AN INVESTIGATION OF FACTORS DETERMINING P1 TRANSDUCTION FREQUENCIES

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A B S T R A C T

The efficiency of recovery of P1 transductants is marker-dependent, and normally varies over a 25-fold range. UV irradiation of either transducing lysates, or recipient cells, results in a selective stimulation of the transduction of markers which are normally transduced poorly. As a result, the range in frequencies of transduction is reduced to about 3-fold, and resembles the gene frequency distribution expected in the donor cells. From these results, it was concluded that P1 packages donor DNA randomly, and the recombination system of the recipient selectively recombines certain regions of DNA. To confirm that P1 packages DNA randomly, restriction fragments of E.coli were used to probe to P1 and to E.coli DNA. It was found that the ratios of markers was approximately the same in both P1 and E.coli DNA, thus substantiating the conclusion that P1 packages DNA randomly.

Possible reasons for selective recombination were examined. Recipients with abnormal chromosome folding (gyr$^-$ mutants), a factor which may influence accessibility of the chromosome to recombination enzymes, showed greatly decreased recovery of transductants, although ratios were similar to wild type. Recipients lacking the RecBC pathway of recombination, and derepressed for the RecF pathway, were found to be better recipients for poorly-transduced markers, although no better for normally well-transduced markers. The role of the nucleases ExoI and ExoV, and of chi sites, in preferential integration of some markers is discussed.

D E C L A R A T I O N

This thesis was composed entirely by myself, and, except for the following experiments, all work was carried out unaided. The construction of the 'RecF' and associated strains, was done by Dr.M.Masters, and the transductions using these strains were performed by Christine Henry. I am indepted to both for their contribution.

The work described in chapter 3 was submitted as a paper to Molecular and General Genetics, and is included as an appendix.

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LIST OF ABBREVIATIONS

Ap^R	ampicillin resistant
ATP	adenosine 5'triphosphate
C	degrees centigrade
cm	centimetre
dATP	deoxyadenosine 5' triphosphate
dCTP	deoxycytidine 5' triphosphate
dGTP	deoxyguanosine 5' triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetra-acetic acid
ExoI	exonuclease I
ExoV	exonuclease V
g	grammes
kb	kilobase pairs
kD	kilodaltons
l	litre
M	molar
mA	milliampere
Md	megadalton
mg	milligramme
min(s)	minute(s)
mm	millimetre
mM	millimolar
moi	multiplicity of infection
nal^R	nalidixic acid resistant
ng	nanogramme
nm	nanometre

OD	optical density
pfu	plaque-forming units
phage	bacteriophage
RNase	ribonuclease
rpm	revolutions per minute
s, sec	second
SDS	sodium dodecyl sulphate; $NaC_{12}H_{26}SO_4$
Sm^R	streptomycin resistant
SSC	saline sodium citrate
t	time
TCA	trichloracetic acid
uCi	micro-Curie
ug	microgramme
ul	microlitre
uM	micromolar
UV	ultraviolet
V	volts
w/v	weight/volume

CHAPTER ONE

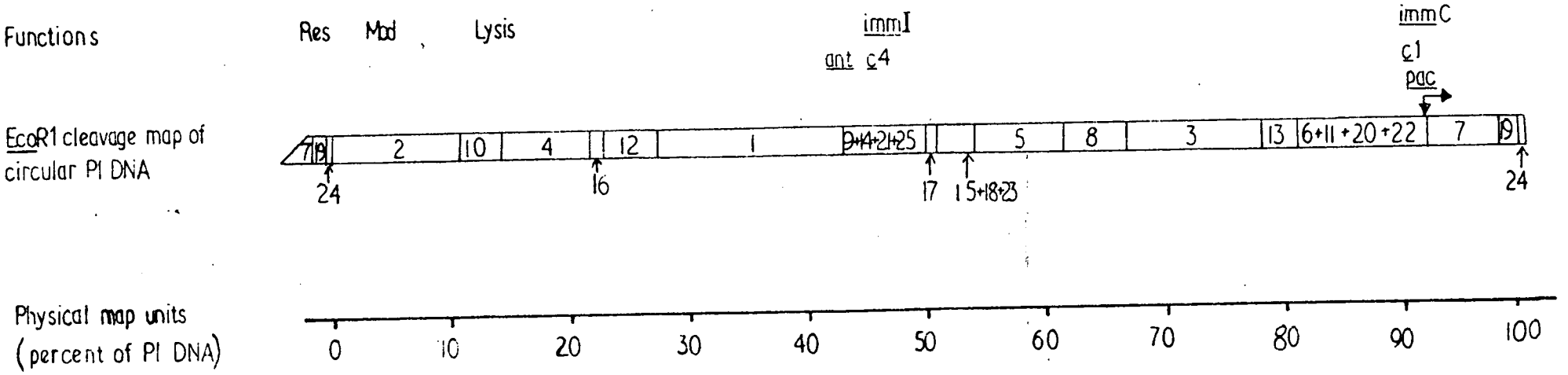
INTRODUCTION

The bacteriophage P1 has proved to be an invaluable tool in *E.coli* genetics, both in strain construction and in gene mapping (Bachmann and Low, 1980) due to its capacity to transmit pieces of host chromosome as a normal part of its life cycle. This process, known as generalised transduction, has been used for many years in the study of *E.coli* genetics (Lennox, 1955; Ikeda and Tomizawa, 1965), but relatively little is known about the process of transduction. In this work, some aspects of the process of transduction are investigated, with a particular focus on events in the recipient cell. The aim of this introduction is firstly to review the present state of knowledge of P1 transduction, and secondly, to outline the processes of recombination and repair in *E.coli* which have a bearing on P1 transduction.

1·1 Physical properties of bacteriophage P1.

P1 is a temperate phage, but whereas most temperate phages in *E.coli* K12 are known to have specific sites for integration on the bacterial chromosome, the P1 prophage exists as an extra-chromosomal element (Ikeda and Tomizawa, 1968) which is maintained as a closed-circular, single-copy plasmid with autonomous replication under stringent control. A complex genetic control maintains the prophage state and prevents superinfection (Scott, 1980).

Figure 1.1



Physical and genetic map of bacteriophage P1.

Important functions and genes referred to in the text only are shown. The fragments generated by EcoR1 cleavage are numbered according to size (1 being the largest). Where several numbers are grouped together, the order of the fragments has not been determined.

The arrows at the right-hand end indicate the origin (pac) and direction of packaging of phage DNA.

The products of two genes, c1 and c4, that are located in separate immC and immI regions of the phage genetic map (fig 1·1) are required for maintenance of the prophage state. c1 represses the viral lytic functions but is not responsible for superinfection immunity, while c4 represses a second immI gene, ant which antagonises c1 mediated repression (Sternberg et al, 1978). There are at least ten other genes involved in control of lysogeny, but as yet, a comprehensive model for P1 lysogeny has not been formulated. Control of replication is precise, maintaining one copy of the plasmid in each cell; prophage loss occurs at a frequency of about 10^{-4} per cell generation (J.L.Rosner, 1972). Wild-type P1 lysogens cannot easily be induced into the lytic cycle in contrast to λ which is often considered to be a typical temperate phage and which is readily induced by treatment of a lysogen with UV and other mutagens. Rare events do occur which result in the breakdown of prophage repression and the expression of viral lytic functions leading to cell death and release of infective particles; thus a culture of a P1 lysogen always contains some free phage particles.

P1 particles contain a single molecule of double stranded infective DNA with a molecular weight of 66×10^6. (Ikeda and Tomizawa, 1965); the infective DNA forms a population of circularly permuted molecules with 9-10% terminal redundancy (Yarmolinsky, 1977). P1 particles adsorb to sensitive recipient cells, in the presence of calcium ions, and inject the infective DNA. On entering

the cell, if lysogeny is to ensue, the DNA immediately circularises, either by RecA-dependent homologous recombination between the redundant ends, or, in *recA*$^-$ cells, by a site-specific recombination event which generates circular molecules at low frequency (Sternberg, 1979b see also section 1·4 below). In either case, the result is a circular prophage molecule of genome size, $58{\cdot}6 \times 10^6$ (Bachi and Arber, 1977). If lysogeny is not established, a lytic infection occurs; phage DNA and proteins are synthesised, host DNA may be degraded (Wall and Harriman, 1974), and finally infective particles are released from the lysed cell.

In addition to infective, plaque-forming particles, a P1 lysate contains a small percentage of transducing particles. Estimates of this proportion vary from 0·3% of the total for a virulent derivative (Ikeda and Tomizawa, 1965) to 2% for a high transducing strain (Sandri and Berger, 1980a). These transducing particles contain no infective P1 DNA, but instead carry a segment of host DNA of equivalent size. When these particles infect a recipient cell, this transducing DNA may become integrated into the recipient chromosome thus giving rise to transductants. This process is described more fully below.

1·2 Packaging of DNA into phage heads.

P1 infective DNA is packaged by a headful mechanism from concatamers synthesised by the rolling circle model (Gilbert and Dressler, 1968). From a restriction analysis

which compared P1 infective and prophage DNA, Bachi and Arber (1977) concluded that packaging is initiated at a site near 92 physical map units, termed pac (Sternberg et al., 1980), then proceeds to the right. This implies some specificity in the enzyme responsible for nicking the DNA prior to packaging.

It is thought that P1 transducing particles arise from the mistaken packaging of host chromosomal DNA during the lytic phase of development. It can be hypothesised that recognition of a similar site to pac on the host DNA will lead to inclusion of bacterial DNA in some phage heads. Harriman (1972) found from a single-burst analysis of P1 that only a fraction of infected cells produced transducing as well as infective particles. However, in individual cells, he found by observing transduction of prophage markers that the chance that two markers which are too far apart to be co-transduced will be packaged in the same cell increases with the proximity of the markers. He hypothesised that infected cells may contain several regions of localised packaging, and that P1 requires a free end to initiate packaging. He suggested that the majority of infected cells would have an intact circular chromosome, and only the few cells whose chromosomes were degraded would provide start sites for encapsidation. In other words, in a few cells, the P1 enzyme responsible for cutting P1 DNA at the pac site, may recognise a similar site on the host chromosome and DNA is then packaged sequentially from this site.

P22, a temperate phage whose host is Salmonella, has been studied in detail by several workers (for review see Susskind and Botstein, 1976). It is capable of generalised transduction, and its mode of transduction may be comparable with that of P1. There is evidence that P22 packages host DNA from certain preferred sites to generate transducing fragments of fairly constant composition (Ozeki, 1959; Pearce and Stocker, 1965). Chelala and Margolin (1974) found that a deletion in the chromosome of a donor for P22 transduction can alter the co-transduction frequencies of a pair of markers located wholly to one side of the deletion even if both donor and recipient carry the deletion, and even if the deletion is not included in the same headful of DNA as the selected markers. They hypothesised that transducing DNA fragments are formed by sequential encapsidation from a small number of preferred starting points on the host chromosome. Schmeiger (1972) isolated mutants of P22 which were altered in their ability to transduce Salmonella DNA. Some mutants (high transducing, or HT) packaged 50% host DNA, compared with 1-5% packaged by the wild type, indicating that some phage functions are responsible for the choice as to whether phage or host DNA is packaged in phage particles. He hypothesised that there was a decrease in the specificity of an enzyme responsible for cutting the DNA prior to packaging. This was supported by the finding that the map position of the mutation responsible for the HT phenotype was

identical with the position of gene 3 on the P22 map (Raj et al., 1974) which seems to be involved in fragmentation of the phage DNA concatamers and possibly codes for the endonuclease necessary for cutting (Botstein et al., 1973).

Transduction mutants of P1 have also been isolated. Wall and Harriman, (1974) isolated classes of mutants with a tenfold increase in transduction (HTF mutants) and a tenfold decrease in transduction (LTF mutants). Their selection technique was based upon the transduction of prophage which does not have to be integrated to be expressed, and thus these mutants were most likely to be altered in the ability to package donor DNA into transducing particles. Burst sizes of some of the mutants did differ from the wild-type, but the differences were not large enough to explain the altered transducing frequencies by altered infective centre production alone. Infection of the recipient by one of the HFT mutants led to an accelerated breakdown of the donor chromosome, supporting Harriman's (1972) hypothesis that packaging of P1 transducing DNA was initiated from free ends of chromosomal DNA.

1.3 P1 transduction frequencies.

If P1 transduction resulted from random packaging of host DNA followed by the integration of a random selection of P1 transducing fragments into the recipient chromosome, typical transduction frequencies for each

Figure 1•2 Transduction frequencies as a function of map position.

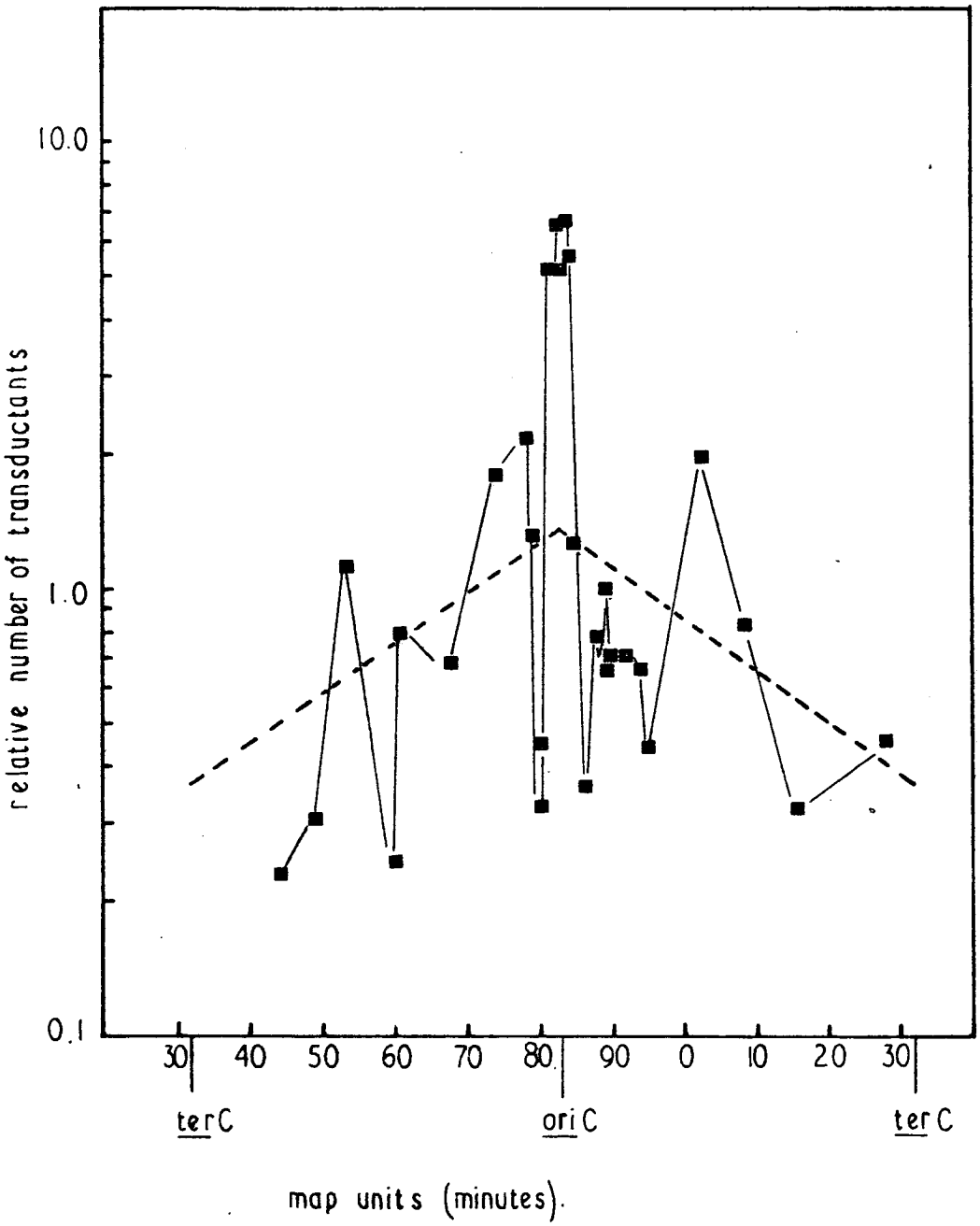
Squares represent transduction frequencies determined by Masters (1977), and are plotted relative to map positions from Bachmann and Low (1980).

Superimposed (broken line) is the gene frequency distribution of an exponential culture similar to those used for frequency determinations.

oriC = origin of replication

terC = terminus of replication

Figure 1.2


10.0
1.0
0.1
relative number of transductants
30
40
50
60
70
80
90
0
10
20
30
terC
oriC
terC
map units (minutes).

marker according to its position on the E.coli map would be expected. Exponentially growing donor cells have two or more replication forks (the number of replication forks is a function of growth rate; Cooper and Helmstetter, 1968) and thus there will be more copies of markers near the origin of replication than of markers near the terminus. A P1 lysate prepared from a culture in the exponential phase of growth, in rich medium, might be expected to yield a ratio of 4:1 origin:terminus markers due to this gene dosage effect. However, observations on the actual frequencies of transduction show much greater differences between markers than would be expected from a gene dosage effect alone (Masters and Broda, 1971; Masters, 1977). Instead of the expected 4-fold difference between origin and terminus markers, there is a 30-fold difference observed between the frequencies of transduction of rbs located near the origin at 83·0 minutes, and his, located near the terminus, at 44·1 minutes on the E.coli genetic map (Bachmann and Low, 1980). The high frequency of transduction of markers in the 2 minute region spanning the origin is particularly noticable, and is accentuated by the very low frequency of transduction of markers flanking this region; for example, pyrE, at 80·7 minutes is transduced with a frequency of only 5% of the frequency of transduction of rbs. Figure 1·2 (Masters, 1977) shows a plot of transduction frequencies of individual markers as a function of map position, and superimposed upon this is a plot of gene frequency

Figure 1·3 Gene clustering on the E.coli chromosome: co-incidence of high-transducing markers with gene-dense regions.

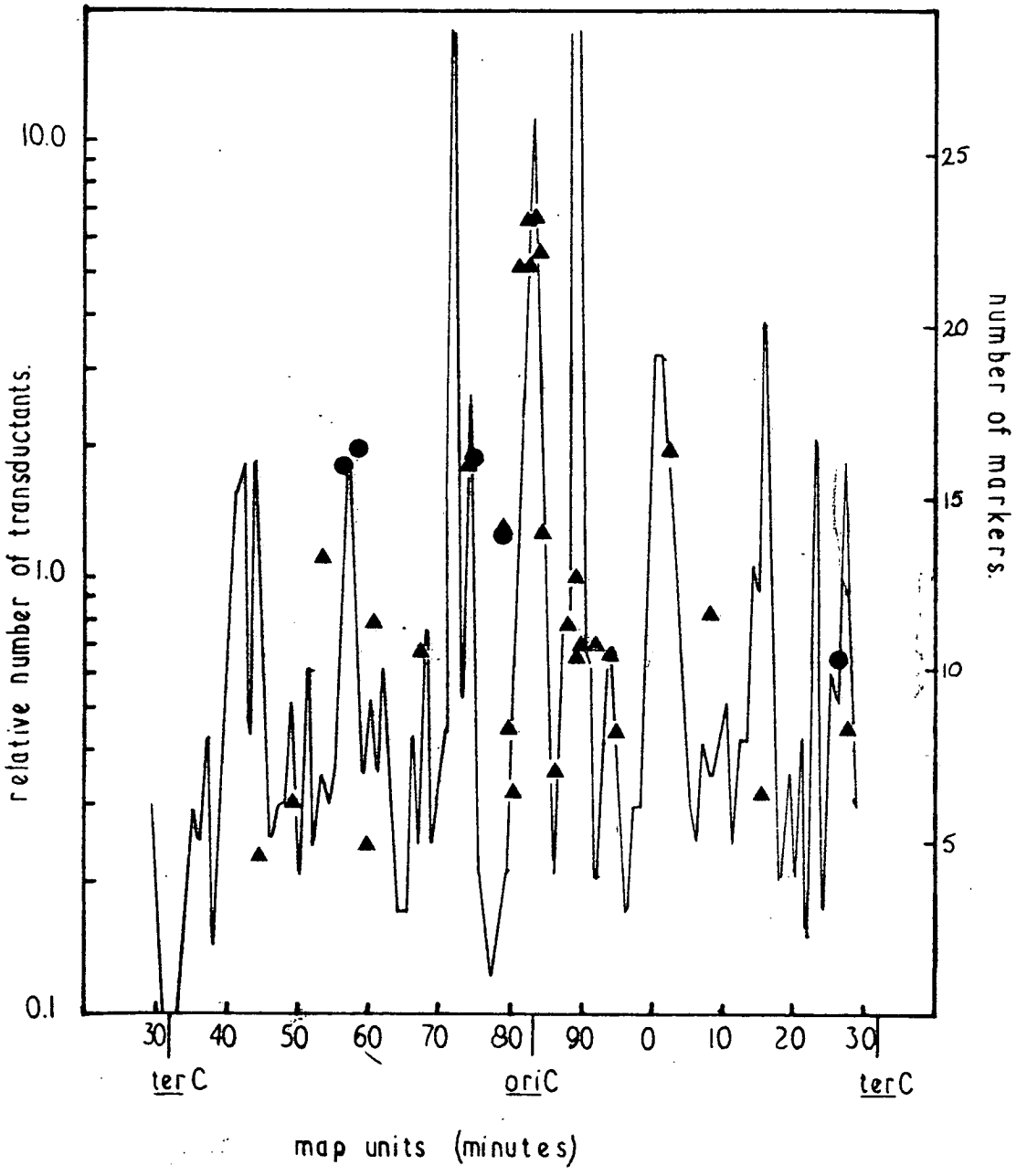
The line joins points representing the number of markers in each minute interval of the chromosome (from Bachmann and Low, 1980). The peaks flanking the origin peak are clusters of ribosomal genes.

Superimposed are frequencies of transduction of individual markers. Triangles are from Masters (1977), and circles from Masters and Broda (1971).

oriC = origin of replication

terC = terminus of replication

Figure 1.3


10.0
1.0
0.1
relative number of transductants.
25
20
15
10
5
number of markers.
30
40
50
60
70
80
90
0
10
20
30
terC
oriC
terC
map units (minutes)

distribution of an exponential culture similar to those used to prepare the phage lysates for frequency determinations. Masters (1977) pointed out a correlation between the frequency of transduction of a marker and the density of genes in the area of the marker. Distribution of genes around the E.coli chromosome is not even; a map of all known genes on the E.coli chromosome (Bachmann and Low, 1980) shows a marked clustering of genes interspersed with relatively gene-sparse regions. A plot of frequency of transduction as a function of map position, and showing gene density, is displayed in figure 1·3, and it can be seen that markers within the gene clusters tend to be transduced at a higher frequency than would be expected from their map position, whereas markers in gene sparse regions tend to be transduced at low frequency. This indicates that there is perhaps some property of these better transduced, gene-dense regions, which makes them more easily packagable by P1, or increases the frequency with which transducing fragments recombine with these regions of the recipient chromosome.

1·4 P1 site-specific recombination.

Sternberg (1979a) demonstrated that P1 has a highly efficient site-specific recombination system encoded by P1 EcoR1 fragment 7, which spans the ends of the genetic map (figure 1·1). By cloning this fragment into a recombination-deficient λ, and by performing phage crosses he found that this recombination was recA and recBC

independent. Not only was the recombination site (*loxP*) located on this fragment, but also on the fragment was a gene encoding the presumptive recombinase function (*cre*), which acted *in trans* to promote recombination. The *loxP* site allows circularisation of P1 infective DNA carrying two *loxP* sites in a *recA*$^-$ background, and may have a role in plasmid segregation (Austin *et al.*, 1981).

Normally, P1 plasmid integrates into the bacterial chromosome of a *recA*$^-$ strain with low effieiency, estimated at about one in every 10^5 cells. However, Chesney *et al.*, (1979) found that P1 could integratively suppress a *dnaA*^{ts} *recA*$^-$ strain by recombination between a site near the ends of the P1 genetic map and a preferred site on the *E.coli* chromosome. Sternberg *et al.* (1980) also observed preferred integration at one site, and sequenced the *loxP* site, and also part of the *E.coli* sequence surrounding an integrated prophage, the *loxB* site its preferred site of integration. They found a 13 base pair perfect inverted repeat hyphenated by an 8 base pair region in the former, and a 10 base pair repeat hyphenated by a 5 base pair region in the latter. There was a striking degree of homology between the two sites, suggesting that perhaps *loxB* is a degenerate *loxP* site left in the bacterial chromosome by a once-integrated P1. Sternberg proposed that integration of the P1 prophage into the bacterial chromosome via site-specific recombination could be responsible for the production of P1 transducing phage. Such an integration event during

the lytic cycle of phage growth would position a normal P1 packaging site (*pac*) adjacent to bacterial sequences. P1 processive headful packaging from this *pac* site would result in the packaging of bacterial DNA into a phage head, and the production of generalised transducing phages. Sternberg *et al.*(1980) demonstrated that if P1 infects a *recA*$^-$ strain in which a hybrid λ phage containing the *pac* site (λP17) is integrated at its preferred site in one orientation, the resulting P1 lysate transduces *tolC*, *metC* and *serA* markers 50 times more efficiently than can a lysate made on a *recA*$^-$ strain without the integrated prophage. Inserted in the opposite orientation, the integrated prophage enhanced transduction of *dnaG* and *argG*, clearly demonstrating unidirectional packaging from *pac*. Thus the integration site for the λP17 prophage lies on the *E.coli* chromosome between *tolC* at 66' and *dnaG* at 67'. It is thus plausible that P1 enzymes recognise sites similar to *pac* on the host chromosome, and that packaging starts preferentially at these sites.

1·5 Fate of transducing DNA in the recipient cells.

When phage DNA enters the cell in order to produce a successful infection, it must resist attack by the host nucleases. The *recBC* product, exonuclease V (Exo V), efficiently degrades linear, double stranded DNA (Goldmark and Linn, 1972) but some forms of DNA are completely resistant. Double stranded circles are not attacked even if they contain single-stranded nicks or gaps of up to

5 nucleotides in length. Different bacteriophages have developed various strategies to avoid degradation, for example, λ circularises on entry into the cell via its cohesive ends to generate a nuclease-resistant double-strand circle (Hershey and Burgi, 1965). Other phage DNAs are linear and do not have the capacity to circularise, and in these cases the ends may be complexed with protein, since transfection of cells with phenol-extracted DNA results in a very low frequency of productive infection (Benzinger, Enquist and Skalka, 1975). P1 infective DNA circularises either by homologous, recA-mediated, or site-specific recombination.

P1 transducing DNA presumably cannot circularise by this method, since it includes no P1 virion DNA, and the chromosomal fragments are unlikely to be able to circularise. However, transducing DNA must be protected from attack to a certain degree, since the recovery of tranductants is many-fold higher than the recovery of recombinants after transformation with linear DNA which presumably is rapidly degraded. Transducing DNA can also persist in cells without recombining into the chromosome for several generations, and, in fact, most transducing DNA fails to recombine to form stable transductants. Cells carrying these extra-chromosomal fragments are known as abortive transductants (Ozeki, 1956; Gross and Englesberg, 1959), and can be detected by their ability to form minute colonies on selective plates. The DNA is expressed, but cannot replicate and is thus inherited unilinearly. The DNA has

apparently escaped degradation by host nucleases which suggests that its structure is modified to a form resistant to ExoV attack.

Ikeda and Tomizawa (1965) showed that there was a protein associated with P1 transducing DNA. Sandri and Berger (1980b) showed that abortively transduced DNA migrated on agarose gels at the same speed as circular P1 prophage DNA; treatment with pronase caused the abortively transduced DNA to migrate at the same speed as linear P1 DNA on agarose gels. This strongly suggests that the abortively transduced DNA circularises with the aid of a protein which renders it resistant to attack by ExoV. This circularisation apparently occurs in the recipient cell, since transducing DNA extracted from phage particles by a non-proteolytic method is linear.

Once formed, the complex of DNA and protein is stable, and does not subsequently recombine with the chromosome at a significant rate; it has been estimated that the frequency of conversion of abortive to stable transductants is as low as 10^{-3} per generation (Ozeki and Ikeda, 1968). The mechanism of this circularisation, and its purpose, is unclear.

The exact mechanism by which P1 transducing DNA recombines with the recipient chromosome had not been elucidated. It is not known whether the integrated DNA circularises and then integrates, or fails to circularise. It is clear that it is dependent upon RecA, and is thus certainly a homologous recombination between donor and

recipient DNA. Possible factors controlling this recombination will be discussed below (1·7, 1·8, 1·9).

Ebel-Tsipis *et al.* (1972) showed that only 2-5% of the total DNA in P22 generalised transducing particles becomes integrated into the host chromosome, the rest forming abortive transductants in a similar manner to P1. Also, they showed that the successfully transduced DNA becomes integrated into the host chromosome as substantial segments of conserved duplex DNA. They estimated the molecular weight of these pieces to be 2×10^6 to 10×10^6, and concluded that integration involves double strand breakage and joining of DNA. In a similar study on P1, Sandri and Berger (1980a) showed that 7-15% of total transducing DNA integrated into the chromosome. The DNA was again found to integrate as a duplex, but the molecular weight of the insert was estimated to be about $5\text{-}10 \times 10^6$, or about one tenth to one sixth of the size of the tranducing fragment. Integration had occurred within one hour after adsorption, and the integrated DNA was replicated by 180'.

These experiments give no indication of the pathway leading to integration of transducing DNA. This is presumably controlled by host functions, and some mutants altered in the ability to integrate transducing DNA have been isolated. Stacey and Oliver (1977) characterised a mutation, *tdi*, which appeared to confer defects in a number of functions including an inability to complete the process of P1 transduction. The *tdi* mutant was a

normal host for P1, since P1 lysates could be made on the strain, and it could by lysogenised by P1, but it could not be transduced for auxotrophic markers, nor could transducing particles be recovered from its lysates. That the transducing DNA was entering the cell, and was not being degraded, was demonstrated by the recovery of Gal^+ transductants after infection of a λ-resistant derivative of the *tdi*$^-$ mutant with a lysate made on a strain carrying the defective prophage λ*dgal*. In this case, the block is circumvented by the λ-coded integrase. Integration of F DNA into the mutant chromosome was normal, and mobilisation was possible, but the mutant could only be transformed at a very much lower frequency than the parental strain. Stacey and Oliver proposed that there could be an additional mechanism required to bring double-stranded DNA into a recombinational pathway, and that this step is defined by the *tdi* mutation. However, the *tdi* mutant is slower growing than the parental strain, and has a lower mutation rate, and thus the *tdi* mutation could have a more general effect on *recA* mediated homologous recombination. The *tdi* mutation has not yet been mapped, and is possibly an allele of one of the known recombination genes. The authors do not comment upon the effect of the mutation on abortive transductants; it is possible that it is involved in the circularisation of transducing DNA, and lack of the function results in degradation of transducing DNA and decreased recovery of abortive transductants.

The presence of certain plasmids in the recipient cell

affects transduction. Oliver and Stacey (1977) showed that the presence of R46 in the recipient greatly reduced the frequency both of P1 transduction and of recombinant formation after conjugation, whilst conferring a slight increase in survival after UV irradiation. Walker (1978) observed a 50% reduction in the recovery of P1 transductants in strains carrying pKM101 or R46. Again, the exact nature of this effect has not been elucidated.

1·6 Three-dimensional structure of the E.coli chromosome.

The E.coli chromosome is about 1 mm in length, and this is packed into a cell of about 1 um in diameter, thus the chromosome must be folded into an organised structure in order to fit into the cell, and yet be accessible for transcription and replication enzymes. Worcel and Burgi (1972) proposed a model for the folded chromosome of E.coli. The chromosome contains negative superhelices in folded E.coli DNA, (extracted by gentle lysis) by measuring its sedimentation coefficient in increasing concentrations of ethidium bromide. Their estimate was consistent with the concentration of superhelices in other naturally-occurring closed-circular DNA molecules. By treating folded E.coli chromosomes with DNase they found that a surprisingly high concentration of DNase was needed to relax the chromosome completely suggesting that more than one nick was necessary; they estimated that 6-40 nicks were required to relax the chromosome completely. Thus it appears that the E.coli chromosome is organised in a series of loops, and a single strand nick in one loop will relax

the superhelicity within that loop without affecting the the superhelix concentration in the rest of the chromosome. Worcel and Burgi proposed that the chromosome was folded into about 50 independent loops stabilised by interaction with a core, probably RNA, since the folded chromosome is resistant to protease treatment, but unfolds in the presence of RNase.

It could be envisaged that regions of the chromosome on the outside of these loops are more accessible to enzymes involved in phage packaging or in recombination; Worcel and Burgi (1972) deduced from the action of DNase that perhaps some stretches of the chromosome were more accessible to DNase. These areas of the chromosome might be expected to carry more coding information since frequently transcribed genes may need to be readily accessible to the transcription machinery. This could be a possible explanation for the clustering of genes around the E.coli map, gene sparse regions may be points of attachment of the E.coli chromosome to the central core, and gene clusters could be on extended loops.

The negative supercoils in all naturally occurring closed circular DNA (Cozzarelli, 1980) points to their fundamental necessity. DNA gyrase of E.coli (Gellert et al., 1976a) introduces negative supercoils into covalently closed duplex DNA. The energy required by DNA gyrase to drive the DNA into the negatively supercoiled form is provided by ATP, and this increases the free energy of the DNA. This free energy promotes the binding of any ligand which unwinds the helix removing the superhelical turns, and

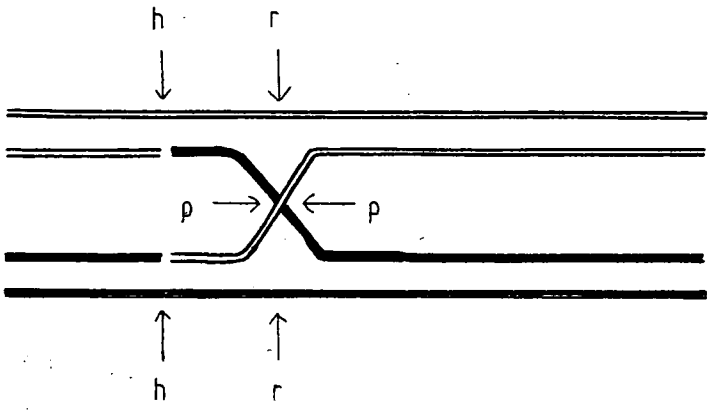
thus predisposes the molecule towards unwinding of the duplex. Botcham *et al*. (1973) found that negative superhelicity increases initiation of RNA synthesis by *E.coli* RNA polymerase *in vitro* probably by facilitating helix unwinding at the promoter site. Superhelical DNA is also required for the initial stages in recombination (see section 1·7 below) and may facilitate unwinding of the helix to expose bases for homologous pairing.

