ASPECTS OF P1 MEDIATED GENERALISED TRANSDUCTION

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PhD. THESIS

EDINBURGH UNIVERSITY

1986
TO MY PARENTS
DECLARATION

This thesis was composed by myself and the experiments presented here were performed entirely by myself.
ACKNOWLEDGEMENTS

I would like to thank my Supervisor, Dr. Millie Masters, for supervising this project and for her help and advice during the last three years.

Dr. David Finnegan, my advisor, has been a constant source of advice and encouragement and I am most grateful to him for his many ideas and suggestions.

I am deeply indebted to Dr. Ken Begg for his advice in all experimental matters relating to studies on the division and replication cycles of E.coli, for his constant good humour and for a number of memorable contests on the squash court. Thanks, Ken.

Dr. Andy Jenkins also deserves my thanks for his interest in my work, for his many suggestions and criticisms, for his application of psychiatric help in times of great stress!

I would also like to thank Dr. Howard Pringle and Dr. Willie Donachie for their help throughout the course of this project.

Ian Oliver contributed significantly to the "ease" with which the experiments in this thesis were performed; his organisational ability and technical expertise ensured that the lab was run as smoothly and efficiently (in the face of almost overwhelming odds!) as was possible.

Sheila Dickson and Kathie Harris are placed high on the Role of Honour for their courage and perseverance in the unenviable and gruesome task for translating my illegible scrawl into this beautiful typescript.

I would also like to thank Graham Brown for producing the A4 sized photographic figures included in this thesis, and Annie Wilson for expertly drawing the graphs.

I am most grateful to Margaret Anderson and the media ladies for providing an excellent and efficient media service.

Finally, I wish to thank my fiancee, Diana, for her support and understanding during the dark months of thesis writing. I hope I can do the same for her when her time comes.
The work in this thesis has concentrated on the P1 transduction process and on the nature of transductional discrimination; events in both donor and recipient cells have been studied.

The levels to which 11 separate markers are packaged into P1 transducing lysates has been measured using quantitative Southern hybridisation. All markers are packaged to equal levels, with the exception of three markers at the 2 minute region of the E.coli map, which are packaged 3 fold higher. These data support the hypothesis that transductional discrimination derives from events in recipient cells and not from marker packaging levels.

A DNA fragment carrying the 160 bp P1 pac site was cloned and used to probe the E.coli chromosome. No homology was seen, suggesting that pac-like sequences are not responsible for initiating chromosomal encapsidation into transducing particles.

P1 infection of a strain of E.coli, whose DNA was previously modified by the introduction of the EcoPl modification gene, produced a lysate exhibiting normal transducing characteristics. This indicates that the EcoPl restriction gene probably plays no part in the generation of transducing particles by the Wall and Harriman (1974) model.

Attempts were made to greatly overpackage specific regions of the E.coli chromosome by forcing the integration of plasmids carrying P1 pac into the chromosome at pre-selected locations. However, multiple copies of the plasmids (in tandem repeat) had occurred and the integrated pac sites were apparently not good substrates for P1 packaging.

Transduction experiments with exponentially growing recipient cells have shown that there is a marker-dependent lag before transductants increase in number. This has led to a novel genetic method to measure C-time and has provided evidence that P1 transductional recombination results in a single strand recombination event.

Finally, it has been demonstrated that transducing DNA
carrying markers closely linked to the *E. coli* chromosomal origin of replication (oriC) is able to undergo limited autonomous replication in recipient cells. Furthermore, evidence is presented which suggests that oriC-linked markers owe their high transduction frequencies to this ability to replicate.
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ABBREVIATIONS

Δ = deletion e.g. resΔ = res-deletion

dTTP = deoxythymidine triphosphate

dUMP = deoxyuridine triphosphate

exo I = exonuclease I

exo V = exonuclease V

e.o.p. = efficiency of plating

NT = Not tested

All other abbreviations are explained in the text
CHAPTER 1

INTRODUCTION
SECTION 1.0 INTRODUCTORY REMARKS

This thesis is concerned with aspects of bacteriophage P1-mediated generalised transduction. The transduction process stems from the ability of the phage, during normal lytic growth, to encapsidate host cell chromosomal DNA into phage heads in place of the phage genome; this results in the formation of generalised transducing particles. DNA from any chromosomal location can be packaged into generalised transducing particles, although not all regions of chromosomal DNA are encapsidated with equal efficiency (see: section 1.2 and chapter 3). Transducing particles, liberated at cell lysis, can then adsorb to other bacterial cells and inject their transducing DNA, which may become stably integrated into the recipient chromosome by homologous recombination. Clearly, this provides an important route for gene transfer, hence the wide use of P1-mediated transduction for laboratory strain constructions and genetic analysis. (Masters 1985 for review).

P1-mediated generalised transduction is a complex process and, as yet, the mechanisms by which it is brought about have not been fully elucidated. However, the processes leading to transduction, as far as are known, are reviewed in this introductory chapter. This chapter can be broadly divided into three parts:

The first part consists of a brief account of P1 molecular biology, together with the possible roles of P1-encoded functions in the generation of transducing particles within donor cells. (sections 1.1-1.5). Part 2 is concerned with the fate of transducing DNA once it enters the recipient cells (sections 1.6-1.7). The third part contains a discussion of recipient cell functions, such as recombination pathways, and their possible roles in determining the fate of transducing DNA after its entry into recipient cells. (sections 1.8-1.11). However, since relatively little is known about the detailed mechanisms of transduction, in some instances the discussion presented in the following sections is highly speculative.
PART 1.
EVENTS IN DONOR CELLS AND THEIR ROLE IN THE TRANSDUCTION PROCESS

SECTION 1.1
BACTERIOPHAGE P1

Bacteriophage P1 is unique amongst coliphages in that virions of at least three distinct sizes are produced at each burst. These are designated P1B (Big), P1S (Small) and P1M (Minute) (Walker 1966, Walker and Andesson 1970). P1B and P1S constitute the majority of particles in a lysate and are present at a ratio of 5:1. (Walker et al. 1979). P1B virions each contain a 90 kb linear double stranded DNA molecule, and are infective at low m.o.i. (Walker et al. 1979, Yun and Vapnek 1977). Collectively, P1B virions encapsidate a population of cyclicly permuted double stranded DNA molecules each consisting of the entire P1 genome, plus 7-12% terminal redundancy (Yun and Vapnek, 1977, Ikeda and Tomizawa, 1968). P1S and P1M virions however are infective only at high m.o.i. and restriction analysis of purified P1S and P1M DNA indicates that collectively they contain the entire P1 genome, but individually they are too small to do so. Hence the requirement for a high m.o.i. to give plaques (Walker et al., 1979). (For simplicity, P1B, P1S and P1M will be referred to as "P1 particles" for the remainder of this thesis.)

P1 virions can adsorb to the surface of Escherichia coli cells, in a reaction requiring Ca^{2+} ions and then inject their genomic DNA. P1 DNA then circularises inside the cell by homologous recombination between the redundant ends (Ikeda and Tomizawa 1968); circularisation can occur independently of the host encoded recombination functions recA and recBC (Rosner 1972), presumably by loxP/cre mediated site specific recombination (see section 1.4). As with other temperate phage there are two possible outcomes for the cell: firstly P1 may enter its lytic, or vegetative, cycle which results in cell lysis and the release of P1 progeny particles, or more rarely P1 lysogenises the recipient cell. In the lysogenic state, P1 lytic functions are repressed and the viral prophage is
FIGURE 1.1  P1 GENETIC MAP.

Reproduced from Yarmolinsky (1984)
Figure 1.1
maintained as an extrachromosomal unit copynumber plasmid, capable of replicating independently of the host chromosome (Austin et al. 1978, Ikeda and Tomizawa 1968, Prentki et al. 1971). The genetic control of P1 lysogeny and prophage maintenance is complex and as yet, poorly understood, so it is not possible to present a comprehensive model for lysogeny. However, work carried out to date indicates that the P1 immunity system, which prevents superinfection and allows establishment of lysogeny, consists of at least three specially distinct regions: (Figure 1.1).

1) The ImmC region which encodes the major P1 repressor, ci (Scott 1975, Scott 1980b)

2) The bof gene, which somehow regulates the activity of ci repressor (Sternberg and Hoess 1983)

3) The immI region, encoding an antirepressor gene ant, together with a repressor of ant, the c4 gene (Scott 1975, Wandersman and Yarmolinsky 1977, Scott 1980). As mentioned above, an adequate model to explain the activities and interactions of these genes and their products has not yet been formulated. However, the activities of some immunity genes are known, at least in part. It is clear that ci protein represses all lytic functions, but that not all promoters are repressed directly by ci (Sternberg and Hoess 1983). CI activity is itself regulated by the actions of at least three other genes coi bof and ant (also called rebA, rebB) (Scott 1980, Tovati-Schwartz 1979, Heilmon et al. 1980) and the ant gene(s) at least, are repressed by c4 repressor. (For a more comprehensive and speculative discussion on P1 immunity, see Sternberg and Hoess 1983). It is clear that there is much scope for further investigation of P1 immunity and the events leading to lysogeny.

Once lysogeny is established, partitioning and copynumber of the P1 prophage are accurately controlled, such that although the copynumber is low, about 1, (ie 1 copy of P1 per copy of the host chromosome) only one in $10^5$ cell divisions results in curing the resident P1 (Rosner 1972). The control of prophage maintenance has been better studied than P1 lysogeny and it is known that P1 encodes its own replication and partitioning functions. P1 DNA can
replicate from two distinct origins of replication, termed oriR (for right) and oriL (left) (Sternberg 1979, Sternberg 1981). However it seems likely that P1 prophage replicates from oriR under the control of a P1-encoded incompatibility determinant incA and the so-called repA gene (Chattoraj et al. 1984, Yarmolinsky et al. 1983, for review see Sternberg and Hoess 1983). OriL, on the other hand, functions during lytic growth and is regulated directly by cI repressor and indirectly (via ant), by the c4 repressor. The above system of replication functions stringently to maintain the unit copynumber status of the prophage.

In order to ensure partitioning of the prophage to daughter cells at division, P1 encodes its own partitioning functions. Three genes appear to be involved: parA, parB and an incompatibility determinant, incB (Sternberg and Hoess 1983). Again, the mechanisms of P1 prophage replication and partitioning are still not fully understood but both are obviously tightly controlled, as indicated by the very low levels of curing.

If lysogeny is not established following the injection of P1 DNA into the E.coli cell, P1 enters the lytic cycle of growth. The genetic control of the events leading to lysis has received scant attention so is poorly understood. However, the effects on the recipient cell are better known; following P1 infection host DNA replication is arrested (at least during P1 vir infection), phage DNA and phage encoded proteins are synthesised, accompanied by degradation of the host chromosome (Wall and Harriman 1974). P1 particles are then assembled in the cytoplasm and infective virions are released as the host cell lyses.

In addition to infective P1 virions, P1 lysis generates a small proportion of generalised transducing particles. These are formed as a by product of the normal P1 life cycle and comprise 0.3% of P1 particles for P1 vir and 2% of particles for P1 Kc (Ikeda and Tomizawa 1965a, Sandri and Berger 1980a). P1 transducing particles contain no phage DNA but instead encapsidate a 90 kb linear molecule of host chromosomal DNA, formed prior to P1 infection (Ikeda and Tomizawa 1965, see also section 1.5). Obviously, this provides an important route for gene transfer, as transducing particles generated
from one E.coli strain can inject their DNA into another strain; the net result being the transfer of 90 kb lengths of chromosomal DNA from one strain to another. Once introduced into the recipient cell, transducing DNA may undergo homologous recombination with the resident chromosome and so stably integrate. For this reason, P1 transduction is widely used by molecular biologists for strain constructions and genetic analysis (For review, Masters 1985).

The mechanism by which P1 generalised transducing particles are formed has not been elucidated. Elegant work by Harriman (1972) showed that transducing and infective P1 particles were liberated from the same cells. Harriman (1972) took non-suppressing E.coli cells which were lysogenic for integrated lambdoid prophages, which carried amber mutations in genes required for excision and prophage replication. These cells were infected with P1, separated from any unadsorbed phage and plated, before lysis, at low concentrations on a lawn of amber suppressing indicator cells. Transducing particles, released at lysis, carrying the integrated prophage, adsorbed to the indicator strain and injected their DNA. Cells receiving such transducing DNA were able to support the lytic cycle of the prophage and so gave rise to large plaques on the indicator plates. These could be distinguished from the small P1 plaques generated by P1 lysis of donor and indicator strains. Analysis of the large plaques revealed a mixture of lambdoid and viable P1 particles. Hence the conclusion that P1 infective and transducing particles are formed together in the same cell.

Aside from this early work, the generation of P1 transducing particles has received relatively little attention, so is still poorly understood. In the following sections, the possible ways by which transducing particles might be formed will be discussed. The models suggested are highly speculative because there is very little firm data available, so the models are implied from circumstantial evidence rather than direct evidence.
SECTION 1.2
PACKAGING OF DNA INTO PHAGE HEADS

An understanding of the mechanisms employed by transducing phage to package DNA is important not only in terms of the phage life cycle, but also to help formulate a model for the encapsidation of transducing DNA in place of the phage genome.

Bachi and Arber (1977) studied P1 packaging by comparing restriction digests of P1 prophage DNA with digests of P1 DNA extracted from virions. They concluded that the packaging substrates were concatamers of P1 DNA, that packaging commenced at a unique site, pac, and proceeded rightwards (on the P1 genetic map) encapsidating sequential "headfulls" of P1 DNA. Furthermore, 4-5 headfulls were encapsidated at any one packaging event. Additional evidence for the unidirectional headfull mechanism of P1 packaging comes, indirectly, from studies in which P1 pac sites were integrated into defined locations on the E.coli chromosome. Sternberg et al. (1981a) constructed an E.coli strain harbouring a site at which the P1 encoded loxP/cre site specific recombination system could act, allowing precise integration and excision of P1 (see section 1.4 for loxP/cre system). During P1 infection, the phage could transiently associate with the E.coli chromosome and thus provide a vehicle for the transient integration of P1 pac. Sternberg et al. (1981b) also lysogenised E.coli by integrating at attB, \( \lambda \) vectors carrying cloned P1 pac sites. If either of the above strains were infected with P1, and the resulting lysates used to transduce suitably marked recipients, markers to one side only of the chromosomally associated pac sites were transduced with raised levels; reversing the orientation of the chromosomally located pac site resulted in increased transduction of markers to the other side. The explanation for the above was that during lysis, P1-encoded packaging machinery recognised the integrated pac sites, which then served as initiation points for packaging. Packaging thus commenced from the integrated pac and proceeded unidirectionally into the E.coli genome resulting in overpackaging, hence increased transduction, of markers to one side of pac.
Recently, the nature of the pac site has been studied in more detail. Sequence analysis of pac suggests that a functional pac may be as small as 150-160 bp and consist of two directly repeated sequences of 30-35 bp which flank a 90 bp spacer region. The repeats each contain 3-4 BclII sites (5' TGATCA 3'), the central 4 bp being the target sequence for E.coli dam methylase (Sternberg 1984, Lacks and Greenberg 1977). Sternberg (1984) observed reduced pac cutting in dam- strains, which cannot methylate the central A residue (GA*TC), i.e. this implies that the 35 bp direct repeats are required for fully functional pac sites. End labelling experiments reveal that pac cutting occurs within the 90 bp spacer region, cleavage occurring at 6 distinct sites. The cutting reaction also requires the activity of a functional P1 gene 9 (pacase) which is located adjacent to pac on the P1 map (Sternberg 1984, Yarmolinsky 1984). The importance of the repeats and spacer region in defining a pac site has not yet been assessed. However mutagenesis of pac sites, in conjunction with pac cutting assays, should provide information as to the sequence requirement for a functional pac site.

In contrast to other phage packaging systems, pacase cleavage of pac occurs early in the P1 cycle. Other phage require head morphogenesis before pac cutting occurs, perhaps suggesting a coupling of pac cleavage with DNA encapsidation (Murialdo and Becker 1978, Sternberg and Hoess 1983). This led Sternberg and Hoess (1983) to propose that P1 pac cutting may play a role in generating a nick, or cut, in P1 DNA from which rolling circle replication initiates; the newly replicated DNA being encapsidated at some later stage.

There are interesting similarities between the packaging system of P1 and that of the other well characterised transducing phage P22. P22 is a temperate phage of Salmonella species and is, in many ways, similar to lambda (For review, see Susskind and Botstein 1978). However, P22 packaging resembles that of P1; i.e. initiates from a defined start signal (again called pac) and sequentially encapsidates headfull amounts of DNA from P22 concatamers (Jackson et al. 1978). As expected, with this type of packaging system, P22 exhibits terminal redundancy (about 2%) (Tye et al. 1974a). Like the P1 packaging system, encapsidation of DNA into P22 heads requires a
defined sequence to initiate. However once packaging commences from pac, no further specific sequences are required for packaging to continue (Tye et al. 1974a and b). This, of course, is in contrast to λ which requires specific sequences, within cos, to initiate packaging and to terminate packaging when the entire λ genome has been encapsidated (Hohn 1975). Kufer et al. (1982) localised the P22 pac site to within P22-gene 3 and Schmeiger (1984a) subsequently demonstrated that such pac sites were indispensible for in vivo P22-mediated packaging of DNA. The product of gene 3 is required for recognition of pac and in conjunction with gene 2, gene 3 is involved in pac specific DNA cleavage (Raj et al. 1974, Jackson et al. 1982). Laski and Jackson (1982), however, demonstrated that pac cleavage occurred without DNA packaging indicating that, as with P1, DNA packaging is distinct from pac cleavage.

P22 can function both as a specialised transducing phage and a generalised transducing phage (Zinder and Lederberg 1952, Smith-Keary 1966, Wing 1968). Similarly to those of P1, P22 generalised transducing particles contain host cell chromosomal DNA packaged in place of the phage genome (Ebel-Tsipis et al. 1972a). The involvement of P22 packaging in the generation of generalised transducing particles has been established and so will be discussed below.

Early work indicated that encapsidation of Salmonella DNA into P22 generalised transducing particles commenced at specific chromosomal locations and generated transducing particles of homogeneous composition (Ozeki 1959, Pearce and Stocker 1965). Chelala and Margolin (1974) showed that deletions in the Salmonella chromosome could drastically alter cotransduction frequencies of markers wholly to one side of the deletion. These observations are consistent with the model for P22 packaging and can be explained in the following manner: if P22 packaging starts from defined chromosomal locations, and ends after the phage head is filled, transducing particles of homogeneous composition will result as each head can encapsidate the same amount of DNA; i.e. packaging has defined end points as well as initiation sites. Hence some markers would always be within the same headful, whereas others would always
be separated by packaging end points and, despite contiguous chromosomal locations, exhibit low cotransduction frequencies. The deletions generated by Chelala and Margolin (1974) would alter the positions of packaging initiation and end points with respect to chromosomal markers. This would result in some markers, which in wild type strains always lie within the same headful, being separated by packaging end points, thus reducing their measured cotransduction frequencies.

Different markers on the Salmonella chromosome are transduced with widely different frequencies due to a 1000 fold range with which markers are packaged into P22 particles (Zinder 1955, Schmeiger 1984b). Highly packaged markers are thought to lie close to the putative chromosomal initiation sites for P22 packaging, whereas the more distal markers are less likely to be packaged by a progressive headful mechanism and so are poorly transduced. Schmeiger (1984b) proposed that sequences closely resembling, or identical to, P22 pac sites occur in the Salmonella genome, which serve as initiation sites for pac cutting and subsequent packaging. Support for this suggestion comes from earlier work with so-called HT (High Transducing) mutants of P22. HT mutants have produced specificity of packaging for P22 DNA, producing lysates with up to 50% transducing particles, as compared to 5% transducing particles in wild type lysates (Schmeiger 1972). Some HT mutations have been localised to P22 gene 3, the gene responsible for pac site recognition and such mutants fail to identify pac sites as initiation points (Tye et al. 1976, Chelala and Margolin 1976, Jackson et al. 1982). Consequently, HT mutants package all markers on the Salmonella chromosome with equal frequency, apparently having lost their specificity for the proposed chromosomal pac-like sites (Schmeiger 1972). The above evidence implies a role for P22 gene 3 in recognising and initiating DNA encapsidation from a number of pac-like sequences, located on the Salmonella chromosome. Such pac-like sequences facilitate the packaging of Salmonella chromosomal DNA into generalised transducing particles via the normal P22 packaging system.

Schmeiger (1984b) measured the packaging of 28 Salmonella markers in a normal P22 lysate. Packaging levels were then plotted
as a function of marker position. The graph showed 5-6 chromosomal regions of maximal packaging levels plus several minor peaks, indicating the existence of about 10 regions of high packaging; Schmeiger (1984b) proposed that these correspond to the chromosomal locations of pac-like sequences. A pac-like site may thus be 9-10 bp in complexity as such a sequence would occur about 10 times on the Salmonella chromosome by chance.

In view of the obvious mechanistic similarities between P22 and P1 packaging, and the similar structure of their generalised transducing particles, it is reasonable to suggest that P1 generates transducing particles in the same manner as P22 i.e. by recognising P1 pac-like sites on the E.coli chromosome from which DNA encapsidatation initiates. However, as yet, there is no evidence for the existence of such pac-like sites and the involvement of P1 packaging in the generation of transducing particles is not clear.

As is the case for P22 mediated transduction, P1 transduction frequencies vary from marker to marker. However, frequencies vary over a much smaller range, only 30 fold, and this is not a reflection of the levels with which markers are packaged (Masters 1977, Newman and Masters 1980, see section 1.7 and chapter 3). P1 can apparently package 4-5 headfuls of DNA (8-10 minutes of the E.coli chromosome) from each initiation event, so at least 10 pac-like sites would be required for packaging of the entire E.coli chromosome (Bachi and Arber 1977). Thus if they exist, P1 pac-like sites must occur at least as frequently in the E.coli genome as P22 pac-like sites in Salmonella. Bearing in mind the possible complexity of P1 pac sites (150-160 bp) (Sternberg 1984) it seems implausible that 10 such sites would occur by chance in the E.coli genome. (The probability of a specific 150 bp sequence arising by chance is $4^{150} = 2 \times 10^{90}$; the E.coli genome is $4 \times 10^6$ bp).

Wall and Harriman (1974) proposed that free ends of chromosomal DNA were all that was required to initiate packaging, supported by their observations that HT mutants of P1 degrade the host chromosome more rapidly than wild type P1 (Wall and Harriman 1973 and 1974), i.e. rapid degradation results in more free ends in chromosomal DNA and hence more packaging substrate. This in turn would cause a
higher proportion of transducing particles. Interestingly, Schmeiger (1984) reports that in vitro DNA packaging by phage P22 can occur independently of pac, providing mature, headful-sized DNA is available. This confirms that P22 at least can utilise free ends of DNA as packaging substrates. However, this process unexpectedly required the pac specific product of gene 3. Similarly, Sternberg (1983) suggests that pac cleavage serves only to generate free ends in P1 DNA, and packaging occurs subsequently by an independent process. These observations, of course, provide no evidence as to the mechanism by which P1 packages the E.coli chromosome, but do raise the interesting possibility that free ends of DNA may be the substrate for packaging. As P1 is known to degrade the host chromosome during lysis, the free ends which are generated may serve as packaging substrates and thus overcome the requirement for P1 pac sites in the E.coli chromosome.

DNA PACKAGING MECHANISMS OF OTHER GENERALISED TRANSDUCING PHAGE

Masters (1985) suggests that for a bacteriophage to be capable of producing generalised transducing particles, the phage must encapsidate its DNA by a similar packaging mechanism to that described for P1 and P22; i.e. must employ a "headful" mechanism whereby once encapsidation commences, no further sequences are required. In addition, Masters (1985) points out that for chromosomal DNA to be encapsidated, it must remain intact during phage lysis. Bacteriophages T1 and T4 both package DNA from concatomers, both encapsidate DNA by a headful mechanism and so both are candidates for generalised transducing phages (McHattie and Gill 1977, Black and Shawe 1983). Both T1 and T4, however, extensively degrade host DNA during lysis and so it would seem unlikely that sufficient host DNA would remain intact to be packaged. However, under appropriate conditions, both phages can generate transducing particles. If T1 phage carrying amber mutations are propagated on amber-suppressing strains and the lysates then used to transduce non-suppressing recipient (this avoids lysis of potential transductants by the virulent T1 phage) transductants can be detected (Drexler,
it seems that encapsidation of host DNA must occur early in the phage lytic cycle, before the host DNA is degraded. T4 transduction, however, requires mutations in the phage which prevent degradation of host cell DNA (Wilson et al. 1979); only under these circumstances are transducing particles formed.

Clearly T1, but not T4, is capable of generating transducing particles as part of its normal life cycle. A detailed description of T1 and T4 packaging mechanisms is beyond the scope of this introductory chapter. It is interesting to note, however, that the mechanism of T1 transduction shows some similarities to that of P22-mediated transduction. T1 transduction frequencies are also marker dependent and vary over a 100 fold range (Drexler 1970). Drexler (1977) reports that T1 transduces bio with greater efficiency than other markers. This is due to the presence of a special site, the nature of which remains unclear, located between gal and att λ. T1 is assumed to recognise this site as a site from which to commence DNA encapsidation, which then proceeds by the sequential headful mechanism, proposed by Gill and MacHattie (1976) and MacHattie and Gill (1977). Hence bio is packaged with higher efficiency than other markers. In addition, Drexler (1977) suggests that the marker dependence of T1 transduction is due to the presence of other packaging "sites", which lie adjacent to highly transduced markers. Clearly, this implies that T1 generates transducing particles by the same mechanism as P22 (Schmeiger 1984b, see "PACKAGING OF DNA INTO PHAGE HEADS", this section).
SECTION 1.3

EcoP1 RESTRICTION/MODIFICATION

P1 encodes its own type III restriction/modification system (EcoP1). The two genes involved termed res (restriction) and mod (modification) are adjacent on the P1 genome (see figure 1.1) and are transcribed together (Iida et al. 1983). The mod gene product is involved in recognition and modification (methylation) of a specific 5 bp asymmetric recognition site (5'AGA*CC 3'), the central A residue being the site of methylation. Modification can occur independently of res gene function (Hadi et al. 1983, Bachi et al. 1979). res mediated restriction, however, requires both functional res and mod genes; mod is necessary for recognition of the above 5 bp site and res for the generation of a double stranded cut, with a 2-4 bp 5' overhang, 24-26 bp 3' to the recognition site (Bachi et al. 1979, Hadi et al. 1983).

The *in vivo* control of res and mod activity is of particular interest. Obviously, when P1 is to establish itself as a lysogen, host DNA must be modified before exposure to the P1 restriction system. Arber et al. (1975) demonstrated that the mod activity was detectable at early times after P1 infection, but res activity only appears much later. More recently, it has been demonstrated that res activity does not reach maximum levels until 3 hours after infection but that mod activity does so after only 15 minutes (Levy et al. 1984). Iida et al. (1983) showed res and mod were transcribed together (mod first) leading Levy (1984) to propose that the UGA stop codon, at the end of the mod gene, is recognised at early times after infection and that the truncated product would be unable to interact with res nuclease, enabling modification but not restriction, to take place. At later times, however, termination at the UGA codon would be suppressed producing a mod methylase which can interact with res; this mod-res complex would then recognise the target site and so allow restriction.

These events have been studied in P1 lysogens. However, it is not clear whether the control of res and mod is similar during lysis. If so, some limited restriction may occur, possibly generating the
double strand breaks in *E. coli* DNA which could serve as packaging substrates. This possibility is discussed and tested in chapter 3.
SECTION 1.4
P1 SITE SPECIFIC RECOMBINATION SYSTEMS LoxP/cre

P1 virion DNA is cyclicly permuted with 7-12% terminal redundancy (Yun and Vapnek 1977). Other phages with similar genome organisation, e.g. T4 and P22, have circular genetic maps, but the P1 map is linear (Epstein et al. 1963, Scot 1968, Suskind and Botstein 1978, Walker and Walker 1975). This apparent anomaly was resolved when Sternberg and Hamilton (1981a) identified a site, LoxP, situated at the end of the P1 genetic map, which was the target for an efficient site specific recombination system. Hence as recombination always occurs between them, the markers flanking LoxP appear unlinked in genetic crosses. Adjacent to LoxP is a gene cre whose product is required for recA-independent recombination at LoxP.

The LoxP site has been well characterised and consists of only 34 bp; two 13 bp inverted repeats flanking an 8 bp spacer region (Hoess et al. 1982). Cre protein, which mediates LoxP recombination, is a 34000 m.w. single polypeptide and binds to LoxP at two cre-binding domains; each consists of the 13 bp repeat plus the adjacent 4 bp of the 8 bp spacer (Abremski and Hoess 1984, Hoess and Abremski 1984). Recombination between two directly repeated LoxP sites results in deletion of the intervening DNA. However, if the LoxP sites are indirectly repeated, the intervening DNA is inverted (Abremski et al. 1983). Therefore, because LoxP sites exhibit a high degree of symmetry, the directionality of LoxP sites must stem from the asymmetric 8 bp spacer.

The LoxP system was originally of interest because of the possibility that it played a role in integrating P1 into the bacterial chromosome, in the same manner as λ site-specific recombination. If several putative chromosomal integration sites existed for LoxP/cre mediated integration of P1, then this could provide a mechanism for the generation of transducing particles; packaging could initiate for the pac site on the integrated P1 and proceed into the E.coli chromosome as subsequent headfuls of DNA are packaged (Sternberg et al. 1981b). However, the available evidence suggests this is probably not the case. LoxP/cre dependent
integration of P1 into the bacterial chromosome can only be detected very rarely and there appears to be only one chromosomal location for integration, loxB, at 66-67 minutes on the E.coli map (Chesney 1979, Sternberg et al. 1981a). This argues that integration of P1 into the chromosome is not of significance. However, the method used to detect integration of P1, involving the integrative suppression of dnaA strains by the P1 prophage, required the integration to be stable. Transient associations of P1 with the E.coli chromosome, which could serve to initiate encapsidation of the chromosome (Sternberg et al. 1981a), could not be detected by the approaches of Chesnay (1979) and Sternberg et al. (1981a) which required integration to be irreversible. If packaging of the E.coli chromosome initiates for P1 integrated via loxP/cre, the first headful would generate a hybrid particle containing 5% P1 sequence, from pac to loxP, with the remainder of the DNA (95% of the molecule) being of chromosomal origin. There is no evidence for the existence of such hybrid particles in wild type lysates but it is not clear whether the techniques used to study the composition of transducing particles (Ikeda and Tomizawa 1965a) would detect the small proportion of P1 DNA encapsidated by the above mechanism. In any case, a major role for loxP/cre in the formation of transducing particles seems unlikely, although it cannot be ruled out, as the loxP/cre system could not be expected to function efficiently during P1 lysis; the substrate for P1 packaging is concatameric P1 DNA (Bachi and Arber 1977) and loxP/cre activity would resolve concatamers into monomers, which of course are not the normal P1 packaging substrate. Instead, the loxP/cre system seems to be of most importance during lysogeny, assuring the stable partitioning of P1 prophage to daughter cells at division. P1 prophage is maintained with a copy number of 1 (Ikeda and Tomizawa 1968), i.e. 2 copies per dividing cell. In dividing cells, recA dependent homologous recombination between the two prophage would result in a P1-dimer which, obviously, can only be partitioned to one of the daughter cells, i.e. the other daughter will have lost the P1 prophage. Austin et al. (1981) showed that stable partitioning of P1 in Rec+ cells is dependent on a functional loxP/cre system; apparently, cre-
dependent recombination between the two loxP sites present, in cis on a P1 dimer is very efficient. The dimers of P1 are quickly resolved to give two monomer P1 prophage which can be segregated, one to each daughter cell.

**P1 cin/cix**

The P1 genome encodes an invertible DNA segment, termed the c-loop, consisting of 3 kb of unique sequence, flanked by 600 bp direct repeats (Lee et al. 1974, Yun and Vapnek 1977, Iida et al., 1982). Inversion of the 3 kb region is mediated by the action of an adjacent DNA invertase gene cin (c inversion), which, independently of recA, promotes recombination between sites, termed cix, located at the ends of the 600 bp repeats (Kutsukake and Iino 1980, Iida et al. 1982). C-inversion controls the host range specificity of P1 (Iida 1984).

Other such invertible systems are known and include:

i) G-loop inversion in bacteriophage Mu, mediated by the product of gin which also controls host range (Chow et al. 1977, Kutsukake and Iino 1980, Giphart-Gossler et al. 1982).


iii) pin gene activity in E.coli which is encoded by and inverts part of the cryptic element el4 (Van de Putte et al. 1984, Kutsukake et al. 1985).

The components of all these systems can substitute for each other in in vivo complementation studies, e.g. P1 cin can mediate G-inversion in Mu and likewise, gin can promote C-inversion. pin can also mediate G-inversion (Chow and Bukhari 1976, Van de Putte et al. 1984, Iino and Kutsukake 1980).

Specialised P1 transducing particles can be generated by cin dependent recombination between P1 and plasmids carrying a cloned cin/cix system, i.e. cin-mediated recombination can cause genome fusion (Kennedy et al. 1983). This raises the interesting possibility that cin or pin dependent recombination could fuse P1 with the E.coli genome during P1 lysis. Packaging could again
commence from the integrated phage and as described in the previous section, the first particle would contain a hybrid genome but this time consisting of 30% P1 sequences (from pac to cix) and 70% of DNA from the chromosome at e14 (25 minutes on the Genetic Map (Bachman (1983)). When considering this idea, the same arguments apply as before i.e. there is no evidence for such hybrid particles but they may be too rare to detect by the methods used (Ikeda and Tomizawa 1965a). However, the possibility that pin or cin do influence transduction of the 25 minute region cannot be ruled out. As yet, no experiments to test this suggestion have been performed.
P1-MEDIATED GENERALISED TRANSDUCTION: ORIGIN OF TRANSDUCING PARTICLES

The derivation of the DNA within transducing particles was elucidated by Ikeda and Tomizawa (1965a). E. coli cells were grown in media containing 5-bromouracil (BU) which was incorporated into the E. coli DNA, so that the chromosomal DNA was density labelled; such density labelled DNA can be separated from normal DNA by centrifugation in caesium chloride equilibrium gradients. The cells were then shifted to non BU-containing media, P1 phage were added immediately and the culture grown until lysis occurred. (Any DNA synthesised after the shift to BU-less media would be of normal density and DNA formed prior to the shift would still be density labelled.) The phage particles, both infective and transducing, were harvested and banded on caesium gradients. The gradients were fractionated and fractions were assayed for P1 infectivity and transduction. Transducing particles banded separately from infective particles and contained fully density labelled DNA, formed prior to P1 infection. P1 infective particles, however, contained light unlabelled DNA which had obviously been synthesised after P1 infection. Ikeda and Tomizawa (1965a) concluded that transducing particles contained no phage DNA but contained instead, 90 kb of host DNA synthesised before P1 infection. Unfortunately this approach would not be able to differentiate between particles with a small amount of P1 DNA linked to host DNA, as might be formed by transient loxp/cre mediated recombination with the chromosome and particles containing no P1 DNA at all. However, it is clear that if any P1 sequences are present they represent a very minor proportion of the DNA within transducing particles. If P1 sequences are present in transducing DNA, however, the nature of the P1 sequences may provide evidence for the derivation of generalised transducing particles.
The preceeding sections have been concerned with the events within donor cells that may influence the generation of transducing particles. The following sections are concerned with the fate of transducing DNA once it enters the recipient cell.

SECTION 1.6
THE TRANSDUCTION PROCESS; EVENTS IN THE RECIPIENT CELL

One of three possible fates awaits transducing DNA following its entry into the recipient cell (Sandri and Berger 1980a):
1) Some molecules will undergo recA-dependent homologous recombination with the recipient chromosome, whereby part of the incoming DNA replaces resident host sequences (termed complete transduction). The remainder of the transducing DNA and the displaced host DNA are then presumably degraded.
2) Other transducing molecules may be totally degraded.
3) The majority of transducing DNA (about 75%) persists as stable, non replicating circular molecules which pass unlinearly to daughter cells (Sandri and Berger 1980b). (Such molecules are termed abortively transduced DNA and cells harbouring them termed abortive transductants).

The above events will be discussed in greater detail below.

Abortive transduction was first recognised following P22-mediated generalised transduction of motility characters in Salmonella and subsequently in P22 and P1-mediated transduction of nutritional markers in Salmonella and E.coli respectively (Stocker 1956, Gross and Englesberg 1959, Ozeki 1959). Abortive transduction of nutritional markers results in the growth of tiny colonies amongst the normal sized colonies formed by complete transductants on selective medium. Analysis of the cells within such tiny colonies reveals only one cell which is again capable of forming another tiny colony. This suggests that abortively transduced DNA is not replicated, so at division, is passed to only one daughter cell. Abortively transduced DNA, however, can be expressed, so at division
the products of that expression pass to both daughter cells; hence, cells which do not receive abortively transduced DNA may nevertheless continue to grow until subsequent divisions dilute the products from the abortively transduced DNA, or normal cellular protein turnover degrades these products. The net result is that abortive transduction results in tiny colonies (see Ozeki and Ikeda 1968 for review).

More recently, Sandri and Berger (1980a and b) investigated the structure and fate of P1-mediated transduced DNA. Using purified transducing particles, encapsidating $^{32}$P, 5-BU labelled transducing DNA, they were able to distinguish incoming DNA from the cold, light DNA resident in recipient cells. Following the introduction of transducing DNA, the recipient cells were lysed gently, using non ionic detergents and the DNA banded on caesium gradients. 7-15% of the incoming DNA banded with the light chromosomal fractions and was shown to have integrated as double stranded regions, about 1/10 the size of the incoming molecules (i.e. about 9 kb). This occurred only in Rec$^+$ cells and so was thought to constitute complete transduction. However, the size of the integrated DNA seems inconsistent with observations that P1 can cotransduce markers up to 90 kb apart, i.e. implies both markers being recombined into the E.coli chromosome together on a long stretch of DNA equivalent in size to the incoming DNA. One possible explanation is that transductional recombination involves multiple crossover events, resulting in several small double insertions, rather than one long double strand region integrated by one crossover at each end. Also Sandri and Berger (1980a) demonstrated that the recombination events in recipient cells occurred rapidly and had reached maximum levels 20 minutes after the introduction of transducing DNA. In addition to the double stranded insertions, 10% of the incoming DNA became associated with the recipient chromosome in a non-specific random manner. Sandri and Berger (1980a) suggested that this was due to degradation of the transducing DNA and reutilisation of its components.

Most of the transducing DNA, some 75%, was shown to persist in recipient cells as a circular protein/DNA complex, the protein
apparently binding the ends of the DNA together to maintain the circular configuration. Such circular molecules were stable for at least 6 hours (the duration of the experiments) and so seemed immune to host recombination and degenerative pathways. Sandri and Berger (1980b) pointed out that such molecules exhibit all the properties of those expected to give rise to abortive transductants, so they were termed collectively "Abortively Transduced DNA".

Circularisation of abortively transduced DNA occurs in the recipient cell, as DNA in transducing particles is linear (Sandri and Berger 1980b). The mechanism of circularisation is not known, nor is the origin of the protein which binds the ends of the DNA. Interestingly, Ikeda and Tomizawa (1965b) demonstrated that DNA in P1 transducing particles, but not in infective particles, is associated with a protein; it is tempting to speculate that this is also the protein which binds the ends of transducing DNA and so may play a role in circularisation.

Abortively transduced DNA is refractory to host cell recombination pathways so it seems plausible that the circular configuration is not adopted by molecules which recombine with the chromosome. Possibly, the two outcomes, abortive or complete transduction, are alternatives and true transductants arise from molecules which fail to circularise before being attacked by host degenerative and recombination pathways. Certainly, linear duplex DNA exhibits some highly recombinogenic features (see section 1.10).
SECTION 1.7
RECOMBINATION TO GIVE COMPLETE TRANSDUCTION: P1 TRANSDUCTION FREQUENCIES

Cells in exponential growth contain approximately 4 chromosomal replication forks (Cooper and Helmstetter 1968), hence the ratio of origin to terminus markers is approximately 4:1. (This effect is termed "Gene dosage"). Therefore, if P1 lysates are made on exponentially growing cells and if packaging of transducing DNA, together with its recombination in the recipient, show no sequence-specific selectivity, origin markers should transduce with 4 times the frequency of terminus markers. This clearly is not the case. Masters (1977) measured the transduction frequencies of 26 markers at different locations on the E.coli chromosome. Transduction frequencies varied over a 30-fold range in a marker-dependent manner.

Transduction frequencies were plotted as a function of map position and the graph showed a series of sharp peaks and troughs, indicating areas of high and low transduction, superimposed over a variation in transduction frequency, possibly due to gene dosage. The highest levels of transduction were for markers in a 4 minute region flanking oriC, i.e. for markers cotransducible with oriC. However, markers just outside this region were poorly transduced e.g. pyrE at 80.7 minutes (see figure 1.2) (Masters and Broda 1971, Masters 1977). Furthermore, Masters (1977) noticed a remarkable correlation between transduction frequencies and gene frequency distribution of markers along the E.coli chromosome. The distribution of genes along the chromosome is not random but markers cluster into regions of relative gene-density and sparsity (Bachman 1983) (see figure 1.3). Masters (1977) showed that markers from gene dense regions tended to be transduced at high levels whereas those from gene sparse regions seemed to be poorly transduced. It should be noted that our knowledge of the number and location of genes on the E.coli chromosome is still incomplete. As more markers are discovered and mapped more may be assigned to the so-called sparse regions and the gene frequency distribution may become more uniform.
Triangles are from Masters and Broda (1971). The dashed line represents the gene frequency distribution due to gene dosage in the exponentially growing culture used for the production of the P1 lysate.
Figure 1.2

MAP POSITION OF SELECTED MARKER
FIGURE 1.3. GENE FREQUENCY DISTRIBUTION OF MARKERS ON THE \textit{E.coli} GENOME

The height of the "blocks" on this circular histogram are proportional to the number of markers in each map unit interval. Marker frequencies were determined from the \textit{E.coli} genetic map published by Bachman (1983).
However, recently discovered genes tend to fall into existing areas of gene density and thus preserve the overall pattern of density and sparsity (Bachman 1983).

Thus P1 transduction is obviously a selective process, some genes being transduced at much higher levels than others. The selectivity could arise at two possible stages: P1 packaging or recombination in the recipient. The role of P1 packaging system has been discussed previously (see section 1.2 and chapter 3) and has been eliminated as the major cause of transductional selectivity. For the remainder (Part 3) of this introduction factors which may be involved in the processes and selectivity of transductional recombination will be reviewed and discussed.
PART 3.
ON THE ORIGIN OF TRANSDUCTION SELECTIVITY

SECTION 1.8
THE THREE DIMENSIONAL STRUCTURE OF THE E.COLI CHROMOSOME

One possible cause of transductional selectivity is apparent from work carried out to study the structure of the bacterial chromosome in vivo.

The E.coli chromosome consists of a double stranded circle of DNA with a diameter of about 1mm (Cairns 1963). Within the cell, however, the chromosome is packed into a nucleoid body about 1μm in diameter (For review see Lyter 1968). This argues that the chromosome must be maintained in an organised, folded structure so that complex cellular processes, such as DNA replication, transcription and chromosome partitioning, may be carried out efficiently. Worcel and Burgi (1972) formulated a model for the folded structure of the E.coli chromosome based on a number of observations. They demonstrated that the chromosome is negatively supercoiled and quantitative estimates of superhelicity were shown to be consistent with those of other naturally occurring DNA molecules. To relax the chromosome in vitro, however, required treatment with surprisingly high concentrations of DNAse; some 6-40 nicks in the DNA were required before complete relaxation of the chromosome was observed. This suggested that the chromosome was organised into a number of distinct loops each of which could maintain its superhelicity independently of adjacent loops. Hence the high requirement for DNAse to relax the entire chromosome. In addition, Stonnington and Pettijohn (1971) showed that the folded structure of the chromosome was a DNA/RNA complex and very sensitive to RNAse; they estimated that RNA comprised 10% by weight of the DNA/RNA complex so Worcel and Burgi (1972) suggested that the chromosome was organised into a series of loops (perhaps as many as 50) which were maintained independently supercoiled by attachment to a central "core" region, possibly consisting of RNA. They also observed that some regions of the chromosomal DNA were less susceptible to DNAse activity, implying a topological protection of the putatively inner
regions of the structure. However, it is not clear whether this putative structure is static, or dynamic; in other words, there is no evidence that fixed areas of the chromosome always constitute the attachment regions whilst other regions always form loops (Pettijohn 1976, Pettijohn and Carlson 1979).

As mentioned previously, the distribution of genes on the E.coli chromosome is not uniform, but markers are clustered into gene-dense regions (Bachman 1983). Perhaps gene-sparse regions represent the inner, or attachment regions of the chromosome structure whereas gene-dense regions constitute the outer, more accessible areas. It seems reasonable to speculate that the outer regions of the chromosome structure are more accessible to cellular processes, such as transcription and recombination and so would encode most information; perhaps the accessibility of these regions to host recombination systems is responsible for the relatively high transduction frequencies in gene-dense regions, observed by Masters (1977).
Abortive transductants are stable, so that the conversion of abortive to complete transduction occurs with a frequency of only $10^{-3}$ per generation (Ozeki and Ikeda 1968). However, the irradiation of P22-transducing lysates or transduced cells with UV-light causes an increase in complete transduction concomitantly with a reduction in abortives; i.e. abortive transductants can be "converted" to complete transductants by UV damage to DNA (Benzinger and Hartman 1962). Likewise, P1 generalised transduction is stimulated by UV-irradiation, although it is not clear if this is accompanied by a drop in abortives (Arber 1960, Wall and Harriman 1974). Helling (1973) showed this effect was dependent on host excision repair genes $uvrA$ and $uvrB$, as well as the $recA$ gene product. Single strand nicks caused by X-rays (Takabe and Hartman 1962) or $^{32}P$ decay (Hartman and Kozinski 1962) were not sufficient to stimulate transduction. Accordingly, it seems that repair mechanisms for removing the photoproducts (e.g. pyrimidine dimers), formed in DNA by UV-irradiation, can also function to bring UV damaged normally non-recombinogenic DNA into a more recombinogenic state.

Newman and Masters (1980) UV-irradiated either P1 transducing phage or recipient E.coli cells and studied the effects on the transduction frequencies of individual markers. They observed that UV-irradiation selectively aided the recovery of these markers which were poorly transduced in unirradiated lysates; indeed, the stimulation of recovery was inversely proportional to the efficiency of recovery in unirradiated lysates. For example, recovery of $i l v$, normally a highly transduced marker was not facilitated by UV-irradiation, whereas recovery of $h i s$, normally poorly transduced was stimulated by UV to such an extent that the difference in recovery between the two markers was reduced from 25 fold, in unirradiated lysates, to only 4 fold. Thus UV-irradiation appeared to suppress the selectivity of transduction observed by Masters (1977) so that differences in transduction frequencies could now be accounted for by
gene dosage effects. (An exception to this general rule was the marker leu, situated at 2 minutes on the E.coli map (Bachman 1983, Newman 1982). leu normally exhibits a high transduction frequency, yet its transduction is stimulated considerably by UV. This will be discussed in detail, in chapter 3). The above observations suggest that selective packaging of markers into P1 transducing particles, if it occurs at all, is not primarily responsible for marker selectivity in transduction. Rather, these data suggest that markers are transferred to recipients with equal probability and that the marker selectivity of transduction stems from discrimination by the host recombination pathways active in recipient cells. UV irradiation may suppress this selectivity indirectly by inducing the host UV-repair pathways which may make all damaged DNA equally recombinogenic.

A study of the pathways for repairing UV-induced lesions in DNA reveals some clues as to how UV-damage could stimulate recombination. However, the subject is complex and an extensive discussion of DNA repair pathways is beyond the scope of this introduction. However, some of the UV-repair processes will be discussed briefly, as will their possible roles in stimulation of recombination (For review of DNA repair, see Hanawalt et al. 1979).

UV-irradiation of DNA generates a variety of photoproducts, such as intrastrand dimers between adjacent pyrimidines. The "pyrimidine dimers" are a major cause of lethal and mutagenic effects (Witkin 1966), exerting their effects by blocking elongation of nascent DNA chains during DNA replication. The E.coli cell has several mechanisms by which pyrimidine dimers are removed which can be broadly categorised into two groups (Clarke and Volkert 1978): extrareplicational repair, which removes the lesion before or after DNA replication and intrareplicational repair which repairs the lesion during DNA replication. Examples of extrareplicational repair include photoreactivation, the light dependent monomerisation of pyrimidine dimers (Rupert 1975), and the so-called short patch repair pathway. Short patch repair is uvrABC dependent and results in excision of the lesion followed by resynthesis of the DNA strand using the undamaged strand as a template (Brown and Grossman 1974, Seeburg et al. 1980); the result is that the repair of lesions by the
above process leads to small patches, ranging in size from 13-30 bp (Rothman 1978, Ben-Ishai and Sharon 1978, Masker 1977). Recently, Sancar and Rupp (1983) have shown that the uvrABC genes provide subunits for an excision nuclease which cuts the 8th phosphodiester bond 5' to the pyrimidine dimer and the 4th or 5th to the 3' side. The resulting oligonucleotide, which contains the lesion, is released and the gap is patched, presumably by DNA polymerase I and ligase. This pathway is recA independent (However, uvr ABC induction via SOS requires recA. For review, see Little and Mount 1982) and requires DNA polymerase I but an alternative excision repair pathway, the "long patch" pathway requires recA, uvrABC but is polA-independent (Cooper and Hanawalt 1972, for review, see Hanawalt et al. and Clarke and Volkert 1978). Long patch repair results in excision and resynthesis of larger regions of DNA flanking pyrimidine dimers. Patches vary in size but may be up to 500 bp.

In addition to the above uvr-dependent extrareplicational excision repair, intrareplicational repair also occurs which requires host recombination functions. Intrareplicational repair is thought to occur in wild type cells, but was first studied in excision-deficient mutants, incubated in the dark after exposure to UV (i.e. no excision or photoreactivation) (Rupp and Howard-Flanders 1968, Rupp et al. 1971, Ganeson 1974). Rupp (1971) showed that intrareplication repair occurred by strand exchanges of limited, but varying lengths, between sister and cousin chromosomes. Apparently, DNA synthesis cannot proceed past pyrimidine dimers so replication terminates and reinitiates downstream from the site of the dimers. The net result is the accumulation of variable length gaps opposite dimers; intrareplicational repair functions to fill these gaps. However, the original lesions still remain in the parental DNA and are gradually removed or diluted to daughter cells (Ganeson 1974, Clark and Margulies 1965).

More recently, in vitro studies with purified RecA protein have shown that RecA protein actively drives the strand exchanges observed in postreplication repair, hence the dependence on host recombination functions; RecA protein transfers a strand from nicked duplex DNA to homologous gapped duplex DNA, commencing with the 3'OH terminus of
the nicked molecule. (Nicked and gapped duplex molecules are both generated when replication of UV damaged DNA takes place). (West et al. 1981, West et al. 1982). In addition RecJ protein can promote strand exchange past pyrimidine dimers and so could displace dimers from a DNA duplex (Livneh and Lehman 1982).

The above is obviously a very brief and incomplete survey of the repair of UV-damaged DNA, and makes no attempt to cover all pathways or the involvement of SOS. It does, however, serve to underline the close relationship of DNA repair and recombination; perhaps recombination occurs via pathways primarily evolved for repair. The mechanism by which UV-damage stimulates recombination is a matter of conjecture, but clearly the action of repair pathways (except perhaps photoreactivation) on UV-damaged transducing DNA may result in integration of that DNA into the chromosome, as a by product of a mechanism designed to repair lesions in chromosomal DNA. This may differ from the mechanism by which unirradiated transducing DNA is recombined into the chromosome and so explain the reduction of marker specificity in transduction following UV-damage to transducing DNA or recipient chromosomal DNA.
As mentioned in the previous section, the reduction of marker selectivity following the use of UV-irradiated lysates, suggests that all markers are packaged into P1-transducing lysates with equal efficiencies, i.e. events in the recipient cells, presumably the host cell recombination pathways, determine transduction frequencies. Therefore, the host cell recombination pathways will be reviewed briefly, below, and their possible roles in transductional selectivity will be discussed.

Homologous recombination between two duplex DNA molecules involves the reciprocal exchange of complementary DNA strands, to generate regions of heteroduplex DNA containing one strand from each parental molecule. Holliday (1965) proposed a model for recombination which requires a symmetric exchange of DNA strands and proceeds via an intermediate structure, termed a Holliday junction. Two cleavage events are possible to resolve the junction; one results in recombinant molecules with DNA flanking the exchange in the parental configuration, whereas the other generates molecules with flanking arms in the recombinant configuration (see figure 1.4).

