AMPLIFICATION OF AN IS1-FLANKED UNIT IN *Escherichia coli* K12

A thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow.

by

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The research reported in this thesis is my own original work, except where otherwise stated, and has not been submitted for any other degree.

Dedicated to my Mum and Dad

for their constant encouragement and love.

CONTENTS

ABBREVIATIONS

(i). Chemicals

Ac - acetate

APS - ammonium persulphate

ATP - adenosine triphosphate

BSA - bovine serum albumin

DNA - deoxyribonucleic acid

d(NTP) - 2'-deoxy (nucleotide)

DTT - dithiothreitol

EDTA - ethylene diamine tetraacetic acid (disodium salt, dihydrate)

EtBr - ethidium bromide

EtOH - ethanol

FSB - final sample buffer

IPTG - isopropyl B-D thiogalactopyranoside

mRNA - messenger ribonucleic acid

PEG - polyethylene glycol 6000

RNA - ribonucleic acid

RNase - ribonuclease

SDS - sodium dodecyl sulphate

TEMED - N N N' N' tetramethylethylenediamine

Tris - tris (hydroxymethyl) amino ethane

tRNA - transfer ribonucleic acid

X-gal - 5-bromo, 4-chloro, 3-indolyl B-D galactoside

(ii). Antibiotics

Ap - ampicillin

Cm - chloramphenicol

Km - kanamycin

rif- rifampicin

str - streptomycin

Tc - tetracycline

(iii). Phenotype

X^r - resistance to X

X^s - sensitivity to X

Cut - citrulline-utilizing mutant

US Cut - unstable Cut

S Cut - stable Cut

(iv). Measurements

mA - milli amps (10^{-3} amps)

bp - base pair(s)

kb - kilobase pair(s)

^oC - degrees Celsius

Ci - Curie

uCi - microcurie (10^{-6} Curie)

kd - kilodaltons (10^3 daltons)

g - centrifugal force equal to gravitational acceleration

g - gramme

mg - milligramme (10^{-3}g)

ug - microgramme (10^{-6}g)

l - litre

ml - millilitre (10^{-3}l)

ul - microlitre (10^{-6}l)

M - molar (Moles per litre)

mM - millimolar (10^{-3}M)

uM - micromolar (10^{-6}M)

m - metre

cm - centimetre (10^{-2}m)

mm - millimetre (10^{-3}m)

mins - minutes

pH - acidity ($-\log_{10}$[molar concentration H^+ ions])

sec - seconds

v - volts

w - watts

(v). miscellaneous

OTCase - ornithine transcarbamylase, ornithine carbamoyl transferase (E.C.2.1.3.3.)

CAT - chloramphenicol acetyl transferase

$\leftarrow$ - direction of transcription

fig - figure

IS - insertion sequence

log - logarithm

no - number

$O.D_{650}$ - optical density at a wavelength of 650nm

Tn - transposon

w/v - weight per volume (all percentage solutions are weight per volume)

v/v - volume per volume

% - percentage

w.t - wild-type

F' - F- prime

Δ - deletion

r.t. - room temperature

m.o.i. - multiplicity of infection

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SUMMARY

The rearrangement associated with US Cut mutants of *E. coli* K12 was shown to be the tandem amplification of an IS1-flanked unit, Tn2901, which contains the *argF* gene. The copy number of Tn2901 in Cut1, a US Cut mutant, was estimated to be 45. A single IS1 element was shown to be present at the novel joint between each amplified unit, indicating that IS1-IS1 recombination is responsible at least for the initial duplication of Tn2901. The amplified unit was cloned as two overlapping restriction fragments: an 11.7kb EcoR1 fragment and a 9.4kb BglII fragment in pUC8/9. The latter fragment expresses the *argF* gene; the former does not. Protein analysis indicated that the *argF* gene product is greatly over-expressed in US Cut mutants. Preliminary northern analysis detected only one transcript (of approximately 2.4kb) from the 11kb chromosomal DNA carried by Tn2901 other than a possible *argF* transcript. Therefore, at least 6kb of Tn2901 is not accounted for in terms of RNA transcripts.

The F factor is required *in cis* for the initial IS1-IS1 recombination event. Two possible functions of the F factor were examined: an F factor IS-mediated rearrangement, and conjugal transfer. Southern hybridization of chromosomal digests to radiolabelled plasmids containing IS3, IS2 and gamma delta indicated that a major rearrangement involving the F factor IS elements had not taken place. The possibility of a minor rearrangement was not ruled out. Conjugal transfer was examined by the use of sodium dodecyl sulphate which depolymerises F pili filaments, and by the construction of a transfer-deficient mutant. The results indicated that conjugal transfer is not required for the amplification of Tn2901.

A technique was developed for the directed integration of a plasmid vector into the bacterial chromosome. Using this technique, the CAT gene was inserted in Tn2901, producing a strain with which unamplified

and amplified colonies can be distinguished by plating on minimal agar containing crystal violet. In the future, the directed integration technique will be of immense value in the replacement of wild-type chromosomal DNA segments with *in vitro* mutagenized sequences. This should allow the localization of the function provided by the F factor and the determination of whether an IS1 gene product is involved in the initial IS1-IS1 recombination event. A chromosomal gene bank was constructed from which a clone was isolated containing the right hand IS1 element of Tn2901 and approximately 2kb of adjacent chromosomal DNA. This clone will allow the *in vitro* construction of mutants of the right hand IS1 element of Tn2901 and their insertion into the bacterial chromosome in place of the wild-type IS1 sequence. The clone will also permit the isolation of overlapping clones from the gene bank extending up to the F factor, and these clones will be of value in the directed integration technique.

CHAPTER 1

Introduction

1.1 General introduction

Genomic rearrangements are of great importance in both prokaryotic and eukaryotic organisms. They are involved in gene expression in a variety of organisms. In eukaryotes this includes antigenic variation in Trypanosoma (Borst and Cross, 1982), generation of a diversity of antibodies (Bernard et al, 1978; Early et al, 1980) and control of mating type in yeast (Nasmyth and Tatchell, 1980). In prokaryotes, pilus expression in Neisseria gonorrhoeae (Hagblom et al, 1985), antigenic variation in Borrelia hermii (Plasterk et al, 1985), phase variation in Salmonella (Simon et al, 1980) and host range in bacteriophage Mu (Van de Putte, 1980) all involve genomic rearrangements.

Gene duplication is of paramount importance in evolution. The creation of a new gene from a redundant copy of an old gene is well documented (Ohno, 1977). For example, it is very likely that the genes for trypsin and chymotrypsin evolved, one from a redundant copy of the other, by the accumulation of mutations which affected the active sites of the enzyme. Natural selection allows the modification of pre-existing genes, but the accumulation of mutations to create an entirely new gene would be lethal without gene duplication since the original function of the gene would be lost. Gene amplification to many copies is associated with certain developmental processes in eukaryotes including chorion gene expression in Drosophila (de Cicco and Spradling, 1984) and DNA puff formation in Rhynchosciara (Glover et al, 1982). Experiments have shown that carcinogens can activate amplification events in mammalian cells (Tlsty et al, 1982; Lavi, 1982) and chromosomal rearrangements have been implicated in the origin of human cancer (Cairns, 1982). In E. coli, amplification of the ampC gene results in resistance to increased levels of ampicillin. Amplification of the lac region of E. coli has also been detected as revertants of certain lac$^-$ mutants.

The results presented in this thesis show that in E. coli K12, Tn2901

can amplify to many copies via IS1-IS1 recombination when the F factor is integrated adjacent to Tn2901. Tn2901 is a 12kb transposon-like structure which contains the argF gene flanked by two copies of IS1 in direct repeat (York and Stodolsky, 1981; Hu and Deonier, 1981a). The argF gene is one of a pair of duplicate genes encoding ornithine transcarbamylase in E. coli K12. Tn2901 is a "silent" transposon in that its transposition has not been reported. This introduction describes in turn the nature of transposable elements and their role in genome rearrangements, the F factor, and gene duplication and amplification.

1.2 Insertion sequences and transposons in prokaryotes

Transposable elements are defined as DNA segments which can insert into several sites in a genome (Campbell et al, 1977). They include simple insertion sequences which contain no known genes unrelated to insertion function and Tn elements which are more complex and do contain additional genes unrelated to insertion function. Prokaryotic transposable elements have been classified into three groups on the basis of structure and mechanism of transposition (Kleckner, 1981):

(1). Class I elements: these include the IS elements and compound transposons derived from them in which a segment of DNA is flanked by two copies of an IS-sequence in either direct or inverted orientation, for example Tn9 (MacHattie and Jackowski, 1977), Tn903 (Grindley and Joyce, 1981). In these compound transposons, all the proteins and sites necessary for transposition are encoded by the IS elements (Tn9 - Rosner and Guyer, 1980; Tn10- Foster et al, 1981). Both of the flanking IS elements are structurally intact, but one is often functionally defective (for example, IS10 left in Tn10- Foster et al, 1981). These IS-flanked segments can transpose as a single unit or one copy of the IS sequence can transpose alone (for example, IS50 in Tn5-

Berg et al, 1982; IS903 in Tn903- Grindley and Joyce, 1980). IS elements range in size from 0.77kb to 1.75kb in length (Kleckner, 1981) and the transposase gene often spans the entire length of the element (for example, IS10 right of Tn10- Halling et al, 1982).

(ii). Class II elements: The Tn3 family. This group contains greater than twenty transposons, including Tn3 and gamma delta, that are closely related both structurally and functionally. These elements are larger than Class I insertion sequences being greater than 5kb in length. They encode all the necessary proteins for their transposition (Kleckner, 1981) and transcription of the genes may be unidirectional (for example, Tn501) or divergent (for example, Tn3- Heffron, 1983). Class I and Class II elements share the feature that their ends are short inverted repeats of between 9bp and 40bp (Kleckner, 1981).

(iii). Class III elements: The transposing bacteriophages. Two closely related bacteriophages, Mu and D108, which use replicative transposition as the normal mechanism for vegetative replication of the bacteriophage genome during lytic growth (Hull et al, 1978). Unlike other temperate bacteriophages they integrate into the host chromosome whether entering the lytic or lysogenic pathway of infection. In contrast to Class I and Class II transposons, the ends of Mu do not have terminal inverted repeats (Kleckner, 1981).

The insertion of transposable elements is not entirely random and different elements show marked variations in specificity of insertion. For example, IS4 has only been found at one integration site, at galT of E. coli (Haberman et al, 1979), whilst Mu shows very little specificity and inserts at many different sites in a target (Bukhari and Zipser, 1972; Daniell et al, 1971).

1.3 IS1

Since IS1 will be shown to be relevant to the work of this thesis, this insertion sequence will be dealt with in detail.

The class I transposable element IS1 is 768bp in length and is the smallest active translocatable DNA element known in bacteria (Calos and Miller, 1980). IS1 is not confined to *E. coli* and is present in the genome of other Enterobacteriaceae species including *Klebsiella* and *Shigella*, but not *Salmonella* (Nyman *et al*, 1981). IS1 can transpose to produce a new copy on the same or a different DNA molecule, and can also mediate the co-integration of a plasmid carrying a copy of IS1 with a different plasmid, resulting in a duplication of IS1 at the co-integration junctions (Ohtsubo and Ohtsubo, 1980).

IS1 can form compound transposons in which two copies of IS1 flank an otherwise non-transposable segment of DNA. The Cm^r transposon Tn9 contains two IS1 elements in direct orientation (MacHattie and Jackowski, 1977). The Cm^r transposons Tn2652 - Tn2656 and the enterotoxin transposon Tn1681 contain two IS1s in an inverted orientation (So *et al*, 1979; Iida *et al*, 1981). Tn2671, a 23kb segment of NR1 is flanked by IS1 elements in direct repeat and is capable of self-promoted transposition at a low frequency (Iida *et al*, 1981b). Laboratory selections for transducing phages have provided two further instances in which large DNA segments are flanked by IS1 elements in direct repeat - the chromosomal *argF* gene (York and Stodolski, 1981; Hu and Deonier, 1981a); and the plasmid *raf* genes (Mattes *et al*, 1978).

IS1 is commonly depicted in a left end (insL) to right end (insR) orientation (Ohtsubo and Ohtsubo, 1978; Johnsrud, 1979; Ohtsubo *et al*, 1980). The IS1 sequence contains at least six open reading frames (Johnsrud, 1979; Ohtsubo and Ohtsubo, 1978; Machida *et al*, 1984b) and it has been suggested that the A and B reading frames (designated *insA* and *insB*) encode polypeptides required for transposition and co-

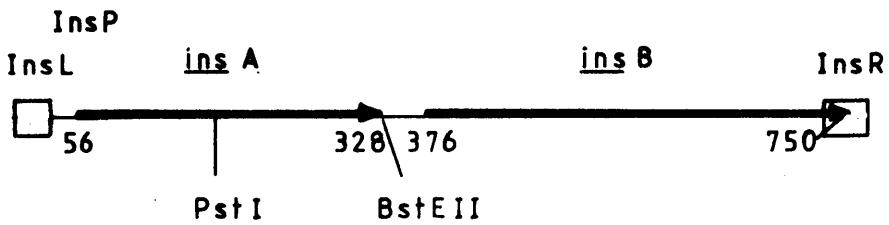


Figure 1.1. Map of IS1 (768bp). The open boxes represent the inverted repeat sequences at the ends of IS1, named InsL and InsR. The arrows on the map show the location and orientation of the two putative open coding frames, insA and insB. InsP denotes a hypothetical promoter for insA and insB. All the numbers represent the coordinates to the nucleotide sequence of IS1.

integration mediated by IS1 (Machida et al, 1984b; Ohtsubo et al, 1980). insA and insB are located side by side in the same orientation (Fig.1.1) and are assumed to be transcribed polycistronically from a promoter within the IS1 sequence (Ohtsubo et al, 1980; Machida et al, 1982). IS1 has inverted repeats of about 35bp at insL and insR ends that have been shown to be required for transposition and co-integration mediated by IS1 (Ohtsubo et al, 1980; Machida et al, 1982). The terminal inverted repeats are promoters: one promoter located at insL is thought to be responsible for synthesis of mRNA of the two genes encoded by IS1, whereas the other at insR is assumed to be involved in the expression of the two genes such that RNA from the latter promoter acts as anti-mRNA of the two genes (Machida et al, 1984a). It has been shown that there is a functional difference between the two ends of IS1 in that insR is more competent than insL in promoting transposition and co-integration (Ishizaki and Ohtsubo, 1984). IS1 left in Tn9, located downstream of the coding sequence for the Cm^r gene, is about 20 times less efficient in its ability to form co-integrates than is the other IS1, located upstream of the Cm^r gene. This has been shown to be due to transcriptional readthrough from the Cm^r gene which may interfere with the transcription of the insA and insB genes encoded by IS1 (Machida et al, 1983).

Sequential deletions of the end sequences of Tn9 (Gamas et al, 1985) indicated that 21-25bp of an isolated extremity is sufficient for transposition of Tn9. Two symmetrical sites 13-23bp from each end of IS1 correspond to the consensus sequence for E. coli integration host factor (IHF) and this sequence is present within the minimal sequence required for transposition (Gamas et al, 1985). The heterodimeric IHF protein, the product of the E. coli himA and hip genes (Friedman et al, 1984; Nash and Robertson, 1981) is involved in several site-specific recombination systems, notably in the integration of lambdoid phages (Nash and Robertson, 1981; Leong et al, 1985) and controls gene expression (Friedman et al, 1984) at the level of transcription (Goosen and Van de Putte, 1984) and translation (Hoyt et al, 1982).

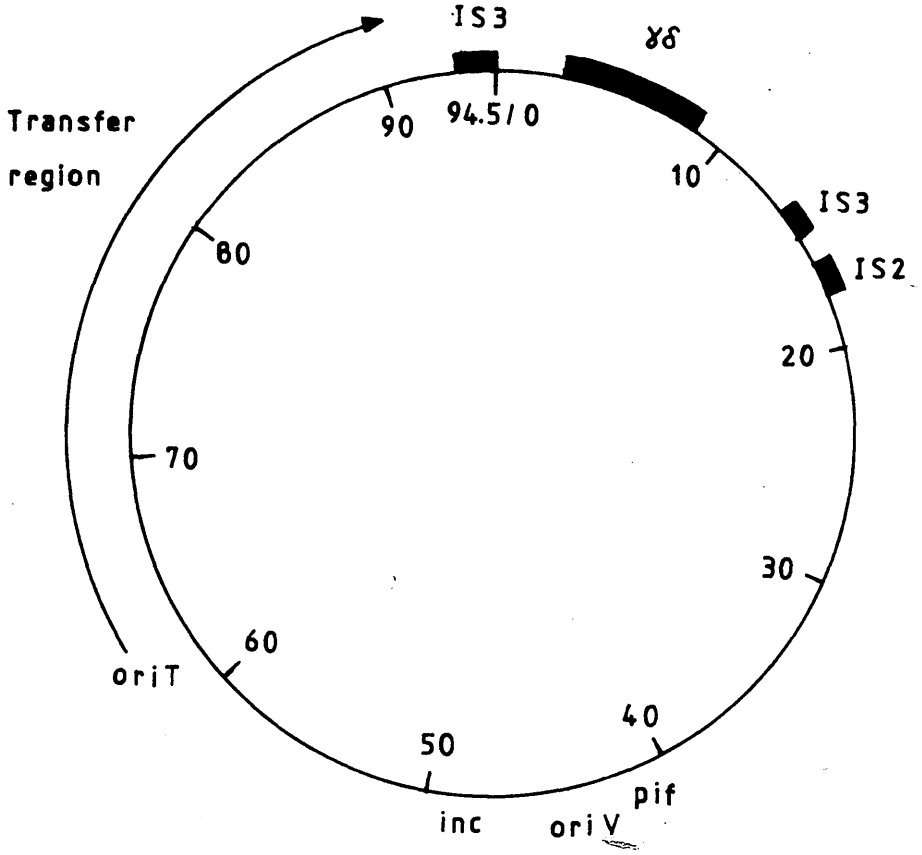


Figure 1.2. A map of the sex factor F. Points are given on the kilobase system of Sharp et al (1972). oriT : origin of transfer; oriV : origin of replication; inc : incompatability; pif : phage infection inhibition (Palchaudhuri and Maas, 1977). The insertion sequences are represented as solid boxes.

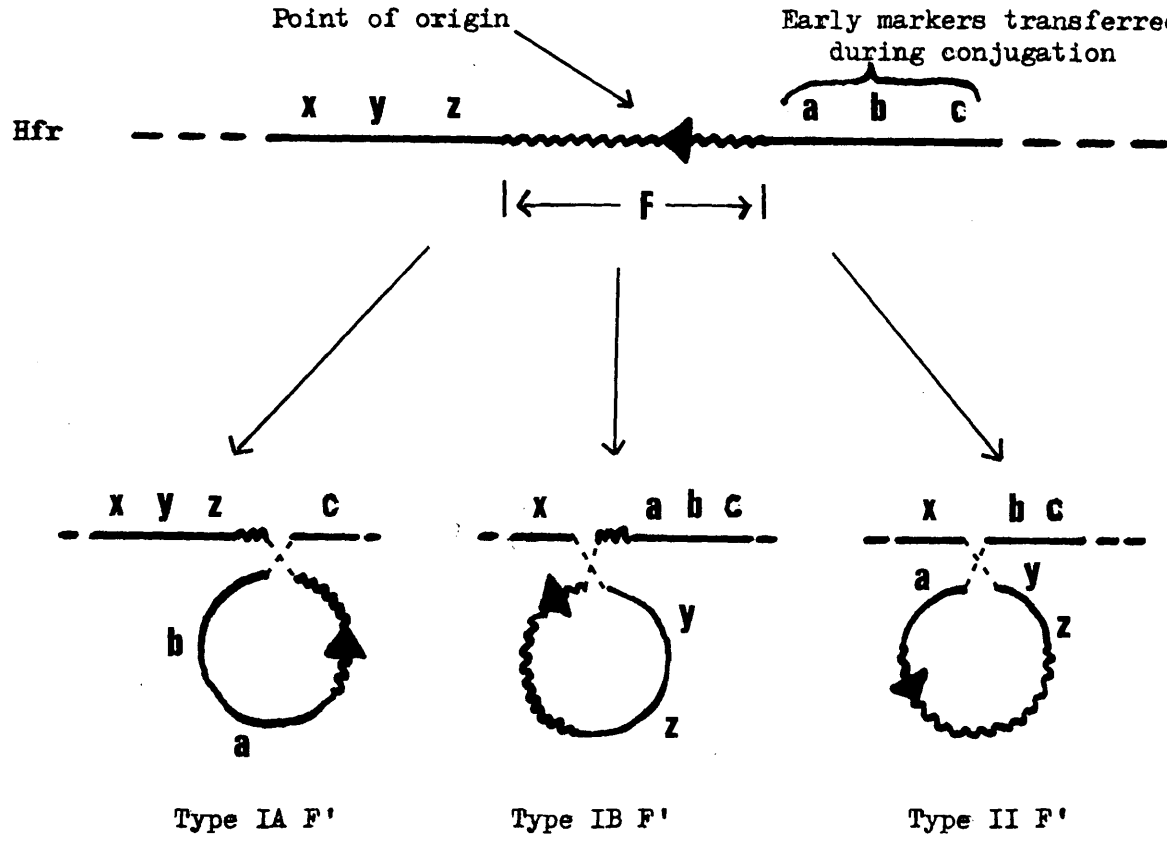


Figure 1.3. Formation of type I and type II F-primes.
The top line in the figure represents part of the chromosome of a hypothetical Hfr strain which transfers the genetic markers a, b and c early, and x, y and z late in conjugation. The lower part of the figure indicates the formation of the three types of F-prime and the orientation of the F plasmid and chromosomal markers.

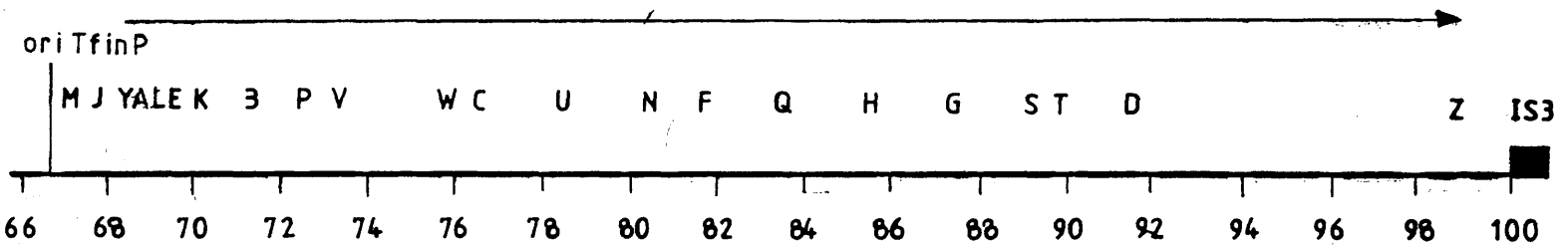
Although IHF may play a role in E. coli physiology, the protein is not an essential function since deletions of the himA gene are not lethal to the bacteria (Friedman et al, 1984). It has been suggested (Gamas, 1985) that IHF or a similar protein could function directly as a cofactor in the transposition reaction (by facilitating or inhibiting interaction of the transposition enzymes with IS1), modulate the expression of IS1-encoded gene products (for example, by repressing transcription from the insL and/or insR promoters), or act at both levels. DNA protection experiments (Gamas et al, 1985) indicate that purified IHF does bind specifically in vitro to both extremities of IS1. Direct evidence for a role of IHF in IS1 transposition has, however, yet to be obtained.

1.4 The F factor

The F sex factor is a conjugative plasmid of 94.5kb in length (Sharp et al, 1972). There are four insertion sequences on F, namely two IS3 elements, IS2 and gamma delta (Fig.1.2) which serve as attachment sites for the recombination between F and the E. coli chromosome leading to the formation of Hfr strains (Davidson et al, 1975). Integration into the chromosome results in F being flanked by a direct duplication of the attachment sequence involved (Davidson et al, 1975) as seen by analysis of type II F-prime plasmids (Davidson et al, 1975; Deonier and Davidson, 1976).

Excision of the F factor from the chromosome of an Hfr strain can occur precisely to regenerate the F plasmid or imprecisely to generate F-prime plasmids which contain bacterial chromosome sequences (Fig.1.3). Two types of F-prime plasmids have been described (Scaife, 1967). Recombination between sites on F and the bacterial chromosome results in a type I F-prime plasmid. Generation of type I F-prime plasmids may occur by aberrant recircularization of conjugally transmitted DNA (Guyer et al, 1977; Hadley and Deonier, 1980) or by

Figure 1.4. Map of the F factor transfer region. The numbers show the kilobase coordinates on the F plasmid map. The traYZ transcript is shown. The direction of transfer, initiated at oriT, is such that the transfer region enters the recipient last. The expression of the traYZ operon is positively controlled by the traJ product.



site-specific recombination events promoted by gamma delta which is present on the F factor (Davidson *et al*, 1975). One class of type I F-prime plasmids, F' *tra*, are deleted for the entire transfer region. Such plasmids have one common endpoint at 16.3F and one variable endpoint, and are thought to be formed by repliconation of the conjugal exogenote in a *rec*A recipient (Guyer *et al*, 1977). The second class of F-prime plasmids, type II factors, results from recombination between flanking bacterial sequences and therefore contains the entire F factor. The generation of type II F-primes can occur by *rec*A-independent recombinational mechanisms or by *rec*A-dependent recombination, for example by recombination between rRNA operons (Blazey and Burns, 1983).

The F plasmid conjugation system has been well-characterized both genetically and biochemically (Willetts and Skurray, 1980). The transfer region (Fig.1.4) covers 33kb and encodes at least 19 genes which are responsible for pilus synthesis and assembly (*tra* A,L,E,K,B, V,W,C,U,F,H,G); cell-cell contact (*tra* N,G); exclusion of donor cells from F-mediated conjugal transfer (*tra* S,T) and DNA transfer and replication (*tra* M,Y,G,D,I,Z). The *tra*Y-Z region is transcribed as a single operon and is subject to positive control by the *tra*J gene product. *tra*M has its own promoter and is positively controlled by the *tra*J gene product (Gaffney *et al*, 1983). Expression of *tra*J is negatively controlled by the FinOP repressor, and positively controlled by the *sfr*A gene product. Since F is a natural *fin*O mutant, *tra*J is constitutively expressed (Finnegan and Willetts, 1971; 1972) unless repressed by complementation *in trans* with the *fin*O gene product from another plasmid such as R100 (Willetts, 1977). Expression of *tra*J is positively regulated by the *sfr*A gene product at a position distal to the transcription and translation starts within the coding region. It has been suggested that a translocational control system analogous to that postulated for *omp*A protein-lipopolysaccharide at the cell membrane could account for the requirement of *sfr*A (Gaffney *et al*, 1983; Behr and Schaitmann, 1981).

oriT is required in cis, being nicked by the traYZ endonuclease (Everett and Willetts, 1980) and transfer proceeds such that the transfer region enters the recipient last. Although it is mainly plasmid encoded, conjugal DNA requires at least five host chromosomal genes: cpxA, cpxB, sfrA, sfrB and fexB in the donor, and ompA in the recipient (Willetts and Skurray, 1980).

The F plasmid is present at a copy number of only 1 or 2 per chromosome equivalent. An understanding of its replication mechanism was facilitated by the demonstration that a 9kb EcoR1 fragment called "mini-F" encoded all of the replication and stability determinants required for autonomous replication (Lovett and Helinski, 1976). The mini-F replicon encodes two replication origins, oriV and oriS (Scott, 1984). An oriV deleted mini-F can replicate solely from oriS but it is unknown whether both origins function in wild-type F.

1.5 Genetic recombination

Genetic recombination can be divided into two types which differ in the extent of the DNA homology in the region of recombination. Homologous recombination generally requires at least several hundred base pairs of homology between regions to be recombined, whereas non-homologous recombination requires little or no homology. Homologous recombination requires the RecA protein, whereas non-homologous recombination is recA-independant.

1.5.1 Homologous recombination

The RecA protein, which is required for homologous recombination, is a DNA-dependant ATPase which unwinds DNA in the presence of ATP. It binds single-stranded DNA, promoting the homologous pairing of duplex molecules with single-stranded or partially single-stranded DNA (Radding, 1982). Thus it catalyses the formation of displacement loops and figure-eight structures- two putative early stages in

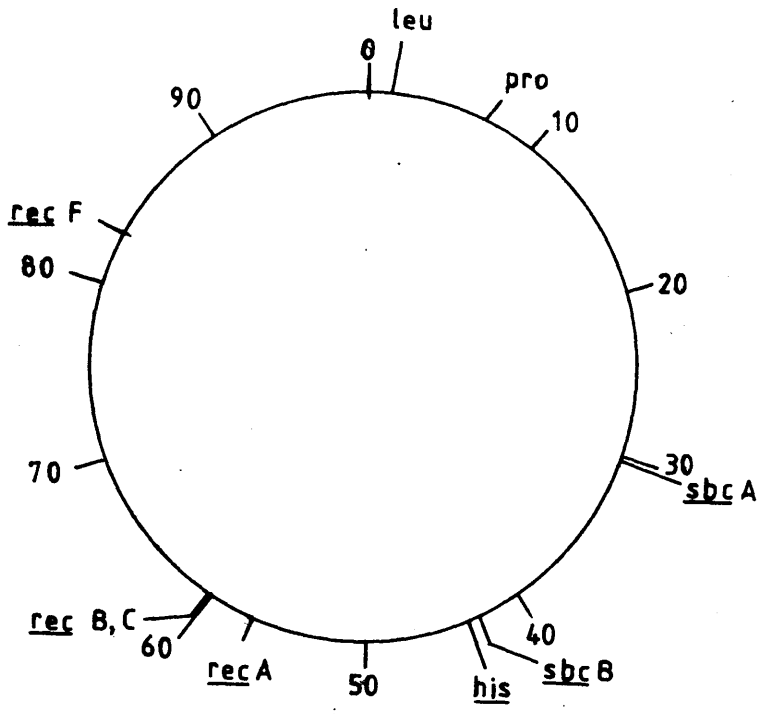


Figure 1.5. Map of the E. coli K12 chromosome showing the positions of genes involved in recombination.

recombination.

General recombination is thought to occur by several alternative pathways, each of which utilizes at least one biochemical function not shared by the others (Clark, 1971). All of these pathways absolutely require the RecA protein. The major pathway operating in wild-type cells is the recBC pathway which is important in post-conjugal and transductional recombination and requires exonuclease V, the product of the recB, recC and recD genes (Amundsen et al, 1986). Exonuclease V binds to double-stranded DNA ends and then rapidly moves along the DNA, unwinding it and producing single-stranded loops (Taylor and Smith, 1980; 1985). During this unwinding which requires ATP hydrolysis, the enzyme cuts the DNA with high frequency at Chi sites, 5'-GCTGGTGG-3', which stimulate genetic recombination in their vicinity (Ponticelli et al, 1985; Taylor et al, 1985). The two other pathways, the recF and the recE pathways, play a major role in the special situation in which the recBC pathway is blocked by mutation. The RecA RecF pathway, which is involved in plasmid recombination, requires exonuclease I to be absent (owing to mutation of the gene sbcB) and also requires at least one additional gene (recF) not required by the recBC pathway (Horii and Clark, 1973). The recE pathway requires mutation in the gene sbcA and the recE gene product, exonuclease VIII, which is encoded by the Rac prophage (Gillen et al, 1981; Kaiser and Murray, 1979). Both the sbcA and sbcB mutations restore recombination proficiency to recBC strains (Barbour et al, 1970) and recBC sbcA strains are hyper-recombinogenic for plasmids. The map positions of recA, recB, recC, sbcB, recE and recF are shown in Figure 1.5.

1.5.2 Non-homologous recombination

(i). Site-specific recombination

This category includes those recombination events which take place in the absence of RecA protein but are limited to particular DNA

sequences present on one or both of the DNA molecules involved. Site-specific recombinases recognise two limited DNA sequences which may be identical (for example, the resolvase of Tn3- Grindley et al, 1982) or different (for example, lambda integration- Mizuuchi et al, 1981; Weisberg and Landy, 1983). In the case of two identical sites, recombination will not change the sequence of these sites, whereas crossing-over between two dissimilar sequences will generate two hybrid sequences. In the lambda system, the Int protein promotes reciprocal recombination between two identical 15bp core sequences which are flanked by unique arm sequences. The resultant hybrid sites cannot undergo further Int-promoted recombination without the help of the phage Xis protein whose function is necessary for excision of the prophage (Abremski and Gottesman, 1982; Guarneros and Echols, 1970). Site-specific recombination is catalysed in vitro by the recombinase protein acting by itself (for example, the resolvases) or with the help of accessory proteins (IHF or Xis for lambda integrase- Abremski and Gottesman, 1982; Nash, 1975; Yin et al, 1985).

(ii) Transpositional recombination

Transpositional recombination is RecA-independent and requires a "transposase" function encoded by the element. This type of recombination is site-specific in that it requires the ends of the element (usually short inverted repeats of 8-40bp). These ends have little or no homology with the target sequence. Transposition can occur via co-integrate formation (for example, Tn3- Arthur and Sherratt, 1979) where two copies of the element are formed and each retains one junction with the donor molecule and forms one with the target. The co-integrate molecule is then resolved to produce a single copy of the element on each molecule. For Tn10, transposition appears to occur by a non-replicative simple insertion (Kleckner et al, 1984). For some IS elements (for example, IS1, IS903), transposition can proceed by a non-replicative, simple insertion or by co-integrate formation (Weinert et al, 1984; Biel and Berg, 1984).

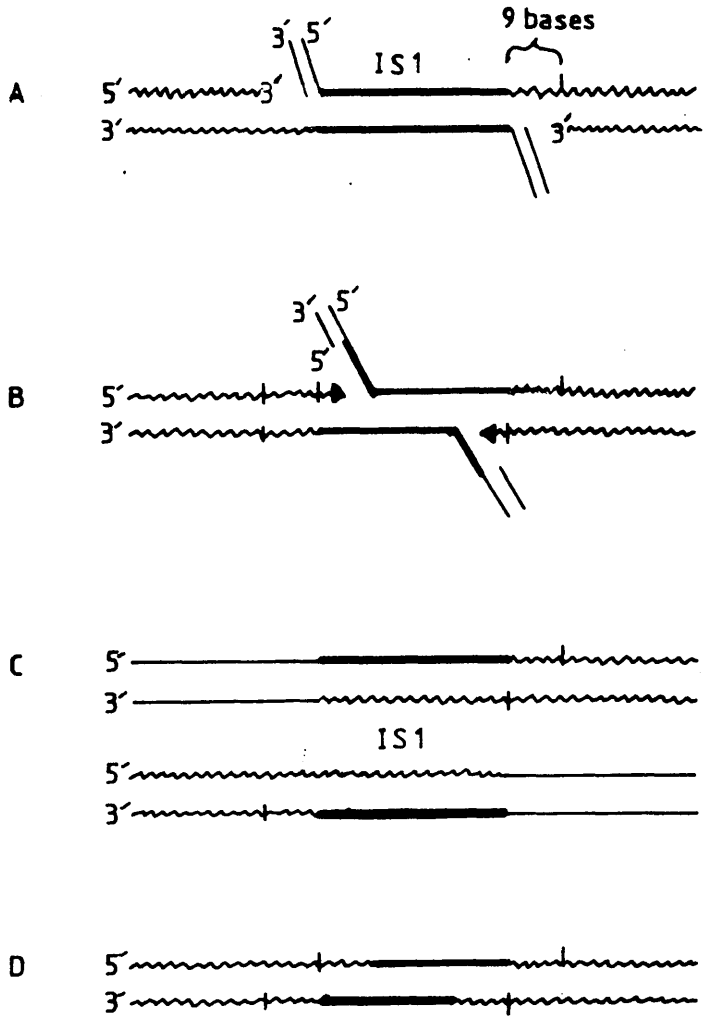


Figure 1.6. (A). Intermediate molecule resulting from recombination of a donor plasmid containing the IS1 sequence with a recipient plasmid (see text). The donor and recipient plasmids are actually circular, but only portions of them are shown here. Note that a 9bp sequence in the recipient strands has been subjected to a staggered cut. (B). Structure formed when displacement DNA synthesis procedes from the 3' ends of the staggered cut of the recipient strands in the 5' 3' direction across IS1. (C). Cointegrate structure formed when the DNA synthesis proceeds completely across IS1 and the newly synthesized strands are joined with the 5' ends of the donor strands. (D). Recipient molecule which has received an IS1 insertion.

A model for the transposition of IS1 (Ohtsubo et al, 1980; Shapiro, 1979) proposes that the initial steps of both insertion of IS1 and co-integration mediated by IS1 share common mechanisms. These mechanisms involve the assembly of the two inverted repeat ends of IS1 at a target site: nicking and joining between donor and recipient strands gives rise to a staggered cut at the target site, with each end of the insertion element covalently linked to each end of the staggered cut. This results in the formation of a forked molecule containing the donor and recipient linked together as shown in Figure 1.6A. If displacement DNA synthesis occurs in the 5'→3' direction from the 3'OH of the primer to completion, a co-integrate molecule will be found as suggested by Shapiro (1979). If DNA synthesis occurs, but terminates prematurely, and this is followed by cleavage of the strands of the donor molecule, the resultant molecule will be the recipient carrying the IS1 element.

The amplification of Tn2901 is recA-dependent, although it is possible that the initial duplication event involves a site-specific recombination system.

1.6 Mechanisms involved in DNA rearrangements

DNA rearrangements can be classified by their structure (for example, deletion, inversion, insertion) or by their mechanism of formation. Since the mechanisms by which many DNA rearrangements occur are now known, and since different rearrangements often share similar mechanisms of formation, this latter method of classification will be used here.

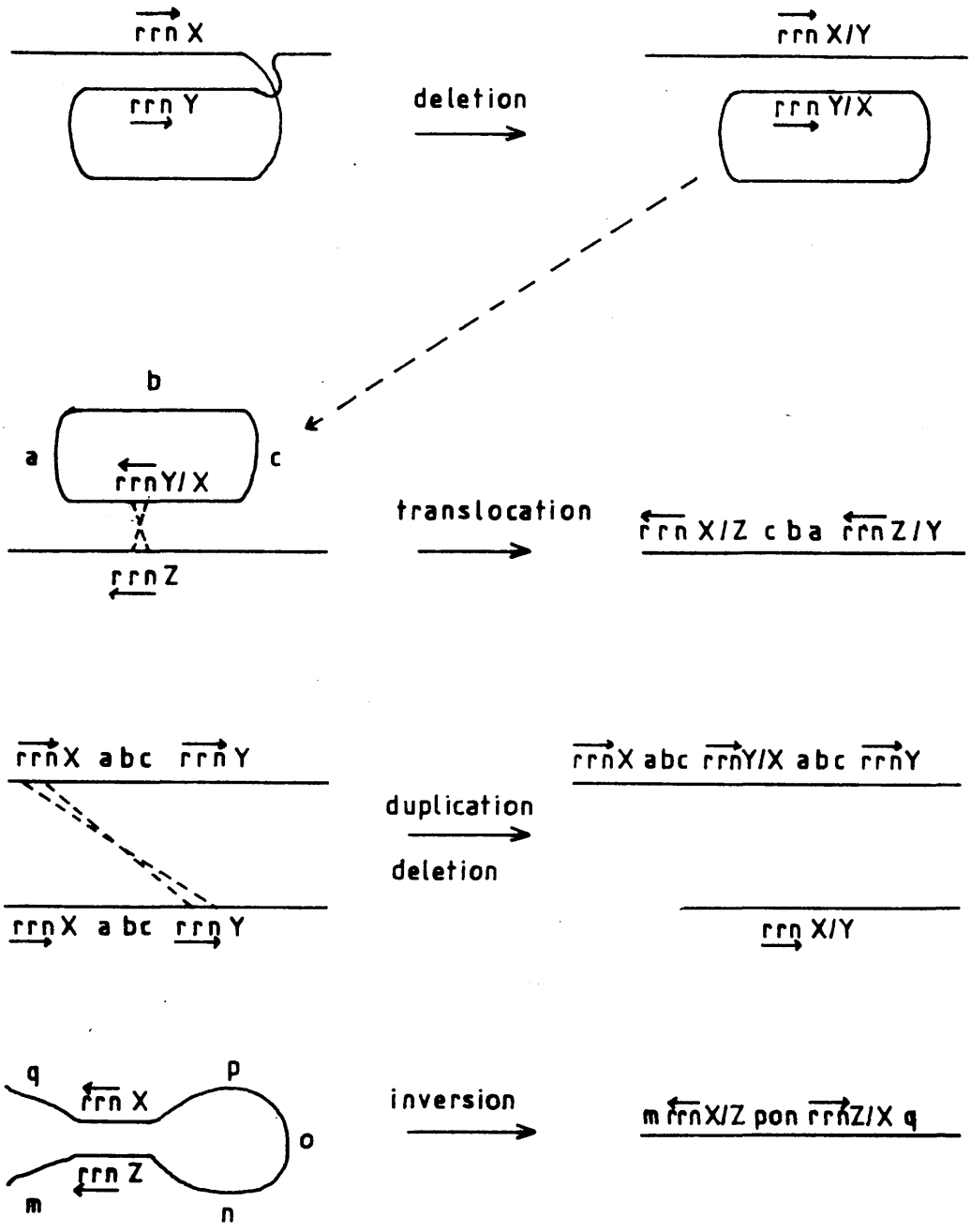


Figure 1.7. Types of chromosomal rearrangements produced by unequal recombination between rrn operons (After Lehner and Hill, 1980).

1.6.1 DNA rearrangements caused by homologous recombination

These rearrangements are recA-dependent and therefore take place by homologous recombination.

(i). Element unrelated

Recombination between homologous segments of euchromosomal DNA can generate various rearrangements. For example, homologous recombination between combinations of the seven rRNA operons in E. coli K12 can lead to rearrangements such as deletions, translocations, duplications and inversions (Lehner and Hill, 1980) as shown in Figure 1.7. Deletion of Tn10 from his-Tn10-his occurs by recombination between the flanking his genes (Chumley and Roth, 1980).

(ii). Element related

The only role of transposable elements in recA-dependant rearrangements is to provide homologous segments in appropriate positions so that homologous recombination between them causes rearrangements. The insertion of the F factor into the chromosome usually involves homologous recombination between insertion sequences present on F and the bacterial chromosome (Davidson et al, 1975). Homologous recombination can occur between directly repeated elements resulting in a deletion or duplication of the intervening material (MacHattie and Jackowski, 1977), or between inversely orientated elements to produce an inversion of the intervening material (Louarn et al, 1985). A compound transposon (element-DNA-element), in addition to transposing to new target sites, can move to a site at which a third element of the same type exists by homologous recombination (for example, Tn10-his-Tn10 - Chumley and Roth, 1980; Tn2671- de Bruijn and Bukhari, 1978; Iida et al, 1978; 1981b).

1.6.2 Rearrangements known to be caused by element specified recombinases and transposases, and by site-specific recombination

Transposable elements, when inserted at a given site, can cause rearrangements of adjacent DNA sequences. Such element-promoted rearrangements include deletions and inversions (for example Tn10- Kleckner et al, 1979) and these can be explained by the mechanism of transposition outlined in section 1.8 for IS1. When two identical insertion sequences are located on the same DNA molecule the two elements plus intervening DNA constitutes a new transposon. If the two elements are in the same orientation, intramolecular recombination at an internal resolution site will delete the intervening DNA (MacHattie and Jackowski, 1977).

1.6.3 Rearrangements thought to involve DNA sequence structure

DNA secondary structures mediated by misaligned hydrogen bonding between repeated or palindromic DNA sequences provide explanations for previously unexplained mutations from base substitutions to larger addition and deletion mutations. The correlation of the endpoints of spontaneous deletions (for example, in the lacI gene- Todd and Glickman, 1982) and the presence of repeated sequences, quasi-palindromic sequences, or both, is far greater than expected by chance (Ripley and Glickman, 1982). Such sequences permit the formation of DNA secondary structures in which the nonpalindromic regions are not hydrogen bonded. Models involving the repair of such mismatches have been proposed to explain a large number of DNA rearrangements (Ripley and Glickman, 1982).

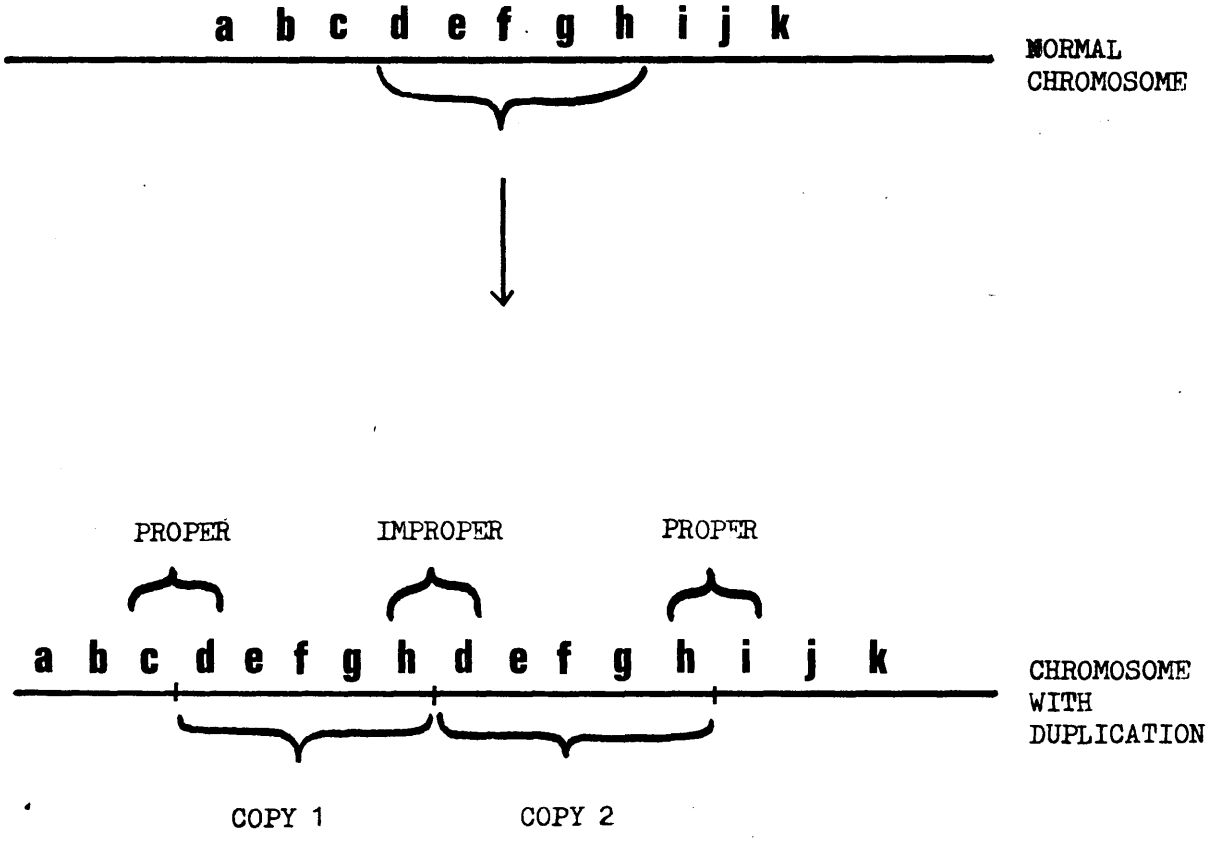


Figure 1.8 Structure of a tandem duplication. a - k represents a chromosomal segment, d e f g is tandemly duplicated.

1.7 Gene duplication and amplification

In both prokaryotes and eukaryotes, gene duplications and amplifications have been observed at a variety of loci and share some similar features. Gene duplication and amplification may increase the gene product of a duplicated or amplified gene, and provides a reservoir of extra genetic material which may be altered through evolutionary processes (Ohno, 1970; Hegeman and Rosenberg, 1970; Horiuchi et al, 1963; Rigby et al, 1974; Folk and Berg, 1970).

1.7.1 Properties of tandem duplications and amplifications

Tandem genetic duplications and tandem amplifications have the following genetic properties:

(i). Tandem duplications may cause no loss of function

The reason why tandem duplications may cause no loss of function can be seen in the diagram of a tandem duplication presented in Figure 1.8. In the chromosome carrying the duplication, the only rearrangement in sequence is the marked point between the two tandemly repeated copies, known as the "novel joint" (h-d). This impropriety does not lead to a loss of genetic information since proper versions of the sequences are present elsewhere (c-d and h-i). For this reason, no loss of function should result from the duplication mutation. Exceptions to this rule are duplications within a single gene, which may inactivate that gene, and duplications of several genes within a single operon, which could generate a polar effect at the novel joint between duplicated segments upon expression of that operon. Also, a duplication may be deleterious to the cell if an increased gene product alters the balance of an important metabolite in the cell.
Since duplications may lead to no loss of function, unequal recombination between any two points on the chromosome could in theory give rise to a viable duplication-carrying recombinant. Thus even very

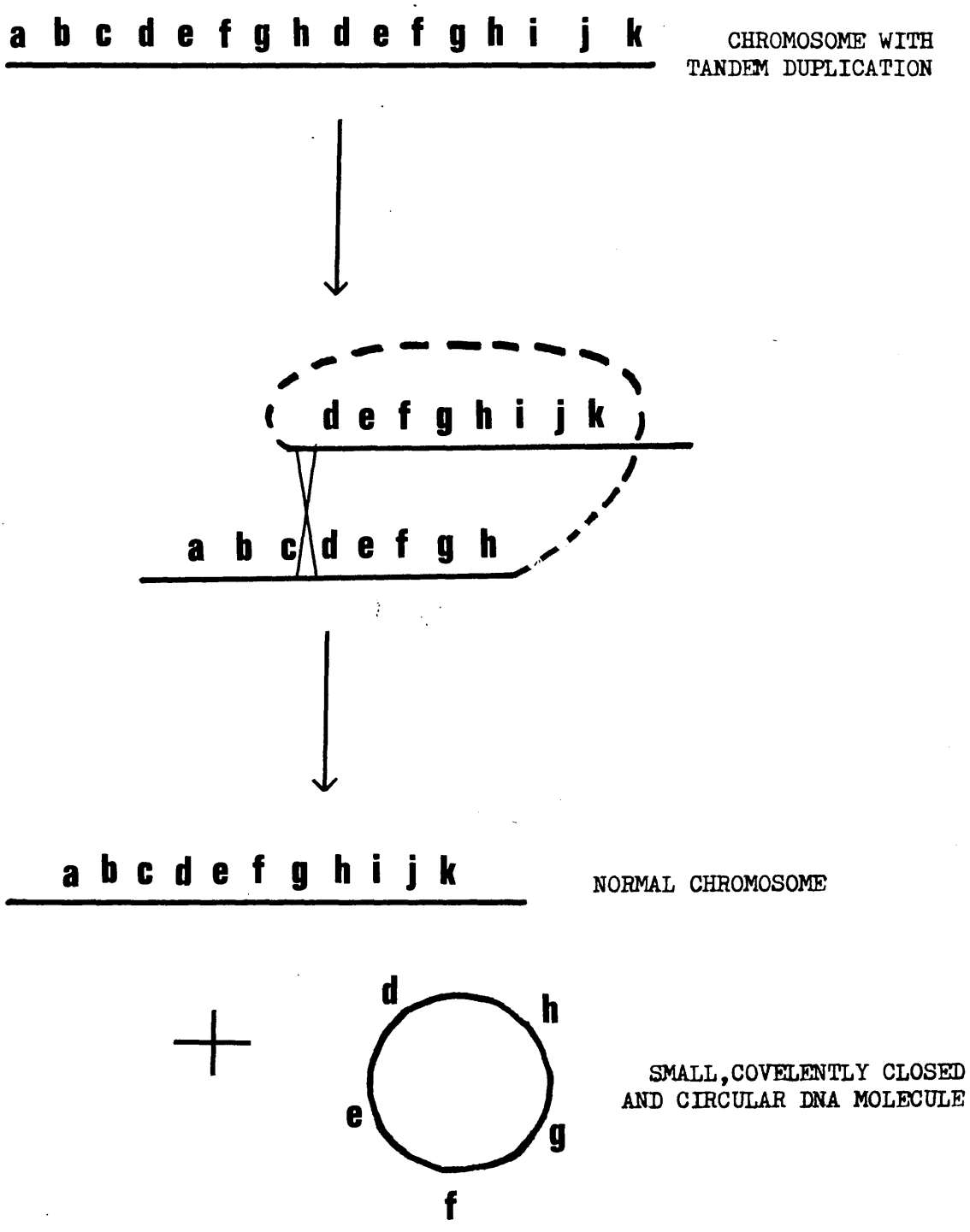


Figure 1.9. Mechanism for the segregation of a tandem duplication.

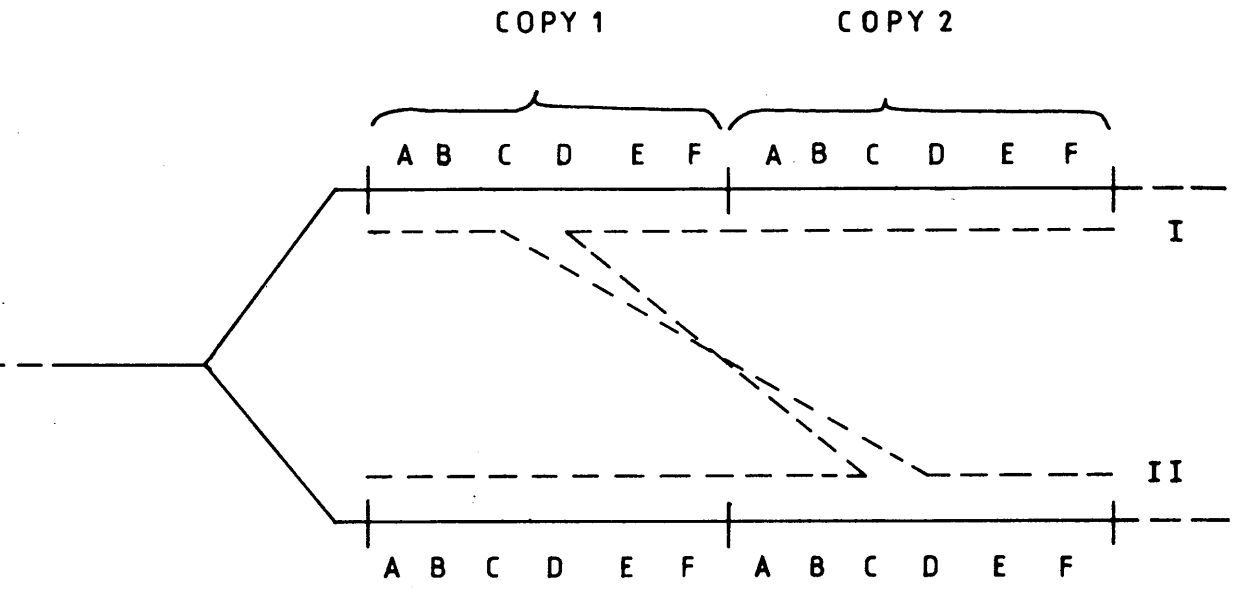


Figure 1.10. Legitimate recombination resulting in a triplication (I) or haploidization (II) of a tandem duplication.

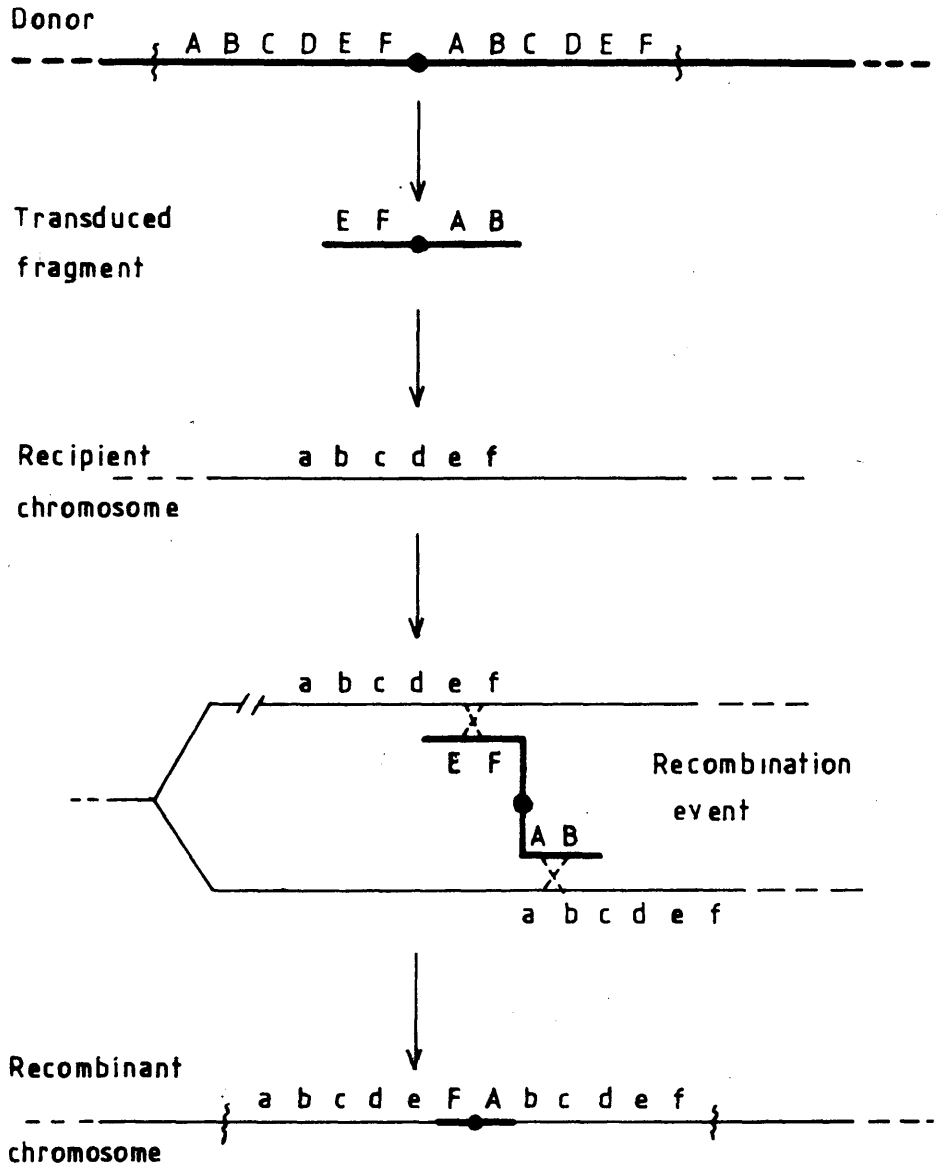


Figure 1.11. A mechanism for transduction of large tandem duplications. Recipient and donor DNAs are represented by light and bold lines respectively. Dotted lines represent reciprocal recombination events. (After Anderson and Roth, 1977).

large duplications could be maintained - the only restriction on the permissible size of a duplication might be the ability of the cell to replicate and segregate this large chromosome faithfully. Indeed, very large duplications of up to one-third of the chromosome have been reported in S. typhimurium (Straus and Hoffmann, 1975) and B. subtilis (Audit and Anagnostopoulos, 1975).

(ii). Duplications are subject to frequent loss and further amplification

Tandem duplications are generally unstable genetic structures. Reciprocal recombination between the two copies of duplicated material serves to excise intervening DNA and yields a single copy of the previously duplicated unit (Fig.1.9). Since this process involves legitimate recombination between homologous sequences, it generally occurs frequently and is recA-dependant. Recombination between the two copies can also lead to further amplification (Fig.1.10).

(iii). Extremely large duplications can be transduced

As mentioned previously, the only novel base sequence in chromosomes carrying a tandem duplication is located at the novel joint between copies of the duplicated region. Transduction of this novel joint into a normal (haploid) recipient can serve to re-establish the donors duplication state in the recipient. This is possible even if the duplicated region is larger than the DNA molecule carried by the transducing phage. It is assumed that transfer of a segment containing the novel joint, followed by its out of register pairing with two sister chromosomes and a double crossover gives rise to the duplication in the recipient as shown in Figure 1.11 (Jamet-Vierny and Anagnostopoulos, 1975; Hill et al, 1969).

1.7.2 Selection of tandem duplications and amplifications

A selection procedure to isolate cells harbouring a duplication or amplification is a prerequisite to the study of tandem duplication and amplification. Examples of such selection procedures are given below. However, many duplications and amplifications may have no selectable phenotype and therefore will not be detected. Similarly, a tandem amplification may have a selectable phenotype whereas the duplication may not. This is the case with the amplification of Tn2901 - the amplification to many copies can be selected for easily, but the initial duplication has no known selectable phenotype and is therefore "silent".

Selections designed to detect cells carrying tandem duplications and amplifications are often based on the increased gene dosage that the duplication or amplification confers. Examples are selection for overproduction of B-galactosidase (Horiuchi *et al*, 1963), ribitol dehydrogenase (Rigby *et al*, 1974), glycyl tRNA synthetase (Folk and Berg, 1970; Straus and Straus, 1976). Chromosomal gene amplification has also been observed after integration of a plasmid bearing a fragment of host chromosomal DNA and stepwise selection in progressively increasing concentration of an antibiotic (Young, 1983; 1984; Albertini and Galizzi, 1985; Gutterson and Koshland, 1983). Properties of the novel base sequence located at the novel joint between tandemly duplicated regions have also ben used to select for duplications and amplifications (for example, *his*D- Anderson and Roth, 1978; *arg*H- Beeftinck *et al*, 1974; Cunin *et al*, 1970; the *trp* operon- Jackson and Yanofski, 1973; *lac*- Tlsty *et al*, 1984). Under appropriate conditions, this novel sequence may confer a selectable phenotype; cells harbouring a duplication or amplification may then be detected. Detection methods of this kind most frequently involve selection for turn-on of genes whose expression have been prevented by either polarity effects or inactivation of promoter elements. Tandem duplication may serve to fuse intact unexpressed genes to functional

promoters. Thus the novel base sequence results from the juxtaposition of a functional promoter and the structural gene whose expression is selected.

Selection for mutations causing gene duplication was first described by Horiuchi and coworkers (1963). By causing *E. coli* to grow in the presence of limiting quantities of lactose in a chemostat they enriched for cells able to hyperproduce B-galactosidase; hyperproducing strains make more B-galactosidase than normal strains because they contain multiple copies of *lac* per genome. Gene duplications can also be obtained by selecting for recessive lethal mutations, for example certain *trpA*36 missense suppressor mutations (su^+_{36}) can only be recovered in strains which have also generated copies of the wild-type (su^-_{36}) gene (Hill *et al*, 1969; Carbon *et al*, 1969). In phage T4, duplications have been selected as sets of complementing rII mutations that cannot recombine because of overlapping deletions in that region (Homyk and Weil, 1974; Parma *et al*, 1972; Parma and Snyder, 1973). In bacteriophage lambda, duplications have been selected for in strains carrying deletions that leave the DNA too short to be effectively packaged into the phage head (Busse and Baldwin, 1972; Emmons *et al*, 1975; Emmons, 1974; Emmons and Thomas, 1975). Duplications have been obtained in transduction experiments with phage P1 in *E. coli* in which one end of the segment to be transduced does not find a pairing partner in the recipient as a result of a deletion. In these cases an illigitimate fusion event (for example, circularisation of the transducing fragment) followed by recombinational insertion can be used to explain the ensuing structures (Rae and Stodolsky, 1974; Stodolsky *et al*, 1972; Stodolsky, 1974).

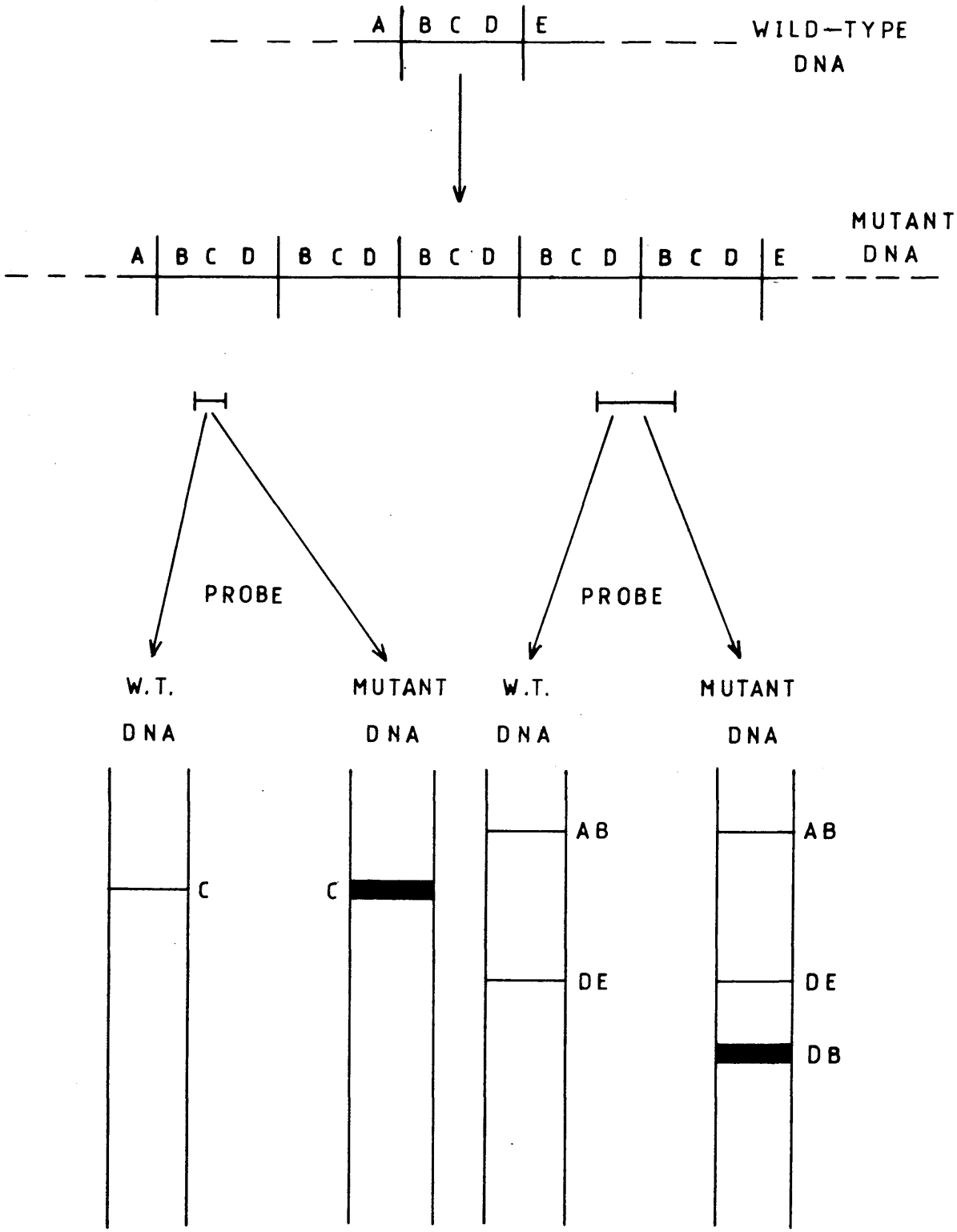


Figure 1.12. Detection of a chromosomal tandem amplification by Southern blotting. A-E represent a DNA sequence, B-D being amplified in the mutant DNA. If restriction digests are probed with a fragment internal to the amplified unit, no novel fragment is detected although an intense band (or bands) is seen. If the probe used contains the novel joint fragment, then a novel intense restriction fragment is seen.

1.7.3 Detection of tandem duplications and amplifications

Chromosomal DNA containing a highly amplified sequence can be detected by digestion with restriction endonucleases. On an agarose gel stained with ethidium bromide the amplified DNA may appear as intense restriction fragments above the background of non-amplified fragments (Tlsty *et al*, 1984; Albertini and Galizzi, 1985; Fishman and Hersheberger, 1983; Altenbuchner and Cullum, 1984). Hybridization of a probe containing a restriction fragment from the duplicated or amplified unit to Southern blotted chromosomal restriction digests will also detect the duplication or amplification (Fig.1.12). Tandem duplications can be detected by heteroduplex analysis where a heteroduplex is prepared comprising one strand carrying the duplication and one strand devoid of it. In this situation the insertion loop is not observed in a fixed position due to the loop being situated at any site in the duplicated region (Busse and Baldwin, 1972). The tandem character of a duplication is inferred from its instability interpreted as recombinational loss of a duplicated segment (Beeftinck *et al*, 1974; Jamet-Vierny and Anagnostopoulos, 1975; Hill *et al*, 1969; Folk and Berg, 1971; Straus and Hoffman, 1975). Tandem duplications of the *glyTpurD* region of *E. coli* have been physically isolated in the form of covalently closed DNA circles which are formed as a result of recombination between ribosomal RNA genes (Hill *et al*, 1977). In non-tandem duplications, recombinational loss of one of the duplicated segments would eliminate the DNA separating these segments, giving rise to a potentially lethal deletion.

A further method to detect duplications is a transduction cross between complementing pairs of mutants (Anderson *et al*, 1976). Their distinction from true recombinants is possible by the instability of the duplications. Unstable transformants have been detected in *Pneumococcus* (Ravin and Takahashi, 1970; Ledbetter and Hotchkiss, 1975; Kashmiri and Hotchkiss, 1975) and in *B. subtilis* (Jamet-Vierney and Anagnostopoulos, 1975; Audit and Anagnostopoulos, 1975; Trowsdale

and Anagnostopoulos, 1976).

1.7.4. Frequency of duplications and amplifications

The wealth of reported tandem duplications and amplifications (Anderson and Roth, 1977) suggests that they are frequent mutational events in bacteria and their phages. Some spontaneous duplications and amplifications are reported to arise at a frequency of around 10^{-4} to 10^{-5} (Folk and Berg, 1970; 1971; Straus and Hoffman, 1975). A lower value of 10^{-6} to 10^{-7} is reported for the argECBH region of *E. coli* (Beeftinck *et al*, 1974). In the *his* region of *S. typhimurium* duplications are found at a frequency of 10^{-4} to 10^{-3} (Anderson *et al*, 1976). In *B. subtilis*, 0.5% of transformants of the *ilv*A4 mutant to *ilv*$^{+}$ are duplications (Jamet-Vierny and Anagnostopoulos, 1975). Miller and Roth (1971) have found that spontaneous duplications of the *sup*R and *sup*S region of *s. typhimurium* may be carried by 10% of the cell population. Mild ultraviolet irradiation of *E. coli* results in duplication of the *glyTpur*D region of *E. coli* in three to five percent of the surviving population (Hill and Combriato, 1973). Duplications are rare in phage T4 (5×10^{-8}) but in this case a deletion has to be selected simultaneously if maturation is to occur.

Although most estimates of chromosomal duplication and amplification are quite high, considerable variation exists among estimates. The diverse techniques used to select tandem duplications make comparisons of estimates difficult. Anderson and Roth (1981) developed a method to study the duplication process and compare the frequency of duplications at 38 sites on the *S. typhimurium* chromosome. Their results suggest that the frequencies of duplications at different chromosomal sites are highly variable, the highest frequency being for duplications of the rRNA cistrons.