1·7 General recombination in E.coli.

General genetic recombination, which takes place between homologous DNA, accounts for most of the essential recombination in the *E.coli* cell. The major pathway for general recombination in *E.coli* is the RecBC pathway which requires functional products of the three genes *recA*, *recB* and *recC*. Other pathways for recombination, for example RecF (see section 1·8) normally play a minor role in general recombination, but also have a requirement for a functional *recA* gene. Mutations in *recB* or *recC* result in severely reduced viability, and reduced recombination frequencies in Hfr x F^- crosses, and in P1 transduction (K.Haefner, 1968), while mutations in *recA* result in a wide variety of pleiotropic effects including deficiency in general recombination, increased sensitivity to radiation, failure of UV-induction of prophage λ, absence of mutability and inability to co-ordinate cell division with DNA synthesis (Clark and Margulies, 1965; Clark, 1973).

The process of homologous recombination involves transfer of homologous regions of single-stranded DNA and

Figure 1.4



The Holliday model for genetic recombination.

(after Meselson and Radding, 1975)

1. Single-strand breaks are made at chemically identical sites on homologous duplexes.
2. Heteroduplex DNA is then formed symmetrically between h and r on both duplexes.
3. The two pairs of like strands at the site of the crossover are considered to be equivalent with respect to recognition by a DNase which terminates the exchange.
4. Cleavages at p produce two molecules with the flanking arms in the parental configuration, whereas cleavage at r produce molecules with flanking arms in the recombinant configuration.

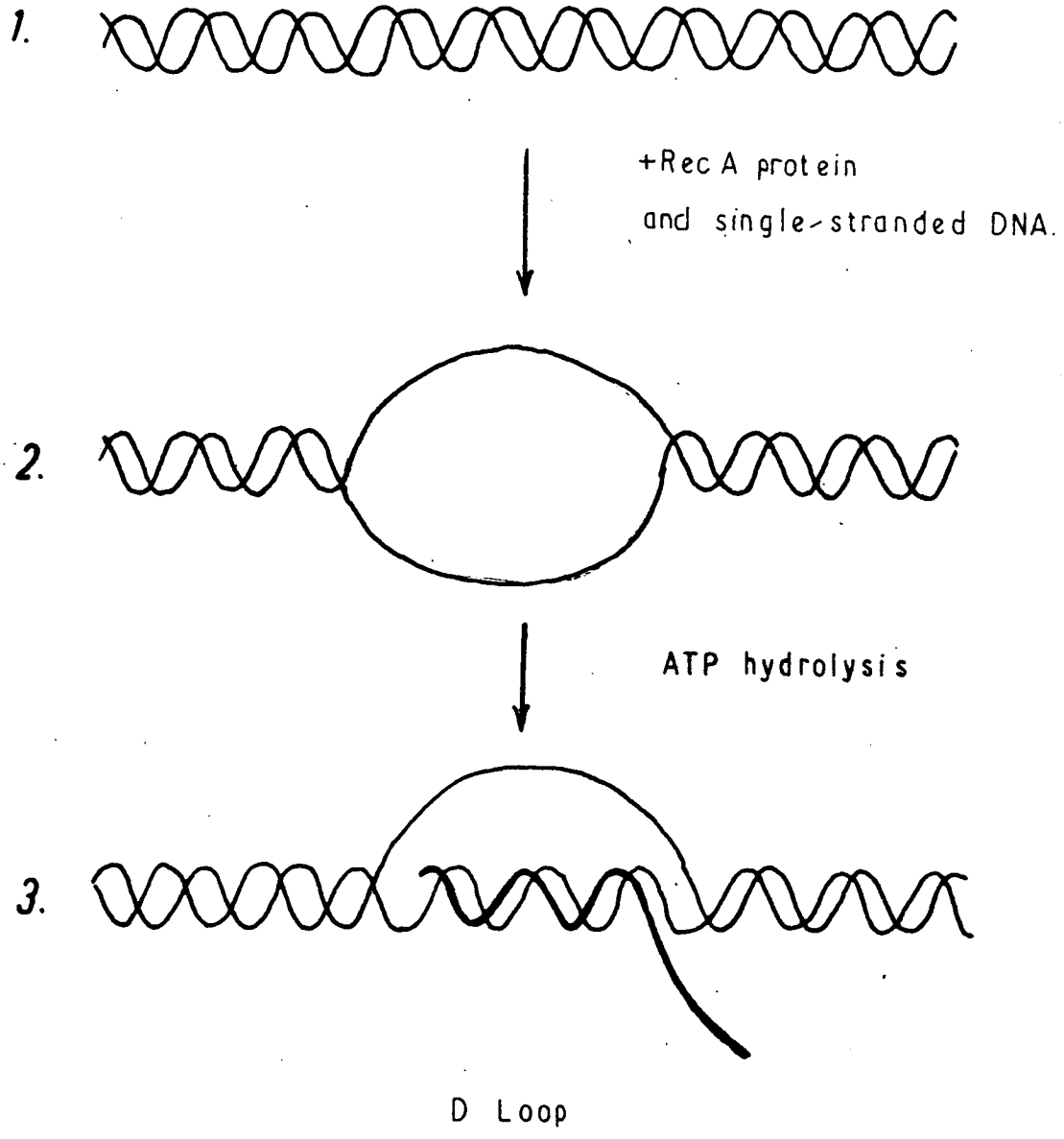
depends upon the annealing of complementary strands to produce, at an early stage in recombination, a region of duplex DNA containing one strand from each parental DNA molecule (Radding, 1978). Holliday (1964) proposed a model to explain recombination and aberrant segregation in fungi (figure 1·4) which results in a symmetric exchange of DNA via an intermediate form, or Holliday structure. This model had been adopted and modified by bacterial geneticists, supported by observations from *in vitro* studies and from electron micrographs which show the postulated Holliday structure (Potter and Dressler, 1978). The importance of the *recA* gene product in strand transfer has been shown by observations that the heteroduplex overlaps required by recombination are not formed in *recA*$^-$ mutants, in contrast to other recombination deficient mutants such as *recB*$^-$ or *recC*$^-$ (Potter and Dressler, 1976).

The cloning of the *recA* gene (McEntee and Epstein, 1977) and the purification of its protein product (Weinstock *et al.*, 1979; Shibata *et al.*, 1979) has allowed detailed studies on the action of the *RecA* protein which have led to some understanding of the mechanism of the early stages of recombination. The product of the *recA* gene is a protein with a molecular weight of 37 800 (Sancar *et al.*, 1980; Horii *et al.*, 1980), which has DNA dependent ATPase activity and a high affinity for single-stranded DNA. Shibata *et al.*, (1979) discovered that purified RecA protein catalysed the homologous pairing of

Figure 1·5 Formation of a D loop.

1. Single-stranded DNA catalyses the binding of supercoiled duplex by RecA protein.
2. The duplex is partially unwound, promoting a search for homology.
3. The single strand (heavy line) invades the duplex in a region of homology to form heteroduplex DNA by pairing to one strand of the duplex, but without stable Watson-Crick binding. The reaction is accompanied by ATP hydrolysis and the resulting structure is a D loop.

Figure 1.5


1.
+Rec A protein
and single-stranded DNA.
2.
ATP hydrolysis
3.
D Loop

superhelical DNA and single-stranded fragments, a reaction which only occurs at a slow rate *in vitro* in the absence of RecA protein (Holloman *et al.*, 1975). The product of this 'strand assimilation' was a D loop, or displacement loop (figure 1·5), which contains a heteroduplex region with a single-stranded fragment paired to one strand of the duplex, and its formation was accompanied by ATP hydrolysis. The D loop resembles a putative recombination intermediate and may represent an early stage in recombination, where a single DNA strand invades a homologous duplex to initiate strand exchange .

Further investigations showed that single-stranded DNA promoted binding of RecA protein to double-stranded DNA, and unwinding of the duplex by RecA protein (Cunningham *et al.*, 1979). The unwinding occurred without topoisomerase activity, and so required a free end of DNA, or superhelical turns, to allow rotation of the DNA strands. Unwinding could be promoted by non-homologous single-stranded DNA, not necessarily with a free end, but strand assimilation only occurred in the presence of homologous DNA. RecA protein also catalysed homologous pairing between two duplex molecules, provided one of them contained one or more short, single-stranded regions (Cunningham *et al.*, 1980). West *et al.* (1981) found that RecA protein could promote pairing between closed circular duplex DNA and gapped double-stranded circular DNA, or between closed-circular duplex DNA and closed-circular DNA with a short annealed fragment, without unwinding of either circle to generate a free end.

It has been suggested that initial pairing occurs first in regions of non-homology at the site of a single-strand gap, and that stable pairing occurs after repeated dissociation and reannealing of the two molecules, or by movement of one DNA molecule relative to the other until regions of homology coincide (West *et al.*, 1981). Cunningham *et al.* (1980) made electron microscope observations of three-stranded joints formed between gapped circular DNA and closed circular DNA, and found that the homologous junction was far removed from the single-strand gap in 31 of 44 joints examined. This supports the theory that single strands simply promote a search for homology.

The strand assimilation reaction was found to be stimulated by single-strand binding protein, or SSB protein (formerly known as helix destabilising protein), the product of the *ssb* (*lexC*) gene (McEntee *et al.*, 1980). The strand assimilation reaction requires, *in vitro* in the absence of SSB protein, one RecA protein monomer per five base pairs, and is inhibited by excess single-stranded DNA, so apparently single stranded DNA competes with duplex DNA for binding to RecA protein resulting in the formation of non-productive single-strand DNA-RecA protein complexes. SSB protein, which is bound to single-stranded DNA *in vivo* thus prevents the non-productive binding of RecA protein, and also prevents degradation by nucleases.

Meselson and Radding (1975) proposed a model for genetic recombination which, with some modifications

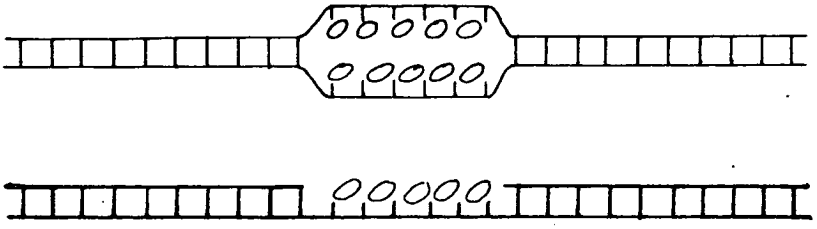
Figure 1·6 Model for the formation of joint molecules by gapped circular DNA and closed circular DNA.

A. Single-stranded regions of DNA promote binding of RecA protein to duplex DNA, and unwinding of the duplex. Single strands are protected by SSB protein (small elipses).

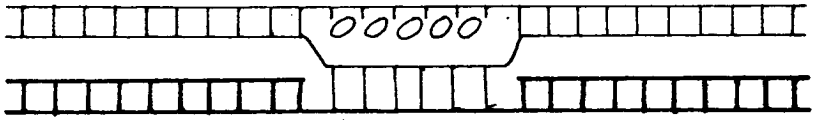
B. RecA protein promotes annealing of the single-strand gap to the duplex, and homologous pairs are aligned by reiterative dissociation, or procession of one molecule relative to the other.

C. RecA protein promotes uptake of a free end of the interrupted strand (strand assimilation) which forms a region of heteroduplex DNA similar to a D loop. This structure is referred to as a joint molecule in the text.

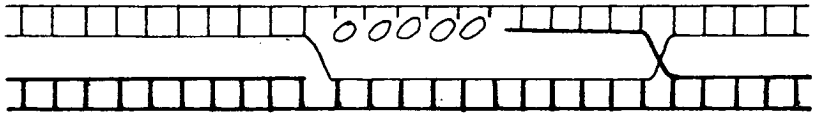
Figure 1.6



A Single stranded DNA promotes local unwinding of DNA duplex catalysed by Rec A protein.



B Search for homology promoted by RecA protein.



C Strand assimilation.

(West et al., 1981) is supported by the observations reported above. The first stages in the formation of a joint molecule are illustrated in figure 1·6.

1. Single-stranded regions of DNA promote binding of RecA protein to DNA, and unwinding of the duplex.
2. RecA protein, bound to duplex DNA promotes non-specific binding of single-stranded DNA to the duplex, catalysed by SSB protein, and promotes a search for homology by reiterative dissociation, or by procession of one DNA molecule, thus aligning homologous pairs.
3. RecA protein catalyses the uptake of a free end of the interrupted strand to produce a structure similar to a D-loop, without normal Watson-Crick heteroduplex structure.

The following stages in recombination are more a matter of conjecture, but one interpretation of the data (Meselson and Radding, 1975) leads to the following steps for maturation of the joint molecule:

1. The joint molecule is acted upon by cutting-in-trans enzymes (Ross and Howard-Flanders, 1977), to allow initiation of exchanges of one or both strands to give, respectively, asymmetrical or symmetrical exchange. The structure can now form a stable, covalently-bonded, molecule.
2. Strand exchange continues by the concerted operation of strand displacement by polymerase action, or strand assimilation by exonucleolytic action.
3. The cross connection is free to migrate in either direction as a consequence of the rotary diffusion of

the two DNA molecules, or branch migration (Lee et al., 1970; Warner et al., 1978), such that strand transfer is now occurring at some distance from the site of initial pairing.

4. Isomerisation of this Holliday structure after dissociation of the recA protein-DNA complex may interchange the two pairs of like strands at the site of exchange, making them both susceptible to exonucleolytic attack that terminates the exchange by cleaving the crossing strands leaving the arms in the parental or the recombinant configuration. The recBC nuclease, which has unwinding as well as nucleolytic activity may be involved in this resolution of the Holliday structure.
5. Mismatched base pairs in heteroduplex regions are subject to enzymic repair (Holliday, 1964; Whitehouse and Hasting, 1965; Radding, 1978).

1·8 The RecF and RecE pathways of recombination.

The RecBC pathway accounts for 99% of general recombination in the wild-type cell. The very low level of recombination in a recB$^-$ or recC$^-$ mutant can be restored almost to wild-type capacity by mutations at sbcA or sbcB. The sbcA$^-$ mutation derepresses the gene recE which is carried by the cryptic prophage rac, and which codes for DNA exonuclease VIII (ExoVIII), an enzyme isofunctional with λ exonuclease. The sbcB$^-$ mutation inactivates DNA exonuclease I (ExoI). It was generally recognised that the sbcA and sbcB mutations controlled two separate pathways

of recombination, the RecE and RecF pathways respectively, which were both inhibited by ExoV, the product of the recB and recC genes. However, evidence that recF$^+$ is required for recombination both in recBC$^-$ sbcB$^-$ and recBC$^-$ sbcA$^-$ strains, led Clark (1980) to propose that there was a single alternative pathway in recBC$^-$ mutants, and that ExoVIII in some way neutralises the inhibitory effect of ExoI on this pathway.

Since this pathway is only effective in the absence of certain wild-type functions, it seems unlikely that it has a major role in cellular recombination. However, there is evidence that the RecF pathway is responsible for mediating recombination between different forms of DNA than does the RecBC pathway. The exact role of ExoV in recombination is not known, other than that it has the ability to unwind linear, double-stranded DNA and nick the single strands produced in the presence of SSB protein (Telander Muskavitch and Linn, 1980). From experiments on conjugational recombination frequencies (DeHaan et al., 1972) it appears that the major contribution of ExoV to conjugational recombination is at a site on the exogenote where its strand unwinding activity is most likely to create single-stranded DNA useful in RecA protein-mediated synapsis with the chromosome. If such synapsis occurs between each end of linear exogenote and the recipient chromosome, the result could be the substitution of a long double-stranded donor segment for its corresponding chromosomal segment. In contrast, the RecF pathway appears to be responsible for closely spaced, double recombination

events in conjugation (Clark, 1980), Mahajan and Datta (1977, 1979), concluded that there was a greater frequency of single-stranded DNA substitution events in conjugational recombination mediated by the RecF pathway than was produced by the RecBC pathway. They deduced that recombination via the RecF pathway involved integration of a single strand only, and that this was subject to mismatch repair resulting in a greater recovery of unselected recipient markers, and a much higher level of clonal heterogeneity.

Porter, McLaughlin and Low (1978) deduced that levels of genetic recombination in E.coli measured between the same two alleles was strongly dependent upon parental configurations of DNA; for example, genes carried by a non-integrated λ recombined much more easily with genes carries by an F-prime factor, than with chromosomal genes. Recombination between the λ genes and the chromosome was elevated in a RecF background.

It can be envisaged that the RecF pathway has a specialised function in some types of recombination, although intermediates of the RecF pathway are probably frequently degraded by ExoV thus excluding a major role in general recombination. However, RecF does have an alternative role in repair (Clark et al., 1979).

1.9 Hotspots for general recombination.

Generalised recombination effects exchanges at any point on homologous DNA, but exchanges may occur more frequently in some regions than in others. In certain

cases, the increased frequency of exchanges has been attributed to special sites at and around which recombination occurs at elevated levels. These sites may be recognition sequences for a protein involved in recombination. Chi sites are well-studied examples of sites that when present in λ enhance recombination in their vicinity (Chattoraj et al., 1978). Chi sites have recently been detected in eukaryotes (Kenter and Birshtein, 1981) and an equivalent site cog in Neurospora was found to influence neighbouring recombination rates (Catcheside, 1974).

Chi sites were first detected in λ where they caused a large-plaque phenotype in red⁻ gam⁻ mutants. In these mutants, the action of the recBC nuclease, which is normally inhibited by the gam product, prevents replication of λ DNA by the rolling circle method. Monomeric DNA, the product of theta replication, is not a substrate for encapsidation, but the presence of chi, a hotspot for recombination, allows formation of dimers which can be packaged into phage heads.

Chi sites stimulate exchange mediated by the RecBC pathway only; they are inactive when only the RecF or RecE pathway of E.coli, or the red pathway of λ , is operating. Each site stimulates recombination within approximately 10^4 base pairs of its locus, and stimulation reaches a maximum of about 10-fold very near the locus and diminishes with distance (Stahl et al., 1975). The effect appears to be assymmetric; stimulation is greater to the

left of chi than to the right (Stahl and Stahl, 1975), and this is supported by the nucleotide sequence at separate chi sites (Smith et al., 1980). Sequences of two chi sites in λ showed a 23 base pair region of homology between the two sites with no inverted repeats. Mutations of chi^+ to chi^- were all found to map within an eight base pair sequence:

GCTGGTGG

which seems to be the active sequence (Schultz et al., 1981).

Chi sites were detected in wild type E.coli chromosomes by cloning separate EcoR1 fragments from a chromosomal digest into λ red^- gam^-, and chi was estimated to occur at a density of one per 5-15 kb (Malone et al., 1978). The importance of chi to the E.coli cell has not been established. It is not essential for RecBC-mediated recombination, but the occurrence of the chi sequence at a higher frequency than would be expected by chance seems to indicate that it does have some role.

1·10 Induction of recombination by damaged DNA.

Non-lethal doses of UV irradiation can cause an increase in genetic exchanges. This stimulation of recombination has been very well documented in phage (Jacob and Wollman, 1955; Rupp et al., 1971; Lin and Howard-Flanders, 1976; Lin et al., 1977) and has also been reported in E.coli (Howard-Flanders et al., 1978). It can be caused by agents other than UV for example, mitomycin C (Shaw and Cohen, 1965), and cross-linking

agents such as psoralen (Lin et al., 1977). The close relationship between repair of damaged DNA and recombination was established by the isolation of Rec$^-$ mutants of E.coli deficient in both functions (Clark and Margulies, 1965). A detailed discussion of the mechanisms of repair is beyond the scope of this introduction, but a brief mention of the best documented pathways is necessary for an understanding of the role of repair in damage induced recombination.

UV irradiation produces a variety of photo-products in DNA, including intrastrand cyclobutane-type dimers between adjacent pyrimidines known as pyrimidine dimers These have been identified as a major cause of lethal and mutagenic effects (Witkin, 1966). Pyrimidine dimers present a complete blockage to the elongation of nascent DNA chains during replication since they cannot form stable nucleotide pairs. This discussion will be limited to the response of the cell to these lesions only. The E.coli cell has a number of mechanisms to circumvent this blockage, which can be placed in two categories as suggested by Clark and Volkert (1978). Extrareplicational repair pathways remove the dimer before it reaches the replication fork, or after it has passed through the replication fork, while intrareplicational repair pathways act on the lesion as it is replicated.

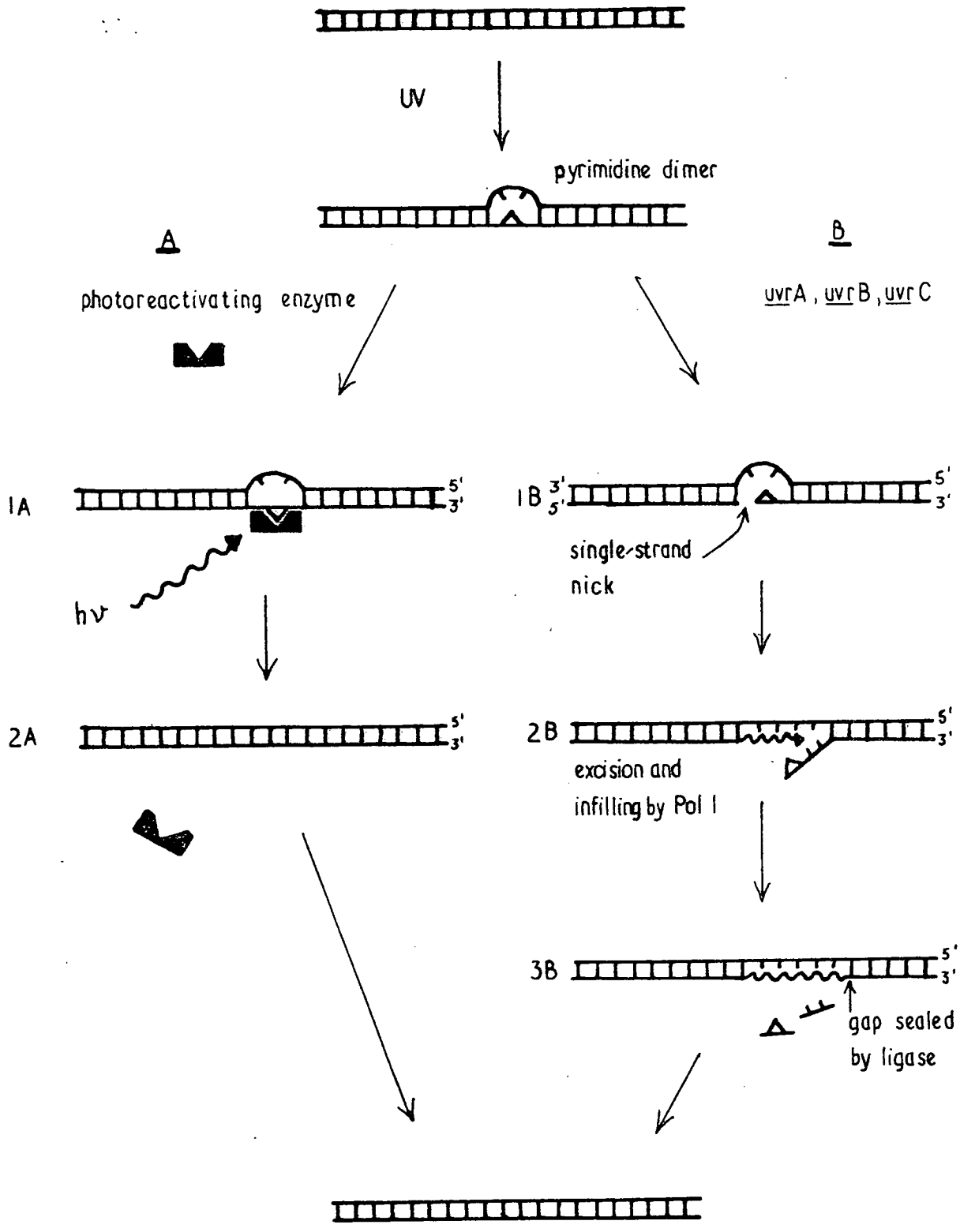
Extrareplicational repair results in the complete excision of a pyrimidine dimer. Photoreactivation is the enzyme-mediated, light-dependent, monomerisation of pyrimidine dimers (Rupert, 1975). The phr gene of E.coli

Figure 1•7 Extra replicational repair.

Action of UV on DNA produces a pyrimidine dimer. Photoreactivating enzyme (pathway A) recognises the dimer and binds to the DNA. The action of light on this comlex (1A) splits the dimer; the enzyme is released leaving the DNA intact (2A).

In the dark (pathway B) uvrA and uvrB products together make a single-strand nick on the 5' side of the dimer (1B). DNA polymerase I (Pol I) excises bases from this nick and fills in the gap (2B). uvrC product is needed for efficient excision. DNA ligase seals the ends (3B) thus completing the repair.

Figure 1.7


UV
pyrimidine dimer
A
B
photoreactivating enzyme
uvrA, uvrB, uvrC
1A
1B
5'
3'
hν
single-strand
nick
2A
2B
excision and
infilling by Pol I
3B
gap sealed
by ligase

codes for a protein, or photoreactivating enzyme, which binds specifically to a pyrimidine dimer. The action of light on this complex splits the dimer and the enzyme is released leaving the DNA intact (figure 1·7a).

Pathways for excision repair in the dark are dependent upon *uvrA*$^+$ and *uvrB*$^+$. These two gene products together make a single strand nick or incision on the 5' side of the dimer (Braun and Grossman, 1974). Seeberg *et al.* (1980) found that the *uvrC*$^+$ product was needed for efficient incision, and Kato (1972) showed that *uvrC*$^+$ product was needed to complete excision of the dimer. DNA polymease I binds at the nick and adds nucleotides to the 3' end, then makes a second nick in the chain to release the damaged region; it may continue to digest nucleotides, thus translating the nick. DNA ligase closes the nick thus completing repair (figure 1·7b). This type of 'short patch' repair is *recA*-independent, but a second excision repair pathway termed 'long patch' has been proposed which is recA$^+$uvrA$^+$ *uvrB*$^+$ dependent (Cooper and Hanawalt, 1972).

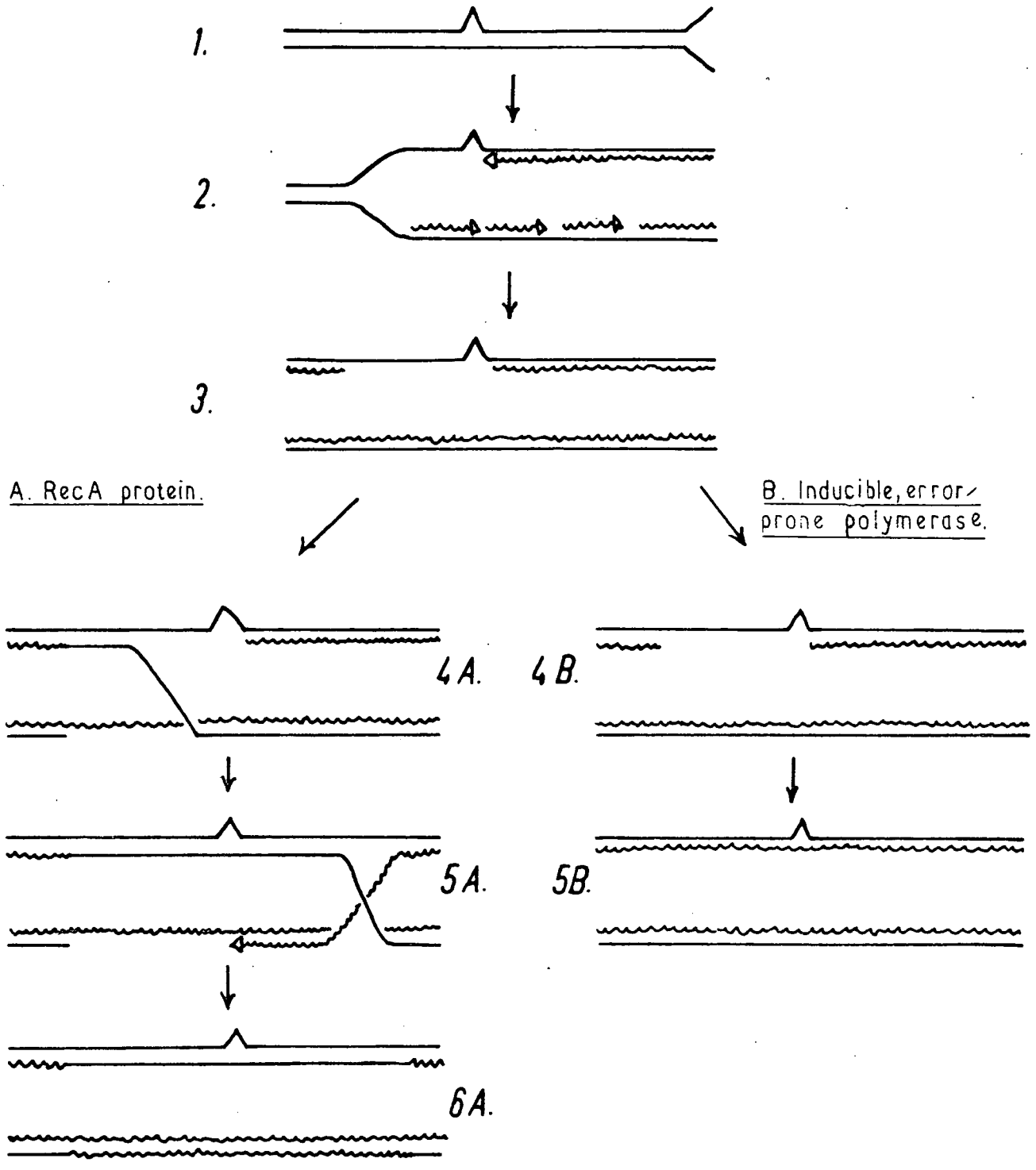
If the replication fork reaches a pyrimidine dimer, the DNA cannot be completely replicated, and single strand gaps 1 500-40 000 nucleotides long are left in the daughter strands (figure 1·8; Johnson and McNeill, 1978). These gaps are subject to intrareplicational repair, by recombination between daughter and parental strands in breakage-reunion gap-filling repair dependent upon either the RecBC pathway, or the RecF pathway for recombination (figure 1·8). An alternative route to accomplish gap filling involves the blocking of the 3'-5' correction endonuclease activity of one or more of the DNA

Figure 1·8 Intrareplicational repair.

The replication of pyrimidine dimer (1 and 2) leaves a single-strand gap (3). RecA protein binds to this gap and aligns it with a homologous region of the sister duplex. When homologous pairing is achieved, an enzyme nicks the duplex and RecA protein initiates strand transfer (4A), producing subsequently a double-strand exchange similar to a Holliday structure (5A). The lower duplex is repaired by DNA polymerase leaving one intact daughter molucule, and one daughter molecule still carrying a dimer which is subject to excision repair (6A).

Alternatively, an inducible, error-prone polymerase activity may continue synthesis across a pyrimidine dimer (transdimer synthesis; 4B), which can later be excised.

Figure 1.8


1.
2.
3.
A. RecA protein.
B. Inducible, error-prone polymerase.
4A.
4B.
5A.
5B.
6A.
parental DNA.
newly synthesised DNA.
pyrimidine dimer.

polymerases, by one or more inducible proteins, or the induction of a separate, error-prone polymerase. This prevents the removal of bases which are not hydrogen-bonded in the normal configuration, and hence DNA synthesis can continue past a pyrimidine dimer. This type of inducible repair could be responsible for the induction of mutagenesis which is a part of the SOS response (see chapter 5, section 1). This pathway was termed transdimer synthesis (Clark and Volkert, 1978; figure 1·8). The result of these intrareplicational repair pathways is to leave the lesion in the DNA, and this must subsequently be excised by one of the extra-replicational repair pathways.

All of these pathways except photoreactivation, generate single-strand gaps and ends as intermediates in repair. Stacey *et al*. (1969) proposed that the formation of single-strand gaps with unpaired bases during the excision and repair of DNA containing photoproducts induced recombination, whilst Rupp and Howard-Flanders (1968) proposed that the replication of DNA and the formation of daughter strands containing gaps opposite the dimers generated free strand ends able to initiate recombination. These theories are compatible with the finding that one of the earliest stages in homologous recombination is probably the invasion of a duplex by a single-stranded DNA molecule. Clark and Volkert (1978) proposed another pathway of repair: incision-promoted recombinational repair which is dependent upon *uvrA*$^+$ *uvrB*$^+$ and *recA*$^+$. The availability of such recombinogenic forms

of DNA, and the inducibility of a number of the repair and recombination pathways (Sedgwick, 1975; Witkin, 1976) underlines the close association between repair of damaged DNA, and the observed increase in recombination.

1.11 Summary.

The aim of this project was primarily to establish at which stage during the process of P1 transduction events leading to the wide variation in recovery of different markers occurs. This introduction has presented several possibilities which may give rise to the observed discrepancies. Briefly these were: 1. Selective phage packaging due to a limited number of initiation sites for packaging for which there is considerable evidence in P22, or reduced accessibility of some regions of the donor chromosome; 2. Selective recombination in the recipient due to problems of accessibility due to the three-dimensional structure of the recipient chromosome, or to a limitation of recombination enzymes, or to a concentration of recombination events in some regions due to the presence of recombination-stimulating sites. The experiments in the following chapters were designed to discriminate between events in the donor and in the recipient, and to attempt to define more clearly events in the recipient leading to the formation of stable transductants.

CHAPTER TWO

MATERIALS AND METHODS

2·1 Bacterial and phage strains.

Bacterial strains are listed in table 2·1. Bacteria were maintained on nutrient agar plates stored at 4 C for regular use, or in stabs of nutrient agar at 4 C. Stocks were stored as frozen cultures which were made by diluting overnight cultures of bacteria with an equal volume of 2x freezing mix which consists of KH_2PO_4, 6·3 g; $Na_3C_6H_5O_7$, 0·45 g; $MgSO_4.7H_2O$, 0·09 g; $(NH_4)_2SO_4$, 0·9 g; KH_2PO_4, 1·8 g; glycerol, 44g; distilled water to 500 ml. Cultures were frozen at -70 C.

Bacteriophage strains are listed in table 2·2. Phage P1kc was a laboratory stock which initially gave titres of about 5×10^8 to 1×10^9 pfu/ml. During the course of this work the titre improved to $1\text{-}5 \times 10^{10}$, and transduction frequencies per phage increased possibly due to the selection of a variant. P1 could be stored in CsCl for a few months, but fresh stocks were made frequently. Phage λ stocks were stored in CsCl solution, or maintained as lysogens.