The existence of Holliday junctions has been confirmed by electron microscopy and their formation in E.coli has been shown to require the product of the recA gene (Potter and Dressler 1976). Thus the recA gene will be discussed in more detail below because its function is directly relevant to the mechanism of transductional recombination.

THE recA GENE OF E.COLI

All generalised homologous recombination (as opposed to site-specific) in E.coli is dependent on RecA protein (with the possible exception of certain recF dependent intraplasmidic recombination events) (Clarke and Margulies 1965, Laban and Cohen 1981). RecA protein is a multifunctional enzyme, hence mutations in recA produce pleiotropic effects, e.g. deficiency in generalised recombination and
FIGURE 1.4. HOMOLOGOUS RECOMBINATION

Figure 1.4 depicts the theoretical model for homologous recombination, first suggested by Holliday (1967).

A: the initiation of a crossover between DNA strands in homologous alignment.

B: strand exchange and branch migration.

C: the characteristic cross shaped Holliday junction can be resolved two ways: A/A gives a short heteroduplex region but with the chromosome arms in the parental configuration, B/B also gives a short heteroduplex region but in this instance, the chromosome arms are in the recombinant configuration.
DNA repair, mutagenesis, impaired cell division and inability to induce SOS (for reviews, see Clarke 1973, Little and Mount 1982).

RecA protein has two main roles in the cell. Firstly RecA protein indirectly controls the expression, via the lexA repressor of a number of din (damage inducible) genes involved in the SOS response (Little and Mount 1982). Secondly, during repair and recombination, RecA protein potentiates synapsis and strand exchange between homologous DNA molecules. A detailed description of the experimental evidence in support of these statements is beyond the scope of this introduction. However, the involvement of RecA protein in strand exchange will be reviewed briefly, below.

The recA gene was cloned by McEntee and Epstein (1977) and RecA protein was subsequently purified by Weinstock et al. (1979) and Shibata et al. (1979) who showed that, as expected, RecA protein facilitates homologous pairing of DNA molecules. In vitro studies showed RecA protein alone could rapidly promote synapsis of homologous DNA molecules and more slowly, could drive strand exchange between DNA duplex and single strand homologous DNA, or could promote the reciprocal exchange of strands between homologous duplex molecules. Thus heteroduplex DNA could be generated in vitro by the action of RecA protein (Das Gupta et al. 1980, Das Gupta et al. 1981, Cox and Lehman 1981). However, for the reactions to occur, one of the participating DNA molecules must be single stranded or be nicked or gapped, as RecA protein cannot facilitate strand exchange between two wholly duplex molecules. These observations, plus many others enabled the formulation of a model for RecA protein-directed in vitro synthesis of recombination intermediates.


1) PRESYNAPSIS
In an ATP-dependent, ADP inhibited reaction, RecA protein slowly polymerises onto single stranded DNA, forming a regular nucleoprotein filament. This step is essential for synapsis to occur later (Dun et al. 1982, Flory and Radding 1982, Howard-Flanders et al. 1984). The E.coli single-stranded binding protein (SSB, formerly called helix destabilising protein), the product of the lexC gene, aids this
reaction by destabilising secondary structure in single stranded DNA, and alleviating the inhibitory ADP effect, hence allowing RecA-polymerisation to proceed more rapidly (McIntee et al. 1980, Flory and Radding, Cox and Lehman 1981, Muniyappa et al. 1984).

2) SYNAPSIS

In the presence of a non-hydrolysable analogue of ATP, RecA protein can bind to duplex DNA forming a regular nucleoprotein filament with 16 bp and 6.4 molecules of RecA protein per turn of the helix. If ATP is present, however, the bound RecA protein can promote extensive unwinding of the duplex, coupled with ATP hydrolysis. Thus the duplex can be unwound and then subsequently invaded by single stranded homologous DNA. In the presence of the single strand DNA, synapsis with the duplex occurs rapidly bringing the molecules into homologous side by side alignment. This "nascent heteroduplex joint" (sometimes called a paranemic joint) forms in a reaction coupled with extensive RecA-mediated unwinding of the duplex, at the site of synapsis (Wu et al. 1983, Stasiak et al. 1982, Ohtani et al. 1982, Radding 1985). Paranemic joints can then be converted to plectonemic joints, in which the molecules are intertwined, by the action of topoisomerase 1 (Cunningham et al. 1981). Consistent with this in vitro observation is the report by Fischel and Kolodner (1984) that top1 mutants, which lack a fully functional topoisomerase 1, exhibit a recombination deficient phenotype. This implies a major role for top1 in in vivo recombination events.

3) STRAND EXCHANGE

RecA protein then actively drives DNA strand exchange in the 5'-3' direction, with respect to the displaced strand, to generate a region of heteroduplex DNA up to several kb long. Strand exchange is thus polar, requires ATP and can even proceed past lesions in DNA, such as pyrimidine dimers, mismatches etc (Kahn et al. 1981, Cox et al. 1981, Bianchi and Radding 1983, Livneh and Lehman 1982).

The RecA protein directed homology search is remarkably efficient. 2005 bp duplex molecules, sharing only 151 bp of homology, can be paired with the same efficiency in vitro, as molecules that are entirely homologous (Gonda and Radding 1983). However, homologous regions must be above a "minimum size" for RecA
protein to facilitate pairing, the amount of homology required is not known, but 30 bp is insufficient (Gonda and Radding 1983) and Singer et al. (1982) showed that a minimum of 50 bp is required for T4 recombination in vivo. The nature and reasons for the "cut off" point, below which RecA protein will not recognise homology, are not known. Likewise the exact mechanism of the homology search is not known. Gonda and Radding (1983) showed, with some very elegant work, that the homology search is a progressive event, rather than a series of random pairing and unpairing steps; with molecules sharing limited homology, increasing the amount of heterologous DNA actually increases the rate at which the homology search is completed, rather than decreasing it. It is not clear whether the search proceeds by RecA driven translocation of the duplex with respect to the invading strand or whether it occurs by facilitated diffusion along the tertiary complex.

Of course, the results discussed above have often been obtained using, as reaction substrates, molecules which probably would not occur in vivo. However experiments have been carried out with molecules more likely to be found in vivo, e.g. linear duplex DNA, gapped duplex DNA and molecules with single strand ends. These molecules undergo similar RecA promoted reactions and the end results are often molecules linked by Holliday junctions (see Radding 1985 for review). For example, West et al. (1983) were able to generate recombinant plasmid molecules in vitro, using nicked or gapped parental molecules as substrates. Incubation of such molecules with RecA protein and subsequent treatment with DNA polymerase I and DNA ligase, generated biologically active biparental plasmid dimers with a figure-of-eight conformation i.e. with one Holliday junction. Such molecules could be resolved to viable monomer progeny in both RecA+ and RecA- hosts. Thus at least for plasmidic recombination, RecA protein is required for the formation of recombination intermediates but not for their resolution to give recombinant progeny.

The mechanism by which Holliday junctions are cleaved in E.coli to give mature recombinant progeny is not known. Mizuuchi et al. (1982) reported that T4 endonuclease can resolve Holliday junctions to give both possible recombinant configurations. However, as yet,
no such E.coli enzyme has been isolated, but it is possible that recBC and sbcB enzymes (exoV and exoI respectively) may be involved. Leach and Stahl (1983) noted the improved viability of λ phage, carrying perfect palindromes in recBC sbcB strains. Palindromes can "fold back" to form cruciform structures which are physically identical to Holliday junctions, so Leach and Stahl (1983) hypothesise that removal of exoV and exoI prevents cleavage of λ at the site of the palindrome and hence increases survival of the phage.

The importance of RecA protein in P1 mediated generalised transduction is obvious; all generalised homologous recombination, including transduction, requires RecA protein. Thus in RecA− cells, transduction is abolished (Clarke and Margulies 1965).

Strand exchange in vitro requires stochiometric amounts of RecA protein, so that increasing the levels of RecA protein within the cell might be expected to improve transduction frequencies (Shibata et al. 1979). Newman (1982) raised the intracellular levels of RecA protein either by transforming strains with pEH13, a high copy plasmid carrying a cloned recA gene (Emmeson et al. 1980) or by using strains which expressed recA at increased levels (LLoyd 1978). If such strains were used as recipients in transduction experiments the effects were marginal with transduction frequencies being altered very little, if at all. Accordingly, it seems that the level of RecA protein within the cell is not a limiting factor for transduction.
PATHWAYS OF GENERALISED RECOMBINATION IN E.COLI

RecBC PATHWAY

The major pathway of recombination in wild type E.coli is dependent on the products of three genes: recA (discussed above), recB and recC (Clark 1973). recBC mutants were initially isolated as recombination deficient strains but were later shown to be lacking in ATP-dependent DNAase activity; both exonuclease and endonuclease activities were found to be lacking (Goldmark and Lim 1970). Accordingly, the major role of RecBC enzyme was assumed to be nucleolytic and so early in vitro studies were carried out in systems designed to maximise RecBC enzyme nucleolytic activity. In appropriate but non-physiological conditions, RecBC enzyme can progressively degrade single or double stranded DNA as well as gapped duplex DNA circles; the products of the degradation being oligonucleotides 5-8 bp long (Goldmark and Lim 1972, Karu et al. 1973). However, under more physiological conditions and in the presence of SSB, the exonuclease activities are suppressed and the enzyme acts to unwind linear duplex DNA (Taylor and Smith 1980) i.e. it appeared that RecBC exonuclease, but not endonuclease activities were artifacts of the in vitro reaction conditions. The unwinding reaction requires free ends of the duplex, allowing entry of the RecBC enzyme, which then tracks along the helix unwinding the duplex. RecBC enzyme is envisaged to have "head" and "tail" regions; as the enzyme tracks along the duplex, DNA strands are fed through the "head" and unwound. The DNA strands then pass through the "tail" of the enzyme and may subsequently rewind. However, DNA passes through the "head" faster than through the tail, so that RecBC enzyme generates growing single stranded regions of DNA ("Rabbit ears") flanked by duplex DNA (see figure 1.5). An alternative loop tail structure is also formed, but not in the absence of SSB (Taylor and Smith 1980, Smith 1985). However, when SSB is present in high concentration, only loop tails are observed.

The relative importance of RecBC endonuclease and unwinding activity in in vivo recombination events is indicated by the
isolation of recBC mutants which lack endonuclease activity, but which can still efficiently unwind DNA (Chaudhury and Smith 1984). Such mutants are as proficient in generalised recombination as their RecBC\textsuperscript{+} parents, indicating that RecBC enzyme nuclease function is not required for generalised recombination. The mechanism by which RecBC mediated unwinding facilitates recombination is a matter of conjecture. Conceivably, the growing regions of single stranded DNA, which are stabilised by SSB, may provide the initial site for RecA polymerisation and subsequent synapsis with homologous duplex DNA. The Rec\textsuperscript{+} phenotype of recBC mutants lacking nuclease activity (Chaudhury and Smith 1984) would tend to rule out RecBC nuclease activity as responsible for resolution of Holliday junctions (Leach and Stahl (1984), see Figure 1.5).

In addition to its role in generalised homologous recombination, RecBC enzyme also recognises so-called chi (crossover-Hotspot-Instigator) sites in DNA and promotes recombination in the vicinity of chi (Lam et al. 1974, For review, see Smith 1985). chi-sites have a unique 8 bp sequence (5' GTGGTGG 3') shown to be present in all chi sites so far sequenced (Smith et al. 1981). Chi-dependent stimulation of recombination is polar, occurring to one side of chi, non reciprocal and in \lambda, chi activity depends on the orientation of the chi sequence with respect to cos i.e. it is recognised in one orientation only (Stahl et al. 1980, Kobayashi et al. 1982).

Interestingly, chi is active when present in only one of the parental molecules and need not even be present in an area of mutual homology; i.e. chi can act when located opposite heterologous DNA. Indeed, chi activity need not always occur in the immediate vicinity of chi but can occur up to 10 kb distant (McMilin et al. 1974, Lam et al. 1974, Stahl and Stahl 1977). The above indicates that chi is not primarily involved in strand pairing, where homology is essential, but must stimulate recombination in some other manner.

There is considerable evidence that chi serves as a target sequence for RecBC enzyme endonuclease activity. RecBC mutants which retain unwinding activity but have lost nuclease activity show no chi dependent stimulation of recombination (Chaudhury and Smith 1984). Other strains, RecBC "pseudorevertants" have been isolated, which are
phenotypically Rec<sup>+</sup>, but again lack chi activity (Shultz et al. 1983). However it is not known whether these mutants have lost RecBC nuclease function. In addition some tex mutants of E.coli, isolated as mutants which excise transposons precisely with high frequency, have lesions in recB or recC. Such strains again are proficient in generalised recombination but lack chi activity because of being unable to recognise chi. Ponticelli et al. (1985) and Taylor et al. (1985) showed that when RecBC enzyme acts, in vitro, on linear duplex DNA containing chi, a nick is generated, 4, 5 or 6 nucleotides 3' to chi. Nicking occurs during unwinding only if chi is approached from the right and single base changes within the 8 bp chi recognition sequence eliminate nicking.

Smith (1985) proposes a model for chi stimulation of recombination: RecBC enzyme enters linear duplex DNA and proceeds to unwind the duplex, generating growing single strand "rabbit ears" of DNA, which are stabilised by SSB (as described above). As RecBC enzyme reaches a chi site, in its active orientation, a nick is generated 3' to chi and as RecBC enzyme passes a single stranded tail of DNA results (see figure 1.5). This single strand is then presumably free to invade a homologous duplex molecule and thus form a heteroduplex joint. This model accounts for all the properties of chi: dependence on recBC pathway, non reciprocality of strand exchange and polarity of chi, all due to the progressive nature of RecBC-mediated unwinding. Likewise, the activity of chi opposite heterologous DNA can be similarly explained; although a heterologous single strand tail cannot stably pair with a duplex, if RecBC unwinding proceeds into a region of mutual homology, the single stranded DNA thus generated can presumably invade a homologous duplex to give stable pairing.

ROLE OF RecBC IN TRANSDUCTION
DOES CHI SITE ACTIVITY INFLUENCE TRANSDUCTION

The RecBC pathway accounts for most homologous recombination in wild type E.coli; recBC strains show only 0.3-20% of the conjugal and transductional recombination observed in their isogenic RecBC<sup>+</sup>
A-C show the current model for the unwinding of duplex DNA by RecBC enzyme. Note the "loop tail" structure in B and the "rabbit ears" shown in C. Parts D-J indicate a possible mechanism for chi-stimulated genetic exchanges; D shows nicking at chi, followed by the strand displacement by the progressive RecBC unwinding (E). F-H depict the possible alignment and strand exchange reactions between the single strand generated by RecBC enzyme and homologous target DNA. Finally, I and J indicate the two possible recombinant structures which would result from the resolution of the Holliday junction, shown in H.

Figure 1.5

A

B

C

D

E

F

G

H

I

J

Chi

Rec BC
parents (Emmerson and Howard-Flanders 1967, Low 1968). In view of this major role in recombination, it seems possible that the marker dependence of transductional recombination is attributable to selectivity by the recBC pathway. How this could occur is a matter of conjecture, but one possible hypothesis is that chi-hotspot activity may determine the relative recombinogenicity of transducing DNA molecules and selectively aid recovery of certain markers near chi sites. Conversely, the presence of chi within a marker may have the opposite effect, i.e. reduce recovery, as the nick caused by recBC enzyme at chi is effectively destroying the integrity of the marker. Support for this suggestion comes from the observations that trp, which is rich in chi sites, is normally poorly transduced in RecBC+ strains. Trp+ transduction increases, however, in recBC sbcB FG strains (Masters et al. 1984). To test the hypothesis that chi sites influence genetic exchanges in transductional recombination, Dower and Stahl (1981) constructed strains lysogenised with λ phage, either with or without chi mutations (wild type λ do not contain chi-sites, so chi mutations generate chi sites). The λ prophages were integrated both in wild type and opposite orientations to alter the relative orientation of chi with respect to the host genome. These strains were used to provide lysates for P1 transduction experiments and the recipients were suitably marked RecBC− or RecBC+ derivatives of isogenic parents. As expected the results confirmed that the presence of chi altered the distribution of crossover events in a polar, non reciprocal recBC-dependent manner. However, the influence of chi on transduction frequencies of markers around the chromosome was not measured in these experiments. Clearly, the isolation of recBC mutants proficient in generalised recombination, but lacking endonuclease activity (and hence lacking chi activity) (Chaudhury and Smith 1984) will be of considerable use in elucidating the role of chi-sites in determining transduction frequencies in RecBC+ recipients.
The role of the recBC pathway in recombination events has been studied by investigating recombination in recBC mutants. Although recBC mutants exhibit a recombination-deficient phenotype, the levels of recombination in such strains can be restored to almost wild-type levels by mutation at the sbcA (suppressor of recBCA) or sbcB (suppressor of recBcb) plus a further mutation at sbcC (suppressor of recBC C) (Barbour et al. 1970, Kushner et al. 1971, Lloyd et al. 1985, submitted for publication).

Mutation at sbcA derepresses the recE gene, the structural gene for Exonuclease VIII, which is located on the cryptic prophage rac (Kushner et al. 1974, Kaiser and Murray 1980).

The sbcB mutation inactivates exonuclease I and allows recombination to proceed via a pathway later found to be dependent on the recF gene (Kushner et al. 1971, Clarke 1973). The recF gene has been recently cloned and sequenced and shown to contain an open reading frame for a 40.5 kd protein. In addition RecF+ plasmids have been shown to produce a 40kd protein in maxicell systems (Blanar et al. 1984). However, the nature and involvement of this putative RecF protein in recombination, remains unknown.

Initially it was thought that the recE and recF-dependent pathways were separate but, as is now known, recombination via recE requires a functional recF-product. Hence Clark (1980) suggests there is a single alternative pathway in recBC mutants.

Very recently, Lloyd et al. (1985) showed that strains thought to have a recBC sbcB genotype had, in fact, an additional mutation at a new locus, designated sbcC. In the absence of sbcC, recBC sbcB strains exhibited low recombination frequencies, low viabilities and poor growth. Such strains rapidly accumulated faster growing variants, due to mutation at sbcC. The nature and role of sbcC has not, as yet, been elucidated.

The relative importance of recBC and recF-mediated recombination in wild type cells is not wholly clear. There is considerable evidence that recF-mediated recombination is inducible and controlled via lexA, the repressor for the SOS response (see Little and Mount...
1982 for review). In recBC sbcB strains, recombination is not only recF-dependent but requires in addition, the products of recJ, recN recQ and ruv genes (Horii and Clark 1973, Lloyd et al. 1983, 1984, Lovett and Clark 1984, Nakayama et al. 1984, 1985). None of these are required for recBC mediated recombination, but recF, recN and ruv products are required for normal repair of uv-damaged DNA (Rothman and Clarke 1977, Lloyd et al. 1984, Picksley et al. 1984). The recently isolated allele, recQ, however has not been well enough characterised to establish its role in repair; certainly recQ confers increased uv-sensitivity in recBC sbcB strains (Nokayama et al. 1984). Also, lexA mutations, which block the SOS response, inhibit recombination in recBC sbcB, but not RecBC+ backgrounds (Lloyd and Thomas 1983, Lovet and Clark 1983). Hence the view that recF mediated recombination is primarily involved in inducible DNA repair, perhaps in conjunction with SOS, and is not of significance in normal cellular recombination (if DNA repair can be considered distinct from recombination). However, there is growing evidence that RecF mediated events play an unexpectedly major role in cellular recombination. Laban and Cohen (1981) and Cohen and Laban (1983) showed certain plasmidic recombination events required a RecF+ genotype even in RecBC+ backgrounds. More recently, Lloyd and Thomas (1984) studied the molecular nature of recombinant molecules formed after Hfr-directed conjugal transfer. They reported that RecBC-dependent and independent mechanisms formed roughly equal proportions of viable recombinant products in wild type strains, indicating a hitherto unexpectedly major role for RecF-mediated events.

It has been known for some time that the recombinant molecules arising from RecBC and RecF pathways differ. In Hfr crosses the RecF pathway catalyses the formation of long heteroduplex recombinant molecules, whereas RecBC-mediated events direct the integration of largely duplex regions of Hfr DNA, flanked by short regions of heteroduplex (Mahajan and Datta 1979, Clark 1980). Also, RecF-dependent recombination is relatively slow and inefficient. However, increasing the intracellular levels of RecA protein dramatically increases the rate of recF-mediated recombination (Lloyd and Thomas
1983). This could be explained by the observations showing strand exchange requires stoichiometric amounts of RecA protein (Cox and Lehman 1981). Thus as recF-mediated recombination generates long heteroduplex molecules, the requirement for RecA protein is higher than for recBC mediated events. (Hence, perhaps the connection between SOS and recF-recombination, as SOS derepresses RecA expression and raises intracellular levels of RecA protein).

ROLE OF recBC IN TRANSDUCTIONAL SELECTIVITY

The role of recBC in transductional recombination is obviously of considerable interest. Sandri and Berger (1980a) reported that transductional recombination results in the integration of duplex regions of transducing DNA. This would argue that transductional recombination occurs via recBC as recF mediated events involve integration of single strand "incoming" DNA. In addition increasing intracellular RecA protein levels has relatively little effect on transduction, again suggesting that recF is not involved, as increasing RecA concentration stimulates recF mediated recombination (Newman 1982, Lloyd and Thomas 1983, Masters et al. 1984).

To investigate the role of the RecBC pathway in determining transduction frequencies of markers around the chromosome, Masters et al. (1984) utilised suitably marked recBC sbcB strains as recipients in transduction experiments. Such strains were slow growing and were transduced with poor efficiency, but markers were still transduced with a 25-fold range in efficiency, i.e. showing a slight reduction from that observed in Rec+ parental strains (Masters 1977). RecBC enzyme cannot therefore be solely responsible for the marker-selectivity of transduction as the above strains utilise the recF pathway yet still exhibit selectivity. Surprisingly, the recB sbcB strains used above spontaneously gave rise to faster growing (FG) derivatives, which were still recB sbcB, i.e. still utilised the recF pathway, but which showed a return to normal, wild type levels of transduction. In addition, marker selectivity was reduced to a 4 fold range in these strains, which was attributable to gene dosage. The nature of the FG mutation is not known. Clearly, FG recBC sbcB
strains have lost the marker selectivity of transductional recombination, implying a role for FG in recombinational discrimination. However, in view of the report by Lloyd et al. (1985) that recBC sbcB strains require the sbcC mutation to exhibit a fast growing, recombination proficient phenotype, it is tempting to speculate that FG exerts a similar effect or may be identical to sbcC.

When Rec+ or sbcB or recB sbcB FG strains are transduced with uv-damaged DNA, maximum levels of transduction are equal for all strains and all show a similar decrease in marker selectivity; recB strains, therefore, show dramatically increased transduction frequencies when transduced with uv-damaged DNA. This supports the hypothesis that uv-damaged DNA is recombined independently of recBC function, implying that uv damage and recB perform a similar role in transduction. However, if heavily irradiated transducing lysates are used, which normally reduces the levels of transduction, FG, recB sbcB and sbc strains show improved recoveries over other strains; perhaps the sbcB product, expB, is involved in degradation of uv-damaged DNA, a process which is alleviated in sbcB strains, allowing the damaged DNA to survive and participate in recombination (Masters et al. 1984).

SUMMARY

In summary, the following salient points arise from genetic studies on the marker dependence of transduction frequencies:

1) P1 transduction frequencies are marker-dependent and vary over a 30-fold range in fully recombinational, or repair, proficient cells.
2) Highly transduced markers are located in gene dense regions of the chromosome or are located within 2 minutes (i.e. 1 P1 headful) of oriC.
3) uv-irradiation of P1 lysates, or recipient cells, alleviates marker selectivity to give a 4-fold range in transduction frequency which can be accounted for in terms of gene dosage in the donor cell. Hence P1 packaging events in the donor cells are unlikely to dictate transduction frequencies by selective overpackaging of highly
transduced markers.

4) uv-stimulation of poorly transduced markers is dependent on uvrAB, suggesting a role for cellular uv-repair pathways in perhaps bringing all transducing DNA to the same recombinogenic potential; the implication being that well-transduced DNA (e.g. ilv) is highly recombinogenic and already transduced at maximum levels. Therefore uv damage will not improve the recombinogenicity so cannot further increase transduction. However if poorly transduced DNA (e.g. his) is poorly recombinogenic, uv-damage may result in the generation of recombinogenic structures (eg. nicks, gaps etc.) by the action of the uv-repair pathways. Hence transduction of poorly transduced markers is improved by increasing the recombinogenic potential of the DNA. This hypothesis explains how uv-repair pathways can bypass the selectivity of the transduction process. Alternatively, as mentioned previously, marker selectivity may be reduced because uv-damaged DNA recombines into the chromosome as a by-product of repair pathways. In other words, the different pathway leading to integration shows no marker selectivity. The two hypotheses differ in that the first implies recombination is distinct from DNA repair pathways.

5) recBC sbcB strains, which utilise the RecF pathway of recombination show decreased levels of transduction and slightly reduced marker selectivity. The acquisition of a further, as yet uncharacterised, mutation FG (sbcC ) increased transduction to normal levels and reduced marker selectivity to 4-fold. Thus recBC cannot be solely responsible for marker selectivity.

However, it is obvious that much work still has to be carried out to elucidate the mechanisms of transductional recombination and the origin of the marker selectivity of the transduction process. Some suggestions as to the work which could be carried out are presented in the "Future Prospects" section at the end of this thesis.
AIMS OF THIS THESIS

The experiments presented in chapters 3-6 of this thesis have been performed to study the transduction process; the aim of the experiments was to investigate the mechanisms which give rise to transductinal selectivity. The experimental work has involved a wide approach to the study of transduction and transductional discrimination, with events in both donor and recipient cells being studied. Accordingly, where appropriate, additional relevant introductory material has been presented in the results chapters.
CHAPTER 2

MATERIALS AND METHODS
SECTION 2.1 BACTERIAL AND PHAGE STRAINS

Bacterial strains are listed in table 2.1. For regular use, bacteria were maintained on L-broth agar plates at 4°C or in 0.7% nutrient agar stabs at room temperature. For long term storage, stationary phase cultures, grown under selection, were harvested by centrifugation and resuspended in 50% glycerol in phosphate buffered saline (table 2.4). Then, the cultures were stored at -20°C.

Strains of bacteriophage are listed in Chapter 3, except for P1; in all experiments PIKc (laboratory stocks) was used for cloning, transductions or packaging assays.

Lysates were stored at 4°C as broth suspensions, unless otherwise stated.

Plasmids are listed in table 2.2.

SECTION 2.2 GROWTH MEDIA AND BUFFERS

Media and buffers are listed in tables 2.3 and 2.4 respectively. Unless otherwise stated, bacteria were routinely cultivated in L-broth or on L-broth agar plates. For propagation of λ or P1 phage, these media were supplemented with 10 mM MgSO₄ or 1 mM CaCl₂ respectively. VB agar, appropriately supplemented, was used for the selection of nutritional markers.

Cultures grown in limiting thymine media were first grown in fully supplemented media, the cells were then washed in PBS and transferred to the thymine-limiting media. Before the experiments were carried out, the cultures were grown for three mass doublings in the limiting medium and diluted 2 times every mass doubling to allow the cells to achieve steady state growth (Begg and Donachie, 1978).

SECTION 2.3 BACTERIAL TECHNIQUES

GROWTH OF BACTERIA

Unless otherwise stated, cultures of E.coli were routinely grown with shaking, at 37°C.
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<td></td>
<td>trpR72</td>
<td></td>
<td>(1981)</td>
</tr>
<tr>
<td>W3110</td>
<td>inv: rrd-rrenE:1</td>
<td>Laboratory stock</td>
<td>Bachman (1981)</td>
</tr>
<tr>
<td>WM1026</td>
<td>ilv trp lac thi</td>
<td>Laboratory stock</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>supD dnaA46</td>
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<td></td>
</tr>
</tbody>
</table>
### TABLE 2.2 PLASMIDS (Phage strains are listed in Chapter 3)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Vector</th>
<th>Insert</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR325</td>
<td>ApCmTr</td>
<td>-</td>
<td></td>
<td>Bolivar et al. 1977</td>
</tr>
<tr>
<td>pMH600</td>
<td>ApCmTcs</td>
<td>pBR325</td>
<td>5.3 kb <em>HindIII</em> fragment from</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>λhisG</td>
<td></td>
</tr>
<tr>
<td>pMH601</td>
<td>ApCmTcs Ilv+</td>
<td>pBR325</td>
<td>7.0 kb <em>HindIII</em> fragment from</td>
<td>This thesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>λilv</td>
<td></td>
</tr>
<tr>
<td>pMH602</td>
<td>ApCmTcs</td>
<td>pBR325</td>
<td>3.0 kb <em>HindIII</em> fragment from</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>λtrp</td>
<td></td>
</tr>
<tr>
<td>pHW001</td>
<td>ApCmTcs ArgH+</td>
<td>pBR325</td>
<td>7.0 kb <em>HindIII</em> fragment (ArgH+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>from W3110</td>
<td></td>
</tr>
<tr>
<td>pBW000</td>
<td>ApCmTcs</td>
<td>pBR325</td>
<td>2.2 kb <em>BamHI</em> fragment (SpyE+)</td>
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<td></td>
<td></td>
<td></td>
<td>from W3110</td>
<td></td>
</tr>
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<td>pBW001</td>
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<td>pBR325</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>from W3110</td>
<td></td>
</tr>
<tr>
<td>pPAC</td>
<td>ApCmTcP1pac+</td>
<td>pBR325</td>
<td>0.65 kb <em>EcoRI</em> fragment 20 from Pl</td>
<td></td>
</tr>
<tr>
<td>pMOD</td>
<td>ApCmTcEcoPlMod+</td>
<td>pBR325Δ</td>
<td>6.6 kb <em>BamHI-HindIII</em> fragment (Mod+) from Pl</td>
<td></td>
</tr>
<tr>
<td>pPAC600</td>
<td>ApHisG+P1pac+</td>
<td>pPAC</td>
<td>5.3 kb <em>HindIII</em> fragment (HisG+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>from pMH600</td>
<td></td>
</tr>
<tr>
<td>pPAC601</td>
<td>ApIlv+P1pac+</td>
<td>pPAC</td>
<td>7.0 kb <em>HindIII</em> fragment from pMH601</td>
<td></td>
</tr>
<tr>
<td>pPAC602</td>
<td>ApP1pac+</td>
<td>pPAC</td>
<td>2.9 kb <em>HindIII</em> fragment from pMH600</td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Vector</td>
<td>Insert</td>
<td>Source/Reference</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>--------</td>
<td>------------------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>pR121600</td>
<td>$A_p^r$ $\text{His}^+$</td>
<td>pR121</td>
<td>5.3 kb $\text{HindIII}$ fragment from pMH600</td>
<td>This thesis</td>
</tr>
<tr>
<td>pR121601</td>
<td>$A_p^r$ $\text{Ile}^+$</td>
<td>pR121</td>
<td>7.0 kb $\text{HindIII}$ fragment from pMH601</td>
<td>&quot;</td>
</tr>
<tr>
<td>pR121602</td>
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<td>pR121</td>
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</tr>
<tr>
<td>pR121</td>
<td>$A_p^r$ $\text{Cm}^+$ $\text{Tc}^r$</td>
<td>pBR325</td>
<td>0.46 kb $\text{EcoRI}$ fragment 21 from P1</td>
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</table>
### TABLE 2.3 GROWTH MEDIA

#### L-Broth (LB)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difco Bacto Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Difco Bacto Yeast Extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>11</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

#### L-broth agar

- LB + 15 g Difco agar per litre

#### BBL Agar

- Baltimore Biological Laboratories
- Trypticase: 10 g
- NaCl: 5 g
- Difco agar: 15 g
- Distilled water: 11

#### BBL Top Agar

- As for BBL agar, but only 6.5 g Difco agar per litre

#### VB Minimal Agar (Vogel and Bonner, 1956)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5% Difco agar in distilled water</td>
<td>400 ml</td>
</tr>
<tr>
<td>20 x VB salts</td>
<td>25 ml</td>
</tr>
<tr>
<td>20% carbon source</td>
<td>5 ml</td>
</tr>
<tr>
<td>Amino acids and vitamins as required</td>
<td></td>
</tr>
</tbody>
</table>

#### VB minimal medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 x VB salts</td>
<td>25 ml</td>
</tr>
<tr>
<td>20% carbon source</td>
<td>5 ml</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>400 ml</td>
</tr>
<tr>
<td>Amino acids and vitamins as required</td>
<td></td>
</tr>
</tbody>
</table>

#### 20 x VB salts

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>4 g</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>40 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>250 g</td>
</tr>
<tr>
<td>NaNH$_4$.HPO$_4$.4H$_2$O</td>
<td>70 g</td>
</tr>
<tr>
<td>Distilled water to 11</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3 (continued)

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>(1)</th>
<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
<th>(5)</th>
<th>(6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Alanine</td>
<td>ALA</td>
<td>H₂O</td>
<td>10</td>
<td>A</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>ARG</td>
<td>&quot;</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>ASN</td>
<td>&quot;</td>
<td>10</td>
<td>A</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>ASP</td>
<td>&quot;</td>
<td>10</td>
<td>A</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>L-Cysteine HCl</td>
<td>CYS</td>
<td>&quot;</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>GLT</td>
<td>&quot;</td>
<td>10</td>
<td>A</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>GLN</td>
<td>&quot;</td>
<td>10</td>
<td>A</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Glycine</td>
<td>GLY</td>
<td>&quot;</td>
<td>10</td>
<td>A</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>L-Histidine HCl</td>
<td>HIS</td>
<td>&quot;</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>ILE</td>
<td>&quot;</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>LEU</td>
<td>&quot;</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>LYS</td>
<td>&quot;</td>
<td>10</td>
<td>A</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>MET</td>
<td>&quot;</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>PHE</td>
<td>0.01N NaOH</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>L-Proline</td>
<td>PRO</td>
<td>H₂O</td>
<td>3</td>
<td>A</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>DL-Threonine</td>
<td>THR</td>
<td>&quot;</td>
<td>10</td>
<td>A</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>TRP</td>
<td>&quot;</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>TYR</td>
<td>0.01N NaOH</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>DL-Valine</td>
<td>VAL</td>
<td>H₂O</td>
<td>4</td>
<td>A</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>L-Isoleucine/</td>
<td>ILV</td>
<td>&quot;</td>
<td>2/4</td>
<td>A</td>
<td>5</td>
<td>20/40</td>
</tr>
<tr>
<td>DL-Valine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casamino acids</td>
<td>CAA</td>
<td>&quot;</td>
<td>100</td>
<td>A</td>
<td>25</td>
<td>5000</td>
</tr>
</tbody>
</table>

**Purines and Pyrimidines**

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>ADE</td>
<td>0.03N HCl</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Thymine</td>
<td>THY</td>
<td>H₂O</td>
<td>2</td>
<td>A</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td>URA</td>
<td>&quot;</td>
<td>2</td>
<td>A</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

**Vitamins**/
Table 2.3 (continued)

<table>
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<tr>
<th>Vitamins</th>
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<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
<th>(5)</th>
<th>(6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>BIO</td>
<td>H₂O</td>
<td>0.1</td>
<td>F</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Niacin (nicotinic acid)</td>
<td>NAD</td>
<td>&quot;</td>
<td>0.5</td>
<td>F</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Thiamine HCl (B₁)</td>
<td>THI</td>
<td>&quot;</td>
<td>1</td>
<td>F</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

(1) Abbreviations
(2) Solvent
(3) Concentration of stock solution (mg ml⁻¹)
(4) Method of sterilization (A: autoclave; F: filter)
(5) Amount of stock solution to be added to medium (ml 500 ml⁻¹ medium)
(6) Final concentration in medium (µg ml⁻¹)
### TABLE 2.4 BUFFERS

#### Phage Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>7 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>MgSO$_4$·0.1M</td>
<td>10 ml</td>
</tr>
<tr>
<td>CaCl$_2$·0.01M</td>
<td>10 ml</td>
</tr>
<tr>
<td>Gelatin solution 1%</td>
<td>1 ml</td>
</tr>
<tr>
<td>Distilled water to 1 litre</td>
<td></td>
</tr>
</tbody>
</table>

#### Phosphate-buffered Saline (PBS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>30 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>70 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>46 g</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>2 g</td>
</tr>
<tr>
<td>Distilled water to 10 litre</td>
<td></td>
</tr>
</tbody>
</table>

#### Tris-EDTA (TE) Buffer (pH 8)

- 0.1M Tris/HCl
- 10 mM EDTA

#### Tris Calcium Buffer

- 0.1 M Tris pH 7.4
- 1 mM CaCl$_2$

#### 20 x SSC (Saline Sodium Citrate)

- 3.0 M NaCl
- 0.3 M Sodium citrate

#### 50 x Tris Acetate Gel Buffer

- 2 M Tris-HCl pH 8.2
- 1 M Sodium Acetate
- 0.05M EDTA
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Routine Concentration in media (µg/ml)</th>
<th>Stock Concentration (mg/ml)</th>
<th>Solvent for stock solution</th>
<th>Storage temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>10</td>
<td>12.5</td>
<td>50% EtOH/Water</td>
<td>-20</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>50</td>
<td>100</td>
<td>Water</td>
<td>-20</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>20</td>
<td>20</td>
<td>EtOH</td>
<td>4</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100</td>
<td>20</td>
<td>Water</td>
<td>4</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>50</td>
<td>10</td>
<td>Methanol</td>
<td>4</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>15</td>
<td>10</td>
<td>Water</td>
<td>4</td>
</tr>
</tbody>
</table>
SELECTION OF ANTIBIOTIC RESISTANCE

Antibiotics made up, stored and included in the growth media, as listed in table 2.5. Trimethoprim selection was carried out in minimal medium only, whereas other antibiotics were included in both rich or minimal media.

MEASUREMENT OF CULTURE OPTICAL DENSITY

O.D. of the cultures was measured in a Perkin-Elmer Colman model SS spectrophotometer at the wavelengths stated in the text (either 650 600 or 540 nm).

MEASUREMENT OF CELL SIZE

Cell size distribution (usually expressed as the median cell volume) were measured using a Coulter Counter model 2B, fitted with a 30 μ diameter orifice, interfaced with a Coulter chanallyser connected to a model XY Recorder II chart recorder (Coulter Electronics Ltd. Harpenden).

Cultures for cell size measurements were grown in filtered media and 0.2 ml samples were withdrawn and mixed with 0.2 ml of filtered formaldehyde buffer (20% formaldehyde in phosphate buffered saline). 100 μl samples were diluted into 8 ml filtered azide-saline solution (36 g NaCl 2 g NaN₃, distilled water to 4 L) and counted.

SELECTION OF ThyA⁻ STRAINS USING TRIMETHOPRIM

Bacteria were grown to stationary phase in L-broth, then harvested by centrifugation and washed with an equal volume of phage buffer. Cells were then pelleted again, and resuspended in 1/10 volume of phage buffer. 100 μl aliquots were spread on minimal plates, supplemented with 50 μg/ml thymine and which contained 50 μg/ml trimethoprim. Plates were incubated at 37°C for 3 days and then colonies were picked and purified by streaking on the same medium. Individual colonies were then picked and checked for the ThyA⁻ phenotype.
TEST FOR ULTRAVIOLET SENSITIVITY OF Po1A^- STRAINS

A loopful of mid log phase culture was inoculated as a single streak across an L-broth plate. Zones of the plate were exposed to 0, 100, 200 and 300 ergs/mm² of UV irradiation from a germicidal lamp. This was achieved by using a piece of card to cover the plate and moving the card across the plate at set time intervals. In this way, successively longer zones of the plate were exposed to the UV.

PURIFICATION OF FILAMENTOUS CELLS BY FILTRATION UNDER VACUUM

10 ml of exponentially growing cells at an OD₅₄₀ of 0.3 were filtered through a 47 mm diameter Millipore nitrocellulose filter (of the appropriate pore size) under vacuum, in a Millipore filtration apparatus (Cat. no. XX10 047 00). The filter was then washed, by passing 300 ml sterile phage buffer through under vacuum. The filter was then removed from the apparatus and placed into a flask containing 10 ml pre-warmed L-broth and the cells were resuspended from the filter by gentle rotary shaking for 2-3 minutes. Normal sized cells were recovered from the filtrate by a similar procedure, but obviously, a smaller pore size filter was used.

SECTION 2.4 PHAGE TECHNIQUES

PREPARATION OF P1 LIQUID LYSATES

Bacteria were grown in L-broth supplemented with 1 mM CaCl₂, with vigorous shaking at 37°C to an OD₅₄₀ of 0.5. P1 phage were then added with a M.O.I of 1.5-2. The cultures were grown shaking until lysis occurred, usually after about 75-90 minutes (depending on the bacterial strain). Then 0.5 ml of chloroform was added to the lysate and after a further 5 minutes shaking at 37°C, the lysate was centrifuged, at maximum speed for 15 minutes, in a bench centrifuge (for small volumes of lysate). Larger volumes were spun for 10 minutes at 10,000 rpm in an appropriate rotor in a Sorval RC5B centrifuge. The supernatant was then removed and titred.

PREPARATION OF P1 PLATE LYSATES

10⁷ pfu of P1 and 200 µl of a fresh overnight culture (grown in
L-broth plus 1 mM CaCl₂) were mixed and incubated at 37°C for 10 minutes. 2.5 ml molten BBL top agar (at 50°C) was added to each mix and then poured on to thickly poured, freshly made L-broth Agar plates supplemented with 1 mM CaCl₂. After 8 hours incubation at 37°C (usually 9 a.m. - 5 p.m. for convenience) or when P1-lysis was clearly observed, 5 ml L-broth CaCl₂ was added to each plate and the plates placed at 4°C overnight. Then the top layer of agar, and the 5 ml L-broth Agar were scraped into a universal bottle and the mixture vortexed vigorously. Then the lysates were centrifuged at maximum speed in a bench top centrifuge for 15 minutes and the supernatant was removed and titred.

P1 TRANSDUCTION

Recipient cells were grown to stationary phase in L-broth CaCl₂. 1 ml of the cells were concentrated 10-fold by centrifugation and resuspension in 1/10 volume of L-broth CaCl₂ approximately 10⁸ pfu of P1 phage were then added (in a volume of not more than 100 μl) and the mix was incubated for 10 minutes at 37°C. The mix was then diluted back to 1 ml by the addition of 0.8 ml phage buffer. Samples were then either spread directly on to selective plates or where appropriate, the transduction mixes were washed first, to remove residual L-broth, before plating on selective plates. Unless otherwise stated 200 μl aliquots were plated on to duplicate selective plates and numbers of transductants shown are the mean of the two plates.

P1 TRANSDUCTION OF EXPONENTIALLY GROWING CULTURES

For the experiments described in Chapters 5 and 6, exponentially growing cultures of recipient bacteria were transduced, according to the following method.

 Cultures were grown, shaking at the growth temperature stated, to OD₅₄₀ of 0.3. Cells from 20 ml of the culture were pelleted by centrifugation in a bench top centrifuge at maximum speed for 15 minutes. The cells were resuspended in 1 ml of P1 lysate with a titre of 2 x 10¹³ pfu/ml, i.e. P1 phage were added with a m.o.i. = 5. Phage were allowed to adsorb for 10 minutes at 37°C before the mix was diluted into 20 ml L-broth plus 10 mM NaCitrate
pre-warmed to the growth temperature. The culture was then diluted 2-fold every mass doubling into pre-warmed L-broth NaCitrate. (NaCitrate was included to chelate any residual Ca\(^{2+}\) ions and so prevent further phage adsorption.) 200 \(\mu l\) samples were withdrawn at intervals and spread on selective plates.

**GROWTH OF PHAGE \(\lambda\).**

**LIQUID LYSATES AND PLATE LYSATES**

These were prepared as described for P1 except that 10 mM MgSO\(_4\) was substituted for CaCl\(_2\).

**UV-INDUCTION OF \(\lambda\) LYSOGENS**

Lysogenic bacteria were grown to mid log phase (OD\(_{540}\) = 0.5), harvested by centrifugation and then resuspended in ½ volume of phage buffer. The cells were irradiated 400 ergs/mm\(^2\) UV light in 10 cm glass petri dishes. No more than 7 ml of cells were placed in each dish and dishes were swirled during the period of irradiation to ensure the maximum uniform irradiation of the cell. Cells were then diluted 4 times into fresh pre-warmed L-broth plus 10 mM MgSO\(_4\). The cultures were then grown with vigorous shaking at 37°C until lysis occurred. Chloroform was then added to the cultures then the lysates were clarified by centrifugation (as above) and subsequently titred.

(a) **CONCENTRATION OF PHAGE (P1 AND \(\lambda\)) BY PEG PRECIPITATION** (Yamamoto et al., 1970)

Phage lysates were prepared and clarified by centrifugation as described above. DNAase 1 (Sigma) and RNAseA (Sigma) were added to give a final concentration of 10 \(\mu\)g/ml (for both). The lysate was incubated for 1 hour at room temperature and then NaCl was added to 40 g/l, together with 10% w/v PEG; both were dissolved by stirring. The lysate was left at 4°C overnight and then the precipitate (PEG + Phage) was pelleted by centrifugation at 15000 rpm for 15 minutes in an appropriate rotor in a Sorval RC5B centrifuge. Pellets were resuspended in about 1/50 of the original volume of phage buffer by gentle rotary shaking at 4°C for several hours.
(b) **PELLETING BY HIGH SPEED CENTRIFUGATION**

Lysates were prepared and DNAse and RNase treated as above. They were then centrifuged at 23000 rpm in a type 21 rotor in an MSE Superspeed 65 ultracentrifuge for 3 hours. Pellets were re-suspended as above, by gentle rotary shaking at 4°C.

**CAESIUM CHLORIDE STEP GRADIENTS**

Phage suspensions (concentrated as above) were loaded on to caesium step gradients. Gradients were composed of three 1 ml steps of density: 1.3, 1.5 and 1.7 g/ml. Gradients were centrifuged at 35000 rpm for 3 hours in a 6x14 Ti swing out rotor in an MSE Superspeed 75 ultracentrifuge. Phage bands were removed by side puncture, using a syringe fitted with a 23 gauge needle, in a volume not exceeding 0.5 ml.

**CAESIUM CHLORIDE EQUILIBRIUM GRADIENTS**

Phage from the step gradients were added to Beckman "quickseal" polyallomer tubes and the tubes then filled with 1.5g/ml density caesium chloride solution. Tubes were sealed and then gradients were formed by centrifugation in a Ti50 rotor, in a OTD50B Sorval ultracentrifuge at 38000 rpm for 48 hours. Bands were removed by side puncture, as above. Phage were stored in CsCl at 4°C until required.

**SECTION 2.5 FLUORIMETRIC MICROASSAY FOR DNA IN PROKARYOTIC CELLS**

(Legros and Kepes, 1985)

This assay was used in conjunction with the chloramphenicol "run out" experiment described in Chapter 5.

1 ml samples of cells (prepared as described in Chapter 5) were washed three times with an equal volume of ice cold phosphate buffered saline and resuspended in a final volume of 1 ml. An aliquot was permeabilised by addition of 1/100 volume of toluene, mixed with a solution of 4'6-Diamidino-2-phenylindole.2HCl (DAPI. Sigma) in water, to give a 3 ml sample with an OD600 between 0.08 and 0.008 and a final DAPI concentration of 90 ng/ml. Fluorescence was measured with a KONTRON SFM25 fluorimeter equipped with a 150 W Xenon high-pressure lamp and an R212 photomultiplier. Excitation and emission wavelengths were 346 nm and
452 nm respectively. Voltage calibration was set at 380V. Entrance and exit slits for the two monochromators were 10 and 11 mm respectively. The change in DNA content of cells was calculated from the change in relative fluorescence and from the OD<sub>600</sub> of the sample (see Chapter 5).

SECTION 2.6 DNA TECHNIQUES

PREPARATION OF PHAGE DNA (P1 and λ)

Phage banded in caesium, as described above, were dialysed against three changes of TE buffer over an 18 hr period. The phage were then transferred to snap-cap tubes, proteinase K (Sigma) was added to a concentration of 50 μg/ml and the tubes incubated for 1 hour at 65°C. The phage were then extracted three times with an equal volume of redistilled phenol and the aqueous layer removed. The aqueous layer was then extracted once with chloroform/isoamyl alcohol (24:1) and three times with ether. At each step, the non-aqueous layer was back-extracted with an equal volume of TE. The aqueous layers were pooled and dialysed against three changes of TE over a 24 hour period. DNA was stored at 4°C in TE buffer.

Note: for the extraction of P1 DNA, but not λ DNA, P1 phage were first recovered from the CsCl step gradient (as above) and then dialysed against three changes of TE buffer over 18 hours. Phage were then transferred to a snap-cap tube and DNase I (Sigma) was added to a final concentration of 50 μg/ml. The phage were incubated for 1 hour at room temperature and then banded on a CsCl equilibrium gradient. This step was included as an additional safeguard to ensure that no host-chromosomal DNA, aside from that within the transducing particles, was contaminating the phage DNA preparations.

EXTRACTION OF E.coli CHROMOSOMAL DNA

(A modification of the methods of Marmur 1961, Cross-Peller et al. 1973)

E.coli cells were grown, in 250 ml L-broth, to OD<sub>540</sub> of 0.7. Cells were pelleted by centrifugation in a GSA rotor in a Sorval RC5B centrifuge for 15 minutes at 10000 rpm. Cells were then washed
with 20 ml 10 mM TRIS, 1 mM EDTA pH8 and again pelleted as above. Cells were resuspended in 6 ml 25% Sucrose 50 mM TRIS pH8. Lysozyme was added to a final concentration of 1.2 mg/ml and the mixtures warmed to 37°C with gentle shaking. 3.25 ml of 0.25 m EDTA pH8 was added, then the cells were placed on ice and incubated for 5 minutes. Then 6.75 ml Triton lysis buffer (2% Triton, 62.5 mM EDTA, 50 mM TRIS pH8) was added, together with SDS to 0.5%, RNase A (Sigma) to 100 µg/ml and proteinase K (Sigma) to 50 µg/ml. (Note RNase was first heated to 80°C for 10 minutes to remove any contaminating DNase activity.) The mixture was mixed very gently by inversion of the tube and then incubated overnight at 37°C, with very gentle shaking. The mix was then extracted three times with an equal volume of redistilled phenol (equilibrated with 0.5M Tris pH8, 10 mM EDTA, 10 mM NaCl 0.5% SDS) and three times with ether equilibrated with TE. The DNA preparation was then dialysed against 3 changes of TE over a 24 hour period. DNA was stored at 4°C until required.

PLasmid DNA EXTRACTION

1. Small scale, Alkaline Lysis

(This method was as described in the BRL NACS prepac instruction manual.)

5 ml stationary phase cells (grown in L-broth with selection if possible) were pelleted by centrifugation in a bench top centrifuge. Cells were resuspended in 100 µl of 25 mM TRIS (pH 8.0), 50 mM EDTA, 1% w/v Glucose. 200 µl of 0.2M NaOH 1% w/v SDS (made up fresh each time) was then added and the contents of the tube mixed gently by inversion of the tube, and the tubes incubated for 10 minutes on ice. 150 µl of ice cold 5M potassium acetate (KAc) (3M KAc + 2M acetic acid to pH 4.8) was then added, the samples mixed gently, as above, and left on ice for five minutes. The dense white precipitate which formed after the addition of the 5M KAc was spun out by centrifuging the tubes at maximum speed, for 10 minutes in a bench-top microfuge. The supernatant was decanted into a fresh tube and 2 volumes of absolute ethanol (chilled to -20°C) was added, the contents mixed and the tubes placed at -20°C for 20 minutes. The precipitate was
pelleted by centrifugation, as above, the supernatant was discarded and the pellet resuspended in 200 µl of TE. 100 µl of ice cold 7.5 M ammonium acetate was then added, the contents of the tubes were mixed well and then placed on ice for 20 minutes. Tubes were then centrifuged, as above, the pellet discarded and the supernatant was decanted to a fresh tube. 0.6 ml of cold (-20°C) absolute ethanol was added to each tube, the contents of the tubes were mixed and then the tubes were placed at -20°C for 15 minutes. Tubes were centrifuged as above and then the supernatant was removed completely by aspiration. Pellets were dried in a vacuum dessicator and then resuspended in 50 µl TE.

This method routinely yielded up to 20 µg of DNA from each preparation.

2. Large Scale Preparation by Hydroxylapatite (HAP) Chromatography (Coleman et al. 1978)

Solutions are listed in table 2.6. Plasmid DNA was prepared from 100 ml stationary phase cells by a proportionately scaled up version of the alkaline lysis method described above. The DNA preparation was then diluted by the addition of 10 times the volume of lysing buffer (see table 2.6). The mix was layered on to 2 g HAP (Biorad DNA grade) which had been suspended in washing buffer and loaded into a glass column with a sintered glass disc. Positive air pressure was applied to force the DNA preparation through the column. The DNA bound to the column was then washed with washing buffer, forced through under pressure, until the OD$_{260}$ of the eluate dropped to less than 0.1. The column was then washed with 15-20 ml low phosphate buffer and then the DNA was eluted with eluting buffer. 1.5 ml fractions were collected and DNA content was assayed by measuring the OD$_{260}$. Samples containing DNA were then pooled, dialysed against these changes of TE over a 24 hour period, then ethanol precipitated (as described below) and resuspended in 200 µl TE buffer.