Since the detection of chromosomal duplications and amplifications depends on a suitable selection procedure, many chromosomal

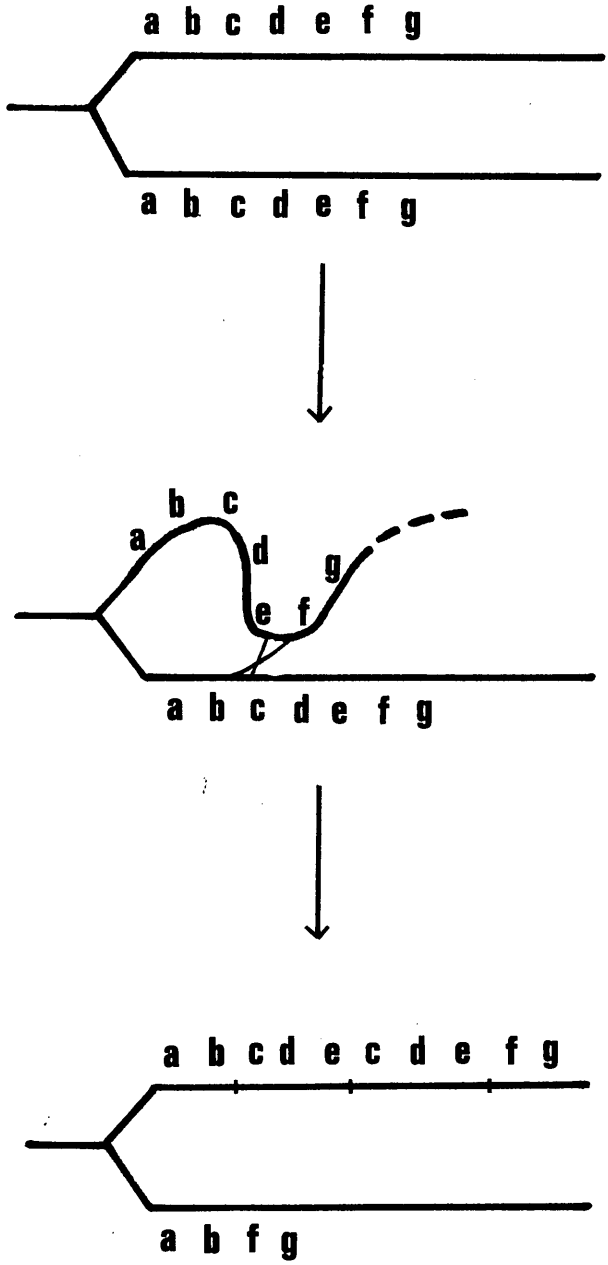


Figure 1.13. Mechanism for the formation of a tandem duplication involving unequal recombination between sister chromatids.

duplications and amplifications will not be detected due to the lack of a selectable phenotype.

1.7.5. Size and extent of the duplicated region

The size of the duplicated region varies in *E. coli* from around the size of a gene (Beeftinck *et al*, 1974), to as much as six percent of the genome (Folk and Berg, 1971; Hill and Combriato, 1973). Duplications of up to one-third of the chromosome have been reported in *S. typhimurium* (Straus and Hoffman, 1975) and *B. subtilis* (Audit and Anagnostopoulos, 1975). The extent of the tandem repitition varies from two copies (a duplication) to around 500 copies in *Streptomyces fradiae* (Fishman and Hersheberger, 1983).

1.7.6. Possible mechanisms for the formation of a tandem duplication and amplification

Several mechanisms have been postulated for the formation of tandem duplications and amplifications and these are outlined below:

Unequal recombination

In this mechanism, postulated by Hill and Combriato (1973), sister chromatids recombine unevenly behind a replication fork with the result that one chromatid retains a tandem duplication and the other ends up with a corresponding deletion (Fig.1.13).

Intrachromosomal recombination followed by migration

This mechanism, postulated by Hill and Combriato (1973), involves an intrachromosomal recombination event, generating a small circle of DNA and a chromosome with a deletion. This small circle could then migrate to another chromosome within the same cell and integrate by a homologous recombination event, generating a tandem duplication

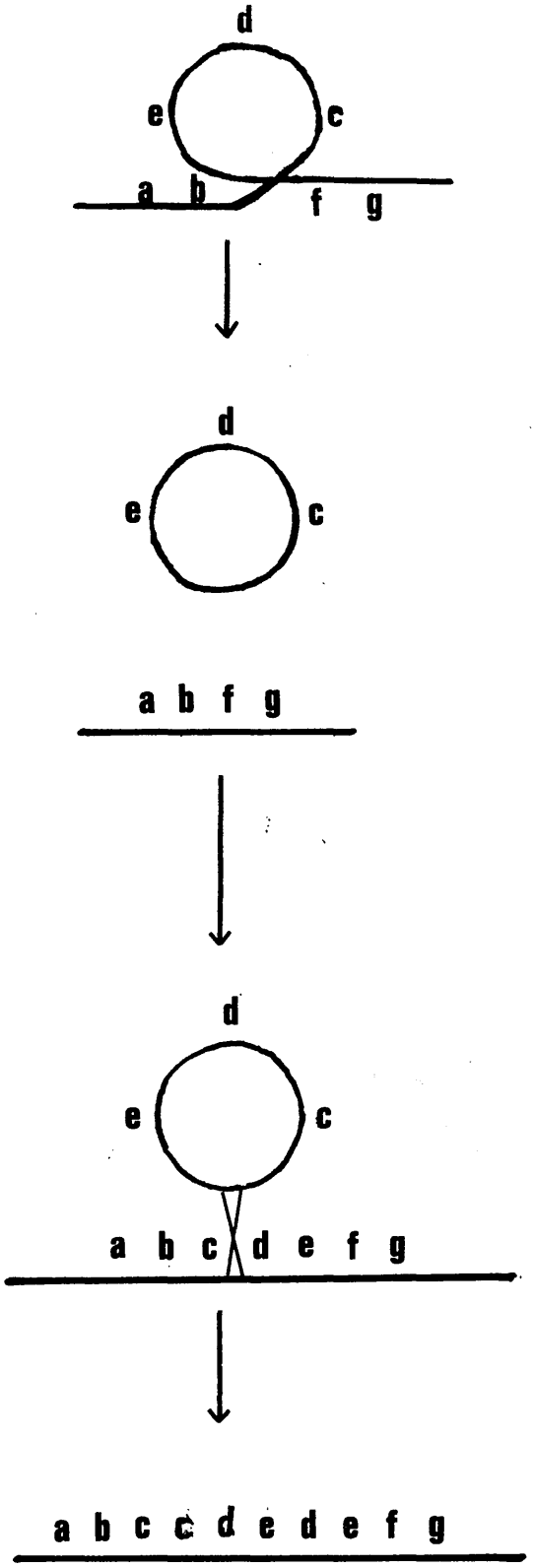


Figure 1.14. Mechanism for the formation of a tandem duplication involving intrachromosomal uneven recombination followed by integration of the resulting small circle of DNA into the second chromosome.

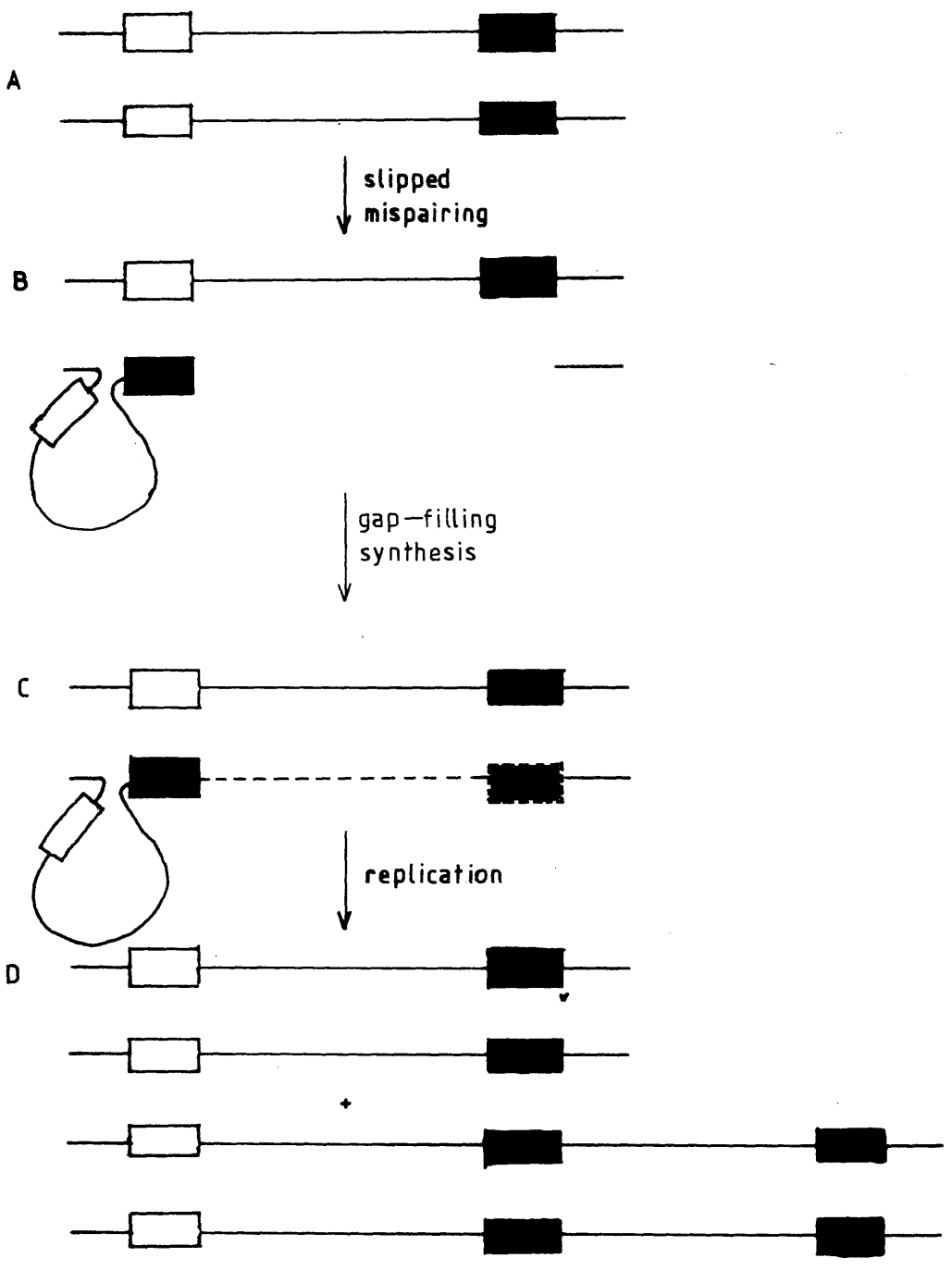


Figure 1.15. Formation of a tandem duplication by slipped mispairing of small repeated sequences.
(a) The open and filled boxes represent two homologous sequences.
(b) Misaligned inter-strand base pairing mediated by the repeated sequences, with the looping out of intervening DNA.
(c) Gap-filling synthesis, templated by the opposite strand.
(d) The resultant duplication following replication.

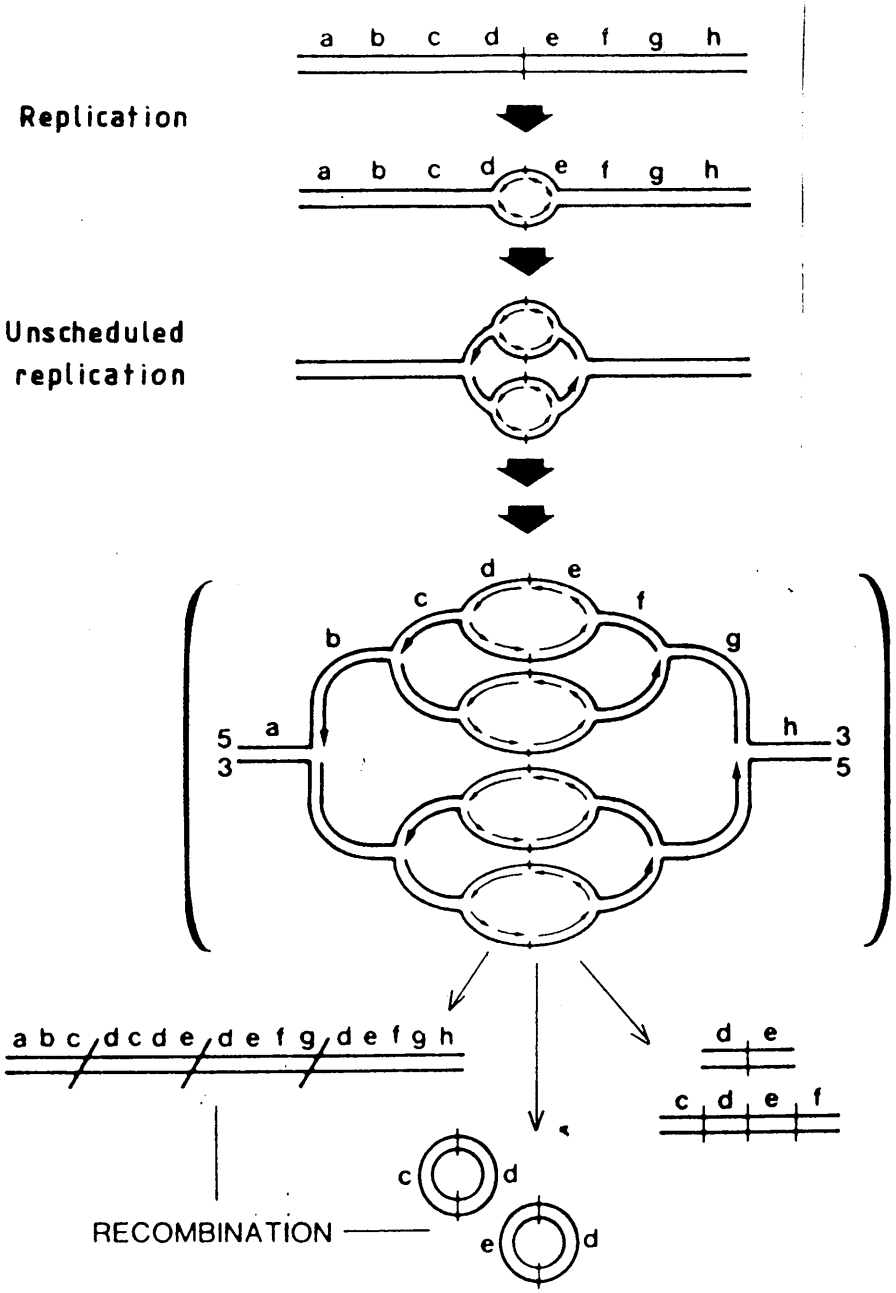


Figure 1.16. Unscheduled DNA synthesis and recombination. Bidirectional replication at an origin generates a bubble that can undergo further rounds of unscheduled replication, resulting in a structure containing partially replicated duplexes. Recombination can generate extrachromosomal circles or linear duplexes. (After Stark and Wahl, 1980).

(Fig1.14). This model, like the one above, requires that regions of DNA at the ends of the duplication participate in an uneven recombinational event.

Slipped mispairing of repeated sequences

Tandem duplication of a 9.8kb unit containing *ampC* has been shown to occur via very short DNA homologies of 12-13bp in length (Edlund and Normark, 1981). Similarly, in the *lac* region of *E. coli* homologous sequences of less than 12bp result in the amplification of regions 7kb-37kb in length (Tlsty *et al*, 1984). Edlund and Normark (1981) suggested that recombination between such small homologies involves the RecA protein although it is not known whether *recA* is required only for subsequent amplification following duplication, or whether it is also required for the initial duplication as well. Such duplication and amplification can also be explained through secondary structures involving misaligned inter-strand base pairing mediated by the repeated sequences (Ripley and Glickman, 1982). The repeated sequences allow the misalignment of the second repeat upon the first, with the resultant looping out of the intervening sequence. This generates a structure in which extension of the strand looped back, templated by the misaligned complement produces a duplication (Fig.1.15).

Saltatory replication

In eukaryotes, a mechanism of saltatory replication has been proposed to explain amplification (Fig.1.16). The central feature of this model is the initiation of replication at the same origin (perhaps within the amplified sequence) more than once in any given S phase of a cell cycle. A second feature of this model (in its most simplistic form) is that DNA replication from this origin slows down or ceases at some site within the chromosome prior to joining with a replication fork proceeding from an adjacent origin of replication (Schimke, 1982).

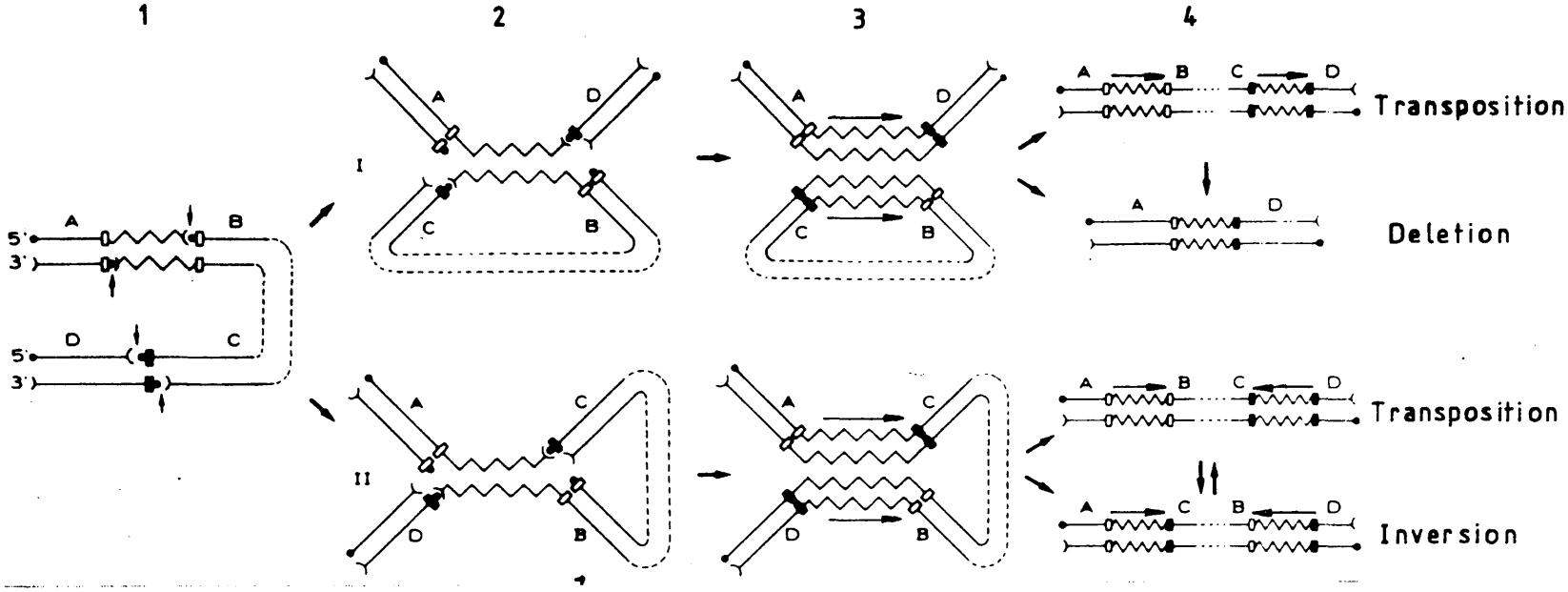


Figure 1.17. Intramolecular model for transposition, deletion formation and inversion (based on Shapiro, 1979). The first step involves singlestrand endonucleolytic cuts at both ends of IS1 and a 9bp staggered nick at the new target site. The resulting free IS1 ends are then joined to the DNA ends produced at the target site; this is possible in either of two orientations (I and II). Replication across IS1 produces the structures shown in the third step. A site-specific recombination across the two IS1 copies then produces transposition in direct or inverse orientation. In the absence of recombination, a deletion or inversion results.

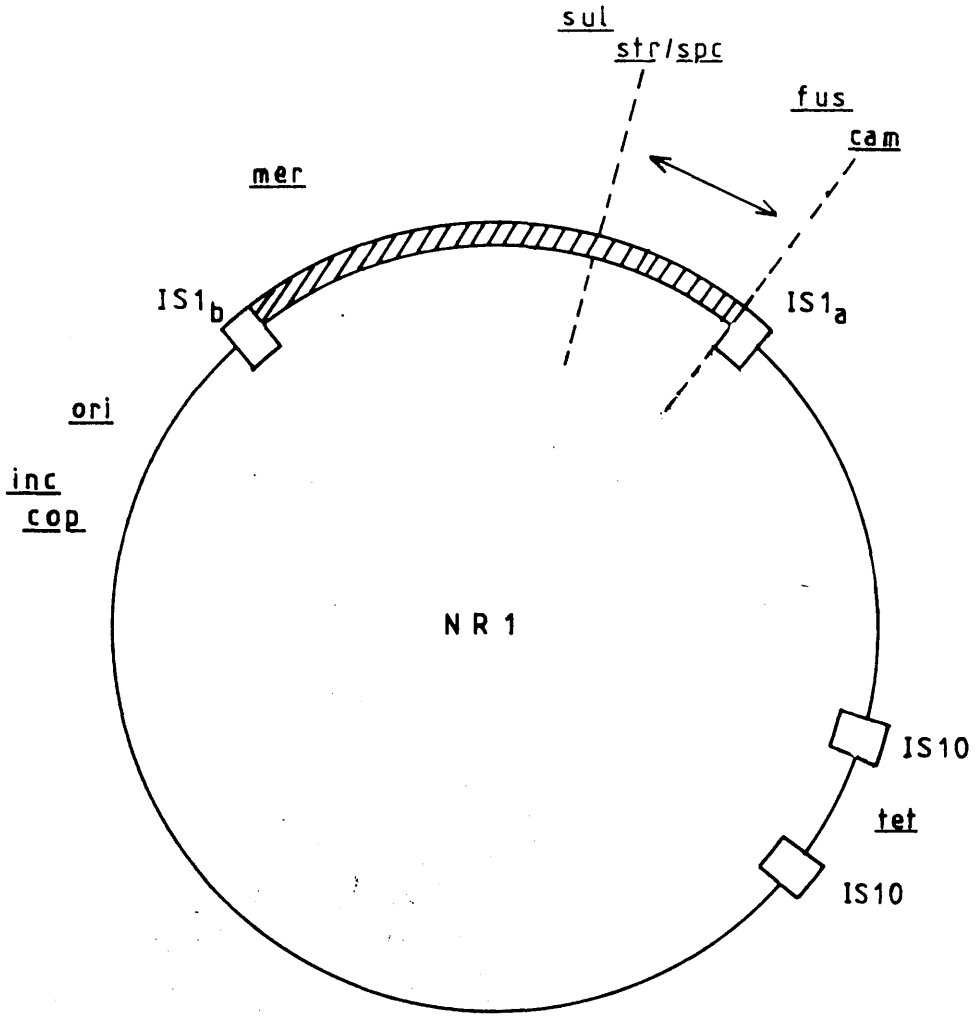


Figure 1.18. Restriction endonuclease map of the composite R plasmid NR1. The portion of the plasmid indicated between the dotted lines extending from $IS1_a$ into the str-spc gene is deleted in derivatives of NR1 which can amplify the r-determinants in E. coli.

This produces a so-called "onion skin" configuration and may constitute the characteristic double minutes of eukaryotic amplification (Schimke, 1982).

1.8 IS1-mediated DNA rearrangements

When IS1 is present in a particular DNA sequence, numerous rearrangements can occur in its vicinity (Reif and Saedler, 1975; 1977; Cornelis and Saedler, 1980; Saedler *et al*, 1980). IS1 can promote deletions of adjacent DNA; IS1 is retained and thus can promote further rounds of increased deletion formation (Reif and Saedler, 1975; 1977). IS1 can also promote inversion of adjacent DNA (Cornelis and Saedler, 1980). This inversion appears to involve transposition of a given IS1 element to a nearby site in inverse orientation with concomitant inversion of the DNA between the two inversely repeated copies of IS1. A model has been proposed to explain the deletions and inversions mediated by IS1 (Saedler *et al*, 1980) and is outlined in Figure 1.17. Also, a stimulation of homologous recombination has been observed in the immediate vicinity (50-100bp) of an IS1 insertion in the cI gene of lambda (Lieb, 1980).

IS1-mediated amplification

Amplification of DNA segments flanked by copies of IS1 in direct repeat have been reported for the r-determinant of NR1 and derivatives (Peterson and Rownd, 1983); bacteriophage P1ApCm (Froehlich *et al*, 1986) and Tn9 on RTF (Chandler *et al*, 1979). These three amplification systems are outlined briefly below.

NR1

The bacterial R plasmid NR1 (Fig.1.18) is one of the most extensively characterised systems of antibiotic resistance gene transfer. NR1 is 90kb in size and consists of two dissociable components- the

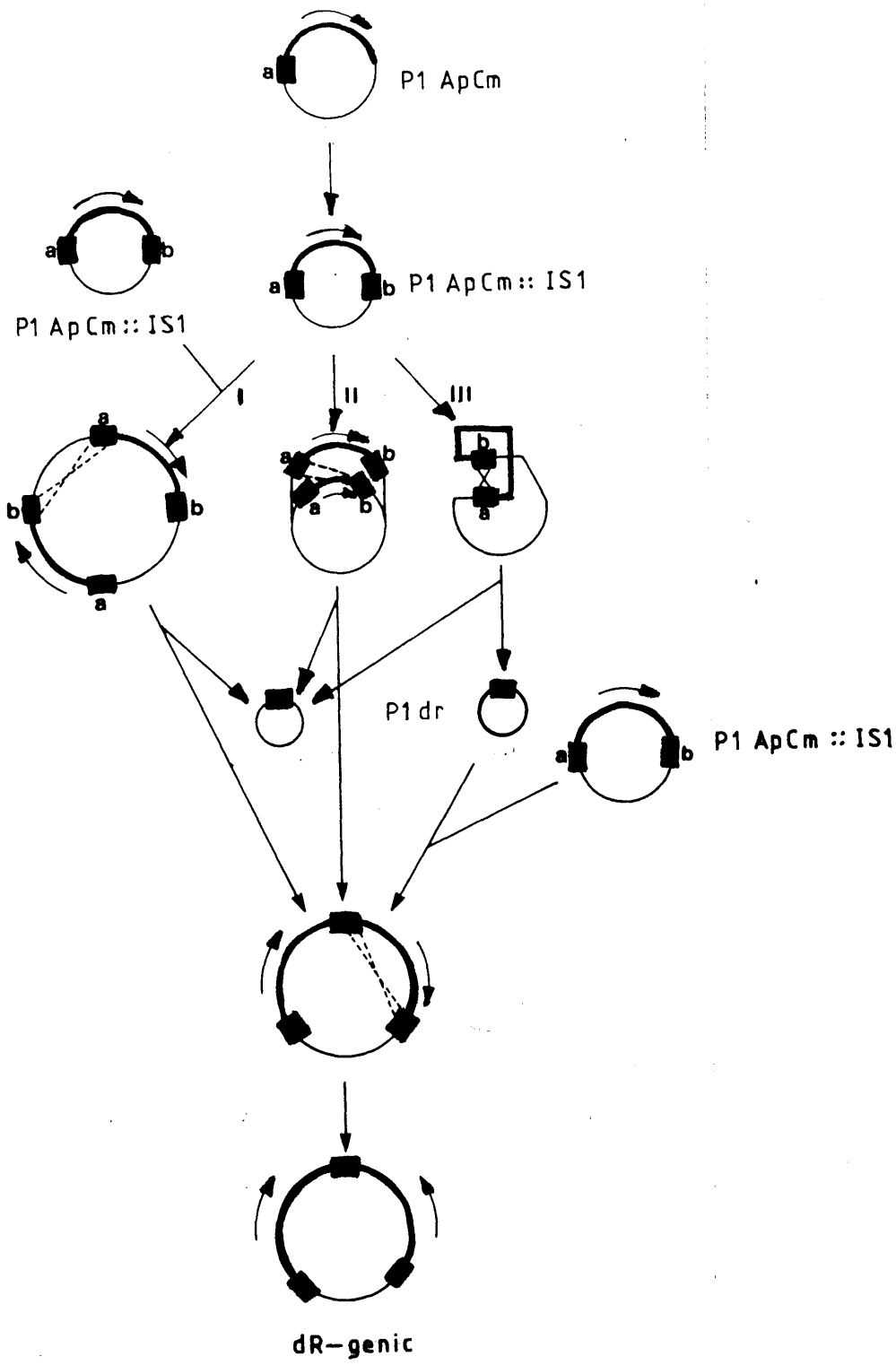


Figure 1.19. Models for the formation of dR-genic plasmids. The boxes labelled a and b represent Tn9 or IS1. Box a represents the Tn9 resident in P1 ApCm, and b represents either IS1 or Tn9. The arrows outside the circles show the orientation of the P1dR region. I, II and III represent three possible pathways of formation of dR-genic plasmids. I involves the formation of a co-integrate molecule by homologous recombination; II involves reciprocal recombination between IS1 elements; III involves the dissociation of P1 ApCm ::IS1 into two circles, followed by recombination of the autonomous P1dR plasmid with a second P1 ApCm::IS1. (After Froehlich et al, 1986).

resistance transfer factor (RTF; 69kb) and the resistance determinants (r-determinants; 20.6kb) which are separated by direct repeats of the insertion element IS1 (Davies and Rownd, 1972; Hu *et al*, 1975; Ptashne and Cohen, 1975). The r-determinant of NR1 confers resistance to chloramphenicol and fusidic acid (*cam fus*), streptomycin and spectinomycin (*str spc*), sulphonamides (*sul*) and mercuric ions (*mer*) (Miki *et al*, 1978). The tetracycline resistance (*tet*) gene on NR1 is encoded by Tn10 on the RTF. The selective amplification of NR1 drug resistance genes is mediated by *rec*-dependant recombination between the two IS1 elements (Peterson and Rownd, 1983). In *Proteus mirabilis*, the whole IS1-flanked region is amplified about 20-fold when the cells are cultured in medium containing high concentrations of appropriate antibiotics (Hashimoto and Rownd, 1975). Strains of *E. coli* do not undergo such a transition unless they are deleted for the *cam fus* and *str spc* regions of the r-determinants. IS1 itself is not deleted (Huffman and Rownd, 1984). IS1 has been shown not to be absolutely necessary for the amplification (Peterson and Rownd, 1983) and *cam* DNA sequences of similar size can also serve as homologous recombination sites (Peterson and Rownd, 1985).

P1ApCm

When cells harbouring the low copy number plasmid P1ApCm are grown in high concentrations of ampicillin and chloramphenicol, a class of small, high copy number plasmids are obtained which are called P1dR plasmids. P1dR plasmids are thought to be formed from another class of plasmids, the largely rearranged P1dR-genic plasmids which contain an inverted duplication of the IS1-flanked P1dR region (Froehlich *et al*, 1986). The formation of P1dR plasmids is *rec*A-dependant since they are not obtained in a *rec*A host. Figure 1.19 shows the proposed models for the formation of dR-genic plasmids. The first intermediate (P1ApCm::IS1) is probably formed by the transposition of Tn9 or IS1,

providing the homology needed for the duplication of the IS1-flanked region.

Tn9 on RTF and P1

Tn9 is 2.6kb long and carries the Cm^r gene bounded by two directly repeated IS1 elements (MacHattie and Jackowski, 1977). Tn9, when inserted in the plasmid RTF, can amplify (to form tandem repeats) in E. coli in response to passage in chloramphenicol (Chandler et al, 1979). Such behavior is also observed when Tn9-like derivatives carried by P1 are subjected to similar treatment (Meyer and Iida, 1979). Neither the position of Tn9 in the replicon, or the type of host replicon employed, plays a role in the amplification process (Chandler et al, 1979). Only a very low level of amplification is detected in a recA host which suggests that two processes may be involved in the amplification: an initial relatively infrequent recA-independant duplication of the transposon, followed by a more efficient recA-dependant amplification (Chandler et al, 1979).

1.9 The argF gene, Tn2901 and the isolation of US Cut mutants

The genes of the arg regulon in E. coli are scattered over several regions of the chromosome. They are subject to repression by arginine, mediated by a common regulatory molecule, the product of the argR gene (Cunin et al, 1983). The argR gene product acts at a basic arginine operator sequence preventing RNA polymerase from initiating transcription.

Among the Enterobacteriaciae, E. coli is the only strain known to possess two structural genes coding for ornithine transcarbamylase (OTCase) (Legrain et al, 1976a). OTCase is a multisubunit enzyme which catalyses the reaction converting ornithine to citrulline in the pathway of arginine biosynthesis. In E. coli K12, the products of each of these two genes, argI and argF, associate randomly to form a

trimer, giving a family of four isoenzymes, consequently designated FFF, FFI, IIF and III. The gene products of argF and argI are very similar- they are indistinguishable by size, each coding for a protein with a molecular weight of approximately 36kd. Sequence analysis of the two genes (Van Vliet et al, 1984) has shown 78.1% overall homology at the nucleotide level and 86% at the amino acid level, with a stretch of 25 codons showing almost perfect homology. The FFF and III isoenzymes differ in their thermolability (Legrain et al, 1976a) and their elution patterns on diethylaminoethyl- Sephadex columns (Legrain et al, 1972).

It has been suggested that the chromosome of E. coli was formed by two successive duplications of a primative genome which was one quarter of the size of the present-day chromosome (Zipkas and Riley, 1975). However, if the duplicate genes argF and argI were created by such an event, they would be expected to be either 90 or 180 degrees apart. This is not the case. The argF gene is situated at 7.5 minutes on the E. coli chromosome, while the argI gene is at 85 minutes. However, it is unlikely that two genes so similar could have arisen independently of one another. The genes involved exhibit much greater similarities than their counterparts in other isoenzyme systems, for example lactate dehydrogenase and aldolase in mammals (Horecker, 1975; Markert, 1963). Moreover, no argF gene has been detected in E. coliW or E. coliB (Legrain et al, 1972) - in the latter case the analysis has been carried far enough to conclude that the gene is absent and not merely nonfunctional. Furthermore, no argF equivalent has been found in Klebsiella pneumonia (Matsumoto and Tazaki, 1970), S. typhimurium (Prozeski, 1968), Enterobacter aerogenes and Serratia marcesens (Legrain et al, 1976a), the genetic linkage maps of which show important similarities to that of E. coli. Legrain et al (1976a) have therefore suggested that argF might be the product of a relatively recent event (duplication or transposition) peculiar to E. coli. Indeed,the argF gene in E. coli K12 is flanked by two IS1 elements in direct repeat in a transposon-like structure termed Tn2901

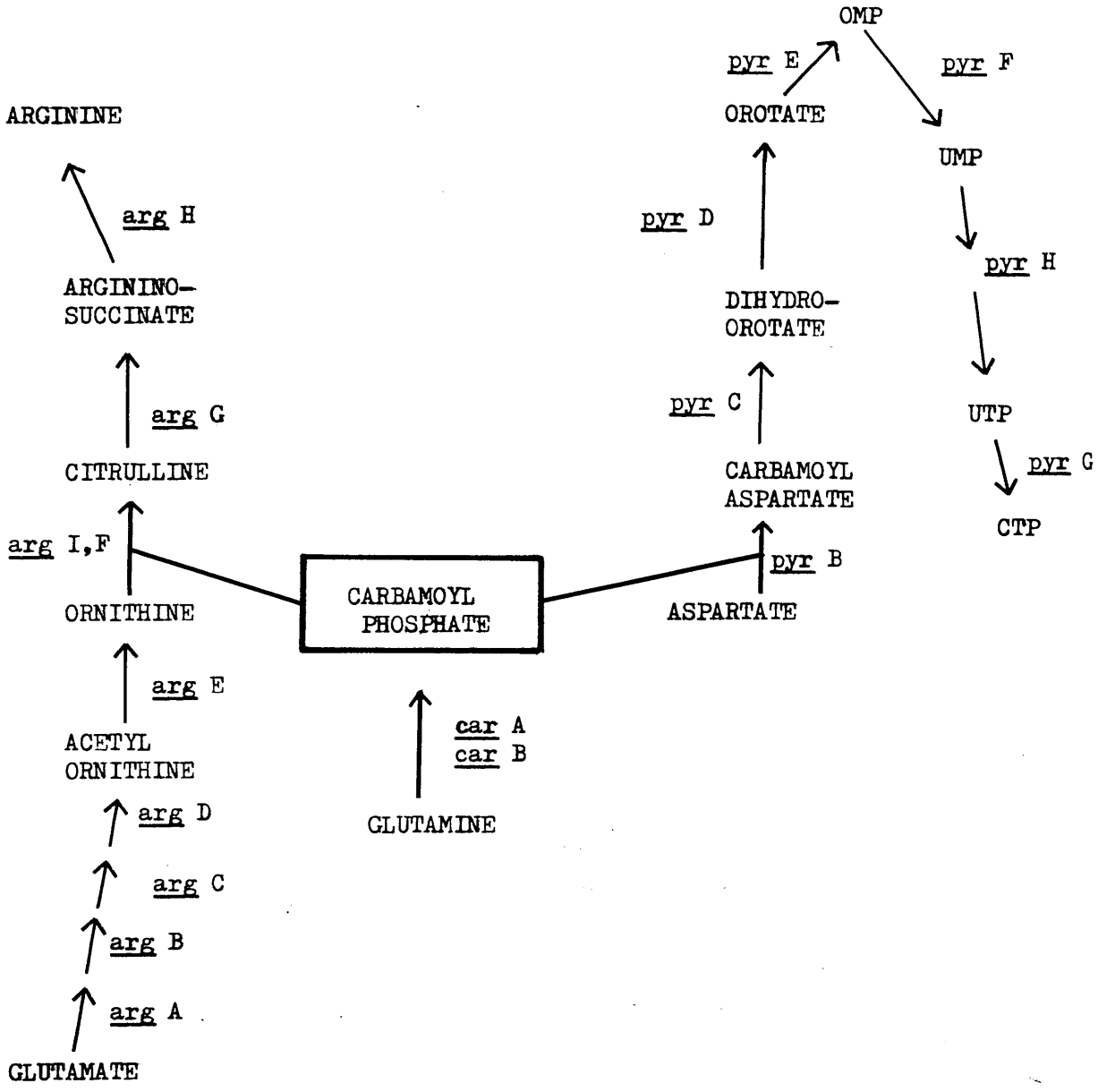


Figure 1.20. Arginine and pyrimidine biosynthesis.

(York and Stodolsky, 1981; Hu and Deonier, 1981a).

The de novo synthesis of arginine and the pyrimidines requires carbamoyl phosphate (Fig.1.20). The carbamoylation of ornithine by OTCase produces citrulline. In E. coli K12, the equilibrium of the carbamoylation is highly favourable to the production of citrulline and the release of inorganic phosphate. The equilibrium constant for the carbamoylation of ornithine at pH8.0 is 2×10^5 (Legrain et al, 1976).

E. coli K12 mutants which lack carbamoyl phosphate synthase, the product of the car genes, require both arginine and uracil for growth. It is possible, however, to obtain further mutants in which carbamoyl phosphate is obtained by the phosphorolysis of citrulline due to an overproduction of OTCase. These mutants can be selected for by plating car strains on minimal medium supplemented with excess citrulline and are called Cut (citrulline-utilizing) mutants (Legrain et al, 1976). Hfr strains in which the F factor is integrated adjacent to argF (HfrP4X) yield many more Cut mutants than F^+ or F^- strains, or strains in which the F factor is integrated at any other site (Jessop and Glansdorff, 1980). Many of the Cut mutants isolated from HfrP4X are unstable (US Cut) and genetic data suggests that these unstable mutants contain a chromosomal rearrangement in the proA argF region (Jessop and Glansdorff, 1980; Legrain et al, 1976b). The formation of US Cut mutants is recA-dependent and the US Cut mutants are to a large extent stabilized in a recA background (Jessop and Glansdorff, 1980).

Aims of this work

The work presented in this thesis has two aims. Firstly, to determine the structure of the rearrangement associated with the US Cut mutants; and secondly, to initiate studies on the mechanism of formation of the rearrangement.

Chapter 2

Materials and Methods

2.1. Chemicals

Chemicals	Source
General chemicals and organic compounds	B.D.H., Koch-light, Hopkin & williams, B.C.L., May & Baker, Formachem Research Int., B.R.L.
Antibiotics	Sigma
Biochemicals	Sigma, Koch-light.
Media	Oxoid, Difco.
Agar	Oxoid, Davis.
Agarose and low melting point agarose	B.R.L.
SDS	Serva
Radiochemicals	Amersham Int., New England Nuclear.
Lambda HindIII DNA	B.R.L.
"Replicote"	Hopkin & Williams
Transfer membranes	Pall (Biodyne A) and Amersham (Hybond-N).

2.2. Enzymes

All enzymes were obtained from B.R.L., with the exceptions of the following:-

Calf-Intestinal Alkaline Phosphatase - B.C.L.

Lysozyme - Sigma

Yeast tRNA was also obtained from B.R.L.

2.3. Bacterial Strains

The bacterial strains used were all derivatives of E.coli K-12 and are listed in table 2.1. Genotype and phenotype symbols are those recommended by Bachman et al (1976) and Novick et al (1976).

2.4. Plasmids and Bacteriophages

Plasmids and bacteriophages are listed in Tables 2.2 and 2.3 respectively. Nomenclature follows Novick et al (1976).

2.5. Culture Media

L Broth : 10g. tryptone, 5g. yeast extract, 5g. NaCl, 1g. glucose, 20mg. thiamine made up to 1 litre with distilled water and adjusted to pH7.0 with NaOH.

L Agar : Above broth containing 1.5% agar.

L Broth without glucose : Above broth without glucose.

Minimal Broth : 7g. K_2HPO_4, 2g.KH_2PO_4, 4g.$(NH_4)_2SO_2$, 0.25g. tri-sodium citrate, 0.1g.$MgSO_4.7H_2O$ made up to 1 litre with distilled water.

Minimal Agar : Above broth containing 1.5% agar.

When required supplements were added to minimal medium at the following concentrations:

Citrulline : 2,500ug./ml.

Table 2.1. Bacterial strains

Strain	Origin	Relevant markers	Reference/Comment
JEF8*	P4X	Hfr *thr*$^-$ *carB*$^-$ *metB*$^-$	Legrain *et al*, 1976 Cut yielder
JEF8 Cut12	Cut from JEF8	Hfr *thr*$^-$ *carB*$^-$ *met*$^-$	Stable Cut
JEF8 Cut1 to 10	Cut from JEF8	Hfr *thr*$^-$ *carB*$^-$ *met*$^-$	US Cut
P678.14	JEF8 x P678	F$^-$ *thr*$^-$ *carB*$^-$ *met*$^-$ *lac*$^-$ str	Legrain *et al*, 1976
D7-8	JEF8 x P678.14 *proA*$^-$	F$^-$ *carB*$^-$ *met*$^-$ *lac*$^-$ str	Jessop and Glansdorff, 1980
NGX2		F$^-$ *argF*$^-$ *proA*$^-$ *argI*$^-$ *pyrB*$^-$ str	Jessop and Glansdorff, 1980
D7-13 *pro*$^-$		F$^-$ *carB*$^-$ *met*$^-$ *lac*$^-$ *pro*$^-$	A.P. Jessop
DS902		*recA thr*$^-$ *leu*$^-$ *thi*$^-$ *pro*$^-$ *his*$^-$ *arg*$^-$ str^r	D.J. Sherratt
DS903		*recF thr*$^-$ *leu*$^-$ *thi*$^-$ *pro*$^-$ *his*$^-$ *arg*$^-$ str^r	D.J. Sherratt
Clr-207		*recA srl* :: Tn10	Kleckner, Roth and Bostein, 1977
D30-1	US Cut from JEF8 x P678.14 *proA*$^-$	*thr*$^-$ *carB*$^-$ *met*$^-$ str	Jessop and Glansdorff, 1980
Δ M15		Δ (*pro-lac*) ∅ 80d*lacZ* M15	Ruther *et al*, 1981

JEF8 pCC7 2,4	pCC7 integrated in Tn2901 in JEF8	*thr*$^-$ *car*B$^-$ *met*$^-$ *thy*$^-$ nal^r Cm^r	Chapter 6
Cut1 pCC7	pCC7 integrated in Tn2901 in JEF8 Cut1	*thr*$^-$ *car*$^-$ *met*$^-$ Cm^r	Chapter 6
JEF8 *tra*	transfer-deficient	*thr*$^-$ *car*$^-$ *met*$^-$	Chapter 5
CSH56 *arg*I		△(*lac-pro*) *thi*$^-$ *arg*I$^-$	A.P. Jessop
CB50		△(*lac-pro*) *thi*$^-$ str^r	C. Boyd
Hfr *pol*A	transfers *pol*A early	Hfr *pol*A1 rif^r	D.J. Sherratt
F-prime strains			
DF30D.2	F' *pro*$^+$ *lac*$^+$		A.P. Jessop
DF30D.2 CSH56		*car*$^-$ *thy*$^-$	A.P. Jessop
II-45	DF30D.2 US Cut		A.P. Jessop
II-23	DF30D.2 S Cut		A.P. Jessop
I-4	DF30D.2 S Cut		A.P. Jessop

* HfrP4X transfers *arg*F and *pro*A first, in a clockwise direction

Table 2.2. Bacterial plasmids

Plasmid	Description	Phenotype	Size(Kb)	Source/reference
pACYC184	vector derived from p15A	Cm^rTc^r	4.0	Chang & Cohen,1978
pUC8/9	vector derived from pBR322	Ap^r	2.67	Vieira & Messing,1982
pUC18	vector derived from pBR322	Ap^r	2.67	Yanisch-Perron *et al*,1985
RSF1341	pMB8::Tn3 *tnpA*$^-$ *tnpR*$^-$ *res*$^-$	Ap^r	6.3	Heffron *et al*,1977
pCB101	Lambda dv vector	Cm^r	5.0	Boyd and Sherratt,1986
pAK100	pAA231 BamH1 deletion Tn3 *tnpA*$^+$ *tnpR*$^-$ *res*$^+$	Cm^r	7.2	Kitts,1982
pACYC	pACYC184::	Cm^r	-	A. Arthur
pBR322	cloning vector	Ap^rTc^r	4.363	Sutcliffe, 1978
pBR322::IS1	IS1 in pBR322	Tc^r	-	G. Coupland
pD115	pBR322 + IS5	Tc^r	-	Schoner and Kahn,1981
pCD4-110	pBR322 + IS3/*argE* fragment	Ap^r	-	Charlier *et al*, 1982
pCD44-17	pK01 + 871bp IS2 fragment	Ap^r	-	N. Glansdorff

pMC23	*argF* in pBR322	Ap^r	-	Crabeel *et al*, 1979
pBE51	cloned *traJ* amber mutant	Tc^r	-	Kennedy *et al*, 1977
pCC1	11.7Kb EcoR1 fragment from Cut1 in pUC9	Ap^r	14.4	Chapter 3
pCC5	as pCC1, but opposite orientation	Ap^r	14.4	Chapter 3
pCC15	9.4Kb BglII fragment from Cut1 in pUC8	Ap^r	12.1	Chapter 3
pCC16	as pCC15, but opposite orientation	Ap^r	12.1	Chapter 3
pCC7	2.3Kb BamH1 fragment from pCC1 in pCB101	Cm^r	7.3	Chapter 6
pCC11 pCC12 pCC13	plasmids obtained from Cut1 pCC7 by P1 transduction	Cm^r	19.0	Chapter 6
pCC14	IS1 EcoR1/BglII fragment from pCC11 in pUC8	Ap^r	3.6	Chapter 6
pCC21	clone isolated from gene-bank in pBR322	Ap^r	10.8	Chapter 6
pCC18	2.7kb HindIII fragment from pCC21 in pCB101 derivative	Cm^r	7.0	Chapter 6

Table 2.3 Bacteriophages

'phage	Relevant characteristics	Source
P1	used in P1 transductions	A.P. Jessop
λvir	mutant in O_L and O_R. grows on lambda lysogens.	D.J. Sherratt
λcI	produces no cI repressor. cannot grow on a lambda lysogen.	D.J. Sherratt

Arginine,Histidine,Methionine,Threonine : 100ug./ml.

Uracil : 50ug./ml.

Proline,Leucine : 40ug./ml.

Thymine : 120ug./ml.

glucose : 2,500ug./ml.

lactose : 2,500ug./ml

xylose : 2,500ug./ml.

Soft Agar : 6g. agar in 1 litre distilled water.

R Agar : 12g agar, 10g tryptone, 1g yeast extract, 8g NaCl made up to 1 litre with distilled water.

All growth media were sterilized by autoclaving at 121^{o}C for 15 mins.

2.6 Buffers and Other Solutions

(a). General Buffers

'Phage Buffer : 7g. Na_2HPO_4, 3g. KH_2PO_4, 5g.NaCl, 0.25g. $MgSO_4.7H_2O$, 15mg. $CaCl_2.2H_2O$, 1ml. 1% gelatin solution made up to 1 litre with distilled water.

Calcium Chloride Solution : 50mM $CaCl_2.2H_2O$ in distilled water.

Magnesium Sulphate Solution : 100mM $MgSO_4.7H_2O$ in distilled water.

(b). Electrophoresis

Buffer E$^-$: 40mM Tris HCl, 20mM NaAc, 1mM EDTA.$2H_2O$, pH adjusted to 8.2 with glacial acetic acid.

1xTBE : 10.8g. Tris HCl, 5.5g. Boric acid, 4ml. 0.5M $Na_2EDTA.2H_2O$ made up to 1 litre with distilled water, pH8.3.

Tris/glycine Running Buffer for Protein Gels : 14.41g. glycine, 3.03g. Tris HCl, 1g. SDS made up to 1 litre with distilled water.

DNA Final Sample Buffer (FSB) : 10% Ficoll, 0.5% SDS, 0.05% Bromophenol blue, 0.06% Orange G in buffer E.

Single Colony Lysis/Gel Buffer : 2% Ficoll, 1% SDS, 0.01% Bromophenol blue, 0.05% Orange G in buffer E.

Protein Final Sample Buffer : 50% glycerol, 5% SDS, 5% B-mercaptoethanol (added fresh before use), 0.01% Bromophenol blue in 50mM Tris HCl, pH6.8.

Upper Buffer for SDS-PAGE Gel (4X) : 0.5M Tris HCl, pH6.8, 0.4% SDS.

Lower Buffer for SDS-PAGE Gel (4X) : 1.5M Tris HCl, pH8.8, 0.4% SDS.

10X MOPS Buffer : 0.2M Morpholinopropanesulphonic acid, 0.05M NaAc, 0.01M Na_2EDTA pH7.0. (Stored in a dark bottle).

(c). DNA Manipulation

1xTE : 10mM Tris HCl, 1mM $Na_2EDTA.2H_2O$, pH 7.6 unless otherwise stated.

Gelatin in TE : 100ug. gelatin/ml. of TE.

Lytic Mix : 1% Triton X-100, 50mM Tris HCl, 60mM $Na_2EDTA.2H_2O$, pH adjusted to 8.0 with HCl.

10X Low salt buffer : 100mM Tris HCl, pH7.4, 100mM $MgSO_4.7H_2O$, 10mM

DTT.

10X Medium salt buffer : 500mM NaCl, 100mM Tris HCl, pH7.4, 100mM $MgSO_4.7H_2O$, 10mM DTT.

10X High salt buffer : 1M NaCl, 500mM Tris HCl, pH7.4, 100mM $MgSO_4.7H_2O$, 10mM DTT.

10X Sma I Buffer : 200mM KCl, 100mM Tris HCl, pH8.0, 100mM $MgSO_4.7H_2O$, 1omM DTT.

10X Ligation Buffer : 660mM Tris HCl,pH7.5, 100mM MgCl, 10mM $Na_2EDTA.2H_2O$, 10mM DTT.

STET Buffer : 50mM Tris pH8.0, 50mM Na_2EDTA pH8.0, 5% Triton X-100, 8% sucrose.

10 x T4 DNA polymerase buffer : 0.33M Tris acetate (pH7.9), 0.66M Potassium acetate, 0.66M magnesium acetate, 5.0M dithiothreitol, 1mg/ml bovine serum albumin (Pentax fraction V).

(d). DNA and RNA Hybridization

20X SSC : 3M NaCl, 0.3M NaCitrate, pH7.0.

20X SSPE : 3.6M NaCl, 0.2M NaH_2PO_4/Na_2HPO_4, 20mM Na_2EDTA, pH7.0.

50X Denhardts' : 1% Ficoll, 1% Polyvinylpyrrolidone, 1% Bovine serum albumin.

Pall Denaturing Buffer : 2.5M NaCl, 0.5M NaOH.

Pall Neutralizing Buffer : 3M Na Ac, pH5.5.

Pall Wash Buffer : 5mM Na Phosphate, pH7.0, 1mM EDTA, 0.2% SDS.

Pall High Stringency Wash Buffer : 5mM Na Phosphate,pH7.0, 1mM EDTA, 0.1% SDS, 0.1X SSC.

All the above solutions were sterilized by filtration or by autoclaving at 108°C for 10mins, except the $CaCl_2$ and $MgSO_4$ solutions which were autoclaved at 114°C for 10 mins.

2.7. Growth Conditions

Liquid cultures for transformations, matings, plasmid and chromosomal DNA preparations were grown up from a single colony inoculum, taken from a fresh plate, with shaking at 37°C. The medium used was either L broth or minimal medium with added supplements according to the strain in use and the experiment being performed. Additional thymine was added to L broth when a thymine-requiring strain was being used.

Stationary phase overnight cultures were grown up at 37°C from a single colony without shaking.

Growth of cells on plates was on L or minimal agar with supplements or antibiotics according to the strain in use. Plates contained about 25ml L agar or 40ml minimal agar. Plates were incubated for 12-18 hours (L agar) or 24-96 hours (minimal agar) at 37°C unless otherwise stated. Dilution and washing of cells were carried out in 'Phage buffer.

Bacterial strains were stored on L agar slopes at room temperature or frozen in the following way : 0.75ml of a stationary phase overnight culture was mixed with 0.75ml of a 40% glycerol, 2% peptone solution

Table 2.4. Antibiotic selections

Antibiotic	**Selective concentration**	**Stock solution**
Ampicillin	50ug/ml	10mg/ml in water
Chloramphenicol	50ug/ml	10mg/ml in EtOH
Streptomycin	250ug/ml	100mg/ml in water (made fresh)
Tetracycline	10ug/ml	1mg/ml in water (made fresh)
Kanamycin	20ug/ml	2mg/ml in water

and the mixture stored at -20°C.

2.8. Antibiotic Selections

The standard concentrations listed in Table 2.4 were used throughout for both liquid and plate selections. Agar solutions were cooled to 50°C before adding the antibiotic solution(s).

2.9. Indicators

X-gal(5-bromo, 4-chloro, 3-indolyl B-D galactosidase) : This gratuitous substance was used in conjunction with the ΔM15 host strain and pUC vectors. When the polylinker region of a pUC plasmid is not interrupted by the presence of a cloned fragment, complementation occurs between the plasmid encoded partial B-galactosidase polypeptide and the partially deleted chromosomal locus to produce a functional polypeptide. This hydrolyses the colourless X-gal and results in blue colouration of colonies possessing pUC vectors without cloned inserts. Consequently, this provides a convenient screening for plasmids possessing cloned inserts in the polylinker region since these colonies are white.

IPTG (Isopropyl B-D thiogalactopyranoside) : This compound is a gratuitous inducer of the *lac* promoter (and the related hybrid *tac* promoter, de Boer *et al*, 1983) and its inclusion in a growth medium ensures that these promoters are fully induced.

X-gal and IPTG were freshly made up at a concentration of 20mg./ml. in methanol and distilled water respectfully. In L agar plates X-gal was used at a concentration of 40ug./ml. and IPTG at a concentration of 20ug./ml.

Crystal Violet : Crystal violet was made up at 4mg./ml. in distilled water and used at 1.8ug./ml in minimal agar plates.

2.10. Plasmid DNA Isolation

Plasmid DNA for all *in vitro* manipulations including nick translations was prepared by the cleared lysate/ CsCl method. Small amounts of plasmid DNA suitable for restriction analysis was prepared by the STET method.

(i). Cleared lysate/ CsCl Method.

100ml of L broth plus appropriate antibiotic(s) was inoculated from a single colony of the plasmid containing strain and grown up overnight (about 16 hours) with shaking. The cells were harvested by centrifugation (12,000g,2mins,4°C) and resuspended in 3.3ml. of an ice-cold 25% sucrose solution in 0.25M Tris HCl, pH8.0. 0.67ml. of a freshly made 10-20mg./ml. lysozyme solution in 0.25M Tris HCl,pH8.0 was thoroughly mixed in and the solution swirled on ice for 5 mins. 1.3ml. of 0.25M $Na_2EDTA.2H_2O$, pH8.0 was added, mixed in, and the solution swirled on ice for a further 5 mins. 5.3ml. of lytic mix was gently added, mixed in, and the mixture incubated at room temperature for 15-30 mins to form a "crude lysate". The cell debris was pelleted (48,000g,45mins,4°C) and the supernatant (the "cleared lysate") carefully decanted. 7ml of this solution was added to 7.50g of CsCl and the CsCl allowed to dissolve. 0.5ml. of a 3mg./ml. solution of ethidium bromide in TE was added, the density of the resultant solution checked and (if neccessary) adjusted to 1.58-1.60g./ml. The solution was poured into a "Quick-Seal" ultracentrifuge tube, the tube filled with paraffin oil and sealed. After ultracentrifugation (200,000g,16hours,15°C) in an angled rotor), the DNA bands were

visualised under long wavelength ultra-violet light (300-360nm) and the lower band (containing the covalently closed, circular plasmid DNA) removed through the side of the tube with a 21 guage needle and syringe. Three volumes of TE buffer were added to this material and the EtBr removed by repeated butanol extraction until the butanol layer was colourless. The DNA was then precipitated with two volumes of ethanol and resuspended in 200ul of TE buffer.

The DNA concentration was estimated by running an aliquot on an agarose gel.

(ii). STET Method

Cells were either harvested by centrifugation (12,000g,2mins,4^{o}C) from 1.5ml. overnight culture or an equivalent mass of cells was scraped from a fresh plate with a toothpick. The cells were thoroughly resuspended in 350ul STET buffer in a large microfuge tube and 25ul 10mg./ml. lysozyme in sterile distilled water was added and mixed in gently. The cells were left for 10mins at either room temperature or 37^{o}C (some strains were difficult to lyse and the 37^{o}C incubation aided lysis). The partially lysed cells were then boiled for 2mins in a water bath, the cell debris pelleted by centrifugation (12,000g,15mins,4^{o}C) and the cell pellet removed with a toothpick and discarded. 1/10 volume of NaAc and 0.6 volume ice-cold isopropanol were added to precipitate the DNA, which was then recovered by centrifugation (12,000g,15mins,4^{o}C). The DNA pellet was carefully washed twice with 70% EtOH in TE and dried. The DNA was resuspended in 20ul TE buffer and 5ul used for restriction analysis.

2.11 Chromosomal DNA Isolation

100ml. of minimal broth plus appropriate supplements was inoculated from a single colony and grown up overnight (about 16 hours) with shaking in a 250ml flask. The cells were harvested by centrifugation (12,000g,2mins,4°C) and resuspended in 3.3ml of an ice-cold 25% sucrose in 0.25M Tris HCl, pH8.0. 0.67ml of a freshly made 5mg/ml lysozyme solution in 0.25M Tris HCl, pH8.0 was added and the mixture swirled on ice for 5mins. 1.3ml of 0.25M Na_2EDTA, pH8.0 was added and the mixture swirled on ice for a further 5mins. 5.3ml of lytic mix was slowly added and gently mixed in and the solution left at room temperature for 15-30mins for lysis to occur. The solution was then incubated with 50ug/ml RNase (made in distilled water and boiled for 5mins before use) at room temperature for 30-45mins or 37°C for 15-20mins, followed by incubation with 50ug/ml proteinase K (made in distilled water) for 20-30mins at 37°C. The proteinase K step was essential to reduce the viscosity of the lysate. The lysate was then phenol extracted three times, or until the mixture became clear at the interphase. To pipette the lysate without shearing the DNA, the end (0.5cm) was cut from all Finpipette tips before use, and the lysate/phenol mixture was inverted gently (never vortexed). Often the phenol extractions were followed by a final extraction with a 1:1 mixture of phenol and chloroform. The lysate was then extracted with chloroform two or three times and the DNA recovered by ethanol precipitation.

The DNA concentration was estimated routinely by running an aliquot on an agarose gel. When accurate quantitation of DNA was required, spectrophotometric determination was used. Readings were taken at wavelengths of 260nm and 280nm. The reading at 260nm was used to calculate the concentration of nucleic acid in the sample. (an OD of 1 corresponds to approximately 50ug per ml of double stranded DNA - Maniatis *et al*, 1982). The ratio between the readings at 260nm and 280nm (OD_{260}/OD_{280}) provided an estimate of the purity of the DNA.

All DNA preparations had OD^{260}/ OD_{280} of between 1.8 and 2.0, indicating that the DNA preparation was reasonably pure.

2.12 Phenol extraction; Precipitation of nucleic acids

(i). Phenol extraction : An equal volume of distilled or salt treated phenol (saturated with 1M Tris HCl, pH8.0) was added to the solution to be extracted, thoroughly mixed in and the two phases separated by centrifugation (12,000g,5mins,4°C). The aqueous phase was carefully removed and the extraction repeated once or twice more. The final aqueous phase was extracted two or three times with an equal volume of chloroform to remove all traces of phenol.

(ii). Precipitation of nucleic acids : This was performed with either isopropanol or ethanol. Unless there was a volume constraint ethanol was used as there is less co-precipitation of non-nucleic acid materials (e.g. salt, sugars) with ethanol and it is also more volatile and therefore more easily removed (Maniatis et al, 1982).

a). With Ethanol : 0.1 volumes of 3M NaAc and two volumes of ethanol were added to the solution to be precipitated, mixed in and the mixture left at -20°C for 20-120 mins.

b). With Isopropanol : 0.1 volumes of 3M NaAC and 0.6 volumes of isopropanol were added, mixed in and the mixture left at room temperature for 20-120 mins.

In either case, if only a small amount of DNA was being precipitated, 0.01 volumes of a 1mg/ml solution of yeast tRNA in TE was also added to aid in the formation of a precipitate.

The precipitated material was spun down (12,000g,25mins,4°C) and

carefully washed in 1ml of 70% EtOH in TE without disturbing the pellet. The pellet was dried at 37°C and resuspended in an appropriate volume of TE buffer.

2.13 RNA isolation by hot phenol extraction

20ml minimal broth with appropriate supplements was inoculated with 0.1ml of an overnight culture and grown to mid-log phase ($O.D_{550}$=0.3). The cells were harvested by centrifugation (12,000g,2mins,4°C), resuspended in ice-cold M9 buffer, reharvested and resuspended in 20ml 0.02M Na Acetate, 0.5% SDS, 1mM Na_2EDTA, pH5.5 (ASE buffer). 40ml hot phenol (saturated with ASE buffer,60°C) was added, mixed by gentle vortexing and shaken in a 60°C water bath for 5 mins. The phenol and aqueous phases were separated by centrifugation (12,000g,2mins,20°C) and the aqueous layer removed. The phenol extraction was repeated, followed by two chloroform extractions. The solution was then adjusted to 0.1M KCl by the addition of 4M KCl and the RNA precipitated with two volumes of EtOH. After at least three hours at -20°C the RNA was pelleted by centrifugation (12,000g,30mins,4°C), rinsed briefly in 70% EtOH in TE, dried and resuspended in distilled water. The RNA was then precipitated by the addition of three volumes Na Acetate, pH6.0 and kept at -20°C for one hour before pelleting the RNA by centrifugation (12,000g, 15mins, 4°C). The salt precipitation step was repeated and the RNA finally resuspended in 10ul distilled water and kept at -20°C.

Treatment of materials used for RNA extraction

Sterile, disposable plasticware was essentially free of RNase and required no pretreatment.

All glassware was baked at 250°C for 16 hours

All solutions were prepared using baked glassware, distilled and autoclaved water.

Where possible solutions were treated with 0.1% diethylpyrocarbonate for at least 12 hours and autoclaved prior to use.

2.14 Gel Electrophoresis

(i). Standard Vertical Agarose Gels

Vertical gel kits were used which held two 16.5 X 15.5cm glass plates 0.3cm apart. Agarose was dissolved in buffer E at 100°C and cooled to 55°C before being poured between the glass plates to fill them. A 10 or 15- toothed "Teflon" comb was quickly inserted into the top of the gel and, once the gel had set, this was removed and the bottom and top reservoirs of the gel apparatus filled with sufficient E buffer to cover the gel. Single colony gel supernatants were loaded directly onto the gel, whilst marker DNAs and restriction digests were made up to 20ul with TE buffer (if neccessary) and mixed with 4ul of DNA final sample buffer before loading. Gels were run at constant voltage - 2volts/cm for about 15 hours or 6 volts/cm for 5 hours - at room temperature and were stained by soaking in an EtBr solution (0.5ug/ml in buffer E) for 15-20 mins. They were viewed on a 260nm ultra-violet transilluminator and photographed on ilford HP-5 film using a 35mm camera fitted with a red filter.

0.8% agarose was used for most purposes: single colony gels, intact plasmid DNAs and analysis of chromosomal and plasmid restriction enzyme digests. 1% agarose was used when small (<5kb) fragments were being separated, and 0.6% agarose when very large fragments (> 9kb) were being separated.

(ii). Low Melting Point Agarose Gels

These gels were used to isolate small particular fragments from a restriction enzyme digest for use in a cloning procedure. A horizontal gel system was used for this type of agarose as it is considerably less robust than general purpose agarose. The agarose concentrations used were 0.8- 1.0%.

Agarose was dissolved in buffer E at 100^{o}C and cooled to 37^{o}C. A 1.5mm thick comb was suspended over a plastic tray (19 X 11.5 X 0.4cm) so that there was a gap of 0.5mm between the bottom of the teeth and the floor of the tray. The agarose was poured in, allowed to set, the comb removed and the gel immersed in buffer E. The samples (15-25ul) were loaded and the gel run at 2volts/cm for 15 hours at room temperature. Staining was as described for standard agarose gels above.

(iii). Horizontal Gels.

A horizontal gel system was used for 0.6% and 0.4% agarose gels because these are much less robust than higher percentage agarose gels. These were made by pouring 100ml of molten agarose into a 11 x 19 cm perspex gel former with a 13-space Teflon comb. After the gel had solidified the wells were wetted with E buffer, the comb removed and the gel transferred to a gel tank with 800-900ml buffer E. Gels were usually run at 5 volts/cm for approximately 3 hours. Staining was as described above for standard vertical gels.

(iii). Standard Polyacrylamide Gels (non-denaturing)

These gels were used to separate restriction fragments less than 1kb in size. The acrylamide concentration could be varied according to the sizes of the fragments being analysed.

Vertical gels were again used, 16.5 X15.5cm with a 13-tooth plastic

comb. The gel was first sealed using 0.8% agarose in TBE and the acrylamide prepared by mixing the following:

	5%	3.5%
distilled water	50ml	42.5ml
10 X TBE	8ml	6ml
Acrylamide	20ml	10.5ml
10% APS	0.96ml	0.72ml
10% TEMED	0.48ml	0.36ml

Stock acrylamide was 20% acrylamide/ 1% bis-methylene acrylamide.

5% acrylamide gels gave good separation over a size range of 80-500bp. 3.5% acrylamide gels gave good separation over a size range of 100-1000bp.

The acrylamide solution was poured between the gel plates, the comb quickly inserted and the gel allowed to polymerise for at least one hour at room temperature. The comb was removed, the top and bottom reservoirs filled with TBE buffer, the wells washed out to remove any unpolymerised acrylamide, the samples (15-25ul) loaded and the gel run at a constant current of 20mA for about 3 hours at room temperature. Staining and photography were as described above for agarose gels.

(iv). SDS-Polyacrylamide gels for analysis of protein samples

The gel system used was a two part SDS-polyacrylamide gel, a modification of that of Laemmli (1970). The upper part was a short stacking gel (acrylamide concentration = 4.5%) and the lower part was a longer separating gel (acrylamide concentration = 10%). The gel was

poured in the same apparatus as used for standard polyacrylamide electrophoresis of DNA samples and was poured in two parts :

a). Separating gel : 10ml of 4X lower gel buffer was mixed with 16.6ml distilled water, 13.4ml 30% acrylamide/ 0.8% N N' bis-methylene acrylamide, 20ul TEMED and 120ul 10% APS and the solution poured between the gel plates to a level 1cm below the bottom edges of the teeth of the comb. The top of the gel was immediately sprayed with a 0.1% SDS solution to stop bubbles forming on it and the gel allowed to polymerise completely. The SDS solution was drained off.

b). Stacking gel : 2.5ml of 4X upper gel buffer was mixed with 6.0ml distilled water, 1.5ml 30% acrylamide/ 0.8% N N' bis-methylene acrylamide, 20ul TEMED and 30ul 10% APS and the solution poured on top of the lower gel, ensuring there were no bubbles at the interphase. The comb was inserted quickly, the gel allowed to polymerise and the comb removed. The gel was installed into the running apparatus, the reservoirs filled with protein gel Tris/glycine running buffer, the wells washed out with buffer to remove any unpolymerised acrylamide and the samples (10-30ul) loaded. The gel was run at a constant current of 25mA for 3 hours or until the blue dye reached the bottom of the gel. The gel plates were carefully separated, the stacking gel portion discarded and the separating gel portion fixed and stained in a solution of 0.1% coomassie brilliant blue, 50% methanol, 10% acetone for at least 60mins with gentle shaking. The stain was then replaced with a solution of 10% methanol, 10% acetone and the gel was destained for up to two days, with at least one change of destain solution, before photographing.

The molecular weight marker proteins used were purchased from Sigma and contained the following proteins:

Protein	Approx. Mol.Wt.
Albumin, Bovine	66,000
Albumin, egg	45,000
Glyceraldehyde-3-phosphate dehydrogenase	36,000
Carbonic anhydrase	29,000
Trypsinogen, bovine	24,000
Trypsin inhibitor	20,100
Lactalbumin	14,200

(v). Formaldehyde gels for electrophoresis of RNA

1.5g or 1.0g agarose was added to 73ml of distilled water, dissolved at 100°C and cooled to 60°C. 10ml 10X MOPS buffer and 16.2ml 37% formaldehyde were added and the gel poured immediately onto a glass plate whose edges were bound with plastic tape. A 10-space plastic comb was inserted and the gel allowed to set completely. The gel was then transferred to a horizontal gel tank, the reservoirs were filled with 1X MOPS buffer, and the samples loaded. The gel was run at 30 volts overnight (about 16 hours) or 100 volts for 4 hours and a sample track removed and stained with acridine orange (1mg/ml).