2·2 Growth media and buffers.

Growth media and buffers are listed in tables 2·3 and 2·4 respectively. L-broth (LB) and L-agar (LA) were used for all phage work; for P1, LBC and LAC, which are LB and LA supplemented with 10^{-3}M CaCl2, were used. VB

Table 2·1 Bacterial strains

STRAIN	GENOTYPE	SOURCE AND REFERENCE
W3110	Prototroph	Laboratory stock.
MM303	*argH metB ilv*^{42} *his trp pyrE thi tna xyl*-4 *mtl malA lacY uhp* Ø80^{s} *rpsL* T6^{R}(P1)	Laboratory stock. Masters, 1977.
MM3031*mal*$^{+}$	MM303 P1 cured, *mal*$^{+}$	Laboratory stock. Newman and Masters, 1980.
MM7	*argG metB leu his ilv pyrE uhp malA xyl lac gal rpsL gyrA*	Laboratory stock. Masters, unpublished.
NF279*recA*$^{+}$	*his argG leu metB ilv pyrB rbs mtl xyl gal lac malA rpsL rpsE*	Laboratory stock.
CP154	*argA his lysA thi rpsL*	Laboratory stock.
LE234	*metB argE ilv tna pro*	E.Orr. Fairweather *et al*., 1980.
LE701	*metB argE ilv tna pro gyrB*	E.Orr. Fairweather *et al*., 1980.
MH5	Prototrophic: Hfr KL16 *gyrA*	Laboratory stock.
DM1187	*thi*-1 *thr*-1 *leu*-6 *proA*2 *his*-4 *ilv*^{ts} *galK*1 *ara*-14 *mtl*-1 *xyl*-5 *tsx*-33 *rpsL tif*-1 *sfiA*11 *lexA*3 *spr*51	R.G.Lloyd. Mount, 1977.

GC3217	thi-1 thr-1 leu-6 proA2 his-4 argE3 ilv^{ts} galK1 ara-14 mtl-1 xyl-5 tsx-33 rpsL tif-1 sfiA11	R.G.Lloyd. Mount, 1977
DM1420	thi-1 thr-1 leu-6 proA2 ilv^{ts} galK1 ara-14 mtl-1 xyl-5 tsx-33 rpsL sifA11 lexA3 spr-51	R.G.Lloyd. Mount, 1977.
N1462(pPE13)	spr sfi $recA^{\Delta}$ (pPE13)	P.T.Emmerson.
N1460	Hfr KL16 argA21 Δ(srl-recA)21 deoB	R.G.Lloyd.
ED2123	sbcB17 recB	N.S.Willetts.
JC7526	recB21 recC22 sbcB15 sfiB103; other markers as AB1157	A.J.Clark.
NEM259	met supE supF r^{-} m^{-}	Laboratory stock.
AA125	$(lacZ)^{\Delta}$ $(trpED)^{\Delta}$ $(gal\text{-}uvrB)^{\Delta}$ his tsx tonA lacI3 sup^{o}	Laboratory stock.
AB1160	proA2 his-4 ilvC7 argE3 thi-1 lacY1 galK2 xyl-5 mtl-1 supE44	B.J.Bachmann.
AB1161	proA2 his-4 ilvA210 argE3 thi-1 lacY1 galK2 xyl-5 mtl-1 supE44	B.J.Bachmann.

AB2070	proA2 trp-3 his-4 ilvE12 metE46 thi-1 ara-9 lac galK2 malA1 mtl-1 rpsL ton-1 tsx-3 supE44	B.J.Bachmann. Marsh and Duggan, 1972.
AB3505	proA2 trp-3 his-4 ilvD188 metE46 argH1 lac galK2 xyl-7 mtl-1 malA1 tsx-3 supE44	B.J.Bachmann. Wechsler and Adelberg, 1969.
AB1157	thi-1 thr-1 leu-6 proA2 his-4 argE3 lacY1 galK1 ara-14 mtl-1 xyl-5 tsx supE44 str-31.	Laboratory Stock.
Gif102	HfrP01 thrA1015 metLM1005 thi-1 relA1	B.J.Bachmann. Thèze et al., 1974.

Table 2·2 Phage Strains

STRAIN	GENOTYPE	SOURCE AND REFERENCE
P1kc		Laboratory stock
λNM 540	*sr1*(1-2)$^{\Delta}$ *att*$^{+}$ *imm*^{21} *nin*	N.E.Murray (Murray and Murray, 1975)
λNM 616	*lac*$^{+}$ *att*$^{+}$ *imm*^{21} *nin*	N.E.Murray (Wilson and Murray, 1979)
λ *trpABC*	λ540*trpABC*	N.E.Murray (Hopkins *et al.*, 1976)
λ *tna*	λ540*tnaA*	R.J.Myers (Borck *et al.*, 1976)
λ *vir*		Laboratory stock
λ NK55	*b*221 *c*III167::Tn10 *c*1857 *ind*$^{+}$ *O*am29	Kleckner *et al.*(1978)

Table 2·3 Growth Media

L-broth (LB)

Difco Bacto Tryptone	10 g
Difco Bacto yeast extract	5 g
NaCl	5 g

Distilled water to 1 litre; pH to 7·2 with NaOH.

L-agar (LA)

Difco Bacto Tryptone	10 g
Difco Bacto yeast extract	5 g
NaCl	10 g
Difco agar	15 g

Distilled water to 1 litre; pH to 7·2 with NaOH.

Nutrient broth (NB)

Oxoid nutrient broth no. 2	25 g

Distilled water to 1 litre.

Nutrient agar (NA)

Oxoid nutrient broth no. 2	25 g
Davis New Zealand agar	12·52 g

Distilled water to 1 litre.

BBL agar

Baltimore Biological Laboratories Trypticase	10 g
NaCl	5 g
Difco agar	10 g

Distilled water to 1 litre.

BBL top agar

As for BBL agar, but only 6·5 g Difco agar per litre.

VB minimal agar (Vogel and Bonner, 1956)

1·5% Difco agar in distilled water, molten	400 ml
20 x VB salts	25 ml
20% carbon source	10 ml

Amino acids and vitamins as required.

VB minimal medium

20 x VB salts	25 ml
20% carbon source	10 ml
Sterile distilled water	400 ml

Amino acids and viatamins as required.

20 x VB salts

$MgSO_4.7H_2O$	4 g
Citric acid	40 g
K_2HPO_4	200 g
$NaNH_4.HPO_4.4H_2O$	70 g

Distilled water to 1 litre.

Amino acids and vitamins

Amino acids	20 ug/ml
B_1	2 ug/ml
Biotin	2 ug/ml
Uracil	5 ug/ml

Difco top agar

Difco agar	6·5 g

Distilled water to 1 litre.

MacConkey agar

Peptone	20 g
Bile salts no.3	1·5 g
NaCl	5 g
Neutral Red	0·03 g
Agar	15 g

Distilled water to 1 litre.

Table 2·4 Buffers

Phage buffer

Na_2HPO_4	7 g
KH_2PO_4	3 g
NaCl	5 g
$MgSO_4$, 0·1 M	10 ml
$CaCl_2$, 0·01 M	10 ml
Gelatin solution, 1%	1 ml
Distilled water to 1 litre	

Tris-EDTA buffer (TE buffer)

0·01 M Tris/HCl pH 8·0

1 mM EDTA

TEN buffer

TE buffer + 5 mM NaCl

1 x SSC

0·15 M NaCl

0·03 M sodium citrate

SSC:$CaCl_2$

3 parts 1 x SSC to 4 parts 0·1 M $CaCl_2$

agar, supplemented with appropriate amino acids, vitamins and sugars, was used as a selective medium.

2·3 Bacterial techniques.

(i) Growth of bacteria. Bacterial cultures were grown in LB at 37 C in a New Brunswick rotary water bath shaker or as overnight cultures in LB incubated at 37 C without shaking.

(ii) Measurement of OD and cell number. Cell mass was measured in a Perkin-Elmer Coleman Model 55 Spectrophotometer at 540 nm (OD_{540}). Cell size distribution was measured in a Coulter Counter Model ZB interfaced with a Coulter Channeliser (Coulter Electronics Ltd., Harpenden, England). 0·2 ml cell samples were mixed thoroughly with 0·2 ml of 0·5% formaldehyde solution. A 50 ul sample was diluted into 8 ml of filtered azide-saline solution (NaCl, 36 g; NaN_3, 2 g; water to 4 litres), and counted. Size distribution was recorded.

(iii) Hfr matings. Hfr donors were diluted 10-fold into pre-warmed LB from overnight cultures and grown at 37 C in static culture with maximum surface area to allow for aeration to a density of 4×10^8 cells/ml. Streptomycin-resistant recipient cultures were grown in shaking cultures at 37 C in LB to a density of 4×10^8 cells/ml, and equal volumes of donor and recipient were mixed then incubated without shaking at 37 C. Matings were stopped at appropriate times by withdrawing aliquots and vortexing, then plating 0·1 ml of a 10 x dilution in phage buffer on selective plates supplemented

with streptomycin (200 ug/ml).

(iv) Isolation of a tetracycline-resistant culture with Tn10 present at undetermined sites. The method of Csonka and Clark (1979) was used to link a marker with an unselectable phenotype to a transposon carrying a drug resistance determinant, thus facilitating its manipulation. Strain W3110 was grown to a density of 5×10^8 cells/ml in LB supplemented with maltose (4 mg/ml). 10 ml of culture was sedimented and resuspended in 1 ml of a lysate of λNK55 (titre 4×10^{10} pfu/ml) and allowed to adsorb for 15 minutes at room temperature. The mixture was diluted into 50 ml of LB supplemented with glucose (4 mg/ml) and sodium citrate (10 mM, pH 7·0) and incubated with aeration at 30 C until the OD began to increase. Tetracycline (1 ug/ml) was added and incubation was continued for 1 hour. At the end of this time, the concentration of tetracycline was increased to 15 ug/ml, and the culture was grown to saturation at 42 C. A 10 ml aliquot was again subcultured into the same medium and grown to saturation at 42 C. The incubation at 42 C ensures that all cells lose the phage which has a temperature sensitive repressor, whilst growth in tetracycline selects for those cells which carry a chromosomal Tn10 insertion. A P1 lysate was made on this culture, which carries random Tn10 insertions, at 37 C and was used to infect suitable recipients and select for the required linkage.

2·4 Phage P1 techniques.

(i) Growth of phage by liquid infection. A P1 sensitive strain (usually W3110) was grown with aeration at 37 C in LBC, in a flask of a capacity at least 5 x greater than the volume of the culture, to an OD_{540} of about 0·5, then infected with P1 at an moi of about 1. Incubation was continued until lysis, which occurred at 1½ to 3 hours after infection. Lysis was completed by the addition of chloroform (1-2 ml/litre) and incubation continued for a further 15 minutes. Lysates were clarified by centrifugation at 10k. rpm in the GSA rotor of a Sorvall RC-5B centrifuge. Phage stocks were titrated by mixing 0·1 ml of phage, diluted in phage buffer, with 0·1 ml of W3110 in mid-log phase and 2·5 ml of molten BBL top agar, pouring on LAC plates, and incubating at 37 C overnight.

(ii) Growth of phage: plate lysates. For small volume lysates, 10^7 p.f.u. of P1 and 0·1 ml of W3110 in mid-log phase were mixed with 2·5 ml of molten BBL top agar and poured on to a freshly made LAC plate. After incubation at 37 C for 5-6 hours, the top agar layer was scraped off with a sterile spatula and vortexed with 1 ml of phage buffer and a drop of chloroform. The suspension was centrifuged in a bench-top centrifuge, and the clear supernatant was removed and titrated.

(iii) P1 transductions. Recipient strains were grown in LBC to late log or stationary phase (1-5 x 10^9 cells/ml). Strains non-lysogenic for P1 were concentrated 10x. to reduce phage killing, by pelleting in a bench-top

centrifuge and resuspending in $^{1}/_{10}$ volume of LBC. P1 lysates were diluted to a concentration of approximately 10^{9} pfu/ml in LBC or phage buffer. 0·1 ml of P1 was mixed with 0·1 ml of recipient cells and incubated at 37 C for 15 minutes. This mix was diluted to 1 ml with phage buffer, and 0·1 ml of this suspension, and also a 10^{-1} dilution, was spread on selective plates. Transductants were scored after 1-2 days' growth at 37 C.

2·5 Phage λ techniques.

(i) Growth of phage. Liquid and plate lysates and titrations were performed as described for P1, except that media were supplemented with 10^{-2} M $MgSO_4$ instead of $CaCl_2$.

(ii) Complementation test. The complementation pattern of specialised transducing phages was tested by spotting a phage lysate, diluted in phage buffer if necessary, on to a lawn of an appropriate recipient. The recipient was grown to stationary phase in LB, then resuspended in 10^{-2}M $MgSO_4$. 0·1 ml of the suspension was mixed with 2·5 ml of molten Difco top agar and poured on to selective plate. 0·02 ml of phage suspension was placed on the surface, and the plate was incubated for 2 days at 37 C. Colonies were picked and checked for λ lysogeny to distinguish complementation from recombination.

(iii) Test for λ lysogeny. The test strain was grown in LB overnight, then resuspended in 10^{-2}M $MgSO_4$, mixed with molten BBL top agar and poured on to an LA plate. 0·02 ml each of λNM540, to test for lysogeny by an *imm*[21]

phage, and λ<u>vir</u>, to test for λ sensitivity, was placed on the lawn which was incubated for 4-5 hours at 37 C. Cross-streak tests were performed by streaking parallel bands of λ<u>vir</u> and λNM540 on a dry LA plate, then cross-streaking with the test strains over first the λNM540, then the λ<u>vir</u>. In both tests, lysis by λ<u>vir</u> but not by λNM540 indicated lysogeny by an <u>imm</u>^{21} phage.

(iv) <u>UV induction of λ from lysogenic strains</u>. Lysogenic bacteria were grown to early log phase (OD_{540}, 0·5; 2 x 10^8 cells/ml) in LB at 37 C, harvested and resuspended in half the original volume of 10^{-2} M $MgSO_4$. The cells were irradiated in 10 cm diameter glass petri dishes with 400 ergs/mm^2 UV, then diluted 4-fold into fresh, warm LB supplemented with 10^{-2} M $MgSO_4$, and grown in the dark at 37 C with good aeration for 2 hours, or until lysis occurred. Chloroform was added (1 ml/litre), and the lysate was clarified and titrated as above.

2·6 UV irradiation of phage P1 lysates and bacterial cultures.

Phage or bacterial suspensions were diluted 10-fold in phage buffer and placed in a 10 cm diameter glass petri dish. The open dish was exposed to UV at a dose rate of 10 ergs/mm^2/second with agitation. Irradiated phage and bacteria were shielded from the light and used as soon as possible after irradiation. Transductions and titrations using irradiated organisms were accomplished with the minimum exposure to light, and incubated in the dark to prevent photoreactivation.

2·7 Concentration of bacteriophage cultures.

Similar methods were adopted for λ and P1.

(i) Concentration of phage by polyethylene glycol precipitation (Yamamoto et al., 1970). Phage lysates were prepared and clarified as above, then NaCl (40 g/litre) was added and dissolved by stirring. Polyethylene glycol 6000 (PEG 6000) was added at a concentration of 10% w/v and dissolved by stirring, then the lysate was left overnight at 4 C. The precipitated complex of PEG 6000 and phage was harvested by centrifugation at 10k rpm for 15 minutes in the GSA rotor of a Sorvall RC-5B centrifuge. Pellets were resuspended in about 1/50 of the original volume of phage buffer by stirring in the cold for a few hours. DNase (DNase 1, Sigma) and RNase (RNase A, Sigma) were added at a final concentration of 10 ug/ml, and the suspension was incubated at 37 C for 1 hour. Debris were sedimented at low speed in a bench-top centrifuge.

(ii) CsCl step gradients. Phage concentrated by PEG precipitation were loaded on to CsCl step gradient composed of three 1 ml steps of 1·7, 1·42, and 1·3% w/v CsCl in phage buffer in a 17 ml polycarbonate tube. The gradient was spun in the AH627 (swing-out) rotor of a Sorvall OTD 50 centrifuge for 3 hours at 22k rpm. Phage bands were removed through the side of the tube with a 23 g needle and syringe.

(iii) CsCl equilibrium gradients. Phage was further purified in equilibrium gradients. Phage bands were mixed with 1·42 w/v CsCl in phage buffer and loaded into 15 ml

polyallomer heatseal tubes. Gradients were formed by spinning in the Ti50 rotor of a Sorvall OTD 50 centrifuge for 30 hours at 33k rpm. Phage bands were removed as above.

Phage λ can be stored indefinitely in CsCl at 4 C, but P1 tends to be unstable so DNA was extracted immediately after banding to obtain the maximum yield.

2·8 DNA techniques.

(i) Extraction of phage DNA. Phage which had been concentrated in CsCl was dialysed against 3 changes of phage buffer over 12 hours, and placed in a siliconised Corex centrifuge tube with an equal quantity of distilled phenol which had been equilibrated with TE buffer and stored at -20 C. The two were mixed gently by inversion for 10 minutes, and then the layers were separated by low speed centrifugation. The aqueous layer was removed with a pasteur pipette and added to a second aliquot of phenol. The extraction was repeated twice more, then the aqueous layer was removed and dialysed for 15 hours against 4 or 5 changes of TE buffer to remove all traces of phenol. DNA was stored in TE buffer at 4 C.

(ii) Preparation of E.coli DNA. W3110 was grown in 1 litre of LB at 37 C with vigorous shaking to an OD_{540} of about 0·5 (log phase). Cells were harvested by centrifugation, and the pellet was resuspended in 50 ml 25% sucrose in 50 mM Tris buffer pH 8·0 with 1·7 ml of a freshly prepared solution of lysozyme (10 mg/ml; Sigma, Grade 1 from egg white), 6·5 ml EDTA (0·25 M, pH 8·0), 13·5 ml lysis buffer (2% Triton X100, 0·0625M EDTA,

50 mM Tris buffer pH 8·0) and 2·8 mg of pre-incubated Protease (Sigma, Type IV). The mixture was digested overnight at room temperature.

The digest was extracted 4x with an equal volume of phenol (as for phage DNA extractions), mixing gently by inversion for 2 minutes. The layers were separated by low-speed centrifugation and the phenol layer saved for re-extraction with 0·5 M Tris buffer pH 8·0.

DNA was precipitated from the aqueous phase by layering 2 volumes of ice-cold ethanol on the surface and spooling the DNA on a clean, acid-washed, glass rod. The DNA was dissolved in 50 ml 0·1 x SSC, and the ethanol precipitation repeated. The DNA was re-dissolved in 50 ml 0·1 x SSC, and treated with RNase (20 ug/ml, boiled for 20 minutes to inactivate contaminating DNase). The phenol extraction was repeated once more, followed by ethanol precipitation. DNA was dissolved in 10 ml of TE buffer and dialysed against 4-5 changes of TE buffer over 1-2 days.

(iii) Small-scale plasmid preparation. (Birnboim and Doly, 1979). The solutions used for this preparation are listed in Table 2·5. 1·5 ml cultures were grown overnight in NB with aeration to give maximum cell density. These were transferred to 1·5 ml polypropylene microfuge tubes and pelleted by spinning for 1 minute in a Quickfit microcentrifuge. The supernatant was discarded, and the pellet resuspended in 0·1 ml of lysis solution by vortexing, then left on ice for 30 minutes. 0·2 ml of alkaline SDS solution was added. After a further 5 minutes on ice, 0·15 ml of high salt solution was added,

Table 2·5 Solutions for Birnboim preparation.

Lysis solution	
Lysozyme	2 mg/ml
Tris/HCl pH 8·0	25 mM
EDTA pH 8·0	10 mM
Glucose	50 mM
Alkaline SDS solution	
NaOH	0·2 M
SDS	1%
High salt solution	
Sodium acetate	3 M
To pH 5 with acetic acid	
Low salt solution	
Sodium acetate	0·1 M
To pH 6 with acetic acid	

and the suspension was mixed by inversion, then left on ice for 60 minutes.with occasional mixing. The precipitate was removed by centrifuging for 5 minutes, and the supernatant was transferred to a fresh microfuge tube together with 1 ml of cold ethanol, then left at -20 C for 30 minutes. Nucleic acids were pelleted by centrifuging for 2 minutes in a microcentrifuge; the supernatant was disgarded and the pellet dissolved in 0·1 ml of 0·1 M sodium acetate, pH6. 0·2 ml of cold ehanol was added, nucleic acids were precipitated at -20 C for 10 minutes, pelleted in a microcentrifuge then dried under vacuum. The final pellet of DNA and RNA was dissolved in 50 ul of TE buffer. This final solution was suitable for transformations.

(iv) Ethanol precipitation of small quantities of DNA. 1/10 volume of 5 M potassium acetate and 2-3 volumes of cold ethanol were added to DNA in TE buffer in a microfuge tube and mixed well, then left at -70 C for 1 hour, or overnight. DNA was pelleted at 4 C in a Quickfit microcentrifuge, the supernatant was decanted, and the pellet dried under vacuum. The DNA was dissolved in 50-100 ul of TE buffer.

(v) Restriction of DNA. DNA was digested with restriction enzyme (1 unit/ug of DNA) for 1 hour at 37 C in a total volume of 10 ul, with 1 ul of 10x reaction buffer. Reaction buffers for Hind III and EcoR1 are shown in table 2·6. The reaction was stopped by incubating at 70 C for 10 minutes to inactivate the enzyme and also to separate the cohesive ends of λ.

Table 2·6 Buffers for restriction and ligation of DNA.

10 x reaction buffers

	HindIII	EcoR1
Tris/HCl pH 7·5	100 mM	1·0 M
$MgCl_2$	100 mM	50 mM
NaCl	500 mM	500 mM
2-mercaptoethanol	60 mM	60 mM

10 x ligase cocktail

1 M tris/HCl pH 7·2	660 ul/ml
0·1 M EDTA	100 ul/ml
1 M $MgCl_2$	100 ul/ml
1 M dithiothreitol	100 ul/ml
0·1 M ATP	10 ul/ml

(vi) Ligation of DNA fragments with cohesive ends. Restricted DNA was diluted to a concentration of 5-30 ug/ml in 10 mM Tris pH 7·5, 0·1 M NaCl. 1/10 final volume of 10x ligase cocktail (Table 2·6) was added together with 1-2 ug of DNA and 1 unit of T4 DNA ligase (New England Biolabs) in a total volume of 20-50 ul. The mixture was incubated at 10 C for 3-6 hours, then at 0 C, during which time samples were removed for transfection.

(vii) Transfection and transformation of E.coli cells (after Lederberg and Cohen, 1974). Cells competent for transfection were obtained by growing a suitable host strain (NEM259 or AA125 for phage DNA) to an OD_{540} of 0·55-0·65 in 50 ml of LB at 37 C. Cells were chilled on ice for 15 minutes, then pelleted and resuspended in 25 ml of ice-cold 0·1 M $MgCl_2$, then immediately re-pelleted and resuspended in 2·5 ml of ice-cold 0·1 M $CaCl_2$. They were then kept on ice for at least 30 minutes.

About 50 ng of ligated DNA, or plasmid DNA, was diluted to 0·1 ml in SSC:$CaCl_2$, and mixed with 0·2 ml of competent cells. The mixture was left on ice for 30 minutes, then heat-shocked at 42 C for 2 minutes, then returned to ice for 30 minutes before plating out. 0·1 ml was mixed with 2·5 ml of molten BBL top agar supplemented with 10^{-2} M $MgSO_4$, poured on BBL plates, and incubated at 37 C overnight.

For transformation with plasmid DNA, after the heat shock the mixture was diluted to 1 ml with NB and incubated at 37 C to allow expression of drug resistance. 0·1 ml

was then spread on selective plates.

(viii) DNA electrophoresis on agarose gels (McDonnell et al., 1977). For a horizontal 0·7% 25 cm x 15 cm gel, 1·4 g of agarose (Sigma, Type II) was dissolved in 200 ml of Tris-acetate buffer (40 mM Tris, 20 mM sodium acetate, 1mM EDTA; pH to 8·2 with acetic acid) by boiling for three minutes. After cooling to 50-60 C, and adding ethidium bromide (0·5 ug/ml) the gel was poured into a 25 cm x 15 cm perspex gel former. A slot former was placed 5 cm from one end giving 12 slots each of capacity 20-30 ul. The gel was allowed to set for 1 hour, then the slot former was removed and the gel immersed in Tris-acetate buffer in an electrophoresis tank.

Restricted DNA was mixed with 2 ul of loading buffer (50% glycerol, 50% Tris acetate buffer, 0·04% bromophenol blue) and loaded into the slots. Electrophoresis was at 30 V, 15 mA, overnight, or 100 V, 50 mA, for 4-5 hours. Bands were illuminated with UV and photographed using Ilford FP4 film with a 15 second exposure and a red filter.

2·9 Polyacrylamide gel electrophoresis of proteins.

(i) Labelling cells with ^{35}S methionine and extraction of proteins (after Clark et al., 1979). Cells were grown overnight in fully supplemented VB minimal medium with glucose, then diluted 10x into fresh, warm medium and grown to an OD_{540} of 0·3-0·4. Cultures for UV treatment were exposed to 400 erg/mm^2 of UV and then wrapped in foil. Incubation of all cultures was continued for a further 40 minutes. 1·5 ml of cells were removed into microfuge

Table 2·7 Solutions for SDS-polyacrylamide gels

Stock acrylamide	
Acrylamide (Sigma, electrophoresis grade)	30 g
NN' methylene bis acrylamide (Sigma)	0·8 g
Distilled water to 1 litre; filter and store in the dark	
Upper tris (x 4)	
Trizma base (Sigma)	6·06 g
10% SDS in water (Sigma, electrophoresis grade)	4·0 ml
Distilled water to 100 ml; pH to 6·8 with HCl	
Lower tris (x 4)	
Trizma base	18·17 g
10% SDS in water	4·0 ml
Distilled water to 100 ml; pH to 8·8 with HCl	
Overlay buffer	
3 M tris/HCl pH 8·8	25 ml
10% SDS in water	2 ml
Distilled water to 200 ml	
Electrophoresis buffer	
Trizma base	3 g
Glycine	14·4 g
10% SDS in water	10 ml
Distilled water to 1 litre	

Sample buffer

Tris/HCl pH 6·8	0·05 M
SDS	1%
EDTA	0·002 M
2-mercaptoethanol	0·5%
Glycerol	10%

In distilled water.

Composition of gradient gel solutions

	20%	7%
Acrylamide/bis	6·7 ml	2·6 ml
Lower tris	5·3 ml	5·3 ml
H_2O	3·0 ml	9·0 ml
10% ammonium persulphate[1]	25 ul	25 ul
TEMED[2]	3-7 ul	3 ul

Stacking gel

Acrylamide/bis	1·0 ml
Upper tris	2·5 ml
H_2O	6·5 ml
10% ammonium persulphate[1]	40 ul
TEMED[2]	15 ul

Sealing gel

3 M tris/HCl pH 8·8	1·26 ml
Acrylamide/bis	6·1 ml
10% SDS in water	100 ul
80% sucrose	1·8 ml
H_2O	0·7 ml
15% ammonium persulphate[1]	100 ul
TEMED[2]	8 ul

1 Ammonium persulphate was freshly prepared for each gel.

2 TEMED: N,N,N',N' - tetramethylethyenediamine
(Koch-Light Laboratories Ltd.)
Both TEMED and ammonium persulphate were added immediately before pouring the gel.

tubes and chilled on ice for 15 minutes. Cells were pelleted, washed once in 1 x VB salts, resuspended in growth medium without methionine, and incubated at 37 C for 30 minutes. 5 uCi ^{35}S methionine (The Radiochemical Centre, Amersham, Bucks.) was added and incubation was continued for 5 minutes. The label was chased with 100 ug/ml unlabelled methionine for 2 minutes. Labelled cells were immediately pelleted, washed once in 1 x VB salts, and resuspended in 0·1 ml of final sample buffer (Table 2·7). Samples were heated for 2½ minutes in a boiling water bath, then cooled on ice, and stored at -20 C.

(ii) Separation of proteins on SDS-polyacrylamide gels (Laemmli, 1970). SDS-polyacrylamide gels were poured between 25 cm x 15 cm glass plates which were thoroughly cleaned before use. 1 mm thick perspex spacers were greased lightly and clamped between the plates. The gel was placed vertically in a 30 ml sealing trough, and sealing gel (table 2·7) was poured into the trough to seal the lower edge. When this had polymerised, 10 ml of 7% and 10 ml of 20% SDS acrylamide solutions (table 2·7) were placed in separate chambers of gradient maker. Mixing was started, and the gel was run slowly between the plates to within 5 cm of the top. Overlay buffer was layered on the surface to assist polymerisation. After polymerisation, which was complete after about 30 minutes, the buffer was poured away, and the top was washed with distilled water. Stacking gel was added to fill the gel, and a comb was set into the stacking gel. When this had set, the comb was removed and the wells were washed and

filled with running buffer (table 2·7). The gel was set in place vertically between two troughs of running buffer and protein samples, mixed with bromophenol blue (0·01%) were loaded into the wells using a 100 ul Hamilton syringe. Standard protein mix (which contained Phosphorylase b, 94·0 kD; bovine serum albumin, 67·0 kD; ovalbumin, 43·0 kD; carbonic anhydrase, 30·0 kD; soybean trypsin inhibitor, 20·1 kD; α-lactalbumin, 14·4 kD) was also loaded. Electrophoresis was at 150 V, 10 mA, for 1 hour, then at 350 V, 20 mA, for 10 hours.

(iii) Fixing, staining and autoradiography of polyacrylamide gels. The gel was removed from between the plates, washed in distilled water, then immersed in fixing solution (45% methanol, 9% acetic acid in distilled water) for 10 minutes on a rotary shaker at 37 C. This was removed and staining solution (0·1% Coomassie brilliant blue in fixing solution) was added for 10 minutes. The gel was finally washed in destaining solution (7% acetic acid, 5% methanol in distilled water). The gel was dried down on to Whatman 3 MM paper using a Bio-Rad gel drier, then autoradiographed for 2-3 days at room temperature using X-ray film (Kodak X-Omat H) which had been pre-flashed to increase sensitivity. Film was developed and fixed with Kodak developer and fixer.

2·10 Preparation of probes for use in hybridisation.

(i) Extraction of DNA fragments from agarose gels by 'freeze-squeeze' (Thuring et al., 1975). Up to 100 ug of DNA was completely restricted with an appropriate enzyme,

and loaded into a single large well in a 0·7% agarose gel which contained a trace of ethidium bromide (1 ug of a 10% solution). The fragments were separated by electrophoresis, and the relevant band was excised under UV illumination. The DNA-containing agarose was wrapped in Parafilm, and frozen at -20 C for 30 minutes. Fluid was squeezed from the gel by pressure from thumb and forefinger. 100 ul of TE buffer was added to the agarose, and the process repeated. Any contaminating agarose was removed from the clear fluid by centrifugation, then the DNA was precipitated with ethanol and potassium acetate, and resuspended in 100 ul of TE buffer.

(ii) Separation of restriction fragments on sucrose gradients. Sucrose was dissolved in sterile TE buffer and boiled for 10 minutes to destroy nucleases. 5% and 20% sucrose solutions were placed in separate chambers of a gradient maker, mixing was started, and the gradient was run slowly into a 17 ml polycarbonate centrifuge tube. 5-20% gradients gave good separation of up to 8 kb fragments from λ; larger inserts could not be separated completely. Restricted DNA (50-100 ug) was layered on the surface of the gradient, then centrifuged for 18 h at 22k rpm in the AH627 rotor of a Sorvall OTD 50 centrifuge. Bands were separated by collecting 0·5 ml fractions dropwise from the bottom after puncturing the tube. The fractions containing DNA were identified by running 10 ul of each fraction on an agarose gel. Fractions containing the desired band were pooled, and dialysed overnight or for 2-3 hours against 2 changes of TE buffer. The DNA was precipitated with ethanol and potassium acetate, and

resuspended in TE buffer.

(iii) Labelling DNA by nick translation (Rigby et al., 1977). 15-20 uCi of ^{32}PdCTP (The Radiochemical Centre, Amersham, Bucks.) was dried down under vacuum and resuspended in 10 ul of 10x reaction buffer (50 mM TrisHCl, pH 7·8; 5 mM $MgCl_2$; 10mM 2-mercaptoethanol) with 1 ul each of 10 mM solutions of dGTP, dATP and dTTP, and 1 ug of DNA in TE buffer. 1 ul of DNase 1 (1 ug/ml stock solution; Sigma Type 1 pancreatic) was added and nicking of the DNA was allowed to proceed at room temperature for 2 minutes. DNA polymerase 1 (1 ul of 1 unit/ul) was added and the reaction was continued for 3-4 hours at 15 C. At the end of this time, 5 ul of orange G dye was added, and the DNA separated from reaction substrates by chromatography on a 10 ml Sephadex G50 column in TEN buffer. Unincorporated label elutes with the dye well after the DNA. 5-drop fractions were collected and counted by Cerenkov radiation in a Packard Tri-Carb Liquid Scintillation Counter. Fractions containing peak activity (approximately 40% of total incorporated counts) were pooled and stored at -20 C.

2·11 Assay of DNA homology by liquid hybridisation.

(i) Hybridisation conditions. DNA for use in reassociation experiments was sonicated for a total of 2 minutes at maximum output using the fine probe of an MSE sonicator in 30 second bursts with 30 second pauses for cooling. This treatment generated fragments of 200-400 base pairs in length. Labelled, sonicated probe

Table 2·8 Solutions for assay of DNA homology.

2·5 x HB (Hybridisation buffer)	
NaCl	3·75 M
Tris/HCl pH 7·5	0·025 M
EDTA pH 7·5	0·0025 M
5 x VB (Vogt buffer; Vogt, 1973)	
Glycerol	25%
Sodium acetate, pH4·6	150 mM
$ZnSO_4$	5 mM
S1 mix	
Double-stranded salmon sperm DNA, 1·5 mg/ml	0·24 ml
Denatured salmon sperm DNA, 1·5 mg/ml	0·24 ml
2·5 x HB	0·27 ml
5 x VB	2·9 ml
H_2O	6·35 ml

DNA was mixed with unlabelled, sonicated, test DNA in a total volume of 150 ul in a microfuge tube. The DNA was denatured by placing in a boiling water bath for 5 minutes. 100 ul of 2·5 x HB (table 2·8) was added, and the tube was immediately brought to 65 C, whilst 10 ul was removed into 65 ul of ice-cold water for the 0 time sample. Reassociation was allowed to proceed at 65 C for up to 36 hours, and 10 ul samples were removed at appropriate intervals. Samples were stored at 4 C until assayed.

