TRANSPOSITIONAL PROPERTIES AND ORGANISATION OF TN7

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Mark Stephen Rogers

Institute of Genetics,
University of Glasgow,
Church St,
Glasgow.

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Contents

Abbreviations

Chemicals

APS - ammonium persulphate
ATP - adenosine triphosphate
BSG - buffered saline gelatin
CIP - calf intestinal phosphatase
CsCl - caesium chloride
DMF - dimethylformamide
DMS - dimethylsulphate
DNA - deoxyribonucleic acid
DTT - dithiothreitol
EDTA - ethylenediaminetetra-acetic acid (disodium salt)
EtBr - ethidium bromide
EtOH - ethanol
IPTG - isopropylthio-B-D-galactoside
NaAc - sodium acetate
RNA - ribonucleic acid
SDS - sodium dodecylsulphate
Tris - tris (hydroxymethyl) amino ethane
XGAL - 5-bromo-4-chloro-3-indolyl-B-galactoside

Antibiotics

Ap - Ampicillin
Cm - Chloramphenicol
Kan - Kanamycin
Nal - Naladixic acid
Rif - Rifampicin
Sp - Spectinomycin
St - Streptomycin
Tc - Tetracycline
Tp - Trimethoprim

Phenotypes

X^r - resistance to X

X^s - sensitive to X

Measurements

bp - base pairs

Kb - Kilo base pairs

A - amps

mA - milliamps

V - volts

mV - millivolts

Ci - curies

mCi - millicuries

uCi - microcuries

^oC - degrees centigrade

g - centrifugal force equal to gravitational acceleration

g - grammes

mg - milligrammes

ug - microgrammes

ng - nanogrammes

l - litres

ml - millilitres

ul - microlitres

cM - centimetres

mM - millimetres

nM - nanometres

M - molar

mM - millimolar

pH - acidity defined as $[-\log_{10}(\text{Molar concentration } H^+ \text{ ions})]$

min - minutes

sec - seconds

Miscellaneous

D&M - Davis and Mingioli

SC - supercoiled

OC - open circular

UV - ultraviolet light

Tn - transposon

WT - wild type

LMP - low melting point

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Summary

The intermolecular transposition of Tn7 was investigated and the products identified as simple inserts. Replicon fusions mediated by recA and recF independent homologous recombination were also recovered.

The frequency of transposition was dependent upon the presence or absence of a cloned hotsite (a specific and preferred point of integration in the E.coli chromosome which is also active when cloned into plasmids) in the target replicon. Transposition to hotsites was more efficient than transposition to plasmids (cold sites). However, the context of the donor (whether Tn7 was integrated in a hotsite or a cold site) also affected the frequency of transposition to hotsites though transposition to cold sites was independent of the donor context or copy number. Transposition from a hotsite to a hotsite was less frequent than transposition from a cold site to a hotsite. The copy number of the donor may influence the magnitude of this effect.

cis-acting and trans-acting functions required for transposition have been defined. Only the ends of the transposon were required in cis for efficient transposition. Three trans-acting functions were mapped and found to be required absolutely for transposition to both hotsites and cold sites. Two further trans-acting functions were localised; one was required for transposition to the hotsite while the other was required for transposition to cold sites. This result confirmed that two different, though probably related, transposition mechanisms are employed for Tn7 transposition. These trans-acting functions were correlated with polypeptides using minicells. Almost all of the available coding capacity of Tn7 which is essential for transposition is required to encode these polypeptides.

Finally, an E.coli strain with a mini-Tn7 inserted at the chromosomal hotsite was used to study the integration of Tn7 into secondary chromosomal sites. A preliminary analysis of ten insertions provided no evidence for the presence of preferred secondary sites. The failure to observe such sites was disscussed.

CHAPTER 1

INTRODUCTION.

1.1 General Introduction

Transposable elements are defined as specific DNA sequences which can move from one location to another in the genome. First proposed by McClintock (1950, 1951) to account for the pattern of variegation in maize kernels and leaves, they have since been found in a wide variety of prokaryotic and eukaryotic systems (for reviews see Shapiro, 1983; Kleckner, 1981).

Mu, the first example of a prokarotic element (Taylor, 1963), was identified because of its ability to cause mutations in E.coli. Some years later, many polar mutations were shown to be caused by the insertion of discrete segments of DNA, distributed throughout the genome (Jordan et al, 1968; Starlinger and Saedler, 1972; Shapiro, 1969). Antibiotic resistance genes, known to spread rapidly, were also shown to be translocated (Hedges and Jacob, 1974; Berg et al, 1975; Kleckner et al, 1975). At present, many transposons have been identified with a variety of phenotypes (Kleckner, 1981). Some are incapable of independent transposition and must be complemented by a functional and presumably related transposon (Ravetch et al, 1979; Miller and Cohen, 1980; Fischoff et al, 1980; Heffron, 1983). Others carry a multitude of accessory determinants such as antibiotic resistance, toxin and sugar utilization genes (So et al, 1979; So and McCarthy, 1980; Cornelius et al, 1978, 1979, 1981; Cornelius and Saedler, 1980).

1.2 General Properties of Transposons

With one exception, Tn554, all transposons studied so far, generate short 3 - 13 base pair direct repeats at the insertion site (Kleckner, 1981; Murphy and Lofdahl, 1984), thought to be caused by replication across staggered nicks in the target replicon (Grindley and Sherratt, 1978). The direct repeats are not required for subsequent transposition (Kleckner and Ross, 1978; Kleckner, 1979).

Nearly all transposons have short inverted repeats at their termini which are required in cis for transposition. The symmetry implied by these repetitions probably explains why insertion of these elements occurs in both orientations (Kleckner, 1981). Mu (and D108) and Tn554

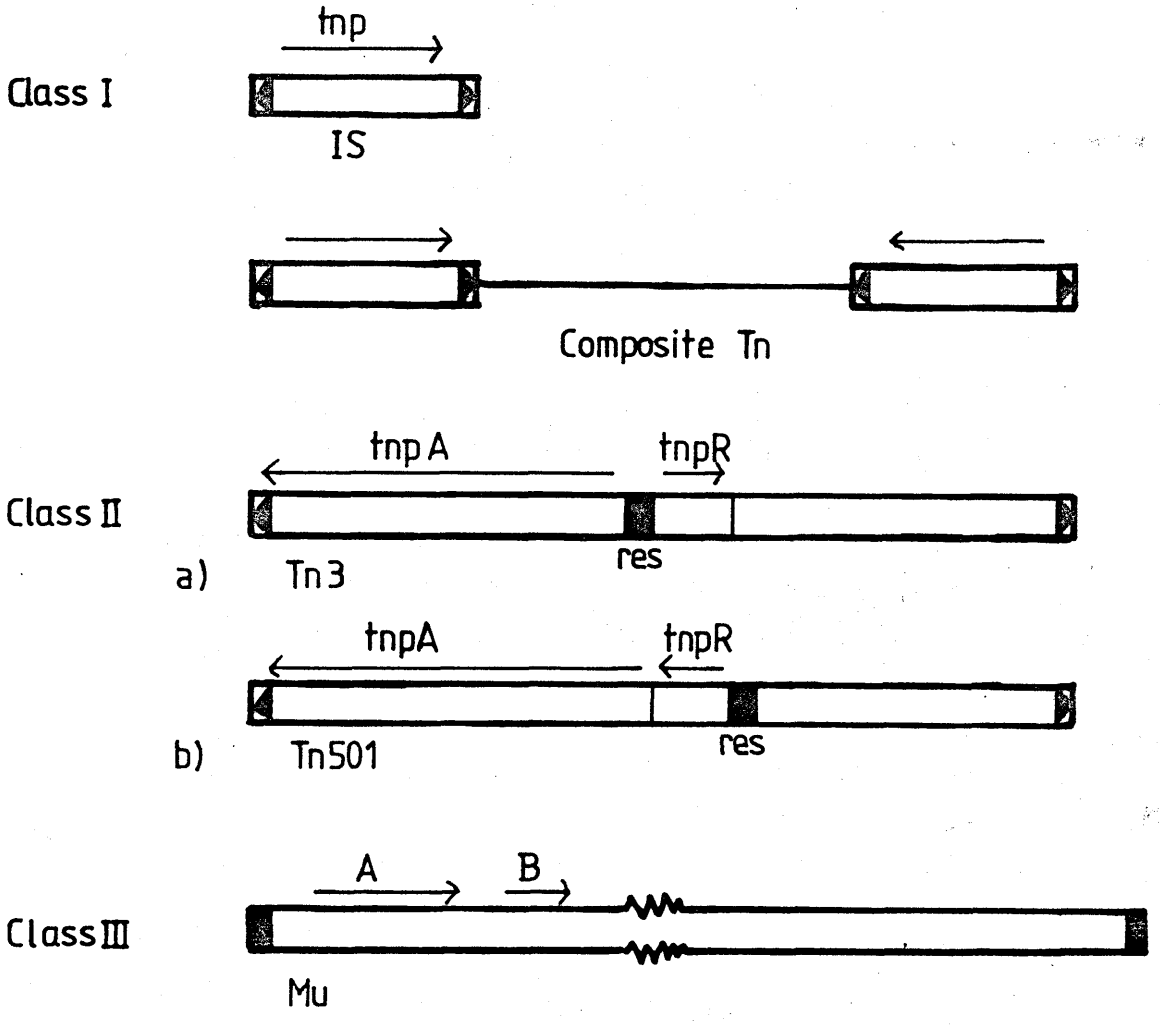

Figure 1.1 Illustration of the organisation of transposons in different classes (from Grindley and Reed, 1985). Arrows indicate direction of transcription and orientation. Inverted repeats are indicated with black triangles.

are exceptions (Kahmann and Kamp, 1979; Murphy and Lofdahl, 1984; Groenen et al, 1985). The ends of Mu are characterised by sequences which, though not defining the boundaries of the element, are required for transposition (Groenen et al, 1985).

1.3 Organisation of Transposons

Transposons have been placed into three classes which reflect their genetic organisation, sequence homology and possibly similar mechanisms of transposition. Figure 1.1 indicates the differences in structure between the different classes of elements, though elements like Tn7 and Tn554 cannot be placed into any of these classes (Kleckner, 1981; Grindley and Reed, 1985). Until recently, it was common to distinguish between transposable elements, like Tn3, and insertion sequences. This is no longer true and these terms are used interchangably.

1.3.1 Class I Elements: Insertion Sequences and Composite Transposons

Class I elements only encode functions related to their transposition. Composite elements encode additional accessory determinants which only transpose when bounded by insertion sequences in inverted or direct repeat. All the information required for transposition is located within the IS modules at either end of the accessory determinants and these modules are capable of transposition independently of the central segment (Kleckner, 1981). Some IS modules like IS50L have lost the ability of independent transposition.

Each insertion sequence encodes at least one protein, referred to as a transposase, required for its transposition, which probably acts at the ends of the element (Foster et al, 1981; Grindley and Joyce, 1980; Isberg and Syvanen, 1981). IS50 encodes a second product using an alternative initiation site 120bp 3' to the transposase start, which acts to regulate transposition (Johnson and Reznikoff, 1981; Johnson et al, 1982; Isberg et al, 1982; Rothstein et al, 1980a, 1980b; Rothstein and Reznikoff, 1981; Rossetti et al, 1984).

1.3.2 Class II Elements: The Tn3 Family

This class of related transposons can be divided into two subgroups according to their organisation and their ability to cross complement each other; Tn3 and Tn501 being examples from each subgroup.

All class II elements encode products which are required for transposition. They generate five base pair duplications at the target. Tn21, and possibly Tn501, encode a third protein which acts as a positive regulator of transposition (Hyde and Tu, 1985). Class II elements have 38 - 40 base pair inverted repeats at their ends and the accessory determinants are not segregated from the transposition functions but form an integral part of the main body of the transposon (see Kleckner, 1981; Grindley and Reed, 1985). Transposition occurs via an obligate cointegrate intemediate (Arthur and Sherratt, 1979; Muster and Shapiro, 1980; McCormick et al, 1981). The tnpA gene product, or transposase, acts to fuse the donor and recipient replicons in the form of a cointegrate (direct repeats of the transposon separated by the donor and recipient replicons (figure 1.2C)) which is resolved via the action of the tnpR gene product, or resolvase, upon the res site (Grindley, 1983; Heffron, 1983; Gill et al, 1978, 1979; Arthur and Sherratt, 1979; Kitts et al, 1981, 1982). The tnpR gene acts as a resolvase and as a repressor of both tnpA and tnpR transcription (Chou et al, 1979a, 1979b).

In the Tn3 subgroup, the transposition genes are transcribed divergently from a single regulatory region called res (Chou et al, 1979a, 1979b; Gill et al, 1979; Heffron et al, 1979) which is also the site of action of the resolvase enzyme (Reed, 1981; Kostriken et al, 1981).

In the Tn501 subgroup, the tnpA and tnpR genes are transcribed in the same direction. These gene products are interchangable between a number of similar elements (Diver et al, 1983; Rogowsky and Schmitt, 1984; Grinstead et al, 1982). Tn21 has a third gene, tnpM, which negatively regulates tnpR and positively regulates tnpM, probably at the level of transcription (Hyde and Tu, 1985).

1.3.3 Class III Elements: The Transposing Bacteriophages

Mu and D108 are similar, though heteroimmune phages (Kleckner, 1981). Figure 1.1 shows the organisation of the transposition functions of Mu. Replication of the Mu genome occurs by repeated cycles of transposition and the two events are genetically indistinguishable. Five base pair duplications of the target site occur during Mu transposition, which is generally replicative. Cointegrates are the major product of transposition; simple inserts are rarely observed (Chaconas et al, 1981; Howe and Schumm 1980). However, the initial integration event after infection is conservative (Liebart et al, 1982; Akroyd and Symonds, 1983; Harshey, 1984).

Mu encodes two proteins required for normal transposition, MuA and MuB which are 77Kd and 33Kd respectively. MuA is essential for transposition while MuB acts as an accessory protein which is required for full activity (Wijffelman and Lotterman, 1977; Toussaint and Faelen, 1973; Faelen and Toussaint, 1973; Faelen et al, 1979).

MuA and MuB lie in the left end but are separated from the boundary by a regulatory region encoding a phage repressor gene and immunity function (van de Putte, 1980; Howe and Bade, 1975).

1.4 Regulation of Transposition.

It is clear that transposition is a tightly regulated process as uncontrolled proliferation of transposons (which are generally capable of over replicating their host) is not observed. Transposition is normally rare, ocurring between 10^{-4} and 10^{-8} per cell per generation (Kleckner, 1981). Precise excision of an element is not a major regulatory mechanism. The frequency of insertion is orders of magnitude greater than the frequency of precise excision. Similarly, the presence of many unoccupied insertion sites within the genome shows that saturation of potential targets does not limit the transposition frequency (Nymann et al, 1981).

For a number of elements control is exerted at the level of transcription and translation (Trinks et al, 1981 (IS4); Casadaban et al, 1982 (Tn3); Raleigh and Kleckner, 1986; Simons et al, 1983

(Tn10)). Transposons from all classes encode regulatory proteins which act to limit transposition. For Mu and the class II elements this occurs by repressing the transcription of genes essential for transposition (Howe and Bade, 1975; Toussaint and Resbois, 1983; Chou et al, 1979a, 1979b; Gill et al, 1979; Kitts et al, 1981). In Tn5 a second polypeptide which is translated from the same reading frame as the transposase gene inhibits transposition, either by complexing with the transposase or competing for the transposon ends (Rothstein and Reznikoff, 1981; Biek and Roth, 1980; Reznikoff, 1982; Johnson et al, 1982; Isberg et al, 1982). Tn21 encodes a regulatory protein, in addition to resolvase, which acts to increase the level of transposition and to decrease the level of resolution (Hyde and Tu, 1985).

Host encoded functions, notably the adenosine methylation gene dam have also been implicated in the regulation of transposition and indicate for Tn10 at least, that the state of the transposon DNA can trigger transposition (Kleckner et al, 1984; Roberts et al, 1985). Such a mechanism probably ensures that transposition only occurs after host replication of the transposon and may allow a conservative 'donor suicide' transposition event to appear replicative (Kleckner et al, 1984; Bender and Kleckner, 1986). Tn5 and Tn903 are also regulated by dam methylation (Kleckner et al, 1984).

Class I elements, exhibit a cis-preference for transposase action; transposition is complemented inefficiently in trans (Machida et al, 1982; Grindley and Joyce, 1980; Foster et al, 1981; Isberg and Syvanen, 1981; Morisato et al, 1983).

Class II elements exhibit a phenomenon termed transposition immunity; insertion of a second copy of a transposon is extremely rare into a replicon already possessing a copy or even a single end of that transposon (Sherratt et al, 1980; Wallace et al, 1980; Robinson et al, 1977). Transposition immunity has been described for Tn7 though it does not belong to this class of elements and there is some dispute over the magnitude of the effect (Hauer and Shapiro, 1984; Hassen and Brevet, 1984; N Craig, pers. comm.).

A regulatory property unique to Mu (and D108) is the stoicheiometric

activity of the MuA protein. Upon release of a DNA replication block at the time of Mu induction and the simultaneous inhibition of protein synthesis, only a single round of Mu replication (and transposition) occurs (Pato and Reich, 1982, 1984). Continuous synthesis of the MuA protein is required for sustained transpositional activity.

1.5 Insertional Specificity of Transposons.

Transposable elements show a large variation in their insertional specificity. IS4, Tn554, have a very limited target specificity. IS4 has only been found in three sites within the E.coli chromosome. Homology between these targets exists but it is not at the site of integration (Klaer et al, 1980, 1981; Klaer and Starlinger, 1980; Pfeifer et al, 1977). Tn554 has a single insertion site in the chromosome of S.aureus (Phillips and Novick, 1979; Krolewski et al, 1981). Tn7 also has an extreme preference for a specific site in the chromosome of E.coli and other genera, called the hotsite, but it inserts into many sites in conjugative plasmids (Lichtenstein and Brenner, 1981, 1982; Barth and Grinter, 1977; Barth and Datta, 1977; Barth et al, 1978; Turner et al, 1984; Ely, 1982; Thomson et al,1981). It will also insert into the E.coli chromosome at other sites when the hotsite is occupied or deleted (C Lichtenstein, pers. comm.; N Craig, pers. comm.; chapter 6).