(vi). Interpretation of restriction data

The size of linear restriction fragments was calculated using the relationship :

$$\log M = c \times 1/D$$

where M = molecular size in base pairs, D = distance migrated on gel, and c = a constant.
Lambda Hind III restriction fragments were used as molecular size standards and the sizes of restriction fragments were determined from a plot of distance D against logM.

2.15 Single colony gel analysis for plasmid DNA

A single colony was streaked out onto a selective plate and grown overnight. Using a toothpick, a mass of cells (about 0.6cm^2 from an area of confluent growth) was removed from the plate and thoroughly resuspended in 200ul of single colony lysis buffer in a small (0.6ml) microfuge tube. The cells were left to lyse for 15-30 mins at room

temperature, the cell debris pelleted by centrifugation (12000g,15mins,4°C) and 50ul of the supernatant loaded onto an agarose gel.

2.16 Extraction of DNA from gels

(i). Low melting point agarose gels.

After staining, the gel was illuminated using long wavelength (300-360nm) ultra-violet light and the gel slice containing the fragment of interest excised. This slice was melted at 65°C and diluted by adding two volumes of buffer E, also at 65°C. After mixing by inversion the solution was cooled to 37°C, phenol extracted three times, chloroform extracted twice, the DNA recovered by ethanol precipitation and resuspended in a small volume of TE.

(ii). Agarose gels.

After staining, the gel was viewed using long wavelength ultra-violet light and the gel slice containing the fragment of interest excised. This slice was placed inside a piece of dialysis tubing (which had been previously boiled in distilled water for 15-20mins) with 250-500ul of E buffer, the ends sealed with dialysis clips and the whole sac placed in a horizontal gel box and covered with buffer E. Electrophoresis was carried out at 50 volts for three hours and at the end of this period the current was reversed for one minute and then reversed again for 30 secs. The fluid from the sac was removed, the DNA recovered by ethanol precipitation and resuspended in TE buffer. Fragments greater than 9kb : TBE buffer was used instead of buffer E and electrophoresis was for 16 hours instead of 3 hours. The TBE buffer covering the sac was replaced once during electrophoresis.

2.17 Genetic transformation

All plasmids, other than F-primes, were introduced into different host strains by transformation.

An overnight culture of the recipient strain was diluted 1 in 100 in 20ml L broth and grown with shaking to a density of 2 X 10^8. The cells were harvested by centrifugation (12000g, 2mins,4°C), resuspended in 10ml ice-cold $CaCl_2$ solution, reharvested and resuspended in 400ul ice-cold $CaCl_2$ solution. 200ul aliquots of this suspension were dispensed into large (1.3ml) microfuge tubes. The transforming DNA (0.1ug in 1-60ul of TE) was added and the tubes kept on ice for 15 mins. A heat shock was then carried out (42°C water bath for 2 mins) and the cells returned to ice for several minutes. 1ml of L broth was added to each tube and the cells incubated at 37°C for 45-90 mins to allow expression of the plasmid genes. 100-200ul aliquots of the transformation mixture were spread onto appropriate selective plates and incubated as appropriate.

chloramphenicol selection : cells were expressed for 90mins and 200ul aliquots were plated. Plates were incubated for 24-72 hours, as appropriate

ampicillin selection : cells were expressed for 45 mins and 100ul aliquots plated. Plates were incubated for no longer than 18 hours as ampicillin-sensitive satellite colonies usually began to appear at this time.

The presence of plasmid DNA in transformed cells was checked by single colony gel analysis.

2.18 Restriction enzyme digestion

1-10ul of DNA in TE was mixed with gelatin in TE to give a total volume of 17ul. 2ul of the appropriate 10X restriction buffer was

added. Restriction enzyme (2units of enzyme per ug plasmid DNA, 5 units per ug chromosomal DNA) was added and the solution incubated at 37°C (30°C for smaI digests) for 1-3 hours. If the sample was to be run on a gel the reaction was terminated by adding 4ul FSB. If the DNA was to be subjected to further manipulations the reaction was terminated by phenol extraction twice, chloroform extraction twice and the fragments recovered by ethanol precipitation.

The restriction buffers used were :

(a). Low salt : Bgl II, HaeII, HpaI, KpnI, SstI, XmaI

(b). Medium salt : BamHI, HindIII, AccI, AluI, HincII, PstI, Sau3A

(c). High salt : EcoR1, PvuI, SalI, XbaI

SmaI was used in its own particular buffer. ClaI and SphI were used in core buffer (supplied by the enzyme manufacturer).

For multiple digests requiring different buffers, the lowest salt buffer was used first and restriction carried out for 1 hour. The salt concentration was then adjusted by adding an appropriate volume of a higher salt buffer and the DNA incubated with the other enzyme(s).

2.19 Calf-intestinal alkaline phosphatase (CIP) treatment

If it was not possible to select or screen for the insertion of a DNA fragment into a plasmid, CIP was used to remove the 5'phosphate groups from the linearised vector to prevent its recircularisation. This was done by adding 1.5 units CIP per ug of DNA to the restriction digest mix at the end of the required restriction period, mixing thoroughly and incubating for a further 10-15mins at 37°C. This procedure was effective in all restriction buffers used. The reaction was terminated by phenol extraction twice, chloroform extraction twice followed by ethanol precipitation to recover the DNA fragments.

2.20 Removal of 3' protruding ends with T4 DNA polymerase

Restriction fragments with 3' protruding ends were made blunt ended by using the 3' exonuclease activity of T4 DNA polymerase. In the presence of all four dNTPs, T4 DNA polymerase will remove unpaired 3' tails from restriction fragments and will stop when it reaches the first paired base if the appropriate complementary dNTP is present. The following were added to a small microfuge tube:

restriction fragment	(up to 0.5ug in 10ul)
10 x T4 polymerase buffer	2ul
H_2O	to 19ul
2mM solution all four dNTPs	1ul

Various amounts of T4 DNA polymerase were added (from 1-10 units) and the reactions incubated for 5 mins at 37°C. 1ul 0.5M EDTA was added and the mixtures extracted once with phenol and chloroform. Following ethanol precipitation and centrifugation, the pellets were washed in 70% EtOH in TE and dissolved in TE. Ligation was then carried out.

2.21 Ligation of DNA fragments

The fragments to be ligated were resuspended together in 16ul of TE buffer and 2ul each of 10X ligation buffer and 4mM ATP (in 4mM Tris HCl,pH7.5) added. T4 DNA ligase was added (0.01 units per ug of DNA for "sticky-ends"; 1 unit per ug DNA for flush ends), all the components mixed and then incubated overnight at 16°C. The ligation mixture was then made up to 200ul by the addition of TE buffer and 20-60ul used to transform competent cells.

2.22 Nick-translation of DNA using ^{32}P

The following were added in order to a small microfuge tube :

10 x Nick translation buffer	5ul
DNA	0.5ug
unlabelled dNTPs	1ul each of a 1mM solution
distilled H_2O	to 40ul

The mixture was chilled on ice, and 0.5ul of DNase added (0.1ug/ml in nick-translation buffer containing 50% glycerol, stored at -20°C). 5 units of E.coli DNA polymerase I were added and the solution mixed by vortexing. 10uCi [α-^{32}P] dATP (1000Ci/mMole) was mixed in and the solution incubated at 16°C + 1°C for 60 mins. The reaction was stopped by the addition of 2ul of 0.5M Na_2EDTA.

Separation of nick-translated DNA from unincorporated dNTPs

The nick-translated DNA was separated from unincorporated dNTPs by using a column of Sephadex G-50 (medium). A 10ml pipette was rinsed with "replicote" and allowed to dry. The tip was blocked with glass wool which had been previously soaked in "replicote" and allowed to dry. The column was filled with Sephadex G-50 in TE, pH8.0 and run through with about 3ml TE buffer, pH8.0. The nick-translation mixture was added to the column together with two marker dyes - phenol red, which runs with the unincorporated dNTPs, and blue dextran, which runs with the nick-translated DNA - and eluted with TE buffer, pH8.0. About 1.5ml "probe" was collected from the column. These conditions usually resulted in incorporation of between 50% and 70% of the [a-^{32}P]dNTP into DNA. The proportion of incorporation was occassionally determined by TCA precipitation assays but this was not done routinely. 0.5ml

nick-translated probe was usually used per hybridization.

2.23 DNA transfer to nylon membrane and detection

(a). Transfer procedures

(i). Southern blotting

Southern transfer was performed by a modification of the procedure of Southern (1979).

After electrophoresis the gel was removed from the apparatus, stained and photographed, then placed in 150ml 0.25M HCl for 25 mins with gentle agitation. The gel was then rinsed briefly in distilled water prior to gentle agitation with 150ml denaturing solution for 30 mins. After rinsing again, the gel was agitated for a further 30-60 mins in 150ml neutralizing solution. Two reservoirs containing 20X SSC were placed side by side, spanned by a glass plate. A sheet of Whatmann 3MM filter paper was placed over the glass plate from one reservoir to the other and any air bubbles formed between the glass plate and the filter paper were expelled using a pipette. The gel was placed on the paper/glass support and flanked by strips of sellotape to ensure that the blotting buffer went through the gel rather than around it. A piece of Pall nylon membrane was placed on top of the gel surface and a clean pipette rolled over the membrane surface to remove trapped air bubbles. Two layers of filter paper were placed on top of the membrane followed by two Boots disposable nappy pads with the outer covers removed. This was covered by a glass plate and a weight of about 1Kg. Transfer was allowed to proceed overnight (about 16 hours) after which the membrane was removed from the gel surface, air dried and baked at 80^{o}C for 1 hour. The gel was restained to check that all the DNA had transferred.

(ii). Colony and patch lifts

Hybond-N nylon membrane (cut to size) was placed on the surface of the colonies or patches to be lifted and a sterile needle was used to make small holes in the membrane for later orientation. After several minutes the membrane was removed and placed colony side up for 5mins on filter paper saturated with denaturing buffer. The membrane was then placed on filter paper saturated with neutralizing solution for 5 mins after which it was air dried and U.V. irradiated for 5 mins on a 260nm transilluminator.

(iii). Dot blots

(b). Prehybridization

The membrane was placed in a plastic bag with 10ml hybridization solution (5X Denhardts solution, 5X SSPE, 0.2% SDS). 1mg sonicated salmon sperm DNA was denatured by the addition of 1/10 volume 1N NaOH and heating at 65°C for 10 mins, then neutralized by the addition of 1/10 volume 1M HCl, and added to the hybridization solution The bag was then heat sealed and placed inside a second bag with a wet paper towel. The bags were incubated in a 65°C water bath for 1-2 hours with gentle agitation.

(c). Hybridization

The bags were opened and the mixture replaced with fresh hybridization solution. 0.5ml nick-translated probe was added to the salmon sperm DNA and was denatured and neutralized as above, then immediately added to the hybridization solution. The double bag was resealed and incubated at 65°C overnight (about 16 hours) with gentle agitation.

(d). Washing membranes

After hybridization the membrane was rinsed briefly in low stringency wash buffer, then sealed in a plastic bag with about 250ml of the same buffer and agitated vigorously for at least 30 mins. This was repeated if only a low stringency wash was required. If a high stringency wash was required the buffer was replaced with high stringency wash buffer and agitated vigorously at 65^{o}C for 20-25 mins. The membrane was then sealed in a fresh plastic bag.

When Hybond-N membrane was used the following changes were made to the above protocols :

Hybridization solution contained 6X SSC, 5X Denhardts solution, 10% SDS.

The membrane was washed twice for 15mins in 2X SSC at 65^{o}C and once for 30 mins in 2X SSC containing 0.1% SDS at 65^{o}C. If a higher stringency wash was required the membrane was additionally washed in 0.1X SSC at 65^{o}C for 10mins.

(e). Autoradiography

The membrane was exposed to Kodak X-Omat film in a light-proof cassette at -20^{o}C using a single Dupont Cronex lightning-plus intensifying screen. (The film was sandwiched between the membrane and the intensifying screen). For quantitative analysis of dot blots and dilution blots the film was pre-flashed and the flashed side placed against the intensifying screen with the membrane on top.

2.24 RNA blotting and detection procedures

(a). Transfer of RNA to Hybond nylon membranes

After electrophoresis in a denaturing gel system transfer of RNA to Hybond-N nylon membrane was set up as for DNA except that the gel was

not treated with any solutions prior to blotting. The gel was blotted for at least 12 hours, air dried and irradiated with U.V. light on a transilluminator to crosslink the RNA (as recommended by the supplier of the membrane).

(b). prehybridization

The membrane was prehybridized in a sealed plastic bag containing 5ml/cm^2 5xSSPE, 50% formamide, 5x Denhardts solution, 10% dextran sulphate and 20ug/ml denatured salmon sperm DNA at 42°C for at least 4 hours.

(c). Hybridization

The hybridization solution was replaced with fresh hybridization solution with the addition of 0.5 ml denatured, nick-translated probe. The bag was resealed and incubated at 42°C with gentle shaking overnight (about 16 hours).

(d). Washing membranes

After hybridization the membrane was incubated in 50ml 5X SSPE for 15 mins at 42°C. This step was repeated. The membrane was then incubated in 50ml 1X SSPE, 0.1% SDS for 30 mins at 42°C. A higher stringency wash was achieved by incubation of the membrane in 0.1X SSPE, 0.1% SDS for 15 mins at room temperature. The membrane was then sealed in a plastic sleeve for autoradiography.

(e). Autoradiography

Autoradiography was as for Southern blots except that when possible

two intensifying screens were used.

(f). Removal of probe and re-use of RNA blots

Membranes were washed for 1-2 hours at 65°C in
0.005M Tris-Hcl, pH8.0
0.002M Na_2 EDTA
0.1 x Denhardts solution
The membrane was then prehybridized and hybridized as described above.

2.25 Preparation of protein samples for gel electrophoresis

Cells from 800ul of a dense overnight culture (minimal medium) were harvested by centrifugation (12000g,2mins,4°C), the supernatant poured off and the cell pellet vortexed to give a thick cell suspension. 80ul of protein final sample buffer was added and the mixture immediately vortexed again for about 10 secs. The mixture was incubated at 100°C for 5-10 mins and 30ul run on a denaturing acrylamide gel. The remainder of the sample was kept at -20°C until required.

2.26 Bacterial matings

(a). Bacterial matings in culture

Overnight cultures of donor and recipient cells (usually in minimal media) were diluted 1 : 100 in L broth and grown with shaking to mid log phase (to a density of about 2-3 x 10^8 cells/ml). Donor and recipient cells were then mixed in a ratio of 1 : 10 in a total volume of 5ml (usually 4.5ml recipient : 0.5ml donor). Aeration during mating was achieved by having a large surface to volume ratio using a 100ml flask and placing the flask on a 33rpm rotor at 37°C (vigorous agitation would disrupt mating pairs). Matings using F-prime donors

were left for 60mins as were matings using Hfr donors to transfer an early chromosomal marker. Hfr donors transferring a late marker were left for 90mins. 100ul aliquots of the mating mixture and several dilutions (usually 1:2,10^{-1}, 10^{-2}) were then spread onto minimal selective plates (containing selection against both the donor and the recipient). Aliquots of both the donor and the recipient cultures were also spread onto the selective plates as controls). The plates were incubated at 37°C for 24-72 hours.

(b). Bacterial mating using replica plating

Freshly growing single colonies of the donor strains were picked and patched onto an L agar plate (with added thymine if required), then incubated at 37°C for three hours. If the replica plating was being used to test donor ability, a known donor was included in the master plate if possible. 10ml L broth was inoculated with 0.1ml of an overnight culture of the recipient strain, grown to log phase and 0.1ml aliquots spread onto two L agar plates. The master plate was then replicated onto one recipient lawn and also onto another fresh L agar plate. The plates were incubated at 37°C overnight (about 16 hours) then all were replicated onto minimal selective plates. The plates of donor and recipient cells alone served as controls to ensure selection against both the donors and the recipient. The plates were incubated at 37°C for 48 hours.

Production of F^- phenocopies

In any population of Hfr, F' or F^+ strains a small percentage of cells have lost both F pili and also F-determined surface components and temporarily behave as recipients. The percentage of phenocopies can be increased by growing the cells under conditions in which the formation of new F-pili and cell surface components is inhibited (Curtiss et al,

1969).

Cells from an overnight culture were harvested by centrifugation (12000g,2mins,4°C) and resuspended in minimal broth containing no glucose or supplements, with the exception of thymine in the case of thymine-requiring strains. The culture was incubated for 4 hours at 37°C without shaking and then treated as a normal recipient in mating experiments.

2.27 Recovery of Citrulline-utilizing mutants from car⁻ strains.

10ml minimal broth plus arginine and uracil (and, of course, any other required supplements) was inoculated with 0.1ml of a 10^{-2} dilution of an overnight culture and grown to mid-log phase (about 2-3 x 10^8 cells/ml). 0.1ml aliquots (10^0, 10^{-1}, 10^{-2} dilutions) were plated onto minimal plates plus citrulline and incubated at 37°C. Plates were scored after 4-7 days.

Cut mutants used for further experiments were purified by streaking for single colonies several times.

2.28 Detection of instability of Cut mutants

A single Cut colony was picked from a citrulline minimal plate and suspended in 0.5ml phage buffer. A 10^{-2} dilution was made from this suspension and 0.1ml used to inoculate 10ml minimal broth plus arginine and uracil (and any other required supplements). This was grown for about 24 hours with shaking to stationary phase and 0.1ml used to inoculate a further 10ml of minimal broth plus arginine and uracil. This was grown to mid-log phase and 0.1ml aliquots of several dilutions plated (usually 10^{-4},10^{-5}) on minimal agar plus arginine and uracil to give single colonies. After about 48 hours these colonies were replicated onto both minimal agar plus arginine and uracil, and minimal agar plus citrulline. Plates were incubated at 37°C and

examined after 24 hours to detect the non citrulline-utilizing segregants. The plates were usually re-incubated for a further 24 hours to check that the segregants were growing, albeit slowly, on the citrulline plates.

2.29 Production of a P1 lysate

10ml L broth was inoculated with 0.1ml of an overnight culture and grown with shaking to late log phase. The cells were harvested by centrifugation (3000g, 10mins, r.t.) and resuspended in L broth containing 0.5mM $CaCl_2$ and 1mM $MgSO_4$. The following were then added to a small capped test tube :

0.1ml $CaCl_2$ solution

0.1ml $MgSO_4$ solution

0.1ml bacterial suspension

0.1ml P1 (diluted 10^{-2} -10^{-8} to give a m.o.i. of 0.1)

The mixture was incubated at 37°C for 25mins then added to 2.5ml soft agar (previously boiled and cooled to 42°C) and quickly poured onto a fresh, very thin R plate. The plates (not inverted) were sealed in a plastic box with wet tissues and a vial of water and incubated at 37°C for about 18 hours. 2.5ml ice-cold phage buffer was added to all plates on which lysis was almost confluent and the plates were incubated at 4°C for 15-30mins. The agar overlay was then scraped off into a glass bottle using a sterile glass spreader and vortexed with a few drops of chloroform for at least 30secs to lyse any remaining bacteria. The mixture was centrifuged (3000g,10mins,r.t.) and the supernatant recovered. If neccessary the centrifugation step was repeated to remove any remaining cell debris. The phage lysate was titred on 903 and routinely contained about 10^9 p.f.u./ml.

2.30 Transduction using P1

A P1 lysate was prepared on the appropriate donor strain. 10ml of L broth was inoculated with 0.1ml of an overnight culture of the recipient strain and grown to late log phase. The cells were harvested by centrifugation (3000g,10mins,r.t.) and resuspended in L broth containing 0.5mM $CaCl_2$ and 1mM $MgSO_4$. The following were added to a small capped test tube and incubated at 37°C for 20 mins:

0.1ml $CaCl_2$
0.1ml $MgSO_4$
0.1ml bacterial suspension
0.1ml phage P1 (at a m.o.i of about 0.1)

0.2ml 1M sodium citrate was added to stop further infection and if necessary the mixture was then incubated with 0.5ml L broth (plus 0.5mM $CaCl_2$ and 1mM $MgSO_4$) for 30 mins at 37°C to allow expression of selective genes. 0.5ml aliquots were then plated with 2.5ml soft agar on selective plates. Control plates of 0.1ml P1 lysate and 0.1ml bacterial suspension alone were also plated. All plates were inverted and incubated at 37°C for 24-72 hours.

2.31 Construction of recA strains

recA strains were constructed by P1 transduction of recA from Clr-207 in which Tn10 is inserted in srl, adjacent to recA. A P1 lysate was made on Clr-207 and used to transduce the required strain. Selection was for tetracycline-resistance (encoded by Tn10) on R plus Tc plates. Single colonies were screened for co-transduction of recA by U.V. sensitivity tests.

2.32 Testing recA phenotype by U.V. sensitivity

The recA phenotype of strains was determined by testing the sensitivity of the strain to ultra-violet light.
Strains to be tested were patched onto two L agar plates using sterile toothpicks (known recA and recA$^+$ strains were always included as controls). The patches were then replicated onto fresh L agar plates. One set of plates (patches and replicas) was not exposed to U.V. light and served as a control. The duplicate set of plates was irradiated with U.V. light for two mins (254nm, 42cm from the source: approx. 120uW cm^{-2}) and all the plates were incubated at 37^{o}C overnight. recA strains characteristically showed little or no growth on the U.V. irradiated plates.

2.33 Isolation of F-prime strains

F-primes were isolated from JEF8 by crossing with a recA recipient, e.g. P678.14, so that homologous recombination between the donor DNA and the recipient chromosome was not possible. Purified F-prime strains were tested for donor ability by crossing them with a F$^-$ recipient (usually by replica plating) and selecting for chromosomal markers (pro$^+$ and lac$^+$) carried by the F-prime. Such markers should be transferred at a high frequency.

2.34 Screening for thy$^-$ mutations with trimethoprim

Trimethoprim depresses the growth of thy$^+$ cells, but not that of thy$^-$ cells, in the presence of high amounts of thymine (Miller,1972). After many generations of growth in thymine and trimethoprim, cultures of E.coli contain predominantly thy$^-$ cells.

0.1ml of an overnight culture was used to inoculate 5ml of minimal medium plus thymine (200ug/ml) and trimethoprim (200ug/ml), and was

grown at 37°C overnight to saturation. This was repeated by subculturing into a further 5ml fresh broth and growing again to saturation. 0.1ml aliquots of diluted culture (10^{-4}, 10^{-5}) were then spread onto L agar plates supplemented with thymine (200ug/ml) and grown overnight at 37°C. The colonies were patched onto L plus thymine plates and, once grown, replicated onto minimal agar plates with and without thymine to identify thy⁻ patches. If no thy⁻ colonies were obtained, the original culture (kept at 4°C) was subcultured into fresh broth and grown to saturation several times before the plating was repeated.

CHAPTER 3.

Determination of the structure of the rearrangement and cloning of the amplified unit

The specific activity of OTCase is extremely high in US Cut mutants (Legrain et al, 1976b). Analysis of the OTCase isoenzyme pattern of four randomly chosen US Cut mutants (Legrain et al,1976b) indicated that altered expression of the argF gene, but not argI, was responsible, and transduction and conjugation data (Jessop and Glansdorff,1980; Legrain et al,1976b) suggested that the US Cut mutants contain an aberration in the region of the argF gene. Therefore it was decided to examine this region for a rearrangement associated with the US Cut phenotype.

The plasmid pMC23 (Crabeel et al, 1979) contains the argF gene and approximately 0.7kb of adjacent chromosomal DNA (Fig.3.1). If this plasmid is used to probe Southern blots of restricted chromosomal DNA, it should be possible to detect any major rearrangement involving the argF gene or adjacent chromosomal restriction fragments which hybridize to the plasmid. However, if the rearrangement does not directly involve the argF gene or adjacent restriction fragments which hybridize to pMC23 it will not be detected by this method. Similarly, those rearrangements which involve this region but which do not result in a noticably different restriction pattern will also be undetected using this approach.

The strains used in this chapter are JEF8, an HfrP4X derivative; various US Cut mutants (isolated from JEF8); two F^- strains, P678.14 and D7-8; and Cut12, a stable Cut mutant (approximately 50% of Cut mutants isolated from JEF8 are stable).

RESULTS

3.1 Recognition of amplified DNA in the US Cut mutants

Chromosomal DNA was extracted from 8 US Cut mutants (previously

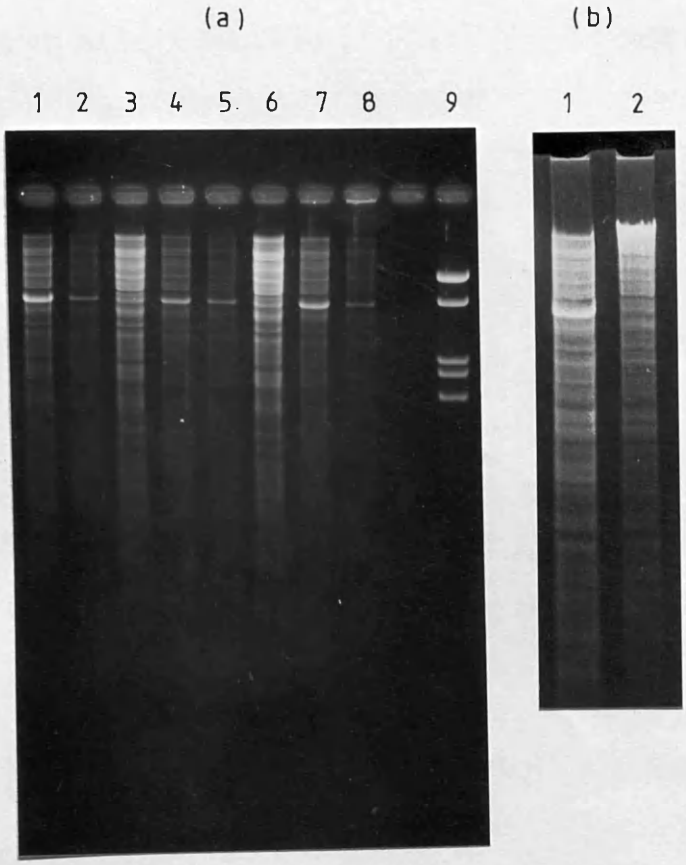


Figure 3.2. (a). 0.7% Agarose gel of EcoR1 restriction digests of chromosomal DNA from JEF8 and US Cut isolates.

Track 1: Cut 1, Track 2: Cut 3, Track 3: JEF8

Track 4: Cut 5, Track 5: Cut 7, Track 6: JEF8

Track 7: Cut 9, Track 8: Cut 11

(b). 0.8% Agarose gel of EcoR1 restriction digests of chromosomal DNA from Cut 1 and a stable Cut isolate, Cut 12.

Track 1: Cut 1, Track 2: Cut 12

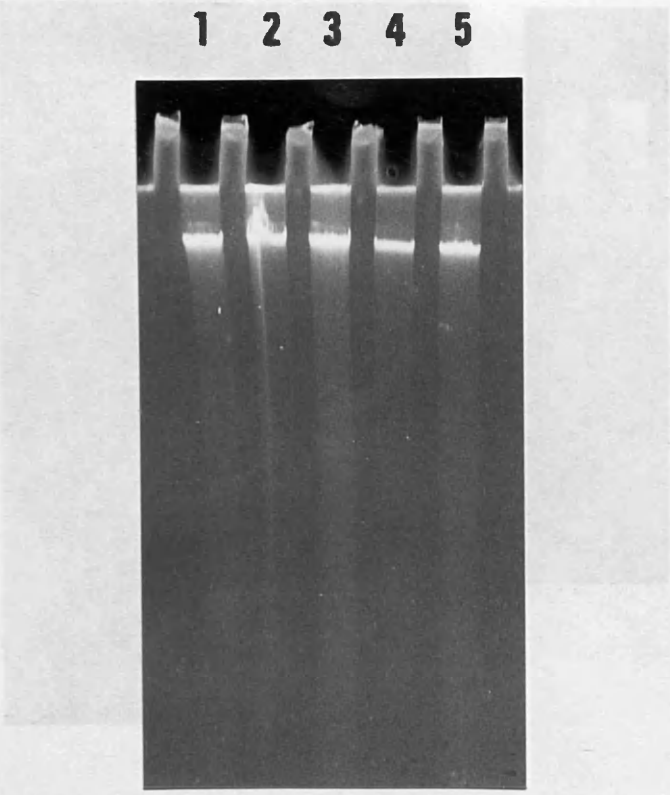


Figure 3.3. 0.8% agarose gel containing unrestricted DNA from five US Cut isolates.

Track 1: Cut 1, Track 2: Cut 3, Track 3: Cut 5,

Track 4: Cut 8, Track 5: Cut 9

isolated from JEF8), JEF8 and Cut12, a stable Cut mutant, for restriction digestion and Southern hybridization.

Restriction enzymes cutting at a 6 base-pair recognition sequence will cut on average once every 4kb with a distribution of fragment sizes that approximates a bell-shaped curve. These fragments are highly suitable for Southern hybridization since good separation is achieved on an 0.8% agarose gel and they are easily blotted onto nylon transfer membrane. Also, fragments of similar size hybridizing to the probe should be well separated and hence their sizes can be easily and accurately calculated. Restriction enzymes cutting at a 4 base-pair recognition sequence will, on the other hand, generate a distribution of fragment sizes that is too small for good separation using standard agarose gel electrophoresis techniques and many of the fragments generated will be less than 1kb in size. Also, the interpretation of an autoradiograph may be easier if several relatively large fragments hybridize to the probe rather than many small fragments. Therefore EcoR1, which recognises a six base-pair sequence, was used to digest equivalent amounts of each of the chromosomal DNAs. These digests were run on an agarose gel (Fig.3.2). The EcoR1 digests of DNA from all the US Cut mutants contain an intense band of approximately 12kb visible above the background of other restriction fragments which is not present in the digest of JEF8 or Cut12, a stable Cut mutant. This intense band may indicate the presence of a 12kb plasmid in the US Cut mutants containing a single EcoR1 site and thus generating a single, intense, 12kb EcoR1 fragment. Alternatively, it may represent a 12kb chromosomal fragment present in multiple copies in the US Cut mutants. To distinguish between these two possibilities undigested DNA from several US Cut isolates was run on an agarose gel (Fig.3.3). No intense bands are seen indicating that the intense band in the chromosomal digests is not due to the presence of plasmid DNA in the US Cut isolates. Furthermore, single colony gel analysis of Cut 1 does not reveal the presence of any plasmid DNA in the strain. This suggests that the chromosome of the US Cut mutants contains a fragment

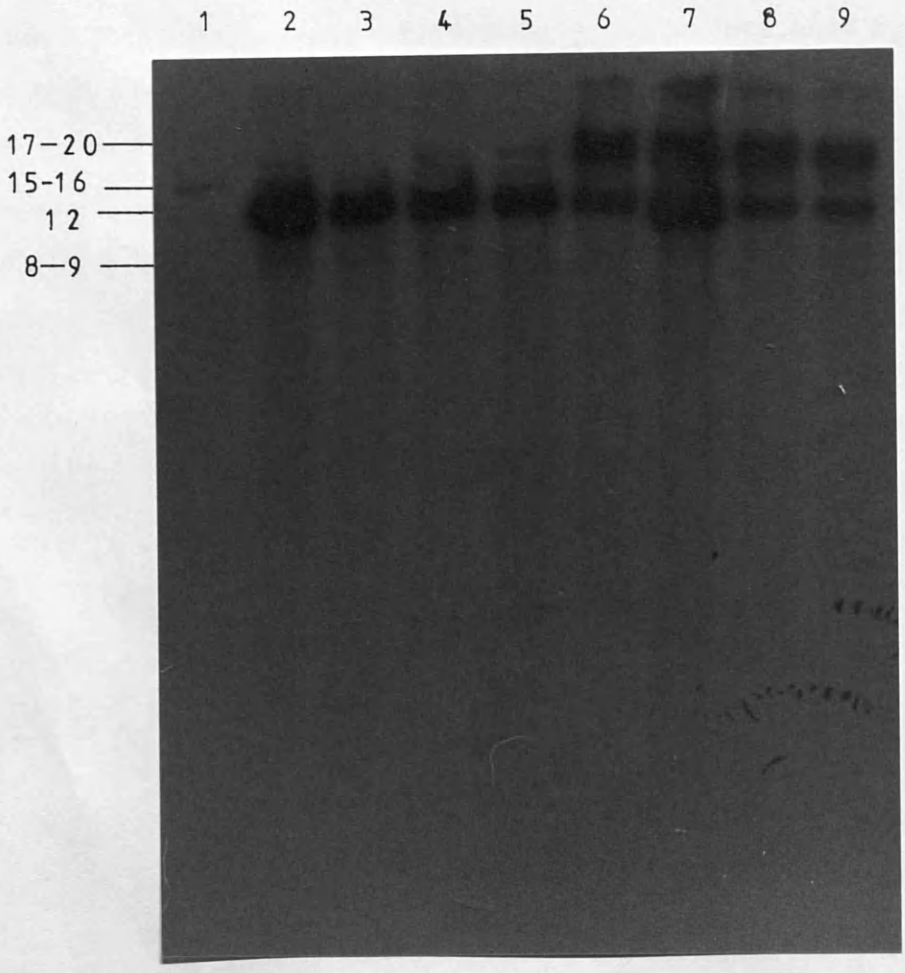


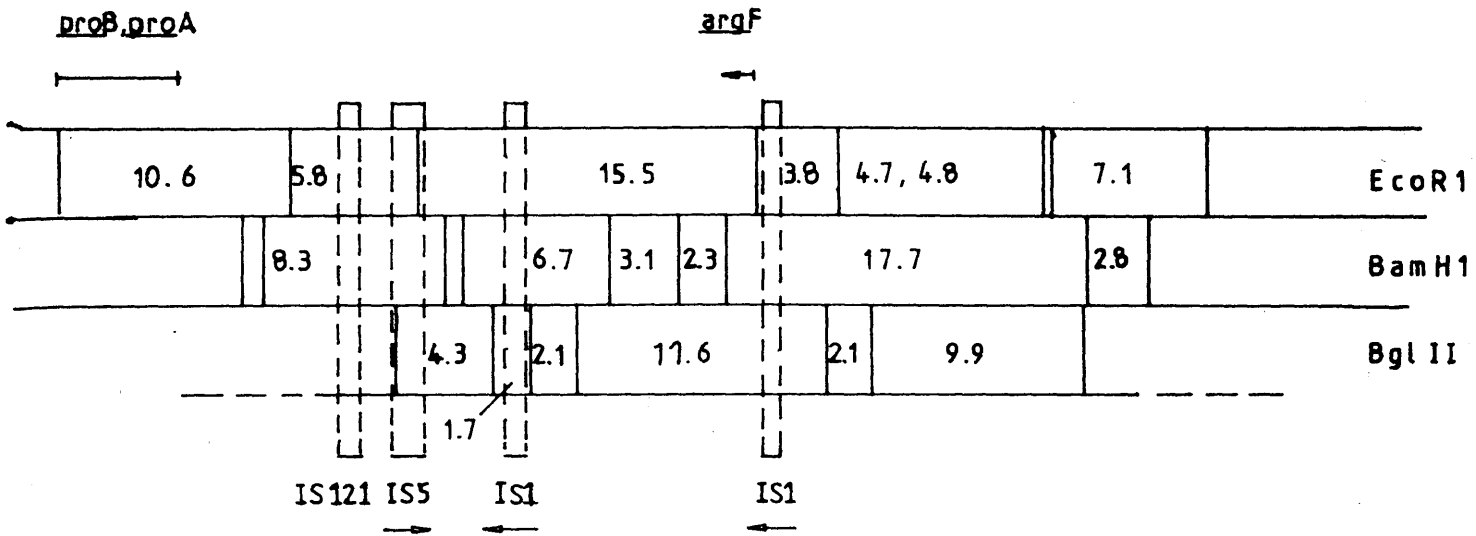
Figure 3.4. Hybridization of ^{32}P-labelled pMC23 to Southern transfers of chromosomal DNA from JEF8 and US Cut isolates. (DNA was digested with EcoR1 and run on an 0.6% agarose gel).

Track 1: JEF8, Track 2: Cut 1, Track 3: Cut 3,

Track 4: Cut 4, Track 5: Cut 5, Track 6: Cut 7,

Track 7: Cut 8, Track 8: Cut 9, Track 9: Cut 11

(a).


proB,proA
argF
10.6
5.8
15.5
3.8
4.7, 4.8
7.1
EcoR1
8.3
6.7
3.1
2.3
17.7
2.8
BamH1
4.3
2.1
11.6
2.1
9.9
Bgl II
1.7
IS121
IS5
IS1
IS1

(b)

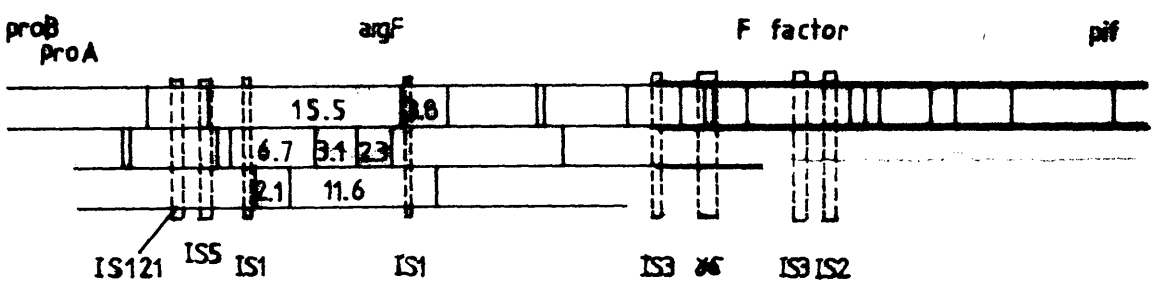

proB
proA
argF
F factor
pif
15.5
3.8
6.7
3.1
2.3
2.1
11.6
IS121
IS5
IS1
IS1
IS3
IS3
IS2

Figure 3.5a. Restriction map showing EcoR1, BamH1 and BglII sites within the argF region of E. coli K12. The positions of the argF gene, IS1, IS5 and IS121 elements are marked. All sizes are given in kilobases. (After Hadley et al, 1983).

b. Restriction map showing the position of Tn2901 in relation to the integrated F factor.

which is present in multiple copies compared with the single copies represented by the fragments with a heterogeneous size distribution.

3.2 Hybridization to pMC23, a plasmid containing the argF gene

A gel containing EcoR1 digests of eight US Cut mutants and of JEF8 was blotted onto nylon transfer membrane. The plasmid pMC23 (Crabeel et al,1979), which contains the argF gene and approximately 0.7kb of adjacent chromosomal DNA, was nick-translated and hybridized to the Southern blot. After hybridization the membrane was washed in conditions of high stringency. The resultant autoradiograph (Fig.3.4) shows that

(1). the probe hybridizes to the 12kb intense fragments seen in the restriction digests of the US Cut isolates. This indicates that the argF gene or adjacent DNA is present on the 12kb intense fragment and hence the argF gene region is amplified in the US Cut mutants.

(2). a faint band of 15-16kb hybridizes to the probe in tracks of both JEF8 and the US Cut mutants.

(3). four of the US Cut mutants contain a second intense band of 17-20kb. This band, which may be comprised of two bands very close together, is of equal intensity to the 12kb band but is not viualised on the agarose gel.

(4). a faint band of 8-9kb hybridizes to the probe in tracks of all the US Cut isolates and JEF8.

Since the whole plasmid (rather than an argF-containing fragment alone) was nick-translated similar digests were probed with the vector DNA, pBR322. No hybridization was detected indicating that all the chromosomal fragments seen in the pMC23 autoradiograph had hybridized to the cloned insert and not to sequences in the vector molecule.

A partial restriction map of the pro-lac region of E. coli K-12 is available (Hadley et al,1983) showing EcoR1, BamH1 and BglII restriction sites within the argF region (Fig.3.5). The restriction

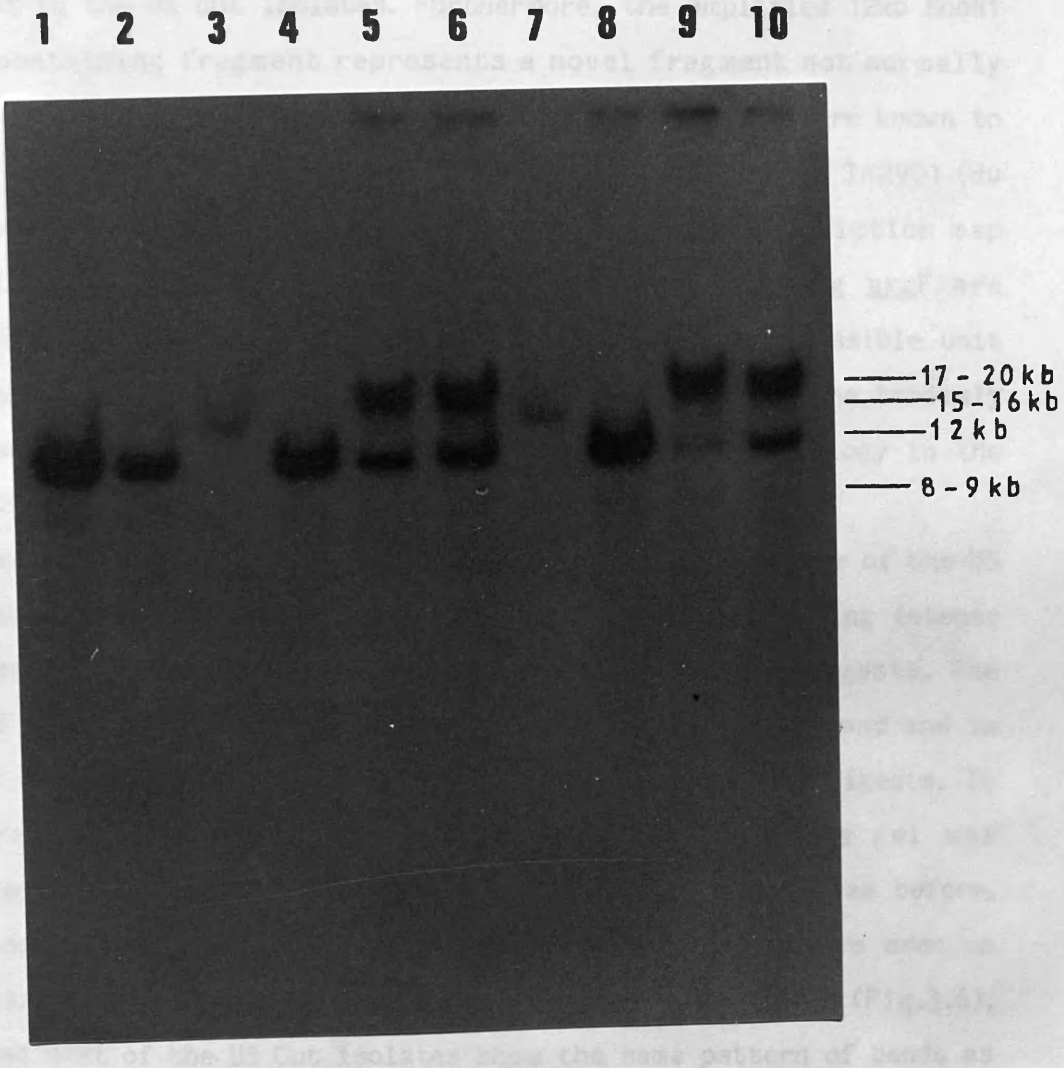


Figure 3.6 Hybridization of ^{32}Plabelled pMC23 to Southern transfers of chromosomal DNA from JEF8 and US Cut isolates. (DNA was digested with EcoR1 and run on an 0.6% agarose gel).

Track 1: Cut 1, Track 2: Cut 3, Track 3: JEF8,

Track 4: Cut 4, Track 5: Cut 5, Track 6: Cut 7,

Track 7: JEF8, Track 8: Cut 8, Track 9: Cut 9,

Track 10: Cut 11

map shows that the argF gene is normally present on a 15.5kb EcoR1 fragment. An EcoR1 fragment corresponding to this size does hybridize to pMC23 in both JEF8 and the US Cut mutants and its presence indicates that the original argF containing EcoR1 fragment is still present in the US Cut isolates. Furthermore, the amplified 12kb EcoR1 argF-containing fragment represents a novel fragment not normally present in E.coli K-12. Two IS1 elements in direct repeat are known to flank the argF gene in a transposon-like structure named Tn2901 (Hu and Deonier,1981; York and Stodolsky,1981) and the restriction map (Fig.3.5) indicates that the two IS1 elements flanking argF are approximately 11kb apart. Therefore Tn2901 represents a possible unit of amplification since it is the correct size and since the tandemly repeated IS1 elements could possibly act as sites of homology in the formation of the amplification.

The second intense band seen in the autoradiograph of four of the US Cut mutants cannot be easily explained since no corresponding intense fragments are seen in agarose gels of the restriction digests. The second bands are of at least equal intensity to the 12kb band and so should also be visible in agarose gels of the restriction digests. To confirm the presence of the second amplified bands the gel was repeated using DNA from the same chromosomal preparation as before. Again no intense fragments other than the 12kb fragment were seen on the agarose gel. The gel was blotted and probed with pMC23 (Fig.3.6). JEF8 and most of the US Cut isolates show the same pattern of bands as the previous Southern blot although the faint 15-16kb and 8-9kb bands are barely visible. However, several of the US Cut isolates show different banding patterns than before. Cut 5 had previously resulted in only a faint 17-20kb band but this band is now marginally more intense than the 12kb amplified fragment. Cut 8 had previously shown a 17-20kb amplified band but now only shows a single intense band of 12kb.

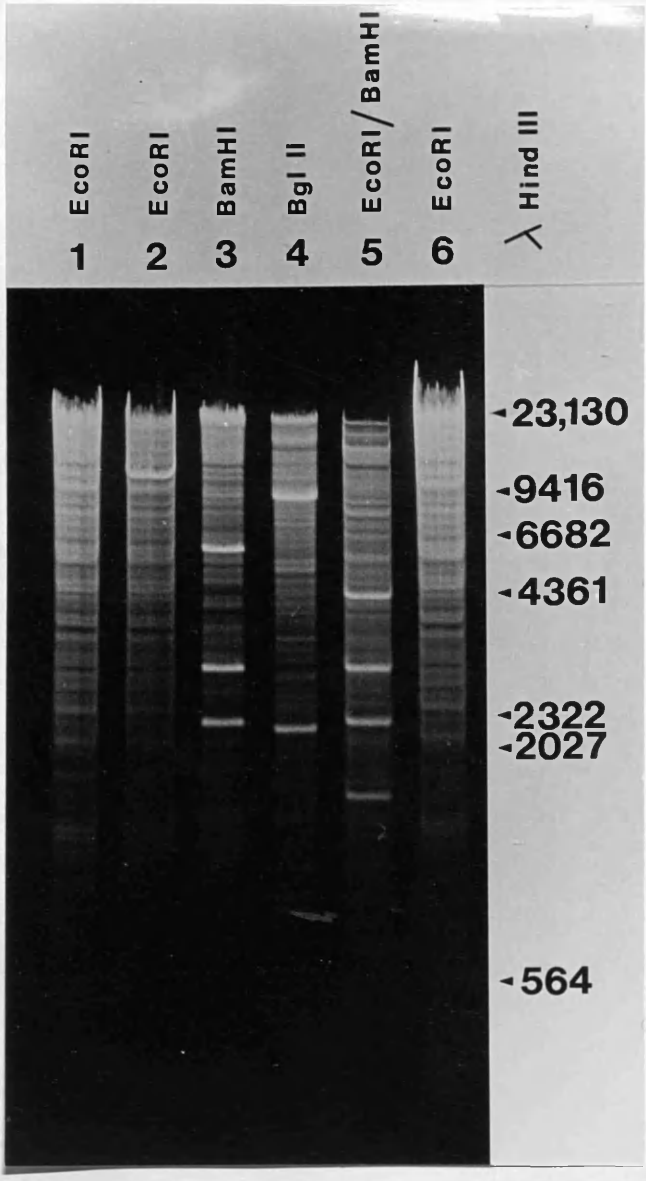


Figure 3.7. 0.6% agarose gel of restriction digests of JEF8 and Cut 1. Sizes given are in base-pairs.

Tracks 1 and 6 : JEF8, Tracks 2 to 5 : Cut 1

Table 3.1.
Approximate sizes of the intense fragments as estimated by agarose gel electrophoresis with known size standards, are given in kb. Fragments hybridizing to pMC23 are in boldface.

Enzyme(s)	Estimated fragment sizes	Sum of fragments
EcoR1	**12.0**	12.0
BamH1	**6.3**, 3.1, 2.3	11.6
BglII	**9.4**, 2.1	11.5
BamH1/EcoR1	4.7, 3.1, 2.3, **1.8**	11.9

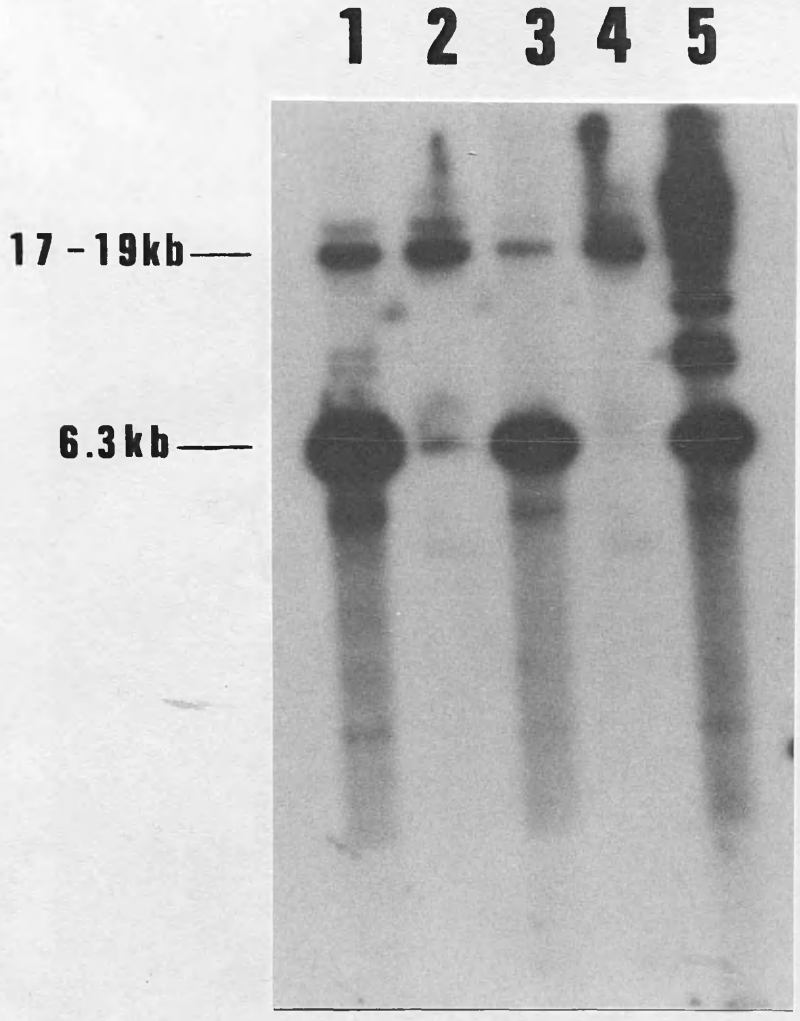


Figure 3.8. Hybridization of ^{32}P-labelled pMC23 to Southern transfers of DNA from JEF8 and several US Cut isolates. (Chromosomal DNA was restricted with BamH1 and run on an 0.8% agarose gel).

Track 1: Cut1 Track 2: JEF8 Track 3: Cut 5

Track 4: JEF8 Track 5: Cut8

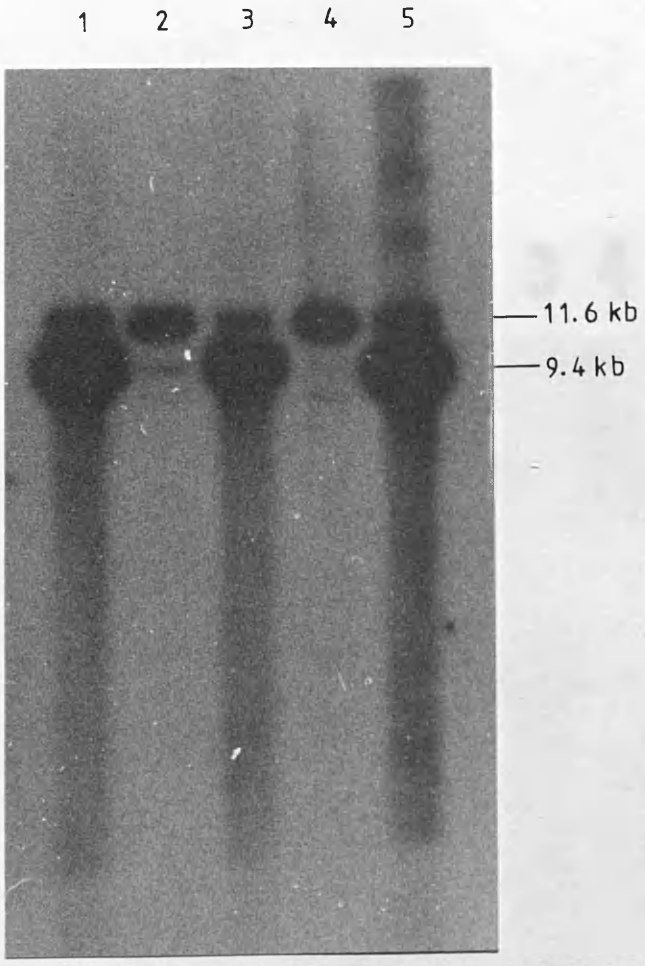


Figure 3.9. Hybridization of ^{32}P labelled pMC23 to Southern transfers of DNA from JEF8 and US Cut isolates. (Total DNA was digested with BglII and run on an 0.8% agarose gel).

Track 1: Cut1 Track 2: JEF8 Track 3: Cut5

Track 4: JEF8 Track 5: Cut8

3.3 Size of the amplified unit

In order to more accurately determine the size of the amplified fragment, chromosomal DNA from Cut 1 was digested with several restriction enzymes and run on an agarose gel (Fig.3.7). Each digest contains a number of intense bands whose sizes were determined from marker fragments on the gel. The sizes of the intense bands in each digest are shown in Table 3.1. The sum of the sizes of the intense fragments in each digest represents the size of the unit amplified in Cut 1. These sizes are also shown in Table 3.1. The mean total size of all the restriction digests is 11.7kb and this represents the size of the amplified unit in Cut1. The presence of intense BamH1 fragments of 2.3kb and 3.1kb further suggests that Tn2901 is the unit of amplification since BamH1 fragments of these sizes are present within Tn2901 (Fig.3.5). The gel was blotted onto transfer membrane and hybridized to the argF-containing plasmid pMC23. The size of the intense band which hybridized to argF in each digest is shown in Table 3.1.

3.4 Confirmation of Tn2901 as the unit of amplification

The location of BamH1 and BglII sites in the argF region of the published restriction map (Fig.3.5) prompted the decision to further examine the US Cut mutants using these restriction enzymes in an attempt to confirm Tn2901 as the unit of amplification.
Three representative US Cut mutants were chosen (Cut 1,5 and 8) for further restriction analysis with BamH1 and BglII. Chromosomal DNA from each of these isolates and JEF8 was digested with BamH1 and BglII, the digests run on agarose gels and Southern blotted onto nylon transfer membranes. The membranes were hybridized to nick-translated pMC23, the argF-containing plasmid (Fig.3.8 and 3.9). The restriction map of the region (Fig.3.5) shows that argF is usually contained

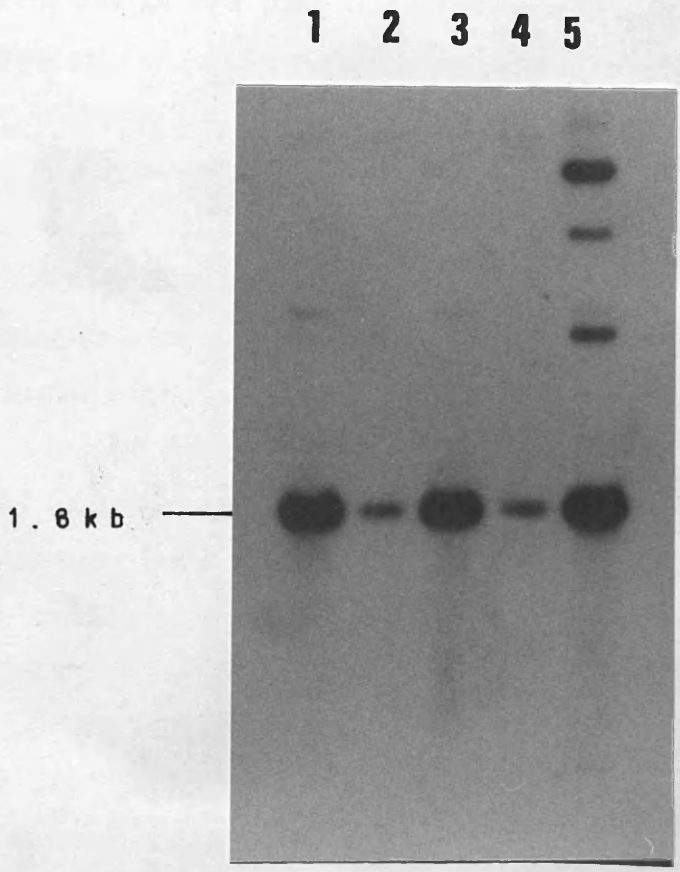


Figure 3.10. Hybridization of ^{32}P-labelled pMC23 to Southern transfers of DNA from JEF8 and US Cut isolates. (Total DNA was digested with EcoR1 and BamH1 and run on an 0.8% agarose gel).

Track 1: Cut1 Track 2: JEF8 Track 3: Cut5

Track 4: JEF8 Track 5: Cut8

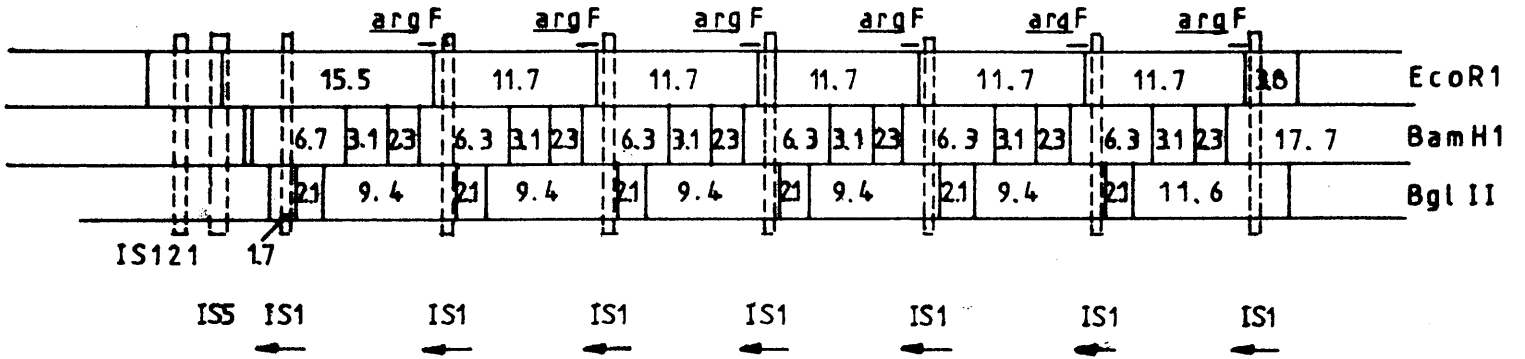


Figure 3.11. Restriction map showing the tandem amplification of Tn2901. Six copies of Tn2901 are shown for convenience. IS elements are represented as thin boxes. All sizes given are in kilobases. The position of the argF gene is shown in each unit.

within a 17.7kb BamH1 fragment and within an 11.6kb BglII fragment. JEF8 and the three US Cut mutants all contain bands corresponding to these fragments indicating that the original argF-containing BamH1 and BglII fragments are present in the US Cut isolates. The US Cut mutants also contain a novel, amplified 6.3kb BamH1 band and a novel amplified 9.4kb BglII band which are not present in JEF8, and which were visible in an agarose gel of the restriction digests (Fig.3.7).The presence of the original argF-containing fragment and a novel, amplified argF-containing fragment in each digest indicates that the amplified DNA consists of a stretch of tandem repeats. When digested with both EcoR1 and BamH1, chromosomal digests of JEF8 and the US Cut isolates should contain a 1.8kb fragment which hybridizes to argF. This was confirmed by hybridizing nick-translated pMC23 to a Southern blot of chromosomal DNA restricted with EcoR1 and BamH1 (Fig.3.10). The 1.8kb band is amplified in the US Cut mutants but not in JEF8.

When Tn2901 is tandemly repeated (Fig.3.11) using the data from the restriction map, novel restriction fragments are obtained which correspond to the sizes of the novel amplified fragments seen in the US Cut mutants indicating that Tn2901 is the unit of amplification.

Several other non-amplified bands were present in the digests of Cut8 probed with pMC23 which were not present in the other US Cut isolates or JEF8. In these digests the bands were larger than the novel, amplified fragment and could therefore possibly represent partially digested DNA. Cut 1 contained two very faint BamH1 bands which corresponded to two of the additional bands in the BamH1 digest of Cut8 and these also suggest the presence of partially digested DNA. Several other faint bands were seen in all tracks but these may be unrelated to the amplification. In several tracks of JEF8 a very faint band was seen which has the same size as the novel amplified band in the US Cut isolates. This suggests that a small proportion of JEF8 cells may carry the novel fragment, most likely in the form of a duplication. The fact that the novel 11.7kb EcoR1 fragment was not present in Southern blots of JEF8 probed with argF (Fig.3.4 and 3.6)

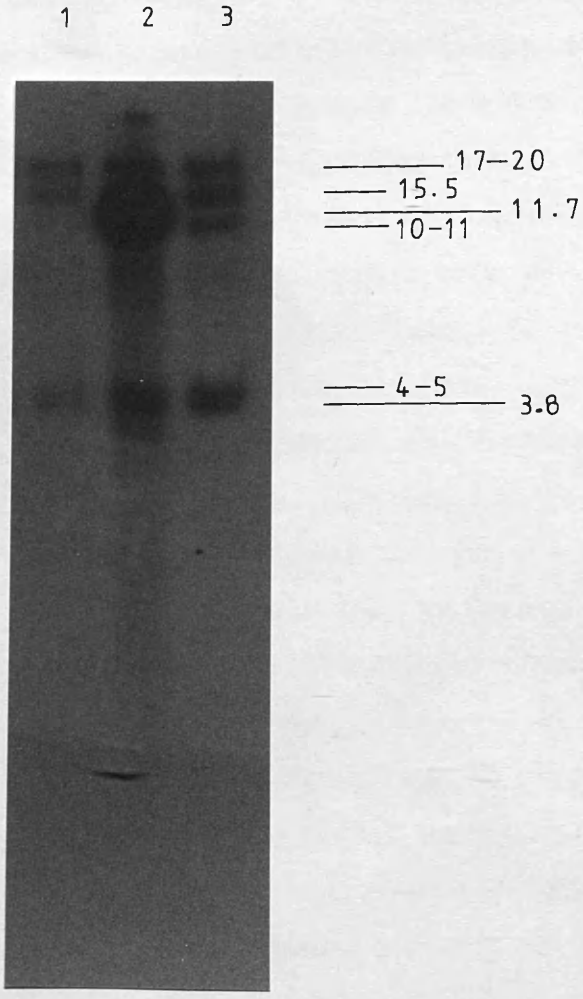


Figure 3.12. Hyoridization of ^{32}P-labelled pBR322::IS1 to Southern transfers of chromosomal DNA restricted with EcoR1. (Digested DNA was run on an 0.8% agarose gel).

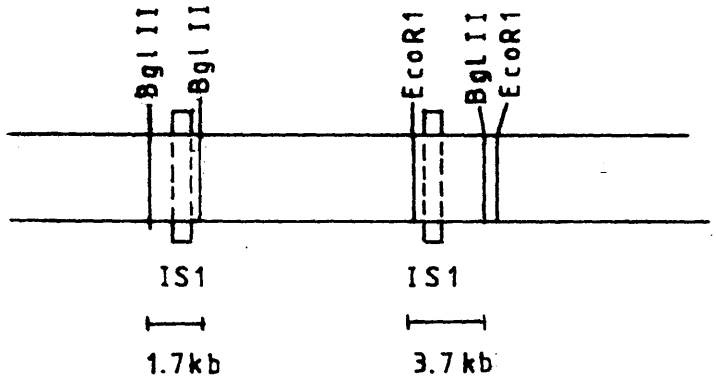
Track 1: D7-8 Track 2: Cut1 Track 3: JEF8

further suggests that the novel fragment is unlikely to be present in all JEF8 cells.

3.5 Hybridization to IS1

To confirm that Tn2901 is the unit of amplification, it was decided to probe a Southern blot containing EcoR1 digests of chromosomal DNA from a US Cut mutant with a plasmid containing IS1. If Tn2901 is the unit of amplification then IS1 should hybridize to the 11.7kb EcoR1 amplified fragment. The restriction map of the argF region (Fig.3.5) shows the two IS1 elements flanking IS1 to be present, one on the 15.5kb argF-containing EcoR1 fragment and the other on an adjacent 3.8kb EcoR1 fragment. Therefore IS1 should also hybridize to these fragments in the EcoR1 chromosomal digest of the US Cut mutant.
DNA from Cut1, JEF8, and an F^- strain, D7-8, was digested with EcoR1, run on an agarose gel and Southern blotted onto nylon transfer membrane. The membrane was probed with pBR322::IS1, a plasmid containing the entire IS1 insertion sequence. The autoradiograph (Fig.3.12) shows that IS1 hybridizes strongly to the 11.7kb amplified unit in Cut1, confirming that Tn2901 is the unit of amplification. IS1 also hybridizes to the expected 15.5kb and 3.8kb EcoR1 fragments in JEF8. In Cut1 the 3.8kb band is present but the 15kb-16kb region of the blot is obscured by the intense signal from the 11.7kb amplified fragment and so the 15.5kb expected fragment is not detected. Several other fragments also hybridize to IS1 of 17-20kb, 4-5kb and 10-11kb and these represent other IS1 elements in the strains since IS1 is not cut by EcoR1. D7-8 contains the 15-16kb band but not the 10-11kb band. A similar blot was probed with pBR322 alone and no hybridization was seen.

(a). unamplified Tn2901



(b). amplified Tn2901

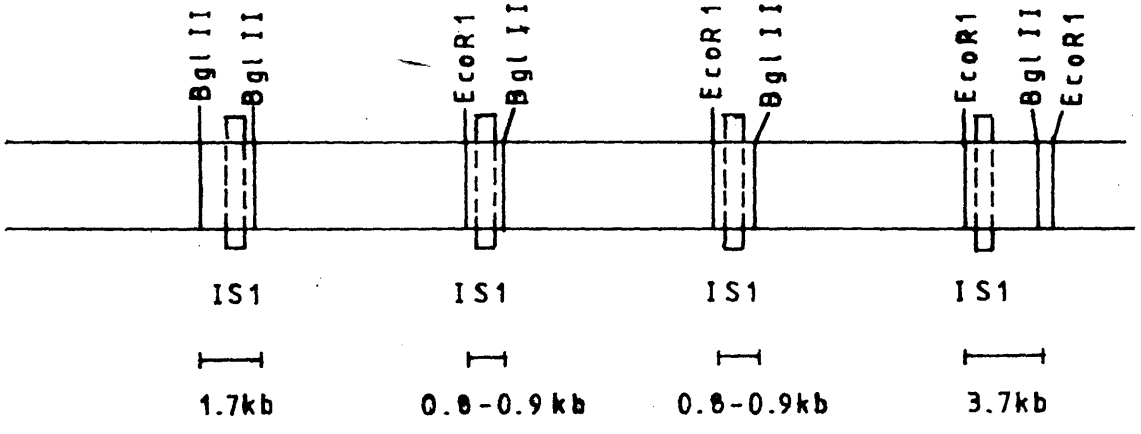


Figure 3.13. Figure showing the IS1-containing restriction fragments of Tn2901 in an amplified and unamplified strain. In the amplified strain three copies of Tn2901 are shown for convenience.

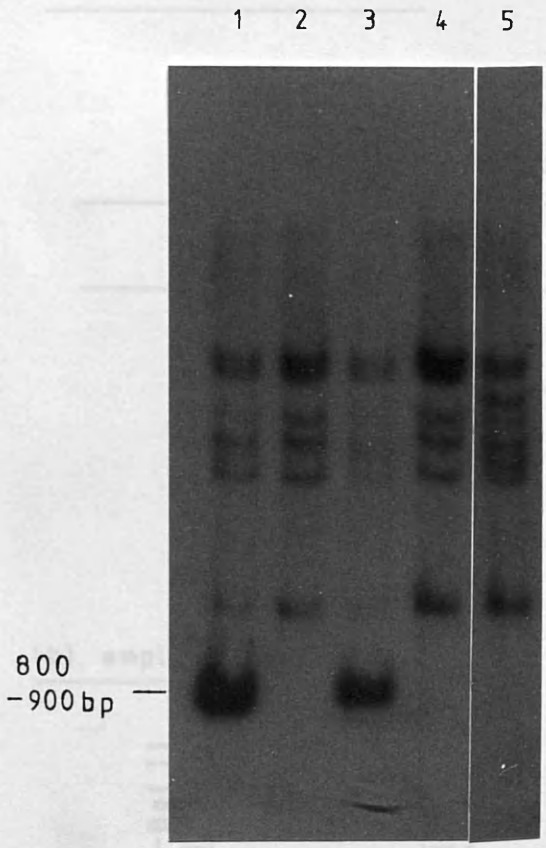


Figure 3.14. Hybridization of ^{32}P-labelled pBR322::IS1 to a Southern blot containing DNA restricted with BglII and EcoR1.

Track 1: Cut1 Track 2: JEF8 Track 3: Cut5

Track 4: JEF8 Track 5: P678.14

3.6 The structure of the novel joint

The most probable structure of the novel joint between each tandem repeat is a single IS1 element obtained by recombination between the two pre-existing IS1 elements on either side of the amplified unit (Fig.3.11). Since other structures are also possible it was neccessary to examine the junction fragments in order to determine their structure.

IS1 is 768bp in length (Ohtsubo and Ohtsubo,1978) and contains no EcoR1 or BglII restriction enzyme cleavage sites. When digested with both EcoR1 and BglII the two IS1 elements flanking argF in E. coli K-12 would be present on a 3.7kb fragment and a 1.7kb fragment (Fig.3.13). However, in the proposed structure of the amplified DNA (Fig.3.11) each novel IS1 element is flanked very closely by an EcoR1 site and a BglII site generating an IS1-containing EcoR1/BglII fragment which should be only slightly larger than the 768bp IS1 element (Fig.3.13). Of course the original 3.7kb and 1.7kb IS1-containing fragments would still be present at the ends of the amplified DNA. The presence of an amplified IS1-containing EcoR1/BglII fragment of approximately 800 or 900bp would indicate that a single IS1 element was present at the novel joints between adjacent units of amplification as proposed. Any other structure should not contain such a fragment. For example, the presence of two IS1 elements at each junction would yield a much larger EcoR1/BglII fragment of at least 1.6kb.

