm from lysates of ^{3}H-labelled uninfected or λΔ(*oriC*)*Oam*-infected cultures, which bound to λ DNA (5 µg) carried on nitrocellulose filters. In order to compare the extent of background hybridisation from one experiment to another, the cpm bound were expressed as a percentage of the input cpm, i.e. they were normalised.

FIG 7.16 **Linearity of λ DNA-DNA hybridisation**

a. Increasing amounts of ^{32}P-labelled purified λ DNA were hybridised to unlabelled λ DNA (5 μg) attached to nitrocellulose filters (See section 2.2.13 for the method). The filters were washed and the radioactivity bound (as cpm) was measured.

b. *Su⁻thy⁻* cells (E777) were cultured at 37°C in M9CAA containing thymine (2μg/ml) and prelabelled with [^{3}H]thymine (20 μCi/2μg/ml) for 3 generations. The culture was divided into 2 and one half was infected with λ*oriC Oam* (moa 1.6). After 35 minutes non-radioactive thymine was added to both cultures at a final concentration of 200 μg/ml. The cultures were lysed by centrifuging the cells and resuspending them in half the volume of TE Sucrose solution. (see Section 2.2.13). Varying volumes (0-100 μl) of the λ *oriC Oam* - infected lysate were taken and the uninfected cell lysate was added to give a volume of 100 μl. The DNA in each sample was heat-alkali denatured and hybridised to λ DNA (5 μg) bound to nitrocellulose filters (See section 2.2.13). Note the volume of the lysate is half the volume of the cellular aliquot. The filters were washed and the radioactivity bound (as cpm) was measured.

The mean of duplicate samples (given by the vertical bars) are plotted.

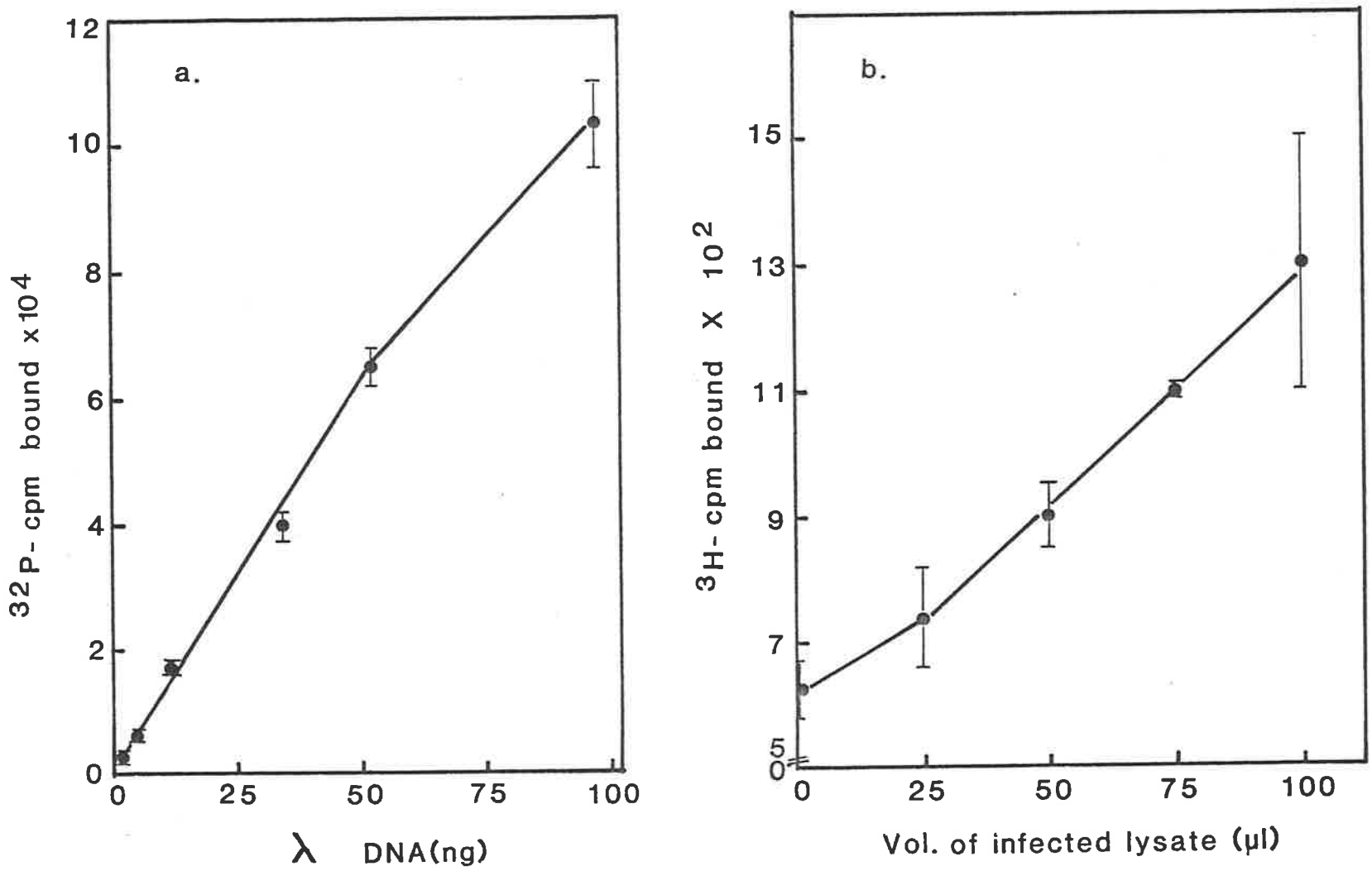

a.
12
10
8
6
4
2
0
^{32}P- cpm bound x10^4
0
25
50
75
100
λ DNA(ng)
b.
15
13
11
9
7
5
0
^{3}H- cpm bound X 10^2
0
25
50
75
100
Vol. of infected lysate (µl)

TABLE 7.2

Background binding of labelled *E. coli* DNA, from unirradiated and UV-irradiated cells, to λ DNA

	No. of samples	Percent cpm bound*	95% confidence limits of μ
unirradiated cells	37	0.195 ± 0.053	0.195 ± 0.017
90 J/m² - UV irradiated cells	12	0.264 ± 0.085	0.264 ± 0.054

* mean ($\bar{x}$) ± standard deviation (S_{n-1})

The percentage cpm bound with *in vivo*, ^{3}H-labelled *E. coli* DNA, from unirradiated cells and 90 J/m² UV-irradiated uninfected cells, to λ DNA (5 μg) were compiled from several independent experiments. A student-T distribution was used to calculate the 95% confidence limits of the population mean (μ) of these values (See section 2.2.18)

When the background cpm from uninfected, unirradiated or UV-irradiated cultures were compared, it was found that the percentage background was significantly higher in irradiated cells (Table 7.2). There was no obvious explanation for this.

Since the backgrounds were rather high compared with the λ-specific hybridisations, further experiments were carried out in an attempt to reduce the overall background, and in particular that obtained with UV-irradiated cultures. The following additions were made to the prehybridisation and/or the hybridisation mixtures:

a. In case the background *E. coli* (E777) binding to the λ DNA was due to the presence of a λ-like element in the chromosome, homologous unlabelled Su^- thy^- cellular DNA (from E777 cells) was added to the prehybridisation and the hybridisation mixtures. This would saturate any λ-like sequences in the *E. coli* DNA, and so prevent them from hybridising to the λ DNA on the filter.

b. Previous experiments had shown that free radioactive thymine increased non-specific cpm binding (data not shown). Therefore excess unlabelled thymine was added to the prehybridisation mix, to dilute the residual radioactive thymine.

c. It was also possible that the increased background obtained with UV-irradiated cells was due to UV-induction of

a λ-like cryptic element. If such an element was induced it would contribute significantly to the background of non-specific hybridisation. To prevent this possibility, unlabelled bacterial cells (E777) were irradiated with 90 J/m² of UV and lysed, the DNA was denatured and added to the prehybridisation mixture at 10x the concentration at which it would normally be present in a hybridisation mix. This treatment should compete out any induced elements present.

d. To reduce non-specific binding of labelled *E. coli* DNA to the nitrocellulose filters, heterologous unlabelled calf-thymus DNA was added to the prehybridisation mixtures.

To test the effects of the above treatments, ^{3}H- labelled *E. coli* DNA was prepared by prelabelling Su^- thy^- cultures (E777) with [^{3}H]thymine (20 μCi/2 μg/ml) in M9CAA medium and portions of the culture were irradiated with 90 J/m² of UV. The cells were lysed and hybridisation mixtures were prepared as described in Section 2.2.13. Aliquots of 100 μl of the lysate were hybridised to λ DNA (5 μg) on nitrocellulose filters. Table 7.3 lists the data obtained in this experiment. The addition of homologous (E777 chromosomal) or heterologous (calf thymus) DNA (treatments a_1 and d), reduced the background hybridisation (by 25%) compared with the standard conditions used (treatment e).