IS5 and Tn10 insert randomly over large regions of the genome but fine mapping of insertion sites reveals a local sequence specificity (Kleckner et al, 1979; Foster, 1977; Noel and Ames, 1978; Halling and Kleckner, 1982; Engler and Van Bree, 1981). Palindromic consensus sequences for these sites have been derived (Engler and Van Bree, 1981; Halling and Kleckner, 1982).

Tn3, Mu and Tn9 insert at many different sites within short preferred regions (Kleckner, 1981; Weinstock et al, 1979; Miller et al, 1980; Bukhari and Zipser, 1976; Saedler et al, 1980). This specificity may reflect a preference for A+T rich regions and/or homology of the target site with the ends of the transposon (Tu and Cohen, 1980; Miller et al, 1980).

The different specificities observed for different transposons

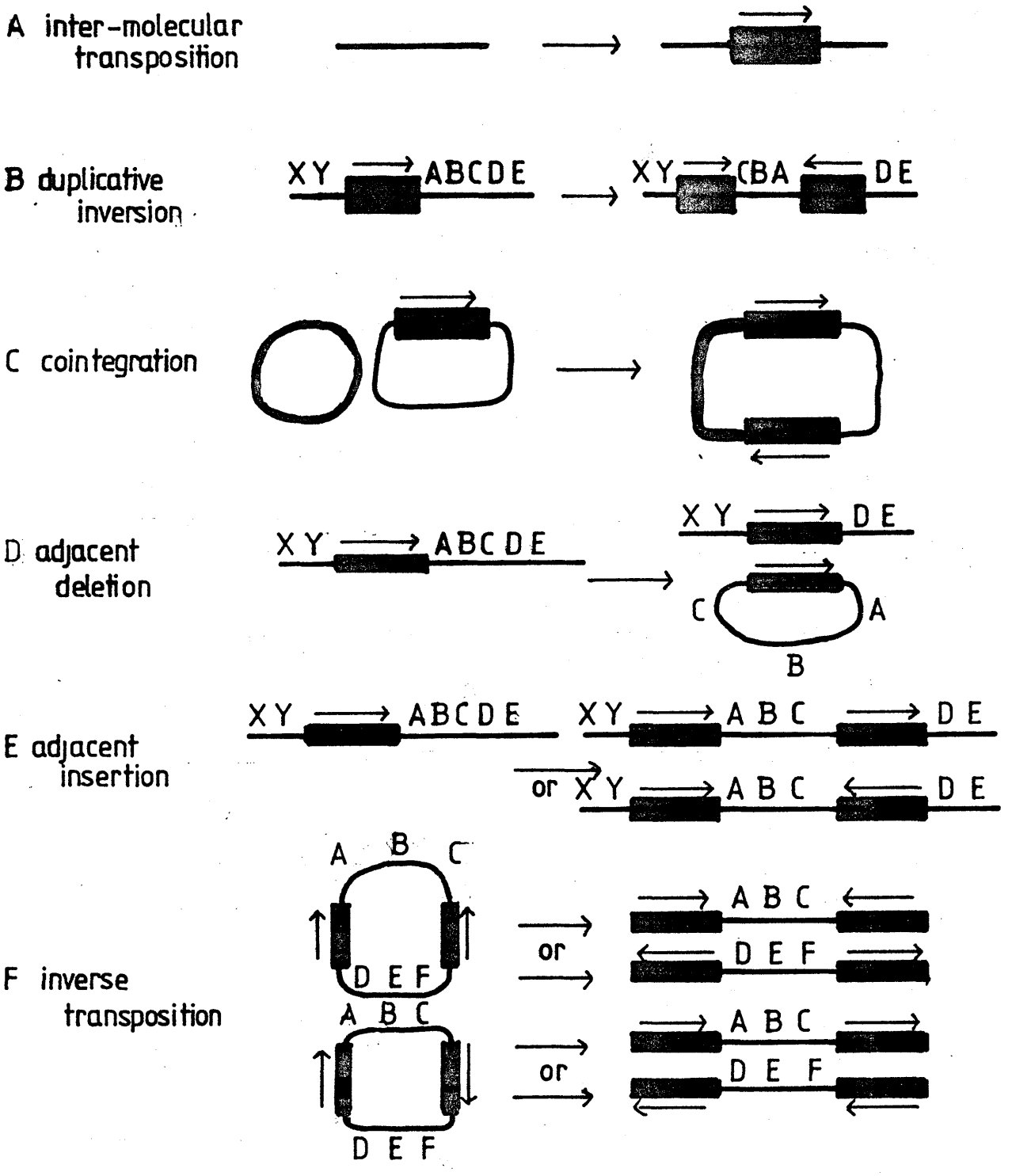

Figure 1.2 Illustration of transposon mediated DNA rearrangements (from Kleckner, 1981). Letters represent genes external to the element. Arrows indicate relative orientation of the element. Thick boxes represent the transposon.

suggests that transposon encoded functions are involved in the recognition of the target site.

1.6 The Consequences of Transposition: Implications for the Mechanism of Transposition

Transposition of an element from one location to another is only one of a number of genetic rearrangements, both intermolecular and intramolecular, mediated by transposons (figure 1.2). The major intermolecular products of transposition are simple inserts (figure 1.2A) and cointegrates (figure 1.2C). Simple insertions result from the integration of a transposon into a new site in a target replicon. Both ends of the element form novel joints with the target. Cointegrates, on the other hand, fuse donor and target replicons via direct repeats of the element. One of the original donor-element boundaries is retained while the other end is joined to new sequences in the target replicon. Resolution of a cointegrate by homologous or site-specific recombination results in products identical to those of a simple insertion. Both of these intermolecular events are mediated by transposons from every class (Kleckner, 1981; Toussaint and Faelen, 1973; Gill et al, 1978; Ohtsubo et al, 1980b; Galas and Chandler, 1982; Chaconas et al, 1981).

Replicative (duplicative) inversions (figure 1.2B) and adjacent deletions (figure 1.2D) are alternative products of an intramolecular cointegration which depends upon the relative orientation of the target to the transposon ends (Cornelius and Saedler, 1980; Faelen and Toussaint, 1978; Chaing and Clowes, 1980; Weinert et al, 1983, 1984; Othsubo et al, 1978; Mickel et al, 1977; Ross et al, 1979; Reif and Saedler, 1977).

A fifth rearrangement, adjacent insertion (figure 1.2E), may be either intermolecular or intramolecular and is the product of a simple insertion event into a replicon which already contains a copy of the transposing element (Saedler et al, 1980; Clowes et al, 1980). If transposition of the element is nonreplicative this product can only arise via intermolecular transposition; donor suicide would prevent the detection of any intramolecular events of this type.

The sixth transposon mediated event is specific to the composite forms of class I elements (or of class II and class III which are known to exist (Dobritsa et al, 1981, 1983; Faelen et al, 1978b)) and is termed inverse transposition (figure 1.2F). It is mediated by a transposition of the replicon excluding those sequences between the IS modules. When the target lies on the same molecule as the donor (actually within the inverse transposon), an inversion deletion occurs (Foster et al, 1981; Chandler et al, 1977; Rosner and Guyer, 1980; Reif, 1980). If the target lies on a different replicon, a cointegrate like structure is formed which lacks the accesssory determinants (Harayama et al, 1984).

All of these products occur in the absence of the recA gene product indicating that they are transposon mediated. However, in a rec^+ background, they can be derived by a combination of transposition and homologous recombination (Kleckner, 1981).

Events which involve the cointegrate pathway must be replicative; the transposon is duplicated. Products generated by a simple insertion process may be either replicative or nonreplicative. The presence of cointegrates, adjacent deletions and duplicative inversions indicate that a replicative cointegrate pathway of transposition is employed. The absence of these events suggests that a simple insertion pathway is used. Some transposons can use either a cointegrate or a simple insertion mechanism (Mu, IS1, IS903).

Cointegrates are not observed during transposition of IS50 (Tn5) and IS10 (Tn10) (Berg, 1983; Harayama et al, 1984; Hirschel et al, 1982a, 1982b; Isberg and Syvanen, 1985). Similarly, deletions are mediated by IS10 and IS50 at low efficiency (Weinert et al, 1984). These deletions are believed to occur via a simple insertion pathway involving inverse transposition (Ross et al, 1979; Weinert et al, 1984; Isberg and Syvanen, 1985). Other insertion sequences such as IS1 and IS903 do promote cointegrates, adjacent deletions and replicative inversions (Grindley and Joyce, 1980; Ohtsubo et al, 1980b; Weinert et al, 1984).

These data suggest that Tn5 and Tn10 transpose by a simple insertion process. For Tn10 at least, this process is generally nonreplicative

(Morisato and Kleckner, 1984; Bender and Kleckner, 1986). IS1 and IS903 must, at least occasionally, transpose using a replicative cointegrate process, though unlike the class II elements this cannot be via a obligate cointegrate intemediate unless resolution occurs only during the transposition event; cointegrates mediated by these elements are stable in recA$^-$ backgrounds but occur at only 1% - 5% of the frequency of simple inserts (Grindley and Reed, 1985; Galas and Chandler, 1982).

Class II elements generally transpose via a replicative cointegrate pathway. Transposition occurs by a two step mechanism. The first, which requires the ends of the transposon and the tnpA gene product, generates a cointegrate. The second, requires the internal resolution site, res, and the product of the tnpR gene which breaks down these cointegrates by site-specific recombination to give products that resemble simple inserts (Grindley, 1983; Heffron, 1983; Arthur and Sherratt, 1979; Gill et al, 1978, 1979; Kitts et al, 1981, 1982). Adjacent deletions and replicative inversions which do not require the tnpR gene product are observed with Tn3 (Bishop and Sherratt, 1984). This is consistent with these deletions being mediated by the cointegrate pathway.

Mu transposition is generally replicative and the end products are those associated with the cointegrate pathway (Chaconas et al, 1981; Howe and Schumm 1980; Ljungquist and Bukhari, 1977; Coelho et al, 1980).

Initial insertion of Mu into the host genome is conservative and is a simple insertion event (Liebart et al, 1982; Akroyd and Symonds, 1983; Harshey, 1984). However, this event is atypical because the infecting DNA is linear though held in circular form by proteins (Puspurs et al, 1983).

Evidence from in vitro transposition of Mu has determined that transposition can be replicative or conservative (with only limited replication of the ends) and that the intermediates obtained are consistent with the symmetrical model of transposition discussed below (Mizuuchi, 1983, 1984; Craigie and Mizuuchi, 1985; Craigie et al, 1984, 1985; Arthur and Sherratt, 1979; Shapiro, 1979;, Ohtsubo et al,

1980b).

In vitro studies of Mu transposition have also provided a mechanism by which the orientation of the ends relative to each other is sensed. The two step synapsis model (Boocock *et al*, 1986), used to explain the orientation dependent nature of resolvase action (it only acts on direct repeats), has been applied to Mu (Craigie and Mizuuchi, 1986). Ends of Mu in inverted orientation form a topological configuration that is required for the reaction to proceed. If the ends are in the wrong orientation, or are on different molecules, this topological configuration is unstable and the reaction aborts. If the correct topology is established prior to the ends being placed on different molecules or in the incorrect orientation, the reaction proceeds.

1.7 Models of Transposition

Models developed to account for the transpositional process may be divided into two major groups; conservative, where replication of the entire element does nor occur, and replicative which does involve replication of the element. Replicative mechanisms are further divided into symmetric and asymmetric depending on whether events are initiated at both ends or at one end of the element respectively. All of these models have been comprehensively discussed recently so that a full description will not be presented here (Grindley and Reed, 1985).

The conservative cut and paste model was proposed to account for the transposition of Tn5 (Berg, 1977). It can be applied to other transposons which do not promote cointegrates or their intramolecular equivalents. This model suggests that double strand breaks occur at the ends of the element and at the target site, which are held in contact by the transposase. These breaks may occur together or at different times. The element is ligated to the target replicon and the remainder of the donor is released as a linear molecule which is degraded.

The second mechanism, an asymmetric replicative model, was developed to account for the presence of both simple inserts and cointegrates (Harshey and Bukhari, 1981; Galas and Chandler, 1981) which in the model are alternative end products of a branched pathway. The model

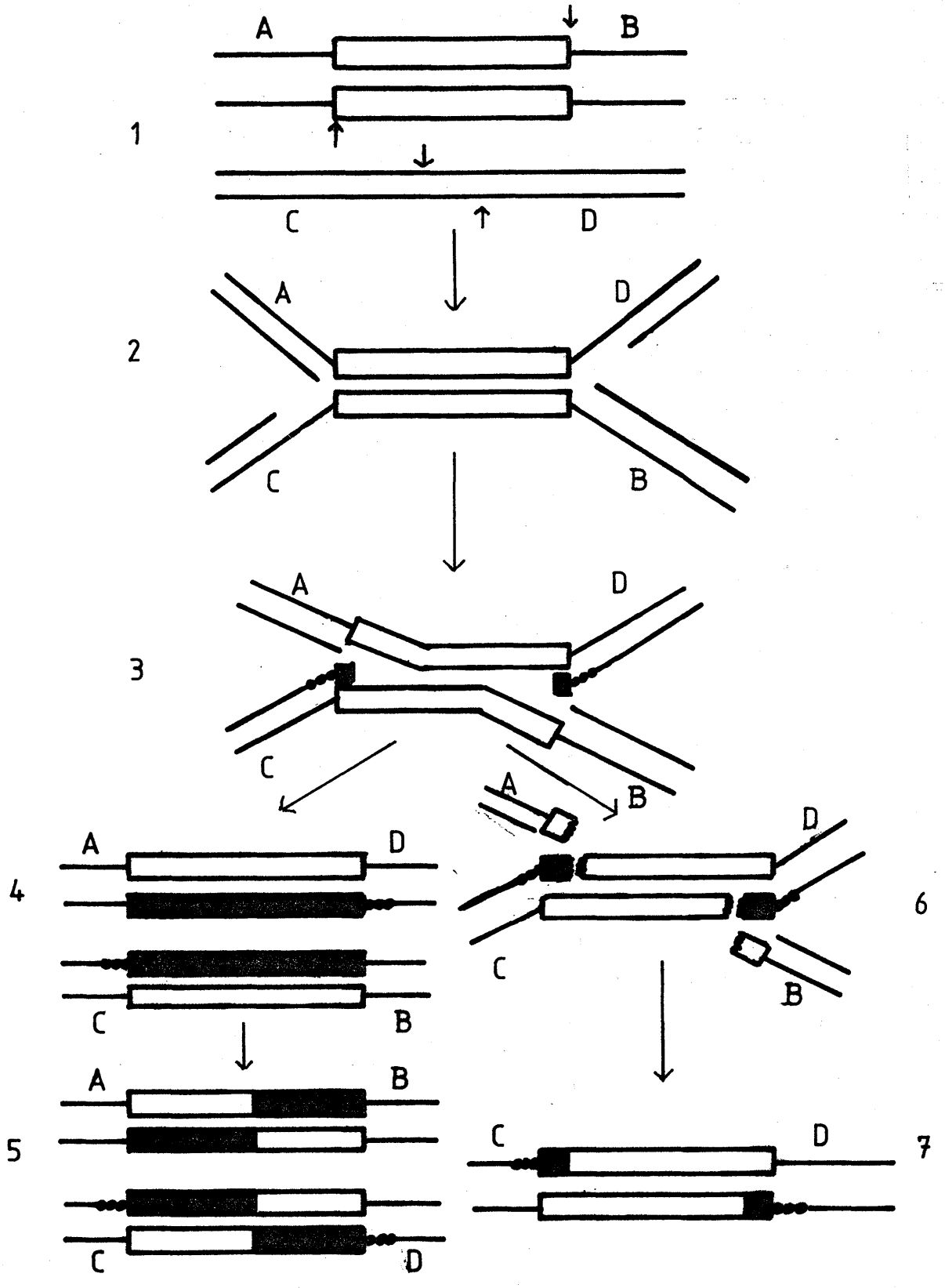
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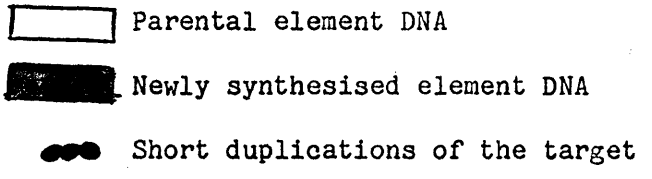
Figure 1.3 Symmetric model of transposition (from Grindley and Reed, 1985). Strand transfer is initiated at the 3' end (though 5' transfer is not excluded, see text). 1) Single strand cleavage at the transposon end and staggered double strand cleavage at the target. 2) Transfer of transposon ends to target forms two replication forks (3). Then either 4) replication across the element followed by sealing of replicated DNA generates a cointegrate which is 5) resolved via site-specific recombination. Alternativily, 6) premature termination of replication forks, after repair of staggered breaks, by breakage and transfer of the second strand of the donor and 7) repair of gaps produces a nonreplicative simple insert.



can be divided into three steps; initiation, replication, and termination. After a single strand of the element has been transferred and joined to the target, rolling circle type replication of the element is envisaged, which carries the transposase to the opposite end of the element. Depending on which strand (parental or nascent) is joined to the remaining target strand, a simple insert or a cointegrate will result.

The third model, the symmetrical replicative model, initially proposed that all transposition events occured via a cointegrate which was subsequently broken down by site-specific or homologous recombination (Arthur and Sherratt, 1979; Shapiro, 1979). It was later modified by Ohtsubo *et al* (1980b) to allow for the direct formation of nonreplicative simple inserts. This model is illustrated in figure 1.3.

A conservative mechanism does not account for the formation of cointegrates and their intramolecular analogs. It cannot apply generally to all transposons. Mu, IS903, IS1 must be capable of replicative transposition and it seems unlikely that these elements can mediate transposition by two separate mechanisms. The data regarding the transposition of IS10 and IS50 are compatible with a conservative cut and paste mechanism.