(ii) S1 nuclease assay. The S1 nuclease assay of Crosa et al., (1973) was used to determine the proportion of single-stranded DNA in each sample of a reassociation experiment. 125 ul of S1 mix (table 2·8) was added to each 75 ul sample, which was then divided into 2 x 100 ul aliquots. 5 ul of a 5-fold dilution of S1 enzyme prepared as described by Sutton (1971) was added to one of each pair of aliquots, and both were incubated at 37 C for 20 minutes. To stop the reaction, the tubes were cooled on ice, and 100 ul of ice-cold 10% TCA was added to both. TCA-precipitable material was collected on 2·1 cm GF/C Whatman filter discs by vacuum filtration, washed with 15 ml of cold 5% TCA, then with 2 ml of ethanol. Filters were dried in air and placed in vials with 5 ml of 0·7% butyl-PBD scintillant in toluene (2(4'-t-Butylphenyl)-5-(4''-biphenylyl)-1,3,4-oxadiazole; Koch-Light Laboratories Ltd. Bucks.) and counted in a Packard Tri-Carb Liquid Scintillation Counter.

CHAPTER THREE

EFFECT OF UV IRRADIATION ON P1 TRANSDUCTION FREQUENCIES.

3·1 Introduction

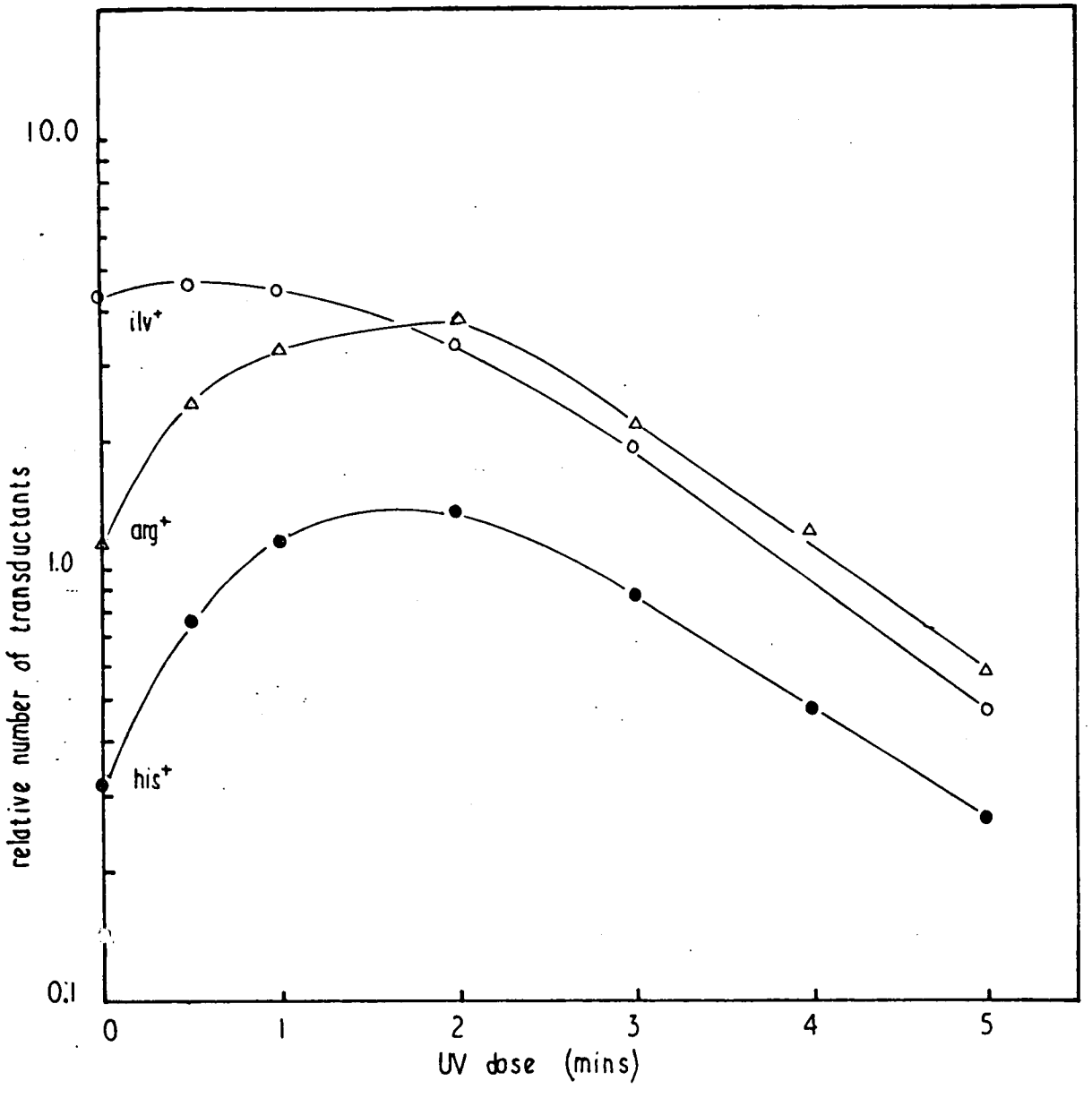
It was pointed out in chapter 1 that there are much greater differences in the recovery of markers after P1 transduction than would be expected from a gene dosage effect alone. Two possible explanations for this were suggested: 1. P1 packages some regions of the *E.coli* chromosome at much higher frequency than other regions or 2. Some transducing fragments are recombined into the recipient chromosome with much greater efficiency than other regions. In order to distinguish between these two possibilities, events occurring in the donor must be dissociated from events occurring in the recipient.

The efficiency with which P1 transducing DNA recombines into the chromosome is quite low: about 85-90% of the transducing DNA fails to recombine into the chromosome and persists as abortively transduced DNA (Sandri and Berger, 1980b). The possibility exists that all markers are packaged by P1 with equal efficiency, and are found among abortive transductants according to their frequency of occurrence in the donor cell. Abortive transductants may be scored for certain markers, particularly those for carbohydrate utilisation, but other markers are almost impossible to score due to minute colony size, or presence of background growth of revertants. Attempts at counting numbers of abortive transductants

Figure 3·1 Transduction of MM303 with irradiated P1.

Liquid lysates of P1 kc made on the prototrophic strain W3110 were irradiated as described in the text, and used to transduce MM303. Numbers of Ilv^+, Arg^+ and His^+ transductants recovered after various periods of irradiation are shown. Irradiation was at a dose rate of 10 ergs/mm^2/sec. Survival of phage is 3×10^{-4} of original titre after 2 minutes irradiation.

Figure 3.1


10.0
1.0
0.1
ilv+
arg+
his+
relative number of transductants
0
1
2
3
4
5
UV dose (mins)

gave variable results and were abandoned.

As already mentioned, treatment of DNA with UV stimulates recombination, increasing the recovery of recombinants in Hfr crosses, and also after P1 transduction using irradiated P1 lysates. It has been proposed that the increased frequency of P1 transduction results from the diversion of transducing DNA from the formation of abortive transductants to the formation of stable transductants (Benzinger and Hartman, 1962). Irradiation of P1 lysates was therefore adopted as an approach to the analysis of the ratio of markers carried by P1 transducing DNA.

3·2 Effect of UV irradiation of phage lysates upon the recovery of transductants.

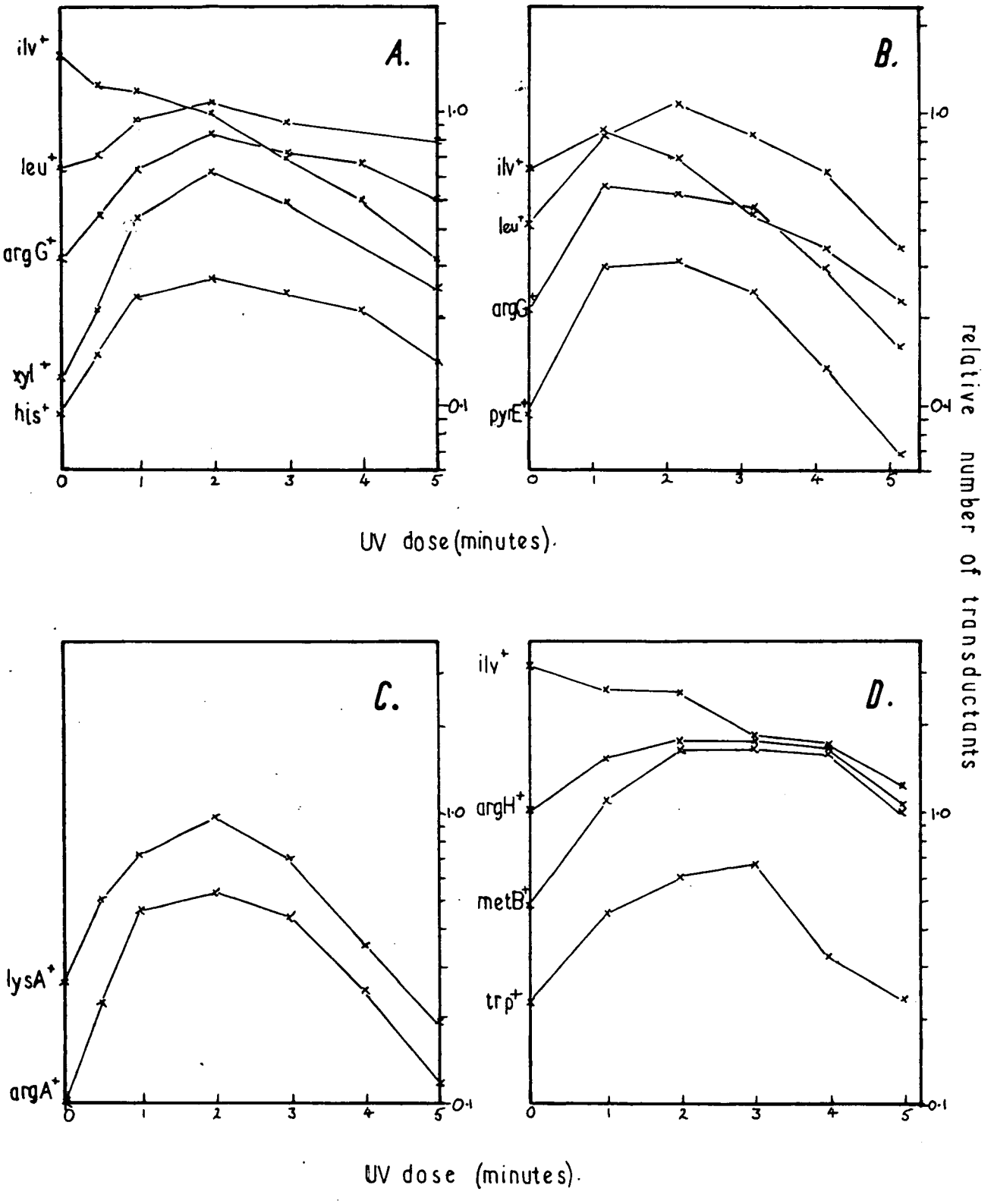
Phage lysates prepared from the prototroph W3110 were irradiated with increasing doses of UV as described in chapter 2, and used to transduce a recipient for a variety of markers. Frequencies of transduction were scored relative to the number of $ArgH^+$ transductants at 0 UV dosage, which was arbitrarily designated as 1.

Figure 3·1 shows the results of an experiment where MM3031 was infected with UV irradiated P1; the frequency of transduction of 3 markers is plotted against UV dosage given to the phage. It can be seen that the stimulation of transduction caused by UV irradiation is not the same for all markers, but does reach a peak for 2 markers at a dose of 2 minutes, or 1 200 ergs/mm^2. The recovery of

Figure 3•2 Frequencies of transduction of a variety of markers into four different recipients using irradiated P1.

Transductions were as described in the text, using P1 irradiated for 0-5 minutes. Recipients used were: (a) NF279 (b) MM7 (c) CP154 (d) MM3031. Transductions in (a) and (b) used identical P1 lysates; (c) and (d) used identical lysates.

Figure 3.2


A.
B.
C.
D.
ilv+
leu+
argG+
xyl+
his+
pyrE+
lysA+
argA+
argH+
metB+
trp+
1·0
0·1
0
1
2
3
4
5
UV dose (minutes).
relative number of transductants

the marker Ilv, which is normally transduced with a very high frequency, is not stimulated to any significant extent. In contrast, recovery of His^+ transductants, which normally occurs at very low frequency, is stimulated ten-fold. Arg^+ transductants are normally recovered at an intermediate frequency, but UV irradiation results in increased recovery such that after 1 200 ergs of UV there is little difference between the recovery of Ilv^+ and Arg^+ transductants.

Figures 3·2 a, b, c and d show similar experiments with the unrelated strains NF279 and CP154, and MM7, which is related to NF279, and other markers from MM303. Some general points can be drawn from these results:

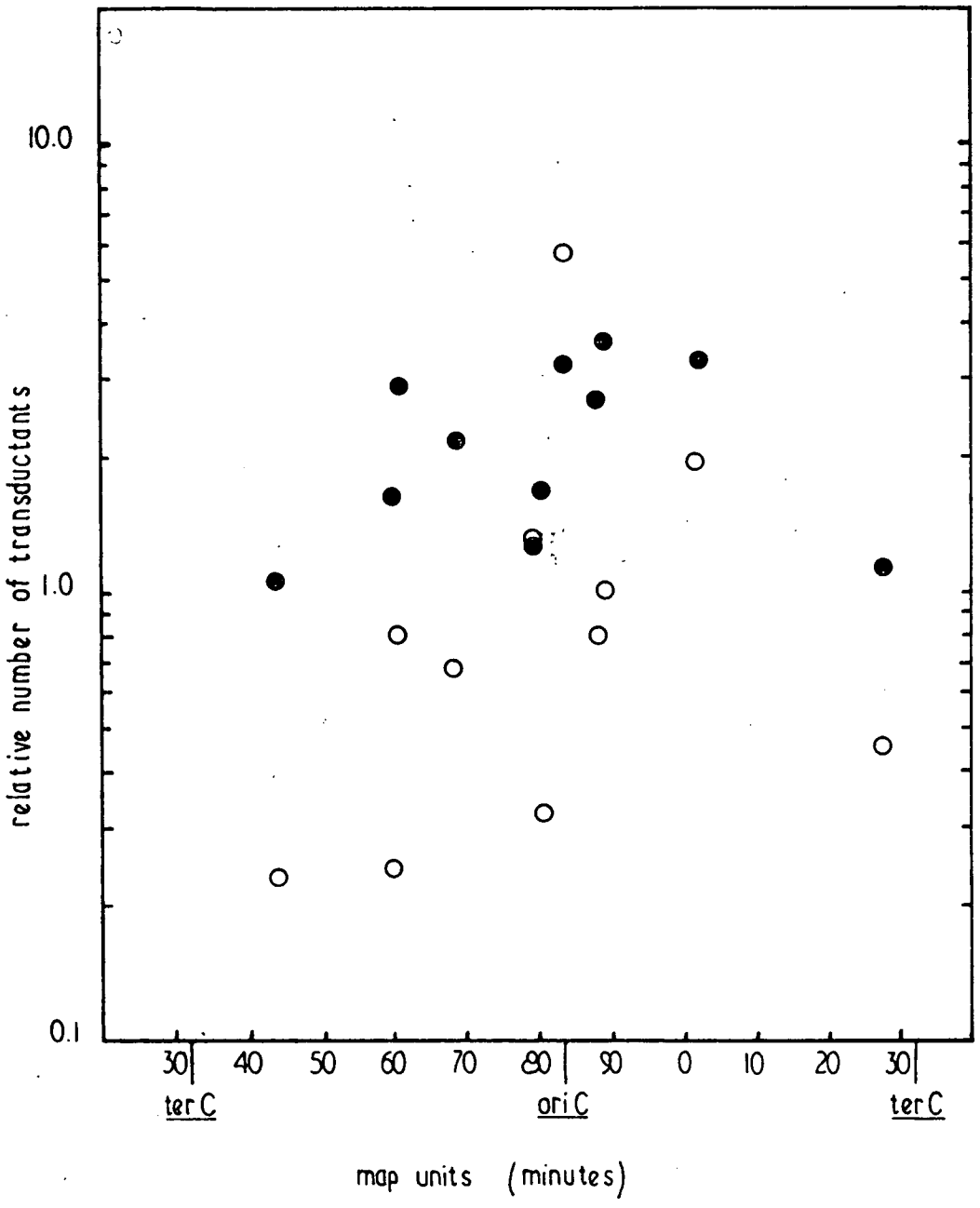
1. Transduction of 'early' markers argH, metB, ilv and xyl become nearly equal; tranduction of ilv is either very slightly stimulated or decreases, presumably depending upon the fine balance between the transduction stimulating and the DNA damaging effects of UV irradiation.
2. Transduction of 'late' markers trp and his are also equalised, and stimulated to a great extent.
3. Transduction of intermediate markers argA and lysA is stimulated, but not to the same level as the early markers.

There is one discrepancy; recovery of Leu^+ transductants, an intermediate marker, is stimulated to exceed the recovery of Ilv^+ transductants.

Figure 3·3 Frequency of transduction as a function of time of replication.

Points represent the number of transductants for individual markers relative to the frequency of transduction of Arg^+ transductants recovered before irradiation of phage, and each was calculated from the average of 2-5 separate experiments. Recipients used were: MM303, MM7, CP154 and NF279. Open circles, before irradiation; closed circles, after 2 minutes UV irradiation to phage.

Figure 3.3


10.0
1.0
0.1
relative number of transductants
30
40
50
60
70
80
90
0
10
20
30
terC
oriC
terC
map units (minutes)

From these collected data a pattern emerges: the recovery of transductants after maximum stimulation with UV can be related to the position of the marker on the E.coli map. Figure 3·3 shows a plot of frequency of transduction before and after 2 minutes of irradiation as a function of the relative time of replication. It can be seen that the initial 25-fold differences in transduction frequencies between well- and poorly-transduced markers is reduced to an approximately 3·5-fold difference. Transduction frequency is, in part, a function of gene frequency in the donor cell; transducing lysates are produced from cells in the log phase of growth and the particular culture used to produce the P1 lysate for these experiments had a generation time of 28'. The cells would be expected to have an origin: terminus ratio of markers of 2·7 due to multiple replication forks. Thus, after UV irradiation, the ratio of origin to terminus markers recovered approaches that expected from a gene dosage effect alone, and the large differences normally observed are greatly diminished. These results strongly suggest that transducing lysates contain bacterial genes in proportions equivalent to their frequencies in the donor cells.

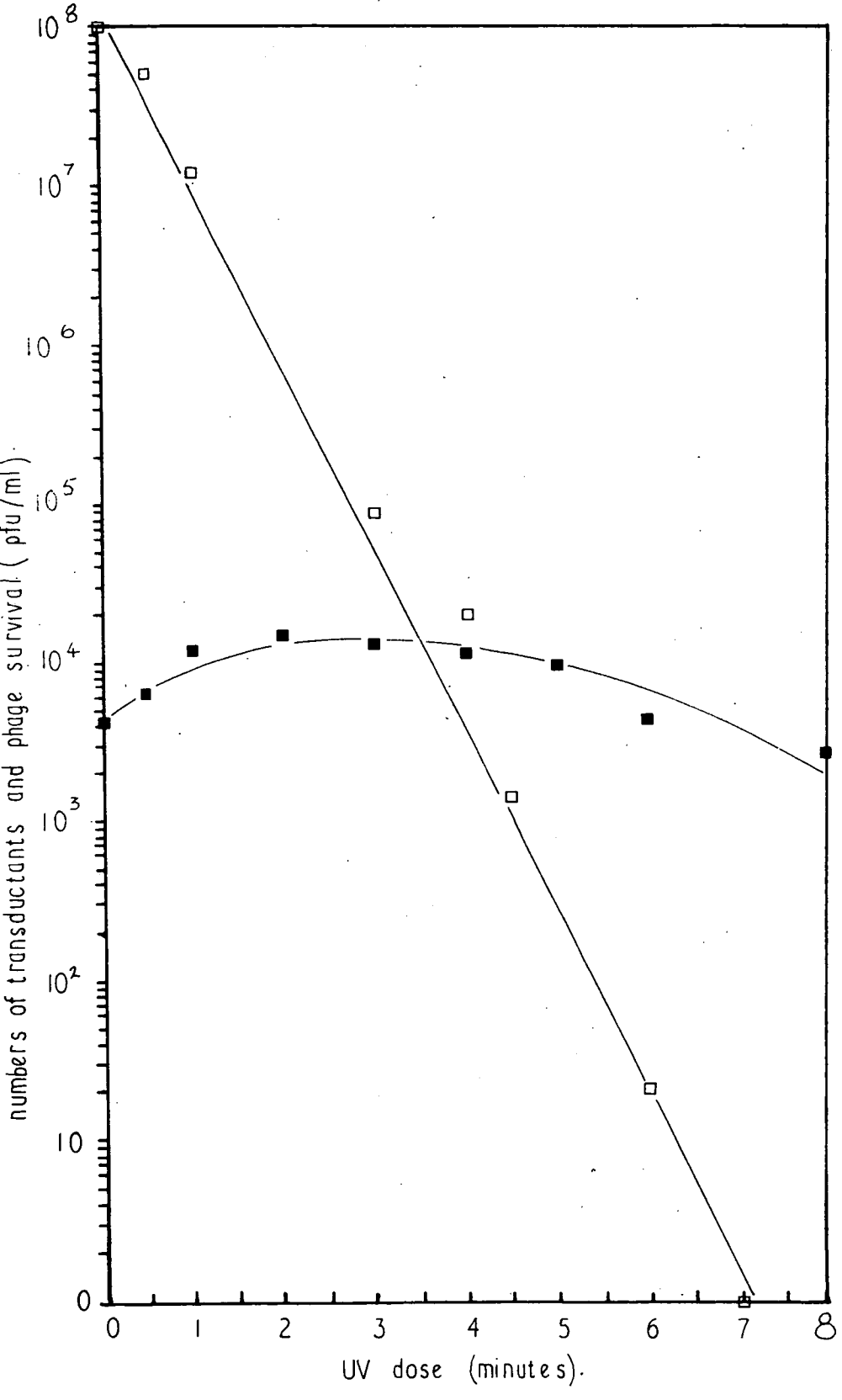
3·3 An inducible factor is not responsible for stimulating transduction.

Irradiation of the P1 transducing particle enables the transducing DNA to bypass some selective process in the recipient. The next experiment was designed to

Figure 3·4 UV survival curve for P1, and numbers of transductants at increasing UV dosage.

P1 was irradiated as described in the text, and pfu/ml was determined by titration. Open squares represent pfu/ml plotted against UV dosage (10 ergs/mm^2/sec). Closed squares represent numbers of Arg^+ transductants obtained from the irradiated lysate.

Figure 3.4


numbers of transductants and phage survival (pfu/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
0 1 2 3 4 5 6 7 8
UV dose (minutes).

distinguish between the following two possible causes of the observed stimulation: 1. The observed stimulation results from an increased concentration in the cell of a factor involved in general recombination which is induced by UV damage in DNA, 2. the tranducing DNA becomes a better substrate for the normal cellular recombination as a result of cleavage caused by UV.

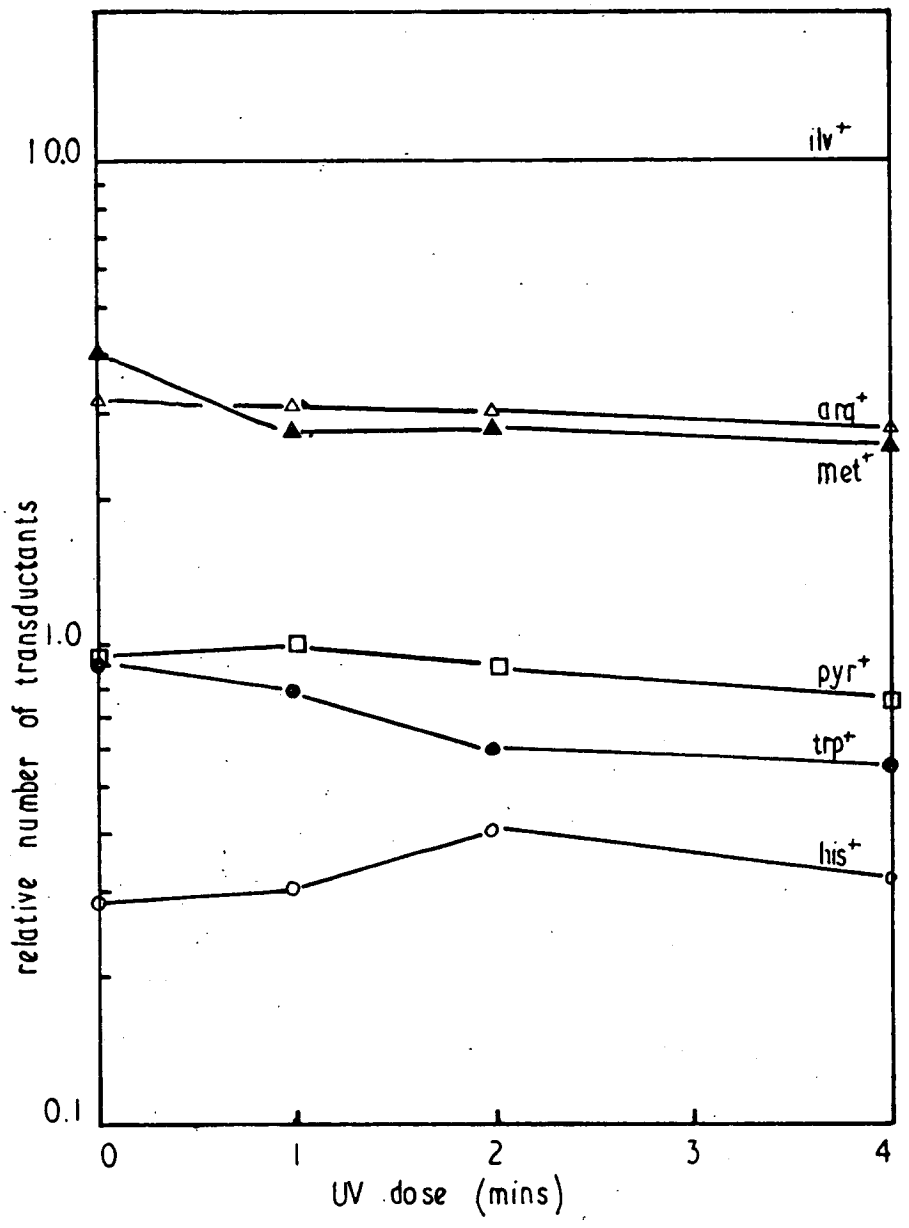
These two possibilities can be distinguished by introducing UV-damaged, non-transducing DNA into the recipient, simultaneously with undamaged, transducing DNA.

Non-transducing phages were grown on MM3031, a derivative of MM303 which was used as the recipient, then concentrated by PEG precipitation and banding on a CsCl step gradient to a titre of $1{\cdot}7 \times 10^{12}$ pfu/ml. An aliquot of this preparation was diluted 100-fold in phage buffer, then irradiated with increasing doses of UV as previously. 0·3 ml of the irradiated phage was mixed with 0·1 ml of phage prepared on W3110 (titre 8×10^{8} pfu/ml) and added to the prepared recipient cells. The non-transducing phage was present at a final multiplicity of 4·5 thus ensuring that almost every cell in the recipient population would be infected with an irradiated phage. The damage caused by irradiation does not impair the ability of the P1 particle to inject DNA into the recipient cell since it is possible to recover transductants when phage have been irradiated such that they are completely inviable (figure 3·4). This, and the use of a P1 lysogen as a recipient to prevent phage killing, ensures that each cell received some damaged DNA without impairing viability. Transductants

Figure 3·5 Transduction of MM303 with a mixture of unirradiated transducing phage and irradiated non-transducing DNA.

P1 grown on MM3031 and concentrated in CsCl to a titre of $1{\cdot}7 \times 10^{12}$ pfu/ml was diluted 100x in phage buffer and irradiated as described in the text. 1 minute of UV at a dose rate of 10 ergs/mm^2/sec reduced phage titre by 50x. 0·3 ml of irradiated phage was mixed with 0·1 ml of P1 prepared on W3110 (8×10^8 pfu/ml) and added to the recipient cells. Numbers of transductants are shown relative to the number of Ilv^+ transductants recovered, and plotted against UV dosage.

Figure 3.5


10.0
1.0
0.1
relative number of transductants
ilv+
arg+
met+
pyr+
trp+
his+
0
1
2
3
4
UV dose (mins)

can only arise as a result of recombination between the chromosome and undamaged transducing DNA from the P1 lysate grown on W3110, since the irradiated lysate carries the same negative alleles as the recipient.

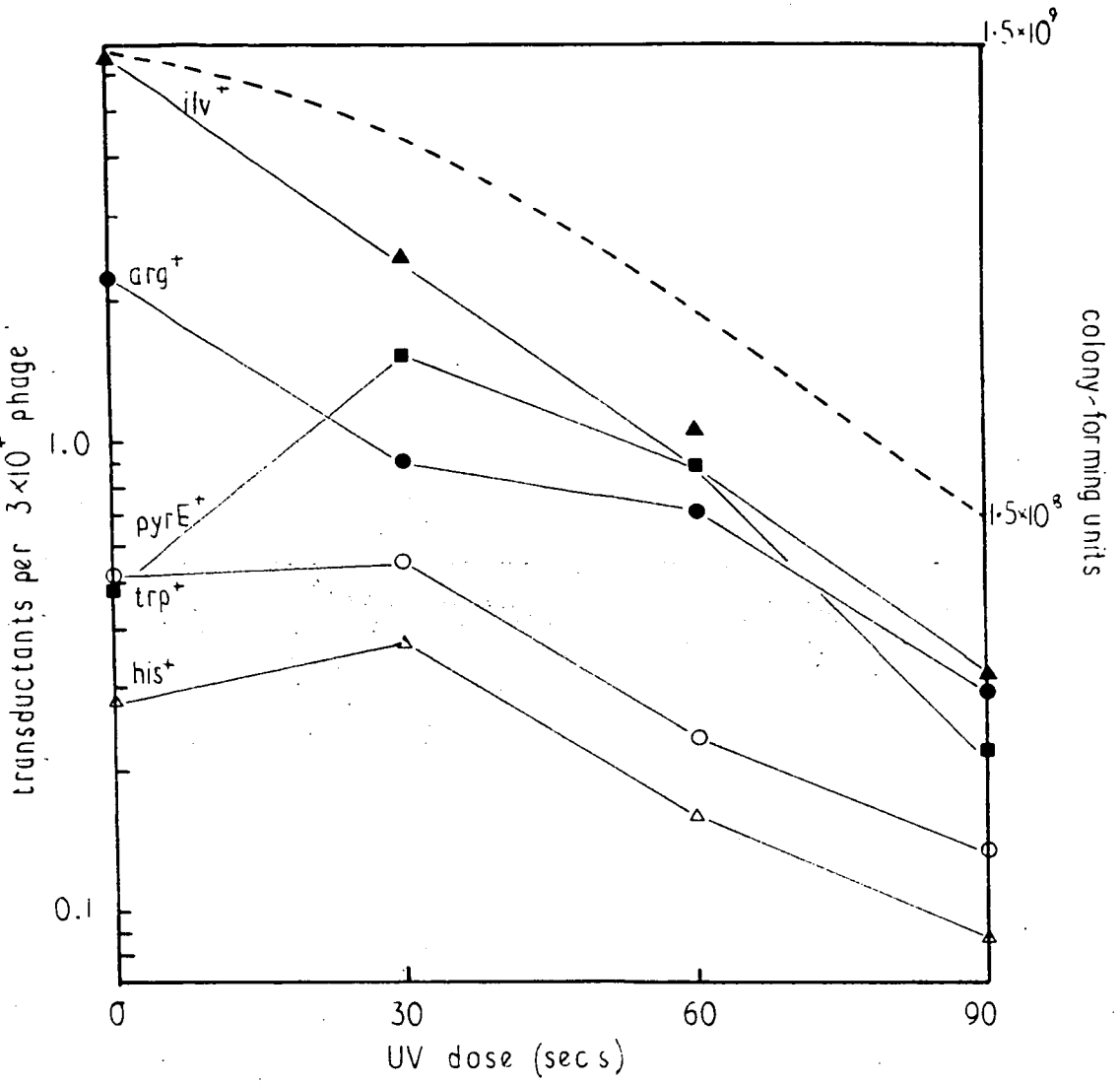
The results of this experiment are shown in figure 3·5. It can be seen that the presence of highly damaged DNA has very little effect upon the recovery of stable transductants. None of the markers tested shows the stimulation in recovery relative to the recovery of Ilv^+ transductants found in previous experiments. Thus it does seem more likely that the stimulation of transduction arises because the recombining DNA is a better substrate for recombination enzymes, rather than because there is an inducible factor in the cell which raises the general level of recombination.

3·4 UV irradiation of recipient cells prior to transduction.

To show that damage to donor DNA is not required for the stimulation of transduction, the recipient cells were irradiated before transduction, rather than the transducing lysate.

Cells were resuspended in phage buffer and irradiated as described, then resuspended in LBC and infected with a P1 lysate grown in W3110 at a multiplicity of about 1. Numbers of transductants for six markers recovered after various periods of irradiation are shown in figure 3·6. It can be seen that numbers of Ilv^+ and Arg^+ transductants fall sharply as the viability of the recipient decreases. However, recovery of poorly transduced markers *his*, *trp* and

Figure 3.6



Transduction of irradiated cells with P1 phage.

Cells of MM303 were irradiated as described in chapter 2 and transduced as described in the text with unirradiated P1. Numbers of transductants for various markers recovered after times of irradiation shown are plotted, together with survival of colony-forming units.

pyrE increases relative to the recovery of Ilv$^+$ at a low UV dosage. At maximum stimulation, as in previous experiments, the 20-fold differences in frequencies of recovery are reduced to about 3-fold differences. Thus, irradiation of the recipient results in a similar pattern of stimulation as does irradiation of the donor phage.