Chromosomal DNA from two US Cut isolates, Cut1 and Cut5, together with JEF8 and P678.14 was digested with EcoR1 and BglII and run on an agarose gel. The gel was Southern blotted onto nylon transfer membrane and hybridized to nick-translated pBR322::IS1. The autoradiograph (Fig.3.14) shows that both Cut 1 and Cut 5 contain an amplified IS1-containing fragment that is not present in either JEF8 or P678.14. The size of this fragment is approximately 800-900bp, indicating that a single IS1 element is present at the novel joint between each adjacent

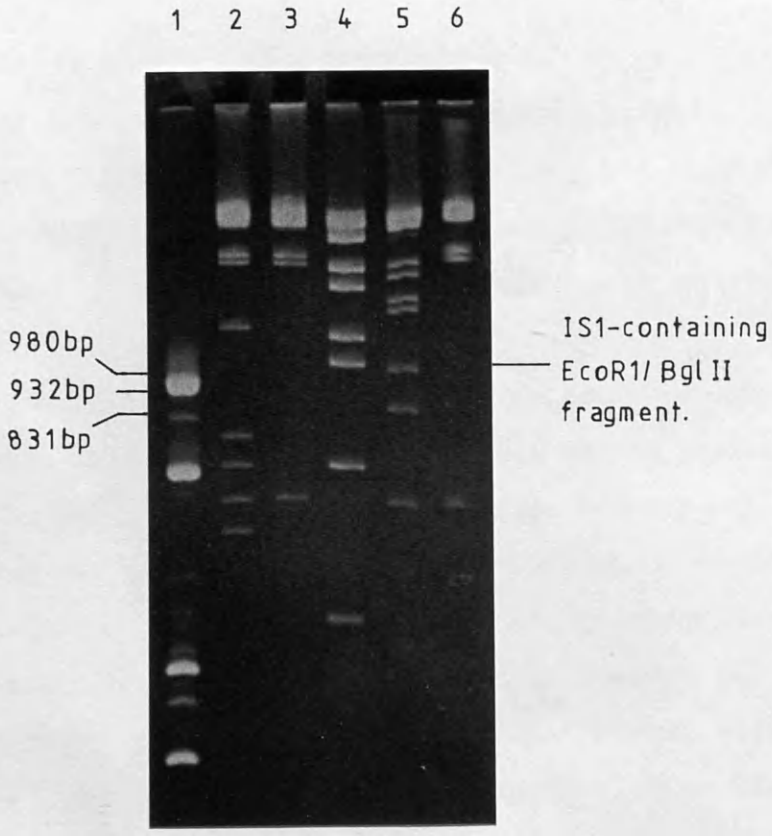


Figure 3.15. Acrylamide gel (3.5 %).

Track 1: pUC8 Sau3A

Track 2: Lambda HindIII/BglII

Track 3: Lambda HindIII

Track 4: pCC11 BglII/EcoR1

Track 5: Lambda HindIII/EcoR1

Track 6: Lambda HindIII

unit of amplification as predicted. Fragments of approximately 3.7kb and 1.7kb also hybridize to the IS1 probe in both JEF8, P678.14 and the US Cut mutants corresponding to the original IS1-containing fragments flanking the argF gene.

Six other bands also hybridized to IS1 representing other IS1 elements present in E. coli K-12. Interestingly, P678.14 appears to have two IS1-containing bands which are not present in the US Cut mutants or JEF8 and these may represent two additional IS1 elements in this strain.

3.7 Accurate sizing and cloning of the IS1-containing BglII/EcoR1 fragment from pCC11

The single IS1 element present in pCC11 (see Chapter 6) was probably formed by recombination between the two IS1 elements of Tn2901. This IS1 element is present on a 0.9kb BglII/EcoR1 fragment similar to the novel joint EcoR1/BglII fragment described above. pCC11 was digested with BglII and EcoR1 and run on a 3.5% acrylamide gel with various size markers (Fig.3.15). The size of the IS1-containing BglII/EcoR1 fragment was calculated as approximately 980-1000bp. Since IS1 is 768bp in length (Ohtsubo and Ohtsubo, 1978) this fragment also contains 200-220bp of adjacent chromosomal DNA. Therefore, the EcoR1 and BglII restriction sites must be very close to the ends of the IS1 element since only 200-220bp additional chromosomal DNA is contained within this fragment.

A plasmid containing IS1 was required to replace pBR322::IS1 as a probe since this latter plasmid appeared to have lost the IS1 element during the course of this work. Therefore, the 980bp fragment from pCC1 was excised from a 1% agarose gel, purified, and ligated to pUC8 which had been digested with BamH1 and EcoR1. The ligation mixture was transformed into ΔM15 and plated on L agar containing Ap, IPTG and X-gal. White colonies were screened by single colony gel analysis to

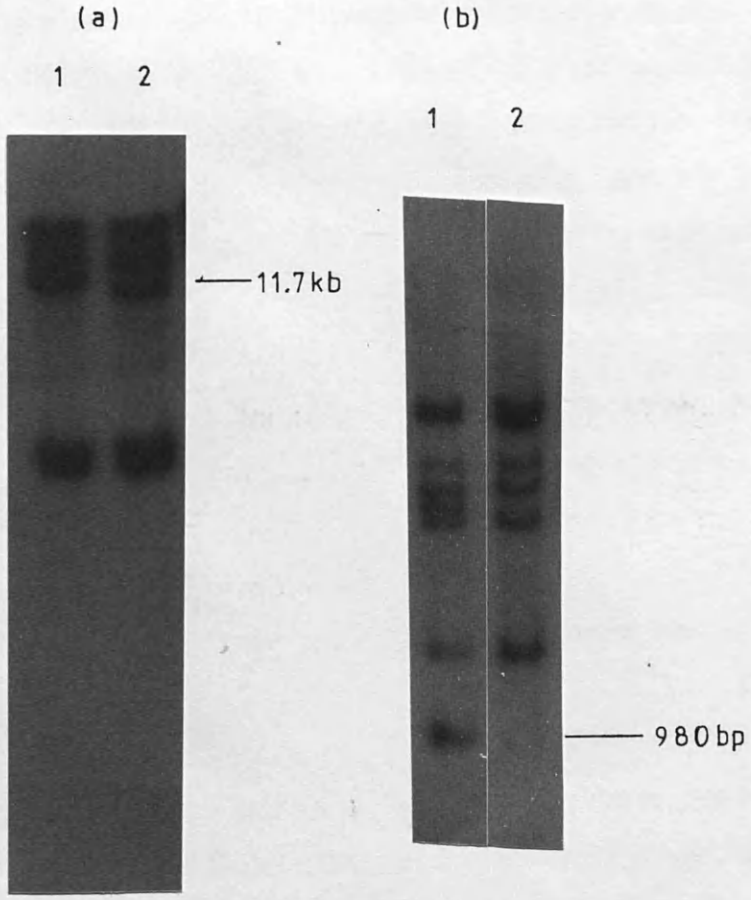


Figure 3.16. Hybridization of ^{32}P-labelled pBR322::IS1 to Southern transfers of chromosomal DNA restricted with EcoR1 and EcoR1/BglII.

(a). EcoR1
Track 1: D30-1 Track 2: JEF8

(b). EcoR1/BglII
Track 1: D30-1 Track 2: JEF8

identify recombinant plasmids and restriction digestion confirmed the presence of the 980bp insert.

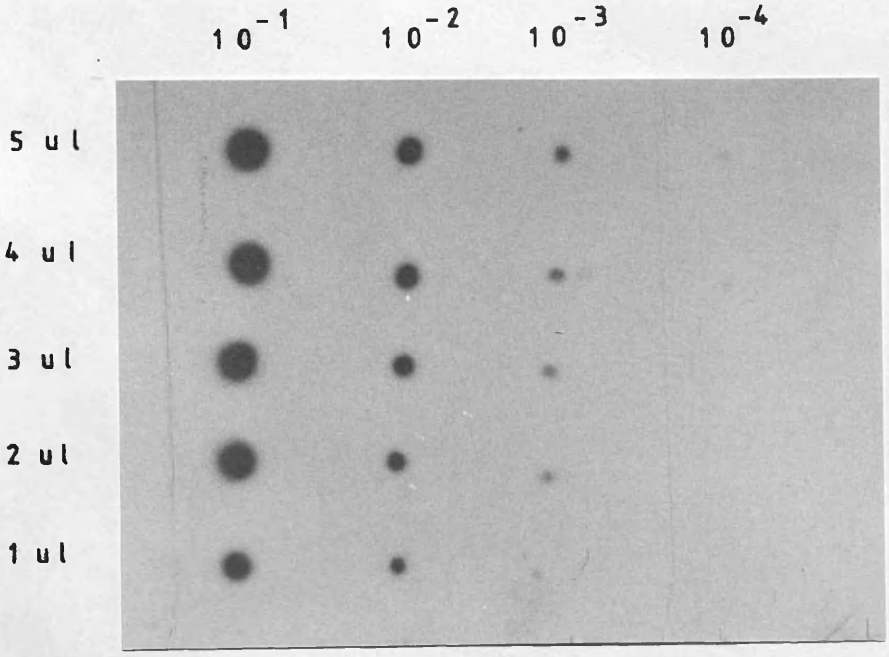
3.8 Analysis of D30-1

D30-1 is a recombinant obtained from a cross between a US Cut mutant (isolated from JEF8) and P678.14. This recombinant yields 1000-fold more US Cut isolates than JEF8 (Jessop and Glansdorff, 1980) and it was suggested (Jessop and Glansdorff, 1980) that D30-1 may be an intermediate in the loss of the US Cut aberration since it resembles a segregant. Chromosomal DNA from D30-1 and JEF8 was digested with EcoR1 and EcoR1/BglII, run on an agarose gel, and Southern blotted onto nylon transfer membrane. The membrane was probed with nick-translated pBR322::IS1. The autoradiographs (Fig.3.16a and b) show that D30-1 contains the 11.7kb EcoR1 and 980bp BglII/EcoR1 novel fragments, indicating that D30-1 contains several copies of the amplified unit. D30-1 differs slightly from JEF8 in the number of other bands hybridizing to IS1. D30-1 is missing a 10-11kb EcoR1 band present in JEF8 and has an additional EcoR1/BglII band not present in JEF8 (and Cut1, see Fig.3.14).

3.9 Determination of the copy number of Tn2901 in US Cut isolates

The copy number of an amplified DNA sequence can be determined by various methods. Altenbuchner and Cullum (1984) estimated the copy number of an amplified sequence in Streptomyces lividans by densitometer scanning of an intense band present in a restriction digest on an agarose gel. This allowed them to determine the percentage of DNA present in the amplified band and hence its copy number.

The copy number may also be determined by hybridization of a probe



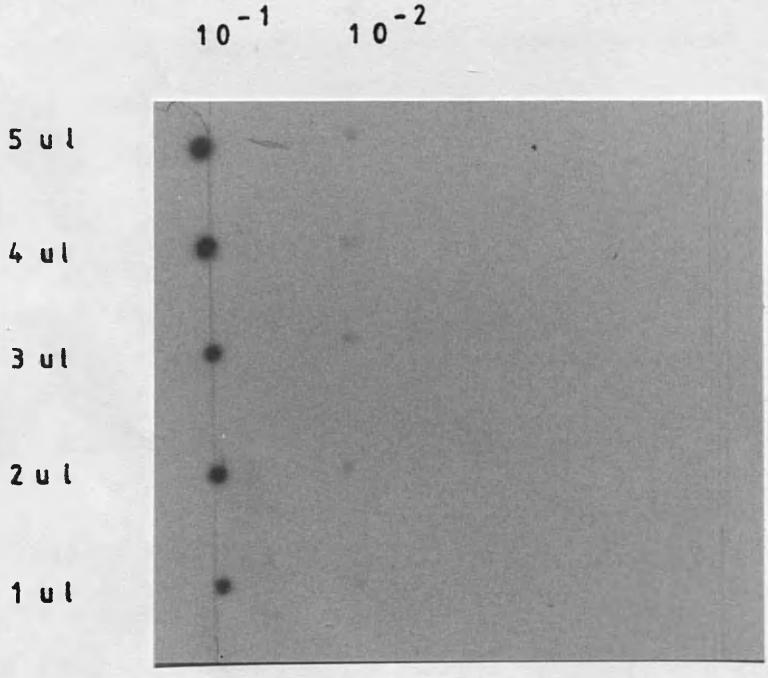


Figure 3.17. Hybridization of ^{32}P-labelled pMC23 to dot blots containing diluted DNA from JEF8 and Cut8. All DNA spots were present on the same nylon membrane filter during hybridization and autoradiography.

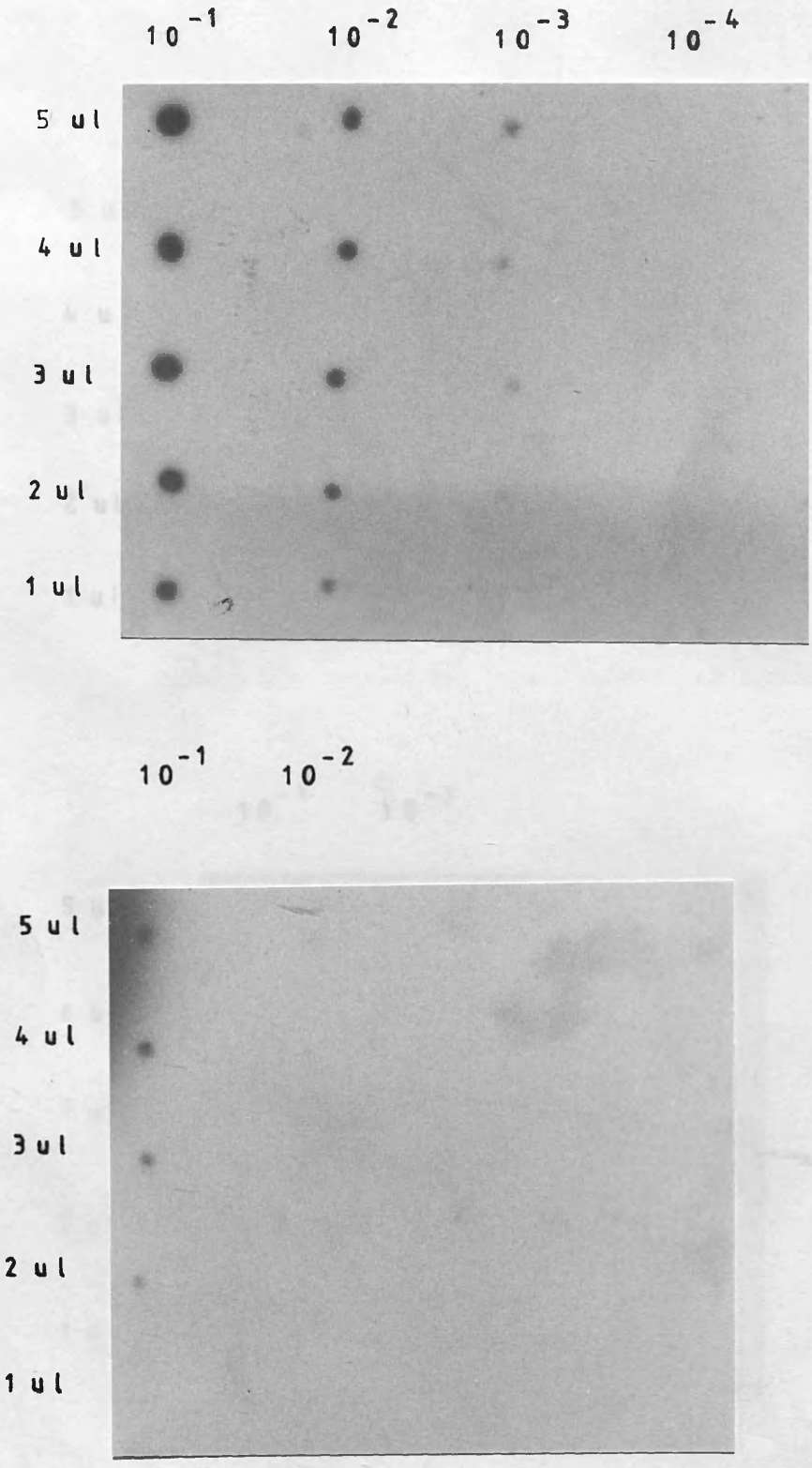


Figure 3.18. Hybridization of ^{32}P-labelled pMC23 to dot blots containing diluted DNA from JEF8 and Cut1. All DNA spots were present on the same nylon membrane filter during hybridization and autoradiography.

Cut1

	10^{-1}	10^{-2}	10^{-3}
5ul	9856	1146	214
4ul	5510	1105	152
3ul	5121	987	129
2ul	3803	616	25
1ul	2341	339	4

JEF8

	10^{-1}
4ul	376
3ul	375
2ul	212
1ul	1

Table 3.2. Scintillation counts from dot blots of Cut1 and JEF8 probed with nick-translated pMC23 (see Figure 3.18). Each spot was cut from the membrane ($1cm^2$) and the radioactivity measured by Cherenkov counting. Each spot was counted three times and the mean value calculated. The background count was subtracted from each value.

which is specific to the amplified sequence to a series of accurate dilutions of the amplified DNA. When the signal intensity of these is compared with that of the parental DNA, which contains only a single copy of the unit, an estimate of the copy number of the amplified unit can be made. The dilutions of DNA can either be spotted directly onto the transfer membrane (dot blots) or digested with an appropriate restriction endonuclease and the fragments separated on an agarose gel and blotted (Tlsty et al,1984; Albertini and Galizzi,1985).

Fishman and Hershberger (1983) used kinetics of hybridization to measure the copy number of an amplified sequence in Streptomyces fradiae. A sample of sheared genomic DNA was denatured and hybridized to a nick-translated probe specific to the amplified sequence. Samples were removed at various times and analyzed to measure the proportion of total trichloroacetic acid-precipitable radioactivity that remained precipitable after digestion of single-stranded DNA with S1 nuclease. Reaction rate constants were then determined and used to calculate the copy number of the amplified sequence present in the genomic DNA.

In order to determine the copy number of the amplified unit in the US Cut isolates, dot blots were prepared using accurately diluted DNA from two representitive US Cut mutants, Cut 1 and Cut 8, and JEF8. The dot blots were hybridized to nick-translated pMC23, the argF-containing plasmid. The resulting autoradiographs (Fig.3.17 and 3.18) were examined visually and a comparison made of the diameter and intensity of signals from the US Cut mutants and JEF8.

The autoradiographs show that the diameter and intensity of the spots produced by JEF8 at the 10^{-1} dilution is greater than that of the US Cut mutants at the 10^{-3} dilution but less than that of the US Cut mutants at the 10^{-2} dilution. Therefore the copy number of Tn2901 in each US Cut isolate lies somewhere between 10 and 100 copies, most likely in the range of 40-60 copies. Each spot from Cut1 and JEF8 was cut from the transfer membrane and the counts emitted from each spot measured by scintillation counting (Table 3.2). The scintillation counts suggest a copy number of between 30 and 60 copies but many of

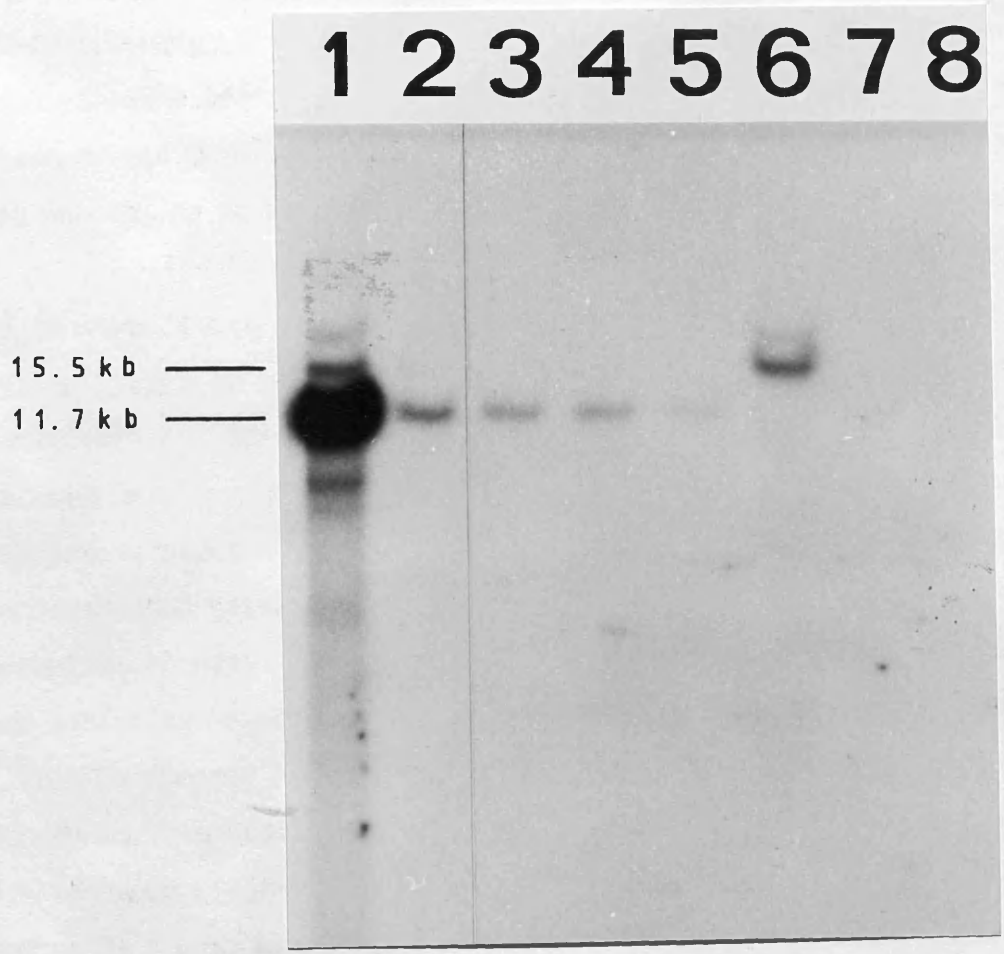


Figure 3.19. Estimation of the extent of the amplification. Samples containing decreasing amounts of DNA from Cut1 were brought to a constant final concentration by the addition of sonicated slmon sperm DNA, and hybridized to nick-translated pMC23. Track 6 contains undiluted DNA from JEF8. The remaining tracks contain the following dilutions of DNA from Cut1:

1. undiluted 2. 1:50 3. 1:75

4. 1:100 5. 1:200 7. 1:400

8. contains only sonicated salmon sperm DNA.

Sizes given are in kilobases.

the counts do not correspond ideally to the dilution of DNA on the membrane. Therefore a more exact copy number value cannot be obtained from the autoradiographs.

In order to obtain a more exact value, samples containing accurately diluted DNA from Cut1 and JEF8 were all brought to the same final DNA concentration by the addition of sonicated salmon sperm DNA. The DNA samples were restricted with EcoR1, run on an agarose gel and Southern blotted onto nylon transfer membrane. The Southern blot was hybridized with nick-translated pMC23. The autoradiograph (Fig.3.19) suggests that the copy number of Tn2901 in Cut1 is slightly less than 50 and has been estimated at 45.

3.10 Cloning of the amplified unit

Prior to molecular analysis of the mechanism of formation of the amplification it was neccessary to clone the amplified unit.The visualization of the amplified DNA as intense restriction fragments on an agarose gel greatly facilitated the cloning of the unit of amplification because it was possible to directly excise the fragments of interest from the gel. EcoR1 has only one recognition site within each tandem repeat and yields a single intense restriction fragment of 11.7kb. Although large DNA fragments are generally considered more difficult to clone into plasmid vectors than small fragments (Maniatis et al, 1982) it was considered more useful to clone the single 11.7kb EcoR1 fragment rather than several smaller fragments produced by another restriction endonuclease such as BamH1. Fishman and Hershberger (1983) cloned five small SalI fragments from an amplified strain in preference to a single BglII fragment. However the production of a composite restriction map of the cloned fragments involved complicated partial restriction digests and many Southern hybridizations which could probably have been avoided if the single

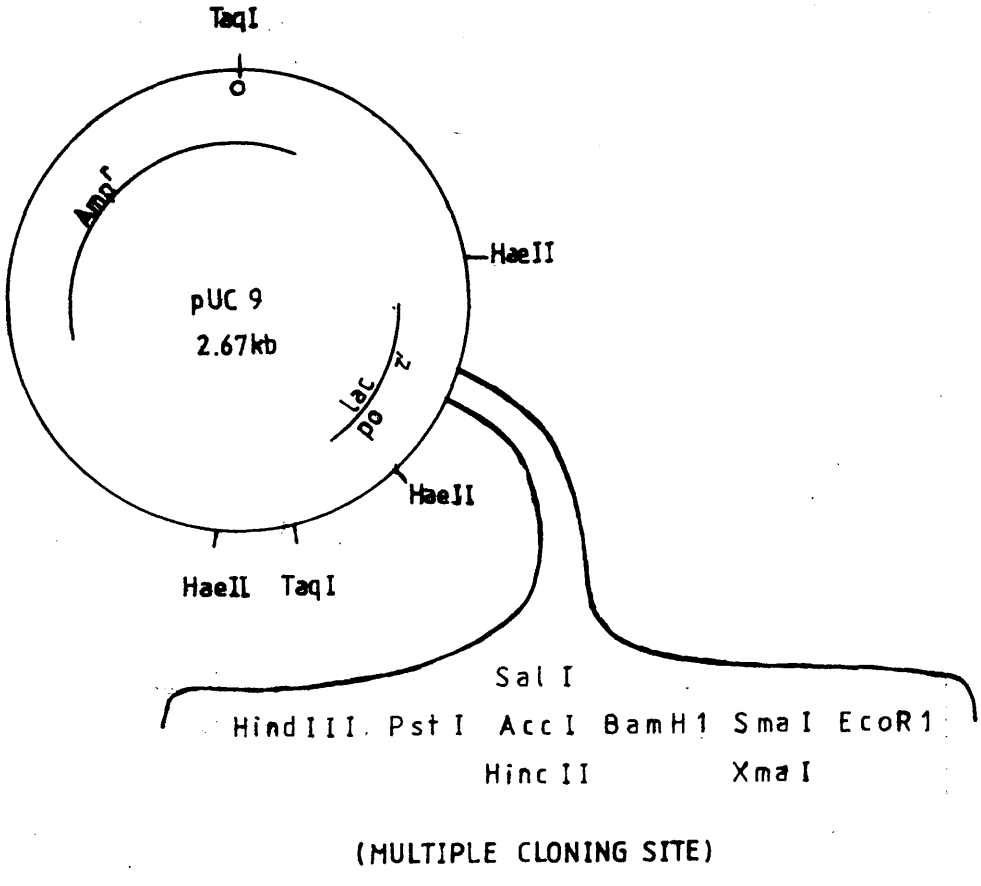


Figure 3.20. Diagram of the cloning vector pUC9. pUC8 contains the multiple cloning site in the opposite orientation

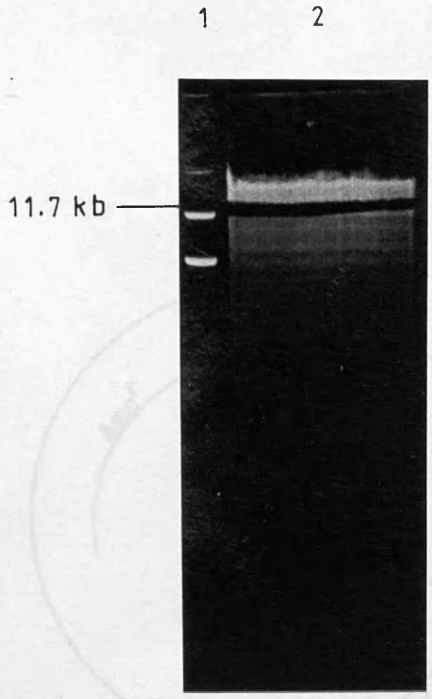


Figure 3.21. 0.7% agarose gel showing Cut1 DNA digested with EcoR1, with the 11.7kb intense band excised (Track 2). Track 1 contains lambda HindIII size markers.

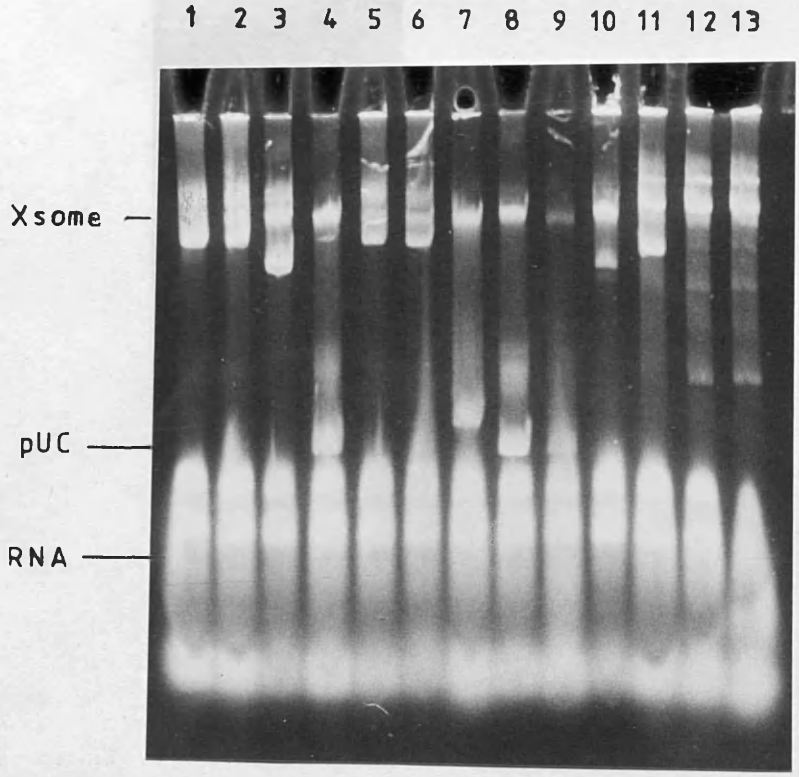


Figure 3.22 Single colony gel analysis of white Ap^r transformants to identify recombinant plasmids. Tracks 1 and 5 contain the recombinant plasmids which were named pCC1 and pCC5 respectively. The large plasmids present in several other tracks were shown by restriction analysis to contain clones of other chromosomal fragments of similar size. (Xsome = chromosome)

BglII fragment had been cloned instead. Therefore it was decided to clone the EcoR1 11.7kb fragment.

In order to clone the amplified fragment a plasmid vector was required which possesed the following :

(i). a replicon which had a high number of copies per cell so that large amounts of the cloned insert could be easily obtained.

(ii). a selectable marker.

(iii). a variety of suitable sites for restriction endonucleases. The EcoR1 site must be in a non-essential region of the plasmid and there should preferably be other restriction sites suitable for further manipulations such as restriction analysis and sub-cloning.

(iv). a suitable method to enable rapid screening for cloned inserts. This was essential if a large fragment was to be cloned (Maniatis et al, 1982).

(v). preferably a small size for easier restriction analysis of the cloned insert.

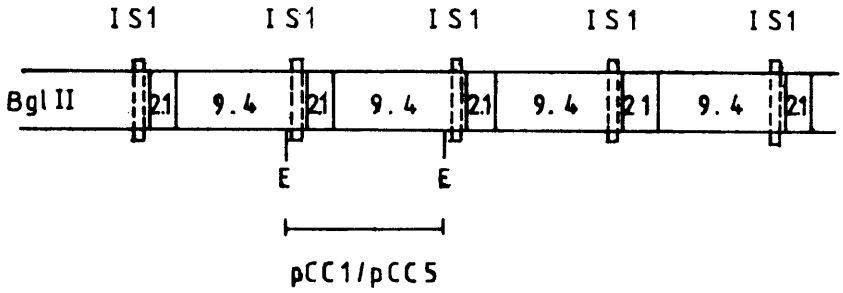
The plasmid vector chosen, pUC8/9 (Fig.3.20), fulfilled all the above requirements. It has a high copy number (about 120 copies per cell), a small size (2.67kb), and encodes resistance to ampicillin. It contains a multiple restriction enzyme cloning site and permits a highly sensitive histochemical screening for recombinants on medium containing IPTG and the chromogenic substrate X-gal.

Approximately 10ug of chromosomal DNA from Cut 1 was digested with EcoR1 and run on an agarose gel. The 11.7kb intense fragment was excised from the gel (Fig.3.21) and the fragment electroeluted from the agarose slice and purified. pUC 9 was digested with EcoR1 and incubated with CIP to prevent re-circularization of the vector. The vector and fragment DNAs were mixed and ligated and used to transform △ M15. The transformed cells were plated on L-agar containing ampicillin, IPTG and X-gal. White colonies which appeared on the plates after incubation were patched and screened by single colony gel analysis to identify recombinant plasmids (Fig.3.22) which appear much larger than pUC9 alone. STET DNA was made from several recombinant

Figure 3.23. Structure and construction of pCC1 and pCC5.

(a). Figure showing the position of the 11.7kb EcoR1 fragment in the amplification. Sizes given are in kilobases.
(b). Figure showing the orientation of the cloned 11.7kb EcoR1 fragment in pCC1. pCC5 contains the fragment in the opposite orientation. The vector used was pUC9.

(a).


IS1
IS1
IS1
IS1
IS1
Bgl II
2.1
9.4
2.1
9.4
2.1
9.4
2.1
9.4
2.1
E
E
pCC1/pCC5

(b).

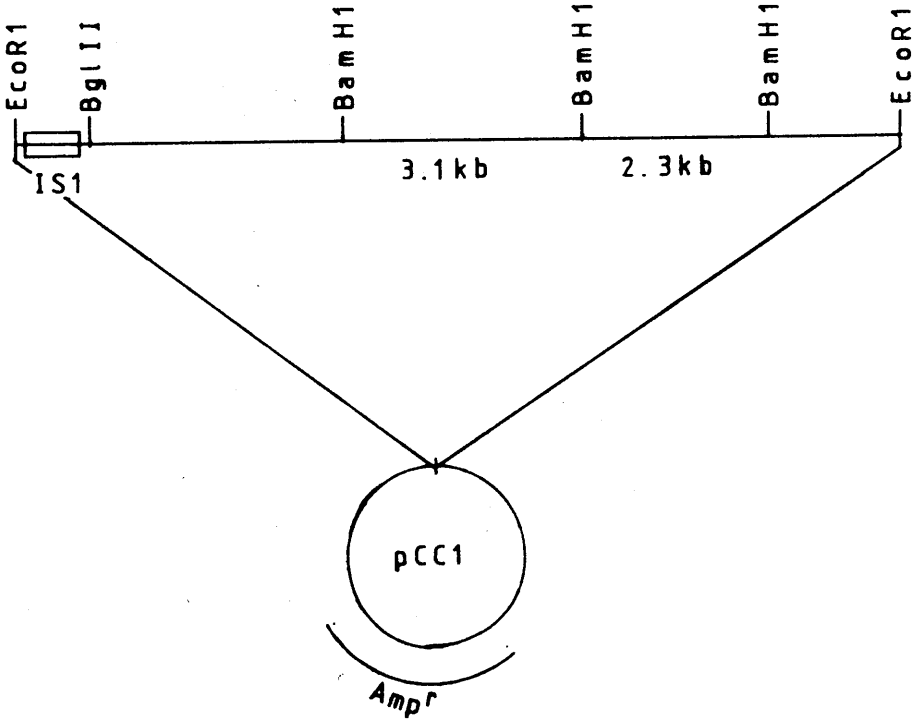
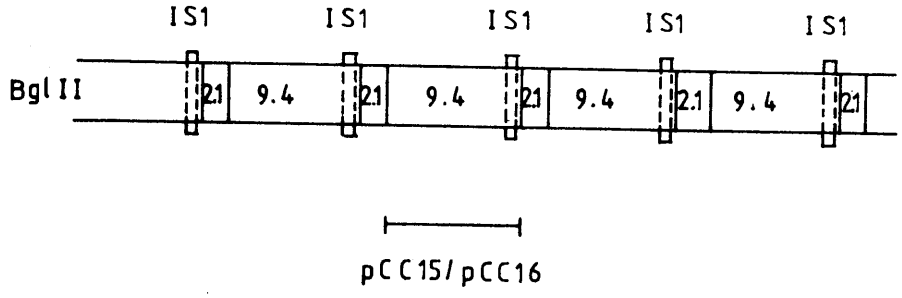

EcoR1
Bgl II
BamH1
BamH1
BamH1
EcoR1
IS1
3.1kb
2.3kb
pCC1
Amp^r

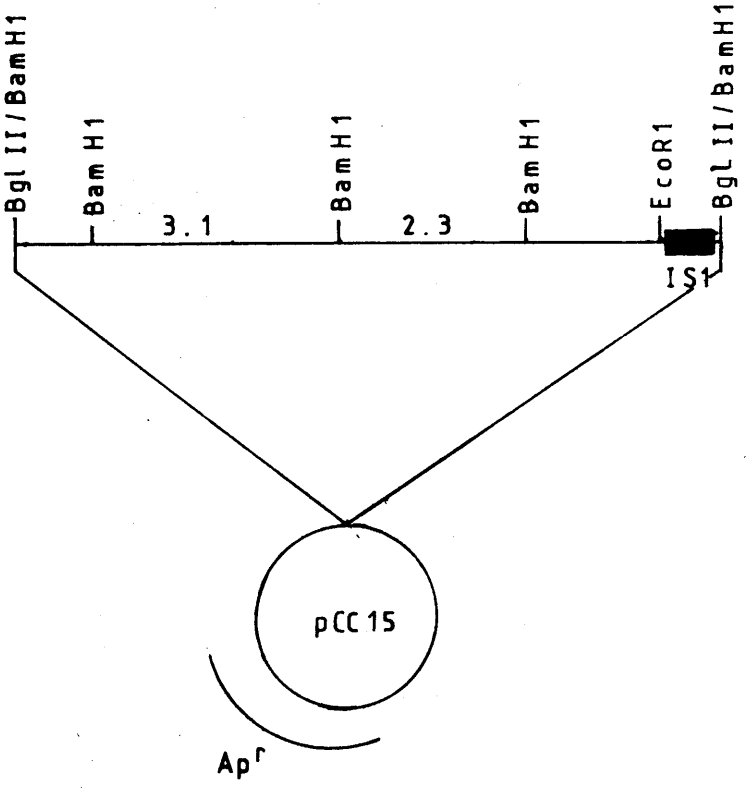
Figure 3.24. Structure and construction of pCC15 and pCC16.

(a). Figure showing the position of the 9.4kb BglII fragment in the amplification. Sizes given are in kilobases.
(b). Figure showing the orientation of the cloned 9.4kb BglII fragment in pCC15. pCC16 contains the fragment in the opposite orientation. The vector used was pUC8.

(a).


IS1
IS1
IS1
IS1
IS1
Bgl II
2.1
9.4
2.1
9.4
2.1
9.4
2.1
9.4
2.1
pCC15/pCC16

(b).


Bgl II/BamH1
Bam H1
3.1
BamH1
2.3
Bam H1
EcoR1
Bgl II/BamH1
IS1
pCC15
Ap^r

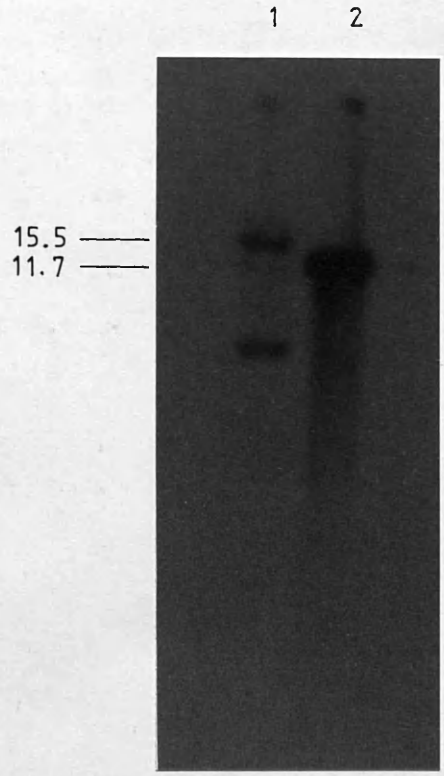


Figure 3.25. Hybridization of ^{32}P-labelled pCC1 to a Southern blot of chromosomal DNA restricted with EcoR1

Track 1: JEF8 Track 2: Cut1

plasmids and digested with EcoR1 and BamH1 to identify clones of the 11.7kb amplified fragment. Although the fragments purified from the agarose gel predominantly contained the 11.7kb amplified fragment, many other fragments of the same or similar size were also present and therefore BamH1 digests were necessary to identify clones that contained the 11.7kb amplified fragment. Two clones, pCC1 and pCC5, were isolated, both of which contained the 11.7kb amplified fragment but in opposite orientations (Fig.3.23).

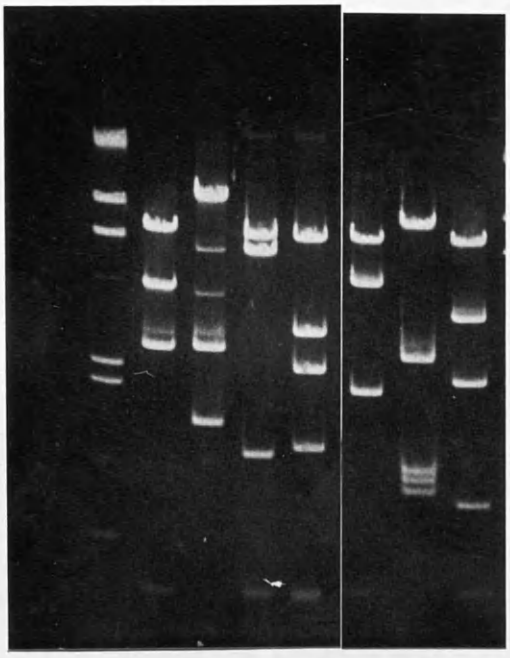
In order to clone the remaining part of the amplified unit which was not contained within the 11.7kb EcoR1 fragment it was decided to clone the 9.4kb amplified fragment generated by restriction with BglII. Chromosomal DNA from Cut1 was digested with BglII, run on an agarose gel and the intense 9.4kb fragment excised and purified. This was ligated into the BamH1 site of pUC8. (The "sticky-ends" produced by restriction with BamH1 are compatible with those generated by restriction with BglII). Plasmids with cloned inserts were analysed by restriction digestion. Two clones were obtained, pCC15 and pCC16, which contained the 9.4kb BglII fragment in opposite orientations (Fig.3.24).

3.11 Confirmation of cloning of the amplified unit

(1). Hybridization of pCC1 to Cut 1 and JEF8

Chromosomal DNA from Cut1 and JEF8 was digested with EcoR1, run on an agarose gel and Southern blotted. pCC1 was nick-translated and hybridized to the Southern blot. The autoradiograph (Fig.3.25) shows that pCC1 hybridizes strongly to the 11.7kb amplified fragment in Cut1 and also to the original non-amplified 15.5kb fragment in both JEF8 and Cut1 confirming that pCC1 contains the 11.7kb EcoR1 amplified fragment. The very strong hybridization of pCC1 to Tn2901 allows its use as a very efficient probe since autoradiographs require significantly shorter exposure times than with similar blots probed

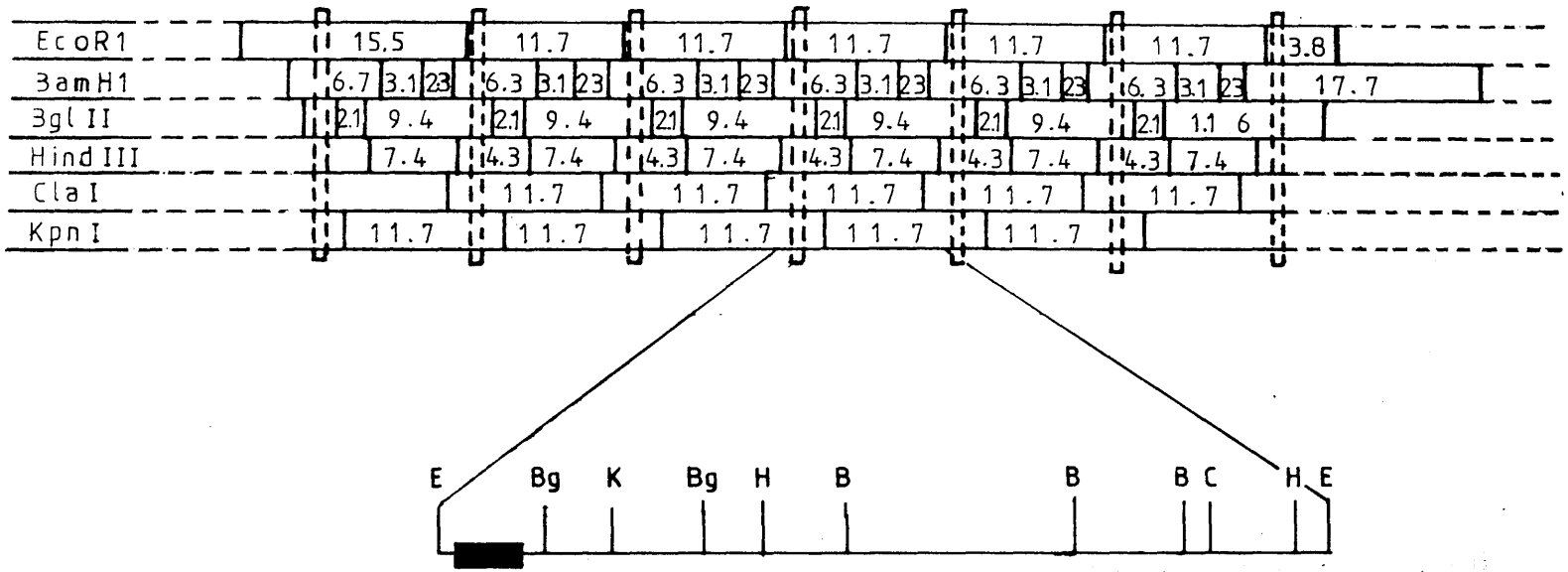
(a).



(b).

Digest	sizes
HindIII + EcoR1	7.4, 4.3, 2.7, 0.4
ClaI + EcoR1	10.0, 2.7, 1.8
ClaI + HindIII	7.0, 6.0, 1.4, 0.4
BamH1 + HindIII	7.0, 3.1, 2.3, 1.9, 0.4
KpnI + HindIII	7.4, 4.5, 2.1, 0.4
KpnI + BglII + EcoR1	8.6, 2.7, 1.2, 1.0, 0.9
BglII + HindIII	7.4, 3.7, 2.2, 0.7, 0.4

(c)



(Scale 1cm = 1kb)

Figure 3.26 . Restriction analysis of pCC1

(a) Restriction digests :

Track 1: Lambda HindIII

Track 2: HindIII + EcoR1

Track 3: ClaI + EcoR1

Track 4: ClaI + HindIII

Track 5: BamH1 + HindIII

Track 6: KpnI + HindIII

Track 7: KpnI + BglII + EcoR1

Track 8: BglII + HindIII

(b). sizes of restriction fragments in (a).

(c).Restriction map showing positions of EcoR1, BamH1, BglII, HindIII, ClaI and KpnI sites within the amplification. All restriction fragment sizes given are in kilobases.

with pMC23.

(2). Do pCC1, pCC5, pCC15 and pCC16 express argF?

The argF gene sequence (Piette et al, 1982; Van Vliet et al, 1984) indicates that the complete argF gene and its control region should be present in the cloned 11.7kb EcoR1 and 9.4kb BglII fragments. To determine whether this is the case, plasmid DNA from each of the four clones was used to transform NGX2, an $argF^-$ $argI^-$ strain. The transformed cells were plated on L-agar containing ampicillin and later patched onto minimal agar containing leucine, proline and uracil to screen for Arg^+ transformants. The transformed cells were also plated directly onto the minimal agar to select directly for Arg^+ transformants. No Arg^+ transformants were obtained using either pCC1 or pCC5. Both pCC15 and pCC16 expressed argF but pCC16 transformants grew very slowly on minimal agar and did not appear until 24 hours after pCC15 transformants.

3.12 Mapping of the KpnI, ClaI and HindIII restriction sites within Tn2901

Molecular analysis of the mechanism of formation of the amplification may be facilitated by the mapping of further restriction sites within Tn2901. Therefore, pCC1 was restricted with various restriction endonucleases to identify restriction enzymes which cut infrequently within Tn2901. ClaI and KpnI have single sites and HindIII has two sites within Tn2901. The positions of the ClaI, KpnI and HindIII restriction sites were mapped using double digests with EcoR1, BamH1 and BglII. Similar digests are shown in Figure 3.26a and the fragment sizes are given in Figure 3.26b. The deduced map positions are shown in Figure 3.26c. Restriction digestion of pCC1 also indicated that the 11.7kb EcoR1 fragment of Tn2901 contains 3 SmaI sites, 3 SalI sites, 3 AvaI sites, and 4 (or more) PvuII and AccI sites (data not shown). The

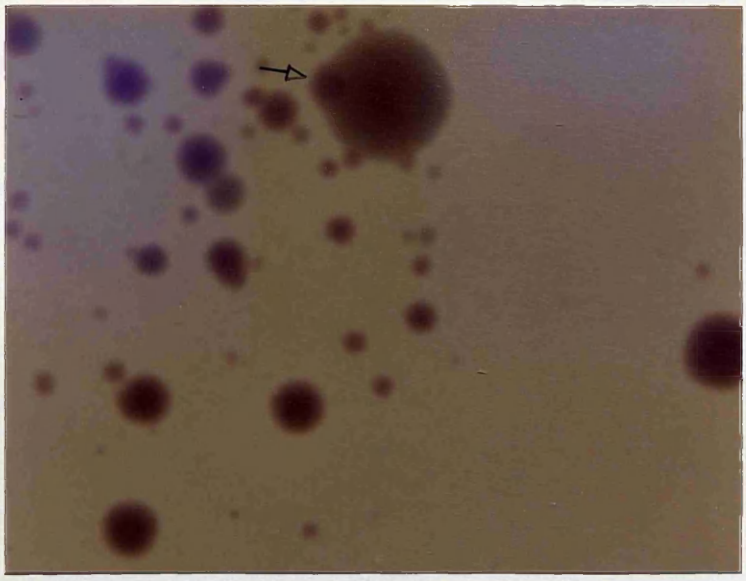
positions of these sites within Tn2901 were not determined.

3.13 An additional method for screening and selection of the amplification

In order to test such things as the effects of genetic background on the formation of the amplification, and to examine the formation of "stable" Cut mutants, it would be very useful to have a quick additional method with which to screen for the presence of the amplification. Stability testing of Cut mutants is very time consuming and the results are variable between US Cut mutants. pCC1 has provided a probe which hybridizes very strongly to patched colonies containing the amplification and is currently used to screen colonies for the presence of the amplification. However, hybridization procedures require a considerable amount of work, and there is a limit to the number of colonies that can be routinely screened. Alternative selection methods for the amplification would also be useful since it may be possible to isolate intermediates of Tn2901 copy number by different selection procedures.

Crystal violet is a rosanilin indicator dye (Proctor and Rownd, 1982) which binds to chloramphenicol acetyl transferase (CAT, Tanaka et al,1971; 1974), the product of the CAT gene. Colonies containing a Cm^r plasmid are dark purple, whereas Cm^s colonies are pale coloured. The 2.3kb BamH1 fragment from pCC1 was subcloned into pCB101, and this plasmid, pCC7, was forced to integrate into the chromosome of JEF8 at Tn2901 (for details of construction, see Chapter 6). This strain, JEF8 pCC7, therefore has the CAT gene inserted internal to Tn2901. It was observed that this Cm^r strain produces pale colonies on minimal agar containing crystal violet (1.8ug per ml), perhaps due to the presence of only a single copy of the CAT gene. Therefore, if JEF8 pCC7 were plated on citrulline and crystal violet it might be possible to

(a). JEF8 pCC7, 2



(b). JEF8 pCC7, 4

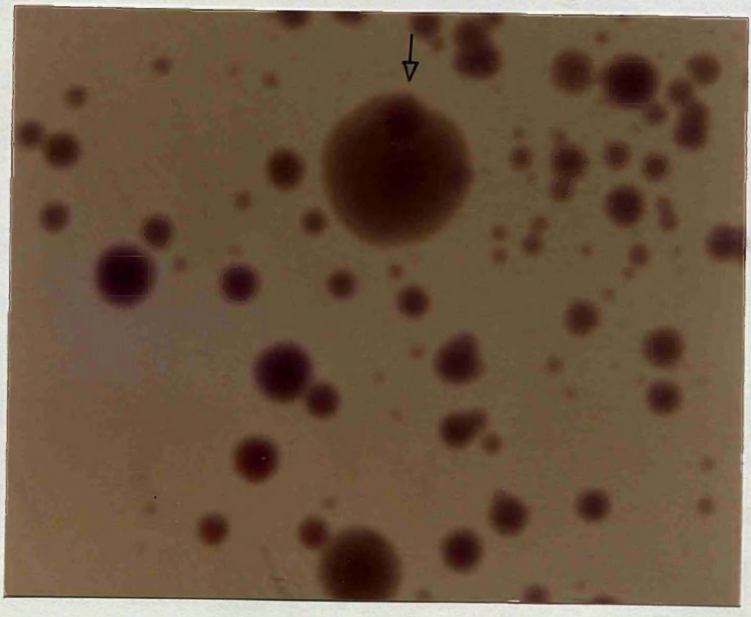


Figure 3.27 The use of crystal violet as an indicator of the amplification of Tn2901.
JEF8 pCC7 strains were plated on minimal agar supplemented with citrulline, methionine, threonine, thymine, glucose and crystal violet. The plates were photographed after 7 days incubation at 37°C. The dark purple colonies contain the amplification; the majority of the pale colonies do not. The dark purple sectors present in pale coloured colonies are marked with an arrow.

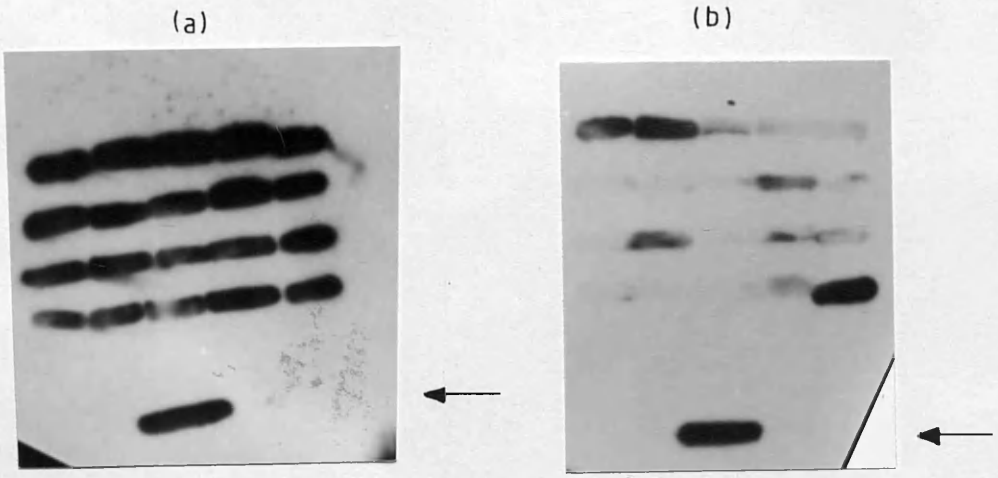


Figure 3.28. Hybridization of ^{32}P-labelled pCC1 to patch transfers of pale and purple colonies taken from minimal medium containing citrulline and crystal violet. The isolated patch on each filter (arrowed) Cut1 as a positive control.

(a) purple colonies (b) pale colonies

Table 3.3 Cut yield from JEF8 pCC7,4

N^o mutants per 10^8 viable cells	
pale coloured	dark purple
1.8×10^3	2.4×10^3

Since approximately $^3/_{20}$ pale coloured colonies contain the amplification, this represents 2.7×10^3 amplified and 1.6×10^3 non-amplified Cut mutants per 10^8 viable cells. JEF8 pCC7,4 was grown in minimal medium supplemented with arginine, uracil, methionine, threonine and glucose. The cultures were inoculated with 0.1ml of a 10^{-2} dilution of an overnight culture. 10^0, 1:2, and 10^{-1} dilutions of cultures were plated on minimal medium supplemented with citrulline, methionine, threonine, glucose and crystal violet. The total number of Cut mutants from four pates was counted (this varied from 52 - 201 colonies).

distinguish between amplified and unamplified colonies due to the difference in copy number of the CAT gene as a result of amplification of Tn2901. However, in JEF8 pCC7 the integration of pCC7 in Tn2901 results in an insertion of 7.3kb and this may prevent amplification of Tn2901 since the two IS1 elements are approximately 18kb apart.

When JEF8 pCC7 is plated on minimal agar supplemented with citrulline, methionine, threonine, thymine and crystal violet, two shades of purple colonies are seen (Fig.3.27). This suggests that colonies of JEF8 pCC7 containing an amplified Tn2901, and therefore an amplified CAT gene , are a darker purple than unamplified colonies. To confirm that dark purple colonies contain the amplification, 20 dark purple colonies and 20 pale coloured colonies were patched onto minimal agar containing citrulline and crystal violet and transferred to Hybond-N nylon membrane. The membranes were hybridized to radiolabelled pCC1. The autoradiograph (Fig.3.28) indicates that all the purple colonies contain the amplification, but only $^{3}/_{20}$ pale coloured colonies contain the amplification. The presence of these pale colonies containing the amplification may be explained by the loss of pCC7 or Cm^{r} from these colonies. Unfortunately, chloramphenicol cannot be added to the medium containing crystal violet since the drug interferes with the colour difference on the agar plates. The number of dark purple and pale coloured colonies obtained from JEF8 pCC7 is shown in Table 3.3. Since approximately $^{3}/_{20}$ pale coloured colonies contain the amplification, this represents 2.7 x 10^{3} amplified and 1.6 x 10^{3} non-amplified Cut mutants per 10^{8} viable cells. Two F^{-} strains, P678.14 and D7-8, yield 3 and 30 Cut mutants per 10^{8} viable cells respectively (Jessop and Glansdorff, 1980). Therefore, the 1.6 x 10^{3} non-amplified Cut mutants per 10^{8} viable cells obtained from JEF8 pCC7 represents a much higher yield than that obtained from the F^{-} strains. Genetic data in progress (A.P. Jessop, pers. comm.) correlates a high yield of non-amplified ("stable" Cut) mutants with the presence of the argF gene, and it is likely that these mutants are in some way related to the US Cut mutants.

Most pale colonies produce dark purple sectors after several days' incubation (Fig.3.27). The majority of pale colonies produce a single dark purple sector, but up to five sectors have been observed within a single colony. These sectors occur in well-separated colonies and cannot simply be due to several colonies growing closely together.
It was hoped that the presence of the CAT gene in Tn2901 would allow amplification of Tn2901 by a different selection procedure, namely that of increased chloramphenicol concentration in the medium. However, the single copy CAT gene was found to be resistant to very high levels of chloramphenicol (up to 500ug per ml), and so this selection procedure was unsuccessful.

DISCUSSION

The results presented in this chapter show that the US Cut phenotype is associated with the amplification of Tn2901, a 12kb unit which contains the argF gene and is flanked by two copies of IS1 in direct repeat. The amplified DNA was visualised on an agarose gel as one or more intense restriction fragments present in restriction digests of chromosomal DNA. No plasmid bands were seen in undigested DNA which indicates that the intense restriction fragments in the US Cut isolates represent chromosomal fragments which are present in multiple copies compared with those in the parental strain, JEF8. All of the US Cut isolates examined produced the same pattern of intense restriction fragments and from these the size of the amplified DNA was calculated to be 11.7kb. A stable Cut isolate, Cut12, did not produce any intense restriction fragments. The size of Tn2901, 12kb, suggested that it may be the unit of amplification since the tandemly repeated IS1 elements could possibly act as sites of homology in the formation of

the amplification. All the restriction data also suggested Tn2901 as the unit of amplification and hybridization of IS1 to the amplified 11.7kb EcoR1 fragment confirmed this. The original argF-containing fragment and a novel amplified argF-containing fragment were present in each restriction digest probed with pMC23 indicating that the amplified DNA consists of a series of tandem repeats. IS1 hybridized to a novel 980bp BglII/EcoR1 amplified fragment which indicates that a single IS1 element is present at each novel joint in the amplification. The insertion of the CAT gene into Tn2901 provides a convenient screen for the amplification. Interestingly, the frequency of amplification is not reduced by the insertion of 7.3kb of DNA into Tn2901 showing that the size of the amplified unit is not critical to the amplification. The presence of dark purple sectors in pale colonies suggests that the amplification can occur spontaneously from unamplified Cut mutants.

Amplification or duplication of regions bounded by direct repeats of IS1 have been described and include the r-determinants of the composite R plasmids NR1, R1 and R100-1 (Huffman and Rownd, 1984; Peterson and Rownd, 1985); bacteriophage P1 Ap Cm (Froehlich et al, 1986) and Tn9 in bacteriophage P1 Cm (Meyer and Iida, 1979). No amplification of IS1-flanked chromosomal genes has been previously described. The sizes of the regions amplified or duplicated varies from 2.7kb (Tn9) to 17-23kb (P1 Cm Ap) and Tn2901 is within this size range. Amplification of the r-determinant of NR1 in E. coli and Salmonella typhimurium, but not in Proteus mirabilis, involves an IS1-mediated deletion (Huffman and Rownd, 1984). Huffman and Rownd have suggested that there may be a gene or region of the plasmid within the 0.6kb deleted region which is an inhibitor of the amplification in E. coli and S. typhimurium but not in P. mirabilis. No deletion extending from either of the directly repeated IS1 elements of Tn2901 has been detected. The BglII and EcoR1 restriction sites closely flanking each IS1 element in the amplification have been shown to be present and it is likely that one of these restriction sites would have been deleted

if an IS1-mediated deletion was involved.

Two types of model have been proposed to explain the tandem amplification of drug resistance genes on R plasmids (Hu et al, 1975; Ptnashneand Cohen, 1975; Clewell and Yagi, 1977; Anderson and Roth, 1977) and these may be relevant to the mechanism of amplification of Tn2901. One model involves the excision of the resistance transposon followed by re-integration into a second R plasmid molecule. In the R plasmid NR1 excision and re-integration occurs by reciprocal recombination between two IS1 elements (Hu et al, 1975; Ptnashe and Cohen, 1975). In the other model, recombination between the daughter chromosomes after replication gives rise to tandem duplication and deletion by recombination between the IS1 elements (Anderson and Roth, 1977; Clewell and Yagi, 1977). Further amplification in both models may result from recombination between homologous sequences within the duplicated region. Amplification of drug resistance genes on R plasmids is recA-dependent (Huffman and Rownd, 1984; Meyer and Iida, 1979) as is amplification of Tn2901 (Jessop and Glansdorff, 1981). A low level of duplication of the R-determinant of R100-1 was detected by hybridization techniques (Chandler et al, 1979) and Chandler et al (1979) have suggested that amplification may involve an initial relatively infrequent recA-independent duplication followed by amplification by a more efficient recA-dependent process.

The copy number of the amplified unit in the US Cut isolates was estimated to be 45. Amplification of the argF gene may result in an increase of the argF gene product, OTCase, and this alone might be expected to be sufficient to produce the US Cut phenotype - the multiple gene copies titrating out repressor molecules and overcoming the normal regulatory control of the argF gene. However, it has been suggested (Legrain et al, 1976b) that the lack of repressibility of OTCase in the US Cut isolates cannot be explained by exhaustive binding of the arg repressor on multiple copies of the argF operator since US Cut mutants exhibit normal repressibility of N-a-acetylornithinase (another enzyme of the arg regulon). It is therefore

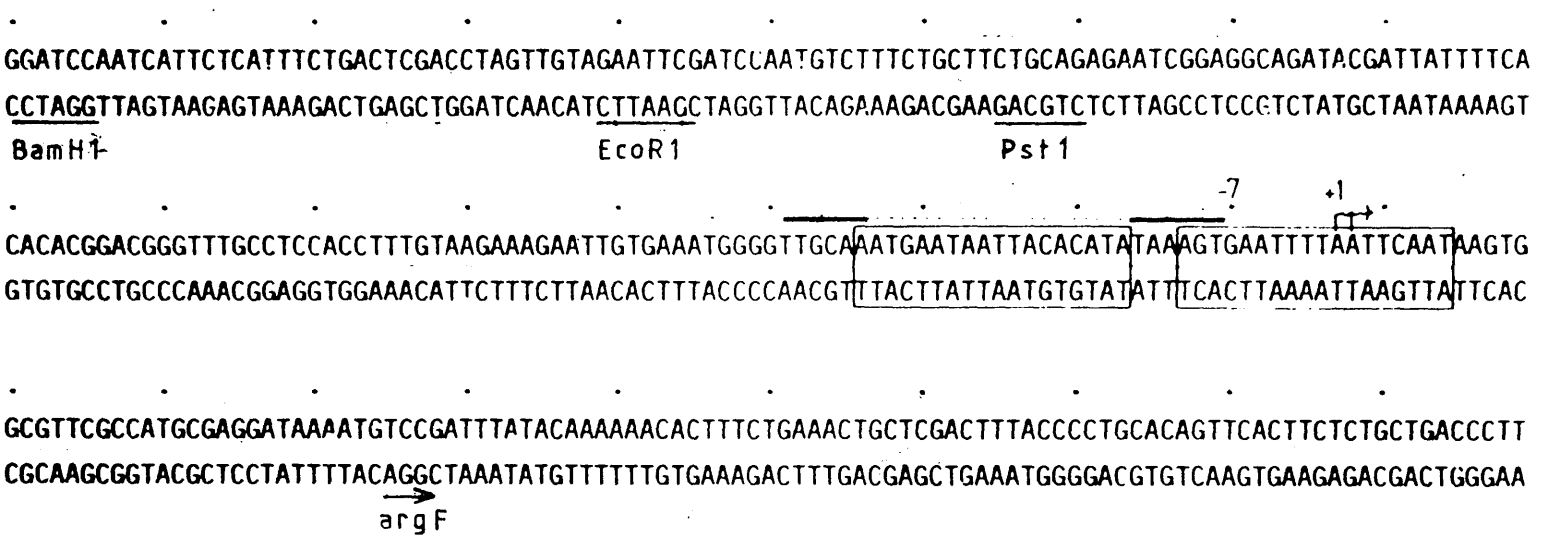


Figure 3.29. Nucleotide sequence of the control region of the argF gene. Restriction sites are underlined. The promoter argFp is indicated by two full lines, corresponding to the -35 and -10 sequences; also indicated are the two start points of transcription. Operator sequences are boxed. (After Piette et al, 1982)

more likely that one or more copies of the argF gene is not under the control of the arg repressor. The question of how many copies of Tn2901 are expressing argF is further discussed in Chapter 4. The dot blots indicated that both of the US Cut isolates examined, Cut1 and Cut8, contain approximately the same number of copies of the amplified unit. No attempt has been made to select for further amplification of Tn2901 and it is therefore unknown whether the 45 copies produced is a result of the selection system used or whether there is some cellular control over the number of copies of Tn2901 being produced.

The 11.7kb EcoR1 fragment was cloned into pUC9 in both orientations to produce plasmids pCC1 and pCC5. Neither orientation of the cloned fragment expressed the argF gene when transformed into NGX2, an argF$^-$ argI$^-$ strain. This suggests that the complete argF gene is not contained within the cloned 11.7kb EcoR1 fragment. However, the argF gene sequence (Fig.3.29) (Piette et al,1982; Van Vliet et al,1984) shows the EcoR1 site to be approximately 200bp proximal to the transcription startpoint of the argF gene and it also shows both the promoter and operator sequences to be entirely contained within the EcoR1 fragment. Furthermore, Moore et al (1981) have cloned a functional argF gene on a fragment delimited by two Pst1 sites - the Pst1 site being approximately 22bp fom the EcoR1 site towards the argF gene. Piette et al (1982) also reported the isolation of a lambda darg transducing phage harbouring a functional repressible argF gene on a 4.1kb fragment terminating between the Pst1 site and the promoter-operator region (Fig.3.29). Therefore the cloned 11.7kb EcoR1 fragment should also contain a functional argF gene.

Crabeel et al (1979) cloned a functional argF gene on a 1.66kb BamH1 fragment. However, an argF-containing BamH1 fragment of this size was not present in JEF8 or the US Cut isolates. Hu and Deonier (1981) also reported that no 1.66kb argF-containing BamH1 fragment was present in their laboratory strains. Sequence analysis of the 1.66kb BamH1 fragment (Van Vliet et al,1984) indicates that the BamH1 site lies 40bp from the EcoR1 site (Fig.3.29). The 1.66kb BamH1 fragment was

cloned in the BamH1 site of pBR322. The cloned fragment expressed argF in both orientations (Crabeel et al,1979) and this was confirmed in NGX2, the argF⁻argI⁻ strain. Readthrough synthesis from the tetracycline promoter produced extremely high levels of OTCase in one orientation and expression in this orientation was only weakly repressible by arginine (Crabeel et al,1979). In the opposite orientation, where no readthrough should occur from the Tc promoter, the argF gene was expressed to a lesser extent and extensive repressibility was observed in the presence of arginine. Therefore both pCC1 and pCC5 should be capable of expressing the argF gene in NGX2. The 11.7kb EcoR1 fragment is orientated in pCC1 such that there should be readthrough from the lacZ promoter in pUC9 into the argF gene and this should result in high levels of OTCase being produced. Readthrough synthesis is known to be relatively insensitive to repression exerted at sites distal to a transcription start (Reznikoff et al,1969; Franklin,1971) and therefore pCC1 should express argF even in the presence of arginine.

The 9.4kb BglII amplified fragment from Cut1 was cloned into the BamH1 site of pUC8 in both orientations producing plasmids pCC15 and pCC16. When transformed into the argF⁻argI⁻ strain NGX2 both plasmids expressed the argF gene although pCC16 was very slow growing. pCC15 is cloned in the orientation such that there may be readthrough from the lacZ promoter towards argF although the argF gene is approximately 980bp from the end of the cloned fragment with a complete IS1 element separating the lacZ promoter from the argF gene. However readthrough from the lacZ promoter to the argF gene would account for the difference in expression of the argF gene in pCC15 and pCC16. Alternatively, the slow growth of pCC16 (in NGX2) on minimal agar without arginine could be due to the over-expression of an unidentified gene on the cloned fragment whose product is deleterious to cell growth. However, pCC15 and pCC16 (in NGX2) both have normal growth rates on L-agar supplemented with ampicillin which suggests that over-expression of such a gene is not responsible for the slow

growth rate of pCC16 on medium lacking arginine.