It has become clear that the asymmetric model cannot account for all the current data (Grindley and Reed, 1985). Firstly, it predicts that both simple inserts and cointegrates are products of a replicative process. *In vitro* studies with Mu and *in vivo* studies with Tn10 indicate that no extensive replication of the element occurs during the formation of simple inserts (Craigie and Mizuuchi, 1985; Bender and Kleckner, 1986). Secondly, simple insertions could be either intermolecular or intramolecular under this model. Intramolecular simple inserts are rarely, if ever, observed. Thirdly, the transposase was proposed to move across the element with the replication fork. Inverse transposition of Tn10 requires that the target site lies within the transposon. Cleavage of the target site would disrupt replication across the element. Also, the length dependence of transposition, which was believed to support this model, indicates that the distance between the ends of the transposon by any

path is critical and not the length of the element itself (Rosner and Guyer, 1980; Chandler et al, 1982; Morisato et al, 1983; Biel and Berg, 1984; Way and Kleckner, 1985). Finally, Tn10 has been shown to distinguish between available ends (Morisato and Kleckner, 1984). It interacts preferentially with the most active termini and the use of a less active terminus is supressed even if it lies between more active ends. Under the asymmetric model, the weaker termini should be used with an efficiency that is dependent only on the position of a third end relative to itself and not on its activity.

The modified symmetrical transposition model (Shapiro, 1979; Arthur and Sherratt, 1979; Ohtsubo et al, 1980b) provides a mechanism which can account for the current data and direct confirmation of this model has been obtained for Mu in vitro (Mizuuchi, 1983; Craigie and Mizuuchi, 1985). Reversal of the polarity of strand transfer shown in figure 1.3 would account for the absence of simple inserts during transposition of the class II elements (Kleckner, 1981; Grindley and Reed, 1985) and the variable ratio of simple inserts to cointegrates seen for different transposons may be due to the variable efficiencies with which replisomes are formed at the transposon termini (Grindley and Reed, 1985). Alternatively, this ratio may be influenced by the ability of the transposase to promote a second cleavage of the single stranded region attaching the donor replicon to the transposon after initial strand transfer.

1.8 Transposable Elements and the Genome

One of the questions still under debate is the role that transposons play within the host cell. It is apparent that transposons which encode accessory determinants like antibiotic resistance can provide a selective advantage to their host. The rapid spread of these genes among enteric bacteria correlates well with the ubiquitous use of antibiotics in medicine and for promoting growth among farm animals (Datta et al, 1980). However, such selection is not directed at the element but rather at the accessory determinant. Maintenance of the element is not required.

Chemostat studies reveal that a number of elements are capable of conferring a growth advantage (Biel and Hartl, 1983; Hartl et al,

1983; Chao et al, 1983). For IS10 this advantage results from mutations caused by insertion of the element into a specific gene (Chao et al, 1983) while in Tn5 the advantage was due to the presence of the transposase gene, under conditions where carbon was limiting, though transposition was not required (Biel and Hartl, 1983; Hartl et al, 1983). Similar effects have been observed for P1, P2 and Mu lysogens (Eldin et al, 1977).

The role of transposons in the generation of rearrangements in the genome has been suggested as the reason for their survival. The ability to reorganise other unrelated sequences either directly via transposition or indirectly by acting as regions of homology for the host encoded recombination system may be a selectable phenotype (see Campbell, 1981)

Generally, the transposition process leads to an overall increase in the number of copies of a transposon within a cell. The ability of transposons to 'over-replicate' their host has led to the proposal that the maintenance of an element in the genome is due solely to the difficulties of eliminating all copies of the element simultaneously (Doolittle and Sapienza, 1980; Orgel and Crick, 1980; Doolittle, 1982). Selection for elements which have developed strategies to prevent their deletion, such as over-replication, may be sufficient to explain their presence in the genome. Comparison of the transposition frequency (where the donor site remains occupied) with the excision frequency indicate that this situation does pertain within a cell (even for transposons which transpose nonreplicatively).

1.9 The Transposon Tn7

Tn7 has only recently been studied with the intent of understanding its organisation and transposition. It is a large 14Kb element with interesting and unique properties. It was first identified as conferring streptomycin (St), spectinomycin (Sp) and trimethoprim (Tp) resistance to the conjugative plasmid R483 (Hedges et al, 1972) and later shown to be capable of translocating these resistances to the E.coli chromosome and other plasmids with a concomitant increase in the target replicons size (Barth et al, 1976). Within the sensitivity of P1 transduction, independent insertions into the E.coli chromosome

appeared to insert into the same site (Barth et al, 1976). Insertions into plasmids were not specific and the transposon was used to generate a map of the transfer regions of RP4 by insertional mutagenesis (Barth and Grinter, 1977; Barth and Datta, 1977; Barth et al, 1978). From this analysis, it was noticed that all (49) insertions into RP4 occurred in a single orientation relative to the restriction maps of the plasmid and transposon. This has been reported for other plasmids and for Tn7 insertions in other strains of E.coli (Hodge, 1983). Only one insertion has been found in the opposite orientation (Moore and Krishnapillai, 1982). Insertion of a transposon in only a single orientation but in many sites is unique among transposable elements. It implies that the transposon can recognise some aspect of the plasmid that is orientation specific. What this property is remains unclear. It cannot be replication; RP4 is replicated bidirectionally.

Insertion of Tn7 into the chromosome of many bacterial strains has been reported and all appear to have a specific insertion site which is usually referred to as a hotsite for Tn7 (Thomson et al, 1981; Ely, 1982; Turner et al, 1984). Cloning and sequencing have revealed that the site of integration between different genera is highly conserved over an extensive region, which is larger than the 70bps required to define the hotsite (C Lichtenstein, pers. comm.). The E.coli chromosomal hotsite has also been cloned, sequenced and identified as the transcriptional terminator of the glmS gene (Lichtenstein and Brenner, 1981, 1982; Gay et al, 1986). A 969 base pair EcoRI fragment is an effective target for the insertion of Tn7 when cloned in multicopy vectors. The orientation of Tn7 into this site is determined by the sequence of the site and not by any plasmid determinant. Inversion of the site within a plasmid results in inversion of subsequent insertions of Tn7 (Lichtenstein and Brenner, 1981). This distinguishes transposition of Tn7 into the hotsite from transposition into plasmids and implies that different, though probably related, transposition mechanisms are employed.

Lichtenstein and Brenner (1982) also sequenced the ends of Tn7 during their analysis of the hotsite. Tn7 generates five base pair duplications of the target site and has characteristic terminal inverted repeats (28 base pairs). However, there are also four tandem

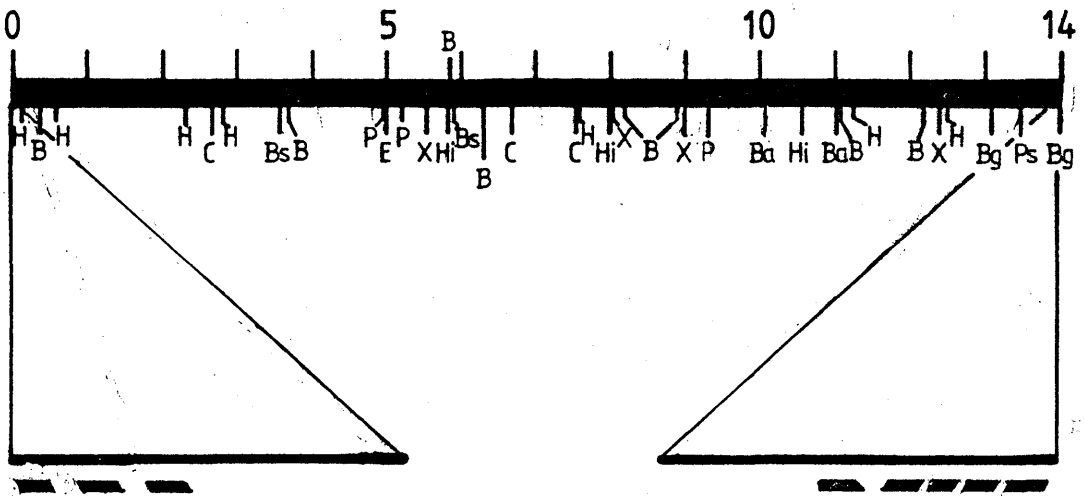


Figure 1.4 Restriction map of Tn7 (Gosti-Testu et al, 1983) and expanded view of the organisation of the ends. The left end contains three copies of a 22bp consensus sequence seperated by unique sequence. The right end contains a further copy in direct repeat and four contiguous copies in inverted repeat.

Abbreviations: B, BclI; Ba, BamHI; Bg, BglII; Bs, BstEII; C, ClaI; E, EcoRI; H, HincII; Hi, HindIII; P, PvuII; Ps, PstI; X, XbaI

are also repeated in the left end but are separated by non-homologous sequences (figure 1.4). Conventionally, the left end of Tn7 contains the antibiotic resistance genes. Numbering of the transposon begins from this boundary (figure 1.4). These repeats give Tn7 asymmetric ends and may provide a mechanism by which the orientation of Tn7 is determined. The ends show differences in their activity. The right end is capable of promoting transposition immunity (see below) while the left end is not (N Craig, pers. comm.). Also, two right ends in inverted orientation can transpose when complemented, while two left ends cannot (N Craig pers. comm.).

Tn7 encodes multiple resistance determinants (St/Sp and Tp) which have been defined to the left end of the transposon and are encoded by two genes (Hodge, 1983; Smith and Jones, 1984). The dihydrofolate reductase (DHFR) gene encoding resistance to trimethoprim has been sequenced (Fling and Richards, 1983; Simonsen et al, 1983). The polypetide encoded by this gene has been observed in minicell and maxicell protein preparations and is 18Kd (Fling and Elwell, 1980; Brevet et al, 1985). The predicted size of the protein from the sequence is 17,577d. Comparison of the restriction map derived from the sequence with the restriction map of Tn7 places this gene between 2.32Kb and 2.8Kb and indicates that it is transcribed from left to right as Tn7 is drawn in figure 1.4.

The adenyltransferase gene encoding resistance to St and Sp has not been sequenced but it is probably, at least partially, encoded within the BstEII fragment between 3.9Kb and 5.8Kb as deletion of this region produces a $St^{S}Sp^{S}$ phenotype (Smith and Jones, 1984). Maxicell analysis of the proteins produced by Tn7 indicate that this gene encodes a protein of 32Kd (Brevet et al, 1985).

The phenomenon of transposition immunity thought to be unique to the class II elements is also observed in Tn7 though the strength of the effect is unclear (Hauer and Shapiro, 1984; Hassan and Brevet, 1983; N Craig, pers. comm.). Hassan and Brevet (1983) reported that the presence of Tn7 in RP4 did not reduce the efficiency with which a second copy of the transposon could insert into this plasmid. However, some of their data does show a small interference effect

which has also been detected by others (Hauer and Shapiro, 1984). Nancy Craig (pers. comm.) has shown that the transposition of Tn7 into pOX38 is dramatically reduced (3 logs) by the presence of a copy of Tn7 or of just the right end in this replicon.

The main approach to the dissection of the fuctions of Tn7 required for transposition has been to construct *in vitro* deletions of Tn7 and assay their ability to transpose. Some complementation of these deletions, using other deletions of Tn7 or using fragments of Tn7 cloned onto multicopy vectors, has led to the identification of three functions required for transposition (Grinter, 1983; Hodge, 1983; Smith and Jones, 1984; Hauer and Shapiro, 1984; Ouartsi *et al*, 1985). One of these functions was encoded entirely within the 2.2Kb HindIII fragment between 5.8Kb and 8.0Kb and was only required for transposition to plasmids (Hauer and Shapiro, 1984; Smith and Jones, 1984). The other two functions were encoded between the BglII at 14.0Kb and the SstI site at 10.9Kb and between 13.1Kb and 7.9Kb (figures 1.4 and 4.1). Ouartsi *et al*, (1985) dispute the presence of a function at the right end though the results presented in chapter 4, and all the published and unpublished work confirm both its presence and requirement for transposition (Hauer and Shapiro, 1984; C Wadell and N Craig, pers. comm.). Also, the sequence of the right end up to the PstI site at 13.5Kb contains an open reading frame starting 135 base pairs in from the end which extends beyond the PstI site.

Recently, Brevet *et al* (1985) have published a maxicell analysis of the proteins produced by Tn7. They identify six polypeptides including the resistance genes. Discussion of this paper will be left until chapter 5.

In this thesis, the structural organisation of Tn7 has been investigated and the polypeptides produced have been determined. A mini Tn7, Tn7-1, has been constructed which was complemented to transpose in *trans* using fragments of Tn7 cloned into three compatible multicopy plasmids. These recombinant plasmids were analysed subsequently for Tn7 specific polypeptides using the minicell strain DS944 which allows the detection of plasmid borne polypeptides (Adler *et al*, 1967; Clark-Curtiss and Curtiss III, 1983). Analysis of the transposition properties and the products of intermolecular

transposition gave data on the type of mechanism that might be employed and a study of the insertion of Tn7 into the chromosome of an E.coli which already contained a copy of Tn7-1 at the hotsite was initiated. No evidence for preferred secondary hotsites in the chromosome was obtained.

CHAPTER 2

MATERIALS AND METHODS.

Table 2.1 Bacterial strains

Strain	Characteristics	Reference/Source
DS903	*rec*F143, *pro*A7, *Str*31, *thr*1, *leu*6 *tsx*33, *mtl*2, *his*4, *arg*E3, *lac*Y1, *gal*K2, *ara*14, lambda$^-$	JC9239; Horii and Clarke (1973)
DS903::Tn7	DS903 with Tn7 in hotsite	Dave Sherratt
DS941	DS903 but *lac*I^q, *lac*ZΔM15 *lac*Y$^+$	Dave Sherratt
DS944	*min*B, *thi*, *lac*I^q, *lac*ZΔM15, *rec*A	Dave Sherratt
BMH 71-18	F'[*pro*$^+$*lac*I^{q}ZΔM15], *Sup*E,	Messing *et al* (1977)
ΔM15	Δ[*lacpro*], *thi*, ϕ80d*lac*ZΔM15	Ruther *et al*, (1981)
CB51	*dam*-3$^-$, *ara*, *thi*, Δ[*lacpro*]	Chris Boyd
DS916	*Rif*^{r}, *rec*A, *his*, *trp*, *Sp*^{r}	Dave Sherratt
MR1	DS916 but *Nal*^{r}	Chapter 3
MR4	DS903::Tn7-1	Chapter 4
MR4::Tn7	Chromosomal Tn7 insert in MR4	Chapter 6
MR5	MR4 F['*pro*$^+$*lac*I^{q}ZΔM15]	Chapter 4
MR6	DS903::Tn7-2	Chapter 4

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

2.2 Plasmids. The plasmids used and constructed in this study are listed in table 2.2 and their nomenclature follows that of Novick et al (1976).

2.3 Chemicals.

CHEMICALS	SOURCE
General chemicals	B.D.H., Hopkins and Williams, Kochlight Laborotories, May and Baker
Media	Difco, Oxoid
General biochemicals	Sigma, Pharmacia
Agarose	BRL
X-gal, IPTG	BRL
Radiochemicals	NEN
10 X core buffer	BRL
Antibiotics	Sigma
Restriction enzymes	BRL, Boerhinger mannheim
En^{3}hance	Dupont

2.4 Culture media.

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

L-Agar: As L-Broth without glucose and the addition of 15g/l agar.

Iso-sensitest Broth: 23.4g Iso-sensitest Broth made up to 1 litre with distilled water.

Iso-sensitest Agar: 31.4g Iso-sensitest Agar made up to 1 litre with distilled water.

Minimal Agar: 7g K_2HPO_4, 2g KH_2PO_4, 4g NH_4SO_4, 0.25g trisodium

Table 2.2 Plasmids

Plasmid[1]	Marker	Compatibility	Comment	Source/reference
R388	Tp^r	IncW	-	Datta and Hedges (1972)
pEN300	Tp^r	IncW	Hotsite cloned in R388	E Nimmo
pUC8	Ap^r	ColE1	-	Vieira and Messing (1982)
pMR80	Ap^r	ColE1	Hotsite cloned in pUC8	Chapter 3
pMR86	Ap^r	ColE1	Hotsite cloned in pUC8	Chapter 3
pMR12	Ap^r	ColE1	Hotsite cloned in pUC8	Chapter 3
pACYC184	Cm^r,Tc^r	P15A	-	Chang and Cohen (1978)
pEAL1	Cm^s,Tc^r	P15A	Hotsite derivative of pACYC184	Lichtenstein and Brenner, (1981)
R388::Tn7	Tp^r,St^r,Sp^r	IncW	Tn7 insertion in R388	Chapter 3
pEN300::Tn7	Tp^r,St^r,Sp^r	IncW	Tn7 insertion into hotsite of pEN300	Chapter 3
pMR80::Tn7	Ap^r,Tp^r, St^r,Sp^r	ColE1	Tn7 insertion into hotsite in pMR80	Chapter 3
pMR86::Tn7	Ap^r,Tp^r, St^r,Sp^r	ColE1	Tn7 insertion into hotsite in pMR86	Chapter 3
pMR12::Tn7	Ap^r,Tp^r, St^r,Sp^r	ColE1	Tn7 insertion into hotsite in pMR12	Chapter 3
$pUC8::Tn7_I$	Ap^r,Tp^r, St^r,Sp^r	ColE1	Tn7 cloned in pUC8	Chapter 3
$pUC8::Tn7_{II}$	Ap^r,Tp^r, St^r,Sp^r	ColE1	Tn7 cloned in pUC8	Chapter 3
$pACYC184::Tn7_I$	Tc^r,Tp^r, St^r,Sp^r	P15A	Tn7 cloned into pACYC184	Chapter 3
$pACYC184::Tn7_{II}$	Tc^r,Tp^r, St^r,Sp^r	P15A	Tn7 cloned into pACYC184	Chapter 3
pEAL1::Tn7	Tc^r,Tp^r, St^r,Sp^r	P15A	Tn7 insertion into hotsite in pEAL1	Chapter 3
ColE1::Tn7	Tp^r,St^r,Sp^r	ColE1	Tn7 insertion in ColE1	Dave Sherratt
pEAL1::Tn7 EcoRI	Tc^r	P15A	EcoRI (5.0Kb) - RE[4]	Derek Hodge
pCB27	Cm^r	λ origin	-	Chris Boyd
pCB22	Cm^r	λ origin	-	Chris Boyd
pMR88	Ap^r	ColE1	RE - PstI (13.5Kb)	Chapter 4
pMR1	Ap^r,Tp^r, St^r,Sp^r	ColE1	same as pMR88 and LE[5] up to PvuII (4.98Kb)	Chapter 4
pNE77	Cm^r	P15A	2.2Kb HindIII fragment (5.8Kb - 8.0Kb)	Nelly Ekaterinaki
pMR11	Ap^r,Cm^r	ColE1	LE - HincII (0.168Kb) PstI (13.5Kb) - RE	Chapter 4
pMR83	Ap^r	ColE1	ClaI (7.6Kb) - RE	Chapter 4

Notes to Table 2.2

1. Plasmids pMR61 - pMR61 are based on pGLW8; plasmids pMR101 - pMR117 are based on pMR100; pMR25 and pMR36 are based on pACYC184.
2. Only vectors used in the complementation studies are listed with promoters.
3. Fragments are described in the direction that transcription or translation would run through them.
4. RE; Includes sequences up to the right end of Tn7.
5. LE; Includes sequences up to the left end of Tn7.
6. Contains two fragments of Tn7 cloned independently

Table 2.2 Cont.