3.5 Role of repair enzymes in the stimulation of P1 transduction by UV.

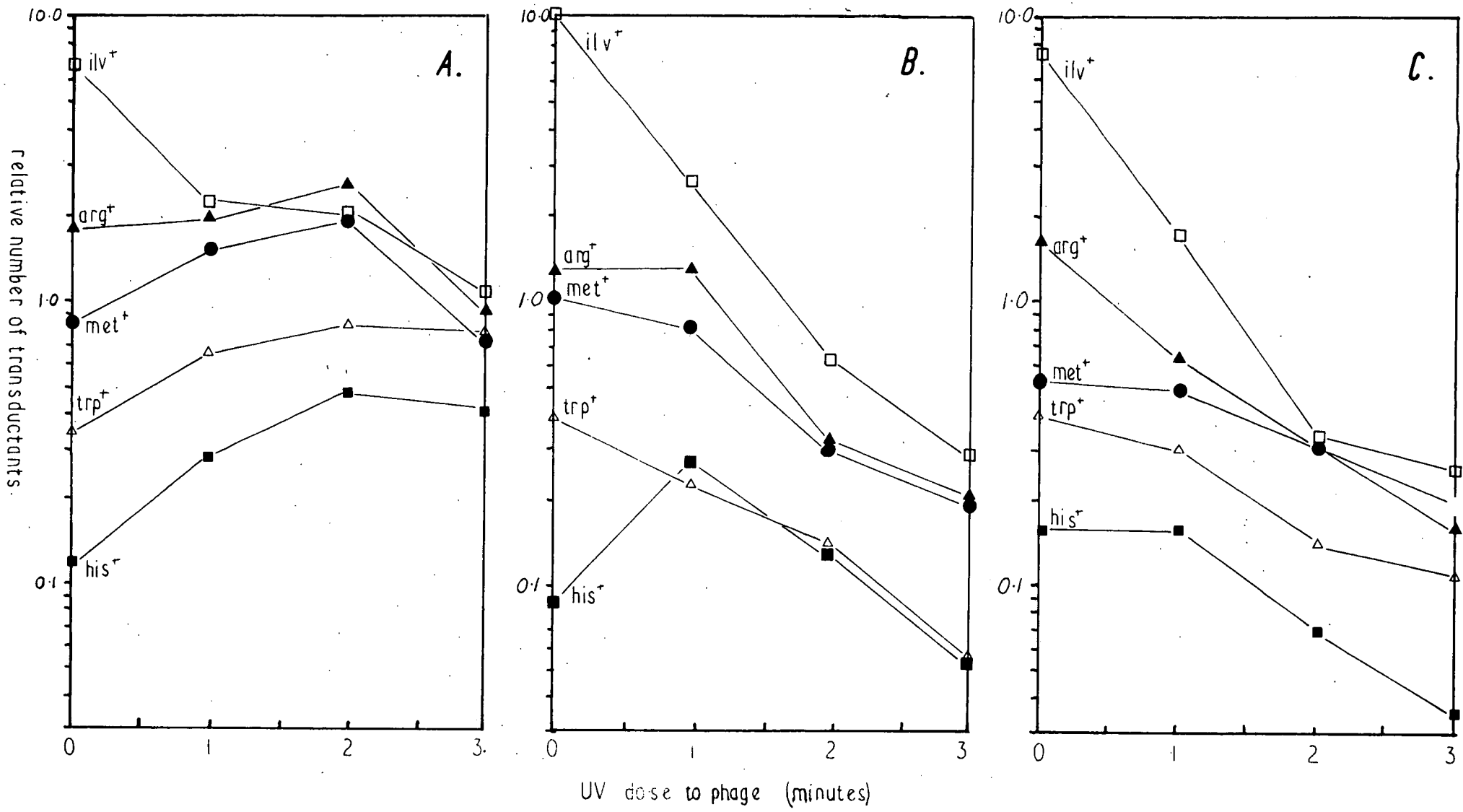
In a further investigation of the mechanism of the stimulation of transduction, recipients deficient in excision repair were used in UV transduction experiments.

Suitable recipients were constructed by introducing uvrA$^-$, uvrB$^-$ and uvrC$^-$ mutations into MM3031. Because these give an unselectable phenotype, the mutations were first linked to the transposon Tn 10 by the method of Csonka and Clark (1979) described in chapter 2. AB1884 (uvrC), AB1885 (uvrB) and AB1886 (uvrC) were infected with P1 (W3110::Tn10), and tetracycline-resistant colonies were selected on LB plates supplemented with 15 ug/ml of tetracycline. 200-300 clones were picked and patched on two LB-tetracycline plates, one of which was exposed to 500 ergs UV. UV resistant, tetracycline resistant clones would have Tn10 closely linked to the uvr$^+$ marker. To link to the negative allele, P1 lysates made on these strains were used to infect the parental, uvr$^-$ strain, and tetracycline resistant clones were selected. These were patched and tested for UV sensitivity. Tetracycline resistant, UV sensitive strains have the Tn10 element linked to the negative

Figure 3·7 The role of excision repair enzymes in UV-stimulated recombination.

P1 transductions were performed as described in the text using P1 which was irradiated with UV for 0, 1, 2 and 3 minutes at a dose rate of 10 ergs/mm^2/second. Recipients used were A. MM3031, B. MM3031 *uvrA*, C. MM3031 *uvrB*.

Figure 3.7


A.
B.
C.
ilv+
arg+
met+
trp+
his+
10·0
1·0
0·1
0
1
2
3
relative number of transductants
UV dose to phage (minutes)

allele, and were used as donors to transduce *uvr*$^-$ mutations into MM3031 by selecting for tetracycline resistance, and screening for UV sensitivity.

The resulting strains MM3031 *uvrA*$^-$ and *uvrB*$^-$ were used as recipients for transduction experiments. The *uvrC*$^-$ mutation could not be transferred to a suitable recipient; although it was apparently linked to Tn10 the Tn10 insertion seemed to render the strain non-transducible by P1. This insertion may have been in a gene similar to *tdi*, described earlier.

The results of transduction of MM3031, MM3031*uvrA*$^-$ and MM3031*uvrB*$^-$ are shown in figure 3·7. Transductions were as previously using P1 lysates exposed to UV at a dose rate of 100 ergs/mm^2 for 0, 1, 2 and 3 minutes.

It can be seen that the *uvrB*$^-$ mutation completely eliminates UV-mediated stimulation of transduction of all markers, while the *uvrA* lesion eliminates stimulation of transduction of the markers *ilv*, *argH*, *trp*, *pyrE* and *metB*, but transduction of *his* is still stimulated at a low UV dose, but not to such an extent as in the parental strain MM3031.

3·6 Discussion.

These results clearly show that there is some discrimination by the recipient in the frequency with which different fragments of transducing DNA are integrated into the chromosome, and that this discrimination can be eliminated by the introduction of UV damage into the recombining DNA. It is possible that some regions of the

chromosome are more susceptible to damage by UV than are others, and that the damage-stimulated recombination is concentrated on these fragments. The susceptibility of each marker to UV is indicated by the rate at which recovery of that marker decreases from the point of maximal stimulation due to inactivation of the relevant gene. Slight differences can be seen between the markers (figures 3·1 and 3·2), but these do not negate the major conclusion that the differences in P1 transduction arises from a selective recombination in the recipient.

Most markers follow a similar pattern; the observed frequency of transduction after irradiation being dictated by the relative time of replication. However, the recovery of some markers diverges from this pattern, most notably the marker leu, at 2 minutes on the chromosome, which is recovered at greater frequency after UV treatment than are origin markers. It is therefore possible that there is some bias towards packaging of certain regions by P1, but the experiments reported here indicate that this is not primarily responsible for the discrepancies in transduction frequencies. It was indicated in chapter 1 that Sternberg et al.(1980) have shown preferential integration at and consequent packaging from one site on the E.coli chromosome. This site was found to be at 67 minutes on the E.coli chromosome, and although this is not a high transducing region, other sites for preferential integration may exist. A more detailed analysis of the DNA content of transducing particles is necessary to confirm these conclusions, and this is

presented in chapter 7.

In section 3·3, it was demonstrated that the damage stimulated transduction is probably not caused by an inducible factor. Introduction of damaged replicons into the E.coli cell is known to elicit a set of co-ordinated responses, the SOS response (Rosner et al., 1968; see also chapter 5), which includes the induction of some repair pathways (Kenyon and Walker, 1981). The introduction of damaged linear DNA in the form of Hfr DNA does not induce this response, and it is probable that damaged transducing DNA will similarly not induce SOS. Damaged transducing DNA, then, is simply a better substrate for the cell's recombination system.

The experiments descibed in section 3·6 show that uvrA$^+$ and uvrB$^+$ gene products are necessary for damage-stimulated transduction, and are in agreement with the results of Helling (1973). In addition, he found that uvrC$^+$ gene product was not essential, and he hypothesised that an initial step in general recombination could be bypassed by the action of uvrA and uvrB nucleases to produce single-strand ends. Complete excision of the damaged DNA, which requires the uvrC gene product, is not essential. Helling found that there was still a slight initial stimulation in the uvrA$^-$ and uvrB$^-$ strains, and he proposed that this was due to the presence of another enzyme which could partially replace the uvrA and uvrB nucleases, but that most UV-induced recombination was dependent upon this function.

Clark and Volkert (1978) proposed an additional

pathway for recombination to explain this phenomenon; incision-promoted repair. Damaged DNA, then, may bypass an initial step in recombination which is responsible for the selective recovery of markers after transduction, and all damaged DNA is repaired, and thus recombined, with equal efficiency. The nature of the initial step is not clear, and this is investigated further as described in subsequent chapters.

CHAPTER FOUR

EFFECT OF REDUCED SUPERHELICITY OF THE RECIPIENT CHROMOSOME ON P1 TRANSDUCTION.

4·1 Introduction.

As stated in chapter 1 the E.coli chromosome is organised into a series of loops stabilized by a core of RNA and protein; each loop forms a domain of supercoiling. The folding of the chromosome may limit the accessibility of some parts of the chromosome to enzymes involved in recombination or repair. If this were so altering or disrupting the three dimensional structure of the E.coli chromosome might selectively alter the frequency of recombination of transducing DNA.

E.coli DNA gyrase (topoisomerase II) introduces negative supercoils into the E.coli chromosome in a reaction dependent upon ATP hydrolysis (Cozzarelli, 1980). The enzyme acts by passing a double helical DNA segment through a transient double stranded break in DNA (Mizuuchi et al., 1980). DNA gyrase activity is produced by a complex of two subunits; molecular weights 100 000 and 90 000 coded for by genes gyrA (nalA) and gyrB (cou) respectively (Mizuuchi et al., 1978). The enzyme is the target of several antibiotics: coumermycin and novobiocin affect the gyrB subunit; and nalidixic and oxolinic acids affect the gyrA subunit. The action of these antibiotics is to reduce the degree of supercoiling of the E.coli chromosome (Drlica and Snyder, 1978; Gellert et al., 1976b). The two subunits appear to function in different steps of the supercoiling reaction: the gyrA

subunit is responsible for the breakage and reunion activity of the enzyme, while the gyrB subunit is involved in the binding of ATP (Mizuuchi et al., 1978).

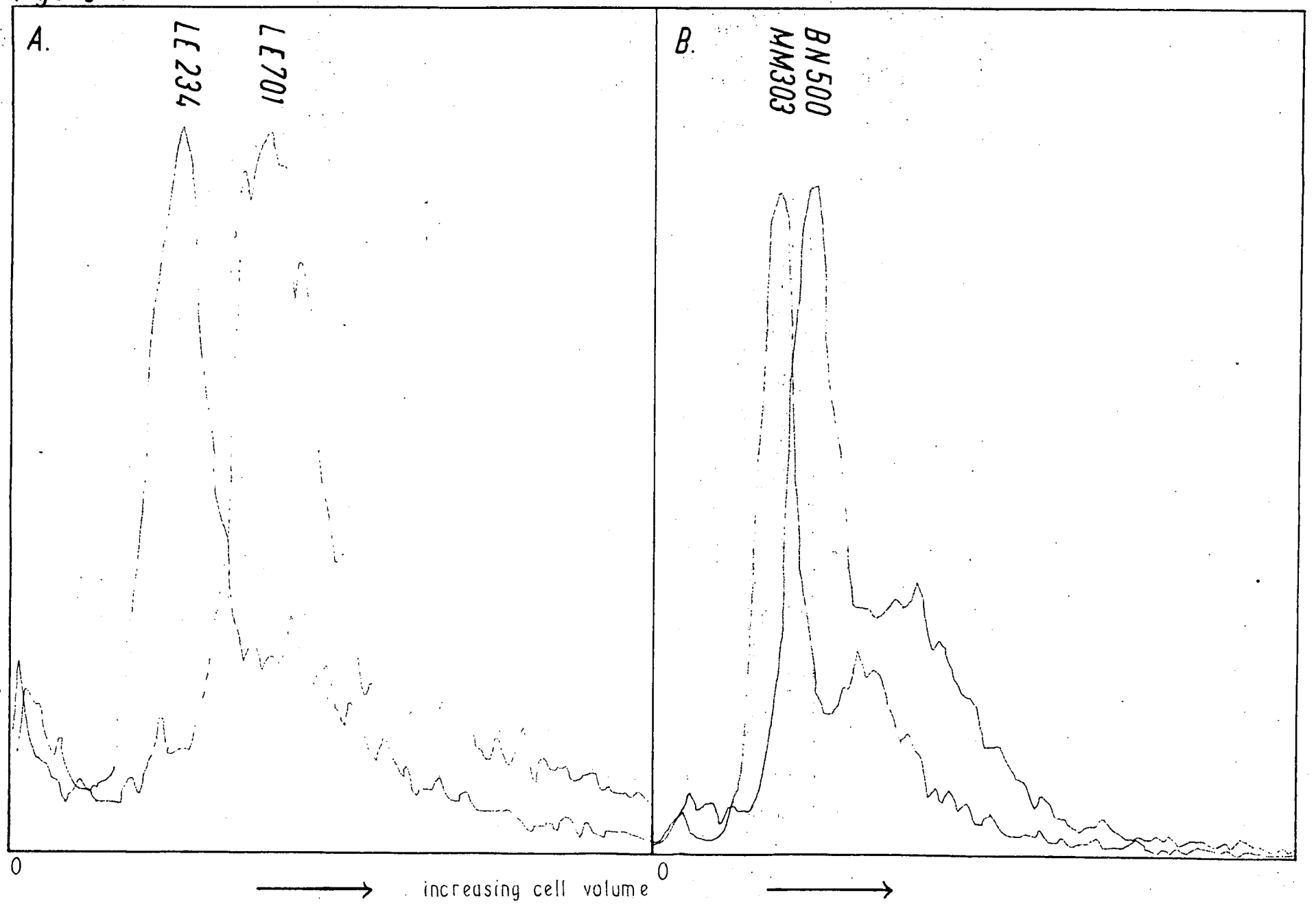
The precise physiological role of DNA gyrase is not known, although the inhibitory effects of nalidixic acid on DNA replication and transcription (Botcham et al., 1973; Kreuzer and Cozzarelli, 1979) and of novobiocin and coumermycin on DNA synthesis and transcription (Fairweather et al., 1980) indicate the necessity of DNA gyrase activity for these processes. Orr et al., (1979) isolated a conditional lethal mutant in the gyrB gene which showed aberrant division and disorganisation of nuclei at the non-permissive temperature, suggesting that DNA gyrase is essential for the structural organization of the chromosome and its normal association with the cell membrane.

It seemed likely that reduced supercoiling in the chromosome of E.coli strains mutant in DNA gyrase activity may result in disruption of the normal three-dimensional organisation of the chromosome. Two mutants were chosen to investigate the effect of reduced supercoiling on recombination of transducing fragments in the recipient cell: LE701, a gyrB mutant (Fairweather et al., 1980) and MH5, a gyrA mutant. LE701 was isolated as a coumermycin resistant mutant and it has a tendency to form chains and filaments during normal growth. It has an approximately 30% reduction in DNA : cell mass ratio compared to the parental strain, and an increased cell mass, which together indicate that initiation of DNA replication is delayed.

Figure 4·1 Comparative cell volume of parental and gyr mutant strains.

Cell volumes were determined using a Coulter counter as described in chapter 2. The figure shows a plot of number of cells against increasing cell volume for the two pairs of strains: A. LE234 (parental) and LE701 (gyrB); B. MM303 (parental) and BN500 (gyrA).

Figure 4.1


A.
LE 234
LE 701
B.
MM303
BN 500
number of cells
0
0
0
increasing cell volume

4·2 Transduction of gyrase mutants.

The effect of the gyrB mutation on P1 transduction was determined by comparing the recovery of stable transductants from the gyrB mutant LE701 and from the parental strain LE234. LE701 is resistant to 70 ug/ml of coumermycin and has a larger average cell volume than the parent (figure 4·1A).

The gyrA mutation in MH5 was in a prototrophic background, so a suitable recipient for transduction was constructed by transducing the mutation from MH5 into MM303 which has a number of auxotrophic markers. Selection was for nal^R on LBC plates containing 70 ug/ml nalidixic acid. The resulting nal^R strain (BN500) had all the auxotrophic markers of MM303, was resistant to 70 ug/ml nalidixic acid and had a larger average cell volume as determined using a Coulter counter (figure 4·1B)

Transduction was performed as outlined in chapter 2 using a P1 lysate of titre $5·3 \times 10^9$ pfu/ml grown on W3110. Transductants were scored on selective plates and the results of experiments using gyr mutants and parental strains are shown in table 4·1. Also shown are the relative frequencies of transduction.

It can be seen that there is little alteration in the relative frequencies of transductions in the two mutant strains when compared to the parental strains. However, absolute frequencies are reduced. The gyrA mutant, MH5, shows approximately 50% reduction in recovery of all markers, while the gyrB mutant shows a 5-6 fold decrease.

Table 4·1 Transduction of gyr mutants.

A. Effect of gyrB on transduction.

Recipient	Number of transductants per 1×10^7 phage, and (below) relative ratio (No. of Pro^+ = 1)			
	Ilv^+	Met^+	Pro^+	Arg^+
LE234	535	59	51	87
	10·5	1·2	1·0	1·7
LE701	130	11	7	17
	18·6	1·6	1	2·4

B. Effect of gyrA on transduction.

Recipient	Number of transductants per 5×10^7 phage, and (below) relative ratio (No. of Arg^+ = 1)					
	Arg^+	Met^+	Ilv^+	His^+	Trp^+	Pyr^+
MM303	695	630	2376	153	252	220
	1·0	0·91	3·4	0·22	0·36	0·32
BN500	308	244	1325	67	113	102
	1·0	0·79	4·3	0·22	0·37	0·33

4·3 Discussion.

Mutations affecting DNA gyrase result in a highly pleiotropic phenotype which includes reduction in the rate of DNA and RNA synthesis and cell division. These may be due to a direct interaction of the enzyme in these processes but are more likely to be caused by a general disruption of DNA metabolism by a loss of supercoiling. The ubiquitous occurrence of negative supercoiling to the same extent in circular DNA isolated from all natural sources (Cozzarelli, 1980) suggests its fundamental importance. Supercoiled DNA is known to be necessary for site-specific recombination (Mizuuchi and Nash, 1976) and also increases the rate of initiation of RNA synthesis by E.coli polymerase *in vitro* probably by facilitating helix unwinding at the promoter (Botcham et al., 1973).

These results show that P1 transduction into a DNA gyrase mutant is severely reduced, and this may point to a general effect upon the mutant cells' ability to perform general recombination. Negative superhelicity is also essential for the activity of RecA protein in unwinding DNA to initiate recombination (Shibata et al., 1979). The reduced P1 transduction frequencies may simply be a manifestation of the reduced ability of RecA protein to initiate recombination with the mutant chromosome.

Since it has not been established that the gyrase mutants have an altered chromosome structure brought about by reduced supercoiling it cannot be concluded that

there is no effect caused by the alteration of the three-dimensional structure of the chromosome upon P1 transduction frequencies.

CHAPTER FIVE

ROLE OF RECA PROTEIN AND CHI SITES IN P1 TRANSDUCTION

5·1 INTRODUCTION

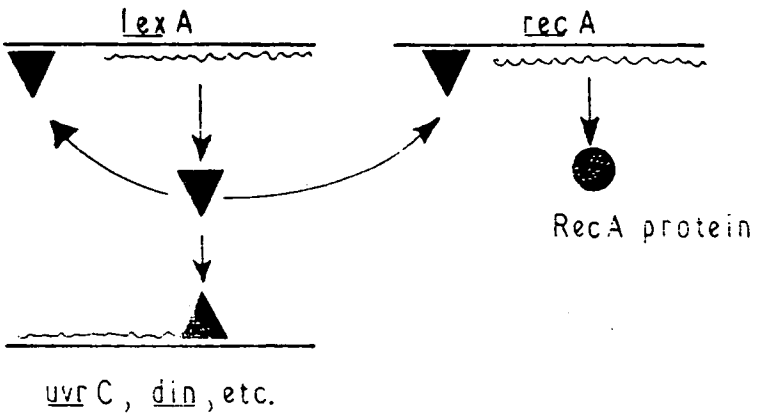
In chapter 3 it was shown that UV irradiation stimulates transduction by P1, both after irradiation of the phage, and of the recipient cell, resulting in a consequent loss of discrimination during integration of transducing DNA. The experiments in this chapter were designed to investigate how this discrimination arises.

RecA protein is essential for homologous recombination and also for efficient P1 transduction, and it could be envisaged that it has a greater affinity for some areas of the chromosome and that these regions would therefore more frequently undergo recombination events. By increasing the concentration of RecA protein in the cell, areas with less affinity for the protein might be expected to undergo recombination with greater frequency. The experiments of Lloyd (1978) encouraged this reasoning; he found that by increasing the concentration of RecA protein in the cell, the number of genetic exchanges in Hfr x F^- crosses is greatly elevated. It was possible that a similar effect might be observed with P1 transduction and so a similar experiment was performed.

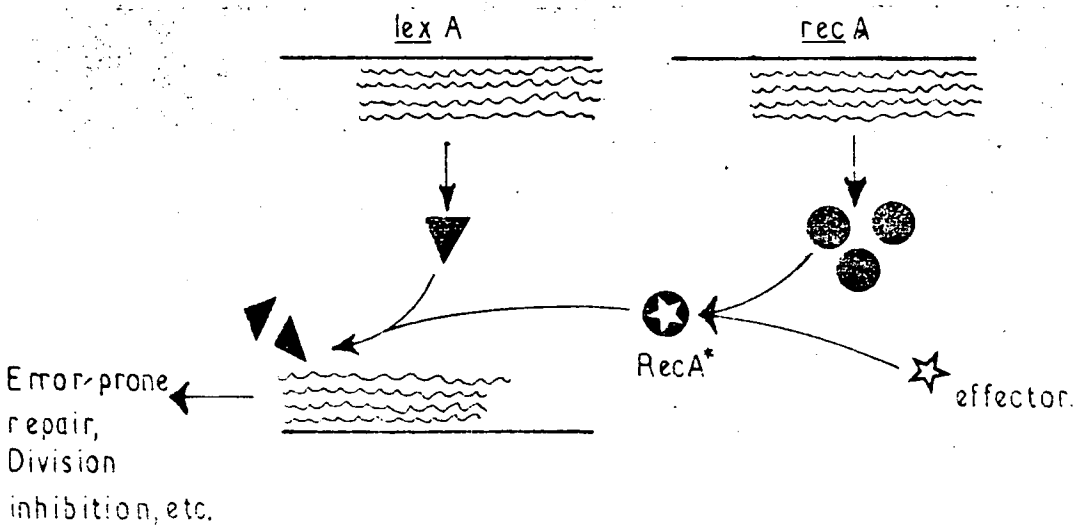
Initially, to boost the concentration of RecA protein in the cell, mutants which over-produce RecA protein described by Mount (1977) and used by Lloyd (1978)

Figure 5.1

A. Repression.



B. Induction. "SOS"



The SOS response. See text for full explanation.

A. lexA protein (large triangles) normally represses recA and other din genes, and also its own promoter.

B. An effector causes the conversion of RecA protein (large circles) to RecA*, a specific protease, which cleaves the lexA repressor thus allowing increased production of RecA protein and increased expression of other din genes which lead to the other manifestations of SOS.

were used as recipients in P1 transduction experiments. These strains are mutants in the control of RecA protein synthesis, which is a complex system elucidated during investigations of the E.coli response to DNA damage. Normally the recA gene is repressed by the lexA product and RecA protein is synthesised at a basal level sufficient for homologous recombination. When E.coli DNA is damaged by such treatments as UV irradiation, the cell reacts by initiating a set of coordinated actions, known collectively as the SOS response (Witkin, 1976). The response begins with the association of RecA protein with an effector which alters its conformation such that it can act as a very specific protease (McEntee, 1977; Craig and Roberts, 1980). A model for control postulates that this protease, or RecA*, cleaves the lexA gene product which represses the recA gene and thus RecA protein synthesis is derepressed to such an extent that it constitutes 3% of total cell protein (figure 5·1).

The lexA gene product also represses a number of other genes, including its own promoter (Brent and Ptashne, 1980) which are consequently expressed as part of the SOS response. These include; uvrA and uvrB, increased expression of which results in increased repair of chromosomal and incoming damaged DNA (Kenyon and Walker, 1981); a cell division inhibitor, which leads to filamentation; and a group of damage inducible or din genes whose function is not yet clear but may account for increased mutagenesis by the induction of an error-prone DNA polymerase (Kenyon and Walker, 1980). RecA* also

cleaves λ repressor protein, and thus lysogens are induced (Roberts et al., 1978).

The nature of the effector is a matter for conjecture; any impediment to DNA synthesis, including thymine starvation, mitomycin C treatment, disruption of the replication fork by denaturation of dnaB, dnaG or polC proteins induces the SOS response (Oishi et al., 1978). One suggestion is that the inducing signal may be oligonucleotides arising from degradation of damaged DNA (Oishi and Smith, 1978).

A mutation at the recA locus, tif, or recA 441 (temperature inducible filamentation; Castellazzi et al., 1972) mimics the SOS response at 42 C in all respects including overproduction of RecA protein. The tif product is temperature sensitive and assumes the RecA* protease conformation at 42 C (Emmerson and West, 1977). The filamentation response leads to poor viability, but a second mutation sfiA (suppressor of filamentation; George et al., 1975) specifically suppresses the filamentation respone at 42 C, but has no effect on the other temperature induced responses. The tif-1 sfiA strain GC3217 overproduces RecA protein at 42 C and synthesis is elevated at 37 C. Mount (1977) isolated another RecA protein overproducing mutant DM1187 from a lexA tif-1 sfiA strain, which is non-inducible and very sensitive to mutagenic treatments. DM1187 expressed SOS functions constitutively at normal temperatures due to a second mutation at the lexA locus, spr which stops production of the lexA repressor. DM1420 is tif$^+$ and

hence is derepressed for RecA protein synthesis but not for other SOS functions.

A second strategy to increase the concentration of RecA protein in the cell was later used. The recA gene has been cloned on multicopy plasmids (Sancar and Rupp, 1979; Emmerson et al., 1980) and one of these plasmids was introduced into a suitable recipient for P1 transduction.

In another series of experiments, the effect of special sites for recombination of P1 transduction frequencies was investigated. The best documented of these are chi sites (see chapter 1) although the role of these in chromosomal recombination has not been determined. Chi sites are only effective when recombination is by the RecBC pathway, and thus if chi sites are responsible for the recombination of high transducing markers, recB$^-$ or recC$^-$ recipients should give lowered transduction frequencies for higher transducing markers only. It was therefore decided to construct mutants lacking the RecBC pathway and to look for effects on P1 transduction.

5·2 P1 transduction of mutants constitutive for RecA protein synthesis.

The strains DM1187, GC3217 and DM1420 which Lloyd (1978) used in experiments which showed that increased RecA protein synthesis resulted in enhanced crossover frequency were used as recipients in transduction experiments. Transductions were as described in chapter 2, and were performed at 37 C at which temperature SOS-controlled functions are elevated in GC3217 (Lloyd, 1978). The results are displayed in table 5·1, together with results

Table 5·1

Recipient	Relevant Genotype	Numbers of transductants per 5 x 10^6 pfu, and ratios relative to numbers of Leu^+ transductants.				
		Thr^+	Leu^+	Pro^+	Arg^+	His^+
AB1157	*tif*$^+$ *sfi*$^+$ *lex*$^+$ *spr*$^+$	1262	2001	597	ND	327
		0·63	1·00	0·30		0·16
GC3217	*tif*-1 *sfiA*11 *lex*$^+$ *spr*$^+$	814	1390	368	909	195
		0·59	1·00	0·26	0·65	0·14
DM1187	*tif*-1 *sfiA*11 *lexA*3 *spr*51	ND	1717	443		205
			1·00	0·26		0·12
DM1420	*tif*$^+$ *sfiA*11 *lexA*3 *spr*51	1232	2112	593		
		0·58	1·00	0·28		

Effect of RecA protein overproduction on frequencies of transduction. 0·1 ml of P1 was mixed with 0·1 ml of recipient cells. 10^{-2} dilutions of this mixture were spread on selective plates which were incubated at 37 C for 2 days. 1 000-4 000 colonies were counted for each marker. Ratios of frequencies relative to numbers of Leu^+ transductants are also displayed; numbers of Leu^+ arbitrarily fixed as 1·00.
ND = not determined due to high reversion rate.

using the parental strain, AB1157 as a recipient. It is clear that RecA overproduction has no marked effect upon transduction; numbers of Thr^+, Leu^+ and Pro^+ transductants are virtually identical when either AB1157, the parental strain, or DM1420, a constitutive overproducer of RecA protein, is used as the recipient. In both GC3217, and DM1187, which are both *tif*$^-$ *sfi*$^-$, numbers are slightly depressed, although not significantly so. In none of the mutant strains is the ratio of numbers of transductants relative to *leu*$^+$ transductants altered, thus the overproduction of RecA protein to the extent found in these strains does not stimulate the transduction of poorly transduced markers.

5·3 Introduction of multicopy recA plasmid into a recipient for P1 transduction.

pPE13 (Emmerson et al., 1980) was constructed by sub-cloning a 1·6 Md *Bst* fragment from λ*recA* (McEntee *et al*., 1976) into pBR322. The plasmid carries an intact *recA*$^+$ gene, and in a host cell directs the synthesis of a large amount of RecA protein. However, this overproduction does not lead to induction of the SOS response (McEntee, 1977) and effects upon recombination caused purely by an increased concentration of RecA protein will be observed without interference caused by increased mutagenesis.

A suitable host for this plasmid, useful in P1 transductions, was constructed from MM3031 by introducing a deletion of the *recA* region to prevent homologous recombination of the plasmid into the chromosome. N1460

Figure 5·2 Autoradiograph of whole cell proteins extracted from ^{35}S labelled cells.

Cells were labelled, proteins extracted and separated on a 7-20% SDS-polyacrylamide gel as described in chapter 2. Proteins were made from: track 1. MM3031, 2. MM3031 treated with 400 ergs UV before labelling, 3. BN56 , 4. BN56 pPE13. Standard proteins (unlabelled) were: α-lactalbumin, 14·4 kD; soybean trypsin inhibitor, 20·1 kD; carbonic anhydrase, 30·0 kD; ovalbumin, 43·0 kD; bovine serum albumin, 67·0 kD and phosphorylase b, 94·0 kD.

Figure 5.2


1.
2.
3.
4.
Standard proteins. (molecular weights).
67 000
43 000
38 000
30000
20 000
67 000
43 000
38000
30 000
20000

is an Hfr donor which transfers a deletion extending from srl to recA from an origin at 61 minutes. N1460 was crossed with MM3031, selecting for His^+ and counter-selecting on plates supplemented with 200 ug/ml streptomycin. His^+ Sm^R clones were checked for sensitivity to UV, and one clone, BN56Δ, was transformed with pPE13, selecting for ampicillin resistance on LB plates supplemented with 50 ug/ml of ampicillin. Ap^R clones were checked for UV resistance, and the presence of a plasmid was confirmed in Birnboim plasmid preparation. To check that RecA protein was being overproduced in this strain (BN56ΔpPE13), which is not derepressed for RecA protein synthesis, cells were labelled with ^{35}S methionine and whole cell proteins were extracted and separated on a 7-20% SDS-polyacrylamide gel. The gel was dried down and autoradiographed; figure 5·2 shows an autoradiograph of whole cell proteins prepared from MM3031, MM3031 treated with 400 ergs of UV before labelling, BN56Δ, and BN56ΔpPE13. The increase in synthesis of a 38 000 moleclar weight protein after UV can clearly be seen; this band is entirely absent from BN56Δ and is thus identified as RecA protein. BN56Δ pPE13 clearly synthesises RecA protein copiously, and although the amounts of protein loaded for each strain is different, it is obvious that it exceeds the amount synthesised during the SOS response (track 2).

BN56Δ pPE13 was used as a recipient for P1 transduction. Transductions were performed as described previously,

Table 5·2

Recipient	UV dose (minutes)	Numbers of transductants per $5·5 \times 10^6$ pfu (before irradiation) and ratios of frequencies relative to numbers of Arg^+ before irradiation.				
		Arg^+	Met^+	Ilv^+	Trp^+	$PyrE^+$
MM3031	0	1030	490	3140	225	395
		1·00	0·48	3·00	0·22	0·38
	2	1800	1660	2570	601	1046
		1·70	1·60	2·50	0·58	1·00
BN56 Δ (pPE13)	0	1040	680	4230	265	460
		1·00	0·65	4·00	0·25	0·44
	2	2390	1890	3010	1021	1562
		2·30	1·80	2·90	0·98	1·50

Comparison of transduction into MM3031 and BN56Δ(pPE13)

Transductions were as described in the text using P1 irradiated for 0 and 2 minutes as indicated.

