ONE-ENDED TRANSPOSITION OF Tn1/3.

A thesis submitted for the degree of Master of science at the University of Glasgow.

by

Elaine Robertson Nimmo.

Institute of Genetics,

Church Street,

Glasgow.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Dedicated

to my parents.

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CONTENTS.

ABBREVIATIONS.

(i) Chemicals.

APS	ammonium persulphate.
ATP	adenosine triphosphate.
BSA	bovine serum albumin.
CTP	cytosine triphosphate.
DNA	deoxyribonucleic acid.
d(NTP)	2'-deoxy (nucleotide).
dd(NTP)	2',3'-dideoxy (nucleotide).
DTT	dithiotreitol.
EDTA	ethylene diamine tetra-acetic acid (disodium salt, dihydrate).
EtBr	ethidium bromide.
EtOH	ethanol.
FSB	final sample buffer.
GTP	guanosine triphoaphate.
IPTG	isopropyl B-D thiogalactopyranoside.
PEG	polyethylene glycol 6000.
RNA	ribonucleic acid.
RNase	ribonuclease.
SDS	sodium dodecyl sulphate.
TEMED	N,N,N',N'tetramethylethylenediamine.
Tris	tris (hydroxymethyl) amino ethane.
TTP	thymidine triphosphate.

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

(ii) Antibiotics.

Ap Ampicillin.

Cm Chloramphenicol.

Rif Rifampicin.

St Streptomycin.

Tc Tetracyclin.

Tp Trimethoprim.

(iii) Phenotype.

bla B-lactamase.

X^r resistance to X.

X^s sensitivity to X.

ori origin of replication.

res resolution site.

(iv) Measurements.

mA. milliamp (10^{-3} amps).

bp. base pair(s).

Kb. kilobase pair(s) (10^3 bp.).

^oC. degrees Celsius.

Ci. Curie.

uCi. microCurie (10^{-6} Curie).

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.

mm. millimetre.

min. minute(s).

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

sec. seconds.

V. volts.

W. watts.

(v) Miscellaneous.

fig. figure.

IR. inverted repeat.

IS. insertion sequence.

no. number.

RF. replicative form.

Tn transposon.

SUMMARY

The plasmids pBR322 and pPAK100 each contain one half of a transposon. pBR322 contains the right half of Tn3 while pPAK100 contains the left half of Tn1. These half transposons were assayed for the ability to transpose and in both cases transposition was detected at frequencies about 1000 times lower than wild type. The same half transposons were found to confer transposition immunity to a plasmid, and the level of immunity was found to be the same whether the whole transposon or only half of it was present.

A series of deletion mutants was constructed, using restriction enzymes and the exonuclease BAL 31. These were assayed for the ability to transpose and to confer immunity to the host plasmid. All the deletion mutants except one were able to transpose and to cause transposition immunity. The exception which did not transpose was sequenced and found to have lost most of its transposon sequences, including all of the inverted repeat. The others all still contained their inverted repeat sequences.

One-ended transposition resulted in the formation of cointegrates between the donor plasmid and the recipient, R388. All experiments were performed in recA strains of E.coli and in the absence of resolvase, therefore no break down of cointegrates

was possible and the transposition event was stopped at the cointegrate stage. Restriction enzyme analysis of these cointegrates revealed that the donor plasmid was inserted at many places into the recipient plasmid and the two plasmids were joined at the junction of the inverted repeat and plasmid sequences. All the cointegrates analysed had the transposon inserted in the same orientation, but the relatively small number analysed means that the possibility of finding inserts in the opposite orientation cannot be excluded.

No duplication of transposon sequences was detected using restriction enzymes, however a duplication of about 100bp. or less would not have been detected using this system. Sequencing work on three examples done in this laboratory (A.Arthur, pers. comm.) has shown that there has been a small duplication of the inverted repeat sequences. In the three examples studied the duplication was 13bp., 32bp. and 100bp. Sequencing was also done to discover whether there had been any duplication of target sequences. It was found that, as in "normal" transposition, a 5bp. duplication had occurred. The data infers that transposons having only one inverted repeat are able to transpose, and that transposition can occur using either of the inverted repeats. It is still unclear whether transposition occurs by a symmetric or asymmetric mechanism, although the evidence of Craigie and Mizuuchi, (1985), strongly suggests that at least in the case of Mu, transposition occurs in a symmetric fashion.

CHAPTER 1

INTRODUCTION.

1.1. A DEFINITION OF TRANSPOSONS AND INSERTION ELEMENTS

Prokaryotic transposable elements are discrete pieces of DNA that are capable of inserting into bacterial genomes, and plasmids, at many sites, leaving a copy of themselves in the donor molecule. Most transposons can be grouped into one of three classes. Class I contains the insertion elements and composite transposons; Class II, the Tn3 family; and Class III the transposing bacteriophages. Some transposons eg. Tn7 have so far not been assigned to any of the three groups.

1.1.1. Class I - Insertion Elements and Composite Transposons.

Insertion elements can be defined as having no detectable genes other than those related to insertion and transposition functions. They were originally detected when DNA was analysed and found to contain extra sequences.(Jordan *et al*, 1968; Shapiro, 1969). Insertion elements are small (750 - 1500bp.) and have short inverted repeats at the ends, of about 15 to 40bp. Different insertion elements transpose at different rates, the transposition frequencies varying between 10^{-5} and 10^{-9} per generation. Transposition events are detected as spontaneous DNA mutational events involving insertions, deletions and inversions. Transposition can also causes polar effects such as activation of gene expression.

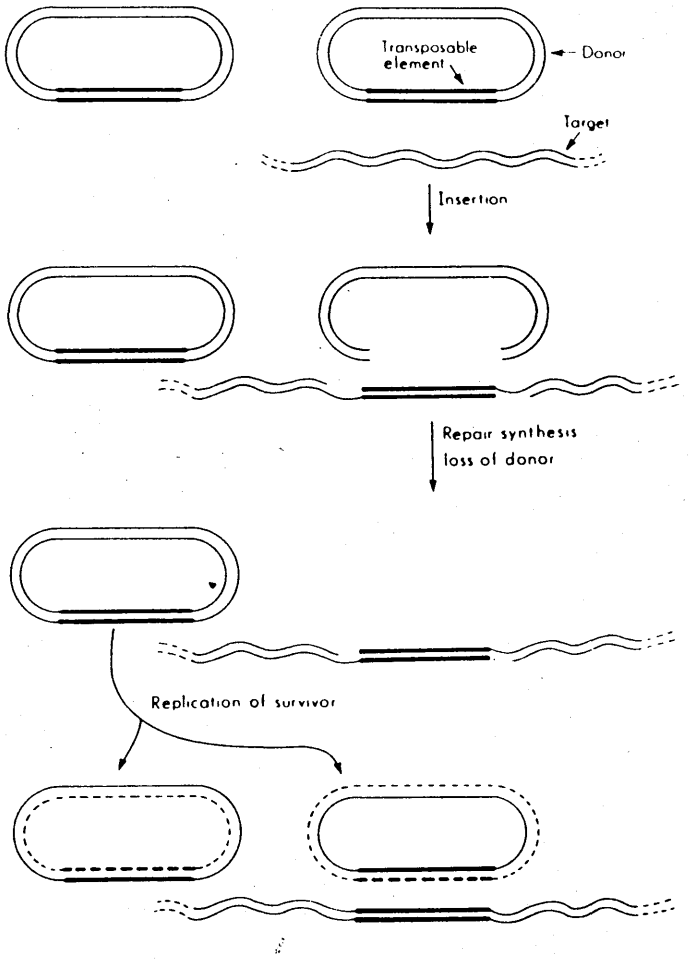

Figure 1.1.

Conservative Transposition (from Berg et al, 1984). Double strand breaks are made at each end of the DNA segment to be transposed, and single strand nicks are made in the target sequences. Single strands at each end of the element are joined to the nicked target sequences and the single strand gaps are filled in, generating target sequence duplications. The vector from which the element is taken is not recircularized, and is lost through degradation. However, sibs of the donor replicon may replicate to replace it.

Composite transposons are made up of two insertion elements, either as direct or inverted repeats, flanking a new piece of DNA. Tn9 is an example of a composite transposon. It has two copies of IS1 flanking the gene for Cm resistance and it contains no other genes. Transposition of large regions of DNA can be mediated by insertion elements. Tn2671 is a 23Kbp. segment of DNA flanked by direct repeats of IS1: It encodes several different antibiotic resistance genes, and can transpose at low frequencies (Iida et al, 1981). Other examples of composite transposons include Tn5 encoding Km resistance and Tn10, encoding Tc resistance. Both of these transposons are flanked by insertion elements, in inverted orientation. In both Tn5 and Tn10, a functional transposase is provided by the right inverted repeat, while the left inverted repeat produces a reduced amount of transposase (Tn10), or prematurely terminates transcription of transposase (Tn5). The differences between the left and right inverted repeats are caused by a single base change in the case of Tn5 (Auerwald et al, 1980).

It has been proposed by Berg et al (1984) that Tn5 transposes in a conservative (non-replicative) fashion. This is shown in figure 1.1. Double strand breaks are made at each end of the transposon and it is inserted into the donor at a staggered nick. The single-stranded gaps are filled in, generating the target sequence duplication. The donor, now without the transposon sequence, is lost, but it can be replaced

by replication of one of its siblings.

1.1.2. Class II - The Tn3 Family.

Tne Tn3 family is made up of transposons that are long (5Kb.or longer) and have 35 to 40bp. inverted repeats. Transposition generates 5bp. duplications in the target DNA (Ohtsubo *et al*, 1978). There are about 20 transposons belonging to this group and they usually code for single or multiple antibiotic resistances. Tn3, the best studied member of this group encodes penicillin resistance. Tn501 does not encode resistance to an antibiotic, it encodes a gene for mercury resistance. Transposons of the Tn3 family, are medically important because they confer antibiotic resistance to many pathogenic bacteria. They are usually found on plasmids, although they can also transpose to the chromosome. Also, they have a similar structure, and use the same transposition mechanisms.

The Tn3 family can perhaps be divided into two subgroups. One contains Tn3 and Tn1000 - in which the *tnp*A and *tnp*R genes are divergently transcribed, and the other includes Tn21, Tn501 and Tn1721 in which the *tnp*A and *tnp*R genes are transcribed in the same direction. For sucessful transposition, the inverted repeats and the *res* site are needed in *cis*, while the *tnp*A and *tnp*R gene products can be provided in *trans*.

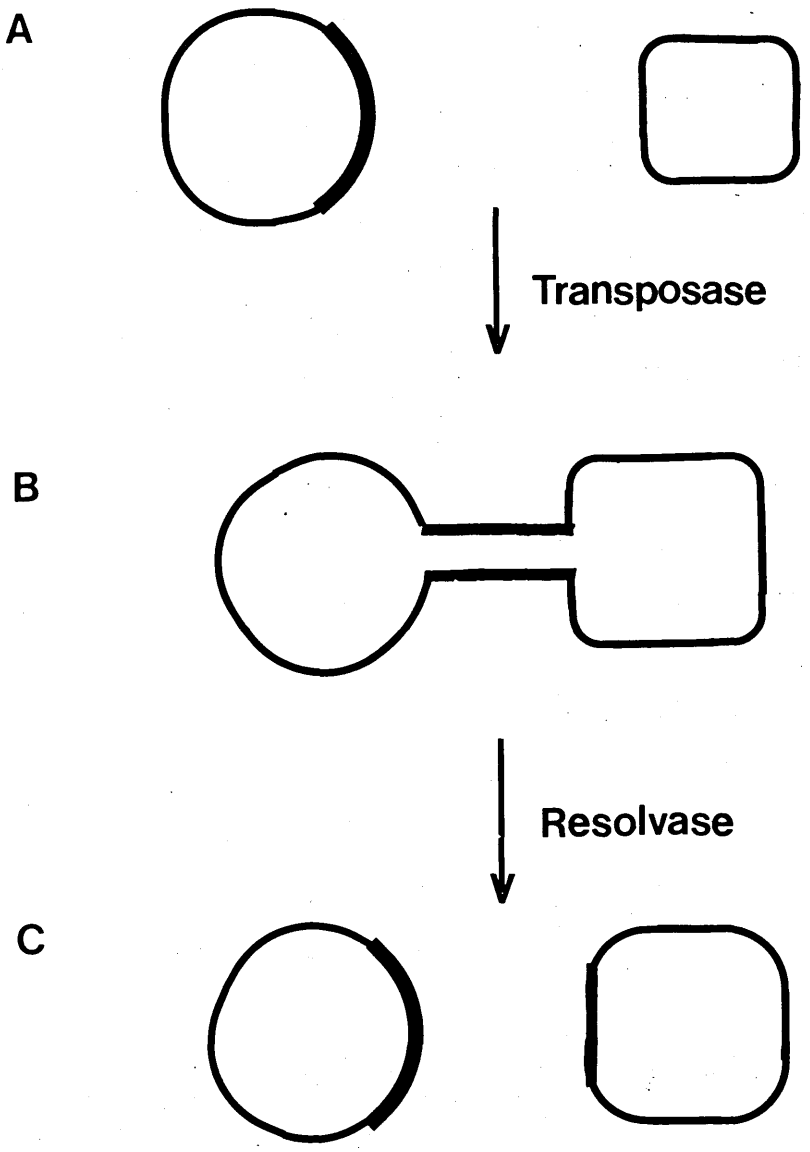

Figure 1.2.

Replicative Transposition.
Transposition of Tn3 is a two step process. Transposase is required for cointegrate formation and then resolvase acts at the res site to break down the cointegrate into donor and recipient, each with a copy of the transposon.

Intermolecular transposition of Tn3 is thought to be a two step process (Arthur and Sherratt, 1979; Shapiro, 1979) and is shown in figure 1.2. First, a 5bp.staggered cut is made at the target site and single stranded nicks are made at the ends of the transposon, presumably by the transposase. Each free end of the transposon is then joined to one of the overlapping ends of the staggered nick. Replication can then repair the duplex 5bp. repeats and complete the rest of the transposon. This produces a cointegrate in which both replicons are fused with one copy of the transposon, in direct repeat, at each junction point. The tnpR gene product, resolvase, completes the transposition process by mediating recombination between the two transposons at the res site (Gill et al, 1978; Reed, 1981; Kostriken et al, 1981). This results in the regeneration of the original replicon with the donor transposon, and the production in the target replicon, of another copy of the transposon. In the absense of resolvase in a recA cell, the transposition process will be stopped at the cointegrate stage. However, in rec+ cells, homologous recombination occurs to break down the cointegrate into the two replicons, each with a copy of the transposon. The target replicon now has 5bp. direct repeats on either side of the integrated transposon, but these do not seem to be involved in subsequenttransposition events. If the 5bp. repeats are altered or removed, there is no effect on further transposition (Ohtsubo et al, 1979; Reed et al, 1979).

1.1.3. Class III - The transposing Bacteriophages.

This class contains the two related phages Mu and D108. Mu is a 39Kb. phage which, upon infection can either enter the lytic cycle, where it is replicated, packaged and released; or can enter the lysogenic state where the viral DNA is integrated into the host DNA and forms a stable association. Mu can also integrate into the host chromosome, via transposition, when it enters either the lytic cycle or the lysogenic state. Mu integrates randomly in the chromosome, and then during the lytic cycle it can transpose to new locations, causing duplication of the 5bp. target sequence (Allet, 1979: Hahmann and Kamp,1979). Transposition of Mu can cause deletions, inversions, duplications etc., like other transposible elements.

1.2. TERMINAL REPEATS.

A feature common to almost all transposable elements is the presence of terminal repeats. These can be in inverted or direct orientation. Insertion elements terminate in short inverted repeats, and some transposons are themselves bounded by insertion elements, either in direct repeat, as in Tn9, or in inverted repeat, as in Tn5. The inverted repeats of the insertion elements and the Class II transposons are small and vary between 15 and 40bp. The terminal repeats of the composite transposons, being IS elements, can be fairly large (up to

1500bp.). Tn7 does not seem to belong either to the composite transposon or to the Tn3 family. It does not have true terminal repeats, but instead has regions of homology at the ends. Each end contains a 22bp. sequence that is repeated several times. In the right end, these repeats are contiguous, while in the left end they are inverted, and separated by other sequences. Similarly, the bacteriophage Mu does not have true terminal repeats: it has inverted repeats of only 2bp., but like Tn7, also has certain homologies in the ends. There is extensive homology between a sequence beginning at one end and a sequence beginning about 80bp. in from the other end. It also contains a small (7 - 9bp.) sequence which occurs once near one end and five times near the other end. This sequence also occurs near the ends of several of the Tn3 family transposons.

1.3. SITE OF INSERTION

Tn554, a transposon of Staphylococcus aureus, does not have repeats at its ends (Murphy and Lofdahl, 1984) and does not generate duplication of host sequences on insertion. This is different to all other known transposition events, which result in the duplication of a small region of the target site. The size of duplication is generally the same for a given transposon, but varies from transposon to transposon. The size of the duplication varies from only 3 or 4bp. (IS3), to 11 or 12bp. (IS4). A duplication size shared by several transposons suggests that they transpose by similar mechanisms and have a common

ancestor (e.g. Transposons belonging to the Tn3 family all cause 5bp. duplications.) The lack of duplication of sequences by Tn554 may point to a different mechanism of transposition.

Some transposable elements are able to integrate at many places in DNA molecules, with no obvious specificity, while others seem to have one or a few preferred sites of insertion. IS4, an insertion element, seems only to have one insertion point in E.coli, in galT (Habermann et al, 1979). Tn7 transposition into the chromosomes of various species occurs at specific sites and in only one orientation (Lichtenstein and Brenner, 1981; Ely, 1982; Fennewald and Shapiro, 1979), however it does seem to be able to transpose into a variety of plasmids with little site specificity (Barth et al, 1976). Other transposable elements show much less site specificity. Tn9, IS1 and Tn3 all show a preference for AT rich regions, although within these regions there are many integration sites (Kretschmer and Cohen, 1977; Kuhn et al, 1979; Johnsrud et al, 1978; Miller et al, 1980). Tn9 insertions have been found grouped within a region containing a sequence identical to the last 7bp. of IS1, and IS1 insertions have also been found within regions showing homology with its ends (Galas et al, 1980). Tn3 insertions have been found to occur near sequences resembling its ends (Tu and Cohen, 1980).

1.4. EUKARYOTIC TRANSPOSABLE ELEMENTS AND RETROVIRUSES

Transposable elements were first discovered in Maize by McClintock (McClintock, 1951). Since then, transposable elements have been found in other eukaryotes such as Yeast and Drosophila as well as in prokaryotes. Retroviruses have been found in vertebrates and these show remarkable similarities to transposons. It has been argued that retroviruses may have evolved from transposable elements (Temin, 1980).

Many transposable elements have been found in Maize; Dissociation elements (Ds) and Activator elements (Ac) being the best known. Many Ds elements differ from Ac elements only by internal deletions; indeed Ds9 differs from Ac by a deletion of 194bp. located in one of the open reading frames (ORFs). Double Ds elements have been found which consist of one copy of a Ds element inserted, in inverse orientation, into an identical copy of itself. A Ds element cannot transpose unless a copy of Ac is present in the same cell; in the absence of Ac, Ds elements are static. The Ds and Ac elements both cause 8bp. duplications at the site of insertion, and many of the Ds elements terminate in inverted repeats of the sequence TAGGGATGAAA. The two Ac elements so far sequenced terminate in almost identical sequences, are both 4563bp. long and have 3 open reading frames. In Southern blotting experiments, approximately 40 bands have been found to hybridize when the Maize DNA is probed with Ac;

however, it is not known how many of these sequences are transposable. (For a review of transposable elements in plants see Doring and Starlinger, 1984).

Another transposon found in Maize is Mutator (Mu), (Robertson,1980). This is a 1.4Kbp. transposon with inverted repeats of about 210bp., and causes 9bp. direct repeats at the site of insertion. (Freeling,1984). It is present at 20-40 copies per cell and it is frequently the cause of mutations in Maize. Elements called Enhancer (En) and Suppressor Mutator (Spm) have also been found in Maize. There are also degenerate elements related to En and Spm which are unable to transpose unless a functional En or Spm is present. En and Spm show some similarities to transposable elements found in the dicotyledonous plants Soybean (le1) and Snapdragon (Tam1 and Tam2). They all produce 3bp. duplications at the insertion site and share some homology at their termini. (Vodkin et al, 1983; Bonas et al, 1984). This indicates either that horizontal transmission of elements is possible between these species, or that convergent evolution has taken place.

In Drosophila melanogaster many transposable elements have been found. Among the best studied are the copia-like elements - copia, 412, 297 and mdg1. These have several properties in common but share no sequence homology. They are present at 20-40 sites throughout the Drosophila genome and cause 4 or 5bp. direct repeats at the site of insertion. They are flanked by direct

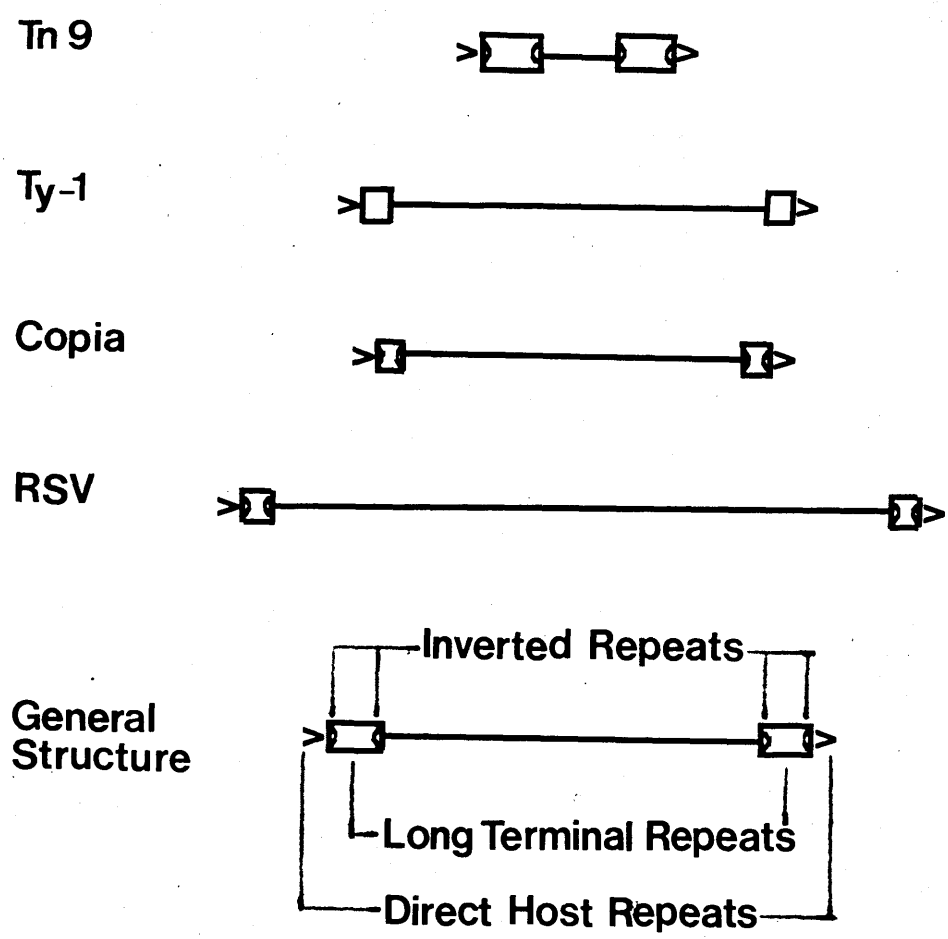

Figure 1.3.

Comparison of the general structure of transposons and retroviruses.

The structure of three transposable elements, Tn9, Ty-1 and Copia is shown along with a retrovirus, Rous Sarcoma Virus (RSV) for comparison. The general structure of each can be seen to be very similar.

repeats of between 276 and 571bp, both of which have short (about 10bp.) inverted repeats at the ends. In Drosophila melanogaster there are about 30 families of copia-like elements.

Retroviruses are single-stranded RNA animal viruses which replicate through a DNA intermediate. They show remarkable similarities to transposable elements including Tn9 in bacteria, Ty1 in Yeast and copia in Drosophila. (See figure 1.3 for a comparison). They are flanked by long terminal repeats (LTRs) in direct orientation, each LTR having a short inverted repeat at each end. The integrated form of the retrovirus is known as the provirus. They cause 4bp. duplications at the site of insertion. Like other transposable elements, retroviruses have been found to cause many mutations. Insertions into genes have been found, as well as deletion formation. Excision of proviruses has been found, although the mechanism is not yet understood. Single copies of the LTRs have also been found, indicating that elimination of proviral sequences may be occurring by normal recombination in some cases.

Three genes (gag, pol and env) are encoded by the retrovirus. Replication defective viruses, with mutations in any of these genes, are commonly found. These require the presence of replication-competent virus in order to replicate. The gag gene codes for the core protein of the virus, the pol gene encodes the reverse transcriptase and the env gene codes for the viral envelope. A fourth gene can be encoded by the retrovirus

and this is known as the onc gene. The presence of the onc gene is associated with transformation of cultured cells and the induction of tumours in animals, but is not involved in the replication of the virus.

Retroviruses are present in most, if not all vertebrates, and can make up a substantial amount of the genome. In primates and rodents this is thought to amount to about 0.1% of the total DNA (Todaro et al, 1980). Cells may have more than one copy of the retrovirus integrated into the genome and the locations may differ between individuals. Upon infection, the RNA is copied using reverse transcriptase to give a linear double stranded DNA with LTRs at both ends. This is converted to a circular molecule which has either one copy of the LTR, or two copies in tandem, which then integrates into the genome. (For discussion see Gilboa et al, 1979). The circular proviral DNA is similar to circular copies of copia which have been found in Drosophila melanogaster embryos and in tissue culture (Flavell and Ish-Horowicz, 1981 ; Sinclair et al, 1983). These circular copies contain a complete copy of copia and they may be able to replicate. This circular copy may arise by reverse transcription of a copia RNA transcript to give a double stranded DNA which circularises, or it may be due to homologous recombination between the terminal repeats of an integrated copia which would result in the excision of the integrated element.

Recently it has been shown (Boeke et al, 1985; Garfinkel et al, 1985) that Ty elements of yeast transpose through an RNA intermediate, and that the translated sequences of open reading frames show amino acid sequence homology to reverse transcriptases (Clare and Farabaugh, 1985; Hauber et al, 1985)

1.5. HOST MUTATIONS AFFECTING TRANSPOSITION

The transposition of insertion elements probably depends to a certain extent on host factors. For example, it is likely that insertion elements depend on the host for replication functions.

In E.coli, one of the first such host functions detected was the polA gene which encodes DNA polymerase I. Mutations in this gene were found to be detrimental to transposition (Sasakawa et al, 1981; Clements and Syvanen, 1980; Syvanen et al, 1982), decreasing the transposition frequency of many insertion elements. Polymerase I has both a 3' to 5' polymerase activity and a 5' to 3' exonuclease activity. Mutations in different alleles of polA affected transposition, indicating that both the polymerase and the exonuclease activity are required. Presumably polymerase I is required during transposition for duplication of the element and filling in gaps.