3. Instant Crude Lysis Technique

[A modification of the method by Barnes (1977)] - a technique for the rapid screening of clones for the presence of plasmid DNA.
<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phosphate Buffer (4.8 M)</strong></td>
<td>Na₂HPO₄·2H₂O 2.4M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Na₂PO₄·2H₂O 2.4M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>In distilled water</td>
<td></td>
</tr>
<tr>
<td><strong>Lysing Buffer</strong></td>
<td>Urea 9.0M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphate buffer 0.270M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SDS 0.876M</td>
<td></td>
</tr>
<tr>
<td><strong>Washing Buffer</strong></td>
<td>Urea 8.0M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphate buffer 0.240M</td>
<td></td>
</tr>
<tr>
<td><strong>Low Phosphate Buffer</strong></td>
<td>Phosphate buffer 0.010M</td>
<td></td>
</tr>
<tr>
<td><strong>Eluting Buffer</strong></td>
<td>Phosphate buffer 0.30M</td>
<td></td>
</tr>
</tbody>
</table>

All solutions made in distilled water.
Cells were harvested either by centrifugation of 1 ml of a fresh overnight culture, grown under selection, or by scraping cells from a "patch" from a selective plate. Cells were resuspended in 50 μl of Tris acetate gel buffer, in a snap cap tube then 50 μl of "instant crude lysis buffer" was added and thoroughly mixed in. (Instant crude lysis buffer: 10% w/v Ficoll, 8% w/v SDS, in Tris Acetate buffer. Plus 0.04% bromphenol blue, 0.04% xylene cyanol.) Tubes were incubated at 65°C for 30 minutes and then vortexed for 30 seconds. 50 μl samples were loaded directly on to Agarose gels and electrophoresed as described below.

NACS PREPAC COLUMN CHROMATOGRAPHY

When DNA samples needed to be highly purified, i.e. free from contaminating protein or chromosomal DNA, the DNA was purified using NACS prepac columns (BRL).

Up to 40 μg of DNA prepared by either of the methods described above, was treated with RNAse T1 (Sigma) at a concentration of 0.05 u/μl in a volume of 400 μl for 15 minutes at 37°C. NaCl was then added to give a final concentration of 0.2M.

NACS prepac columns were prepared by first washing the column with 3 x 1.0 ml aliquots of 2.0 M NaCl in TE forced through the column under pressure. Then the columns were washed with 3 x 1.0 ml aliquots of 0.2M NaCl in TE, again forced through under pressure.

The DNA sample was loaded on to the column and allowed to run through the column under gravity. The bound sample was washed with 10 ml 0.2M NaCl in TE which was also allowed to run through under gravity, and then the DNA was eluted stepwise with one 100 μl aliquot and two 200 μl aliquots of 1.0M NaCl in TE. The eluate was collected in a snap cap tube and the DNA precipitated by the addition of 1 ml of cold (-20°C) absolute ethanol and the tube was placed at -20°C for 20 minutes. The DNA precipitate was pelleted, as described above, the supernatant removed by aspiration and the pellet dried in a vacuum dessicator. The pellet was then resuspended in 50 μl TE.

The above method was used to purify both supercoiled plasmids and DNA restriction fragments isolated from agarose gels (method
described below). In the case of DNA fragments, the RNAse T1 step was omitted.

The solutions used for the above procedure were all made up volumetrically, in accordance with the manufacturers recommendations.

**ETHANOL PRECIPITATION OF DNA**

1/10 volume of 3M sodium acetate pH 5.2 was added to the DNA solution together with 2 volumes of cold (-20°C) absolute ethanol. The contents were thoroughly mixed and then the solution placed at -20°C for at least 20 minutes. The DNA precipitate was pelleted by centrifugation (either in a microfuge for 10 minutes, or in an SS34 rotor in a Sorval RC5B centrifuge at 10,000 rpm for 10 minutes, depending obviously on the size of the sample), the supernatant was discarded and the pellet dried in a vacuum dessicator. The pellet was then resuspended in an appropriate volume of TE buffer.

**RESTRICTION OF DNA**

1/10 volume of the appropriate restriction buffer was added to the DNA (see table 2.7) and digestions were routinely performed with 5 units of enzyme/μg DNA, at 37°C for 2 hours. The exception was with *E.coli* genomic DNA, which was incubated for 4-5 hours at 37°C to achieve complete digestion. Where necessary, enzymes were removed by phenol extraction, chloroform/isoamy1 alcohol extraction and then the DNA was ethanol precipitated.

**BACTERIAL ALKALINE PHOSPHATASE (BAP) TREATMENT OF DNA**

For the cloning procedures to construct the recombinant plasmids described in Chapters 3 and 4, pBR325 vector DNA was routinely treated with BAP to remove 3' and 5' phosphate groups and so prevent vector reconstitution during ligation. 50 units of BAP was added for each μg of restricted DNA and the mix was incubated at 65°C for 1 hour.

Restriction enzyme and BAP were removed from the DNA by extractions with firstly an equal volume of redistilled phenol equilibrated with TE and then with chloroform/isoamy1 alcohol (24:1) extraction. Both non aqueous phases were back extracted with
### TABLE 2.7

(a) **BUFFERS FOR DIGESTION OF DNA BY RESTRICTION ENDONUCLEASES**

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Working Concentration of 10 x buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris-HCl, pH 7.5</td>
<td>0.20M</td>
</tr>
<tr>
<td>50 mM NaCl</td>
<td>0.50M</td>
</tr>
<tr>
<td>6 mM MgCl$_2$</td>
<td>0.06M</td>
</tr>
<tr>
<td>0.01% BSA (bovine serum albumen)</td>
<td>0.1%</td>
</tr>
<tr>
<td>10 mM 2-mercaptoethanol</td>
<td>0.10M</td>
</tr>
</tbody>
</table>

**Universal type 2:**

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Working Concentration of 10 x buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>33 mM Tris-acetate, pH 7.9</td>
<td>0.33M</td>
</tr>
<tr>
<td>66 mM CH$_3$COOK</td>
<td>0.66M</td>
</tr>
<tr>
<td>10 mM CH$_3$COOMg</td>
<td>0.10M</td>
</tr>
<tr>
<td>0.01% BSA</td>
<td>0.1%</td>
</tr>
<tr>
<td>10 mM 2-mercaptoethanol</td>
<td>0.10M</td>
</tr>
</tbody>
</table>

**EcoR1 type:**

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Working Concentration of 10 x buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM Tris-HCl, pH 7.5</td>
<td>1.00M</td>
</tr>
<tr>
<td>50 mM NaCl</td>
<td>0.50M</td>
</tr>
<tr>
<td>6 mM MgCl$_2$</td>
<td>0.06M</td>
</tr>
<tr>
<td>0.01% BSA</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

**Bam HI/Sal type:**

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Working Concentration of 10 x buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris-HCl, pH 8.0</td>
<td>0.01M</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>1.50M</td>
</tr>
<tr>
<td>6 mM MgCl$_2$</td>
<td>0.06M</td>
</tr>
<tr>
<td>0.01% BSA</td>
<td>0.1%</td>
</tr>
<tr>
<td>10 mM 2-mercaptoethanol</td>
<td>0.10M</td>
</tr>
</tbody>
</table>

(b) **BUFFER FOR LIGATION OF DNA FRAGMENTS**

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Working Concentration of 10 x buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl, pH 7.8</td>
<td>0.05M</td>
</tr>
<tr>
<td>10 mM MgCl$_2$</td>
<td>0.10M</td>
</tr>
<tr>
<td>1 mM Spermidine tri-HCl</td>
<td>0.01M</td>
</tr>
<tr>
<td>0.01% BSA</td>
<td>0.10%</td>
</tr>
<tr>
<td>20 mM DTT (dithiothreitol)</td>
<td>--</td>
</tr>
<tr>
<td>1 mM ATP</td>
<td>--</td>
</tr>
</tbody>
</table>
TE, and the total aqueous phases were mixed. DNA was then ethanol precipitated and resuspended in an appropriate volume of TE.

**LIGATION OF DNA FRAGMENTS**

Ligation reactions were performed in 1 x ligase cocktail (Table 2.7) with a concentration of 30-50 ng/μl of participating DNA molecules. Approximately 20 units of T4 DNA ligase was added to each ligation, and the reaction was incubated overnight at 15°C, before being used to transform competent cells.

Note, when the vector molecule had been treated with BAP prior to ligation, the ratio of vector molecules to clonable fragments in the ligation mix was adjusted to 3:1. This was because BAP treatment invariably led to the loss of about 50% of ligation efficiency when compared to cloning experiments when the vector was not treated with BAP.

**PREPARATION OF TRANSFORMATION-COMPETENT CELLS**

(A modification of the method of Lederberg and Cohen, 1974)

A fresh overnight of the strain was diluted 1:50 into L-broth plus 20 mM MgCl₂/SO₄ (with selection if appropriate) and the culture grown at 37°C with vigorous aeration until the OD₅₄₀ reached 0.5. The culture was chilled on ice and the cells pelleted by centrifugation for 10 minutes at 10,000 rpm in an appropriate rotor, in a Sorval RC5B centrifuge at 4°C. The supernatant was discarded and the cells were resuspended in 1/4 volume of ice cold 0.1M MgCl₂. The cells were pelleted again, as above, and the supernatant discarded. Cells were resuspended in 1/20 of the original volume of ice cold 0.1M CaCl₂ and cells were kept on ice for 30-45 minutes. Cells were again pelleted, as above, and resuspended in 1/20 of the original volume of ice cold 0.1M MOPS (pH 6.5) 50 mM CaCl₂, 20% glycerol. Cells were kept on ice until required or placed at -70°C for long term storage.

**TRANSFORMATION OF COMPETENT CELLS**

50 ng DNA (from ligation mix, etc.) was added to a 200 μl aliquot of competent cells (prepared as above) in a snap cap tube and then the mix was kept on ice for 30 minutes. (Care was taken to ensure that the DNA was thoroughly mixed in with the competent
cells.) The transformation mix was then given a 3 minute heat-shock at 37°C and then placed on ice for a further 15 minutes. After this period, 1 ml of L-broth was added to the mix, which was then incubated at 37°C for 1 hour before 100 μl aliquots were spread on to selective plates.

AGAROSE GEL ELECTROPHORESIS OF DNA (McDonnell et al. 1977)

Except where stated electrophoresis was carried out using 1% Agarose gels (Sigma low EQ) in Tris Acetate gel buffer. Horizontal agarose slab gels were cast in BRL "midi" (Model H5) and "baby" (Model H6) gel apparatus. Appropriate amounts of the DNA sample were mixed with 1/4 volume of loading buffer (50% glycerol, 50% Tris acetate, 0.04% Bromophenol blue, 0.04% xylene cyanol) and loaded on to the gels. DNA samples were electrophoresed with the gel submerged with buffer circulation, usually overnight at 20V or during the day at an appropriate voltage. Gels were stained for 30 minutes in 0.5 μg/ml Ethidium bromide in Tris Acetate buffer and destained for 30 minutes in buffer alone. DNA in the gels was visualised with a uv-transilluminator and gels were photographed using large format Ilford HP5 negatives. The camera was fitted with both UV and red filters, the lens aperture set at f4.5 and the exposure time was usually 15 seconds.

ISOLATION OF DNA FRAGMENTS FROM AGAROSE GELS

DNA samples were size fractionated by electrophoresis as above, except that the highly purified Miles "Seakem LE" agarose was used for the gels. The gels were lightly stained (10 minutes only in the stain), viewed with the UV transilluminator and photographed as above. The required DNA fragment was cut from the gel in the smallest segment of agarose that was possible, using a sterile scalpel blade, and transferred to a sterile plastic petri dish. The piece of agarose was sliced thinly and placed in a dialysis bag with 1 ml Tris acetate gel buffer. The bag was submerged in an electrophoresis tank holding Tris acetate buffer (the bag was placed crosswise to the direction of the current) and electrophoresed at 200 V for 2 hours. The current was reversed for 10 minutes and then again for 2 minutes. The buffer in the dialysis bag was transferred to a fresh bag, without the fragments of agarose, and
dialysed against 3 changes of 0.2M NaCl in TE over an 18 hour period. This sample was then purified and concentrated by NACS prepac column chromatography and ethanol precipitation (described above).

TRANSFER OF DNA FROM AGAROSE GELS TO NITROCELLULOSE

[A modification of the methods described by Southern (19/5) and Smith and Summers (1980)]

DNA samples were electrophoresed on an agarose gel, stained and photographed as described above. The DNA was depurinated by soaking the gel (with gentle agitation) in 250 ml of 0.25 M HCl for 15 minutes. This step was then repeated using fresh 0.25M HCl. The gel was then rinsed with distilled water and the DNA was denatured by soaking the gel (with agitation) in 250 ml of 0.5M NaOH, 1.5M NaCl for 20 minutes. Again, this was repeated with fresh solution. The gel was then neutralised by soaking in 250 ml of 1.0M ammonium acetate, 0.02M NaOH, with agitation, for 20 minutes. (This was also done twice.)

The treated gel was then placed face downwards on a clean glass plate and a sheet of nitrocellulose (0.25 μm pore size, Schleicher and Schuell) cut to exactly the same size as the gel and soaked in the above ammonium acetate/NaOH solution, was placed carefully on to the gel. Care was then taken to ensure that no bubbles remained between the gel and the nitrocellulose. Then 6 sheets of Whatman 3MM paper, cut to the same size as the gel and soaked in the above buffer, were placed on to the nitrocellulose; again care was taken to ensure no bubbles were trapped. On top of this was placed a 2 inch thick stack of blotting paper, cut to approximately the size of the gel. Finally, a sheet of perspex was placed on top of the stack and the whole stack was evenly weighted, usually with a 500 ml blood bottle full of water. Transfer was allowed to take place overnight and then the filter lifted from the gel, rinsed for 2 minutes in 2 x SSC (Table 2.4) and blotted dry. The DNA was bound irreversibly to the nitrocellulose by baking the filter at 80°C under vacuum for 2 hours.

Filters were stored at room temperature until required.
DNA probes were \(^{32}\text{P}\)-labelled either by nick translation, according to the method of Rigby et al. (1977), or by the oligonucleotide priming method described by Feinberg and Vogelstein (1984).

Routinely 100 ng of probe DNA was labelled. Labelled DNA was separated from unincorporated label by passing the "Labelling reaction mixture" through a 15 cm column of Sephadex G-50 (medium); the probe eluting in the first peak of radioactivity. Probes were then further purified by Elutip (Schleicher and Schuell) column chromatography. (Note, this procedure was identical to that described for NACS prepac column chromatography except, of course, Elutip columns were used.)

**HYBRIDISATION OF DNA PROBES TO DNA IMMOBILISED ON NITROCELLULOSE FILTERS**

Filters, prepared as above, were first prehybridised in a solution identical to the hybridisation solution, for 2 hours at room temperature.

Probes were boiled for 5 minutes, to separate the DNA strands, and then added to usually 15-20 ml of hybridisation solution. The filters were sealed in plastic bags together with the probe in the hybridisation solution and hybridisation carried out overnight at 37°C with rotary shaking.

Routinely, \(1-1.5 \times 10^7\) cpmp of probe were included in each hybridisation.

After hybridisation, the filters were removed from the bag and given 4 washes, each of 30 minutes: 2 in 250 ml of 2 x SSC 0.1% SDS and 2 in 250 ml of 2 x SSC.

Filters were then blotted dry and autoradiographed.

The hybridisation solution used was normally that shown in Table 2.8a. However, for the hybridisations to \(P1\)-transducing DNA described in Chapters 3 and 4, the hybridisation was carried out in the solution shown in Table 2.8b.
### TABLE 2.8 HYBRIDISATION SOLUTIONS

<table>
<thead>
<tr>
<th>Table</th>
<th>Solution Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>Formamide (AnalaR) 50% v/v</td>
</tr>
<tr>
<td></td>
<td>SSC 2 x</td>
</tr>
<tr>
<td></td>
<td>Denhardt's solution 1 x</td>
</tr>
<tr>
<td>(b)</td>
<td>Formamide (AnalaR) 50% v/v</td>
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<tr>
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<tr>
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<tr>
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<td>Dextran Sulphate 3% w/v</td>
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<td>SDS 0.1%</td>
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<td>1% w/v Bovine serum albumen (Sigma, Fraction V, 89-99%)</td>
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<tr>
<td></td>
<td>1% w/v Ficoll (Sigma Type 400, MW 400 000)</td>
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<tr>
<td></td>
<td>1% w/v Polyvinylpyrrolidone (PVP, MW 40 000)</td>
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SECTION 2.7 AUTORADIOGRAPHY OF FILTERS

Filters were autoradiographed according to the recommendations set out in Amersham review 23: "Radioisotope detection by Fluorography and Intensifying Screens".

Autoradiography was carried out using Cronex (Du Pont) Grade 4 blue based x-ray film, preflashed to an OD540 of 0.15. Filters were autoradiographed in cronex (Du Pont) cassettes fitted with "xtra-life" intensifying screens and the cassettes placed at -70°C for the exposure. Exposure times depended on the strength of the signal from the bound probe, but ranged from 30 minutes to 7 days as appropriate.

SECTION 2.8 DENSITOMETRIC ANALYSIS OF HYBRIDISATION SIGNALS

Hybridisation was measured quantitatively by scanning the autoradiographs with a Vitatron TLD100 densitometer, attached to a chart recorder. A 100 μm slitpiece and a grey (U60) filter were used. The machine was calibrated to give a baseline of 10% and a maximum deflection, for each scan, of about 75%. Intensities of bands were calculated by integrating the trace derived from the densitometer scan. Peaks (due to the absorbance of the bands on the autoradiogram), were integrated by tracing around them on a Summagraphics magnetic data tablet, attached to a Ferranti Cetec digitiser and an Olivetti P640 microcomputer: area calculations were performed by a program written by Dr. A. F. W. Coulson.

Quantitation of the amount of homologous sequence in the test tracks was made by comparing the intensity of hybridisation in those tracks with a standard curve derived from the intensities of hybridisation to tracks containing known quantities of homologous sequence (see Chapter 3).
P1 transduction frequencies vary over a 30-fold range in a marker dependent manner. Marker selectivity, in contrast to the situation during P22-mediated generalised transduction is probably not due to selective packaging of preferred regions of the donor chromosome. Instead, marker selectivity is thought to arise from recombinational discrimination in recipient cells (Newman and Masters 1980, Masters et al. 1984, Schmeiger 1984b, see chapter 1). The above conclusions stem from the observation that uv-irradiation of transducing lysates, prior to transduction, results in the selective stimulation of the recovery of poorly transduced markers; the transduction of markers which are normally transduced well is stimulated little, if at all. Indeed, the range in transduction frequency is reduced to 3-4 fold and can be accounted for by gene dosage effects. This implies that all markers are packaged into P1 particles with equal efficiency and that uv-irradiation of the transducing DNA results in suppression of the marker-selectivity in transduction. There is, however, one known exception to this general rule. The marker, \textit{leuB}, situated at 2 minutes on the \textit{E.coli} map (Bachman 1983) is normally highly transduced. However, following uv-irradiation of P1 lysates, \textit{leu} transduction is unexpectedly stimulated above even that of efficiently transduced \textit{oriC}-linked markers. Transduction of markers cotransducible with \textit{oriC}, e.g. \textit{ilv}, is very high when unirradiated lysates are used and is not stimulated by uv-irradiation. If the levels of transduction observed with irradiated lysates are a reflection of the packaging levels of individual markers in the lysate (Newman and Masters 1980), gene dosage in donor cells dictates that markers near \textit{oriC} should be present in excess of \textit{leuB}, some 18-20 minutes from \textit{oriC}. Hence, if all markers are packaged equally efficiently by P1, \textit{ilv} transduction should exceed that of \textit{leuB}. This is clearly not the case (Newman 1982), so it is possible that \textit{leuB} may be packaged with greater efficiency than \textit{ilv}, or other markers. In view of the fact that P1 packages DNA by sequential encapsidation of headful amounts of DNA (Bach and Arber 1977), it seems likely that other markers from the 2
minute region, in which \textit{leuB} is located, will also be overpackaged.

A study of the packaging levels of individual markers in P1 lysates had not been performed. Accordingly, there is no direct evidence that markers (other than those from the 2 minute region) are packaged with equal efficiency; the work with uv-irradiated lysates, however, strongly suggests that this is so (Newman and Masters 1980, Newman 1982).

The packaging levels of markers have been easily calculated when studying P22-mediated transduction of \textit{Salmonella} as both complete and abortive transductants form colonies on selective plates (complete and abortive transduction can account for 90\% of incoming transducing DNA (Sandri and Berger 1981a)); colony size distinguished between the two as abortives formed tiny colonies (Ozeka and Ikeda 1968 for review). Unfortunately, this does not seem to be so for P1-mediated transduction of \textit{E.coli} (at least in our hands). Newman (1982) reported that for most nutritional markers, abortive transductants either did not form colonies, or that the tiny colonies they formed were obscured by background growth on selective plates. Hence packaging of markers into P1 transducing particles can not easily be assayed by the simple technique of counting complete and abortive transductants. Instead Newman (1982) attempted to measure the packaging levels of markers by liquid DNA-DNA hybridisation. Cloned chromosomal DNA fragments (probes) were denatured and mixed with denatured DNA extracted from P1 lysates. Under appropriate reaction conditions the probes were allowed to anneal to homologous transducing DNA, present in the DNA extracted from the P1 lysate. The amount of hybridisation was assayed quantitatively and so should have enabled estimation of the levels to which sequences, homologous to the probes, were represented in the lysate. However, this approach proved technically difficult, and the results were inconclusive. Accordingly, a different approach to quantitative hybridisation was employed here.

The total amount of transducing DNA present in DNA extracted from P1 transducing lysates can be calculated from the observation that transducing particles make up about 2\% of the particles in a lysate (Sandri and Berger 1980a). Because transducing and infective
particles encapsidate equivalent amounts of DNA (Ikeda and Tomizawa 1965a) 2% of the DNA extracted from a P1 lysate will be transducing, i.e. the ratio of transducing to P1 DNA is 1:50. Assuming that packaging of the E.coli genome is not marker-selective then the amount of any specific 4 kb E.coli sequence (4 kb is chosen as an example because this is the average size of fragment generated by digestion with a restriction enzyme recognising a 6 bp target site) present in 1 μg of DNA from a P1 lysate can be calculated as follows:-

\[
\frac{1 \times 10^{-6} \text{g} \times 50}{4 \times 10^{3} \text{bp}} \times \frac{4 \times 10^6 \text{bp}}{1 \text{pg sequence/μg DNA from lysate}} = 2 \times 10^{11} \text{ g of sequence/μg DNA from lysate}
\]

Sequences at such low levels can be detected and visualised using DNA/DNA hybridisation techniques such as Southern Hybridisation (Southern 1975), in conjunction with autoradiography. The amount of hybridisation can be quantitatively assessed by analysing the exposure of the X-ray film by the bound probe; band intensities can be measured using a scanning densitometer, which assesses the absorbtion of the band, followed by integration of the densitometer-trace with a digitiser. This method is described in more detail in section 3.3.

The above approach was chosen as a simple and convenient method of assessing packaging levels of individual markers in P1 lysates. However, the initial steps required the isolation and characterisation of specific E.coli chromosomal fragments to be used as hybridisation probes for the detection and quantitisation of homologous sequences in transducing DNA.
SECTION 3.2
DERIVATION AND CONSTRUCTION OF HYBRIDISATION PROBES
RECOMBINANT LAMBDA PROBES

In vitro constructed recombinant λ phage were available and were initially used as hybridisation probes. The phage, consisting of chromosomal HindIII fragments inserted into λNM540 cloning arms (Murray and Murray 1975) are listed, together with their derivations, in Table 3.1.

RECOMBINANT PLASMID PROBES

Recombinant plasmids used as probes are listed in Table 3.1 and the construction of those not previously described is detailed below.

CONSTRUCTION OF pMH600, pMH601 and pMH602

pMH600 (hisG), pMH601 (ilvABC) and pMH600 (trp) were constructed, as described in chapter 2, by subcloning the HindIII chromosomal inserts from λhis, λilv and λtrp, respectively (see Table 1.1 for details of these recombinant phage) into the unique HindIII site of the multicopy plasmid vector pBR325 (Bolivar et al. 1978). Since the HindIII cloning site lies within the promoter for the plasmid encoded tetracycline (Tc) resistance gene (Sutcliffe 1978), recombinant plasmids confer an ampicillin (Ap⁺) resistant, chloramphenicol (Cm⁺) resistant tetracycline-sensitive (Tc⁵) phenotype on the host cell. The above recombinant plasmids were introduced into suitable transformation-competent recipient cells by the method described in chapter 2. Plasmid DNA was extracted by alkaline lysis from cells receiving recombinant plasmids, the DNA was restricted with HindIII and visualised by agarose gel electrophoresis. Recombinant plasmids were confirmed to contain inserts of the same size as those contained in the parental recombinant λ phage.

In addition, to confirm that pMH600 (hisG) and pMH601 (ilv) had received the correct inserts, complementation studies were performed:
competent MM303-1 (Ilv-) cells and HfrG6 (HisG-) cells were prepared and transformed with pMH601 and pMH600 DNA respectively. pMH601 was able to complement the ilv lesion in MM303-1. Likewise, pMH600 was able to complement the hisG lesion of HfrG6 enabling the cells to grow on -his minimal plates. pMH602, however, received only one of the two HindIII inserts from \( \lambda \)trp, so complementation was not expected nor sought.

HindIII digests of pMH600, pMH601 and pMH601 are shown in figure 3.1. Diagrams indicating the sizes of the inserts are in figure 3.2. No attempts were made to construct detailed restriction maps of the cloned inserts.

CONSTRUCTION OF W3110 HindIII and BamHI CHROMOSOMAL LIBRARIES

Since probes for some markers of interest, such as \( \text{LeuB} \), were not already easily available it was decided to construct libraries of \( \text{E.coli} \) chromosomal DNA cloned into pBR325, which could then be screened for \( \text{LeuB} \)-containing plasmids and plasmids carrying other markers of interest.

Accordingly, genomic DNA was isolated from the prototrophic strain W3110, by the method described in chapter 2. HindIII-restricted or BamHI-restricted W3110 was ligated with HindIII or BamHI-restricted pBR325 vector DNA respectively and the ligation mix transformed into competent W3110 cells (see chapter 2). Insertion of DNA fragments into the BamHI or HindIII cloning sites of pBR325 results in inactivation of the \( \text{Tc}^r \) gene, so that the total number of clones in each library and the proportion of recombinant clones, could be assessed by comparing the number of clones with \( \text{Apr}^r\text{Cm}^r\text{Tc}^r \) (parental) and \( \text{Apr}^r\text{Cm}^r\text{Tc}^5 \) (recombinant) phenotypes. The BamHI library contained in total about \( 6 \times 10^3 \) clones, 98% of which contained recombinant molecules. Hence, the BamHI library contained enough clones to cover the \( \text{E.coli} \) genome about 6 times. (The \( \text{E.coli} \) genome is 4000 kb. As the average restriction fragment size for BamHI digestion is 4 kb, \( 10^3 \) clones should contain the entire \( \text{E.coli} \) genome). The HindIII library was slightly less successful, containing \( 4.5 \times 10^3 \) clones, 95% of which
were recombinants and so could cover the *E. coli* genome 4-5 times.

Once the number of recombinants in each library had been established, the competent cells transformed with each of the library DNAs were grown, to stationary phase, in 100 ml L-broth CmAp liquid media. The plasmid DNA in each library was extracted from the culture by large scale alkaline lysis. The DNA was dispensed into 4-5 μg aliquots and stored at -70°C as a precipitate in 70% ethanol. In addition both restricted and unrestricted DNA from the libraries was run on as gel, and is shown in figure 3.3.

There are obviously some disadvantages in preparing a library of *E. coli* DNA by the above method. Clearly, the choice of a high copynumber vector may select against the recovery of sequences which are detrimental to the cell in high copy. In addition, the amplification step may enhance this effect or may result in the loss of sequences which reduce plasmid copynumber or stability. However, this method was employed because plasmids isolated from the DNA library could be used directly as probes, without the need for further subcloning. In addition, the ease with which high copy plasmid DNA can be prepared and purified, especially with the aid of column chromatography (see chapter 2) is a great advantage. Finally, amplification and subsequent aliquoting of library DNA ensures that the library is maintained and can be used many times.

In any case, since the purpose of constructing the libraries was to isolate specific nutritional markers and not to obtain a representative sample of chromosomal markers, the above method was considered suitable.

**ISOLATION OF LeuB, PyrE and ArgH COMPLEMENTING PLASMID CLONES FROM W3110 DNA LIBRARIES**

**LeuB-COMPLEMENTING CLONE**

Competent MM18 cells were transformed with DNA from both BamH1 and HindIII libraries. Cells were plated on -leucine ApCm plates and incubated overnight at 37°C. No growth was observed on plates spread with cells transformed by the HindIII library DNA, but approximately 100 colonies per plate grew on plates spread with cells
transformed by the BamHI library DNA. It was observed that after 2-3 days on the bench, or 1-2 days at 37°C, the large Leu+ colonies became surrounded by small satellite colonies. Such colonies could not grow alone on -leu plates. Accordingly it seemed likely that leu+ colonies were excreting leucine, or a precursor, which was diffusing into the -leu agar medium. Surrounding Leu clones could then presumably take up this substance to allow their growth on the -leu plates. 25 leu-complementing colonies were picked, and streaked on the same selective plates. Single colonies were picked, grown up in -leuApCm liquid medium and their plasmid DNA was extracted by alkaline lysis. All 25 clones contained a large 14 kb plasmid so DNA from 5 was restricted with BamHI and visualised by agarose gel electrophoresis. All clones contained a 6 kb vector plasmid (pBR325) carrying a cloned 8.5 kb BamHI chromosomal fragment. It is not possible to compare this fragment with a comprehensive restriction map of the leu region of the chromosome, as none has been published. Wessler and Calvo (1981) however, report that the control region of the leu operon contains an internal HindIII site, explaining the failure to obtain leu-complementing clones for the HindIII library. The HindIII library DNA was nonetheless used for the attempted isolation of leu-complementing clones, as some partial digestion of the chromosome could have occurred during the construction of the library, so it was possible that the leu region might have been cloned intact on a partially digested HindIII fragment. However, as mentioned above, no leu-complementing clones were obtained from cells transformed with HindIII-library DNA. To establish that the leu-complementing plasmid (pBWO01) did indeed carry the leu region and not a suppressing sequence, the following marker rescue experiment was performed: MM20, a polA derivative of MM18, was transformed with pBWO01 plasmid DNA. Stable leu+ clones arose at very low frequency and only 15 were obtained from transformation with a total of 2 µg pBWO01 DNA. As polA strains cannot support replication of ColE1-derived replicons, such as pBR325 (Kinsbury and Helinsky 1970), stable leu+ clones could arise by three possible routes (see figure 3.4).
1) revertants of the original leuB mutation could yield leu+
colonies. However, no growth was observed on -leu control plates spread with equivalent amounts of MM20 cells transformed with pBR325 alone, so eliminating this possibility.

2) Reversion of the PolA\(^-\) mutation would allow replication of pBW001 within the recipient cell and so give a Leu\(^+\)Ap\(^-\)Cmr\(^+\) phenotype. However, the 15 Leu\(^+\) clones obtained were screened for the presence of free plasmid DNA by the instant crude lysis technique (chapter 2). None were found to contain detectable amounts of free plasmid DNA.

3) Integration of pBW001 into the MM20 genome by a single homologous or illegitimate recombination event would allow the plasmid to be stably maintained in the PolA\(^-\) background; such clones would exhibit a Leu\(^+\)Ap\(^-\)Cmr\(^+\) phenotype. However, it would not be possible to distinguish whether the recombination event was homologous (i.e. between leu sequences on the plasmid and on the chromosome) or illegitimate (i.e. independent of extensive mutual homology between the participating molecules), so Leu\(^+\)Ap\(^-\)Cmr\(^+\) clones could arise if the plasmid pBW001 carried either a suppressing sequence of LeuB\(^-\) or the leuB gene. Of the 15 Leu\(^+\) clones obtained above, 9 were Leu\(^+\)Cm\(^+\)Ap\(^-\) (Figures 3.4a, 3.4b).

4) A double crossover event between the BamHI insert carried on pBW001 and homologous chromosomal sequences, could "marker rescue" the leuB gene from pBW001 to give Leu\(^+\)Ap\(^-\)Cm\(^+\)S clones. This could only occur if pBW001 carried leuB and homologous flanking sequences (figure 3.4c). Of the 15 Leu\(^+\) clones obtained, 6 were of this type, i.e. Leu\(^+\)Ap\(^-\)Cm\(^+\)S. Since this double crossover event requires extensive homology between participating molecules, the occurrence of Leu\(^+\)Ap\(^-\)Cm\(^+\)S clones indicated that pBW001 does indeed carry leuB and flanking sequences, rather than a suppressing sequence.

It seems likely that the 8.5 kb BamHI insert contains the leu operon promoter/operator region as the leuB gene, at least, is expressed in pBW001. It is, however, possible that leuB expression occurs via transcriptional readthrough from the tc gene promoter or from other plasmid encoded promoters.

pBW001 is shown in figures 3.1 and 3.2.
ArgH COMPLEMENTING CLONE

(MM18) competent cells were transformed with DNA from the BamHI and HindIII libraries and spread onto -ArgCMAP selective plates. Only cells transformed with HindIII library DNA formed colonies after overnight incubation at 37°C. As with the leu-complementing clones after incubation for 1-2 days at 37°C, satellite colonies appeared around the large clones growing on the selective plates. 10 large clones were picked, purified on the same selective plates and single colonies which grew overnight at 37°C were picked. These clones were grown to stationary phase in -Arg CmAp selective medium and their plasmid DNA was extracted by alkaline lysis. The DNA, both uncut and restricted with HindIII was visualised by agarose gel electrophoresis. The results (figure 3.5a) showed that Arg⁺ MM18 cells contained a number of different recombinant plasmids, presumably because the concentration of DNA in the transformation mix was too high and so competent cells had taken up more than one DNA molecule. To identify which of these, if any, carried the argH gene, the gel was transferred to nitrocellulose by Southern blotting (Southern 1975). The filter was probed by hybridisation with ³²p-labelled λ arg DNA. Subsequent autoradiography revealed that each clone contained a common 7.0 kb HindIII fragment homologous to λarg (see figure 3.5). (The picture was complicated, however, by the presence of partially digested DNA molecules which resulted in the detection of a number of higher molecular weight bands which are also homologous to λarg. However, the common 7.0 kb band was assumed to be the same fragment as the 7.0 kb HindIII fragment carried on λarg). To purify the putative Arg⁺ plasmid away from the other plasmids in the cell, the DNA from the Arg⁺ clones was transformed at low concentration into competent MM18 cells which were then spread on -ArgCmAp plates. Colonies appeared on selective plates after overnight incubation at 37°C and 5 were picked and restreaked on -ArgApCm selective plates. DNA was extracted from the purified clones by alkaline lysis and visualised by agarose gel electrophoresis. The gel showed the presence of a 12.9 kb plasmid, carrying a 7.0 kb HindIII insert which could complement the argH mutation in MM18. This plasmid was thus carrying the same insert as λarg and was named
pHW001. A HindIII digest of pHW001 is shown in figure 3.1 and the plasmid is diagrammed in figure 3.2.

**pyrE SUPPRESSING PLASMID (spyE) (pBW000)**

Competent 303-1 cells were transformed with DNA from both libraries and were spread onto -Ura (pyrE cells required uracil) ApCm plates. No growth was observed. However, in addition, an aliquot of the transformed cells were inoculated in -Ura ApCm selective liquid media and after 2 days shaking at 37°C, growth was observed in the flask containing cells transformed with the BamH1 library DNA. A sample of the culture was taken and streaked on -Ura ApCm plates. After 2-3 days incubation at 37°C, small colonies grew. DNA prepared from these clones by alkaline lysis revealed an 8.2 kb plasmid (pBW000) consisting of the pBR325 vector carrying a cloned 2.2 kb BamH1 fragment. This result was surprising, as An et al. (1979) isolated specialised transducing \( \lambda \) phage carrying pyrE together with flanking chromosomal DNA. They restriction mapped the chromosomal DNA and showed pyrE contained one BamH1 site in the coding region but contained no internal HindIII sites. Accordingly, the lack of growth from cells transformed with HindIII library DNA and the growth, though poor, of BamH1 library transformants, were both surprising. The plasmid pBW000, contained only one BamH1 insert, so ruling out the possibility of the pyrE gene having been cloned intact on a partially digested BamH1 fragment. Also, as there are no internal HindIII sites in pyrE, HindIII library DNA would be expected to yield recombinant plasmids carrying a complete pyrE gene. This obviously, was not the case.

It was possible, however, that the pyrE complementing 2.2 kb BamH1 fragment contained some form of suppressor of pyrE, rather than an intact pyrE gene. To establish whether pBW000 carried pyrE or a suppressor of pyrE, marker rescue experiments were carried out in the same manner as for pBW001: Competent MM20 cells were transformed with pBW000 DNA and spread onto -Ura ApCm or L-broth ApCm plates. No growth was observed on -Ura ApCm plates, and only 9 colonies appeared in total on the L-broth ApCm plates. None of the 9 were Ura\(^+\), so the integrated plasmid cannot suppress pyrE, and so obviously could
not have carried the intact pyrE gene. There was, however, the possibility that pBW000 could be carrying part of the pyrE gene which, when fused to the Tc gene during the plasmid construction, resulted in a hybrid polypeptide which retained some of the pyrE activity. Hence the poor growth of pyrE strains harbouring pBW000, when grown on -Ura plates. In this case, integration of the plasmid in MM20 may not necessarily regenerate an active pyrE gene so the strain would still be unable to grow on -Ura plates. However, the recombination would have introduced the Ap"Cm" genes into the E.coli chromosome adjacent to the location of pyrE, at 82 minutes on the E.coli map. The ApCm genes should thus be cotransducible with tnaA, at about 83.4 minutes on the E.coli map (Bachman 1983), as tna and pyrE exhibit about 12% cotransduction, when tna is the selected marker (Masters 1977). To test this hypothesis, P1 lysates were made on 5 of the MM20 Ap"Cm" clones, obtained after transformation with pBW000. The lysates were used separately to transduce MM303 to Tna+ or Ap"Cm". 100 Tna+ transductants obtained from each lysate, were screened for cotransduction of the Ap"Cm" phenotype. In the reciprocal experiment, 100 Ap"Cm" transductants were screened for Tna+ cotransduction. However the above experiments failed to detect any Tna+Ap"Cm" clones, suggesting that pBW000 was probably not cotransducible with tna, so therefore, unlikely to be integrated at pyrE. However, the above results do not conclusively prove that pBW000 does not encode pyrE or part of pyrE. Jenkins (personal communication) suggests that measuring cotransduction of integrated CoIE1-derived replicons with chromosomal markers, is not a reliable method of mapping the site of plasmid integration, when the recipient used to score transduction is PolA+ (as is MM303). In addition, the observations reported in chapter 4 suggest that integration of pBR325 plasmids into the chromosomes of PolA strains causes, or results in multiple inserts. This would reduce cotransduction of markers flanking the insertion site (see chapter 4 for discussion). Accordingly, the lack of cotransduction between integrated pBW000 and Tna cannot be taken as conclusive evidence that pBW000 does not carry pyrE. However, the report by An et al. (1979) that pyrE contains an internal BamH1 site, makes it
most unlikely that pBW000 does carry an intact $\text{pyrE}$ gene. However, the above observations suggest that pBW000 perhaps carries a form of extragenic, high copy suppressor of $\text{pyrE}$ (designated $\text{spyE}$).

No further attempts were made to characterise the 2.2 kb BamH1 insert of pBW000, nor to determine its chromosomal location. pBW000 was, however, used out of interest as a hybridisation probe in a packaging assay.
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pGS17 was a generous gift from John Guest.
FIGURE 3.1. RECOMBINANT PLASMID PROBES

A. 1. HindIII digested pBR325  
2. HindIII digested pMH602  
3. HindIII digested pMH601  
4. EcoRI digested W3110 genomic DNA  
5,6,7. EcoRI digested P1 DNA -as size standards.

B. 1. EcoRI digested P1 DNA  
2,3,4. pBW000: undigested, restricted with BamHI and with EcoRI respectively.

C. Independent isolates of pMH600, either unrestricted (1,3,5 and 7) or restricted with HindIII (2,4,6 and 8). 9) shows λhis CBAHFI restricted with HindIII.

D. Independent isolates of pBW001, either undigested (2,4 and 6) or restricted with BamHI (1,3 and 5). 7 shows P1 DNA restricted with EcoRI.

E. 1. HindIII restricted λargH.  
2. unrestricted pBR325  
3. pBR325 restricted with HindIII

Independent isolates of pHWO01 are shown either restricted with HindIII (4,6 and 8) or undigested (5 and 7). P (track 8) is the product of partial digestion and is about 13 kb in size.

NOTE: all P1 EcoRI-restriction fragment sizes are shown in figure 3.9.

The largest molecular weight fragment in the restricted-λ tracks (C9, E1) is due to annealing of the two cloning arms at cos.
Figure 3.1

A

B

C
3.1 contd.

D

E

8.5
6.0

P
7.0
6.0
amp, cap and tet show the positions of the ampicillin resistance, chloramphenicol resistance and tetracycline resistance genes respectively and the arrows indicate the direction of transcription. ori denotes the origin of replication. Cloned chromosomal sequences are represented by blocks and pBR325 sequences are indicated by a single line.

H, E and B show the presence of HindIII, EcoRI and BamHI sites respectively.
Figure 3.2

A

pMH600
11.5 kb

B

pMH601
13 kb

C

pMH602
9 kb
Figure 3.2 contd.

D

E

F

pBW000
8.2 kb

pBW001
14.5 kb

pHW001
13 kb
FIGURE 3.3. LIBRARY DNAs

1. Unrestricted pBR325 vector DNA.

2. HindIII library DNA restricted with HindIII. Note the ladder pattern of DNA fragments above and below the vector band (shown by the arrow).

3. Unrestricted HindIII-library DNA.
   Note the ladder pattern of plasmid bands, which are all greater in size than the vector plasmid (track 1), but there is a predominance of small sized plasmids.

4. BamHl-library DNA restricted with BamHl.

5. Unrestricted BamHl library DNA.

6. EcoRI-digested P1 DNA.
FIGURE 3.4 SCHEMATIC DIAGRAM INDICATING THE THREE MECHANISMS OF INTEGRATION OF pBWO01 INTO THE CHROMOSOME (see text for discussion)

1. Single homologous recombination event, i.e. 1 crossover.

2. Illegitimate recombination

3. "Marker rescue", i.e. homologous recombination involving 2 crossovers which replaces the mutant allele (indicated by the small circle/line) with the wild type allele.

The thick line represents plasmid sequences and the boxes represent regions of homology between plasmid and chromosome. (See text for discussion)
FIGURE 3.5 DETECTION OF ArgH+ PLASMIDS

A. 2-tier gel (a and b indicate the position of the wells) on which was loaded undigested (tracks 5,7,9,11,13) and HindIII-restricted (tracks 4,6,8,10,12) plasmid DNA's isolated from ArgH+ -complementing clones (see text for discussion). Tracks 1,2 and 3 contain HindIII restricted λarg, HindIII-restricted pBR325 and unrestricted pBR325 respectively.

3.5B shows an autoradiogram of the above gel, transferred to nitrocellulose and probed with λarg. Note the common 7.0 Kb homologous band in the HindIII restricted tracks.
Figure 3.5

A

B
SECTION 3.3
PROTOCOL FOR ESTIMATION OF PACKAGING LEVELS OF MARKERS IN P1 TRANSUDING LYSATES

A quantitative approach to Southern Hybridisation (Southern 1975) was the method of choice in estimating packaging levels. The protocol was as follows:

Three independent P1 lysates were grown on the prototrophic strain W3110, and the phage lysates harvested as described in chapter 2, except for the following modification: to ensure there was no contamination from host cell chromosomal DNA, after Caesium step-gradient centrifugation, phage were dialysed against 3 changes of phage buffer, overnight, and then treated with 5 \( \mu \)g/ml DNAse for 1 hour at room temperature. Control experiments where extraneous plasmid DNA was added prior to treatment indicated that DNA not protected by encapsidation within phage heads was totally degraded. DNAse-treated phage were then rebanded on CsCl equilibrium gradients to remove DNAse. Phage were harvested by side puncture, dialysed to TE and the phage DNA extracted as described in chapter 2.

Genomic DNA was extracted from stationary phage W3110 E.coli cells by the method described in chapter 2; stationary phase cells were used as the ratio of origin to terminus markers should be 1:1 (Cooper and Helmstetter (1968), see section 3.4, table 3.4).

Both P1 and E.coli genomic DNAs were restricted to completion with EcoRI. Restricted P1 DNA (the amount varied from gel to gel but was always accurately measured) was run on a 1% agarose gel, alongside tracks containing restricted E.coli genomic DNA as standards. 2% of the DNA in the P1 track was expected to be transducing DNA (Sandri and Berger 1980a) and the transducing DNA was assumed to consist, collectively, of the entire E.coli genome, but with markers represented according to their packaging levels. Hence, to quantitate the levels at which markers were packaged into P1, the chromosomatal standards were loaded with 1% to 10% of the amount of DNA in the P1 track so that at later stages in the experiment, when a standard curve was plotted of hybridisation (as measured by
chromosomal band intensities) against the amount of chromosomal DNA in the track, the curve would span the amount of hybridisation in the P1 track (as measured by the corresponding band intensity in the P1 track). In this way, the packaging of different markers could be assessed by comparing the amount of hybridisation in the P1 track with a standard curve obtained by measuring the hybridisation with increasing amounts of E.coli genomic DNA.

Accordingly, gels were run, as described above, and transferred to nitrocellulose by Southern blotting.

Probes were prepared from the recombinant plasmids and lambda phage, listed in table 3.1 and 3.2, hybridised to filters in appropriate conditions and the filters autoradiographed as detailed in chapter 2.

Autorads revealed homologous bands of equivalent molecular weight in chromosomal and P1 tracks (see figures 3.6 and 3.7). As expected, the bands in chromosomal tracks increased in intensity with the increasing amount of DNA loaded. To obtain a linear relationship between band intensity and the amount of sequence in the track, the X-ray film was preflashed before autoradiography, to give an absorbance of 0.15. In addition, exposure of the film by the probe bound to the filter was carried out at -70°C, with two intensifying screens (see chapter 2).

Band intensities were calculated by first measuring the exposure of the film with a scanning densitometer and subsequently integrating the densitometer trace. E.coli genomic DNA band intensities were then plotted against the amount of DNA banded in the track, to give the standard curve. Packaging levels of markers in P1 transducing DNA were then estimated by comparing P1 band intensities with the standard curve and reading from the graph the quantity of sequence needed to give an equivalent level of hybridisation.

With this approach to quantitate hybridisation, it was important that exposure of the X-ray film during autoradiography always remained in the range where intensity was directly proportional to the amount of sequence homologous to the probe; hence, the above conditions for autoradiography. In addition, exposures were never allowed to saturate the film such that increasing exposure time
caused a non linear increase in band intensity.

**MARKER PACKAGING LEVELS**

Initially recombinant \( \lambda \) phage were used as hybridisation probes; the entire phage DNAs were nick translated and probed to test filters. Packaging of \( \text{ilv, arg, trp and his} \) was measured using \( \lambda \text{ilv, arg, trp and his} \) respectively. Packaging levels are shown in table 3.3 and typical quantitative Southern blots shown in figure 3.6. Clearly, a problem associated with the use of \( \lambda \) probes was the number of bands lit up due to homology between the \( \lambda \) cloning arms and cryptic lambdoid prophage present in the *E.coli* genome (Kaiser 1980). In order to simplify the hybridisation pattern and as confirmation of the packaging data, recombinant plasmids were constructed as previously described and all the plasmids listed in table 3.2 were used as hybridisation probes, both singly and in combination with other probes. A total of 12 markers were tested in this way. The results of typical hybridisations are shown in figure 3.8 along with the standard curves obtained from the autorads. Obviously, this study involved a large number of individual hybridisations and for reasons of space the majority are not shown. However, a summary of the packaging levels estimated by this procedure is presented in table 3.3, and the chromosomal locations of the test markers is shown in figure 3.8. The error calculations shown in table 3.3 were obtained simply by taking the value with the maximum deviation from the mean as the limit of the error. The maximum error obtained was for the packaging of a \( \lambda \) band, at about 50%. Hence, the results show remarkable consistency. The source of errors could be from small inaccuracies when loading gels or estimating concentrations of DNA in preparations.

The results of the packaging assays (table 3.3) clearly show that, with the exception of markers from the 2 minute region (\( \text{leu, fts and ace} \)), markers are packaged into transducing particles with similar efficiencies. \( \text{arg} \) packaging was arbitrarily designated at 1.0 and clearly, packaging levels of other markers (excepting these from the two minute region) do not differ significantly from 1.0.
FIGURE 3.6  P1-PACKAGING ASSAYS USING RECOMBINANT \( \lambda \) PROBES.

AUTORADIOGRAMS OF FILTERS:

A. \( \lambda \)hisG PROBE (Borck and Murray, 1977)
1.  5 \( \mu \)g EcoRI digested P1 DNA
2.  0.4 \( \mu \)g EcoRI restricted W3110 DNA
3.  0.3 " " " "
4.  0.2 " " " "
5.  0.1 " " " "

B. \( \lambda \)arg PROBE (Newman 1982)
1.  5 \( \mu \)g EcoRI digested P1 DNA
2.  0.4 \( \mu \)g EcoRI restricted W3110 DNA
3.  0.3 \( \mu \)g " " " "
4.  0.2 \( \mu \)g " " " "
5.  0.1 \( \mu \)g " " " "

Band intensities on the above autoradiograms and marker packaging levels were estimated as described in the text. In both 3.6A and 3.6B the 15 kb, 7.8 kb (7.9 kb) and 4.9 kb (5.0 kb) bands were due to homology between the \( \lambda \) cloning arms and, presumably, cryptic prophage present in the W3110 genome. Other bands were due to homology between W3110 and the chromosomal inserts of the \( \lambda \) probes.

Packaging levels are shown in Table 3.3.
FIGURE 3.1. PACKAGING LEVELS OF \textit{ftsQA}, \textit{galK}, \textit{ace}, \textit{trp}, \textit{terC}, \textit{spyE}, \textit{leuB}, \textit{ilv} and \textit{oriC}

Packaging levels of these markers are shown in Table 3.3.

Figure 3.7 depicts examples of quantitative hybridisations which were performed as described in the text. For reasons of space, the standard curves derived from the autoradiograms are not shown.

FIGURE 3.7A PACKAGING LEVELS OF \textit{ftsQA} AND \textit{galK}

A1. Agarose gel loaded with:
   1, 7 & 8) 3μg of \textit{EcoRl} restricted \textit{P1} DNA's (independently isolated).
   2. 0.075 μg \textit{EcoRl} restricted \textit{P1} DNA
   3. 0.15 μg " " "
   4. 0.30 μg " " "
   5. 0.45 μg " " "
   6. 0.60 μg " " "


A3. Densitometer scan of the 13 kb \textit{galK} bands. (Track numbers correspond to those above).

A4. Densitometer scan of the 2.2 kb \textit{ftsQA} bands. (Track numbers correspond to those above).
3.7 contd.