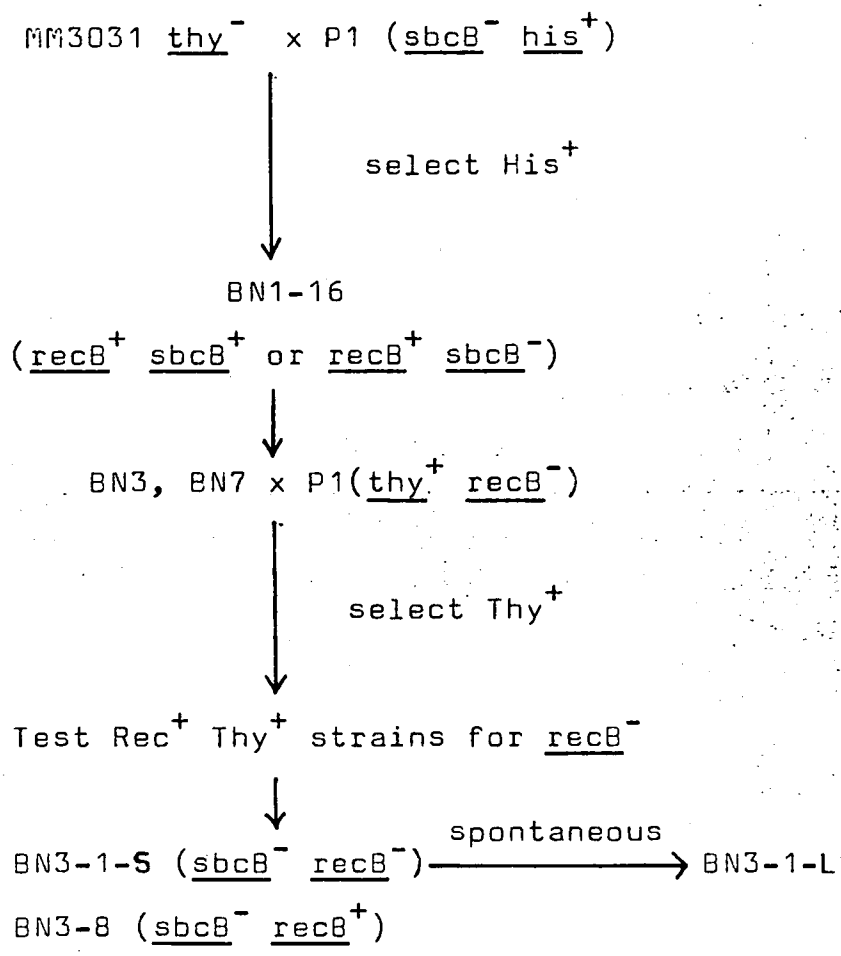
using a P1 lysate grown on W3110, and selecting for five auxotrophic markers. Results are displayed in table 5·2, together with a simultaneous experiment on MM3031. It is apparent that increasing the concentration of RecA protein in the cell does not significantly affect the level of transduction, or alter the ratio of markers recovered. However, the elevated synthesis of RecA protein does appear to have an effect upon the recovery of markers after UV irradiation. There is a 30-50% improvement in the recovery of all markers, particularly the low-transducing markers trp and pyrE.

5·4 Role of chi site activity and the RecF pathway of recombination in P1 transduction.

To eliminate chi site activity, strains were constructed which lacked the RecBC pathway of recombination, but which were derepressed for the RecF pathway and hence recombination proficient. Two mutations were introduced: recB$^-$ or recC$^-$ which eliminates the recBC nuclease, ExoV, and sbcB$^-$, which eliminates ExoI, which is a suppressor of the RecF pathway. Neither of these mutations can be selected for easily, and sbcB$^-$ alone confers no obvious phenotype. The following strategy, outlined in figure 5·3, for constructing 'RecF' strains was used.

The parental strain, MM3031 thy$^-$ was infected with P1 prepared from a his$^+$ sbcB$^-$ strain (ED2123) and His$^+$ transductants were selected. Approximately 50% of these would be expected to be sbcB$^-$ also (Wu, 1966). BN1-16 were isolated at this stage. Next, P1 prepared on a thy$^+$,

Figure 5·3 Derivation of 'RecF' strains. (see text for further explanation).



recB$^-$ strain (JC7526 or ED2123) was used to transduce BN1-16 and Thy$^+$ transductants were selected. About 50% would also be expected to be recB$^-$. Those strains which were sbcB$^-$ would be expected to yield only recombination-proficient Thy$^+$strains; sbcB$^+$ his$^+$ strains would yield a mixture of Rec$^+$ and Rec$^-$ transductants. Thy$^+$ clones originating from BN1-16 were checked for recombination proficiency in Hfr crosses, and also for the presence of the recB$^-$ marker as follows. P1 lysates grown on Rec$^+$ putative sbcB$^-$ strains were used to infect CP154 (argA lysA), and Arg$^+$ Lys$^+$ transductants were selected and tested for recombination proficiency in Hfr crosses. Recovery of recombination deficient clones indicated that the donor was recB$^-$.

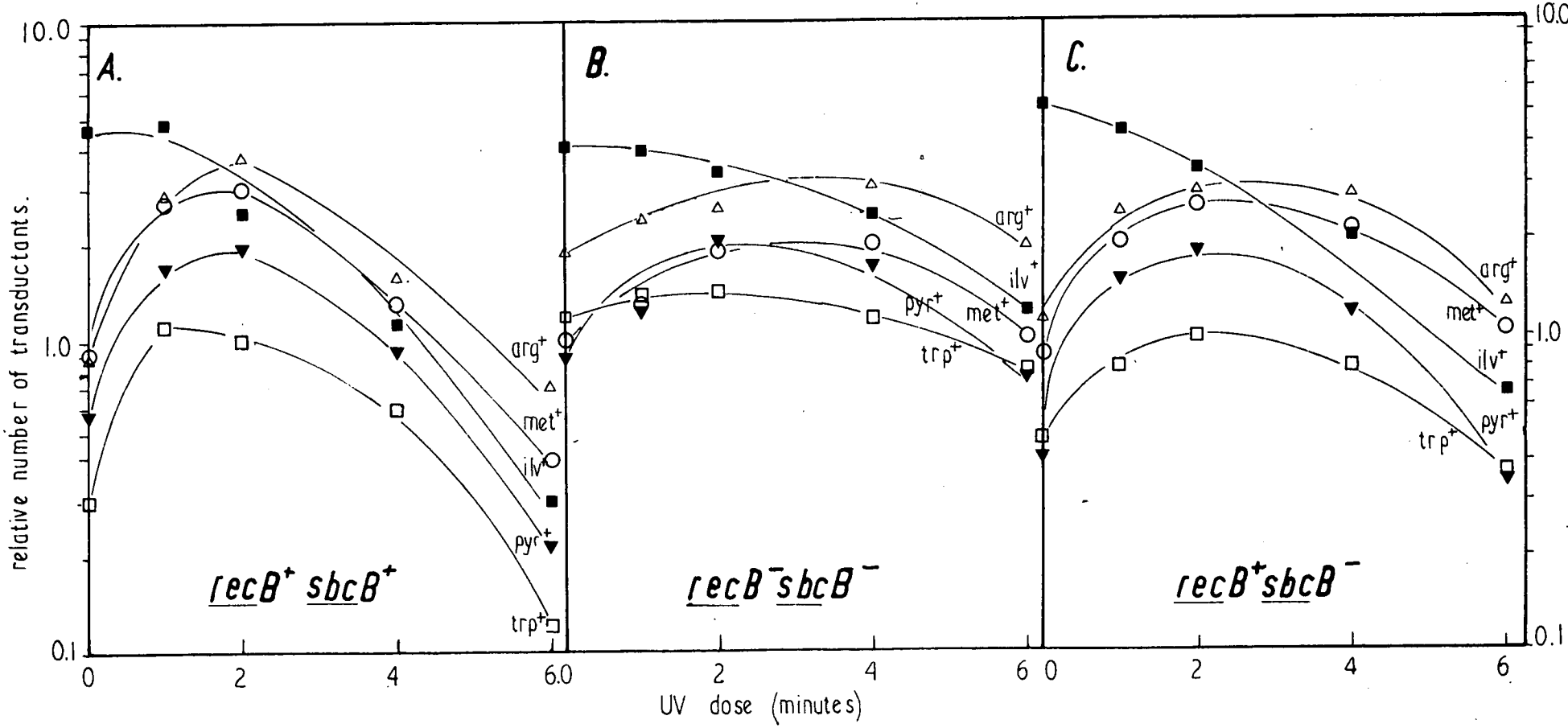
The first recB$^-$ sbcB$^-$ isolates grew slowly and were poor recipients for transduction, but they spontaneously changed to faster growing, large colonies on LB plates which were good recipients for P1 transduction. One of these segregants, BN3-1-L, was used in a transduction experiment, together with BN3-8, one of the sbcB$^-$ recB$^+$ isolates. Transductions were performed as previously using MM3031, BN3-1-L and BN3-8 as recipients for P1 prepared on W3110, and irradiated with UV for 0, 1, 2, 4 and 6 minutes. The results of these experiments are plotted in figure 5·4.

From these graphs it is clear that the recB$^-$ sbcB$^-$, or RecF strain, is a better recipient for low-transducing markers trp and pyrE, although no better for high-transducing markers. It is also a better recipient for

Figure 5•4 Frequency of transduction into sbcB$^-$ strains using irradiated P1 lysates.

P1 lysates grown on W3110 were irradiated with UV at a dose rate of 10 ergs/mm^2/second for 0-6 minutes, and used to transduce A. MM3031 (*recB*$^+$ *sbcB*$^+$); B. BN3-1-L (*recB*$^-$ *sbcB*$^-$); C. BN3-8 (*recB*$^+$ *sbcB*$^-$).

Figure 5.4


A.
B.
C.
recB+ sbcB+
recB- sbcB-
recB+ sbcB-
arg+
met+
ilv+
pyr+
trp+
relative number of transductants.
UV dose (minutes)
10.0
1.0
0.1
0
2
4
6.0
6

UV damaged DNA. The sbcB⁻ strain BN3-8 also shows a marked improvement in the recovery of all markers after UV damage over the recovery in the wild-type strain.

5·5 Discussion

The experiments in this chapter represent two approaches to the problem of bias in the recovery of some markers after P1 transduction. The experiments in the first two sections were an attempt to decrease the discrimination by increasing the concentration of an essential participant in homologous recombination, RecA protein, by various means. The overall level of recombination resulting in the formation of stable transductants is unaltered in a strain which constitutively synthesises RecA protein, DM1420, and virtually unchanged in two other RecA protein overproducers, GC3217 and DM1187, both of which are tif mutants. Lloyd (1978) suggested that the frequency of crossover between Hfr and F⁻ chromosomal DNA is strongly dependent upon the level of recA gene product in merozygotes, although integration of Hfr DNA per se is relatively independent of RecA protein level. He pointed out a possible enhancement of recombination by the tif-1 mutant protein. It is possible that P1 transducing DNA was subject to a higher frequency of crossover, but this was not measured in these experiments. Also, Hfr DNA enters the cell as a single strand whereas P1 DNA is integrated as a double strand (Sandri and Berger, 1980a), and thus is possibly the

substrate for different enzymes, as discussed in chapter 1.

In order to avoid the mutator effects of two of these strains, and the high reversion rates of some of the markers in these strains, a different method was adopted to obtain increased levels of RecA protein. The plasmid pPE13 which directs the synthesis of a copious amount of RecA protein, unaccompanied by any of the SOS responses was introduced into MM3031. Again, the effects were small.

It can thus be concluded that the differences observed in transduction frequency are not due to differing affinities for a limiting amount of RecA protein. It seems evident that the variations result from some other aspect of the structure of the transduced DNA.

Homologous recombination usually occurs by the RecBC pathway. Recombination by the RecF pathway was examined, both in order to compare the specificities of the two pathways, and to gain information about the possible role of chi sites in transductional recombination. Results of P1 transductions into the RecF strain shows a striking difference from <u>recB</u>$^{+}$ strains in that it integrates poorly transduced DNA at a higher frequency than wild-type indicating that a selective process in recombination is not operating in this strain. Perhaps the activities of ExoI and ExoV, which are lacking in the RecF strain, destroy the poorly transduced DNA selectively either because it is delayed in integration, or because its recombination intermediates are better substrates for

these enzymes. BN3-8 lacks one of these enzymes, ExoI, and it is a better recipient for damaged DNA than the wild-type; ExoI may be selectively destroying damaged DNA.

Another factor to be considered is the role of chi site activity. Whether chi sites have any effect upon general recombination is not known. The experiments in this chapter neither prove nor disprove the theory that chi site activity is responsible for selectivity in P1 transduction. It is possible that the recB⁻sbcB⁻ strain which lacks chi activity is a better recipient for all markers due to the lack of exonuclease activity, but that in a wild-type strain, chi site activity causes rapid and preferential integration of the high-transducing DNA. The low-transducing DNA may lack chi sites, may not be so rapidly integrated, and may be more susceptible to nuclease attack. A model based on these results is further discussed in the final chapter.

The fact that the initial recB⁻sbcB⁻ isolates, which grew poorly, rapidly developed faster growing derivatives indicated that these may have gained another highly advantageous mutation. The nature of this further mutation has not been investigated, but it may be that it is this mutation, rather than the recB⁻sbcB⁻ mutations, which is resposible for the alteration in transduction frequencies. In any case, it is clear that the discrimination between markers in P1 transduction is genetically controlled in the recipient.

CHAPTER SIX

CLONING OF RESTRICTION FRAGMENTS FROM CHROMOSOMAL DNA INTO PHAGE λ VECTORS.

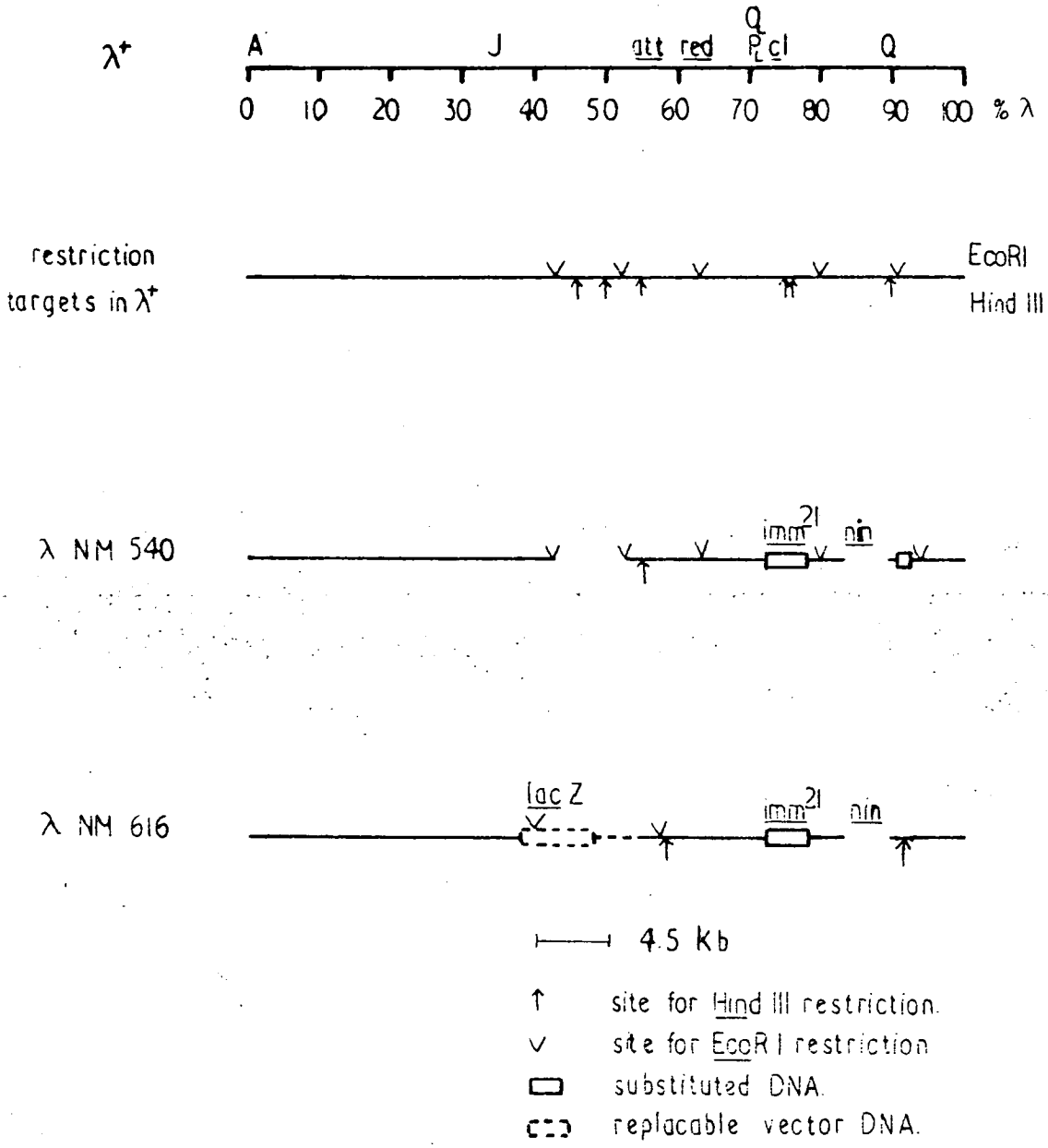
6·1 Introduction.

The development of *in vitro* systems for creating specialised λ transducing phages (Murray and Murray, 1975; Borck *et al.*, 1976) provides a convenient means for isolating and preparing large quantities of particular fragments of E.coli chromosome free of vector DNA. A bank, or pooled lysate, of recombinant phages is first prepared as described below, from which phages can be isolated which carry selected markers by complementation of particular mutations.

6·2 Phage λ cloning vectors.

Two cloning vectors were used. These were λNM540 (Murray and Murray, 1975) and λNM616 (Wilson and Murray, 1979), which are illustrated in figure 6·1. λNM540 (*sr1*(1-2)$^{\Delta}$, *att*$^{+}$, *imm*^{21}, *nin*) was constructed for use as a receptor for fragments generated by *Hin*dIII. The chromosome is deleted for about 21% of the wild-type complement, and it has a capacity for restriction fragments of up to 10 kb which are inserted into the single *Hin*dIII site between gene J and the attachment site to generate an integration and recombination proficient phage. It also carries the wild-type allele of *imm*^{21}, derived by replacing the λ immunity region with the phage 21 immunity region.

Figure 6.1



Physical maps of λ wild-type, λ NM540, and λ NM616.

Relevant genes are marked on λ wild-type, which is calibrated in % of λ genome.

λNM616 (*lac*$^+$, *att*$^+$, *imm*^{21}, *nin*) is a derivative of λ*plac*5. It is an integration and recombination proficient displacement vector in which donor DNA is inserted between two *Eco*R1 sites to the left of the attachment site, displacing the fragment containing most of the *lacZ* gene. It has a capacity for a 12 kb insert, and the two arms of λ cannot rejoin to form a viable phage since the complement of DNA is too low. This, and the presence of the *lacZ* gene in the vector, facilitates recovery of recombinants since only phages carrying an insert are viable, and the original vector forms red plaques on MacConkey-lactose plates, whereas recombinants form white plaques.

6·3 Construction of pooled lysate.

Receptor phage and *E.coli* W3110 DNA were digested to completion separately with *Eco*R1 (New England Biolabs) or *Hin*dIII (Boehringer Mannheim Corporation). The reaction was terminated by inactivating the enzymes at 70 C for 15 minutes, then cooling on ice.

Restricted donor and receptor DNAs were mixed in the proportion of 3-4 donor to 1 of recipient, then incubated at 30 C for 15 minutes to separate pre-annealed fragments. The mixture was ligated with T4 DNA ligase at 10 C for 6 hours, then kept on ice for 2-6 days. During the incubation period, samples (about 50 ng) of the ligating mixture were diluted to 0·1 ml with SSC:$CaCl_2$ and used to transfect 0·2 ml of competent cells (NEM259 or AA125). After transfection, cells were

mixed with molten BBL top agar and plated on BBL plates. Plaques were harvested after overnight incubation at 37 C as for a plate lysate. A total of about 1 000 plaques was pooled into one lysate.

Numbers of recombinant phages could be estimated directly when λNM616 was used as the receptor. AA125 was used as the transfection strain; it is *lacZ*$^{\Delta}$, and thus infection with the original *lac*$^{+}$ vector gives red plaques on MacConkey lactose plates, whereas infection with recombinant, *lac*$^{-}$, phages gives white plaques. Maximum numbers of recombinant phages were recovered after 48 hours incubation at 0 C.

The resulting pooled lysates had a titre of 10^5-10^6 pfu/ml, and this was boosted by re-infecting NEM259, plating on BBL plates at 37 C, and harvesting the lysate. The boosted lysate had a titre of 10^9-10^{10} pfu/ml.

6·4 Selection for transducing phages carrying particular segments of the E.coli chromosome.

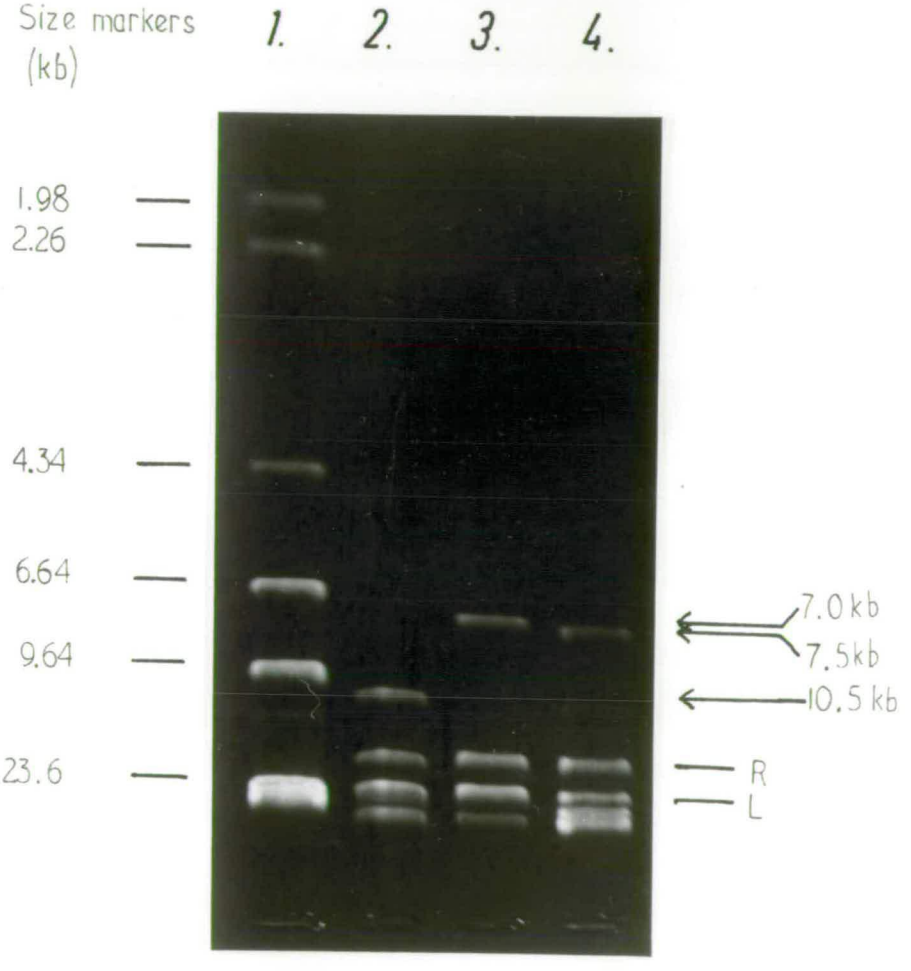
Transducing phages were selected from the pooled lysate by their ability to complement mutations in appropriate bacterial strains. 10^6-10^7 pfu in phage buffer were mixed with about 10^8 recipient cells grown to late log phase and resuspended in 10^{-3} M $MgSO_4$. After adsorption for 15 minutes at 37 C, ten-fold dilutions were spread on selective plates.

Colonies appeared after 1-2 days; these were restreaked on selective plates and checked for lysogeny by cross-streaking against λ*vir* and λNM540 on LB plates.

Figure 6·2 Restriction pattern of λ transducing phages.

DNA was prepared and restricted as described in chapter 2. Restricted DNA was loaded on a 0·7% agarose gel which was electrophoresed at 30 v, 15 mA overnight then stained with ethidium bromide. Bands were visualised with UV and photographed through a red filter. Track 1, λC1857 DNA digested with HindIII (size markers); track 2, λBN2 digested with HindIII; track 3, λBN1 digested with HindIII; track 4, λBN74 digested with HindIII. R and L indicate the right and left arms of λNM540 from the recombinant phages. Bands larger than R and L are incompletely digested.

Figure 6.2


Size markers
(kb)
1.
2.
3.
4.
1.98
2.26
4.34
6.64
9.64
23.6
7.0 kb
7.5kb
10.5 kb
R
L

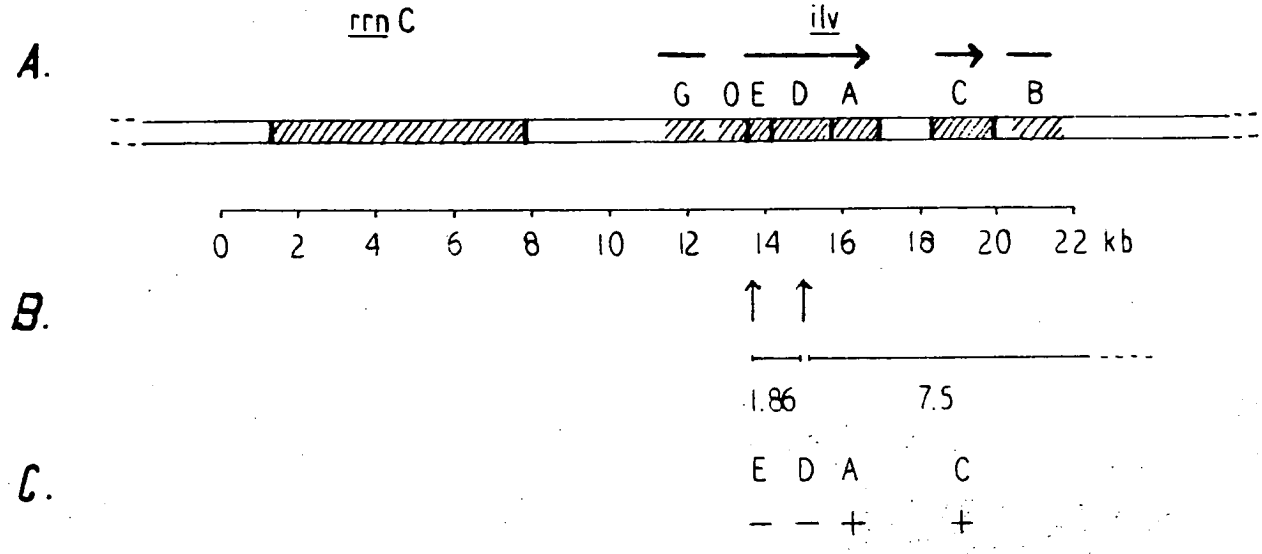
Free phage was prepared from lysogens by UV induction, and DNA was prepared and analysed on agarose gels.

6·5 Isolation and characterisation of λ ilv phage.

The λNM540 pooled lysate was used to complement MM3031 *mal*$^+$ on VB minimal plates, selecting for Ilv$^+$ clones. Ilv$^+$ colonies were checked for λ lysogeny, and phage was prepared by UV induction. From one clone, a λ*ilv* phage, λBN74, was isolated. DNA was prepared, and restricted with *Hin*dIII. The restriction pattern is shown in figure 6·2 lane 4; the chromosomal insert is a 7·5 kb *Hin*dIII fragment. The complementation pattern of the phage was also checked using AB1160 (*ilvC*$^-$), AB1161 (*ilvA*^{ts}), AB2070 (*ilvE*$^-$) and AB3505 (*ilvD*$^-$). BN74 complements *ilvA* and *ilvC* (figure 6·3), but not *ilvE* or *ilvD*. A restriction pattern of the *ilv* gene cluster at 84·1 minutes (McCorkle *et al*., 1978) is shown in figure 6·3, and from this data, and the complementation pattern, it was deduced that λBN74 carries a 7·5 kb *Hin*dIII fragment which extends from a *Hin*dIII site in the *ilvD* gene rightward through the *ilvA*, *ilvC* and *ilvB* genes, and beyond the *ilv* gene cluster.

The *ilv* gene cluster is organised into four transcriptional units as shown in figure 6·3; *ilvEDA*, *ilvB*, *ilvC* and *ilvG*. *ilvO* is a regulatory locus affecting *ilvEDA* and *ilvG*. Thus *ilvA*, which is carried on the 7·5 kb *Hin*dIII fragment and which complements the *ilvA*$^-$ lesion in AB1161 must be transcribed either from the P_L promoter, which can promote transcription beyond *att*

Figure 6.3



Physical organisation of the ilv gene cluster at 83 minutes, and mapping of λ BN74.

(McCorkle et al., 1978; Baez et al., 1979).

A. The gene cluster is organised into four transcriptional units, and these are shown (heavy lines), together with the direction of transcription (where known).

B. HindIII sites within the cluster are marked (↑), together with fragment size in kb.

C. The complementation pattern of λ BN74 was established using the strains described in the text (+, complementation; -, no complementation).

into the central region of λ including transcription from genes inserted at shnλ3 in the l orientation (Hopkins et al., 1976), or from a minor promoter situated between the ilvD and ilvA genes (MacCorkle et al., 1978).

6·6 Isolation of λarg, λmet, and λpro phages.

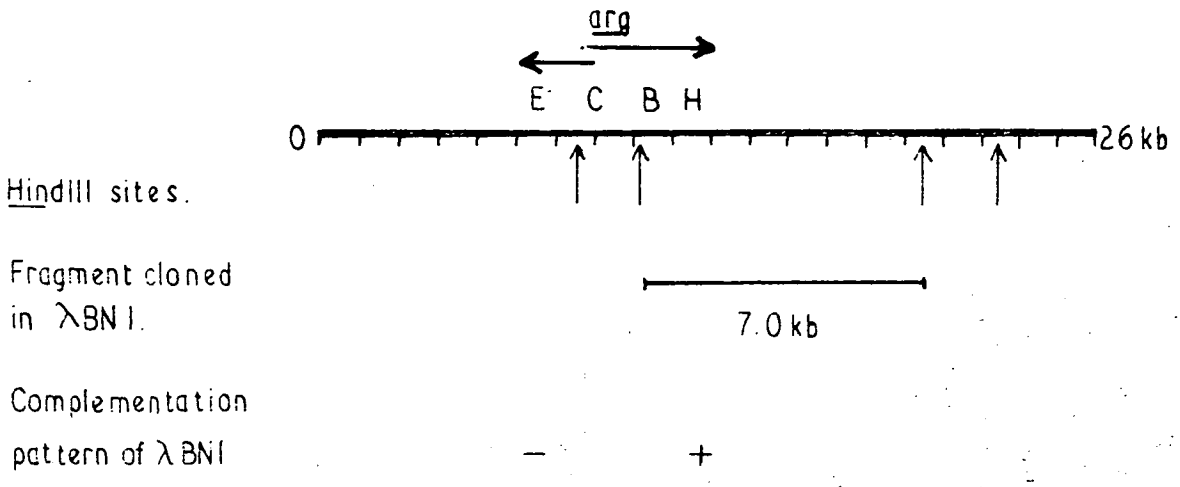
Transducing phages carrying the argH and metB genes were isolated by complementation of MM3031 mal$^+$ with the λNM540 pooled lysate. Digests of the λ argH phage, λ BN1, and the λ metB phage, λ BN2, with HindIII are shown in figure 6·2.

λ BN1 carries a 7·0 kb HindIII fragment, which can be identified with the 7·0 kb fragment mapped by Crabeel et al. (1977), and the 7·35 kb fragment mapped by Kreuger et al. (1981). This is confirmed by the complementation pattern shown in figure 6·4. λ BN1 complements the argH mutation in MM3031 mal$^+$, but not the argE mutation in AB1157. A physical map of the argECBH cluster is shown in figure 6·4. It is probable that the argH gene in λBN1 is being transcribed from the secondary promoter which was identified between argH and argB (Crabeel et al., 1977).

λBN2 contains a 10·5 kb HindIII fragment which complements metB but not the metLM mutation in Gif102. A comprehensive restriction map of the metBJLF cluster has not been published, so this fragment cannot be related to existing maps.

A phage carrying the proA gene was also isolated by complementation of AB1157 with the λNM616 pooled lysate.

Figure 6.4



Physical organisation of the arg gene cluster at 89 minutes, and mapping of λBN1.

Directions of transcription of the genes are shown (heavy lines) and HindIII sites. The complementation pattern was established using the strains described in the text.

The chromosomal insert was very small, less than 1 kb, and unfortunately, reassociation experiments using this fragment were not satisfactory as the probe reassociated at a rate barely distinguishable from the reassociation driven by test DNA under the conditions used.

CHAPTER SEVEN

COMPARISON OF THE RATIOS OF MARKERS IN P1 AND LOG-PHASE E.COLI DNA USING KINETICS OF REASSOCIATION.

7·1 Introduction.

The experiments described in chapter 3 strongly suggested that P1 does not selectively package DNA, but that the recipient selectively recombines some regions. From this hypothesis it would follow that markers would be represented in transducing DNA in the same ratios as in the DNA of donor cells. In order to substantiate the earlier results, an experiment was designed to estimate the relative ratios of several markers in P1 transducing DNA and in E.coli W3110 DNA.

Estimates of the percentage of transducing particle in a P1 lysate vary from 0·3% (Ikeda and Tomizawa, 1965) to 2% (Sandri and Berger, 1980a), and, since P1 can package 2% of the chromosome into a phage head, a given sequence will be represented by, on average, 2% of transducing particles or about 0·02% of total particles. Therefore, a very sensitive method is needed to enable the estimation of the concentration of different sequences. Nucleic acid hybridisation techniques have been used for many years to detect and quantify homologous sequences in DNA (Britten and Kohne, 1968). Filter techniques (Denhardt, 1966) have certain limitations; particularly that a sufficient excess of DNA cannot be loaded on to filters. However, perfection of techniques for hybridisation in solution (Britten et al., 1974) has led to measurements of

very low concentrations of sequences in test DNA. Gelb et al. (1971) could detect less than one SV40 DNA molecule (3×10^6 daltons) per mammalian cell genome (4×10^{12} daltons) by using a high ratio of mammalian DNA to viral DNA to compensate for the large differences in their respective molecular weights.

Similar methods could therefore be used to analyse the content of P1 transducing DNA. Fragments of chromosomal DNA were isolated from the λ transducing phages described in chapter 6, and used to probe P1 DNA; because of the sensitivity of the method it was unnecessary to separate the transducing particles from the infective particles. The relative concentration of each marker was compared with that obtained when W3110 log phase DNA was used as the test DNA. In the next section, the process of renaturation is described, and the calculation of reassociation rates, and hence DNA concentration, is explained.

7·2 DNA-DNA reassociation kinetics.

DNA renaturation is the process by which two single-stranded molecules of DNA with complementary sequences meet and form a stable duplex structure. The rate of renaturation is dependent upon several parameters (Wetmur and Davidson, 1968). Briefly, these are:

1. DNA-phosphate concentration. The reaction is approximately second order in DNA-phosphate concentration.
2. Temperature. The reaction rate increases as the temperature decreases below T_m (the temperature for

50% denaturation of DNA) reaching a broad, flat maximum from 15-30 C below T_m, and then decreases with a further decrease in temperature.

3. Single-strand length. For a given DNA at a fixed DNA-phosphate concentration, a decrease in single-strand length results in a decrease in the rate of renaturation. For this reason, the DNA used in reassociation experiments was sheared to the same average length.

4. Complexity. Complexity is defined as the total number of DNA base pairs in non-repeating sequence, and the rate is inversely proportional to complexity. The rate of renaturation of DNA of simpler organisms such as viruses is faster than that of more complex organisms such as bacteria.