Plasmid[1]	Marker	Compatibility	Promoter[2]	Comment	Source
pMR84	Ap^r	ColE1	lac	RE - ClaI (7.6Kb)	Chapter 4
pMR85	Ap^r	ColE1	-	LE - PvuII (4.98Kb) ClaI (7.6Kb) - RE	Chapter 4
pKK223-3	Ap^r	ColE1	tac	-	Chris Boyd
pGLW8	Ap^r	ColE1	tac	-	Fiona Stuart
pMR18	Cm^r, Km^r	λ origin	-	-	Chapter 4
pMR100	Km^r	λ origin	tac	-	Chapter 4
pMR25	Cm^s, Tc^r	P15A	-	RE - HincII (12.5Kb)	Chapter 4
pMR36	Cm^s, Tc^r	P15A	-	RE - PvuII (9.3Kb) PvuII (5.2Kb)-EcoRI (5.0Kb)	Chapter 4
pMR41	Cm^s, Tc^r	P15A	-	BglII (14.0Kb - 13.1Kb)	Chapter 4
pMR9	Ap^r	ColE1	lac	RE - EcoRI (5.0Kb)	Chapter 4
pMR63	Ap^r	ColE1	tac	HindIII (8.0Kb - 5.8Kb)	Chapter 4
pMR59[6]	Ap^r	ColE1	tac	RE - BamHI (11.05Kb) HindIII (8.0Kb - 5.8Kb)	Chapter 4
pMR68[6]	Ap^r	ColE1	tac	PstI - BamHI (11.05Kb) HindIII (8.0Kb - 5.8Kb)	Chapter 4
pMR69[6]	Ap^r	ColE1	tac	BglII - BamHI (10.05Kb) HindIII (8.0Kb - 5.8Kb)	Chapter 4
pMR51	Ap^r	ColE1	tac	BamHI (11.05Kb) - ClaI (7.6Kb)	Chapter 4
pMR56	Ap^r	ColE1	tac	BamHI (10.1Kb) - ClaI (7.6Kb)	Chapter 4
pMR57	Ap^r	ColE1	tac	pMR51 with XbaI deletion (8.05Kb-9.0Kb)	Chapter 4
pMR58	Ap^r	ColE1	tac	pMR56 with XbaI deletion (8.05Kb-9.0Kb)	Chapter 4
pMR55	Ap^r	ColE1	tac	PvuII (9.3Kb) - ClaI (7.6Kb)	Chapter 4
pMR66	Ap^r	ColE1	tac	pMR51 with BclI deletion (8.2Kb-8.9Kb)	Chapter 4
pMR64	Ap^r	ColE1	tac	EcoRV (9.8Kb) - ClaI (7.6Kb)	Chapter 4
pMR61	Ap^r	ColE1	tac	BamHI (10.1Kb) - HindIII (8.0Kb)	Chapter 4
pMR62	Ap^r	ColE1	tac	pMR61 but with XbaI deletion (8.05Kb-9.0Kb)	Chapter 4
pMR106	Km^r	λ origin	tac	same insert as pMR51	Chapter 4
pMR113	Km^r	λ origin	tac	same insert as pMR57	Chapter 4
pMR101	Km^r	λ origin	tac	RE - BamHI (11.05Kb)	Chapter 4
pMR102	Km^r	λ origin	tac	PstI (13.5Kb) - BamHI (11.05Kb)	Chapter 4
pMR117	Km^r	λ origin	tac	BglII (13.1Kb) - BamHI (11.05Kb)	Chapter 4
pMR112	Km^r	λ origin	tac	pMR101 with BclI deletion (11.1Kb-12.2Kb)	Chapter 4
pMR118	Km^r	λ origin	tac	same insert as pMR56	Chapter 4
pMR115	Km^r	λ origin	tac	same insert as pMR55	Chapter 4

citrate, 0.1g $MgSO_4.7H_2O$, 17.5g agar, made up to 1 litre in distilled water.

Davis-Mingioli (D&M) Salts (X4): 28g K_2HPO_4, 8g KH_2PO_4, 4g $(NH_4)_2SO_4$, 1g sodium citrate, 0.4g $MgSO_4.7H_2O$, made up to 1 litre with distilled water.

D&M Minimal Medium: 25ml D&M salts, 5ml 20% casamino acids, 250ul 20% glucose, 0.5ml vitamin B1 (1mg/ml), made up to 100ml with distilled water.

M9 Salts (X10): 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl in 1 litre of distilled water.

M9 Minimal Medium: 10ml M9 salts, 2ml glucose (20% w/v), 0.1ml 1M $MgCl_2$, 0.1ml 100mM $CaCl_2$, 0.1ml (1mg/ml) vitamin B1, 2.5ml 20% casamino acids, made up to 100ml with distilled water.

3XD Minimal Media: 150ml phosphate concentrate, 10ml 10% NH_4Cl, 6ml 10% $MgSO_4.7H_2O$, 10ml 0.1% Glycerol, 3ml 0.05M $CaCl_2$, 15g casamino acids, 797ml distilled water. Phosphate concentrate and $CaCl_2$ added slowly after autoclaving.

Brain Heart Infusion Media: Add 37g Brain Heart Infusion media powder to 1 litre of distilled water.

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

Phosphate concentrate: 30g KH_2PO_4 (anhydrous), 70g Na_2HPO_4 (anhydrous) made up to 1 litre in distilled water.

Supplements: When required, supplements were added to minimal media at the following concentrations:

glucose 2mg/ml	amino acids 40ug/ml	thymine 50ug/ml
thiamine vitamin B1 20ug/ml		casamino acids 1%

2.5 Sterilisation. All growth media were sterilised by autoclaving at 120°C for 15 minutes; supplements, gelatin solution and buffer solutions at 108°C for 10 minutes and $CaCl_2$ at 114°C for 10 minutes.

2.6 Buffer solutions.

Electrophoresis

10 X E Buffer: 48.4g Tris-HCl, 16.4g NaAc, 3.6g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water, pH adjusted to 8.2 with acetic acid.

10 X TBE Buffer pH8.3: 109g Tris-HCl, 55g Boric Acid, 9.3g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water; pH is 8.3.

10 X Laemmli gel buffer: 144g Glycine, 30g Tris-HCl made up to 1 litre in distilled water. When diluted, 10ml 10% SDS (w/v) was added for every 1 litre of 1 X concentration.

Single Colony Gel Loading Buffer: 2% Ficoll, 1% SDS, 0.01% Bromophenol blue, 0.01% Orange G in Buffer E.

Horizontal Agarose and Polyacrylamide Gel Loading Buffer: 25% (w/v) sucrose, 0.06% Bromophenol Blue, 10mM Tris-HCl (pH8.0).

Vertical Agarose Gel Loading Buffer: 1% Ficoll, 0.5% SDS, 0.06% Bromophenol Blue, 0.06% Orange G in 1 X TBE.

Restriction and Ligation Buffers:

10 X Low salt: 100mM Tris-HCl pH7.5, 100mM $MgSO_4$, 10mM DTT. Stored at 4°C.

10 X Medium salt: 500mM NaCl, 100mM Tris-HCl pH7.5, 100mM $MgSO_4$, 10mM DTT. Stored at 4°C.

10 X High salt: 1M NaCl, 500mM Tris-HCl pH8.0, 100mM $MgCl_2$, 10mM DTT. Stored at 4°C.

10 X SmaI Buffer: 200mM KCl, 100mM Tris-HCl pH8.0, 100mM $MgCl_2$, 10mM

DTT. Stored at 4°C.

10 X Ligation Buffer: 660mM Tris-HCl pH7.6, 66mM $MgCl_2$, 100mM DTT. Stored at -20°C.

4mM ATP: Dissolve 60mg of ATP in 0.8ml distilled water. Adjust to pH7.5 with 0.1M NaOH, made up to 1ml with distilled water; Stored at -20°C in aliquots and thawed once only.

TE Buffer: 10mM Tris-HCl, 1mM EDTA; pH8.0.

All these buffers were stored for long term at -20°C.

Protein sample buffer: 10% Glycerol, 0.01% Bromophenol blue, 5% B-mercaptoethanol, 3% SDS, 0.625M Tris-HCl pH8.0. Stored at room temperature.

10 X Nick Translation Buffer: 500mM Tris-HCl pH7.2, 100mM $MgSO_4$, 1mM DTT, 500ug/ml BSA; stored at -20°C

20 X SSC: 3M NaCl, 300mM trisodium citrate pH7.0

Wash Buffer: 5mM NaH_2PO_4, 1mM Na_2EDTA, 0.2% SDS pH7.0

Phenol All phenol used in the purification of DNA contained 0.1% 8-hydroxyquinoline, and was buffered against 0.5M Tris-HCl pH8.0.

Birmboin-Doly I: 50mM Glucose, 25mM Tris-HCl pH8.0, 10mM EDTA; add lysozyme to 1mg/ml immediately before use.

Birmboin-Doly II: 0.2M NaOH, 1% SDS; made fresh.

Birmboin-Doly III: 5M KAc pH4.8; mix equal volumes of 3M CH_3COOK and 2M CH_3COOH, pH should be 4.8.

STET Buffer: 8% Sucrose, 5% Triton X-100, 50mM EDTA, 50mM Tris-HCl pH8.0

Buffered Saline Gelatin (BSG): 0.85% NaCl, 0.03% KH_2PO_4, 0.06%

Na_2HPO_4, 100ug/ml Gelatin.

5% Sucrose BSG: BSG made in the presence of 5% Sucrose.

20% Sucrose BSG: BSG made in the presence of 20% Sucrose.

2.7 Antibiotics. The antibiotic concentrations used throughout for both liquid and plate selections were as follows:

Name	Source of resistance	Selective conc^n	Stock sol^n
Ampicillin (Ap)	plasmid	50ug/ml	5mg/ml (water)
Streptomycin (St)	chromosomal	300ug/ml	30mg/ml (water)
Streptomycin	Tn7	5ug/ml	0.5mg/ml (water)
Rifampicin (Rif)	chromosomal	50ug/ml	5mg/ml (methanol)
Tetracycline (Tc)	plasmid	10ug/ml	1mg/ml (10mM HCl)
Chloramphenicol (Cm)	plasmid	25ug/ml	2.5mg/ml (ethanol)
Kanamycin (Kan)	plasmid	50ug/ml	5mg/ml (water)
Trimethoprim (Tp)	plasmid/Tn7	50ug/ml	5mg/ml (50% water/ 50% ethanol)
Spectinomycin (Sp)	Tn7	25ug/ml	2.5mg/ml (water)
Naladixic acid (Nal)	chromosome	20ug/ml	2mg/ml (1M NaOH)

All stock solutions were stored at 4°C and when required added to molten agar pre-cooled to 55°C.

2.8 Indicators. X-gal (5-Bromo-4-chloro-3-indolyl-B-galactoside) was used in conjuction with the host strains ΔM15 and DS941 and the pUC vectors providing a screen for plasmids with inserts in the polylinker region. Clones containing inserts are generally white; clones lacking inserts are blue. X-gal was stored at a concentration of 20mg/ml in dimethylformamide (DMF) at -20°C and added to L-agar plates to a final concentration of 20ug/ml.

2.9 Growth conditions. Liquid culture for transformation, DNA preparations or conjugations were routinely grown in L-broth, or when Tp selection was required in isosensitest broth, at 37°C with vigorous shaking. Growth on plates was on L-agar, minimal medium plus supplements, or on isosensitest agar for selection on Tp. Antibiotics

were used as required. Plates contained 25ml of agar solution and were incubated at 37°C overnight unless otherwise stated. All dilutions were carried out in D&M salts.

Bacterial strains were stored in 50% L-broth, 20% glycerol and 1% peptone at -20°C. Inocula from these stocks were applied to L-agar plates and grown, prior to replating on selective plates.

2.10 Plasmid DNA preparation. Two methods were used to obtain DNA from cells.

Birmboin and Doly (1979) DNA preparation: 200 ml cultures of stationary phase cells were harvested by centrifugation (12000g, 5min at 4°C). The pellet was resuspended in 4ml of Birmboin-Doly I solution and incubated on ice for 5min. 8ml of Birmboin-Doly II solution were added and the solution left on ice for 5min before 6ml of cold Birmboin-Doly III solution was added, gently mixed and left on ice for a further 5min. The cell debris and most chromosomal DNA was removed by centrifugation (32000g, 5min at 4°C) and the plasmid DNA precipitated by addition of an equal volume of isopropanol followed by centrifugation at 39200g for 15min. This DNA was further purified by banding on a CsCl/EtBr gradient. The DNA was resuspended in 2.09ml of water and added to 270ul of a 15mg/ml EtBr solution. 5g of CsCl were dissolved in 3ml of water and added to the DNA/EtBr solution. The gradients were centrifuged in a Beckman Ti70 fixed angle rotor at 200,000g for 16 hours at 20°C. Two bands were visible, a lower supercoiled plasmid band and an upper chromosomal and relaxed plasmid DNA band. The lower band was removed using a 1ml syringe and the EtBr removed by repeated butanol extractions (using water saturated butanol). The salts were removed by dialysis in 2 X 500ml 1 X TE. The DNA was then ready for use.

Mini DNA preparation using the method of Holmes and Quigley (1981): 1.5ml of an overnight culture containing the plasmid of interest was harvested by centrifugation in a 1.5ml eppendorf tube and resuspended in 350ul of STET buffer. 25ul of STET buffer containing lysozyme at a concentration of 10mg/ml was added and the tube vortexed briefly. This solution was boiled for 40sec and centrifuged in an eppendorf microfuge for 15min at 4°C. The pellet was discarded using a

toothpick and 40ul of 3M NaAc and 400ul of cold isopropanol was added, followed by microcentrifugation for 7min which precipitated the nucleic acid. The pellet was washed twice in 70% ethanol and dried briefly in a vacuum drier before being resuspended in 20ul - 50ul 1 X TE. This DNA was suitable for digestion and other *in vitro* manipulations.

2.11 Chromosomal DNA preparation. 100ml of stationary phase culture was harvested by centrifugation (12000g, 10min, 4°C) and resuspended in 3.3ml 25% sucrose. 0.67ml of a 5mg/ml lysozyme solution in 0.25M Tris-HCl pH8.0 was added and the mixture left on ice for 5min. 1.3ml of 0.25M EDTA was added followed by 5.3ml of 2% Sarkosyl (made up in 50mM Tris-HCl pH8.0 and 50mM EDTA pH8.0). Lysis was allowed to proceed for 5min on ice. Treatment of the lysed cells with RNase (final concentration 50ug/ml) and then with Proteinase K (final concentration 50ug/ml) broke down the RNA and proteins. The DNA was purified initially by repeated phenol extractions until the interface was clear. The addition of 1.5ml of 5M NaCl to every 20ml of solution prior to phenol extraction helped clear the interface. Chloroform and ether extractions removed any traces of phenol or chloroform. The DNA was precipitated using ethanol, washed in 70% ethanol and further purified by banding in a CsCl/EtBr gradient (see above for this procedure).

2.12 Ethanol precipitation of DNA. The DNA solution was made 0.3M in NaOAc and 2 volumes of cold ethanol added. After mixing, the DNA was precipitated by cooling on ice for up to 1 hour and pelleted by centrifugation (27000g, 15min, 4°C for large volumes or 12000g, 15min, 4°C for small volumes in eppendorf centrifuge tubes). The pellet was usually washed in 70% ethanol and dried briefly in a vacuum drier.

2.13 Restriction of DNA. Restrictions were usually performed in a total volume of 20ul containing between 0.25ug and 1ug of DNA, 2ul of 10X restriction buffer and 1 unit/ug DNA of enzyme, the volume being made up with distilled water. For larger scale restrictions the volumes were scaled up accordingly. The reactions were allowed to proceed for 1 to 2 hours at the appropriate temperature. Reactions were stopped either by the addition of gel loading buffer or by rapid heating to 70°C for 5 min followed by rapid cooling on ice.

2.14 Ligation of DNA fragments. The restriction fragments to be ligated were mixed such that the insert was in 3 times excess over the vector (10 times excess for blunt end ligations) and made up to 20ul by the addition of 2ul 10 X ligation buffer, 2ul 4mM ATP and distilled water. T4 DNA ligase was added (0.01 units/ug DNA for 'sticky' end ligation and 1 unit/ug DNA for 'Blunt' end ligation) and the solution ligated for 1 hour at room temperature (overnight at 16°C for 'blunt' end ligations). Aliquots of the ligation mix were used to transform competent cells.

2.15 Calf Intestinal Phosphatase (CIP) treatment. To increase cloning efficiency, CIP was used to remove the 5'-terminal phosphate groups from the linearised vector to prevent recircularisation of the vector. This technique is particularly useful if there is no direct selection for the insert. CIP works in high, medium or low salt buffers and was used at a concentration of 1-2 units/ug DNA. It was added directly to the restriction digest for the final 15min of the incubation and was heat killed in the manner described for restriction enzymes.