Further experiments were carried out, to determine whether treatments a_1 and d consistently reduced the background, obtained with irradiated and unirradiated cells, while not reducing the λ-specific DNA-DNA hybridisation. To do this, Su^- *thy*$^-$ cultures (E777) were prelabelled for three generations with [^{3}H]thymine (20μCi/2μg/ml) in M9CAA medium and irradiated with 0 or 90 J/m² of UV. A portion of the unirradiated culture was infected with λ*oriC Oam* (moa 5), 35 min later samples were removed from the unirradiated, λ*oriC Oam* - infected culture (A), from the unirradiated, uninfected culture (B), and from the UV-irradiated, uninfected culture (C). Hybridisation mixtures were prepared from these and hybridised to λ DNA on filters which had been prehybridised in 10 x Denhardts and 4 x SSC, with no addition (x) or with chromsomal E777 DNA (y), or calf thymus DNA (z) added.

The data obtained are listed in Table 7.4. Addition of E777 DNA to the prehybridisation mix (y) did not significantly alter the cpm hybridised in any sample (A, B or C). Addition of calf thymus DNA (z) reduced the background binding obtained with unirradiated (B) but not with UV-irradiated (C) cultures . However, subsequent experiments showed that the effect of calf thymus DNA was not consistent (data not shown).

TABLE 7.3

Effect of altered prehybridisation and hybridisation solutions on binding of *E. coli* DNA to λ DNA

Sample No.	Conditions Used	UV dose J/m²	cpm bound	mean % hybridisation *
a_1.	Su^-thy^- in the prehybridisation mix	0	418 405 425	0.179 ± .004
		90	487 498 451	0.219 ± .011
a_2.	Su^-thy^- DNA in the hybridisation mix	0	582	0.250
		90	542 486 407	0.216 ± .031
b.	thymine (200μg/ml) in the prehybridisation mix	0	878 664 671	0.317 ± .052
		90	588 671 611	0.285 ± .019
c.	Su^-thy^- irradiated cellullar lysate in prehybridisation mix	0	1007 872	0.403 ± .039
		90	539 790 338	0.254 ± .103
d.	Calf-thyus (CT) DNA in the prehybridisation mix	0	352	0.151
		90	490	0.224
e.	10 x Denhardt	0	577 497	0.230 ± .024
	4 x SSC (standard mix)	90	725 576 615	0.292 ± .035

* mean of the cpm bound calculated as a percentage of the input cpm ± standard deviation.

³H-labelled DNA from cells of *E. coli* strain E777 was tested for the ability to bind to filters carrying 5 μg of unlabelled λ DNA as described in the text. Different factors involved in prehybridisation and hybridisation were varied see columns 2 and the text. The input cpm /200μl aliquot of cells were, 0J/m² = 2.32 x 10^5, 90 J/m² = 2.18 x 10^5

TABLE 7.4

The effect of addition of calf-thymus (CT) DNA and *E. coli* DNA to the prehybridisation mixture, on background and λ DNA hybridisation

Sample and input cpm	Prehybridisation treatment	cpm bound			mean % cpm bound #
A.					
λ*oriC Oam* infected	x. D/SSC *	1448	856		0.488 ± .177
unirrad[d] cells	y. Su⁻*thy*⁻DNA	1337	798	649	0.393 ± .154
2.36 x 10^5	z. CT DNA	906	762	481	0.306 ± .091
B.					
Unirradiated	x. D/SSC	518	330	339	0.151 ± .040
Su⁻*thy*⁻ cells	y. Su⁻*thy*⁻ DNA	391	388	270	0.133 ± .026
2.61 x 10^5	z. CT DNA	189	214	180	0.074 ± .006
C.					
90 J/m² irrad[d] Su⁻	x. D/SSC	232	285	236	0.162 ± .019
thy⁻ cells	y. Su⁻*thy*⁻ DNA	261	206	259	0.157 ± .020
1.54 x 10^5	z. CT DNA	346	311	256	0.197 ± .029

mean of the percentage cpm bound ± standard deviation.

* D/SSC is 10x Denhardts/4x SSC solution.

Aliquots were prepared for hybridisation as described in the text, non-specific and λ specific hybridisation was estimated

Since none of the treatments used consistently reduced the background, to any great extent it was decided to omit the additions and use the original method devised for studying hybridisations (Section 7.4.4(i)). As this method had a relatively high standard deviation for each sample, each subsequent experiment was repeated several times before making a conclusion.

7.4.5 Estimation of *oriC*-dependent replication in unirradiated cells

λ DNA replication in λ*oriC Oam*-infected Su^- cells initiates mainly from *oriC* but if the *Oam* mutation is leaky some initiation may occur from *ori*λ. In order to allow the extent of oriC-dependent replication to be assessed the contribution (if any) of *ori*λ-dependent replication had therefore to be measured.

Su^-thy^- cells (E777) were prelabelled with [^{3}H]thymine and infected with either λ*oriC Oam* or λΔ(*oriC*)*Oam*, or were left uninfected. Aliquots of 200 µl were removed 15 and 35 min after infection and hybridised to λ DNA bound to nitrocellulose filters. The radioactivity which was incorporated and hybridised due to replication of λ DNA from *ori*λ (i.e. *Oam* leak) was measured (Table 7.5b) by subtracting the radioactivity (cpm) derived from uninfected cells, from that derived from λΔ(*oriC*)*Oam* infected cells (Table 7.5a). It was found that no substantial replication occurred from *ori*λ, up to 35 min after infection (Table 7.5).

TABLE 7.5

Estimation of λ *oriC Oam* replication from *oriλ* and *oriC*

a.	cpm/200 µl bound to λ filters*		
Time after infection	0-1 min	15 min	35 min
Phage used			
uninfected	425 ± 54	535 ± 44	646
λ Δ (*oriC*) *Oam*	N.D.	438 ± 42	726 ± 0.76
λ *oriC Oam*	406 ± 1.4	836 ± 51	1213 ± 16
b.	cpm due to replication from *oriλ*		
λ Δ (*oriC*) - uninfected	N.D.	-97 ± 60	80
c.	cpm due to replication from *oriC*		
λ*oriC Oam* - uninfected	-19 ± 54	301 ± 67	567
λ*oriC Oam* - λ Δ (*oriC*)*Oam*	N.D.	398 ± 66	487 ± 16

* mean of duplicates ± standard deviation

Su⁻*thy*⁻ cells (E777) were cultured in M9CAA and prelabelled or 3 generations with [³H]thymine (20 µCi/ml). At an O.D. of 0.4 the culture was divided into 3 portions which were infected with λ*oriC Oam*, or λΔ(*oriC*)*Oam*, or were not infected. At the times indicated aliquots (200 µl) were removed and hybridised to λ DNA (5 µg) attached to filters as described in Section 2.2.13. The filters were washed and the radioactivity bound (as cpm) was measured.

From the data in Table 7.5a the *oriC*-dependent λ DNA replication was measured (Table 7.5c). The background cpm, determined using either uninfected or λΔ(*oriC*)*0am*-infected cultures were subtracted from the radioactivity (cpm) hybridised from the λ*oriC 0am*-infected culture to give the *oriC*-specific cpm. No *oriC*-specific cpm were detected 0 min after infection, but a significant number of *oriC*-specific cpm were found at 15 and 35 min after infection. The *oriC*-specific cpm were similar whether uninfected or λΔ(*oriC*)*0am*-infected cells were used to estimate the background.

Therefore the radioactivity that was incorporated, into λ DNA due to replication from *oriC*, and hybridised to λ DNA, (referred to as *oriC*-specific cpm for brevity), was measured by two means :

oriC-specific cpm/200 µl = cpm bound from 200 µl of a λ*oriC 0am* infected culture

MINUS

cpm bound with 200 µl of an uninfected

or

λΔ(*oriC*)*0am* infected culture

oriC-specific cpm were used as a measure of *oriC*-dependent replication.

7.4.6 Effect of UV-irradiation on *oriC*-specific cpm

The effect of irradiation of cells, prior to infection, on *oriC*-dependent replication was studied.

One problem with comparing cpm hybridised in unirradiated cultures with cpm hybridised in UV-irradiated cultures is the fact that host DNA replication is depressed after UV-irradiation so that background cpm are lower. Therefore the background binding from irradiated cultures, infected with λΔ*(oriC)0am* or uninfected, was measured for each sample in order to calculate the *oriC*-specific cpm in irradiated cultures. Thus, this value is a measure of the absolute amount of λ DNA which has incorporated label in the irradiated cells. This quantity, for the short period of time studied, should be the same as that obtained with unirradiated cells if UV has no effect on replication from *oriC*.