Supercoiling of DNA in bacterial cells is controlled by two enzymes, DNA gyrase, which puts in negative supercoils, and topoisomerase I, which removes them. Mutations in gyrA or gyrB

(DNA gyrase) reduce supercoiling, while mutations in top1 (topoisomerase I) increase negative supercoiling. A decrease in supercoiling reduces transposition frequencies (Isberg and Syvanen, 1982). himB, a mutation in gyrB, has a decreased level of integration and also a decreased rate of transposition of Tn3 and Tn5. Sternglanz et al (1981) claimed that top1 mutants also reduced the level of transposition of Tn5, and the level of Tn3 transposition in the presence of a top1 mutation was reduced in some cases. This seemed to depend on the recipient plasmid. Isberg and Syvanen, however, claimed the opposite. They found that top mutants transposed at slightly higher frequencies than in the wild type. They suggest this is due to the slightly higher supercoil densities and that supercoiling promotes transposition at high frequencies. Both gyrase and topoisomerase mutants alter the transcription of genes, so it is possible that transposition is affected by this. Isberg and Syvanen have suggested that the recipient molecule is a poor substrate for transposition if it is not supercoiled. Topoisomerase may participate in the breakage and joining of DNA during transposition.

polA, gyrA, gyrB, and top1 are the best studied host mutations involved in transposition, but many others have also been found. Recombination of homologous sequences of some IS elements, such as Tn903, may require "recA" for cointegrate formation (Young et al, 1980; Grindley and Joyce, 1981). Tn5 is dependent on "recA" for cointegrate formation but not for

transposition. This strongly suggests that Tn5 does not transpose via an obligate cointegrate intermediate and that cointegrate formation can occur during the normal transposition pathway. "recA" is not involved in the transposition process of Tn3, unless the transposons own site-specific recombination system has been inactivated. In this case "recA" is involved in the breakdown of cointegrates rather that in their formation.

The E.coli sex factor F codes for two genes that affect genetic rearrangements. These are ferA and ferB (Hopkins et al, 1980). FerA mutants reduce the precise excision of Tn5 and Tn10 from the F factor, or from the bacterial chromosome, while ferB mutants enhance precise excision in the presence of ferA^{+}. FerB mutants also increase the rate of recombination between two IS3 elements on F'lac-pro when the recA product and the ferA product are present. This suggests that ferB is a repressor gene acting on ferA.

Mu has been found to require the host functions dnaC, dnaB, dnaE, dnaZ and dnaG for replication (Toussaint and Faelen, 1974), and the host protein HU for transposition (Craigie et al, 1985).

1.6. CONTROL OF TRANSPOSITION.

1.6.1. Transposition Immunity.

Transposition immunity can be defined as a transposon-encoded mechanism that prevents the acquisition of a second copy of that transposon. Immunity is a *cis*-acting phenomenon; i.e. the presence of a transposon on a plasmid can inhibit further transposition onto that plasmid, but not onto another transposon-free plasmid. Transposition immunity is not due merely to the instability of plasmids with two copies of a transposon, since plasmids with two copies of a transposon can be constructed or obtained by simultaneous transposition, and these are apparently always stable either when in inverted orientation, or when in direct repeat in a *rec*A strain, providing the *tnp*R gene is inactive.

This phenomenon was first described by Robinson *et al* (1977). It is unique to the Tn3 family and has been seen in Tn1, Tn3, Tn501, Tn21 and Tn1721. Tn21, Tn501 and Tn1721 all show a cross immunity - they are immune to each other, but not to Tn3 (Schmitt *et al*, 1981). These transposons are very closely related to each other, and their transposition functions are interchangeable. This suggests that functions involved in transposition are also involved in immunity. However, rather

than show immunity to Tn3 transposition, Tn501 has been found to be a hot spot for Tn3 insertion, although it does not increase the transposition frequency (Grinsted et al, 1978). Lee et al (1983) discovered that the presence of an immune region could direct insertions into certain regions of plasmids. This may suggest that transposase can track around a recipient molecule before the insertion takes place. In this way it would be able to recognise a good target sequence (like Tn501) or an immune sequence (like another copy of Tn3).

Immunity to Tn3 transposition has also been seen for some naturally occurring plasmids, which do not contain a copy of the transposon. R100-1 and R391 seem to show immunity to Tn3 transposition (Bennett and Richmond, 1976); also, the E.coli chromosome is resistant to Tn3 transposition (Kretschmer and Cohen, 1977). It is not clear whether this natural immunity is related to transposon-mediated immunity.

Wallace et al (1981) proposed that sequences within the terminal 900bp. of the left end of Tn3 were necessary for immunity, but were not always sufficient. It was suggested that the right end of the element showed no immunity. In contrast to these results, Lee et al (1983) showed that the 38bp. inverted repeat was sufficient on its own to confer immunity to a plasmid, and that no other parts of Tn3 were involved. One inverted repeat was found to confer as much immunity as two. Another finding of Lee et al was that certain deletions, which removed a

region between tnpA and tnpR, were immune in most plasmids, but not in some insertions of pMB8. The reason for this is not understood.

Transposable elements must be capable of regulating their transposition in some way so that they ensure their own survival and limit the possibility of causing lethal insertions in the host chromosome. In the case of the Tn3 family, transposition immunity acts to limit the number of copies present on a plasmid, while transposition is also regulated at the transcriptional level by the resolvase protein (Chou et al, 1979; Heffron et al, 1979) In this way, all cells can carry a copy of Tn3, ensuring the transposons survival, but insertions will be limited, ensuring the survival of the host cell.

Tn21, a member of the Tn3 family which exhibits transposition immunity, is also regulated by a gene, tnpM, that encodes a modulator protein. This can enhance transposition and suppress resolution of cointegrates (Hyde and Tu,1985). Tn501 is also thought to encode a modulator protein.

1.6.2. Limited Number of Insertion Sites.

Other transposons employ different methods of regulation. Tn554, a transposon in Staphylococcus aureus, exhibits a high preference for a single site in the chromosome. Once this site is occupied by a copy of Tn554, further transposition of another

Tn554 to the chromosome is inhibited 100-1000 fold (Murphy, 1983). This effect is thought to be caused by a sequence in the terminal 89bp. of the left end of the transposon which may act to 'mop up' a transposase or other protein factor required for transposition.

Tn7, a 14Kbp. transposon encoding resistance to trimethoprim, spectinomycin and streptomycin, transposes at a very high rate into the chromosomes of many species including E.coli and Caulobacter crescentus (Barth et al,1976; Ely, 1982), but it only has one insertion site in each chromosome. Once the insertion site is occupied there is no more transposition. Tn7 transposes at a high frequency and into many locations in the plasmid RP4, but does not transpose easily into other plasmids. It does not posess transposition immunity like that exhibited by the Tn3 family, as two Tn7 transpositions can be shown to occur into RP4 (Hassan and Brevet, 1983). Therefore it appears that Tn7 regulation, at least into bacterial chromosomes and most plasmids is due to the limited number of insertion sites available.

1.6.3. Regulation by Negative Control.

The transposition of Tn5 and Tn10 have been found to be regulated by negative control (Isberg et al, 1982; Johnson et al, 1982; Simons and Kleckner, 1983; Simons et al, 1983). In both cases this is caused by the element itself. Both are composite

transposons, Tn5 is flanked by two copies of IS50, while Tn10 is flanked by two copies of IS10. Tn5 transposase is encoded by the two insertion sequences, however IS50-L contains a point mutation (Auerswald et al, 1980) which results in the premature termination of the two proteins encoded. IS50-R encodes the two genes, one is the transposase while the other acts to inhibit transposition. The transposition of Tn10 is regulated by a phenomenon called multicopy inhibition (Foster et al, 1981). IS10-R encodes a long open reading frame (ORF) which specifies a function essential for transposition. A promoter, p-IN, has been located just upstream of this open reading frame and is responsible for its expression. Another promoter, p-OUT, has been found which lies just inside the start of the ORF, but reading in the opposite orientation. The region of overlap includes the ATG start codon for the ORF. It is thought that the transcript specified by p-OUT pairs with the transcript for the presumptive transposase and in this way inhibits translation of the ORF (Way and Kleckner, 1984). This pairing of complementary RNA molecules encoded by opposite strands has been shown to regulate replication, copy number and incompatibility in ColE1 (Tomizawa and Itoh, 1981a,b).

The aim of this study is to investigate the transposition and immune properties of transposons with only one inverted repeat.

Chapter 2.

MATERIALS AND METHODS

2.1 Chemicals.

Chemical.	Source.
Agar.	Oxoid, Davis.
Agarose.	B.R.L.
Antibiotics.	Sigma.
Biochemicals.	Sigma, Koch-light.
General chemicals and organic compounds.	B.C.L., B.D.H., B.R.L., Formachem Research Int., Hopkins and Williams, Koch-light, May and Baker.
Lambda DNA.	Gift from M.Burke.
Media.	Difco, Oxoid.
Radiochemicals.	New England Nuclear.
S.D.S.	Serva.

2.2 Enzymes.

AhaIII	P&S Biochemicals Ltd.
Ligase	E. Nimmo.
Lysozyme	Sigma.

M13 Universal primer and all other enzymes were obtained from B.R.L.

Table 2.1 Plasmids.

Plasmid	Description	Phenotype	Size (Kb.)	Reference.
R388	Naturally occurring	Tp^rTra^+	33.0	Datta and Hedges, 1972
pBR322	Derived from pMB8	Tc^r Ap^r	4.36	Bolivar et al, 1977
pEN200	BAL 31 deletion of pBR322	Tc^r	3.34	Chapter 3
pEN201	BAL 31 deletion of pBR322	Tc^r	3.76	Chapter 3
pEN202	AhaIII deletion of pBR322	Tc^r	3.76	Chapter 3
pEN203	AhaIII deletion of pBR322	Tc^r	4.34	Chapter 3
pACYC184	Vector derived from p15A	Cm^r Tc^r	4.0	Chang and Cohen, 1978
pPAK100	pAA231 BamHI deletion	Cm^r	7.2	Kitts, 1982
pEN113	ClaI deletion of pPAK100	Cm^r	5.2	Chapter 3
RR17	pBR322 with $tnpR^+$, $tnpA^-$, gamma delta insertion.	Ap^r Tc^r	10.06	Kitts et al, 1982
M13mp19	Phage sequencing vector.	-	7.23	Yanish-Perron et al, 1985
pMB9::Tn103	Insertion of Tn103 into pMB9	Tc^r	9.5	L.Symington

2.3 Bacterial strains.

Strain.	Relevant markers.	Source or reference.
DS902	thr leu his arg recA rpsL(=Str^r)	D.J.Sherratt
DS910	recA1 thy$^+$ minA minB	D.J.Sherratt
DS916	trp his recA rpsE rpoB(=rif^r)	D.J.Sherratt
JM101	lac pro supE thi F'traD36 proAB lacI^q Z M15	Messing, 1983

2.4 Plasmids and bacteriophages.

Plasmids and bacteriophages are listed in Table 2.1. Symbols for phenotype are those recommended by Bachman et al, 1976 and Campbell, 1981.

Table 2.2 Transposons

Common Name	Systematic Name	Derivation	Phenotype	Reference
Tn1	Tn1	Naturally occurring on RP4	*tnpA*$^+$ *tnpR*$^+$ *res*$^+$ Ap^r	Hedges and Jacob, 1974
Tn3	Tn3	Naturally occurring on R1	*tnpA*$^+$ *tnpR*$^+$ *res*+ Ap^r	Kopecko and Cohen, 1975
Gamma Delta	Tn1000	Naturally occurring on F	*tnpA*$^+$ *tnpR*$^+$ *res*$^+$	Guyer, 1978
Tn103	Tn1 Ap	Spontaneous deletion of Tn1	*tnpA*$^+$ *tnpR*$^-$ *res*$^+$ Ap^s	Heffron *et al*, 1977

2.5 Transposons.

Transposons are listed in Table 2.2. The genotype of the Tn1/3 derivatives is based on the nomenclature of Heffron *et al*, 1979.

2.6 Culture media

Isosensitest broth.

23.4g. Isosensitest broth made up to 1 litre in distilled water.

Isosensitest agar.

As above with 1.25% agar.

L broth.

10g. tryptone, 5g. yeast extract, 5g.NaCl, 1g. glucose, 20mg. thiamine, made up to 1 litre with distilled water and adjusted to pH 7.0 with NaOH.

L agar.

As above with 1.5% agar.

Minimal agar.

7g.K_2HPO_4, 2g.KH_2PO_4, 4g.$(NH_4)2SO_4$, 0.25g. tri-sodium citrate, 0.1g.$MgSO_4.7H_2O$, 15g. agar, made up to 1 litre in distilled water.

Soft agar.

6g. agar in 1 litre of distilled water.

2-YT.

16g. tryptone, 10g. yeast extract, 5g. NaCl made up to 1 litre in distilled water.

Supplements.

When required suppliments were added to minimal medium at the following concentrations.

glucose - 2mg./ml.

thiamine (vitamine B1) - 20ug./ml.

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

2.7 Buffers and other solutions.

5 x BAL 31 buffer.

60mM $CaCl_2$, 60mM $MgCl_2$, 1M NaCl, 100mM Tris HCl pH 8.1, 5mM EDTA.

E buffer.

40mM Tris HCl, 20mM NaAc, 1mM $Na_2EDTA.2H_2O$, pH adjusted to 8.2 with glacial acetic acid.

Formamide dye (for sequencing gels).

100ml. formamide stirred with 5g. Amberlite MB1 resin for 30 min. and then filtered to remove resin. 0.03g. xylene cyanol FF, 0.03g. bromophenol blue and 0.75g. $Na_2EDTA.2H_2O$ added and dissolved.

Final sample buffer (FSB).

10% ficoll, 0.5% SDS, 0.05% bromophenol blue, 0.06% orange G in buffer E.

Gelatine/TE.

100µg. gelatine/ ml. TE.

10 x Klenow buffer.

100mM Tris HCl, pH 8.0, 100mM $MgCl_2$

10 x Ligation buffer.

660mM Tris HCl pH 7.6, 100mM $MgCl_2$, 10mM EDTA.$2H_2O$, 100mM B-mercaptoethanol.

PEG/NaCl solution.

2.5M NaCl, 20% polyethylene glycol 6000.

Restriction buffers.

10 x low salt buffer. 100mM Tris HCl, pH 7.4, 100mM $MgSO_4.7H_2O$, 10mM DTT.

10 x medium salt buffer. 500mM NaCl, 100mM Tris HCl, pH 7.4, 100mM $MgSO_4.7H_2O$, 10mM DTT.

10 x high salt buffer. 1M NaCl, 500mM Tris HCl, pH 7.4, 100mM $MgSO_4.7H_2O$, 10mM DTT.

10 x AhaIII buffer. 250mM NaCl, 60mM Tris HCl, pH 7.6, 60mM $MgCl_2$, 60mM B-mercaptoethanol, 100µg./ml. BSA.

Single colony final sample buffer (SCFSB).

2% ficoll, 1% SDS, 0.01% bromophenol blue, 0.01% orange G in E buffer.

Table 2.3 Antibiotics

Antibiotic	Selective concentration	Stock solution
Ampicillin (Ap)	50μg./ml.	5mg./ml. in water.
Chloramphenicol (Cm)	50μg./ml.	5mg./ml. in EtOH.
Rifampicin (Rif)	50μg./ml.	5mg./ml. in MeOH
Streptomycin (Sm)	50μg./ml.	5mg./ml. in water
Tetracyclin (Tc)	10μg./ml.	1mg./ml. in 0.1M NaOH
Trimethoprim (Tm)	50μg./ml.	5mg./ml. in 50% EtOH

1 x TBE.

10.8g. Tris HCl, 5.5g. boric acid, 0.93g. $Na_2EDTA.2H_2O$, made up to 1 litre with distilled water.

1 x TE (general use).

10mM Tris HCl, 1mM $Na_2EDTA.2H_2O$, pH 7.6.

1 x TE (for sequencing reactions).

10mM Tris HCl, 1mM $Na_2EDTA.2H_2O$, pH 8.0.

Most of the above solutions were sterilised by filtration or by autoclaving at 108°C for 10 min. The $CaCl_2$ and $MgSO_4$ solutions were autoclaved at 114°C for 10 min.

2.8 Antibiotic selections.

The standard concentrations of antibiotics used are shown in Table 2.3. These were used for both liquid and plate selections. Isosensitest agar or broth was used when selecting with trimethoprim as trimethoprim is not effective in media containing thymine (Stacey and Simpson, 1965). All other antibiotics were used in L broth or agar.

2.9 Use of X-gal and IPTG.

Cells containing B-galactosidase are able to hydrolyse X-gal (5-bromo, 4-chloro, 3-indoyl B-D galactosidase), producing a blue colour. Cells lacking an active B-galactosidase enzyme produce white colonies. In the phage vector M13, this colour difference is used as a screen for detecting clones with inserts.

IPTG (isopropyl B-D thiogalactopyranoside) is an inducer of the lac promoter.

X-gal and IPTG were made up at a concentration of 20mg./ml. in methanol and water respectively.

2.10 Genetic transformation.

(i) Plasmid DNA.

An overnight culture of recipient cells in L broth was diluted 1 in 20, in 20ml. L broth and grown with shaking for about 90 min. (2×10^8 cells/ml.). The cells were harvested by centrifugation (12,000g, 2 min., 4^oC) and washed by resuspending in 10ml. ice cold $CaCl_2$. The cells were pelleted again and reuspended in ice cold $CaCl_2$. A 0.2ml. aliquot of the cell suspension was mixed with 0.1-0.5µg. DNA (in TE buffer) and put on ice for 15 min.. These were heat shocked (37^oC, 5 min) and put back on ice for a further 10 min. 0.2ml. L broth was added to the cells and this was incubated at 37^oC for 90 min. to allow expression of antibiotic resistance. A 0.1ml. aliquot of this transformation mix was plated out on selective plates and incubated overnight at 37^oC. Where the plasmid being transformed

expressed resistance to ampicillin the antibiotic expression step was omitted.

(ii) M13 RF DNAs.

The recipient strain was always JM101 and cells were prepared as above until the stage of adding the DNA. When the DNA was added, the cells were left on ice for 2-3 hours, heat shocked at 37°C for 5 min., then returned to ice for a further 15-30 min. This transformation mixture was then added to 2.5ml. molten (46°C) 0.6% agar along with 200ul. log phase JM101 cells (with a density of about 2 x 10^8 cells/ml), 20ul. of a 20mg./ml. X-gal solution and 20ul. of a 20mg./ml. IPTG solution. These were mixed and poured on to a minimal agar plate containing thiamine and glucose. Once the overlay had set the plates were incubated at 37°C overnight.

2.11 Plate matings.

Plasmids of the IncW class like R388 produce fragile, rigid pili and therefore conjugal transfer is more efficient when conjugation occurs on a solid surface than in a liquid culture (Bradley et al, 1980). R388 matings were always carried out on the surface of a L agar plate.

Overnight cultures of donor and recipient cells in L broth were diluted 1 in 20 into 20ml. L broth and grown for 90 min. (to a density of about 2 x 10^8 cells/ml). 5ml. of each were mixed

The transposition frequency was calculated by comparing the number of cointegrates transferred with the total number of plasmid R388 transferred. One plate of each dilution was counted. An overnight culture of E.coli undergoes approximately 25-30 generations, therefore the frequency given is the frequency of transposition per 25-30 generations.

$$\text{Transposition frequency} = \frac{\text{cointegrates transferred/ml.}}{\text{R388 transferred/ml.}}$$

Example

R388 transfer. 159 colonies on 10^{-5} dilution $= 1.59 \times 10^{8}$
Coint.transfer. 69 colonies on 10^{-2} dilution $= 6.90 \times 10^{4}$

$$\text{Transposition frequency} = \frac{6.90 \times 10^{4}}{1.59 \times 10^{8}}$$

$$= 4.3 \times 10^{-4}$$

and pelleted (12,000g, 5 min.) and this was resuspended in 0.5ml. L broth and spread on a well dried L agar plate. This was then incubated at 37°C for 90 min. to allow transfer to occur. The cells were washed off the plate in 10ml. phage buffer, pelleted and resuspended in 1ml. phage buffer. The appropriate dilutions were made and 0.1ml. of each was plated out onto selective media and incubated overnight at 37°C. For Ap selection, the cells were washed twice after removing from the agar plate to ensure that the β-lactamase had been removed from the surrounding medium.

2.12 Plasmid and M13 RF DNA isolation.

(i) Cleared lysate.

250ml. of L broth was inoculated with a plasmid containing strain from an overnight culture. The appropriate antibiotic was added and the culture grown at 37°C for 16-20 hours. The cells were harvested by centrifugation (12,000g, 5 min.) and resuspended in 3.3ml. cold 25% sucrose, 0.05M Tris pH 8.0. 0.67ml. of a freshly made up lysosyme solution (10mg./ml in 0.25M Tris pH 8.0) was added and after mixing, was left on ice for 10 min. 5.3ml. lytic mix was added, gently mixed, and left on ice 20-40 min. till the cells had lysed. The cell debris was pelleted by centrifugation (48,000g, 30 min., 4°C) and the supernatent carefully decanted. RNA was removed by the addition of RNAse (10μg./ml., 15 min., 37°C) and protease K was added (10μg./ml., 15 min., 37°C) to remove proteins. This cleared

lysate was then further purified by CsCl/EtBr equilibrium centrifugation.

(ii) CsCl/EtBr equilibrium centrifugation.

4.83ml. cleared lysate was added to 5g. CsCl, 0.1ml. of 0.2M Na_2HPO_4 and 0.33ml. of 3mg./ml. EtBr in TE buffer. The density of this solution was checked and adjusted to 1.58g./ml. This solution was put in a Beckman 'quick seal' tube, the tube filled with parafin oil and heat sealed. After centrifugation (200,000g, 16hr., 15°C) DNA bands were visualised by long-wave UV light (300-360nm.) and the lower, plasmid, band extracted through the side of the tube with a syringe. EtBr was removed from the DNA by repeated butanol extraction until the butanol layer was colourless. The solution was diluted with 2 volumes TE buffer and the DNA precipitated by the addition of two volumes of ethanol. The DNA was resuspended in 0.2-0.5ml. TE buffer and quantified by running on an agarose gel.

(iii) Alkaline/SDS method.

A modification of the method described by Birnboim and Doly (1979).

A single colony was patched out on a selective plate and incubated overnight. The next day a match-head size lump of cells was removed from the plate, resuspended in 100µl. 50mM Glucose, 25mM Tris pH 8.0, 10mM EDTA and left for 5 min. at room temperature. 200ul. 0.2M NaOH 1% SDS was added and this was left on ice for 5 min. 150µl. pre-cooled 3M KAc/2M acetic acid pH 4.8

was added and the solution left a further 5 min. on ice before spinning for 1 min. (12,000g) to pellet the cell debris and chromosomal DNA. 2 volumes EtOH were added to the supernatent and the mixture left at -20°C for 30 min. to precipicate the DNA. This mix was centrifuged (12,000g, 10 min., 4°C), the supernatent removed and the pellet washed twice with 65% EtOH. The pellet was then dried and resuspended in 45ul. TE buffer and 5µl. of a 1mg./ml. solution of RNAse in TE.

2.13 Gel electrophoresis.

(i) Agarose gels.

Vertical gel kits were used which held two 16cm. x 16cm. glass plates 3mm. apart. Agarose was dissolved in buffer E at 100°C and cooled to 50°C. The gel plates were filled with molten agarose and a 10 or 15 toothed teflon comb was inserted into the top. When the gel had set, the top and bottom reservoirs were filled with enough buffer E to cover the gel, and the comb was removed. Single colony gel supernatants were loaded directly onto the gel, while plasmid DNA and restriction digests were made up to 20ul. with TE buffer (if necessary) and 5ul. of DNA final sample buffer before loading. Gels were run at constant voltage - 25 Volts for 16 hours or 90 Volts for 4-5 hours. Gels were stained by soaking in EtBr (0.5µg./ml.) for 30 min. and then viewed on a 260nm. UV transilluminator. Gels were photographed on Ilford HP5 film using a 35mm camera fitted with a red filter.

Interpretation of gels was based on Dugaiczyk et al, 1975. The fastest moving band was the supercoiled monomeric plasmid, which was usually the most abundant species. Behind this was another band containing monomeric open circle and supercoiled dimeric plasmid. Open circle dimers and other higher forms ran behind this but were not always visible. Plasmid linears could sometimes be seen running between the monomer supercoils and dimers. Sheared fragments of chromosomal DNA ran as a thick band towards the top of the gel.

0.8% agarose was used for single colony gels while for the analysis of restriction enzyme digests 1.0% agarose was used.

(ii) Polyacrylamide gels

Polyacrylamide gels were used to separate fragments smaller than 1Kb. in size. 5% acrylamide gels were used asthese give good separation over a size range of 80-500bp. The gel was prepared by mixing 10ml. 20% acrylamide/1% bisacrylamide, 4ml. 10 x TBE buffer, 25ml. distilled water, 480µl. 10% $(NH_4)2S_2O_8$, 240µl. 10% TEMED. This acrylamide solution was poured into vertical gel kits (16cm. x 16cm. x 1.5mm.) which were first sealed using 0.8-1.0% agarose. A 12 tooth teflon comb was inserted and the gel was allowed to polymerise for 1 hour at room temperature. The comb was removed and the top and bottom reservoirs filled with 1 x TBE buffer. The wells were washed out to remove any unpolymerised acrylamide and the samples (10-25µl.) loaded and the gel run at a constant current of 20mA for 2-3 hours. Staining and photography were as described for agarose

gels.

2.14 Single colony analysis

Single colony analysis is a rapid method of analysing plasmid DNA. Using a toothpick, a mass of cells ($0.5cm^2$ from an area of confluent growth) was removed from the plate and resuspended in 150µl. of single colony final sample buffer (SCFSB) in a small (0.6ml.) microfuge tube. The cells were spun at 12,000g (15 min, 4°C) and 50µl. of the supernatent was loaded onto an agarose gel.

2.15 Restriction enzyme digests.

2-10µl. of a DNA solution was mixed with 2µl 10 x restriction enzyme buffer, 0.1-1µl. restriction enzyme (1-2 units/µg. DNA), and made up to 20µl. with a 100µg./ml. gelatin in TE solution. This was mixed and incubated for 1-3 hours at 37°C (or 67°C for *TaqI*). If the digest was to be run on a gel, 5µl FSB was added which stoped the digestion and the sample was loaded onto a gel. If the DNA was to be subject to any further manipulation, the enzyme was inactivated by phenol extraction and the DNA recovered by ethanol precipitation.

The restriction buffers used were:

(i) Low salt: HaeIII, HpaII, TaqI.

(ii) Medium salt: BamHI, ClaI, HhaI, HincII, HindIII, HinfI, PstI, PvuII, Sau3A.

(iii) High salt: EcoRI

AhaIII was used in its own buffer.

2.16 Phenol extraction and precipitation of DNA.

(i) Phenol extraction.

An equal volume of distilled phenol (saturated with 1M Tris HCl, pH 8.0) was added to the solution to be extracted, mixed thoroughly mixed and the two phases resolved by centrifugation (12,000, 5 min., 4°C).

the aqueous phase was carefully removed and extracted once or twice again. 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 DNA.

This was done with either ethanol or isopropanol.

(a) Ethanol: 0.1 volumes of 3M NaAc and 2 volumes of ethanol were added to the solution to be precipitated. This was mixed and left at -20°C for 1-2 hours.

(b) Isopropanol: 0.1 volumes 3M NaAc and 0.54 volumes of isopropanol were added. This was mixed and left at room

temperature for 1-2 hours.

In either case the DNA was spun down (27,000g, 20 min., 4°C), washed in 1-3ml. 70% ethanol in TE, re-spun (27,000g, 20 min., 4°C), and the pellet dried under vacuum. The DNA was resuspended in an appropriate volume of 1 x TE.

2.17 Ligation of DNA fragments.

Fragments to be ligated were resuspended together in 16µl. of TE buffer, 2µl 10 x ligation buffer and 2µl. 4mM ATP (in 4mM Tris HCl, pH 7.5). T4 DNA ligase was added (0.01 units/µg. DNA for 'sticky' ends, 1 unit/µg. DNA for flush ends), the solution mixed and incubated overnight at 18°C. The ligation mixture was used diluted 1 in 10 and 1 in 20 to transform competent cells.

2.18 BAL 31

BAL 31 is a nuclease which simultaneously degrades the 3' and 5' ends of duplex DNA. It can be used to create deletions of varying lengths in DNA. 2.5µg. restricted DNA was resuspended in 40µl. H_2O and 10µl. 5 x BAL 31 buffer. 0.5µl. BAL 31 was added and the solution incubated at 30°C for 1 min. The reaction was stopped by the addition of EDTA to a final concentration of 30mM. The enzyme was removed by phenol extraction and the DNA precipitated with ethanol. The DNA could then be ligated.