Expression of argF from the 9.4kb BglII fragment but not from the 11.7kb EcoR1 fragment in pCC1 or pCC5 further suggests that the complete argF gene is not present on the 11.7kb EcoR1 fragment. One possible explanation for the non-expression of the 11.7kb EcoR1 fragment is that the EcoR1 site in JEF8 is in a different position from the EcoR1 site in the published sequence (Piette et al, 1982; Van Vliet et al, 1984). An alternative explanation for the non-expression of argF in pCC1 and pCC5 is that not all the copies of the argF gene in the amplification are functional. Therefore a non-functional argF gene could perhaps have been cloned in pCC1 and pCC5 although this is only speculative.

D30-1, a recombinant produced by crossing a US Cut mutant with P678.14, yields 1000-fold more US Cut mutants than JEF8 (Jessop and Glansdorff, 1980). This strain was shown to contain the 11.7kb EcoR1 and 980bp EcoR1/BglII novel joint fragments, indicating that D30-1 contains at least two copies of Tn2901. The exact number of copies of Tn2901 has not been determined although it is unlikely to be greater than five. The unusually high yield of Cut mutants obtained from this strain can be explained by unequal crossing over between copies of Tn2901 to produce the amplification. The few copies of Tn2901 present in D30-1 could either have originated from a segregant present in the donor culture, or several copies of Tn2901 could have been recombined into the recipient chromosome from a donor containing the amplification. The number of copies of Tn2901 in D30-1 is not sufficient to produce the US Cut phenotype since D30-1 is not a US Cut mutant. Further segregation to produce a single copy Tn2901 has not been observed (A.P. Jessop, pers. comm.).

Differences were noted in the pattern of hybridization to IS1 of two strains - P678.14 and D30-1. These strains may contain one or more extra IS1 elements not present in JEF8 or the US Cut isolates. However, since no differences were noted between JEF8 and the US Cut isolates it is unlikely that these IS1 elements are involved in the

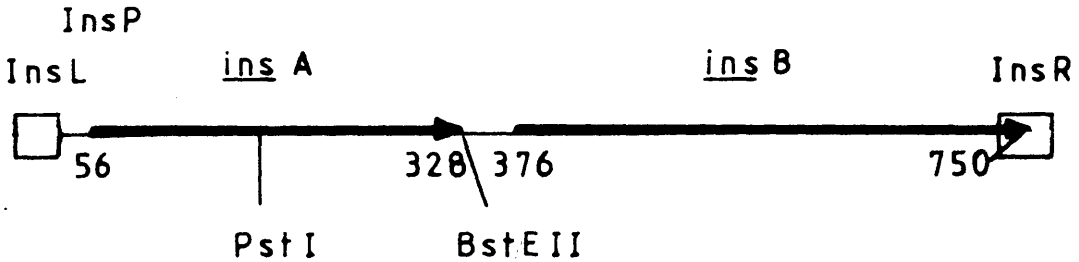
amplification. The possibility of secondary rearrangements involving other insertion sequences is discussed in Chapter 5.

Several US Cut isolates produced second amplified bands when hybridized to argF which were not visualized on the corresponding agarose gels of EcoR1 restriction digests and these second amplified bands were not reproducible when the Southern hybridization was repeated. The most likely explanation for these conflicting results is that the DNA contains some partially restricted fragments, and that the second intense fragment seen in several of the US Cut isolates represents partially digested DNA which, when fully restricted, would yield a single intense fragment of 12kb. This would explain the varying results of the two Southern blots while using the same DNA preparation. Alternatively, the second intense band may represent a second amplified fragment present in several of the US Cut isolates. The questions then remain of why the corresponding intense fragment was not seen on an agarose gel and why the band patterns differed between the two Southern blots. The latter can perhaps be explained by uneven hybridization such that the two amplified fragments each bound different amounts of probe. This may be theoretically possible where the probe DNA concentration was limiting. This however seems unlikely since artefacts of this type were not seen on any other Southern blots of chromosomal DNA containing amplified bands. To avoid such artefacts in future hybridizations it was decided to use a single US Cut isolate, Cut1, which gave consistent results in the two Southern blots probed with argF, for further molecular analysis and cloning.

Another faint band of 8-9kb hybridized to argF although it appears not to be related to the structure of the amplification. Sequence analysis has shown the duplicate gene for OTCase, argI, to be approximately 76% homologous to the argF gene (Kikuchi and Gorini,1975) and therefore the 8-9kb argF-containing band may represent the argI gene.

CHAPTER 4

Preliminary RNA and Protein Analysis of Tn2901



Calculated transcript length = 750 - 56 bp = 694 bp

polypeptides (M.W.): Ins A 10,000 (91 amino acids)

Ins B 15,000 (125 amino acids)

Figure 4.1. Figure showing the two putitive coding frames of IS1, insA and insB. These genes are assumed to be transcribed polycisronically, and hence should produce a transcript of approximately 694 bp. insA encodes a polypeptide of 91 amino acids (approximately 10,000 m.w.) and insB encodes a polypeptide of 125 amino acids (approximately 15,000 m.w.). Polypeptide sizes are from Ohtsubo et al (1981).

This chapter attempts to determine whether the 45-fold amplification of Tn2901 in the US Cut mutants results in a similar increase in the argF gene product, whether the stable Cut mutants over-express argF, and whether any other polypeptides are encoded by Tn2901.

(1). Are all copies of Tn2901 expressing argF?

One might expect that a 45-fold amplification of the argF gene would result in the US Cut phenotype by simply titrating out the arg repressor and allowing expression of one or more copies of the argF gene. However, Legrain et al (1976b) have suggested that this is not the case since another enzyme of the arg regulon, N-a-acetylornithinase, remains repressible in US Cut mutants and so free repressor must be present. It is perhaps more likely that one or more copies of the argF gene is not under the control of the arg repressor. Therefore, a 45-fold increase in Tn2901 may not necessarily result in a 45-fold increase in OTCase.

(2). Are any other polypeptides encoded by Tn2901?

Tn2901 consists of approximately 11kb of chromosomal DNA flanked by two IS1 elements. This 11kb region contains only one known protein-coding gene, argF. argF is approximately 1kb long and hence the other 10kb represents a so-called silent region of the E. coli chromosome. However, this region may contain as yet unidentified protein-coding genes which will also be amplified in the US Cut mutants. IS1 is thought to encode two proteins, InsA and InsB, whose genes lie side by side in the same orientation and are assumed to be transcribed polycistronically (Fig.4.1). The total transcript length should therefore be approximately 0.7kb and the two proteins approximately 10kd and 15kd respectively (see Fig.4.1 for calculations).

(3). Do stable Cut mutants over-express the argF gene?

Patch hybridization of nick-translated pCC1 to Cut mutants obtained from JEF8, and the use of crystal violet as an indicator of the amplification in JEF8 pCC7, has revealed the presence of a class of non-amplified "stable" Cut mutants. These may be related in some way to the US Cut mutants since they are obtained at a much higher frequency from JEF8 than from F^- strains. It was decided to examine stable Cut mutants for over-expression of the argF gene.

It was decided to examine both the RNA transcripts produced by Tn2901 and the total cell protein in an attempt to provide answers to these questions.

RESULTS

4.1 Protein analysis

Protein samples were prepared from the following strains:

JEF8 grown in minimal medium supplemented with citrulline, uracil, methionine, threonine and glucose,
Cut5, Cut1 and Cut12 grown in minimal medium supplemented with citrulline, methionine, threonine and glucose.

F-prime strains
DF30D2 CSH56 grown in minimal medium supplemented with citrulline, uracil, glucose and thymine.
DF30D2 CSH56 II-45 (US Cut), I-4 (S Cut) and II-23 (S Cut) grown in minimal medium supplemented with citrulline, glucose and thymine.

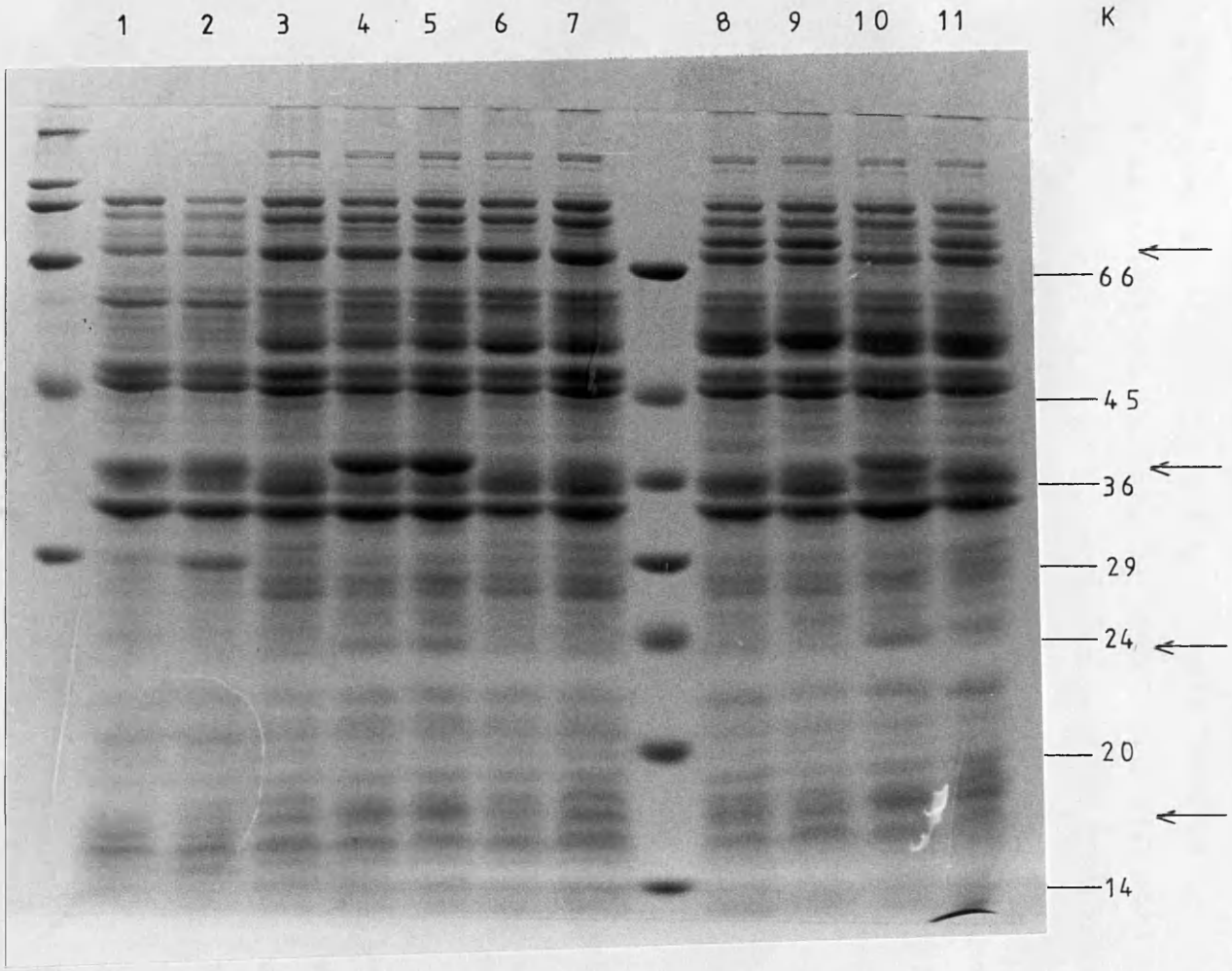

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Figure 4.2. SDS-polyacrylamide gel (for gel conditions see materials and methods).

Track 1: pCC15 in NGX2

Track 2: pCC21 in NGX2

Track 3: JEF8

Track 4: Cut5 (US Cut)

Track 5: Cut1 (US Cut)

Track 6: Cut12 (stable Cut)

Track 7: JEF8

Track 8: DF30D.2 CSH56 I-4 (stable Cut)

Track 9: DF30D.2 CSH56

Track 10: DF30D.2 CSH56 II-45 (US Cut)

Track 11: DF30D.2 CSH56 II-23 (stable Cut)

The other tracks contain molecular weight size standards. Molecular weights (in kilodaltons) are shown; differences in band patterns are indicated with arrows (see text for details).

Plasmid strains

pCC15 and pCC21 in NGX2 grown in minimal medium supplemented with leucine, proline, uracil and glucose.

The protein samples were run on an SDS-polyacrylamide gel and stained with coomassie brilliant blue (see materials and methods). The gel (Fig.4.2) shows an intense band present in the samples from Cut1 and Cut5 which is not present in JEF8, Cut12 or either of the plasmid tracks. This band is also present, although not so intense, in the sample from DF30D2 CSH56 II-45 (US Cut) but not in any of the other F-prime strains. This band is approximately 37kd in size which corresponds to the expected size of the argF gene product (36.8kd- Van Vliet et al, 1984). Several other differences in the banding patterns are observed (marked by arrows):

(1). an intense band of approximately 67kd is present in DF30D2 CSH56 and the two S Cut F-prime strains but is not present in the US Cut F-prime strain or any other tracks.

(2). two faint bands of approximately 24kd and 17kd appear to be present in the three US Cut tracks (Cut1, Cut5 and DF30D2 CSH56 II-45) but not in any other tracks.

(3). a strong band of approximately 29kd is present in the sample from pCC21. However, this band is also present in pBR322 alone (data not shown) and therefore is a vector-encoded polypeptide.

4.2 Northern analysis

RNA was prepared from cultures of JEF8 and Cut1 grown in minimal medium (as for protein analysis). The RNA samples were run on formaldehyde gels (1% and 1.5% agarose), blotted and hybridized to ^{32}P-labelled pCC1. The blot from the 1% gel was then washed and re-probed with ^{32}P-labelled pCC14 (IS1-containing 980bp BglII/EcoR1 fragment from pCC12).

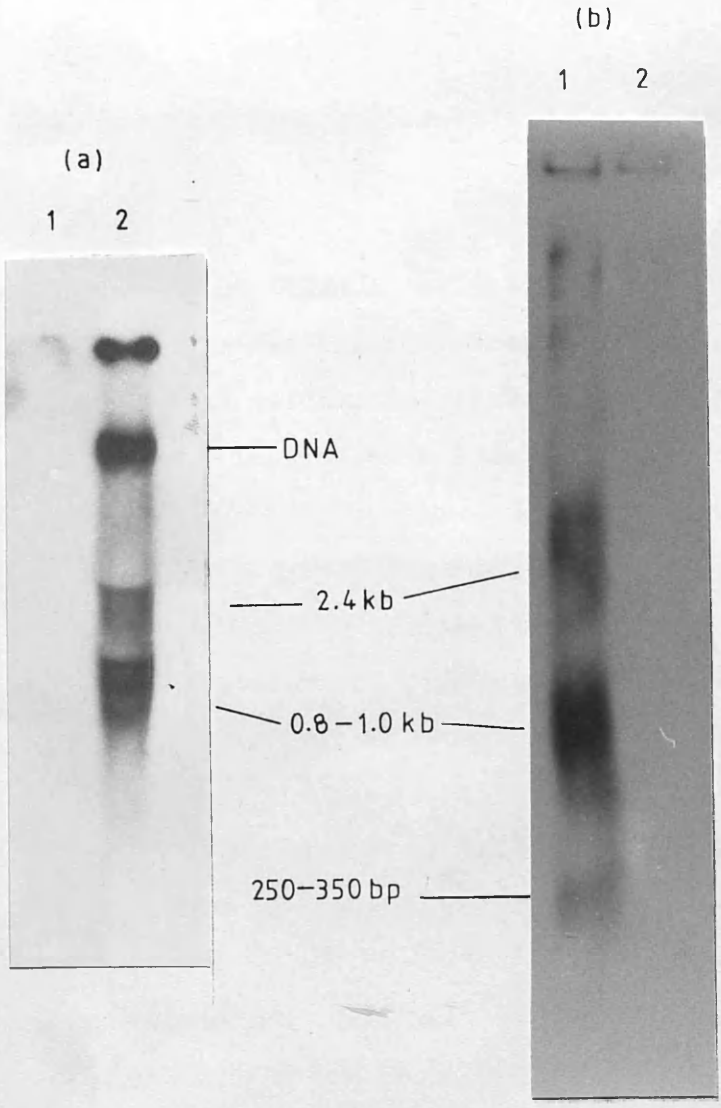


Figure 4.3. Hybridization of ^{32}P-labelled pCC1 to northern transfers of RNA isolated from JEF8 and Cut1.

(a) 1.5% agarose/formaldehyde gel

Track 1: JEF8 Track 2: Cut1

(b) 1% agarose/formaldehyde gel

Track 1: Cut1 Track 2: JEF8

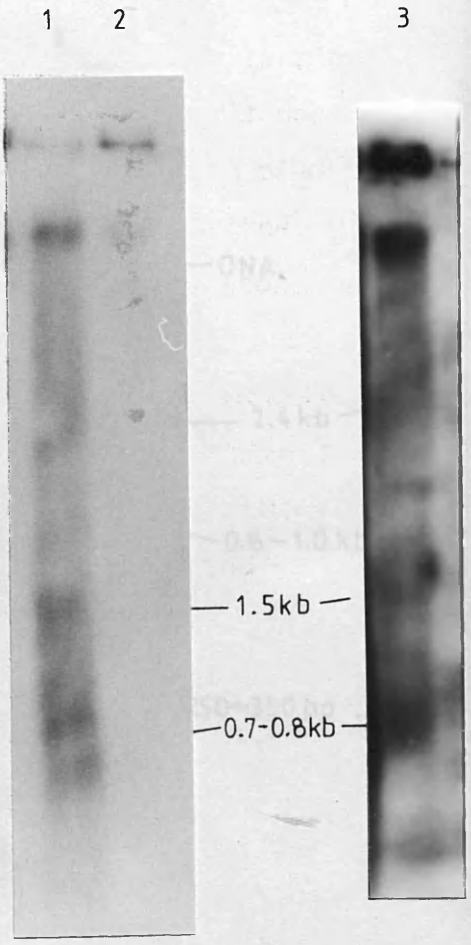


Figure 4.4. Hybridization of ^{32}-labelled pCC14 to northern transfers of RNA from JEF8 and Cut1. (The 1% agarose/formaldehyde gel previously probed with pCC1 was washed and reprobed).

Track1: Cut1 Track 2:JEF8

Track 3:JEF8(over-exposed)

The 1.5% agarose gel probed with pCC1 (Fig.4.3a) shows a minimum of three bands present in the Cut1 track but none in the track containing RNA from JEF8. Identical tracks of JEF8 and Cut1 stained with acridine orange appeared visually to contain similar amounts of RNA although no other method was used to determine the concentration of RNA in each track. The largest band represents contaminating chromosomal DNA. The second band is approximately 2.4kb. The third band is approximately 0.8-1.0kb and may be composed of several bands close together. The 1% agarose gel probed with pCC1 (Fig.4.3b) contains RNA prepared from different cultures. However, again no hybridization is seen in the track containing RNA from JEF8. RNA from Cut1 hybridizes to two bands of approximately 2.4kb and 0.8-1.0kb corresponding to the bands seen in the 1.5% agarose gel. A third band (which may be an artifact) is also seen in the 1% gel and is approximately 250-300bp in size. The northern blot re-probed with ^{32}P-labelled pCC14 (Fig.4.4) shows possible bands of 0.7-0.8kb and 1.5kb in the track containing RNA from Cut1 although the background is quite high. Two bands of similar size also appear to be present in the track containing RNA from JEF8. However, the JEF8 track required to be greatly over-exposed and hence it also has a very high background.

DISCUSSION

Expression of argF in the US Cut mutants

The results presented in this chapter suggest that at least two transcripts are produced from Tn2901 in the US Cut isolates, of approximately 2.4kb and 0.8-1.0kb. This latter transcript is probably the transcript from the argF gene. No transcripts were detected in RNA isolated from JEF8 probed with pCC1 which may be the result of poor hybridization techniques or of a low level of transcripts from Tn2901 in JEF8. The protein gel indicated an intense band of approximately 37kd (the expected size of the argF gene product) present in Cut5 and

Cut1 but not in JEF8. Therefore, the argF gene product is obviously greatly increased in the US Cut isolates compared to JEF8 but the extent cannot be determined from these preliminary results. The two plasmids pCC15 and pCC21 in NGX2 were grown in minimal medium lacking arginine to ensure expression of the cloned argF gene. However, both these plasmids showed no noticeable increase in the argF gene product on the protein gel. Northern analysis may be of further value in determining the relative increase in the argF gene product in the US Cut mutants. Growth of JEF8 in minimal medium containing ornithine and citrulline should ensure expression of the argF gene and this transcript should then be detected by northern analysis. It will be necessary to continue to run RNA samples on formaldehyde gels if pCC1 is used as a probe since other transcripts from Tn2901 would complicate the results of dot blots. Also the RNA concentration in different samples requires better quantitation and this may be achieved by running a non-denaturing gel to give better visualisation of the RNA and hence better quantitation.

Expression of argF in stable Cut mutants and F-prime strains

The protein gel shows no noticeable increase in the argF gene product in Cut12 or the stable Cut F-prime strains, DF30D2 CSH56 I-4 and II-23 indicating that stable Cut and US Cut mutants differ in their expression of argF. Also the US Cut F-prime strain, DF30D2 CSH56 II-45, contains a less intense 37kd band than Cut1 and Cut5. This suggests that either the amplification carried by the F-prime contains less copies of Tn2901 than the chromosomal amplification, or that expression of argF is altered on the F-prime.

Are any other polypeptides encoded by Tn2901?

The protein gel shows two faint bands of 17kd and 24kd present in the US Cut isolates but not in any other strains. These bands may simply be artifacts caused by, for example, differences in sample concentration or they may represent polypeptides present in greater

quantity in the US Cut isolates than in other strains. The 24kd polypeptide is probably too large to be encoded by IS1 and may represent the product of an unknown gene in Tn2901. The 17kd polypeptide could possibly be encoded by IS1 since the *insB* gene encodes a polypeptide of 15kd. A higher percentage SDS-polyacrylamide gel would give better visualisation of the bands and would also show polypeptides smaller than 14kd.

The northern blots of Cut1 probed with pCC1 suggest that Tn2901 produces two transcripts of 2.4kb and 0.8-1.0kb, the latter probably representing the *argF* transcript. Hybridization to pCC14 suggested possible bands of 1.5kb and 0.7-0.8kb. This latter band may represent the IS1 transcript, although the region of 0.7-1.5kb has a high background.

Both the northern and protein analysis suggests that the 11kb of chromosomal DNA in Tn2901 does not encode more than two or three polypeptides. If this is the case, then the question arises of whether the rest of Tn2901 is simply "junk" DNA, and if so then why is Tn2901 maintained in *E. coli* K12? The genome size of an organism can be thought of as the result of an evolutionary balance between forces tending to increase it, such as duplications, and those tending to reduce it, such as deletions. The arguments for and against the retention of "junk" DNA in a highly economic prokaryotic organism such as *E. coli* have been well documented (Cavalier-Smith, 1985). It has been suggested that one possible function for non-genic DNA is that of "protection" of the genic DNA against mutagens and transposable elements. However, there is extensive experimental evidence showing that large genomes are proportionally more sensitive to radiation and are under continuous powerfull selection to minimize such damage (Maynard Smith, 1978). Extra non-functional DNA will therefore always be harmfull and will also place extra demands on the cell for energy and nutrients such as phosphate. Spare non-coding DNA is unlikely to be required for progressive evolution since new genes are far more likely to evolve by modification of old ones following gene

duplication than they are to arise de novo from non-coding DNA (Ohno, 1970; Cavalier-Smith, 1985).

If the 11kb chromosomal DNA carried by Tn2901 does not consist of "junk" DNA, then possible functions of the DNA must be considered. No argF gene has been detected in E. coli B, E. coli W, Klebsiella pneumoniae, Enterobacter aerogenes and Serratia marcesens (Legrain et al, 1972; 1976a; Matsumoto and Tazaki, 1970; Prozeski, 1968). Legrain et al (1976a) have therefore suggested that argF might be the product of a relatively recent transposition event peculiar to E. coli K12. If this is the case, it is possible that Tn2901 contains coding DNA functional in another organism but not in E. coli K12. This is perhaps unlikely since the argF gene is expressed in E. coli K12. Alternatively, Tn2901 could contain a silent gene or operon which is only activated under certain conditions similar to the cryptic bgl operon of E. coli K12.

Non protein-coding DNA can have several important functions:

(1). encoding RNA genes specifying functional RNA molecules, notably tRNA and rRNA

(2). DNA sequences that specify sites for replication initiation and termination without themselves coding for proteins

(3). DNA sequences that provide specific recognition sites for site-specific recombinases

(4). "segregator" sequences that provide specific sites for attachment of the bacterial chromosome to the cell membrane (Hendrikson et al, 1981)

Therefore, one or more such sequences may be present in Tn2901. However, it is unlikely that such sequences can account for the total non protein-coding DNA in Tn2901.

CHAPTER 5.

Possible roles of the F factor in the formation of the amplification.

F^- strains are unable to produce the amplification from a single copy of Tn2901. However, it has been shown (A.P.Jessop, unpublished results) that F^- cells can further amplify several copies of Tn2901. This implies that the F factor is required for the initial IS1-IS1 recombination but not for the further amplification of the duplicated copies. The F factor is required *in cis* for the formation of the amplification and so the involvement of an F factor-encoded trans-acting protein is unlikely.

Several reports in the literature are pertinent to the initial IS1-IS1 recombination event "activated" by the F factor. Braedt (1985) found that the IS1 elements of Tn9 could recombine in certain *E. coli recA* strains but not in others. Braedt (1985) proposed that an IS1 gene product is involved in the recombination event and that this recombinase is repressed in certain strains. The recombination was not detectable in Tn9 derivatives containing a mutation in the *insA* and *insB* open reading frames, suggesting that an IS1 gene product is involved in the recombination. Reif and Arber (1980) suggested that IS1 contains an internal resolution site analogous to that of Tn3 (Arthur and Sherratt, 1979) since insertion into the Pst1 site reduces transposition frequency without affecting IS1-mediated co-integration. Therefore, it is possible that the F factor could activate a recombinase necessary for the initial IS1-IS1 recombination event.

Recombination between the directly repeated IS1 elements flanking the r-determinants of NR1 in *P. mirabilis* occurs readily. However, in *E. coli* the transition requires a deletion extending from $IS1_b$ (IS1 itself is not deleted) and covering the *cam fus* and *str spc* regions of the r-determinants (Huffman and Rownd, 1984). It is not known why this deletion is necessary for the transition in *E. coli*; the deleted region may contain a gene which encodes an inhibitor of the transition in *E. coli* (Huffman and Rownd, 1984). Therefore, an analogous deletion or rearrangement mediated by the F factor may be required for the

initial IS1-IS1 recombination event in Tn2901.

Transposable genetic elements and insertion sequences, when inserted at a given locus, can cause large-scale rearrangements (e.g. deletions, inversions, F-prime plasmid excisions) of adjacent DNA sequences (Nevers and Saedler, 1977). The F factor contains four IS elements - IS2, two IS3 sequences and gamma delta. If an F factor IS element is involved in the initial IS1-IS1 recombination event, this would explain the requirement of the F factor in cis. Two types of IS-mediated rearrangement could be involved in the initial IS1-IS1 recombination event:

(A). a major rearrangement resulting in the physical fusion of two DNA sequences necessary for the initial IS1-IS1 recombination event. For example, a deletion extending from the F factor to Tn2901 may fuse an F factor promoter with a gene encoding an IS1-specific recombinase which is normally silent. Such a major rearrangement would result in a noticeably different restriction pattern on a Southern blot of chromosomal digests probed with an appropriate chromosomal fragment. The first part of this chapter investigates whether such a major rearrangement has taken place in the US Cut mutants.

(B). a minor rearrangement such as an inversion of a small IS element to activate a normally silent gene. Minor rearrangements are not fully investigated in this chapter because :-

(1) cloned fragments of chromosomal and F factor sequences were not available to probe the entire region between (and including) Tn2901 and the F factor.

(2) only a partial restriction map of the region has been published. The probes available - namely IS2, IS3 and gamma delta elements- often hybridize to large restriction fragments within which a minor rearrangement would not be easily detected. The use of restriction enzymes with unknown sites within the region was not desired since any minor rearrangement detected would not necessarily be adjacent to an F factor IS element.

The second part of this chapter attempts to determine whether conjugal transfer is required for the initial IS1-IS1 recombination event. Conjugation is the process by which DNA is transferred from a donor bacterium to a recipient bacterium by a mechanism involving contact between the donor and recipient cells. The transfer operon of F encodes and regulates all the proteins required for the transfer of DNA to the recipient cell. Transfer of chromosomal DNA by an Hfr strain has been shown to be responsible for several chromosomal mutations in E. coli (Berg et al, 1983; Syvanen et al, 1986; Kunz and Glickman, 1983). Since transfer of chromosomal DNA requires the presence of the F factor in cis, conjugal transfer may be required for amplification of Tn2901 which would explain the requirement of the F factor in cis adjacent to the unit of amplification.

Conjugal transfer enhances excision of Tn5 from sites in the lac operon when F and Tn5 are in cis in the same DNA molecule (Berg et al,1983). This excision event is recA-independent, is not correlated with transposition and does not require the Tn5-encoded transposase (Berg, 1977; Egner and Berg, 1981). A single strand of DNA is known to be transferred during conjugation (Gross and Caro, 1966; Cohen et al, 1968). This has lead Berg et al (1983) to propose a model of excision which involves the intramolecular reannealing of complementary sequences in Tn5's inverted repeats following single stranded transfer and slippage of nascent DNA strands during the copying of the single-stranded template. Presumably a similar mechanism could operate with the tandemly repeated IS1 elements in Tn2901 although whether this would result in a tandem duplication of Tn2901 is unknown.

Syvanen et al (1986) also found that stimulation of precise excision of Tn5 and Tn10 from sites within the bacterial chromosome is dependant upon conjugal transfer. They showed that the transposon did not need to be present on the transferred DNA molecule and that stimulation of excision occurred readily from the recipient chromosome. It is possible that the recipient bacteria are signalled to increase genetic recombination perhaps by the presence of single-

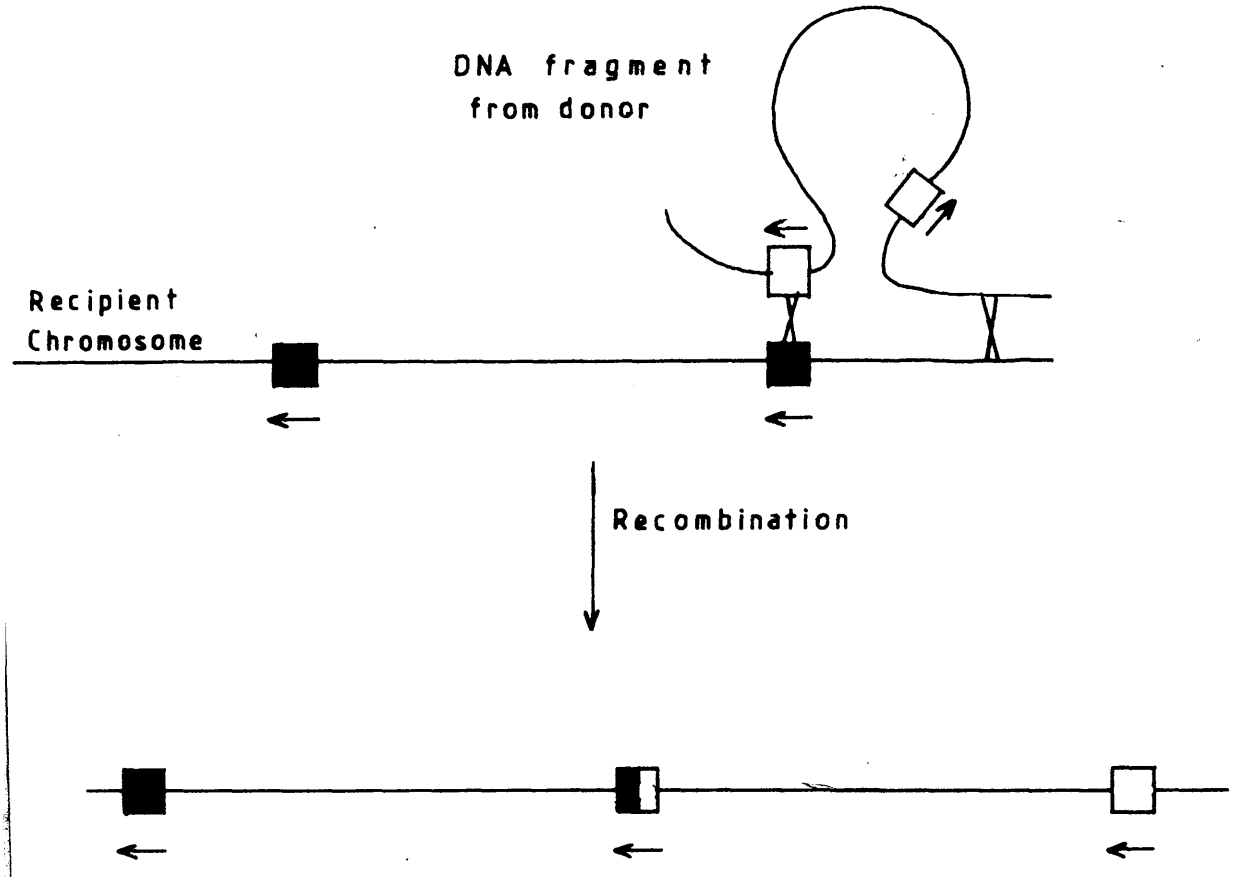


Figure 5.1. A possible mechanism by which conjugal transfer could result in the duplication of Tn2901. The IS1 elements of the donor molecule are represented as open boxes; those of the recipient chromosome as shaded boxes. Unequal recombination between the donor and recipient DNA results in a duplication of Tn2901. The hybrid IS1 element is shown as a partly shaded box.

stranded DNA in the cytoplasm. Such an increase in general recombination as a result of conjugal transfer could possibly result in the duplication of Tn2901 and would provide a role for the F factor *in cis*. An incoming strand of DNA carrying Tn2901 may be unequally recombined into the chromosome via the directly repeated IS1 elements so as to produce a duplication of Tn2901 (Fig.5.1) which could then be further amplified. Alternatively, a general increase in recombination stimulated by the conjugation process may result in the initial IS1-IS1 recombination event producing a duplication of Tn2901 and thereafter may also enhance the further amplification of the duplicated copies.

Kunz and Glickman (1983) detected a small but reproducible increase (1.8 -fold) in the frequency of *lacI*$^-$ mutations following conjugal transfer of an F'*lac*$^+$ plasmid. The frequency of nonsense mutations was however 15-fold higher than in the absense of transfer corresponding to a 300-fold increase in the rate of base substitutions per round of replication compared with that of normal vegetative DNA replication. This shows that the fidelity of conjugal DNA replication is considerably lower than that of vegetative DNA replication.

The stable Cut mutants isolated from JEF8 could be *argF* or *argI* constitutive mutants, or *argG* leaky strains (Legrain *et al*, 1976b). However, it is likely that the majority of stable Cut mutants isolated from JEF8 share a common mechanism of formation with the amplified Cut mutants since the stable mutants are isolated at a much lower frequency from P678.14, an F$^-$ strain, than fom JEF8 (Jessop and Glansdorff, 1980). One stable Cut mutant examined contained a single G:C→T:A transition in the control region of *argF* which resulted in a 12-fold drop in *argF* repressibility (Van Vliet *et al*, 1984). Kunz and Glickman (1983) found an increase in G:C→T:A transitions following conjugal transfer, particularly at several hotspots within the *lacI* gene. This suggests that the infidelity of conjugal transfer may be responsible for the high frequency of stable Cut mutants isolated from JEF8 by increasing the frequency of mutations at hotspots within the

argF control region.

Conjugation is often assumed to occur exclusively between male and female cells. However, microscopy has revealed that Hfr and F-prime cultures contain aggregated cells which morphologically resemble mating aggregates (Achtman, 1975). Coulter counter measurements (Achtman et al, 1978a) showed that 15%-45% of the cells in Hfr or F-prime cultures were aggregated, compared with 60%-75% of the cells in standard mating mixtures. DNA transfer is known to occur during such a cell surface contact stage in F factor-mediated bacterial conjugation (Panicker and Minkley, 1985) and DNA transfer has been shown to occur within populations of male cells. Syvanen et al (1986) found that mating occurred between about 0.5% of cells in an F-prime culture within two hours, demonstrating that surface exclusion by the F factor is not perfect.

In any population of Hfr or F^+ strains a small percentage (usually 0.1% - 1%) of the cells have lost both F pili and F-determined surface components and temporarily behave as recipients (Miller, 1972). These phenotypic recipients are referred to as F^- phenocopies. The percentage of phenocopies can be increased by growing the cells in conditions under which the formation of new F pili and cell surface components is inhibited , for example by continued aeration of saturated cultures or starvation in the absence of a required amino acid (Curtiss et al, 1969). Therefore, although F factors encode surface exclusion factors which impede conjugation between F-containing strains, the barrier to conjugation is incomplete and is easily overcome by altering the growth conditions of the strain. In fact, JEF8 acts as a recipient even without starvation or altered growth conditions (A.P. Jessop, pers. comm.). Berg et al (1983) found that when two F-prime strains were grown together on an agar surface to mimic crowding between cells in colonies, more than a quarter of the F-primes were transferred. Therefore conjugal transfer may be responsible for the formation of both the US Cut and stable Cut mutants isolated fom JEF8.

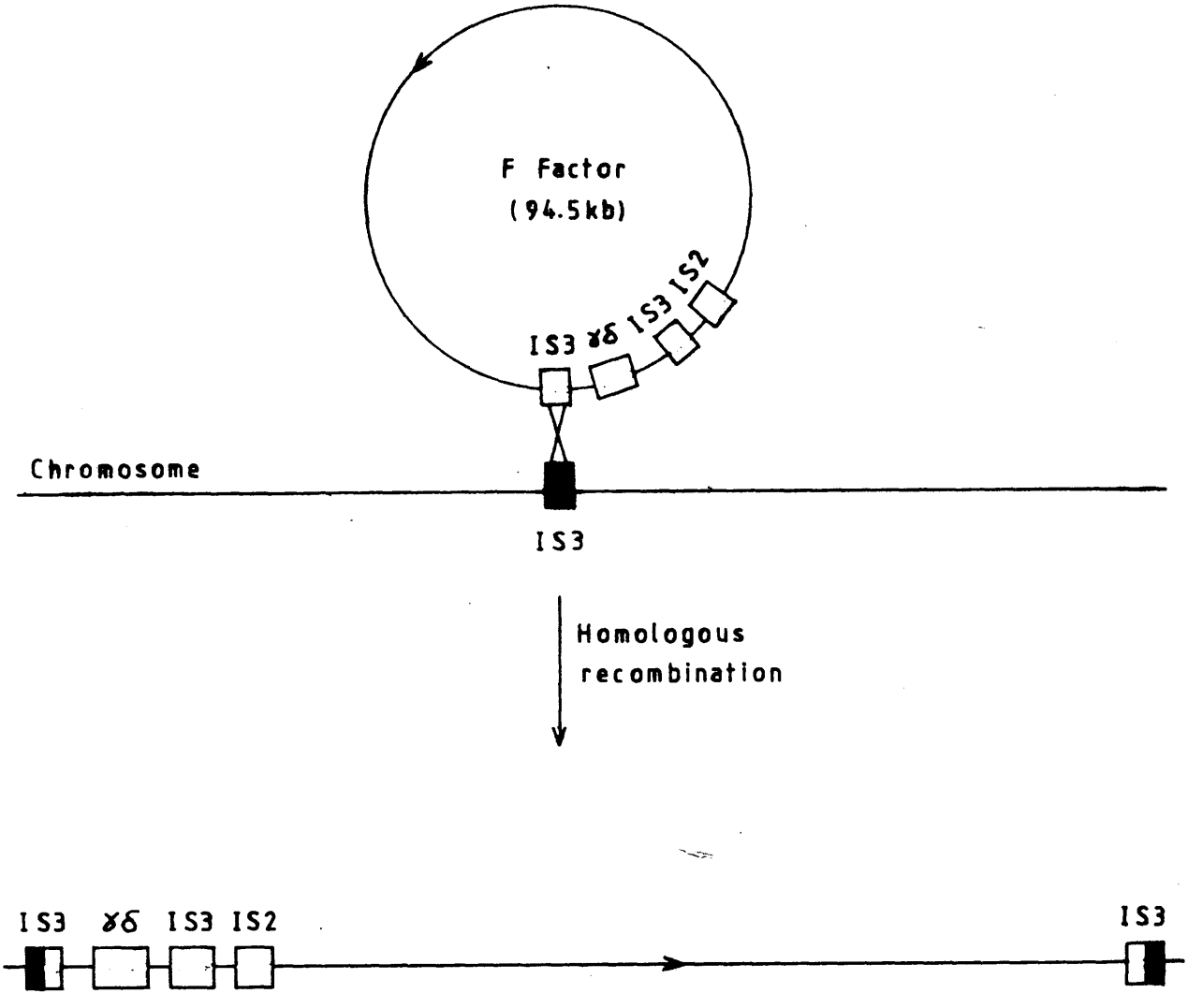


Figure 5.2. Formation of HfrP4X. In HfrP4X, the F factor is integrated via homologous recombination between IS3 sequences present on the F factor and on the bacterial chromosome. Direction of transfer is indicated by an arrow.

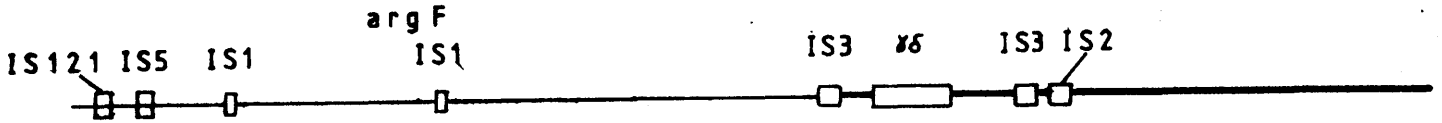


Figure 5.3. Insertion sequences present in the argF region of the E.coli K12 chromosome. The chromosome is represented by a thin line; the F factor by a thick line; and the IS elements by open boxes.

5.1 An IS-mediated rearrangement

The F factor contains four IS elements - IS2, two IS3 sequences and gamma delta. In HfrP4X the F factor is integrated via an IS3 sequence present on the E. coli chromosome and an IS3 sequence present on the F factor (Deonier and Davidson, 1976). Therefore in HfrP4X the integrated F factor is flanked by two IS3 elements (Fig.5.2). The pro-lac region of E. coli K12 is extremely rich in insertion sequences (Fig.5.3) having approximately one per 20kb of genomic DNA compared to approximately one per 120kb over the entire chromosome. (The pro-lac region, excluding the elements of the integrated F factor, contains two IS1 elements, one IS3 element, one IS5 element and one IS121 element -Timmons et al,1984). Plasmids were obtained which contained the insertion sequences IS2, IS3, IS5 and gamma delta. By probing southern blots containing restriction digests of chromosomal DNA with these plasmids it should be possible to detect any major rearrangements, e.g. deletions or inversions, involving these elements.

5.1.1 IS2

The F factor contains a single IS2 element which is known to mediate certain F factor integrations in the proA-purE region of the E. coliK12 chromosome (Davidson et al,1975). The insertion of IS2 into a gene or operon can have various important effects on transcriptional regulation. For example, the insertion of IS2 in the gal operon in one orientation (orientation I) causes polar effects whereas its insertion in the other orientation (orientation II) leads to constitutive

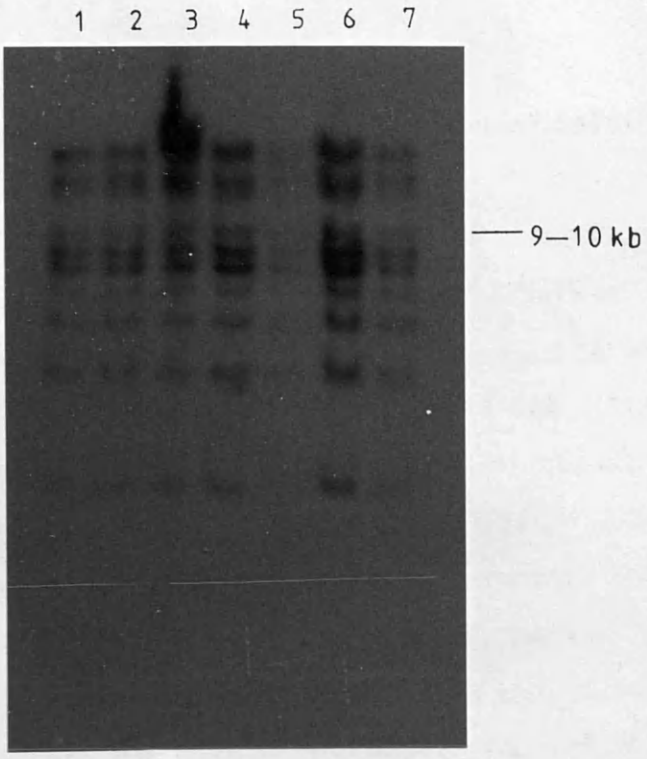


Figure 5.4. Hybridization of ^{32}P-labelled pCD44-17 to Southern transfers of chromosomal DNA. (DNA was restricted with EcoR1 and run on an 0.8% agarose gel).

Tracks 1 and 2 : Cut 1, Tracks 3 and 4 : JEF8,

Track 5: Cut 5, Track 6: JEF8, Track 7: Cut 8

expression of adjacent genes (Saedler et al,1974; Starlinger and Saedler,1976). However insertion of IS2 in orientation II does not always activate adjacent genes. Based on this observation, Glansdorff et al (1981) proposed that rather than moving around active promoters (which could be potentially harmful), IS2 (and IS3) may carry a sequence that is inactive by itself but can become part of a promoter when joined to an appropriate target sequence. If this is the case then one can imagine ways in which IS2 could have a cis-acting effect on the formation of the amplification, either by itself or in conjunction with another IS-mediated rearrangement. For example a change in orientation of the IS2 element in the F factor could perhaps activate a silent F factor gene required for the amplification of Tn2901. Activation of poorly expressed genes by IS2 has been described for yeast genes in E. coli (Brennan and Struhl, 1980; Walz et al,1978) and an archaebacterial gene in E. coli (Wood and Konisky, 1985). Similarly the transposition of IS2 to a nearby region or a deletion endpoint occurring at IS2 may form a novel promoter due to the correct positioning of the IS element and target DNA sequences (Glansdorff et al, 1981) and hence activate a silent or poorly expressed gene required for the amplification.

Chromosomal DNA from three US Cut isolates- Cut1, Cut5 and Cut8- together with JEF8 was restricted with EcoR1, Southern blotted from an agarose gel and hybridized to radiolabelled pCD44-17, a plasmid containing part of the IS2 insertion sequence. The autoradiograph (Fig.5.4) shows approximately 10 bands hybridizing to IS2. The IS2 sequence (Ghosal et al,1979) contains no EcoR1 recognition sites and so each band represents a single IS2 element present in the strain. The F factor IS2 element should be present on a 9kb-10kb EcoR1 fragment (Ohtsubo and Ohtsubo, 1977) and a fragment of this size does hybridize to pCD44-17. The published number of IS2 elements present in E. coli K12 varies from 4 to 13 (Saedler and Heiss, 1973; Hu et al,1975) which agrees with the 10 copies noted in JEF8 and the US Cut isolates. All the US Cut isolates and JEF8 produced the same pattern

of bands, therefore any major rearrangement involving IS2 in the US Cut isolates appears unlikely.

5.1.2. IS3

IS3 is very similar to IS2 in the effect it has on adjacent chromosomal DNA sequences. Like IS2, IS3 is known to mediate integration of the F factor in the proA-purE region (Davidson et al, 1975) (in fact, HfrP4X has the F factor integrated via an IS3 element present on the F factor and an IS3 element adjacent to argF on the chromosome) and is able to activate gene expression (Glansdorff et al, 1980; Zafarullah et al, 1981). The above discussion on possible roles for IS2 in the formation of the amplification therefore also applies to IS3.

IS3 elements have also been reported to generate chromosomal inversions in the lac region of E. coli K12 (Savic et al, 1983) most likely via homologous recombination between two inversely repeated IS3 elements. Three IS3 elements are associated with the integrated F factor in HfrP4X. It is possible that an inversion between two inversely repeated IS3 elements could result in the IS3-mediated activation of a silent gene by the formation of a promoter at a novel joint created by the inversion event. The activation of a silent gene could result in the production of a protein required for the formation of the amplification. Since all three IS3 elements associated with the integrated F factor are in direct repeat, another chromosomal IS3 element in the opposite orientation might be involved. Alternatively, a recombination event between two IS3 elements in direct repeat might result in a deletion of the intervening DNA. If such an IS3-mediated rearrangement is involved in the formation of the amplification this would provide an explanation for the requirement of the F factor in cis.

Chromosomal DNA from several US Cut isolates, JEF8, and a stable Cut isolate, Cut 12, was digested with BamH1, Southern blotted from an

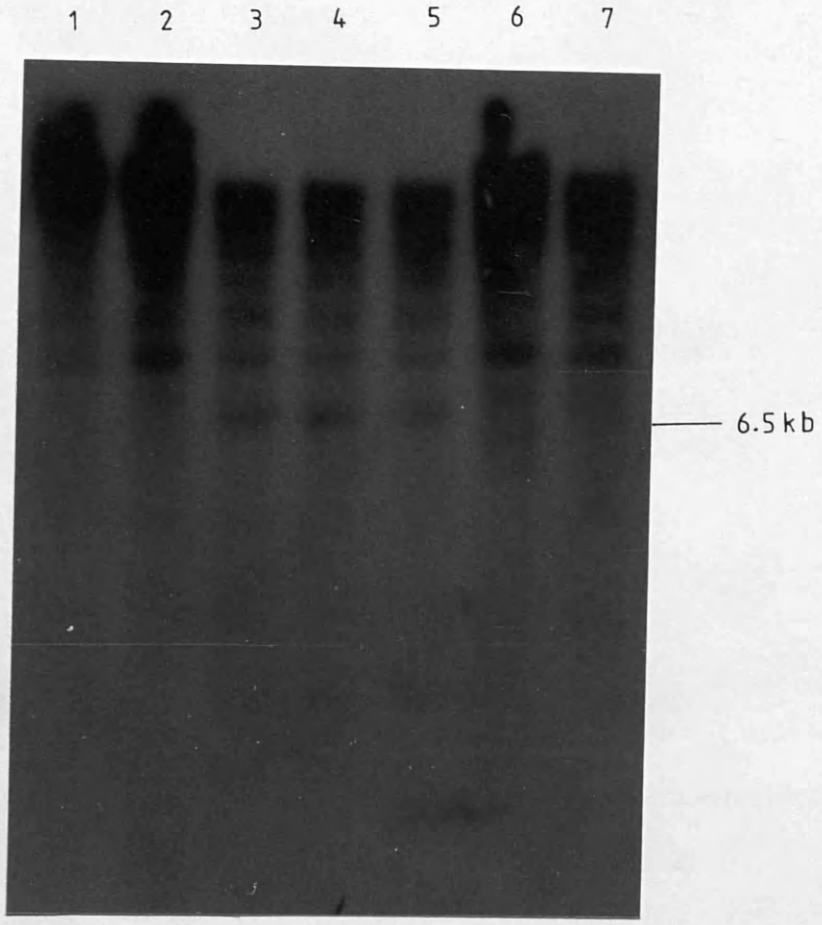


Figure 5.5. Hybridization of ^{32}P-labelled pCD4-110 to Southern transfers of chromosomal DNA. (DNA was restricted with BamH1 and run on an 0.8% agarose gel).

Tracks 1 and 2 : JEF8, Tracks 3 and 4 : Cut 5

Track 5: Cut 7, Track 6: P678.14, Track 7: Cut 12

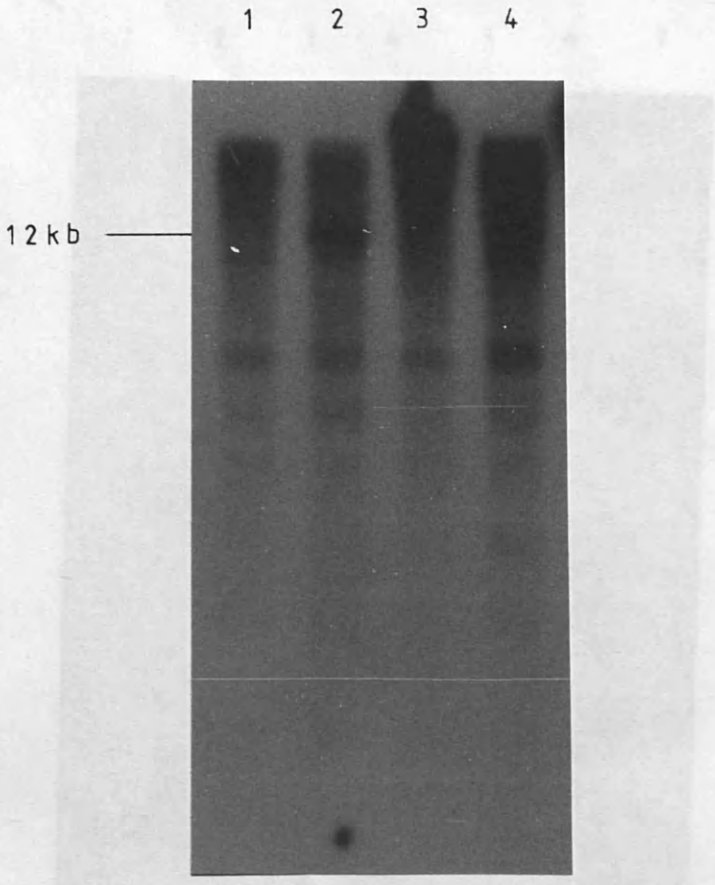


Figure 5.6. Hybridization of ^{32}P-labelled pCD4-110 to Southern transfers of chromosomal DNA. (DNA was restricted with EcoR1 and run on an 0.8% agarose gel).

Track 1: JEF8, Track 2: Cut 1, Tracks 3 and 4: P678.14

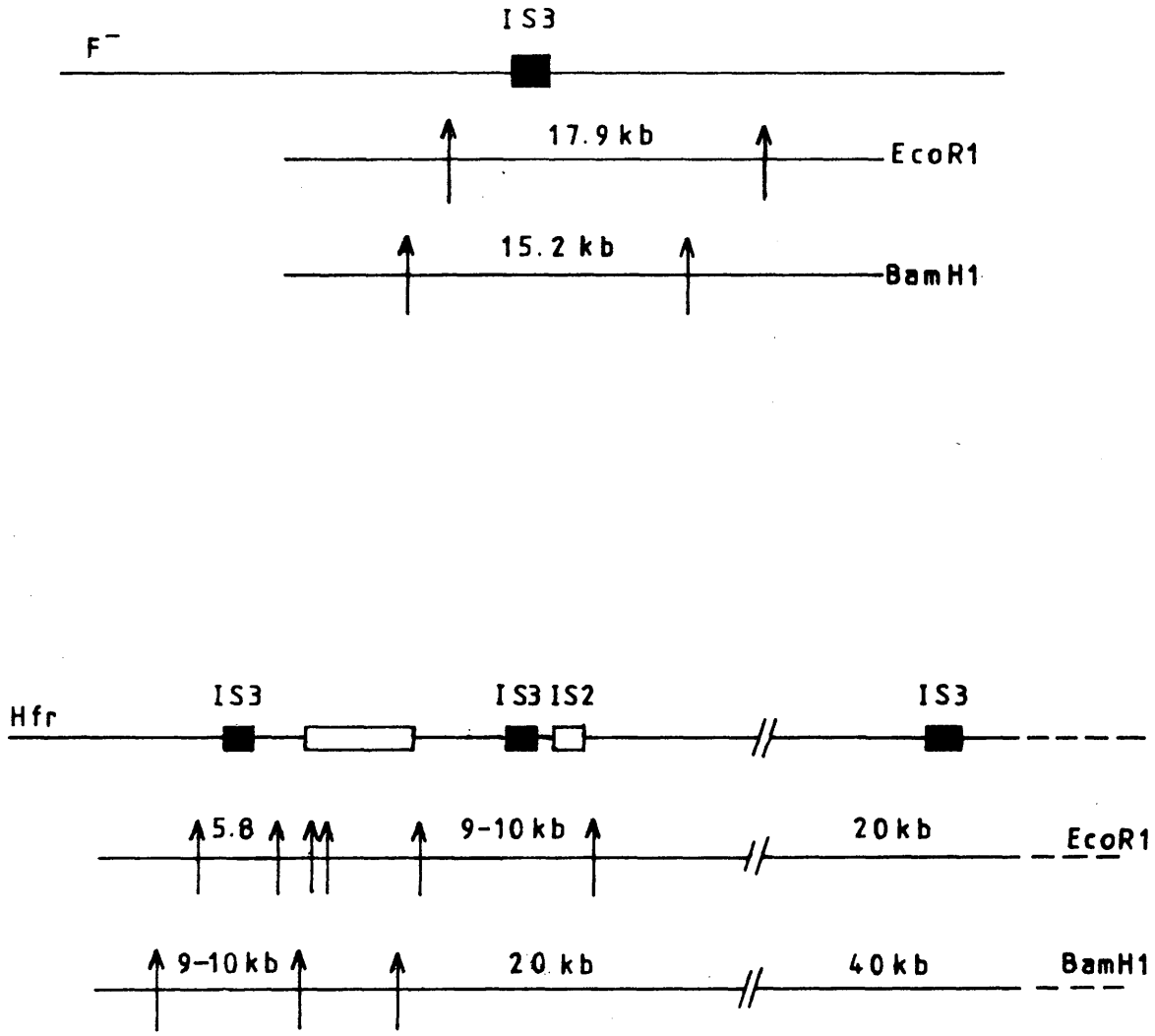


Figure 5.7. Restriction maps of F⁻ and HfrP4X strains showing IS3-containing EcoR1 and BamH1 restriction fragments. IS3 insertion sequences are represented as filled-in boxes. All sizes given are in kilobases.

agarose gel and hybridized to radiolabelled pCD4-110, a plasmid containing part of the IS3 insertion sequence (Charlier et al, 1982). The autoradiograph (Fig.5.5) shows a pattern of bands above a background smear even although the membrane had been washed in conditions of high stringency. The US Cut isolates contain approximately six bands, all larger than 6.5kb. BamH1 does not cut within the 1.4kb IS3 sequence and so each band represents a different IS3 element. This number of copies of IS3 agrees with the 5 to 6 copies of IS3 previously reported to be present on the E. coli K12 chromosome (Hu and Deonier,1981b). JEF8, P678.14 and Cut12 all contain one less band than the US Cut isolates, of approximately 6.5kb. A band of approximately 10kb also appears to be missing from P678.14.

DNA from JEF8, Cut1 and P678.14 was restricted with EcoR1, Southern blotted from an agarose gel and hybridized to the IS3-containing plasmid (Fig.5.6). Again the US Cut isolate, Cut1, contains a band, of approximately 12kb, which is not present in the other strains. P678.14 has an additional band missing of approximately 5kb. However, it appears that the probe has hybridized to the 11.7kb EcoR1 and the 6.3kb BamH1 argF-containing amplified bands, producing the extra band seen in the US Cut isolates in each digest. The IS3 sequence in pCD4-110 was cloned from the argECBH operon and contains a small piece of the argECBH control region which accounts for its hybridization to argF (Charlier et al,1982). The IS3 sequence is not present on a convenient restriction fragment which would separate it from the adjacent DNA and therefore the Southern blot was not probed with the IS3 sequence alone.

The two bands missing from P678.14 (an approximately 5kb EcoR1 fragment and an approximately 9kb BamH1 fragment) may represent F factor IS3 elements. P678.14 is an F^- strain and therefore should not contain the two IS3 elements present in Hfr strains which are part of the F factor. Also the chromosomal IS3 element, adjacent to argF, which is involved in Hfr formation will possess a changed restriction pattern in the Hfr strains (Fig.5.7). This IS3 element in an F^- strain

is present on a 15.2kb BamH1 fragment and on a 17.9kb EcoR1 fragment (Hadley et al, 1983). Fragments of these sizes were not clearly distinguishable above the background in the Southern blots probed with IS3 and so differences in these fragments would not be detected. When the F factor is integrated via this IS3 element new restriction fragments are created at the novel joints of integration, generating an IS3-containing approximately 9-10kb BamH1 fragment and an approximately 5.8kb EcoR1 fragment. These fragments correspond in size to the BamH1 and EcoR1 bands missing from P678.14. The IS3 element present at the other end of the integrated F factor would be present on a novel approximately 20kb EcoR1 fragment and on an approximately 40kb BamH1 fragment and both of these fragments are too large to be distinguished in the Southern blots.

The IS3 sequence within the integrated F factor is present on a 9-10kb EcoR1 fragment and on an approximately 20kb BamH1 fragment. This latter fragment is too large to distinguish in the Southern blot. The 9-10kb EcoR1 fragment is approximately the same size as the EcoR1 band missing from P678.14 although this may also be explained by the change in restriction pattern of the IS3 element nearest to Tn2901. These two EcoR1 fragments may be similar in size and are therefore only detected as a single band in the Southern blot.

No differences were noted between JEF8 and the US Cut mutants in either digest which could not be explained. Therefore it is unlikely that an IS3-mediated rearrangement is involved in the formation of the duplication or amplification.

5.1.3. Gamma delta

One copy of the insertion sequence gamma delta (Guyer, 1978) is present on the F factor. It is the largest of the known insertion elements being 5.7kb long and is more complex than the other insertion sequences. Gamma delta shows a close structural and functional

relationship with the Ap^r transposon Tn3 (Kleckner, 1981). Gamma delta has been shown to be the major cause of deletions in the F'*lac*$^+$*pro*C$^+$ plasmids ORF203 and F13 (Deonier *et al*,1983). Endpoints of deletions mediated by gamma delta were distributed non-randomly within a 210kb (5 min) region between *pro*C and *lac*. It is possible that a large deletion extending from gamma delta and ending at Tn2901 is necessary for the initial IS1-IS1 recombination event in US Cut mutants. Deonier *et al* (1983) found several deletion endpoints adjacent to Tn2901 and at least one of these was associated with a more complex genome rearrangement. However, although the distribution of deletion endpoints was non-random, there was no marked tendency for the deletion endpoints to fall at IS1, IS3 or IS5 elements present in the chromosomal region of the F' plasmids.

Possibly when the F factor is integrated at a different chromosomal location, as in HfrP4X, a different set of deletion endpoints will be obtained. Deonier *et al* (1983) suggested that the deletion endpoints may be associated with the organization of supercoiled domains on the F-primes (Sinden and Pettijohn, 1981). It is possible that these may begin and end at different sites on the bacterial chromosome resulting in a different set of deletion endpoints. However several of the results presented in Chapter 3 indicate that a large deletion extending from gamma delta has not occurred in the US Cut mutants. Hybridization of the *arg*F gene to BamH1 digests of chromosomal DNA from US Cut isolates indicated that the 17.7kb *arg*F-containing fragment had not been deleted. Southern hybridization also showed the *arg*F-containing 3.8kb EcoR1 and 11.6kb BglII fragments, and the IS1-containing 6.7kb BamH1 fragment to be all still present in the US Cut isolates. Therefore a deletion extending to Tn2901 is not involved in the formation of the amplification. However the possibility of a smaller gamma delta-mediated deletion still exists or, perhaps less likely, a deletion extending in the opposite direction away from Tn2901.

Chromosomal DNA from several US Cut isolates and JEF8 was restricted

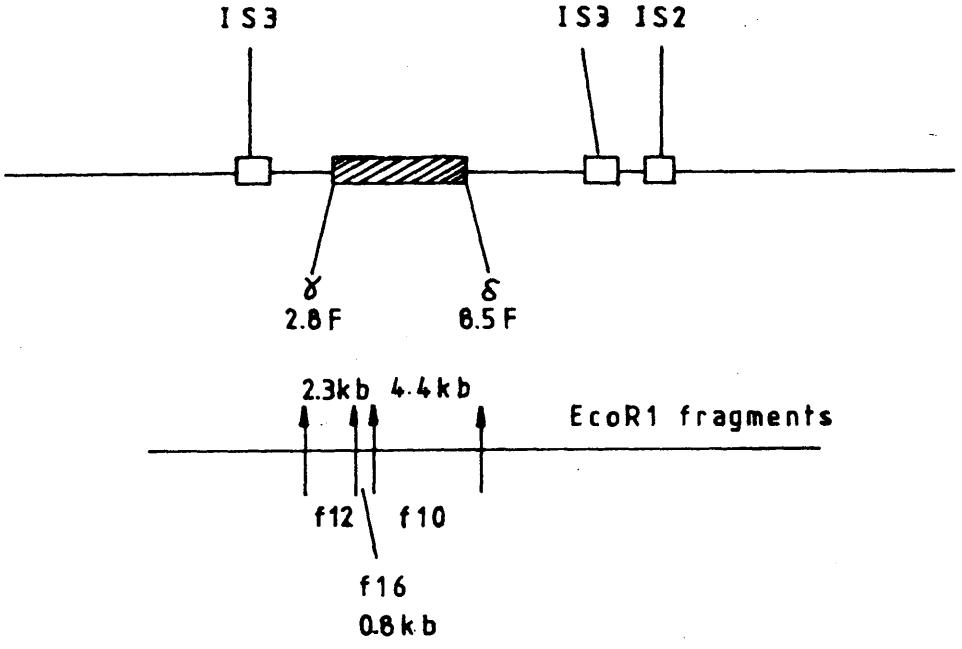


Figure 5.8. Restriction map showing gamma delta-containing EcoR1 fragments. Gamma delta is represented as a hatched box. All sizes given are in kilobases.

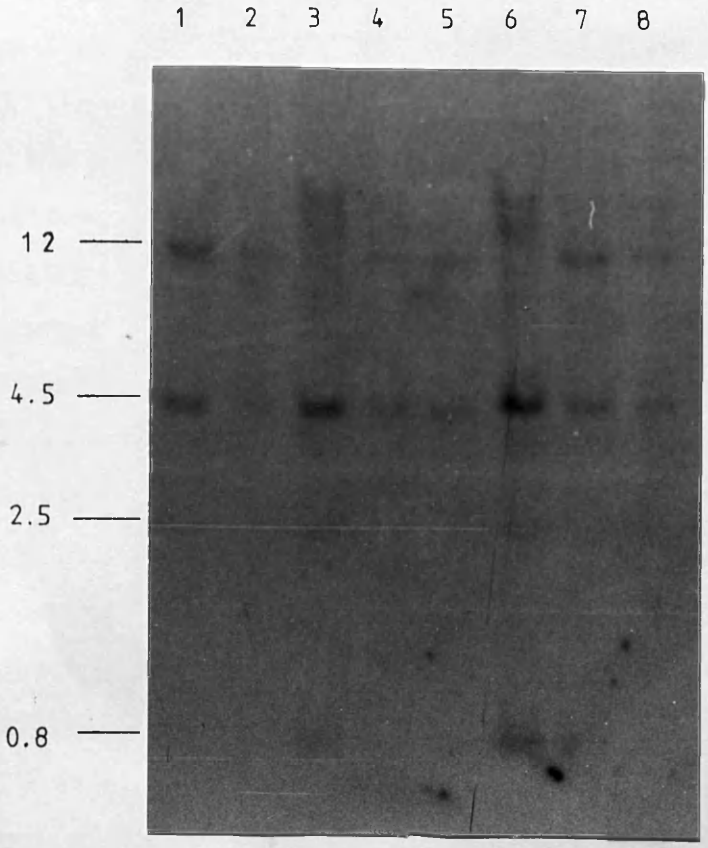


Figure 5.9. Hybridization of ^{32}P-labelled pACYC$\chi\delta$ to Southern transfers of chromosomal DNA. (DNA was restricted with EcoR1 and run on an 0.8% agarose gel).

Track 1: Cut 1, Track 2: Cut 3, Track 3: JEF8,

Track 4: Cut 5, Track 5: Cut 7, Track 6: JEF8,

Track 7: Cut 8, Track 8: Cut 9

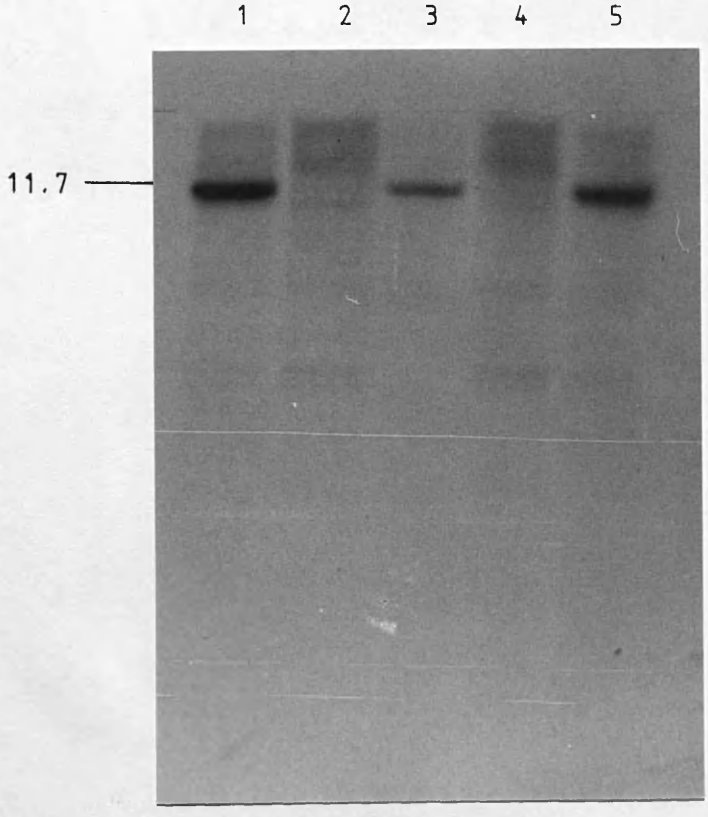


Figure 5.10. Hybridization of ^{32}P-labelled pACYC184 to Southern transfers of chromosomal DNA. (DNA was restricted with EcoR1 and run on an 0.8% agarose gel).

Track 1: Cut 1, Track 2: JEF8, Track 3, Cut 5,

Track 4: JEF8, Track 5: Cut 8

with EcoR1, Southern blotted from an agarose gel and hybridized to radiolabelled pACYC$\gamma\delta$, a plasmid containing the entire gamma delta insertion sequence. EcoR1 cuts the gamma delta sequence twice at sites which are close together generating three gamma delta-containing EcoR1 fragments- f10, f12 and f16 (Fig.5.8). These fragments are known to be 4.38kb, 2.32kb and 0.80kb respectively (Ohtsubo and Ohtsubo, 1977). The autoradiograph (Fig.5.9) shows two distinct bands and two faint bands hybridizing to pACYC$\gamma\delta$ in tracks containing the US Cut isolates. The larger of the two distinct bands is not present in JEF8 and is approximately 12kb. The other three fragments appear to be present in both the US Cut isolates and JEF8 although the smaller two fragments are very faint. The three smaller fragments have sizes corresponding to the three expected gamma delta-containing fragments from the F factor sequence (4.5kb, 2.5kb and 800bp) and this indicates that the complete F factor gamma delta sequence is present with no detectable rearrangements in the two external gamma delta-containing fragments. If a deletion had been present extending from gamma delta in either direction then a change in the size of one of these external EcoR1 fragments would have been expected.