In the experiments described below, the effect of UV-irradiation has been expressed as the ratio of cpm from unirradiated cells to cpm from irradiated cells. If the ratio is 1.0, UV has no effect on replication, if it is <1.0, UV stimulates *oriC* replication, and if it is >1.0, UV inhibits *oriC* replication.

Two doses of UV, 45 J/m² and 90 J/m² were studied for their effect on *oriC*.

TABLE 7.6

a. Su$^-$*thy*$^-$ cells (E777) were cultured in M9CAA containing thymine (2 µg/ml) and continuously labelled for 3 generations with [^{3}H]thymine (20 µCi/ml). At an O.D. o.4, cells were irradiated with either 0 or 45 J/m^2 of UV and were then infected with λ*oriC Oam* (moa 2-3), uninfected cultures were used as controls. At 15 and 35 min aliquots (200 µl) were removed and hybridised to λ DNA (5 µg) attached to filters. Filters were washed and the radioactivity bound per filter was measured and is expressed as cpm bound/200 µl. The data from 3 independent experiments are shown.

b. *oriC* - specific cpm were measured by substracting the mean cpm bound with the uninfected control culture, from the mean cpm bound with the λ *oriC Oam* infected culture. The data in a. were used to calculate this (See section 2.2.18 for the calculation of the standard deviation).

TABLE 7.6

Effect of 45 J/m² of UV on *oriC*-dependent replication of λ *oriC Oam*

a.	cpm bound */200 µl			
time after infection	15 min		35 min	
UV dose J/m²	0	45	0	45
Sample				
I λ*oriC Oam*	1189 ± 124	975 ± 146	2301	1682 ± 94
uninfected	836 ± 12	632 ± 78	1064 ± 41	830 ± 50
II λ*oriC Oam*	1327 ± 53	1454 ± 589	1950 ± 22	1687 ± 75
uninfected	1001 ± 140	763 ± 3.5	769 ± 157	616 ± 3
III λ*oriC Oam*	906 ± 101	717 ± 37	1583 ± 197	1500 ± 105
uninfected	667 ± 71	537 ± 130	670 ± 139	553 ± 118

b.	*oriC* - specific cpm				RATIO #	
TIME	15 min		35 min		15 min	35 min
UV dose Jm²	0	45	0	45	0/45 Jm²	
I	353 ± 124	343 ± 165	1237 ± 41	852 ± 106	1.02	1.45
II	326 ± 149	691 ± 589	1181 ± 158	1071 ± 75	0.47	1.10
III	239 ± 123	180 ± 135	913 ± 241	947 ± 157	1.32	0.96

* mean of duplicates ± standard deviation

ratio of the mean values

With a UV fluence of 45 J/m², the ratios of unirradiated to irradiated *oriC*-specific cpm was not significantly different from 1.0 both 15 and 35 min after infection (Table 7.6b). Therefore this fluence did not depress replication from *oriC* up to 15 min after infection. (The counts bound ± standard deviation for the background and λ*oriC Oam*-infected culture are shown in Table 7.6a).

The cells were then irradiated with a higher fluence (90 J/m²) and *oriC*-specific cpm estimated in the irradiated and unirradiated cultures. The data from 5 independent experiments are compiled in Table 7.7. Samples were removed 0-1 min and 35 min after infection. The cpm bound from the background and λ*oriC Oam* infected cultures are shown in Table 7.7a. The *oriC*-specific cpm were calculated from these data and are expressed in Table 7.7b.

As expected, no significant *oriC*-specific cpm were present in either the unirradiated or irradiated culture immediately (0-1 min) after infection, but at 35 min, *oriC*-specific cpm were present in both the unirradiated and 90 J/m²-irradiated cultures. However, the *oriC*-specific cpm were reduced in every case in the irradiated cells relative to the values obtained with unirradiated cells. The ratio of unirradiated to 90 J/m² irradiated *oriC*-specific cpm varied between 2.7 and 6.7. The 95% confidence limits of the population mean (μ) of the ratios is 4.688 ± 1.338 (see Section 2.2.18) i.e. replication from *oriC* is from 3.35 to

TABLE 7.7

a. Su$^-$*thy* cells (E777) were cultured in M9CAA containing thymine (2 µg/ml) and prelabelled for 3 generations with [^{3}H]thymine (20 µCi/ml). At an O.D. of 0.4, the cells were irradiated with either 0 or 90 J/m^2, and a part of the cultures was infected with λ*oriC Oam* or λ0$^+$ (λ68). The control cultures were either kept uninfected or were infected with λ Δ (*oriC) Oam*. The moa of phage varied between 1 and 3 for different experiments. Samples were removed immediately (0-1 min) and 35 min after infection, cells were lysed and the DNA hybridised to λ DNA (5 µg) attached to nitrocellulose filters. The filters were washed and the radioactivity bound was measured. This is expressed as cpm bound/200 µl in the table.

Data from 6 independent experiments are shown. For expts. I, II and III the background was determined using λΔ *oriC) Oam* infected cells. For expts IV, V and λ0$^+$ the background was determined using uninfected cells.

b. The data in a. were used to calculate *oriC*- and *ori* λ - specific cpm by substracting the mean background cpm bound (control) from the mean cpm bound with the test cultures.

TABLE 7.7 (a.)

Effect of 90 J/m² of UV on *oriC*- and *oriλ*-dependent replication

		cpm bound */200 µl			
Time after infection		0-1 min		35 min	
UV dose J/m²		0	90	0	90
λ *oriC Oam*					
I	test	360 ± 17	297 ± 0	1380 ± 129	589 ± 93
	control	326 ± 66	310 ± 22	510 ± 68	375 ± 3
II	test	336 ± 5	292 ± 7	896 ± 4	426 ± 3
	control	315 ± 2	285 ± 16	565 ± 98	350 ± 8
III	test	397 ± 17	402	1139 ± 159	479 ± 48
	control	361 ± 75	364 ± 11	716 ± 71	416 ± 58
IV	test	ND	ND	1635 ± 346	838 ± 15
	control			884 ± 169	562 ± 89
V	test	ND	ND	2183 ± 152	783 ± 49
	control			858 ± 231	529 ± 28
λ 0^+	test	ND	ND	27300 ± 2404	24750 ± 2474
	control			667 ± 24	868 ± 148

* mean of duplicates ± standard deviation

ND - Not determined

TABLE 7.7.b

Effect of 90 J/m² of UV on *oriC*- and *oriλ*-dependent replication

	oriC-specific cpm *				RATIO #
Time after infection	0-1 min		35 min		35 min
UV dose J/m²	0	90	0	90	0/90 J/m²
λ *oriC Oam*					
I	34 ± 68	−13 ± 22	870 ± 145	214 ± 93	4.0
II	21 ± 5	7 ± 17	331 ± 98	76 ± 9	4.3
III	36 ± 76	38 ± 11	423 ± 174	63 ± 75	6.7
IV	ND	ND	751 ± 385	276 ± 90	2.7
V	ND	ND	1325 ± 276	254 ± 56	5.2
	ori λ - specific cpm *				
λ 0^+	ND	ND	26633 ± 2404	23882 ± 2478	1.1

* mean λ *oriC*- or λO^+- cpm bound$_{(1)}$

− mean background cpm bound$_{(2)}$

$\pm \sqrt{S.D.^2_{(1)} + S.D.^2_{(2)}}$

\# - ratio of the mean values obtained above.

95% confidence limits of the population mean (μ) of the ratios are 4.688 ± 1.338 (See Section 2.2.18)

6.02 times less in the UV-irradiated culture than in the unirradiated. The 95% confidence limits of μ were calculated using the 1-tailed test described in Section 2.2.18, assuming that the ratios are normally distributed.

To ensure that the depression of replication from *oriC* was not somehow an artifact of the experimental procedure, replication from *ori*λ was studied under the same conditions. λ*O*$^+$ (λ68) phage were used to infect unirradiated and 90 J/m^2 UV-irradiated cells at an moa of 0.5. (A λ phage not carrying *oriC* was used, to avoid complications with inhibition of replication from that origin. By using a λ*O*$^+$ phage, replication from only one origin *ori*λ was tested). Phage production by this phage was unaffected by UV-irradiation of the cells (Fig. 7.17). λ DNA replication was studied by hybridisation as described for λ*oriC*. The radioactivity bound (as cpm) using an uninfected background sample and the λ-infected sample 35 min after infection are recorded in Table 7.7a. The replication from *ori*λ i.e. *ori*λ-specific cpm were calculated by subtracting the background cpm bound from the cpm bound with the λ-infected culture (Table 7.7b) in both the unirradiated and the irradiated cultures. Since the ratio between *ori*λ-specific cpm in unirradiated and UV-treated cultures is 1.1 it was concluded that replication from oriλ was not inhibited by UV-irradiation of the cells. Therefore, the depression of replication of λ*oriC Oam* in UV-irradiated cells is specifically due to an effect on

FIG 7.17 Effect of UV on λO^+ phage production

$Su^- thy^-$ cells (E777) were cultured in M9CAA containing thymine (2μg/ml) at 37°C to 3 x 10^8 cfu/ml and infected with λO^+ phage (λ68) phage (moa 0.5). Incubation was continued at 37°C. After 5 minutes the unabsorbed phage were inactivated with antiserum. After a further 5 minutes incubation the culture was diluted into fresh medium and phage production was assayed on C600 indicator bacteria at intervals.