2.19 DNA Sequencing.

In this work the Sanger method of chain terminating sequencing was used (Sanger et al, 1977). The book 'M13 Cloning and Sequencing Handbook', published by Amersham International plc, gives a more detailed account of the theory and practice of sequencing and was the principal source of reference for this work.

(i) Preparation of single-stranded template DNA.

20ml. of 2-YT medium was inoculated with 200ul. of an overnight culture of JM101 cells and 1.5ml. aliquots of this mixture dispenced into 10ml. cultute tubes. Each tube was inoculated with a single M13 plaque and grown at 37°C for 16 hours with vigorous shaking. The cells (containing all chromosomal, plasmid and M13 RF DNAs) were removed by centrifugation (12,000g, 5 min., 4°C), the supernatent removed and re-centrifuged to remove all traces of host cells. 0.8ml. of this supernatent was added to 200ul. of 2.5M NaCl/20% PEG solution in a large microfuge tube and allowed to stand on ice for 1 hour to precipitate viral particles. The solution was centrifuged (12,000g, 15 min., 4°C), the supernatent discarded, the remaining material re-centrifuged and all traces of supernatent removed, leaving only the white viral pellet. The pellet was resuspended in 100µl. TE buffer (pH 8.0) and this suspension phenol extracted twice, chloroform extracted once, and the DNA recovered by ethanol precipitation at -20°C. The DNA was

spun down, washed in 70% EtOH, and dried under vacuum before being resuspended in 100µl. TE buffer and the phenol and chloroform extractions repeated. After being spun down, washed and dried again, the DNA was resuspended in 30µl. TE buffer and was ready for sequencing.

(ii) Annealing of primer to template.

5µl. single-stranded template DNA, 1µl (2ng.) M13 universal primer, 1.5µl. 10 x Klenow buffer and 2.5µl. distilled water were mixed together, incubated at 60°C for 30-60 min and then allowed to cool slowly (over a period of 2-3 hours) to room temperature. This provided sufficient material for the four sequencing reactions for each clone.

(iii) Dideoxy sequencing reactions.

All solutions were made up in distilled water and kept frozen at -20°C. Stock solutions at a concentration of 10mM of all four dNTPs were prepared and these used to make up working solutions at a concentration of 0.5mM. A cold chase solution was prepared which was a uniform mix of all four dNTPs, each at a concentration of 0.5mM. dNTP (N^{o}) mixes were then prepared from the working solutions as shown below.

	A°	C°	G°	T°
0.5mM dCTP.	20µl.	1.0µl.	20µl.	20µl.
0.5mM dGTP.	20µl.	20µl.	1.0µl.	20µl.
0.5mM dTTP.	20µl.	20µl.	20µl.	1.0µl.
1x TE buffer.	20µl.	20µl.	20µl.	20µl.

Stock solutions, also at a concentration of 10mM, of all four ddNTPs were prepared and used to make up the working solutions shown below.

ddATP	0.025mM
ddTTP	0.50mM
ddGTP	0.15mM
ddCTP	0.025mM

Equal volumes of each N° mix and the corresponding ddNTP working solution were mixed to produce the N°/ddNTP reaction mix.

The radio-labelled nucleotide used was a-^{35}S-dATP of specific activity 1000-13000 Ci/mmole. The concentration of the solution supplied varied from 7.9-9.22µm. and this was adjusted by the addition of an appropriate volume of a 100µm. cold dATPaS solution to give a final overall concentration of 16µM dATPaS.

1.5µl. of this solution was used, so that each reaction contained 7.60-8.87pmoles (8.07-11.54µCi) of a-^{35}S-dATPaS

For the sequencing reactions, 1.5µl. of the above labelled nucleotide mix was added to the annealed primer/template mix and 1µl. of Klenow enzyme solution (1 unit/µl.). This was mixed by pipetting in and out, and a 2.8µl. aliquot was placed into each of four small microfuge tubes labelled A, T, G and C. These each contained 2µl. of the appropriate N^{o}/ddATP reaction mix. Again this was mixed by pipetting in and out. The reaction was allowed to proceed for twenty min. at room temperature before 2µl. of the chase mix was added. This was mixed and allowed to stand for a further 15 min. at room temperature. The reaction was stopped by the addition of 4µl. of formamide dye mix to each tube. Samples were then prepared for loading onto a gel or stored at -20^{o}C until required.

(iv) High resolution polyacrylamide gel electrophoresis.

The gel aparatus used consisted of two 45 x 23cm. glass plates held apart by 0.4mm. thick 'Plasticard' spacers along each long side and sealed with vinyl tape. Combs were also cut from 0.4mm. thick 'Plasticard'.

For a 50ml. 6% gel, the following components were mixed together: 21g. 'Ultrapure' urea, 5ml. 10 x TBE buffer, 7.5ml. 38% acrylamide/2% NN' bis-methylene acrylamide (deionised by the addition of 5g. of 'Amberlite' MB1 resin and stirring at room temperature for 30 min., followed by filtration to remove the resin) and distilled water up to a total volume of 50ml. These

were stirred together till the urea was dissolved, 300µl. of 10% APS and 50µl. of TEMED mixed in, the gel poured, the comb inserted, the plates clamped together firmly and polymerisation allowed to proceed to completion (1-2 hours at room temperature.).

The gel was installed in the running aparatus, the reservoirs filled with TBE buffer, the comb removed and the wells washed out with buffer to remove and unpolymerised acrylamide. The gel was pre-run for 30-60 min. and just before loading the wells were again washed out to remove any urea that may have leached into them.

The samples were heated to 90-95°C for 3 min. and 3-4µl. of each was loaded immediately onto the gel. The gel was run at constant power (40 Watts) for 2 or 4 hours. After running, the gel was fixed in a 10% acetic acid (v/v) solution for 10-15 min. at room temperature, carefully transferred to Whatman 3mM. filter paper and dried down under vacuum. Bands were visualised by autoradiography of a sheet of Kodak 'X-Omat' S1 film for 1-4 days at room temperature.

CHAPTER 3

CONSTRUCTION AND ANALYSIS OF PLASMIDS

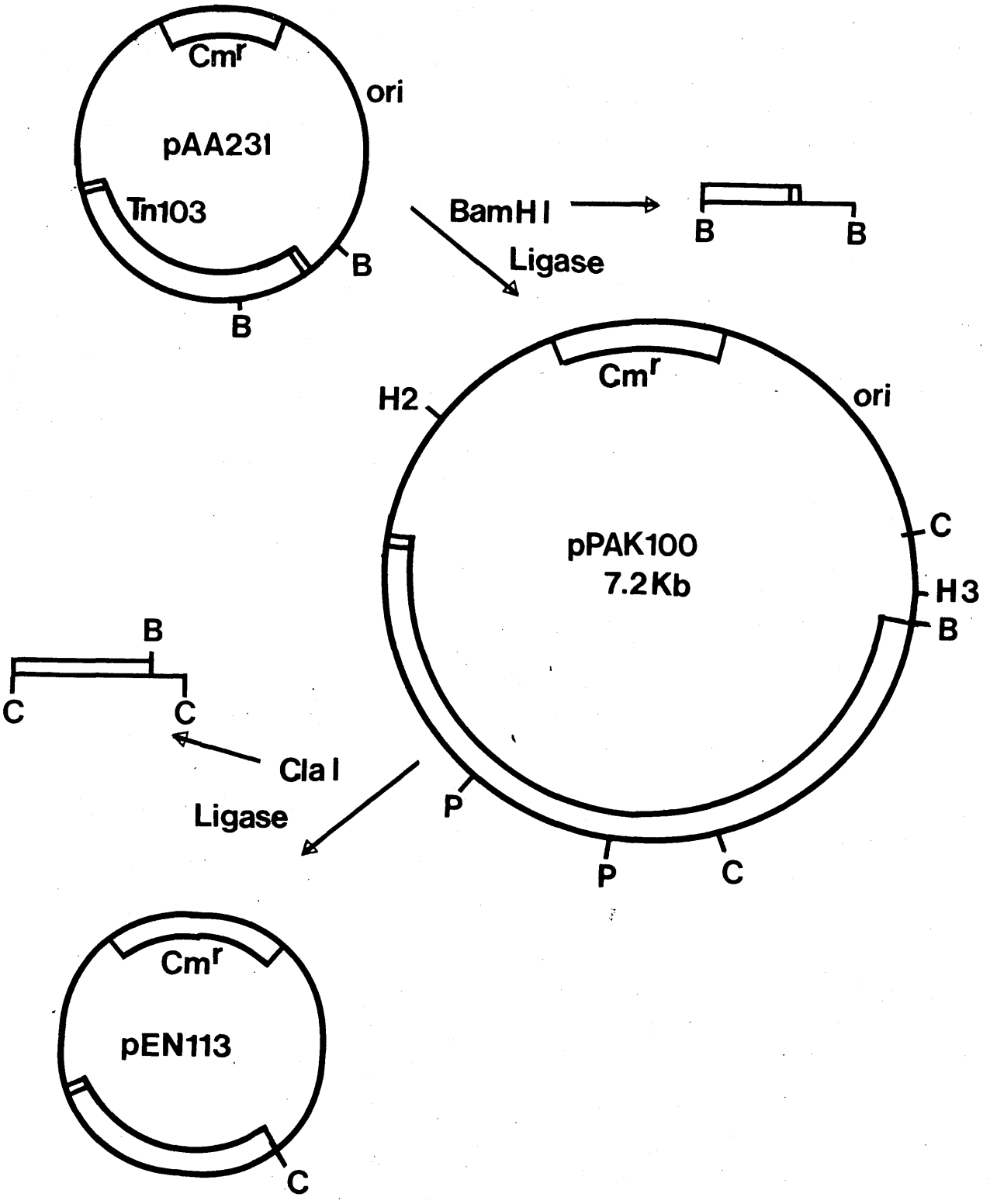

Figure 3.1.

Structure and derivation of plasmids pPAK100 and pEN113.

B BamHI
C ClaI
H3 HindIII
P PstI
H2 HincII

3.1. INTRODUCTION

This chapter describes the construction and analysis of plasmids used to study the transposition and immune properties of one-ended transposons. The two main plasmids used in this study are pBR322 and pPAK100. These are both small, high copy number plasmids which contain the left or the right half of a transposon.

3.2. CONSTRUCTION OF tnpA⁻ DERIVATIVE OF pPAK100.

The plasmid pPAK100 is 7.2Kbp. and is derived from pACYC184::Tn103, it confers resistance to Cm. It contains the left 3.65Kb. of Tn1, which includes the left inverted repeat, the tnpA gene, the res site, and about 360bp. of the tnpR gene. Its construction is shown in Figure 3.1, and is described fully in Kitts (PhD Thesis). It is a small, non-conjugative, non-mobilisable plasmid.

A tnpA⁻ deletion derivative of pPAK100 was constructed by digesting with ClaI, which gives two fragments, and re-ligating the larger fragment. This is shown in Figure 3.1. Purification of the larger fragment was not necessary since a simple recircularisation was required. The ligated plasmid was transformed into strain DS902. Transformants were analysed by gel electrophoresis (Figure 3.2), and one colony which appeared to be smaller than pPAK100 was selected and analysed by digestion

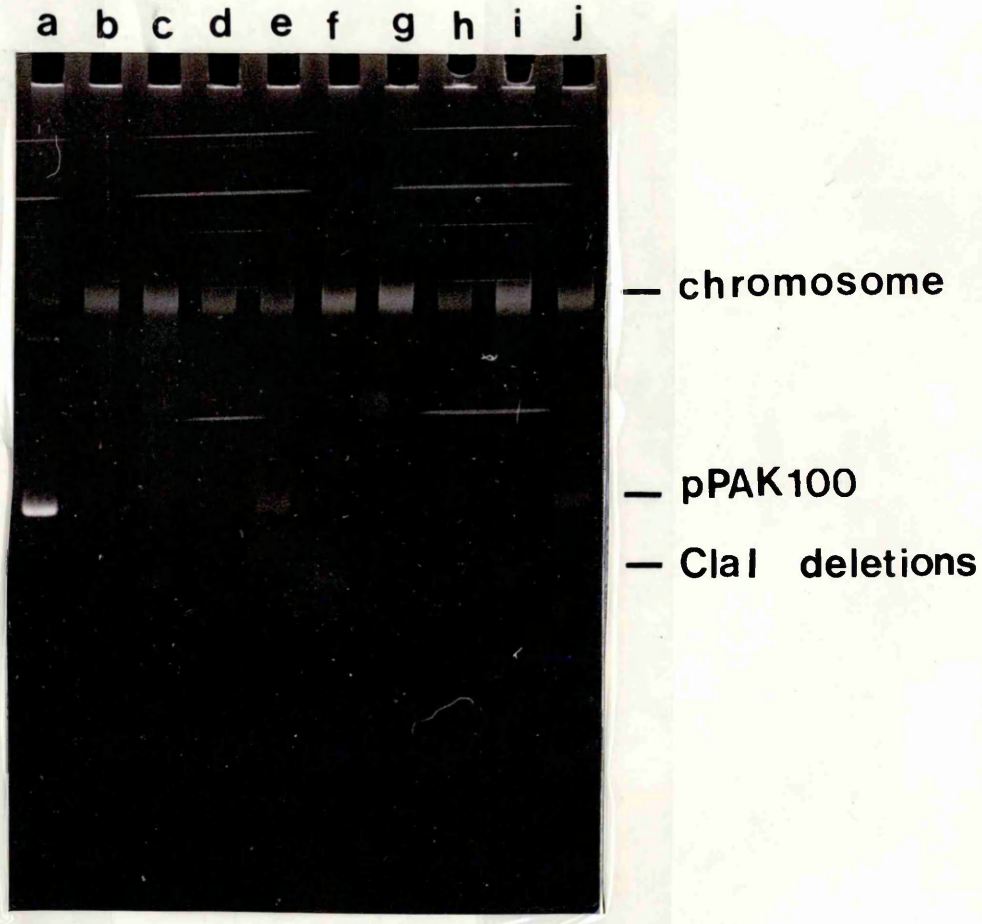

Figure 3.2.

Single colony gel of ClaI deletion mutants of pPAK100.

Lane a. pPAK100 standard.
Lanes b. to j. Presumptive deletion derivatives.

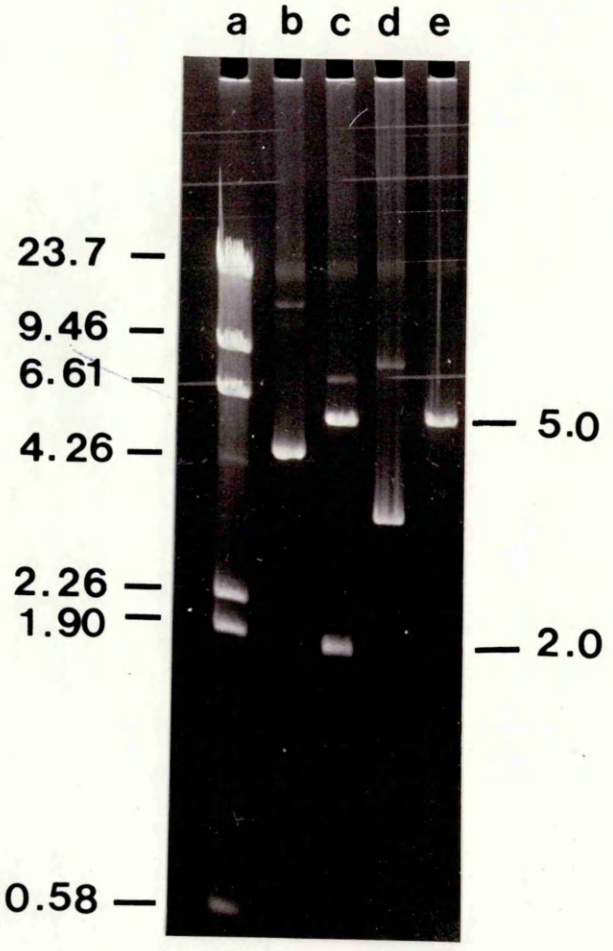

Figure 3.3.

Restriction digests of pPAK100 and pEN113.

Lane a. Lambda HindIII size markers.
Lane b. Uncut pPAK100.
Lane c. pPAK100 cut with ClaI.
Lane d. Uncut pEN113.
Lane e. pEN113 cut with ClaI.

with ClaI. Figure 3.3 shows pPAK100 and the deletion derivative (pEN113) digested with ClaI. Digestion of pPAK100 with ClaI gives bands of 5Kb. and 2Kb. while digestion of pEN113 with ClaI gives only one band of 5Kbp.. The plasmid pEN113 contains 2.0Kb. of Tn1, comprising of the left inverted repeat and 1967bp. of tnpA. This does not now produce a functional transposase since much of the tnpA gene is not present. The plasmid pEN113 was used in the following chapters on the transposition and immunity of 'one-ended' transposons.

3.3. CONSTRUCTION OF DELETION DERIVATIVES OF pBR322.

The plasmid pBR322 is 4363bp. in length, it confers resistance to Ap and Tc, and is present at a high copy number in cells. It contains the B-lactamase gene, giving resistance to Ap, from Tn3; the Tc resistance gene from pSC101; and the origin of replication of pMB1. (For details of construction see Bolivar et al 1977). It is very useful because it has been completely sequenced (Sutcliffe 1979) and therefore all the restriction sites are known (Figure 3.4). pBR322 contains the right 1.26Kb. of Tn3 which consists of the right inverted repeat, the B-lactamase gene, and 27bp. of the tnpR gene. It does not contain any tnpA sequences.

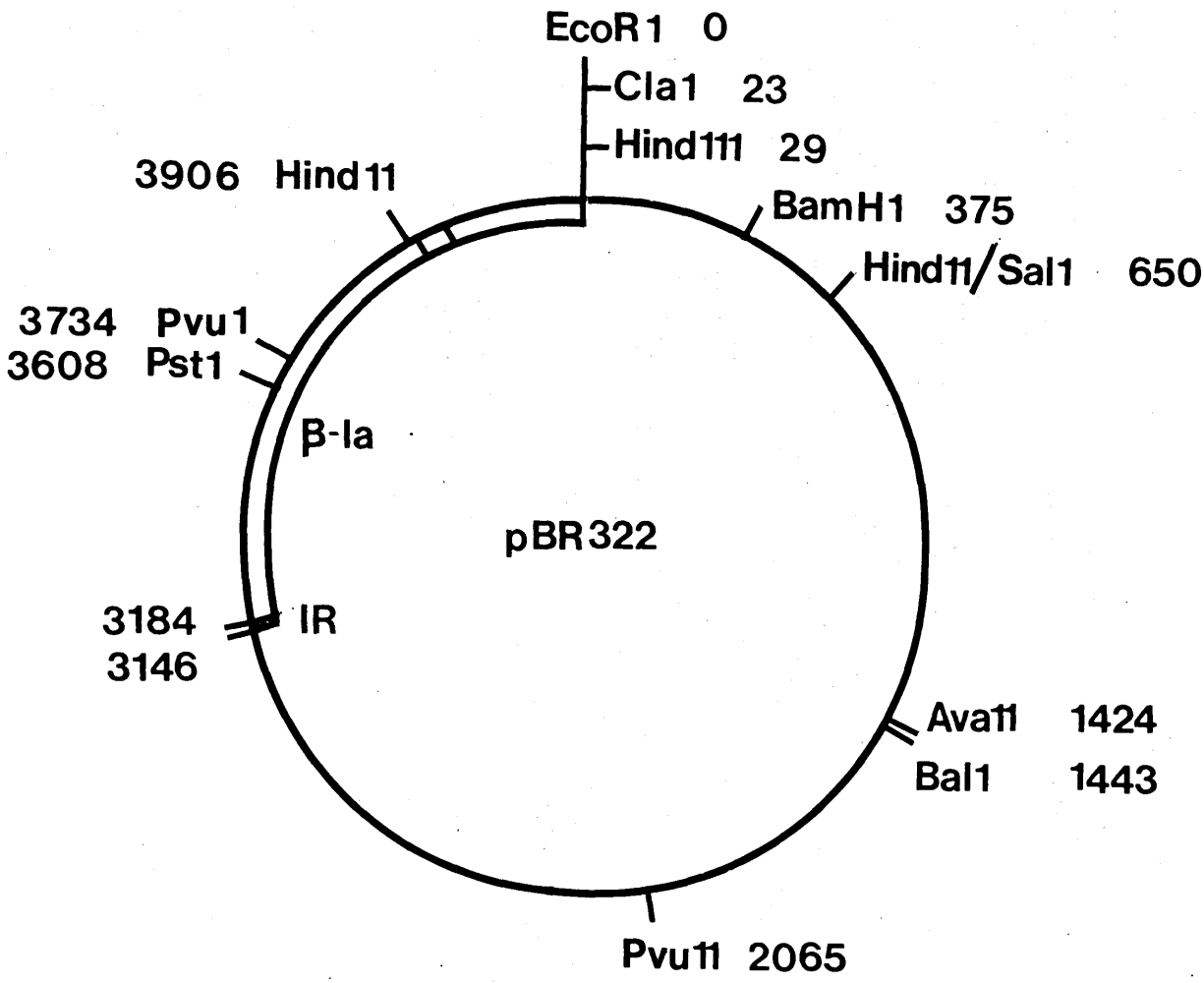

Figure 3.4(a).

(a) Restriction map of pBR322 showing unique sites and their positions.

Transposon sequences (▭) are shown along with the inverted repeat (▯).

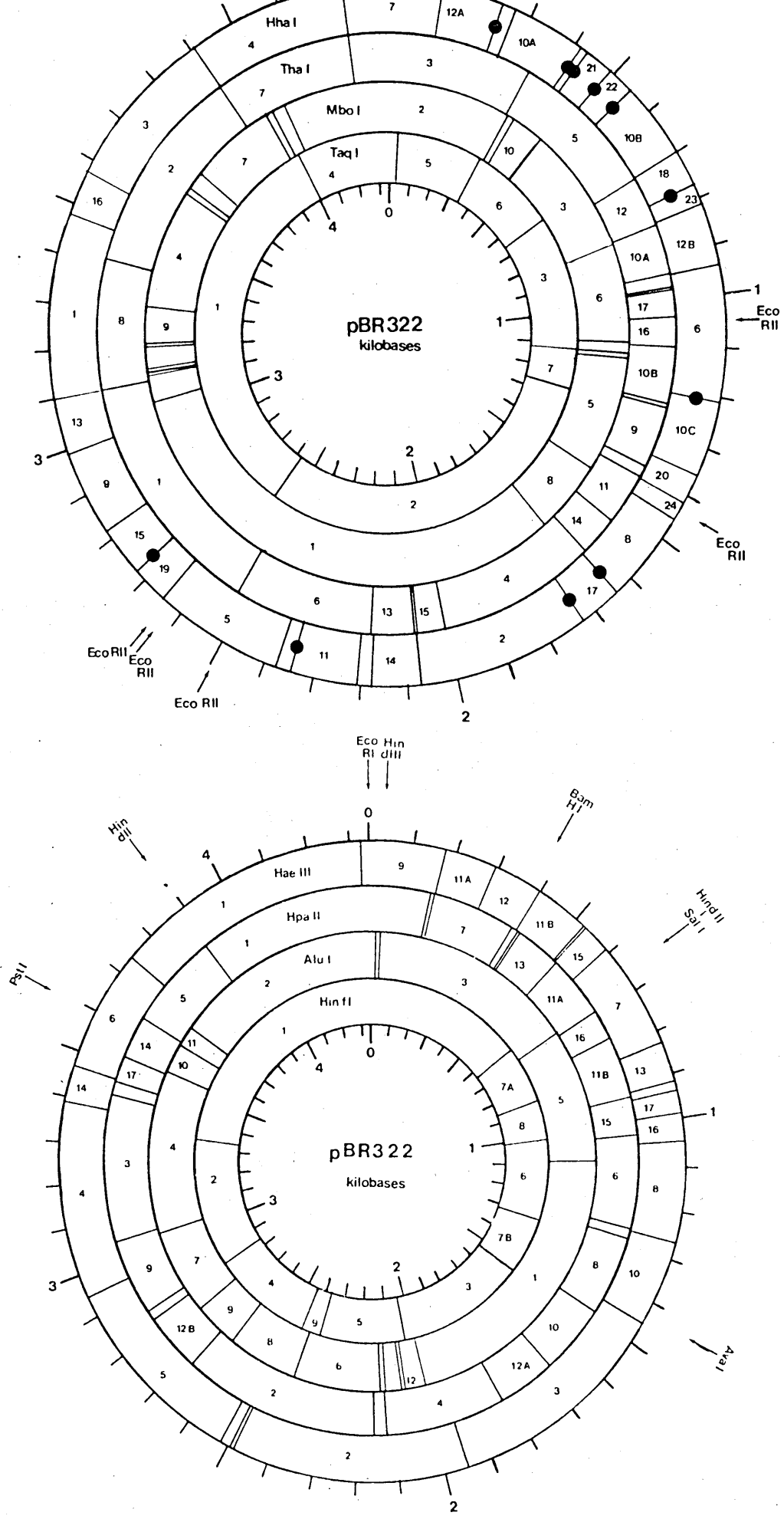

Figure 3.4(b).

(b) Restriction maps of pBR322 showing frequently

The results of chapter 4 show that pBR322 shows immunity to accepting further copies of Tn1/3, while the results of chapter 5 show that it is able to transpose when complemented with a plasmid producing transposase. To study the immune and transposition properties more closely, deletion mutants which approached the inverted repeats of pBR322 were constructed. These were then analysed for immunity and transposition.

3.3.1 CONSTRUCTION OF pEN200 AND pEN201

From studying the available maps of pBR322, it seemed that there were no suitable restriction sites that would delete parts of the transposon, but leave the rest of the plasmid intact. Figure 3.4 shows the unique sites in pBR322. Since there is a unique *Pst*1 site in the B-lactamase gene, it was decided to cut with *Pst*1, and then use BAL 31 to remove transposon sequences. BAL 31 is an exonuclease which is able to 'chew back' both strands of double stranded DNA, producing deletions of varying sizes depending on the time the reaction is left. 2μg of pBR322 DNA was linearised with *Pst*1 and then digested with BAL 31. The DNA was phenol extracted, ethanol precipitated, and resuspended in 20 μg 1xTE. This was ligated overnight at 16°C and then the ligation mix was diluted 1 in 20 and transformed into DS902. Colonies that grew on Sm Tc plates were screened for Ap sensitivity. Those that were sensitive to Ap were analysed by agarose gel electrophoresis. Two colonies appeared to be Ap

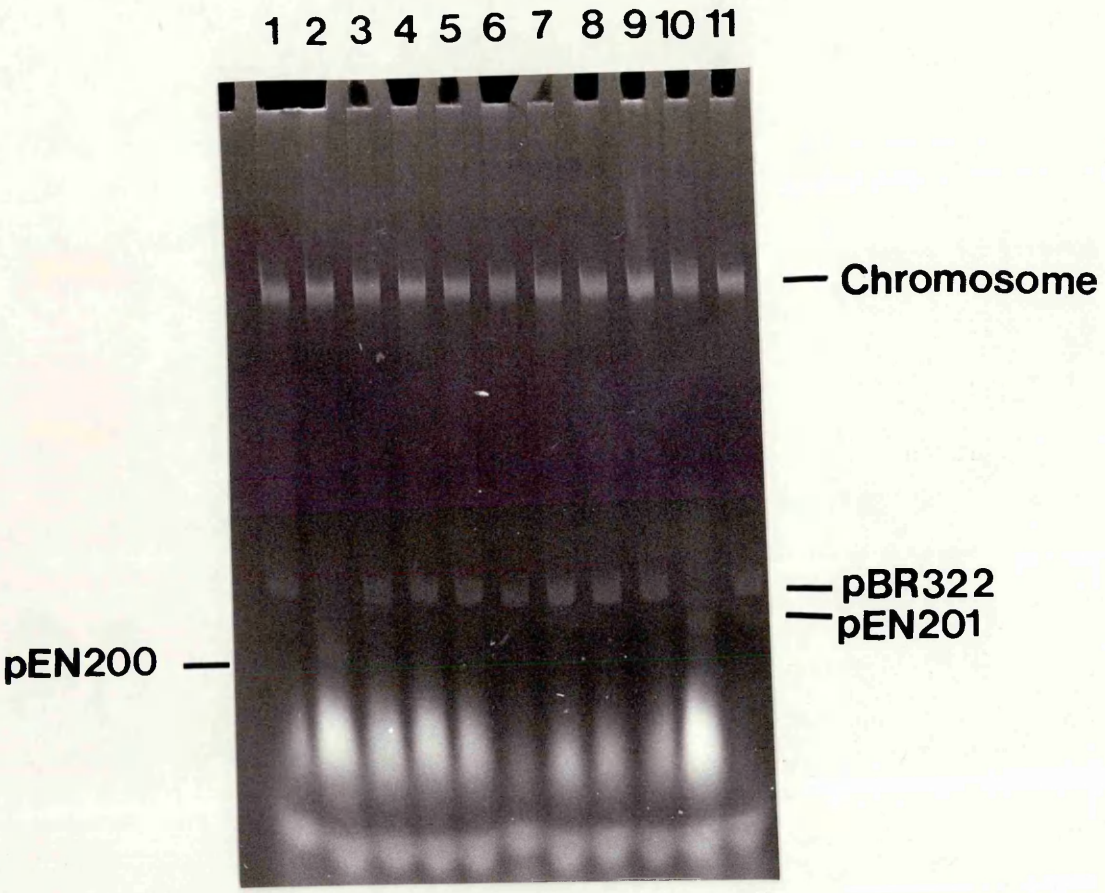

Figure 3.5.