Examination of the pACYC184 plasmid sequence (Alton and Vapnek, 1979; C. Boyd, pers. comm.) indicated that pACYC184 contains approximately 140bp of the insertion sequence IS1. Hybridization to IS1 would obviously account for the 12kb EcoR1 band present in the US Cut isolates but not in JEF8. To test whether the 11.7kb amplified EcoR1 fragment was hybridizing to the IS1 sequence in pACYC184, a Southern blot containing chromosomal DNA from several US Cut isolates and JEF8 which had been restricted with EcoR1 was hybridized to radiolabelled pACYC184. The autoradiograph (Fig.5.10) indicates that the 11.7kb EcoR1 fragment does hybridize to pACYC184. Having accounted for the 12kb fragment, the presence of the three EcoR1 fragments hybridizing to gamma delta indicates that only one gamma delta sequence is present in JEF8 and the US Cut isolates. This agrees with the published 0 to 3 gamma delta elements present in E. coli K12 (Davidson et al,1975;

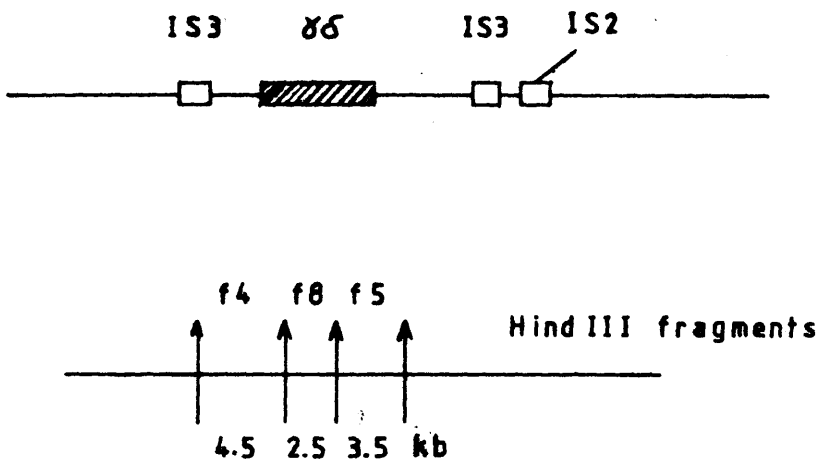


Figure 5.11. Restriction map showing gamma delta-containing Hind III fragments.Gamma delta is represented as a hatched box. All sizes given are in kilobases.

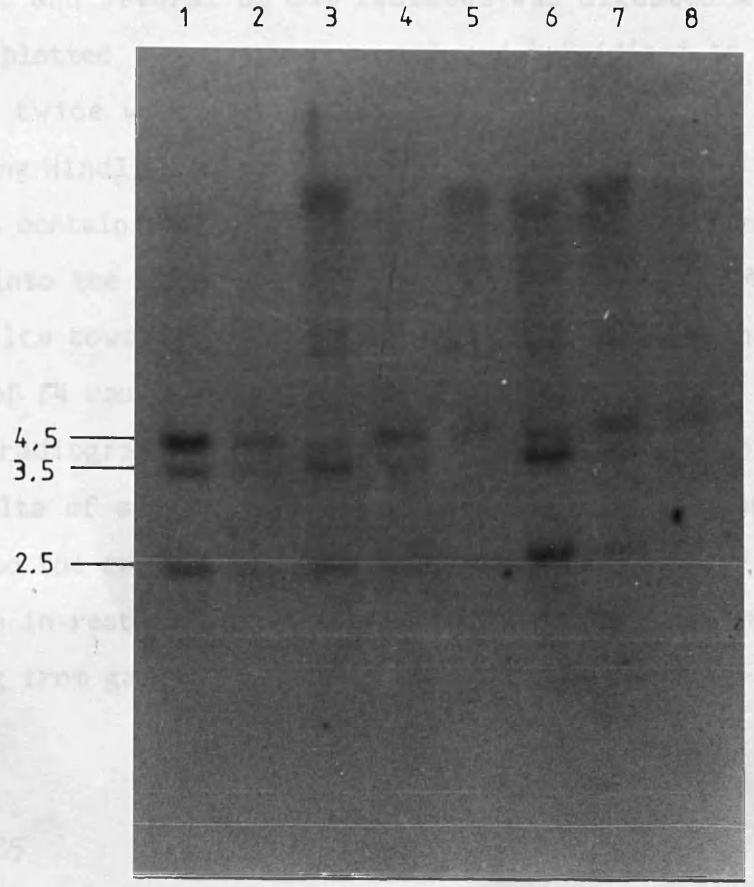


Figure 5.12. Hybridization of ^{32}P-labelled pACYC$\gamma\delta$ to Southern transfers of chromosomal DNA. (DNA was restricted with Hind III and run on an 0.8% agarose gel).

Track 1: Cut 1, Track 2: Cut 3, Track 3: JEF8,

Track 4: Cut 5, Track 5: Cut 7, Track 6: JEF8,

Track 7: Cut 8, Track 8: Cut 9

Guyer,1978; Guyer et al,1981).

To confirm that the chromosomal region adjacent to gamma delta had not been deleted or rearranged, as suggested by the EcoR1 digests, DNA from JEF8 and several US Cut isolates was digested with Hind III, Southern blotted from an agarose gel and hybridized to pACYCγδ. Hind III cuts twice within gamma delta generating three gamma delta-containing HindIII restriction fragments - f4, f8 and f5 (Fig.5.11). f4 and f5 contain chromosomal DNA on either side of gamma delta and f4 extends into the adjacent IS3 sequence. If a deletion extending from gamma delta towards Tn2901 exists then a change in the restriction pattern of f4 would be expected.

The autoradiograph (Fig.5.12) shows three fragments hybridizing to gamma delta of approximately 4.5kb, 3.5kb and 2.5kb. These correspond in size to the expected HindIII fragments f4,f5 and f8 indicating that no change in restriction pattern has taken place and hence no deletion extending from gamma delta in either direction.

5.1.4. IS5

Although the insertion sequence IS5 is not present on the F factor it is present adjacent to Tn2901 and is known to have various effects on adjacent DNA sequences. Therefore IS5 is relevent to any discussion of IS-mediated rearrangements.

IS5 is 1.2kb long and both its ends are known to be deletogenic (Chow and Broker,1978) and to stimulate general recombination within adjacent DNA sequences (Lieb, 1980). IS5 also exerts polar effects in at least one orientation (Crabeel et al,1979) and insertion of IS5 in the lpp gene of Serratia marcesens caused inactivation of the gene (Nakamura and Inouye, 1981). It has been reported that spontaneous mutations which activate the cryptic bgl operon of E. coli are due to insertion of IS5 or IS1 into a specific region in the operon (Reynolds et al, 1981). In contrast to IS2 and IS3, which can activate adjacent

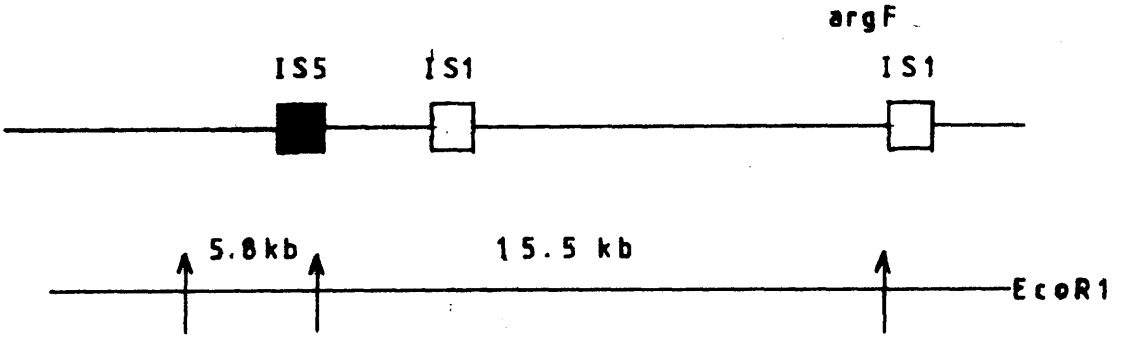


Figure 5.13. Restriction map showing IS5-containing EcoR1 fragments. IS5 is represented as a filled-in box.

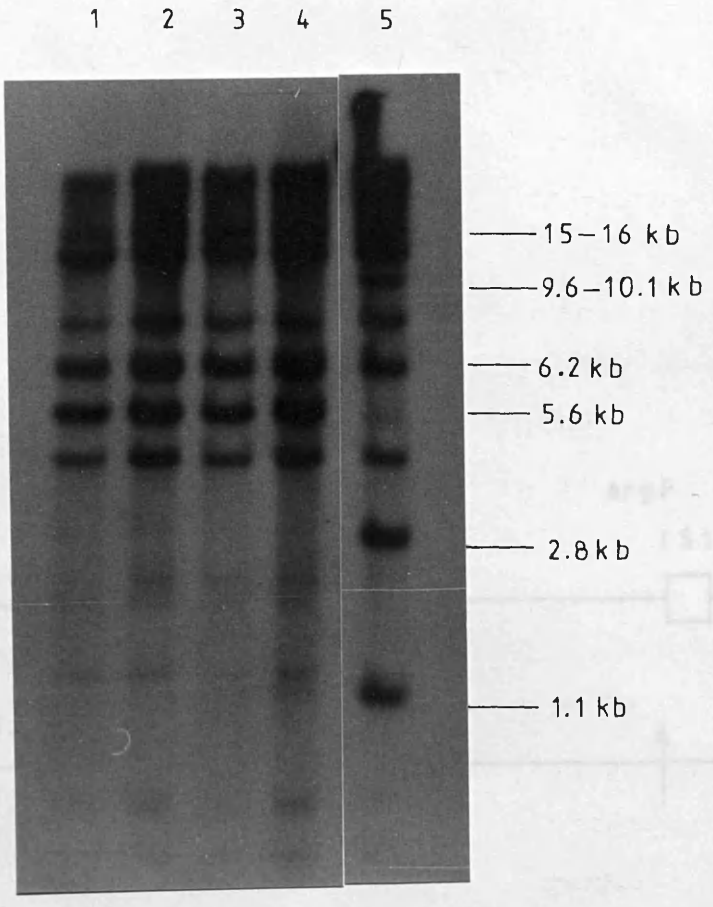


Figure 5.14. Hybridization of ^{32}P-labelled pDII5 to Southern transfers of chromosomal DNA. (DNA was restricted with EcoR1 and run on an 0.8% agarose gel).

Track 1: Cut 1, Track 2: JEF8, Track 3: Cut1,

Track 4: JEF8, Track 5: P678.14

genes in a constitutive manner, expression of the insertion-activated bgl operon is inducible and is dependent on cyclic AMP. Two models have been developed to explain this form of cis-activation of the bgl operon : (1) Insertion of IS5 or IS1 into the specific region activates a pre-existing, inactive promoter or provides promoter structure, or (2) the operon contains an operator site that is disrupted by the insertion sequence (Reynolds et al,1981). Transcriptional activity of a yeast gene in E. coli by IS5 insertion has also been reported (Jund and Loison, 1982). Therefore a rearrangement, e.g. a transposition or deletion involving IS5, could possibly activate a gene involved in the amplification or inactivate a gene coding for a repressor of the amplification.

DNA from Cut1, JEF8 and P678.14 was digested with EcoR1, Southern blotted from an agarose gel and hybridized to pD115 (Schoner and Kahn,1981), a plasmid containing the entire IS5 sequence. EcoR1 cuts within the IS5 sequence once, approximately 100bp from the end of the sequence. The IS5 element (IS5A) adjacent to Tn2901 is known to be present on a 5.8kb EcoR1 fragment with approximately 100bp extending into the argF-containing 15.5kb EcoR1 fragment (Fig.5.13). The autoradiograph (Fig.5.14) shows six intense bands hybridizing to both JEF8 and Cut1 indicating that no detectable rearrangement has taken place in the US Cut isolate. A band of approximately the same size as the 15.5kb fragment is seen, as are two others of approximately 5.6kb and 6.2kb respectively, both of which might represent the 5.8kb fragment expected. The 5.6kb band in P678.14 is missing, which probably represents the IS5 element adjacent to Tn2901. This is likely since the P678.14 used was a spontaneous pro mutant and may therefore contain a deletion in this region. Bands of approximately 9.6-10.1kb, 2.8kb and 1.1kb are present in P678.14 but not in JEF8 or Cut1. Schoner and Kahn (1981) probed chromosomal digests of three E. coli K12 strains with pD115 to determine the number of copies of IS5 and their locations. The number of insertion sequences found (approximately 10 copies) and their positions in the genome remained

Table 5.1

(a). Effect of SDS on conjugal transfer

	N° recombinants obtained per 10^8 viable cells		ratio B/A
cross	with SDS (A)	without SDS (B)	
JEF8 x D7-13 *pro met*	4.1×10^1	2.8×10^3	68.3
JEF8 x NGX2	2.4×10^2	2.9×10^3	12.0
JEF8 x P678.14 *pro nal*	3.2×10^0	2.3×10^3	718.7

(b). Effect of SDS on Cut yield from JEF8

N°. mutants per 10^8 viable cells		ratio B/A
with SDS (A)	without SDS (B)	
1.7×10^2	2.9×10^2	1.7

(c). Cut yield from JEF8 *tra*

Strain	N° mutants per 10^8 viable cells
JEF8	1.7×10^2
JEF8 *tra*	4.2×10^2

(a). Bacterial crosses.
Bacterial crosses were carried out in liquid culture as described in Materials and Methods. The same donor culture was used for all crosses; the same recipient culture was used for each pair of crosses. SDS was added at a final concentration of 0.01% to the flask before the donor and recipient cultures. Each cross was plated after 60 mins incubation. A total of four plates were counted for each cross and the mean value taken.
The crosses were plated on the following media:

JEF8 x D7-13 pro met: arginine, uracil, threonine, glucose, Sm ± SDS
(Selection: pro^+, Counter-selection: Sm)

JEF8 x NGX2 : arginine, uracil, leucine, glucose ± SDS
(Selection: pro^+, Counter-selection: met, thr)

JEF8 x P678.14 pro nal: arginine, uracil, methionine, threonine, glucose naladixic acid ± SDS
(Selection: pro^+, Counter-selection: nal)

(b). Cut yield.
JEF8 was grown in minimal medium supplemented with arginine, uracil, methionine, threonine, glucose +/- SDS (at a final concentration of 0.01%). The cultures were inoculated with 0.1ml of a 10^{-2} dilution of an overnight culture. 10^0 and 10^{-1} dilutions of cultures were plated on citrulline, methionine, threonine, glucose +/- SDS. The total number of of Cut isolates from four plates was counted (this varied from 129 - 569 colonies). The experiment was repeated three times with similar results.

(c). Cut yield from JEF8 tra
JEF8 and JEF8 tra were grown in minimal medium supplemented with arginine, uracil, methionine, threonione and glucose (inoculated with 0.1ml of a 10^{-2} dilution of an overnight culture). 10^0 and 10^{-1} dilutions of cultures were plated on citrulline, methionine, threonine and glucose. A total of four plates were counted for each strain.

constant in strains that had been separately maintained in the laboratory for many years (Schoner and Kahn,1981). There is a clear difference in the number of copies of IS5 present in JEF8 and P678.14. However, since no differences were detected between JEF8 and Cut1 it is unlikely that the difference in the number of copies of IS5 found in P678.14 is related to the formation of the amplification.

5.2 Conjugal transfer

This section investigates the possible involvement of conjugal transfer in the formation of the Cut mutants by JEF8. Two approaches were taken, namely:- (1) treatment of cells with SDS, and (2) construction of a transfer-deficient mutant of JEF8.

(1). Treatment of cells with SDS

F pili are extremely sensitive to low concentrations of sodium dodecyl sulphate, a detergent which is known to depolymerise F pili filaments. Extended F pili filaments quickly disappear from the cell surface of bacteria treated with the detergent, thereby effectively destroying donor ability (Tomoeda et al, 1975). Achtman et al (1978a) found that most of the spontaneous Hfr-Hfr and F'-F' aggregates disappeared within 1 min of SDS treatment. The effect of SDS on several bacterial crosses was determined (Table 5.1a). The addition of SDS to the conjugation flasks results in a 12-fold to 700-fold decrease in the recombinants obtained from these crosses. Since conjugal transfer between male cells may be involved in the formation of the amplification and also stable Cut mutants, JEF8 was grown in the presence of SDS to determine whether the resultant decrease in donor ability would affect the yield of Cut isolates from the strain. The results are shown in Table 5.1b. The results show that SDS treatment only results in a 1.7-fold decrease in Cut yield, suggesting that conjugal transfer is not required for the formation of the

amplification. These results are however inconclusive, and it was therefore decided to use a different approach to investigate the possible role of conjugal transfer in the formation of the Cut mutants.

(2). Construction of JEF8 *tra*

It was decided to construct a mutant of JEF8 which is unable to transfer DNA. A cloned *traJ* gene which contains an amber mutation is available (pBE51-Kennedy *et al*, 1977). The *traJ* gene product is a positive regulator of the *traYZ* operon (Willetts and Skurray, 1980). *traJ* mutants are therefore pleiotropic, being defective in transfer and pilus formation, and they are also surface exclusion-deficient since *traJ* also controls expression of *traS* and *traT* (Kingsman and Willetts, 1978; Achtman *et al*, 1971; 1972). JEF8 was transformed with pBE51 and plated on L-agar containing Tc. Approximately half of the resultant transformants were exposed to U.V. light (254nm, 42cm from the source, approximately 120uW cm^{-2}) for 2-3 seconds and, together with transformants which had not been exposed to U.V. light, were streaked for single colonies several times on L-agar containing Tc. Single colonies were then patched onto L-agar, plate mated with NGX2 and replicated onto minimal agar containing leucine, proline, methionine, threonine and glucose to select for Arg^+ recombinants. Out of 112 colonies screened, one was obtained (from a U.V.-irradiated plate) which did not produce any Arg^+ recombinants. This strain was tested for donor ability in liquid culture with NGX2, and again no Arg^+ recombinants were obtained. Therefore the strain, JEF8 *tra*, was tested for Cut yield. The results (Table 5.1c) show that JEF8 *tra* produces no decrease in the yield of Cut mutants compared with JEF8.

DISCUSSION

The results presented in this chapter did not detect any rearrangement in the US Cut mutants other than the amplification of Tn2901. Chromosomal restriction digests probed with plasmids containing IS2, IS3, gamma delta and IS5 did not suggest that any of these elements, or chromosomal DNA adjacent to them, were deleted or rearranged in any way and therefore a major rearangement mediated by these elements is clearly not involved in the initial IS1-IS1 recombination event.

The results in this chapter do not rule out the involvement of a minor rearrangement in the IS1-IS1 recombination event. For example, a rearrangement entirely within a single restriction fragment may have been undetected if it was small enough not to change the position of the restriction fragment on the Southern blot. Obviously the limits of detection vary with the size of restriction fragment: a change in size of a small restriction fragment would be more easily detected than a similar change in size of a large restriction fragment.

The F factor IS2 element and the internal IS3 element are present on a 9kb-10kb EcoR1 fragment and a change in size of this fragment of up to several hundred base-pairs would not have been detected. Likewise, an inversion of the IS2 or IS3 elements entirely within the EcoR1 fragment would have been undetected on the Southern blot. The IS3 element nearest to Tn2901 is present on a 5.8kb EcoR1 fragment and a deletion or addition of approximately 200bp or more should result in a noticeably different fragment size on the Southern blot. However, a rearrangement such as an inversion not resulting in a change of size of this 5.8kb EcoR1 fragment would not have been detected. The IS3 element furthest from Tn2901 is present on an approximately 20kb EcoR1 fragment and an approximately 40kb BamH1 fragment. Changes in size of these fragments of up to several kilobases would not result in a noticeably different restriction pattern on the Southern blot and so would have been undetected.

The gamma delta sequence is cut twice by HindIII generating three gamma delta-containing fragments of 4.5kb, 3.5kb and 2.5kb. Only very small deletions (of less than 150bp) would have been undetected on the Southern blot. The EcoR1 gamma delta-containing fragments are also small (2.3kb, 0.8kb, 4.4kb) and similar small deletions or additions within any of these fragments would also have been detected. Therefore it is highly unlikely that a deletion extending from the gamma delta sequence has taken place in the US Cut isolates.

The IS5 element adjacent to Tn2901 is present on a 5.8kb EcoR1 fragment with approximately 100bp extending into the argF-containing 15.5kb EcoR1 fragment. A deletion or other rearrangement resulting in a change in size of the 5.8kb fragment of greater than 200bp would have been detected in the Southern blot. A second insertion sequence, IS121, is also present in the 5.8kb IS5-containing fragment (Timmons et al, 1984). No probe for this element was available. However, since IS121 is contained within the same restriction fragment as IS5, any IS121-mediated rearrangement would have been detected by the IS5 probe. Obviously, any small rearrangement or an inversion of IS121 or IS5 entirely within the 5.8kb EcoR1 fragment would not have been detected.

With the possible exception of a very small rearrangement or a rearrangement within a very large restriction fragment, the results indicate that no IS2, IS3, IS5 or gamma delta-mediated rearrangement has taken place in the US Cut mutants and hence the formation of the amplification appears not to involve a major F factor IS-mediated rearrangement. Minor rearrangements could be investigated further by using a variety of restriction endonucleases and varying the gel conditions to allow better separation of fragments within a similar size range. However, it was decided not to pursue minor rearrangements since only a partial restriction map of the region is available and no probes for other restriction fragments in the region are available; furthermore, the development of molecular techniques should provide more conclusive results.

Cultures of JEF8 were treated with SDS in an attempt to determine whether conjugal transfer is required for the formation of the amplification. Treatment with SDS has several limitations. Firstly, SDS treatment does not totally inhibit bacterial mating since recombinants were obtained from each of the three crosses carried out in the presence of SDS. If conjugation is required for the amplification, the presence of a few conjugation-proficient cells in the SDS-treated culture may give rise to many recipient cells harbouring a duplication of Tn2901. However, the number of recombinants obtained from these crosses was 12-fold to 700-fold less than the number of recombinants obtained from similar crosses not treated with SDS. Therefore, if conjugation is required for the amplification, one might expect a similar decrease in the yield of Cut mutants from a culture of JEF8 grown in the presence of SDS. A small (1.7-fold) decrease in Cut yield was observed in cultures of JEF8 grown in medium containing SDS which suggests that conjugation is not a prerequisite for the formation of the amplification although conjugal transfer may enhance the formation of the amplification, perhaps by one of the mechanisms suggested above. The second limitation of the SDS treatment is that it may have unknown effects on the bacterial cell. If, for example, the uptake of citrulline was affected, this might affect the yield of Cut mutants. Therefore, since an unknown number of cells retain donor ability and since the SDS treatment may have unknown effects on the cell, no definite conclusions can be drawn regarding the role of conjugal transfer in the amplification of Tn2901 from these results, although they suggest that conjugal transfer is not an absolute requirement.

The construction of JEF8 tra provided more conclusive results on the role of conjugal transfer. JEF8 tra shows no decrease in the yield of Cut mutants compared with JEF8 and it is therefore extremely unlikely that the formation of the amplification involves conjugal transfer. While it is likely that JEF8 was produced as a result of homogenotization with pBE51, the traJ mutation in JEF8 has not yet

been verified. Therefore, the possibility remains that JEF8 tra is a transfer-deficient mutant resulting from the brief exposure of JEF8 to U.V. light. If JEF8 tra does contain the traJ amber mutation, this implies that no genes of the transfer operon are required for the initial IS1-IS1 recombination event since the traJ gene product is a positive regulator of the traYZ operon (Willetts and Skurray, 1980). If, however, JEF8 tra contains a different mutation then the involvement of transfer operon genes cannot be ruled out. It is clear that conjugal transfer is not required for the initial IS1-IS1 recombination event since JEF8 tra is transfer-deficient. A certain number of amplifications or duplications could, arguably, exist in the population due to previous conjugal transfer and these might result in the isolation of Cut mutants. However, it is likely that the tra mutation would result in a significant decrease in the yield of Cut mutants if conjugal transfer were required for the initial IS1-IS1 recombination event.

The results presented in this chapter clearly indicate that conjugal transfer is not required for the amplification of Tn2901. Several alternative approaches could have been used. Ideally, an Hfr strain identical to HfrP4X with the one exception of polarity of chromosomal transfer could have been used since this strain should yield little or no US Cut mutants if conjugal transfer was required. However, no Hfr strain with opposite polarity to HfrP4X is known. The chromosome of an F^- cell can be mobilized from a chosen origin without the F factor being present. Boyd and Sherratt (1986) have mobilized the E. coli chromosome by integrating a derivative of pCB101, a lambda dv-based vector, at the site of the lambda prophage in the chromosome. When provided with suitable mobilizing plasmids, strains in which pCB101 is integrated are able to transfer the chromosome in a polar way. Using this technique it would be possible to mobilize the chromosome of an F^- strain from a chosen site of pCB101 integration adjacent to Tn2901. Of course, the polarity of the integrated vector would be critical since Tn2901 would only be mobilized efficiently by one orientation of

the integrated vector. Therefore, US Cut isolates should be obtained from one orientation of the integrated vector if conjugal transfer of Tn2901 is involved in the formation of the amplification.However, conjugal transfer may stimulate the IS1-IS1 recombination event in the recipient chromosome, in which case conjugal transfer of Tn2901 itself would not be required.

If neither an IS-mediated rearrangement nor conjugal transfer is the function provided by the F factor for the formation of the amplification, then other possible functions of the F factor must be examined. Since the F factor is required *in cis*, the F factor must provide something other than a *trans*-acting protein, for example a protein binding site. Several examples are known of prokaryotic genes whose transcription can be controlled by regulatory proteins that bind to sites at considerable distances from the genes. The activation of the *E. coli glnALG* promoter requires two binding sites for NR_1 located 110 and 140bp upstream of the start of transcription. Moving these binding sites more than 1000bp does not diminish the ability of NR_1 to stimulate transcription (Reitzer and Magasanik, 1986). Similarly, two *deoR* binding sites are required for *deoR* repression of the *deo* operon of *E. coli* (Dandanell and Hammer, 1985) and these sites can be separated by up to almost 1kb without any loss of repression. However these examples of protein binding sites, although acting over a considerable distance must involve a relatively small distance compared to the possible distance involved if the F factor provides a protein binding site to regulate transcription of a distant gene. Since the gene must be outwith the integrated F factor (F cannot simply provide a *trans*-acting protein), the distance from the gene to the binding site must be at least equal to the distance from the binding site to the end of the F factor. The gene involved may, for example, activate a recombinase which acts at the IS1 elements of Tn2901. Such a recombinase could possibly be encoded by the IS1 elements themselves, in which case the protein binding site would be at a distance of at least 23,000bp from the gene.

Various site-specific recombination systems can recognise the orientation of two sites on the same DNA molecule separated by many thousands of base-pairs. This requires that proteins acting at separated sites on the same molecule communicate with each other. Examples of such site-specific recombination systems include the bacteriophage Mu and Tn3. A tracking model was originally proposed for such phenomena whereby a protein recognises a specific sequence on the DNA and then moves along the DNA to another specific sequence where, perhaps by interacting with another protein, it initiates transcription. However, Craigie and Mizuuchi (1986) have eliminated tracking as a mechanism for site-specific recombination involving the ends of bacteriophage Mu. An alternative model involves the interaction between distant DNA-bound proteins with the intervening DNA looping out to allow the protein-protein interaction (Ptashne,1986). Boocock *et al* (1986) have also described experiments which suggest that the ends of Tn3 find themselves by such a looping mechanism. Therefore, the F factor could provide a protein binding site that interacts with another site, perhaps on Tn2901, many thousands of base-pairs away to allow the initial IS1-IS1 recombination event to occur.

If the F factor does provide a protein binding site, it should be possible to isolate a restriction fragment of F which carries the site and allows amplification of Tn2901 when integrated adjacent to Tn2901 in the chromosome of an F^- strain. Such an experiment is described in Chapter 6. The constitutive expression of the *tra* regulon of F42*lac* has been found to be required for enhanced recombination with lambda p*lac*5 (Porter,1981; Seifert and Porter,1984). The *tra* dependence involves a *cis*-acting component thought to be *oriT* on F42*lac*. It has been suggested that the nicking reaction at *oriT* allows the *recBC* enzyme to enter the F42*lac* molecule which could then provide single-stranded DNA substrates to facilitate the initiation of recombination (Yancey and Porter, 1985). Since the amplification of Tn2901 is *recBC*-independent (A.P.Jessop, pers. comm.) this mechanism cannot apply.

However, oriT may be required for the initial IS1-IS1 recombination event by providing a site of entry for a different enzyme required for the IS1-IS1 recombination. If this is the case then insertion of a cloned oriT sequence (or an analogous nicking site, e.g. bom of ColE1 - Boyd and Sherratt, 1986) into an F⁻ strain adjacent to Tn2901 should be sufficient for the IS1-IS1 recombination event if the nicking endonucleases were provided in trans. An additional F-encoded function may also be required. A cloned restriction fragment containing oriT is available (Achtman, 1978b) although limited time did not permit its use.

Alternatively, the F factor could provide an origin of replication from which repeated rounds of localized replication could originate. This could frequently provide a replication fork at Tn2901 which would allow unequal recombination between the IS1 elements, the formation of a duplication and the further amplification of the duplicated copies. The frequency with which the F factor produces such replication forks would have to be much greater than the normal vegetative replication fork to account for the lack of US Cut mutants obtained from F⁻ strains.

Alternatively, the integrated F factor may produce a conformational change in the argF region to allow access of an essential protein to an otherwise inaccessible region of the chromosome, for example access of a recombinase to the IS1 elements of Tn2901. It is known that the topological state of DNA plays a crucial role during replication, transcription and recombination (Gellert, 1981). In eukaryotes, DNA supercoiling is an essential step in the formation of DNA-histone complexes and thus in the folding and organization of the chromosome. In prokaryotes, DNA supercoiling results in torsional strain and puts the DNA into an energetically active state important for DNA replication, transcription and genetic recombination. The normally silent bgl operon of E. coli K12 can be activated by a decrease in negative supercoiling of the chromosomal DNA (DiNardo et al,1982) caused by compensatory mutations in DNA gyrase genes in strains

lacking DNA topoisomerase I. This decrease in negative supercoiling may allow more effective binding of RNA polymerase or less effective binding of a repressor. The lacI gene may be another example of a gene activated by a decrease in negative supercoiling (Sanzey, 1979).

The transposable element gamma delta has been shown to greatly reduce expression of a cloned E. coli ebgA gene when present in cis with respect to the ebgA gene (Stokes and Hall, 1984). This effect is independant of both orientation and position of gamma delta within the replicon but is dependant on the orientation of the ebgA gene and the total size of the replicon. It has been suggested that gamma delta alters the local supercoiling in the region of the ebg promoter in such a way as to inhibit transcription (Stokes and Hall, 1984). Gamma delta can be positioned as much as 4 to 5kb from the ebgA gene without any loss of repression. Since gamma delta is present on the F factor, it is possible that it may alter the supercoiling of adjacent chromosomal DNA resulting in the activation of a gene required for the initial IS1-IS1 recombination event, or in repression of a gene encoding a repressor of the IS1-IS1 recombination event. A change in supercoiling could allow access of a recombinase to the IS1 elements or could prevent the binding of a repressor molecule to Tn2901. Insertion of a transposable element can prevent expression of the bacterio-opsin (bop) gene in Halobacterium halobium when the element is inserted up to 1.4kb upstream of the bop gene (Pfeifer et al, 1983). In one case insertion of a second element distal to the first element restored the bop^+ phenotype which may be the result of the same mechanism that permits gamma delta to act on ebgA (Stokes and Hall, 1984). Therefore, the integration of the F factor adjacent to Tn2901 could possibly alter the supercoiling of adjacent chromosomal DNA to "activate" the initial IS1-IS1 recombination event.

Note added in proof

JEF8 *tra* has now been confirmed by complementation as a *traJ* mutant (Fiona Russell, this laboratory).

CHAPTER 6

Development of a system for molecular analysis of the amplification

The results presented in the previous chapter suggest that neither a second IS-mediated rearrangement nor conjugal transfer is the function provided by the F factor required for the formation of the amplification. To investigate the function provided by the F factor, deletion analysis of the F factor should be very useful since mutants may be constructed which are unable to amplify Tn2901 and this would allow the identification of regions of F which are essential for the amplification. IS1-IS1 recombination in Tn9 has been shown to occur on plasmid constructs and this has provided a convenient system for testing the effects of deletions on recombination in Tn9 (Braedt, 1985). However, since IS1-IS1 recombination in Tn2901 requires the presence of the integrated F factor and does not occur detectably in F^- strains, it is possible that deletion analysis of Tn2901 on a plasmid vector would not give a true representation of IS1-IS1 recombination in the chromosome. Therefore, deletions must either be made in the chromosome *in vivo*, or be constructed *in vitro* and transferred into the chromosome by directed integration.

(1). *in vivo* deletions: Various methods are available for creating deletions *in vivo* which could be used for deletion analysis of the F factor. For example, in a strain deleted for the *pro-lac* region, an F'*pro*$^+$ *lac*$^+$ could be forced to integrate at the *ton*B locus adjacent to the Ø80 prophage by selecting for bacteriophage T1 resistance. By inducing the Ø80 lysogen, it may be possible to obtain deletions extending into the F factor. Alternatively, deletions could be made by transductional shortening of an F-prime using phage P1. A fragment which can recircularise and replicate autonomously in the recipient bacteria would be selected. It should be possible to select for *arg*F$^+$ and include the F factor origin of replication; size constraints would require that the transfer region of F be deleted. These techniques may be of value in creating large deletions extending into the F factor.

However, the endpoints of such deletions will be variable and may be difficult to determine. Nevertheless, several techniques for creating *in vivo* deletions have already been attempted (A.P. Jessop, pers. comm.) but these were unsuccessful due to strain problems.

(2). *in vitro* deletions: Specific deletions or other mutations may be constructed by *in vitro* techniques, and these could be introduced back into the chromosome to determine their effects. *In vitro* constructed deletions could be introduced into the integrated F factor of JEF8 and their effects on Cut yield determined. This should allow the identification of the F factor region required for the amplification. *In vitro* mutagenesis (e.g. the introduction of deletions or point mutations) could also be applied to the IS1 elements of Tn2901 and these could be used to replace the wild-type IS1 elements in the chromosome. This would allow the involvement of an IS1-encoded gene product to be determined and the crossover site in the IS1-IS1 recombination event to be identified. Obviously, *in vitro* techniques would be much more specific than *in vivo* techniques since defined mutations would be introduced.

The use of *in vitro* mutagenesis would obviously require the development of a technique for directed integration of cloned chromosomal fragments into the bacterial chromosome. Techniques for the replacement of genes in the bacterial chromosome have been described (Gutterson and Koshland, 1983; Scherer and Davis, 1979; Winans *et al*, 1985; Raibaud *et al*, 1984; Stahl and Ferrari, 1984) and such techniques require cloned chromosomal DNA adjacent to the mutagenized sequence to permit recombination of the sequence into the chromosome. Therefore, it would be necessary to clone chromosomal DNA adjacent to Tn2901 to allow the replacement of the wild-type IS1 sequences with the mutant sequences.

The development of a technique for directed integration would be useful to identify the function provided by the F factor. Clones containing fragments of the F factor could be forced to integrate into an F^- strain at chromosomal sites homologous to a cloned restriction

fragment. This would require cloning of chromosomal fragments to allow directed integration. Using this technique, it may be possible to isolate the region of the F factor required for the amplification on one or more cloned restriction fragments. Detailed molecular analysis of the cloned fragments carrying the F factor function would then be possible. If the cloned fragment containing the F factor function was integrated at different chromosomal locations, it would be possible to determine the effect of such things as increasing or decreasing the distance between Tn2901 and the cloned F factor function; changing the orientation of the F factor function, or even integrating the cloned fragment adjacent to Tn2901 but on the opposite side from the usual site of F factor integration. In vitro mutagenesis could also be applied to the cloned F factor fragment to further define the function that it provides for the formation of the amplification.

This chapter describes the development of a technique for the integration of cloned DNA at specific chromosomal restriction fragments, the various approaches used to clone a region of chromosomal DNA adjacent to Tn2901, and the construction of a gene bank from Hfr JEF8.

6.1 Directed integration of cloned DNA into the bacterial chromosome

(1). Attempted integration of pCC1 into JEF8 polA1

Greener and Hill (1980) forced a ColE1-type replicon carrying a cloned insert to integrate into the E. coli chromosome at its region of homology. Replication of colE1, and hence pBR322, is dependant upon host DNA polymerase I (Kingsbury and Helinski, 1970). Therefore, colE1-type plasmids cannot be transformed into a polA1 recipient since the incoming plasmid cannot replicate and is unable to establish itself as an autonomous replicon. However, Greener and Hill (1980) have shown that if an incoming plasmid contains an area of homology with the host chromosome, it can integrate by homologous recombination

at that site. Such integrants are obtained at a very low frequency : when a plasmid carrying 11.6kb of homologous DNA was used, 23 transformants per ug of DNA were obtained compared with approximately 10^6 transformants per ug in a *polA*$^+$ strain (Greener and Hill, 1980). pCC1 consists of the 11.7kb amplified EcoR1 fragment cloned into pUC9 (Chapter 3). The pUC vectors were derived from pBR322 (Vieira and Messing, 1982) and therefore pCC1 should be unable to replicate in a *polA* strain. It was therefore decided to attempt to integrate pCC1 into Tn2901 in JEF8. It was neccessary to construct a derivative of JEF8 which was *polA* to enable the forced integration of pCC1. An Hfr strain carrying the *polA1* allele was crossed with a nal^r derivative of JEF8 and plated on minimal agar containing threonine, arginine, uracil and naladixic acid to select for *metB*$^+$ recombinants. *metB* and *polA* are very close together on the *E. coli* K12 chromosome, being at 88 min and 86 min respectively. Therefore, the majority of *metB*$^+$ recombinants should also be *polA1*. Several hundred *metB*$^+$ recombinants were obtained. The *polA1* allele confers U.V. sensitivity on the host strain and therefore the U.V. sensitivity of the *metB*$^+$ recombinants was determined. Of the 48 *metB*$^+$ recombinants screened, 40 were U.V. sensitive. One of these was tested to ensure that the strain had remained *car*$^-$, which it had.

JEF8 *polA1* *metB*$^+$ nal was transformed with pCC1, and also with pUC9 as a control, and plated onto L agar containing ampicillin. Approximately 400 transformants per ug were obtained from both pCC1 and pUC9. Single colony gel analysis of several transformants indicated that integration of pCC1 has not taken place since plasmid DNA was visible. Several transformants were screened for sensitivity to U.V. light and they were found to be U.V. sensitive indicating that they were still *polA1*. A different approach was therefore used to force integration of a vector into the chromosome.

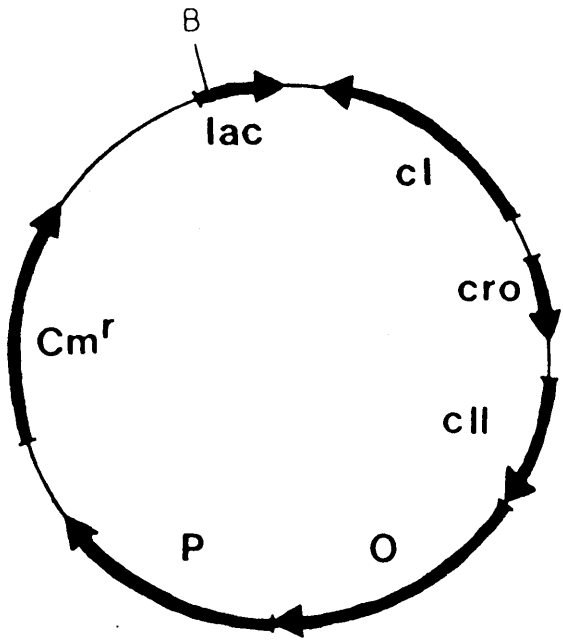


Figure 6.1. Map of the lambda origin vector pCB101 (5.0kb). All genes except cI are transcribed in a clockwise direction. The unique BamH1 site is indicated (B). (After Boyd and Sherratt, 1986).

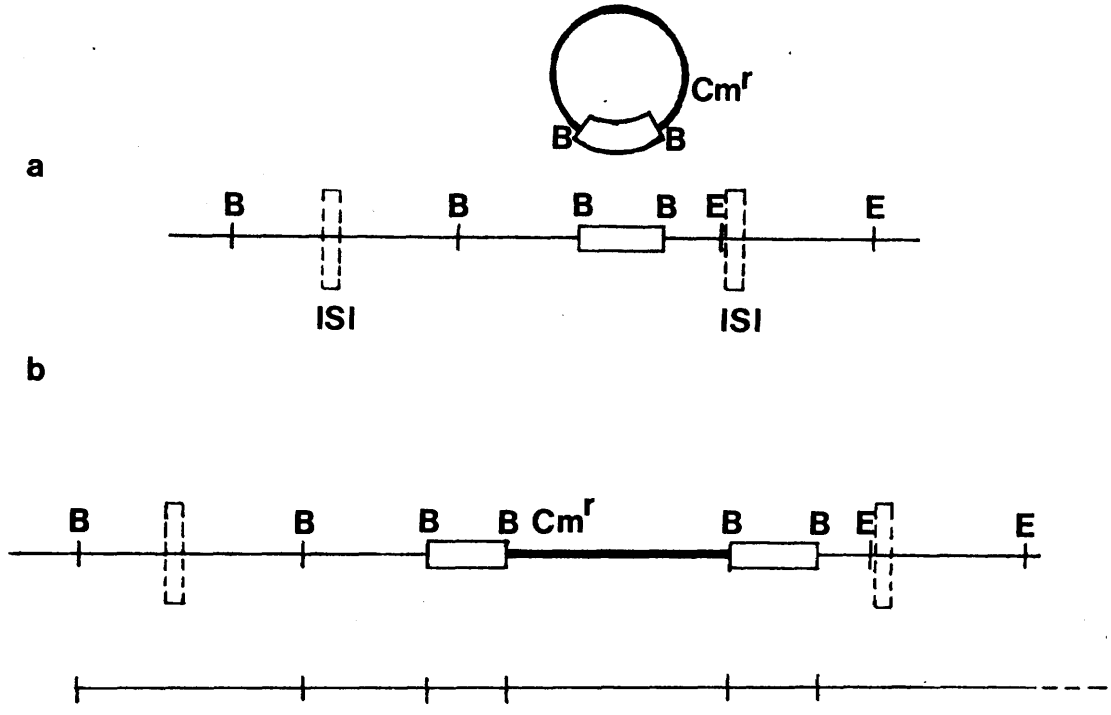


Figure 6.2. Forced integration of pCB101 containing a cloned fragment of homology with Tn2901 in the bacterial chromosome (a). The cloned region of homology is represented as an open box; the bacterial chromosome as a thin line; and the vector DNA as a heavy line. (b) shows the resultant chromosomal structure containing a duplication of the cloned fragment flanking the integrated vector. BamH1 (B) and EcoR1 (E) sites are shown.

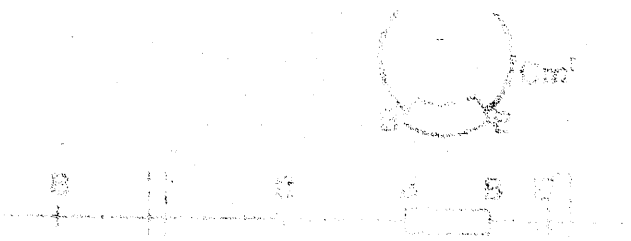


Figure 6.3. The construction of pCC7 and pCC9.

(a). Restriction map showing BamH1 fragments internal to Tn2901. The sizes given are in kilobases.

(b). The 2.3kb BamH1 fragment, purified from pCC1, is inserted at the BamH1 site of pCB101 to produce plasmids pCC7 and pCC9. The orientation of the cloned fragment in these plasmids is unknown.

(c). Single colony gel analysis of two white transformants (Tracks 1 and 2), and DNA from pCB101 (Track 3) indicating that the transformants contain plasmids with inserts.

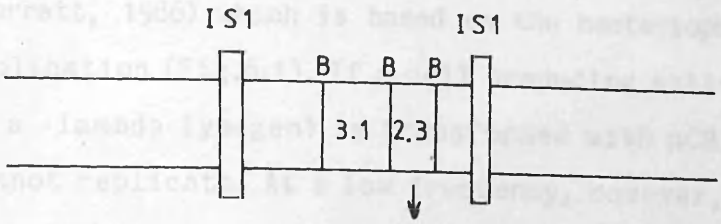
(d). BamH1 restriction analysis of pCC7 and pCC9 indicating that they both contain the 2.3kb insert.

Track 1: Lambda HindIII Track 2: pCC7 BamH1

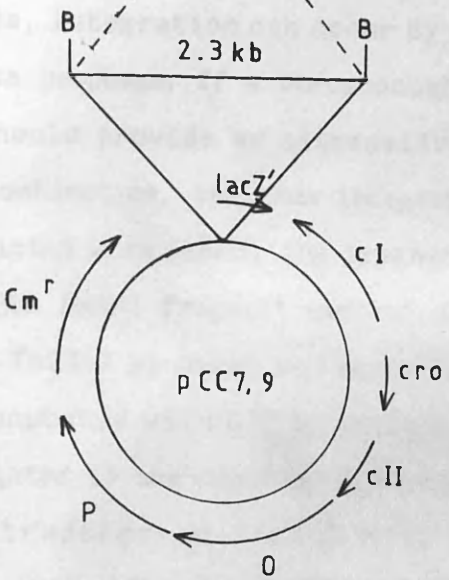
Track 3: pCC7 uncut Track 4: pCC9 BamH1

Track 5: pCC9 uncut

(a)


IS1
IS1
B
B
B
3.1
2.3

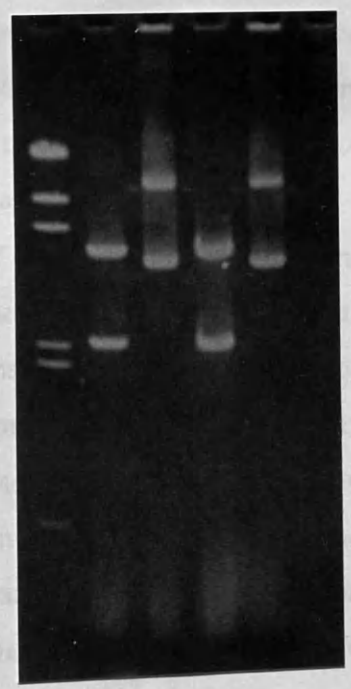
(b)


B
B
2.3 kb
lacZ'
cI
Cm^r
pCC7.9
cro
cII
P
O

(c).


1
2
3
5.0 kb

(d).


1
2
3
4
5
2.3kb

(2). Construction of pCC7 and integration into Cut1

An integrative plasmid vector, pCB101, has been developed (Boyd and Sherratt, 1986) which is based on the bacteriophage lambda origin of replication (Fig.6.1). If a cell producing active cI repressor (such as a lambda lysogen) is transformed with pCB101 then the plasmid cannot replicate. At a low frequency, however, (in a rec$^+$ cell) the plasmid DNA can survive by integrating directly into the chromosome via homologous recombination. Since pCB101 contains the cro, O and P genes of lambda, integration can occur by homologous recombination with the lambda prophage. If a chromosomal fragment was cloned into pCB101 this should provide an alternative region of homology for homologous recombination, and thus integration, to occur (Fig.6.2).

pCC1 was restricted with BamH1, the fragments separated on an agarose gel and the 2.3kb BamH1 fragment excised and purified. This fragment is internal to Tn2901 as shown in Figure 6.3a. pCB101 was restricted with BamH1, incubated with CIP to prevent recircularization of the vector, and ligated to the purified 2.3kb BamH1 fragment. The ligation mixture was transformed into Δ M15, and plated onto L agar supplemented with Cm, X-gal and IPTG. 30 transformants were obtained of which 4 were white. Single colony gel analysis of two white tranformants indicated that they contain plasmids with inserts because they contain a larger plasmid band than pCB101 alone (Fig.6.3c). Plasmid DNA was prepared from these two clones which were named pCC7 and pCC9. Restriction with BamH1 confirms the presence of the 2.3kb BamH1 fragment in both of these plasmids (Fig.6.3d).

JEF8 and Cut1 were tested for the presence of a lambda prophage using lambda cI and lambda vir phages, and both were found to be lambda lysogens. This made them suitable host strains for the integration of pCC7 or pCC9. JEF8 and Cut1 were each transformed with approximately 1ug of pCC7 DNA and plated onto L agar supplemented with Cm. It should be noted that Cut1 was not kept under continuous selection for the amplification and probably contained fewer than 45 copies of Tn2901 as a result. Four transformants were obtained with Cut 1 but no

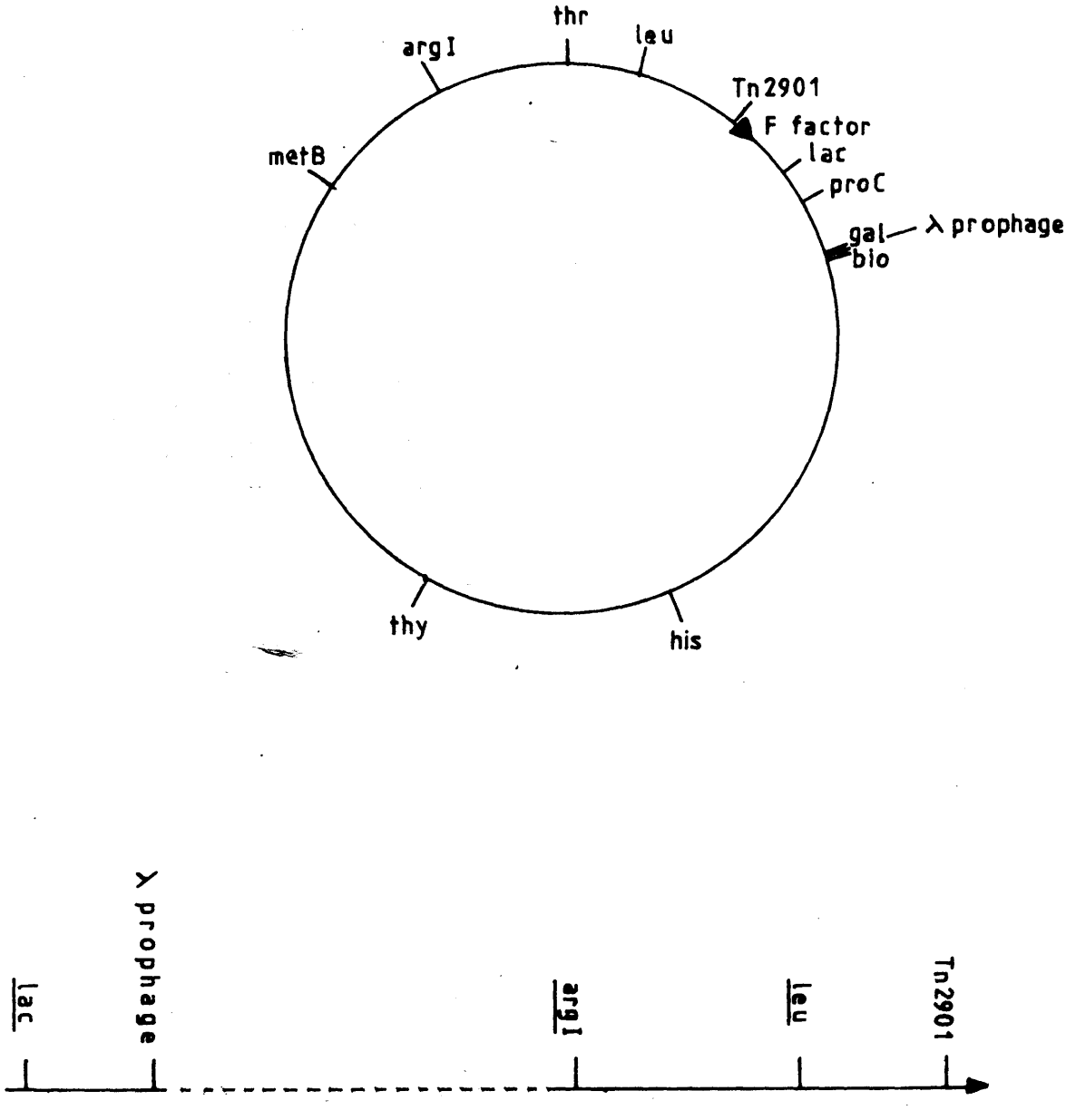


Figure 6.4. Diagram showing position of the integrated F factor in HfrP4X and its direction of transfer. Tn2901 is transferred early; the lambda prophage is transferred late.

transformants were obtained with JEF8. (Repeated transformation of JEF8 did eventually result in transformants and these are dealt with in section 6.2). Control transformations with pACYC184 DNA resulted in approximately 10^4 transformants per ug of DNA. Single colony gel analysis of the four Cut1 transformants indicated that only one of these contains plasmid DNA, suggesting that pCC7 is integrated in the chromosome in the other three transformants. Each of the three Cm^r transformants in which pCC7 was possibly integrated were crossed with CB50 for 50 min and plated on minimal agar supplemented with proline, methionine, arginine, Sm and Cm. This selected for Cm^r recombinants. If pCC7 were integrated in the lambda prophage it should be transferred with very low frequency since the lambda prophage resides at 17 min on the bacterial chromosome between gal and bio and hence would be transferred as a very late marker by JEF8 and JEF8 Cut1 (Fig.6.4). However, each of the three strains transferred Cm^r at a very high frequency consistent with the integration event having taken place within the 2.3kb BamH1 fragment of Tn2901.

6.2 Attempted cloning of chromosomal DNA adjacent to Tn2901 by "chromosomal walking"

It would be useful to develop a technique which would allow the isolation of chromosomal DNA specifically from the pro-lac region for several reasons:

(1). Data presented in Chapter 3 revealed the presence of a second class of Cut mutants, referred to as "stable" Cut mutants. The isolation of Tn2901 and adjacent chromosomal DNA from several of these mutants will be necessary for molecular analysis of the structure of these mutations. The isolation of chromosomal DNA specifically from the pro-lac region would obviously be more convenient than the construction of gene-banks from each mutant.

(2). Mutants have been isolated which are unable to amplify Tn2901,

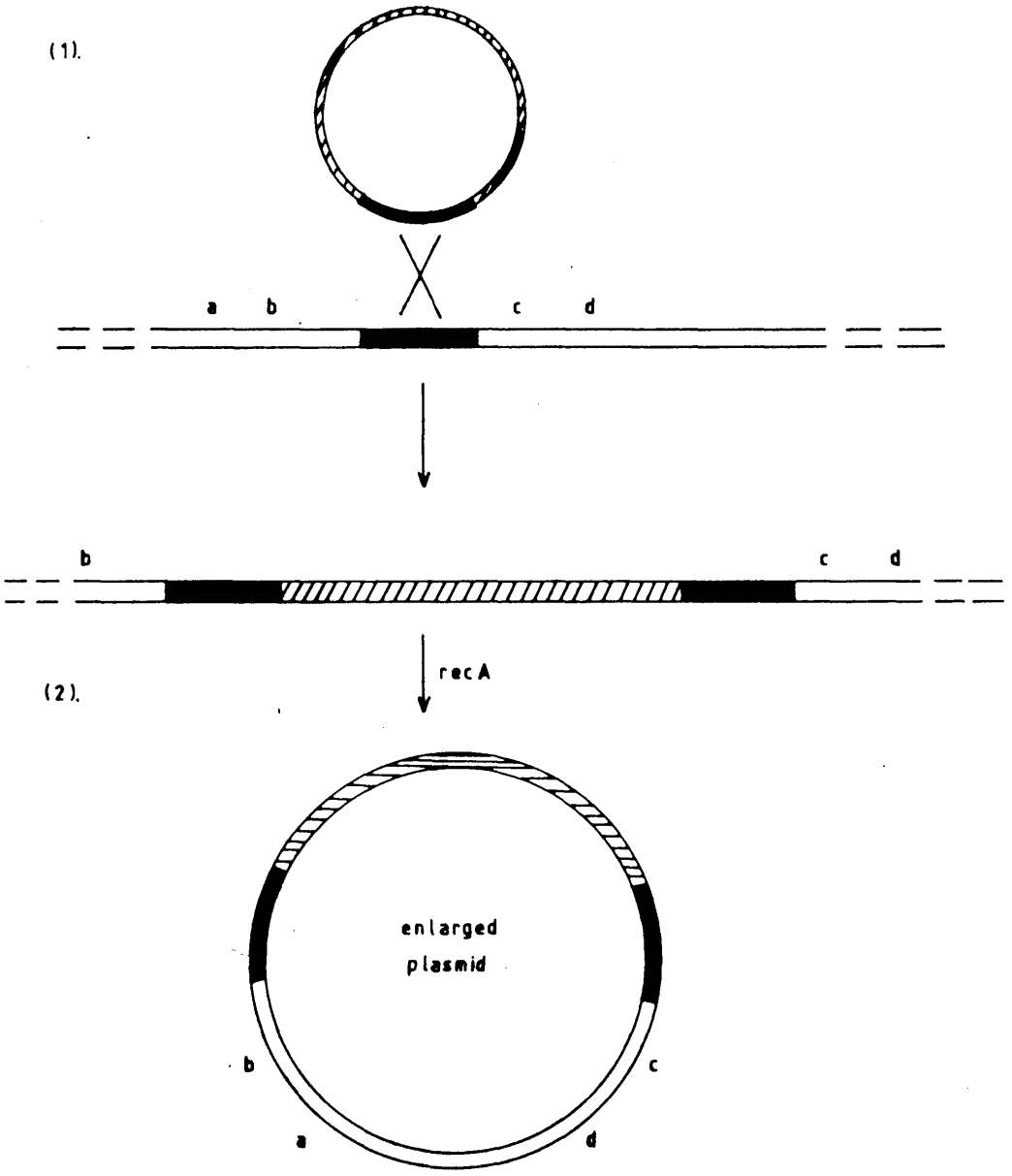


Figure 6.5. Diagram of the "transductional walking" technique. The first step (1) involves the forced integration of a vector (hatched line) into the chromosome by homologous recombination between a cloned fragment (shaded line) and a homologous region (open line). a, b, c and d represent adjacent chromosomal DNA. The second step (2) selects for an enlarged plasmid carrying the integrated vector and flanking portions of the bacterial chromosome by transduction into a recA strain.

and at least three of these map in the argF region (A.P. Jessop, pers. comm.). Cloned chromosomal fragments from the pro-lac region could be transformed into each of the mutant strains to isolate clones of the wild-type genes (which allow amplification of Tn2901). Since no selection is available for the mutations which prevent the amplification, the mutations themselves could not be isolated from gene-banks constructed from the mutant strains. Therefore, a technique which would quickly allow the isolation of chromosomal DNA specifically from the pro-lac region would be of great value.

(3). Cloned chromosomal DNA adjacent to Tn2901 and extending up to the F factor is required for the directed integration technique as mentioned previously. Cloning of this region is complicated by having no direct selection and no cloned DNA which could be used as a probe to screen for clones containing DNA from this region. Therefore, if a chromosomal gene-bank is constructed the only probes available with which to screen the gene-bank are argF and IS1, both of which may hybridize to fragments not extending past Tn2901 and IS1 will hybridize to fragments not even containing Tn2901. Obviously any fragment obtained which did extend past Tn2901 could be used to re-probe the gene-bank and hence "walk" along the chromosome in the required direction. However, many clones may have to be analysed in order to obtain the required fragments.

A technique termed "transductional walking" has been developed by Lin et al (1984) by which chromosomal DNA adjacent to a previously cloned DNA fragment can be cloned through genetic procedures. This method is an extension of directed integration and may provide a simple method by which to clone the chromosomal DNA adjacent to Tn2901. The strategy for transductional walking is outlined below and is shown diagramatically in Figure 6.5.

The first step of the transductional walk involves directed integration of a vector into the chromosomal region of interest. The second step selects for an enlarged plasmid carrying the integrated vector and flanking portions of the host chromosome. This is achieved

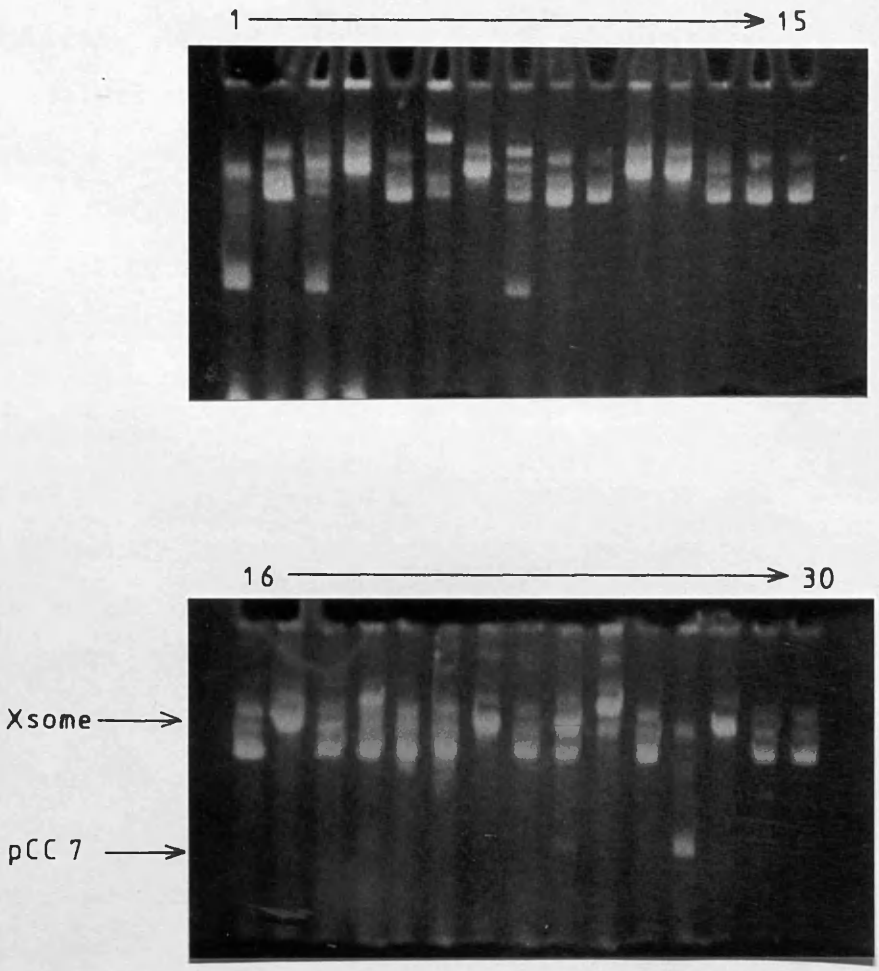


Figure 6.6. Single colony gel analysis of 29 P1 transductants from JEF8 Cut1 pCC7 into 902, a recA strain. The single colony lysates were run on a horizontal 0.8% agarose gel which had two sets of wells. Track 27 contains a single colony lysate of pCC7 in Δ M15 as a size marker. The chromosome is indicated (Xsome).

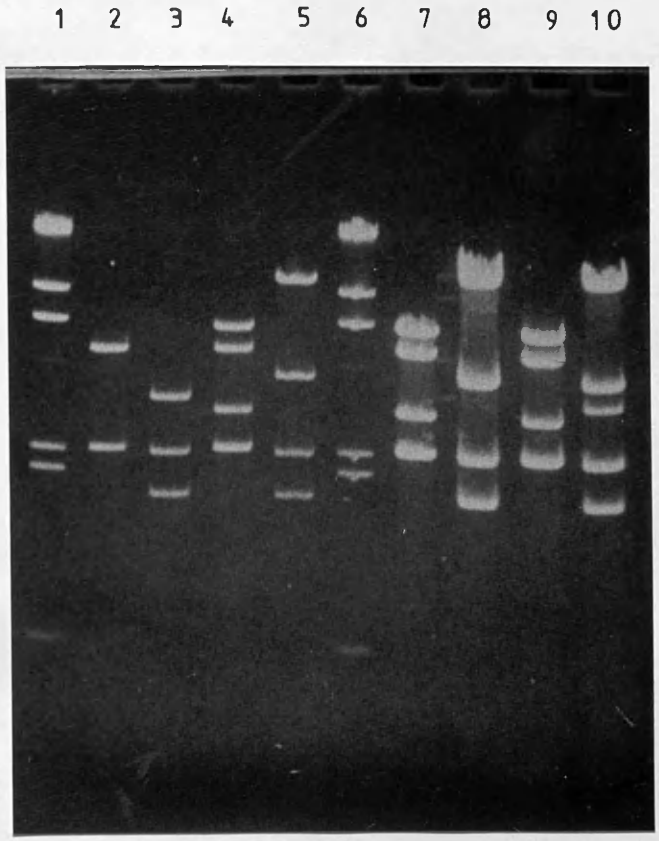


Figure 6.7. Restriction analysis of pCC7, pCC11, pCC12 and pCC13 on an 0.8% agarose gel.

Track 1: Lambda HindIII

Track 2: pCC7 BamH1

Track3:pCC7 EcoR1

Track 4:pCC11 BamH1

Track 5: pCC11 EcoR1

Track 6: Lambda HindIII

Track 7: pCC12 BamH1

Track 8: pCC12 EcoR1

Track 9: pCC13 BamH1

Track 10: pCC13 EcoR1

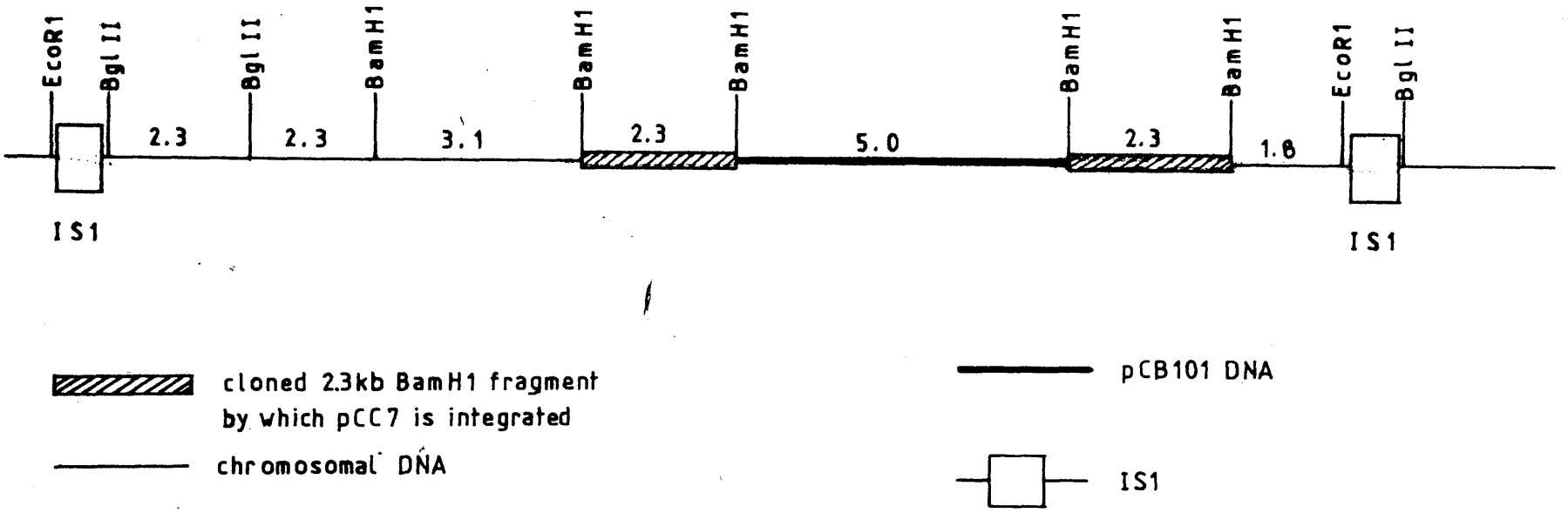


Figure 6.8. Probable structure of Cut1 pCC7 showing the duplicated 2.3kb BamH1 fragment flanking the integrated vector. BglII, BamH1 and EcoR1 sites are indicated; all figures are in kilobases.

by transducing the integrated plasmid into a recA recipient. Normally, a recA mutant cannot be used as a recipient in a P1 tranduction since incorporation of the transduced fragment into the chromosome requires two homologous recombinational events (Hertman and Luria, 1967). However, if a transduced fragment containing the integrated vector circularizes by an illegitimate recombination event it may produce an enlarged plasmid that can replicate using the vector origin. By such a transductional walking technique Lin et al (1984) obtained a transductant carrying a plasmid of approximately 50kb which contained approximately 20kb of flanking chromosomal DNA.

(1). P1 Transduction of Cut1 pCC7

A P1 lysate was made on one of the strains which contained pCC7 integrated at the 2.3kb BamH1 fragment of Tn2901. This lysate was used to transduce 902, a recA strain which is not a lambda lysogen and the mixture was plated on L agar supplemented with Cm. 148 Cm^r transductants were obtained. Single colony gel analysis of 29 transductants (Fig.6.6) indicates that 26 contain large plasmids whilst 3 contain plasmids the same size as pCC7. Plasmid DNA was isolated from 10 of the transductants carrying large plasmids. Each plasmid was restricted with BamH1 and EcoR1 and the fragments separated on an agarose gel. Each plasmid results in the same pattern of EcoR1 and BamH1 fragments. Three such plasmids (named pCC11, pCC12 and pCC13) are shown in Figure 6.7 together with pCC7. BamH1 produces four fragments, two of which are the same size as the fragments obtained in the BamH1 digest of pCC7. EcoR1 also produced four fragments , two of which were the same size as the fragments obtained in the EcoR1 digest of pCC7. The sizes of the fragments, calculated from lambda HindIII size markers, are shown in Table 6.1. The sum of the sizes of the EcoR1 fragments from the large plasmids is 18.8kb, and the sum of the sizes of the BamH1 fragments is 17.6kb. However, the chromosomal 2.3kb BamH1 fragment will be duplicated as a result of pCC7 integration (Fig.6.8), and therefore there may be two 2.3kb

fragments present in the BamH1 digest resulting in a total size of 19.9kb. Therefore the plasmid is much smaller than the 50kb plasmid obtained by Lin et al (1984) although it must contain 11kb to 12kb of chromosomal DNA flanking the integrated plasmid.

The EcoR1, BamH1 and BglII sites of pCC11 were mapped using double digests of these enzymes. The agarose gel containing the restriction digests is shown in Fig.6.9 and the fragment sizes in Table 6.1. Since the two 2.3kb BamH1 fragments flanking pCB101 in Cut1 pCC7 (Fig.6.8) are not cut by either BglII or EcoR1, double digests of BamH1 with BglII or EcoR1 may contain two 2.3kb BamH1 fragments which would only be visualized as a single band on an agrose gel. This may explain why the calculated total plasmid size is always smaller using BamH1 than when other enzymes are used. However, even if an additional 2.3kb was added to the BamH1/BglII digest the total plasmid size would still only be 12.1kb which suggests that at least one other band in the restriction digest must represent two or more restriction fragments of similar size.