2.16 Transformation with plasmid DNA. Plasmids were introduced to different strains by genetic transformation. An overnight culture of the recipient was diluted 1 in 100 into 20ml L-broth and was grown to a density of approximately 10^8 cells/ml (about 90min - 2 hours). The cells were harvested (12000g, 5min, 4°C) and resuspended in 10ml of cold 50mM $CaCl_2$. The cells were pelleted again, resuspended in 1ml of cold 50mM $CaCl_2$ and kept on ice for at least 15min before use. 200ul aliquots of the competent cells were added to the plasmid DNA, mixed gently and left on ice for up to 1 hour. The cells were heat shocked (2min, 42°C) and returned to the ice for a further 15min. An equal volume of L-broth was added and the cells incubated at 37°C for 1 hour to allow expression of the plasmid resistance genes. The cells were plated out on the appropriate selections. For transformation to ampicillin resistance, no expression time was necessary.

The presence of the new plasmid in the transformed colonies was confirmed by single colony gel electrophoresis.

2.17 Single colony gel analysis. This technique enables the plasmid content of a colony to be observed without the purification of plasmid DNA. A single colony was patched out (1cm square) and grown overnight. The patch was scraped off the plate using a toothpick and suspended in 150 - 250ul of single colony gel buffer. The cells were left to lyse at room temperature and centrifuged in a microfuge (12000g, 4°C) for at least 15min. 30ul of the supernatant were loaded onto an agarose gel which did not contain ethidium bromide.

2.18 Curing strains of F. An overnight culture of the strain to be cured was diluted to 10^5 cells/ml and 50ul inoculated into 2.5ml L-broth (pH7.6) with 7.5, 15, 30 and 75ug/ml Acridine orange. These cultures were grown shaking at 37° overnight in the dark. 10^{-2}, 10^{-4} and 10^{-6} dilutions were plated on L-agar and the resulting colonies tested for their F phenotype by conjugation using a replica plating method. Colonies were patched out and replica plated onto an L-agar plate and onto a minimal medium plate spread with a suitable recipient and supplemented with the recipients requirements which allow only transconjugants to grow.

2.19 Gel electrophoresis. Both vertical and horizontal agarose gels were used. Vertical gels were used mainly for single colony analysis while horizontal agarose gels were used for restriction analysis of plasmids and for southern analysis. Unless otherwise stated 1% agarose gels were used.

Horizontal gels. Two types were commonly run.

(1) 100ml gels - 100ml of molten agarose was poured into a 11 X 19cm perspex gel former with a 13 space teflon well former. After the gel had set, the comb was removed and the gel placed in a horizontal gel tank, submerged in E buffer and loaded with 20 - 30ul of sample. Gels were usually run overnight at 20V and stained in ethidium bromide (0.5ug/ml) for 30mins. The stained DNA was photographed on a 254nM wavelength UV transilluminator. Some gels were made up with ethidium bromide to a concentration of 0.5ug/ml.

(2) 200ml gels - These gels were made by pouring 200ml of molten agar into a 16.5 X 23cm gel former with a 20 space well former. The gels

were run in E buffer in a gel tank with a buffering capacity of 3 litres overnight at 20V. The gels were stained with ethidium bromide (0.5ug/ml) and visualised on a 254nM wavelength transilluminator.

Vertical gels: The gel kits held two 16 X 15cm glass plates separated by 3mm spacers. After sealing the edges with molten agar the agar was precooled to 55°C and poured between the glass plates. The comb (10 or 15 teeth) was inserted and the gel allowed to set. The top and bottom wells were filled with E buffer and the comb removed. Samples were loaded and the gel run, generally at 5 V/cm for about 4 hours prior to staining in 0.5ug/ml ethidium bromide. The gel was photographed on a 254nM wavelength transilluminator.

Vertical gels were used mainly for single colony analysis and the interpretation of the DNA band seen followed that of Dugaiczyk et al (1975). The fastest migrating and generally most abundant band was the supercoiled plasmid monomer. Behind this ran the open cicular plasmid band often comigrating with the supercoiled plasmid dimer. Open circular dimers and other higher forms ran higher up the gel. Plasmid linears could sometimes be detected running between the supercoiled monomer and the open circle monomer. Sheared fragments of chromosomal DNA ran as a thick band toward the top of the gel. Large plasmids like R388 run above the chromosomal band.

Low melting point agaose gels. These gels were used to separate fragments which were to be purified from the agarose and used in ligation reactions. The agarose was dissolved in 100ml 1 X E buffer at 100°C, precooled to 37°C and poured as a 100ml gel described above.

Photographing of gels. Ethidium bromide stained gels were viewed on a 254nm UV transilluminator and photographed using Polaroid type 67 land film or using a Pentax 35mm SLR loaded with Ilford HP5 film. Both cameras were fitted with a Kodak Wratten filter No.9.

Sizing of restriction fragments. The size of linear restriction fragments was estimated from graphs of the $\log_{10}$ molecular size plotted against the distance migrated in the gel according to;

$\log M = C \times 1/D$ (Helling et al, 1974)

M = Molecular size in base pairs.

D = Distance migrated

C = Arbitary constant

Molecular weight standards were obtained by restriction of Lambda cI857Sam7 (Philippsen et al, 1978; Haggerty and Scheif, 1976) or the pUC plasmids (Yanish-Perron et al, 1985).

2.20 Extraction of DNA from low melting point agarose gels. After staining, the gel was placed on a long wave transilluminator (300nM - 360nM) and the band of interest excised. The agarose chip was added to 3 volumes of E buffer and heated to 65°C. The solution was cooled to 37°C and an equal volume of room temperature phenol added, mixed throughly by vortexing and the phases separated by centrifugation (12000g, 2min). The supernatant was recovered, phenol extracted 3 times, chloroform extracted twice and ether extracted once. The DNA was precipitated and dried. This DNA was used for ligation.

2.21 UV sensitivity test. This technique was used to rapidly confirm the recombination status of strains. Stationary phase cultures of the strain to be tested and control strains were spotted on to L-agar plates at 10^{-2}, 10^{-4} and 10^{-6} dilutions. These plates were exposed to UV radiation (17.5ergs/sec/M^2) for 30 and 60 seconds and incubated overnight in the dark. *rec*$^+$ strains usually grow after 60 seconds exposure while *rec*A$^-$ strains show reduced growth after 30 seconds and do not grow after 60 seconds exposure. *rec*F$^-$ strains show an intermediate phenotype. Periodically all strains were checked for their recombination status.

2.22 Preparation of polypeptides produced from purified minicells. The plasmid to be examined was transformed into the minicell strain DS944. 200ml of L-Broth was inoculated with this transformed strain and grown upto stationary phase at 37°C. The minicells were easier to purify if the culture was in stationary phase though it did reduce the level of incorporation acheived. Partially purified minicells were obtained by low speed centrifugation (5min, 2000g at room temperature) which preferentially pelleted the whole cells. The supernatant was

collected and the minicells pelleted (12000g, 10min, room temperature) and resuspended in 1ml of BSG. This was loaded onto a 5%-20% sucrose BSG gradient, poured using a peristaltic pump and a gradient former. The sucrose gradient was centrifuged at 2000g for 20min at room temperature. The minicells form a creamy band in the top third of the gradient. These were extracted, pelleted (12000g, 10min, room temperature), and resuspended in 0.5ml BSG. The sucrose gradient purification step was repeated three times. After the third sucrose gradient the minicells were harvested and resuspended in about 600ul of M9 minimal media supplemented with glucose and vitamin B1. The minicells were split into 2 aliquots and IPTG added to one at a final concentration of 0.1mM to derepress the tac promoter (except for minicells containing the plasmids pMR25, pMR26 and pMR41). After incubation at 37°C with shaking for 1 hour, 20uCi of ^{35}S-methionine was added and the minicells incubated at 37°C with shaking for a further hour. 1.5ml of L-Broth was added and the tubes incubated at 37°C for 30 minutes. The minicells were centrifuged at 12000g at room temperature for 5min and washed once in phage buffer prior to being lysed with about 50ul of protein sample buffer. Whole cell extracts were processed in the same manner as a control for contamination.

2.23 Protein Gel Electrophoresis: The electrophoresis of proteins followed the procedure of Laemmli (1970). Unless specifically stated all SDS-polyacrylamide gels were 10% running gel with a 4% stacking gel. The gel plates were separated by 0.8mm spacers and the gel was pre-sealed using molten 0.6% agarose in H_2O. The gels were poured according to the table below;

	Running Gel	Stacking Gel
Acrylamide/Bis (30 ; 0.8)	10ml (10%)	2ml (4%)
Tris-HCl	11.25ml (1M; pH8.8)	1.9ml (1M; pH6.8)
10% SDS	0.3ml	0.15ml
TEMED	0.01ml	0.01ml
APS (made fresh; 100mg/ml)	0.15ml	0.15ml
dH_2O	8.3ml (10%)	10.9ml (4%)

The running gel was poured leaving about 1.5-2.0cm between the top of the running gel and the bottom of the comb. Isopropanol was layered on top of the running gel to create a sharp interphase. Once the

running gel had set, the stacking gel was poured after removing the isopropanol and washing away any residue with distilled water. The comb was pushed into the stacking gel firmly. After the gel had set the comb was removed, and running buffer poured into the gel tank covering the wells. Residue acrylamide was washed out of the slots using running buffer prior to loading the samples. The gel was run overnight at about 6mA until the bromophenol blue dye had reached the bottom of the gel.

The gel was fixed in 30% methanol/10% glacial acetic acid for 1 hour before being soaked in EN^{3}HANCE for a further hour. The EN^{3}HANCE was removed and the gel soaked in water for a further 30 minutes before being dried in a Biorad vacuum drier at 80^{o}C for at least 1 hour. The dried gel was placed against Blue sensitive film (Kodak Xomat S) and kept in the dark at -70^{o}C for a variable time (up to 1 month) to allow exposure of the film.

2.24 Southern hybridisation. Hybridisation of labelled DNA to filter bound chromosomal or plasmid borne DNA followed the procedure of Southern (1975) as modified by Reed and Mann (1985). Horizontal agarose gels were run with the required samples. The gel was photographed after ethidium bromide staining. The gel was soaked in 0.25M HCl for 15 minutes, soaked in 0.4M NaOH for 15 minutes, then placed on a glass plate covered by 2 layers of 3MM paper which overhung the edges and dipped into a tray containing the transfer buffer (0.4M NaOH). Pall Biodyne Nylon membrane cut to the size of the gel was pre-soaked in water and placed on top of the gel ensuring that no air bubbles were trapped. This was covered by further layers of 3MM paper and by paper towels until an 8cm stack was formed. The stack was topped with a glass plate weighed down with a 500ml bottle of water. Transfer was allowed to proceed overnight. The nylon membrane was briefly washed in 2 X SSC and was then ready for pre-hybridisation and hybridisation.

Pre-hybridisation was carried out using a solution of 1.5 X SSC, 0.5% Blotto (w/v non fat powdered milk) and 1% (w/v) SDS. Heat denatured sheared salmon sperm DNA was added at a concentration of 100ug/ml. About 1ml per 4cm^2 of filter was added, sealed in a plastic bag, and gently agitated for 1 hour at 65^{o}C.

Hybridisation was also carried out at 65°C and in the same solution. The probe was labelled by nick translation according to Rigby et al (1977) and denatured by boiling for 5 minutes in 1% SDS and 1.5 X SSC and chilling on ice. The probe was added to the filter by opening up the bag and resealing. The solution was mixed by agitation prior to incubating with gentle agitation overnight at 65°C.

The filter was washed in wash buffer sucessivily at room temperature, 42°C, 55°C and 65°C until no, or very few, counts were found in the wash buffer. The filter was sealed wet in a plastic bag and autoradiographed for a variable length of time depending on the number of counts retained on the filter.

2.25 Nick Translation of DNA. Labelling of plasmid DNA with ^{32}P followed the procedure of Rigby et al (1977). The nick translation reaction was set up according to the following table:

10 X Nick translation Buffer	5ul
DNA	1ug
unlabelled dNTP's	1ul of 1mM stock
[gamma - ^{32}P] dATP	25uCi (2.5ul)
dH_2O	up to 44ul

This solution was chilled to 0°C

A 10^{-4} dilution of a 1mg/ml DNase I solution was prepared in ice cold nick translation buffer and 50% glycerol (stored at -20°C). 0.5ul of this solution was added to the nick translation reaction along with 5 units of E.coli DNA polymerase I. The reaction was incubated at 16°C for 1 hour and stopped with 2ul 0.5M EDTA pH8.0.

The unincorporated counts were separated from the labelled probe using a spun minicolumn (Maniatis et al, 1982). Sephadex G-50 was prepared in 1 X TE buffer and used to fill a 1ml syringe, one end of which had been blocked using siliconised glass wool. The syringe was spun for 4 minutes at 1500g at 4°C. 100ul of TE was added to the column which was centrifuged at 1500g for 4 minutes at 4°C. THe nick translation reaction was diluted to 100ul with TE and spun through the column in the same manner. The void volume was collected in a 1.5ml eppendorf

tube and contained the incorporated label. The unincorporated counts remained in the column.

2.26 Conjugation and transposition assays: To construct strains and assay transposition frequencies, a number of methods were employed:

Liquid mating - This technique was used to rapidly move conjugative plasmids between strains. 200ul of exponentially growing donor and recipient cells were mixed and left at 37°C for 1 hour. Dilutions of this mix were plated on selective media which allowed only recipients containing the conjugative plasmid to grow.

Streak mating - When a conjugative plasmid had to be mated into a number of different strains, this method was used. The donor was spread onto half an agar plate containing antibiotics or lacking supplements that would both counter select the donor strain and select for the plasmid. Streaks of the recipient were made, starting on the clean half of the plate and crossing into the area containing the donor. Only at the boundary between these strains should colonies grow. These were picked and purified.

Plate matings - This technique was employed to measure the transposition frequency between the chromosome or a non mobilisable plasmid containing Tn7 and a conjugative plasmid. Donor and recipient cell were grown overnight and 0.5ml of donor was mixed with 1.5ml of recipient ensuring an excess of the recipient. These cells were concentrated 10 fold and spread on a well dried agar plate. The plate was incubated inverted for 3 hours at 37°C. The cells were washed off the plate using D&M salts and concentrated 10 fold. This was the 0 dilution. Serial dilutions were made and plated out on the relevant selective media which selected for recipients with the plasmid or for recipients which had received the conjugative plasmid and the transposon. The proportion of recipients receiving both plasmid and transposon compared to those receiving only the conjugative plasmid was termed the transposition frequency. The standard errors of transposition assays were less than 1/5 of the frequency of transposition unless otherwise stated.

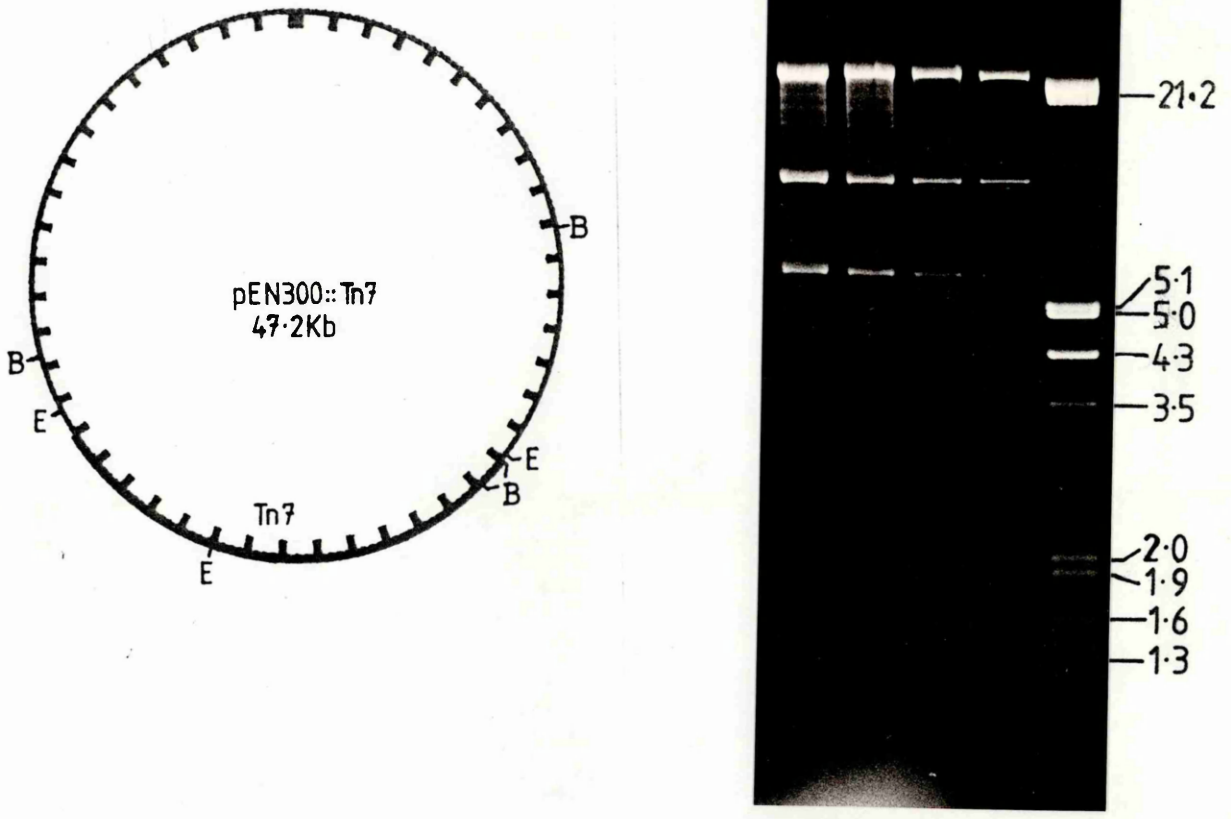
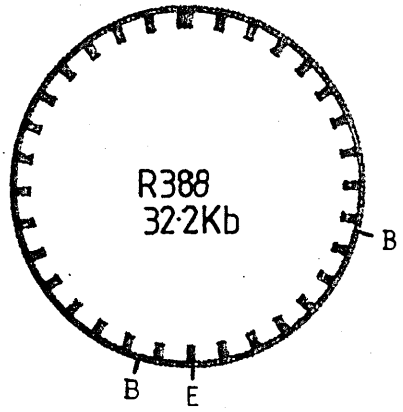
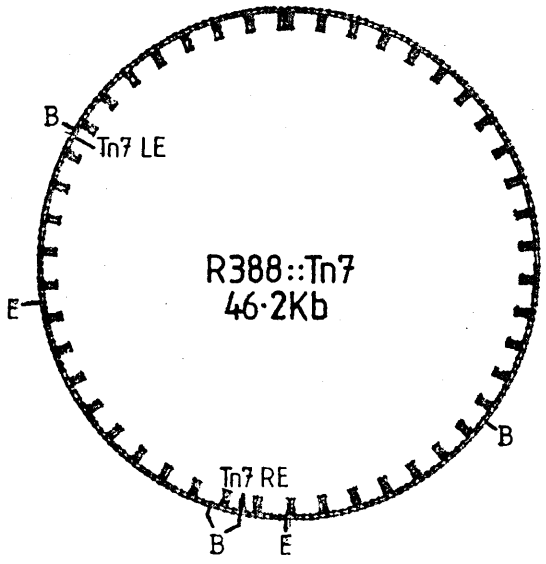


Figure 2.1 Agarose gel of EcoRI restricted pEN300::Tn7 confirming the presence of Tn7 within the hotsite fragment and an illustration of these plasmids.