5. Ionic strength. The rate is dependent upon the ionic strength of an electrolyte such as NaCl below 0·4 M, but is almost independent of ionic strength above this concentration.

6. The rate is dependent upon solvent viscosity.

Because the reaction proceeds with second order kinetics, it is thought that the rate limiting step is the formation of a small region of duplex, which can serve as the nucleation site for the subsequent zippering process (Wetmur and Davidson, 1968).

All other things being equal, then, the rate of reassociation of any given sequence of DNA is a function only of the concentration of that sequence in solution. Thus, when one sequence reassociates at a higher rate than

another in the presence of an excess of test DNA (this is defined as a DNA-driven reaction), under identical conditions of temperature, ionic strength etc., it is clear that that sequence is present at higher concentration. By determining rates of reassociation of small sequences of DNA in the presence of a large excess of test DNA, the concentration of that sequence in the test DNA can be calculated as follows:

The second order reassociation of DNA can be expressed as an ideal 2nd order equation:

$$f_{ss} = \frac{1}{1 + kC_o t}$$

where: f_{ss} = fraction remaining single stranded at time t.

t = time (seconds).

k = reaction rate constant.

C_o = DNA concentration at t = 0 (moles of nucleotides per litre).

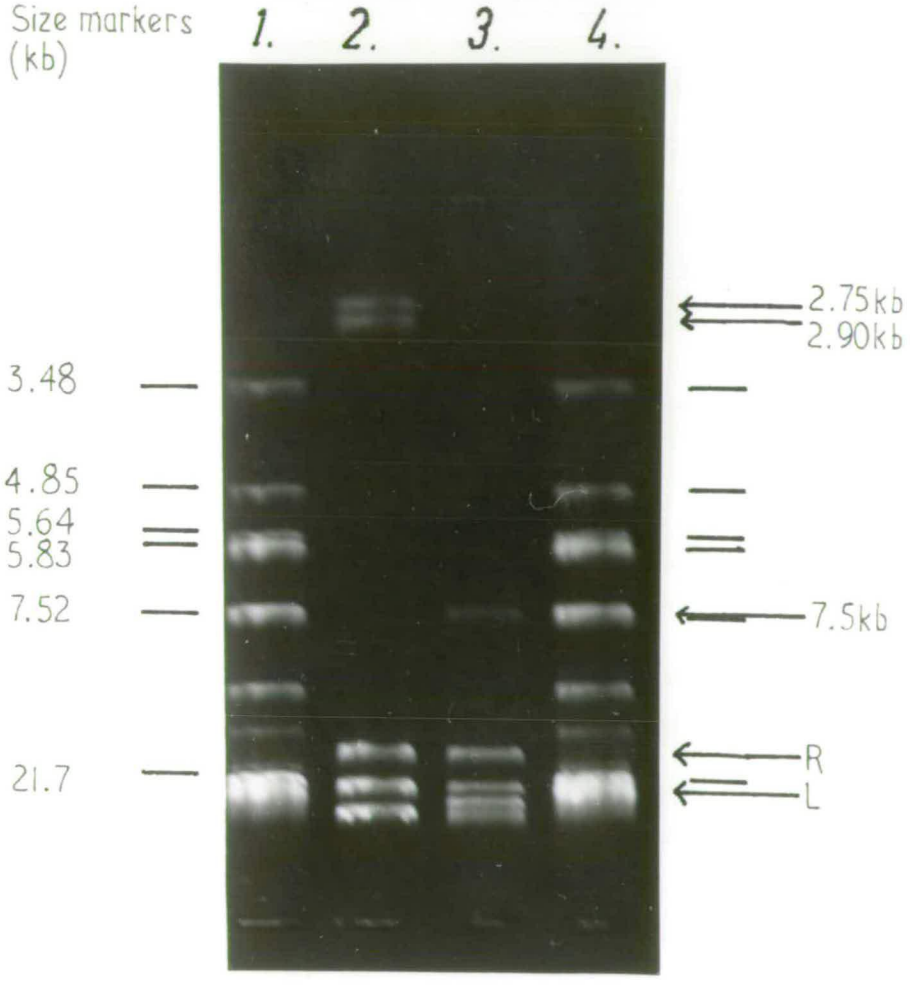
(from Britten et al., 1974). A plot of $\frac{1}{f_{ss}}$ against t will result in a straight line of slope kC_o.

To apply this method to the analysis of P1 transducing DNA, short fragments of E.coli DNA isolated from λ transducing phages were allowed to reassociate in the presence of a large excess of P1 DNA. The rates of reassociation of fragments from different parts of the chromosome driven by P1 DNA were compared with the rates of reassociation of the same fragments driven by DNA extracted from log-phase E.coli W3110 which should have the same gene dosage as the cells on which the P1 was

Figure 7·1 Restriction patterns of λtrpABC and λtna.

DNA was restricted and run on a 0·7% agarose gel as described in the legend to figure 6·2. Tracks 1 and 4, λc1875 DNA digested with EcoR1; track 2, λtrp digested with HindIII; track 3, λtna digested with HindIII. R and L indicate the right and left arms of λNM540 from the recombinant phages. Bands larger than these, and bands in tracks 1 and 4 other than those marked, are incompletely digested.

Figure 7.1


Size markers
(kb)
1.
2.
3.
4.
3.48
4.85
5.64
5.83
7.52
21.7
2.75kb
2.90kb
7.5kb
R
L

grown. From this comparison, it should be possible to see whether P1 transducing DNA carries the same ratio of markers as is found in late log phase E.coli.

7·3 Description of probes used in hybridisation studies.

1. ilv probe. 7·5 kb HindIII fragment isolated from λBN74 separated on sucrose gradient.
2. ftsA probe. 2·2 kb HindIII fragment isolated from pGH4, a pBR325 derivative (G.Hatfull, Ph.D. thesis, University of Edinburgh, 1981). The ftsA gene is situated at 2·25 minutes on the E.coli chromosome, about 0·65 minutes from the leu gene cluster which is of interest due to its anomolous behaviour in the transduction experiments described in chapter 3.
3. metB probe. 10·5 kb HindIII fragment isolated from λBN2 by freeze-squeeze.
4. tna probe. 7·5 kb HindIII fragment isolated from a λtna phage made in vitro from λNM540 (figure 7·1; Borck et al., 1976). The fragment was separated by freeze-squeeze.
5. trp probe. 2·75 and 2·90 HindIII fragments isolated from λtrpABC (figure 7·1; Hopkins et al., 1976), which was made in vitro from λNM540. The fragments were separated from the two arms of λ on a sucrose gradient, and could be considered as a single probe of 5·65 kb for the reassociation experiments.

7·4 Reassociation of isolated fragments of DNA driven by P1 DNA, and by log phase E.coli DNA.

Restriction fragments of E.coli DNA isolated from specialised λ transducing phages or, in one case, from a pBR325 clone, were purified and labelled with α^{32}PdCTP by nick translation as described in chapter 2. The probe was sonicated to generate fragments of 300-400 base pairs, and mixed with an excess of test DNA, also sonicated. The DNA was denatured, and allowed to reassociate at 65 C as described in chapter 2. A typical reassociation mixture consisted of: 1 ug/ml sonicated, denatured, labelled probe DNA; 85-380 ug/ml sonicated, denatured, unlabelled test DNA; and sufficient sonicated, denatured, unlabelled salmon sperm DNA to give a total final concentration of DNA of 500 ug/ml. DNA, in a total volume of 150 ul in water was mixed with 100 ul of 2·5 x HB to give a final volume of 250 ul.

Samples were withdrawn at appropriate intervals, divided into two aliquots, and assayed with S1 nuclease. The percentage remaining single stranded was calculated for each pair of samples as:

$$100 - \frac{\text{TCA precipitable counts after digestion with S1}}{\text{Total TCA precipitable counts}} \times 100$$

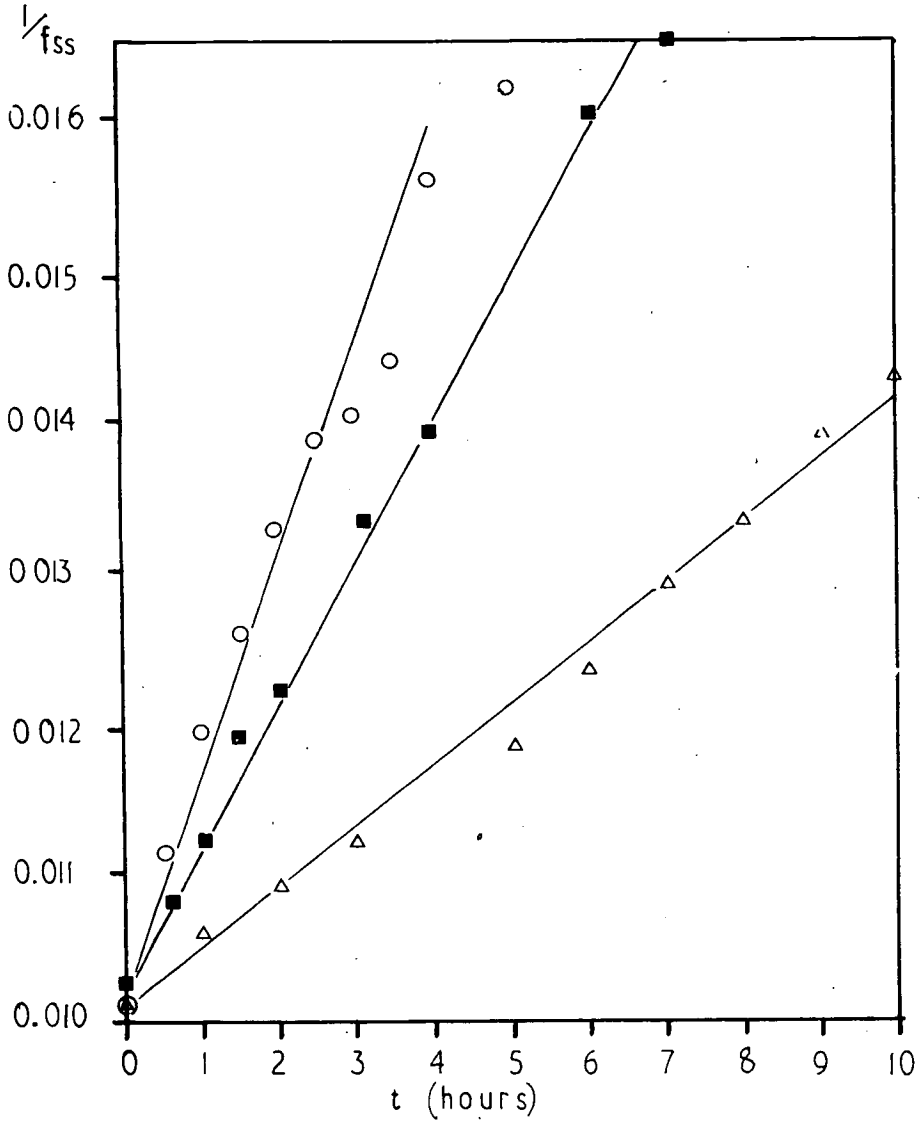
The counts were corrected for background obtained by counting blank filters. The reciprocal of this value was plotted against time.

For each fragment, the reassociation driven by P1 DNA and W3110 log phase DNA (extracted from cells grown in

Figure 7·2 Reassociation of trp probe in the presence of W3110, P1, and salmon sperm DNA.

(a) W3110 DNA (squares), (b) P1 DNA (triangles) and (c) an excess of probe DNA (open circles). $1/f_{ss}$ is plotted against t as described in the text. DNA concentrations in reaction mixtures were (a) 0·76 ug/ml sheared, labelled, probe DNA, 32·5 ug/ml sheared W3110 DNA, 460 ug/ml sheared salmon sperm DNA; (b) 0·76 ug/ml sheared, labelled, probe DNA, 78·4 ug/ml sheared P1 DNA, 420 ug/ml sheared salmon sperm DNA; (c) 7·6 ug/ml sheared, labelled, probe DNA, 500 ug/ml sheared salmon sperm DNA. All reactions were carried out in a total volume of 250 ul at 65 C as described in the text.

Figure 7.2


1/fss
0.016
0.015
0 014
0 013
0 012
0.011
0.010
0
1
2
3
4
5
6
7
8
9
10
t (hours)

broth to an OD_{540} of 0·5) was measured. Also, reassociation of the fragment alone with added salmon sperm DNA to a final concentration of 500 ug/ml was measured as a control reaction to enable the calculation of the rate constant for a known concentration of probe DNA. This rate constant was then used to calculate the concentration of probe sequences in the test DNA.

Figure 7·2 shows the results obtained for the reassociation of trp probe in the presence of salmon sperm DNA, P1 DNA, and W3110 log phase DNA displayed as plots of $\frac{1}{f_{ss}}$ against t. Similar plots were drawn for each probe (figure 7·3) and the slopes of each, together with DNA concentrations in each reaction mix, are displayed in table 7·1.

7·5 Calculation of concentration of probe sequences in P1 and W3110 DNA.

From the plots in figure 7·2, slopes for the renaturation rate of the trp probe in the presence of, respectively, E.coli DNA, P1 DNA, and excess probe DNA were: $2{\cdot}413 \times 10^{-7}$ sec^{-1}, $1{\cdot}032 \times 10^{-7}$ sec^{-1}, $3{\cdot}819 \times 10^{-7}$ sec^{-1}. The second order rate constant, k, for the probe was calculated from the last slope. In this reaction, C_o was 7·60 ug/ml, or $5{\cdot}81 \times 10^{-5}$ moles nucleotides l^{-1}.

From the formula $f_{ss} = \frac{1}{1 + kC_o t}$, a plot of $\frac{1}{f_{ss}}$ against t has a slope of kC_o. Thus:

$$k = \frac{3{\cdot}819 \times 10^{-7}}{5{\cdot}81 \times 10^{-5}} = 6{\cdot}57 \times 10^{-3} \text{ l mol}^{-1} \text{ sec}^{-1}$$

Figure 7·3 Reasssociation of ilv, tna, ftsA and metB probes in the presence of W3110, P1 and salmon sperm DNA.

DNA concentrations in reaction mixtures are shown in table 7·1. All reactions were carried out in a total volume of 250 ul at 65 C. Other methods and reagents were as described in the text. For each probe, $1/f_{ss}$ is plotted against t; slopes of each line are shown in table 7·1.

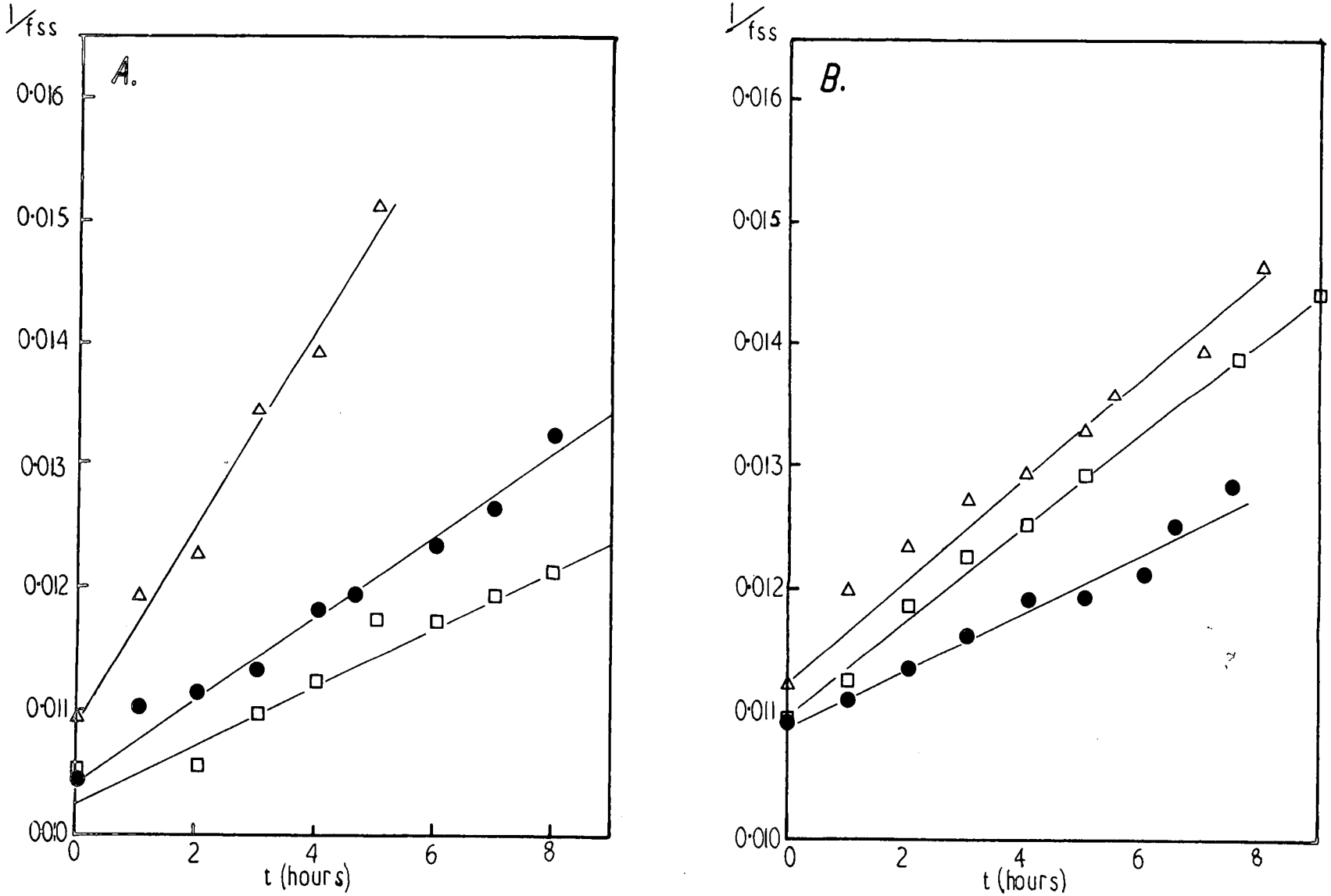
A. Reassociation of ilv probe in the presence of W3110 DNA (triangles), P1 DNA (squares), and salmon sperm DNA (circles).

B. Reassociation of tna probe in the presence of W3110 DNA (triangles), P1 DNA (squares), and salmon sperm DNA (circles).

C. Reassociation of ftsA probe in the presence of W3110 DNA (triangles), P1 DNA (squares), and salmon sperm DNA (circles).

D. Reassociation of metB probe in the presence of W3110 DNA (triangles), P1 DNA (squares), and salmon sperm DNA (circles).

Figure 7.3


1/fss
A.
0·016
0·015
0·014
0·013
0·012
0·011
0·010
0
2
4
6
8
t (hours)
1/fss
B.
0·016
0·015
0·014
0·013
0·012
0·011
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2
4
6
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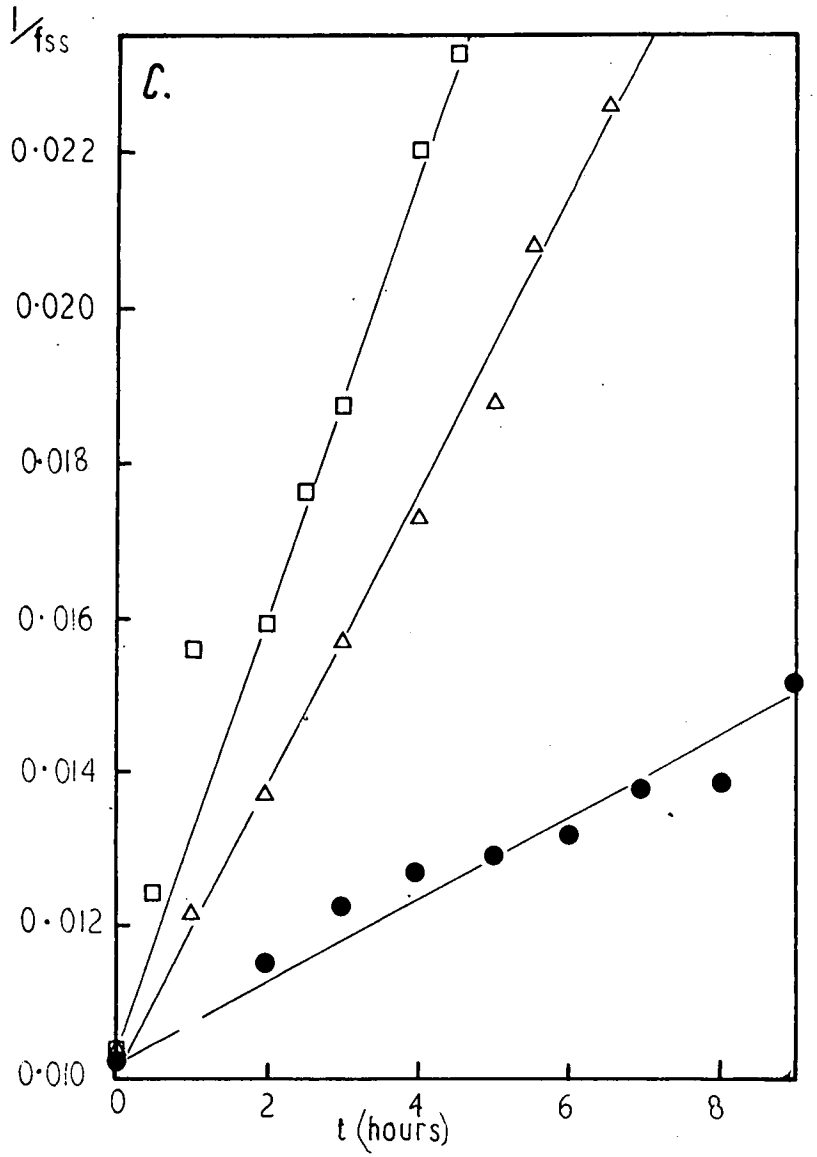
t (hours)


C.
1/fss
0·022
0·020
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0·016
0·014
0·012
0·010
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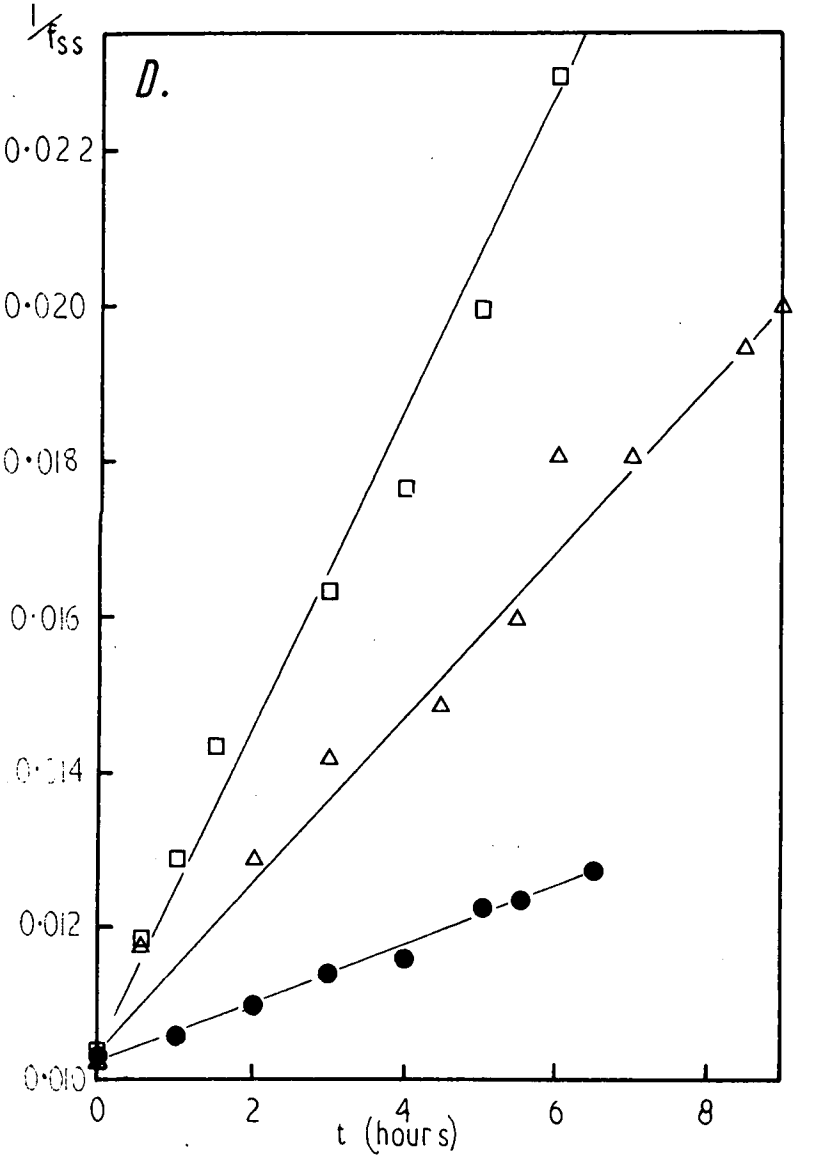
6
8
t (hours)


D.
1/fss
0·022
0·020
0·018
0·016
0·014
0·012
0·010
0
2
4
6
8
t (hours)

Using this value for k, C_o for the trp probe in P1 and W3110 DNA was calculated as follows:

$$C_o(W3110) = \frac{\text{slope}}{k} = \frac{2{\cdot}413 \times 10^{-7}}{6{\cdot}573 \times 10^{-3}}$$

$$= 3{\cdot}67 \times 10^{-5} \text{ moles nucleotides } l^{-1}$$

similarly:

$$C_o(P1) = \frac{1{\cdot}032 \times 10^{-7}}{6{\cdot}573 \times 10^{-3}} = 1{\cdot}57 \times 10^{-5} \text{ moles nucleotides } l^{-1}$$

The labelled probe contributed $6{\cdot}05 \times 10^{-6}$ moles nucleotides l^{-1}; this figure was subtracted, and the concentrations were calculated as umoles trp sequence per ug of test DNA. For W3110 DNA, this was calculated as $9{\cdot}44 \times 10^{-4}$ umoles/ug, and for P1 DNA, $1{\cdot}23 \times 10^{-4}$ umoles/ug.

Similar calculations were made for each probe, and values for k, and concentration of probe sequence in W3110 and P1 DNA, are displayed in table 7·2. These figures do not take into consideration the length, or complexity, of the probes used. It was stated earlier that the rate of renaturation is inversely proportional to complexity. To compare the reassociation rates of probes of different complexity the formula

$$k_n = k \frac{N}{L^{\frac{1}{2}}}$$ where k_n = nucleation rate constant,

N = complexity, and L = molecular weight (Hutton and Wetmur, 1973). The molecular weight was not accurately determined in these experiments, although all samples were reduced to the same average molecular weight by sonication. Instead, to give comparable values for the concentration

Table 7·1 Rates of reassociation of 5 probes driven by E.coli and P1 DNA.

The composition of each reaction mixture (ug/ml DNA) is shown. Reassociation was at 65 C; other reagents and methods are described in the text. Rate of renaturation was determined from a plot of $1/f_{ss}$ against t.

Probe	DNA concentration (ug/ml)				Rate of renaturation (sec^{-1})
	^{32}P labelled probe	W3110 DNA	P1 DNA	salmon sperm DNA	
trp	7·6	-	-	500	$3·82 \times 10^{-7}$
	0·76	32·5	-	460	$2·41 \times 10^{-7}$
	0·76	-	78·4	420	$1·03 \times 10^{-7}$
ilv	10·0	-	-	500	$4·72 \times 10^{-7}$
	1·0	32·5	-	460	$3·78 \times 10^{-7}$
	1·0	-	78·4	420	$1·32 \times 10^{-7}$
metB	1·68	-	-	500	$1·08 \times 10^{-7}$
	0·17	32·5	-	460	$3·06 \times 10^{-7}$
	0·17	-	380.8	120	$3·64 \times 10^{-7}$
tna	4·5	-	-	500	$6·61 \times 10^{-8}$
	0·45	32·5	-	460	$9·92 \times 10^{-8}$
	0·45	-	380·8	120	$1·16 \times 10^{-7}$
ftsA	0·86	-	-	500	$1·48 \times 10^{-7}$
	0·09	32·5	-	460	$5·14 \times 10^{-7}$
	0·09	-	380·8	120	$8·06 \times 10^{-7}$

Table 7·2 Concentration of probe sequences in P1 and W3110 DNA.

The first column shows k, the rate constant, for each probe, which was calculated from the data in table 7·1 as described in the text. The next two columns show concentrations of each of the probe sequences in W3110 and P1 DNA respectively, calculated as described in the text. For easier comparison, the last column shows the concentration divided by complexity of the probe (the units are arbitrary).

Probe	k ($lsec^{-1}mole^{-1}$)	Concentration of probe in test DNA (μmoles nucleotides/ug)		concentration / complexity	
		W3110	P1	W3110	P1
trp	$6·57x10^{-3}$	$9·44x10^{-4}$	$1·23x10^{-4}$	1·67	0·22
ilv	$6·18x10^{-3}$	$1·64x10^{-3}$	$1·71x10^{-4}$	2·19	0·23
tna	$1·92x10^{-3}$	$1·48x10^{-3}$	$1·50x10^{-4}$	1·97	0·20
metB	$5·14x10^{-3}$	$1·79x10^{-3}$	$1·82x10^{-4}$	1·70	0·17
ftsA	$2·25x10^{-2}$	$6·81x10^{-4}$	$9·21x10^{-5}$	3·10	0·42

Table 7·3 Relative ratios of each sequence in W3110 and P1 DNA.

Relative ratios were determined from the final column in table 7·2; *ilv* was arbitrarily designated as 1·0.

Sequence	*ilv*	*tna*	*metB*	*ftsA*	*trp*
Relative ratio in:					
W3110 DNA	1·0	0·90	0·78	1·42	0·76
P1 DNA	1·0	0·87	0·74	1·83	0·96

Omitted from the data are control experiments to show that each reaction was driven by the test DNA. The rates of reassociation of the same amount of probe in the presence of salmon sperm alone, or together with test DNA were compared, and in all cases except one, the presence of test DNA greatly increased the rate of renaturation of the probe. In the case of the λBN3 fragment no difference in rate was detected in the presence of test DNA as mentioned earlier (page 137). These data could not be used.

However, the concentration of DNA used for the reassociation experiments as determined by measurement of the OD at 260 nm in a Zeiss spectrophotometer (table 7·1) would be expected to give too high a ratio of probe to test DNA for the reaction to have been driven by the test DNA. It can only be assumed that there was an error in the measurement of DNA concentration, and that therefore the results obtained are invalid.

of each probe, the concentration, in umoles nucleotide/ug test sequence, was divided by the complexity of the probe. These values are shown in the final column of table 7·2. The ratios of these values for each sequence with respect to the value for ilv probe were calculated for P1 and W3110 DNA, and are shown in table 7·3.

7·6 Discussion

The results displayed in table 7·3 show that the ratio of each of the five markers tested are very similar in E.coli log phase DNA, and in P1 DNA although the ratios are not those which would be expected; a culture at OD_{540} = 0·5 growing in broth should have an origin : terminus marker ratio of 2·3 (Cooper and Helmstetter, 1968). The concentrations of ftsA and trp relative to ilv are much higher in W3110 DNA, which was extracted from a culture of OD_{540} = 0·5, than would be expected from this assumption. The reasons for this are not clear, but there is a similar trend in P1 DNA. Possibly the major pitfall in the interpretation of these results is the use of probes with such a wide range in complexity; the ftsA probe which gave the most anomolous value is much smaller than the other probes. A valid method for compensating for complexity was not applied to these results.

The treatment of the results here is, in the author's opinion, a valid indication that the ratios of the five sequences tested in similar in P1 and W3110 DNA, although the absolute values are not comparable with one another.

The similarity between the two sets of ratios is further evidence that P1 does not package E.coli DNA selectively; selective packaging to the extent suggested by the poor recovery of some markers should result in obvious discrepancies in the two sets of ratios. Also since the size of probes used was much smaller than the size of a transducing fragment packaged by P1 (about 90 kb), the predominance of one or more species of fragment should certainly have been detectable.

A second unexpected aspect of these data is the surprisingly high ratio of each sequence in P1 DNA compared to that in E.coli DNA. These figures indicate that there is between 1/6 and 1/10 the amount of each sequence in P1 DNA compared to the amount in W3110 DNA. Unless there is a property of the two different samples of DNA which causes them to renature at a different rate, such as contaminating EDTA or salts, which would make the two sets of figures not directly comparable, there appears to be a much higher percentage of E.coli DNA packaged into P1 particles than previous estimates have shown. Sandri and Berger (1980a) made their estimate of 2% of the total DNA by growing transducing lysates on ^{32}P labelled bacteria, a method which ought to result in a highly accurate determination. There may of course be strain differences; it was noted at the beginning of chapter 2 that the strain of P1 used in these determinations had a very high frequency of transduction. It is therefore possible that this strain of P1 packages host DNA into 1/6 - 1/10 of phage particles, although determinations

using different samples of P1 and W3110 DNA should be made to substantiate or contradict these results. A repeat of the determination of the fraction of transducing particles made by Sandri and Berger (1980a) should also reveal any strain differences.

CHAPTER EIGHT

DISCUSSION.

The primary conclusion of this work is that P1 transducing DNA is selectively recombined into the recipient chromosome. Two types of evidence were presented: firstly, genetic; it was shown that UV irradiation could selectively improve the recovery of some markers. The second line of evidence was physical; this should have been easier to interpret than the first type of evidence, but in fact it proved to be rather more difficult, possibly because of the wide range in size of probes used. However, the results indicate that the conclusion drawn from genetic evidence - that there is no, or very little, preferential packaging of donor DNA by P1, is valid.

In chapter 1, some evidence for selective packaging in P22 was discussed, and also Sternberg's hypothesis that P1 recognises packaging sites in chromosomal DNA and packages from these. It was possible that high-transducing regions were adjacent to these recognition sites, but this theory was contradicted by the results of the hybridisation experiments. Sternberg observed preferential packaging from a λ prophage carrying the *pac* region of P1, and hypothesised that this had integrated at a site on the chromosome which was homologous with the *pac* site. This was perhaps a rather artificaial situation.

If P1 does not selectively package some regions of the chromosome, it is apparent that transducing DNA must be selectively recombined in the recipient in order to give rise to the large differences in the recovery of markers which is observed. UV irradiation only stimulated the recombination of some markers, which indicates that other regions, such as the origin, are normally integrated at high efficiency. The stimulation of transduction is caused by the presence of single-strand ends generated by uvrA and uvrB gene products in either the donor or the recipient (Helling, 1973; this work). Whether single-strand ends are necessary for integration of transducing DNA was not established. Zieg et al., (1978) used a variety of mutants, including lig, polA and uvrD, in an attempt to define those which are necessary for the processes of recombination, transduction, and conjugation. From these experiments, it was not clear whether strand breaks were limiting, or necessary, for transduction. In any case, if single-strand breaks are the cause of the high efficiency of transduction of some regions, it is not apparent why some regions of the chromosome are more susceptible to single-strand breaks than others.