(● - ●) unirradiated cells

(o - o) 90 J/m²-irradiated cells

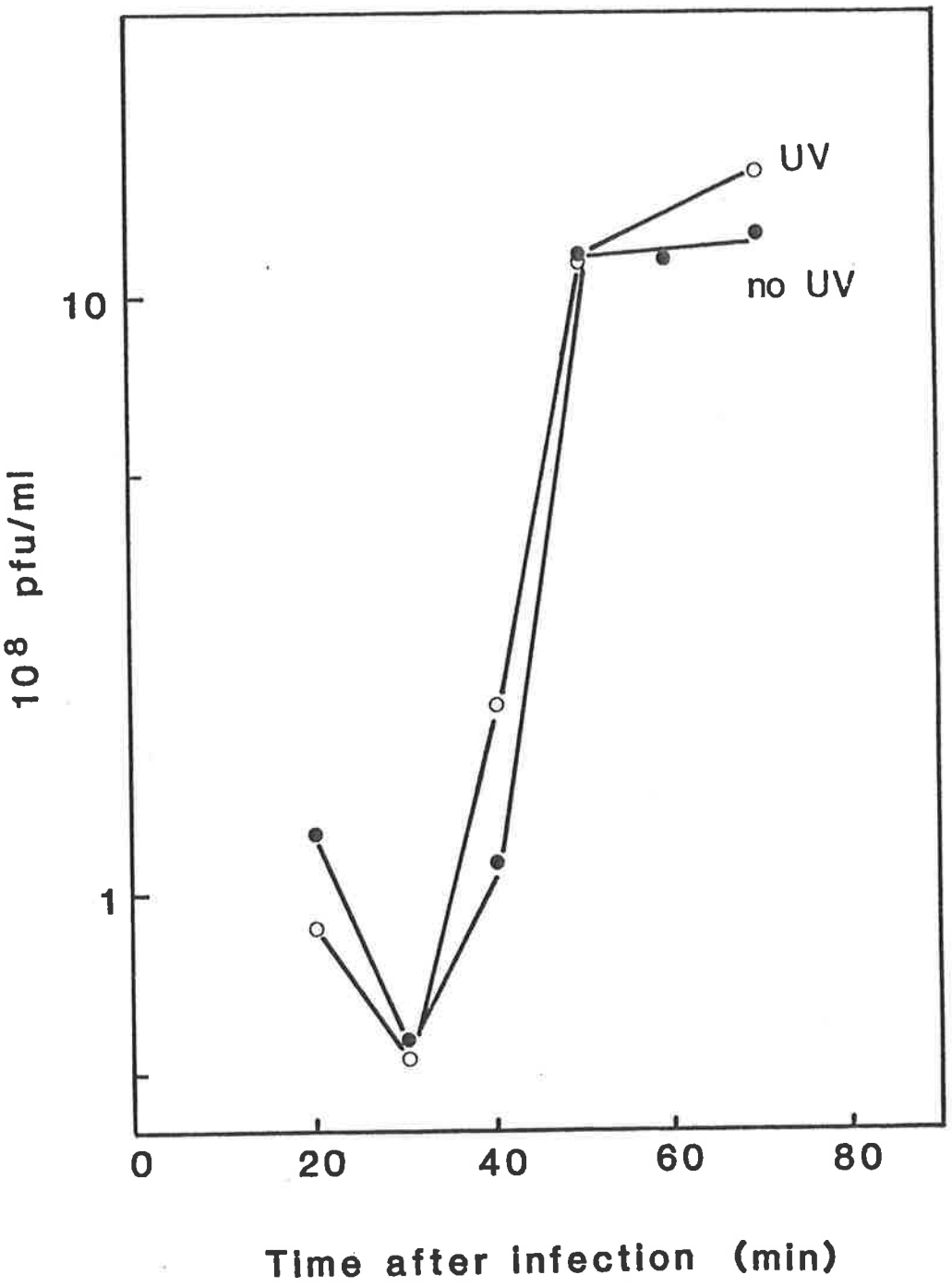
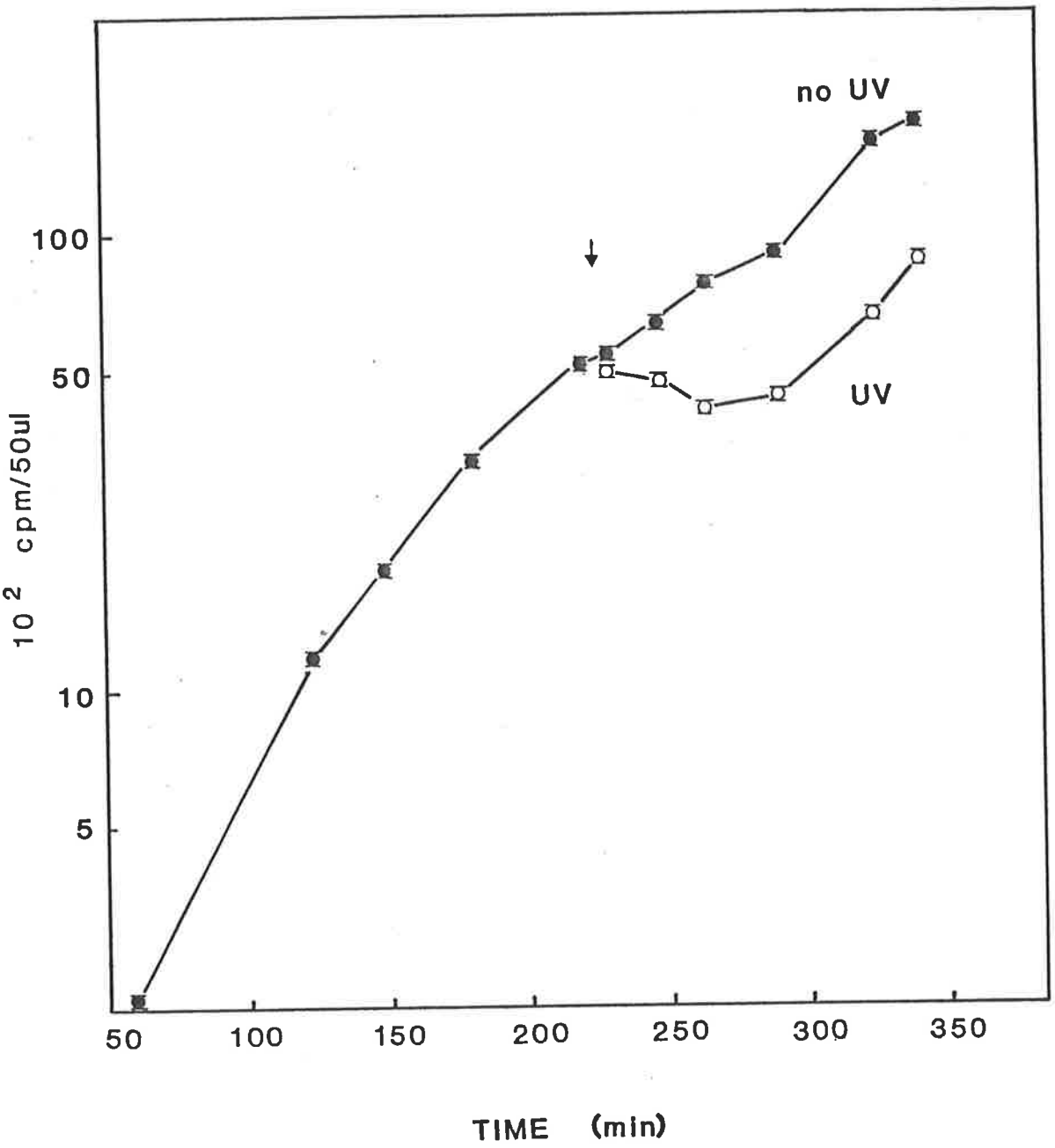

UV
no UV
10
1
10^8 pfu/ml
0
20
40
60
80
Time after infection (min)

FIG 7.18 DNA replication in Su⁻*thy*⁻ cells after UV-irradiation

Su⁻*thy*⁻ cells (E777) were cultured in M9CAA and thymine (2μg/ml), [³H]thymine (2μCi/ml) was added at 0-min and aliquots were TCA precipitated at intervals with time. At the time indicated (↓) cells were irradiated with 0 and 90 J/m² and incorporation of [³H]thymine into TCA precipitable medium was followed. The horizontal bars indicate that the duplicate samples fall within the area of the symbols.