A

![Graph Image 1](chart_no:brwnor)

![Graph Image 2](chart_no:brwnor)
FIGURE 3.7B  PACKAGING OF ace
Autoradiogram of filter probed with pGS17
1. 3 μg EcoRI restricted P1 DNA
2. 0.75 μg EcoRI restricted W3110 DNA
3. 0.60 μg " " " "
4. 0.45 μg " " " "
5. 0.30 μg " " " "
6. 0.15 μg " " " 

FIGURE 3.7C  PACKAGING OF trp
Autoradiogram of filter probed with pMH602.
5. 0.075 μg EcoRI restricted W3110 DNA
4. 0.15 μg " " " "
3. 0.30 μg " " " "
2. 0.45 μg " " " "
1. 0.6 μg " " " 
6. 3.0 μg EcoRI restricted P1 DNA
7. 3.6 μg " " " 

FIGURE 3.7D  PACKAGING LEVEL OF TerC
Autoradiogram of Filter probed with pTH51
1. 5 μg EcoRI restricted P1 DNA
2. 0.9 μg EcoRI restricted W3110 DNA
3. 0.6 μg " " " "
4. 0.3 μg " " " "
5. 0.15 " " " 

Note: the additional bands in the P1 track, which are not present in the W3110 tracks, of 12 kb, 9.5 kb and 8.0 kb.
FIGURE 3.7E  PACKAGING OF spyE

Autoradiogram of filter probed with $^{32}$P-labelled pHW000 (spyE)

1. 0.075 µg BamHI restricted DNA
2. 0.15 µg
3. 0.3 µg
4. 0.45 µg
5. 0.6 µg
6. 3 µg BamHI restricted P1 DNA

FIGURE 3.7F  PACKAGING OF LeuB

Autoradiogram of filter probed with $^{32}$P-labelled pDWO01

1. 3 µg EcoR1 restricted P1 DNA
2. 0.60 µg EcoR1 restricted P1 DNA
3. 0.45 µg
4. 0.30 µg
5. 0.15 µg
FIGURE 3.7G  PACKAGING OF ilv

Autoradiogram of filter probed with $^{32}$P-labelled pMH601 (ilv)

1, 2, & 8) 5.0 µg EcoR1 restricted P1 DNA
3. 0.075 µg EcoR1 restricted W3110 DNA
4. 0.15 µg " " " "
5. 0.30 µg " " " "
6. 0.45 µg " " " "
7. 0.60 µg " " " 

Note the additional 7.2 kb bands and 4.0 kb bands, present in the P1 tracks but not the W3110 tracks. Bands could be the product of a partial digestion consisting of the 4 smallest bands.

FIGURE 3.7H  PACKAGING OF oriC

Autoradiogram of filter probed with pCM959 (oriC)

1. 5.0 µg EcoR1 restricted P1 DNA
2. 0.45 µg EcoR1 restricted W3110 DNA
3. 0.30 µg " " " "
4. 0.15 µg " " " "
5. 0.075 µg " " " "
<table>
<thead>
<tr>
<th>Marker</th>
<th>Probes</th>
<th>No. of Independent Estimations</th>
<th>No. of P1 Lysates Tested</th>
<th>Mean Packaging Levels</th>
<th>Maximum Error</th>
<th>Chromosomal Location (Bachman 1983)</th>
</tr>
</thead>
<tbody>
<tr>
<td>leuB</td>
<td>pBW001</td>
<td>6</td>
<td>4</td>
<td>2.9 (2.1)</td>
<td>0.4</td>
<td>1.8</td>
</tr>
<tr>
<td>ace</td>
<td>pGS17</td>
<td>3</td>
<td>3</td>
<td>3.5 (2.0)</td>
<td>0.3</td>
<td>2.7</td>
</tr>
<tr>
<td>fts</td>
<td>pDK302</td>
<td>6</td>
<td>4</td>
<td>2.9 (2.0)</td>
<td>0.5</td>
<td>2.3</td>
</tr>
<tr>
<td>ilv</td>
<td>pMH601, λilv</td>
<td>4</td>
<td>4</td>
<td>0.7 (6.0)</td>
<td>0.2</td>
<td>84.6</td>
</tr>
<tr>
<td>arg</td>
<td>pHW001, λarg</td>
<td>4</td>
<td>4</td>
<td>1.0 (1.0)</td>
<td>0.3</td>
<td>89.5</td>
</tr>
<tr>
<td>trp</td>
<td>pMH602, λtrp</td>
<td>4</td>
<td>4</td>
<td>0.5 (0.6)</td>
<td>0.2</td>
<td>27.6</td>
</tr>
<tr>
<td>his</td>
<td>pMH600, λhis</td>
<td>4</td>
<td>4</td>
<td>0.8 (0.3)</td>
<td>0.3</td>
<td>44.0</td>
</tr>
<tr>
<td>oriC</td>
<td>pCM959</td>
<td>5</td>
<td>4</td>
<td>1.0</td>
<td>0.2</td>
<td>84.0</td>
</tr>
<tr>
<td>terC</td>
<td>pTH51</td>
<td>1</td>
<td>1</td>
<td>1.0</td>
<td>-</td>
<td>32.5</td>
</tr>
<tr>
<td>7.8 kb</td>
<td>NM540</td>
<td>8</td>
<td>3</td>
<td>0.6</td>
<td>0.3</td>
<td>10.0</td>
</tr>
<tr>
<td>galK</td>
<td>pDK302, PK01</td>
<td>7</td>
<td>4</td>
<td>0.9</td>
<td>0.1</td>
<td>17.0</td>
</tr>
<tr>
<td>spyE</td>
<td>pBW000</td>
<td>2</td>
<td>1</td>
<td>1.0</td>
<td>-</td>
<td>90.0</td>
</tr>
</tbody>
</table>
FIGURE 3.8. MAP POSITION AND PACKAGING LEVELS OF MARKERS LISTED IN TABLE 3.3

Error bars depict the level of errors shown in Table 3.3.
Figure 3.8

MARKER AND MAP POSITIONS IN MINUTES
FIGURE 3.9  **EcoRI** DIGESTED DNA PREPARED FROM THREE INDEPENDENTLY PREPARED P1 LYSATES

Fragment sizes were obtained from Bachi and Arber (1977).

**EcoRI** restriction of P1 DNA generates 26 **EcoRI** fragments but only the first 17 are shown here. Note that fragments 4, 5 and 6 are of the same molecular weight (6.7 kb) and run as a single band. Likewise fragments 12 and 13 are of a similar size (2.9 kb). (see figure 1.1 for a comprehensive restriction map of P1 DNA)
Figure 3.9
These packaging assays thus provide direct physical evidence that the 30-fold range in transduction frequencies observed by Masters (1977) is not a reflection of marker packaging efficiencies, but that the marker selectivity of the transduction process is a consequence of recombinational discrimination in recipient cells (Newman and Masters 1980, Newman 1982).

Markers from the two minute region of the chromosome (ftsQA, leuB and ace) are packaged to about 3-fold the level of other markers (table 3.3); this clustering of highly packaged markers in the two minute region is illustrated in figure 3.8.

Since leuB is over represented in the P1-lysate, these observations explain why leuB-transduction is stimulated above ilv following uv-irradiation of lysates (Newman 1982), when gene dosage predicts that this should not occur.

Gene dosage effects should also have given rise to a 3-fold spread in packaging levels (Cooper and Helmsteller 1968) with oriC and ilv packaged at about 3-4 times that of trp or terC. This clearly did not occur. Stationary phase W3110 E.coli cells were used as the source for the genomic DNA in the standard tracks on the gel. Thus the origin to terminus ratio in the standard tracks should have been 1:1. If not, and the ratio of oriC to terC was in fact 3:1, rather than 1:1 this could explain why 3-4-fold spread in packaging was not observed by the packaging assays. Accordingly this ratio was checked by comparing the relative hybridisation of galK to ftsQA sequences in the chromosomal digests; both markers are carried on the same plasmid, pDK302. The extent of ftsQA homology between pDK302 and the chromosome is 2.3 kb (Robinson et al. 1984) and between galK and the chromosome is 1.3 kb (McKenney et al. 1981, McKenney 1982). Hence, if the ratio of fts to gal K is 1:1 in the chromosomal standards, the ratio of hybridisation of fts: gal K should be 2.3:1.3 = 1.76. Accordingly, intensities of the galK and ftsQA chromosomal bands were measured, as before (see figure 3.7) and the ratios of hybridisation are shown in table 3.4. Clearly, the ratios do not differ significantly from fts:galK = 1.76. Hence, origin: terminus ratio must be 1:1 otherwise fts, at 2 minutes, would be represented in excess of galK, at 17 minutes (Bachman 1983).
TABLE 3.4

HYBRIDISATION INTENSITIES OF *galK* AND *ftsQA* BANDS.

<table>
<thead>
<tr>
<th>SIZE (kb)</th>
<th>% HOMOLOGY WITH CHROMOSOMAL FRAGMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ftsQA</em></td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td><em>galK</em></td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

CHROMOSOMAL HYBRIDISATION INTENSITIES:

<table>
<thead>
<tr>
<th>INTENSITY</th>
<th>RATIO OF INTENSITIES</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ftsQA</em></td>
<td><em>galK</em></td>
</tr>
<tr>
<td>18.63</td>
<td>11.57</td>
</tr>
<tr>
<td></td>
<td>1.61</td>
</tr>
<tr>
<td>15.78</td>
<td>8.89</td>
</tr>
<tr>
<td></td>
<td>1.75</td>
</tr>
<tr>
<td>11.50</td>
<td>6.70</td>
</tr>
<tr>
<td></td>
<td>1.71</td>
</tr>
</tbody>
</table>

CHROMOSOMAL TRACK:

<table>
<thead>
<tr>
<th>(figure 3.7a)</th>
<th>INTENSITY</th>
<th>RATIO OF INTENSITIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>18.63</td>
<td>11.57</td>
</tr>
<tr>
<td>4</td>
<td>15.78</td>
<td>8.89</td>
</tr>
<tr>
<td>3</td>
<td>11.50</td>
<td>6.70</td>
</tr>
</tbody>
</table>
It seems likely, therefore that cells undergoing P1 lysis also have an origin to terminus ratio of about 1:1 otherwise packaging or oriC would be expected to exceed terC by 3:1. This in turn suggests that the 3-4 fold spread in transduction frequencies observed when P1 lysates are uv-irradiated result either from gene dosage variation in the recipient cell and not from the donor, or that origin DNA is always more recombinogenic than DNA nearer terC. The above observations are in contrast to those of Masters and Broda (1971) who concluded that gene dosage in donor cells could indeed influence transduction frequencies; markers near the origin had higher transduction frequencies when the P1 transducing lysates were grown on rapidly growing cells (which have an origin to terminus ratio of 3-4:1) than when produced from slow growing cells (which have origin: terminus ratio of < 2:1). How these observations tie in with those discussed above is not clear.

One interesting and unexpected observation, arising from the packaging assays, was the presence of bands in P1 tracks which were homologous to the probe (figure 3.7(d)) which were absent from chromosomal digests. For example, when pCM959 is used as a probe, to measure the packaging level of oriC, 5 bands are lit up in the P1 track whereas only one is lit up in the chromosomal digest. pCM959 is 4.012 kb and should share homology with only one 10 kb EcoR1 fragment from the E. coli origin region (Von Meyerburg and Hansen 1984); this is the band lit up in the chromosomal digest by the pCM959 probe (figure 3.7). In the P1 digest, however, in addition to the 10 kb homologous band, 4 much weaker bands were also detected. The two larger bands (about 20 kb and 18 kb) could have arisen by partial digestion of P1 transducing DNA but the two smaller bands (6.5 kb and 3.5 kb) obviously could not. Interestingly, the location of oriC is about 3.5 kb from the right end and 6.5 kb from the left end of the EcoR1 fragment homologous to pCM959, so cleavage at oriC, during P1 lysis could generate two novel bands of 3.5 kb and 6.5 kb; whether this observation is of significance is a matter of conjecture. Clearly, it may be coincidence that two of the extra bands present in the P1 digest are 3.5 kb and 6.5 kb, so the observation may not indicate that cleavage occurs at oriC during P1

\[
\text{\textit{oriC}}
\]

\[
\text{\textit{terC}}
\]
lysins.

Extra bands in P1 tracks are also detected using other probes, such as PAM001, PAM001 or pOK302 (figure 3.7), but not with all probes, e.g. pAM1. For example, it shares homology with only one band.

In some instances, such extra bands may be due to partial digestion of the P1 transducing DNA, but, in other cases, as described above for the pOK235 probe, the extra bands cannot be due to incomplete digestion as they are smaller than the expected size. This will be discussed more fully in section 3.6.

**ESTIMATE OF THE PROPORTION OF TRANSUDCING DNA IN P1 LYSATES**

The quantity of arg sequence present in P1 transducing DNA was taken to be representative of other markers, i.e. it was assumed that the majority of markers are packaged to the same levels as arg. Hybrdisation to arg sequences in P1 transducing DNA was compared to the hybridisation to arg sequences in chromosomal DNA as described above; it was estimated that the amount of arg sequence present in 1 µg P1 DNA is equivalent to the amount of arg sequence present in 0.06 µg chromosomal DNA. In other words, 1 µg of P1 DNA (P1 sequences + transducing DNA) must contain 0.06 µg transducing DNA. Transducing DNA must therefore constitute about 6% of the DNA in P1 lysates. However, since the error involved in the measurement of arg hybridisation was about 30% (see table 3.3), the quantity of transducing DNA in a P1 lysate (if all chromosomal DNA is represented to the same extent as arg) is between 4% to 8% (6% ± 2%) of the total DNA. These estimates are higher than those previously reported for the proportion of transducing DNA in P1 Kc lysates; Sandri and Berger (1980a) estimated the proportion of transducing DNA in P1 Kc lysates; Sandri and Berger (1980a) estimated that 2% of the DNA from a P1 lysate was transducing. However, the method employed in this thesis to estimate the amount of transducing DNA in the lysate may be giving a misleading result; since the packaging levels of only 13 markers was assessed (table 3.3), this sample may be too small to indicate if arg packaging really is representative of the majority of markers. Moreover, the method used here was primarily designed to measure the
packaging levels of individual markers and not of transducing DNA as a whole. The method of Sandri and Berger (1980a), however, was designed to estimate the total amount of transducing DNA in a P1 lysate; transducing and infective P1 DNAs were differentially labelled, the former with $^{32}$P prior to infection and the second with $^3$H after P1 infection. By comparing the amount of $^{32}$P-label to the amount of $^3$H label in the P1 lysate an estimate was made of the amount of transducing DNA. Possibly Sandri and Berger’s method would give a more reliable estimate. It is possible, however, that the propagation of P1 phage in our laboratory has resulted in isolating a high-transducing derivative of P1. Since our work is primarily the study of transduction, lysates which have given poor transduction tended to be discarded, whereas those giving good transduction have been propagated. Perhaps this process has resulted in enriching for a high transducing P1 phage. This could account for the apparent high proportion of transducing particles in our lysates.

**POSSIBLE CAUSES OF VARIATIONS IN P1-PACKAGING EFFICIENCY OF TRANSDUCING DNA**

The mechanism by which P1 generates transducing particles remains obscure. It is also unclear why P1 should selectively overpackage DNA from the two minute region of the *E.coli* chromosome. There have been several possible mechanisms suggested, by which P1 packaging machinery could be directed to encapsidate *E.coli* DNA. All could conceivably give rise to preferred regions of packaging; these putative mechanisms have been discussed in chapter 1, but briefly, they are the following:

1) The presence of P1 pac-like sites in the *E.coli* genome, as for P22 pac-like sites in *Salmonella*.

2) Transient associations, during lysis, between P1 and the *E.coli* chromosome, which effectively result in the integration of a P1 pac site.

3) The generation of double stranded cuts in *E.coli* genomic DNA which can then serve as initiation points for chromosomal encapsidation.
The first two mechanisms could cause selective packaging if there was a sparse or, alternatively, non random distribution of pac-like sites (as for P22 transduction of Salmonella) or "association sites" such that packaging would initiate from a limited number of chromosomal locations, or perhaps more frequently from specific areas encoding a high proportion of such sites. The third mechanism mentioned above could also cause packaging specificity if more chromosomal breaks occurred in some regions that in others; packaging might occur more frequently in areas with more breaks.

Alternatively, it might be argued that a high proportion of breaks would interfere with packaging and so reduce encapsidation.

Accordingly, experiments were performed to investigate the first and third hypothesis and these are discussed below.

SECTION 3.4. DOES THE E.COLI GENOME ENCODE P1 pac-LIKE SITES

Salmonella DNA is packaged into P22 generalised transducing particles by the P22 packaging machinery which can initiate packaging from P22 pac-like sites in the Salmonella chromosome (Schmager 1984, see chapter 1.). Regions of the chromosome close to pac-like sites are packaged with 1000 fold the frequency of more distal regions. If P1 transducing particles are generated by a similar mechanism, i.e. by recognition of P1 pac-like sites, then such sites must either be more widely distributed than P22 pac-like sites in Salmonella, or exert their influence over a larger region of the chromosome as chromosomal packaging by P1 is fairly uniform; so far, P1 packaging of markers has been shown to vary over only a narrow range (section 3.3). As discussed in chapter 1, approximately 10 evenly spaced P1 pac-like sites would be required in E.coli DNA to assure uniform chromosomal packaging.

P1 pac is approximately 160 bp in size and consists of two 35 bp direct repeats separated by a 90 bp spacer region (Sternberg 1984). The pac site is thus of sufficient size to be used as a specific hybridisation probe to probe for homologous pac-like sites in the E.coli chromosome. Accordingly, in an attempt to detect P1 pac-like sites in the E.coli genome, pac was cloned from P1, into a multicopynumber vector and then used to probe digests of E.coli DNA.
immobilised on nitrocellulose.

**CONSTRUCTION OF pPAC**

P1 DNA encodes 26 EcoRI sites and hence contains 26 EcoRI fragments (Bachi and Arber 1977) (see figure 3.4). P1 pac is located on the small 650 bp EcoRI fragment 20 (R120) (see figure 1.1). As there is no convenient selection for a recombinant plasmid containing pac, R120 was purified away from other P1 EcoRI fragments to simplify the cloning procedure; EcoRI-restricted P1 DNA was run on a preparative 2% agarose gel and fragment R120 was purified from the gel by the procedure described in chapter 2 (figure 3.10). The purified fragment was ligated with EcoRI-restricted pBR325 vector DNA, as previously described and the ligation mix used to transform competent MM18 cells. Since insertion of a DNA fragment into the EcoRI cloning site of pBR325 inactivates the Cm gene. MM18 clones harbouring recombinant plasmids were identified by their ApR TcR Cms phenotype. 10 recombinant clones were picked and their plasmid DNA was prepared by alkaline lysis. The plasmid DNA was restricted with EcoRI and visualised by 2% agarose gel electrophoresis: 6 of the recombinants carried a 650 bp insert, another a 530 bp insert and the remaining three carried no detectable insert, although the insert may have been too small to see on the gel.

A problem associated with cloning fragments of P1 is that the phage DNA cannot easily be separated from transducing DNA. Hence in any preparation of P1 DNA, some 6% of the DNA is chromosomal DNA derived from the donor cell. Preparations of purified P1 restriction fragments, as described above, will thus be contaminated with chromosomal restriction fragments of a similar size and, which comigrate with the P1 fragment on the preparative gel. Thus to ensure that the above recombinant plasmids carried inserts derived from P1 and not the donor chromosome, the 7 plasmids with detectable inserts were restricted with EcoRI and run on a 2% agarose gel, alongside EcoRI-restricted P1 DNA (figure 3.11a). The gel was transferred to nitrocellulose by Southern blotting. One of the
FIGURE 3.10

A. PREPARATIVE 2% AGAROSE GEL LOADED WITH EcoRl RESTRICTED P1 DNA.

The position of the 0.65 kb fragment 20 is marked by the arrow head. Since this gel was lightly stained before photography, this small band is not clearly visible here. Each track (1-4) contains 2.5 μg P1 DNA.

B. 2% AGAROSE GEL SHOWING EcoRl RESTRICTED pPAC DNA

1. EcoRl restriction of plasmid with 530 bp insert (see text)
2. EcoRl digested pPAC DNA
3. EcoRl digested P1 DNA

C. DIAGRAM OF pPAC

H = HindIII site
E = EcoRl site
cap, amp and tet indicate the positions of the chloramphenicol resistance, ampicillin resistance and tetracycline resistance genes respectively. ori shows the position of the origin of replication.

P1 EcoRl fragment 20 is represented by the block between the two EcoRl sites and the arrows indicates the direction of packaging (see Chapter 4).
Figure 3.10c

pPAC
6.6 kb
recombinant plasmids with a 650 bp insert (the same size as R120) was selected, and the DNA purified on a NACS prepac column. 200 ng of the DNA was $^{32}$P-labelled by nick-translation and used to probe to the above filter. The autorad (figure 3.11b) shows that all the 650 bp inserts are homologous and all share homology with R120, the P1 band encoding pac. The 530 bp insert (figure 3.11b, track 5) shared no homology with the probe and thus may have been derived from the donor chromosome. This clone, although containing an insert different in size from R120, was included on the blot because of the possibility that the insert derived from a crossover between the two 35 bp direct repeats in pac; such an event would delete about 120 bp of R120 to give a fragment of about 530 bp (Figure 3.10b, 3.12). Since this had obviously not occurred, no further attempts were made to characterise this plasmid.

The plasmid used for the above probe was taken as the pac-containing plasmid and named pPAC. A diagram of pPAC is shown in figure 3.10c.

PROBING FOR P1pac HOMOLOGY IN THE E.COLI CHROMOSOME

5 µg EcoR1 restricted chromosomal DNA was run on an agarose gel alongside 20 ng EcoR1 restricted P1 DNA and about 10 ng EcoR1-restricted pPAC. Also included on the gel were 0.2 µg EcoR1-digested P1 and pPAC DNA, to serve as size standards. The small amount of restricted P1 DNA was included on the gel as a hybridisation control to ensure that the method is sensitive enough to detect a single copy of pac in the E.coli genome:

The P1 genome is 0.02 the size of the E.coli genome. Thus, for the P1 and chromosomal tracks to contain equivalent amounts of a shared, homologous, DNA sequence, the amount of P1 DNA loaded = 5 µg (the amount of chromosome) x 0.02. However, while pPAC shares 650 bp homology with P1 (fragment R120), but pac consists of only 160 bp, i.e. about 0.24 of R120. To obtain equivalent hybridisation between P1 and the chromosome, which is expected to carry only the 160 bp pac sites, the amount of P1 DNA needed to obtain an equivalent amount of pac sequence, would be:
5 μg (the amount of chromosomal DNA) \times 0.02 \times 0.25 = 25 ng P1 DNA.

The above gel was run overnight and transferred to nitrocellulose by Southern blotting.

200 ng of NACS prepac-purified pPAC DNA was $^{32}$P-labelled, by nick translation, to high specific activity; 4 \times 10^7 cpm were incorporated into the 200 ng. 2 \times 10^7 cpm were used as a probe and hybridised to the test filter overnight. The hybridisation conditions employed here differed from those described in chapter 2 in that a salt concentration of 6 x SSC was used to lower the stringency of the reaction and so allow pairing between pPAC and sequences which did not share 100% homology. The filter was then autoradiographed. Homology between the pPAC probe and P1 fragment R120, in the P1 and pPAC tracks was clearly visible after only 2 hours exposure. No homology, however, was seen with chromosomal DNA at the time. A longer exposure of 38 hours and 7 days were carried out to detect any weak hybridisation to the chromosomal DNA. After 38 hrs, weak bands were visible in the chromosomal track and such bands became clearly visible after 7 days exposure (figure 3.13). However, the observed homology between pPAC and the chromosomal DNA is not necessarily due to shared weakly homologous pac-sites. The above crude approach made use of intact pPAC as the probe, so the homology could stem from shared sequences located on any part of the plasmid probe. If the probe was contaminated with small amounts of chromosomal DNA which became $^{32}$P labelled, this could also account for the weak hybridisation observed. It was thus necessary to distinguish between the above possibilities and so establish whether homology between pPAC and the E.coli genome was due to shared P1 sequences. Accordingly the above experimental procedure was refined such that a more specific probe could be used.

As above, 5 μg EcoRI-restricted DNA was run on an agarose gel, alongside 25 ng EcoRI-restricted P1 DNA as a hybridisation control. 0.2 ng EcoRI-restricted P1 DNA was also run, to give size standards, but this track was cut from the gel before transfer to nitrocellulose. The probe was made using a different procedure than described above.
10 µg pPAC DNA was restricted to completion with EcoR1 and the entire sample loaded onto a preparative low melting temperature agarose gel. The 650 bp fragment, about 1 µg in total, was cut from the gel and 100 ng was $^{32}$P labelled by the modified "random primer" method (Feinberg and Vogelsten 1983, 1984). This procedure allows purified restriction fragments to be labelled to very high specific activity. Obviously, by preparing purified R120 as the probe, only shared P1 sequences can then cause hybridisation between probe and E.coli genomic DNA. Also, the method should improve the sensitivity of the experiment as the label is no longer dispersed amongst 5 kb of heterologous DNA, but is concentrated in 650 bp. So, R120 was $^{32}$P labelled by random primer method and a total of $4 \times 10^7$ cpm incorporated. $2 \times 10^7$ cpm of probe was hybridised to the test filter in 6 x SSC salt. In addition, the hybridisation was performed, in the presence of 50 µg/ml heparin in place of 1 x Denhardts solution (Singh and Jones 1984, see chapter 2). The filter was washed, autoradiographed and the result shown in figure 3.14. The probe hybridised strongly to P1 fragment 20, as expected, indicating that the technique was sufficiently sensitive to detect a single copy of pac in the chromosomal track. However, no R120 homology was detectable with the E.coli chromosome even after 7 days exposure. Accordingly it seems that the homology between intact pPAC and E.coli chromosome was due to pBR325 vector sequences, or the presence of contaminating chromosomal DNA in the probe.

An experiment similar to the above was performed which differed in that a BamH1 digest of E.coli DNA was probed with $^{32}$P labelled R120. This experiment was carried out to eliminate the possibility that restricting the chromosome with EcoR1 fortuitously resulted in all putative pac sites residing on very small restriction fragments; very small DNA fragments are not efficiently transferred to nitrocellulose and so would not have been detected by the above procedure. The result of the BamH1 blot, (gels not shown) was identical to the EcoR1 blot, i.e. no detectable homology between P1 fragment R120 and E.coli chromosomal DNA.

The above observations suggest that either pac-like sites do not exist in E.coli DNA or the above procedure was not adequate to detect
FIGURE 3.11  

**EcoR1 RESTRICTED PLASMIDS ISOLATED FROM THE LIGATION OF P1 FRAGMENT 20 TO EcoR1 RESTRICTED pBR325**

A.  **2% AGAROSE GEL**

1.  EcoR1 restricted P1 DNA
2-8  Independent isolates of putative pPAC plasmids.  
   Note the small 5.30 bp insert in track 5.

B.  **AUTORADIOGRAM OF THE ABOVE GEL, TRANSFERRED TO NITROCELLULOSE AND PROBED WITH $^{32}$P-LABELLED DNA FROM CLONE 2**

All inserts, except in track 5, share homology with the probe, which also shares homology with P1 R120

The faint 6 kb band in track 1 is probably caused by spill over from track 2 (see text).
FIGURE 3.12  SCHEMATIC DIAGRAM OF THE GENERATION OF A 125 bp DELETION AT THE P1 pac SITE (a)

Homologous recombination between the two 35 bp repeats (b) would generate a 125 bp deletion plus a 125 bp circle (c).
Figure 3.12

P1 pac

a

35 bp

90 bp

35 bp

b

125 bp Circle

c

125 bp Circle
FIGURE 3.13 PROBING FOR P1 pac SITES IN THE E.coli CHROMOSOME

A. 1. 0.2 μg EcoRI restricted pPAC
   2. 0.2 μg EcoRI restricted P1 DNA (size standards)
   3. 50 ng EcoRI restricted pPAC
   4. 5 μg EcoRI restricted W3110 DNA
   5. 25 ng EcoRI restricted P1 DNA (hybridisation control)

B. Tracks 1 and 2 were cut from the above gel. Tracks 3, 4 and 5 were transferred to nitrocellulose and probed with $^{32}$P-labelled pPAC. 3.13 shows a 7-day exposure of the film. Note the intense 0.65kb R120 bands in tracks 3 and 5, the very intense 6.0 kb vector band in track 3 and the weakly intense bands in track 4; a number of pPAC homologous bands are visible in 4; the most notable of which is about 2.0 kb in size (see text for discussion).
FIGURE 3.14 PROBING FOR P1 pac HOMOLOGY IN THE E.coli CHROMOSOME

A. 1. 25 ng EcoRI restricted P1 DNA (hybridisation control).
   2. 5 µg EcoRI restricted W3110 DNA
   3. 0.5 µg EcoRI restricted P1 DNA (size standards).

B. Track 3 was cut from the above gel. Tracks 1 and 2 were transferred to nitrocellulose and probed using 32P-labelled P1 EcoRI fragment 2G. (see text)

No bands were visible in the chromosomal track 2.
A 3-day exposure is shown here although exposure up to 2 weeks were carried out.
such sites. It is possible that the putative pac-like sites share very limited homology with P1 pac, perhaps only in certain essential regions of the sequence. Therefore, R120 may not share sufficient homology with chromosomal pac-like sequences to allow their detection by Southern hybridisation. As discussed in chapter 1, there is still considerable doubt as to the sequence requirement for a functional P1 pac. The sequence data and pac-cutting assays carried out by Sternberg (1984) imply that the 35 bp repeats are required. However, whether the sequence of the 90 bp spacer region must be conserved is less clear. Certainly, pacase cutting at pac occurs in at least 6 specially distinct sites (Sternberg 1984), arguing perhaps, that the 90 bp spacer need not be accurately conserved, as no defined cleavage point exists. If the putative pac-like sites have significant sequence for pac on R120, then mismatches or areas of non homology may reduce hybridisation (Beltz et al. 1983). However, assuming that the 35 bp repeats are conserved and bearing in mind the expectation that at least 10 pac-like sites would need to reside in the E.coli genome (i.e. 20 copies of the 35 bp repeat), the R120 probe should have hybridised to E.coli chromosomal DNA with sufficient strength to allow detection of such pac-like sites; certainly smaller probes can be used to detect homologous sequences in similar experiments, but again, mismatches etc. reduce hybridisation considerably (Gillam et al. 1975, Wallace et al. 1981).

The results discussed above are thus inconclusive, as failure to detect pac-like sites in E.coli genomic DNA may stem from two possibilities.

1) There are no pac-like sites in E.coli DNA.
2) P1 pac-like sites do not share sufficient homology with the R120 probe to be detected by Southern hybridisation.

The little evidence available supports the former suggestion. The study of P1 packaging levels of markers from different chromosomal locations (this chapter) reveals at most, a 7 fold range in packaging. This differs from the range in packaging levels observed with P22, which recognises P22-pac-like sites in Salmonella DNA, giving a 1000 fold range in packaging. As mentioned before, for P1 to package the E.coli genome evenly, at least 10 pac-like sites
would be required (Bachi and Arber 1977, Sternberg and Hoess 1983, see chapter 1). Bearing in mind the probable complexity of the P1 pac (150-160 bp) (Sternberg 1984) and even if only the 35 bp repeats are required, the probability of such sites occurring by chance is very small; as mentioned in chapter 1, the probability of any specific 150 bp sequence or 35 bp sequence occurring by chance can be calculated as $4^{150}$ and $4^{35}$ respectively.

$$4^{150} = 2 \times 10^{90} \text{ i.e. once in every } 2 \times 10^{90} \text{ bp.}$$

$$4^{35} = 1.2 \times 10^{21} \text{ i.e. once in every } 1.2 \times 10^{21} \text{ bp.}$$

as the E.coli genome is only $4 \times 10^6$ bp, neither are likely to occur, even once, by chance.

The possibility exists, however, that the requirement for P1 pac cutting is not a specific sequence; perhaps structural or topological features in the DNA are recognised, by pacase, to constitute a pac-site. In this case, the use of P1 R120 as a probe for pac-like sites in the E.coli chromosome would be unsuitable as no sequence homology may exist between P1 and E.coli pac sites.

SECTION 3.5

P1 RESTRICTION/MODIFICATION:
POSSIBLE INFLUENCES ON TRANSDUCTION

Wall and Harriman (1974) hypothesised that packaging of the E.coli chromosome into P1 phage heads commenced from double stranded breaks in E.coli DNA, which arose during normal P1 lysis. More recent evidence supports the idea that P1 and P22 both recognise DNA with double stranded breaks, as the packaging substrate (Sternberg and Hoess 1983, Schmeiger 1984). Based on his experiments with P1 packaging system, Sternberg (1983) speculated that pacase-dependent cleavage of pac serves to generate a double strand cut, providing an end at which subsequent and presumably, independent replication or packaging events occurred. Likewise, Schmeiger (1984) reported that P22, which has a very similar packaging mechanism to P1, can encapsidate mature, linear, headful sized DNA molecular in vitro without any requirement for pac cutting. It seems plausible that, as suggested by Wall and Harriman (1984), P1 packaging machinery may seize on double strand ends in chromosomal DNA and commence
sequential encapsidation of the chromosome into generalised transducing particles.

The P1 genome has not been fully characterised as yet, but a comprehensive restriction map of known P1 genes has been compiled (Yarmolinsky 1984). P1 restriction endonuclease, the product of the res gene is, as yet, the only known nuclease encoded by P1 which could produce the double strand cuts in DNA required for encapsidation by the above model.

The regulation of res and mod activity is complex (see chapter 1) and it seems that during the establishment of lysogeny, mod activity reaches a maximum rapidly whereas res activity does not achieve maximum levels until 3 hours post infection (Levy et al. 1984). Whether this pattern of regulation occurs during lysis is not known but as P1 DNA is modified before lysis, it seems likely that res activity must be low and mod activity must predominate. This would be in accordance with a model proposing that restriction is involved in generating ends in DNA which are packaging substrates; P1 restriction enzyme cleaves DNA close to a 5 bp assymmetric recognition sequence (5' AGACC 3') (Bachi et al. 1979). Any specific 5 bp sequence will occur, at random, at a frequency of $4^5 \text{ bp} = 1$ in 1024 bp. If restriction proceeded at maximum levels immediately after P1 infection and before host DNA was modified to any great extent, both the P1 and host DNA would be fragmented into small molecules. In other words no large chromosomal DNA molecules would survive and so no transducing particles would be formed which contain 90 kb continuous stretches of DNA. On the other hand, if limited restriction occurs during lysis, as would be expected (Levy et al. 1984), the "few" breaks in the DNA would allow encapsidation of large uninterrupted stretches of chromosomal DNA by the sequential headful mechanism of P1.

Two hypotheses can be proposed for the influence of res on transduction:
1) EcoP1 restriction is involved in the generation of transducing particles in a non specific manner; the random cutting of host DNA allowing encapsidation to commence randomly.
2) EcoP1 restriction is responsible for the differences in packaging
efficiencies observed in section 3.4; a non random distribution of EcoRI recognition sites may alter the likelihood of certain regions being packaged.

Both these hypotheses have been tested in this section. It should be noted that the above hypotheses do not assume that res is solely responsible for generating transducing particles, but may be simply a contributing factor.

Since it is very difficult to assay P1 res activity during P1 lysis, the role of res in formation of transducing particles was tested by premodifying host DNA before P1 infection. Hence, as resident DNA would be fully modified, (Iida et al. 1983) the involvement of res could be assessed by preventing its activity.

CLONING P1 MOD GENE

P1 res and mod genes are contiguous and have been localised to within P1 BamHI fragment 4 (Heilman et al. 1980) (see figure 3.16). res and mod can be expressed without the requirement for other phage functions, and independently of each other, so can be cloned onto vectors independently of other P1 sequences (Iida et al. 1983). The res and mod genes have been restriction mapped (Iida et al. 1983) and the map shows that res, but not mod contains a single HindIII site and a single EcoRI site. Hence the mod gene can be cloned intact, on either a 12 kb EcoRI-HindIII fragment or a 6.6 kb BamHI-HindIII fragment, but the clone would not contain a functional res gene. Hence cells carrying such clones would have their DNA fully modified, with no possibility of EcoP1 restriction occurring, if infected with P1.

Initially, attempts were made to clone mod on the 12 kb EcoRI-HindIII fragment described above, by double digesting P1 DNA with EcoRI and HindIII and ligating the restricted DNA with EcoRI, HindIII double digested pBR325 vector DNA. Competent W3110 cells were transformed with the ligation mix. 6 possible EcoRI-HindIII fragments of P1 were generated by the above restriction; plasmid DNA was extracted from 100 clones containing recombinant AprCm8Tc5 plasmids by the instant crude lysis technique (chapter 2) and
FIGURE 3.15 CONFIRMATION OF THE PRESENCE OF THE P1-mod GENE ON pMOD

A. AGAROSE GEL LOADED WITH RESTRICTED P1 DNA
1. _EcoR1_ restricted P1 DNA
2. _EcoR1_ + _HindIII_ restricted P1 DNA
3. _BamHI_ restricted P1 DNA
4. _BamHI_ + _HindIII_ restricted P1 DNA

B. AUTORADIOGRAM OF THE ABOVE GEL, TRANSFERRED TO NITROCELLULOSE AND PROBED WITH $^{32}$P-LABELLED pMOD

P1 mod is located on _BamHI_ fragment 4 (8.6 kb) and _EcoR1_ fragment 2 (10.52 kb) (see figure 1.0 and figure 3.16). The putative pMOD lights up bands of these sizes in tracks 3 and 1 respectively.

Double digestion of P1 DNA with _EcoR1_ and _HindIII_ results in the mod carrying fragment being cleaved to about 9 kb in size (figure 1.1). A band of this size is visible in track 2. The _BamHI-HindIII_ fragment carrying mod (track 4) should be 6.6 kb in size: such a band is present in track 4.

These observations confirm that pMOD carries the 6.6 kb Mod$^+$ ResΔ 6.6 kb BamHI-HindIII fragment.

C. RESTRICTION OF pMOD
1. _HindIII_ + _BamHI_ restricted P1 DNA (size standards)
2. _HindIII_ + _BamHI_ restricted pMOD DNA. (Note the 6.6 kb Mod$^+$ band and the 5.6 kb deletion derivative of pBR325 (see text).
3. pMOD restricted with _EcoR1_ (cleaved only once, at the pBR325 _EcoR1_ site).
Figure 3.15

(A) Image with bands at 10.5, 8.6, and 6.6.

(B) Image with bands at 10.5, 8.6, and 6.6.

(C) Image with bands at 12.2, 7.6, 6.6, 5.6, 5.1, and 1.8.
FIGURE 3.16  DIAGRAM OF pMOD AND THE EcoP1 SYSTEM CARRIED ON P1 BamHI FRAGMENT 4 (see also Figure 1.1)

pBR325 \( \Delta \) represents the deletion derivative of pBR325 generated by removal of the 400 bp HindIII-BamHI fragment. B, H and E indicate the positions of BamHI, HindIII and EcoRI sites respectively.
Figure 3.16

pMOD

pBR325Δ

mod

res

P1 Bam-4

5.6 kb

6.6 kb
plasmids were identified which contained, amongst them, 5 of the 6 possible P1 EcoR1-HindIII fragments. However the fragment containing mod was not detected. Inspection of the P1 genetic map (Yarmolinsky 1984) revealed that this P1 EcoR1-HindIII fragment contained a large uncharacterised region. Possibly this encoded genes which were lethal to the E.coli host cell in high copy number. (This would be consistent with our previous observations that P1 EcoR1 fragment 2 (which contains mod) did not seem to be clonable in pBR325 (Myself and Howard Pringle, unpublished observations).

Accordingly, an alternative cloning strategy was adopted. mod can be cloned, intact, on a 6.6 kb BamH1-HindIII fragment (Mod\textsuperscript{+} Res\textsubscript{A} fragment) (both sites lying within EcoR1 fragment 2) which lacks a large part of the uncharacterised P1 EcoR1-2 sequences. Accordingly, P1 DNA was double-digested with BamH1 and HindIII (generating 4 possible BamH1-HindIII P1 fragments) and ligated to BamH1-HindIII restricted pBR325 DNA. Competent W3110 cells were transformed with the ligation mix, and clones receiving recombinant plasmids (Ap\textsuperscript{r}Cm\textsuperscript{r}Tc\textsuperscript{s}) were detected by their Ap\textsuperscript{r}Cm\textsuperscript{r}Tc\textsuperscript{s} phenotype. DNA from 50 such clones was extracted by the instant crude lysis technique and visualised by agarose gel electrophoresis. Plasmids of the correct size to be carrying each of the possible P1 HindIII-BamH1 fragments were detected and those thought to contain the 6.6 kb Mod\textsuperscript{+}, Res\textsubscript{A} fragment were picked. The plasmid DNA was extracted from these clones by alkaline lysis, then restricted with BamH1 and HindIII and visualised by agarose gel electrophoresis; the gel confirmed that the recombinant plasmids contained a 6.6 kb BamH1-HindIII fragment which comigrated with the BamH1-HindIII Mod\textsuperscript{+}Res\textsubscript{A} fragment (figure 3.15a). As additional confirmation that the cloned fragment was indeed the Mod\textsuperscript{+}Res\textsubscript{A} fragment derived from P1 and not a spurious insert derived from transducing DNA, one putative Mod\textsuperscript{+}Res\textsubscript{A} plasmid was chosen, \textsuperscript{32}P - labelled by nick translation and used as a probe to a nitrocellulose filter onto which restricted P1 DNA had been transferred by Southern blotting (Southern 1975). Autoradiographing the filter (figure 3.15b) showed the pattern of hybridisation that had been expected, from a probe carrying the P1-derived 6.6 kb BamH1-HindIII Mod\textsuperscript{+}Res\textsubscript{A} fragment.
The Mod⁺ResΔ plasmid (pMOD) is shown in figure 3.15.

CHECKING pMOD MODIFICATION ACTIVITY

Unmodified λvir plates on a P1 lysogen with an e.o.p. of $10^{-3}$ - $10^{-4}$ (Heilman et al. 1980). To establish that the mod gene carried on pMOD is functional, λvir lysates were prepared on W3110 (pMOD) and W3110. The e.o.p. of the two lysates was measured on isogenic P1 lysogenised and non lysogenic indicator strains; EcoP1 modified λvir should plate with equal efficiency on both indicator strains, whereas unmodified λvir should show an e.o.p. reduced by $10^{3}$ to $10^{4}$ on the P1 lysogen.

PRODUCTION OF EcoP1-MODIFIED AND UNMODIFIED λvir

λvir was plaque purified on W3110 and W3110 (pMOD). Single plaques growing on each strain were picked and propagated on the strain from which they had originated. Lysates were harvested and titred, again using the same parental strain on which they were made.

CONSTRUCTION OF ISOGENIC INDICATOR STRAINS

To assess whether W3110 (pMOD)-grown λvir is EcoP1 modified, two λ sensitive indicator strains were required, one a P1 lysogen and the other a non lysogen. MM303 is a P1 lysogen and MM303-1, a P1-cured derivative of MM303. Unfortunately, both are Mal⁻, so unable to synthesise the α receptor and are thus α resistant. To render the strains α-sensitive, a P1Kc lysate, prepared on W3110, was used to transduce MM303 and MM303-1 to Mal⁺. Maltose⁺ transductants were selected on minimal plates which contained 0.2% maltose in place of glucose. Mal⁺ transductants were checked to ensure they remained identical to parental strains in all other respects.
EcoPi MODIFICATION ASSAY

\( \lambda \text{vir} \) (W3110-grown) and \( \lambda \text{vir} \) (MOD) (W3110 (pMOD)-grown) were titred on both MM303 Mal\(^+\) and MM303-1 Mal\(^+\). The results, shown in table 3.5, show clearly that the e.o.p. for \( \lambda \text{vir} \) (mod) was equal for both P1 lysogenic and non lysogenic indicator strains, i.e. \( \lambda \text{vir} \) (mod) was resistant to EcoPi restriction and thus must have been modified. \( \lambda \text{vir} \) (unmodified) however, showed an e.o.p. of about \( 10^{-3} \) on MM303 Mal\(^+\) when compared to the non-lysogenic MM303-1 Mal\(^+\) strain. Clearly, the resident P1 prophage is reducing the e.o.p., confirming that EcoPi restriction was active.

From the above, it was concluded that P1 mod gene cloned on pMOD was indeed fully expressed.

AFFECT OF PREMODIFYING DONOR CHROMOSOMAL DNA ON TRANSDUCTION FREQUENCIES

P1 transducing lysates were produced from W3110 pMOD and W3110, then titred on W3110. The lysates were used to transduce MM303 and AB1157 and transduction of ilv, arg, his, trp and leu were measured. The results, shown in table 3.6 indicate that the formation of transducing particles in donor cells does not depend on the activity of the P1 restriction system, because transduction frequencies for lysates produced on W3110 (pMOD), where the DNA is premodified and thus resistant to P1 restriction, is not different from the unmodified control lysate, or previously reported transduction frequencies (Masters 1977). However since no direct assay for res activity was carried out on cells infected with P1, the formal possibility still exists that restriction may still have occurred in P1-infected W3110 (pMOD) cells. However, this was considered unlikely as the cloned mod gene was clearly actively expressed in such strains, and presumably the resident DNA was fully modified.
### TABLE 3.5

MODIFICATION ASSAY:

<table>
<thead>
<tr>
<th>INDICATOR STRAIN</th>
<th>( \lambda \text{vir} )</th>
<th>( \lambda \text{vir (mod)} )</th>
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</thead>
<tbody>
<tr>
<td>MM303-1 Mal(^+)</td>
<td>(2.6 \times 10^{10})</td>
<td>(8.6 \times 10^{10})</td>
</tr>
<tr>
<td>MM303 (P1) Mal(^+)</td>
<td>(2.3 \times 10^{7})</td>
<td>(8.5 \times 10^{10})</td>
</tr>
</tbody>
</table>

\( \lambda \text{vir} \) - grown on W3110
\( \lambda \text{vir (Mod)} \) grown on W3110 (pMOD).
<table>
<thead>
<tr>
<th></th>
<th>1ly 192</th>
<th>argH</th>
<th>trpA</th>
<th>hisG1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MM303</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x5 dilution)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P1 (pMOD)</strong></td>
<td>TOTAL NO.</td>
<td>1131</td>
<td>1003</td>
<td>691</td>
</tr>
<tr>
<td></td>
<td>RATIO</td>
<td>5.6</td>
<td>1</td>
<td>0.7</td>
</tr>
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<td>0.6</td>
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<td>0.6</td>
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<td></td>
<td>leuB6</td>
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<tr>
<td></td>
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The data presented in this chapter have demonstrated unequivocally that the 30 fold range in frequencies with which markers are recovered following P1-mediated transduction, is not due to selective packaging by P1 phage. Rather, the marker selectivity of P1 transduction must result from recombinational discrimination in recipient cells, as first hypothesised by Newman and Masters (1980) (Newman 1982, Masters et al. 1984). Newman (1982), however, also presented genetic evidence that implied leuB was packaged at higher levels into P1 transducing particles, than other markers. The packaging assays which measured leuB packaging, and the packaging levels of other markers from the 2 minute region, have confirmed that leuB is indeed overrepresented in the P1 lysate, as are the other markers from the same location (Table 3.3, figure 3.8). There is, as yet, no indication as to why the markers from the 2-minute region are overpackaged into P1 transducing particles. Attempts to measure transduction frequencies of markers, other than leuB, situated in the 2 minute region have not been possible; cotransduction frequencies of markers from this location clearly do not reflect their map positions, so the transduction data have proved almost impossible to interpret (M. Masters and K.J. Begg, personal communications). It is clear, however, that the transductional properties of DNA from the 2 minute region differs from that of other regions in both donor cells (as indicated by the high packaging levels) and recipient cells (as indicated by the anomalous transduction of markers from this region).

The data presented in this chapter have also led to the observation that additional faint bands, not present in digests of chromosomal DNA, were detected in P1 transducing DNA by probing P1 DNA with labelled fragments derived from the chromosome. Some of these bands were obviously not due to partial digestion of P1 transducing DNA as they were smaller than the expected band sizes (present in the chromosomal tracks) (figure 3.7d). It appeared, therefore, that during P1 lysis some cleavage of the full length
fragment was occurring, at distinct sites, to generate novel sized fragments which were homologous to the probe. Such cleavage events occur during P22-mediated packaging of transducing DNA (Chelala and Margolin 1965, see chapter 1) and are caused by the packaging mechanism of the phage; since homogeneous transducing particles are formed by P22, cleavage occurs at defined sites along the host chromosome at intervals of 1 P22" headful" of DNA. It is possible that P1-mediated packaging of the chromosome may produce the same effect, which would account for novel bands appearing in the P1 tracks. However this is considered unlikely because the observations of Bachi and Arber (1977) indicate that the P1 "headful" mechanism is not as accurate as the P22 system, (i.e. DNA molecules packaged into P1 heads are not of uniform size) so homogeneous transducing particles would not be expected to occur. Hence P1-mediated packaging of the chromosome would not generate defined cleavage points in the DNA. Another possible mechanism which may produce defined cuts in the chromosomal DNA is the EcoP1 restriction system. Again this was considered unlikely because P1-restriction probably does not function efficiently during P1-infection (Levy et al. 1984, see chapter 1 and section 3.5). However, to test the possibility that cleavage may occur, during lysis, at EcoP1 restriction sites, the sequence of the 2.3 kb EcoR1 ftsQ fragment (carried on pDK302) (see table 3.2) was scanned for the presence of EcoP1 restriction sites (Robinson et al. 1984). One was found 1.878 kb from one of the EcoR1 sites so EcoP1 cleavage adjacent to this site should have generated two novel bands: one of about 1.8 kb and the other of about 0.5 kb. No such bands were observed (figure 3.7a) so it was concluded that EcoP1 restriction activity was not likely to be responsible for generating the novel bands observed in P1 tracks. Thus, the origin of the novel bands, described above, is still a matter of conjecture and it is not clear which, if any, P1-encoded functions are responsible for their generation.

Attempts to detect sequences residing in the E.coli chromosome, which share homology with P1 pac have proved unsuccessful. Should they exist, such pac-like sequences could obviously provide initiation points for encapsidation of chromosomal DNA into
transducing particles. These results have been discussed previously.

The role of EcoP1 restriction, during lysis in providing double strand breaks from which DNA encapsidation could occur (Wall and Harriman 1974), was assessed by premodifying chromosomal DNA before P1 infection. Clearly, the results indicated that EcoP1 restriction is not of importance in the generation of transducing particles. Again, these results have been discussed previously.

There is obviously much scope for investigation of the mechanism by which P1 generates transducing particles and indeed, why markers from the 2 minute region are overpackaged. The work presented in this chapter emphasises the complexity of the problem; as yet P1 is poorly characterised, so at the moment it is not clear which, if any, P1 genes are essential for generating transducing particles. All that can be said is that P1 transducing particles are probably not formed by the same mechanism as P22-generated transducing particles, which involves recognition of specific pac-like sites at which chromosomal encapsidation commences. This is perhaps surprising as P1 and P22 have, superficially, very similar packaging mechanisms (see chapter 1 and section 3.1).
CHAPTER 4

TARGETING OF P1 pac SITES INTO THE E.coli GENOME
best way to put pac into chemotherapy

PBZ

get for gene

alert the person for x
SECTION 4.1

INTRODUCTION

P1 encapsidates chromosomal DNA into transducing particles with little marker specificity, with the result that all markers are packaged to roughly equal levels (chapter 3, Newman and Masters 1980, Masters et al. 1984). Complete transduction frequencies, however, vary in a marker specific manner over a 30 fold range (Masters 1977).

The majority, some 75%, of transducing DNA does not recombine with the recipient genome but becomes abortively transduced (Sandri and Berger 1980a, b). Abortively transduced DNA adopts a stable circular conformation, its ends bound by protein, hence the speculation that transducing DNA destined to become recombined into the chromosome, does not adopt this conformation (Masters 1984, see chapter 1). So a study of the marker specificity of abortive transduction should provide evidence for the origin and nature of the transductional discrimination observed by Masters (1977). For example, if the fate of transducing DNA is related to the ability of the DNA to circularise, so that DNA which efficiently circularises is not available for recombination, a higher proportion of poorly transduced markers than those which are well transduced, would be expected to occur in abortively transduced DNA, i.e. the opposite to complete transduction (assuming both well and poorly transduced DNAs are equally susceptible to degradation). UV irradiation of cells after transduction occurs (i.e. after abortives have formed) would then be expected to remove the marker specificity in abortively transduced DNA concomitantly with that in complete transduction, by perhaps rendering abortive molecules recombinogenic, as occurs in P22-mediated transduction. Newman and Masters (1980) and Newman (1982) uv-irradiated lysates or recipient cells prior to transduction but as yet, the effects of uv-irradiating cells immediately after P1 transduction has not been assessed (see chapter 1). If, on the other hand, the distribution markers in abortive transductants did not show a predominance of poorly transduced markers, this would suggest perhaps, that poorly transduced DNA was more susceptible to
nucleolytic degradation in the recipient cell. The marker dependence of transduction could then perhaps be explained by proposing that poorly transduced DNA was degraded before recombination could occur.

To test the above hypotheses, the levels of individual markers in abortively transduced DNA would need to be measured. One possible method would be to use a similar quantitative hybridisation procedure to that described in chapter 3. However, it would be necessary to prepare assayable amounts of abortively transduced DNA. Abortively transduced DNA can be prepared by first purifying transducing particles (see chapter 1), using the purified transducing particles to infect recipient cells and then extracting the abortively transduced DNA from recipient cells (Sandri and Berger 1980b). This procedure, however, is technically difficult and yields of abortively transduced DNA are very poor. The main obstacle lies in the difficulty of preparing large amounts of transducing particles; even after pouring several preparations, Sandri and Berger (1980b) were only able to produce in total about $10^{10}$ transducing particles of which only 25-40% would adsorb to recipient cells. Once the transducing DNA was injected about 40% could be reextracted as abortive DNA and subsequently assayed. Hence, the amount of any given 4 kb donor-chromosomal fragment (the average size of a restriction fragment generated by an enzyme with a 6 bp recognition site) present in the total recovered abortively transduced DNA, can be roughly estimated as follows.