Single colony gel of Bal 31 deletion derivatives of pBR322.

Lanes 1, 3, 4, 5, 6, 7, 8, 9and 11. pBR322 or small deletions.
Lane 2. pEN200.
Lane 10. pEN201.

sensitive and smaller than pBR322, these were picked and analysed in detail,(Figure 3.5).These are named pEN200 and pEN201.

Since BAL 31 digests each strand independently, only a fraction of DNA fragments will have blunt ends, which are suitable for ligation. The rest will be a mixture of 3' and 5' non homologous extensions. This probably explains the the fact that only two deletions were picked up after Bal 31 digestion. A greater number of deletions may have been isolated if, after digesting with BAL 31, any single stranded ends had been filled in, or if they had been removed with the nuclease S1.

3.4. ANALYSIS OF pEN200

An estimation of the size, and the end points of the deletions was made by studying the restiction data from the two plasmids. The plasmids were cut with frequently cutting enzymes, and using the restriction data of Sutcliffe (1978),(Figure 3.4) the end points of the deletions were mapped to within 100bp.

Figure 3.6 shows HinfI, HaeIII, TaqI, and HpaII digests of pEN200. The plasmid pBR322 has 10 HinfI fragments, and digestion of pEN200 with HinfI shows fragments 1 and 2 are missing. This indicates that the outer limits of the deletion are from 2845bp. to 631bp. on the pBR322 map. A new junction fragment of approximately 1200bp. is present, implying that the deletion is

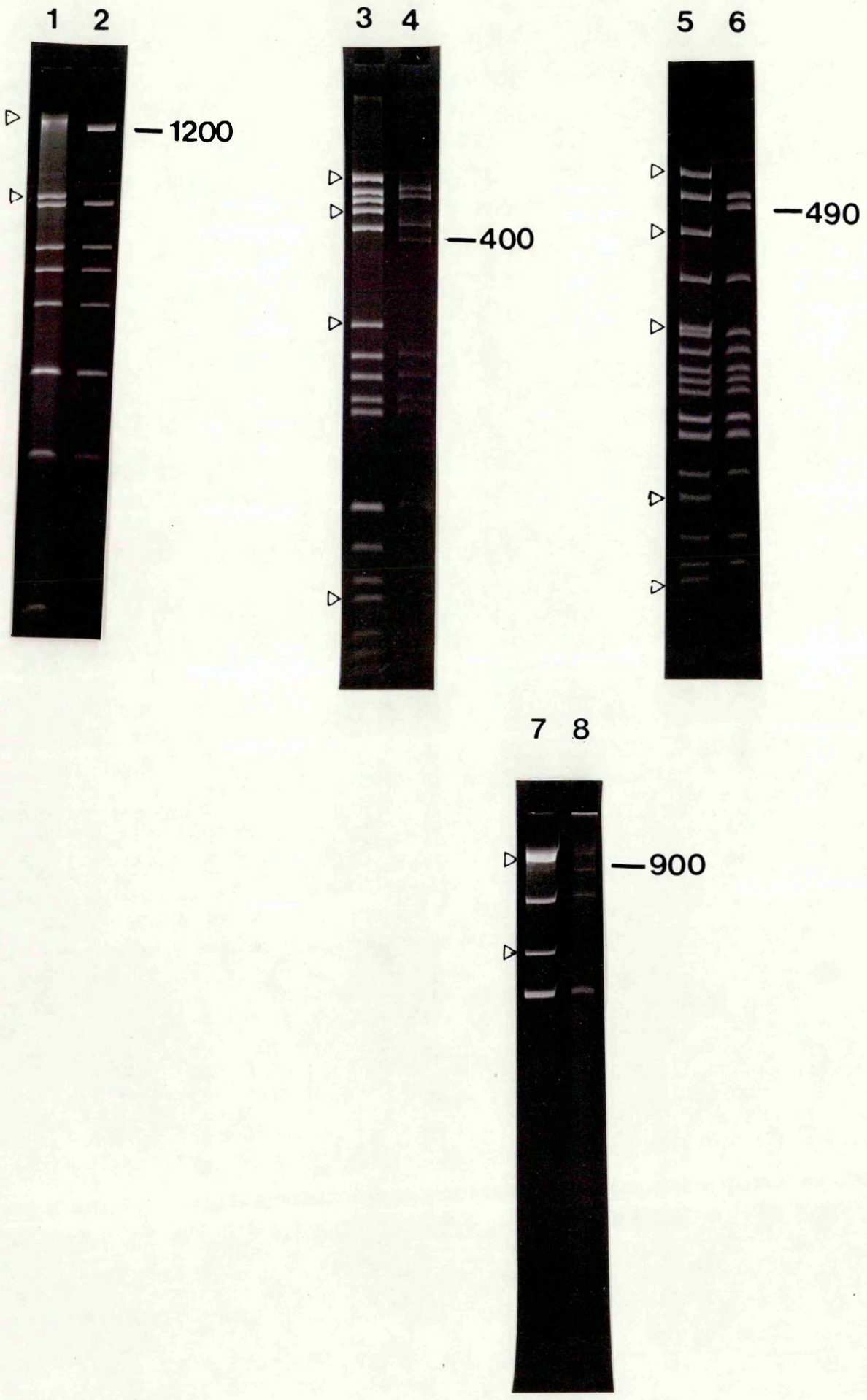
1
2
1200
3
4
400
5
6
490
7
8
900

Figure 3.6.

Comparison of digests of pEN200 and pBR322.
Missing bands are marked (▷), new bands (—), and the approximate size of the new band is shown at the side.

Lane 1. pBR322 HinfI.
Lane 2. pEN200 "
Lane 3. pBR322 HaeIII.
Lane 4. pEN200 "
Lane 5. pBR322 HpaII.
Lane 6. pEN200 "
Lane 7. pBR322 TaqI
Lane 8. pEN200 "

FIGURE 3.7 SUMMARY OF INFORMATION DERIVED FROM RESTRICTION ENZYME DIGESTS OF pEN200

ENZYME	MISSING FRAGMENT No.	SIZE OF NEW FRAG.	SIZE OF DELETION	DELETION ENDPOINTS	
HinfI	1, 2	1,200bp	950bp	2845-3362	3362-631
HaeIII	1, 4, 6, 14	400bp	910bp	2952-3410	3757-4344
HpaII	1, 3, 5, 14, 17, 18	490bp	955bp	3044-3448	3901-161
TaqI	1, 4	900bp	910bp	2573-4017	4017-23

The deletion extends from between 3044bp and 3410bp, to between 3901bp and 4344bp.

Maximum possible size of deletion (from information above) 1300bp
Minimum possible size of deletion (from information above) 489bp

approximately 950bp. Figure 3.7 gives a summary of all the restriction enzyme information on pEN200.

The HaeIII digest of pEN200 reveals that bands 1, 4, 6, and 14 are missing. The new band appearing is approximately 400bp., indicating that the deletion is about 910bp. The missing bands imply that the deletion starts between 2952bp. and 3410bp., and ends between 3757bp. and 4344bp.

The HpaII digest of pEN200 shows the loss of bands 1, 3, 5, 14, and 17. From the restriction map, this infers that band 18 is also missing since it lies between bands 3 and 17. The new band present is approximately 490bp., implying that the deletion is about 955bp. long. The missing fragments indicate that the deletion starts between 3044bp. and 3448bp. and ends between 3901bp. and 161bp.

The TaqI digest of pEN200 shows the loss of bands 1 and 4, and the appearance of a new band of 900bp., indicating that the deletion is 910bp. One of the TaqI sites is methylated, so bands 3, (475bp.), and 7, (141bp.), appear as one band of 616bp. The missing bands reveal that the deletion does not start before 2573bp. or end after 23bp.

If the summarised information in Figure 3.7 is analysed, the deletion appears to be approximately 934bp. long and it starts between 3044bp. and 3410bp., and ends between 3901bp. and 4344bp.

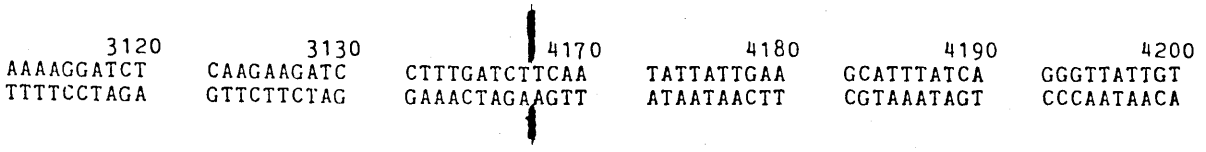

Figure 3.8.

Sequence around the ends of the deletion mutant pEN200. Deletion is 1028bp. long (Nucleotide numbers from Sutcliffe, 1979).

3.5. SEQUENCING OF pEN200

The restriction enzyme analysis of pEN200 gave an approximate idea of the ends of the deletion. However, since it was found that pEN200 had lost the ability to transpose, or to show immunity to further transposition, it was necessary to sequence it to tell whether or not the inverted repeat had been removed by the BAL 31. It was sequenced using the dideoxy sequencing method (Sanger *et al*, 1977). To clone the required fragment into the vector M13, pEN113 and M13 were linearised with *Eco*R1, and the enzyme was then inactivated by phenol extraction. The two digestions were then mixed and ligated together overnight at 16°C. The ligation mix was diluted 1:10 and 10µl of this was transformed into JM101. White plaques were picked and single stranded DNA was made of several of these. This DNA was sequenced and the deletion was found to be 1028bp., extending from co-ordinate 3139, to co-ordinate 4167 on the pBR322 map. This deletion completly removes the inverted repeat and the *B-la* gene, leaving only 196bp. of Tn3 sequence. The sequence around the ends of the deletion is shown in figure 3.8. and a restriction map of pEN200 is shown in figure 3.9.

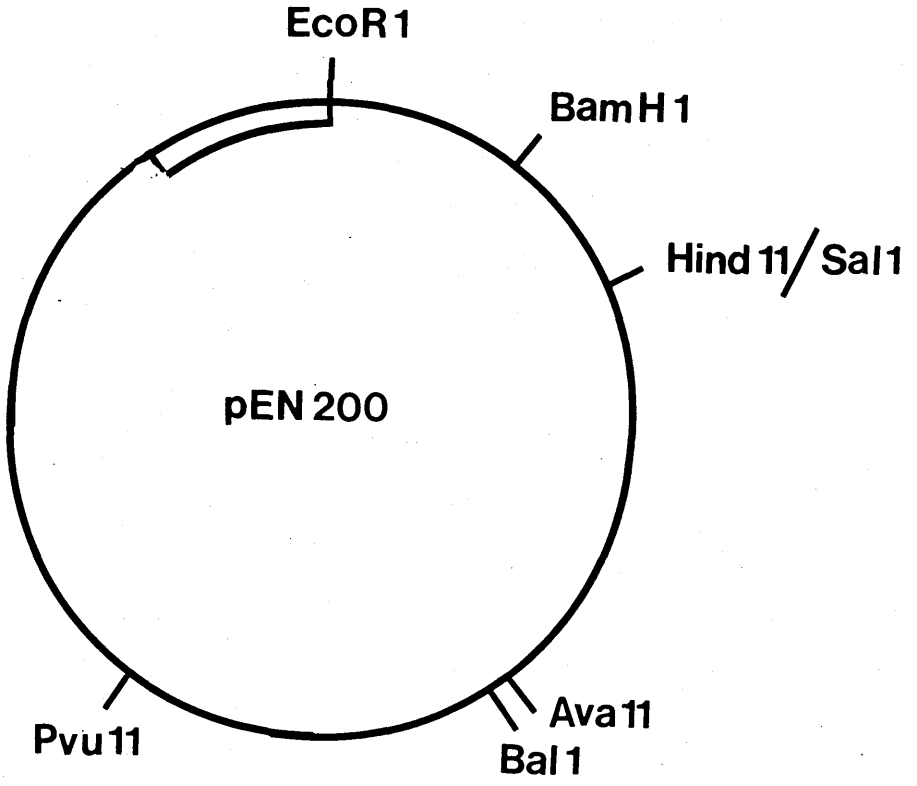

Figure 3.9.

Map of pEN200 showing unique restriction enzyme sites. The remaining transposon sequences are shown (▭).

3.6. ANALYSIS OF pEN201

The plasmid pEN201 was analysed, like pEN200, by restricting with enzymes that cut frequently. The enzymes used were HinfI, HaeIII, HpaII, TaqI, and Sau3A, and this enabled the endpoints of the deletion to be mapped to within 100bp. The gels of these restrictions are shown in Figure 3.10 , and the information gained from them is summarised in Figure 3.11.

The HinfI digest of pEN201 reveals the loss of bands 1 and 2, and the appearance of a new band of about 1600bp.. The loss of these bands and the appearance of the new band implies that the deletion is about 550bp. It starts after 2845bp. and ends before 631bp. on the pBR322 map.

The HaeIII digest shows the loss of bands 1, 4, and 6, and from the pBR322 map, band 14 must also be missing as it is bounded by bands 4 and 6. A new band of approximately 820bp. can be seen and from this, the size of the deletion can be estimated to be about 580bp. The deletion must start between 2957bp. and 3410bp., and end between 3757bp. and 4344bp.

The HpaII digest reveals the loss of bands 1, 3, 5, and 14. From the pBR322 map, bands 17 and 18 must also be missing as they are bounded by bands 3 and 14. The new band appears to be about 740bp., implying that the deletion is approximately 740bp., starting between 3044bp. and 3448bp., and ending between 3901bp.

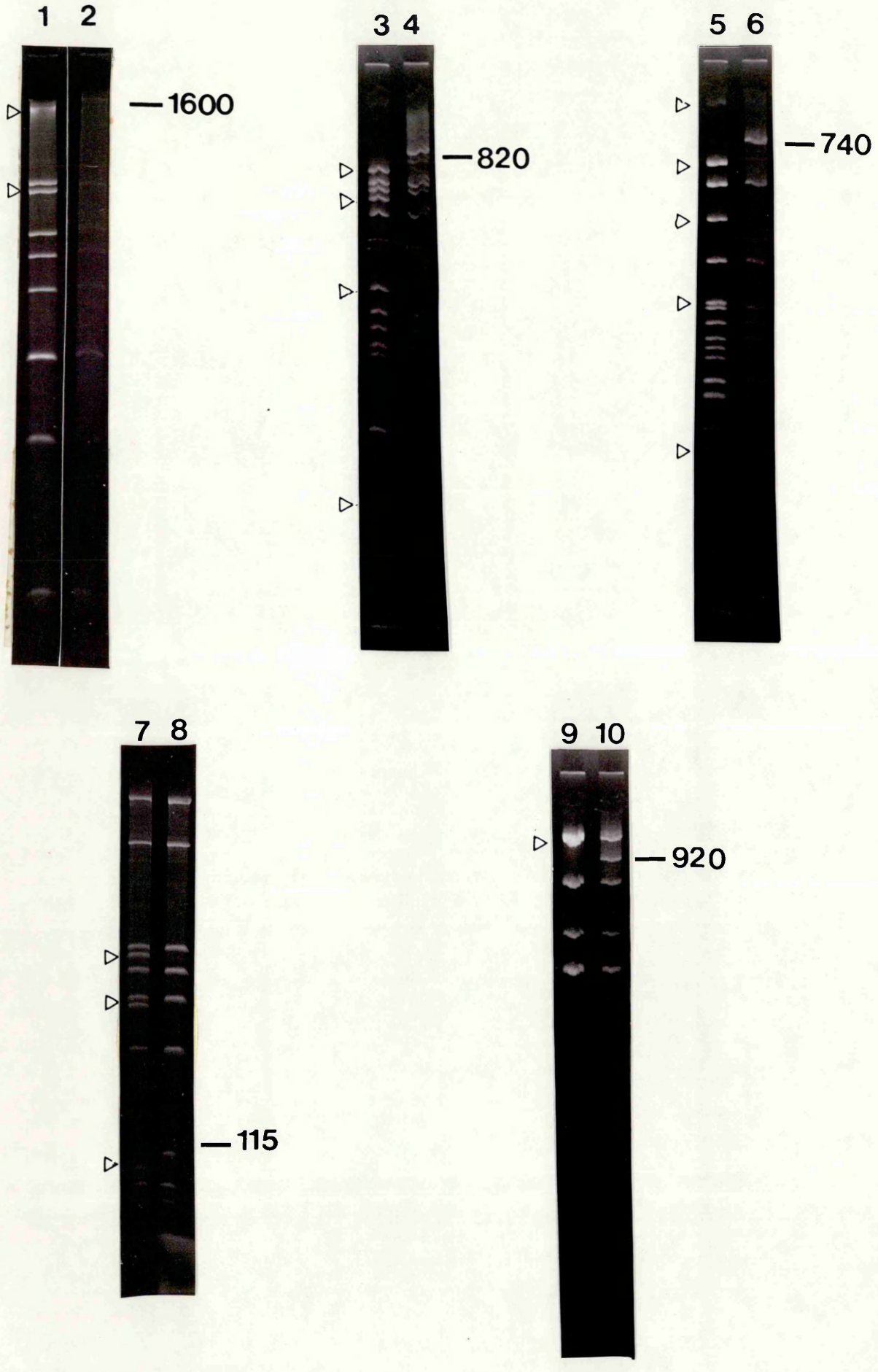
1 2
1600
3 4
820
5 6
740
7 8
115
9 10
920

Figure 3.10.

Comparison of digests of pEN201 and pBR322
Missing bands are indicated (▷) and new bands (—). The approximate size of the new bands is shown at the side.

Lane 1.	pBR322	HinfI
Lane 2.	pEN201	"
Lane 3.	pBR322	HaeIII
Lane 4.	pEN201	"
Lane 5.	pBR322	HpaII
Lane 6.	pEN201	"
Lane 7.	pBR322	Sau3A
Lane 8.	pEN201	"
Lane 9.	pBR322	TaqI
Lane 10.	pEN201	"

FIGURE 3.11 SUMMARY OF RESTRICTION ENZYME DATA ON pEN201

ENZYME	MISSING FRAGMENT No.	SIZE OF NEW FRAG.	SIZE OF DELETION	DELETION ENDPOINTS	
HinfI	1, 2	1,600bp	550bp	2845-3362	3362-631
HaeIII	1, 4, 6, 14	820bp	580bp	2952-3410	3757-4344
HpaII	1, 3, 5, 14, 17, 18	740bp	740bp	3044-3448	3901-161
Sau3A	4, 7, 9, 13, 17,(14, 18, 20)	115bp	720bp	3212-3329	3734-4045
TaqI	1	920bp	525bp	2573-4017	2573-4017

The deletion extends from between 3212bp and 3329bp, to between 3901bp and 4017bp

Maximum possible size of deletion (from information above) 805bp
Minimum possible size of deletion (from information above) 572bp

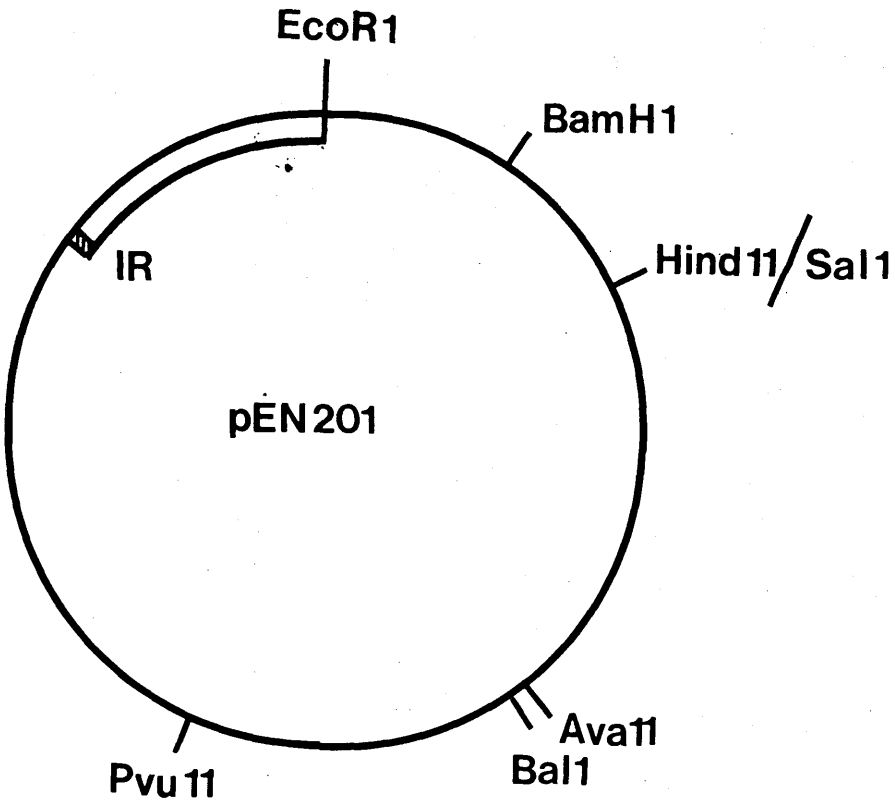

Figure 3.12.

Map of pEN201 with unique restriction sites shown. Transposon sequences (▭) are shown along with the inverted repeat (▨).

and 161bp.

The TaqI digest shows the loss of only one band, 1 (1444bp.), and the appearance of a new band of about 920bp., indicating that the deletion is about 525bp. long. Its endpoints must be between 2573bp. and 4017bp.

In the Sau3A digest (marked MboI in fig.3.4b) bands 4, 7 and 9 are missing, and from the information in the pBR322 map, bands 13 and 17 must also be missing. At one end of the deletion, band 11 is present while band 9 is absent. There is a small band, 20, between them which cannot be detected on these gels, so it may or may not be present. At the other end of the deletion, band 2 is present while band 7 is absent. Between these bands are bands 14 and 18 which are too small to be seen on these gels, so these may or may not be present. The new band seen is about 115bp., making the deletion up to 720bp. long. It starts between 3212bp. and 3329bp., and ends between 3734bp. and 4045bp.

By analysing these results (see Figure 3.11 for summary) the end points of the deletion can be defined as being between 3212bp. and 3329bp. at one end, and between 3901bp. and 4017bp. at the other end. The size of the deletion is approximately 623bp. and this deletion would remove most of the B-la gene but would not extend into the inverted repeat. A restriction map of pEN201 is shown in figure 3.12.

3.7. CONSTRUCTION OF pEN202 AND pEN203

After the construction of deletion mutants of pBR322 using PstI and BAL 31, a new restriction enzyme, AhaIII, became available. AhaIII recognises the sequence TTTAAA and cuts pBR322 three times, leaving blunt ended fragments. All of the sites are within the Tn3 region of pBR322; at 3233bp., 3252bp., and 3944bp.; giving fragments of 3651bp., 692bp., and 19bp. These deletions would not however remove the inverted repeat which is situated between bases 3146 and 3184 on the pBR322 map.

To obtain a deletion mutant which had lost both smaller AhaIII fragments, pBR322 was digested completly with AhaIII, the restriction enzyme denatured by heat inactivation at 70°C and then the cleaved DNA religated overnight. The ligated DNA was used to transform DS902 and transformants were grown on Tc plates. Colonies that grew were screened on Ap, and DNA from Ap sensitive colonies were analysed by agarose gel electrophoresis to identify clones that had lost 711bp. DNA from plasmids that looked the correct size were further analysed with HpaII, and the expected pattern was seen. This plasmid was named pEN202 and the HpaII digest is shown in figure 3.13.

Partial digestion with AhaIII was also used to try to remove only the small 19bp. fragment which is only 49bp. from the inverted repeat. DNA from colonies which were Ap^s Tc^r were

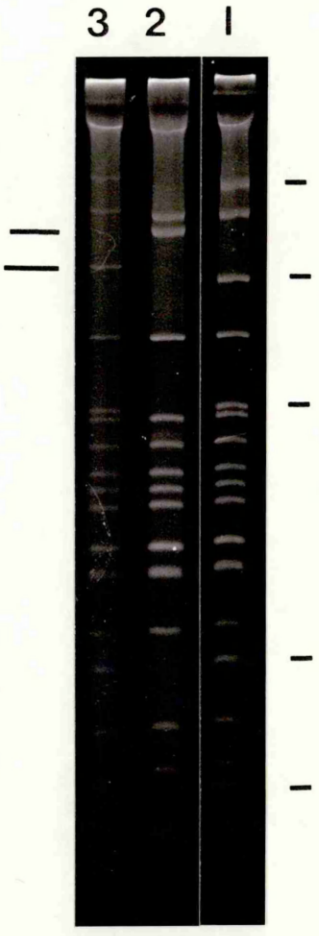

Figure 3.13.

HpaII digest of deletion mutants of pBR322.
Missing bands (—) and new bands (——) are indicated.