Lanes 1-5 Different clones of pEN300::Tn7 cut with EcoRI
lane 6 Lambda cut with EcoRI/HindIII, used as markers.
DNA sizes are given in Kb. Abbreviations: B, BamHI; E, EcoRI

Predicted sizes of an EcoRI digest of pEN300::Tn7 are 32.2Kb, 9.0Kb and 5.8Kb.


R388
32·2Kb
B
B
E


B
Tn7 LE
R388::Tn7
46·2Kb
E
B
Tn7 RE
B
E

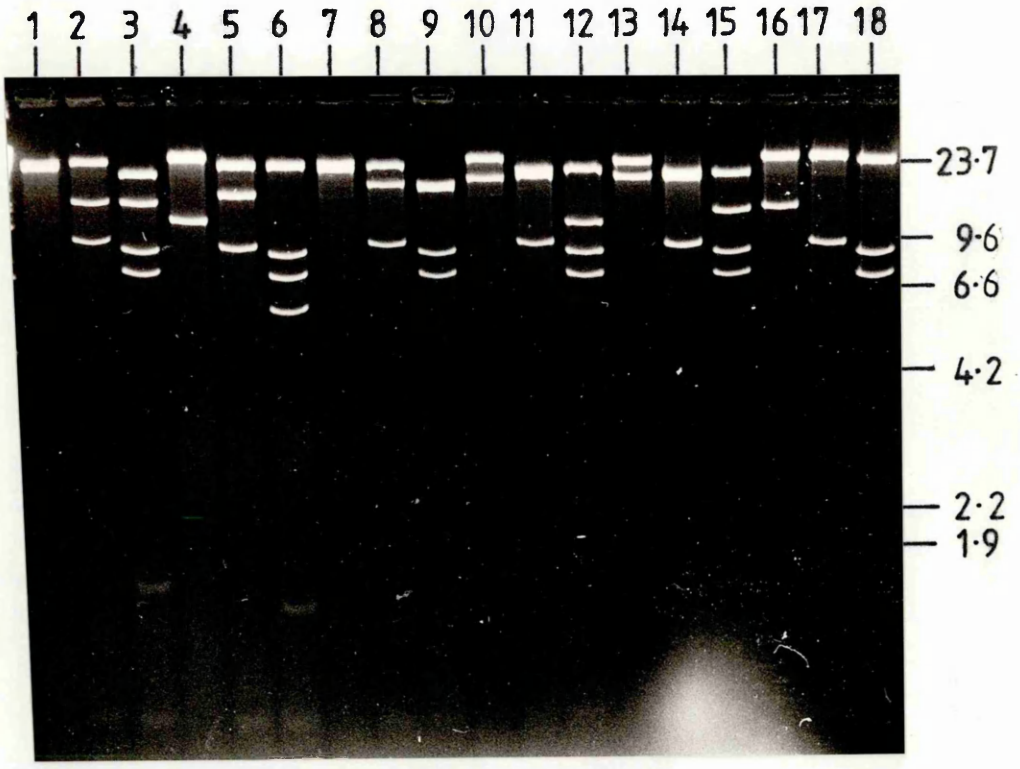


Figure 2.2 Examples of the digestion patterns used to determine the orientation of insertion of Tn7 into R388 and an illustration of one of the R388::Tn7 plasmids shown.

1	R388::Tn7$_1$ (EcoRI)	10	R388::Tn7$_4$ (EcoRI)
2	R388::Tn7$_1$ (BglII)	11	R388::Tn7$_4$ (BglII)
3	R388::Tn7$_1$ (EcoRI/BglII)	12	R388::Tn7$_4$ (EcoRI/BglII)
4	R388::Tn7$_2$ (EcoRI)	13	R388::Tn7$_5$ (EcoRI)
5	R388::Tn7$_2$ (BglII)	14	R388::Tn7$_5$ (BglII)
6	R388::Tn7$_2$ (EcoRI/BglII)	15	R388::Tn7$_5$ (EcoRI/BglII)
7	R388::Tn7$_3$ (EcoRI)	16	R388::Tn7$_6$ (EcoRI)
8	R388::Tn7$_3$ (BglII)	17	R388::Tn7$_6$ (BglII)
9	R388::Tn7$_3$ (EcoRI/BglII)	18	R388::Tn7$_6$ (EcoRI/BglII)

DNA sizes are shown in Kb.
Abbreviations: B, BamHI; E, EcoRI; LE, left end; RE, right end.
Sizes of fragments:

Digest	R388	R388::Tn7 (illustrated)
EcoRI	32.2Kb	10.5Kb, 35.7Kb
BglII	23.9Kb, 8.3Kb	13.2Kb, 0.9Kb, 8.2Kb, 23.9Kb
EcoRI/BglII	23.9Kb, 6.8Kb, 1.5Kb	23.9Kb, 5.1Kb,8.1Kb 0.9Kb, 1.4Kb, 6.8Kb

2.27 Determining the orientation of insertion of Tn7 in R388 and pEN300. The insertion of Tn7 into pEN300 or into any hotsite containing plasmid can be readily examined by digestion of DNA minipreps with EcoRI. This digest cuts the hotsite fragment of 969bp out of the plasmid and if Tn7 is present gives two diagnostic bands of 5.8Kb and 9.0Kb approximately. This digest is also suitable for the analysis of Tn7-1 insertions, giving two band of 2.0Kb and 1.7Kb. EcoRI digestion of Tn7-2 inserts is not as revealing because Tn7-2 does not contain any EcoRI sites. Digestion with BamHI or HindIII can be used giving diagnostic fragments depending on the recipient plasmid.

Transposition into R388 was analysed for the orientation of the Tn7 insertions relative to both the R388 and Tn7 restriction map. Digestion of R388::Tn7 with BglII, EcoRI and a double digestion with both these enzymes allows the orientation and a rough position of the point of insertion to be determined. BglII cuts R388 twice giving 8.3KB and 23.9Kb fragmnts. The absence of one of these fragments in a digestion of R388::Tn7 DNA reveals which of these fragments Tn7 has inserted into. The asymmetric nature of the BglII site in Tn7 (one is only 43bp from the right end (14.0Kb); the other is at 13.1Kb) simplifies the identification of the orientation. In a double digest of R388::Tn7 with EcoRI and BglII, if a BglII site in R388 is closest to the right end of Tn7 then the fragment obtained will be the same size as the fragment obtained in a digestion with BglII alone. This allows the insertion site to be located to one of two positions in R388. A comparison of the predicted and observed fragments for these locations usually defines the insertion site and the orientation. The precise insertion point in R388 cannot be mapped accurately because of a lack of useful restriction sites and the poor restriction map of R388. Figures 2.1 and 2.2 show an example of the patterns obtained from these restrictions for both cold site and hotsite insertions in R388 and pEN300 in an assay maeasuring the frequency of transposition from pEAL1::Tn7.

CHAPTER 3

PROPERTIES OF TN7 TRANSPOSITION.

3.1 INTRODUCTION

Previous work which examined the transposition of Tn7 suggested that the target replicon could influence the transposition frequency (Barth et al, 1976; Hodge, 1983; Lichtenstein and Brenner, 1981). Transposition to plasmids appeared to be much less efficient than transposition to the hotsite, when it was present in the chromosome or when cloned onto plasmids (Hauer and Shapiro, 1984; Hodge, 1983). (The chromosomal insertion site is referred to as the hotsite and insertions into plasmids which are not in this site are referred to as cold site insertions.) However, all of these studies were carried out in a manner that did not allow a direct comparison of these events. Different donor and target replicons and different methods of determining the transposition frequency made any discussion of these results difficult. In this chapter the effect of the recipient upon transposition frequency was determined in a manner that permitted direct comparison between transposition to cold sites and transposition to cloned hotsites.

There was conflicting evidence regarding the ability of Tn7 to promote cointegrates. Hodge (1983) presented data that cointegrates represented a significant proportion of all transposition products and that the ratio of simple insertions to cointegrates varied considerably depending on the donor replicon employed. This implied that Tn7 was capable of replicative transposition and that the donor replicon influenced the ratio between cointegration and simple insertion. Copy number effects were ruled out because no correlation was found between the frequency of cointegrate formation and the donor plasmid copy number. Others, reported a complete absence of any cointegrates during transposition (Smith and Jones, 1984; P Barth, pers. comm. to D Hodge) which suggested that Tn7 could transpose by a nonreplicative mechanism. An investigation was initiated to determine if Tn7 could promote cointegrates. This study indicated that the replicon fusions formed in assays carried out by D Hodge were not a function of the transposition mechanism but resulted from the way the assays were performed. The replicon fusions resulted from a recA and recF independent homologous recombination across the homology created by the insertion of Tn7.

The effect of the donor replicon and the donor context (whether the transposon was transposing from a hotsite or a cold site), was also examined. There was some evidence that transposition of Tn7 can be triggered by the state of the donor replicon and that therefore Tn7 must be able to sense this state (Hauer and Shapiro, 1984). Also, Lichtenstein and Brenner (1981) have suggested and presented some supporting data that the orientation of the hotsite in the recipient replicon affects the transposition of Tn7 to that site. They found a five fold difference in the transposition frequency between two plasmids differing only in the relative orientation of the hotsite. This observation was re-examined using another replicon.

Finally, using plasmids which had Tn7 inserted in both orientations (by cloning) the effect of the orientation of the donor transposon on the frequency of transposition was examined. These Tn7's are surrounded by the same sequence and only differ in the relative orientation of the fragment containing Tn7 (and hence the orientation of Tn7) within the plasmid.

RESULTS AND DISCUSSION

3.2 The Transposition Assay

Two assays were employed to examine the transposition frequency of Tn7; the conjugative, or mate out, transposition assay and the transformation transposition assay. Transposition frequencies determined by each method can be compared internally, but comparison between frequencies determined by different methods is not meaningful.

All the assays described thoughout this thesis measure the transposition frequency from hotsites or plasmids to hotsites or cold sites. In all assays that do not involve the chromosomal hotsite, this site is free and is a target for insertion of Tn7. Competition between the free chromosomal hotsite and the target for the assay may occur. However, if the chromosomal site was a 'sink' for transpositions, it might be expected that the frequency of transposition in cells where this site was free would be lower than when this site was occupied. While this does not appear to be true, assays from the chromosome are difficult to compare with assays from

plasmids because of the possible effects of plasmid copy number. R388 probably has a copy number similar to that of a replicating chromosome. Comparison of the frequency of transposition of pEN300::Tn7 to pMR80 or pEAL1 (table 3.9) and from a chromosomal copy of Tn7 to these plasmids (table 3.7) shows no significant difference suggesting that the chromosomal site does not act as a sink for Tn7.

Alternatively, the frequencies of transposition measured in these assays may not be simply an indication of the transposition from the donor to the recipient directly but also reflect transposition to the chromosome and subsequent transposition from there to the target. If these events were independent, the frequency at which the overall event occurred would be the product of the two separate events. Transposition from plasmids to the chromosome is reported to occur at a high frequency (Barth et al, 1976; Hauer and Shapiro, 1984; Lichtenstein and Brenner, 1981). However, these measurements required the exclusion of the donor replicon by introduction and selection of a second plasmid incompatible with it. Hauer and Shapiro (1984) have found that the process of exclusion itself can stimulate the frequency of transposition so that the frequency of transposition to the chromosome under the conditions used below might be different. Analyses using a strain containing a chromosomal copy of Tn7-1 (see chapter 4) demonstrate that transposition from plasmid to plasmid does occur at about the frequency obtained using DS903 (data not shown). This confirms that the presence of a free chromosomal hotsite does not seriously affect the measured frequency of transposition.

3.2.1 The Conjugative Transposition Assay

The main transposition asssay employed for this and the other studies presented in this thesis was the conjugative or mate out assay (see materials and methods). This assay involves growing up cultures of E.coli containing a conjugative plasmid (R388 or its hotsite derivative pEN300) and the donor plasmid which must not be mobilisable or conjugative. The donor may also be a chromosomal insertion of Tn7. A mating is carried out which samples the conjugative plasmid population and the proportion of these plasmids which also carry the transposon marker is termed the transposition frequency. This does not represent an absolute measure of transposition but does allow

Table 3.1 Growth rate of DS903 containing either R388, R388::Tn7, pEN300 or pEN300::Tn7.

	Specific Growth rate
pEN300	1.49
pEN300::Tn7	1.54
R388	1.48
R388::Tn7	1.48

The specific growth rate is given by 0.699/(Doubling time) hours^{-1}
These growth rates were determined twice and the rates obtained were identical for each experiment.

Table 3.2 Efficiency of conjugation of pEN300, pEN300::Tn7, R388 and R388::Tn7.

	Frequency of Mating
pEN300	0.23
pEN300::Tn7	0.41
R388	0.22
R388::Tn7	0.37

These frequencies were derived as the mean of two experiments.

meaningful comparisons between assays.

Before examining the transposition frequencies and the frequency of replicon fusion, the possible sources of error in the assay were studied. Two areas where errors might occur were immediately apparent. Firstly, the growth rate of cells containing a transposon insertion in pEN300 or R388 might be different from cells containing R388 or pEN300 without any insertion. The growth rates of strains containing one of R388, pEN300, R388::Tn7 or pEN300:Tn7 were determined under conditions similar to those used for measuring the frequency of transposition (table 3.1) . The difference observed between pEN300 and pEN300::Tn7 represents less than a 1 minute difference in doubling time which is within the sensitivity of the measurement. These results show that the presence of Tn7 in a plasmid has no effect on the growth rate of the host.

Secondly, it seemed likely that there might be a difference in the efficiency of mating between a conjugative plasmid containing Tn7 and its parent. Often, the insertion of Tn7 into a plasmid (not in the hotsite) will interrupt a transfer gene making it transfer deficient. This will not affect the transposition frequency because such a defect can be complemented in *trans* by a fully functional copy of the plasmid. Interruption of the DHFR gene will not affect the assay because Tn7 codes for resistance to trimethoprim as well, though these genes are not homologous (Swift *et al*, 1981; Datta *et al*, 1980; Broad and Smith, 1982). The efficiencies of transfer of R388 and R388::Tn7 or pEN300 and pEN300::Tn7 (table 3.2) were compared. The efficiencies of transfer were similar in both cases though both plasmids containing Tn7 transferred with about twice the efficiency of their respective parent plasmids. This difference is within the sensitivity of the assay and is therefore not significant. Insertion into a *cis*-dominant region of the target replicon, such as the origin of replication, would be an event not sampled by this assay. However, the *cis*-dominant regions of R388 represent a small proportion of the entire replicon. Insertions into these regions should be infrequent and should not distort the observed transposition frequency.

3.2.2 The Transformation Transposition Assay

Transformation assays are also susceptible to error. It has been reported that the transformation frequency drops with increasing size of plasmid (Hanahan, 1983). From his data the reduction in the efficiency of transformation for a 4Kb plasmid due to the presence of Tn7 (14Kb) would be about five fold. This will cause the measured transposition frequency to be an underestimate. Secondly, the plasmid molecules containing the transposon insertion will be rare compared to the target replicon itself and care must be taken to avoid double transformants which receive two copies of the target plasmid. Such events will increase the apparent transposition frequency compared to the real transposition frequency. Dilution of the transforming DNA and the use of a large volume of competent cells reduces the number of double transformants considerably.

3.3 Analysis of Replicon Fusion Formation in DS903

Prior to the start of this analysis, Hodge, (1983) obtained evidence that Tn7 generated cointegrates at frequencies which depended upon the donor replicon and varied between <1% and 63% of the frequency of transposition. This result was inconsistent with the available published data. No reports of cointegrate formation by Tn7 had been or have been published though only one group specifically state that they examined the possibility (Smith and Jones, 1984; Hauer and Shapiro, 1984). The ability of Tn7 to generate cointegrates during transposition is important in any discussion of the mechanism employed. The formation of cointegrates implies a replicative transposition mechanism is operating while the absence of cointegrates may indicate that a nonreplicative mechanism is employed (Berg 1983). It was important to analyse the production of cointegrates by Tn7 and determine if they represented true products of the transposition process.

There are three formal possibilities which account for the presence of cointegrates. Firstly, cointegrates might represent obligate intermediates in the process of transposition and are therefore transient (Shapiro, 1979; Arthur and Sherratt, 1979; Galas and Chandler, 1982). In this case, the most important factor in the

observation of cointegrates will be the rate at which they decay to give simple inserts. If this process is slow relative to the generation time of the cell, the frequency of cointegrates will be greater than the frequency of simple inserts. Galas and Chandler (1982) have shown that the ratio of cointegrates to simple inserts will be a factor (the characteristic half life of the cointegrate) which is dependent on the stability of the cointegrate. Cointegrate structures obtained from transposition assays involving Tn7 are stable in recA$^-$ backgrounds but are present at frequencies that are lower than the frequency of simple insertions (Hodge, 1983) excluding this hypothesis as an explanation for the presence of cointegrates. However, these results are compatible with this hypothesis if the breakdown of cointegrates could occur only at, or shortly after, the process of transposition. If breakdown did not occur, resolution of the cointegrates might be blocked. This modification makes the original hypothesis effectively equivalent (for the purpose of this analysis) to the second hypothesis and arguements outlined below apply equally well to both.