Some possible reasons for selective recombination were investigated. Three-dimensional structure was suggested as a possibility but the experiments on gyrase mutants were inconclusive. It is known that supercoiling is important for transcription, site-specific recombination, and for P1 transduction. The chromosome is divided into separate domains of supercoiling, and it has been

suggested (Sinden and Pettijohn, 1981) that different states of torsional tension may be maintained in separate domains. In any case, the domains are independent, and it could be envisaged that there is a structural basis in chromosome folding which permits regulation of DNA-dependent processes (transcription, recombination and replication) in different domains, allowing for perhaps different levels of transcription and recombination in the separate domains.

A variable affinity for RecA protein may be a cause of variation in recombination frequency, but when the RecA protein concentration in recipient cells was greatly increased, no stimulation of transduction, either selective or otherwise, was observed. However, many other proteins are involved in recombination, and a variable affinity for these may lie in the primary structure of the DNA. It has been suggested that chi sites may be recognition or binding sites for a recombination protein (Schultz et al., 1981), and perhaps clustering of these may result in more initiation of crossovers in certain regions. Other binding sites, of course, may exist, and the role of chi sites in chromosomal recombination has not been established.

The results of experiments on a strain without chi sites ('RecF' strain) were rather ambiguous, possible due to the lack of some nuclease activity, as well as chi site activity, and could be interpreted in several ways. The fact that two nucleases, ExoI and ExoV, are missing from the RecF strain strongly suggests that the altered

transduction frequencies observed (figure 5·4) were due to a decrease in the breakdown of recombination intermediates, and thus increased recovery of low-transducing markers. However, this leads to the question of why recombination intermediates from some regions should be more susceptible to nuclease attack than from other regions. Supposing that some fragments are more rapidly integrated than others due to the presence of recombination-stimulating sites, or for some other reason, they would perhaps be less susceptible to nuclease attack. Integration of some fragments via a different pathway may lead to different intermediates, which are less susceptible to nuclease degradation. For example, the RecF pathway only functions in the absence of ExoI and ExoV, suggesting that some intermediates of this pathway are more susceptible to degradation than are intermediates of the RecBC pathway.

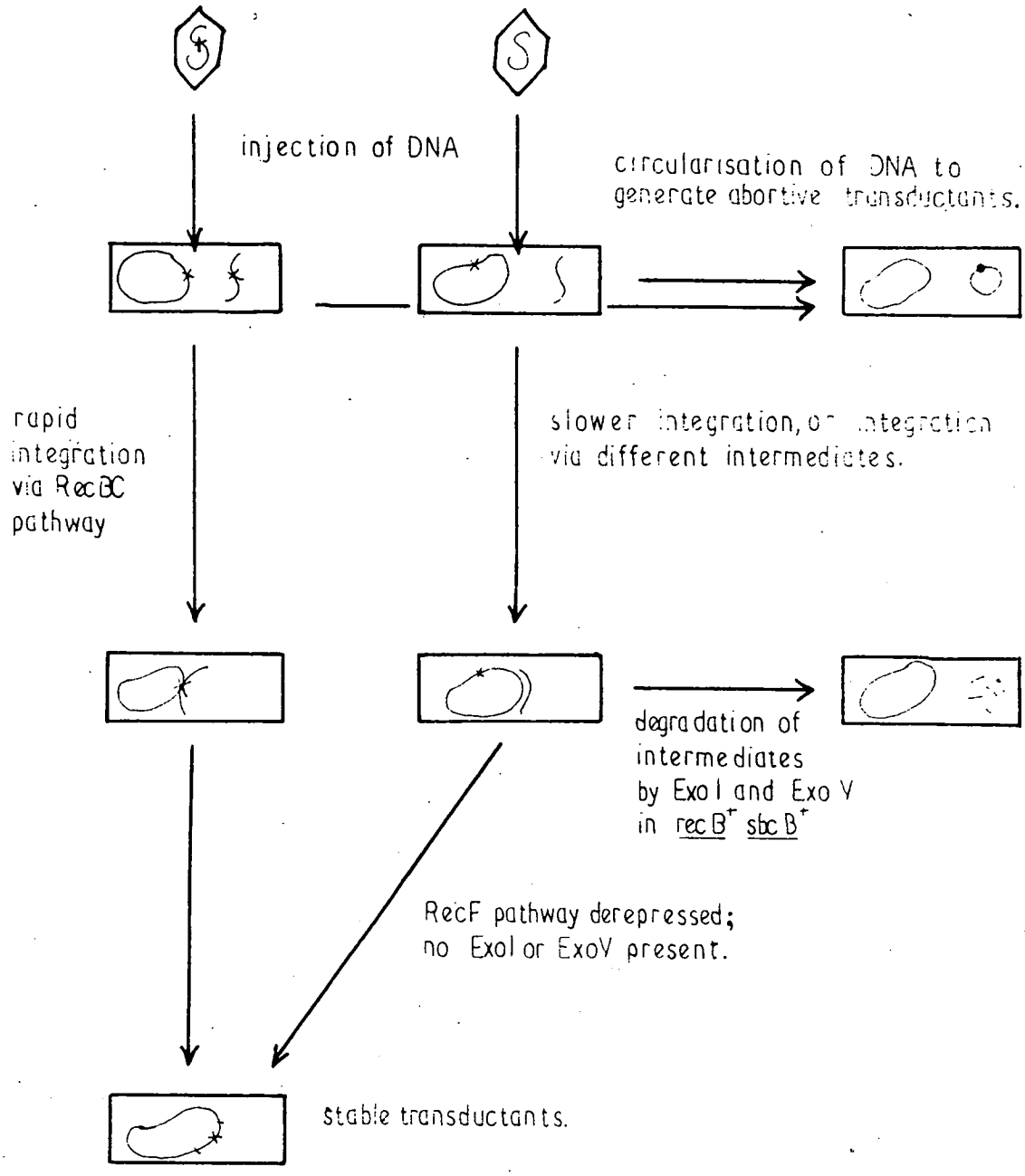
A complex pattern of recombination pathways has been emerging in the last few years. Different recombination pathways appear to favour different substrates. For example, Porter *et al.*, (1978) showed that the RecE/RecF pathway was much more active in recombination between λ p *lac* and chromosomal *lac* than the RecBC pathway. This result was also found by Basu and Oishi (1975), who stressed the role of ExoV in preventing recombination by the RecF pathway. Recently, Howard-Flanders and Bardwell (1981) showed that the double mutations *recB*21*recF*143 and *recB*21*uvrD*152 caused very marked deficiencies in Hfr x F^- crosses, whereas they had only moderate effects (a 3-fold

reduction and a 3-fold increase respectively) upon the frequencies of both spontaneous and damage-induced recombination in homoimmune λ phage-prophage crosses (using λ red⁻ gam⁻). This suggests that there is another pathway, independent of recB, recC and recF operating in these crosses.

By changing the major pathway of recombination from RecBC to RecF transduction frequencies were altered such that increased recombination of poorly transduced markers was observed (figure 5·4), indicating that recombination pathway is important in transduction; though why different markers should be differently affected is not obvious. Again, structural variations could be invoked; the RecBC pathway efficiently integrating DNA with chi or other active sites whilst other regions are inefficiently integrated via the RecF pathway. Loss of the RecBC pathway and exonucleases I and V would result in more equal integration of all regions.

Another factor to be considered is the formation of abortive transductants, which are generated by circularisation of the DNA with a protein. UV treatment causes decreased recovery of abortive transductants, whilst the recovery of stable transductants increases, thus presumably the DNA is more prone to recombination than circularisation. It was stated earlier that once transducing DNA has circularised, it is extremely stable, and recombination with the chromosome is rare. However, presence of UV damage may increase this frequency, and recombination may occur after circularisation. If it is

Figure 6.1



Model for alternative fates of transducing DNA.

Hexagons represent transducing phage particles; the one on the left has a recombinational hotspot (X). Rectangles represent Rec^+ recipient cells. For further explanation see text.

assumed that there is an initial choice between circularisation and recombination, it is possible that this is affected by the pathway of recombination,,or the presence or absence of exonucleases, and discrimination may arise at this stage, rather than at the stage of recombination.

Figure 8·1 shows a tentative model for the role of the recombination pathways, and hotspots for recombination, in the integration of transducing DNA. Transducing fragments entering a cell have an initial choice between circularisation with the aid of a protein to generate abortive transductants, or recombination with chromosomal DNA to generate stable transductants. Recombination may be favoured over circularisation if the DNA is damaged, or if it contains a hotspot for recombination. The bias in recombination may occur solely at this stage; once the DNA has circularised, it is protected from nuclease attack.

A fragment which fails to circularise and contains one or more hotspots for recombination,which may be sites for the initiation of recombination,may be rapidly integrated; if the hotspot is chi, recombination is via the RecBC pathway. Fragments without hotspots may be less efficiently integrated via the RecBC or an alternative pathway, and intermediate structures may be more susceptible to exonuclease degradation either because their resolution is slower, or because their structure is different.

When the RecF pathway is derepressed and ExoI and ExoV are eliminated, integration of previously poorly transduced markers is improved. This is either because

the RecF pathway integrates these markers more efficiently than the RecBC pathway, or because the slowly resolved fragments without hotspots are recombined via intermediates which are no longer degraded by nucleases, or a combination of the two. In this model, rapid integration via chi sites is eliminated in RecF strains, but the general level of recombination is increased due to the lack of exonucleases and/or derepression of the RecF pathway .

This model is by no means proven, but it does go some way to beginning to provide an explanation of some of the phenomena observed in the experiments described here.

There are further possibilities which may be important in recombination. For example, opening of the double helix during active transcription may provide a stimulus for recombination. Transcription would be more active in gene-dense areas, which could explain the coincidence of high transducing frequency within gene-dense regions. However, it is not certain how active transcription is at the stage when transducing DNA is integrated. This appears to occur within 1 hour after infection (Sandri and Berger, 1980a), and although there does seem to be a significant lag in DNA replication after infection, transcription could be sufficiently active to initiate recombination at active sites.

Further work is necessary to confirm and extend the results described in the preceeding chapters. The results

of the hybridisation experiments are particularly tentative, and more extensive determinations of reassociation rates should be made on the probes already described to try to find any sources of error. It should be possible to show that the rate is proportional to the concentration of probe sequence under the conditions used. Further experiments could also be performed, using markers from other chromosomal locations.

As it is apparent that recombination can favour certain pieces of DNA over others, and the experiments described only begun to investigate this, further experiments are possible in this area. For example, a more detailed analysis of chi site density and its possible correlation with areas of high transduction by cloning restriction fragments into recombination-deficient λ, would provide stronger evidence for or against the importance of chi in selective recombination. Since the hypothesis regarding chi sites assumes a specificity of pathway, more experiments on the genetics of P1 transduction should reveal some important facts. These could include transductions using *recF*$^-$ strains which may show poorer integration of damaged or low-transducing markers, and more experiments similar to those of Zieg *et al*. (1978) on *lig*, *pol*, and *uvrD* strains. It was also suggested that poorly transduced markers were slower to recombine, and thus more susceptible to nuclease degradation. It should be possible to detect a delay in the time of integration of some markers by using a *recA*^{ts}

recipient strain, and shifting to the non-permissive temperature at appropriate times after infection.

This work has revealed a hitherto unrecognised possibility: that the E.coli recombination system can selectively integrate certain pieces of DNA. The mechanism by which this occurs is not clear, but the experiments suggested above may shed some light on the problem. They may also reveal further facts about generalised recombination in E.coli, which is emerging as an increasingly complex process.

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The Variation in Frequency with which Markers are Transduced by Phage P1 is Primarily a Result of Discrimination During Recombination

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Summary. The efficiency of recovery of P1 transductants is marker dependent and normally varies over a 25-fold range. UV irradiation of either transducing lysates or recipient cells results in a selective stimulation of the transduction of markers which are normally transduced poorly. As a result the range in frequency of transduction is reduced to about 3-fold and resembles the gene frequency distribution expected in the donor cells. We conclude that P1 transducing lysates are likely to contain a random sample of donor DNA but that the recombination system of the recipient cell exhibits a preference for the DNA of some regions over that of others. Damage to DNA presumably overrides this specificity.

Introduction

The frequency with which recombinants are recovered after transduction of *E. coli* by the generalised transducing phage P1 varies greatly from marker to marker (Masters 1977). Markers close to the chromosomal origin of replication are transduced best, at frequencies up to 30 times that of the most poorly transduced markers (located in general near the terminus). Gene frequency in the donor cell has been shown to be an important factor in determining relative transduction frequencies (Masters and Broda 1971). Since, however, gene frequency in the donor population does not vary more than 4-fold between origin and terminus (Cooper and Helmstetter 1968), and since transduction frequencies do not, in any case, form a smooth gradient between origin and terminus, donor gene frequency cannot be the sole determinant of transduction frequency.

Other factors, such as for instance, the presence of chromosomal DNA sequences which resemble the sites of action of phage endonucleases involved in packaging might result in certain regions being packaged more efficiently than others. There is considerable evidence that packaging specificity is the major determinant of the frequency with which the Salmonella phage, P22, transduces (Susskind and Botstein 1978) and it has been suggested that it may also be an important factor in determining P1 transduction frequency (Low and Porter 1978). Alternatively, although genes might in fact be packaged into transducing phages more or less in proportion to frequency, the proportion of these genes which are eventually integrated so as to yield progeny might well be influenced by factors in the recipient cell. We will present evidence in this paper that the latter alternative is in fact correct: that is, that discrimination occurs during recombination in favour of DNA from certain regions of the chromosome.

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Methods and Materials

Bacterial Strains. W3110 was used as prototrophic donor for the preparation of transducing lysates. MM303 (*thi argH metB xyl malA his lacY tna strA tsx trp tonB pyrE uhp ilv* (P1)) was most often used as transductional recipient. Its derivation is described in Masters (1977). MM303-1 is a P1 cured derivative. Curing was accomplished by replacing the resident P1 prophage with $P1Cm_{ts}$ and selecting Cm^s survivors at 42°. MM7 is derived from JC411 and has the genotype *argG leu metB his malA xyl lac gal strA nalA pyrE uhp ilv*. NF279 *recA*$^+$ is also derived from JC411 and is described in Masters (1977).

Media and Transduction. Plkc lysates were made and used for transduction as described in Masters (1970) and Masters (1977). Media were as described in Masters (1970).

Irradiation of Phage and Bacteria. Phage were suspended at ca. 10^9 pfu/ml in phage buffer (KH_2PO_4 3 g/l, Na_2HPO_4 7 g/l, NaCl 5 g/l, $MgSO_4$ 10^{-3} M, $CaCl_2$ 10^{-4} M, gelatin 0.01 g/l) and 10 ml aliquots placed in a petri dish and irradiated with UV light at a rate of 10 ergs/sec/mm^2. Samples were agitated during irradia-

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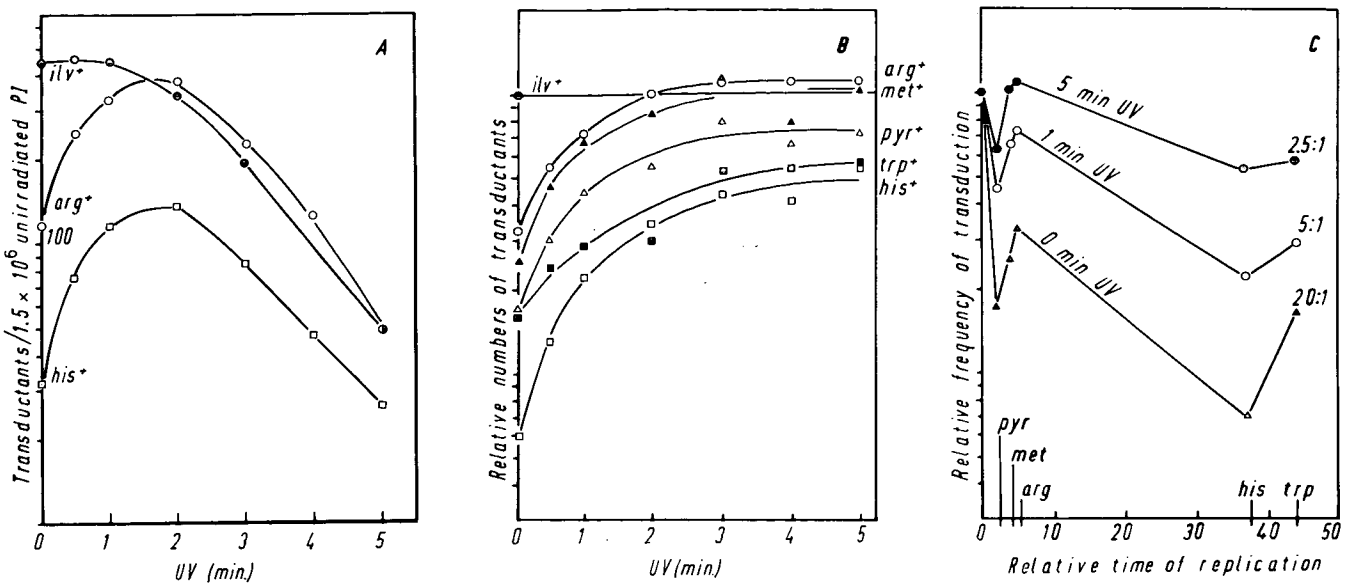

Fig. 1A–C. Transduction of MM 303 with irradiated P1 phage. Liquid lysates of P1kc made on the prototrophic strain W3110 were irradiated as described in Methods and used to transduce MM303. **A** Number of transductants to Ilv^+, Arg^+ and His^+ after various periods of irradiation. Survival of phage pfu is 3×10^{-4} after 2 min of irradiation. **B** Numbers of transductants relative to Ilv^+ after various periods of irradiation. Results are the average of three separate experiments. **C** Frequency of transductants as a function of relative time of replication assuming that the origin is at 83 min and the terminus at 33 min on the standard genetic map

tion. Bacteria were grown to stationary phase in L-broth, washed and resuspended in phage buffer. 10 ml aliquots were irradiated as above. After irradiation, cells were resuspended in L-broth and used for transduction. Irradiated phage and bacterial suspensions were kept in the dark until use.

Results

Irradiation of P1 Transducing Lysates Selectively Alters the Recovery of Transductants

It has long been known that irradiation of P1 transducing lysates results in a stimulation of transduction (Arber 1960; Wall and Harriman 1974). This, as does the occurrence of abortive transduction (Gross and Englesberg 1959), indicates that only some of the transducing DNA transferred to recipient cells is normally integrated to yield recombinant progeny. We reasoned that, if the DNA coding for poorly transduced markers is normally integrated inefficiently, stimulation of the level of integration by UV might selectively aid the recovery of these markers.

The marker *ilv* is normally transduced with very high frequency. Its recovery is not stimulated to any significant extent by irradiation (Fig. 1a). In contrast, the transduction of all other markers tested, each of which is normally transduced at a lower level than is *ilv*, is stimulated by irradiation (Fig. 1a, 1b), and reaches, for each, a characteristic level relative to *ilv*. The transduction of markers such as *his*, which are normally transduced most poorly, is stimulated most. As a result, the 25-fold range in transduction frequencies observed with unirradiated phage is reduced to between 2- and 3-fold.

Transduction frequency is in part a function of gene frequency in the donor cell; that is, origin genes are transduced with higher frequencies than terminus genes (Masters and Broda 1971). As the cells used to prepare the transducing lysates were growing with a generation time of 28 min, an origin/terminus ratio of 2.7 would be expected in these cells (Cooper and Helmstetter 1968). This compares well with the ratio approached after UV irradiation. This approach of transduction frequency to calculated gene frequency can be seen in Fig. 1c, in which the relative number of transductants for each marker is plotted as a function of the time at which the marker replicates. Transduction of the early markers *arg*, *ilv* and *met*, becomes nearly equal, as does that of the late markers *his* and *trp*. The transduction of *pyrE* is less markedly stimulated by this treatment but is effectively stimulated by irradiating recipient cells before transduction (see below).

That the degree to which UV stimulates transduction of a marker is indeed inversely proportional to how well it is normally transduced can be seen by examining the data from nine separate transduction experiments compiled in Fig. 2. In this figure the maximum observed stimulation of transduction for 12

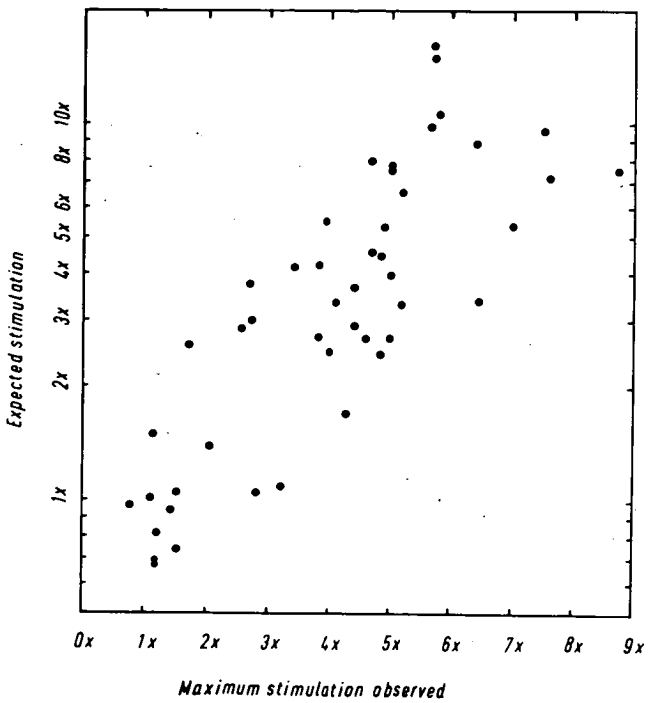

Fig. 2. Relationship between maximum observed UV-stimulation of transduction and stimulation expected (see text). Transductions were carried out for 12 separate markers in strains MM303 (*argH, pyrE, ilv, trp, his* and *metB*), MM7 (*argG, pyrE, ilv, leu, his* and *metB*) and NF279 *recA*$^+$ (*argG, his, leu, metB, ilv, rbs, xyl* and *pyrB*) with irradiated (5 samples per experiment, 30 sec–5 min irradiation) and unirradiated W3110 lysates. Expected stimulation was calculated by dividing the transduction frequency expected on gene dosage considerations (*ori* = 3, *ter* = 1) by that obtained using unirradiated phage (*argH* = 1). Maximum stimulation is calculated by dividing the maximum number of transductants obtained for each marker by the initial number. Maximum stimulations of less than 1 are the ratio of the number of transductants observed for unstimulated markers at UV doses which give maximum stimulation of other markers and the number obtained using unirradiated phage. Values from nine experiments were normalised; generally by setting the maximum stimulation for *metB* equal to 4.1, and individually plotted

separate markers in two unrelated strains is plotted against the stimulation anticipated on the hypothesis that UV is capable of stimulating the transduction of any marker up to a level proportional to its gene dosage in the donor cell. It is assumed that gene frequencies in the donor cell vary from 3:1 between origin and terminus, as would be predicted from the donor growth rate on broth of g = 27 min (Cooper and Helmstetter 1968). The average level of stimulation observed varies from experiment to experiment, presumably as a result of a fine balance between the cell killing and transduction stimulating actions of UV. The values for maximum stimulation were therefore normalised to allow the presentation of the data as a single line rather than as a family of lines. It is clear that transduction of markers transduced with high frequency is stimulated only slightly or not all by UV (points at lower left) while that of those normally transduced poorly is stimulated most (points at upper right). Markers transduced with intermediate frequencies are stimulated to an intermediate degree.

An alternative method of treating the data is to extrapolate the parts of the curves in Fig. 1 a in which inactivation of transducing activity appears exponential, back to their intercepts at 0 min irradiation. If we assume that inactivation of transducing ability by UV is exponential and superimposed on the stimulating activity which occurs at low doses, then such an extrapolation should give a measure of the maximum stimulation possible in the absence of UV inactivation (Arber 1960). Although such an analysis is limited in our experiments by the low numbers of transductants obtained after higher UV doses, our data is sufficient to show a range in extrapolated values of approximately 3X between origin and terminus (data not shown).

These results taken together strongly suggest that transducing lysates contain bacterial genes in proportions equivalent to their frequencies in the donor cells, but that some DNA sequences are normally integrated more efficiently than others.

Selective Stimulation of Transduction Results from the Irradiation of Transducing Fragments

The stimulation of transduction that we observe may be due to the fact that irradiated transducing DNA is itself improved as a substrate for recombination. Alternatively, it may be a consequence of a general stimulation of recombination function resulting from the introduction into the cell of irradiated P1 replicons along with the transducing DNA. Damaged replicons induce, for example, the synthesis of additional *recA* protein (Inouye and Pardee 1970) which could presumably circumvent a rate-limiting step in transductional recombination.

In order to distinguish between these possibilities we infected a recipient population with a mixture of unirradiated transducing phage and irradiated non-transducing P1. Non-transducing phage were obtained by preparing a lysate on MM303-1, a derivative of the strain to be used as the transductional recipient. The lysate was irradiated in the usual way and then mixed 100:1 with an unirradiated lysate of the prototrophic donor W3110. The mixture was used to transduce MM303 at a multiplicity of 4.5, thus ensuring that almost every cell in the recipient population would be infected with an irradiated phage. Since the irradiated lysate carries the same negative alleles as the recipient population, transductants can arise only as a result of recombination between the chro-

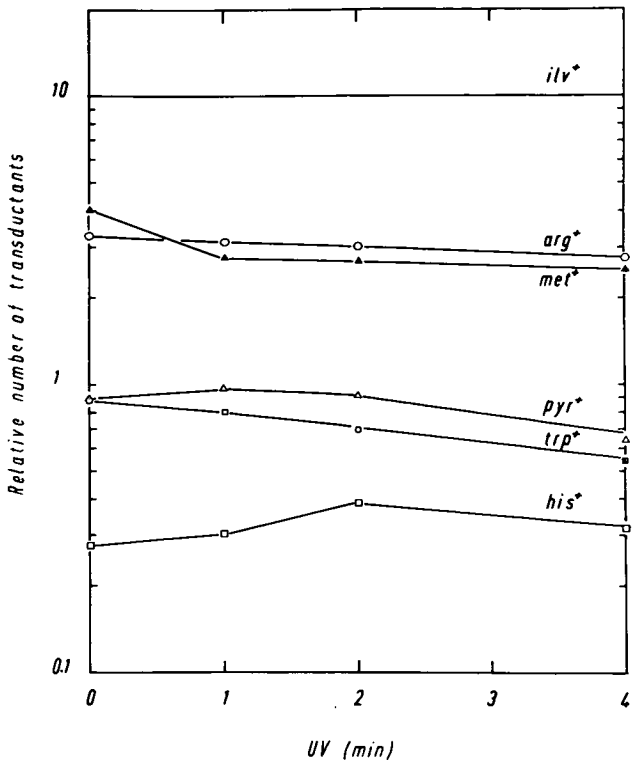

Fig. 3. Transduction of MM303 with a mixture of unirradiated transducing phage and irradiated non-transducing phage. P1 grown on MM303-1 and concentrated in CsCl to a titre of 1.7×10^{12}/ml was diluted 100× in phage buffer and irradiated as described. 1 min of UV reduced phage titre by 50×. 0.3 ml of irradiated phage were mixed with 0.1 ml of phage prepared on W3110 (8×10^8/ml) and added to the recipient cells

mosome and unirradiated DNA. It can be seen (Fig. 3) that relative numbers of transductants are almost unaffected by this treatment; certainly no stimulation of transduction of other markers relative to *ilv* is observed. Thus it seems probable that the stimulation of transduction found on irradiating a transducing lysate is due to the alteration in structure suffered by the transducing DNA fragment rather than to indirect effects mediated by the irradiated P1 DNA.

Irradiation of Recipient Cells Before Transduction Also Alters the Recovery of Transductants Selectively

To exclude the possibility that efficiency of integration varies because of variation in some aspect of transducing DNA structure which can be altered by irradiation, we irradiated the recipient cells before transduction, rather than the transducing phages.

When the recipient cells are irradiated and then transduced with unirradiated phage (Figs. 4a, b) the number of *ilv* and *arg* transductants immediately falls as the recipient cells are killed. In contrast, markers transduced with relatively low frequencies show an initial stimulation in frequency of transduction. This is particularly marked in the case of *pyrE*, which, although very near to *ilv*, is normally transduced poorly. Irradiation of recipient cells stimulates *pyrE* transduction greatly, such that it becomes comparable to that of other early replicated markers (Fig. 4b).

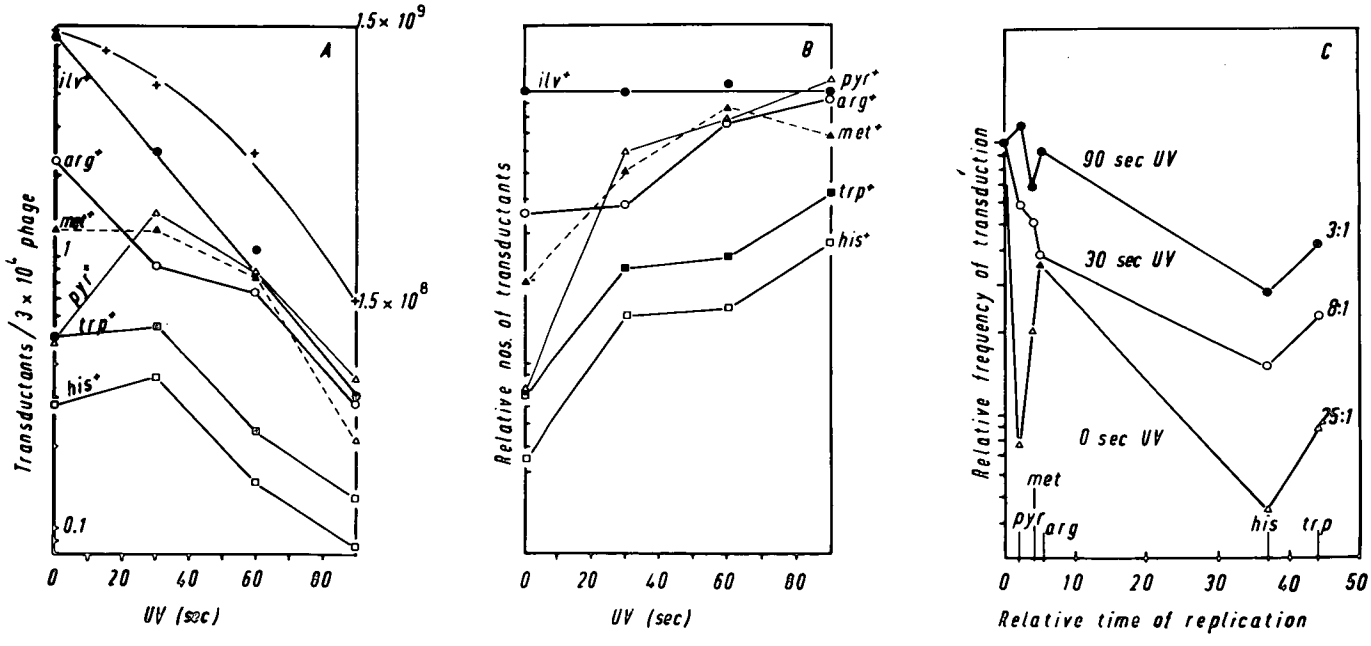

Fig. 4A–C. Transduction of irradiated cells with P1 phage. Cells of MM303 were irradiated as described in Methods and transduced in the usual way with lysates prepared on W3110. **A** Numbers of transductants for a variety of markers after various periods of irradiation; (+) survival of colony-forming units. **B** Numbers of transductants relative to numbers of Ilv^+ after various periods of irradiation. **C** Frequencies of transduction as a function of relative time of replication for 0, 30 and 90 second doses of UV

The total range in frequency of transduction is again reduced to the range expected in gene frequency, from 25- to 3-fold (Fig. 4c).

Thus we see that irradiation of the recipient cells before transduction selectively stimulates the transduction of poorly transduced markers in a manner similar to that observed after the irradiation of transducing lysates.

Discussion

We have shown that irradiation of P1 transducing lysates or of recipient cells selectively stimulates the transduction of markers normally transduced poorly such that the range in frequencies of transduction approximates the range calculated for gene frequencies in the donor cell. The fact that the transduction of poorly transduced markers can be stimulated proves that the corresponding DNA is packaged into phage heads and injected into the recipient cell. This lends support to the hypothesis that P1 encapsidates transducing DNA relatively unselectively and that variations in transduction frequency occur mainly as a result of events in the recipient cell. We hope to confirm this hypothesis by conducting a physical analysis of the gene content of transducing lysates.

That selective stimulation of transduction results from a stimulation of recombination in the recipient cell can be inferred from the observation that the transduction of complete replicons is not stimulated by UV (Arber 1960). Two possible mechanisms which could lead to selective stimulation of recombination after irradiation come to mind. The first is that certain DNA sequences are poor substrates for recombination and generally fail to be recombined because enzymes, such as *recA* protein, are present in low concentrations and fail to interact with them. Selective stimulation might then be expected to occur as a concomitant of treatments which would induce *recA* and other proteins active in recombination, such as irradiation of the cell or the introduction into it of a damaged replicon. We think that this is probably not the mechanism at work here since we have shown that the introduction of irradiated P1 virion DNA into a cell at the time of transduction is not in itself sufficient to stimulate transduction.

Another possibility is that transduced fragments which are normally poor substrates for recombination are rendered better substrates because of direct UV damage to DNA. Gapped or otherwise damaged DNA is well-known to undergo recombination with increased efficiency (Benbow et al. 1974; Konrad 1977). This seems the more likely explanation of our results as irradiation of the transducing DNA or, presumably, of its chromosomal homologue seems to be necessary in order for transduction to be stimulated.

The most intriguing question which arises from this work is that of why some regions of the chromosome are apparently better substrates for recombination than are others. In a previous paper (Masters 1977) we noted that, in general, gene dense regions are transduced better than are gene sparse ones. Since then a new edition of the genetic map has been published (Bachmann and Low 1980) incorporating some 300 new loci. Although this represents a close to 50% increase in the number of mapped genes, the new loci are distributed quite similarly to those already known. Thus the gene dense and gene sparse regions noted in 1976 by Bachmann et al. remain relatively unaltered in relation to one another and it seems likely that they will prove to represent a genuine variation in gene density. If so, there is presumably some factor correlated with gene density, either in DNA sequence or genome organisation, that facilitates transductional recombination.

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