Lane 1. pBR322 HpaII
Lane 2. pEN202 HpaII
Lane 3. pEN203 HpaII

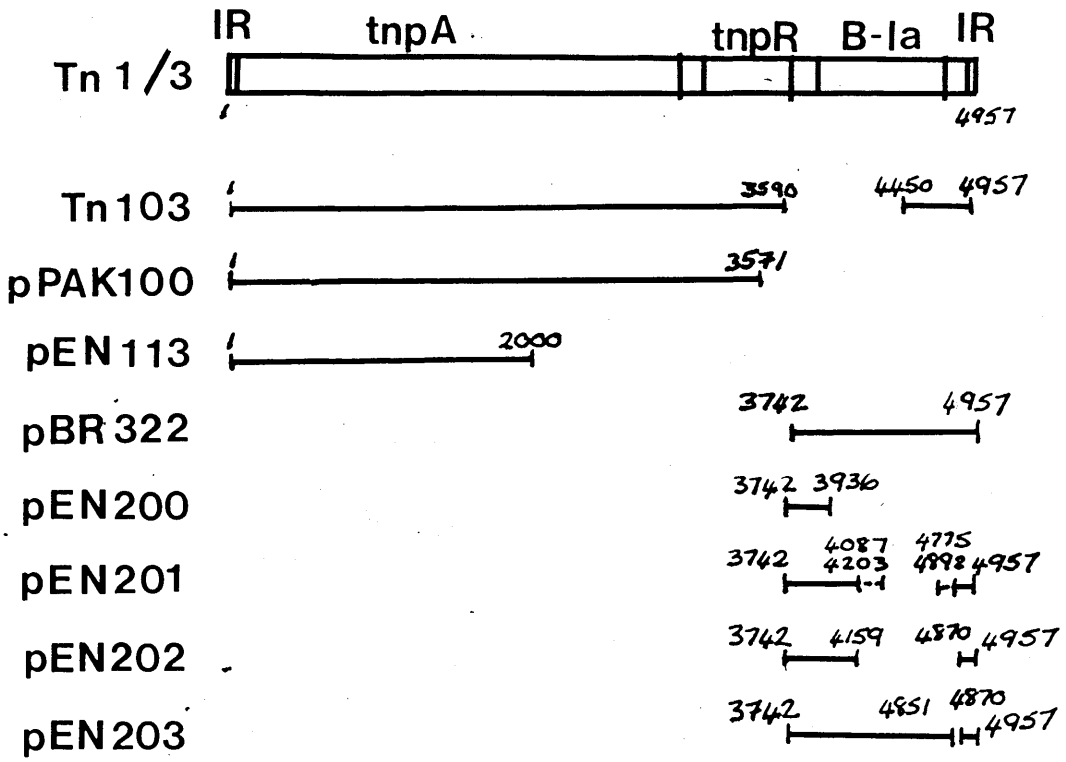

Figure 3.14.

Black lines show the transposon sequences present in the plasmids used in this study.

analysed by restricting with HpaII and running on an acrylamide gel. A 19bp. deletion would not be seen, so colonies which looked similar in size to pBR322 were assumed to have lost the 19bp. fragment. Figure 3.13 shows this digest and the plasmid is called pEN203.

The transposon sequences present in each of the plasmids used in this study are shown in figure 3.14.

Chapter 4

TRANSPOSITION IMMUNITY

4.1. INTRODUCTION

Transposition immunity is a phenomenon employed by some transposons to limit their numbers, and thereby to limit the damage done to host cells. It has been shown to occur in the related transposons Tn1, Tn3, Tn501, and Tn1721 (Robinson *et al*, 1977; Stanisich *et al*, 1977) but not for other transposons, e.g., Tn10 (Bennett *et al*,1977). It is thought not to be limited to the Tn3 family, however, as there have been reports of Tn7 showing transposition immunity (Hauer and Shapiro, 1984; M.Rogers, pers. comm.).

Wallace *et al* (1981a) have proposed that a sequence of 300-450bp near the left inverted repeat is required for transposition immunity. This is said not always to be sufficient on its own; sometimes sequences at the right inverted repeat are also required. Wallace *et al*, (1981b) also suggest that the *tnpR* gene product is required for immunity to be expressed, but this has been subsequently disproved both by this work and that of Lee *et al* (1983). The work described in this chapter shows that one half of Tn1 or Tn3 is sufficient for the establishment of immunity, and either half of the transposon can perform this function.

To study the transposition immunity of various plasmids, each plasmid being tested was transformed into a *recA* strain of *E.coli* containing R388::Tn103. After growth for several

FIGURE 4.1 TRANSPOSITION IMMUNITY OF PLASMIDS WITH ONE LEFT INVERTED REPEAT

RECIPIENT PLASMID	TRANSPOSITION FREQUENCY FROM R388::Tn103 INTO RECIPIENT PLASMID			
	A	B	C	D
pACYC184	4.3×10^{-3}	1.0×10^{-2}	3.4×10^{-3}	3.1×10^{-3}
pACYC184::Tn103	2.7×10^{-4}	2.0×10^{-3}	-	-
pPAK100	6.3×10^{-5}	4.7×10^{-4}	1.3×10^{-4}	4.8×10^{-4}
pEN113	-	-	1.3×10^{-4}	7.6×10^{-4}

Columns A, B, C, and D represent experiments done at different times.
Where it is marked (—) this experiment was not done.

generations at 37°C, the plasmid R388::Tn103 was mated out into another recA strain of E.coli. The exconjugants were analysed and the ratio of cointegrates of R388::Tn103 with the test plasmid, to R388::Tn103, was used as a measure of the transposition frequency of Tn103 into the test plasmid. This gave an indication of the immunity of the plasmid since a low level of transposition into a plasmid indicates a high level of immunity. These assays were done in recA strains, and with Tn103 which is a tnpR$^-$ derivative of Tn1. The strains used were DS902 (Sm^r) and DS916 (Rif^r). I used a recA strain and a mutant of Tn1 which lacked resolvase to insure that when transposition occurred, the result was a cointegrate and the number of cointegrates would accurately reflect the transposition frequency.

4.2. IMMUNITY OF THE LEFT END OF Tn1

The transposition immunity of pPAK100, which contains the left half of Tn1, was studied. The plasmid pPAK100 is described fully in Chapter 3. It is tnpA$^+$, res$^+$, tnpR$^-$, it has the left inverted repeat and is Cm resistant. Figure 4.1 shows the transposition frequencies of Tn103 into various plasmids. The columns A,B,C, and D represent experiments done on four separate occasions. The plasmid pACYC184 was used as a control as it contains no transposon sequences. Transposition of Tn103 from R388 into pACYC184 occurred at frequencies between 3.1×10^{-3} and 1.0×10^{-2}. This tenfold variation in transposition frequency

FIGURE 4.2 TRANSPOSITION FREQUENCY OF Tn103

Transposition frequency of Tn103 between plasmids.

	R388::Tn103 pACYC184	R388 pACYC184::Tn103	R388::Tn103 pACYC184::Tn103
A	4.3×10^{-4}	1.4×10^{-2}	1.1×10^{-4}
B	1.3×10^{-3}	2.5×10^{-2}	2.7×10^{-5}
C	4.3×10^{-3}	1.8×10^{-2}	2.7×10^{-4}
D	1.6×10^{-3}	1.7×10^{-2}	1.3×10^{-4}
E	1.9×10^{-3}	7.4×10^{-2}	1.3×10^{-4}

A, B, C and D are separate experiments. E shows the average of the four experiments.

was observed when experiments were performed on different days. The transposition frequencies into pACYC184::Tn103, pPAK100 and pEN113 were measured. Transposition into pPAK100 and pEN113 occurred at about the same frequency - between 6.3 X 10^{-5} and 7.6 X 10^{-4}. The transposition rate into pACYC184::Tn103 was also measured and is shown in columns A and B. It was found to be slightly higher than into pPAK100, 4.3 times higher in each case. This apparent higher rate of transposition into pACYC184::Tn103 (and therefore lower level of immunity) is probably due to transposition occurring both from R388::Tn103 and from pACYC184::Tn103. Figure 4.2 shows transposition occurring at a higher rate from pACYC184::Tn103 than from R388::Tn103. If cointegrates between pACYC184::Tn103 and R388::Tn103 were analysed it would be seen that most of the transposition occurred from the pACYC184::Tn103 molecule. It therefore seems likely that the apparent lower level of immunity of pACYC184::Tn103 is due to transposition occurring in both directions.

From these results it can be concluded that the left end on its own is sufficient to confer transposition immunity to a plasmid and that the level of immunity is the same whether one or both ends of the transposon is present. Figure 3.14 shows the regions of Tn1 present in Tn103, pPAK100 and pEN113. The information required for transposition immunity must therefore be contained in this left 2Kb.

FIGURE 4.3 TRANSPOSITION IMMUNITY OF PLASMIDS WITH ONE RIGHT INVERTED REPEAT.

RECIPIENT PLASMID	TRANSPOSITION FREQUENCY FROM R388::Tn103 INTO RECIPIENT PLASMIDS				
	A	B	C	D	E
pACYC184	3.4×10^{-3}	3.1×10^{-3}	-	-	-
pBR322	8.9×10^{-6}	3.0×10^{-5}	2.7×10^{-6}	1.7×10^{-5}	7.3×10^{-5}
pEN200	1.2×10^{-3}	6.1×10^{-2}	4.8×10^{-4}	3.7×10^{-3}	1.9×10^{-2}
pEN201	1.3×10^{-5}	3.0×10^{-5}	4.8×10^{-6}	3.8×10^{-5}	2.8×10^{-5}
pEN202	-	-	6.6×10^{-5}	1.1×10^{-4}	8.7×10^{-5}
pEN203	-	-	5.9×10^{-6}	3.3×10^{-5}	7.3×10^{-5}

Columns A, B, C, D and E represent experiments done at different times.
Where it is marked (—) this experiment was not done.

4.3. IMMUNITY OF THE RIGHT END OF Tn3

Plasmids with the right inverted repeat of Tn3 were analysed in the same manner as those with the left inverted repeat. The plasmids tested in this study were pBR322 and its deletion derivatives (described in Chapter 3). The experiments were done on five separate occasions and the results are shown in Figure 4.3. Although the frequencies varied from experiment to experiment, the differencies in immunity, measured as the frequency of transposition into a plasmid, remained constant.

The frequency of transposition of Tn103 into pACYC184 was again used as a control since pACYC184 is similar to pBR322, in that it is a small, high copy number plasmid. It does not however have any transposon sequences. Transposition from R388::Tn103 into pACYC184 occurred at frequencies of 3.4×10^{-3} and 3.1×10^{-3}.

Transposition from R388::Tn103 into pBR322 occurred at frequencies ranging from 2.7×10^{-6} to 7.3×10^{-5}. This is two to two and a half orders of magnitude less than transposition into pACYC184, so the presence of the right half of Tn3 appears to be making a significant difference to the immunity of this small plasmid. Deletion derivatives of pBR322 were constructed using BAL 31 to remove sequences required for immunity. The construction and analysis of these mutants, pEN200 and pEN201, is described in Chapter 3, and Figure 3.14 shows the extent of the

transposon sequences.

Transposition from R388::Tn103 into pEN200, the plasmid with the largest deletion , occurs at frequencies similar to those into pACYC184 (4.8×10^{-4} to 6.1×10^{-2}), indicating that pEN200 has completely lost its transposition immunity. This deletion extends beyond the inverted repeat and the plasmid has only 196bp. of transposon sequences remaining.

Transposition into pEN201 occurs at similar frequencies as transposition into pBR322 (4.8×10^{-6} to 3.8×10^{-5}), so this plasmid is still immune and shows the same level of immunity as pBR322.

Another two deletion mutants of pBR322 were constructed using the restriction enzyme AhaIII. These were named pEN202 and pEN203. The construction of these is described in Chapter 3. Figure 3.14 shows the extent of the transposon sequences. The transposition frequency from R388::Tn103 into pEN202 is similar or slightly higher than the transposition frequency into pBR322 (6.6×10^{-5} to 1.1×10^{-4}). In one case this is up to a twenty-fold difference, which may meen that pEN202 is slightly less immune than pBR322. Transposition into pEN203 occurs at similar frequencies as transposition into pBR322 (5.9×10^{-6} to 7.3×10^{-5}), indicating that it is still immune.

4.4. DISCUSSION

Transposition immunity is a phenomenon whereby a plasmid with one copy of a transposon is 'immune' to receiving a second copy of that transposon. It is necessarily a *cis* acting phenomenon as if it were to act in *trans* further transposition would by prevented in a cell containing one copy of that transposon. It may not be restricted to the Tn3 family as there has been a report of a similar effect with Tn7 (Hauer and Shapiro, 1984; M Rogers, pers. comm.). Within the Tn3 family it is very specific and is limited to other closely related transposons. Tn501 and Tn1721, which have almost identical terminal repeats and can complement one another for transposition functions, show immunity to each other. However, Tn3, which differs from Tn501 at 19 out of 38bp. in the inverted repeats, does not share cross immunity and does not complement for transposition functions. It has also been found that Tn501 insertions can be a hot spot for Tn3 insertion, although they act to direct insertions rather than to increase their frequency (Grinsted *et al*, 1978).

Lee *et al* (1983) have concluded that only the 38bp. inverted repeats of Tn3 are required to confer immunity to a plasmid and that only one inverted repeat is sufficient. The work presented in this chapter agrees with these findings and suggests that either half of the transposon is sufficient to render the plasmid immune to further transposition.

Transposition immunity is thought not to completely prevent transposition to the 'immune' plasmid; it merely reduces the frequency of transposition to that plasmid. We were therefore still able to pick up transposition events to an 'immune' plasmid, although at a greatly reduced frequency.

In figure 4.1 the transposition frequency into derivatives of pACYC184 containing part of Tn1 was measured. The presence of one or two copies of the Tn1 terminal repeats reduced the transposition frequency by one or two orders of magnitude. In the same experiment transposition was found to be slightly higher between R388::Tn103 and a plasmid with two copies of the inverted repeats than into a plasmid with one inverted repeat. This may be due to the ability of the plasmid with two inverted repeats (pACYC184::Tn103), to transpose to R388::Tn103 as well as for R388::Tn103 to transpose to pACYC184::Tn103. The plasmids pPAK100 and pEN113 were found to be equally immune to further transposition, although pPAK100 contains about 1.5Kbp. more of transposon sequence.

The immunity of the right half of Tn3 was measured and the results are shown in figure 4.3. Here the frequency of transposition from R388::Tn103 into pBR322 was measured and compared to the frequency of transposition into pACYC184. Although these plasmids are not related, they are both small and have a high copy number. Transposition into pBR322 was two

orders of magnitude down on transposition into pACYC184, and the presence of the Tn3 sequences on pBR322 is assumed to be the cause of this difference. In constructing pBR322, the Ap resistance gene of Tn3 was used (Bolivar et al, 1977) and so pBR322 contains 1.2Kb. of sequence from the right end of Tn3.

Various deletion derivatives of pBR322 were made and one of these, pEN200, was found to have lost its immunity to transposition. Upon sequencing it was found that this deletion derivative had lost its inverted repeat, unlike the other derivatives. The only remaining transposon sequence is a 196bp. region from between the bla gene and the tnpR gene. Those plasmids still containing the inverted repeat remained as immune to further transposition as pBR322, despite losing some transposon sequences.

All the experiments in this study, and in the work of Lee et al, were done with transposon mutants lacking a functional tnpR gene. Other studies (Sherratt et al, 1981; Muster et al, 1983) have also shown that resolvase is not involved in the phenomenon of transposition immunity.

So far no protein has been found to be involved in transposition immunity. Since tnpA is essential for transposition, it does not seem too unlikely that it may also be involved in the recognition of immune or non-immune sequences. It may be, as suggested in the paper of Lee et al, that a

'transposition complex' may scan the recipient molecule before the initiation of transposition, and if a copy of the transposon is encountered, the complex may dissociate. It is likely that transposition immunity acts at this step in transposition, since it has been shown that in a recA strain it is possible to have two copies of a transposon stably maintained if resolvase is not present (Kitts et al, 1982b).

Intra-molecular transposition has been found not to be limited by immunity (Bishop and Sherratt, 1984). Where there is only one copy of a transposon on a plasmid it is able to transpose in an intra-molecular fashion. However, if an additional Tn1/3 sequence is present this will confer transposition immunity to the plasmid and intra-molecular transposition will be inhibited. As in the case of inter-molecular transposition, immunity can be caused by the presence of only a small region of transposon sequences. The Tn3 sequences present in pBR322 are sufficient to mediate such immunity. In the case of intra-molecular transposition it is possible to envisage a transposition complex of the donor transposon and tnpA, perhaps bound to the inverted repeats, scanning the rest of the molecule. If another transposon sequence is recognised, the complex dissociates; if no transposon sequence is encountered, then an intra-molecular transposition event may occur.

As yet it is too early to speculate on the topology of transposition immunity and the molecular interactions that may occur. However it is hoped that defining the minimum requirements for immunity will lead to a greater understanding of transposition. It appears that all that is required for transposition immunity is the presence of one inverted repeat on the recipient plasmid.

CHAPTER 5

ONE-ENDED TRANSPOSITION

5.1. INTRODUCTION

Transposons of the Tn3 family all share the same two-step mechanism for intermolecular transposition (Kitts et al, 1982a). The tnpA gene encodes a transposase which acts at the terminal inverted repeats and on the target DNA, causing the formation of a cointegrate molecule. The tnpR gene encodes resolvase which acts at the res site to break down cointegrates into the donor and recipient molecules, each with a copy of the transposon. In the absence of resolvase, transposition stops at the cointegrate stage. It was originally thought that both inverted repeats were required for transposition (Heffron et al,1977; Heffron et al, 1979) but the results of this chapter indicate that transposition can occur with only one inverted repeat.

The models of transposition that have been proposed to explain Tn3 transposition can be divided into two classes - symmetric and asymmetric. In the symmetric model, transposition begins with transposase nicking at both inverted repeats, while in the asymmetric model nicking occurs at only one inverted repeat at the start of the transposition process. The existence of one-ended transposition may require an asymmetric transposition mechanism, at least in some cases.

The work of this chapter investigates the phenomenon of one-ended transposition.

FIGURE 5.1 TRANSPOSITION OF Tn1 WITH ONE LEFT INVERTED REPEAT

PLASMID	TRANSPOSITION FREQUENCY INTO R388			
	A	B	C	D
pACYC184	$< 10^{-7}$	$< 10^{-7}$	$< 10^{-8}$	$< 10^{-8}$
pPAK100	2.0×10^{-6}	2.8×10^{-6}	4.2×10^{-6}	5.0×10^{-5}
pEN113	$< 10^{-7}$	$< 10^{-7}$	$< 10^{-8}$	$< 10^{-8}$
pEN113(a)	ND	3.3×10^{-6}	3.5×10^{-6}	ND

A, B, C and D are separate experiments done at different times. ND means that that particular assay was not done. For plasmid pEN113(a) pMB9::Tn103 was used to provide transposase.

5.2. Transposition using one left inverted repeat.

To study the transposition of one-ended transposons containing the left end of Tn1, DNA of pPAK100 was transformed into strain DS902 which contained R388, a large conjugative plasmid, encoding trimethoprim resistance. After growing for several generations at 37°C, the R388 plasmid was mated out into strain DS916 selecting for Rif resistance. Since all the one-ended transposons studied were tnpR$^-$, they were unable to break down cointegrates formed between the donor and recipient plasmids. The ratio of cointegrates to R388 transferred was used as a measure of the transposition frequency. The transposition frequency of a tnpA$^-$ derivative of pPAK100, pEN113, was also measured when complemented by a transposase producing plasmid.

Figure 5.1 shows the results of these transposition assays. The experiments were done on four separate occasions and these results are shown in columns A, B, C and D. The plasmid pACYC184 was used as a negative control since it does not contain any Tn1 or Tn3 sequences, and no transposition was detected when using this plasmid as the donor. The plasmid pPAK100 was found to transpose at frequencies ranging from 2.0×10^{-6} to 5.0×10^{-5}, which is a thousand times less than the transposition frequency of a transposon with two intact inverted repeats (pACYC184::Tn103) into R388.

The transposition of the tnpA⁻ derivative of pPAK100 pEN113 could not be detected in these assays and is shown as less than 10^{-7} and less than 10^{-8}. When this plasmid was complemented in trans for transposase using the plasmid pMB9::Tn103, cointegrates were detected between pEN113 and R388 at frequencies of approximately 3×10^{-6}. Tn103 on the plasmid pMB9::Tn103 was also able to transpose and form cointegrates with R388. Cointegrates between pEN113 and R388 were screened for the presence of pMB9::Tn103 , by streaking out on Tc plates, to eliminate the possibility that Tn103 had transposed from pMB9 to R388 and then to pEN113, giving false transposition frequencies. Some cointegrates were found to be Tc^r and this has been taken into account when working out the transposition frequencies.

Cointegrates between pPAK100 and R388 were run on agarose gels, and were found to be larger than R388 and have higher copy numbers. Figure 5.2 shows a single colony gel of several cointegrates. Within the limits of resolution of the gel system these all appear to be the same size.

FIGURE 5.3 TRANSPOSITION OF Tn3 WITH ONE RIGHT INVERTED REPEAT

PLASMID	TRANSPOSITION FREQUENCY INTO R388			
	A	B	C	D
pBR322	4.4×10^{-4} (8.6×10^{-4})	2.7×10^{-3} ($<10^{-7}$)	1.2×10^{-5}	1.6×10^{-3} (3.0×10^{-5})
pEN200	$<10^{-7}$ (5.5×10^{-4})	$<10^{-7}$ (5.3×10^{-3})	$<10^{-7}$	$<10^{-7}$ (2.9×10^{-5})
pEN201	2.4×10^{-3} (8.6×10^{-4})	7.0×10^{-4} (2.5×10^{-5})	1.8×10^{-5}	1.3×10^{-4} (2.7×10^{-4})
pEN202	ND	7.0×10^{-5} ($<10^{-7}$)	1.7×10^{-5}	ND
pEN203	ND	1.3×10^{-2} (1.0×10^{-5})	2.2×10^{-4}	8.5×10^{-3} (3.7×10^{-4})

pBR322 and its derivatives were complemented for tnpA by pPAK100, when not complimented no transposition was detected. The transposition frequency of the complementing plasmid was also measured and this is shown in brackets. Where the assay was not done this is marked ND. A, B, C and D were done on four separate occasions.

5.3. Transposition using one right inverted repeat.

The ability of one-ended transposons with the right inverted repeat to transpose was also assayed in the same way. The plasmid pBR322 was chosen as a suitable plasmid as it contains the right inverted repeat and is similar in size and copy number to pACYC184. It also contains the B-lactamase gene which confers resistance to ampicillin, and 27bp. of the tnpR gene from Tn3. Tne transposition assay was carried out in the same way as for pPAK100, but since pBR322 does not have the tnpA gene, transposase had to be provided in trans. The plasmid pPAK100 was used as the source of transposase for pBR322. pBR322 and pPAK100 were transformed into strain DS902 and were grown for several generations. R388 was then mated into this strain and the three plasmids were grown up together for several generations to allow transposition to occur. R388 was then mated out into another recA strain, DS916, and the transposition frequency was measured as the ratio of cointegrates to R388. Cointegrates of pBR322 and R388 were checked for the presence of pPAK100 by streaking out on chloramphenicol plates. This ensured that the cointegrate frequency did not include any double transposition events involving pPAK100.

Figure 5.3 shows the results of the transposition assays. When not complemented by pPAK100, no cointegrate formation was ever detected. In most cases the frequency of cointegrate formation between pPAK100 and R388 was also measured. This was

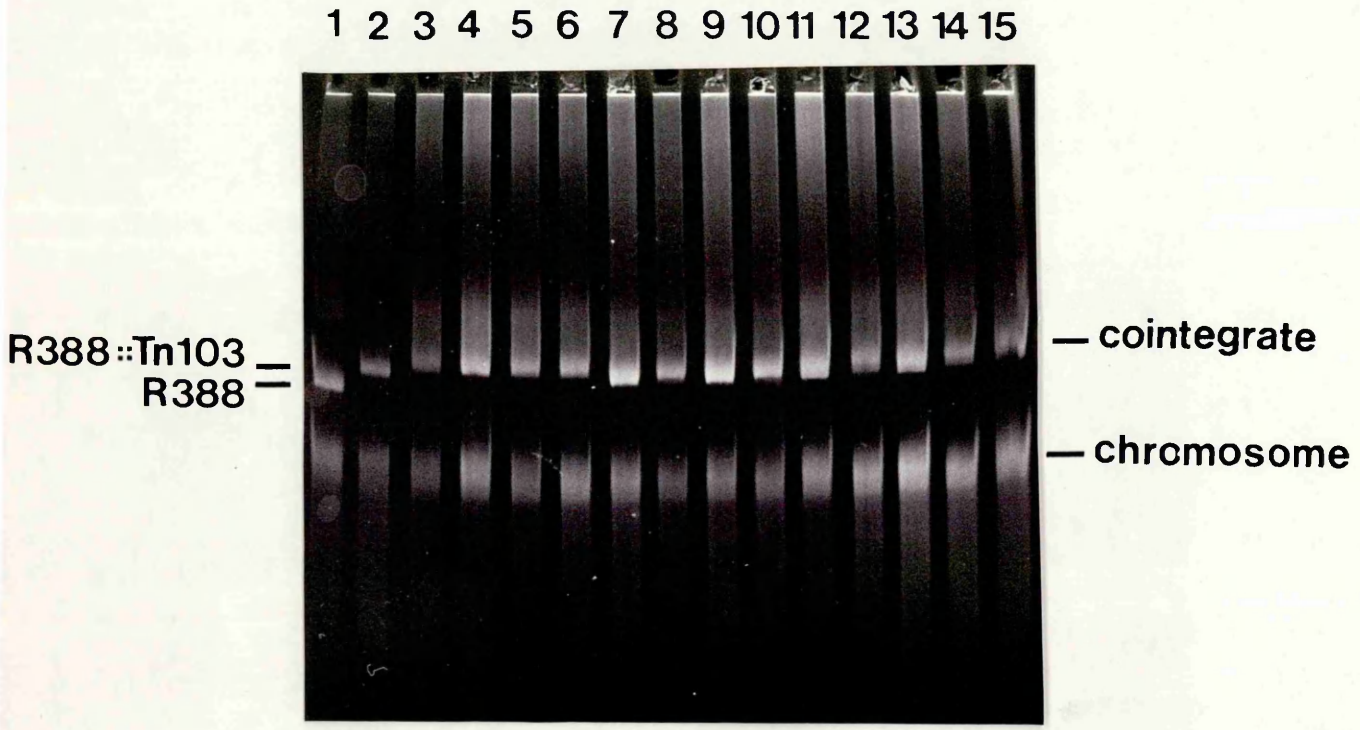

Figure 5.4.

0.8% agarose, single colony gel of cointegrates between pBR322 and R388.

Lane 1. R388.
Lane 2. R388::Tn103.
Lanes 3-15. Cointegrates between pBR322 and R388.

found to range from less than 10^{-7} to 5.6 X 10^{-3}. pBR322 transposition was detected at frequencies of between 1.2 X 10^{-5} and 2.7 X 10^{-3}.

The deletion mutants, pEN200, pEN201, pEN201 and pEN203 were also assayed for one-ended transposition. The construction and analysis of these is described in Chapter 3. The transposition assays were carried out in the same way as for pBR322, with the complementing plasmid, pPAK100, providing transposase in *trans*. Table 5.3 shows that pEN201, pEN202 and pEN203 were able to form cointegrates when complemented but pEN200 was not. No transposition was detected with the deletion derivative pEN200, although in each case the complementing plasmid, pPAK100, formed cointegrates with R388, indicating that transposase was still being produced. In all of these assays, the rate of transposition of the pBR322 derivative was found to be as good, if not better than the rate of transposition of the pPAK100 plasmid. This perhaps indicates that transposase functions equally well in *trans* as in *cis*. It may also indicate that the right end of the transposon is a better target for transposase.

Agarose gels showing the cointegrates of pBR322 and R388 were run. Figure 5.4 shows one of these. The cointegrates can readily be seen to be larger than R388 and R388::Tn103 and they have a higher copy number, indicating that they are fusions of pBR322 and R388 replicating from the pBR322 origin.