(● - ●) unirradiated

(o - o) 90 J/m²-irradiated cells


no UV
UV
100
50
10
5
10^2 cpm/50ul
50
100
150
200
250
300
350
TIME (min)

replication from *oriC*. It can thus be concluded that UV does induce an inhibition active against unirradiated *oriC*.

An important point to note is that 90 J/m² of UV causes a 50-60 min inhibition in chromosomal replication in the Su^- thy^- cells, (Fig. 7.18). The length of this inhibition allows for replication from *oriC* to be inhibited for at least 35-40 min after irradiation before recovery occurs.

7.5 CONCLUSION

UV-irradiation of cells inhibits the replication from unirradiated *oriC*. This was demonstrated both, by following *oriC*-dependent phage production and by measuring *oriC*-dependent replication of λ by DNA-DNA hybridisation.

In both cases *oriλ* was not similarly inhibited, so that the effect was specific to *oriC*. Replication from this origin requires the *dnaA* and *dnaC* products.

CHAPTER 8

GENERAL DISCUSSION

As discussed previously in Chapter 1, cellular DNA appears to be the primary target for UV damage. As a consequence DNA synthesis is inhibited. The cause of this inhibition has long been considered to be a block in elongation of replication forks by pyrimidine dimers or other photoproducts. However the data of Hooper *et al.* (1981), Hooper (1979), and Woods and Egan (1974) showed that the synthesis of undamaged phage 186 DNA was inhibited in UV-irradiated cells. This gave rise to the interesting possibility that UV-irradiation of a cell may in some way change it's physiology, so that the cell becomes refractory to replication from certain origins. This meant that in addition to the *cis*-acting effect the UV-induced photoproducts had on replication, UV may also induce a *trans*-acting inhibition of replication.

It was with a view to answering this question that the study with 186 phage was undertaken. As has been mentioned UV-irradiated cells lose the capacity to produce 186 phage and replicate phage DNA. The loss in capacity occurs at UV-fluences at which λ and P2 are unaffected. Therefore these fluences of UV do not appear to affect general cellular mechanisms such as transcription, translation and DNA elongation which would be used by all these phage. Instead, something which specifically affected some replicons and not others must be affected by UV. A likely target for this inhibition was initiation of DNA replication.

Hooper *et al.* (1981) showed that 186 DNA synthesis was inhibited in UV-irradiated cells. However, DNA synthesis was measured in those experiments by pulse-labelling irradiated cells. This method has the disadvantage that UV-induced breakdown of DNA can cause a dilution of the radioactive precursor pool (Smith and O'Leary, 1968), resulting in depression in the amount of DNA synthesis measured, which is then mistakenly interpreted as being a depression in DNA replication. I confirmed that 186 DNA synthesis was inhibited in UV-irradiated cells using the protocol recommended by Smith and O'Leary (1968). This protocol avoids the possibility of an apparent depression in DNA replication due to dilution of the labelled precursor pool in UV-irradiated cells. The protocol recommends that pre-labelled cells be irradiated, and that the radioactivity incorporated be plotted on a log scale.

Study of the loss in 186 capacity: Three things were considered as possible causes of the loss in the capacity of UV-irradiated cells to produce 186 phage.

(i) 186 DNA synthesis may require concomitant host DNA replication. If this was the case, then a block in the elongation of *E. coli* DNA would suffice to explain the block in 186 replication. However, it was found that phage production could occur when *E. coli* DNA synthesis was inhibited. Phage production was the same in irradiated *uvrA6* mutant cells, where there was no detectable DNA synthesis, as in *uvrA* cells where DNA synthesis recovered

normally. Therefore there was no apparent correlation between the recovery of *E. coli* DNA synthesis and 186 phage production. From this it was concluded that the inhibition in 186 DNA synthesis, observed in UV-irradiated cells, was unlikely to be a consequence of the transient inhibition of host DNA synthesis. These data do not allow one to conclude that 186 replication has no requirement for host synthesis, since a small quantity of replication undetected by TCA-precipitation can occur in UV-irradiated excision-deficient cultures (Billen and Carreira, 1971). However, the observation that inhibition of host synthesis does not correlate with the inhibition in 186 DNA synthesis suggests strongly that the loss in 186 capacity is due to a *trans*-acting inhibition induced in UV-irradiated cells.

(ii) Given that the loss in 186 capacity was due to a trans-acting effect induced by UV, the most obvious question was whether or not it was an SOS controlled function, as several UV-induced functions are part of the SOS regulon. If there was a gene or function in the SOS regulon which inhibited 186 replication, then mutations in either the *recA* or *lexA* genes which abolish the induction (expression) of the regulon should restore completely the irradiated cells capacity to produce 186 phage.

However, a problem in designing these experiments was the dose of UV to be used, since the *recA* and *lexA*(Ind$^-$) mutants are both UV-sensitive compared with the control wild

type cultures. If the same dose of UV was used to irradiated the UV^S (*recA*, *lexA*) and UV^r (wild-type) cultures, then the UV^S mutants would have a much lower survival than the UV^r cultures. But if the strains were irradiated to obtain the same survival, the UV^S mutants would receive a significantly smaller dose of UV than the control. This low dose of UV was known not to induce the loss in 186 capacity in wild type cells and may also be incapable of inducing the response in the UV^S cultures. Therefore if no loss in 186 capacity was obtained on irradiating the *recA* and *lexA* mutants with the lower dose of UV it could have been mistakenly attributed to the presence of the mutations, when in fact the dose of UV was insufficient to induce the loss in capacity. It was decided therefore to irradiate control and mutant cultures with the same dose of UV.

lexA3(Ind^-): This mutation renders the LexA repressed functions non-inducible. UV-irradiated C600 *lexA3*(Ind^-) mutant cells suffered the same loss in capacity to produce 186 phage as did the wild type cells. Therefore the loss in capacity to produce 186 is not due to a LexA repressed function.

recA441(tif-1): This mutation allows activation of the *recA* protease at high temperatures causing the induction of the SOS regulon in the absence of DNA damage. In the AB1157 background the *recA441* mutation did not induce a loss

in the capacity of the cells to produce 186 phage at 41°C. Therefore activation of the *recA* protease is not sufficient to cause the loss in 186 capacity.

recA56: A strain carrying this mutation is deficient in *recA* function. However, UV-irradiation of such a mutant still caused a loss in the capacity of the cells to produce 186 phage. Therefore functional *recA* protein was not required for the loss in 186 capacity.

On the basis of the above data it was concluded that the loss in 186 capacity was not merely due to an induced SOS function.

The conclusion concerning the *recA56* mutant is however subject to a qualification. The percentage of infectious centres obtained with the irradiated *recA56* mutant compared with its unirradiated control was much lower than that obtained with the wild type cells, i.e. the loss in capacity to host infection by 186 was more marked than in the wild-type cells. *recA*-mutants are pleiotropic, affecting many functions, and it could be one of these 'side-effects' which causes the reduction in capacity to host 186. Thus, it is possible that recovery does occur in the absence of RecA, but the recovery is masked by the secondary effect of the *recA* mutation. One possible secondary effect is the *recBC* (exonuclease V)-mediated degradation of DNA. In a UV-irradiated *recA* mutant *recBC* exonuclease-induced DNA

degradation is increased (Swenson, 1976), since the *recA* protein normally represses the *recBC*-exonuclease activity. This exonuclease could be responsible for the drop in the number of productive 186 infections (infective centres) in the UV-irradiated *recA* mutant cells. However, it is unlikely that exonuclease V degrades 186 DNA to a greater extent in the irradiated *recA* mutant compared with the unirradiated mutant, for the following reasons:

(a) *recBC* acts predominantly on damaged DNA at the site of the lesion (Marsden and Ginoza, 1974a; b), it attacks DNA from ends generated by double stranded breakage, but does not attack circular double-stranded DNA with or without single-strand breaks (Wright *et al.*, 1971).

(b) recBC-dependent DNA degradation of *Eco*R1 restricted λ DNA is the same in unirradiated and UV-irradiated (54 J/m²) cells. Therefore UV does not activate or induce exonuclease V (Thoms and Wackernagel, 1982).

(c) *recBC*-dependent DNA degradation is the same in cellular lysates (supplemented with 0.2 or 1.0 mM ATP) of unirradiated and UV-irradiated *recA*$^+$ cells (Barbour and Clark, 1970) and in *recA*$^+$ and *recA13* (RecA$^-$) lysates (Oishi, 1969).