The restriction map of the integrated pCC7 (Fig.6.8), together with the various restriction digests of pCC11, allowed a restriction map of the large plasmids to be made. Briefly, the BamH1 digest of pCC11 produces four fragments of 5.0kb, 2.3kb, 3.1kb and 7.2kb. The 5.0kb fragment almost certainly corresponds to pCB101, and the 2.3kb fragment to the two 2.3kb fragments flanking pCB101 in the integrated pCC7. The presence of a 3.1kb fragment suggests that the 3.1kb BamH1 fragment adjacent to the 2.3kb BamH1fragment of Tn2901 is present, and the 7.2kb fragment represents cloned DNA extending further to either side of pCB101 but not containing any BamH1 sites. It is this fragment that also must contain the novel joint produced by the illegitimate recombination event which resulted in the formation of the enlarged plasmid. Digestion with BamH1 and EcoR1 indicates that this 7.2kb BamH1 fragment contains an EcoR1 site since it is not present in the BamH1/EcoR1 digest. This digest also confirms the presence of the 2.3kb and 3.1kb BamH1 fragments of Tn2901 (which are not cut by EcoR1)

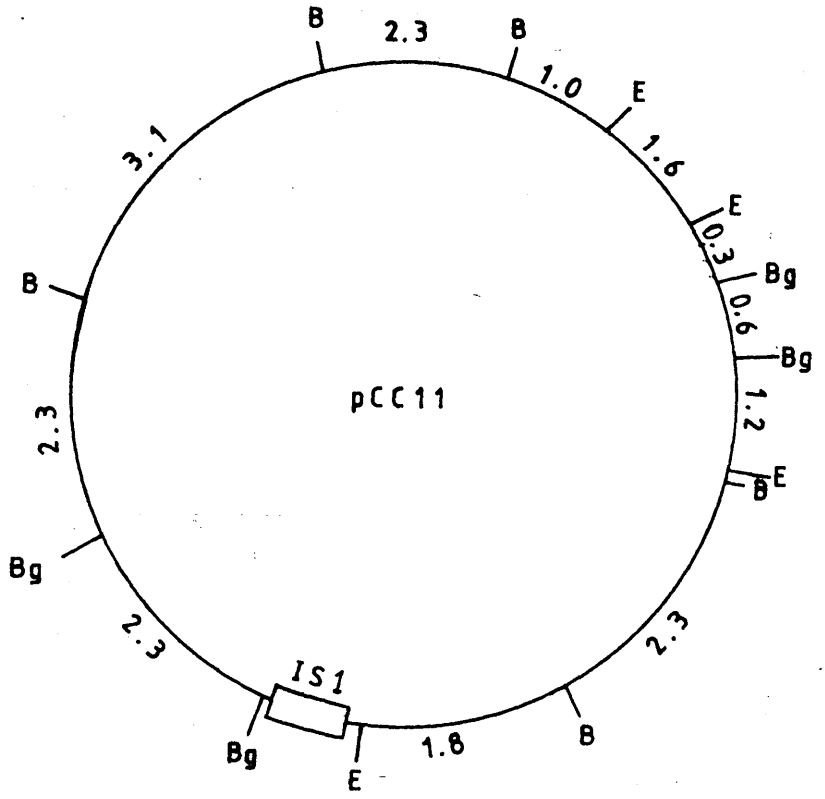


Figure 6.10. Restriction map of pCC11. The restriction map was deduced from restriction digests (see text). BamH1 (B), EcoR1 (E) and BglII (Bg) sites are shown. All sizes are in kilobases.

and also produces the expected internal EcoR1 fragment of 1.6kb from pCB101. A 1.8kb fragment is also produced which is the same size as the BamH1/EcoR1 fragment within Tn2901 suggesting that this fragment is also present in pCC11. BglII produces four fragments including the expected 0.6kb BglII fragment internal to pCB101. This indicates that the flanking chromosomal DNA must contain two BglII sites and the presence of a 2.1kb fragment suggests that the two BglII sites are those within Tn2901.

Restriction digestion therefore indicates that the chromosomal DNA of pCC11 extends from the integrated vector as far as the EcoR1 site adjacent to the right-hand IS1 element and the BglII site adjacent to the left-hand IS1 element of Tn2901. Taking the size of pCC11 to be approximately 19kb (calculated from the sums of the fragments in each digest) and deducting the size of pCC7 (7.3kb), then the size of the flanking chromosomal DNA must be approximately 11.7kb. The distance from the BglII site adjacent to the left-hand IS1 element to the EcoR1 site adjacent to the right-hand IS1 element in Tn2901 is approximately 11kb. This suggests that the recombination event generating pCC11 must have occurred within the IS1 elements of Tn2901 to result in an enlarged plasmid of approximately 19kb. This is confirmed by the presence of an 0.9kb fragment in the BglII/EcoR1 digest which is the size of the amplified BglII/EcoR1 fragment in the US Cut mutants. The restriction map of pCC11 is shown in Figure 6.10 and is consistent with all the restriction fragment sizes in Table 6.1.

(2). P1 transduction of JEF8 pCC7

Since no chromosomal DNA flanking Tn2901 was obtained by P1 transduction of Cut1 pCC7, it was decided to re-attempt to integrate pCC7 into JEF8 in the hope that recombination between the IS1 elements would not occur in this strain.

Approximately 10ug of pCC7 DNA was used to transform competent cells of JEF8. The transformations were plated onto L agar supplemented with

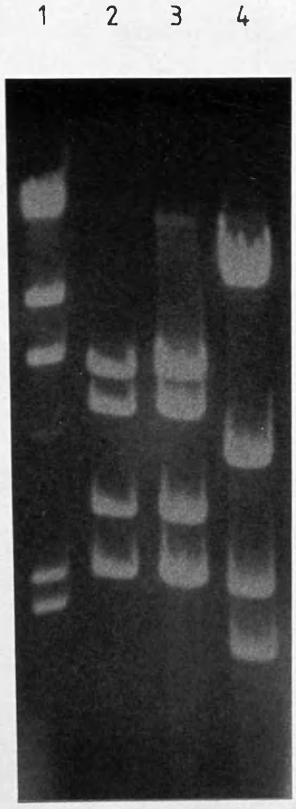


Figure 6.11. Restriction analysis of the large plasmid obtained by P1 transduction of JEF8 thy nal pCC7.

Track 1: Lambda HindIII Tracks 2 and 3: BamH1 digest

Track 4: EcoR1 digest

Cm. Five Cm^r transformants were obtained and single colony gel analysis indicated that three of these did not contain any visible plasmid bands. Of these strains, two (JEF8 pCC7, 2 and 4) produced two shades of purple colonies when plated on minimal medium containing citrulline and crystal violet (see Chapter 3), indicating that pCC7 is inserted in Tn2901. The third strain did not produce dark purple colonies, indicating that pCC7 is not integrated in Tn2901 in this strain.

P1 lysates were made from the two strains in which pCC7 is integrated in Tn2901. The P1 lysates were used to transduce 902, the recA strain. The transduction mixtures were plated on L agar supplemented with Cm. The lysate from JEF8 pCC7,2 produced two colonies and that from JEF8 pCC7,4 produced no colonies. Single colony gel analysis indicated that the transductants from JEF8 pCC7,2 contain plasmid DNA of the same size as pCC7. The P1 lysates made from JEF8 pCC7,2 and JEF8 pCC7,4 were repeatedly used to transduce 902 selecting for Cm^r transductants. A total of 12 transductants were obtained from 8 transductions and 11 of these contained plasmid DNA the same size as pCC7. One transductant was obtained which did contain a large plasmid but restriction analysis of this plasmid using BamH1 and EcoR1 (Fig.6.11) indicates that it is the same as the large plasmids obtained from Cut1 pCC7 generated by recombination between the two IS1 elements of Tn2901.

Lin et al (1984) selected for transduction of a chromosomal gene, mtl, which was adjacent to the integrated vector and mtl^+ transductants were screened for the co-transduction of the Ap^r vector. It was decided, therefore, to select for pro^+ transductants and screen any obtained for the co-transduction of Cm^r. The JEF8 pCC7,2 lysate was used to transduce 902 and the transduction mixture was plated on minimal agar supplemented with threonine, leucine, histidine, arginine and B_1 to select for pro^+ transductants. However, no pro^+ transductants were obtained despite the transduction being repeated several times. Transduction of 903, a rec^+ strain, with the same lysate produced several thousand pro^+ transductants, indicating that

the lysate did contain *pro*$^+$ transducing phages although no *pro*$^+$ plasmids were obtained in the *rec*A strain.

The JEF8 pCC7,2 P1 lysate was also used to tranduce NGX2 *rec*A. The transduction mixture was plated on minimal agar supplemented with leucine, arginine and uracil to select for *pro*$^+$ transductants; leucine and uracil to select for *pro*$^+$ *arg*$^+$ transductants, and leucine, uracil and Cm to select for *pro*$^+$ *arg*$^+$ Cm^r transductants. However, no transductants were obtained despite the transductions being repeated several times.

Since the transductional walking technique had not resulted in a plasmid carrying chromosomal DNA adjacent to Tn2901 it was decided to use a different approach.

6.3 Isolation of F-prime DNA

Since F-primes which contain the chromosomal DNA between *pro*A and *lac* can be obtained easily from JEF8, it was decided to attempt to extract F-prime DNA and use this to clone the required fragments from the region between Tn2901 and the F factor. Since restriction maps are avaiable of both the *pro* - *lac* region (Hadley *et al*, 1983) and the F factor (Ohtsubo and Ohtsubo, 1977) it should be possible to purify and clone the restriction fragments of interest from an agarose gel. If the required restriction fragments could not be easily identified on an agarose gel, a gene-bank could be made from the F-prime DNA and each clone analysed separately by restriction digestion.

6.3.1 Isolation of F-prime DNA by the method of Hansen and Olsen (1978)

There are two problems associated with the isolation of large plasmids using standard procedures. Firstly, with methods that depend on the

Table 6.2 Steps of plasmid isolation

Step	Operation	Conditions
1.	Cell growth	40ml cells grown in minimal broth to about 2 x 10^8 cells/ml.
2.	Cell lysis	Washed cells resuspended at high osmolarity, addition of lysozyme, Na_2EDTA, SDS to 4%, intermittent 55°C pulses.
3.	Alkaline denaturation	pH 12.1 to 12.3, 3 min.
4.	Neutralization	Addition of 2M Tris (pH7.0) to lower pH to 8.5 to 9.0.
5.	Removal of membrane-chromosome complexes	Addition of SDS to 4%, NaCl to 1.0M; 4°C for 6h, centrifugation 30min 12,000 x g.
6.	Concentration of plasmid DNA from supernatant	Addition of PEG to 10%, 4°C for 6h; centrifugation 2,500 xg 5 min ;resuspension in one-fortieth volume
7.	Agarose gel electrophoresis	

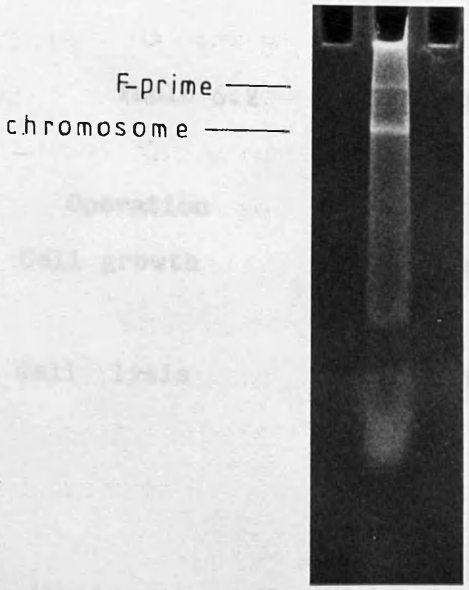


Figure 6.12. 0.7% agarose gel showing F-prime DNA isolated from DF30D.2 in P678.14 recA by the method of Hansen and Olsen (1978).

selective precipitation of chromosomal complexes by either high gravity centrifugation (e.g. Clewell and Helinski, 1969) or high salt precipitation (Guerry et al, 1973), large plasmids do not remain in the supernatant and are therefore lost (Hansen and Olsen,1978). Secondly, some methods employ a shearing step to break chromosomal DNA, which may act to help release plasmid (Bazaral and Helinski, 1968). However, shearing appears also to break much of the large plasmid DNA (Currier and Nester, 1976), which necessitates an increase in the input of cells to provide sufficient plasmid yield. Increasing the input, however, requires a further step to remove excess linear DNA before caesium chloride - ethidium bromide density equilibrium centrifugation. This is done in several protocols by denaturing the linear DNA and selectively removing the single-stranded DNA fragments (Currier and Nester, 1976; Sharp et al, 1972; Palchaudhuri and Chakrabarty, 1976). Hansen and Olsen (1978) developed a method for the rapid isolation of large plasmids which does not require a large input volume of cells. Their method is in part comprised of portions of three previously reported methods (Currier and Nester, 1976; Guerry et al, 1973; Humphreys et al, 1975). It also incorporates changes designed to help release plasmid from the folded chromosome since it has been suggested that in vivo a supercoiled plasmid makes a non-integrative association with the bacterial chromosome which is important for both replication and segregation of the plasmid (Kline and Miller, 1975; Kline et al, 1976).

The method of Hansen and Olsen (1978) was chosen to isolate DNA from an F'*pro*$^+$*lac*$^+$ plasmid, DF30D.2, in P678.14 *rec*A. Since Currier and Nester (1976) had found that the loss of plasmid DNA due to mechanical shearing was considerable, great care was taken to keep this shearing to a minimum. Therefore, manipulations were all very gentle, consisting only of decanting and inversions and there was no pipetting or vortexing of solutions. A brief outline of the steps involved in the isolation of F-prime DNA is shown in Table 6.2. Samples of the crude lysate preparation were run on an agarose gel (Fig.6.12). The

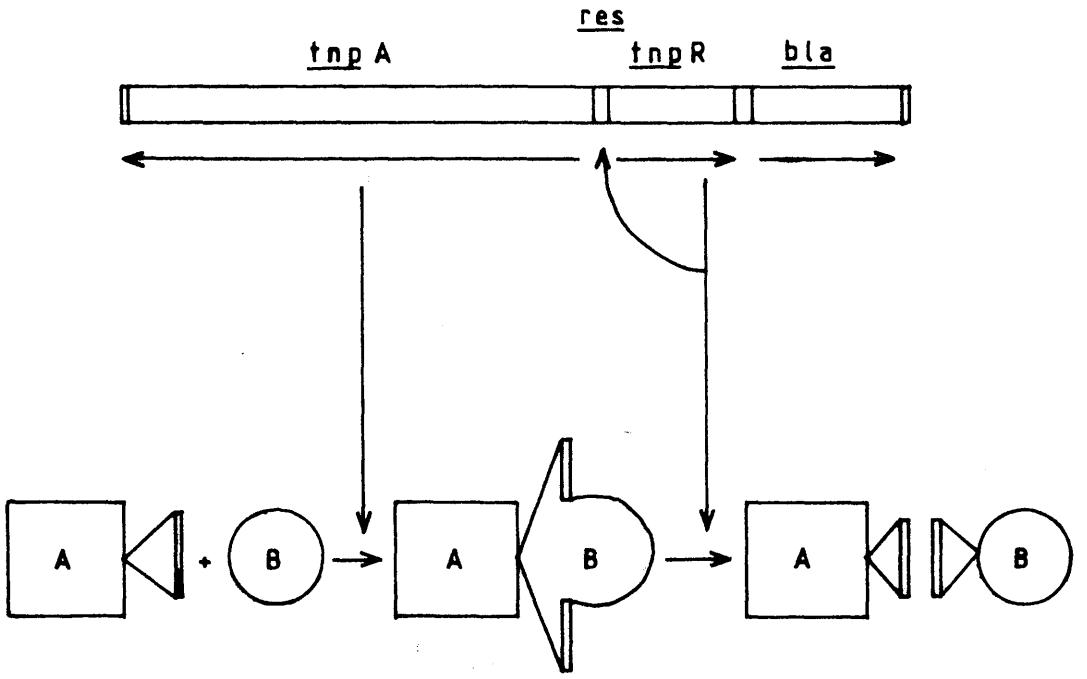


Figure 6.13. A general scheme of the transposition process of Tn3
The tnpA gene product mediates the formation of a co-integrate molecule, which is then resolved by the tnpR product (at res) to generate the normal transposition end products.

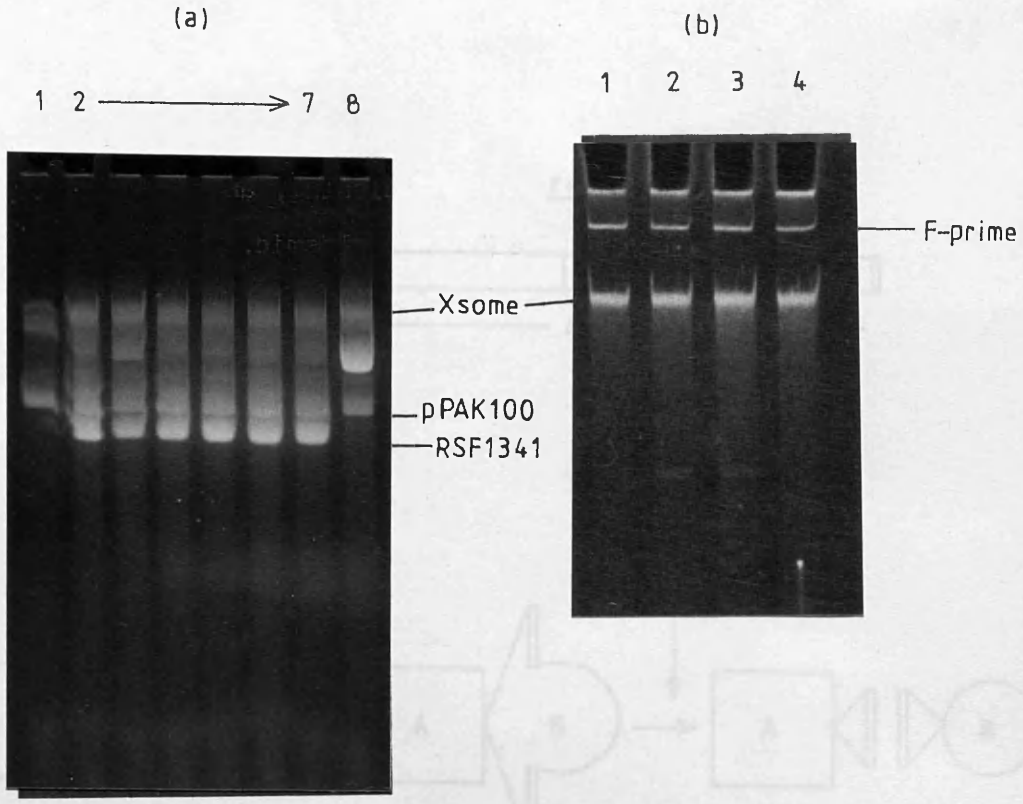


Figure 6.14. Co-integrate formation with Tn3

(a) Single colony gel of transformants of DF30D.2 in P678.14 recA containing pPAK100 and RSF1341. Tracks 1 and 8 contain pPAK100 alone. No F-prime DNA is seen.

(b) Single colony gel of recombinants obtained by crossing the transformants in (a) with NGX2 (selection for pro$^+$ arg$^+$ Ap^r recombinants). An F-prime band is seen in all of the recombinants.

gel shows the presence of F-prime DNA but only a very small yield has been obtained.

Since the method of Hansen and Olsen (1978) did not yield sufficient F-prime DNA for restriction analysis and cloning, an attempt was made to increase the copy number of the F-prime plasmid.

6.3.2 Attempted amplification of F-prime copy number

(1). Co-integrate formation with Tn3

The Ap^r transposon Tn3 transposes via a co-integrate intermediate whose formation requires the tnpA gene product and whose resolution requires the tnpR gene product (Fig.6.13). Plasmids containing Tn3 derivatives which are $tnpR^-$ can form co-integrates with the large conjugative plasmid R388 but cannot resolve such co-integrates to form the normal transpositional products. Such R388 co-integrates have a far higher copy number than R388 alone (Symington, 1982). It may be possible to construct a similar co-integrate using an F-prime instead of R388, resulting in a high copy number F-prime co-integrate. This would overcome the problem of a small yield when isolating F-prime DNA.

Since the gamma-delta element present on the F factor can resolve Tn3 co-integrates (Symington, 1982) it was necessary to employ a $tnpR^-$ derivative of Tn3 which was also res^-. RSF1341 contains a $tnpA^-$ $tnpR^-$ res^- derivative of Tn3. Since this plasmid cannot transpose, another plasmid, pPAK100, was also transformed into the F-prime strain to provide transposase *in trans*. Several F'pro^+ lac^+ strains were transformed with these plasmids giving similar results. Single colony gel analysis shows no F-prime DNA in either the F-prime strain containing the two plasmids (Fig.6.14a) or in the F-prime strain alone. DF30D.2, an F'pro^+ lac^+ plasmid, in P678.14 recA containing both RSF1341 and pPAK100 was crossed with NGX2 recA and plated on minimal agar supplemented with leucine, uracil and Ap to select for

pro$^+$ *arg*$^+$ Ap^r co-integrates. Since pPAK100 and RSF1341 are both *mob*$^-$, any Ap^r exconjugants obtained must contain a co-integrate of the F-prime plasmid and RSF1341. Single colony gel analysis of the *pro*$^+$ *arg*$^+$ Ap^r recombinants obtained (Fig.6.14b) indicates the presence of an F-prime band which is not present in DF30D.2 P678.14 *rec*A. Very faint plasmid bands are seen in the exconjugants but when single colonies were further purified these bands disappeared indicating that they probably represented background growth of the donor strain.

Whether the F-prime co-integrate has a higher copy number than the F-prime alone is unclear. Certainly, the F-prime co-integrate is visible in NGX2 *rec*A whereas the F-prime is not visible in P678.14 *rec*A although this may not be due to a difference in copy number, e.g. the F-prime may be closely associated with the chromosome in P678.14 *rec*A but not in NGX2 *rec*A. However, this is purely speculative. Clearly, even if the F-prime co-integrate has a higher copy number, its copy number is much lower than a similar co-integrate with R388 (Symington, 1982). Therefore, this approach was abandonned since it did not result in a significant increase in the copy number of the *pro*$^+$ *lac*$^+$ F-primes.

(2). Attempted F-prime isolation from JEF8 pCC7

Since pCC7 is integrated at Tn2901 in JEF8 pCC7, an F-prime derived from this strain should contain two replication origins, namely those of the F factor and pCB101. It is possible that the copy number of this F-prime plasmid would be increased due to the pCB101 replication origin, and this would overcome the problem of low yield encountered with F-prime DNA extraction.

JEF8 pCC7, 4 was crossed with two strains. Firstly P678.14 *pro rec*A and plated on minimal agar supplemented with methionine, threonine, arginine, uracil, Sm and Cm to select for *pro*$^+$ Cm^r F-primes. This cross did not yield any recombinants despite being attempted several times. Secondly, JEF8 pCC7, 4 was crossed with CSH56 *arg*I, which

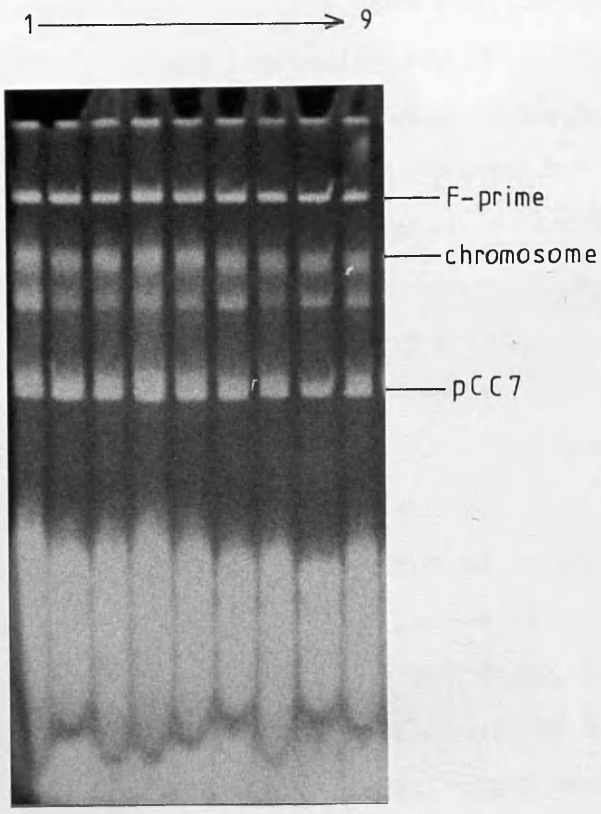


Figure 6.15. Single colony gel analysis of recombinants obtained by crossing JEF8 thy nal pCC7,4 with CSH56 argI and selecting for pro$^+$ arg$^+$ Cm^r. Bands corresponding to the F-prime, chromosome and pCC7 are indicated.

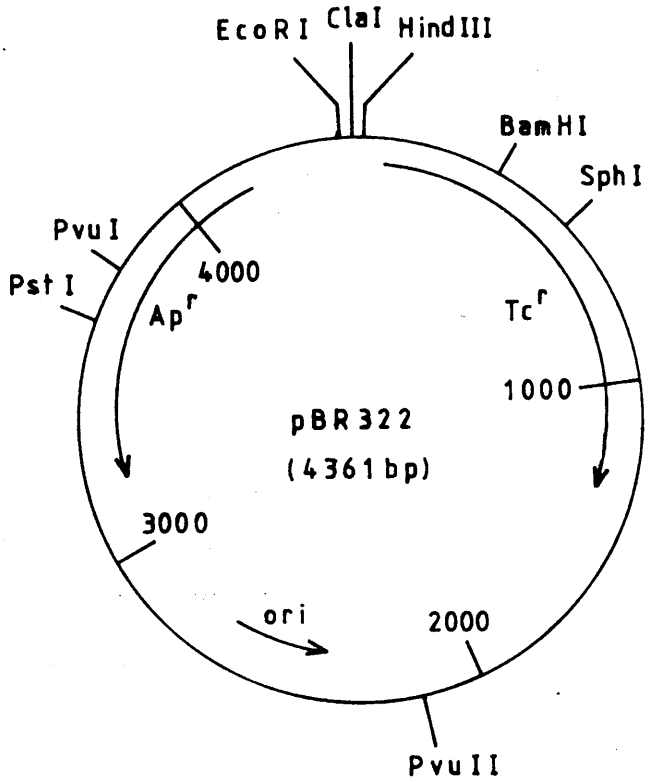


Figure 6.16. Map of the cloning vector pBR322

contains a deletion of the pro-lac region, and plated on minimal agar supplemented with thymine and Cm to select for pro^+ arg^+ Cm^r F-primes. Single colony analysis of the recombinants obtained from this cross (Fig.6.15) indicates the presence of an F-prime band. However pCC7 appears to have recombined out of the bacterial chromosome and therefore the copy number of the F-prime cannot be increased.

6.4 Construction of a gene-bank and cloning of DNA adjacent to Tn2901

Since the above approaches failed to isolate chromosomal DNA fragments adjacent to Tn2901 it was decided to construct a chromosomal gene-bank and probe this with the 11.7kb EcoR1 amplified fragment from Cut1. This should allow the isolation of fragments containing Tn2901 and adjacent chromosomal sequences. Any clones isolated containing DNA adjacent to Tn2901 could be used as probes to walk along the chromosome in the required direction.

6.4.1 Construction of a chromosomal gene-bank from JEF8

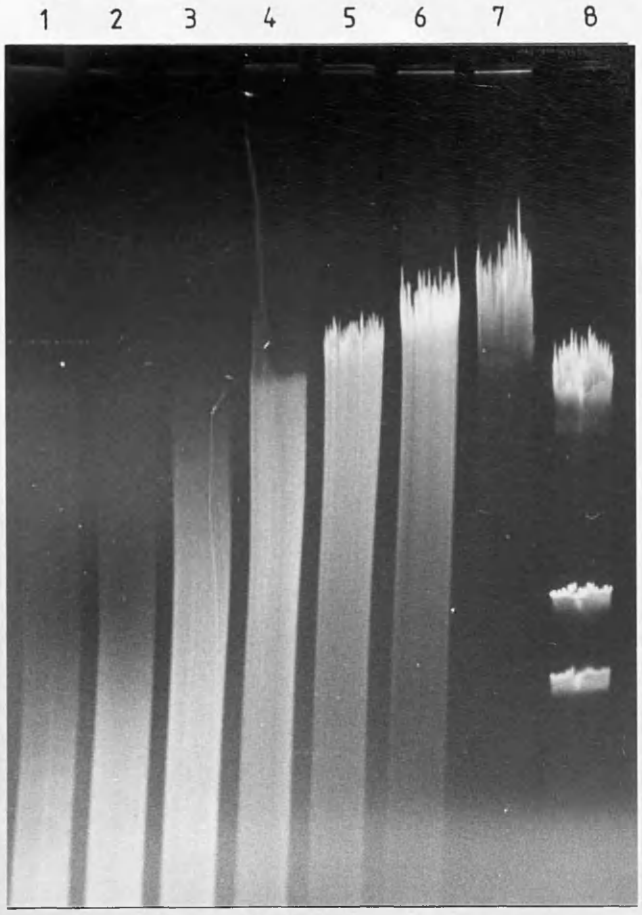
The construction of a gene-bank required a suitable vector with which to clone fragments of chromosomal DNA. The size of the fragments to be cloned influenced the choice of vector since cosmid and lambda vectors are generally more suitable than plasmid vectors for cloning fragments greater than 20kb. It was decided that fragments of 15kb to 20kb would be a suitable size for cloning since these should allow chromosomal "walking" from Tn2901 to the F factor by the isolation of two or three overlapping clones. This size of insert would also be small enough to allow its identification by restriction mapping which was neccessary since no probes are available for this region.
The plasmid vector pBR322 (Fig.6.16) was chosen with which to clone fragments of 15-20kb. pBR322 has been used previously to subclone the

11.7kb EcoR1 amplified fragment (data not shown) and it was therefore hoped that this vector would be able to carry inserts of 15-20kb. (Johnson and Willetts (1980) have reported the use of pBR322 to clone F factor fragments of up to 55.9kb). The copy number of pBR322 is between 30 and 40 and this is more suitable than a very high copy number vector such as pUC8/9 since some genes cannot be cloned on very high copy number vectors. Examples include genes which encode surface structural proteins, e.g. ompA (Beck and Bremer,1980) or proteins that regulate cellular metabolism. Therefore it was appropriate to use a fairly low copy number vector because the region between Tn2901 and the F factor may contain a gene whose product when over-expressed is lethal or deleterious to the host. However a very low copy number vector was also undesirable since this would not facilitate the isolation of large quantities of DNA and the analysis of recombinant plasmids by single colony gel electrophoresis. pBR322 encodes resistance to Ap and Tc and various restriction enzyme sites within these genes are especially suitable for cloning purposes since an insert of DNA at one of these sites will disrupt the resistance gene and provide a suitable screen for the presence of cloned DNA.

Two approaches can be used to prepare a genomic library. The first involves digestion of genomic DNA to completion with a restriction enzyme and insertion of the resulting fragments into a suitable vector. This method, however, has several disadvantages. If the sequence of interest contains recognition sites for the particular enzyme chosen, it will be cloned in two or more pieces. There is also a chance that the sequence may not be cloned at all if, for example, it is contained in a larger DNA fragment than the vector can accept. Also, many small fragments will be generated by this method and an entire library will contain a very large number of recombinant plasmids which could make screening very laborious.

The second approach involves generating genomic fragments of a certain size by random shearing or partial digestion with a frequently cutting restriction enzyme. This method ensures that there is no exclusion of

(a).



(b).

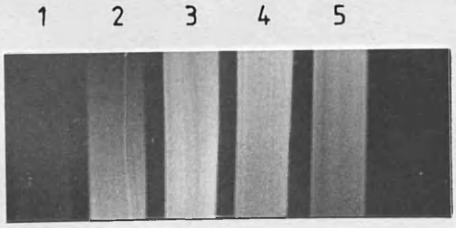


Figure 6.17. Partial Sau3A restrictions of chromosomal DNA from JEF8.

(a). units Sau3A per ug DNA

Track 1: 1
Track 2: 0.5
Track 3: 0.25
Track 4: 0.125
Track 5: 0.0625
Track 6: 0.03125
Track 7: 1ug undigested DNA
Track 8: lambda HindIII

DNA was digested at 37°C for 1 hour then run on an 0.4% horizontal agarose gel at 60v for 9 hours. 1ug of DNA is assumed to be that shown in track 7.

(b).Comparison of fluorescence in the 15-20kb size range. (units per ug DNA)

Track 1: 0.5
Track 2: 0.25
Track 3: 0.125
Track 4: 0.0625
Track 5: 0.03125

Tracks 3 and 4 show the greatest fluorescence.

sequences from the cloned library due to the distribution of restriction sites. Also, this method will generate overlapping clones and therefore provides an opportunity to "walk" along the chromosome which is impossible with a non-overlapping library of fragments as is obtained by complete digestion of the genomic DNA.

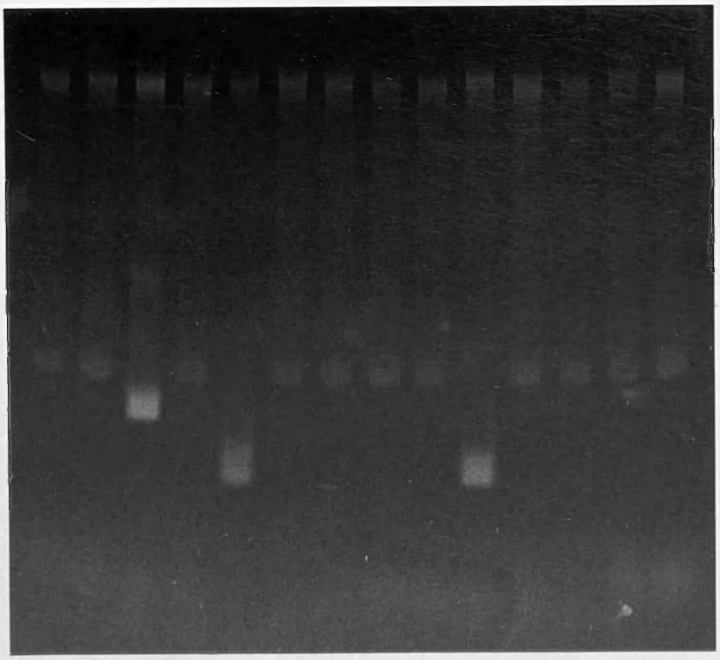
It was decided to use the enzyme Sau3A to generate partially digested restriction fragments of 15kb to 20kb. This enzyme recognises a 4bp sequence and therefore is a very frequent cutter of genomic DNA. Sau3A generates "sticky" ends which are compatible with the ends generated by BamH1. Sau3A fragments can therefore be cloned into the BamH1 site of pBR322 generating Tc^S recombinant plasmids.

Samples containing approximately 1ug of chromosomal DNA isolated from JEF8 were digested with decreasing amounts of Sau3A to obtain a series of partially digested restriction fragments which were separated on a low percentage agarose gel (Fig.6.17a). Since fragments of 15kb to 20kb were required, other fragment sizes in the photograph were covered to allow a comparison of the intensity of fragments in this size range generated by the various dilutions of enzyme (Fig.6.17b). The gel tracks resulting in the greatest fluorescence in this size range are those containing DNA digested with 0.125 and 0.0625 units of enzyme per ug of DNA.

5ug DNA from JEF8 was digested using 0.0625 units Sau3A ug^{-1}; 3ug DNA was digested using 0.125 units ug^{-1}; 2ug DNA was digested using 0.03125 units ug^{-1}, and the total 10ug digested DNA was mixed and run on an agarose gel to separate the partially digested fragments. A slice of agarose containing partial restriction fragments of between 15kb and 23kb was excised and the fragments extracted and purified. The Sau3A digest was repeated using 10ug DNA but 5ug aliquots were digested using 0.125 and 0.0625 units ug^{-1}. Fragments of 15kb to 23kb were again excised and purified from the gel.

2ug of pBR322 DNA was digested to completion with BamH1. The linear molecules were incubated with CIP to prevent recircularization of the vector during the ligation. The partial Sau3A restriction fragments

(a). 1 → 14



(b). 1 → 15

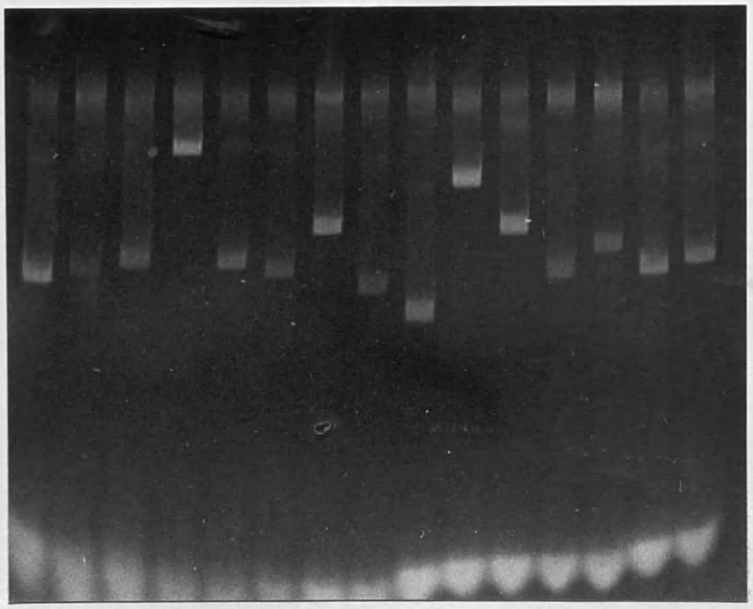


Figure 6.18. Single colony gel analysis of Ap^r transformants. (Single colony lysates were run on an 0.8% agarose gel).

(a). ligation of pBR322 + 15-23kb fragments. Plasmids do not appear to contain inserts and several are smaller than pBR322 itself.

(b). ligation of pBR322 + 5-10kb fragments. All plasmids appear to contain inserts.

were ligated to the linear pBR322 and the ligation transformed into 902, a recA strain.

Approximately 1 x 10^3 transformants were obtained from the ligation and replica plating of the colonies onto L agar containing Tc indicted that approximately one-third of the transformants were Tc^S. Single colony gel analysis, however, reveals that the plasmids in these Tc^S transformants do not contain inserts (Fig.6.18a). Rather several are smaller than pBR322 suggesting that they contain a deletion of all or part of the Tc resistance gene. Transformation of ligated pBR322 alone resulted in approximately 6 x 10^2 transformants and none of these were Tc^S which suggests that pBR322 is unable to accept such a large insert as 15kb - 23kb. Therefore, partially digested Sau3A fragments from JEF8 were prepared of 5kb -10kb and these were ligated to pBR322 in the hope that these smaller fragments would be accepted by pBR322. Approximately 3 x 10^4 transformants were obtained from the ligation. Single colony gel analysis of 15 random transformants indicates that they all contain plasmids with inserts (Fig.6.18b).

Plasmid DNA was prepared from the pooled transformants by washing the colonies off the plates with phage buffer, pelleting the cells by centrifugation and then treating the pellet as that obtained from a normal large scale plasmid preparation.

The probability of having any DNA sequence represented in a gene-bank can be calculated from the formula:

$$N = \frac{\ln (1-P)}{\ln (1-f)}$$

where P is the desired probability, f is the fractional proportion of the genome in a single recombinant, and N is the necessary number of recombinants (Clarke and Carbon, 1976). Therefore to achieve a 99% probability (P=0.99) of having a given DNA sequence represented in the gene-bank with an average insert size of 7kb :

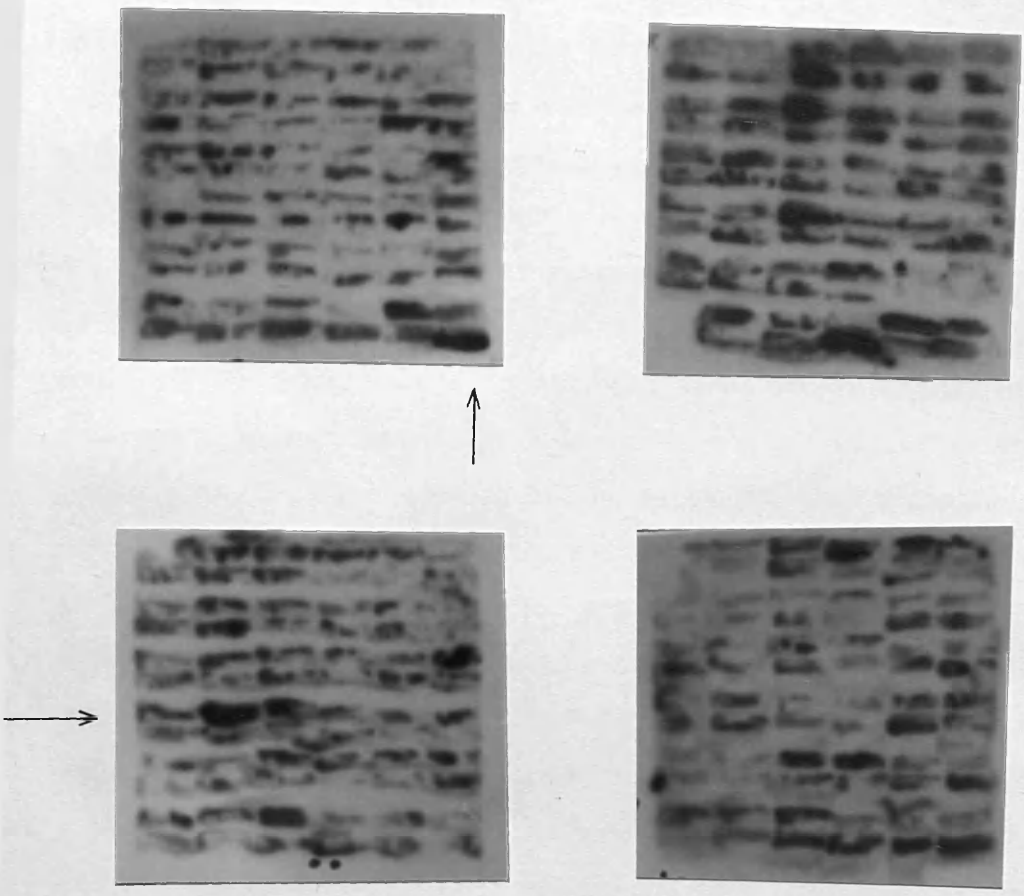


Figure 6.19. Patch hybridization of gene-bank clones to ^{32}P-labelled 11.7kb EcoR1 fragment from pCC1. Four separate nylon membrane filters are shown; two positive clones are indicated by arrows.

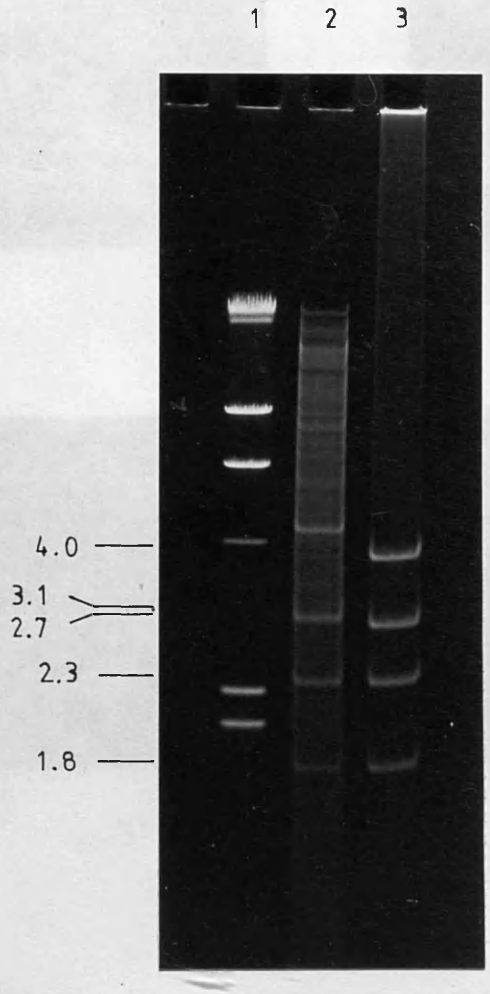


Figure 6.20. BamH1/EcoR1 restriction analysis of Cut5 and pCC21 showing the presence of a 2.3kb and a 1.8kb band common to both. All sizes are in kilobases.

Track 1: Lambda HindIII

Track 2: Cut5

Track 3: pCC21

$$N = \frac{\ln (1-0.99)}{\ln\ 1 - \frac{[7 \times 10^3]}{[4.2 \times 10^6]}}$$

where 4.2×10^6 represents the approximate size of the E. coli K12 chromosome.

Therefore approximately 2760 recombinant plasmids would have to be screened to have a 99% probability of obtaining any particular chromosomal DNA sequence.

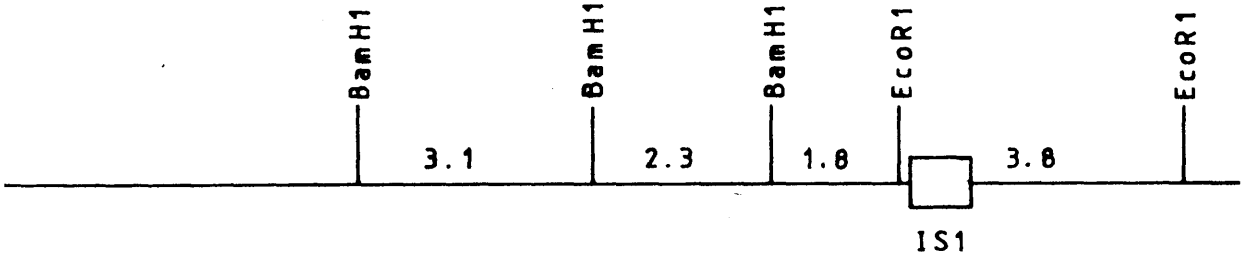
6.4.2 Screening the gene-bank for Tn2901 and adjacent chromosomal DNA

1000 Ap^r transformants from the gene-bank were patched onto L agar supplemented with Ap and the patches transferred to Hybond-N nylon membrane. The 11.7kb EcoR1 fragment from pCC1 was excised from a low melting point agarose gel and purified. The fragment was nick-translated with ^{32}P and hybridized to the membranes, which were washed stringently. 13 patches hybridized strongly to the 11.7kb EcoR1 fragment (Fig.6.19).

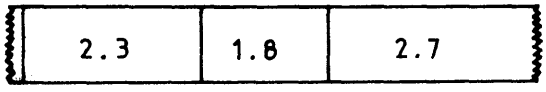
Plasmid DNA isolated from each positive clone was restricted with BamH1 and plasmids producing restriction patterns totally unrelated to Tn2901 and the adjacent chromosomal DNA were discounted. Plasmids producing fragments characteristic of Tn2901, e.g. a 2.3kb BamH1 fragment, or an appropriate number of BamH1 fragments were further digested with EcoR1 and BglII. These digests allowed plasmids which contained too large a number of sites for these enzymes to be discounted. Finally, a single recombinant plasmid, pCC21, was isolated which produced several restriction fragments characteristic of being derived from Tn2901.

pCC21 and Cut5 DNA were restricted with BamH1 and EcoR1 and the digests run on an agarose gel (Fig.6.20). The gel indicates that pCC21 contains both the 2.3kb BamH1 fragment and the 1.8kb BamH1/EcoR1

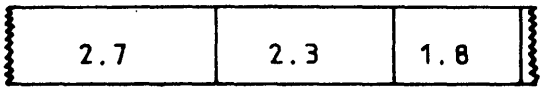
1. Possible structures of cloned fragment



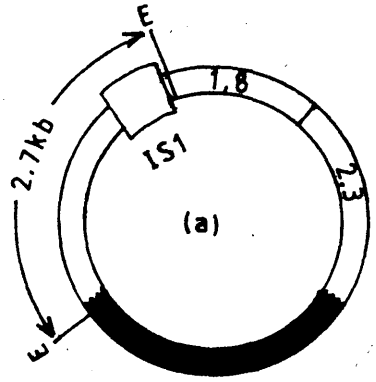
(a).



(b).



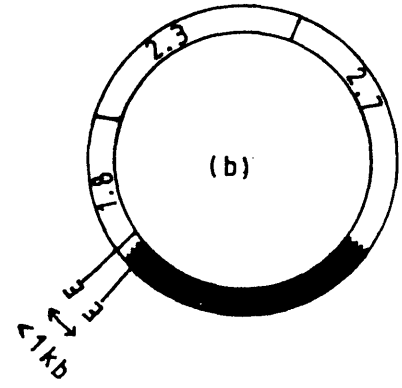
2. EcoR1 fragments



opposite orientation

↓

4.0 kb



opposite orientation

↓

6.6 kb

3. pCC21

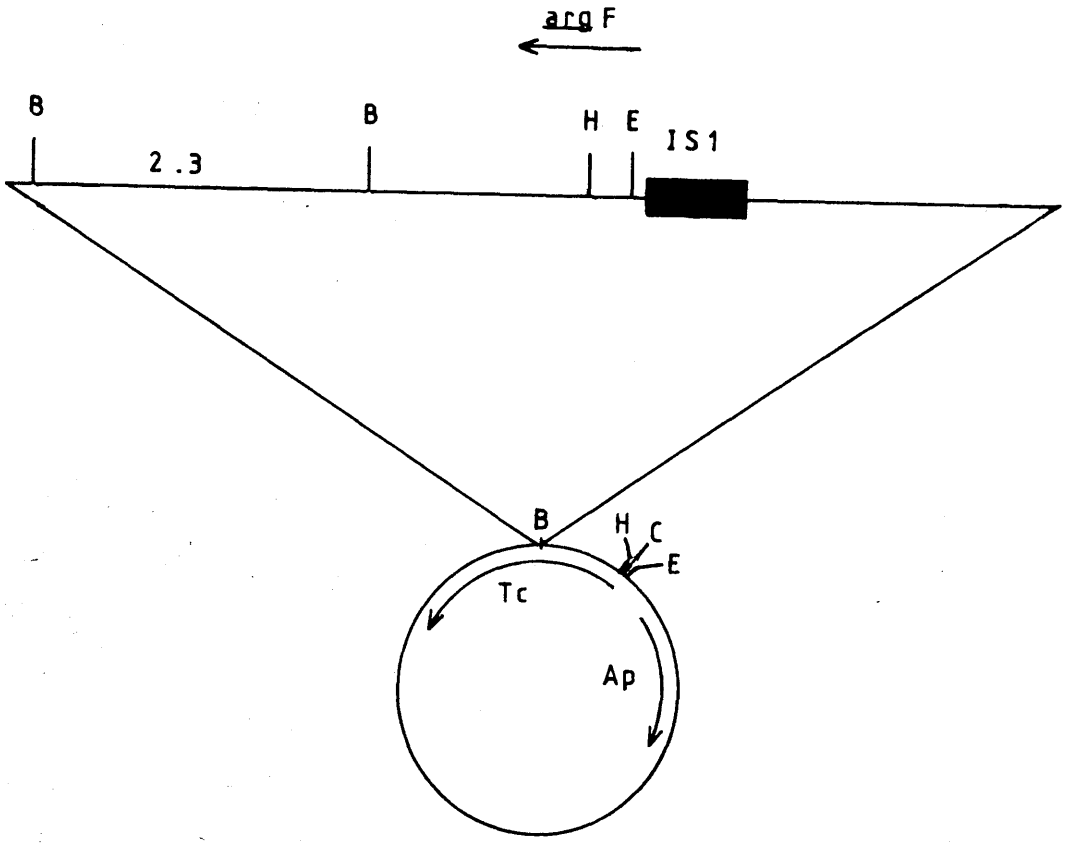


Figure 6.21. Structure of pCC21
(1). Figure showing the possible structure of the cloned DNA in pCC21. The 2.7kb fragment could lie to either side of the 2.3kb and 1.8kb EcoR1/BamH1 fragments.

(2). EcoR1 restriction maps of the two possible structures of pCC1 shown above. The solid line represents vector DNA; the open line represents cloned DNA. IS1 is represented as an open box.

(3). The deduced structure of pCC21 (see text). IS1 is represented as a solid box.

fragment from Tn2901. The BamH1/EcoR1 digest also produces two other fragments, of 2.7kb and 4.0kb. The sum of the BamH1/EcoR1 fragments is 10.8kb which represents the total size of pCC21. Deducting the size of the vector (4.3kb) and the 2.3kb and 1.8kb BamH1/EcoR1 fragments, leaves 2.4kb of cloned chromosomal DNA which contains no BamH1 or EcoR1 restriction sites. This 2.4kb could lie to either side of the known 2.3kb and 1.8kb fragments (Fig.6.21). If the 2.4kb fragment is adjacent to the 1.8kb BamH1/EcoR1 fragment, an EcoR1 fragment of 2.7kb or 4.0kb will be obtained depending on the orientation of the insert in the vector since the EcoR1 site in the vector is 375bp from the BamH1 site (Fig.6.21). Neither of these fragments will be obtained if the 2.4kb fragment is adjacent to the 2.3kb BamH1 fragment (when a < 1kb or a 6.6kb fragment will be obtained (Fig.6.21). The EcoR1 digest produces two fragments of 2.7kb and 8kb, indicating that the 2.7kb EcoR1 fragment is adjacent to the 1.6kb BamH1/EcoR1 fragment and therefore extends past the right-hand IS1 element of Tn2901. The restriction map of pCC21 is shown in Figure 6.21.

Each recombinant plasmid was also transformed into NGX2 and plated on minimal agar supplemented with leucine, proline and uracil to select for *arg*$^+$ transformants. Only cells transformed with pCC21 were able to grow on this medium which confirms that pCC21 contains the *argF* gene.

6.4.3 A preliminary attempt at *in vitro* deletion mutagenesis of IS1

Two types of deletions are possible within IS1 which could be used to determine whether an IS1 gene-product is involved in the initial IS1-IS1 recombination event, and to identify the crossover site in the IS1-IS1 recombination. Firstly, progressive deletions could be made from suitable restriction sites at either end of IS1, terminating at various endpoints within IS1. Restriction analysis could be used to determine the endpoints of such deletions. Secondly, small deletions of one or more restriction sites within IS1 could be made. Small

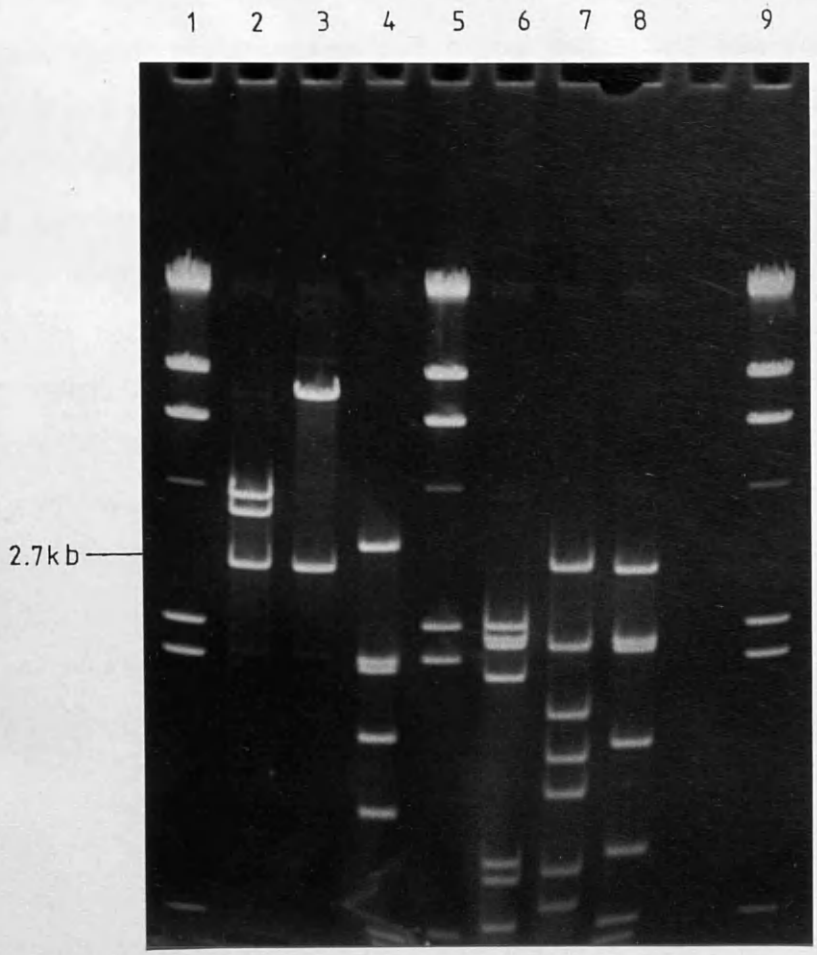


Figure 6.22. Restriction analysis of pCC21.

Track 1: Lambda Hind III

Track 2: SphI/EcoR1

Track 3: EcoR1

Track 4: HincII/EcoR1

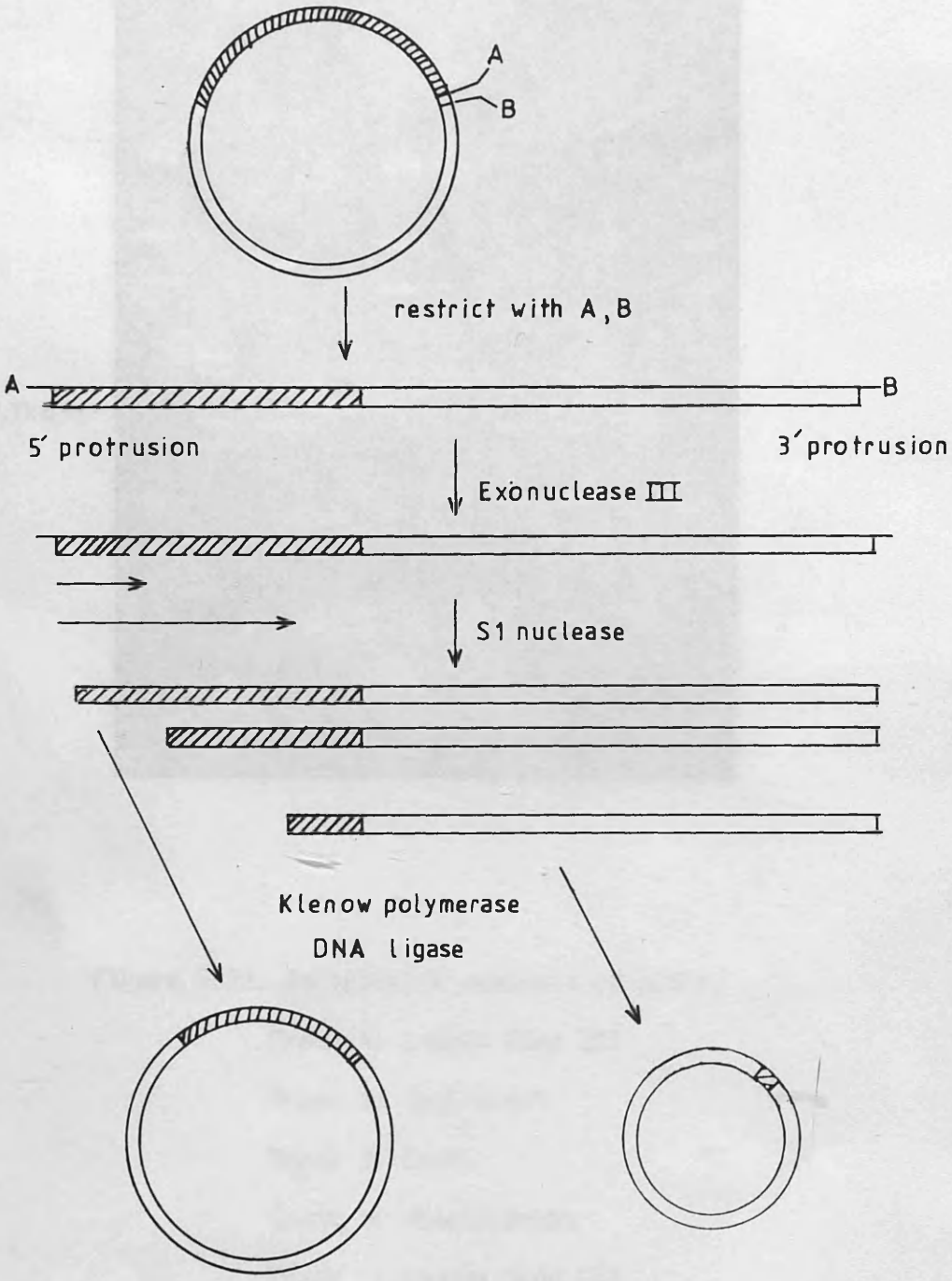
Track 5: Lambda Hind III

Track 6: PvuII/EcoR1

Track 7: AccI/EcoR1

Track 8: AvaI/EcoR1

Track 9: Lambda Hind III


A
B
restrict with A, B
A
B
5′ protrusion
3′ protrusion
Exonuclease III
S1 nuclease
Klenow polymerase
DNA ligase

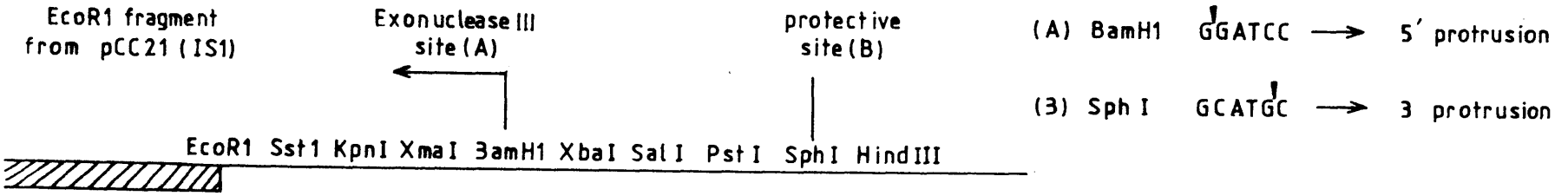


Figure 6.23. Strategy for construction of progressive deletions in IS1 using exonuclease III. The 2.7kb EcoR1 fragment (containing IS1) is cloned into the EcoR1 site of the pUC18 polylinker (above). Exonuclease III will not initiate digestion at DNA ends with four-base 3' protrusions. Therefore digestion with SphI will provide a protective end (B). Restriction with BamH1 (A) provides a 5' protrusion to allow digestion to proceed unidirectionally into the cloned fragment represented as a hatched box (opposite). Aliquots are removed at uniform intervals, treated with S1 nuclease, Klenow DNA polymerase, DNA ligase, and used to transform competent cells. This should produce progressive deletions extending into the IS1 element present in the cloned fragment.

(a).

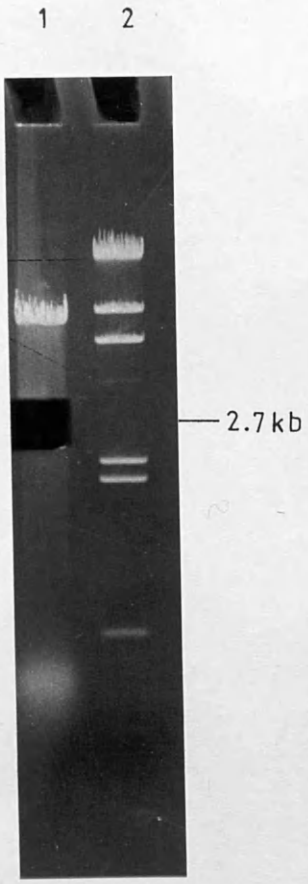
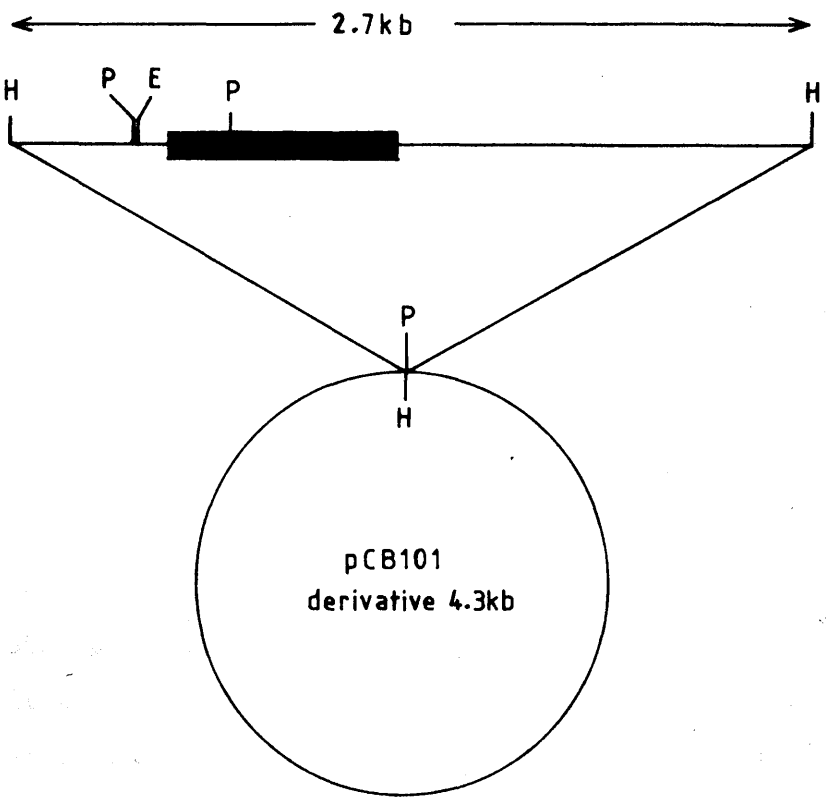


Figure 6.24. (a). Excision of the 2.7kb HindIII fragment from pCC21 digested with HindIII.
Track 1: pCC21 HindIII. The 2.7kb HindIII fragment has been excised from the agarose gel.
Track 2: Lambda HindIII.

(b).



(b). Diagram of pCC18. The 2.7kb HindIII fragment was cloned into the unique HindIII site of a derivative of pCB101 (4.3kb). E-EcoR1, P-Pst I, H-Hind III.

deletions could be made in specific IS1 open reading frames and this would allow the involvement of an IS1 gene-product to be determined.

(1). Preliminary restriction analysis of pCC21

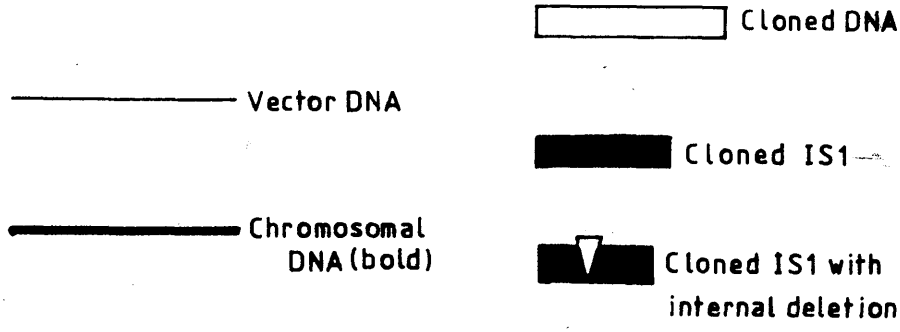
pCC21 was restricted with HindIII, ClaI, KpnI, EcoRI, SphI, HincII, PvuII, AccI and AvaI to identify restriction sites within the 2kb chromosomal region adjacent to the IS1 element. Any unique restriction site within the region would facilitate subcloning of the IS1 element, and may be useful for the subsequent construction of deletions in IS1. Fig.6.22 shows that the 2.7kb EcoR1 fragment is not cut by EcoR1, HindIII, SphI, AccI or AvaI, but is cut by PvuII and HincII (probably at several sites). Similar digests show that the 2.7kb EcoR1 fragment is also not cut by ClaI and KpnI (data not shown).

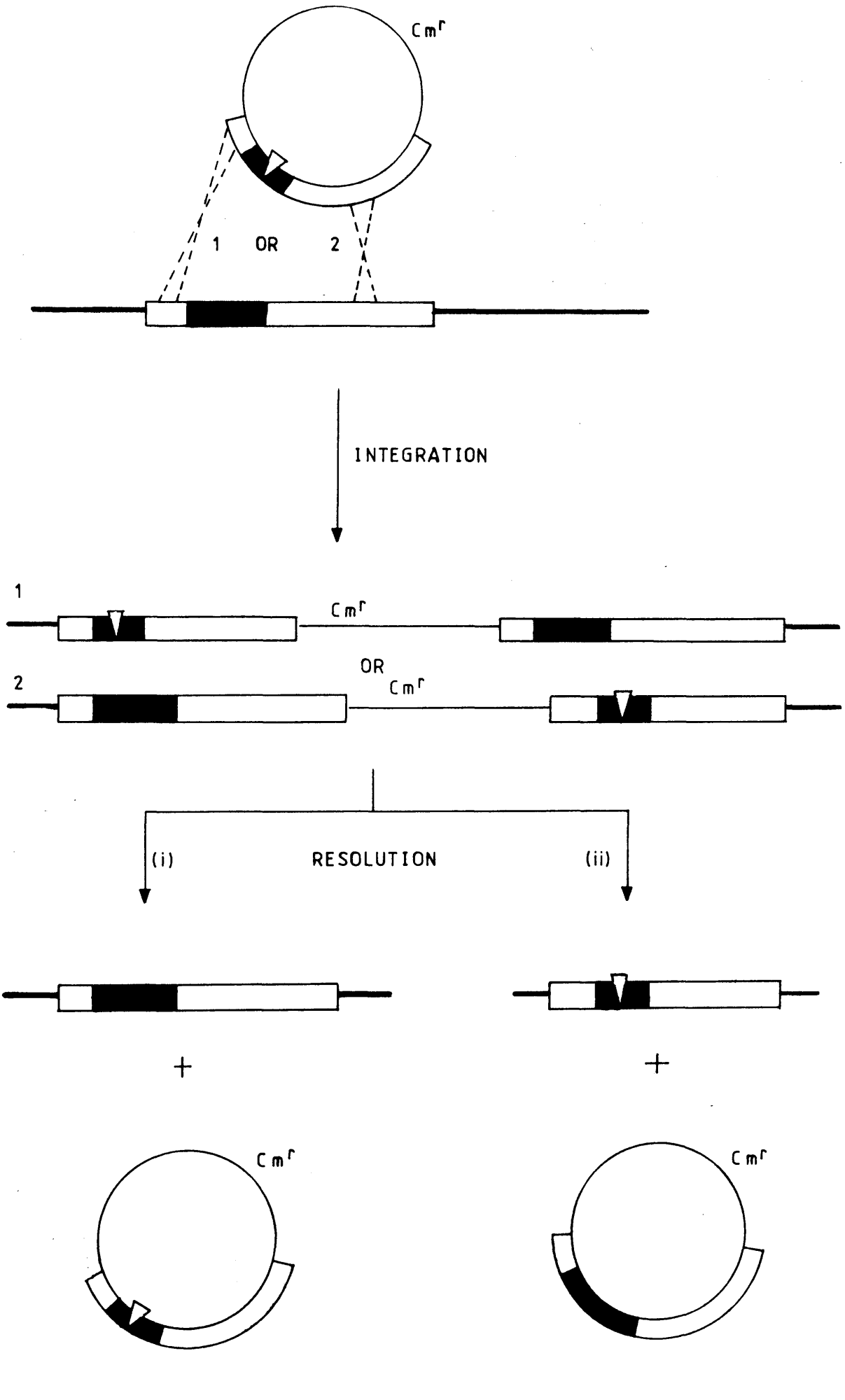
(2). Subcloning of the HindIII fragment from pCC21 into pCB101 and attempted construction of initial deletions

It would be useful to subclone the 2.7kb EcoR1 fragment from pCC21 into pUC18 since the pUC18 polylinker has appropriate restriction sites for unidirectional digestion with exonuclease III (Fig.6.23). This could provide a series of progressive deletions in IS1 which would be valuable for studying the initial IS1-IS1 recombination event (as described below). However despite several attempts this cloning was not successful since only greatly rearranged plasmids were obtained. No attempt was made to determine the structure of these rearrangements since they produced a completely different restriction pattern to that expected. Therefore, this subcloning was abandoned.