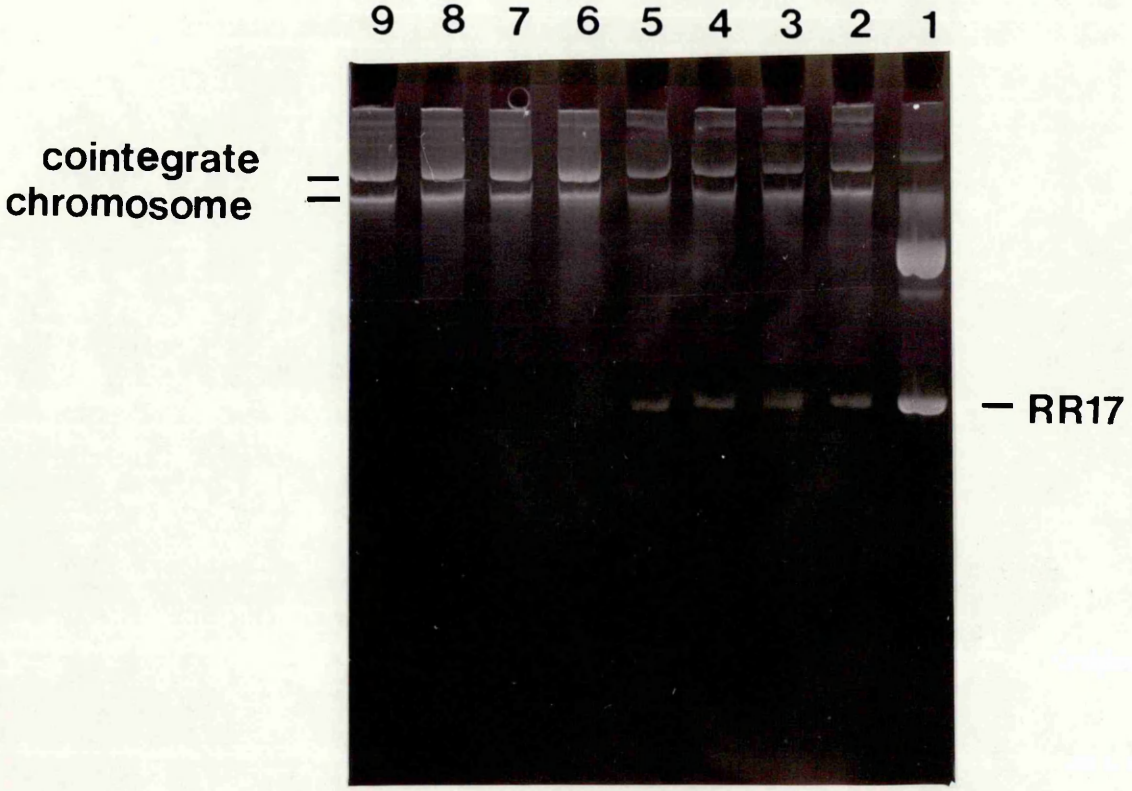

Figure 5.5.

0.8% agarose, single colony gel of cointegrates between pPAK100 and R388.

Lane 1. RR17 marker (source of resolvase).
Lanes 2-5 Cointegrates of pPAK100 and R388, + RR17.
Lanes 6-9. Cointegrates of pPAK100 and R388.

Cointegrates between pBR322 and R388, and between pPAK100 and R388 were found not to break down in recA cells. When the cointegrates were mated into another recA strain (DS902 or DS910), the markers for pPAK100 or pBR322 were always co-transfered.

Because of the large size of these cointegrates, it is not possible to tell from the agarose gels whether these cointegrates are true transpositions, resulting in the duplication of all or part of the transposon, or simple fusions of donor and recipient plasmids with no duplication of DNA. In the case of pPAK100 transposition, if the whole of the transposon sequence has been duplicated then the res site would also be duplicated, and cointegrates could be broken down by the addition of a resolvase producing plasmid. To test this hypothesis, RR17, a plasmid producing resolvase from the related transposonTn1000, was transformed into the cells containing cointegrates of pPAK100 and R388. Tn1000 resolvase is able to work on the Tn1/3 res site (Kitts et al, 1982b). RR17 was transformed into cells containing cointegrates of pPAK100 and R388, and these were grown up at 37°C overnight. Single colonies were patched out, grown up and extracts run on agarose gels. Figure 5.5 shows a small sample of these. Lane 1 shows the plasmid RR17, lanes 2 - 5 show RR17 and the cointegrates and lanes 6 - 9 show the cointegrates alone. From this gel it can be seen that there has been no apparent breakdown of the cointegrate in the presence of the resolvase-providing plasmid. In a separate experiment (not shown) the

presence of RR17 caused cointegrates with Tn103 to break down, indicating that RR17 is producing a functioning resolvase. From this it is concluded that the whole of the transposon has not been duplicated. The res site lies between the tnpA gene and the start of the tnpR gene and is about 3.2Kb. from the left end of Tn1, so any duplication must be less than this. Since the region of Tn3 DNA present in pBR322 does not include the res site, it was not possible to repeat this experiment for the pBR322 derivatives.

5.4. RESTRICTION ENZYME ANALYSIS.

5.4.1. pPAK100 Cointegrates.

The DNA of four cointegrates of pPAK100 and R388 was analysed using the four restriction enzymes BamHI, PstI, HindIII and EcoRI, and using the information derived from these digests their structure was worked out. The DNA for these digests was made by the Birmboim/Doly method (Birmboim and Doly ;1979), and after digestion with the restriction enzymes, the sample was run on a 1% agarose gel alongside known size markers. Figures 5.6, 5.7, 5.8 and 5.9 show the restriction patterns obtained for each of these cointegrates and also a diagram of the structure of the cointegrate. as deduced from the digests. From these figures it can be seen that pPAK100 appears to have inserted at different sites in the R388 plasmid each time, but that the 'break point' in pPAK100 appears to be the same each time, as far as it is possible to tell by restriction analysis. It is not possible to tell from these digests how much, if any, of the transposon has been duplicated during the transposition event.

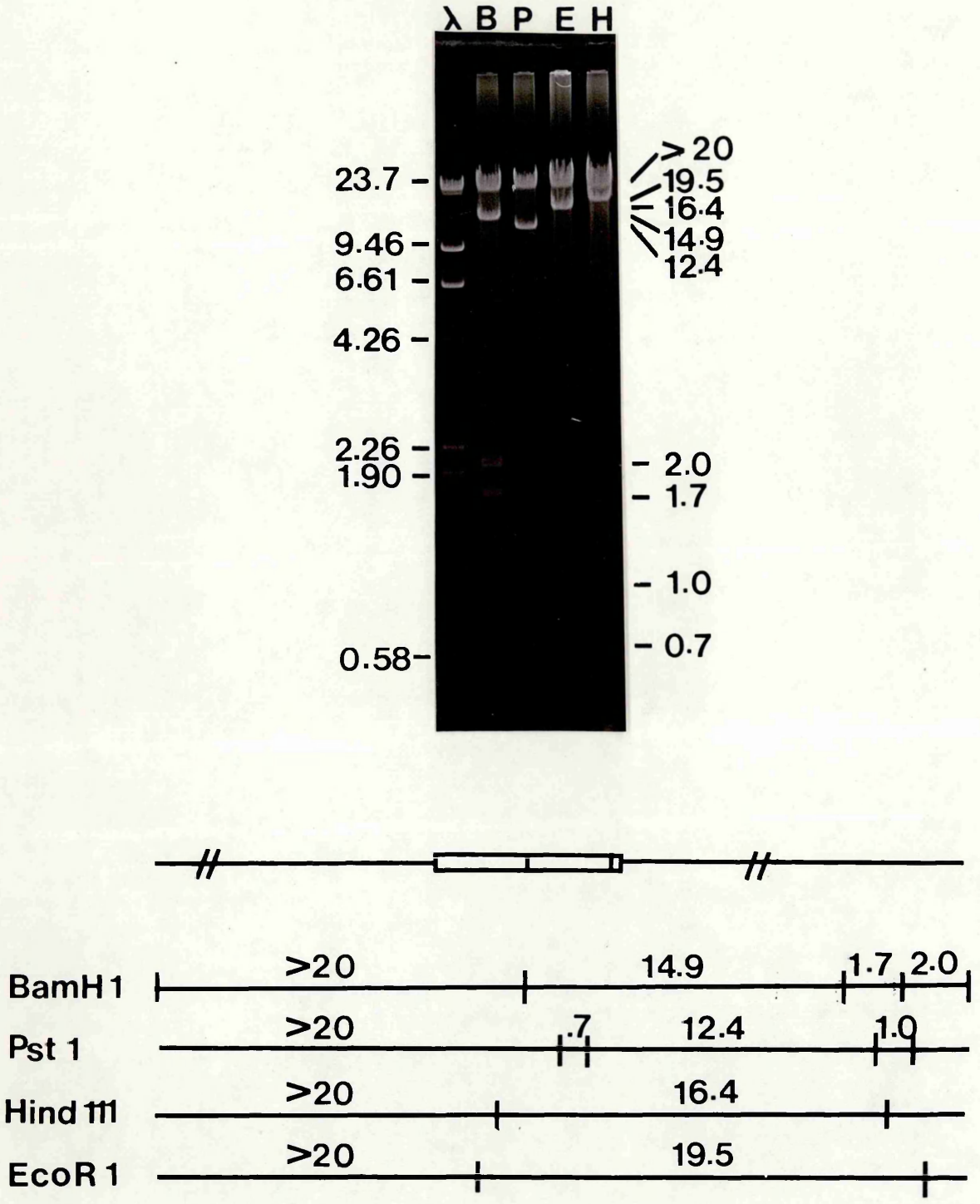

Figure 5.6.

Restriction digests of cointegrate between pPAK100 and R388 - run on a 1% agarose gel.

λ	lambda HindIII size markers.
B	BamHI digest of cointegrate.
P	PstI digest of cointegrate.
E	EcoRI digest of cointegrate.
H	HindIII digest of cointegrate.

The size of fragments, approximate position and orientation of insert is shown in the diagram.

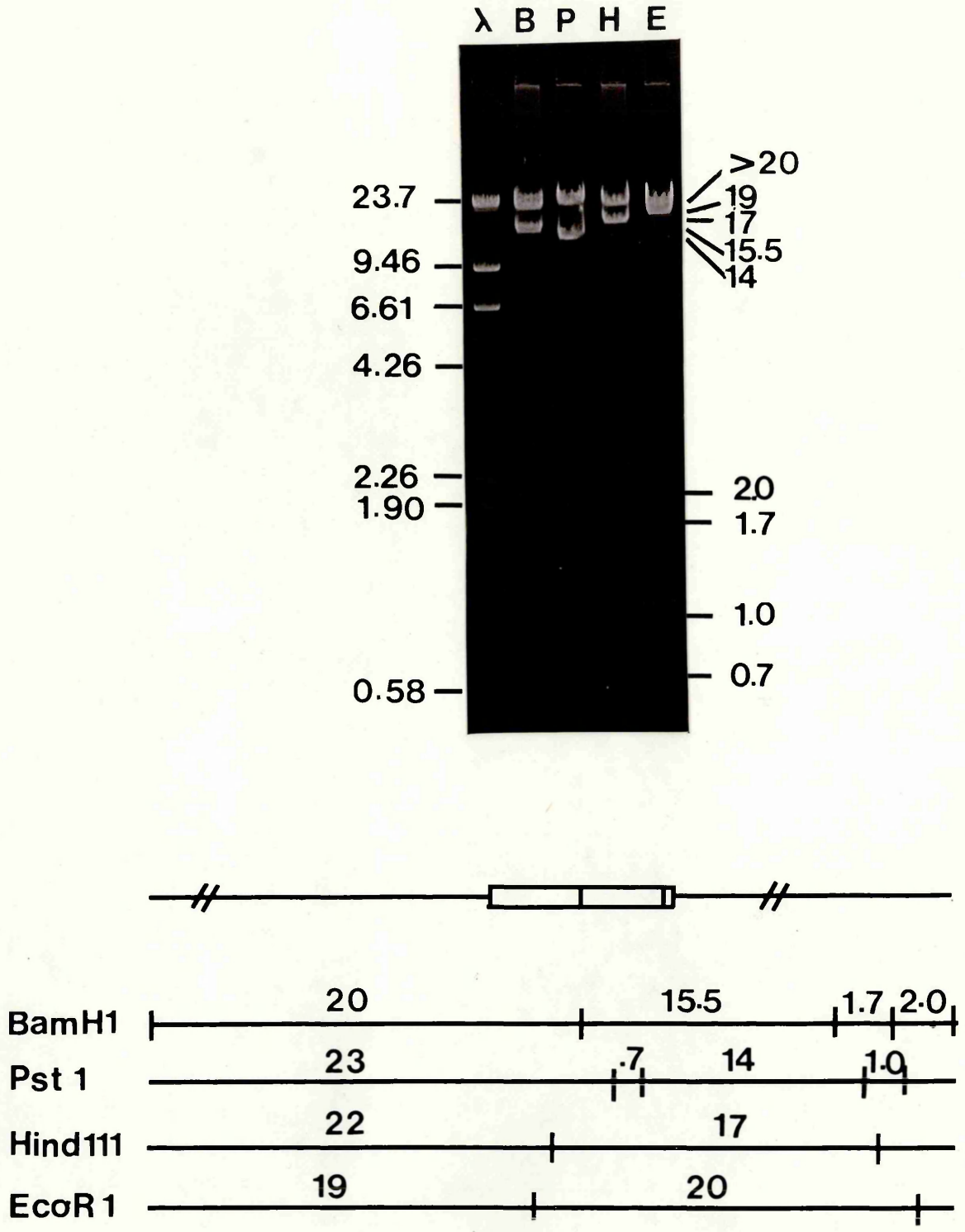

Figure 5.7.

Restriction digests of cointegrate between pPAK100 and R388 - run on a 1% agarose gel.

λ	lambda HindIII size markers.
B	BamHI digest of cointegrate.
P	PstI digest of cointegrate.
H	HindIII digest of cointegrate.
E	EcoRI digest of cointegrate.

Size of fragments, approximate position and orientation of insert is shown in the diagram.

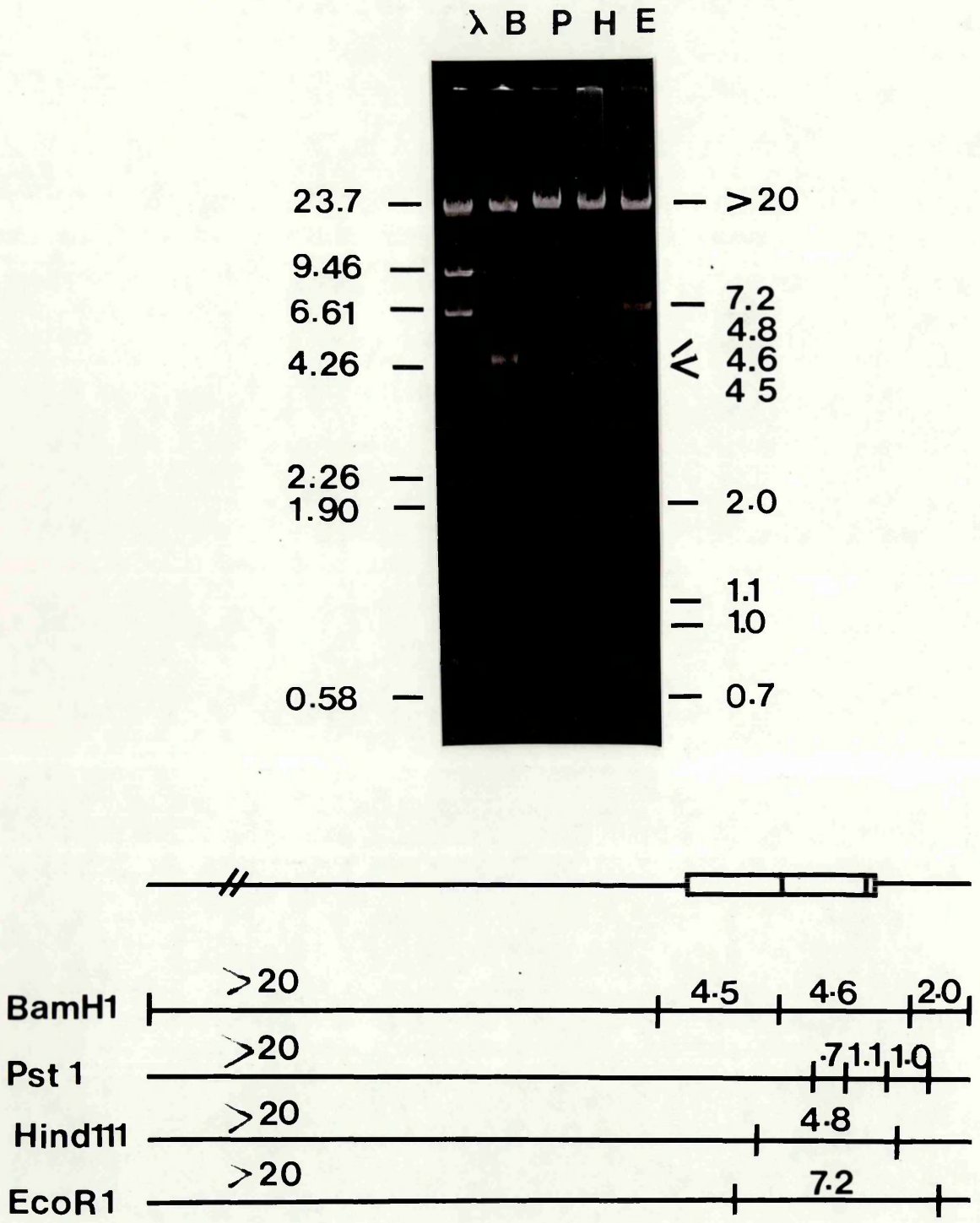

Figure 5.8.

Restriction digests of cointegrate between pPAK100 and R388 - run on a 1% agarose gel.

λ	lambda HindIII size markers.
B	BamHI digest of cointegrate.
P	PstI digest of cointegrate.
H	HindIII digest of cointegrate.
E	EcoRI digest of cointegrate.

Size of fragments, approximate size and orientation of insert is shown in the diagram.

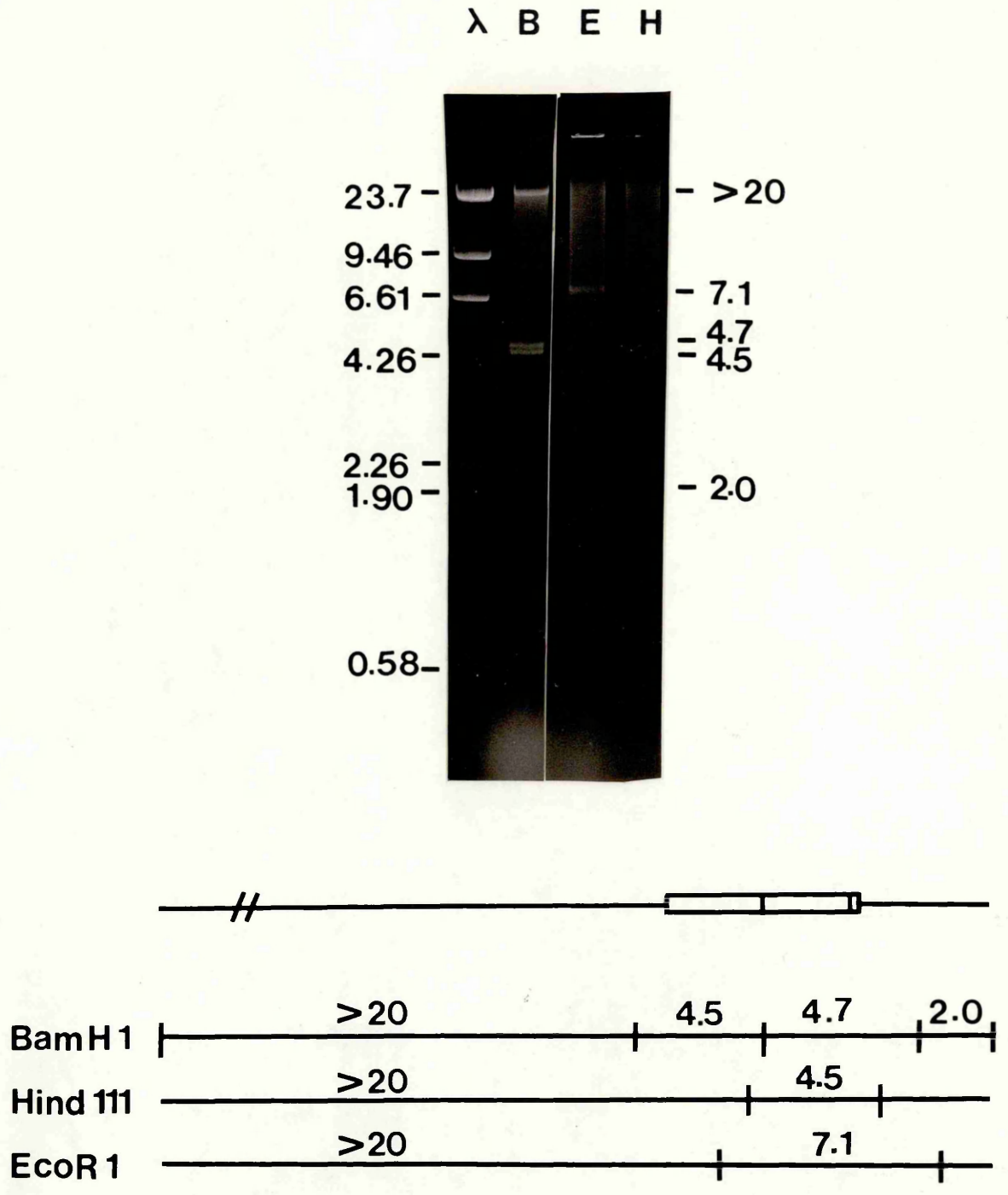

Figure 5.9.

Restriction digests of cointegrate between pPAK100 and R388 - run on a 1% agarose gel.

λ lambda HindIII size markers.
B BamHI digest of cointegrate.
E EcoRI digest of cointegrate.
H HindIII digest of cointegrate.

Size of fragments, approximate position and orientation of insert is shown in the diagram.

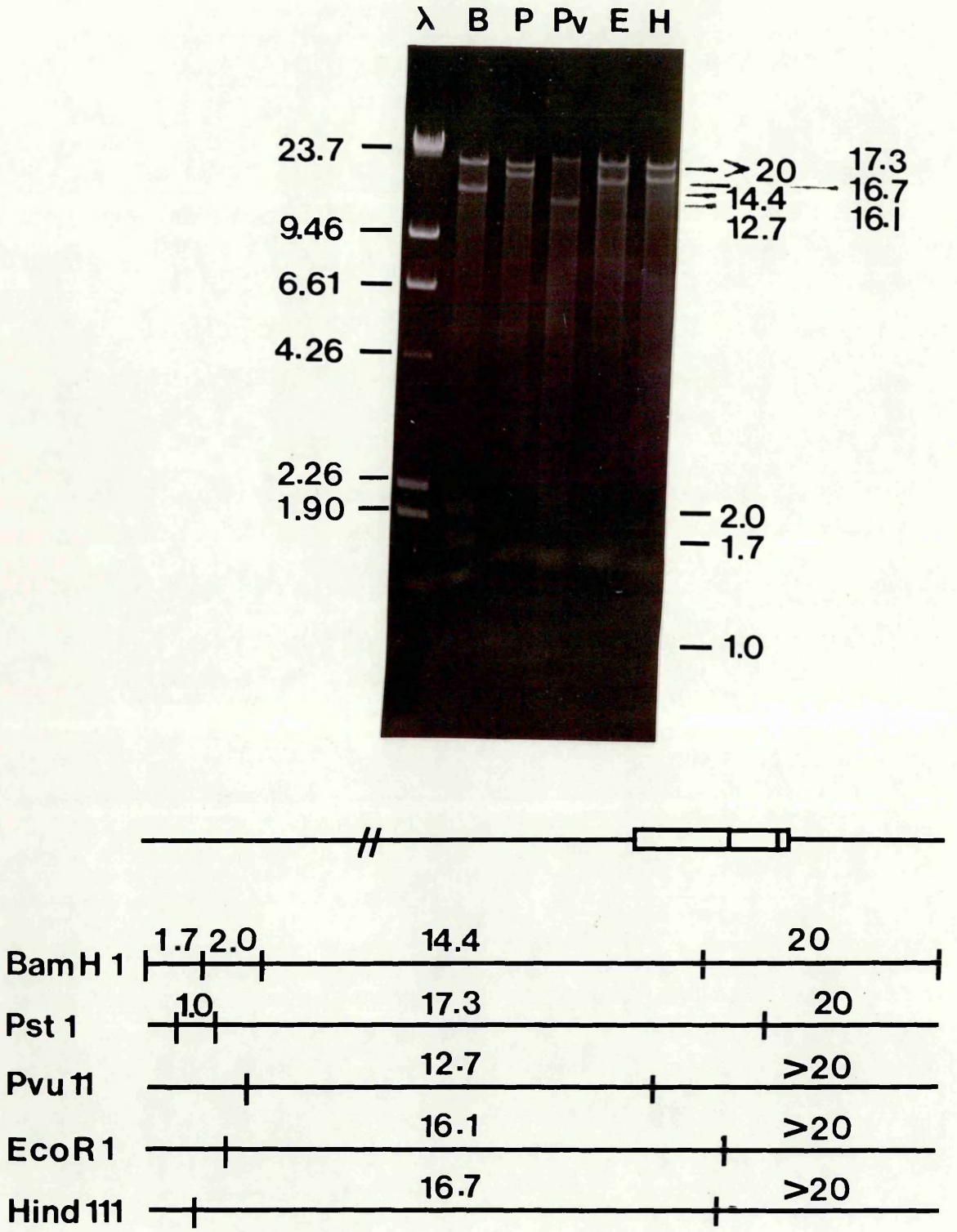

Figure 5.10.

Restriction digests of cointegrate between pBR322 and R388 - run on a 1% agarose gel.

λ	lambda HindIII size markers.
B	BamHI digest of cointegrate.
P	PstI digest of cointegrate.
Pv	PvuII digest of cointegrate.
E	EcoRI digest of cointegrate.
H	HindIII digest of cointegrate.

Size of fragments, approximate position and orientation

5.4.2. pBR322 Cointegrates.

Cointegrates between pBR322, or its derivatives, and R388 were also analysed using restriction enzymes. The DNA was prepared using the Birmboim Doly method, and after digestion with the appropriate enzymes, digests were run on a 1% agarose gel. The restriction enzymes used were again BamHI, PstI, HindIII and EcoRI. Digests of the pBR322 cointegrates are shown in figures 5.10 to 5.14, along with a diagram of the cointegrate deduced from the restriction information. In these cointegrates the pBR322 plasmid, or its derivative, have inserted at different points in the R388 plasmid, but the 'break point' in pBR322, like that in pPAK100, appears to be the same each time, as far as it is possible to tell. Again it is not possible to tell from these digests whether there has been any duplication of the transposon sequences.

The information from figures 5.6 to 5.9 and figures 5.10 to 5.14 is summarised in figure 5.15. This shows the positions of all the inserts of pPAK100 , pBR322 and their deletion derivatives into R388. All of the inserts appear to be in the same orientation.