However, no data were found on the extent of degradation of undamaged DNA in irradiated and unirradiated

intact *recA*$^-$ cells, so that the possibility remains that *recBC*-dependent DNA degradation could cause the lower number of 186 infectious centres in the *recA*$^-$ mutant culture. This possibility should be tested in a *recA recB* double mutant and its isogenic *recB* control. If the *recBC* nuclease was degrading 186 DNA and the loss in capacity was caused by *recA*, then the double mutant should not have any UV-induced loss in capacity to produce 186. This has not yet been tested, but for reasons given above it seems very unlikely.

Despite difficulty in interpreting results with the *recA56* mutant, the data obtained with the *lexA3*(Ind$^-$) mutant show clearly that the loss in capacity is not due to a *lexA* repressed function and those obtained with the *recA441*(tif-1) mutant make it unlikely that the loss in capacity is caused by the *recA* protease.

(iii) Another possible cause of the loss in 186 capacity was that UV-irradiation of the cell may lead to depletion or inactivation of host functions required by some replicons and not by others. For reasons discussed in Chapter 1, it was proposed that the *dnaC* protein was a candidate for such a function. It was reasoned that an increase in the cellular *dnaC* protein should therefore compensate for the UV-induced depletion, so that no loss in phage capacity should occur. To test this, multicopy plasmid (pMOB45, pBR322) clones of *dnaC* which had been shown to overproduce the protein several fold (Kobori and

Kornberg, 1982), were introduced into cells. The presence of the *dnaC* plasmid clones did not restore the irradiated cells capacity to produce 186 phage.

The pMOB45 vector alone decreased the capacity of the irradiated cells to produce 186 phage, which caused difficulties in interpreting the results. The vector did not however, have an adverse effect on 186 phage production in the unirradiated cells. The pMOB45 plasmid may require *dnaC* protein as it is derived from plasmid R1 (Bittner and Vapnek, 1981; Uhlin *et al.*, 1979), which belongs to the FII incompatibility group (Scott, 1984) and plasmids from this group (R100, R1*drd*16) have been shown to require *dnaC* (Scott, 1984; Womble and Rownd, 1979; Goebel, 1973; Rownd, 1978; Ryder *et al.*, 1981). If it does, then irradiation of the plasmid in the cell during the experiment could lead to a further depletion in the amount of cellular *dnaC* protein. Depletion of the cellular pool of *dnaC* protein, following irradiation of the cell carrying pMOB45 plasmid, could then explain the pMOB45-induced loss in the capacity of the cells to host 186. If this explanation was correct, the presence of the *dnaC* clone should have restored to some extent the capacity of irradiated cells to produce 186 phage. This did not occur, a result which suggests that the *dnaC*-depletion hypothesis is incorrect. The conclusion assumes that the *dnaC* protein is overproduced from the plasmid clones in the irradiated cells, to the levels that were obtained in unirradiated cells by Kobori and Kornberg (1982).

It is also possible that pMOB45 in an irradiated cell interferes with 186 phage production for reasons independent of *dnaC*, so that the presence or absence of the *dnaC* gene on the plasmid would have little effect on the loss in capacity. In other words, overproduction of the *dnaC* protein in this experiment may be alleviating the UV-induced loss in capacity but the pMOB45/UV interference prevents normal 186 phage production, masking the effect of the excess *dnaC* protein. This seems unlikely, but it cannot be ruled out as a possibility. However, it is more probable that the *dnaC* protein is not involved in the UV-induced loss in 186 capacity.

Thus, the work with 186 discussed above established that the replication of phage 186 DNA is inhibited in UV-irradiated cells, substantiating the results of Hooper *et al*. (1981). It has ruled out the possibility that the inhibition of 186 replication in irradiated cells is a consequence of the inhibition of *E. coli* DNA replication, making it more likely to be due to a *trans*-acting function induced by UV. The loss in the capacity of irradiated cells to produce 186 phage reflects this inhibition of phage DNA synthesis. The loss in capacity does not appear to be an SOS function, and is also unlikely to be due to a depletion of the *dnaC* protein.

The target for inhibition of replication was considered as mentioned above. Both phage λ and P2, the replication of which is not inhibited in UV-irradiated cells, require *E. coli* elongation functions but not the initiation functions *dnaC* and *dnaA* (Furth and Wickner, 1983; Bowden *et al.*, 1975), so that it is most unlikely that the elongation functions are affected by UV-irradiation. In contrast 186, P1 and Mu, all of which are transiently inhibited in irradiated cells, require host initiation functions (Hooper and Egan, 1981). It therefore seemed most likely that 186 replication was inhibited at initiation rather than at elongation in an irradiated cell. It was anticipated that such an inhibition would act primarily on the initiation of *E. coli* replication in the cell and would fortuitously inhibit replicons like 186 which shared common requirements with *E. coli* initiation. This premise, that replication from the *E. coli* origin is inhibited in UV-irradiated cells, was then tested. The difficulty in testing the premise was to differentiate between *trans*-effects on initiation of replication and the *cis*-effects of lesions (which could affect initiation and/or elongation). Therefore, it was decided to study DNA synthesis from the *E. coli* origin of replication, *oriC*, carried by a phage vector. The use of a phage vector made it possible to introduce the unirradiated origin into irradiated cells, thereby avoiding any problems with *cis*-acting inhibition due to UV-induced lesions in the replicon.

UV-induced inhibition of replication from *oriC*: A λ*oriC* clone carrying both *oriC* and *ori*λ (the phage origin of replication) was obtained from L. Soll (Soll, 1980a) and conditions were established which made phage production dependent on initial replication from *oriC*. The *oriC*-dependent phage burst was absent in cells irradiated with 45 J/m². In contrast a phage burst dependent on *ori*λ was not affected under the same conditions. This data indicated that the initial round of replication from *oriC* but not subsequent λ phage production was specifically inhibited in the UV-irradiated cells. Therefore, UV-irradiation of the cells caused a loss in their capacity to host an *oriC*-dependent phage burst from λ*oriC*, in a manner reminiscent of the loss in 186 capacity. This implied that UV induced a *trans*-acting inhibition of replication from *oriC*.

To confirm that replication from *oriC* was indeed inhibited in UV-irradiated cells, λ DNA replication from *oriC* and *ori*λ was studied in unirradiated and UV-irradiated cells. λ DNA synthesis was measured by DNA-DNA hybridisation. In cells irradiated with 45 J/m² of UV, *oriC*-dependent λ replication was unaffected 15 min after irradiation. However, in cells irradiated with 90 J/m² oriC-dependent λ replication was between 2 to 6 times less than in unirradiated cells, 35 min after irradiation. Under the same conditions *ori*λ dependent replication was unaffected. Therefore, replication from *oriC* was specifically inhibited in UV-irradiated cells. It was

demonstrated that the phage-borne *oriC* required *dnaC* and dnaA, just as is required by the chromosomal *oriC*, thus, the behaviour of *oriC* in the λ*oriC* phage reflects the normal behaviour of *oriC*. Therefore it was concluded that UV does induce a *trans*-acting inhibition of replication from the *E. coli* origin.

It is not yet clear why *oriC*-dependent λ DNA replication was not inhibited in cells irradiated with 45 J/m² when the same dose of UV eliminated *oriC*-dependent phage production. Possible reasons for this may be that:

(i) *oriC*-dependent phage production was studied in an excision repair deficient *uvrB* mutant (Δ*H1*λ*c*I857 strain), and the inhibition may be induced at a lower dose of UV in this strain than in the wild type strain. For example, the SOS functions, which are induced at 10-fold lower doses in excision deficient mutants than in wild type cells.

(ii) The duration of the inhibition may be shorter for the lower dose (45 J/m²), so that by 15 min replication may be back to normal. This is not likely, since the DNA was labelled cumulatively and even a short inhibition should be detected as a decrease in the amount of labelled DNA in the irradiated culture as compared with the unirradiated culture.

If it is confirmed that the inhibition of *oriC* in wild type cells is only induced by relatively high doses of UV, it is interesting to speculate that it may be a part of the heat-shock response. This is an extrapolation from the observation of Krueger and Walker (1984) that the heat-shock proteins *groEL* and *dnaK* are induced only by UV doses > 50 J/m^2. However merely transferring the cells to a high temperature (41°C) did not induce an inhibition of DNA synthesis (see Chapter 7) as would be predicted.