Assuming the maximum values for recovery attained by Sandri and Berger (1980b), the number of 90 kb abortive molecules that can be recovered =

\[
10^{10} \times 0.4 \times 0.4 = 1.6 \times 10^9 \text{ abortive 90 kb molecules.}
\]

MW of a 90 kb abortive DNA molecule = 650 (approximate MW of 1 bp) \times 9 \times 10^4

\[
= 5.85 \times 10^7
\]

Avogadro no. of 90 kb molecules (6.023 \times 10^{23})

weighs 5.85 \times 10^7 g.
\[
\text{weight, in grams, of } 1.6 \times 10^9 \text{ 96-kb molecules} = 1.6 \times 10^9 \times 5.85 \times 10^7 \times 6.023 \times 10^{23} = 1.55 \times 10^{-7} \text{ g} = 155 \text{ ng}
\]

As chromosomal DNA is 50x the size of abortive DNA, the quantity of any 4 kb chromosomal fragment present in the total abortive DNA
\[
= \frac{1.55 \times 10^{-7} \text{ g}}{50} = 3.1 \times 10^{-9} \text{ g}
\]

As mentioned above, this small amount of abortively transduced DNA is the maximum expected yield from \(10^{10}\) transducing particles; to obtain even this amount requires technically difficult and very time-consuming techniques (Sandri and Berger 1980a).

In order to study the distribution of markers in abortively transduced DNA it would be most desirable to develop a means of improving the yields of transducing particles; clues as to how this might be achieved come from P22 and P1 packaging studies. It is difficult to devise a procedure to increase the total amount of transducing particles other than by isolating HT mutants of P1 as did Wall and Harrison (1974). The increase in transduction frequencies obtained from such mutants varied in a marker-dependent manner; for example, leu was stimulated about 20 fold but other markers by only 4-15 fold; such mutants would not, therefore, be of use in this instance since transduction frequencies are altered. In any case, such mutants are extremely difficult to isolate. An alternative strategy would be to direct the overpackaging to high levels of specific, but limited, chromosomal regions such as is observed for Salmonella markers proximal to P22 pac-like sites. Sternberg et al. (1981a) demonstrated that during lysis, transient associations of P1 (and hence P1 pac) with a defined chromosomal location, produced lysates giving 50 fold increased transduction of markers to one side of the association site (see chapter 1). Sternberg and Hoess (1983) also reported that P1 pac, cloned into a \(\lambda\) vector and integrated into \(\text{att } \lambda\), was recognised and cleaved with 60% efficiency during P1 lysis. In other words, P1 pac sites when integrated into the E.coli chromosome were recognised by P1 packaging machinery and so initiated
Thus if P1 pac sites could be stably integrated into the E.coli chromosome at defined locations, flanking DNA should be overpackaged by at least 50 fold during P1 lysis. If so, the increased yield of marker-specific transducing DNA would enable the fate of individual markers to be followed more easily after its entry into recipient cells. In addition, Sternberg et al. (1981a) and Sternberg and Hoess (1983) reported that P1 packaging of the chromosome from integrated pac sites proceeds for 4-5 headfuls (8-10 minutes of the chromosome) before any drop in packaging efficiency. Hence, markers which exhibit very different transduction frequencies, but are located within 8-10 minutes of each other on the E.coli map (e.g. ilv and pyrE) could be overpackaged from the same pac site and the fates of the two (or more) markers could be compared directly against each other.

Three possible strategies were considered as a means of directing P1 pac into the chromosome and hence obtain overpackaging of specific markers. Sternberg's method (Sternberg and Hoess 1983) involving lysogenisation of a strain with λ carrying P1 pac was not initially thought to be suitable; λ normally integrates by λ inter- dependent recombination between a phage site att P.P and att B.B (att λ), located at about 17 minutes on the E.coli map (Campbell 1962, Bachman 1983). To obtain integration at other locations, att λ must be altered or deleted (Campbell 1965, Adhya et al. 1968). Then λ prophage insertion is reduced by about 200 fold and the phage may integrate into many possible locations: e.g.: leu, pro, purE his pheA ays and ilv (Shimada et al. 1972). The low efficiency of this process, the sparsity of attachment sites and the uncertainty of the location of the prophage, suggested initially that the use of a λ pac vector was probably not suitable as a means of detecting the integration of pac into a number of different chromosomal locations. (However, as will become clear later, it now seems that this method may actually be the most suitable).

The second possibility considered was the cloning of P1 pac into a non essential region of a transposable element. This hybrid pac- carrying transposon could then be introduced into the E.coli
chromosome. The advantage of this system would be the possibility of inserting \textit{pac} into any chromosomal location. The random location of the insertion, however, brings with it the problem of then locating the hybrid transposon in the chromosome.

The third possible strategy, and the one which was adopted, was devised from the observation that \textit{polA} strains of \textit{E.coli} cannot support replication of \textit{ColE1} derived replicons, such as \textit{pBR325} (Kingsbury and Helinsky 1970). As described in section 3.2, homologous recombination between a \textit{ColE1}-derived plasmid replicon carrying cloned chromosomal DNA and the chromosome of a \textit{polA} strain, can give a stably integrated plasmid, located at the site of mutual chromosomal homology. If such a plasmid also carries \textit{pac}, then obviously the \textit{pac} site also would be integrated at the same location (figure 4.1). A family of "\textit{pac} vehicle" plasmids can be constructed \textit{in vitro}, each containing \textit{pac} and a specific chromosomal fragment. By transforming such \textit{pac} vehicles into \textit{polA} strains and selecting for plasmid integration, \textit{P1 pac} sites can be targeted into precisely defined chromosomal locations, dependent obviously on the chromosomal fragment present on the plasmid. Suitable DNA fragments have been cloned from most chromosomal locations, but in the initial experiments described below, \textit{ilv} from \textit{pMH601}, \textit{his} from \textit{pMH600} and \textit{trp} from \textit{pMH602} were the chromosomal fragments used, since these were already available in our laboratory. The experiments were performed as a preliminary investigation to establish the following:

1) That the above strategy was suitable for targeting \textit{P1 pac} sites into defined chromosomal sites of \textit{polA} strains.

2) The integrated \textit{pac} sites are recognised and utilised efficiently by \textit{P1} packaging machinery during \textit{P1} lysis.

3) The integrated \textit{pac} sites direct the overpackaging of adjacent markers (but to one side only, see chapter 1) such that these markers are present in the lysates with at least 50 fold higher levels than normal.

If these three criteria were met, the strains harbouring \textit{pac} could be used to produce high yields of specifically enriched transducing particles. These could then be used, as previously described, to infect recipient cells from which the abortively
transduced DNA could be extracted. **Sufficient** sequence of the overpackaged markers should then be available such that they would be easily detectable in abortively transduced DNA by Southern or dot-blot hybridisations (Thomas 1980).
SECTION 4.2
CONSTRUCTION OF pac VEHICLES AND CONTROL PLASMIDS

Microgram quantities of the 6.9 kb HindIII ilv fragment from pMH601, the 5.3 kb HindIII his fragment from pMH600 and the 30 kb trp fragment from pMH602 (described in chapter 3) were prepared by the following procedure:

3 μg of plasmid (pMH600, pMH601, pMH602) was restricted to completion with HindIII and the HindIII inserts separated from vector DNA by preparative agarose gel electrophoresis (figure 4.2). The bands were cut from the gel and the fragments purified by electoelution and NACS prepac column chromatography (chapter 2). pPAC (described in chapter 3) was restricted to completion with HindIII, BAP-treated and aliquots mixed with each of the purified fragments. The mix was ligated overnight and then used to transform competent 303-1 cells. Cells receiving plasmids were selected on L-broth Ap plates. About 300 colonies per plate grew (for each ligation mix) after overnight incubation at 37°C. 100 of each type were purified by patching onto L-broth Ap plates and recombinants screened by patching onto L-broth Ap, L-broth Cm and L-broth Tc. pPAC has an Ap' Cm5 Tc' phenotype and recombinants were detected by an Ap' Cm5 Tc5 phenotype resulting from insertional inactivation of the tc gene (figure 4.3). 10 clones of each recombinant type were picked, grown in L-broth Ap and their DNA extracted by alkaline lysis. HindIII digests of this DNA, confirming the pac vehicles contained the correct inserts, are shown for pPAC600 (his) pPAC601 (ilv) and pPAC602 (trp) (figure 4.4).

In order to provide suitable pac- control plasmids, similar constructions to those described above were carried out using plasmid pR121 as the vector. pR121 was constructed at the same time as pPAC (chapter 3) and by the same procedure. However, instead of the 650 bp fragment R120, present in pPAC, pR121 carries the 420 bp EcoR1 fragment 21 (R121) from P1, cloned into the unique EcoR1 site of pBR325; R121 carries part of the P1 oriL region (figure 1.1). HindIII digests of pR121600 (his), pR121601 (ilv) and pR121602 (trp) are also shown in figure 4.4.
FIGURE 4.1 SCHEMATIC DIAGRAM SHOWING THE INTEGRATION OF A PAC VEHICLE PLASMID

H = \textit{HindIII} site
R = \textit{EcoRI} site

Integration by homologous recombination results in duplication of the region of shared homology; the directly repeated DNA flanks the plasmid sequences. Packaging from the integrated pac site results in cleavage of the plasmid sequences and generates a novel band if the packaged DNA is restricted with \textit{HindIII}: 1.5 kb (direction a) or 5.1 kb (direction b) (see text for full discussion).
Figure 4.1

-pac-

Vehicle

H H R R

H H

H H

H H

a b

P1 PACKAGING

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P1 pac

HOMOLOGOUS SEQUENCES

CHROMOSOMAL DNA

PLASMID DNA
FIGURE 4.2  PREPARATIVE GEL OF HindIII RESTRICTED pMH600, pMH601, pMH602.

1. HindIII restricted pMH600 (5.3 kb insert)
2. HindIII restricted pMH601 (7.0 kb insert)
3. HindIII restricted pMH602 (3.0 kb insert).

5 μg of each plasmid was digested with HindIII. The cloned inserts were cut from this gel, purified as described before and used to construct pac vehicles and control plasmids (see text).
Figure 4.2
FIGURE 4.3  PAC VEHICLE PLASMIDS AND CONTROL PLASMIDS

H = HindIII site
B = BamHI site
E = EcoRI site

Inserts are shown as blocks and plasmid sequences as a single line. tet, cap and amp refer to the tetracycline resistance, chloramphenicol resistance and ampicillin resistance genes respectively. ori denotes the plasmid origin of replication.
Figure 4.3

A) pPAC600
12.1 kb

B) pR121600
11.9 kb

C) pPAC601
13.6 kb

D) pR121601
13.4 kb
4.3 contd.

**E**

![Diagram of pPAC602 9.6 kb](image)

**F**

![Diagram of pR121602 9.4 kb](image)
FIGURE 4.4  HINDIII DIGESTS OF PAC-VEHICLE AND CONTROL (pR121) PLASMIDS

A.
A. HindIII restricted pMH600 (his)
B. HindIII restricted pR121
C-G. Independent isolates of pR121600 restricted with HindIII.
H-L. Independent isolates of pPAC600 restricted with HindIII
M. HindIII restricted pPAC
N. EcoRI restricted P1 DNA as size markers.

B.
A. EcoRI restricted P1 DNA as size standards.
B. HindIII restricted pPAC
C-G. Independent isolates of pPAC601 restricted with HindIII
H-L. Independent isolates of pR121601 digested with HindIII.
(The derivation of the small 3 kb insert into clone J is not known.)
M. HindIII digested pR121
N. HindIII digested pMH601 (i1v)

C.
A. EcoRI digested P1 DNA
B-F. Independent isolates of pPAC602, restricted with HindIII.
G-K. Independent isolates of pR121602 restricted with HindIII.
L. HindIII restricted pPAC
M. HindIII restricted pMH602

Note: the high molecular weight fragments present in some of the tracks (e.g. A: F,G,J) are the products of incomplete restriction.)
Figure 4.4 contd.
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SECTION 4.3
INTEGRATION OF pac VEHICLES AND pR121 CONTROL PLASMIDS INTO THE
CHROMOSOME OF A polA STRAIN

ND113 (polA, KmR) (A. Jenkins 1985) was the recipient chosen
for integration of the plasmids. The strain is largely prototrophic
and so suitable as a donor strain for the production of P1 lysates
and subsequent P1 transduction experiments.

Competent ND113 cells were transformed with large quantities of
pac and R121 vehicle DNAs; approximately 1 \( \mu \)g of each vehicle DNA was
added to separate 100 \( \mu \)l aliquots of competent cells. Cells were
spread onto L-broth Km Ap (15 \( \mu \)g/ml) plates to select those
maintaining the plasmid. Only 3-4 colonies per transformation were
obtained after overnight incubation at 37\( ^0 \)C, i.e. the recovery of
transformants was about \( 10^4 \) lower than would have been expected if
the competent cells were PolA+. Clones were purified on the same
selective plates and checked for the Ap\( ^r \) Km\( ^r \) Tc\( ^S \) phenotype
expected for ND113 carrying pac or R121 vehicles. To ensure that
clones still carried the polA lesion and that the transformation had
not resulted in selecting polA revertants, individual clones were
tested for uv-sensitivity, since Po1A strains exhibit sensitivity to
high levels of uv-irradiation (Cooper and Hanawalt 1972). All the
clones exhibited the same uv-sensitivity as ND113, which was killed
by 200 ergs/mm\(^2\) uv, while the isogenic PolA\(^+\) parent strain
survived > 300 ergs/mm\(^2\). Hence all the recombinant clones
maintained the polA lesion. In addition, to establish that the
transformation and selection procedure had not selected a polA
suppressor, which allowed the incoming plasmids to replicate,
attempts to isolate free plasmid DNA from the clones were performed.
No free plasmid DNA could be obtained from any of the clones. Hence,
the above tests confirmed that pac or R121 vehicles were unable to
replicate autonomously and were being maintained by integration into
the host chromosome.

It was necessary to establish that the plasmids had integrated
by homologous recombination between the chromosome and cloned
chromosomal DNA carried on the plasmid. Plasmids should thus have
integrated at his (44 minutes) for pPAC600 and pR121600, at ilv (84.6 minutes) for pPAC601 and pR121601 and at trp (27.6 minutes) for pPAC602 and pR121602.


As already mentioned, both PAC601 and R121601 should contain a plasmid integrated by homologous recombination, at the chromosomal ilv sequences (84.6 map units). If the above strains were infected with P1 to produce a transducing lysate, recognition of the pac site carried in PAC601 (but not in R121601) would be expected to stimulate the packaging and hence transduction of markers to one side of ilv only, as P1 packaging is a progressive unidirectional progress (Bachi and Arber 1977). ilv (84.6 minutes) is flanked by a number of suitable selective markers; with tna (83.3 map units) and pyrE (82.3 map units) to one side and rha (87.7 map units), argH (89.5 map units) and purDH (90.5 map units) to the other side (Bachman 1983). (purDH is located some 6 minutes from ilv, but since the recognition of a chromosomally located pac site can stimulate packaging for up to 5 P1 headfuls from pac (about 10 map units) without a drop in packaging efficiency (Chesney and Scott 1979, Sternberg and Hoess 1983), this marker, although relatively distant from ilv, would still be suitable) (figure 4.5).

P1 lysates were on PAC601 and R121601 and the lysates were used to transduce suitable marked strains to Ilv+, Trp+, PyrE+, Rha+, ArgH+ and PurDH+ and Trp+. The results of these transductions are shown in table 4.2.

Clearly, using the lysate mode on PAC601, the transduction of Rha+, ArgH+ and PurDH+ was about three fold greater than the frequencies obtained from the P1 lysate prepared on R121601. The transduction of PyrE+, to the other side of ilv, however occurred with a similar frequency for both lysates. Ilv+ transduction for both lysates was lower than expected from the previously published reports (Masters 1977). (However, a possible explanation for this low Ilv+ transduction became apparent later and will be discussed
(Note: this diagram, for simplicity, shows only one inserted plasmid. In fact there are multiple insertions at this site. See text for discussion.)
Figure 4.5

CHROMOSOMAL DNA

1 MAP UNIT

PLASMID INSERT NOT TO SCALE
Transduction of Tna+ was clearly anomalous (table 4.2a) since no Tna+ transductants were recovered when MM304 or MM321 were the recipient strains and in addition, the apparent levels of Tna+ transduction were unusually low when MM327 was the recipient.

It was possible that transduction of the origin region is anomalous when an ND113 derivative is used as the donor strain. To eliminate this possibility P1 lysates were prepared on PAC600, R121600 PAC602 and R121601 and the lysates were used to transduce MM304 to Ilv+, PyrE+, Tna+ and Arg+. The results (not shown) indicated that transduction of these markers occurred at normal levels (Masters 1977), except for Tna+ where once again no Tna+ transductants were obtained. (Note. The above strains all contain integrated plasmids, but the sites of integration should have been near terC, i.e. much too far from oriC to influence the transduction of origin markers). It was not clear why Tna+ transductants were only recovered when MM327, but not MM304 or MM321 were the recipients for the transduction. In spite of the anomalous Tna+ transduction, the above results are consistent with the hypothesis that PAC601 and R121601 both contain plasmids integrated at ilv, as indicated by the low Ilv+ transduction when only PAC601 or R121601 are used as donor strains. The activity of the pac site present in pPAC601 (integrated at ilv in PAC601), but absent from R121601, was confirmed by the selective 3-fold increase in transduction of markers to one side of ilv when PAC601 was used as the donor in a transduction experiment; transduction of Rha+, ArgH+ and PurDH+ were stimulated three fold but PyrE+ transduction (PyrE is situated to the other side of ilv (see figure 5.4)) was not stimulated.

The results were disappointing in one respect. As already mentioned, the introduction of P1 pac into the E.coli chromosome had been shown to stimulate transduction of markers (to one side of pac only) by up to 50 fold (Chesney and Scott 1979, Sternberg et al. 1983). The three fold stimulation obtained here was therefore much lower than expected. This suggested that the integrated pac site in PAC601 was not being utilised efficiently to commence the encapsidation of neighbouring DNA. However once the encapsidation
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PUBLISHED RATIOS (Masters 1977)

5.6 5.5 0.3 0.4 1.0 0.7 0.6

(NOTE - Published ratios were obtained using wild type donor strains, without integrated plasmids).
## TABLE 4.2b

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(see text for discussion of these figures)
process had commenced, it seemed that encapsidation could proceed for at least 3 P1 "headfuls" of DNA without any drop in packaging efficiency; this was indicated by the similar levels with which the transduction of Rha+ (2 headfuls from oriC), ArgH+ and PurDH+ (3 headfuls from Ilv) were stimulated.

Similar genetic tests were carried out to map the location of pPAC602, and PRI21602; both these plasmids should be located at trp (27.7 map units). The transduction of Trp+ and flanking markers was measured using P1 lysates constructed on PAC602, R121602 and (as a control) PAC601.

The transduction of Arg+ (89.5 map units) Trp+ (27.7 map units), His+ (44 map units) PurB+ (25.2 map units) and PyrF+ (28.3 map units) (Bachman 1983) were scored using each of the above lysates and the results are shown in table 4.2b. The results are not easy to interpret unequivocally in view of the low numbers of transductants that were obtained, but it seems that Trp+ transduction is reduced in the lysates made on the strains thought to have plasmids integrated at trp. The markers to one side of the putative integration site (Pyr+ and His+) were transduced with three times the frequencies observed when PAC602 was the donor strain as compared to lysates made on PAC601 or R121602. (This observation is clear for His+ transduction, although less so for PyrF+ transduction because the inability to recover Trp+ transductions from the PAC602 lysate, when MM312 was the recipient, makes it impossible to estimate the relative efficiency of PyrF+ transduction when this lysate was used to transduce PLK831 to PyrF+). However a marker to the other side of the putative integration site (purB) was transduced with equal efficiency for all three lysates.

These results are consistent with the pattern of transduction observed for markers flanking ilv, when lysates made on PAC601 and PAC602 were used to transduce suitable recipients. Firstly, transduction of the marker into which the plasmids are expected to have integrated was reduced. Secondly markers to one side only of the putative integration site were transduced with three fold increased efficiency, when the lysate was made on a strain harbouring
an integrated P1 pac site.

The above results are therefore consistent with the hypothesis that PAC601, R121601, PAC602 and R121602 harbour plasmids which have integrated into the chromosomes of the host cells by homologous recombination. The pac sites in PAC601 and PAC602 appear to be recognised during P1 infection and can direct the overpackaging of adjacent markers; this process is inefficient and leads to only a 3 fold improvement in transduction. However, the effect of the pac site is exerted over a large distance; His+ transduction is stimulated in a lysate from PAC602 even though His+ is about 17 map units from the insertion site of pac (about 8.9 P1 headfuls).

The insertion sites of the plasmids integrated in PAC600 and R121600 were not mapped using the genetic techniques described above.

**PHYSICAL DETECTION OF INTEGRATED pac AND R121-VEHICLE SEQUENCES IN CHROMOSOMAL DNA**

In addition to the genetic evidence described above, physical evidence was required to confirm the presence of the integrated pac and R121-vehicles in the chromosomes of the host strains.

Chromosomal DNA was prepared from the strains listed in table 4.1. DNAs restricted with EcoR1 and DNAs sheared to > 50 kb were run on adjacent tracks of a 1% agarose gel (figure 4.6a). The gels were transferred to nitrocellulose and the filters were probed with 32p-labelled pPAC DNA. The autoradiograms are shown in figure 4.6b.

Hybridisation to tracks containing sheared DNA was exclusively to the high molecular weight sheared DNA, which provided additional confirmation that no free plasmid was present in the strains.

Hybridisation to the EcoR1-digested chromosomal DNAs gave a surprising result, there was intense hybridisation to bands which corresponded in size to those obtained by EcoR1 restriction of the plasmid DNA alone (figure 4.6a). This was expected for PAC601 and R121601, where the Ilv sequences on the plasmid contain internal EcoR1 sites but not for the other strains, which carry inserts with no internal EcoR1 sites. For these plasmids, integration of the
FIGURE 4.6. DETECTION OF INTEGRATED PLASMIDS.

A. 1. EcoRI restricted R121600 DNA
2. Sheared R121600 DNA
3. EcoRI restricted R121601 DNA
4. Sheared R121601 DNA
5. EcoRI restricted R121602 DNA
6. Sheared R121602 DNA
7. EcoRI restricted P1 DNA (size standards)
8,9,10. EcoRI restricted pR121600, pR121601, pR121602 DNA respectively.

B. 1. EcoRI restricted PAC600 DNA
2. Sheared PAC600 DNA
3. EcoRI restricted PAC601 DNA
4. Sheared PAC601 DNA
5. EcoRI restricted PAC602 DNA
6. Sheared PAC602 DNA
7. EcoRI restricted P1 DNA (size standards).
8,9,19. EcoRI restricted pPAC600, pPAC601, and pPAC602 DNA respectively.

C. Tracks 7-10 were cut from the gels shown in 4.6 A and B. These gels were transferred to nitrocellulose and probed with $^{32}$P-labelled pPAC DNA. The autoradiogram is shown in 4.6C. Tracks 1-6 correspond to tracks 1-6 in figure 4.6A and tracks 7-12 correspond (respectively) to tracks 7-10 in figure 4.6B.

Note the presence of the intense plasmid bands in the EcoRI restricted tracks and the presence of novel sized fragments (see text for discussion); novel bands are indicated by unlabelled arrowheads, and the fragment sizes are given in the text.
Figure 4.6

A

B
4.6 contd.
FIGURE 4.7  SCHEMATIC DIAGRAM OF THE INTEGRATION OF pPAC600 INTO THE ND113 CHROMOSOME

Large blocks indicate the duplicated homologous sequences. E = EcoRI site. Plasmid sequences are indicated by the dark line and chromosomal DNA by thin lines. The small arrow represents the P1 pac site.

A. INSERTION OF 1 COPY

Restriction of the DNA with EcoRI could generate two novel sized bands, A and B, homologous to the plasmid sequences (see text).

B. INTEGRATION OF 3 COPIES

Restriction with EcoRI generates the same two novel bands, described above, together with two fragments of 11.3 kb i.e. pPAC600 without the 650 bp. pac fragment, which would be removed by the EcoRI digestion (see text).
Figure 4.7

A

B

-650

11.3

-650

650

11.3

650

11.3

650

11.3

650

11.3

650

11.3

650
plasmid sequences by homologous recombination should have produced two novel EcoR1 "junction" fragments whose sizes could be predicted in the following manner.

pMH602 (which contains the same 2.9 kb trp fragment as both pPAC602 and pR121602) shares homology with a large 30 kb EcoR1 chromosomal fragment (chapter 3). Insertion of pPAC602 (9.5 kb in size), which contains 2 internal EcoR1 sites in the plasmid sequences (figure 4.3), into this fragment and subsequent restriction of the DNA with EcoR1 should generate 3 bands: one of 650 bp (the P1 pac fragment) plus two of novel size. The sum of these two novel bands (the bands would be identical for both pPAC602 and pR121602) would equal 30 kb + 9 kb (i.e. the size of the original band plus the size of the plasmid sequences without the small P1 fragment) = 39 kb. Likewise, integration of pPAC600 and pR121600 by homology should generate 2 novel EcoR1 bands. In this case, since the plasmids are of about 11.3 kb and the chromosomal fragment homologous to the his G fragment (present on pMH600, pPAC600 and pR121600, see chapter 3) is about 20 kb, the sum of the sizes of the two novel bands would be about 31 kb (not including the small P1 fragment which would be cleaved out by EcoR1 digestion) (see figure 4.7).

The novel bands described above should be detected by the pPAC probe used in the hybridisation experiment shown in figure 4.6b.

In tracks containing EcoR1-digested DNA from PAC600 and R121600, the probe hybridises weakly to an 18 kb fragment in addition to the strong hybridisation to the 11.3 kb plasmid-sized band. This may be one of the predicted novel bands but if so, the other band would be expected to be about 12-13 kb. If present, this other band would be obscured by the intense approximately 12 kb plasmid-size band.

In tracks containing EcoR1-digested DNA from PAC602 and R121602 four novel bands were apparent, in addition to the intense plasmid-sized band (about 9 kb). These were of about > 23 kb, > 20 kb, 5.5 kb and 3.6 kb. The first two bands were candidates for the novel bands caused by insertion of the pPAC602 or pR121602 vehicles by homologous recombination at trp since they total about 43 kb; since it is difficult to accurately estimate the sizes of very large restriction fragments, the sum of these two novel bands is probably
not significantly different from the 39 kb total predicted above. The origin of the two smaller novel bands is not clear. These may indicate that pPAC602 and pR121602 have inserted at a site other than trp.

In any case, the combined physical and genetic evidence are not totally conclusive that all the plasmids have indeed integrated by homologous recombination. The transduction data suggest that pPAC601 and pR121601 have integrated by homology as have the integrated pPAC602 and pR121602 plasmids. The physical evidence is less clear, but is not inconsistent with the hypothesis that pPAC600, pR121600, and at least some of the pPAC602 and pR121602 insertions had occurred by homologous recombination.

The unexpected occurrence of intense bands of the same sizes as the integrated plasmids led to the conclusions that, since no free plasmid DNA is present (which could also have given the above pattern), the insertion of pac or R121 vehicles must have resulted in multimerisation of the plasmid inserts to give repeating units (figure 4.7). This suggestion is also supported by the occurrence of strong bands in the chromosomal ladder patterns (figures 4.6a, 4.8a), clearly visible after restriction of the DNA with EcoR1 and Ethidium Bromide staining of the gel; these bands correspond in size to those lit up by the pPAC probe. Such sequences must be heavily overrepresented in the chromosome to be so clearly visible in the chromosomal ladder patterns.

ESTIMATION OF THE NUMBER OF PAC OR R121-VEHICLE INSERTS IN EACH STRAIN

A quantitative Southern hybridisation was used to estimate the number of plasmid inserts present in each strain. 2 µg of chromosomal DNA from each of the 6 strains containing integrated plasmids was restricted with HindIII (to cleave the pBR325 sequence plus P1 fragment from the chromosomal DNA) and run on an agarose gel. Alongside the chromosomal DNAs were run tracks containing increasing amounts of HindIII-restricted pBR325 DNA; the amounts loaded ranged from 1.2 ng to 24 ng (figure 4.8a). The gel was then transferred to
nitrocellulose and the filter was probed with $^{32}$P-labelled pHWO01 (pBR325::argH). The autoradiogram of this filter is shown in figure 4.8b.

The intensities of the bands on the autoradiogram were measured, as before, with a scanning densitometer and digitiser. The band intensities in the pBR325 tracks were used to plot a standard curve of intensity against the amount of sequence loaded. Then the intensity of the pBR325-homologous bands in the chromosomal tracks were compared with the standard curve, to obtain an estimate for the quantity of pBR325 sequence present in each chromosomal track. The number of inserts in each track was then calculated as follows:

Assuming one insert only, the quantity of pBR325 sequence in 2 μg chromosomal DNA =

\[
2 \mu g \times \frac{\text{size of pBR325}}{\text{size of the E.coli genome}} \times \frac{6 \text{ kb}}{4000 \text{ kb}} = 3.0 \text{ ng}
\]

The number of inserts in each strain must therefore equal:

the amount of pBR325 sequence (as measured from the standard curve)

3.0 ng (amount expected from 1 insert)

The band intensities, quantities of pBR325 sequence and the number of inserts are shown in table 4.3. Unfortunately, the number of inserts in both pPAC602 and R121602 was greater than expected, and the values lie outside the standard curve. However, the standard curve was extrapolated to accommodate these values and used to obtain an approximate estimate for the number of inserts in these two strains.

The probe, pHWO01, (chapter 3) consists of pBR325 with a 7.0 kb chromosomal insert containing argH. This plasmid was used for the probe because hybridisation to both the pBR325 and argH sequences, present in the chromosomes of the strains with plasmid inserts, would have provided a useful internal control. However, the difference in size between the argH-containing HindIII band and the pBR325-homologous chromosomal fragments is small; about 400 bp for pac
vehicles and about 600 bp for R121 vehicles. In the experiment described above, the pBR325-homologous bands are very intense and have largely obscured the argH-homologous bands. The argH bands, therefore, could not be scanned independently. However, the faint argH bands are just visible in the PAC600, R121600, pAC601 and R121601 tracks (figure 4.8b). Their low intensity confirms that pBR325 sequences are represented several fold in excess of similarly sized chromosomal sequences. Also, the low intensity of the argH bands indicates that the bands will have contributed little to the measured intensities of the overlapping pBR325 bands in the chromosomal tracks and therefore will not have caused a significant overestimate of the number of plasmid inserts.

The following estimation of the number of plasmid inserts has not been corrected for gene dosage variation between origin and terminus. This is because the strains carrying the integrated plasmids grow very slowly, doubling every 75-90 minutes depending on the strain (data not shown). The origin to terminus ratios within these cultures would not, therefore, differ greatly from 1:1 (Cooper and Helmstetter, 1968). The cause of these very slow growth rates is not clear, but did not correlate with the location of the plasmid insertion. In any case, table 4.3 and figure 4.8b show clearly that the strains carry multiple inserts of the pac or R121 vehicles. Surprisingly, the number of inserts appeared to depend on the chromosomal location of the plasmid (assuming that all the plasmids had indeed integrated by homologous recombination; this seems likely because, in practice, transformation of PolA strains with pBR325 derivatives is not detected unless the plasmid contains DNA which share homology with the recipient cell chromosome i.e. integration by illegitimate recombination or other mechanisms is not detectable (Jenkins et al. in press)). In addition, the data from the previous experiments are consistent with the hypothesis that pac or R121-vehicles had integrated by homology. Plasmid integration at ilv resulted in 6 inserts (for both PAC601 and R121601), integration at his resulted in about 4 inserts of both pPAC600 and pR121600 and integration at trp resulted in 14 inserts of PAC602, and 15 inserts of R121602. The reason why the number of inserts appeared to depend
on the chromosomal location of the integration event is not clear. However, as already mentioned, it is possible that pPAC602 and pR121602 had each integrated at 2 chromosomal locations. If this had indeed occurred, the surprisingly high number of plasmid copies may not be due to 14 or 15 inserts at one location, but perhaps 6 or 7 copies integrated at two separate positions i.e. the number of inserted plasmid copies may not be dependent on the position of insertion.
FIGURE 4.8 QUANTITATION OF THE NUMBER OF PLASMID INSERTS IN STRAINS CARRYING INTEGRATED PLASMIDS

A. 1. 2 μg HindIII restricted PAC600 DNA
   2. 2 μg HindIII restricted PAC601 DNA
   3. 2 μg HindIII restricted PAC602 DNA
   4. 2 μg HindIII restricted R121600 DNA
   5. 2 μg HindIII restricted R121601 DNA
   6. 2 μg HindIII restricted R121602 DNA
   7-10. 2.4 ng, 6.8 ng, 12 ng, 24 ng (respectively of HindIII restricted pBR325.

B. The gel shown in A was transferred to nitrocellulose and probed with 32P-labelled pHWO01 (arg); the 7.0 kb arg-homologous band is just visible in tracks 1, 2, 4 and 5 (see text for discussion).

Note the presence of distinct plasmid-sized bands in the chromosomal ladder patterns (A).
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>BAND INTENSITY</th>
<th>AMOUNT OF pBR325 SEQUENCE (in ng)</th>
<th>NO. OF INSERTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR325</td>
<td>0.5</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>pBR325</td>
<td>2.5</td>
<td>6.8</td>
<td>-</td>
</tr>
<tr>
<td>pBR325</td>
<td>5.2</td>
<td>12.0</td>
<td>-</td>
</tr>
<tr>
<td>pBR325</td>
<td>10.0</td>
<td>24.0</td>
<td>-</td>
</tr>
<tr>
<td>PAC600</td>
<td>5.8</td>
<td>13</td>
<td>4.3</td>
</tr>
<tr>
<td>PAC601</td>
<td>8.4</td>
<td>18</td>
<td>6.0</td>
</tr>
<tr>
<td>PAC602</td>
<td>18.5</td>
<td>42</td>
<td>14.0</td>
</tr>
<tr>
<td>R121600</td>
<td>5.7</td>
<td>13</td>
<td>4.3</td>
</tr>
<tr>
<td>R121601</td>
<td>8.8</td>
<td>19</td>
<td>6.3</td>
</tr>
<tr>
<td>R121602</td>
<td>19.5</td>
<td>45</td>
<td>15.0</td>
</tr>
</tbody>
</table>
SECTION 4.4
EFFICIENCY WITH WHICH INTEGRATED pac SITES ARE UTILISED

Integration of pac and R120 vehicles into the E.coli chromosome results in duplication of the chromosomal DNA, flanking the plasmid sequences (figure 4.1). There is evidence from the previous section that these units are themselves repeated, to give several integrated copies (Table 4.3). However, considering for the moment one unit only, it can be predicted that during P1 lysis, packaging should commence at the integrated pac site and so cleave the plasmid sequences. If no pac cutting occurs, restriction of transducing DNA with HindIII should result in precise excision of the intact plasmid DNA. Cutting at pac, however, will generate novel bands in the HindIII digest, either 1.5 kb (direction a, figure 4.1) or 5.1 kb (direction b, figure 4.1), depending on the orientation of pac. Such novel bands should, of course, be absent from control (R121) lysates.

However, as already mentioned, more than one insert is present at each location and it is difficult to anticipate what effect this may have on the efficiency with which the integrated pac sites are used to initiate chromosomal packaging; note that this is distinct from P1 pacase-directed cleavage at pac, since cleavage can obviously occur without packaging (Sternberg and Hoess 1983, Sternberg 1984, see chapter 1). It is possible that all the integrated pac sites are cleaved, in which case the DNA would become fragmented into small plasmid-sized fragments which would presumably be unsuitable as packaging substrates. In this instance, packaging could presumably only commence from the "end" pac site at the multiple insert.

Alternatively, relatively few of the pac sites may be cleaved and then subsequently initiate encapsidation. In this case, a number of full length plasmid molecules would also be encapsidated together with the novel band.

To test these predictions, P1 lysates were prepared on PAC600, PAC601, PAC602, R121600, R121601 and R121602. P1 DNA was prepared from these lysates by the modified procedure described in chapter 3. P1 DNA, from all the lysates, was restricted with EcoRI and HindIII.
(separately), the digests run on a 1% agarose gel and the gel transferred to nitrocellulose by Southern blotting (figure 4.9a). The filter was prepared with $^{32}$P-labelled pHWO01 (arg) to visualise plasmid sequences and to detect the expected overpackaging of arg by P1 (PAC601). The filter was autoradiographed and the result shown in figure 4.9b.

Clearly, apart from the large >25 kb fragment present in the HindIII digested tracks (see footnotes to table 4.4), the bands are derived either from homology with plasmid sequences (see figure 4.1a,b) or from homology to chromosomal Arg sequences. However, the novel 1.5 kb band present in HindIII digests of P1 (PAC600) P1 (PAC601) and P1 (PAC602) but not in HindIII restricted P1 (R121600), P1 (R121601) and P1 (R121602) tracks, is due to P1-directed cleavage at the integrated pac sites (figure 4.9b). In accordance with the model for the unidirectional nature of P1 packaging and with the observations of Sternberg and Hoess (1983) the other possible novel band (5.1 kb) was not detected.

By comparing the relative intensities of the novel 1.5 kb band to the intact 6.6 kb vector band, an estimate was made for the relative abundance of novel fragment to full length fragment in each lysate.

Size ratio of intact 6.6 kb vector to novel 1.5 kb band = 6.6:1.5 = 4.3:1

If equivalent amounts of both bands are present, because both share total homology with the probe, the ratio of band intensities should therefore be 4.3:1. Accordingly, the band intensities were measured for both novel bands and intact vector bands (figure 4.9b) and the ratio of vector band to novel band calculated as above. The results are shown in table 4.4.

Since the number of inserts at each location is known, it is possible to make predictions as to the expected ratios of novel 1.5 kb bands to intact vector. Consider, for example, the multiple insertions of pPAC600 (at his). About 4 copies of the plasmid are present (figure 4.7, table 4.3). Assuming that if more than one pac site is cleaved, during P1 lysis, only the cleaved pac site furthest downstream (with respect to the direction of packaging) will be able
to initiate encapsidation (since additional cleavage upstream of this pac would generate a molecule too small to be packaged). Also, assuming each pac site has an equal chance of being recognised during the P1 infection, and bearing in mind that packaging from an integrated pac site will generate only 1 novel band but up to 3 full length vector bands, the ratio of novel 1.5 kb fragment to full length vector fragment would be the following:

\[ n: (n - 1) + (n - 2) + (n - 3) \text{ etc. to } (n - n) \]

where \( n \) is the number of inserts.

For pPAC600, \( n = 4 \).

\[ \text{ratio of novel bands to full length vector} = 4: (4 - 1) + (4 - 2) + (4 - 3) + (4 - 4) = 4: 6 = 1: 1.5 \]

The measured ratio (Table 4.3) is 1: 1.8 novel 1.5 kb fragment to full length vector fragment for pPAC600. This value is in good agreement with the predicted value.

For pPAC601, there are about 6 inserts. The predicted ratio of novel band to vector band is therefore

\[ 6: (6 - 1) + (6 + 2) + (6 - 3) + (6 - 4) + (6 - 5) + (6 - 6) = 6: 15 = 1: 2.5 \]

The measured ratio was 1: 0.8 (table 4.3), suggesting that more novel band was present than expected. This indicates, perhaps, that packaging commences preferentially from "downstream" pac sites (with respect to the direction of packaging) which would mean that less full length vector DNA is packaged than might be expected.

pPAC602 is represented about 15 times in the chromosome (Table 4.3) so the predicted ratio of novel to vector bands is (assuming one site of insertion only)

\[ 15: (15 - 1) + (15 - 2) + (15 - 3) \text{ etc. to } (15 - 15) = 15: 105 = 1: 7 \]

The measured value was 1: 2.4 (table 3.4) so there is considerably more novel band than anticipated. Again, this may indicate that packaging prefers "downstream" pac sites.
Alternatively, in this instance, upstream pac sites may be used; 15 inserts of pPAC602, a 9 kb plasmid, totals about 135 kb. Therefore two pac-cleavage events from this region, one upstream and one downstream, may both result in production of a transducing particle since they could be separated by up to 100 kb, which is greater than the capacity of one P1 headful. If so, this would increase the ratio of novel band to vector band in the transducing DNA. Alternatively, if (as has been suggested before) there were two different sites of insertion and assuming half of the inserts occurred at each location, (i.e. about 7 at each site) the predicted ratio of novel to vector bands would be:

\[ \frac{7: (7 - 1) + (7 - 2) + (7 - 3) \text{ etc.}}{7 - 7} = 7: 21 = 1: 3 \]

This value is closer to the observed value of 1: 2.4.

Also of particular interest was the high intensity of the 7.0 kb arg band in the HindIII restricted P1 (PAC601) track (figure 4.9b, track 4), as compared to the vector band and the corresponding bands in the control P1 (R121601) track (figure 4.9b, track 10). Clearly, arg is overpackaged in the P1 (PAC601) lysate by about three-fold over the P1(R121601) lysate. This is in agreement with the transduction date (table 4.2) which showed that the transduction of Arg\(^+\), using the P1(PAC601) lysate, was three-fold greater than that obtained from the P1 (R121601) lysate.
FIGURE 4.9 ESTIMATION OF THE EFFICIENCY WITH WHICH THE INTEGRATED PAC SITES ARE RECOGNISED.

A. 1,2. his P1 (PAC600) DNA restricted with EcoRl (1) or HindIII (2).
3,4. P1 (PAC601) DNA restricted with EcoRl (3) or HindIII (4).
5,6. P1 (PAC602) DNA restricted with EcoRl (5) or HindIII (6).
7,8. P1 (R121600) DNA restricted with EcoRl (7) or HindIII (8).
9,10. P1 (R121601) DNA restricted with EcoRl (9) or HindIII (10).
11,12. P1 (R121602) DNA restricted with EcoRl (11) or HindIII (12).

B. Autoradiogram of the gel, shown above, transferred to nitrocellulose and probed with $^{32}$P-labelled pHW001 (arg). Bands due to arg homology are 7.0 kb and >20 kb (partial digestion product) in HindIII restricted tracks and 20 kb, 3.1 kb and 0.7 kb in EcoRl restricted tracks (see also figure 3.6B). Novel sized fragments (1.5 kb) generated by cleavage at the integrated pac sites are shown by the unlabelled arrow (see text for full discussion). In addition to the arg-homologous bands, the tracks also contain bands due to vector plasmid homology (see figures 4.6A, B and C).
Figure 4:9

A

B

1 2 3 4 5 6 7 8 9 10 11 12

1 2 3 4 5 6 7 8 9 10 11 12

11.3 9.0 5.7 3.5

11.3 9.0 5.7 3.5

20 7.0 6.6 3.1 1.8 0.7
TABLE 4.4  RATIO OF NOVEL 1.5 kb FRAGMENT TO INTACT 6.6 kb VECTOR BAND

<table>
<thead>
<tr>
<th></th>
<th>P1</th>
<th>P1</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(PAC600)</td>
<td>(PAC601)</td>
<td>(PAC602)</td>
</tr>
<tr>
<td>INTENSITY OF 6.6 kb VECTOR</td>
<td>10.53</td>
<td>16.89</td>
<td>10.31</td>
</tr>
<tr>
<td>INTENSITY OF 1.5 kb NOVEL FRAGMENT</td>
<td>1.33</td>
<td>4.99</td>
<td>0.99</td>
</tr>
<tr>
<td>INTENSITY RATIO OF VECTOR: NOVEL BAND</td>
<td>7.9:1.0</td>
<td>3.4:1.0</td>
<td>10.4:1.0</td>
</tr>
<tr>
<td>RATIO OF VECTOR FRAGMENT: NOVEL FRAGMENT</td>
<td>1.83:1</td>
<td>0.79:1</td>
<td>2.41:1</td>
</tr>
</tbody>
</table>

FOOTNOTES TO TABLE 4.4

Ratio of vector: novel fragment was estimated by dividing the measured intensity ratio by that predicted from the relative sizes of the two fragments.
SECTION 4.5
SUMMARY AND DISCUSSION

In this chapter, the suitability of recombinant pac vehicle plasmids has been studied as a means of targeting P1 pac sites into precisely defined chromosomal locations; three criteria were stated in section 4.1, all of which must be met for the method to be considered suitable. The first was that the strategy was suitable for directing pac integration into defined chromosomal locations. Mapping the insertions was carried out for pPAC601 and pR121601. The evidence suggested that the plasmids had indeed integrated at \textit{ilv} due to homologous recombination between plasmid and chromosomal \textit{ilv} sequences. Likewise, the insertion sites of pPAC602 and pR121602 were mapped and the genetic evidence indicated that integration had occurred by homologous recombination at \textit{trp}. The physical evidence, however, suggested that both pPAC602 and pR121601 may have each integrated at two separate locations; one at \textit{trp} and one elsewhere. The sites of pPAC600 and pR121600 insertion were not mapped genetically, but the physical evidence in section 4.3 is not inconsistent with the hypothesis that integration had occurred by homologous recombination at the predicted chromosomal location. Thus, it seemed that the first criterion (section 4.11) was met in that the plasmids tested had probably integrated into the expected chromosomal sites. However, an unexpected complication was the multimerisation of inserted plasmid sequences.

The mechanism which gave rise to the multimerisation of the inserted plasmids is not known. One possible mechanism would be by a form of unequal crossing over between the direct repeats (caused by the plasmid insertion) located on daughter chromosomes within the cell (figure 4.10) (Roth and Schmid 1981).

Perhaps the most mysterious and interesting observation arising from these experiments was the suggestion that the number of plasmid inserts present in the host cell chromosome may have been dependent on the location of the integration event (assuming that the plasmids had all integrated by homologous recombination, since strictly speaking, only the plasmids at \textit{ilv} and \textit{trp} had been shown with any
A: depicts a pac-vehicle plasmid integrated into the host chromosome and which is present on both daughter chromosomes following replication. (Blocks represent the duplications generated at integration which flank the plasmid sequences.) Homologous recombination between the duplicated sequences, as indicated by the arrows, (A) will generate a tandem duplication of the plasmid in one daughter chromosome and a deletion of the plasmid from the other (B).
great degree of certainty to have integrated by homology and even then, pR121601 and pPAC602 may have inserted into two different locations). The reason for the position-dependence of multimerisation is not known.

Multimerisation of pPAC601 and pR121601 could have been responsible for the decreased Ilv⁺ transduction observed when PAC601 and R121601 were the donor strains (section 4.3). Possibly the insertion of 6 copies of the plasmids (about 70 kb of DNA), which would contain substantial regions of non homology with the recipient chromosome, had resulted in reduced recombination at this location. Alternatively (or perhaps in addition to the above) the sequences normally situated to the side of ilv distal to oriC, which would be required for the integration of ilv in the recipient cell, would have been moved some 70 kb further from oriC by the insertion of 6 copies of the plasmids. This would reduce the cotransduction of oriC with sequences flanking ilv and so perhaps reduce transduction of ilv (see chapter 6).

The second and third criteria stated in section 4.1, required that the integrated pac sites be efficiently utilised and would direct the encapsidation of adjacent markers to about 50-fold greater levels than normal. Neither of these two criteria have been met fully; certainly, some but not all of the integrated pac sites have been recognised and cleaved, but the efficiency with which the pac sites are used is difficult to estimate because of the presence of multiple copies of the pac vehicles. The overpackaging and hence increased transduction of markers to one side of pPAC601 have been measured; three markers, Rha⁺, ArgH⁺ and PurDH⁺ have been shown to be packaged and transduced at about 3 fold greater values than normal. Likewise, PurF⁺ and His⁺ were transduced with 3 fold greater efficiency when using the P1 (PAC602) lysate when compared with the P1 (R121602) lysate (section 4.3, Masters 1977). This obviously does not approach the 50-fold levels of increased transduction which have previously been reported (Sternberg et al. 1984, Chesney and Scott 1979).

The experiments reported in this chapter suggest that the use of pac-vehicle plasmids is probably not a good method for directing the
overpackaging of specific chromosomal sequences into transducing particles. Therefore, since this approach apparently does not greatly improve the yield of transducing particles, this method would not be of use in the role for which it was devised, i.e. as a means of improving the yields of specific transducing particles so that the fate of individual markers could be followed after their entry into recipient cells (see section 4.1). The reasons for the apparent failure of this method are not clear. Perhaps the presence of several, closely spaced pac sequences in some way interferes with, or inhibits P1 packaging.

In any case, the use of hybrid pac-containing transposons or λpac vehicles may be a better alternative to pac-vehicle plasmids, for obtaining insertions of pac into the chromosome; presumably only single insertions would then occur in any one location. Once the site of insertion had been mapped, and assuming the insertion was stable, the host strain may be more suitable to direct the P1-mediated overpackaging of markers adjacent to the chromosomal pac.
CHAPTER 5

EVENTS IN THE RECIPIENT CELL FOLLOWING

P1-MEDIATED TRANSDUCTION
The previous two results chapters have been concerned with events in donor cells which influence P1-mediated transduction. This chapter, however, concentrates on events within recipient cells which occur after the introduction of transducing DNA. The marker selectivity of transduction clearly arises from events within recipient cells (Newman and Masters, 1980, Newman, 1982, Masters et al. 1984, Chapter 3, this thesis). Hence studying the fate of transducing DNA (Sandri and Berger 1980a, b) and its effect on the recipient cell, may provide information as to how transductional selectivity arises. Bender and Sambucetti (1983) published an account of the behaviour of exponentially growing Klebsiella cells, in the period immediately following transduction. From their data, they concluded that immediately after transduction cells grew without dividing (i.e. exhibited filamentous growth) for some 6-8 generations before commencing normal division. Bender and Sambucetti (1983) proposed that transductional recombination caused a recombination induced suppression of cell division. In support of this hypothesis it was shown that filamenting cells, purified from a transduced culture, exhibited 1000 fold higher transduction frequencies (as measured by the number of transductants/viable cell) than the culture as a whole.

These observations were of particular interest because, if transductional recombination elicits the same response in E.coli, i.e. results in filamentous growth, this may give an insight into the intracellular processes which accompany transductional recombination and perhaps give an indication of the mechanism of transductional recombination (this will be discussed below). In addition enriching for filamenting cells, after transduction, could provide a simple means of improving the efficiency with which transductants are recovered; this would be of considerable practical use when trying to recover transductants receiving "leaky" or poorly transduced markers.

Initial experiments were carried out with E.coli to repeat Bender and Sambucetti's experiments (described below), but it became clear that after transduction, E.coli was not behaving in the same
fashion as was reported for Klebsiella. The observations derived from E.coli prompted a re-evaluation of Bender and Sambucetti's (1983) data and it is necessary to give a fairly detailed account of their experimental evidence and to discuss, critically, their conclusions.

As already mentioned, Bender and Sambucetti (1983) investigated the effects of P1 transduction on exponentially growing cultures of Klebsiella. They found that transductants did not increase in number for 3-4 hours (6-8 generations) after P1 adsorption and injection of transducing DNA. Transductants also exhibited a transient (15-20 minutes) resistance to penicillin (5000 µg/ml) in the period immediately following transduction. The remainder of the culture, however, including cells which became lysogenised with P1, grew exponentially from the time of P1 adsorption and exhibited penicillin sensitivity at all times. Bender and Sambucetti (1983) explained these results by proposing there was an initial 15-20 minutes post-transductional lag before growth of transductants, hence the period of penicillin resistance, followed by about 6 generations of filamentous growth, resulting in renewed penicillin sensitivity, despite no increase in the number of transductants for a further 3-4 hour period. This hypothesis was supported by transducing exponentially growing Klebsiella with P1, allowing 3 hrs growth, post transduction, and then purifying filamenting cells on sucrose gradients. Such purified filaments showed 1000 fold greater transduction frequencies than the normal culture, consistent with the hypothesis that transductants filamented. However, an analogous experiment was also performed where no time was allowed for growth to occur after the addition of phage. Thus in this instance filaments could not have formed after transduction. When filaments were purified from this culture and assayed for transduction, as above, a similar 1000 fold enrichment of transduction was obtained. Despite these results Bender and Sambucetti (1983) neglect the possibility that pre-formed filaments, already present in the culture before P1 was added, are simply better recipients for transduction; a trivial
explanation for this observation may be that filaments are much larger targets for P1 phage, hence more likely to recover transducing particles and form transductant colonies. Bender and Sambucetti (1983), however, interpreted these data as indicating that transductional recombination inhibits cell division in E.coli. The observations presented in their publication, however, do not prove this hypothesis. Bender and Sambucetti (1983) also speculated that the SOS response was induced by degradation of non-recombined transducing DNA and that SOS-mediated inhibition of cell division was responsible for transduced cells filamenting.