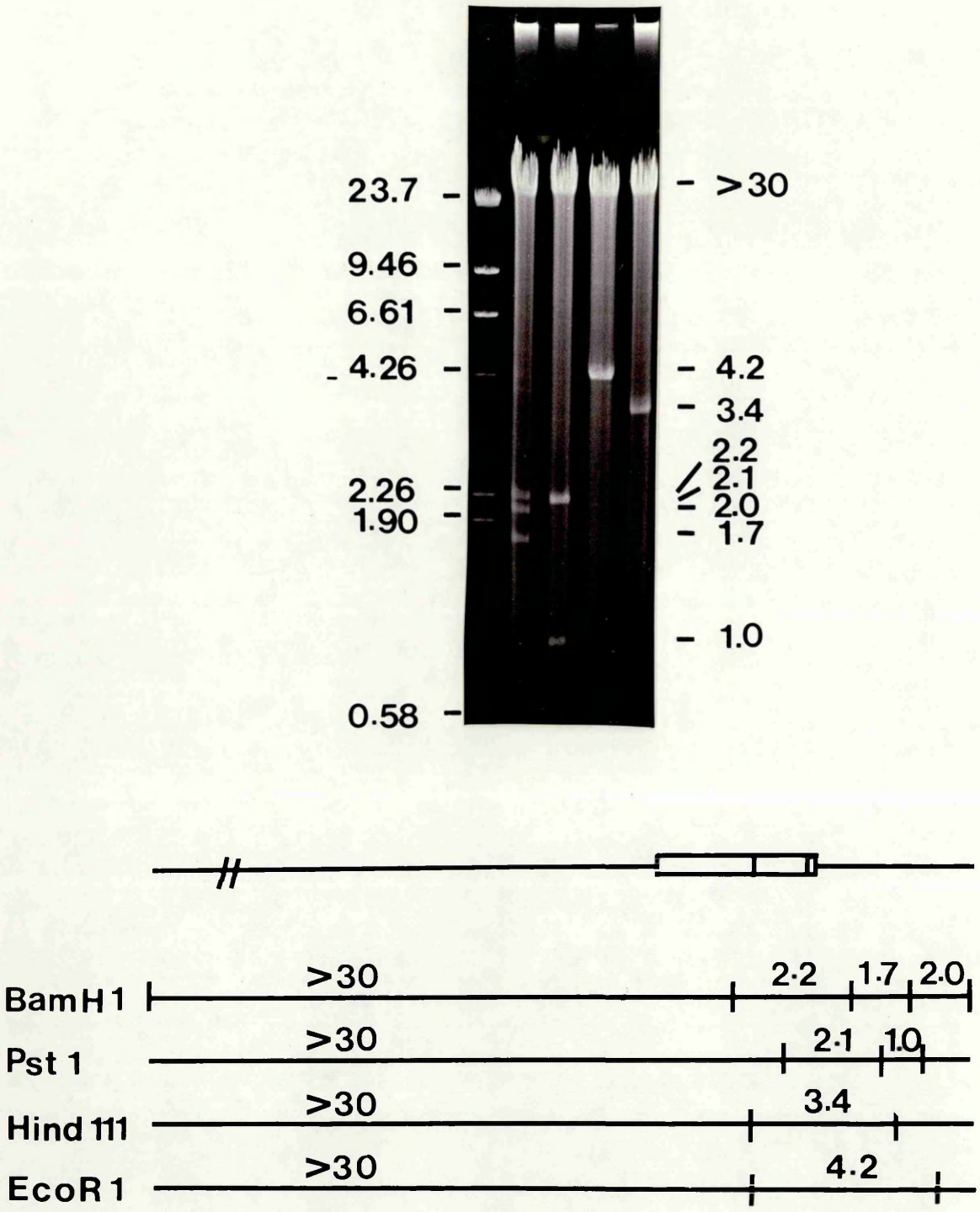

Figure 5.11.

Restriction digests of cointegrate between pBR322 and R388 - run on a 1% agarose gel.

λ	lambda HindIII size markers.
B	BamHI digest of cointegrate.
P	PstI digest of cointegrate.
H	HindIII digest of cointegrate.
E	EcoRI digest of cointegrate.

Size of fragments, approximate position and orientation of insert is shown in the diagram.

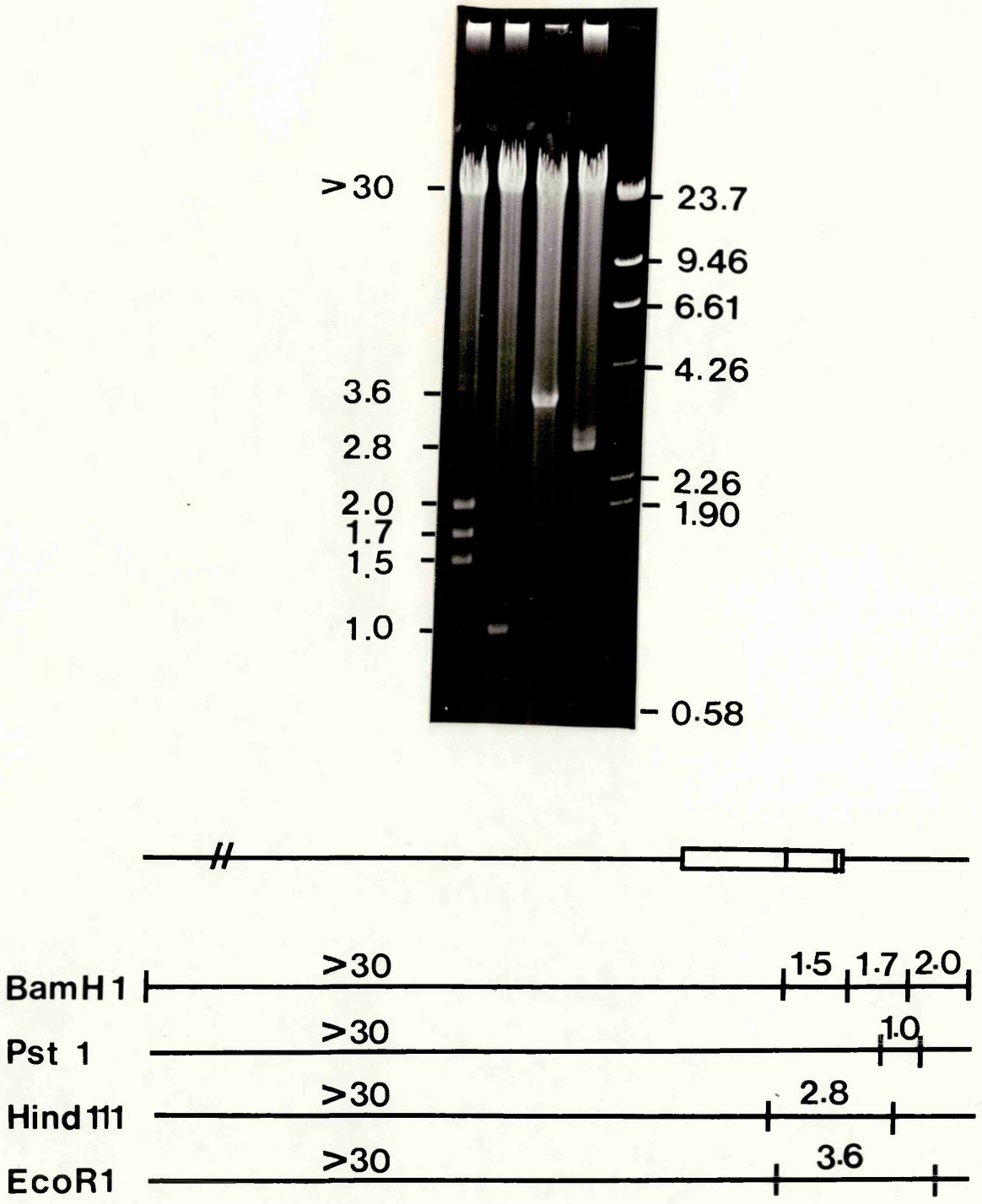

Figure 5.12.

Restriction digests of cointegrate between pEN201 and R388 - run on a 1% agarose gel.

λ lambda HindIII size markers.
B BamHI digest of cointegrate.
P PstI digest of cointegrate.
H HindIII digest of cointegrate.
E EcoRI digest of cointegrate.

Size of fragments, approximate position and orientation of insert is shown in the diagram.

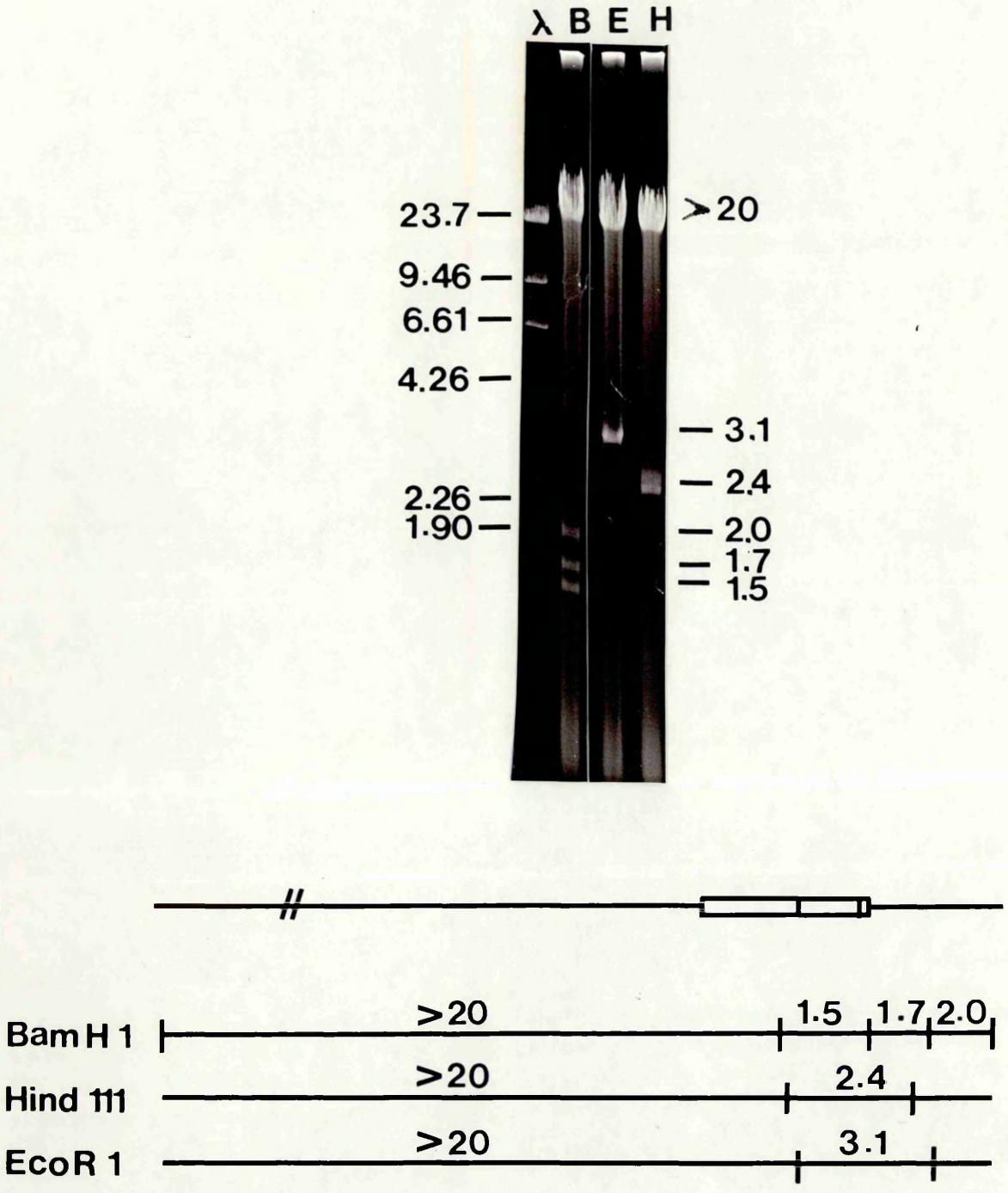

Figure 5.13.

Restriction digests of cointegrate between pEN201 and R388 - run on a 1% agarose gel.

λ lambda HindIII size markers.
B BamHI digests of cointegrate.
H HindIII digest of cointegrate.
E EcoRI digest of cointegrate.

Size of fragments, approximate position and orientation is shown in the diagram.

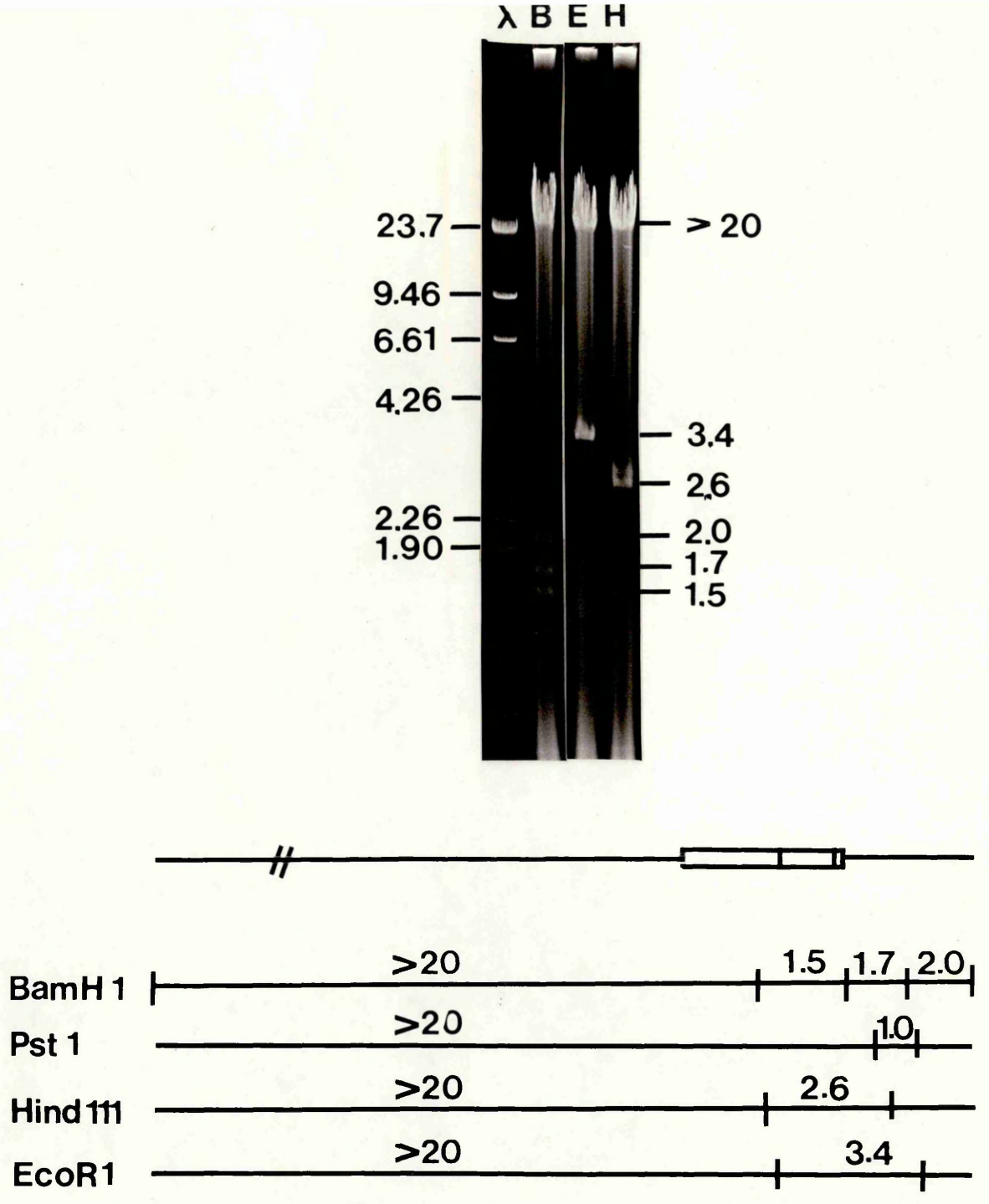

Figure 5.14.

Restriction digests of cointegrate between pEN202 and R388 - run on a 1% agarose gel.

λ lambda HindIII size markers.
B BamHI digest of cointegrate.
P PstI digest of cointegrate.
H HindIII digest of cointegrate.
E EcoRI digest of cointegrate.

Size of fragments, approximate position and orientation of insert is shown in the diagram.

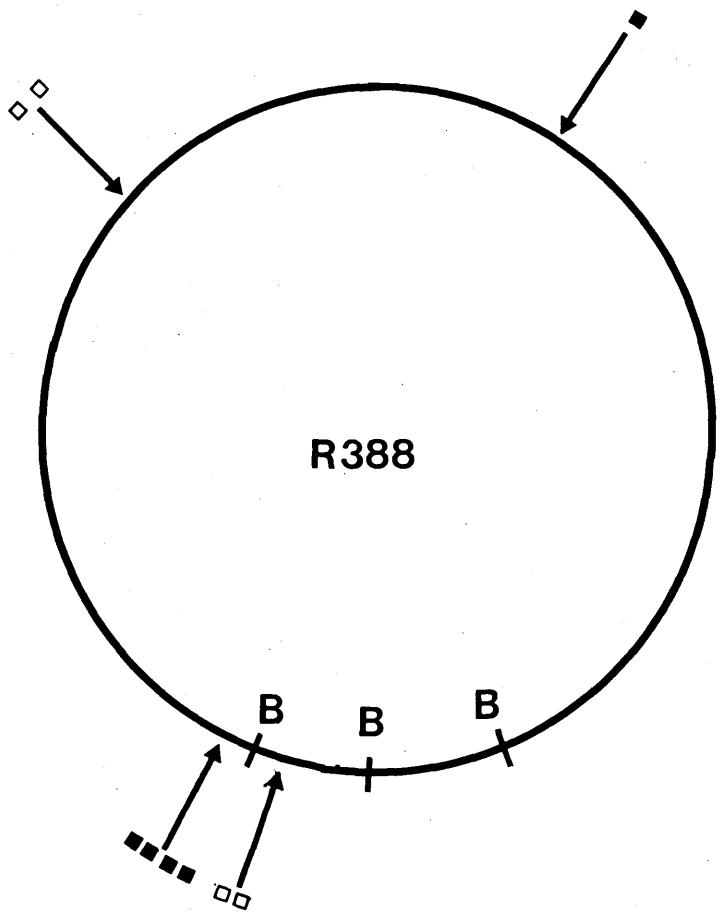

Figure 5.15.

Diagram showing approximate position of inserts of pPAK100, pBR322 and their derivatives into R388. All inserts are thought to be in the same orientation.

□ pPAK100 + pEN113
■ pBR322 + pEN201 + pEN202
B **BamHI**

Along with the cointegrates that were routinely seen when pPAK100 transposed, were another class of cointegrate which was larger than usual (see figure 5.16). These were only occasionally seen. Figure 5.17 shows one of these digested with four restriction enzymes. When cut with BamHI, EcoRI and HindIII they were in each case found to contain one band more than the standard one-ended cointegrate and it can be seen that the extra band is about 7.2Kb., which is the size of pPAK100. The PstI digest gives one new band of about 6.6Kb. and an extra copy of the 0.7Kb. band, adding up to the size of pPAK100. From this digest it appears that two copies of pPAK100 have been inserted into R388 in tandem array.

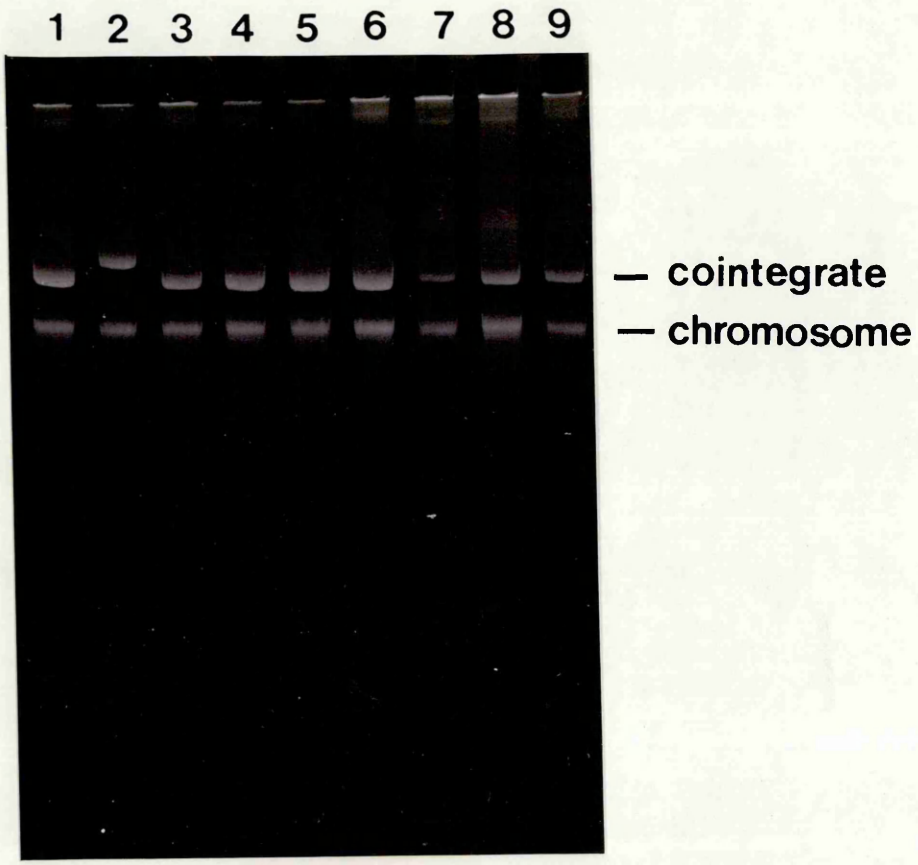

Figure 5.16.

0.8% agarose gel of cointegrates between pPAK100 and R388. The cointegrate band in lane 2. is higher, and therefore larger, than the others.

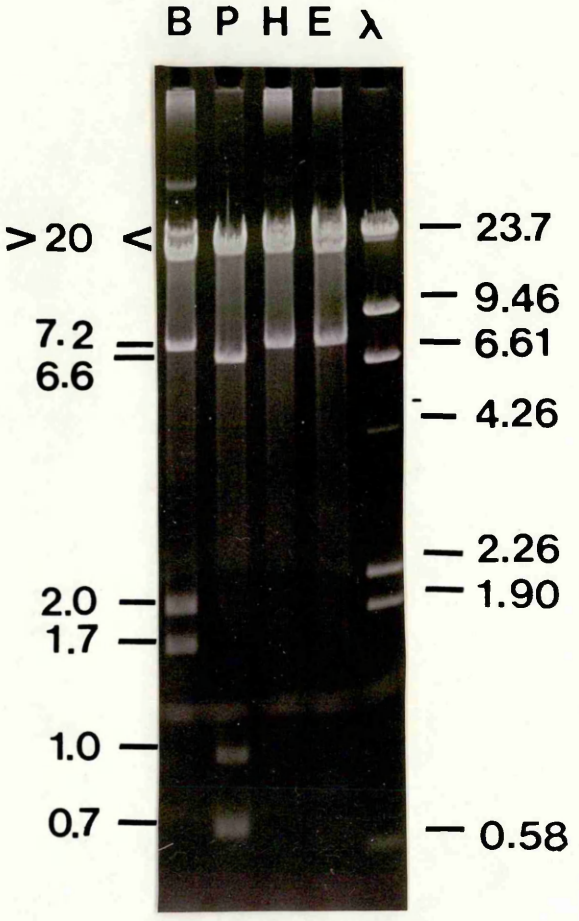

Figure 5.17.

Restriction enzyme digests of the large cointegrate between pPAK100 and R388.

B BamHI digest of cointegrate.
P PstI digest of cointegrate.
H HindIII digest of cointegrate.
E EcoRI digest of cointegrate.
λ lambda HindIII size markers.

In each of the BamHI, PstI, HindIII and EcoRI tracks there are two bands of a size greater than 20Kb., also there appear to be two copies of the PstI 0.7Kb. band.

5.5. DISCUSSION.

Models of Tn1/3 transposition have required the presence of both inverted repeats in *cis* and the *tnpA* gene product, transposase, in *cis* or in *trans*. Plasmids used in this chapter contain only one inverted repeat, but were able to transpose when supplied with transposase. The plasmids used were pBR322, pPAK100 and their deletion derivatives. (See chapter 3 for details) All plasmids are small and have a high copy number. The plasmid pBR322 has the right inverted repeat, the B-lactamase gene, and 27bp. of the *tnpR* gene of Tn3; while pPAK100 has the left inverted repeat, the *tnpA* gene, the *res* site, and 360bp. of the *tnpR* gene of Tn1. All of the deletion derivatives of these plasmids except pEN200 still have the inverted repeat.

In the presence of transposase, it was found that all of the plasmids containing an inverted repeat were able to transpose. Only pEN200 and pACYC184, which contain no transposon sequences, were not observed to transpose. The plasmid pPAK100 encodes its own transposase, but its deletion derivative pEN113, and all the pBR322 based plasmids, required transposase to be supplied in *trans*. When transposase was not provided, no transposition was ever detected. Figures 5.1 and 5.3 show the frequencies of transposition detected. In figure 5.3 the transposition frequencies of pBR322 and pPAK100 in the same cell are compared. Here the transposition frequency of pPAK100 varies from less than 10^{-7} to 8.6×10^{-4}, while the transposition frequency of pBR322

varies from 1.2×10^{-5} to 2.7×10^{-3}. In each case the transposition frequency of pBR322 is as good as or higher than that of pPAK100, which is providing the transposase. This may be due to several factors: the right inverted repeat may be a better substrate for transposase than the left inverted repeat; transposase may work better in *trans* than in *cis*; or Tn3 may be a better substrate for transposase than Tn1 (unlikely since the transposase has been produced by Tn1). The only sequence homology between the left and right halves of the transposon is at the inverted repeats.

The products of the one-ended transposition events are all cointegrates. These are seen in figures 5.2 and 5.4. They are larger than R388 and have a high copy number, which indicates that a fusion of the donor and recipient molecules has taken place. This is the first step in Tn1/3 transposition. In wild-type transposons, this cointegrate is then broken down by the action of resolvase on the *res* site. These one-ended transposition assays, however, are done in the absence of resolvase and in a *recA* strain, which results in the transposition process stopping at the cointegrate stage. Only pPAK100 contains the *res* site, and figure 5.5 shows cointegrates in the presence of a Tn1000 resolvase, which is able to break down Tn3 cointegrates. As can be seen from the gel, no breakdown of the cointegrate occurs, indicating that if any duplication of the transposon has taked place, then it has not proceeded as far as the *res* site, which is over 3Kb, from the end of the

transposon.

Figures 5.6 to 5.14 show digests of cointegrates of one-ended transposition events. The position of inserts of the donor into the recipient plasmid was mapped using the restriction enzymes BamHI, PstI, EcoRI and HindIII. From these results it was deduced that the donor molecule had inserted into many sites in the R388 plasmid. The cointegrates analysed were not sibs, as they were independently isolated from many separate mating experiments, but several inserts could be found clustered around the one region of R388. Because of the method of assaying for transposition, inserts will not be found in regions encoding Tp resistance or in genes required for transfer of R388, so this limits the sequences available for insertion of the donor plasmid. It is known that Tn1/3 has a preference for insertion into AT- rich regions although it does not seem to recognise any specific sequence. This preference for AT-rich regions and the limited number of sites available may account for the clustering of some inserts at the same site.

The 'break point' in the donor molecule appears to be the same in each case, between the inverted repeat and the plasmid DNA. No duplication can be detected by these restriction digests. If any duplication has occurred then it is not very extensive. All of the inserts into R388 have occured in the same orientation, but, since only nine cointegrates have been analysed it is not possible to entirely exclude the possibility

of insertions in the opposite orientation.

Tn1721, another member of the Tn3 family, has also been shown to transpose when only a single end is present (Motsch and Schmitt, 1984). Tn1/3 one-ended transposition has much in common with that of Tn1721, in which transposition occurs at frequencies 100 times lower than wild type; insertion occurs at many sites in the target molecule; and the cointegrates are joined at (or close to) the inverted repeat. The same phenomenon is also reported for Tn21. Avila et al (1984) have found one-ended transposition occurring in Tn21, and have found that most of the inserts contain the whole donor molecule and terminate after passing through the inverted repeat for the second time. They have proposed that termination could occur at sequences that are related to inverted repeat sequences. Machida et al (1982) have proposed that a transposon generated by IS102 has used a sequence found randomly in a pSC101 derivative as a terminal inverted repeat. In Tn3 there are several regions that show some homology (17 or 18/38bp.) to the inverted repeat sequence (by computer, Chris Boyd, pers. comm.). Although Tn1/3 transposase cannot cause transposition of Tn1000, which has inverted repeats that share 28bp. homology, it may be able to use a region of homology to complete the first step of transposition.