Reports in the literature on UV-induced inhibition of DNA synthesis also indirectly support the inhibition of initiation from *oriC*. It has been concluded by some workers that after the UV-induced inhibition in *E. coli* synthesis, the reinitiation of replication occurs synchronously from a site other than that active at the time of irradiation (Hewitt and Billen, 1965; Doudney, 1973b). This site is probably the *E. coli* origin of replication (Billen, 1969). Hewitt and Billen (1965) investigated the reinitiation of *E. coli* chromosomal replication after irradiation. They used a culture uniformly labelled with [^{14}C]thymine and pulse-labelled it with [^{3}H]thymidine for 3 min. After the pulse half the culture was irradiated. The cells were then transferred to bromouracil-substituted medium so that replicating DNA was density-labelled. Samples were taken at intervals and the density-labelled DNA was separated from the light (unsubstituted) DNA on gradients. The amount of ^{14}C- and ^{3}H-labelled DNA in each fraction were measured,

showing the total amount of DNA (^{14}C-) replicated and the amount of pulse-labelled (^{3}H-) DNA replicated after irradiation and transfer to the substituted medium. The percentage of ^{3}H-labelled DNA was plotted versus the total DNA replicated (%). In the control (unirradiated cells) about 50% of the total (^{14}C-labelled) DNA was replicated before a small amount of the ^{3}H-labelled DNA was replicated. This was consistent with the expectation that the replication cycles in progress at the time of pulsing would continue to completion. In the irradiated cells the replication of the pulse-labelled (^{3}H-) region occurred simultaneously with total chromosome replication, after a 20 min delay. This means that the DNA replicated just prior to the irradiation (i.e. ^{3}H-labelled DNA) can be replicated as soon as DNA synthesis resumes. The authors concluded that the reinitiation of replication occurred from a site other than the site that was active prior to exposure, and that protein synthesis was required for this restoration of replication. This would happen if reinitiation during the recovery synthesis occurred from the origin, *oriC*.

Later Billen (1969) concluded that reinitiation after UV-irradiation occurred preferentially at the *E. coli* origin of replicationn. He synchronised a population of cells by starving it of amino-acids. The origin sequences were then labelled with [^{3}H]thymidine for 15-20 min in the presence of amino-acids, so that a new round of replication could initiate. Cells were again starved for amino-acids to

disallow the start of any new replication cycles and the continuing cycle was labelled 40-45 min after initiation with [^{14}C]thymidine, to label its terminus. The cells were then subjected to one of two treatments. Firstly, to show that the chromosomes were differentially-labelled, the cells were again aligned by amino-acid starvation, then transferred to medium containing 5-bromouracil, and the DNA studied by density-gradient centrifugation in CsCl. ^{3}H-labelled (origin) DNA was the first to appear in the hybrid density band and 20 min later ^{14}C-labelled (terminus) DNA began to be replicated. This showed that the chromosomal origin and terminus regions were differentially labelled. Secondly, the cells were irradiated with UV (60 J/m^{2}) immediately after the differential labelling, and the DNA was density-labelled with bromouracil. DNA replication resumed after a delay, with the ^{3}H-labelled DNA (origin) being replicated prior to ^{14}C-labelled DNA (terminus). If replication from the origin was not inhibited then replication could occur and the replication forks would bypass thymine dimers, so that they would have been distributed around the chromosome (out of the ^{3}H-labelled region). At the time of recovery, ^{14}C-labelled terminus DNA would have been replicated prior to or simultaneously with the ^{3}H-labelled origin DNA, but this did not occur. Therefore, it can be inferred that replication from the origin was inhibited for the duration of the delay and resumed subsequently from the origin. The long labelling times for each region and the size of the UV dose,

unfortunately makes it a little difficult to interpret this data conclusively as several forks may be stalled within the ^{3}H-labelled region until the time of recovery. If this happened the resumption of synthesis from these sites would result in ^{3}H-labelled DNA being replicated prior to the ^{14}C-labelled DNA whether or not inhibition of initiation had occurred.

Doudney (1973b) concluded that his findings, on the effect of rifampicin on post-irradiation replication, supported the concept of synchronous initiation of replication of chromosomes in different cells during the recovery phase after UV-irradiation (20 J/m^{2}). Rifampicin is an inhibitor of RNA synthesis, and it also inhibits the initiation of replication from the *E. coli* origin of replication (Messer, 1972). Rifampicin was added to the cultures at various times after UV-irradiation, and DNA synthesis was measured up to 120 or 150 min after irradiation. Rifampicin added 13 min after UV-irradiation of a non-aligned culture, completely blocked recovery of DNA synthesis. If it was added 16 min after irradiation, a 50% (maximum) increase in the amount of DNA occurred. This showed that an initiation event had occurred in at least 50% of the cells within a 3 min period. Addition of the inhibitor between 16 and 50 min did not further increase the amount of DNA before replication stopped, indicating that no new cycles of replication started during this time. However rifampicin added 60-70 min after UV resulted in an increase

in DNA to 100% over the amount of DNA present at the time of UV exposure showing that new rounds of replication initiated between 50-60 min. Therefore, it appears as if an initiation event occurs in the irradiated culture at fixed times. This interpretation is dependent on rifampicin inhibiting reinitiation of replication from the chromosomal origin, as it is known to do in unirradiated cells (Messer, 1972), and not just inhibiting induction of other functions that may be required for recovery DNA synthesis.

Synchronous resumption of replication after UV-irradiation is consistent with the UV-induced inhibition of initiation, since such an inhibition would prevent the start of new cycles of replication during the delay period. During the recovery phase, when the inhibition is released, resynthesis would presumably, start with all the forks aligned at the origin, resulting in a few cycles of synchronous replication. This explanation of the data presupposes that the 20 J/m² dose of UV used by Doudney (1973b) did induce the inhibition of *oriC*-dependent replication under the conditions used. The scenario described above may also explain the requirement for protein synthesis in order that the recovery DNA synthesis may occur (Doudney, 1973a; Kogoma *et al.*, 1979; Hewitt and Billen 1965). The requirement for protein synthesis could reflect the initiation of the new cycle of replication during the recovery phase.

The concept of gap-synthesis, i.e. reinitiation after dimers (Rupp and Howard-Flanders, 1968; Rupp *et al.*, 1971; Ganesan and Smith, 1971) does not preclude inhibition of replication from *oriC* after UV-irradiation. The small amount of replication measured in the UV-irradiated *uvrA* mutant (25% that of the unirradiated control, Rupp and Howard-Flanders, 1968) as short newly synthesised inter-dimer fragments could represent the completion of the cycle in progress at the time of irradiation, without the initiation of new cycles from *oriC*. In the experiments showing gap-synthesis inhibition of *oriC*-dependent replication may not have occurred since most of those experiments were performed in excision-deficient strains irradiated with low doses of UV (< 10 J/m^2). The inhibition of *oriC*-dependent replication may not be induced by this dose.

The presence of an inhibitor of initiation also offers an alternative explanation for two other points raised in the Introduction (Chapter 1):

(i) the induction by high doses of UV of non-photoreactivated damage in cells (Doudney, 1974), and

(ii) DNA synthesis in *uvrA* mutants is not completely inhibited by low doses of UV but continues at a depressed rate.

If the inhibitor of initiation is induced by higher doses of UV and acts in addition to the pyrimidine dimers to inhibit replication, this would explain the occurrence of both photo-reactivated (dimers) and non-photoreactivated inhibition (depression of replication from *oriC*), and it would also explain the absence of inhibition in Uvr- mutants at low doses of UV where the dimers are too few in number to cause a significant delay in synthesis and the *oriC*-inhibition may not be induced.

It is premature to attempt to fit the observed inhibition of replication from *oriC* into a model on DNA replication in a UV-irradiated cell, since a lot more work needs to be performed on the kinetics and dose-dependence of the inhibition. However it is interesting to speculate as to the role of such an inhibition in the UV-irradiated cell. It could be of value to the cells to delay any further initiation until repair is accomplished to prevent attempted replication of gapped DNA and other lesions. Replication forks may also interfere with the repair of lesions. In fact, it has been shown that transient inhibition of replication seems to allow more efficient excision repair (Radman *et al.*, 1970). These workers showed that a λ*cI0ts28* phage mutant (unable to replicate λ DNA at 42°C), had a higher survival in UV-irradiated, wild type and *recA*$^-$ *uvr*$^+$ cells if the phage-host complexes were kept at 42°C for 30-100 min. *uvr*$^-$ mutants, whether *rec*$^+$ or *rec*$^-$ did not show this effect. It was therefore concluded that inactivation

of replication of the phage had resulted in more efficient *uvr*-dependent removal of lesions. Thus in *E. coli* inhibition of new cycles of chromosomal DNA synthesis may allow more efficient repair to occur. Removal of the damage before replication may also result in a lower frequency of targeted mutagensis, which could result in higher survival than would otherwise occur.