The second possibility is that the two events, simple insertion and cointegrate formation, represent alternative end products of a single transposition pathway (Galas and Chandler, 1981; Harshey and Bukhari, 1981). This does not predict anything about the relative frequency at which cointegrates must be observed.

Thirdly, cointegrate formation may be wholly dependent on the formation of a simple insertion. When a simple insertion event has occurred, this may be converted into a cointegrate. This process does not need to be mediated by the transposon but might occur via a recA and recF independent homologous recombination pathway.

These hypotheses can be distinguished by examining the frequency of cointegrate formation and by looking at the frequency of formation of replicon fusions in reconstruction experiments.

Table 3.3 Transposition of pEAL1::Tn7 to pEN300.

	Frequency of transposition	Proportion of transpositions that were replicon fusions
pEN300	2.2×10^{-4} $\pm 2.1 \times 10^{-6}$	4.3×10^{-1}

The values presented are the averages of 18 assays and the spread is given as the standard error. The proportion of replicon fusions ranged from 6% to 96% of the transposition frequency.

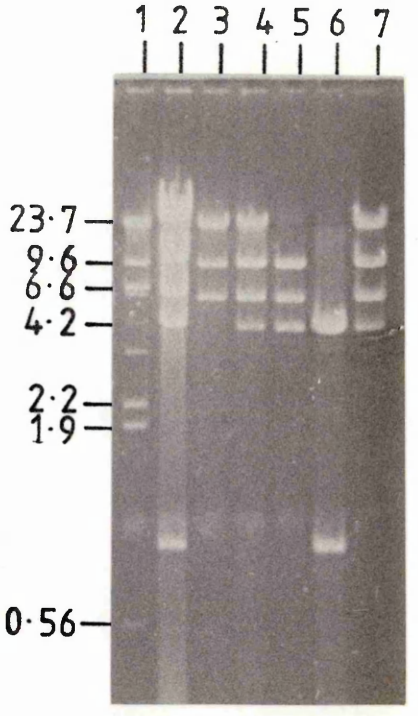


Figure 3.1 EcoRI digest of replicon fusions obtained from assays described in the text.

1 Lambda restricted with HindIII, used as size markers
2 Replicon fusion with one copy of Tn7. pEN300::pEAL1::Tn7
3 pEN300::Tn7
4 Replicon fusion with two copies of Tn7. pEN300::Tn7::pEAl1::Tn7
5 pEAL1::Tn7
6 pEAL1
7 same as lane 4

The sizes of the fragments obtained are those expected for the type of replicon fusion described in the text. The restriction fragments are given in Kb.

3.3.1 Transposition of Tn7 from pEAL1::Tn7 to pEN300

To analyse cointegrate formation, transposition assays similar to those used by Derek Hodge (1983) were carried out. He measured the transposition frequency and frequency of replicon fusion between pEAL1::Tn7 and R388 in a recA$^-$ background. The target replicon employed here was pEN300, a hotsite derivative of R388 in a recF$^-$ strain, DS903. In replicate assays, the frequency of transposition did not vary significantly (table 3.3). However, the frequency of replicon fusion varied enormously from assay to assay ranging from 6% to 96% of the transposition frequency.

When the assay mixes were plated to select for transposition, two size classes were apparent. The larger and faster growing colonies contained replicon fusions which also fell into two size classes. Both were of a higher copy number than pEN300 and both were larger than pEN300::Tn7. The largest plasmids were the same size as pEAL1::Tn7::pEN300::Tn7 cointegrates while the smaller of the replicon fusions were intermediate in size between a simple pEN300::Tn7 and a cointegrate. The slower growing colonies contained plasmids of similar copy number to pEN300 and were the same size as pEN300::Tn7 control plasmids.

Restriction analysis of these plasmids confirmed that the slow growing colonies contained simple insertions of Tn7 into pEN300. The faster growing colonies contained cointegrates (the largest plasmid) and replicon fusions (intermediate in size between the simple insertions and cointegrates) which were not true cointegrates because they contained only a single Tn7 molecule (figure 3.1). The simplest explanation for this third class is a recF independent recombination event across the homology created by the hotsite. Hodge (1983) assayed transposition between pEAL1::Tn7 and R388, so that this third class of replicon fusion was not seen. The cointegrate like molecule could be derived either by transposition or by homologous recombination across two Tn7 molecules, one in the donor plasmid and the second having transposed into pEN300. These two hypotheses can be distinguihed by examining the frequency of replicon fusion caused by recombination alone.

Table 3.4 Replicon fusion in the absence of Tn7.

Plasmids present in DS903		Frequency of replicon fusions
pEN300	pACYC184	$< 7.0 \times 10^{-8}$
pEN300	pEAL1	2.6×10^{-5}
R388	pACYC184	$< 6.0 \times 10^{-8}$
R388	pEAL1	$< 7.0 \times 10^{-8}$

This experiment was carried out once.
The recipient strain was MR1.

Table 3.5 Replicon fusions in the presence of Tn7.

Plasmids present in DS903		Frequency of replicon fusion
pEN300::Tn7	pEAL1::Tn7	1.4×10^{-3}
R388::Tn7	pEAL1::Tn7	2.0×10^{-3}

This experiment was carried out once.
The recipient strain was MR1.

3.3.2 Replicon Fusions in the Absence of Tn7

Strains were constructed which contained either R388 or pEN300 in the presence of either pACYC184 or pEAL1. Each was mated out into MR1 selecting for Tp or TpTc (table 3.4). These results show that recF independent homologous recombination was acting in DS903 to fuse pEAL1 to pEN300. No recombination was observed between R388 and pEAL1 or pACYC184 nor between pEN300 and pACYC184. This suggests that recombination occurs accross the free hotsites in pEAL1 and pEN300. The frequency at which this recombination occurs is about three fold lower than the total frequency of replicon fusions observed. While this is consistent with the proportion of replicon fusions containing one copy of Tn7 from the assays presented in table 3.3, only a limited number of colonies were examined and the frequency of replicon fusions in individual assays from table 3.3 was itself highly variable. The presence of this type of event further suggests that the cointegrates seen are not derived by transposition but are a consequence of a recF independent homologous recombination event subsequent to an initial simple insertion.

3.3.3 Replicon Fusions in the Presence of Tn7

To examine the frequency of replicon fusion caused by recombination across Tn7, a mate out assay was performed in a recF$^-$ strain, DS903, containing pEAL1::Tn7 and one of either R388::Tn7 and pEN300::Tn7. The frequencies of replicon fusions obtained (table 3.5) implies a background frequency of replicon fusion of about 3×10^{-7} for the assay in table 3.3 (about 3 logs lower than the transposition frequency). This is too low to explain the frequency of replicon fusions in terms of post transpositional homologous recombination. However, it was still possible that the transposition process might enhance the recombination between copies of Tn7. During transposition the target and donor molecules must come into close contact. The chance for recombination either during or soon after transposition may be increased. The level of general recombination may also be enhanced by the strand breakages which must occur during the transposition process. To test this, the recombination frequency was measured between Tn7 sequences immediately after transposition had occurred.

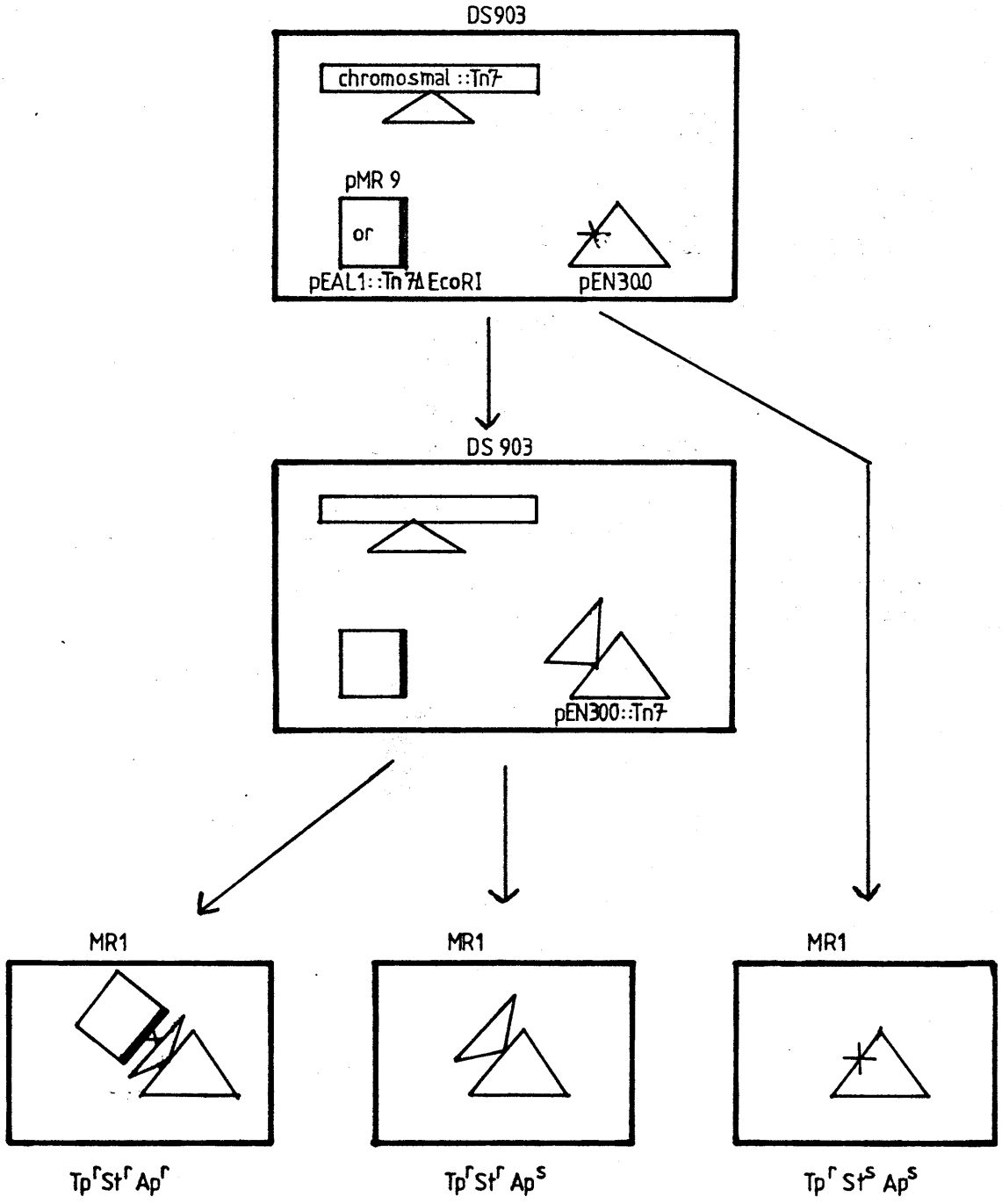

DS903
chromosmal ::Tn7
pMR 9
or
pEAL1::Tn7ΔEcoRI
pEN300
DS 903
pEN300::Tn7
MR1
MR1
MR1
Tp^r St^r Ap^r
Tp^r St^r Ap^s
Tp^r St^s Ap^s

Figure 3.2 Flow diagram of experiment to determine frequency of recombination after transposition.

DS903::Tn7 containing a plasmid with homology to Tn7 (but incapable of transposition or conjugation) and a conjugative target plasmid (pEN300) was constructed (Top). A mate out transposition assay can produce three classes of colony.

a) colonies containing pEN300 ($Tp^rSt^sAp^s$). These result from the conjugation of pEN300 without any transposition or recombination.

b) colonies containing pEN300::Tn7 ($Tp^rSt^rAp^s$). These are the product of transposition and conjugation but not recombination.

c) colonies containing both pEN300::Tn7 and the plasmid with Tn7 homology ($Tp^rSt^rAp^r$). The two plasmids may be fused or seperate. Both must have been fused prior to conjugation in order for the transfer of the non-conjugative plasmid. These are the product of transposition, recombination and conjugation.

Colonies with the same combination of resistances could arise by a cointegrate transposition of Tn7 from pEN300::Tn7 into the non-conjugative plasmid. Such events were not observed and would be rare.

 = Hotsite

 = Homology to Tn7

 = Tn7 insertion

Table 3.6 Reconstruction of post-transpositional homologous recombination.

Strain	Transposition frequency	Frequency of replicon fusions	Ratio
DS903::Tn7 (pEN300, pMR9)	6.8×10^{-6}	7.0×10^{-7}	9.7 : 1
DS903::Tn7 (pEN300, pEAL1::Tn7 *Eco*RI)	3.1×10^{-6}	2.2×10^{-7}	14.1 : 1

The frequencies shown are a mean of two experiments.

3.3.4 Post Transpositional Recombination of Tn7 Sequences

DS903::Tn7 was transformed with pMR9 (a pUC8 derived plasmid containing the right end 9Kb of Tn7; see figure 4.7) or pEAL1::Tn7ΔEcoRI (a similar construct but with a pACYC184 origin of replication; see Hodge, 1983). pMR9 and pEAL1::Tn7ΔEcoRI are incapable of independent transposition but have 9Kb of homology with Tn7.

pEN300 was introduced into these strains by conjugation from MR1(pEN300) using a donor to recipient ratio of about 20:1. Single colony gel analysis confirmed the presence of pEN300 in these strains. A mate out assay was performed selecting for pEN300, pEN300::Tn7 and pEN300::Tn7 linked to either pMR9 or pEAL1::Tn7ΔEcoRI. The strategy for this experiment is given in figure 3.2 and the results are presented in table 3.6.

The transposition frequencies in these assays were lower than expected. The presence of an end of Tn7 in trans and in high copy number is known to depress the transposition frequency (N Craig, pers. comm.). It is unclear if this has occurred in these assays because the relevant control was not performed, and both pMR9 and pEAL1::Tn7ΔEcoRI encode all functions required for transposition in trans. These results confirm that the frequency of replicon fusion caused by recombination after transposition is greater than suggested by the results from the previous section. The frequencies seen in these assays are still lower than that seen in in a normal transposition assay. In this situation recombination occurs subsequent to transposition and involves an entirely seperate molecule. In a 'normal' assay the donor transposon, in intimate contact with the target because of transposition, may be more likely to recombine forming an apparent cointegrate which is generated by recombination and not by transposition.

Two sizes of colony were observed during the assay described in section 3.3.1. The faster growing colonies were replicon fusions with a higher copy number than the slower growing colonies. Possibly the high copy number of the replicon fusions allowed cells containing these plasmids to grow better at a streptomycin concentration of

25ug/ml. MR1, MR1(pEAL1::Tn7) and MR1(R388::Tn7) were plated onto L-agar containing various concentations of streptomycin ranging from 0 to 25ug/ml. MR1 was incapable of growth at concentrations greater than 2ug/ml and grew poorly at 1ug/ml St. MR1(pEAL1::Tn7) grew on all plates. MR1(R388::Tn7) grew well on plates containing up to about 8ug/ml St but much more poorly at concentrations above this. pEAL1::Tn7 is a higher copy number plasmid than R388. Our group are the only group (I know of) that measure transposition using St as a selective marker. All other groups use Tp selection for transpositions. When conjugation into MR1 has occurred, plasmids with simple insertions are at a considerable disadvantage to plasmids of a higher copy number derived by replicon fusion. The plates were grown up for variable times (between 16 and 24 hours) and some of the smaller simple insertion colonies may have been missed in some assays. This might account for the large variation in the proportion of replicon fusions. In all of the assays that followed, a lower concentration of streptomycin (5ug/ml) was used. No replicon fusions were seen in assays using streptomycin at this reduced concentration. Also, the apparent frequency of transposition increased at this reduced concentrations (compare the frequency in table 3.3 with the frequency in table 3.9 for pEAL1::Tn7 to pEN300), supporting the suggestion that the use of streptomycin at a high concentration reduced the total number of simple insertion events observed.

All of the results in this section indicate that the high frequency of replicon fusions can be explained by post transpositional homologous recombination and by the ability of these replicon fusions, once formed and selected for, to overgrow the simple insertions. The absence of cointegrates, (<1% of the frequency of transposition) using other donors such as ColE1::Tn7, pUC8::$Tn7_I$, pUC8::$Tn7_{II}$, R388::Tn7, pEN300::Tn7 and pMR80::Tn7 and in the published papers on Tn7 both before and subsequent to this work adds futher support to this hypothesis. These results imply that replicative transposition mechanisms are not required to explain Tn7 transposition; conservative transposition can explain all of the available data.