It has been suggested that transposition could occur by a rolling circle method (Harshey and Bukhari, 1981; Motsch et al, 1984), and this has been suggested by Avila et al to explain the

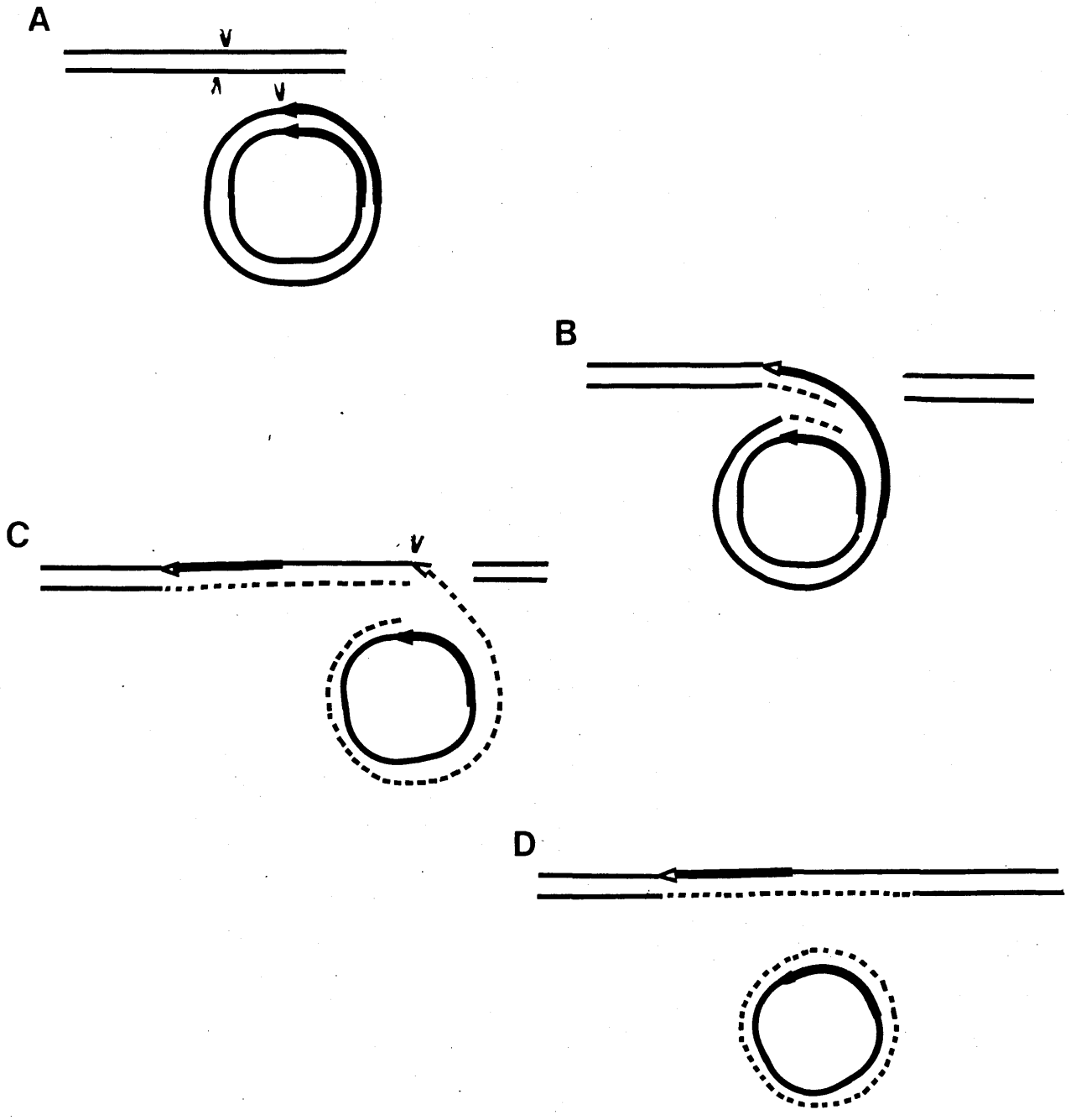

Figure 5.18.

Rolling circle model of transposition (from Motsch et al, 1985).

◀ inverted repeat
▬▬ transposon sequence
——— plasmid
------- newly synthesised DNA
∧ Sites of cleavage

one-ended transposition of Tn21. This model of transposition is shown in figure 5.18 , and could account for the one-ended transposition of Tn1/3. Alternatively, one-ended transposition could occur (in some cases) in a symmetric fashion, with transposase cutting at the inverted repeat and at a region that shared homology with the inverted repeat. Transposition could then proceed in the usual manner.

The rolling circle model could also account for the existence of the larger cointegrates occasionally found with Tn1. If the inverted repeat was not recognised by transposase the first time , then another round of replication occurring would result in the insertion of two contiguous copies of pPAK100. An alternative way of obtaining the larger cointegrates would be if a dimer of pPAK100 had transposed in a one-ended fashion, using one of its ends, resulting in the insertion of the whole dimer molecule. It seems very likely that transposase would have difficulty in recognising an inverted repeat in the wrong orientation since it has been shown that a transposon with two inverted repeats in direct orientation is unable to transpose normally, (Arthur et al, 1984).

Some Tn21 fusions are thought to contain one whole copy of the donor molecule, and for these it has been suggested that transposition occurs by the rolling circle method (Avila et al, 1984). Here transposase is thought to nick at the inverted repeat and then after one round of replication to nick again at

the same site. In many ways this is an attractive idea since it does not involve transposase having to recognise a sequence with poor homology to an inverted repeat, or having to cut at the 'wrong' side of the inverted repeat, and may account at least for some of the one-ended transposition events seem.

Sequencing studies done in this laboratory (A.Arthur pers.comm.) have shown that during one-ended Tn3 transposition, a 5 base pair duplication of the target DNA occurs, perhaps indicating that one-ended transposition occurs by the same mechanisms as 'normal' transposition. These sequencing studies have also shown that small regions of the transposon have been duplicated during one-ended transposition. Of the three examples sequenced, in one 13bp., in the second 32bp. and in the third 100bp. were duplicated. So in each case, all or part of the inverted repeat was duplicated during transposition.

Whether transposition occurs by a symmetric or asymmetric mechanism is so far impossible to tell. Where a large part of the transposon is duplicated, it is easy to see how transposase could cut at the inverted repeat and, either simultaneously or sequentially, cut at a region that has homology to an inverted repeat. However in the cases where the one-ended transposition events result in the duplication of only part of the inverted repeat, it is difficult to understand how transposase could simultaneously cut at two places that are only about 13bp. apart. In this case it seems easier to explain this transposition by the

asymmetric model - where transposase cuts first at one site and then after replication has occurred, cuts at the second site.

Further experiments need to be done to clarify whether Tn1/3 transposition occurrs by a symmetric or asymmetric pathway. Experiments designed to overproduce transposase have been done by Wishart et al (1985) and these show that transposase is able, in the presence of 8mM ATP, to bind specifically to fragments containing the inverted repeats of Tn3. In the absence of ATP transposase binds non-specifically to linear double-stranded DNA. Further experiments to show the exact site of binding and whether transposase binds to both inverted repeats with the same efficiency would be very informative. Another useful experiment would be to look at the products of transposition if DNA replication could be switched off. This experiment has been done by Craigie and Mizuuchi (1985) on the transposing bacteriophage Mu and it strongly suggests that Mu transposes by a symmetric pathway as transposition intermediates were found which resulted from DNA strand transfer at each end of the transposon. Structures resulting from strand transfer at only one end of the transposon were not seen.

CHAPTER 6

CONCLUDING REMARKS

Work by Lee *et al* (1983), has shown the 38bp. inverted repeats to be sufficient on their own for the establishment of transposition immunity. The results of this study have confirmed these findings, indicating that either of the inverted repeats is sufficient on its own to decrease the frequency of transposition into a plasmid by about two orders of magnitude. The removal of the inverted repeat, seen in plasmid pEN200, results in the complete abolition of transposition immunity. As well as maintaining normal levels of immunity, transposons with one end deleted are still able to transpose, albeit at a much reduced frequency. One-ended transposition occurs at frequencies 2-3 orders of magnitude less than "two-ended" transposition in *tnpR*$^-$ cells. Studies done in this laboratory (A. Arthur, pers. comm.) have shown that one-ended transposition results in 5bp. duplications in the target DNA, and in the duplication of small regions of transposon DNA.

Several of the one-ended transposition events were analysed and the inserts into R388 were mapped using restriction enzymes. The inserts occurred at many sites in the R388 plasmid, but the transposon sequences were always inserted in the same orientation. However, since the number of inserts studied was fairly small, it is not possible to exclude the existence of inserts in the opposite orientation.

The question of the mechanics of one-ended transposition still remains. The work of Craigie and Mizuuchi,(1985) strongly supports the theory that Mu transposition occurs via a symmetric

pathway and it would be very informative to repeat these experiments on Tn1/3.

One-ended transposition could be the result of either symmetric or asymmetric transposition. Some of the resulting structures are difficult to explain by the symmetric pathway as in these only a small part of the inverted repeat has been duplicated and transposase would have to cut at two sites close together in the inverted repeat. As yet it is not known if this is possible, but it may be, as transposase is also responsible for creating the 5bp. staggered nicks in the recipient DNA. In vitro studies on Tn1/3 transposition may help our understanding of the mechanics of transposition.

In conclusion, it would appear that the presence of one of the 38bp. inverted repeats is all that is required for the establishment of transposition immunity, and that one inverted repeat is also all that is required, when transposase is supplied in trans, for transposition to occur.

REFERENCES

Allet B. (1979)
Mu insertion duplicates a 5 base pair sequence at the host inserted site.
Cell Vol.16 123-129.

Arthur A., Nimmo E., Hettle S. and Sherratt D. (1984)
Transposition and transposition immunity of different transposon Tn3 derivatives having different ends.
E.M.B.O. Journal. Vol.3 1723-1729.

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

Auerswald E.A., Ludwig G. and Schaller H. (1980)
Structural analysis of Tn5.
Cold Spring Harbor Symp. Quant. Biol. Vol.45 107-114.

Avila P., de la Cruz F., Ward E. and Grinsted J. (1984)
Plasmids containing one inverted repeat of Tn21 can fuse with other plasmids in the presence of Tn21 transposase.
Mol.Gen.Genet. Vol.195 288-293.

Bachman B.J., Low K.B. and Taylor A.L. (1976)
Re-calibrated linkage map of Escherichia coli K-12.
Bacteriol.Rev.Vol.40 116-167.

Barth P.T., Datta N., Hedges R.W. and Grinter N.J. (1976)
Transposition of a deoxyribonucleic acid sequence encoding trimethoprim and streptomycin resistance from R483 to other replicons.
J.Bacteriol. Vol.125 800-810

Bennett P.M., Grinsted J. and Richmond M.H. (1977)
Transposition of TnA does not generate deletions.
Mol. Gen. Genet. Vol.154 205-212.

Bennett P.M. and Richmond M.H. (1976)
The translocation of a discrete piece of DNA carrying the Amp gene between replicons in Escherichia coli.
J.Bacteriol. Vol.126 1-6.

Bishop R. and Sherratt D. (1984)
Transposon Tn1 intra-molecular transposition.
Mol.Gen.Genet. Vol.196 117-122.

Birnboim H.C. and Doly J. (1979)
A rapid alkaline extraction procedure for screening recombinant plasmid DNA.
Nucl.Acids Res. Vol.7 1513-1523

Boeke J.D., Garfinkel D.J., Styles C.A. and Fink G.R. (1985)
Ty elements transpose through an RNA intermediate.
Cell Vol.40 491-500.

Bonas U., Sommer H. and Saedler H. (1984)
The 17Kb. Tam 1 element of Antirrhinum majus induces a 3bp.
dupiccation upon integration into the chalcone synthase gene.
E.M.B.O. Journal. Vol. 3 1015-1019.

Bolivar F., Rodriguez R., Greene P.J., Betlach M., Heynecker H.L., Boyer H.W., Crosa J. and Falkow S. (1977)
Construction and characterisation of new cloning vehicles:II: a multipurpose cloning system.
Gene Vol.2 95-113.

Bradley D.E., Taylor D.E. and Cohen D.R. (1980)
Specification of surface mating systems among drug resistance plasmids of Escherichia coli K-12.
J.Bacteriol. Vol.143 1466-1470.

Campbell A.(1976)
Evolutionary significance of accessory DNA elements in bacteria.
Ann.Rev.Microbiol. Vol.35 55-83.

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

Clements M.B. and Syvanen M. (1980)
Isolation of a polA mutation that affects transposition of insertion sequences and transposons.
Cold Spring Harbor Symp.Quant.Biol. Vol.45 201-204.

Clare J. and Farabaugh P. (1985)
Nucleotide sequence of a yeast Ty element: Evidence for an unusual mechanism of gene expression.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.82 2829-2833.

Craigie R., Arndt-Jovin D.J. and Mizuuchi K. (1985)
A defined system for the DNA strand-transfer reaction at the initiation of bacteriophage Mu transposition: Protein and DNA substrate requirements.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.82 7570-7574.

Craigie R. and Mizuuchi K. (1985)
Mechanism of transposition of bacteriophage Mu: structure of a transposition intermediate.
Cell Vol.41 867-876.

Datta N. and Hedges R.W. (1972)
Trimethoprim resistance conferred by W plasmids in Enterobacteriacae.
J.Gen.Microbiol. Vol.72 349-355.

Doring H.P. and Starlinger P. (1984)
Barbara McClintocks controlling elements: Now at the DNA level.
Cell Vol.39 253-259.

Garfinkel D.J., Boeke J.D. and Fink G.R. (1985)
Ty element transposition: Reverse transcriptase and virus-like particles.
Cell Vol.42 507-517.

Dugaiczyk A., Boyer H.W. and Goodman H.M.(1975)
Ligation of EcoR1 endonuclease generated DNA fragments into linear and circular structures.
J.Mol.Biol. Vol.96 171-184.

Ely B. (1982)
Transposition of Tn7 occurs at a single site on the Caulobacter Crescentus chromosome.
J.Bacteriol. Vol.151 1056-1058.

Fennewald M.A. and Shapiro J.A. (1979)
Transposition of Tn7 in Pseudomonas aeruginosa and isolation of alk Tn7 mutations.
J.Bacteriol. Vol.139 264-269.

Flavell A.J. and Ish-Horowicz D. (1981)
Extrachromosomal circular copies of the eukaryotic transposable element copia in cultured Drosophila cells.
Nature Vol.292 591

Foster T.J., Davis M.A., Roberts D.E., Takeshita K. and Kleckner N. (1981)
Genetic organisation of transposon Tn10.
Cell Vol.23 201-213.

Freeling M. (1984)
Plant transposable elements and insertion sequences.
Ann.Rev.Plant Physiol. Vol.35 271-298.

Galas D.J., Calos M.P. and Miller J.H. (1980)
Sequence analysis of Tn9 insertions in the LacZ gene.
J.Mol.Biol. Vol.144 19-42.

Gilboa E.,Mitra S.W., Goff S. and Baltimore D. (1979)
A detailed model of reverse transcription and a test of crucial aspects.
Cell Vol.18 93-100.

Gill R., Heffron F., Dougan G. and Falkow S. (1978)
Analsis of sequences transposed by complementation of two classes of transposition-deficient mutants of Tn3.
J.Bacteriol. Vol.136 742-756.

Grindley N.D.F. and Joyce C. (1981)
Analysis of the structure and function of the Kanamycin-resistance transposon Tn903.
Cold Spring Harbor Symp.Quant.Biol. Vol.45 125-133.

Grinsted J., Bennett P.M., Higginson S. and Richmond M.H. (1978)
Regional preference of insertion of Tn501 anf Tn801 into Rp1 and its derivatives.
Mol.Gen.Genet. Vol.166 313-320.

Guyer M.S. (1978)
The gamma-delta sequence of F is an insertion sequence.
J.Mol.Biol. Vol.126 347-365.

Habermann P, Klaer R, Kuhn S and Starlinger P (1979)
IS4 is found between 11 or 12 base pair duplications.
Mol.Gen.Genet. Vol.175 369-373.

Harshey R.M. and Bukhari A. (1981)
A mechanism of DNA transposition.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.78 1090-1094.

Hassan D.M. and Brevet J. (1983)
Absence of cis-acting transposition immunity with Tn7.
Plasmid Vol.10 31-44.

Hauber J., Nelbock-Hochsteffer P. and Feldman H. (1985)
Nucleotide sequence and characteristics of a Ty element from yeast.
Nuc.Acids Res. Vol.13 2745-2758.

Hauer B. and Shapiro J.A. (1984)
Control of Tn7 transposon.
Mol. Gen. Genet. Vol.194 149-158

Hedges R.W. and Jacob A.E. (1974)
Transposition of ampicillin resistance from RP4 to other replicons.
Mol.Gen.Genet. Vol.132 31-40.

Heffron F., Bedinger P., Champoux J.J. and Falkow S. (1977)
Deletions affecting the transposition of an antibiotic resistance gene.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.74 702-706.

Heffron F.,McCarthy B.J., Ohtsubo J.J. and Ohtsubo E. (1979)
DNA sequence analysis of the transposon Tn3: three genes and three sites involved in transposition of Tn3.
Cell Vol.18 1153-1163.

Hopkins J.D., Clements M.B., Liang T-Y., Isberg R.R. and Syvanen M. (1980)
Recombination genes on the E.coli sex factor specific for transposable elements.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.77 2814-2818.

Hyde D.R. and Tu C-P.D. (1985)
tnpM: A novel regulatory gene that enhances Tn21 transposition and suppresses cointegrate resolution.
Cell Vol.42 629-638.

Iida S, Hammi C, Echarti C, and Arber W. (1981)
Is the IS1 flanked r-determinant of the R-plasmid NR-1 a transposon?
J.Gen.Micro. Vol.126 413-425.

Iida S., Marcoli R. and Bickle T.A. (1981)
Variant insertion element IS1 generates 8-base pair duplications of the target sequence.
Nature Vol.294 374.

Isberg R.R., Lazaar A.L. and Syvanen M. (1982)
Regulation of Tn5 by the right-repeat proteins: Control at the level of the transposition reaction?
Cell Vol.30 883-892.

Isberg R.R. and Syvanen M. (1982)
DNA gyrase is a host factor required for transposition of Tn5.
Cell Vol.30 9-18.

Johnson R.C., Yin J.C.P. and Reznikoff W.S. (1979)
Control of Tn5 transposition in Escherichia coli is mediated by protein from the right repeat.
Cell Vol.30 873-882.

Johnsrud L., Calos M.P., and Miller J.H. (1978)
The transposon Tn9 generares a 9bp. repeated sequence during integration.
Cell Vol.15 1209-1219.

Jordan E, Saedler H and Starlinger P. (1968)
O^{o} and strong polar mutations in the gal operon are insertions.
Mol.Gen.Genet. Vol.102 353-363.

Kahmann R. and Kamp D. (1979)
The nucleotide sequences of the attachment sites of bacteriophage Mu.
Nature Vol.280 247-250.

Kitts P.A. (1982)
The transposition mechanism of Tn3.
Ph.D. Thesis. University of Glasgow.

Kitts P.A., Lamond A and Sherratt D.J. (1982a)
Inter-replicon transposition of Tn1/3 occurs in two sequential, genetically separable, steps.
Nature Vol.295 626-628

Kitts P.A., Symington L., Burke M., Reed R.R. and Sherratt D. (1982b)
Transposon-specified site-specific recombination.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.79 46-50.

Kopecko D.J. and Cohen S.N. (1975)
Site-specific recA independent recombination between bacterial plasmids: involvement of palindromes at the recombinational loci.
Proc. Nat. Acad. Sci. (U.S.A.) Vol.72 1373-1377.

Kostriken R., Morita C. and Heffron F. (1981)
Transposon Tn3 encodes a site-specific recombination system: Identification of essential sequences, genes, and actual site of recombination.
Pro.Nat.Acad.Sci.(U.S.A.) Vol.78 4041-4045.

Kretschmer P.J. and Cohen S.N. (1977)
Selected translocation of plasmid genes: Frequency and regional specificity of translocation of the Tn3 element.
J.Bacteriol. Vol.130 888-899.

Kuhn S., Fritz H-J. and Starlinger P. (1979)
Close vicinity of IS1 integration sites in the leader sequence of the gal operon of E.coli.
Mol.Gen.Genet. Vol.167 235-241.

Lee C-H., Bhagwat A. and Heffron F. (1983)
Identification of a transposon Tn3 sequence required for transposition immunity.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.80 6765-6769.

Lichtenstein C, and Brenner S. (1981)
Site specific properties of Tn7 transposition into the E.coli chromosome.
Mol.Gen.Genet. Vol.183 380-387.

Machida Y., Machida C. and Ohtsubo E. (1982)
A novel type of transposon generated by insertion element IS102 present in a pSC101 derivative.
Cell Vol.30 29-36.

McClintock B. (1951)
Chromosome organisation and gene expression.
Cold Spring Harbor Symp. Vol.16 13-47.

Messing J. (1983)
New M13 vectors for cloning.
Methods Enzymol. Vol.101 20-78.

Miller J.H., Calos M.P., Galas D.J., Hofer M., Buchel D. and Muller-Hill B. (1980)
Genetic analysis of transposition in the Lac region of Escherichia coli.
J.Mol.Biol. Vol.144 1-18.

Motsch S. and Schmitt R. (1984)
Replicon fusion mediated by a single-ended derivative of transposon Tn1721.
Mol.Gen.Genet. Vol.195 281-287.

Motsch S., Schmitt R., Avila P., de la Cruz F., Ward E. and Grinsted J. (1985)
Junction sequences generated by one-ended transposition.
Nucl.Acids Res. Vol.9 3335-3342.

Murphy E. (1983)
Inhibition of Tn554 transposition; Deletion analysis.
Plasmid Vol.10 260-269.

Murphy E. and Lofdahl S. (1984)
Transposition of Tn554 does not generate a target duplication.
Nature Vol.307 292-294.

Muster C.J., MacHattie L.A. and Shapiro J.A. (1983)
pλCM system: Observations on the roles of transposable elements in formation and breakdown of plasmids derived from bacteriophage lambda replicons.
J.Bacteriol. Vol.153 976-990.

Ohtsubo H., Ohmori H. and Ohtsubo E. (1979)
Nucleotide sequence analysis of Tn3(Ap): Implications for insertion and deletion.
Cold Spring Harbor Symp.Quant.Biol. Vol.43 1269-1277.

Reed R.R. (1981)
Resolution of cointegrates between transposons δδ and Tn3 defines the recombination site.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.78 3428-3432.

Reed R.R., Young R.A., Steitz J.A.,Grindley N.D.F. and Guyer M.S. (1979).
Transpositionof the Escherichia coliinsertionelement generates a five-base-pair repeat.
Proc.Nat.Acad.Sci.(U.S.A.). Vol.76 4882-4886.

Robertson D.S. (1980)
The timing of Mu activity in Maize.
Genetics Vol.94 969-978.

Robinson M.K., Bennett P.M. and Richmond M.H. (1977)
Inhibition of TnA translocation by TnA.
J.Bacteriol. Vol.129 407-414

Sanger F., Nicklen S. and Coulsen A.R. (1977)
DNA sequencing with chain-terminating inhibitors.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.74 5463-5467.

Sasakawa C., Uno Y. and Yoshikawa M. (1981)
The requirement for both DNA polymerase and 5' to 3' exonuclease activities of DNA polymerase I during Tn5 transposition.
Mol.Gen.Genet. Vol.182 19-24.

Schmitt R., Altenbuchner J.,Wiebauer K., Puhler A and Schoffl F. (1981)
Basis of transposition and gene amplification by Tn1721 and related tetracyclin-resistance transposons.
Cold Spring Harbor Symp.Quant.Biol. Vol.45 59-65.

Shapiro J.A. (1969)
Mutations caused by the insertion of genetic material into the galactose operon of Escherichia Coli.
J.Mol.Biol. Vol.40 93-105.

Shapiro J.A. (1979)
A molecular model for the transposition and replication of bacteriophage Mu and other transposable elements.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.76 1933-1937.

Sherratt D., Arthur A. and Burke M. (1981)
Transposon-specified site-specific recombination systems.
Cold Spring Harbor Symp. Quant. Biol. Vol.45 275-281.

Simons R.W., Hoopes B.C., McClure W.R. and Kleckner N. (1983)
Three promoters near the termini of IS10: pIN, pOUT and pIII.
Cell Vol.34 673-682.

Simons R.W. and Kleckner N. (1983)
Translational control of IS10 transcription.
Cell Vol.34 683-691.

Sinclair.,Sang J.H., Burke J.F. and Ish-Horowicz D. (1983)
Extrachromosomal replication of copia-based vectors in cultured Drosophila cells.
Nature Vol.306 198-200.

Stacey K.A. and Simpson E. (1965)
Improved method for the isolation of thymine requiring mutants of Escherichia coli.
J.Bacteriol. Vol.90 554-555.

Stanisich V.A., Bennett P.M. and Richmond M.H. (1977)
Characterisation of a translocation unit encoding resistance to mercuric ions that occurs on a nonconjugative plasmid in Pseudomonas cevosinosea.
J.Bacteriol. Vol.129 1221-1223

Sternglanz R., Dinardo S., Voelkel K.A., Nishimura Y., Hirota Y., Becherer K., Zumstein L. and Wang J.C. (1981).
Mutations in the gene coding for Escherichia coli DNA Topoisomerase I Affect transcription and transposition.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.78 2747-2751.

Sutcliffe J.G. (1978)
pBR322 restriction map derived from the DNA sequence : accurate DNA size markers up to 4361 nucleotide pairs long.
Nucl. Acids Res. Vol.5 2721-2728.

Sutcliffe J.G. (1979)
Complete nucleotide sequence of the Escherichia coli plasmid pBR322.
Cold Spring Harbor Symp. Quant.Biol. Vol.43 77

Vodkin L.O., Rhodes P.R. and Goldberg R.B. (1983)
A lectin gene insertion has the structural features of a transposable element.
Cell Vol.34 1023-1031.

Syvanen M., Hopkins J.D. and Clements M. (1982)
A new class of mutants in DNA polymerase I that affects gene transposition.
J.Mol.Biol. Vol.158 203-212.

Temin H.M. (1980)
Origin of retroviruses from cellular moveable genetic elements.
Cell Vol.21 599-600.

Todaro G.J., Callahan R., Rapp U.R. and DeLarco J.E. (1980)
Genetic transmission of retroviral genes and cellular oncogenes.
Proc.R.Soc.Lond. B210 367-385.

Tomizawa J-I. and Itoh T. (1981a)
Inhibition of ColEI RNA primer formation by a plasmid-specified small RNA.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.78 1421-1425.

Tomizawa J-I. and Itoh T. (1981b)
Plasmid ColEI incompatibility determined by interaction of RNAI with primer transcript.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.78 6096-6100.

Toussaint A. and Faelen M. (1974)
The dependance of temperate phage Mu-1 upon replication functions of E.coli K12.
Mol.Gen.Genet. Vol.131 209-214.

Tu C-P.D. and Cohen S.N. (1980)
Translocation specificity of Tn3: characterisation of sites of multiple insertions.
Cell Vol.19 151-160.

Wallace L.J., Ward J.R. and Richmond M.H. (1981a)
Thelocation of sequences of TnA required for the establishment of transposition immunity.
Mol.Gen.Genet. Vol.184 80-86

Wallace L.J., Ward J.R. and Richmond M.H. (1981b)
The tnpR product of TnA is required for transposition immunity.
Mol.Gen.Genet. Vol.184 87-91

Way J.C. and Kleckner N. (1984)
Essential sites at transposition Tn 10 termini.
Proc.Nat.Acad.Sci.(U.S.A.) Vol.81 3452-3456

Wishart W.L., Broach J.R. and Ohtsubo E. (1985)
ATP-dependent specific binding of Tn3 transposase to Tn3 inverted repeats.
Nature Vol.314 556-558.

Young R., Grillo D.S., Isberg R., Way J. and Syvanen M. (1980).
Transposition of the kanamycin-resistance transposon Tn903.
Mol.Gen.Genet. Vol.178 681-689.

Yanisch-Perron C., Vieira J. and Messing J. (1985)
Improved M13 'phage cloning vectors and host strains:
nucleotide sequences of the M13mp18 and pUC19 vectors.
Gene Vol.33 103-119.