Khidhir *et al.* (1985) recently reported that the inhibition in DNA synthesis induced by low doses of UV (10 J/m^2) is not a *recA lexA* dependent function. This contradicts an earlier study by Trgovcevic *et al.* (1980) who concluded that the inhibition in DNA synthesis was *recA*-dependent. However Trgovcevic *et al.*, (1980) irradiated the UV^r and UV^s mutants with different doses to obtain the same survival. Therefore the UV^s *recA recB* mutant was irradiated with a dose 1/5 to 1/15 of the dose used to irradiate the *recB* control strain, whereas Khidhir *et al.* (1985) irradiated both strains with the same dose of UV. As discussed earlier, low doses of UV may be insufficient to induce a complete inhibition in DNA synthesis, as seen in the *uvr* mutants (Ganesan and Smith, 1971; Williams, 1982). Therefore I think the procedure adopted by Khidhir *et al.* (1985) is more correct and that the inhibition of DNA synthesis induced by irradiation with 10 J/m^2 of UV is not an induced SOS function. In light of the work done with 186 it is possible that the inhibition of initiation (*oriC*-dependent replication) is also independent of *recA*, provided

of course that both 186 and *oriC* are inhibited for the same reasons.

Recovery of DNA synthesis after UV-irradiation: Khidir *et al.* (1985) concluded, that the recovery of DNA synthesis after the UV-induced inhibition was dependent on functional *recA*. Earlier (Chapter 1) it was mentioned that stable replication is *recA*-dependent (Kogoma *et al.*, 1979). It may therefore be that the recovery after UV-irradiation occurs via a form of stable-DNA replication. This may be a second reason why recovery synthesis of DNA has a requirement for protein synthesis. Protein synthesis is required just after the inducing signal for the induction of both SOS functions and induced-stable replication (Kogoma *et al.*, 1979). This is consistent with Doudney's (1973a, 1978) finding that chloramphenicol (a protein synthesis inhibitor) added immediately after UV-irradiation inhibits recovery, but if added just 35-40 min after irradiation, it does not decrease the amount of initial recovery synthesis. Thus the first cycle of recovery DNA synthesis after UV-irradiation may occur by an induced novel form of replication which does not need protein synthesis (Kogoma *et al.*, 1979; Doudney, 1978) or *dnaA* (Jonczyk and Ciesla, 1979), and is *recA*-dependent (Khidir *et al.*, 1985).

Future Work: Several aspects of the inhibition of *oriC*-dependent replication need to be studied further before a model for the inhibition of replication from *oriC* can be proposed.

(i) The onset and duration of the inhibition of replication from *oriC* after irradiation with different doses of UV needs to be examined.

(ii) It would be of interest to determine whether the inhibition of *oriC*-dependent replication or the recovery from this inhibition is a *recA*-dependent SOS function especially in view of the findings of Khidhir *et al.* (1985) discussed earlier. If the inhibition of *oriC* is found to be a function restricted to larger doses of UV (>50 J/m²) it may well be caused by an SOS controlled gene which is tightly regulated by the *lexA* repressor and is induced only by large doses of UV. With regard to this, the *recA* protein is produced maximally at doses >50 and <100 J/m² in wild type cells (Salles and Paoletti, 1983), i.e. the *recA* promoter is maximally derepressed and (by inference) the *lexA* protein completely inactivated only at doses >50 J/m².

(iii) A pseudo-virulent mutant of λ*oriC* phage called λ*oriC*^{c} (*oriC* constitutive) was isolated by Soll (1980b) and was found to be capable of forming plaques on a λ*cI*$^{+}$ lysogen. Replication was not from *ori*λ (Soll, 1980), so that it must have been occurring from *oriC*^{c}. Therefore, it appears that replication from *oriC*^{c} is free from copy number control so that sufficient replication occurs from the *oriC*^{c} origin to allow phage production and plaque formation, even in the presence of λ*cI* repressor. This phage carries two

mutations, one maps within the *oriC* region and the other in the left operator of λ (Bradley, 1983). Both mutations are necessary for the pseudo-virulence phenotype (Bradley, 1983), but the role of the mutation in the left operator of λ is not indicated. Williams (1982) reported a preliminary finding that *oriC*^{c}-dependent plaque formation from λ*oriC*^{c} was not inhibited by 45 J/m². So that the *oriC*^{c} mutation may make the origin insensitive to the UV-induced inhibition, as well as copy number control. However, as mentioned earlier, *oriC*-dependent replication of λ*oriC* was not inhibited by irradiation with 45 J/m² of UV, except in a *uvr* mutant. Therefore, the *oriC*^{c}-dependent phage production and replication need to be examined in cells irradiated with larger doses of UV in order to confirm that *oriC*^{c} is not inhibited in irradiated cells. If replication from *oriC*^{c} is indeed unaffected by UV-irradiation, then identification of the *oriC*^{c} mutation could identify either the inhibitor itself or the site of action of the UV-induced inhibition on the wild type origin *oriC*.

(iv) Identification of the function causing the inhibition of *oriC*-dependent replication: This function could be essential to the cell if it is involved in normal cell cycle control. If this were so, it would not be possible to mutate it in a normal cell. Instead an *E. coli* mutant in which *oriC* is deleted (Δ*oriC*) (von Meyenburg and Hansen, 1980), and replication occurs from Hfr or P2*sig*5 DNA inserted into the host chromosome (integrative suppression),

could be used. Such a mutant is independent of *oriC* so that functions controlling *oriC* are nonessential. *din* (damage-inducible, Kenyon and Walker, 1980) functions could then be isolated in these strains by random insertions of the Mud*lac* phage (Casabden and Cohen, 1979) (*din* mutants isolated previously were derived from *oriC*-requiring strains, so that mutations in essential genes have not been identified). These mutants can then be tested for the ability to exhibit UV-induced inhibition of *oriC*-dependent replication. If the function is shown previously to be independent of the SOS regulon, the background of SOS *din* functions can be removed by introducing a *lexA*(Def) mutation into the Δ*oriC* strain. This would render all the LexA repressed *din* genes constitutive and any further damage-inducible (*din*) functions would be independent of the SOS regulon.

Additional observations: During the course of this study some interesting but unrelated observations were made.

The first concerns the interference between 186 and λ phage. It was found that cells actively replicating 186 phage were depressed in their ability to produce λ phage. This was not due to 186-dependent lysis of the cells before λ packaging, since a 186 phage which was mutant in the ability to express late functions (head, tail and lysis genes), also interfered with the cells ability to produce λ phage. The cause of this interference was not determined.

Another finding was that the AB1157 strain has two cultivars. Isolates of the strain from the east (ABE) and west (ABW) coasts of the U.S.A. differ in their capacity to produce 186 phage after UV-irradiation. The strains also differ in their ability to host a 186 infection under two separate conditions not involving UV-irradiation. Firstly, in LB broth treated with activated charcoal, ABE did not produce a 186 burst but ABW did, and secondly a 186 *tum*9 mutant (non-inducible) by UV and mitomycin C) did not produce a burst in the ABE strain but was capable of doing so in ABW.

The ABE and ABW cultivars were identical in the genetic markers tested. They are also very similar in their response to UV. It would be of interest to locate the marker causing the difference between the two cultivars, but this has not yet been done.

This finding means that mutants derived from strain AB1157 cannot be treated as isogenic strains unless the parental cultivar from which they were derived is known. The significance of this finding lies in the fact that AB1157 and it's mutant derivatives are often used as isogenic strains in the study of the effects of UV on *E. coli* and this could result in misleading conclusions.

While developing a system to study replication of λ*oriC* from *oriC*, a plasmid separation technique was used to isolate labelled λ*oriC* and λ DNA from *E. coli* chromosomal DNA. The isolated DNA was electrophoresed on agarose gels and stained with ethidium bromide. The gel was subsequently photographed and fluorographed. The plasmid DNA was clearly visible and usually some contaminating chromosomal DNA was also present. If however the cells were irradiated prior to infection with λ, the amount of DNA extracted in both the plasmid and chromosomal bands were reduced. This reduction occurred whether or not the DNA was replicating in the irradiated cells. A previous study (Smith, 1962) reported the loss of extractability of DNA from UV-irradiated cells. This was observed as a decrease in the amount of DNA extracted using detergent, from UV-irradiated compared with unirradiated cells. Smith (1962) concluded that the effect was caused by UV-induced cross-links between cellular proteins and DNA. Alexander and Moroson (1962) made a similar observation. The situation I observed was a little different, in that unirradiated DNA introduced into UV-irradiated cells was being retained by the denatured mass created by detergent treatment. It is not known whether this was due to UV-induced protein-DNA cross-links. Protease K treatment was not attempted to free the plasmid DNA as it was anticipated that this would result in unacceptably high levels of chromosomal contaminationon. Therefore an alternative method was used to study *oriC*-dependent replication (Section 7.4).

Conclusion: The UV-induced loss in 186 capacity initiated an investigation into the possibility that UV-irradiation induced an inhibition of the initiation of *E. coli* replication in the absence of photoproducts. It was found that UV does induce a *trans*-acting inhibition of replication from *oriC* carried by an unirradiated replicon. The nature of this inhibition is not known, nor is it known whether it is also the reason for the inhibition of 186 synthesis. Future experiments will be directed towards characterizing the inhibition against initiation from the *E. coli* origin generated by UV-irradiation.

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