It is necessary to give a brief summary of the SOS response such that the relationship between SOS induction and filamentation is clear:

The SOS system of E.coli involves the repression of a number of unlinked genes by a protein repressor molecule, the product of the *lexA* gene (Mount et al., 1972, Gudas and Pardee 1975, Little and Mount 1982). During normal exponential growth, the *lexA* repressor is envisaged to efficiently repress these unlinked genes, which include: *recA*, *lexA* itself, *uvrAB*, *sfiA* plus a number of other genes (Brent and Ptashne 1980, Gudas and Pardee 1975, Huisman and d'Ari 1981, Little et al. 1981, Little and Mount, 1982). However, interruption of normal DNA replication, the presence of DNA-degradation products, or UV damage to DNA (or a number of other treatments) constitute an "inducing" signal which acts, either directly or indirectly, on RecA protein: RecA protein becomes "activated" and either protolytically cleaves or catalyses the autodigestion of *lexA* and *λ* repressors (Little et al., 1980, 1981. Horii et al. 1981, Little and Mount 1982, Yarmolinsky and Stevens 1983, Little 1984). Removal of *lexA* repressor by activated RecA protein allows derepression of *lexA* repressed genes and the resultant effects on the cell include: inhibition of division, induction of *λ* prophage, induction of stable DNA replication, induction of RecA protein, hyper-recombination, increased mutability and enhanced capacity for DNA repair (Little and Mount, 1982). The removal of the inducing signal allows *lexA* repressor to accumulate in the cell, which then returns to normal growth and
division, over some 30-60 minutes (Little and Hanawalt 1977). As mentioned above, one of the features of the response is the inhibition of cell division, resulting in filamentous growth. Filamentation is dependent on the sfiA gene, which as already stated, is lexA repressed during normal growth. SOS induction, however, derepresses sfiA, the product of which accumulates in the cell and which is thought to interact with theftsZ (sfiB) gene product to inhibit cell division (Huisman and d’Ari 1981, Lutkenhaus 1983). Once SOS induction is alleviated, sfiA product is degraded by Lon protease activity within the cell, and normal cell division resumes (Mizusawa and Gottesman 1983).

Bender and Sambucetti (1983) proposed that the degradation of unrecombined transducing DNA, present in transduced cells as a by-product of recombination, could provide an "inducing" signal to activate RecA protein and so elicit the SOS response; this being the cause of transductional filamentation. However, aside from the objection already mentioned above, their observations are not consistent with the hypothesis that transductional recombination in Klebsiella results in filamentation via SOS induction. Work with E. coli indicates that filamentous cells have severely reduced viability on solid media (Donachie and Hobbs, 1967, Donachie et al. 1968). Hence if new transductants grow for 6 generations (3-4 hours) without dividing, the resultant filaments should show greatly reduced viability on selective plates and so the apparent transduction frequency should decrease in samples plated over that 3-4 hour period due to plating death. However, Bender and Sambucetti (1983) observe no such decrease. In addition, when SOS induced filaments do commence division, instead of dividing once every generation time to give two daughters, they divide rapidly into normal sized cells; this is simply because the filament has achieved many times the initiation mass required for initiation of chromosomal replication. Hence rapid initiations occur, and the filaments quickly accumulate many copies of the entire chromosome, so can divide into normal sized cells (Donachie 1968, K. Begg, unpublished observations). Accordingly, if transductants filament via SOS induction, once division
commences, the number of transductants should transiently increase faster than the rate of increase of non-transduced cells. After this initial burst of division, transductants should then increase in number exponentially, at the same rate as the non-transduced cells. Again, this effect is not observed by Bender and Sambucetti (1983).

A note of caution must be added, however, in assuming that Klebsiella behaves in the same manner as E.coli, but in view of the very close relationship between the two organisms (Breed and Murray, 1955), it seems valid to assume that data derived from work on E.coli division is applicable to Klebsiella.

In spite of the above criticism of the conclusions drawn from their observations, Bender and Sambucetti (1983) clearly showed that Klebsiella transductants did not increase in number immediately after P1 transduction and also that enriching for Klebsiella filaments (either formed before or after transduction) following transduction with P1 results in 1000 fold increase in transduction frequency; their observations, however, could be most economically interpreted to mean that filaments are better recipients for transduction. One trivial hypothesis to explain this has already been mentioned; filaments are much larger than normal sized cells so would constitute larger targets for phage adsorption. An alternative hypothesis would be that filamenting cells are in a more recombinogenic physiological state. For example, SOS induced cells show increased levels of RecA protein and increased genetic exchanges opposite UV-generated lesions in DNA (Rupp 1971, LLoyd 1978, Little and Mount 1982). Such cells, of course, would be filamenting due to sfiA mediated inhibition of cell division. In support of this suggestion, work by Yarmolinsky and Stevens (1984) showed, by fusing in frame a lexA repressed promoter to the assayable lacZ gene, that in any culture a certain proportion of cells are SOS induced. If such cells, containing UV-induced repairable lesions in their DNA received transducing DNA, then levels of transductional recombination may be increased, since it is clear that UV-damage to DNA of transducing particles, or recipient cells, prior to transduction results in enhanced transduction (Benzinger and
Hartman 1962, Arber 1960, Newman and Masters 1980). If this is the explanation for increased transduction of filamenting cells, the marker selectivity of transduction, in a population of purified filaments, should be reduced; Masters and Newman (1980), showed that UV-damage to recipient cells or transducing DNA, resulted in selective stimulation of markers which were poorly transduced with unirradiated lysates or cells (see Chapter 1).

The experiments described in this Chapter were carried out to establish the following:

1. Do E.coli transductants filament or are E.coli filaments better recipients for transduction than normal sized cells?
2. Do E.coli transductants show a post-translational lag before increasing in number and if so what is the cause of the lag?

There was already published data suggesting that E.coli transductants do indeed exhibit a lag before increasing in number. Sandri and Berger 1980a, reported a lag of about 180 minutes before the number of Arg\(^+\) or Thr\(^+\) transductants increased following P1-mediated transduction of E.coli.

SECTION 5.2 PURIFICATION OF FILAMENTING CELLS FROM EXPONENTIALLY GROWING CULTURES OF E.coli FOLLOWING P1 MEDIATED TRANSDUCTION

It was decided to repeat the experiments of Bender and Sambucetti (1983) to establish whether E.coli filaments, formed after P1 transduction, were enriched for transductants.

Exponentially growing MM303 cells were transduced with P1, as described in Chapter 2. After phage adsorption, cells were diluted into L-broth citrate (10 mM). Immediately after dilution, and at 30 minute intervals thereafter, 10 ml samples were taken from the culture and filamentous cells were enriched by filtering the sample, under vacuum, through Millipore membrane filters with pore size large enough to allow passage of normal sized cells, but too small to permit filamentous cells to pass through also; pore
sizes of 12µ, 10µ, 8µ, 5µ and 3µ were tried. The filters were then washed with 200-300 ml filtered phage buffer. Cells were resuspended from the filter by transferring the filter, aseptically, to a flask containing 10 ml L-broth and shaking gently for 2-3 minutes. Cells were recovered from the filtrate by passage through a 0.45 pore diameter Millipore filter, which retains all cells and cells were resuspended from this filter as described above. Transduction frequencies for Arg⁺ were measured in samples taken from: the original culture, from the filtrate and from the purified filamentsing cells. The proportion of filamentous cells in each of the above was estimated by viewing samples by phase contrast light microscopy and counting the proportion of filaments. The results are presented in Table 5.1.

Passing the cultures through 3µ and 5µ membrane filters did not enrich filaments much. Almost all cells were retained by the filter, so the proportion of filaments within the culture remained low (Table 5.1). (Filaments are defined in this instance, as: abnormally large non-dividing cells of greater than 2 cell lengths.) However, the 8µ, 10µ and 12µ pore diameter filters were more successful and only small numbers of cells were retained; viable counts/ml were reduced to about 10⁻³ of the unfiltered cultures. However, after filtration, the proportion of filaments in the population of cells retained by the filter was increased by about 400 fold (Table 5.1). The Arg⁺ transduction frequencies observed for the cells retained by the filter, however, was enriched by only 5-10 fold over the unfiltered cultures. Although Bender and Sambucetti (1983) give no indication of how successful their procedure was in purifying filaments, it seems that in contrast to their observations, enriching for E.coli filaments does not proportionately enrich for transductants. Hence it seems unlikely that all transductants filament.

Other evidence implies that not all (if any) transductants filament. Since samples were taken at 0, 30 and 60 minutes post transduction, if transduction caused cells to filament, then the filtration procedure should result in more efficient enrichment for transductants at each subsequent time point, because
<table>
<thead>
<tr>
<th>Table 5.1</th>
<th>PURIFICATION OF FILAMENTOUS CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t = 0</td>
</tr>
<tr>
<td></td>
<td>Arg⁺</td>
</tr>
<tr>
<td>A 153</td>
<td>2.2 x 10⁸</td>
</tr>
<tr>
<td>3μ B 41</td>
<td>0.5 x 10⁸</td>
</tr>
<tr>
<td>C 162</td>
<td>2.3 x 10⁸</td>
</tr>
<tr>
<td>A 182</td>
<td>2.4 x 10⁸</td>
</tr>
<tr>
<td>5μ B 26</td>
<td>0.3 x 10⁸</td>
</tr>
<tr>
<td>C 171</td>
<td>2.0 x 10⁸</td>
</tr>
<tr>
<td>A 130</td>
<td>2.3 x 10⁸</td>
</tr>
<tr>
<td>8μ B 10</td>
<td>1.3 x 10⁵</td>
</tr>
<tr>
<td>C 122</td>
<td>2.1 x 10⁸</td>
</tr>
<tr>
<td>A 123</td>
<td>2.0 x 10⁸</td>
</tr>
<tr>
<td>10μ B 0.7</td>
<td>1.1 x 10⁵</td>
</tr>
<tr>
<td>C 131</td>
<td>2.3 x 10⁸</td>
</tr>
<tr>
<td>A 127</td>
<td>1.8 x 10⁸</td>
</tr>
<tr>
<td>12μ B 0.3</td>
<td>0.7 x 10⁶</td>
</tr>
<tr>
<td>C 141</td>
<td>2.2 x 10⁸</td>
</tr>
</tbody>
</table>
Table 5.1 (continued)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Arg⁺/VC</th>
<th>Arg⁺/VC</th>
<th>%</th>
<th>Methylation of Filaments of Filaments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NT</td>
<td></td>
<td></td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>3μ B</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>NT</td>
<td></td>
<td></td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>5μ B</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>A</td>
<td>37</td>
<td>2.1 x 10⁸</td>
<td>1.7 x 10⁻⁷</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8μ B</td>
<td>0.3</td>
<td>1.1 x 10⁵</td>
<td>2.7 x 10⁻⁶</td>
<td>69.0</td>
<td>345</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>42</td>
<td>2.2 x 10⁸</td>
<td>1.9 x 10⁻⁷</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>50</td>
<td>2.6 x 10⁸</td>
<td>1.9 x 10⁻⁷</td>
<td>0.2%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10μ B</td>
<td>0.6</td>
<td>0.8 x 10⁵</td>
<td>7.5 x 10⁻⁶</td>
<td>79.0</td>
<td>395</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>43</td>
<td>1.9 x 10⁸</td>
<td>2.2 x 10⁻⁷</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>32</td>
<td>2.2 x 10⁸</td>
<td>1.4 x 10⁻⁷</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12μ B</td>
<td>0.3</td>
<td>0.9 x 10⁵</td>
<td>3.3 x 10⁻⁶</td>
<td>77</td>
<td>385</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>34</td>
<td>2.1 x 10⁸</td>
<td>1.6 x 10⁻⁷</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A = culture before filtering
B = cells resuspended from test filter
C = cells passing through test filter
Arg⁺ = no. of transductants per 100 μl culture
VC = no. of viable cells/ml culture
t = time in minutes, after transduction at which the sample was taken
Table 5.1 (continued)  

<table>
<thead>
<tr>
<th>t = 30</th>
<th>Arg+</th>
<th>VC</th>
<th>Arg+</th>
<th>% Enrichment of Filaments</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Arg+</td>
<td>VC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3μ B</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5μ B</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>70</td>
<td>2.5 x 10^8</td>
<td>2.8 x 10^{-7}</td>
<td>0.2</td>
</tr>
<tr>
<td>8μ B</td>
<td>0.5</td>
<td>1.0 x 10^5</td>
<td>5.0 x 10^{-6}</td>
<td>74.0</td>
</tr>
<tr>
<td>C</td>
<td>80</td>
<td>2.1 x 10^8</td>
<td>3.8 x 10^{-7}</td>
<td>0.0</td>
</tr>
<tr>
<td>A</td>
<td>82</td>
<td>2.1 x 10^8</td>
<td>3.9 x 10^{-7}</td>
<td>0.2</td>
</tr>
<tr>
<td>10μ B</td>
<td>0.5</td>
<td>0.9 x 10^5</td>
<td>5.5 x 10^{-6}</td>
<td>78.0</td>
</tr>
<tr>
<td>C</td>
<td>73</td>
<td>1.9 x 10^8</td>
<td>3.8 x 10^{-7}</td>
<td>0.0</td>
</tr>
<tr>
<td>A</td>
<td>61</td>
<td>1.9 x 10^8</td>
<td>3.2 x 10^{-7}</td>
<td>0.2</td>
</tr>
<tr>
<td>12μ B</td>
<td>0.5</td>
<td>0.6 x 10^5</td>
<td>8.3 x 10^{-6}</td>
<td>73.0</td>
</tr>
<tr>
<td>C</td>
<td>57</td>
<td>1.6 x 10^8</td>
<td>3.5 x 10^{-7}</td>
<td>0.1</td>
</tr>
</tbody>
</table>
FIGURE 5.1 TRANSDUCTION OF MM303 to Arg$^+$

■ = viable counts/ml

▲ = Arg$^+$ transductants (per 200 µl culture)

See text for discussion.
Figure 5.1

![Graph showing the relationship between time and number of transductants/viable counts per ml](image_url)
<table>
<thead>
<tr>
<th>t (minutes)</th>
<th>Viable counts/ml</th>
<th>Arg⁺ Transductants</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$9.1 \times 10^6$</td>
<td>64</td>
</tr>
<tr>
<td>15</td>
<td>$1.5 \times 10^7$</td>
<td>69</td>
</tr>
<tr>
<td>30</td>
<td>$2.2 \times 10^7$</td>
<td>70</td>
</tr>
<tr>
<td>45</td>
<td>$2.7 \times 10^7$</td>
<td>68</td>
</tr>
<tr>
<td>60</td>
<td>$4.5 \times 10^7$</td>
<td>66</td>
</tr>
<tr>
<td>75</td>
<td>$6.9 \times 10^7$</td>
<td>75</td>
</tr>
<tr>
<td>90</td>
<td>$1.2 \times 10^8$</td>
<td>88</td>
</tr>
<tr>
<td>105</td>
<td>$1.6 \times 10^8$</td>
<td>128</td>
</tr>
<tr>
<td>120</td>
<td>$2.5 \times 10^8$</td>
<td>192</td>
</tr>
<tr>
<td>135</td>
<td>$1.9 \times 10^8$</td>
<td>256</td>
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<tr>
<td>150</td>
<td>$4.7 \times 10^8$</td>
<td>272</td>
</tr>
<tr>
<td>165</td>
<td>$5.8 \times 10^8$</td>
<td>504</td>
</tr>
<tr>
<td>180</td>
<td>$1.0 \times 10^9$</td>
<td>960</td>
</tr>
<tr>
<td>195</td>
<td>$1.2 \times 10^9$</td>
<td>1280</td>
</tr>
<tr>
<td>210</td>
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<td>134</td>
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<tr>
<td>225</td>
<td>$3.9 \times 10^9$</td>
<td>1936</td>
</tr>
<tr>
<td>240</td>
<td>$9.5 \times 10^9$</td>
<td>200</td>
</tr>
</tbody>
</table>
FIGURE 5.2  TRANSDUCTION OF MM303 TO $\text{Trp}^+$

$\circ = \text{viable counts/ml}$

$\bullet = \text{Trp}^+ \text{ transductants/200 } \mu\text{l culture}$
Figure 5.2

[Graph showing the relationship between number of transductants and viable counts over time in minutes.]
<table>
<thead>
<tr>
<th>t</th>
<th>Viable counts/ml</th>
<th>TRP⁺ Transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1.6 \times 10^8$</td>
<td>107</td>
</tr>
<tr>
<td>15</td>
<td>$1.8 \times 10^8$</td>
<td>118</td>
</tr>
<tr>
<td>30</td>
<td>$2.8 \times 10^8$</td>
<td>111</td>
</tr>
<tr>
<td>45</td>
<td>$3.2 \times 10^8$</td>
<td>104</td>
</tr>
<tr>
<td>60</td>
<td>$4.3 \times 10^8$</td>
<td>123</td>
</tr>
<tr>
<td>75</td>
<td>$5.4 \times 10^8$</td>
<td>131</td>
</tr>
<tr>
<td>90</td>
<td>$9.2 \times 10^8$</td>
<td>160</td>
</tr>
<tr>
<td>105</td>
<td>$1.3 \times 10^9$</td>
<td>210</td>
</tr>
<tr>
<td>120</td>
<td>$1.6 \times 10^9$</td>
<td>260</td>
</tr>
<tr>
<td>135</td>
<td>$2.5 \times 10^9$</td>
<td>404</td>
</tr>
<tr>
<td>150</td>
<td>$3.0 \times 10^9$</td>
<td>592</td>
</tr>
<tr>
<td>165</td>
<td>$4.4 \times 10^9$</td>
<td>768</td>
</tr>
</tbody>
</table>
as the filaments became larger they should be more efficiently retarded by the membranes. In fact the opposite effect is observed; at subsequent time points the number of transductants decreases, concomitantly with the dilution of the culture. This does indicate, however, in agreement with Bender and Sambucetti (1983) that there is a lag immediately following transduction, before transductants increase in number.

The above observations indicate that filamentous cells are 5-10 fold better recipients for transduction. This could be accounted for in terms of the size of filaments; larger cells are simply better targets for phage adsorption, and so more likely to receive a transducing particle.

SECTION 5.3 DO E.coli TRANSDUCTANTS INCREASE IN NUMBER IMMEDIATELY AFTER TRANSDUCTION?

To confirm that E.coli transductants show a post-transductional lag before increasing in number, MM303 cells, growing exponentially in L-broth at 37°C were transduced, as described before. Samples were taken at timed intervals after transduction and the numbers of viable counts and Arg⁺ transductants were scored. The above experiment was also carried out, separately, scoring Trp⁺ transductants and viable counts. The results (Tables 5.2, 5.3 and figures 5.1 and 5.2) show clearly, in both the above experiments that there was a lag before the number of transductants increased, whilst the viable counts of the culture increased exponentially from the time the transduction mixture was diluted into L-broth citrate. However, in contrast to the data of Bender and Sambucetti (1983) who reported that the duration of the lag was marker independent, it appeared that Trp⁺ transductants began to increase in number after about 60 minutes lag but that Arg⁺ did not increase until about 90 minutes. In addition, the duration of the lag was considerably shorter than the 3-4 hour delay reported by Bender and Sambucetti (1983).

To confirm that Trp⁺ transductants increased before Arg⁺, the above time-course transduction of exponentially growing MM303 was repeated but in this case Arg⁺ and Trp⁺ transductants were
FIGURE 5.3  TRANSDUCTION OF MM303 TO Arg⁺, Trp⁺

A. ■ = viable counts
   ● = Arg⁺ transductants
   ▲ = Trp⁺ transductants

B. Graph in 5.3A replotted showing the number of transductants per initial number against time. The 34 minute lag between the increase of Trp⁺ and Arg⁺ transductants can be clearly seen.
5.3 contd.

![Graph B](image)

- Y-axis: Number of Transductants per Initial Number
- X-axis: Time in Minutes
- 34 MINS
Table 5.4

TRANSDUCTION OF MM303 TO TRP\(^+\), ARG\(^+\)

<table>
<thead>
<tr>
<th>t (minutes)</th>
<th>Viable counts/ml</th>
<th>Arg(^+)</th>
<th>Trp(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(4.9 \times 10^7)</td>
<td>178</td>
<td>118</td>
</tr>
<tr>
<td>15</td>
<td>(5.8 \times 10^7)</td>
<td>188</td>
<td>117</td>
</tr>
<tr>
<td>30</td>
<td>(1.3 \times 10^8)</td>
<td>193</td>
<td>119</td>
</tr>
<tr>
<td>45</td>
<td>(2.1 \times 10^8)</td>
<td>219</td>
<td>124</td>
</tr>
<tr>
<td>60</td>
<td>(3.3 \times 10^8)</td>
<td>178</td>
<td>120</td>
</tr>
<tr>
<td>75</td>
<td>(5.9 \times 10^8)</td>
<td>206</td>
<td>152</td>
</tr>
<tr>
<td>90</td>
<td>(5.6 \times 10^8)</td>
<td>224</td>
<td>174</td>
</tr>
<tr>
<td>105</td>
<td>(9.4 \times 10^8)</td>
<td>234</td>
<td>180</td>
</tr>
<tr>
<td>120</td>
<td>(1.3 \times 10^9)</td>
<td>264</td>
<td>368</td>
</tr>
<tr>
<td>135</td>
<td>(1.8 \times 10^9)</td>
<td>376</td>
<td>464</td>
</tr>
<tr>
<td>150</td>
<td>(-)</td>
<td>425</td>
<td>608</td>
</tr>
<tr>
<td>165</td>
<td>(3.7 \times 10^9)</td>
<td>688</td>
<td>840</td>
</tr>
<tr>
<td>180</td>
<td>(5.6 \times 10^9)</td>
<td>864</td>
<td>992</td>
</tr>
</tbody>
</table>
scored from the same transduced culture (Table 5.4; figure 5.3a). When the data are plotted as the number of transductants/initial number against time (Figure 5.3b) the difference in lag is clearly observed. Trp<sup>+</sup> transductants increase some 34 minutes before Arg<sup>+</sup>.

**THE SEGREGATION MODEL**

The marker dependence of the lag before transductants increase in number can be explained very simply, by considering the nature of the *E. coli* DNA replication and division cycle:

The time required to replicate the entire *E. coli* chromosome (called the C-time) = the time taken for replication forks to travel from oriC to terC = 41 minutes at 37°C (Cooper and Helmsstetter 1968). After the replication fork arrives at the terminus, a delay (the D-time) of about 20 minutes is observed before the cell divides. A division, therefore, always follows an initiation of replication by C+D minutes (= 60 mins at 37°C). Cells in exponential growth, dividing every 20-30 minutes must therefore contain multiple replication forks (Cooper and Helmsstetter 1968). If an exponentially growing, non synchronous, population of *E. coli* cells is considered, replication forks are evenly distributed over the chromosomes of the population. Consider transductional recombination events occurring within exponentially growing cells which will result in integration of wild type alleles adjacent to oriC. Before such transductants can give rise to two wild-type daughter cells, the transduced DNA must be duplicated. This will happen first, in the case of oriC transductants, for cells which have initiated immediately after the transductional event (i.e. the replication fork is immediately upstream of the recombination site). Then, before two recombinant daughter cells can be produced, replication and division must be completed, i.e. the replication fork must travel from oriC to terC (= C-time) and then division occurs after the D-time, i.e. oriC transductants will increase in number after a minimum of 60 minutes (C+D). However, if in other cells transductional recombination events occur close to terC, the minimum time before the transductants give rise to two wild type
daughters will be shorter, and approach the D-time. This will occur in cells in which the replication cycle is nearly complete (i.e. replication forks are immediately upstream from the site of recombination) and so take very little time to replicate through the wild type allele and reach terC. Division would then occur after the D-time (20 minutes). (Note: this assumes that P1-transductional recombination results in a double strand replacement reaction and not a single strand exchange. This will be discussed later. Sandri and Berger (1983).) Hence, transductants should increase in numbers in a marker dependent manner, with terC markers first, followed in succession, by markers replicated late, midway and early in the chromosomal replication cycle. In other words, the delay before transductants increase in number is due to the time taken to segregate wild type and mutant alleles to daughter cells; the delay will therefore be referred to as the segregation lag. If this above model is correct, the difference in segregation lags between Trp⁺ and Arg⁺ transductants can be predicted in the following manner. arg and trp are situated at map positions of 89.5 and 27.6 respectively on the E.coli map (Bachman 1983). Thus replication forks at arg are 5.5 map units downstream from oriC and those at trp are 4 minutes upstream from terC. The segregation model predicts that the difference in segregation lags between trp (first) and arg (second) will be equal to the time taken for replication forks to pass from arg to trp.

Velocity of replication forks at 37°C =

\[
\frac{50 \text{ (distance in map units from oriC to terC)}}{41 \text{ minutes (C-time at 37°C)}} = 1.22 \text{ map units/minutes}
\]

Separation of arg and trp in map units = 41.5 map units

\[
time \text{ for replication forks to travel from arg to trp} = 41.5 \times 1.22 = 34 \text{ minutes}
\]

The measured difference in segregation lag between Trp⁺ and Arg⁺ transductants = 34 minutes (figure 5.3b).

The experimental data are thus in total agreement with the
predicted difference in segregation lags between \( \text{Trp}^+ \) and \( \text{Arg}^+ \) transductants.

SECTION 5.4 TESTING THE SEGREGATION MODEL

The segregation model predicts that segregation lags depend on the chromosomal position of the selected markers with transductants receiving markers replicated late in the cycle increasing before those replicated midway and early (see Section 5.3). The model can thus be tested by establishing the segregation lags of transductants for markers at different locations around the \( \text{E.coli} \) chromosome. In addition if the proposed segregation model is correct then cell growth, and transduction, in conditions which alter the velocity of \( \text{E.coli} \) replication forks, and hence C-time, should alter segregation lags in a predictable manner. C-time can be altered in the following way:

The chromosomal replication velocity in thymine auxotrophs was shown to be determined by the concentration of thymine in growth media (Pritchard and Zaritsky 1970, Zaritsky and Pritchard 1971, Begg and Donachie 1978). The growth rate of a culture in limiting thymine is independent (within certain limits) of replication velocity, so cells growing in limiting thymine which have a decreased replication velocity show an increased origin to terminus ratio i.e. compensate for increased C-time by more replication forks per dividing cell (Zaritsky and Pritchard 1971). Since initiation is dependent on cell mass, such cells exhibit increased cell mass (Donachie 1968, Pritchard and Zaritsky 1971, Zaritsky and Pritchard 1971, Begg and Donachie 1978). Pritchard and Zaritsky (1970) derived a formula for the relationship linking mean cell mass, \( \bar{M} \), of a culture growing at steady state, with the C-time, D-time and the generation time \( \tau \)

\[
\bar{M} = k2 \frac{(C+D)}{\tau}
\]

where \( k \) is a constant. Since median cell volume is proportional to \( \bar{M} \) (Begg and Donachie, 1978), since alteration of thymine concentration does not affect D (within certain limits) in Thy strains and since \( \tau \) is also unaffected by thymine limitation (Pritchard and Zaritsky (1970), Zaritsky and Pritchard (1971))
the median cell volume of cultures grown at steady state in limiting thymine must be proportional to C-time. This provides a simple means to test the segregation model: a suitably marked thymine requiring strain could be grown at steady state, in which growth rate and median cell volume are constant, in different concentrations of thymine and the median cell volumes of the cultures measured using a Coulter channeliser. Since cell volume is proportional to C-time, the difference in cell volumes between cells in the two cultures should indicate the difference in their C-times. Such cultures could be transduced with PI, as described before, and the segregation lags of markers in the different cultures should be altered in a manner predictable from their different C-times.

A less satisfactory method of testing the segregation model can be devised from the observations of Cooper and Helmistetter (1968) who reported that C-time was constant (41 minutes) at 37°C over a wide range in growth rates. Hence the segregation lags for markers would be independent of growth rate at 37°C. The effect of temperature on C-time has received little attention and values for C-time at 30°C have not been published. Common sense, however, would suggest that lowering the temperature should reduce the rate of any physiological or chemical process. Hence at 30°C it can be expected that replication velocity will fall, i.e. C-time will increase. Accordingly, cells grown and transduced at 30°C should show an increased spread in segregation lags between origin and terminus markers, than cells which have been grown and transduced at 37°C.

THE LAG BEFORE TRANSDUCTANTS INCREASE IN NUMBER IS DEPENDENT ON THE CHROMOSOMAL LOCATION OF THE SELECTED MARKER

As described above, the segregation model predicts that the lag before transductants increase in number is dependent on the chromosomal location of the selected marker; terminus markers should increase first and origin markers last, as has been observed for Trp+ and Arg+. To test this hypothesis further, a suitably marked strain (MM327) was constructed which contained the markers of
MM303 plus purF (50 minutes on the map. Bachman, 1983), and lysA (61.4 mins.).

CONSTRUCTION OF MM327

MM323, a purF derivative of MM303, was made ThyA by the trimethoprim selection procedure described in Chapter 2. To introduce the lysA mutation, MM323 ThyA (MM326) was then transduced to Thy+ using a P1 lysate propagated on CP154 (argA, ThyA lysA). Since MM323 is argH, the introduction of argA would not have allowed Arg+ transductants to be scored in the following experiment. Thus Thy+ transductants were screened for lysA and ArgA+; the lysA genotype was confirmed by showing that MM327 was unable to grow on -lys plates and ArgH ArgA+ genotype confirmed by demonstrating that the P1 lysate grown on CP154 (ArgH ArgA-) could transduce MM327 to the Arg+ phenotype. Since CP154 is ArgH ArgA-, MM327, obviously, could only be transduced to Arg+ if possessing the ArgH ArgA+ genotype. One such clone was picked, and checked to ensure it still possessed the same phenotype (except for lysA) as the parental strain MM323. This strain was called MM327, and contains the following lesions (plus others not mentioned): argH (89.5 map units) trp (27.6 map units) lysA (61.4 map units) and purF (50 map units). The segregation model predicts that Trp+ transductants should increase first, followed by PurF+, LysA+ and ArgH+ last.

TRANSDUCTION OF MM327 TO ArgH+, Trp+, LysA+ PurF+

Log phase MM327 cells, grown in L-broth Ca2+ at 37°C were transduced with P1 and grown as described before. Immediately on dilution of the transduction mix into L-broth citrate and at 15 minute intervals thereafter, samples were taken and viable counts, together with Arg+ Trp+ Lys+ Pur+ transductants were scored. The results, corrected for the dilution of the culture are shown in Table 5.5 and the graph showing transduction frequencies against time is plotted in Figure 5.4a. When the graph is replotted as the number of transductants per initial number, against time (Figure 5.4b) the relative segregation lags
FIGURE 5.4  TRANSDUCTION OF MM327 TO Arg⁺, Trp⁺, Lys⁺ PurF⁺

A.  

- = Lys⁺ transductants
• = viable counts
▼ = PurF⁺ transductants
○ = Trp⁺ transductants
▲ = Arg⁺ transductants
T = time in minutes

B.  

Graph in 5.4A replotted, showing the number of transductants per initial number against time. For clarity, only the curves are shown. The segregation lags of the markers can be clearly seen (see text). Segregation lags for LysA⁺ and PurF⁺ transductants can be predicted in the same way as for Arg⁺ (see text).

\[ \text{Trp} \text{ is } 3.8 \text{ map units from } \text{terC} \]
\[ \text{LysA} \text{ is } 31.5 \text{ map units from } \text{terC} \]

\[ \text{lag between } \text{Trp}⁺ \text{ and } \text{LysA}⁺ \text{ transductants} \]
\[ = \frac{31.5 - 3.8}{\text{replication velocity}} = \frac{31.5 - 3.8}{1.25 \text{ map units/minute}} \]
\[ = 22.4 \text{ minutes.} \]

\[ \text{purF} \text{ is } 18.5 \text{ map units from } \text{terC} \]

\[ \text{lag between } \text{Trp}⁺ \text{ and } \text{purF}⁺ \text{ transductants} \]
\[ = \frac{18.5 - 3.8}{1.25 \text{ map units/minute}} \]
\[ = 12 \text{ minutes.} \]

These predicted values are in close agreement with those shown in Figure 5.4 B.
Figure 5.4

Graph A shows the number of transductants per 0.2 ml against time (T) on a logarithmic scale, ranging from $10^2$ to $10^4$. The viable counts per ml are also plotted on the same graph, ranging from $10^8$ to $10^9$. The data points are indicated with different symbols for comparison.
5.4 contd.

![Graph showing the number of transductants per initial number over time.](image)
<table>
<thead>
<tr>
<th>t (minutes)</th>
<th>VC/ml</th>
<th>Arg⁺</th>
<th>Trp⁺</th>
<th>Lys⁺</th>
<th>Pur⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.2 x 10³</td>
<td>395</td>
<td>282</td>
<td>501</td>
<td>138</td>
</tr>
<tr>
<td>15</td>
<td>NT</td>
<td>400</td>
<td>261</td>
<td>510</td>
<td>140</td>
</tr>
<tr>
<td>30</td>
<td>NT</td>
<td>407</td>
<td>300</td>
<td>568</td>
<td>155</td>
</tr>
<tr>
<td>45</td>
<td>NT</td>
<td>408</td>
<td>360</td>
<td>564</td>
<td>155</td>
</tr>
<tr>
<td>60</td>
<td>1.14 x 10⁹</td>
<td>450</td>
<td>328</td>
<td>542</td>
<td>152</td>
</tr>
<tr>
<td>75</td>
<td>NT</td>
<td>394</td>
<td>332</td>
<td>586</td>
<td>158</td>
</tr>
<tr>
<td>90</td>
<td>2.05 x 10⁹</td>
<td>504</td>
<td>448</td>
<td>696</td>
<td>168</td>
</tr>
<tr>
<td>105</td>
<td>NT</td>
<td>525</td>
<td>586</td>
<td>786</td>
<td>244</td>
</tr>
<tr>
<td>120</td>
<td>NT</td>
<td>600</td>
<td>676</td>
<td>888</td>
<td>336</td>
</tr>
<tr>
<td>135</td>
<td>4.81 x 10⁹</td>
<td>864</td>
<td>1032</td>
<td>1028</td>
<td>424</td>
</tr>
<tr>
<td>150</td>
<td></td>
<td>1058</td>
<td>1232</td>
<td>1680</td>
<td>544</td>
</tr>
</tbody>
</table>

*These values must be corrected*
can be seen. Clearly, the transductants increase in number in the predicted manner; Trp⁺ first, then PurF⁺, LysA⁺ and last, Arg⁺. The difference in lag between Arg⁺ and Trp⁺ is again 34 minutes, giving as before, a value for C-time = 41 minutes. The delays between: Trp⁺ and PurF⁺, Trp⁺ and LysA⁺ and LysA⁺ and ArgH⁺ are consistent with the hypothesis that the difference in lag between markers is directly proportional to their separation on the chromosome (see figure 5.4b). These observations clearly support the segregation model.

The experimental data, however, depart from the predicted results in one obvious manner. The segregation model predicts that the number of Trp⁺ transductants should increase after about 23 minutes post transduction. (3 minutes for replication forks to pass through trp and reach terC, plus the D-time (= 20 minutes).) However, Trp⁺ transductants do not increase until about 55-60 minutes after transduction; MM327 divides every 36 minutes in L-broth at 37°C so Trp⁺ increase is delayed by about 1 generation time, as are all the other markers. (This will be discussed in detail in section 5.6.)

**EFFECT OF SLOWING REPLICATION VELOCITY IN A THY⁻ STRAIN BY LIMITING THYMINE IN THE GROWTH MEDIUM**

As mentioned above, growth of thymine requiring auxotrophs in media containing limiting thymine results in decreased replication velocity, hence increases in C-time, origin to terminus ratio and cell volume (Pritchard and Zaritsky 1970, Zaritsky and Pritchard 1971, Begg and Donachie 1978). The increase in C-time has been shown to be proportional to the increase in cell volume (described above). As described before, this relationship can be used to test the segregation model.

Thy⁻ (thymine requiring) strains can be broadly categorised into 3 classes: high thymine requirers, low thymine requirers and super-low thymine requirers (O'Donovan 1978). High thymine requirers typically require 20-50 µg/thy/mL medium and have the thyA genotype; the thyA mutation leads to a deficiency in thymidylate synthetase, which prevents de novo synthesis of dTTP,
resulting in an accumulation of dUMP; dUMP is then degraded to deoxyuridine and then to deoxyribose-1-phosphate by a phosphatase and thymidine phosphorylase respectively. This leads to a source of deoxyribose-1-phosphate which is required for the incorporation of thymine (O'Donovan and Newhard 1970, Beacham and Pritchard 1971). However, deoxyribose-1-phosphate is readily degraded by two inducible enzymes, deoxyribomutase (drm or deoB) and deoxyriboaldolase (dra or deoC). Hence the high requirement for exogenous thymine in thyA strains to compete incorporation against degradation (O'Donovan 1978). Low thymine requiring derivatives can be obtained from thyA strains by mutation at deoB or deoC, which alleviates the intracellular degradation of deoxyribose-1-phosphate. Such strains typically require 1-2 μg thymine/ml (O'Donovan and Newhard 1970, Beacham et al. 1968, Alilehanian et al. 1966, O'Donovan 1978). Super-low thymine requiring strains will not be dealt with here.

Experiments carried out to investigate the effects of limiting thymine on replication velocity of thy− strains have employed low-thymine requiring strains. (Begg and Donachie 1978). Hence, to duplicate the conditions giving steady state growth in limiting thymine, MM326 was made low-thy by spreading cells onto plates containing 2 μg/mL thymine, hence selecting spontaneous deoB or deoC mutants. The resulting strain (MM326-1) was checked to ensure it still carried the other parental (MM326) markers.

GROWTH OF MM326-1 IN LIMITING THYMINE

MM326-1 was grown in media supplemented with 2.0 μg, 5.0 μg, 10 μg and 50 μg thymine/mL, as described by Begg and Donachie (1978) (see Chapter 2) in conditions expected to allow steady state growth. The growth rate of the cultures was measured by following the OD540 and samples were viewed by phase contrast light microscopy. The results (not shown) indicated that in all thymine concentrations, except 50 μg/mL, the culture was thymine starved, with cells forming long filaments and exhibiting abnormal shapes, presumably due to the induction of SOS
by thymine starvation (Little and Mount, 1982).

Begg and Donachie (1978) reported that certain strains never achieve steady state growth in limiting thymine and it seemed that this was the case for MM326-1 (also K. Begg, personal communication). Accordingly, MM326-1 was unsuitable as a recipient with which to test the segregation model using limiting thymine to increase C-time. However, Begg and Donachie (1978) reported that the strain OV2, although not achieving steady state in glucose based medium, did achieve steady state growth in a medium containing glycerol as the carbon source. Thus OV2 was lysogenised with P1, to prevent P1-killing during transduction, and the growth characteristics of both OV2 and OV2(P1) were assessed in glucose or glycerol media containing 1.5 µg, 2.0 µg, 5.0 µg and 10 µg thymine/ml, as described before; the OD$_{540}$ was followed for each culture and the median cell volumes of the cultures were measured with a Coulter channelyser. The results (tables 5.6, 5.7, 5.8, 5.9 and figures 5.5, 5.6, 5.7 and 5.8) indicated that, in agreement with Begg and Donachie (1978), neither OV2 nor OV2(P1) achieved steady state growth in any of the tested thymine concentrations in a glucose based medium. The periodic fluctuations in median cell volume observed for OV2 and OV2(P1), in glucose medium, were presumably caused by dilution of the culture with fresh medium, at every mass doubling; presumably the fast growth rate of cells in glucose medium was depleting thymine from the medium. However, in contrast to the observations of Begg and Donachie (1978) OV2 failed to achieve steady state growth in any of the above thymine concentrations when glycerol was the carbon source. OV2(P1), however, achieved steady state growth in glycerol media supplemented with 5 µg thymine/ml and 10 µg thymine/ml; cells in the two cultures differing in volume by some 25% (figure 5.8). Since cells grown in glycerol medium plus 5 µg thymine/ml are 25% larger than those grown in the same medium supplemented with 10 µg thymine/ml, C-time of cells in 5 µg thymine/ml is expected to be 25% greater than that of cells grown in 10 µg thymine/ml. The segregation model predicts that cells with a 25% increase in C-time will show a 25% increase in the difference between segregation lags of two separate markers.
TABLE 5.6 (a)  
MEDIAN CELL VOLUMES OF OV2(P1) CULTURES GROWN IN GLUCOSE MEDIUM WITH LIMITING THYMINE

<table>
<thead>
<tr>
<th>[Thymine] μg/ml</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>22</td>
<td>26</td>
<td>26</td>
<td>34</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>24</td>
<td>24</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>22</td>
<td>21</td>
<td>25</td>
<td>29</td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>17</td>
<td>13</td>
<td>18</td>
<td>19</td>
</tr>
</tbody>
</table>

TABLE 5.6 (b)  
OD₅₄₀ OF OV2(P1) CULTURES GROWN IN GLUCOSE MEDIUM WITH LIMITING THYMINE

<table>
<thead>
<tr>
<th>[Thymine] μg/ml</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0.116</td>
<td>0.161</td>
<td>0.216</td>
<td>0.253</td>
<td>0.280</td>
</tr>
<tr>
<td>2</td>
<td>0.128</td>
<td>0.189</td>
<td>0.278</td>
<td>0.335</td>
<td>0.402</td>
</tr>
<tr>
<td>5</td>
<td>0.151</td>
<td>0.233</td>
<td>0.328</td>
<td>0.406</td>
<td>0.514</td>
</tr>
</tbody>
</table>
TABLE 5.7 (a)
MEDIAN CELL VOLUME OF OV2 GROWN IN GLUCOSE MEDIUM WITH LIMITING THYMINE

<table>
<thead>
<tr>
<th>t</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Thymine] µg/ml</td>
<td>1.2</td>
<td>22</td>
<td>24</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>20</td>
<td>21</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>16</td>
<td>17</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>11</td>
<td>13</td>
<td>15</td>
<td>NT</td>
</tr>
</tbody>
</table>

TABLE 5.7 (b)
OD$_{540}$ OF OV2 GROWN IN GLUCOSE MEDIUM IN LIMITING THYMINE

<table>
<thead>
<tr>
<th>t</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Thymine] µg/ml</td>
<td>1.5</td>
<td>0.145</td>
<td>0.169</td>
<td>0.174</td>
<td>0.203</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.150</td>
<td>0.178</td>
<td>0.201</td>
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<td>5.0</td>
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<td>0.241</td>
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</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.189</td>
<td>0.241</td>
<td>0.306</td>
<td>0.418</td>
</tr>
</tbody>
</table>

$t =$ time in minutes
Table 5.8(a)
MEDIAN CELL VOLUMES OF OV2(P1) GROWN IN GLYCEROL MEDIUM WITH LIMITING THYMINE

<table>
<thead>
<tr>
<th>[Thymine] µg/ml</th>
<th>Time in Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0 15 30 45 60</td>
</tr>
<tr>
<td>2.0</td>
<td>21 26 29 35 NT</td>
</tr>
<tr>
<td>5.0</td>
<td>17 19 18 18 18</td>
</tr>
<tr>
<td>10.0</td>
<td>13 13 13 14 13</td>
</tr>
</tbody>
</table>

Table 5.8(b)
OD$_{540}$ OF OV2(P1) IN GLYCEROL MEDIUM WITH LIMITING THYMINE

<table>
<thead>
<tr>
<th>[Thymine] µg/ml</th>
<th>Time in Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0 15 30 45 60</td>
</tr>
<tr>
<td>2.0</td>
<td>0.133 0.155 0.170 0.205 0.226</td>
</tr>
<tr>
<td>5.0</td>
<td>0.160 0.208 0.253 0.316 0.390</td>
</tr>
<tr>
<td>10.0</td>
<td>0.155 0.197 0.248 0.324 0.400</td>
</tr>
</tbody>
</table>
Table 5.9(a)

MEDIAN CELL VOLUMES OF Ov2 GROWN IN GLYCEROL MEDIA WITH LIMITING THYMINE

<table>
<thead>
<tr>
<th>[Thymine] μg/ml</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>20</td>
<td>24</td>
<td>30</td>
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<td>30</td>
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<td>17</td>
<td>19</td>
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<tr>
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<td>13</td>
<td>15</td>
<td>16</td>
<td>19</td>
<td>20</td>
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</tbody>
</table>

Table 5.9(b)

OD_{540} OF Ov2 GROWN IN GLYCEROL MEDIUM WITH LIMITING THYMINE

<table>
<thead>
<tr>
<th>[Thymine] μg/ml</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0.050</td>
<td>0.057</td>
<td>0.060</td>
<td>0.071</td>
<td>0.072</td>
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<tr>
<td>2.0</td>
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<td>0.066</td>
<td>0.073</td>
<td>0.085</td>
<td>0.087</td>
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<tr>
<td>5.0</td>
<td>0.088</td>
<td>0.096</td>
<td>0.113</td>
<td>0.135</td>
<td>0.151</td>
</tr>
<tr>
<td>10.0</td>
<td>0.097</td>
<td>0.109</td>
<td>0.131</td>
<td>0.172</td>
<td>0.191</td>
</tr>
</tbody>
</table>
Table 5.10(a)

**MEDIAN CELL VOLUMES OF OV2(P1) IN GLYCEROL MEDIUM WITH LIMITING THYMINE, AFTER P1 TRANSDUCTION**

<table>
<thead>
<tr>
<th>t</th>
<th>-30</th>
<th>-15</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Thymine]</td>
<td>5</td>
<td>17</td>
<td>16</td>
<td>17</td>
<td>18</td>
<td>20</td>
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<td>10</td>
<td>13</td>
<td>13</td>
<td>12</td>
<td>14</td>
<td>15</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 5.10(b)

**OD$_{540}$ OF OV2(P1) GROWN IN GLYCEROL MEDIUM AFTER P1 TRANSDUCTION**

<table>
<thead>
<tr>
<th>t</th>
<th>-30</th>
<th>-15</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Thymine]</td>
<td>5</td>
<td>0.222</td>
<td>0.260</td>
<td>0.305</td>
<td>0.351</td>
<td>0.392</td>
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<td>10</td>
<td>0.212</td>
<td>0.255</td>
<td>0.310</td>
<td>0.333</td>
<td>0.399</td>
<td>0.460</td>
</tr>
</tbody>
</table>
FIGURE 5.5

A. GROWTH OF OV2 IN GLUCOSE MEDIUM WITH LIMITING THYMINE

○ = 1.5 µg Thymine/ml
▼ = 2 µg Thymine/ml
▲ = 5 µg Thymine/ml
■ = 10 µg Thymine/ml

B. MEDIAN CELL VOLUMES OF OV2 CULTURES GROWN IN THE ABOVE MEDIA.
Figure 5.5

Graph showing the OD540 over time in minutes.
5.5 contd.

- Time in minutes vs. Med. Cell Volume chart

- Key: •, ▲, ▼, ■
FIGURE 5.6

A. GROWTH OF OV2 IN GLYCEROL BASED MEDIUM WITH LIMITING THYMINE

▲ = 1.5 μg thymine/ml
● = 2 μg thymine/ml
▼ = 5 μg thymine/ml
■ = 10 μg thymine/ml

B. MEDIAN CELL VOLUMES OF OV2 CULTURES GROWN IN THE ABOVE MEDIA
Figure 5.6
B

5.6 contd.

MEDIAN CELL VOLUME

TIME IN MINUTES

10 20 30 40

15 30 45 60
Figure 5.7
FIGURE 5.7

A. GROWTH OF OV2(P1) IN GLUCOSE BASED MEDIUM WITH LIMITING THYMINE

- = 1.5 µg Thymine/ml
▼ = 2 µg Thymine/ml
▲ = 5 µg Thymine/ml
■ = 10 µg Thymine/ml

Note: the Y-axis represents OD_{540}

B. MEDIAN CELL VOLUMES OF OV2(P1) CULTURES GROWN IN THE ABOVE MEDIA.
5.7 contd.

![Graph showing changes in median cell volume over time in minutes.](image)

- **Y-axis:** Median Cell Volume
- **X-axis:** Time in Minutes
- The graph illustrates the increase in median cell volume over time, with different markers indicating different conditions or groups.
FIGURE 5.8

A. GROWTH OF OV2(P1) IN GLYCEROL BASED MEDIUM WITH LIMITING THYMINE

▲ = 1.5 μg thymine/ml
● = 2 μg thymine/ml
▼ = 5 μg thymine/ml
■ = 10 μg thymine/ml

B. MEDIAN CELL VOLUMES OF OV2(P1) CULTURES GROWN IN THE ABOVE MEDIA.

Note: OV2(P1) achieves steady state growth in 5 μg thymine/ml and 10 μg thymine/ml
Figure 5.8

A

0.5

0.1

OD540

TIME IN MINUTES

0 15 30 45 60
5.8 contd.

B

MEDIAN CELL VOLUME

TIME IN MINUTES

10

20

30

40

50

15 30 45 60
As a preliminary experiment, to investigate the effects of the P1-transduction procedure on steady state growth, exponentially growing OV-2 cells, grown in glycerol medium supplemented with 5 \( \mu \text{g/ml} \) and 10 \( \mu \text{g thymine/ml} \) and with glycerol as the carbon source were transduced with P1 as described before. Samples were taken at intervals, and the median cell volumes of the cultures were measured with the Coulter channelyser. The results (table 5.10 a,b) showed that OV2(P1) in both thymine concentrations come out of steady state growth after the addition of P1 phage and median cell volumes started to increase. This experiment was repeated, with the same result (data not shown); i.e. the cells started to increase in volume. These results showed that following the transduction procedure, the cultures were not in a suitable physiological condition to be used to test the segregation model.

It was not clear why the addition of the P1 phage to the cultures should have caused the cultures to come out of steady state growth. One possible hypothesis is that some replication of the incoming phage DNA may have occurred. If so, presumably the internal thymine pool would have been depleted, and so the cells would have come out of steady state growth. Although OV2(P1) is lysogenic for P1, and so incoming P1 phage should not replicate, the high M01 required for these experiments (see Chapter 2) would have resulted in cells receiving many copies of P1 phage; the introduction of many copies of P1 may have titrated replication-repressor molecules to allow some replication of the incoming phage DNA (Sternberg and Hoess 1983 for review of P1 replication). However, since exponentially growing cultures transduced by the above procedure did not lyse, the replication of P1 DNA, if it occurred at all must have been transient.

No further attempts were made to test the segregation model by increasing the C-time of thymine-requiring cells in limiting thymine.

**EFFECT OF SLOWER GROWTH RATE AT 37°C ON SEGREGATION LAGS**

Cooper and Helmstetter (1968) reported that C-time at 37°C was independent of growth rates provided cells were doubling at
least once every 60 minutes. By growing cells in minimal medium at 37°C, the growth rate drops but C-time should remain constant. The segregation model thus predicts that the difference in lag between origin and terminus markers is also independent of growth rate at 37°C. Accordingly MM327 was grown exponentially in minimal medium (see Chapter 2), supplemented with appropriate amino acids, at 37°C. The culture was transduced, as described before; viable counts, Arg⁺ and Trp⁺ transductants were scored. The growth rate of the culture was followed by measuring the OD₅₄₀. Clearly, the growth rate of MM327 was reduced in minimal medium when compared to rich medium (L-broth) (figure 5.9). However, very poor transduction frequencies were obtained, at about 10⁻² of the frequencies observed for L-broth grown cultures. One possible cause of this was that the Phosphate concentration in the VB salts, present in the minimal medium, was too high, precipitating the Ca²⁺ ions required for PI adsorption as insoluble Ca₃(PO₄)₂. The experiment was repeated but in this case, a low phosphate concentration salt solution used in place of VB. The results were identical; very poor transduction was obtained and the numbers of transductants per selective plate were too small to give meaningful results (typically 2-3 per plate). Attempts to increase the numbers, per selective plate, by concentrating cells by centrifugation before spreading were unsuccessful; even with washed samples, which prevents the transfer of growth medium to the selective plates and so reduces background growth, the large number of cells plated seemed to inhibit the development of transductant colonies. This method of testing the segregation model was therefore abandoned.

EFFECT OF TEMPERATURE ON SEGREGATION LAGS

As mentioned before, decreasing the growth temperature was expected to increase C-time and hence to extend the difference in segregation lag between terminus and origin markers.