The 2.7kb HindIII fragment from pCC21 was excised from an agarose gel (Fig.6.24a) and cloned into the HindIII site of pCB101, and the ligation transformed into Δ M15. The transformation was plated on L-agar containing Cm, X-gal and IPTG. The resultant white colonies contained recombinant plasmids with the HindIII fragment inserted in pCB101 in only one orientation. This plasmid was named pCC18 and is shown in Figure 6.24b. pCC18 contains three PstI sites resulting in

Figure 6.25. Expected pathway for the replacement of a wildtype IS1 element with an *in vitro* altered element. A recombinant plasmid derived from pCB101 carrying a cloned IS1 element with an internal deletion and adjacent chromosomal sequences is transformed into JEF8. Recombination can occur to either the left or right of the deletion giving rise to one of two possible chromosomal DNA structures with integrated plasmid. The plasmid integrates are obtained by selection for chloramphenicol resistance. After removal of chloramphenicol from the medium, chloramphenicol-sensitive segregates are obtained when plasmid DNA resolves from the chromosome via homologous recombination. The segregates retain either the original IS1 sequence or the altered IS1 sequence in the chromosome. Replacement of the original sequence occurs when when the plasmid resolves via recombination on the opposite side of the deletion from the integration event.




Cm^r
1
OR
2
INTEGRATION
1
Cm^r
OR
2
Cm^r
(i)
RESOLUTION
(ii)
+
+
Cm^r
Cm^r

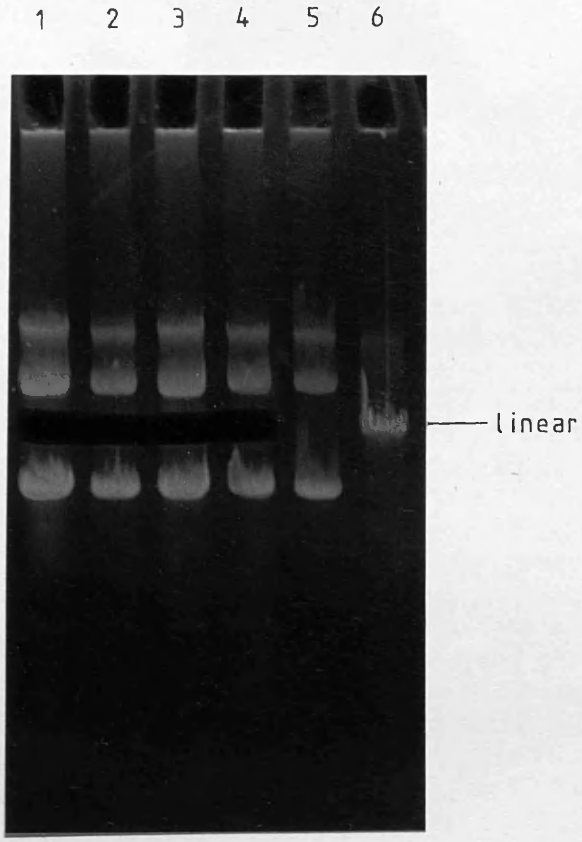


Figure 6.26. Partial digestion of pCC18 with PstI.
pCC18 was incubated with 0.05-0.1 units PstI per ug DNA for 30 mins to produce partially digested molecules. pCC18 was also digested to completion with BamH1 (which has one site in pCC18) to produce a linear size marker (Track 6). Tracks 1-4 show the partially digested DNA with the linear fragment excised. Track 5 contains uncut pCC18 DNA.

three PstI fragments when fully restricted. It was decided to use this plasmid to delete the PstI site within IS1 since the resulting plasmid could be directly integrated into JEF8. If this plasmid excises it may result in the replacement of the wild-type IS1 element of Tn2901 with the deleted IS1 element (Fig.6.25). Since deletion of the PstI site is known to affect IS1-mediated co-integrate formation (Ohtsubo et al, 1981), deletion of this site may also affect the initial IS1-IS1 recombination event.

pCC18 was partially digested with PstI to produce linear molecules (Fig.6.26). A BamH1 digest of pCC18 was used as a size marker for linear molecules which were excised from the gel and purified, treated with various concentrations of T4 DNA polymerase and ligated. The ligation mixture was transformed into M15 and the resultant Cm^r transformants analysed by restriction digestion. A total of 30 transformants were obtained from 12 ligations, but restriction analysis of these indicates that they still contain three PstI sites. Time did not permit any further attempt at deleting the PstI site of IS1.

DISCUSSION

The technique developed in this chapter for the integration of pCB101 at any cloned restriction fragment will be of immense value in the analysis of the initial IS1-IS1 recombination event and the role of the F factor in the "activation" of this event. As mentioned previously, this technique will allow the insertion of cloned F factor restriction fragments adjacent to Tn2901. Cloned F factor restriction fragments are available (Achtman et al, 1978b) and these may be cloned into pCB101, together with a chromosomal fragment to allow integration

to occur. By forcing integration in an F^- strain and and testing these strains for Cut yield, it should be possible to isolate the function provided by the F factor on a cloned restriction fragment (unless, for example, F has a topological role). The integration technique will allow the construction of specific F factor deletions and this also should allow the identification of the region of F required for the amplification.

A gene-bank was prepared from which a clone was isolated containing approximately 2kb of chromosomal DNA adjacent to the right-hand IS1 element of Tn2901. This chromosomal DNA should allow the replacement of the right-hand IS1 element of Tn2901 with various mutant IS1 sequences and it may also be used to re-probe the gene-bank in order to "walk" along the chromosome towards the F factor by isolating a series of overlapping clones. Since only 1000 clones have been screened, it should also be possible to isolate chromosomal DNA adjacent to the left-hand IS1 element of Tn2901 by probing a further set of clones.

The role of the two IS1 elements of Tn2901 may be simply to provide homology for RecA-dependent recombination, or alternatively, an IS1-encoded gene product may be involved in the initial recombination event. IS1 is reported to encode two polypeptides (InsA and InsB) whose genes are located side by side in the same orientation and are assumed to be transcribed polycistronically (Ishizaki and Ohtsubo, 1984; Machida et al, 1984b). Various mutations within IS1 markedly reduce or abolish the frequency of co-integrate formation and transposition of IS1 (Ohtsubo et al, 1981; Reif and Arber, 1980). Deletion of either the unique PstI or BstEII site of IS1 results in a 100-fold to 1000-fold reduction in IS1-mediated co-integration (Ohtsubo et al, 1981). Reif and Arber (1980) have shown that an insertion in the PstI site reduces transposition by 100-fold in a recA background although IS1-mediated co-integration is not affected. Reif and Arber (1980) have suggested that the insertion damages a presumptive resolution site in IS1 analogous to that of Tn3. It

should now be possible to replace the chromosomal IS1 sequences of Tn2901 with *in vitro* deleted IS1 sequences. This will be useful in several ways:

(1). Deletions within IS1 which are known to affect transposition and co-integrate formation may be used to determine whether an IS1-encoded gene-product is required for the initial IS1-IS1 recombination event. It has been suggested (Machida *et al*, 1982) that the IS1 encoded proteins can function *in trans* but act preferentially on the IS1 or transposon sequence from which they are produced. Therefore, the presence of other IS1 elements in the strain should not interfere significantly with the *in vitro* deleted elements. If an IS1-encoded gene product is involved, it is probable that both IS1 elements of Tn2901 will have to be replaced to affect the recombination event. This should be possible once the left hand IS1 element and adjacent chromosomal DNA have been isolated from the gene-bank. However, it is possible that only one of the two IS1 elements present in a transposon is active in promoting transposition and co-integrate formation (Ishizaki and Ohtsubo, 1984) and this may also be true of the IS1-IS1 recombination event. An initial attempt at deleting the PstI site in the right hand IS1 element of Tn2901 with T4 DNA polymerase was unsuccessful, perhaps due in part to the complication of having three PstI sites present in the molecule. Time did not permit this deletion to be attempted again although it should be relatively straightforward to delete the PstI site internal to IS1 using T4 DNA polymerase, S1 nuclease or mung-bean nuclease. The unique BstEII site in IS1 could likewise be deleted with S1 or mung-bean nuclease, or filled-in using Klenow or T4 DNA polymerase.

(2). Whether or not an IS1-encoded gene product is involved, it will be useful to determine the crossover site of the initial IS1-IS1 recombination event. If the IS1 elements simply provide regions of homology for homologous recombination, then the crossover should occur at any site within the IS1 sequence. If, on the other hand, an IS1-encoded gene product (or another site-specific recombinase) is

involved, then the crossover could possibly occur at only one site within the IS1 sequence. By progressively deleting an IS1 sequence from one or both ends, it should be possible to determine whether one crossover site exists or whether the crossover can occur at any point in the IS1 sequence. Progressive deletions can be made using exonuclease III which degrades DNA unidirectionally when certain restriction enzymes are used to linearise the DNA. An attempt was made to subclone the EcoR1 fragment from pCC21 into pUC18 which has suitable sites for unidirectional digestion using exonuclease III. However, this subcloning was unsuccessful, perhaps due to the high copy number of pUC18. The HindIII fragment from pCC21 was only cloned into pCB101 in one orientation. This suggests that an unknown gene, whose product is deleterious to the cell, may be over-expressed in this orientation (due to readthrough from the lacZ promoter), and at high copy number in pUC18. Time did not permit the construction of exonuclease III deletion mutants of IS1, but the construction of such mutants and their insertion into the bacterial chromosome in place of the wild-type IS1 sequence should provide much information about the crossover site and hence the nature of the initial IS1-IS1 recombination event. Likewise, small deletions of restriction sites within a single IS1 element would provide markers with which to analyse the hybrid IS1 elements in the amplification. Such markers should also provide information about the crossover site within the IS1 elements.

The plasmids obtained by transducing Cut1 pCC7 into 902 all contained a single IS1 element and had a restriction map indicative of a recombination event having occurred between the IS1 elements of Tn2901. It is unlikely that these plasmid molecules are present in Cut1 pCC7 since these have not been observed on an agarose gel of single colony lysates. These plasmids were obtained at a very high frequency from Cut1 pCC7 but only a single such plasmid has been obtained from JEF8 pCC7. This represents at least a 1000-fold difference between the two strains and this cannot be explained by the

difference in copy number of Tn2901. These results suggest that the IS1 elements of Tn2901 in Cut1 may differ in some way from those in JEF8 since they apparently recombine at such a high frequency. Therefore, it is important that any *in vitro* deletions made in IS1 to study the initial IS1-IS1 recombination event are made in the IS1 elements of JEF8 and not those of Cut1 since the elements of Cut1 are already "activated" by the F factor.

The isolation of F-prime DNA from DF30D.2 P678.14 did not yield sufficient quantities of DNA for restriction analysis and cloning purposes. However, this F-prime has not been observed by single colony gel analysis which suggests that it may be closely associated with the bacterial chromosome. Two attempts to increase F-prime copy number were unsuccessful. The results suggest that an F-prime containing an additional origin of replication (from RSF1341) still uses its own replication origin, unlike R388 (Symington, 1982). The inability to isolate an F-prime plasmid containing pCC7 suggests that this F-prime may be deleterious to the host since F-primes were readily isolated from JEF8 pCC7 in a *recA*$^+$ host which allows pCC7 to recombine out.

CHAPTER 7

Concluding remarks

The aims of this thesis were firstly to determine the structure of the rearrangement associated with the US cut phenotype, and secondly to initiate studies on the mechanism of formation of the rearrangement.

The structure of the rearrangement

The rearrangement associated with the US Cut phenotype was shown by restriction analysis and Southern blotting to be a tandem amplification of Tn2901, a 12kb IS1-flanked unit containing the argF gene. The copy number of Tn2901 in Cut1, a US Cut isolate, was estimated to be approximately 45. A single IS1 element was shown to be present at the novel joint between each amplified unit, indicating that IS1-IS1 recombination was responsible at least for the initial duplication event. The subsequent amplification to many copies is known to be recA-dependant and therefore need not necessarily involve the IS1 elements; rather recombination could occur at any point in Tn2901. Protein analysis indicated that the argF gene product is over-expressed in US Cut mutants; preliminary northern analysis detected only one transcript (of approximately 2.4kb) from Tn2901 other than a possible argF transcript leaving at least 6kb of Tn2901 not accounted for in terms of RNA transcripts.

Stable Cut mutants

A second class of Cut mutants was identified by the use of crystal violet as an indicator of the amplification in JEF8 pCC7 and by patch hybridization to nick-translated pCC1. Although these "stable" Cut mutants were not of primary importance in this work, several observations were made regarding them. Stable Cut mutants do not contain the amplification, and protein analysis indicated that the argF gene product in stable Cut mutants is not overexpressed to the same extent as it is in the US Cut mutants. Since stable Cut mutants are obtained at a very high frequency from JEF8 and at a low frequency from F^- strains, it is possible that the formation of stable Cut mutants is in some way related to the formation of the amplification

of Tn2901.

The role of the F factor in the IS1-IS1 recombination event

Two possible roles for the F factor in the formation of the initial duplication were examined: an F factor IS-mediated rearrangement, and conjugal transfer. No evidence was found to suggest that either of these are required for the duplication of Tn2901, although a minor F factor IS-mediated rearrangement was not ruled out. Therefore, it is likely that the F factor provides one of the following for the initial IS1-IS1 recombination event:

(1) a protein binding site

(2) a topological effect, possibly mediated by gamma delta

(3) a site of entry for a required enzyme

(4) an origin of replication to produce frequent localized replication forks at Tn2901 to facilitate unequal recombination between the IS1 elements.

The technique developed in this work for the integration of pCB101 at any cloned restriction fragment will be of immense value in determining the role of the F factor in the amplification. Firstly, cloned F factor restriction fragments may be integrated adjacent to Tn2901 and their effect on Cut yield determined. Secondly, directed integration and *in vitro* mutagenesis could be combined to construct specific deletions within the integrated F factor, and their effect on Cut yield determined. Both these techniques may allow the function provided by the F factor to be localized to one or more cloned restriction fragments.

Is an IS1-encoded gene product involved?

Recombination between pairs of IS elements can produce inversions (Savic *et al*, 1983; Louarn *et al*, 1985), additions (Davidson *et al*,1975) or deletions of large DNA segments (Timmons *et al*, 1983). It is not known whether an IS-encoded gene product is involved in these IS-IS recombination events, or whether the IS elements simply provide

regions of homology for rec-dependant recombination. In a recA$^+$ host, amplification of the drug resistance genes on NR1 proceeds as readily between two directly repeated non-IS sequences as by recombination between two directly repeated IS1 elements (Peterson and Rownd, 1985). In a recA$^-$ host, intramolecular recombination between two directly repeated IS50 sequences on a plasmid proceeds with a frequency only slightly higher than with two directly repeated segments of nontransposable lambda DNA (Isberg and Syvanen, 1985). These findings suggest that an IS-encoded gene product is not involved in these recombination events.

The IS1 elements of Tn2901 do not detectably recombine to produce a duplication in the absence of the integrated F factor. Therefore, it is tempting to speculate that the F factor allows expression of a recombinase specific for the IS1 elements, perhaps encoded by the IS1 elements themselves. The P1 transduction data from JEF8 pCC7 and Cut1 pCC7 indicated that recombination occurs more frequently between the IS1 elements of Cut1 than those of JEF8. It would be interesting to construct a recA derivative of Cut1 pCC7 to determine whether the IS1-IS1 recombination which results in the large P1 transduced plasmids is recA-dependant. The technique developed for directed integration should allow the replacement of the IS1 elements of Tn2901 in JEF8 with in vitro mutagenized elements, or with similar sized segments of non-IS DNA. This should allow the determination of whether an IS-encoded gene product is involved in the initial IS1-IS1 recombination event.

In conclusion

The amplification of Tn2901 has already given some insight into the control of chromosomal gene amplification at the molecular level. This system provides an opportunity to study gene amplification both at the level of the initial IS1-IS1 recombination event, and at the level of the subsequent amplification to many copies. Most other reported amplification systems do not facilitate such a study. Also, this

system has the advantage of being a genomic amplification rather than an amplification of plasmid DNA sequences. Although several other chromosomal amplification systems have been reported, the amplification of Tn2901 is the only reported amplification of IS1-flanked chromosomal genes.

The technique developed in this work for the directed integration of pCB101 should be very valuable in the further study of the mechanisms involved in the initial IS1-IS1 recombination event. The replacement of wild-type chromosomal segments with <u>in vitro</u> mutagenized sequences should allow the determination of whether an IS1-encoded gene product is involved in the initial IS1-IS1 recombination, and the role of the F factor in this recombination event.

Abremski,K. and Gottesman,S. (1982). Purification of the bacteriophage lambda xis gene product required for lambda excisive recombination. J. Biol. Chem. 257, 9658-9662.

Achtman,M. (1975). Mating aggregates in E. coli conjugation. J. Bacteriol. 123, 505-515.

Achtman,M., Willetts,N. and Clark,A.J. (1971). Beginning a genetic analysis of conjugational transfer determined by the F factor in E. coli by isolation and characterization of transfer-deficient mutants. J. Bacteriol. 106, 529-38.

Achtman,M., Willetts,N. and Clark.,A.J. (1972). Conjugal complementation analysis of transfer-deficient mutants of Flac in E. coli. J. Bacteriol. 110, 831-842.

Achtman,M., Morelli,G. and Schwuchow,S. (1978a). Cell-cell interactions in conjugating E. coli: role of F pili and fate of mating aggregates. J. Bacteriol. 135, 1053-1061.

Achtman,M., Skurray,R.A., Thompson,R., Helmuth,R., Hall,S., Beutin,L. and Clark,A.J. (1978b). Assignment of tra cistrons to EcoR1 fragments of F sex factor DNA. J. Bacteriol. 133, 1383-1392.

Albertini,A.M. and Galizzi,A. (1985). Amplification of a chromosomal region in B. subtilis. J.Bacteriol. 162, 1203-1211.

Altenbuchner,J. and Cullum,J. (1984). DNA amplification and an unstable arginine gene in Streptomyces lividans 66. Mol. Gen. Genet. 195, 134-138.

Alton,N.K. and Vapnek,D. (1979). Nucleotide sequence of the chloramphenicol transposon Tn9. Nature 282, 864-869.

Amundsen,S.K., Taylor,A.F., Chaudhury,A.M. and Smith,G.R. (1986). recD: The gene for an essential third subunit of exonuclease V. Proc. Natl. Acad. Sci. USA. 83, 5558-5562.

Anderson,R.P. and Roth,J. (1977). Tandem genetic duplications in phage and bacteria. Ann. Rev. Microbiol. 31, 473-505.

Anderson,P. and Roth,J. (1978). Tandem chromosomal duplications in S. typhimurium. Fusion of histidine genes to novel promoters. J. Mol. Biol. 119, 147-166.

Anderson,P. and Roth,J. (1981). Spontaneous tandem genetic duplications in S. typhimurium arise by unequal recombination between rRNA (rrn) cistrons. Proc. Natl. Acad. Sci. USA. 78, 3113-3117.

Anderson,R.P., Miller,C.G. and Roth,J.R. (1976). Tandem duplications of the histidine operon observed following generalized transduction in S. typhimurium. J. Mol. Biol. 105, 201-218.

Arthur,A. and Sherratt,D.J. (1979). Dissection of the transposition process: a transposon-encoded site-specific recombination system. Mol. Gen. Genet. 175, 267-274.

Audit,C. and Anagnostopoulos,C. (1975). Studies on the size of the diploid region in B. subtilis merozygotes from strains carrying the trpE26 mutation. Mol. Gen. Genet. 137, 337-351.

Bachman,B.J. and Low,K.B. (1980). Linkage map of E. coli K12. Microbiol. Rev. 44, 1-56.

Bachman,B.J., Low,K.B. and Taylor,A.L. (1976). Recalibrated linkage map of E. coli K12. Bacteriol. Rev. 40, 116-167.

Barbour,S.D., Nagaishi,H., Templin,A. and Clark,A.J. (1970). Biochemical and genetic studies of recombination proficiency in E. coli II Rec^+ revertants caused by indirect suppression of Rec^- mutations. Proc. Natl. Acad. Sci. USA. 67, 128-135.

Bazaral,M. and Helinski,D. (1968). Circular DNA forms of colicinogenic factors E1, E2 and E3 from E. coli. J. Mol. Biol. 36, 185-194.

Beck,E. and Bremer,E. (1980). Nucleotide sequence of the gene ompA encoding the outer membrane protein II of E. coli K12. Nucleic Acids Res. 8, 3011-3024.

Beeftinck,F., Cunin,R. and Glansdorff,N. (1974). Arginine gene duplications in recombination proficient and deficient strains of E. coli K12. Mol. Gen. Genet. 132, 241-253.

Behr,M.G. and Schaitman,C.A. (1981). Regulation of the ompA outer membrane protein of E. coli. J. Bacteriol. 147, 972-985.

Berg,D.E. (1977). Insertion and excision of the transposable kanamycin resistance determinant Tn5. In Bukhari,A.I., Shapiro,J.A. and Adhya,S.L. (Eds), DNA insertion elements, plasmids and episomes. Cold Spring Harbour Press, Cold Spring Harbour, NY, pp 205-212.

Berg,D.E., Johnstrud,L., McDivitt,L., Ramabhadran,R. and Hirschel,B.J. (1982). The inverted repeats of Tn5 are transposable elements. Proc. Natl. Acad. Sci. USA. 79, 2632-2635.

Berg,D.E., Egner,C. and Low,J.B. (1983). Mechanism of F factor-enhanced excision of transposon Tn5. Gene 22, 1-7.

Bernard,O., Hozumi,N. and Tonegawa,S. (1978). Sequences of mouse immunoglobulin light chain genes before and after somatic changes. Cell 15, 1133-1144.

Biel,S.W. and Berg,D.E. (1984). Mechanism of IS1 transposition in E. coli: choice between simple insertion and co-integration. Genetics 108, 319-330.

Blazey,D.L. and Burns,R.O. (1983). recA-dependant recombination between rRNA operons generates type II F' plasmids. J. Bacteriol. 156, 1344-1348.

Boyd,A.C. and Sherratt,D.J. (1986). Polar mobilization of the E. coli chromosome by the ColE1 transfer origin. Mol. Gen. Genet. 203, 496-504.

Boocock,M.R., Brown,J.L. and Sherratt,D.J. (1986). Structural and catalytic properties of specific complexes between Tn3 resolvase and the recombination site res. J. Biochem. Soc. Trans. 14, 214-216.

Borst,P. and Cross,G.A.M. (1982). Molecular basis for trypanosome antigenic variation. Cell 29, 291-303.

Braedt,G. (1985). Recombination in recA cells between direct repeats of insertion element IS1. J. Bacteriol. 162, 529-534.

Brennan,M.B. and Struhl,K. (1980). Mechanisms of increasing expression of a yeast gene in E. coli. J. Mol. Biol. 136, 333-338.

Bukhari,A.I. and Zipser,D. (1972). Random insertion of Mu-1 DNA within a single gene. Nature New Biol. 236, 240-243.

Busse,H.G. and Baldwin,R.L. (1972). Tandem genetic duplications in a derivative of phage lambda. II. Evidence for structure and location of endpoints. J. Mol. Biol. 65, 401-412.

Cairns,J. (1981). The origin of human cancers. Nature 289, 353-357.

Calos,M.P. and Miller,J.H. (1980). Transposable elements. Cell 20, 579-595.

Campbell,A. (1981). Evolutionary significance of accessory DNA elements in bacteria. Ann. Rev. Microbiol. 35, 55-83.

Campbell,A., Berg,D., Bostein,D., Lederberg,E., Novick,R., Starlinger,P. and Szybalski,W. (1977). Nomenclature of transposable elements in prokaryotes. In Bukhari,A.I., Shapiro,J.A. and Adhya,S.L. (Eds), DNA insertion elements, plasmids and episomes. Cold Spring Harbour Press, Cold Spring Harbor, NY, pp 15-22.

Capage,M. and Hill,C.W. (1979). Preferential unequal recombination in the glyS region of the E. coli chromosome. J. Mol. Biol. 127, 73-87.

Carbon,J., Squires,C. and Hill,C.W. (1969). Genetically altered $tRNA^{GLY}$ subspecies in E. coli. Cold Spring Harbour Symp. Quant. Biol. 34, 505-512.

Cavalier-Smith,T. (1985). The evolution of genome size. A Wiley-Interscience publication.

Chandler,M., Boy de la Tour,E., Willems,D. and Caro,L. (1979). Some properties of the chloramphenicol resistance transposon Tn9. Mol. Gen. Genet. 176, 221-231.

Chang,A.C.Y. and Cohen,S.N. (1978). Construction and characterization of amplifiable, multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134, 1141-1156.

Charlier,D., Piette,J. and Glansdorff,N. (1982). IS3 can function as a mobile promoter in E. coli. Nucleic Acids Res. 10, 5935-5948.

Chow,L.T. and Broker,T.R. (1978). Adjacent insertion sequences IS2 and IS5 in bacteriophage Mu and an IS5 in a lambda darg bacteriophage. J. Bacteriol. 133, 1427-1436.

Chumley,F.G. and Roth,J.R. (1980). Rearrangement of the bacterial chromosome using Tn10 as a region of homology. Genetics 94, 1-14.

Clark,A.J. (1971). Toward a metabolic interpretation of genetic recombination of E. coli and its phages. Ann. Rev. Microbiol. 25, 438-464.

Clark,S.P., Yoshikai,Y., Taylor,S., Siu,G., Hood,L. and Mak,T.W. (1984). Identification of a diversity segment of human T-cell receptor B-chain and comparison with the analogous murine element. Nature 311, 387-389.

Clarke,L. and Carbon,J. (1976). A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire E. coli genome. Cell 9, 91-99.

Clewell,D. and Helinski,D. (1969). Supercoiled circular DNA-protein complex in E. coli: purification induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. 62, 1159-1166.

Cornelis,G. and Saedler,H. (1980). Deletions and an inversion induced by a resident IS1 of the lactose transposon Tn951. Mol. Gen. Genet. 178, 367-374.

Crabeel,M., Charlier,D., Cunin,R. and Glansdorff,N. (1979). Cloning and endonuclease restriction analysis of argF and of the control region of the argECBH bipolar operon in E. coli. Gene 5, 207-231.

Craigie,R. and Mizuuchi,K. (1986). Role of DNA topology in Mu transposition: mechanism of sensing the relative orientation of two DNA segments. Cell 45, 793-800.

Cunin,R., Elseviers,D. and Glansdorff,N. (1970). De novo gene duplication versus reactivation of cryptic genes in E. coli K12. Mol. Gen. Genet. 108, 154-157.

Cunin,R., Eckhardt,T., Piette,J., Boyen,A., Pierard,A. and Glansdorff,N. (1983). Molecular basis for modulated regulation of gene expression in the arginine regulon of E. coli K12. Nucleic Acids Res. 11, 5007-5019.

Currier,T. and Nester,E. (1976). Isolation of covalently closed circular DNA of high molecular weight from bactria. Anal. Biochem. 76, 431-441.

Curtiss,R., Caro,L.G., Allison,D.P. and Stallions,D.R. (1969). Early stages of conjugation in E. coli. J. Bacteriol. 100, 1091-

Dandanell,G. and Hammer,K. (1985). Two operator sites separated by 599bp are required for deoR repression of the deo operon of E. coli. EMBO J. 4, 3333-3338.

Daniell,E., Roberts,R. and Abelson,J. (1971). Mutations in the lactose operon caused by bacteriophage Mu. J. Mol. Biol. 69, 1-8.

Davidson,N., Deonier,R.C., Hu,S. and Ohtsubo,E. (1975). Electron microscope heteroduplex studies of sequence relations among plasmids of E. coli. X.DNA sequence organisation of F and F-primes, and the sequences involved in Hfr formation, p. 56-65. In D. Schlessinger (ed.), Microbiology-1974. American Society for Microbiology, Washington, D.C.

Davies,J.E. and Rownd,R.H. (1972). Transmissible multiple drug resistance in Enterobacteriaceae. Science 176, 758-768.

deBruijn,F.J. and Bukhari,A.I. (1978). Analysis of transposable elements inserted in the genomes of bacteriophages Mu and P1. Gene 3, 315-331.

de Boer,H.A., Comstock,L.J. and Vasser,M. (1983). The tac promoter: a functional promoter derived from the trp and lac promoters. Proc. Natl. Acad. Sci. USA. 80, 21-25.

de Cicco,D.V. and Spradling,A. (1984). Localization of a cis-acting element responsible for the developmentally regulated amplification of Drisophila chorion genes. Cell 38, 45-54.

Deonier,D. and Davidson,N. (1976). The sequence organisation of the integrated F-plasmid in two Hfr strains of E. coli. J. Mol. Biol. 107, 207-222.

Deonier,R.C., Hadley,R.G. and Hu,M. (1979). Enumeration and identification of IS3 elements in E. coli strains. J. Bacteriol. 137, 1421-1424.

Deonier,R.C., Yun,K. and Kuppermann,M. (1983). Gamma delta-mediated deletions of chromosomal segments on F-prime plasmids. Mol. Gen. Genet. 190, 42-50.

DiNardo,S., Voelkel,K.A. and Sternglanz,R. (1982). E. coli DNA topoisomerase I mutants have compensatory mutations in DNA gyrase genes. Cell 31, 43-51.

Early,P., Huang,H., Davis,M., Calame,K. and Hood,L. (1980). An immunoglobulin heavy chain variable region is generated from three segments of DNA: V_H, D and J_H. Cell 19, 981-992.

Egner,C. and Berg,D.E. (1981). Excision of transposon Tn5 requires the inverted repeats, but not the transposase functions of Tn5. Proc. Natl. Acad. Sci. USA. 78, 459-463.

Edlund,T. and Normark,S. (1981). Recombination between short DNA homologies causes tandem duplication. Nature 292, 269-271.

Emmons,S.W. (1974). Bacteriophage lambda derivative carrying two copies of the cohesive end site. J. Mol. Biol. 83, 511-525.

Emmons,S.W., MacCosham,V. and Baldwin,R.L. (1975). Tandem genetic duplications in phage lambda. III. The frequency of duplication mutants in two derivatives of phage lambda is independant of known recombination systems. J. Mol. Biol. 91, 133-146.

Emmons,S.W. and Thomas,J.O. (1975). Tandem genetic duplications in phage lambda. IV. The locations of spontaneously arising tandem duplications. J. Mol. Biol. 91, 147-152.

Everett,R. and Willets,N.S. (1980). Characterization of an *in vivo* system for nicking at the origin of conjugal DNA transfer of the sex factor, F. J. Mol. Biol. 136, 129-150.

Finnegan,D.J. and Willetts,N,S. (1971). Two classes of F*lac* mutants insensitive to transfer inhibition by an F-like R factor. Mol. Gen. Genet. 111, 256-264.

Finnegan,D.J. and Willetts,N. (1972). The nature of the transfer inhibitor of several F-like plasmids. Mol. Gen. Genet. 127, 306-307.

Fishman,S. and Hershberger,C. (1983). Amplified DNA in *Streptomyces fradiae*. J. Bacteriol. 155, 459-466.

Fishman,S.E., Rosteck Jr,P.R. and Hersheberger,C.L. (1985). A 2.2kb repeated DNA segment is associated with DNA amplification in *Streptomyces fradiae*. J. Bacteriol. 161, 199-206.

Folk,W.R. and Berg,P. (1970). Characterization of altered forms of glycyl tRNA synthetase and the effects of such alterations on amino-acyl transfer RNA synthesis *in vivo*. J. Bacteriol. 102, 204-212.

Folk,W.R. and Berg,P. (1971). Duplication of the structural gene for glycyl tRNA synthetase in *E. coli*. J. Mol. Biol. 58, 595-610.

Foster,T.J., Davis,M., Roberts., Takeshita,K. and Kleckner,N. (1981). Genetic organisation of the transposon Tn10. Cell 23, 201-213.

Franklin,N.C. (1971). The N operon of lambda: extent and regulation as observed in fusions to the tryptophan operon of *E. coli*. In Hershey,A.D. (Ed) The bacteriophage Lambda. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY. pp 621-638.

Friedman,D., Olsen,E.J., Carver,D. and Gellert,M. (1984). Synergistic effect of *him*A and *gyr*B mutations: evidence that *him* functions control expression of *ilv* and *xyl* genes. J. Bacteriol. 157, 484-489.

Froehlich,B.J., Watkins,C. and Scott,J.R. (1986). IS1-dependant generation of high copy number replicons from bacteriophage P1ApCm as a mechanism of gene amplification. J. Bacteriol. 166, 609-617.

Gaffney,D., Skurray,P. and Willetts,N. (1983). Regulation of F conjugal genes studies by hybridization and *tra*-*lac*Z fusion. J. Mol. Biol. 168, 103-122.

Gamas,P., Galas,D. and Chandler,M. (1985). DNA sequence at the end of IS1 required for transposition. Nature 317, 458-460.

Gellert,M. (1981). DNA topoisomerases. Ann. Rev. Biochem. 50, 879-910.

Ghosal,D., Sommer,H. and Saedler,H. (1979). Nucleotide sequence of the transposable DNA element IS2. Nucleic Acids Res. 6, 1111-1122.

Gillen,J.R., Willis,D.K. and Clark,A.J. (1981). Genetic analysis of the RecE pathway of genetic recombination in E. coli K12. J. Bacteriol. 145, 521-532.

Glansdorff,N., Charlier,D. and Zafarullah,M. (1981). Activation of gene expression by IS2 and IS3. Cold Spring Harbor Symp. Quant. Biol. 45, 153-156.

Glass,R.E. (1982). Gene function : E. coli and its heritable elements. Croom Helm, London.

Glover,D.M., Zaha,A., Stocker,A.J., Santelli,R.V., Pueyo,M.T., de Toledo,S.M. and Lara,F.J.S. (1982). Gene amplification in Rhynchosciara salivary gland chromosomes. Proc. Natl. Acad. Sci. USA. 79, 2947-2951.

Goosen,N. and Van de Putte,P. (1984). Regulation of Mu transposition. I. Localization of the presumed recognition sites for HimD and Ner functions controlling bacteriophage Mu transposition. Gene 30, 41-46.

Greener,A. and Hill,C.W. (1980). Identification of a novel genetic element in E. coli K12. J. Bacteriol. 144, 312-321.

Grindley,N.D.F. and Joyce,C.M. (1980). Genetic and DNA sequence analysis of the kanamycin resistance transposon Tn903. Proc. Natl. Acad. Sci. USA. 77, 7176-7180.

Grindley,N. and Joyce,C. (1981). Analysis of the structure and function of the kanamycin resistance transposon Tn903. Cold Spring Harbor Symp. Quant. Biol. 45, 125-133.

Grindley,N.D.F., Lauth,M.R., Wells,R.G., Wityk,R.J., Salvo,J.J. and Reed,R.R. (1982). Transposon-mediated site-specific recombination: identification of three binding sites for resolvase at the res sites of gamma delta and Tn3. Cell 30, 19-27.

Gross,J.D. and Caro,L. (1966). DNA transfer in bacterial conjugation. J. Mol. Biol. 16, 269-284.

Guarneros,G. and Echols,H. (1970). New mutants of bacteriophage lambda with a specific defect in excision from the host chromosome. J. Mol. Biol. 47, 565-574.

Guerry,P., LeBlanc,D.J. and Falkow,S. (1973). General method for the isolation of plasmid DNA. J. Bacteriol. 116, 1064-1066.

Gutterson,N.I. and Koshland,D.E. (1983). Replacement and amplification of bacterial genes with sequences altered in vitro. Proc. Natl. Acad. Sci. USA. 80, 4894-4898.

Guyer,M.S. (1978). The gamma delta sequence of F is an insertion sequence. J. Mol. Biol. 126, 347-361.

Guyer,M.S., Davidson,N. and Clark,A.J. (1977). Heteroduplex analysis of tra F' plasmids and the mechanism of their formation. J. Bacteriol. 131, 970-980.

Guyer,M.S., Reed,R.R., Steitz,J.A. and Low,K.B. (1981). Identification of a sex factor affinity site in E. coli as gamma delta. Cold Spring Harbor Symp. Quant. Biol. 45, 135-140.

Habermann,P., Klaer,R., Kuhn,S. and Starlinger,P. (1979). IS4 is found between 11 or 12bp duplications. Mol. Gen. Genet. 175, 369-373.

Hadley,R.G. and Deonier,R.C. (1980). Specificity in the formation of tra F-prime plasmids. J. Bacteriol. 143, 680-692.

Hadley,R.G., Hu,M., Timmons,M., Yun,K. and Deonier,R.C. (1983). A partial restriction map of the proA-purE region of the E. coli K-12 chromosome. Gene. 22, 281-287.

Hagblom,P., Segal,E., Billyard,E. and So,M. (1985). Intragenic transposition leads to pilus antigenic variation in Neisseria gonorrhoeae. Nature 315, 156-158.

Halling,S., Simons.R., Way.J., Walsh,R. and Kleckner,N. (1982). DNA sequence organisation of Tn10's IS10-right and comparison with IS10-left. Proc. Natl. Acad. Sci. USA. 79, 2608-2612.

Hansen,J. and Olsen,R. (1978). Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J.Bacteriol. 135, 227-238.

Hashimoto,H. and Rownd,R. (1975). Transition of the R factor NR1 in Proteus mirabilis: level of drug resistance on non-transitioned and transitioned cells. J. Bacteriol. 123, 56-68.

Hayday,A.D., Saito,S.D., Kranz,D.M., Tanigawa,G., Einsen,H.N. and Tonegawa,S. (1985). Structure, organisation and somatic rearrangement of T-cell gamma genes. Cell 40, 259-269.

Heffron,F. (1983). Tn3 and its relatives. In Mobile genetic elements. Ed. Shapiro,J.A. Academic press. NY. pp 223-260.

Heffron,F., Bedinger,P., Champoux,J.J. and Falkow,S. (1977). Deletions affecting the transposition of an antibiotic resistance gene. Proc. Natl. Acad. Sci. USA. 74, 702-706.

Hegeman,G.D. and Rosenberg,S.L. (1970). The evolution of bacterial enzyme systems. Ann. Rev. Microbiol. 24, 429-462.

Henikoff,S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28, 351-359.

Hertman,I. and Luria,S.E. (1967). Transduction studies on the role of a rec$^+$ gene in the ultraviolet induction of prophage lambda. J. Mol. Biol. 23, 117-133.

Hill,C.W. and Combriato,G. (1973). Genetic duplications induced at very high frequency by ultraviolet irradiation in E. coli. Mol. Gen. Genet. 127, 197-214.

Hill,C.W., Foulds,J., Soll,L. and Berg,P. (1969a). Instability of a missense suppressor resulting from a duplication of genetic material. J. Mol. Biol. 39, 563-582.

Hill,C.W., Schiffer,D. and Berg,P. (1969b). Transduction of merodiploidy: induced duplication of recipient genes. J. Bacteriol. 99, 274-278.

Hill,C.W., Grafstrom,R.H., Harnish,B.W. and Hillman,B.S. (1977). Tandem duplications resulting from recombination between ribosomal RNA genes in E. coli. J. Mol. Biol. 116, 407-428.

Homyk,T. and Weil,J. (1974). Deletion analysis of two non-essential regions of the T4 genome. Virology 61, 505-523.

Horecker,B.L. (1975). Biochemistry of isozymes. pp11-38. In C.L. Markert (ed.) Isozymes vol I. Academic press.

Horii,Z.I. and Clark,A.J. (1973). Genetic analysis of the recF pathway of genetic recombination in E. coli K12: isolation and characterisation of mutants. J. Mol. Biol. 80, 327-344.

Horiuchi,T., Noriuchi,S. and Novick,A. (1963). The genetic basis of hyper-synthesis of B-galactosidase. Genetics 48, 157-169.

Hoyt,M.A., Knight,D.M., Miller,H.I. and Echols,H. (1982). Control of phage lambda development by stability and synthesis of cII protein: role of the viral cIII and host hflA, himA and himD genes. Cell 31, 565-573.

Hu,M. and Deonier,R.C. (1981a). Mapping of IS1 elements flanking the argF gene region on the E. coli K12 chromosome. Mol. Gen. Genet. 181, 222-229.

Hu,M. and Deonier,R.C. (1981b). Comparison of IS1, IS2 and IS3 copy numbers in E. coli strains K12, B and C. Gene 16, 161-170.

Hu,S., Ohtsubo,E., Davidson,N. and Saedler,H. (1975). Electron microscope heteroduplex studies of sequence relations among bacterial plasmids: identification and mapping of the insertion sequences IS1 and IS2 in F and R plasmids. J. Bacteriol. 122, 764-775.

Huffman,G.A. and Rownd,R. (1984). Transition of deletion mutants of the composite resistance plasmid NR1 in E. coli and S. typhimurium J. Bacteriol. 159, 488-498.

Hull,R.A., Gill,G.S. and Curtiss,R. (1978). Genetic characterisation of Mu-like bacteriophage D108. J. Virol. 27, 513-518.

Humphreys,G.O., Willshaw,G.A. and Anderson,E.S. (1975). A simple method for the preparation of large quantities of pure plasmid DNA. Biochem. Biophys. Acta. 383, 457-463.

Iida,S., Meyer,J. and Arber,W. (1978). The insertion element IS1 is a natural constituent of coliphage P1 DNA. Plasmid 1, 357-365.

Iida,S., Meyer,J. and Arber,W. (1981a). Genesis and natural history of IS1-mediated transposons. Cold Spring Harbor Symp. Quant. Biol. 45, 27-37.

Iida,S., Nanni,C., Echarti,C. and Arber,W. (1981b). Is the IS1-flanked r-determinant of the plasmid NR1 a transposon? J. Gen. Microbiol. 126, 413-426.

Iida,S., MeyerJ., Linder,P., Goto,N., Nakaya,R., Reif,H-J. and Arber,W. (1982). The kanamycin resistance transposon Tn2680 derived from the R plasmid Rts1 and carried by phage P1Km has flanking 0.8kb long direct repeats. Plasmid 8, 187-198.

Ishizaki,K. and Ohtsubo,E. (1984). Functional difference of the two ends of insertion sequence IS1 in transposition and cointegration. Cold Spring Harbor Symp. Quant. Biol. 49, 207-213.

Jackson,E.N. and Yanofsky,C. (1973). Duplication-translocations of tryptophan operon genes in E. coli. J. Bacteriol. 116, 33-40.

Jamet-Vierny,C. and Anagnostopoulos,C. (1975). Induction and transmission of a merodiploid condition near the terminal area of the chromosome of B. subtilis. Genetics 81, 437-458.

Jessop,A.P. and Glansdorff,N. (1980). Genetic factors affecting recovery of non-point mutations in the region of a gene coding for ornithine transcarbamylase: involvement of both the F factor in its chromosomal state, and the recA gene. Genetics 96, 779-799.

Johnstrud,L. (1979). DNA sequencing analysis of the transposable element IS1. Mol. Gen. Genet. 169, 213-218.

Johnson,D.A. and Willetts,N.S. (1980). Construction and characterization of multicopy plasmids containing the entire F transfer region. Plasmid 4, 292-304.

Jund,R. and Loison,G. (1982). Activation of transcription of a yeast gene in E. coli by an IS5 element. Nature 296, 680-681.

Kaiser,K. and Murray,N.E. (1979). Physical characterisation of the "Rac prophage". Mol. Gen. Genet. 175, 159-174.

Kashmiri,S.V.S. and Hotchkiss,R.D. (1975). Evidence of tandem duplications of genes in a merodiploid region of Pneumococcal mutants resistant to sulphonamide. Genetics 81, 21-31.

Kennedy,N., Beutin,L., Achtman,M. and Skurray,R. (1977). Conjugation proteins encoded by the F sex factor. Nature 270, 580-585.

Kikuchi,A. and Gorini,L. (1975). Similarity of genes argF and argI. Nature 256, 621-623.

Kingsbury,D.T. and Helinski,D.R. (1970). DNA polymerase as a requirement for the maintenance of the bacterial plasmid ColE1. Biochem. Biophys. Res. Commun. 41, 1538-1544.

Kingsman,A. and Willetts,N. (1978). The requirements for conjugal DNA synthesis in the donor strain during Flac transfer. J. Mol. Biol. 122, 287-300.

Kitts,P. (1982). Ph.D. thesis. University of Glasgow.

Kleckner,N. (1981). Transposable elements in prokaryotes. Ann. Rev. Genet. 15, 341-404.

Kleckner,N., Moisato,D., Roberts,D. and Bender,B. (1984). Mechanism and regulation of Tn10 transposition. Cold Spring Harbor Symp. Quant. Biol. 49, 235-244.

Kline,B. and Miller,J. (1975). Detection of non-integrated plasmid DNA in the folded chromosome of E. coli : physiochemical approach to studying the unit of segregation. J. Bacteriol. 121, 165-172.

Kline,B., Miller,J., Cress,D., Wlodarczyk,M., Manis,J. and Otten,M. (1976). Nonintegrated plasmid-chromosome complexes in E. coli. J. Bacteriol. 127, 881-889.

Kunz,B.A. and Glickman,B.W. (1983). The infidelity of conjugal DNA transfer in E. coli. Genetics 105, 489-500.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lavi,S. (1982). Carcinogen-mediated activation of SV40 replicons: a model system for initiation of carcinogenesis. In gene amplification. Schimke, R.T. (ed). Cold Spring Harbor Laboratory, CSH, NY. pp225-230.

Ledbetter,M.L. and Hotchkiss,R.D. (1975). Chromosomal basis of the merozygosity in a partially diploid mutant of Pneumococcus. Genetics 80, 667-678.

Legrain,C., Halleux,P. and Glansdorff,N. (1972). The dual genetic control of ornithine carbamoyl transferase in E.coli : a case of a bacterial hybrid enzyme. Eur. J. Biochem. 27, 93-102.

Legrain,C., Stalon,V. and Glansdorff,N. (1976a). E. coli ornithine carbamoyltransferase isoenzymes. Evolutionary significance and the isolation of lambda argF and lambda argI transducing bacteriophages. J. Bacteriol. 128, 35-38.

Legrain,C., Stalon,V., Glansdorff,N., Gigot,A., Pierard,A. and Crabeel,M. (1976b). Structural and regulatory mutations allowing utilization of citrulline as a source of carbamoyl phosphate in E. coli K-12. J. Bacteriol. 128, 39-48.

Lehner,A.F. and Hill,C.W. (1980). Involvement of rRNA operons in S. typhimurium chromosomal rearrangements. J. Bacteriol. 143, 492-498.

Leong,J.M., Nunes-Duby,S., Lesser,C.M., Youderain,P., Susskind,M.M. and Landy,A. (1985). The 0 80 and P22 attachment sites: primary structure and interaction with E. coli integration host factor. J. Biol. Chem. 260, 4468-4477.

Lin,R-J., Capage,M. and Hill,C.W. (1984). A repetitive DNA sequence, rhs, responsible for duplications within the E. coli K12 chromosome. J. Mol. Biol. 177, 1-18.

Lieb,M. (1980). IS5 increases recombination in adjacent regions as shown for th repressor gene of coliphage lambda. Gene 12, 277-280.

Louarn,J.M., Bouche,J.P., Legendre,F., Louarn,J. and Patte,J. (1985). Characterization and properties of very large inversions of the E. coli chromosome along the origin-to-terminus axis. Mol. Gen. Genet. 201, 467-476.

Lovett,M.A. and Helinski,D.R. (1976). Method for the isolation of the replication region of a bacterial replicon: construction of a mini-F'Km plasmid. J. Bacteriol. 127, 982-987.

MacHattie,L.A. and Jackowski,J.B. (1977). Physical structure and deletion of the chloramphenicol resistance element in Tn9 in bacteriophage lambda. In Bukhari,A.I., Shapiro,J.A. and Adhya,S.L. (Eds), DNA insertion elements, plasmids and episomes. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Machida,Y., Machida,C., Ohtsubo,H. and Ohtsubo,E. (1982). Factors determining frequency of plasmid integration mediated by insertion sequence IS1. Proc. Natl. Acad. Sci. USA. 79, 277-281.

Machida,C., Machida,Y., Wang,H-C., Ishizaki,K. and Ohtsubo,E. (1983). Repression of cointegration ability of insertion element IS1 by transcriptional redthrough from flanking regions. Cell 34, 135-142.

Machida,C., Machida,Y. and Ohtsubo,E. (1984a). Both inverted repeat sequences located at the ends of IS1 provide promoter functions. J. Mol. Biol. 177, 247-267.

Machida,Y., Machida,C. and Ohtsubo,E. (1984b). Insertion element IS1 encodes two structural genes required for its transposition. J. Mol. Biol. 177, 229-245.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982). Molecular cloning. Cold Spring Harbor Laboratories, Cold Spring Harbour, NY.

Markert,C.L. (1963). Epigenetic control of specific protein synthesis in differentiating cells. pp66-84. In Locke, M. (ed). Cytodifferentiation and macromolecular synthesis. Academic Press.

Matsumoto,M. and Tazaki,T. (1970). Genetic recombination in Klebsiella pneumoniae: an approach to genetic linkage mapping. Jpn. J. Microbiol. 14, 129-141.

Mattes,R., Altenbuchner,J. and Schmitt,R. (1978). The translocation of the raffinose operon occurs by insertion sequence mediated site-specific recombination. Hoppe-Seylers. Z. Physiol. Chem. 359, 1118-1119.

Maynard-Smith,J. (1978). The evolution of sex. Cambridge University press. Cambridge.

Meyer,J. and Iida,S. (1979). Amplification of chloramphenicol resistance transposons carried by phage P1Cm in E. coli. Mol. Gen. Genet. 176, 209-219.

Meyer,T.F., Mlawer,N. and So,M. (1982). Pilus expression in Neisseria gonorrhoeae involves chromosomal rearrangement. Cell 30, 45-52.

Miki,T., Easton,A.M. and Rownd,R.H. (1978). Mapping of the resistance genes of the R plamid NR1. Mol. Gen. Genet. 158, 217-224.

Miller,J.H. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratories, Cold Spring Harbor Press, Cold Spring Harbor,NY.

Mizuuchi,K., Weisberg,R., Enquist,L., Mizuuchi,M., Buraczynska,M., Fueller,C., Hsu,P-L., Ross,W. and Landy,A. (1981). Structure and function of the phage lambda att site: size, Int-binding sites, and location of the crossover point. Cold Spring Harbor Symp. Quant. Biol. 45, 429-437.

Moore,S., Garvin,R., and James,E. (1981). Nucleotide sequence of the argF regulatory region of E. coli K12. Gene. 16, 119-132

Morisato,D., Way,J.C., Kim,H.J. and Kleckner,N. (1983). Tn10 transposase acts preferentially on nearby transposon ends in vivo. Cell 32, 799-807.

Nakamura,K. and Inouye,M. (1981). Inactivation of the Serratia marcesens gene for the lipoprotein in E. coli by insertion sequences, IS1 and IS5; sequence analysis of junction points. Mol. Gen. Genet. 183, 107-114.

Nash,H.A. (1975). Integrative recombination of phage lambda DNA in vitro. Proc. Natl. Acad. Sci. USA. 72, 1072-1076.

Nash,H. and Robertson,C. (1981). Purification and properties of the E. coli protein factor required for lambda integrative recombination. J. Biol. Chem. 256, 9246-9253.

Nevers,P. and Saedler,H. (1977). Transposable genetic elements as agents of gene instability and chromosomal rearrangements. Nature 268, 109-115.

Novick,R.P., Clones,R.C., Cohen,S.N., Curtiss,R., Datta,N. and Falkow,S. (1976). Uniform nomenclature for bacterial plasmids: a proposal. Bacteriol. Rev. 40, 168-189.

Nyman,K., Nakamura,K., Ohtsubo,H. and Ohtsubo,E. (1981). Distribution of the insertion sequence IS1 in gram negativ bacteria. Nature 289, 609-612.

Ohno,S. (1977). Evolution by gene duplication. Berlin-Heidelberg. New York. Springer.

Ohtsubo,H. and Ohtsubo,E. (1977). Repeated sequences in plasmids, phages and bacterial chromosomes. In, Bukhari,A.I., Shapiro,J.A. and Adhya,S.L. (Eds), DNA insertion elements, plasmids and episomes. Cold Spring Harbour Press, NY, pp49-63.

Ohtsubo,H. and Ohtsubo,E. (1978). Nucleotide sequence of an insertion element IS1. Proc. Natl. Acad. Sci. USA. 75, 615-619.

Ohtsubo,E., Zenilman,M., Ohtsubo,H., McCormick,M., Machida,C. and Machida,Y. (1980). Mechanism of insertion and cointegration mediated by IS1 and Tn3. Cold Spring Harbor Symp. Quant. Biol. 45, 283-295.

Ohtsubo,H., Nyman,K., Doroszkiewicz,W. and Ohtsubo,E. (1981). Multiple copies of iso-insertion sequences of IS1 in Shigella dysenteriae chromosome. Nature 292, 640-643.

Palchaudhuri,S. and Chakrabarty,A. (1976). Isolation of plasmid DNA from Pseudomonas putida. J. Bacteriol. 126, 410-416.

Panicker,M.M. and Minkley Jr,E.G. (1985). DNA transfer occurs during a cell surface contact stage of F sex-factor mediated bacterial conjugation. J. Bacteriol. 162, 529-534.

Parma,D.H. and Snyder,M. (1973). The genetic constitution of tandem duplications of the rII region of bacteriophage T4D. Genetics 73, 161-183.

Parma,D.H., Ingraham,L.J. and Snyder,M. (1972). Tandem duplications of the rII region of bacteriophage T4D. Genetics 71, 319-335.

Peterson,B.C. and Rownd,R.H. (1983). Homologous sequences other than insertion elements can serve as recombination sites in plasmid drug resistance gene amplification. J. Bacteriol. 156, 177-185.

Peterson,B.C. and Rownd,R.H. (1985). Recombination sites in plasmid drug resistance amplification. J. Bacteriol. 164, 1359-1361.

Pfiefer,F., Betlach,M., Martienses,R., Friedman,J. and Boyer,H.W. (1983). Transposable elements of Halobacterium halobium. Mol. Gen. Genet. 191, 182-188.

Piette,J., Cunin,R., Van Vliet,F., Charlier,D., Crabeel,M., Ota,Y., and Glansdorff,N. (1982). Homologous control sites and DNA transcription starts in the related argF and argI genes of E. coli K12. EMBO. 1, 853-857.

Plasterk,R.H.A., Simon,M.I. and Barbour,A.G. (1985). Transposition of structural genes to an expression sequence on a linear plamid causes antigenic variation in the bacterium Borrelia hermii. Nature 318, 257-263.

Ponticelli,A.S., Schultz,D.W., Taylor,A.F. and Smith,G.R. (1985). Chi-dependant DNA strand cleavage by RecBC enzyme. Cell 41, 145-151.

Porter,R.D. (1981). Enhanced recombination between F42lac and lambda plac5: dependance on F42lac fertility functions. Mol. Gen. Genet. 184, 355-358.

Proctor,G.N. and Rownd,R.H. (1982). Rosanilins: indicator dyes for chloramphenicol resistant enterobacteria containing chloramphenicol acetyl transferase. J. Bacteriol. 150, 1375-1382.

Prozeski,O.W. (1968). Transductional analysis of arginine-less mutants in Proteus mirabilis. J. Gen. Microbiol. 54, 127-143.

Ptashne,M. (1986). Gene regulation by proteins acting nearby and at a distance. Nature 322, 697-701.

Ptashne,K. and Cohen,S.N. (1975). Occurrence of insertion sequence (IS) regions on plasmid DNA as direct and inverted nucleotide sequence duplications. J. Bacteriol. 122, 776-781.

Radding,C.M. (1982). Homologous pairing and strand exchange in genetic recombination. Ann. Rev. Genet. 16, 405-438.

Rae,M.E. and Stodolsky,M. (1974). Chromosome breakage, fusion and reconstruction during P1dl transduction. Virology 58, 32-54.

Raibaud,O., Mock,M. and Schwartz,M. (1984). A technique to integrate any DNA fragment into the chromosome of E. coli. Gene 29, 235-245.

Ravin,A.W. and Takahashi,E.A. (1970). Merodiploid ribosomal loci arising by transformation and mutation in Pneumococcus. J. Bacteriol. 101, 38-52.

Reif,H.J. and Arber,W. (1980). Analysis of transposition of IS1kan and its relatives. Cold Spring Harbor Symp. Quant. Biol. 45, 40-43.

Reif,H.J. and Saedler,H. (1975). IS1 is involved in deletion formation in the gal region of E. coli K12. Mol. Gen. Genet. 137, 17-

Reif,H.J. and Saedler,H. (1977). Chromosomal rearrangements in the gal region of E. coli K12. pp 81-91. In DNA insertion elements, plasmids and episomes. Bukhari,A.I., Shapiro,J.A. and Adhya,S.L. (ed). Cold Spring Harbor Laboratory, CSH, NY.

Reitzer,L.J. and Magasanik, B. (1986). Transcription of glnA in E. coli is stimulated by activator bound to sites far away from the promoter. Cell 45, 785-792.

Reznikoff,W., Miller., Scaife,J. and Beckwith,J. (1969). A mechanism for repressor action. J. Mol. Biol. 43, 201-213.

Reynolds,A.E., Felton,J. and Wright,A. (1981). Insertion of DNA activates the cryptic bgl operon in E. coli K12. Nature 293, 625-629.

Rigby,P.W.J., Burleigh,B.D. and Hartley,B.S. (1974). Gene duplication in experimental enzyme evolution. Nature 251, 200-204.

Ripley,L.S. and Glickman,B.W. (1982). Unique self-complementarity of palindromic sequences provides DNA structural intermediates for mutation. Cold Spring Harbor Symp. Quant. Biol. 47, 851-861.

Rosner,J.L. and Guyer,M.S. (1980). Transposition of IS1-lambda bio-IS1 from a bacteriophage lambda derivative carrying the IS1-cat-IS1 transposon Tn9. Mol. Gen. Genet. 178, 111-120.

Ruther,W., Koenen,M., Otto,K. and Muller-Hill,B. (1981). pUR222, a vector for cloning and rapid chemical sequencing of DNA. Nucl. Acids. Res. 9, 4087-4098.

Saedler,H. and Heiss,B. (1973). Multiple copies of the insertion-DNA sequences IS1 and IS2 in the chromosome of E. coli K12. Mol. Gen. Genet. 122, 267-277.

Saedler,H., Reif,H.J., Hu,S. and Davidson,N. (1974). IS2, a genetic element for turn-off and turn-on of gene activity in E. coli. Mol. Gen. Genet. 132, 265-289.

Saedler,H., Cornelis,G., Cullum,J., Schumacher,B. and Sommer,H. (1980). IS1-mediated DNA rearrangements. Cold Spring Harbor Symp. Quant. Biol. 45, 93-98.

Sanzey,B. (1979). Modulation of gene expression by drugs affecting DNA gyrase. J. Bacteriol. 138, 40-47.

Savic,D.J., Romac,S.P. and Ehrlich,S.D. (1983). Inversion in the lactose region of E. coli K-12 : inversion termini map within IS3 elements and . J. Bacteriol. 155, 943-946.

Scaife,J. (1967). Episomes. Ann. Rev. Microbiol. 21, 601-638.

Scherer,S. and Davis,R.W. (1979). Replacement of chromosome segments with altered DNA sequences constructed in vitro. Proc. Natl. Acad. Sci. USA. 76, 4951-4955.

Schimke,R.T. (1982). Summary. pp 317-333. In Gene amplification. Cold Spring Harbor Laboratory, CSH, NY.

Schmitt,R., Altenbuchner,J., Wiebauer,K., Arnold,W., Puhler,A. and Schoffl,F. (1981). Basis of transposition and gene amplification by Tn1721 and related tetracycline-resistant transposons. Cold Spring Harbor Quant. Biol. 45, 59-65.

Schoner,B. (1983). E. coli K12 F' plasmids carrying insertion sequences IS1 and IS5. Gene 21, 203-210.

Schoner,B. and Kahn,J. (1981). The nucleotide sequence of IS5 from E. coli. Gene. 14, 165-174.

Schoner,B. and Schoner,R.G. (1981). Distribution of IS5 in bacteria. Gene 16, 347-352.

Scott,J.R. (1984). Regulation of plasmid replication. Micobiol. Rev. 48, 1-23.

Seifert,S. and Porter,R.D. (1984). Enhanced recombination between lambda plac5 and mini-F-lac: the tra regulon is required for recombination enhancement. Mol. Gen. Genet. 193, 269-274.

Shapiro,J.A. (1979). Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. Proc. Natl. Acad. Sci. USA. 76, 1933-1973.

Sharp,P.A., Hsu,M-T., Ohtsubo,E. and Davidson,N. (1972). Electron microscope heteroduplex studies of sequence relations among plasmids of E. coli. J. Mol. Biol. 71, 471-497.

Scherer,S. and Davis,R.W. (1979). Replacement of chromosome segments with altered DNA sequences constructed in vitro. Proc. Natl. Acad. Sci. USA. 76, 4951-4955.

Simon,M., Zieg,J., Silverman,M., Mandel,G and Doolittle,R. (1980). Phase variation in Salmonella: evolution of a controlling element. Science 209, 1370-1374.

Sinden,R.R. and Pettijohn,D.E. (1981). Chromosomes in living E. coli cells are segregated into domains of supercoiling. Proc. Natl. Acad. Sci. USA. 78, 224-228.

So,M., Heffron,F. and McCarthy,B.J. (1979). The E. coli gene encoding heat stable toxin is a bacterial transposon flanked by inverted repeats of IS1. Nature 277, 453-456.

Sommer,H., Schumacher,B. and Saedler,H. (1981). A new type of IS1-mediated deletion. Mol. Gen. Genet. 184, 300-307.

Southern,E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Stahl,M.L. and Ferrari,E. (1984). Replacement of the B. subtilis subtilisin structural gene with an in vitro-derived deletion mutation. J. Bacteriol. 158, 411-418.

Stark,G.R. and Wahl,G.M. (1984). Gene amplification. Ann. Rev. Biochem. 53, 447-492.

Starlinger,P. and Saedler,H. (1976). IS elements in micro-organisms. Curr. Top. Microbiol. Immunol. 75, 111-153.

Stodolsky,M. (1974). Recipient gene duplication during generalized transduction. Genetics 78, 809-822.

Stodolsky,M., Rae,M.E. and Mullerbach,E. (1972). The addition of lac^+ chromosome fragments to the E. coli proA-proB-lac deletion XIII chromosomes. Genetics 70, 495-510.

Stokes,H.W. and Hall,B.G. (1984). Topological repression of gene activity by a transposable element. Proc. Natl. Acad. Sci. USA. 81, 6115-6119.

Strauss,D. and Hoffmann,G.R. (1975). Selection for a large genetic duplication in S. typhimurium. Genetics 80, 227-237.

Straus,D.S. and Straus,L.D. (1976). Large overlapping tandem genetic duplications in S. typhimurium. J. Mol. Biol. 103, 143-154.

Sutcliffe,J.G. (1979). Complete nucleotide sequence of the E. coli plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. 43, 77-90.

Symington,L. (1982). Ph.D. thesis, Glasgow University.

Syvanen, J.M. (1980). Tn903 induces inverted duplications in the chromosome of bacteriophage lambda. J. Mol. Biol. 139, 1-17.

Syvanen,J.M. and Roth,J.R. (1972). The structural genes for ornithine transcarbamylase in S.typhimurium and E.coli K-12. J. Bacteriol.110, 66-70.

Syvanen,M., Hopkins,J.D., Giffin IV,T.J., Liang,T-Y., Ippen-Ihler,K. and Kolodner,R. (1986). Stimulation of precise excision and recombination by conjugal proficient F' plasmids. Mol. Gen. Genet. 203, 1-7.

Tanaka,H., Kudo,O., Sato,K., Izaki,K. and Takahashi,H. (1971). Inhibition of chloramphenicol O-acetyl transferase of E. coli by basic triphenylmethane dyes. J. Antibiotic. 24, 324-325.

Tanaka,H., Izaki,K. and Takahashi,H. (1974). Some properties of chloramphenicol acetyl transferase, with particular reference to the mechanism of inhibition by basic triphenylmethane dyes. J. Biochem. 176, 1009-1019.

Taylor,A.F. and Smith,G.R. (1980). Unwinding and rewinding of DNA by the recBC enzyme. Cell 22, 447-457.

Taylor,A.F. and Smith,G.R. (1985). Substrate specificity of the DNA unwinding activity of the recBC enzyme in E. coli. J. Mol. Biol. 185, 431-443.

Taylor,A.F., Schultz,D.W., Ponticelli,A.S. and Smith,G.R. (1985). RecBC enzyme nicking at Chi sites during unwinding: location and orientation-dependance of the cutting. Cell 41, 153-163.

Timmons,M.S., Spear,K. and Deonier,R.C. (1984). Insertion element IS121 is near proA in the chromosomes of E. coli K-12 strains. J. Bacteriol. 160, 1175-1177.

Tlsty,T., Brown,P.C., Johnston,R. and Schimke,R.T. (1982). Enhanced frequency of generation of methotrexate resistance and gene amplification in cultured mouse and hamster cell lines. In Gene amplification. Schimke,R.T. (ed). Cold Spring Harbor Laboratory, CSH, NY. pp 231-238.

Tlsty,T.D., Albertini,A.M. and Miller,J.H. (1984). Gene amplification in the lac region of E. coli. Cell 37, 217-224.

Todd,P.A. and Glickman,B.W. (1982). Mutational specificity of UV light in E. coli: indications for a role in DNA secondary structure. Proc. Natl. Acad. Sci. USA. 79, 4123-

Trowsdale,J. and Anagnostopoulos,C. (1975). Evidence for the translocation of a chromosome segment in B. subtilis carrying the trpE26 mutation. J. Bacteriol. 122, 609-618.

Van Vliet,F., Cunin,R., Jacobs,A., Piette,J., Gigot,D., Lauwereys,M., Pierard,A. and Glansdorff,N. (1984). Evolutionary divergence of genes for ornithine and aspartate carbamoyl-transferases - complete sequence and mode of regulation of the E.coli argF gene; comparison of argF and argI with pyrB. Nucleic Acids Res. 12, 6277 -6289.

Van de Putte,P., Cramer,S. and Giphart-Gassler,M. (1980). Invertible DNA determines host specificity of bacteriophage Mu. Nature 286, 218-222.

Van de Vate,C. and Symonds,N. (1974). A stable duplication as an intermediate in the selection of deletion mutants of phage T4. Genet. Res. 23, 107-113.

Van de Vate,C., Van den Ende,P. and Symonds,N. (1974). A stable duplication of the rII region of bacteriophage T4. Genet. Res. 23, 107-113.

Vieira,J. and Messing,J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19, 259-268.

Walz,A., Ratzkin,B. and Carbon,J. (1978). Control of expression of a cloned yeast (Saccaromyces cerevisiae) gene (trp5) by a bacterial insertion mutant (IS2). Proc. Natl. Acad. Sci. USA. 75, 6172-6176.

Weinert,T.A., Derbyshire,K., Hughson,F.M. and Grindley,N.D.F. (1984). Replication and conservative transpositional recombination of insertion sequences. Cold Spring Harbor Symp. Quant. Biol. 49, 251-260.

Weisberg,R.A. and Landy,A. (1983). Site-specific recombination in phage lambda. pp211-250. In Hendrix,R.W., Roberts,J.W., Stahl,F.W. and Weisberg,R.A. ed., Lambda II. Cold Spring Harbor laboratory, NY.

Willetts,N. (1977). The transcriptional control of fertility in F-like plasmids. J. Mol. Biol. 112, 141-148.

Willetts,N. and Skurray,R. (1980). The conjugation system of F-like plasmids. Ann. Rev. Genet. 14, 41-76.

Winans,S.C., Elledge,S.J., Krueger,J.H. and Walker,G.C. (1985). Site directed insertion and mutagenesis with cloned fragments in E. coli. J. Bacteriol. 161, 1219-1221.

Wood,A.J. and Konisky,J. (1985). Activation of expression of a cloned archaebacterial gene in E. coli by IS2, IS5 or deletions. Mol. Gen. Genet. 198, 309-314.

Yancey,S.D. and Porter,R. (1985). General recombination in E. coli K12: in vivo role of the RecBC enzyme. J. Bacteriol. 162, 29-34.

Yanisch-Perron,C., Vieira,J. and Messing,J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33, 103-119.

Yin,S., Bushman,W. and Landy,A. (1985). Interaction of the lambda site-specific recombination protein Xis with attachment site DNA. Proc. Natl. Acad. Sci. USA. 82, 1040-1044.

York,M.K. and Stodolsky,M. (1981). Characterisation and P1argF derivatives from E.coli K-12. I. IS1 elements flank the argF gene segment. Mol. Gen. Genet. 181, 230-240.

Young,M. (1983). The mechanism of insertion of a segment of heterologous DNA into the chromosome of B. subtilis. J. Gen. Microbiol. 129, 1497-1512.

Young,M. (1984). Gene amplification in B. subtilis. J. Gen. Microbiol. 130, 1613-1621.

Zafarulla,M., Charlier,D. and Glansdorff,N. (1981). Insertion of IS3 can "turn on" a silent gene in E. coli. J.Bacteriol. 146, 415-417.

Zerez,C.R. and Weiss,R.L. (1986). Effect of chloramphenicol and ethidium bromide on the level of ornithine carbamoyl-transferase in Neurospora crassa. J. Bacteriol. 166, 679-682.

Zipkas,D. and Riley,M. (1975). Proposal concerning mechanism of evolution of the genome of E. coli. Proc. Natl. Acad. Sci. USA. 72, 1354-1358.

Zissler,J., Mosharaffa,E., Pilacinski,W., Fiandt,M. and Szybalski,W. (1977). Position effects of insertion sequences IS2 near the genes for prophage lambda insertion and excision. In Bukhari,A.I., Shapiro,J.A. and Adhya,S.L. (Eds), DNA insertion elements, plasmids and episomes. Cold Spring Harbor Press, NY, pp381-388.