MM327 cells were grown exponentially at 30°C in L-broth and transduced with PI, as described previously. Samples were taken at time intervals after PI transduction and scored for viable
FIGURE 5.9  GROWTH OF MM327 IN L-BROTH AND MINIMAL MEDIUM

□ = growth in L-broth

Δ = growth in minimal medium
<table>
<thead>
<tr>
<th>t (minutes)</th>
<th>Viable Counts</th>
<th>Arg$^+$</th>
<th>Trp$^+$</th>
</tr>
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<td>0</td>
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<td>95</td>
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<td>$7.5 \times 10^7$</td>
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<td>$9.7 \times 10^7$</td>
<td>503</td>
<td>246</td>
</tr>
<tr>
<td>60</td>
<td>$1.2 \times 10^8$</td>
<td>653</td>
<td>304</td>
</tr>
<tr>
<td>80</td>
<td>$1.6 \times 10^8$</td>
<td>756</td>
<td>348</td>
</tr>
<tr>
<td>100</td>
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<td>811</td>
<td>424</td>
</tr>
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<td>822</td>
<td>448</td>
</tr>
<tr>
<td>140</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>160</td>
<td>$4.0 \times 10^8$</td>
<td>922</td>
<td>538</td>
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<tr>
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<td>200</td>
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<td>1362</td>
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<td>1504</td>
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<tr>
<td>280</td>
<td>-</td>
<td>2250</td>
<td>1884</td>
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</table>
FIGURE 5.10 TRANSDUCTION OF MM327 TO Arg⁺ Trp⁺ AT 30°C

A.

▲ = Trp⁺ transductants per 200 μl culture
■ = Arg⁺ transductants per 200 μl culture

(viable counts are not shown here but the culture was doubling in mass every 60 minutes)

B.

Graph shown in A replotted with Trp⁺ transductants multiplied by a factor of 2. A lag of 46 minutes is seen between Trp⁺ and Arg⁺ transductants.
Figure 5.10

A

10^3

10^2

5 \times 10^1

0 20 40 60 80 100 120 140 160 180 200 220 240 260 280

TIME IN MINUTES

NUMBER OF TRANSCLUSANTS
5.10 contd.

![Graph showing the number of transductants over time. The x-axis represents time in minutes ranging from 0 to 280, and the y-axis represents the number of transductants ranging from $10^2$ to $10^3$. The graph includes two curves, one indicated by triangles and the other by squares. A dashed line indicates a time difference of 46 minutes between the two curves.](image-url)
counts, Arg\(^+\) and Trp\(^+\) transductants. The results (table 5.11, figure 5.10) were surprising. Instead of a lag before the number of Arg\(^+\) or Trp\(^+\) transductants increased in number, there was an initial rapid increase, faster than the growth rate of the culture, which was identical for both Arg\(^+\) and Trp\(^+\). However, the rate of increase slowed after some 40 minutes. Then, at later time points, the numbers of transductants once more increased, this time at the same rate as the non transduced cells and with Trp\(^+\) increasing before Arg\(^+\) transductants. The cause of the initial rapid increase in transductants was not clear but was assumed to result from the low (30°C) growth temperature, perhaps slowing down a phage directed process such as injection of DNA. Presumably, plating early samples, before this putative process had occurred, was disrupting the phage/bacterium interaction, so preventing completion of the process. Clearly, if this was indeed the case, the curves in figure 5.10a showing transduction of Arg\(^+\) and Trp\(^+\) against time, are composite curves, resulting from two independent processes. The initial part of the curves, showing the rapid increase in the numbers of transductants, was presumably due to the interruption of a phage/bacterium interaction and the latter part of the curves was thought to be caused by the expected segregation lags. These assumptions were made to enable the segregation lags of Trp\(^+\) and Arg\(^+\) transductants and hence, C-time at 30°C to be estimated.

At early points, segregation effects would not have altered the ratio of Arg\(^+\) transductants to Trp\(^+\). At t=0, t=20 and t=40 minutes the ratio of Arg\(^+\) to Trp\(^+\) transductants remains constant at 2:1. This is in agreement with previously reported Arg\(^+\) and Trp\(^+\) transduction frequencies (Masters 1977). Thus, to estimate the difference in segregation lag between Arg\(^+\) and Trp\(^+\) transductants, the values for Trp\(^+\) transductants were multiplied by a factor of 2 and the curve replotted (Figure 5.10b); the difference in lags could then be clearly seen and it was estimated that Trp\(^+\) transductants increased in number some 46 minutes before Arg\(^+\) transductants (see figure 5.10b). This is, of course, in agreement with the prediction that cells grown at 30°C would
have slower replication velocities than those grown at 37°C and so the difference in segregation lags between Trp<sup>+</sup> and Arg<sup>+</sup> would be greater at 30°C than at 37°C. By applying the same formula as before, an estimate for C-time at 30°C can be made.

Replication velocity at 30°C =
Distance from Arg to Trp = 41.6 map units
Difference in segregation lags between Arg<sup>+</sup> and Trp<sup>+</sup> = 46 minutes

C-time at 30°C =

distance from oriC to terC (map units)
replication velocity (map units/minute)

= 50 map units
0.9 map units/minute

= 55.6 minutes.

Although no published data exist for C-time at 30°C, with which to compare the above value, the results obviously are in agreement with the predictions of the segregation model; clearly, growth of the cells at 30°C had increased the difference in segregation lag between Trp<sup>+</sup> and Arg<sup>+</sup> transductants, above that observed at 37°C. Assuming the segregation model is correct, the C-time at 30°C must be 55.6 minutes.

Interestingly, the growth rate of MM327 at 30°C was 1.6 times that at 37°C (Figures 5.3a, 5.10a), but the C-time at 30°C was only 1.4 times that at 30°C. The significance of this is not known. Nor is it clear what effect the drop in temperature would have had on D-time, though for the purpose of the following discussion, D-time at 30°C was assumed to have increased by about 1.5 times that measured at 37°C. However, since the effect of temperature on D-time is not known, it is obvious that the conclusions drawn below must be somewhat tentative.

From the data shown in Figure 5.10a, it is difficult to
estimate the time at which transductants first increase in number due to segregation lags, rather than the putative slow phage/bacterium interaction. After the initial rapid increase, the number of transductants plateau, then start to go up again, but at a gradually increasing rate. However, since in figure 5.10b, the early part of the curves, for both Trp+ and Arg+ transductants are nearly identical, it was assumed that Trp+ transductants first increased in number at a time corresponding to the end of the trp segregation lag, at the point where Trp+ transductants increased above Arg+ transductants, in figure 5.10b, i.e. after some 90-95 minutes. Assuming D-time is 30 minutes at 30°C (i.e. 1.5 x 20 minutes at 37°C - see above) this again suggests that Trp+ transductants increase in number, about 1 generation time after the time predicted by the segregation model; Trp+ transductants should have started multiplying after the time required for replication forks to pass through trp to terC (about 5 minutes at 30°C) plus the D-time (assumed to be about 30 minutes at 30°C), i.e. after a total of about 35 minutes. However, figure 5.10b indicates that the duration of the Trp+ segregation lag is actually about 90-95 minutes, i.e. as observed at 37°C, segregation lags appear to be 1 generation time longer than expected. This will be discussed in section 5.6.

However, the observations presented above still support the hypothesis that the time at which transductants increase in number after the introduction of transducing DNA into the recipient cell, is marker dependent; the marker dependence of the process is due to the time taken to segregate mutant and wild type alleles to daughter cells at division.

ESTIMATION OF C-TIME AT 30°C AND 37°C

As already mentioned, the segregation model predicts that the difference in segregation lags between two specially separated markers, at a given growth temperature, depends on the DNA replication velocity at that temperature. Accordingly, the segregation lags of Arg+ and Trp+ have been used to estimate the C-time at 37°C and 30°C, to be 41 and 55.6 minutes respectively.
The above value for C at 37°C is in total agreement with the published value (Cooper and Helmstetter 1968) of 41 minutes. However, as yet no published data exist giving a value for C at 30°C. Therefore it was decided to obtain an independent estimate for C at 30°C, to compare with the value obtained from the segregation lags of Arg⁺ and Trp⁺ at 30°C and so to further test the validity of the segregation model.

Martin (1970) derived a mathematical relationship between the generation time of a culture, the C-time and the residual DNA synthesis in the culture after the prevention of initiation of replication (but not of chain elongation); a graph of this function is presented in figure 5.11. Therefore, if the generation time of a culture and the residual DNA synthesis in the culture, after blocking initiation, are measured, the C-time of the culture can be estimated.

Initiation can be halted immediately by the addition of chloramphenicol to the culture; chloramphenicol inhibits protein synthesis and so prevents further initiations of replication. However, elongation of the nascent DNA chains is independent of protein synthesis so on-going rounds of replication proceed to completion. (Billen 1959, Lark et al. 1963, Maaloe and Hannawalt 1961). A convenient means of measuring the DNA content of a culture (before and after CAP treatment), is afforded by the recent development of a fluorimetric microassay for DNA in prokaryotic cells (Legros and Kepes 1985). This assay obviates the need for radiolabelling of DNA. Thus the above procedures were used to estimate the residual DNA synthesis in CAP treated cultures, grown both at 30°C and 37°C (included as a control to ensure the method was reliable) and so enabled C-time to be estimated at both these temperatures.

MM327 was grown, shaking, in L-broth at both 30°C and 37°C. At an OD₆₀₀ of approximately 0.15, CAP was added to these cultures to final concentration of 200 µg/ml and the cultures were grown for a further 100 minutes. From the start of the experiment, 40 minutes before the CAP addition, and at 20 minute intervals thereafter, samples were taken and the OD₆₀₀ and DNA content of the
FIGURE 5.11  RELATIONSHIP BETWEEN RESIDUAL DNA SYNTHESIS AND THE RATIO OF THE C-TIME TO THE GROWTH RATE

Redrawn from the data of Martin (1970)
(See text)
Figure 5.11

Residual synthesis as a percentage of the existing DNA

Ratio of the replication time to the growth rate
### Table 5.12: Residual DNA Synthesis After Prevention of Initiation

#### 37°C

<table>
<thead>
<tr>
<th>Flask 1</th>
<th>Flask 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>t mins</strong></td>
<td><strong>OD&lt;sub&gt;600&lt;/sub&gt;</strong></td>
</tr>
<tr>
<td>0</td>
<td>0.045</td>
</tr>
<tr>
<td>20</td>
<td>0.081</td>
</tr>
<tr>
<td>40</td>
<td>0.144</td>
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<tr>
<td>60</td>
<td>0.231</td>
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<tr>
<td>80</td>
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</tr>
<tr>
<td>120</td>
<td>0.707</td>
</tr>
<tr>
<td>140</td>
<td>0.957</td>
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</tbody>
</table>

% increase in DNA after CAP addition = 51.8% (±5.3% error)

#### 30°C

<table>
<thead>
<tr>
<th>Flask 3</th>
<th>Flask 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>t mins</strong></td>
<td><strong>OD&lt;sub&gt;600&lt;/sub&gt;</strong></td>
</tr>
<tr>
<td>0</td>
<td>0.054</td>
</tr>
<tr>
<td>20</td>
<td>0.086</td>
</tr>
<tr>
<td>40</td>
<td>0.114</td>
</tr>
<tr>
<td>60</td>
<td>0.157</td>
</tr>
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<td>120</td>
<td>0.384</td>
</tr>
<tr>
<td>140</td>
<td>0.469</td>
</tr>
</tbody>
</table>

% increase in DNA after CAP addition = 58% (± 6.5% error)
FIGURE 5.12

A. GROWTH OF MM 3C3 IN L-BROTH AT 37°C

● = Control culture
■ = Culture to which 200 µg/ml chloramphenicol was added at t = 40 minutes.

B. RESIDUAL DNA SYNTHESIS (EXPRESSED AS RELATIVE FLUORESCENCE) OF THE ABOVE CULTURES
RELATIVE FLUORESCENCE vs TIME IN MINUTES

5.12 contd.
FIGURE 5.13

A. GROWTH OF MM303 IN L-BROTH AT 30°C

- = Control culture

■ = Culture to which 200 μg/ml chloramphenicol was added at t = 40 minutes.

B. RESIDUAL DNA SYNTHESIS (EXPRESSED AS RELATIVE FLUORESCENCE) OF THE ABOVE CULTURES
Figure 5.13

A

OD 600

-1.00

0.10

0.01

0 20 40 60 80 100 120 140

TIME IN MINUTES
5.13 contd.

![Graph showing relative fluorescence over time in minutes. The x-axis represents time in minutes (0 to 140), and the y-axis represents relative fluorescence (%). There are two lines on the graph, one for 100% and another for 10%.]
cultures were measured. (Also included in the experiment were control cultures, at both temperatures, to which CAP was not added.)

The results, shown in Table 5.12 and figures 5.12 and 5.13, showed that, as expected, the addition of CAP caused a rapid cessation of growth and a cessation of DNA synthesis. The residual DNA synthesis at 30°C and 37°C after CAP addition was estimated to be 58% (± 6.5% error) and 51.8% (± 5.3% error) respectively. The standard curve (figure 5.11) of the relationship between residual DNA synthesis, C-time and generation time (Martin 1970) shows that:

1) at 30°C: 58% residual DNA synthesis indicates that 
   $C = 1.5 \pm 6.5\% \times \text{generation time}$.
   Generation time at 30°C = 40 minutes (Figure 5.11)
   Therefore $C$ at 30°C = 1.5 x 40 mins = 60 minutes ± 4 minutes.

2) at 37°C: 51.8% residual DNA synthesis indicates that 
   $C = 1.3 \pm 5.3\% \times \text{generation time}$.
   Generation time at 37°C = 30 minutes.
   Therefore $C$ at 37°C = 1.3 x 30 = 39 minutes ± 2 minutes.

The value for $C$ at 37°C (39 ± 2 minutes) is in good agreement with the previously reported value of 41 minutes, which indicates that the method employed here was yielding accurate results. The value for $C$ at 30°C (60 minutes ± 4 minutes) is also in good agreement with the value estimated from the segregation of Arg⁺ and Trp⁺ at 30°C (55.6 minutes). These results, therefore are strongly supportive of the segregation model.

SECTION 5.5 SUMMARY AND DISCUSSION

The results presented in this chapter contradict the conclusions of Bender and Sambucetti (1983), who reported that P1-mediated transduction of an exponentially growing culture of Klebsiella caused a recombination induced inhibition of cell division and hence transduced cells formed long filaments; their conclusions have already been discussed in Section 5.1. The
observations presented in this chapter have clearly demonstrated
the following points, in contrast to those of Bender and Sambucetti
(1983):

1. Pl-mediated transduction of exponentially growing
E.coli cells does not seem to cause cells to filament; rather it
seems likely that preformed filamentous cells are 5-10 fold better
recipients for transduction (Section 5.2).

2. The lag before transductants increase in number is
dependent on the chromosomal location of the selected marker:
Terminus markers increase first, followed in order, by markers
replicated late, midway and then early in the replication cycle. A
model to explain the marker dependence of the lags has been
proposed (Section 5.4) and the model states that the post
transductional lag before transductants increase in number depends
on the position dependent time of segregation of mutant and wild
type alleles to daughter cells. The model predicts that the
delay between the increase of terminus and origin markers is
due to the additional time taken (before division can occur),
for replication forks to pass from origin markers to terC, than
for replication forks to pass from the terminus markers to terC.
Support for the model comes from the demonstration that the lag
between increase in the number of transductants does indeed
depend on the position of the selected markers, with terminus
markers increasing first, and origin markers last (section 5.3
and 5.4). In addition, the model predicts that growth of
cells in conditions which alter C-time, (and hence replication velocity)
will alter the spread of the lag between increase of terminus
and origin markers, i.e. if C-time is increased (and so replication
velocity has decreased) the lag between the increase of terminus
and origin markers should increase concomitantly. Attempts were
made to test this prediction by growing low-thymine requiring strains,
at steady state, in different limiting thymine concentrations, so
as to increase C-time in a predictable fashion. However, the
cultures did not remain in steady state growth after the trans-
duction procedure, so this approach had to be discarded (section 5.5).
However, cells grown and transduced at 30°C, i.e. in conditions expected to increase C-time, showed, as predicted, the difference in segregation lag between terminus (Trp\textsuperscript{+}) and origin (Arg\textsuperscript{+}) transductants was greater than that which had been measured at 37°C.

Since the segregation model demands that the spread in lags between origin and terminus markers depends on replication velocity, the difference in lag between markers of known location can be used to estimate C-time. Such calculations were performed (Sections 5.3, 5.5) and C-time at 37°C, estimated by the above method was shown to be 41 minutes; this value is in complete agreement with the measurements of Cooper and Helmstetter (1968) who estimated C-time at 37°C to be 41 minutes. Assuming the segregation model is correct, the spread in segregation lags, at 30°C was used to estimate C-time at 30°C, the difference in segregation lag between Trp\textsuperscript{+} and Arg\textsuperscript{+} transductants was 46 minutes, giving an estimate for C-time at 30°C to be 55.6 minutes.

An independent estimate for C-time at 30°C was obtained, by measuring residual DNA synthesis after inhibition of initiation of replication. The value of C = 60 ± 4 minutes at 30°C is in good agreement with that obtained from the segregation lags of Arg\textsuperscript{+} and Trp\textsuperscript{+} at 30°C, so these results strongly support the validity of the segregation model.

Measuring the segregation lag of an unmapped marker with respect to the lags of other known markers, would allow its time of replication and hence, chromosomal location to be assigned to one of two positions either side of oriC; measurement of segregation lags may therefore be of practical use in the initial mapping of markers. However, the use of segregation lags as a means of mapping markers has not yet been tested.

The data presented in figures 5.1, 5.2, 5.3, 5.4 and 5.10 indicate that the measured segregation lags depart from those predicted by the model in an obvious manner; as mentioned before, the time at which transductants increase in number is delayed by a period equal to 1 generation time in excess of the duration of
the lag predicted by the model. (This observation is clearly seen at 37°C, but it must be noted that the result is not so clear cut at 30°C, where the time at which transductants first increase in number is not easy to estimate (figure 5.10a).)

There are at least three possible hypotheses to explain the above observation. Firstly, cells which receive transducing DNA (just P1 DNA is not sufficient to provoke the response) fail to grow or divide for a period equal to their generation time (both at 37°C and 30°C). This is considered most unlikely because it is difficult to envisage a normal physiological response which prevents cell growth for 1 generation after the introduction of homologous transducing DNA; to obtain such an effect would, for example, require the addition of several antibiotics, simultaneously, to the growth medium. Clearly, the cells do not filament after receiving transducing DNA (Section 5.2) so eliminating the possibility that filamentous growth is responsible for the increased lag. It was noted, however, that Klebsiella transductants exhibited a very short period of penicillin resistance, (15-20 minutes), immediately following transduction, but this period was considerably shorter than the generation time of 40 minutes (Bender and Sambucetti 1983).

The second hypothesis is that, in contrast to the reports of Sandri and Berger (1980a), the P1 mediated recombination event results in a single strand exchange reaction, rather than a double strand replacement. The passage of not one, but two replication forks through the newly recombined DNA would then be required before two wild-type daughter transductants were produced at division (figure 5.14); one fork would be needed to replicate the heteroduplex allele (which was formed by the transductional recombination event) to give one wild type and one mutant allele and the second fork, arriving one generation time after the first, would then produce two copies of the wild type allele which would segregate, one to each daughter, at division. However, if true, this hypothesis contradicts the work of Sandri and Berger (1980a) (mentioned above) and also is not consistent with the other observations (Chapter 1) which
suggest that transductional recombination is a double-stranded replacement reaction. (see Chapter 1, Mahyeran and Datta, 1979, Newman 1982, Masters et al. 1984). (This second hypothesis will be discussed in greater detail in the next Chapter (Chapter 6).)

The third hypothesis is that the transductional recombination process requires the passage of a replication fork through the region of recombining DNA (or through DNA about to recombine), resulting in the integration of one double stranded wild type allele immediately after the passage of the replication fork (Figure 5.15). As before, in order to produce two wild type daughters at division, two replication forks must pass through the region. The first would be necessary for the recombination process to occur and the second (arriving one generation time later) to replicate the double strand wild type allele to give 2 copies which can segregate at division, one to each daughter.

Of the three hypotheses described above, the third is the most attractive since it explains the additional generation time required before new transductants increase in number and is consistent with the observations on the nature of the recombinant molecules arising from transductional recombination. In addition, since markers co-transducible with oriC exhibit very high levels of transduction, (Masters 1977) and presumably have the capability to replicate from the oriC present on the transducing DNA, it is not implausible, by any means, to speculate that replication of DNA will influence its recombinogenicity. This will be discussed in more detail in Chapter 6.
FIGURE 5.14 INTEGRATION OF SINGLE STRANDED DNA DURING TRANSDUCTIONAL RECOMBINATION

Dark lines represent wild type transducing DNA and the open lines represent recipient chromosomal DNA. Circles represent the replisomes and the arrow gives the direction of DNA replication.

A. Transducing DNA and recipient DNA come into homologous alignment and crossovers are initiated.

B. The crossovers result in the replacement of a single strand region of recipient DNA with a single strand of transducing DNA.

C. Replication of the heteroduplex gives a homoduplex "transductant" daughter strand.

D. A further replication fork is required to give two "transductant" daughter chromosomes (see text for discussion).
Figure 5-14

A

B

C

D
FIGURE 5.15 INTEGRATION OF DOUBLE STRANDED TRANSDUCING DNA AT A REPLICATION FORK

Dark lines indicate transducing DNA. Open lines represent recipient DNA, circles represent replisomes and the arrows give the direction of DNA replication.

A. Transducing DNA and recipient DNA come into homologous alignment.

B. As a replication fork passes through the region, double stranded transducing DNA becomes integrated.

C. The passage of a second replication fork is required to give 2 "transductant" daughter chromosomes.
Figure 5.15
CHAPTER 6

THE ROLE OF DNA REPLICATION IN TRANSDUCTION
In the previous chapter, the possibility was raised that DNA replication may directly influence transduction. Of course, the suggestion that DNA replication and recombination are in some way coupled, is not without precedent and has been demonstrated to be true for both T4 and \(\lambda\)-mediated events (Mosig 1983, Stahl 1979 for reviews).

The role of recombination in T4 DNA replication has been the subject of intensive study and although a detailed review of this topic will not be attempted here, a brief outline of the coupling of the two processes is relevant.

Replication of T4 DNA, early in the phage lytic cycle, initiates from defined T4 origins of replication and requires a fully functional E. coli RNA polymerase (Kojinski 1983, Macdonald et al. 1983). However, since E. coli RNA polymerase is modified during T4 development, and cannot then recognise early origin promoters, initiation of T4 DNA replication must employ a different mechanism (Rabussay 1982, Mosig 1983). Apparently, the ends of newly replicated T4 DNA are highly recombinogenic and will pair efficiently with homologous sequences; since T4 is a terminally redundant phage, i.e. has homologous sequences at each end of the genome, the ends of the infective T4 DNA molecule can pair with each other and a circular structure may be formed (figure 6.0) (Mosig 1983). To proceed efficiently, this process requires certain T4-encoded functions, such as the product of T4 gene 32, a DNA helicase (Mosig et al. 1981, Mosig, 1983). Once the strand exchanges have occurred, the 5' end of the invading strand, located in the heteroduplex region, can serve as a primer for the initiation of DNA replication, a process Mosig (1983) refers to as recombinational initiation; i.e. late in T4 development, recombination is a prerequisite for the initiation of replication.

Conversely, studies with \(\lambda\) red -mediated recombination suggested that \(\lambda\)-recombinant formation is generally dependent on replication. It has been shown that in the absence of phage replication, \(\lambda\)-red-mediated recombination in RecA\(^-\) cells results
A and B show the products of RNA polymerase-dependent initiation and replication from the origin. Without RNA polymerase, initiation occurs via recombination. Two types of structures result: either circles (C') or arrays of molecules (C,D) and both are substrates for efficient T4-directed packaging of DNA (E).

Reproduced from Mosig (1983).
Figure 6.0

Origin

Initiation Functions

Replication Functions

RNApol

Replication Functions

Recombination Functions

Packaging Functions

Recombination and Recombination
in a skewed cross-over distribution, with exchanges occurring near the right end on the \( \lambda \) chromosome. If replication is allowed, replicated \( \lambda \) progeny show a much more even distribution of exchanges, with cross-overs distributed over the entire \( \lambda \) chromosome (Stahl and Stahl 1971, Stahl et al. 1972, 1973, Stahl et al. 1974).

More recent studies have shown that red-mediated recombination in non-replicating \( \lambda \)-crosses occurs because \( \lambda \cos \) constitutes a hot spot (or is very close to a hot spot) for red-mediated recombination (Stahl et al. 1985). Genetic exchanges are limited to the neighbourhood of \( \cos \), hence the skewed distribution of cross-overs in non-replication crosses. Stahl et al. (1985) attribute the hot spot activity of \( \cos \) to cleavage, by the \( \lambda \)-terminase, of the \( \lambda \) genome at \( \cos \). This apparently occurs even without packaging, and the resultant double stranded "ends" are thought to be highly recombinogenic substrates for the red pathway. However, to recover \( \lambda \) recombinants arising from exchanges at sites distal to \( \cos \) the phage must be allowed to replicate. In other words, recombinant formation is dependent on \( \lambda \) DNA replication, except in the special case where DNA ends are generated by site specific cleavage. The mechanism by which replication brings about recombination is at the moment, a matter of conjecture. One possible hypothesis suggested by Stahl et al. (1985) is that replication of the \( \lambda \) genome, by the rolling circle mechanism, generates tails of DNA with double strand ends; such double strand ends are thought to exhibit highly recombinogenic features, in both the \( \lambda \)red and host recBC pathways of recombination (Kobayashi et al. 1982, Stahl 1979).

There is also evidence to suggest that the E.coli origin of replication, ori\( C \), is involved in the initiation of recombination. Masters et al. (1979) reported that recombination occurred with high frequency between pairs of molecules which each contained ori\( C \), e.g. plasmids carrying ori\( C \) integrated with high frequency into the chromosomally located ori\( C \) in both Rec\( A^+ \) and Rec\( A^- \) cells. Likewise, cointegrate plasmids were formed, with high frequency, between plasmids carrying ori\( C \), in a recombination event that was also Rec\( A^- \)-independent. The mechanism of these recombination events has
not been elucidated but, clearly, these observations suggest that either oriC sequences themselves, or the process of replication at oriC, confer recombinogenic properties on DNA molecules.

In addition to the above, indirect evidence from work with Pl-mediated transductional recombination points to a link between replication, or the presence of oriC sequences, and recombination. Masters (1977) reported the preferential transduction of markers, such as ilv, rbs and tna, which were co-transducible with the origin of replication, oriC (i.e. located within 2 map units, or 1 Pl "headful" of oriC). Markers situated just outside this four minute region, such as pyrE and rha, however, were transduced with much poorer efficiencies. Since it has now been established unequivocally that transductional discrimination does not stem from Pl packaging efficiencies, (Chapter 1, Chapter 3, Newman and Masters 1980, Newman 1982, Masters et al. 1984) selective recovery of origin transductants must stem from events occurring in the recipient cell. The most obvious difference between origin transducing DNA and transducing DNA derived from other chromosomal regions is the presence of oriC, which leads to two hypotheses as to why origin transducing DNA may be preferentially recombined:

Firstly, it is possible that the local DNA topology of the origin region exhibits highly recombinogenic features; certainly the nucleotide sequence of the 450 bp surrounding oriC reveals the presence of many inverted repeats in the DNA. Presumably such repeats confer the potential for the DNA to form complex secondary structures, such as stem loops or cruciform structures (Meyer et al. 1979). Whether such structures are recombinogenic is not clear, but Leach and Stahl (1983) (see chapter 1) suggest that cruciform structures are physically identical to Holliday junctions and may be recognised and resolved, by host recombination functions such as recBC enzyme, or by the product of the sbdB gene. If such events occur at oriC, then perhaps recombinogenic double strand breaks or nicks are generated during the "resolution" process.

The second obvious possibility is that oriC sequences present in transducing DNA, confer on that DNA, the ability to replicate once
it enters the recipient cell. Whether such replication events take place is not known, but if they do, this might result in enhanced transduction of origin DNA since, for example, more copies of this DNA would be available to participate in recombination, or recombinogenic ends may become available during the process of replication.

It was mentioned in Chapter 1 that transducing DNA in phage heads is linear, and that after injection into recipient cells, the DNA destined to form recombinants possibly remains linear rather than adopting the circular protein/DNA conformation of abortive DNA (Sandri and Berger 1980a, b, Masters 1985). Fuller et al. (1981) reported that initiation at oriC, in vitro, is inefficient when the substrate DNA, containing oriC, is linear. Assuming these in vitro observations are a reflection of the in vivo processes, it might be predicted that linear origin transducing DNA would not be an efficient substrate for initiation of DNA replication and therefore may not replicate. However, such linear DNA is probably highly recombinogenic or susceptible to nucleolytic activity (see Chapter 1 and above) and so will recombine or degrade rapidly.

If, on the other hand, the origin DNA adopts the supercoiled circular conformation characteristic of abortively transduced molecules (Sandri and Berger 1980b) initiation of replication at oriC may well take place; Fuller et al. (1981) reported that in vitro initiation at oriC occurs efficiently when substrate DNA is covalently closed and negatively supercoiled. If so, two outcomes are immediately apparent: multiple copies of origin transducing DNA sequences may result, although whether such copies will separate from each other or remain attached, is difficult to anticipate. Since abortive DNA is not continuous covalently closed circular DNA, but is a DNA circle with the ends linked by a protein (Sandri and Berger 1980b), it is difficult to predict whether DNA replication will terminate "correctly" to give complete, separate, daughter abortive molecules. Intuitively it would seem likely that "correct" termination does not take place and that the abortive structure may well be disrupted by the arrival of replication forks at the DNA ends held by the DNA binding protein.
This may result in the structure becoming broken, or nicked or
gapped and so force the newly replicated stable abortive DNA into
a more recombinogenic form. Alternatively, the putative replication
forks may abort before reaching the ends of the DNA molecule. This
would give more than one copy of the origin DNA, but these would be
physically linked at the unreplicated region.

Of course the above suggestions are highly speculative
but do indicate possible mechanisms by which replication at oriC
carried on transducing DNA might stimulate recombination:

(1) replication may increase the number of copies of oriC-
containing transducing DNA and so increase the likelihood
of a recombination event;

(2) replication may increase the number of copies and
concomitantly convert stable abortive DNA to a more
recombinogenic form.

Other circumstantial evidence suggesting that replication
may influence transductional recombination was implied from the
results presented in the previous chapter (section 5.6 for
discussion); one possible interpretation of these results was
that replication of DNA was a prerequisite for transductional
recombination. (However, this was not the only possible inter-
pretation of the observations (Section 5.6).)

It was of considerable interest, therefore, to study the
effects of DNA replication on the transduction of markers linked to
oriC (i.e. where both resident and transducing DNA have the
potential to replicate) and also to explore the possibility that
transductional recombination occurring at any chromosomal location
requires replication at least of resident DNA.

A convenient method of testing the above hypotheses was to
use mutant strains temperature sensitive for the dnaA gene,
one of the genes required for initiation of DNA replication at oriC.
A number of such dnaAts strains have been isolated and accordingly,
a number of different dnaAts alleles are available (Carl 1970,
1970, Abe and Tomizawa 1971). (However, for the purposes of the
experiments described below only dnaA167, dnaA5, and dnaA46 were tried).

The growth characteristics of dnaAts strains, when shifted from the permissive (30°C) temperature to the restrictive temperature (42°C) can be briefly summarised as follows:

If dnaAts mutants are shifted from 30°C to 42°C, initiation stops, but ongoing rounds of replication continue to completion. The cells continue to grow and divide normally for the period, after shift up, until all replication rounds in progress have completed and the cells have divided after termination of replication, i.e. C-time + D-time = 60 minutes, (Zyskind et al. 1977, Hirota et al. 1968). With continued incubation normal division ceases resulting in an increase in cell size, but some anucleate cells are produced by an abnormal asymmetrical division process (Hirota et al. 1986). In addition, cells accumulate "initiation potential" such that if they are returned to 30°C, because the dnaA product is not irreversibly denatured at 42°C, initiation occurs immediately and synchronously in all cells. This initial initiation can be rapidly followed by further initiation events if sufficient cell growth has taken place at the restrictive temperature (Eberle et al. 1982).

The above behaviour of dnaAts strains was made use of to determine whether a correlation exists between the replication of particular chromosomal markers and their transductional recombination. The rationale for the experiments described below was as follows:

Consider a dnaAts mutant grown at 30°C. The replication forks of the culture (as a whole) would be distributed over the chromosomes of the population of cells. If such cells were shifted to 42°C, no further initiations would occur and the distribution of replication forks in the population would start to change; since the dnaA protein is not required for chain elongation, the forks would continue to travel towards the terminus, so replication would cease, firstly for origin markers and then, in sequence, for markers replicated early, midway and, finally, those replicated late in the cycle. (Figure 6.1).
Arrows indicate the direction of travel by the replication forks. 1, 2 and 3 represent the chromosomes of three exponentially growing cells, at the time of shift up to 42°C (A), at a time equal to C-time/2 after shift up (B) and at a time equal to C-time after shift up (C). (Note, the lines indicate one half only of the replicating chromosomes.)
His process would reach completion after a period equal to the C-time. If transductional recombination of a marker occurs at the time it is replicated, then if transducing phage were added shortly before, or immediately on shift up to 42°C, the relative transduction frequencies of markers located at various parts of the chromosome would be unaltered since the relative positions of replication forks in the population are unaltered. (Assuming transductional recombination occurs rapidly, as suggested by Sandri and Berger, 1980a and Ebel Tsipis et al. 1972ab). If phage were added (to separate cultures shifted to 42°C simultaneously) at subsequent time points, transduction first of origin markers, then in sequence, of later-replicated markers would be expected to fall, concomitant with the change in distribution of replication forks in the population, i.e. at any time point, transduction of markers downstream of replication forks should be unaffected, whereas transduction of markers upstream of the position of the replication forks would be expected to fall.

An alternative (or additional) means of testing the above hypothesis would be to shift a dnaAts strain to 42°C, and allow all replication and "normal" division to complete. The culture could then be shifted to 30°C for 5 minutes (as described above) and returned to 42°C, thus allowing a synchronous initiation of DNA replication in the culture. The replication forks would then travel synchronously from oriC to terC and the culture would then divide synchronously, some 60 minutes after initiation (Eberle et al. 1982, A. Jenkins, unpublished observation). Thus if transductional recombination requires replication, only replicating markers would constitute substrates for recombination, i.e. transduction of individual markers should correlate with the position of the replication forks in the culture and so will depend on the time, after initiation, at which P1 transducing phage are added.

As already mentioned, the experiments suggested above assume that after entering the recipient cells, transducing DNA either recombines, is degraded, or forms abortive molecules rapidly and does not remain in the cell in a recombinogenic state
for long periods of time. Certainly the observations of Sandri and Berger (1980a) and Ebel-Tsipis (1972a,b) suggest that the recombination events following both P1 and P22-mediated transduction occurs rapidly and are complete within 20 minutes.

SECTION 6.2 CONSTRUCTION AND CHARACTERISATION OF MM327 dnaA46 AND MM327 AND dnaA167

MM327 dnaA46 and MM327 dnaA167 were constructed by an identical protocol: The dnaA gene is located at 83.1 map units and tnaA at 83.3 map units on the E.coli chromosome (Bachman, 1983). The two markers are thus closely linked. Accordingly, P1 transducing lysates were prepared on WM1026 (dnaA46, TnaA+) and N167 (dnaA167, TnaA+) and used, separately to transduce MM327 (DnaA+, TnaA-) to TnaA+. TnaA+ transductants were screened for template sensitive growth at 42°C and checked to ensure that they still retained the other MM327 parental markers (except, of course, TnaA-).

TRANSDUCTION OF MM327 (dnaA167) and MM327 (dnaA46) TO Ilv+ Arg+ Lys+ Trp+ AFTER INHIBITION OF INITIATION OF REPLICATION

MM327 (dnaA167), MM327 (dnaA46) and MM327 (dnaA+) were grown, shaking in L broth (Ca2+) at 30°C, to an OD540 of 0.3. The cultures were each subdivided into 3 flasks which were shifted simultaneously at 42°C. P1 transducing phage were added to the cultures either at the time of shift up, 10 minutes after shift up, or 30 minutes after the shift to 42°C. 5 minutes after the addition of the phage, Na citrate was added to the cultures, to a final concentration of 10 mM, to prevent further adsorption of the transducing phage. The cultures were grown, shaking at 42°C for a further 35 minutes after the addition of phage, to ensure all recombination, degradation or formation of abortives would have occurred. (Sandri and Berger (1980a) showed these processes were complete within the first 20 minutes after adsorption and probably occurred even before this 20 minutes period.) Samples
### TABLE 6.1: TRANSDUCTION OF MM327, MM327 dnaA46 AND MM327 dnaA167 AFTER TEMPERATURE SHIFT

<table>
<thead>
<tr>
<th></th>
<th>t = 0 minutes</th>
<th>t = 10 minutes</th>
<th>t = 30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ilv&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Arg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Lys&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>MM327</strong></td>
<td>2003</td>
<td>392</td>
<td>583</td>
</tr>
<tr>
<td><strong>Ratio</strong></td>
<td>5.1</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>MM327 dnaA46</strong></td>
<td>3212</td>
<td>403</td>
<td>601</td>
</tr>
<tr>
<td><strong>Ratio</strong></td>
<td>8.0</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>MM327 dnaA167</strong></td>
<td>3111</td>
<td>396</td>
<td>591</td>
</tr>
<tr>
<td><strong>Ratio</strong></td>
<td>7.8</td>
<td>1.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Footnotes**

The number of transductants for each marker was calculated as the sum of the number of colonies on duplicate selective plates, spread with a 200 μl aliquot of culture. The time given above each table is the time, after shift up to 42°C, at which phage were added to the culture.
were then taken, washed with phage buffer and plated on appropriate selective plates which were incubated at 30°C to score transductants. During the experiment, the OD$_{540}$ of the cultures were followed and the cultures were diluted 2 fold at each mass doubling to ensure that they remained in exponential growth and the culture OD$_{540}$ never increased above 0.4. For each sample, Ilv$^+$ Arg$^+$ Lys$^+$ and Trp$^+$ transductants were scored and viable counts were estimated (Table 6.1). The results indicated that there was no demonstrable correlation between the location of replication forks and the position at which transductional recombination takes place; had this been the case, the transduction frequencies of Ilv$^+$, Arg$^+$, Lys$^+$, and Trp$^+$ would have been expected to vary during the experiment, according to the following predictions:

Assuming C-time at 42°C is about 40 minutes (this has not been established, but for a number of reasons it seems likely that 40 minutes is the minimum attainable C-time. This will not be discussed here, since an approximate estimate for C-time at 42°C will suffice for the purposes of the discussion.) The velocity of the replication forks will be

$$\frac{50 \text{ map units (distance from oriC to terC)}}{40 \text{ minutes}} = 1.25 \text{ map units/minute.}$$

After a dnaA strain has been shifted to the non-permissive temperature, no further initiations occur, but existing replication forks would still continue to travel from oriC to terC at 1.25 map units/min, i.e. replication of the markers in the culture would cease, first for origin markers and then for early, midway and late markers, as the replication forks pass to terC (see Section 6.1). The time at which replication of a specific marker will cease, in the population, can be estimated from the map position of the marker and the velocity of the replication fork. Ilv at 84.6 minutes, is 0.6 minutes from oriC. Therefore the time at which Ilv will cease to be replicated after the shift to 42°C
distance from oriC to ilv = 0.6 map units
replication velocity = 1.2 map units/minute

= 0.48 minutes.

argH, lysA and trp are 5.5 map units, 22.6 map units and 46.2 map units from oriC respectively, so, in the population, replication of these markers would cease after:

\[
\begin{align*}
\frac{5.5 \text{ map units}}{1.25 \text{ map units/minute}} &= 4.4 \text{ minutes for argH} \\
\frac{22.6 \text{ map units}}{1.25 \text{ map units/minute}} &= 18.1 \text{ minutes for lysA} \\
\frac{46.4 \text{ map units}}{1.25 \text{ map units/minute}} &= 36.9 \text{ minutes for trp}
\end{align*}
\]

Thus, for the experiment described above, if recombination required replication, addition of phage at time of shift up should not have altered transduction frequencies of any markers in the dnaAts or DnaA+ strains. Addition of phage after 10 minutes should have resulted, in the dnaAts strains, Ilv+ and Arg+ transduction being reduced, but Lys+ and Trp+ would have been unaffected. Addition of phage after 30 minutes should have caused a reduction of Ilv+ Arg+ and Lys+ transduction in the dnaAts strains with only Trp+ being unaffected.

Clearly, the results presented in Table 6.1 demonstrate that there is no correlation between the time at which phage are added, after shift up, and the transduction of Ilv+ Arg+ Lys+ or Trp+, which suggests that transductional recombination does not occur at the time of replication of the resident homologous DNA. However, there still remains the possibility that recombinogenic intermediates were much more stable than had been expected and remained in the recipient cells during the period of growth at 42°C. Then, once the cells were plated at 30°C, (in order to score transductants) initiation of replication could again commence, so recombination might have occurred subsequently at replicating markers. This, however, was considered unlikely since,
as already mentioned, transducing DNA destined to recombine, probably remains linear and linear DNA is very susceptible to degradation within E.coli cells (see Chapter 1).

The results presented in Table 6.1 were anomalous in one respect. The ratios of Ilv⁺:Arg⁺:Lys⁺:Trp⁺ transductants in the isogenic control strain MM327 were 5.1:1:1.5:0.4 respectively whereas the ratios of these same markers in the dnaAts strains were 7.8:1:1.5:0.4 respectively. Clearly, Ilv⁺ transduction is stimulated, in the dnaAts strains, using the experimental protocol described above. One possible explanation for these results was that the inactivated dnaA protein might, in some way, be stimulating transduction of markers closely linked to oriC; there are a number of dnaA protein binding sites at oriC (Fuller and Kornberg 1983) so perhaps the denatured dnaA protein was binding to the transducing DNA at oriC and causing nicks or local topological changes in the DNA which resulted in increased recombinogenicity. However, the experiments presented later in this chapter eliminate this possibility, and these are discussed in Section 6.6.

Another possible explanation for the stimulation of Ilv⁺ transduction is afforded by the observations of Eberle et al. (1982) (described above) who reported that initiation mutants, grown at the non-permissive temperature accumulate initiation potential. Presumably if oriC transducing DNA entered dnaAts cells grown at 42°C, it would achieve and maintain the abortive configuration with the same efficiency as other transducing DNA, since no initiation of DNA replication could occur. After incubation at 42°C, for 40 minutes, cells were plated at 30°C and then would be able to initiate replication. Eberle et al. (1982) reported that after only 1.5 generation equivalents at the non-permissive temperature, 4-5 initiation events could occur in the cell once it was returned to the permissive temperature. Since, during the experiment described above the dnaAts strains remained at 42°C for a minimum of 40 minutes (about 1.5 generation equivalents), it is reasonable to assume that rapid initiations would have occurred at both resident oriC sequences and those present on the putative oriC-abortive DNA. As a consequence, Ilv⁺ transduction
might be stimulated by increasing the number of ilv copies available for recombination, or disruption of the stable abortive configuration, or possibly the initiation event itself renders DNA more recombinogenic.

In order to investigate the role, if any, of DNA replication at oriC in influencing the transduction of markers linked to oriC, three approaches were adopted, and these are described in the following sections.

SECTION 6.3 SEGREGATION OF MARKERS COTRANSDUCIBLE WITH oriC

As mentioned previously, transducing DNA carrying oriC sequences may be able to replicate once it enters the recipient cell. Hence, markers carried on the same transducing molecule may be replicated, after injection of the transducing DNA, independently of the resident chromosome. Origin markers may therefore be expected to exhibit different segregation lags from those predicted from their map locations. For example, if such markers replicated autonomously and could segregate to daughter cells at division independently of the resident chromosomes, the number of "origin" transductants may increase immediately after transduction, i.e. in this instance, no segregation lags would be observed since the lags stem from the chromosomal position-dependent segregation of wild type and mutant alleles, to daughter cells.

However, assuming origin markers do exhibit lags, the duration of the expected lags for origin markers can be estimated, as before, by the following procedure:

Consider the lags between the increase of Trp+ transductants and Ilv+ or Tna+ transductants.

Ilv (84.6 map units) is located 0.6 map units from oriC. Tna (83.3 map units) is located 0.6 map units from oriC (Bachman 1983).

Velocity of replication forks at 37°C = 1.25 map units/minute (see Chapter 5).
Therefore time required for replication forks to travel from ilv to trp (trp is at 27.7 map units) =

\[
\text{distance from ilv to trp} = \frac{46.2 \text{ map units}}{1.25 \text{ map units/minute}} = 37 \text{ minutes}
\]

Therefore, time required for replication forks to travel from tna to a position an equivalent distance as trp from the terminus is also approximately 37 minutes.

i.e. Ilv\(^+\) and Tna\(^+\) transductants would be expected to exhibit a segregation lag about 37 minutes longer than for Trp\(^+\). (Assuming that replication does not affect segregation.)

To test the possibility that oriC-linked markers, which may be able to replicate, exhibit different segregation lags from those predicted above, MM303 was grown exponentially in L-broth Ca\(^{2+}\) to OD\(_{540}\) = 0.3 and P1-transduced as described before. The transduction mix was diluted into L-broth citrate and grown for a further 180 minutes as described previously. Samples were taken at the time of dilution and at 15 minute intervals thereafter and viable counts/ml, Ilv\(^+\), Tna\(^+\), Arg\(^+\) and Trp\(^+\) transductants were scored (Tables 6.2, figures 6.2A, 6.2B).

Clearly, transductants receiving either of the two origin markers Ilv\(^+\) and Tna\(^+\) did not exhibit the predicted lags before increasing in number, whereas Arg\(^+\) and Trp\(^+\) transductants showed the segregation lags predicted from the segregation model (i.e. Trp\(^+\) increased 34-35 minutes before Arg\(^+\) transductants, see Chapter 5). Instead, Ilv\(^+\) and Tna\(^+\) transductants increased from the time the transduction mix was diluted into the growth medium. The rate of initial increase of Tna\(^+\) or Ilv\(^+\) transductants was slow, with a doubling time of about 220 minutes (which was about 8 times that of the untransduced cells (27 minutes)) and this period lasted for about 90-100 minutes. After this period of slow increase, at about the time predicted by the segregation model, (see above) the rate at which Ilv\(^+\) or Tna\(^+\) transductants were multiplying, increased sharply, to that of the growth rate of the culture (Figures 6.2A and B).
<table>
<thead>
<tr>
<th>Time in Minutes</th>
<th>Viable counts/ml</th>
<th>Tna&lt;sup&gt;+&lt;/sup&gt;/5</th>
<th>I1v&lt;sup&gt;+&lt;/sup&gt;/5</th>
<th>Arg&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Trp&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.9 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>569</td>
<td>467</td>
<td>389</td>
<td>263</td>
</tr>
<tr>
<td>15</td>
<td>1.2 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>518</td>
<td>504</td>
<td>384</td>
<td>277</td>
</tr>
<tr>
<td>30</td>
<td>1.5 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>583</td>
<td>425</td>
<td>388</td>
<td>266</td>
</tr>
<tr>
<td>45</td>
<td>2.7 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>653</td>
<td>510</td>
<td>403</td>
<td>264</td>
</tr>
<tr>
<td>60</td>
<td>3.7 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>658</td>
<td>528</td>
<td>-</td>
<td>328</td>
</tr>
<tr>
<td>75</td>
<td>4.9 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>701</td>
<td>584</td>
<td>460</td>
<td>408</td>
</tr>
<tr>
<td>90</td>
<td>7.8 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>750</td>
<td>600</td>
<td>480</td>
<td>476</td>
</tr>
<tr>
<td>105</td>
<td>1.2 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>-</td>
<td>640</td>
<td>524</td>
<td>592</td>
</tr>
<tr>
<td>120</td>
<td>2.2 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1049</td>
<td>792</td>
<td>6811</td>
<td>952</td>
</tr>
<tr>
<td>135</td>
<td>2.6 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1787</td>
<td>1112</td>
<td>1112</td>
<td>1088</td>
</tr>
<tr>
<td>150</td>
<td>4.5 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2705</td>
<td>1392</td>
<td>1392</td>
<td>1456</td>
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<tr>
<td>165</td>
<td>5.2 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2886</td>
<td>2160</td>
<td>1696</td>
<td>2288</td>
</tr>
<tr>
<td>180</td>
<td>6.8 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Footnotes**

For each marker, the number of transductants was calculated as the mean number of transductants on 2 duplicate plates.

Time zero was the time at which the transduction mix was diluted into the growth medium.
FIGURE 6.2  TRANSDUCTION OF MM303 TO Ilv⁺, Arg⁺, Trp⁺, Tna⁺

A.

○ = viable counts
△ = Trp⁺ transductants
● = Arg⁺ transductants
■ = Ilv⁺ transductants
▼ = Tna⁺ transductants

B. B depicts the graph, shown in 6.2A, replotted with transduction frequencies plotted as the number of transductants per initial number. For clarity, the curves are shown here and the points have been omitted.
Figure 6.2

A

TIME IN MINUTES

NUMBER OF TRANSDUCTANTS

10^2

10^3

10^4

Viable counts/mL

10^7

10^8

10^9

0 15 30 45 60 75 90 105 120 135 150 165
6.2 contd.

**Diagram B**

- **Y-axis:** Number of transductants per initial number
- **X-axis:** Time in minutes

Graph showing the increase in number of transductants over time, with data points at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, and 165 minutes.
The processes which give rise to the slow increase in origin transductants are a matter of speculation.

The most attractive and economical explanation for the early slow increase of Tna\(^+\) and Ilv\(^+\) transductants is that, in the 90-100 minute period after the transduction (i.e. when the origin transductants were increasing slowly), some of the transducing DNA carrying oriC, plus Tna\(^+\) or Ilv\(^+\) sequences had escaped recombination or degradation and was undergoing replication. The slow increase could thus be accounted for in one of at least two ways:

Replication of origin transducing DNA could have provided more than one physically separate copy of the transducing DNA which could segregate to both daughter cells even at the earliest divisions. Recombination would later occur in each cell.

An alternative (and perhaps more plausible) explanation would be that replication of origin DNA was providing more than one copy of the transducing DNA, but as already mentioned, it seems doubtful that replication of oriC transducing DNA would generate physically separate molecules since it is not clear whether replication would be able to proceed to completion (see section 6.1). However, if replication did begin, the resultant daughter molecules even if unable to separate, might be able to recombine with more than one of the resident homologous oriC regions. (Note that at the fast growth rates used here, the recipient cells would have been replicating dichotomously (Cooper and Helmstetter, 1968) so there would be more than 1 recipient origin present in the cells, see also Chapter 5.) In other words, if more than one copy of the wild type Ilv\(^+\) or Tna\(^+\) allele were present in each recipient (in contrast to the "normal" situation where recipient cells, receiving transducing DNA which could not replicate, would have contained only one copy) recombination would have been possible with more than one of the resident sequences. If so, no segregation lags would have been observed since there would have been no requirement for replication of the "recombined" wild type allele before two transductant cells could arise.

At the latter part of the experiment, about 90 minutes post transduction, Ilv\(^+\) and Tna\(^+\) transductants started to increase at
the same rate as the culture. This occurred, for Ilv* transductants, at the time predicted by the segregation model but for Tna* transductants, this seemed to occur slightly earlier than predicted (Figure 6.2B). However, it was difficult to accurately estimate the time at which Tna* transductants start increasing rapidly, since the points are rather scattered at the relevant part of the Tna* curve (figure 6.2A). In spite of this, it seems likely that this period of rapid increase was indeed due to the completion of the Ilv* or Tna* segregation lags.

The above results, therefore, suggest that in the period immediately following transduction, some oriC-Tna* and oriC-Ilv* transducing molecules underwent recombination immediately, but some of these molecules were able to persist and replicate within the recipient cells for a period of at least 90 minutes. However, since the initial increase in origin transduction was very slow, it would suggest that either replication of origin transducing DNA was very inefficient or that relatively few such molecules survived in the recipient cells long enough to be replicated. After this 90 minute period, origin transductants increased rapidly, at the same growth rate as the culture as a whole, presumably due to the end of the segregation lags for origin DNA which had recombined immediately after entering the recipient.

SECTION 6.4 EFFECT ON TRANSDUCTION OF DELETING oriC FROM RECIPIENT CELLS

The E.coli strain DK201 (Kogoma and Von Mayenberg, 1983) harbours a small deletion (Δ1071) which removes: \(\text{atpB, gidA, oriC and asnA,}\) i.e. is about 5-10 kb in extent (Bachman 1983). This strain, however, is viable since it harbours the sdrA mutation and so is derepressed for constitutive stable DNA replication. The sdrA lesion maps to the \(\text{rnh (RNAseH)}\) gene and it is thought that removal or inactivation of RNAseH allows initiation of replication to commence at least 4 or 5 "secondary" origins of replication on the E.coli genome, located at positions 95, 45, 20 and two close to terC, at position 32 on the E.coli map (De Massey et al. 1984, Ogewa et al. 1984); indeed, it now seems likely that
RNAseH is involved in ensuring that in normal (OriC+, SdrA+) cells, initiation occurs specifically at OriC and not at the secondary origins of replication (Kogoma et al. 1985).

In addition to 1671, sdrA, DK201 also contains the temperature sensitive dnaA5 allele, but since constitutive stable DNA replication is independent of the dnaA protein, DK201 will grow at both 30°C and 42°C (Lindahl and Lindahl 1984, Kogomá and Kornberg 1984).

DK201, therefore, seems ideal to test the effects of replication from OriC sequences on the transduction of origin markers, such as ilv. For example, if DK201 were grown and transduced with P1 at 30°C, incoming OriC-transducing DNA would presumably be able to initiate replication from OriC since at 30°C, the recipient cells dnaA5 product is active; in this instance, only the incoming DNA would be able to replicate since the small OriC deletion in the recipient would prevent the initiation of replication from the corresponding 84 map unit region in the recipient (Bachman 1983). However, if DK201 was grown and transduced at 42°C, then the dnaA protein would be inactive, so neither OriC-transducing DNA nor the corresponding region of the recipient chromosome could initiate replication from the 84 map unit region of the genome. Thus by growing and transducing DK201 at 30°C and 42°C and scoring transduction of markers linked to OriC, or distant from OriC, it should be possible to determine whether replication from resident OriC sequences, or from incoming OriC sequences, or both, is responsible for the high levels of transduction of OriC-linked markers.

In addition, DK201 harbours the his22 mutation (at position 44 on the E.coli map, Bachman, 1983) which is close to one of the putative sdrA-dependent origins (located at position 45, Ogawa et al. 1984). If replication from the putative secondary origins in sdrA strains also stimulates transduction of linked markers, then His+ transduction might well rise, both at 30°C and 42°C.
DK201 was grown shaking at 30°C, in a low phosphate minimal medium, appropriately supplemented containing 26 µg/ml Km, to OD$_{540}$ = 0.3. (A minimal medium was used because Kogoma et al. (1985) reported that sdrA strains are sensitive to rich media and even in the minimal medium DK201 grew very slowly, requiring 3 days growth at 30°C to reach the required OD$_{540}$ (data not shown). The culture was divided into 2 parts, and 1 ml cells from each sample were concentrated 10-fold by centrifugation and then placed at 30°C or 42°C for 30 minutes (to ensure that all the dnaA5 protein was denatured at 42°C). The two samples were then transduced with a P1 lysate (prewarmed to the required temperature) as described previously.

Ilv$^+$, Arg$^+$ Trp$^+$ His$^+$ and Met$^+$ transductants were scored by plating aliquots of the transduction mix on to appropriate selective plates; the 42°C transduction mix was spread on to prewarmed (42°C) plates and incubated for 3 days at 42°C. Similarly, the 30°C transduction mix was spread on plates prewarmed to 30°C and incubated at 30°C for 3 days. The results are shown in Table 6.3.

The P1 lysate utilised for the above transduction was prepared from W3110 grown in L-broth Ca$^{2+}$ as already described. To ensure that no L-broth was added to the transduction mixes (since DK201 is broth sensitive), it was necessary to pellet the P1 phage by high speed centrifugation (Chapter 2) and then resuspended the phage gently into 100 mMTRIS pH 7.3 10 mM CaCl$_2$. (Phage buffer was not used because the phosphate in phage buffer (see Chapter 2) was found to be precipitating calcium from the transduction mix and so preventing adsorption.)

The results (Table 6.7) showed that similar levels of transduction were obtained at both 30°C and 42°C for ArgH$^+$, MetB$^+$, Trp$^+$, His$^+$; the ratios with which these transductants were recovered were also similar at 1.0:0.8:0.6:0.3 respectively. These ratios are very similar to those reported by Masters (1977) for transduction of OriC$^+$ DnaA$^+$ strains at 37°C.
### Table 6.3A: Transduction of DK201 at 30°C

<table>
<thead>
<tr>
<th></th>
<th>Ilv⁺</th>
<th>ArgH⁺</th>
<th>Trp⁺</th>
<th>MetB⁺</th>
<th>His⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>173</td>
<td>180</td>
<td>92</td>
<td>140</td>
<td>62</td>
<td></td>
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<tr>
<td>179</td>
<td>167</td>
<td>107</td>
<td>132</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>188</td>
<td>173</td>
<td>101</td>
<td>153</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>540</strong></td>
<td><strong>520</strong></td>
<td><strong>300</strong></td>
<td><strong>425</strong></td>
<td><strong>170</strong></td>
</tr>
<tr>
<td><strong>RATIOS</strong></td>
<td>1.0</td>
<td>1.0</td>
<td>0.6</td>
<td>0.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>

### Table 6.3B: Transduction of DK201 at 42°C

<table>
<thead>
<tr>
<th></th>
<th>Ilv⁺</th>
<th>ArgH⁺</th>
<th>Trp⁺</th>
<th>MetB⁺</th>
<th>His⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>133</td>
<td>76</td>
<td>101</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>142</td>
<td>63</td>
<td>111</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>150</td>
<td>65</td>
<td>121</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>0</strong></td>
<td><strong>425</strong></td>
<td><strong>204</strong></td>
<td><strong>333</strong></td>
<td><strong>132</strong></td>
</tr>
<tr>
<td><strong>RATIOS</strong></td>
<td>-</td>
<td>1.0</td>
<td>0.5</td>
<td>0.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Since His\(^+\) transduction did not differ in the sdrA strain from that reported for OriC\(^+\) SdrA\(^+\) recipients (Masters 1977), sdrA dependent replication from the secondary origin at 45 map units on the chromosome (Ogawa et al. 1985) if it occurred in this experiment, obviously did not affect transduction of a marker (his) cotransducible with the putative origin. However, Ilv\(^+\) transductants were recovered with the same frequency as ArgH\(^+\) transductants, so Ilv\(^+\) transduction was reduced to about 20% of the level reported by Masters (1977). Surprisingly, at 42\(^\circ\)C, no Ilv\(^+\) transductants were recovered at all. The above experiment was repeated a further three times in an attempt to recover Ilv\(^+\) transductants at 42\(^\circ\)C; the results (not shown) were identical to those above; i.e. normal levels of transduction for ArgH\(^+\) MetB\(^+\) Trp\(^+\) and His\(^+\) at both temperatures, with Ilv\(^+\) transduction equal to ArgH\(^+\) at 30\(^\circ\)C, but none of the experiments succeeded in recovering Ilv\(^+\) transductants at 42\(^\circ\)C.

The normal transduction of ArgH\(^+\), MetB\(^+\) Trp\(^+\) and His\(^+\) and the inability to recover Ilv\(^+\) transductants at 42\(^\circ\)C could be explained in a number of ways.

1. The inability of both the oriC-transducing DNA and the resident origin region DNA (which harbours the small oriC) to initiate replication at 42\(^\circ\)C had reduced the transduction of origin-linked markers to undetectably low levels. This seems implausible since, without the ability to initiate replication the transduction of oriC-linked markers would be expected to fall to that of non oriC-linked markers and not to be abolished totally.

2. Recipient cells receiving Ilv\(^+\) sequences (or sequences flanking oriC) were inviable at 42\(^\circ\)C on -ilv selective medium; again, this explanation seems implausible since there is no apparent reason as to why this should occur.

3. The most plausible explanation is that at 42\(^\circ\)C, the denatured dnaA5 product was specifically interfering with the transduction of oriC-linked markers.
To investigate the second possibility, 10 Ilv\textsuperscript{+} transductants were picked from a selective plate spread with cells which had been transduced and incubated at 30\degree C. These were patched on to a -ilv selective plate and incubated at 42\degree C. All grew after 2-3 days, indicating that transductants receiving Ilv\textsuperscript{+} sequences were viable on -ilv selective plates at 42\degree C. This in turn suggested that the inability to recover Ilv\textsuperscript{+} transductants at 42\degree C was not due to the introduction of sequences which were lethal to the recipient at 42\degree C, when grown on -ilv selective plates.

It was not possible to establish whether the inability to recover Ilv\textsuperscript{+} transductants at 42\degree C was due to the inactive dnaA5 product interfering with origin-specific transduction or due to the prevention of replication from oriC sequences at 42\degree C.

Similarly, the results of the 30\degree C transductions, described above, were also difficult to interpret unequivocally, since the reduced Ilv\textsuperscript{+} transduction may also stem from more than one course:

1. The small oriC deletion at the origin region, may perhaps be preventing homologous pairing of the recombinating molecules. Alternatively, the removal of oriC sequences from the recipient may have deleted the putative recombinogenic sequences or structural features which may be responsible for initiating the high levels of transductional recombination at oriC (see Section 6.1).

2. The dnaA5 product, although capable of initiating DNA replication at 30\degree C was in some way defective and was inhibiting origin transduction.

3. Prevention of replication from the resident oriC region was resulting in reduced transduction of flanking markers such as Ilv.

The above results are therefore somewhat inconclusive as to the role of oriC-specific DNA replication in influencing the transduction of markers linked to oriC. Also, the strain DK201 was very slow growing and difficult to work with so its use was dropped and an alternative approach was adopted to study the role of oriC-dependent DNA replication on the transduction of oriC-linked markers.
SECTION 6.5  TRANSDUCTION OF DnaAts INTEGRATIVELY SUPPRESSED STRAINS

As already mentioned, to study the effects of replication from oriC on the levels of recombination at flanking markers, it was necessary to prevent replication from oriC during both the transduction and growth on selective plates. In the previous experiment this had been achieved using DK201, on oriCΔ, dnaAts sdrA strain. An alternative approach would be to use oriC⁺ strains, temperature sensitive for the dnaA gene, but containing an additional origin of replication that could be used during growth at 42°C.

Two such strains were constructed from MM327 (dnaA167) and MM327 (dnaA46).

CONSTRUCTION OF MM328 (MM327 dnaA46, pKN500) AND MM329 (MM327, dnaA167, pKN500)

CM1843 (Von Mayenberg and Hansen 1980) harbours the same oriC deletion as DK201, but can replicate its DNA because it also contains pKN500, a Km⁺ derivative of the plasmid R1, integrated into its chromosome (Molin and Nordstrom 1980). Masters (unpublished observations) localised the site of insertion to near MetB, which maps at position 89 on the E.coli map (Bachman 1983).

A P1 lysate was prepared on CM1843 and the lysate used to transduce both MM327 (dnaA46) and MM327 (dnaA167) to Km⁺ and temperature resistant growth at 42°C. 10 Km⁺ clones were picked (for each strain) from the 42°C incubated plates and checked to ensure they still retained the parental MM327 (dnaAts) auxotrophic markers; 1 clone (of each) was picked (called MM328 and MM329) and checked to ensure they still harboured, respectively, the dnaA46 and dnaA167 alleles. This was accomplished by making P1 plate lysates on both MM328 and MM329 and using the lysates to transduce MM327 to Tna⁺ (Tna is closely linked to dnaA · Bachman 1983). 10 Transductants from each lysate were screened for temperature sensitive growth at 42°C. All Tna⁺ transductants were unable to grow at 42°C, indicating that the donor strains MM328 and MM329 still retained their dnaAts alleles.
TRANSDUCTION OF MM328 AND MM329 TO Ilv\textsuperscript{+} Arg\textsuperscript{+} Lys\textsuperscript{+} Trp\textsuperscript{+} His\textsuperscript{+} AT 30°C AND 42°C

MM328 and MM329 were both grown, shaking, in L-broth Km at 42°C to OD\textsubscript{540} of 0.4. Cells were concentrated 10-fold by centrifugation. Aliquots were placed at 30°C and at 42°C and incubated for 30 minutes, before transduction with P1 lysates, prewarmed to 30°C and 42°C respectively. (The 30 minute period of incubation at 30°C and 42°C was to ensure that the thermosensitive dnaA proteins had fully denatured before transduction.) The cultures grown and transduced at 30°C and 42°C were spread on to selective plates, prewarmed to 30°C and 42°C respectively to score transductants.

Viable counts/ml, Ilv\textsuperscript{+} Arg\textsuperscript{+} Lys\textsuperscript{+} Trp\textsuperscript{+} transduction were measured (Tables 6.4 A and B) for both MM328 and MM329 both at 30°C and 42°C.

The results show clearly that at 30°C, Ilv\textsuperscript{+} Arg\textsuperscript{+} Lys\textsuperscript{+} Trp\textsuperscript{+} and His\textsuperscript{+} are transduced with ratios of 4:1.2:0.6:0.3 respectively, which are very similar to the ratios reported for transduction of these markers in OriC\textsuperscript{+} DnaA\textsuperscript{+} recipient strains at 37°C (Masters 1977, Chapter 5). At 42°C transduction of Arg\textsuperscript{+} Lys\textsuperscript{+} Trp\textsuperscript{+} and His\textsuperscript{+} is reduced by about 2-fold for each marker but the ratios are still 1.0:2:0.6:0.3 respectively. Ilv\textsuperscript{+} transduction at 42°C is dramatically reduced by about 30-fold when MM329 is the recipient and by about 90 fold when MM328 is the recipient. Thus at 42°C but not 30°C, the transduction of Ilv\textsuperscript{+} is specifically reduced when compared to other markers (Tables 6.4 A and B). These results, therefore, are highly suggestive that when DNA replication from oriC is prevented, the high transduction characteristic of markers close to oriC is reduced, but other markers, more distal to oriC, are unaffected. Therefore these results support the hypothesis that replication from oriC is responsible for the high levels of origin transduction. Unfortunately, the strains used here did not harbour any other suitable origin markers apart from ilv, so it is not possible to say whether transduction of other markers closely linked to oriC would also have been reduced. Clearly, it would be desirable to repeat the above experiment using strains harbouring
### TABLE 6.4A  
TRANSDUCTION OF MM328 AT 30°C AND 42°C

<table>
<thead>
<tr>
<th></th>
<th>Ilv⁺</th>
<th>Arg⁺</th>
<th>Lys⁺</th>
<th>Trp⁺</th>
<th>His⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>30°C</strong></td>
<td>1975</td>
<td>455</td>
<td>819</td>
<td>231</td>
<td>136</td>
</tr>
<tr>
<td>Ratio</td>
<td>4.3</td>
<td>1.0</td>
<td>1.8</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>42°C</strong></td>
<td>21.5</td>
<td>142</td>
<td>308</td>
<td>102</td>
<td>42.5</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.15</td>
<td>1.0</td>
<td>2.1</td>
<td>0.7</td>
<td>0.3</td>
</tr>
</tbody>
</table>

### TABLE 6.4B  
TRANSDUCTION OF MM329 AT 30°C AND 42°C

<table>
<thead>
<tr>
<th></th>
<th>Ilv⁺</th>
<th>Arg⁺</th>
<th>Lys⁺</th>
<th>Trp⁺</th>
<th>His⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>30°C</strong></td>
<td>1462</td>
<td>373</td>
<td>-</td>
<td>232</td>
<td>128</td>
</tr>
<tr>
<td>Ratio</td>
<td>4.0</td>
<td>1.0</td>
<td>-</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>42°C</strong></td>
<td>49.5</td>
<td>179</td>
<td>408</td>
<td>91</td>
<td>50.5</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.27</td>
<td>1.0</td>
<td>2.3</td>
<td>0.5</td>
<td>0.3</td>
</tr>
</tbody>
</table>
other markers such as rbs (at 84.4 map units on the chromosome, Bachman 1983).

**SECTION 6.6 SUMMARY AND DISCUSSION**

The work detailed in this chapter was carried out as a preliminary investigation into the possible link between DNA replication and transductional recombination: firstly considering recombination at all chromosomal locations and secondly, recombination occurring at the highly transduced region spanning the E. coli origin of DNA replication.

As already discussed in the previous chapter, it was noted that after transduction of an exponentially growing culture, transductants exhibited a marker-dependent segregation lag before increasing in number. A model was proposed to account for the spread in segregation lags and three hypotheses were put forward to explain the fact that segregation lags for all markers were 1 generation time longer than expected. Briefly, these are the following (see also Chapter 5):

1. Transductional recombination occurs at, or closely following, replication forks in the target DNA and results in a double stranded replacement of resident DNA. A second replication fork would then be required to replicate the transduced DNA before two daughter transductants were produced.

2. Transduced cells fail to grow or divide for exactly one generation time after receiving transducing DNA.

3. Transductional recombination results in a predominantly single stranded exchange reaction, so again two replication forks must pass through the transduced marker before two daughter transductants were formed.

The results presented in Section 6.2 would tend to eliminate the first possibility since there was no demonstrable correlation between the replication of and transduction of the specific markers that were tested i.e. it seemed that recombination could occur without concomitant replication of the target DNA. (However, since the cells were plated at 30°C to recover transductants, the
possibility remains that transductional recombination requires replication of the target sequence; possibly "recombinogenic" transducing DNA was more stable than had been expected from Sandri and Berger's (1980a) data, and so remained within the recipient cells until the cells were plated at 30°C. Recombination might then have occurred at replication forks formed after the initiation of replication from oriC.)

The second possible explanation (above) for the unexpected length of the segregation lags was almost certainly eliminated by the experiment in Section 6.3 which showed that cells receiving origin-transducing DNA, at least, could divide from the time immediately after transduction, when the transduction mix was diluted into the growth medium. So, unless only cells receiving origin specific transducing DNA are able to grow in the period immediately post-transduction, (which seems most implausible) then these observations tend to suggest that the second hypothesis (above) is probably incorrect.

The third hypothesis to explain the additional generation time before transductants started to multiply proposes that transductional recombination was predominantly a single stranded exchange reaction (see above). Previous reports, however, have concluded that both P1 and P22-mediated transduction results in, at least, some double stranded insertion of transducing DNA into the host chromosome (Sandri and Berger, 1980a,b, Ebel-Tsipis et al. 1972a,b). Certainly, it is clear from their data that double strand insertions do occur but their data also can be interpreted to mean that substantial heteroduplex regions may also be present (i.e. regions where single stranded exchange may have occurred). Figure 6.3 shows a reproduction of part of a figure from the paper by Sandri and Berger (1980a) who infected recipient cells (containing "Light" 3H-labelled chromosomal DNA) with purified transducing particles encapsidating 32p-labelled 5-Bromouracil density labelled "heavy" transducing DNA. The cells were lysed some 30 minutes after infection and the chromosomal DNA was extracted and purified away from unrecombined transducing DNA. Samples were then banded on caesium chloride density gradients: high molecular
Closed circles show $^{32}$P,S-BU labelled "Heavy" transducing DNA. Open circles represent $^3$H-labelled "Light" recipient chromosomal DNA. Parts D, E, F and G are not mentioned in the text and these represent the following:

D and E are control gradients containing heavy and light DNA which were mixed together and loaded on to the gradient (i.e. no transduction had taken place) and these gradients indicate the positions of the heavy and light fractions.

F and G are similar to B and C respectively, but show DNA isolated 90 minutes post-transduction. In these cases, the transducing DNA has been replicated so no $^{32}$P label is found in the heavy region of the gradient.

Part H indicates the two ways of producing hybrid density double strand DNA (see text for discussion): 1 and 4. ($H = ^{32}$P-labelled "heavy" DNA strand, $L = "light"$ DNA strand). If such DNA's were to be spun on strand separating gradients, label would remain in the hybrid density region if structure 4 was present initially (5 and 6) but would shift to the heavy region if structure 1 (i.e. heteroduplex heavy/light DNA) was present (2 and 3) before denaturation.
weight DNA (Figure 6.3A), chromosomal DNA sheared to about 10% the size of the incoming transducing molecule (Figure 6.3B) and DNA sonicated to 1% of the size of the transducing molecule (Figure 6.3C) were banded and the gradients were fractionated. Clearly, in the high molecular weight sample, the $^{32}$P-label is predominantly associated with the "light" fractions, indicating that the transducing DNA is associated with the recipient chromosome (Figure 6.3A). In the sheared sample (Figure 6.3B), some $^{32}$P-label is found in the heavy fractions, showing that double stranded transducing DNA had been released from the recipient chromosome by the shearing process. In addition, some label is found in the fractions of "hybrid" density. When the sample has been sonicated to 1% of the size of the incoming transducing DNA, the $^{32}$P-label shifts, predominantly, to the fractions of "hybrid" density (Figure 6.3C).

$^{32}$P-labelled hybrid density DNA could have arisen in two ways (Figure 6.3H), either by double stranded heavy DNA linked directly to double stranded light DNA or by regions of heteroduplex DNA with one light and one heavy strand. In the former case, if molecules were broken into ever decreasing sizes, the $^{32}$P-label would shift from the hybrid to the heavy region concomitantly with a decrease in molecular weight. In the latter case, label would remain in the hybrid region. Sandri and Berger (1980a), however, noted that with a decrease in sample molecular weight to 1% of the transducing molecule (Figure 6.3C), label shifts towards the hybrid region, which suggests that heteroduplex DNA is indeed present. Surprisingly, the peak of $^{32}$P-labelled heavy DNA observed in Figure 6.3B was lost in Figure 6.3C. This was unexpected, since a decrease in molecular weight of the double stranded heavy DNA would not have been expected to alter its density.

In any case, the observations of Sandri and Berger (1980a) are highly suggestive of the presence of substantial amounts of heteroduplex DNA, with one strand derived from the transducing DNA and the other from the resident host chromosome. Sandri and Berger (1980a) do not discuss this possibility, but if heteroduplex DNA was present, this could explain the apparently anomalous observation that the size of the (double stranded) insert resulting from
transductional recombination was only about 1/10 the size of the transducting DNA. As already mentioned in Chapter 1, it is difficult to reconcile this observation with the ability of P1 to co-transduce markers which are up to 2 map units apart on the E. coli genome (the size of the intact transducing molecule is also about 2 map units = 90-100 Kb) (Ikeda and Tomigawa 1968, Sandri and Berger, 1980a,b). One possibility (already mentioned) was that transductional recombination resulted in many small double stranded inserts but this would not be consistent with the observed patterns of cotransduction; consider three linked theoretical markers, in the order A, B and C on the chromosome. If multiple inserts occur, the frequency with which $A^+B^+C^+$ transductants are recovered should not differ from the frequency of $A^+B^-C^+$. In practice, however, $A^+B^+C^+$ occurs far more frequently than $A^+B^-C^+$ (Lennox, 1955). Possibly, transductional recombination generates extensive regions of heteroduplex DNA, flanked by double stranded regions of transducing DNA; the formation of such recombinant structures would explain the data of Sandri and Berger (1980a), who proposed that the result was a double stranded insertion of low molecular weight transducing DNA and the work presented in this thesis, which suggests that the recombination event is predominantly single stranded. Recombination could initiate from the linear ends of transducing DNA or from nicks or gaps in the transducing molecule. As described in Chapter 1, recA-promoted strand exchange would then be expected to occur, generating regions of heteroduplex DNA to each side of the crossover; certainly, such events are thought to occur in other forms of recombination, such as conjugal recombination, which results in the formation of long heteroduplex molecules (Lloyd and Thomas 1984). As described in Chapter 1, resolution of the heteroduplex joints could occur in two ways, but both would result in recombinant molecules containing both heteroduplex and homoduplex regions, and which may or may not contain double stranded insertions of transducing DNA. This model, of course, is purely speculative, but would explain the anomalous results described above.
A possible means of directly testing the nature of the recombinant molecules will be given in Chapter 7.

In addition to testing the link between replication and recombination of target DNA, at all chromosomal locations, in this chapter, the role of replication at oriC was investigated in determining the high transduction of oriC-linked markers. Clearly, the results presented in Section 6.3 showed that origin transducing DNA was able to replicate within recipient cells and the results in Section 6.5 were highly suggestive of a role for replication from oriC in influencing transduction in the origin region. For example, under conditions where neither the resident oriC sequences or the incoming transducing oriC sequences could initiate replication, transduction, specifically of ilv (0.6 map units from oriC, Bachman 1983) was reduced dramatically. Other markers, located at other chromosomal regions, were unaffected.

In addition, the above results indicate that oriC sequences per se are not the cause of high origin transduction, as was suggested in Section 6.1, but that initiation of replication at oriC is the crucial event. This is suggested by the above results because oriC sequences were present in both recombining molecules yet transduction of Ilv⁺ was reduced when initiation was prevented. A note of caution must be added since Ilv⁺ transduction was the only origin marker tested. This experiment should therefore be repeated and transduction of other origin markers should also be studied in conditions where initiation cannot occur at oriC. Also, the unlikely possibility still remains that reduction of origin transduction was not caused by the prevention of initiation at oriC, but instead, the inactive dnaA product was in some way interfering with origin transduction. This was a significant worry in the interpretation of the results, since dnaA protein has a high affinity for oriC (Fuller and Kornberg, 1983).

Additional experiments to test the role of replication from oriC in stimulating recombination will be proposed in Chapter 7.
CHAPTER 7

SUMMARY AND FUTURE PROSPECTS
INTRODUCTORY REMARKS

The results chapters of this thesis (Chapters 3-6) contain both a presentation of the experimental work and a discussion of the results. Since the data in the results chapters have already been discussed in detail it would be repetitive to do so again here. Instead, this concluding chapter concentrates mainly on briefly summarising the conclusions drawn from the experimental results (so far as is possible) and in suggesting experiments perhaps to be carried out at a later date, which may prove useful in helping to elucidate more fully the events leading to P1-mediated generalised transduction.

In addition, a model for the transduction process as a whole will be given. This serves both to summarise the transduction process and highlight the areas in which much work is still required before the mechanisms of transduction are fully understood.

SECTION 7.1

ON THE PACKAGING OF TRANSDUCING DNA INTO PHAGE HEADS

The data presented in Chapter 3 suggested that transducing DNA was packaged into phage heads with little or no marker specificity. Therefore, in accordance with the genetic evidence of Newman and Masters (1980) and Masters et al. (1985), these data indicate that the marker specificity of transduction (Masters, 1977) is not primarily due to selective packaging by phage P1. However, Newman (1982) presented genetic evidence that the marker leuB, situated at 2 map units on the E.coli map (Bachman 1983) was perhaps packaged by P1 with a higher efficiency than other markers. In support of this hypothesis, I have shown that leuB and nearby markers (ftsQA and ace) are packaged with threefold greater efficiency than the other markers measured.

The mechanism by which P1 encapsidates transducing DNA, or indeed overpackages markers from the leuB region has not been elucidated. As indicated in Chapter 3, there is no evidence that P1 pac-like sites are involved and are present in the E.coli genome.
Nor is there any evidence as to which P1 genes are involved in initiating the encapsidation of host chromosomal DNA; however, it is clear that the EcoP1 system is not involved in generating DNA "ends" to serve as packaging substrates by the Wall and Harriman (1974) model (Chapter 1, Section 3). Although it is clear that P1 over-packages markers near leuB, the extent of this highly packaged region is not known nor indeed is the locus of maximal packaging levels. Defining the limits of this region may help elucidate the mechanisms of this selective overpackaging.

One approach would be to employ combined genetic and physical techniques to construct a detailed "packaging map" of the region. Packaging levels could be estimated genetically using UV-irradiated lysates (Newman and Masters 1980) or physically as in Chapter 3. This would require the construction of suitably marked strains to serve as recipients for markers in and around the 2 minute region and the isolation (or acquisition) of cloned DNA fragments also from this region. This should allow the extent of the highly packaged region to be determined and the locus, if any, of maximum packaging to be identified.

Detailed examination of the most efficiently packaged DNA, perhaps by sequencing, may yield information as to the mechanism resulting in high packaging of the 2 minute region.

Of course, the packaging levels of relatively few markers has been studied, either genetically or physically. Therefore it is possible that, in addition to the 2 minute region, there are other chromosomal locations which exhibit high packaging levels. However, to construct a detailed packaging map of the E.coli chromosome using the techniques described above would be extremely time consuming and impractical.

The major difficulty with studying P1 packaging of transducing DNA lies with the fact that relatively little is known about the mechanism of P1 packaging. In addition, the phage genome has not been very well characterised and there have been no reports as yet of mapped mutations in the P1 genome which alter the transducing ability of the phage. The HT mutants isolated by Wall and Harriman (1974) were never fully characterised and in our hands, proved difficult
to work with. They were reported to be highly unstable and did not exhibit increased levels of transduction when tested in this laboratory (Masters, unpublished observations).

In addition to the Wall and Harriman (1974) HT mutants, it was reported that a deletion derivative of P1 which lacked the vad and gta and teu genes (figure 1.1) exhibited increased transducing activity (Lida, Meyer and Arber, 1980, cited in Yarmolinsky 1984). Again, we failed to demonstrate this (Masters, unpublished observations).

Further elucidation of the role of P1-encoded functions in the transduction process will probably require further information on the mechanism of P1 packaging and identification of which, if any, P1 encoded functions (in addition to those involved in packaging) play a role in the production of transducing particles.

SECTION 7.2
INTRODUCTION OF P1 pac -SITES INTO THE E.COLI CHROMOSOME

In Chapter 4, an attempt was made to selectively overpackage specific regions of the E.coli chromosome into transducing particles; the method involved the use of recombinant pac "vehicle" plasmids which were directed into preselected chromosomal locations by homologous recombination, thus integrating P1 pac sites into defined locations on the E.coli chromosome. However, it appeared that the integrated pac sites were not efficiently utilised when the cells carrying the plasmids were infected with P1. These results were disappointing since it had been hoped that this approach would have allowed the packaging of specific markers to be stimulated to high levels. This, in turn, would have allowed the fates of these individual markers to be followed once they were transduced into recipient cells (Section 4.1).

The reason for the poor success of this approach was not clear. One possible explanation was afforded by the unexpected and mysterious observation that the integrated "vehicle" plasmids had multimerised, i.e. there was more than one copy of the P1 pac site at each chromosomal location. Perhaps in some unknown fashion, the presence of multiple copies of pac sites in close proximity
interferes with the P1 packaging process.

The mechanisms and causes of the multimerisation of the plasmid insertions remains unclear. It was suggested in Section 4.5 that a form of unequal crossing over may have been responsible for the multimerisation (figure 4.9). However, the factors which caused the multimerisation to occur are a matter of speculation. Although the cells containing plasmid insertions were selected using Ampicillin resistance, the levels of ampicillin which were used were low (15 μg/ml). This should not have been sufficient to necessitate the multimerisation of the inserts to give the required level of ampicillin resistance, since a single copy of the Ap\textsuperscript{r} gene on the plasmid should confer resistance to at least 50 μg Ampicillin/ml. (Bolivar et al. 1977, John March, unpublished observations).

In addition, the observation that the number of plasmid inserts may depend on the chromosomal location of the integrated plasmids is most interesting. The reasons for this are not clear. One possible explanation for the position dependence of multimerisation may be that the molecular environment of the Ap\textsuperscript{r} gene alters its expression. For example, when present on pBR325, a single copy of the gene would confer resistance to high levels of Ampicillin (as described above). When integrated into the chromosome, however, the level of expression may be altered by the flanking chromosomal sequences; for example, convergent transcription or topological constraints may alter the expression. Hence multimerisation of the insert may be required to raise the levels of Ampicillin resistance to meet the levels of ampicillin in the selective medium. The putative effects from flanking DNA would not be expected to be identical at different chromosomal locations, so in this case the multimerisation would be expected to be position dependent. This suggestion, of course, is highly speculative but presumably could be tested by assaying the levels of β-lactamase produced by the strains or by testing whether the number of inserts in the strains decreases or increases with decreased or increased selection pressure, i.e. by altering the levels of Ampicillin in the medium.

In addition, it would be most interesting to obtain other integration events at different chromosomal locations; this could
be achieved by using a number of plasmids each carrying the Ap\(^r\) gene and also chromosomal fragments from different locations. It should then be possible to investigate further the correlation between the site of insertion and the number of plasmid inserts.

There are, of course, many such experiments which could be carried out to further investigate causes of the multimerisation of the plasmid inserts.

With regard to the overpackaging of defined chromosomal locations (which was the purpose of the work carried out in Chapter 4) it seems certain that a different approach to that described in Chapter 4, must be adopted. Two alternative approaches were mentioned in Section 4.1: the use of recombinant Pl pac-carrying transposons or \(\lambda\)pac attP\(\Delta\). As already mentioned in Section 4.1, both these approaches have disadvantages: e.g. the need to map the inserted molecule after the integration and, for \(\lambda\)pac attP\(\Delta\), the limited number of integration sites. However, both these approaches would probably give only single copy insertions, so they may be more suitable than the method used in Chapter 4.

For example, the transposon Tn5 may constitute a suitable vehicle for the integration of Pl pac into the E.coli chromosome. Tn5 has inverted repeats at each end which are essential for transposition (Rothstein and Reznikoff 1981, Reznikoff 1982). However the unique sequences between the repeats, including the Kmr\(^r\) gene, are not required for transposition (Jongensen et al. 1979). Pl pac could presumably be inserted into this non-essential region and then the recombinant transposon allowed to transpose into the E.coli genome. However, the insertions would occur at random so the sites of insertion would then need to be mapped.

Alternatively, a pac site could be introduced specifically into the E.coli origin region if carried on transposon Tn7; although Tn7 will insert randomly into plasmids, it will transpose with high efficiency into one location and in one orientation only in the E.coli chromosome. The single insertion site is at about map position 83.3, between glmS and uncA (Lichtenstein and Brenner 1981, Bachman 1983). Tn7 is large, about 14 kb, and encodes a number of
antibiotic resistance markers, any one of which would probably be suitable for the insertion of the P1 pac site. Tn7 may therefore be a useful vehicle to specifically integrate P1 pac into the origin region and so allow the overpackaging of origin DNA, during P1 lysis. Other transposons, of course, may also be suitable for the purpose of directing P1 pac into the E.coli chromosome (Calos and Miller, 1980 for review) and with careful choice of the vehicle, it should be possible to obtain insertions of P1 pac into many chromosomal locations.

It is possible, however, that the insertion of P1 pac into the E.coli chromosome may not provide sufficient stimulus for the packaging of adjacent markers to high levels. Previously, the high levels of host chromosomal packaging by P1 (or P7) have been achieved by integrating the entire phage genome, and hence the pac site, into the host chromosome. (Note, the experiments described by Sternberg and Hoess 1983, who integrated λ-pac hybrids into att λ (see Chapter 1), did stimulate chromosomal packaging, but it is not clear to what level packaging was stimulated). Phage were integrated, either transiently during P1 infection (Steinberg et al. 1981a, b), or by isolating stable insertions of the prophage and superinfecting with a virulent phage. For example, Chesney and Adler (1982) integrated P7 into the att P7 site in E.coli, which lies between dnaG and tolC (i.e. at about 66.7 minutes on the E.coli map). Superinfection of this strain with P1 vir (note that P1 and P7 are very similar, sharing about 90% homology, Walker and Walker 1976) resulted in cell lysis; the transducing lysate produced from this showed a 275-fold stimulation of tolC transduction.

It has always been assumed that the overpackaging of markers to one side of the phage integration site was due to the presence of the integrated pac sites (Sternberg et al. 1981a, b, Sternberg and Hoess 1983). However, it is not clear how effective a pac site alone (i.e. without the accompanying phage genome) would be in stimulating the packaging of adjacent markers to high levels; the data presented in Chapter 4 suggest, perhaps, that pac sites alone may not be sufficient. It is possible that in order to package specific chromosomal regions to high levels, the entire phage
must be integrated adjacent to this region, as described by Chesney and Adler (1982).

In spite of the possible problems in achieving high packaging levels of specific markers, to do so would be most desirable. Clearly, this may pave the way for a direct study on the fate of individual markers once they enter the recipient cells. This, in turn, may provide additional information on the causes of transductional selectivity.

SECTION 7.3.

THE SEGREGATION MODEL

As discussed in Section 5.1, Bender and Sambucetti (1983) reported that if an exponentially growing culture of Klebsiella was transduced with P1, there was a marker-independent lag before transductants increased in number. In addition, their data suggested that Klebsiella filaments were up to 1000 fold better recipients for transduction than were normal sized cells. Accordingly, the experiments presented in Chapter 5 were carried out, initially, to investigate if E.coli cells behaved similarly to Klebsiella. The results presented in Chapter 5 differ from those of Bender and Sambucetti (1985): E.coli filaments were only about 5-fold better recipients for transduction than were normal sized cells. This was perhaps attributable to the larger size of the filamentous E.coli cells; the larger the cell, the more likely to receive a phage particle, since large cells are bigger targets than normal cells. In addition, transduction of exponentially growing E.coli cells was followed by a short lag before the transductants (but not the viable counts of the culture) increased in number. The lag, however, was marker dependent. The duration of the lag was shown to depend on the chromosomal position of the selected marker; terminus markers increased first, followed in succession by those replicated late, midway and then early in the replication cycle. A model, the "segregation" model, has been put forward to explain the duration of the lags and the model states that:

The lag before transductants increase in number results from the position dependent segregation of the wild type and mutant alleles
(of the selected marker) to daughter cells at division. In addition the difference in segregation lags between markers is dependent on the replication velocity of the chromosome (see Chapter 5 for a full discussion of the model). Support for the model comes from the clear demonstration that the segregation lags of markers are as predicted from their location on the E.coli map. Furthermore, the differences in segregation lags between markers are exactly as predicted from their spatial separation on the E.coli chromosome and from the chromosomal replication velocity at the growth temperature. The differences in segregation lags between Arg^+ and Trp^+ transductants was used to estimate C-time at 37°C and 30°C, giving values of 41 minutes and 55.6 minutes respectively. The above value for C, at 37°C, is in total agreement with the value reported by Cooper and Helmstetter (1968) of 41 minutes. As an independent check, C was measured at 37°C and 30°C, using the fluorimetric method described in Section 5.5 and Chapter 2. The values for C at 37°C and 30°C obtained from this method were 39 ± 2 minutes and 60 ± 4 minutes respectively. These values are in good agreement with those estimates from the segregation lags and therefore lend further support to the validity of the segregation model.

The experimental data differed in one respect from the predictions of the segregation model: the time at which transductants first increased in number was delayed by one generation time longer than expected. However, the segregation model was based on the assumption that transductional recombination resulted predominantly in a replacement of resident double strand DNA with double strand transducing DNA (Sandri and Berger 1980a). If so, then one explanation for the unexpectedly long lags was that transductional recombination occurred at, or was dependent on, chromosomal replication forks (Section 5.6 for discussion), i.e. transductional recombination required replication of the target sequences. However, the experiments in Section 6.2 indicated that there was no demonstrable correlation between recombination and replication of the target molecules, which suggests that the above explanation for the long segregation lags was probably not correct. The alternative hypothesis that transductional recombination results in
predominantly a single strand exchange reaction, rather than the small double strand insertions indicated by the data of Sandri and Berger (1980a). (Sections 5.6 and 6.6 for discussion.) There is, however, no easy method to determine the structure of the recombinant molecule generated by the transductional recombination event. Perhaps the density labelling experiments performed by Sandri and Berger (1980a) (discussed in Section 6.6) could be repeated in an attempt to elucidate the structure of the hybrid density DNA which was detected in their gradients. As already mentioned in Section 6.6, hybrid density DNA could arise in two ways: from heteroduplex DNA with one heavy and one light chain, or alternatively, from the presence of duplex heavy DNA and duplex light DNA on the same molecule (Figure 6.3). As already mentioned, if such DNA were run on strand separating gradients, in the former case described above, label would shift to the heavy region of the gradient. In the latter case, the label would remain in the hybrid region.

Using a very similar experimental procedure, Ebel Tsipis et al. (1972a) carried out just such experiments to elucidate the structure of recombinant molecules formed by P22-mediated generalised transduction in Salmonella. They found that on strand separating gradients, label shifted to the heavy region, as compared with non-strand denaturing gradients indicating that heteroduplex light/heavy DNA was probably present in transduced cells. This suggested that single strand replacement reactions may have occurred during the transduction process. Ebel Tsipis et al. (1972a), however, do not discuss this possibility.

However, such experiments are technically very difficult to perform and the interpretation of the above results was difficult since it was not clear whether the shift to the heavy region, described above, was accompanied by a reduction in label at the hybrid region. It was thus not clear whether heteroduplex heavy/light DNA resulted from transductional recombination in Salmonella.

Since this type of experiment is difficult to perform, the elucidation of the structure of recombinant molecules, resulting from transductional recombination, may not be possible unless
efficient in vitro recombination experiments can be performed. In this instance easily assayable amounts of the recombinant progeny molecules may be available and so allow their structure to be determined.

SECTION 7.4
ON THE ROLE OF INITIATION OF DNA REPLICATION AT oriC IN THE TRANSDUCTION OF oriC-LINKED MARKERS

As mentioned in Chapter 1 and Section 6.1, markers closely linked to oriC exhibit very high levels of transduction (Masters 1977). One hypothesis put forward to account for this was that oriC sequences themselves, or the initiation of replication at oriC, confer highly recombinogenic properties when oriC is present in cis on transducing DNA. Support for the involvement of replication in high origin transduction comes from the clear demonstration that oriC- transducing DNA, but not transducing DNA from the other regions that were tested, was capable of (limited?) autonomous replication once it entered the recipient cell (Section 6.3).

The transduction experiments presented later in Chapter 6 were less easy to interpret. The experiments, involved the transduction of dnaAts recipient strains; either oriCΔ, sdrA or integratively suppressed at the restrictive temperature by the integration of pKN500 (Sections 6.4, 6.5). The above strains were transduced at 30°C and 42°C, (i.e. the permissive and non permissive temperatures for the thermolabile dnaA protein). At the restrictive temperature, in all cases, the transduction of the oriC-linked marker Ilv+ was reduced whereas the transduction of markers unlinked to oriC was unaffected. These results are consistent with the hypothesis that, in the absence of dnaA protein at 42°C, (i.e. when replication was unable to initiate from oriC), the prevention of replication at either the resident, target, oriC sequence or the incoming oriC had reduced the transduction of a marker linked to oriC; this suggests that replication is required for high origin transduction. However, it is possible that the denatured dnaA protein still retained its high affinity for the origin of replication and was, in some
unknown fashion, "poisoning" the transduction events involving oriC (Section 6.6 for discussion). Although unlikely, this possibility cannot be eliminated by the above experiments. In order to overcome this problem, similar experiments to those described in Sections 6.4 and 6.5 could be performed; in this case, however, instead of using thermolabile dnaAts alleles to prevent replication at oriC, dnaA amber mutations, coupled with a temperature sensitive amber suppressor could be used to achieve the same effects. Suitably marked strains harbouring dnaA amber mutations, plus SupFts alleles (Schaus et al. 1981) and which were intergratively suppressed at the non permissive temperature by pKN500, may constitute suitable recipients. At the permissive temperature, normal dnaA protein would be produced, allowing the resident and incoming oriC sequences to initiate replication. In this instance, the transduction of all markers should occur at normal levels. At the non-permissive temperature the supFts allele could be inactive, so would not suppress the dnaAmber mutation. Therefore no initiation could occur at oriC and if the above hypothesis is correct (i.e. replication initiating at oriC is responsible for high origin transduction) the transduction specifically of oriC-linked markers would be expected to fall.

Presumably, if this kind of approach is adopted, there should soon be further evidence to support or contradict the hypothesis that replication at oriC directly influences the transduction of oriC-linked markers.

SECTION 7.5
A POSSIBLE "TRANSDUCTION" PROTEIN

As described previously, following P1-mediated transduction only 15% of the incoming transducing DNA becomes associated with the host chromosome. 75% of the DNA circularises to form large (about 90 kb) circles, the ends of which are linked by protein. This is so-called abortively transduced DNA (Sandri and Berger 1980a,b). The circular structure confers immunity to host cell recombination and degradative pathways, so abortively transduced
DNA is stable. Masters (1985) suggests that transducing DNA destined to undergo recombination with the host chromosome may not adopt this configuration.

The nature and origin of the protein which binds abortively transduced DNA is not known. It is not clear whether the protein is of cellular or phage origin. Nor is it known if the protein binds exclusively to transducing DNA or whether it has a role in the P1 life cycle and binds to P1 DNA as well. Ikeda and Tomijawa (1968b) reported that transducing particles, but not infective particles, contained a protein bound to the DNA; it is tempting to speculate that this may be the same protein that is found binding the ends of abortively transduced DNA.

Other viruses, such as adenovirus, Bacillus phage 22 and the linear plasmids of Streptomyces are found to bind protein at their ends, and this appears to be involved in replication (Rekash et al. 1977, Salers et al. 1978, Hirochika et al. 1984). In view of this, it would be interesting to study the protein binding to the ends of abortively transduced DNA, to clone the gene(s) coding for it and ultimately to determine the role of the protein in influencing transduction and the P1 life cycle.

As described in Chapter 1 and Chapter 2, abortively transduced DNA can be prepared by density labelling host cell DNA before P1 infection. The abortively transduced DNA can then be extracted from recipient cells and visualised by agarose gel electrophoresis (Sandri and Berger, 1980a,b). In conjunction with this technique host cellular proteins could be radioactively labelled before P1 infection, the host cells shifted to non-radioactive medium and simultaneously infected with P1. After lysis, the lysate could be used to infect recipient cells, then abortively transduced DNA could be extracted from these cells and the DNA run on an agarose gel. Autoradiography of the gel would reveal if the protein complexed with the abortively transduced DNA was radioactively labelled, i.e. originated in the donor cell before P1 infection. By labelling cellular proteins before or during P1 infection or by labelling proteins in the recipient cells, it should be possible to
establish the origin of the protein which binds to abortively transduced DNA.

Once the origin of the DNA-binding protein has been established it may be possible to clone the gene(s) which encode it. Antibodies to the entire protein/DNA complex could be prepared by injecting abortively transduced DNA into mice. These may provide a "probe" with which to screen libraries of \textit{E. coli} or P1 DNA, to detect clones expressing the DNA binding protein.

In this way, it should be possible to determine the origin of the protein found complexed with abortively transduced DNA, and to clone the gene(s) expressing the protein. This would enable studies to be carried out on the protein itself and its role, if any, in transduction and the P1 life cycle.

Clearly, the above suggestions give only a brief outline of the approach that would be used to characterise the DNA binding protein. The above procedures would almost certainly be technically difficult, especially since it has not proved possible to isolate large amounts of abortively transduced DNA (Sandri and Berger 1980a). However, if successful, this approach would yield interesting data since the protein itself is of interest, as well as its possible role in determining transductional selectivity (see Chapter 1).

SECTION 7.6
A MODEL FOR THE TRANSDUCTION PROCESS

The preceding sections have concentrated on summarising the experimental results, presented in Chapters 3, 4, 5 and 6 and in proposing specific experiments which perhaps could be performed to further test the hypotheses put forward in these chapters. In this section, however, I will attempt to present, briefly, an overall model for the process of transduction, i.e. summarising the process, and pointing out the general areas where further research is needed.

A simple diagram of the transduction process is shown in Figure 7.1.
FIGURE 7.1 SCHEMATIC DIAGRAM OF THE TRANSDUCTION PROCESS

A. Adsorption of an infective P1 particle to the cell wall.

B. Lysis of the cell, releasing progeny P1 infective particles, plus a small proportion (0.3%-6%, Ikeda and Tomizawa 1965a, Chapter 3) of transducing particles (indicated here by the thick line representing the genome. The black closed circle represents the protein associated with transducing particles, Ikeda and Tomizawa, 1965b).

C. A transducing particle adsorbs to the surface of a recipient cell and injects its transducing DNA. The DNA may either recombine into the recipient chromosome (depicted by the thick line within the cell) (D) or form abortively transduced DNA (E) or be degraded (not shown).

(This figure is not drawn to scale)
Figure 7.1
The transduction process initiates in donor cells, when P1 infective particles adsorb to P1-sensitive recipient cells and inject their DNA. If lysogeny does not ensue, the phage enters the lytic cycle of growth, which results in donor cell lysis (Figure 7.1A, 7.1B). At lysis, P1 progeny particles are released, and among them, a small proportion of transducing particles; estimates of the proportion of transducing particles vary: 0.3% for P1 vir (Ikeda and Tomizawa 1965a), 2% or 6% for P1 Kc (Sandri and Berger 1980a, Chapter 3). Transducing particles apparently contain no phage DNA (see Chapter 1 for discussion of this point) but instead contain host chromosomal DNA associated with a protein (Ikeda and Tomizawa 1965a,b).

The mechanism by which P1 generates transducing particles remains one of the most mysterious steps in the process. Several indirect observations, however, indicate that the mechanism of P1 encapsidation of host DNA is not similar to that of P22 (see Chapter 1), i.e. suggest that P1 does not commence encapsidation of host DNA from pac-like sites located in the chromosome:

1. P1 packages the E.coli chromosome with little marker specificity, in contrast to P22 packaging of the Salmonella chromosome (Newman and Masters (1980), Schmeiger (1984), Chapter 3).

2. With P1 transduction in E.coli there is no evidence for defined chromosomal packaging initiation sites (as have been shown to be formed during P22 transduction in Salmonella) because unlike P22, P1 does not generate homogeneous transducing particles.

3. The P1 pac site is complex, about 160 bp in size (Sternberg, 1984), so pac-like sites are unlikely to occur by chance in the E.coli genome even once. Since P1 can only package 8-10 headfuls of DNA from one initiation event, it would require the presence of at least 10 pac-like sites in the E.coli chromosome to obtain even chromosomal packaging (see Chapters 1 and 3).
(4) An attempt to detect P1 pac-like sites in the chromosome by probing chromosomal DNA with $^{32}\text{P}$-labelled P1 pac DNA was not successful (Chapter 3).

The most plausible model for the mechanism of P1-mediated packaging of the host chromosome was proposed by Wall and Harriman (1974) who suggested that packaging might commence from double stranded breaks in host DNA. Such breaks are thought to occur, presumably at random, during the P1 lytic cycle.

Obviously this is an area in which there is much scope for further investigation. A fuller understanding of the packaging process and the identification of P1 functions which affect transduction may be of use in unravelling the mysteries of host chromosomal encapsidation into transducing particles.

The second stage of the transduction process occurs following the injection of transducing DNA into the recipient cell (Figure 7.1C). As has been mentioned already, the majority of the transducing DNA, some 75%, adopts the abortive configuration and forms a circular protein/DNA complex (Figure 7.1E). The mechanism of circularisation is a matter of conjecture. It is possible that the protein associated with transducing DNA (Ikeda and Tomizawa 1965a) is the protein which binds the ends of the abortive DNA and plays a role in the initiation of circularisation (to illustrate the point, in figure 7.1E, the protein carried in transducing particles is shown as being the same as that which binds the abortive DNA). In addition, it is possible that the marker selectivity of transduction is due to selectivity in the circularisation process; since DNA destined to become recombined into the chromosome (Figure 7.1D) may not adopt the stable abortive configuration, it is possible that highly transduced markers are poor substrates for circularisation, whereas markers exhibiting low transduction are good substrates.

As described before, the protein which binds the ends of abortive DNA is of particular interest. Thus a study of abortively transduced DNA and its binding protein may prove helpful in understanding the transduction process.
Transducing DNA which does not adopt the circular abortive configuration is either degraded (this will not be discussed) or some 10-15% becomes associated with the host chromosome by homologous recombination via host recombination pathways (Sandri and Berger 1980a). The nature of the recombination event is not clear, and the structure of the resultant recombinant molecule is open to debate. Sandri and Berger (1980a) suggest that transducing DNA is integrated in a double stranded replacement reaction whereas the data presented in Chapter 5 indicates that the recombination event results predominantly in a single strand replacement.

It is clear, however, that the marker selectivity of the transduction process arises at this stage in the process.

Transductional selectivity is another topic of considerable interest, since it is clearly dependent on host recombination pathways. Masters et al. (1984) demonstrated that the marker specificity of transduction is eliminated in a recBC sbcB FG recipient background (see Chapter 1). The mechanism by which the products of these genes bring about the marker selectivity of transduction is not known; indeed, this is another area in which much work still has to be carried out. As described in Chapter 1, the in vivo and in vitro activities and substrate requirements of both RecA protein and RecBC enzyme are becoming much better understood. This may enable the development of efficient in vitro recombination systems in the foreseeable future. Clearly, such systems would prove invaluable in determining the roles of host recombination functions in the mechanism of transductional recombination. Indeed, an in vitro recombination system may resolve the question of the nature of the recombinant structure arising from transductional recombination.

The above comments and observations serve to summarise, very briefly, the transduction process and indicate the general directions in which future research into the transduction process may, perhaps, be expected to proceed. Broadly, these areas include:

(1) The mechanism of P1 encapsidation of host DNA into transducing particles.
The formation and composition of abortively transduced DNA and an investigation of the protein which binds at the ends of abortively transduced DNA.

The roles of host cell recombination genes in determining the marker selectivity of the transduction process.

The mechanism of transductional recombination and the nature of the recombinant molecule.

It is obvious that much has still to be done before the mechanisms of P1-transduction, one of the most widely used genetic techniques, has been fully understood. With the continuing development and improvement of techniques it is to be hoped that experiments which have proved technically difficult (or impossible) in the past (e.g. the study of abortively transduced DNA, or recombination in vitro) may become considerably easier to perform and so shorten our path to an understanding of the transduction process as a whole.
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