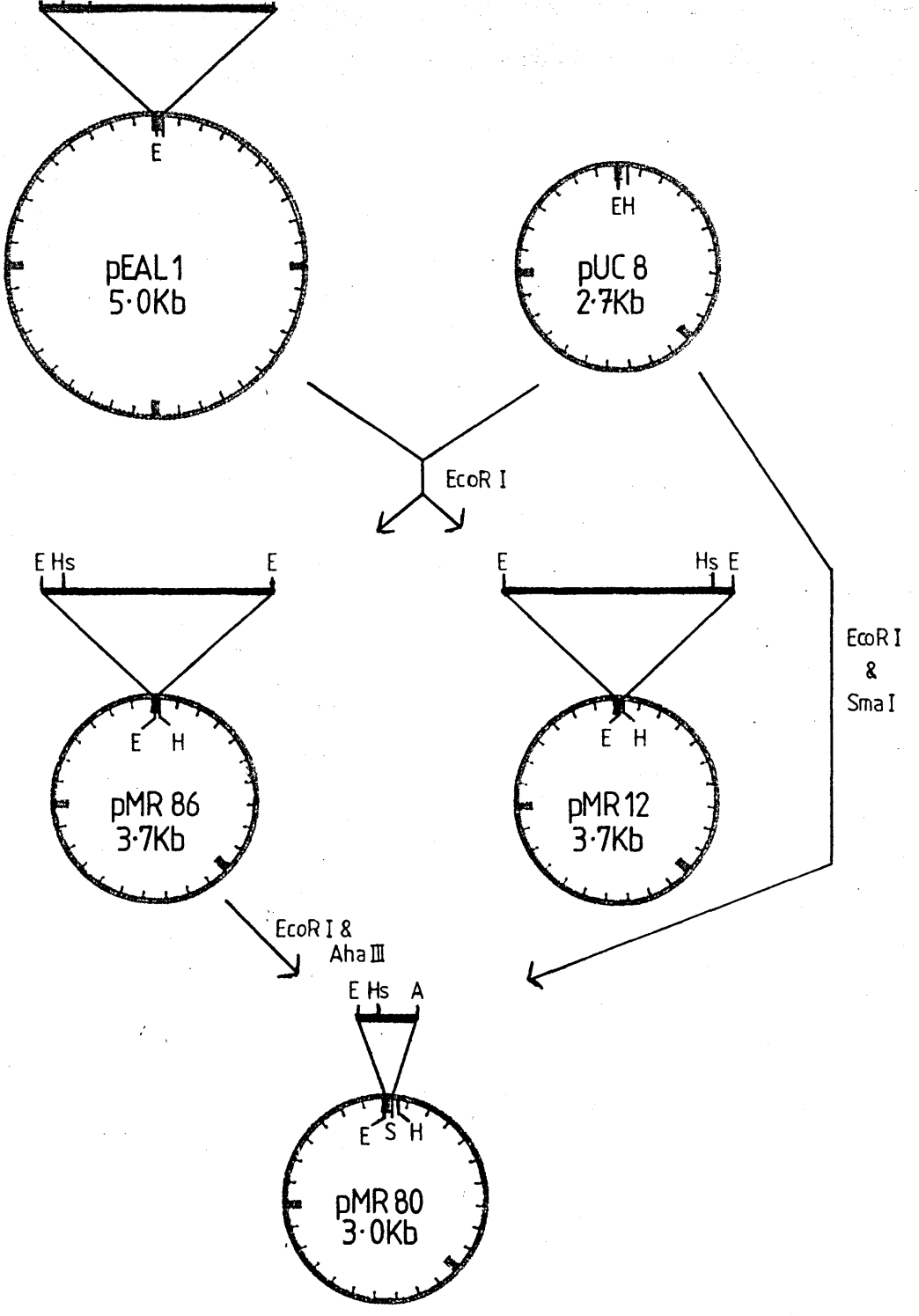


Figure 3.3 Strategy for the construction of pMR86, pMR12 and pMR80.

The cloning is described in the text. All the plasmids are drawn to scale. The hotsite fragment is represented by a horizontal line. Abbreviations: E, EcoRI; A, AhaIII; H, HindIII; S, SmaI; Hs, Hotsite (point of Tn7 insertion)

Table 3.7 Transposition of Tn7 from DS903::Tn7 to pUC8, pMR80, pMR86, pMR12, pACYC184 and pEAL1.

Plasmid	Frequency of transposition
pUC8	$< 2.8 \times 10^{-4}$
pMR80	5.5×10^{-3}
pMR86	1.2×10^{-3}
pMR12	2.3×10^{-3}
pACYC184	$< 2.9 \times 10^{-6}$
pEAL1	6.8×10^{-3}

This experiment was repeated three times.

3.4 Construction of Plasmids Containing the Hotsite

pEAL1 was available from previous work and pEN300 was constructed by Elaine Nimmo in this laboratory. A hotsite derivative of pUC8 was required; pMR86 and pMR12 were constructed by digestion with EcoRI and ligation of pEAL1 and pUC8. pMR80 was constructed by digestion of pMR86 with EcoRI and AhaIII and ligation to pUC8 digested with EcoRI and SmaI. This constructed a hotsite derivative of pUC8 containing a 246bp fragment encoding all of the sequence required to specify the hotsite. pMR12 and pMR86 contain 967bp of hotsite sequence. Figure 3.3 diagrams these clones.

3.5 Transposition of Tn7 to pUC8, pACYC184, pMR80, pMR86 and pMR12

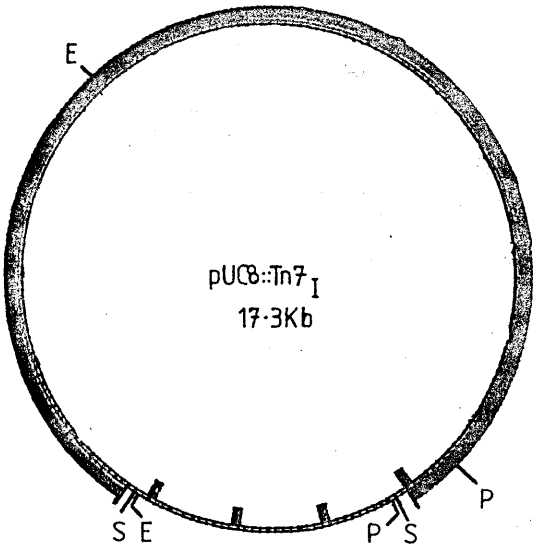
Tn7 had to be transposed into these constructs if they were to be used as donors of Tn7 in transposition assays. Each plasmid was transferred into DS903::Tn7 and grown up in L-broth. Rapid DNA minipreps were made and the DNA diluted to 0.5ml. Between 10ul and 50ul of this DNA was transformed into DS903 selecting for either the vector or the vector containing a transposon. All of the plasmids containing hotsites were efficient targets for Tn7 transposition but no transposition was observed into pUC8 or pACYC184 (table 3.7).

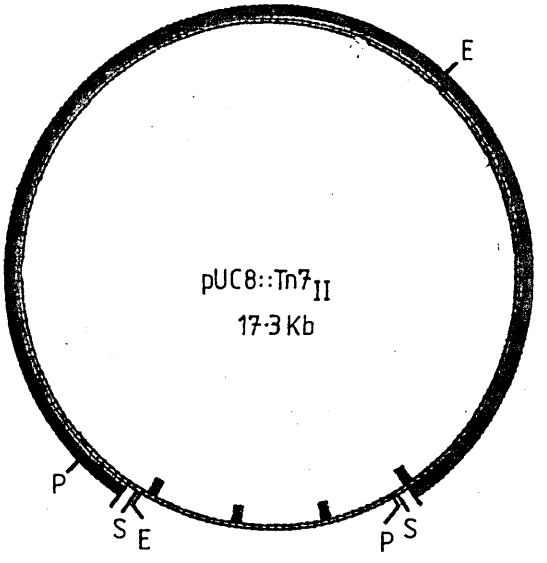
It was possible that pUC8 or pACYC184 had little free space into which Tn7 could insert without disrupting an essential function. To discount this, dimers of pACYC184 and pUC8 were used as targets in a similar assay. No transposition was detected. Neither could any transposition be detected afer growing DS903::Tn7 containing either pACYC184 or pUC8 for many generations in the presence of Tn7.

Rather than continue to try and insert Tn7 into pUC8 and pACYC184 via transposition, a complete Tn7 was cloned into these plasmids.

3.6 Construction of pUC8::Tn7

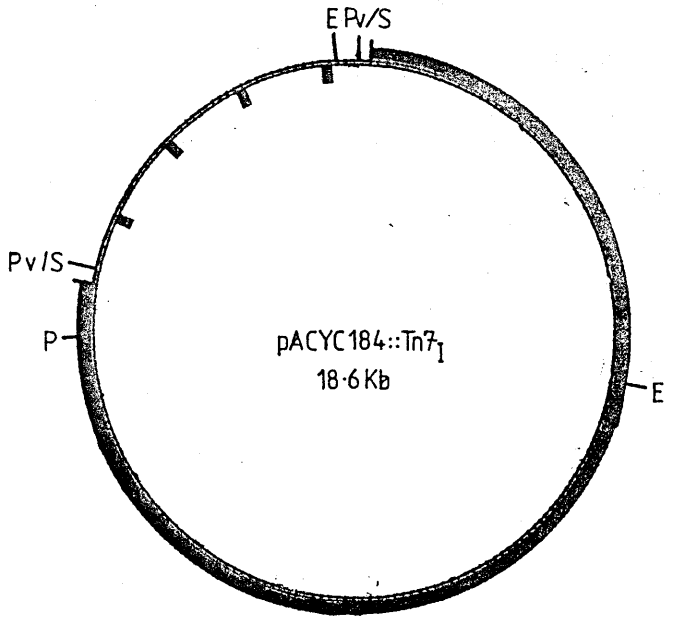
The plasmid pRP71 was obtained from Peter Barth (Barth and Grinter, 1977). This plasmid is a Tn7 insertion derivative of RP4 which is Tc^S. Tn7 has inserted between two SmaI sites seperated by about 600bp. There are no SmaI sites in Tn7 so that digestion of pRP71 and

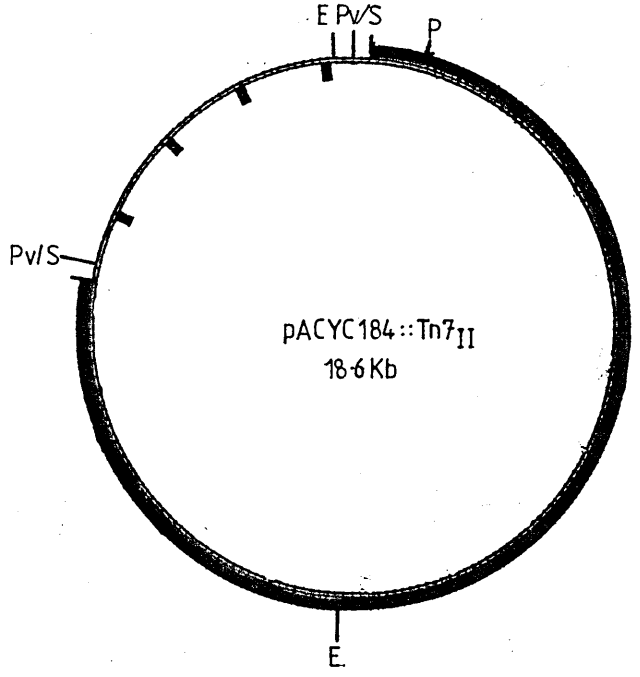




Figures 3.4 Representations of the plasmids pUC8::Tn7$_I$ and pUC8::Tn7$_{II}$.

The thick lines represent Tn7. The thin lines represent plasmid sequences. The positions of closely spaced restriction sites are not drawn to scale. Abbreviations: P, PstI; S, SmaI; E, EcoRI;





Figures 3.5 Representations of the plasmids pACYC184::Tn7$_I$ and pACYC184::Tn7$_{II}$.

The thick lines represent Tn7. The thin lines represent plasmid sequences. The positions of closely spaced restriction sites are not drawn to scale. Abbreviations: P, PstI; S, SmaI; E, EcoRI; Pv, PvuII

Table 3.8 Properties of the plasmids derived from R388, pACYC184 and pUC8.

Plasmid	Hotsite	Tn7
pUC8	-	-
pMR80	+	-
pUC8::$Tn7_{II}$	-	+
pMR80::Tn7	+	+
R388	-	-
pEN300	+	-
R388::Tn7	-	+
pEN300::Tn7	+	+
pACYC184	-	-
pEAL1	+	-
pACYC184::$Tn7_{II}$	-	+
pEAL1::Tn7	+	+

Table 3.9 Transposition of Tn7 from and to hot and cold sites.

RECIPIENT	DONOR pEN300::Tn7	R388::Tn7	pMR80::Tn7	pUC8::$Tn7_{II}$	pEAL1::Tn7	pACYC184::$Tn7_{II}$
R388	-	-	1.8×10^{-5}	8.9×10^{-6}	2.5×10^{-5}	2.8×10^{-5}
pEN300	-	-	1.2×10^{-3}	1.7×10^{-3}	9.2×10^{-4}	5.1×10^{-3}
pUC8	$<2.2 \times 10^{-6}$	$<3.4 \times 10^{-5}$	-	-	$<6.4 \times 10^{-5}$	-
pMR80	2.4×10^{-3}	3.6×10^{-2}	-	-	6.8×10^{-3}	-
pACYC184	$<2.2 \times 10^{-6}$	$<2.0 \times 10^{-4}$	-	-	-	-
pEAL1	5.0×10^{-3}	4.2×10^{-2}	-	-	-	-

The standard error for pACYC184::$Tn7_{II}$ to R388 was less than 1/3 the frequency transposition. These assays were repeated between 3 and 5 times.

pUC8 with SmaI and ligation of a mixture of these produced the plasmids diagrammed in figure 3.4 after transformation of the ligation mix. These plasmids were called pUC8::$Tn7_I$ and pUC8::$Tn7_{II}$.

3.7 Construction of pACYC184::Tn7

pACYC184 was restricted with PvuII and pUC8::$Tn7_I$ was digested with SmaI. These digests were mixed, ligated and transformed, selecting for resistance to both tetracycline and trimethoprim. A few of the colonies obtained were examined and the plasmids pACYC184::$Tn7_I$ and pACYC184::$Tn7_{II}$ were identified (figure 3.5).

3.8 Transposition of Tn7

The cloning and transposition of Tn7 into R388, pEN300, pMR80, pUC8, pACYC184 and pEAL1 produced a series of vectors based on three compatible plasmids, pUC8, pACYC184 and R388. For each of these vectors, there is a family of plasmids that contain the hotsite and/or Tn7. Table 3.8 gives the properties of all these plasmids. R388 and its derivatives are conjugative plasmids, while the pUC8 and pACYC184 derived plasmids are non-conjugative.

Table 3.9 gives the frequencies of transposition of all of these reciprocal assays. Cointegrates were not observed in any of these assays (<1% of the frequency of transposition for all assays except for assays using pEAL1::Tn7 where the frequency of cointegrate formation was <2% of the frequency of transposition). While most of the effects were small (though reproducible) and hence must be treated with considerable caution, a number of factors appeared to affect the transposition frequency. Firstly, the frequency of transposition to plasmids was less than the frequency of transposition to the hotsite.

Secondly, the context of the donor transposon, ie whether it was in a plasmid site or in a hotsite, affected the frequency of transposition to hotsites though not to plasmids. The transposition frequency from a hotsite to a hotsite was less than the frequency obtained using a cold site transposon as a donor (compare pEN300::Tn7 and R388::Tn7 transposition to pMR80 and pEAL1). To ensure that this result was

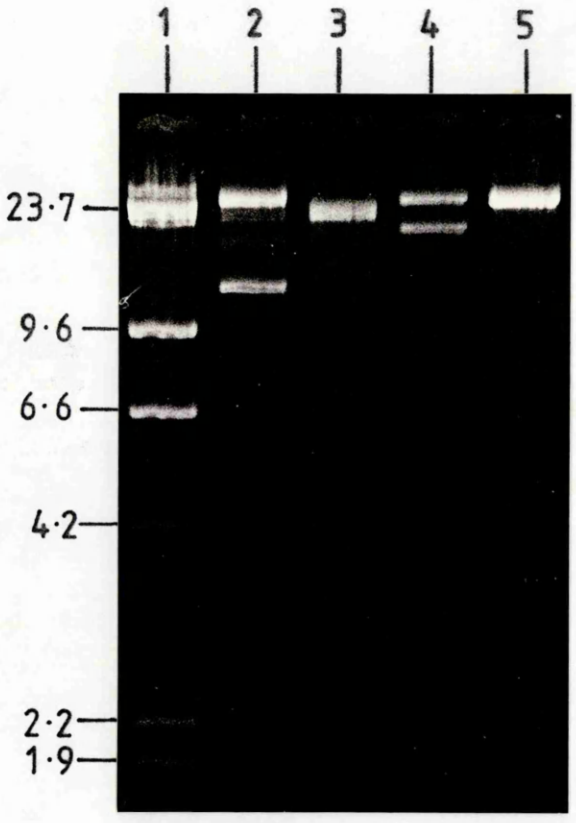


Figure 3.6 EcoRI digest of three independent R388::Tn7 clones derived from assays described in the text.

1 Lambda DNA digested with HindIII, used as size markers.
2 R388::Tn7
3 R388::$Tn7_1$
4 R388::$Tn7_2$
5 R388

The gel confirms that the three insertions of Tn7 into R388 have occurred at different sites. The digests give distinct patterns for each of the plasmids. The restriction fragments are marked in Kb.

Table 3.10 Frequency of transposition from R388::Tn7 is not due to the sequence around the insertion point.

Donor	Recipient	Frequency of transposition
R388::Tn7	pEAL1	4.2×10^{-2}
R388::$Tn7_1$	pEAL1	1.6×10^{-2}
R388::$Tn7_2$	pEAL1	1.5×10^{-2}

These assays were repeated twice.

independent of the cold site donor Tn7 used, the frequency of transposition was measured for two other R388::Tn7's, which had Tn7 inserted in different positions within R388 (figure 3.6). The transposition frequencies to pEAL1 from these different donors were the same (table 3.10), suggesting that there is a more fundamental reason for the difference in transposition frequency. Perphaps Tn7 is inefficient at transposing to homologous sequences (such as between two hotsites), or that the proteins involved are affected in some unknown way by the close proximity of two hotsites during this type of transposition.

Thirdly, the transposition frequency from either R388::Tn7 or pEN300::Tn7 to pEAL1 and pMR80 was the same, which indicates that the recipient plasmid in which the hotsite is resident does not affect the transposition frequency. The difference between the transposition frequency from pEALI::Tn7 to pEN300 and pMR80 appears to contradict this. However, the assay used to measure transposition to pEN300 was a conjugative assay while a transformation assay was used to measure transposition to pMR80. This makes any comparison of these frequencies impossible.

The difference in transposition frequency to a hotsite from either a hotsite or a cold site was not a simple effect but was influenced by other factors such as the copy number of the donor replicon. While the effect was about 10 fold between R388::Tn7 and pEN300::Tn7 as donors, it was only about 5 fold from pACYC184 derived donors and was not observed in assays involving pUC8 derived donors. This appears to correlate inversely with the copy number of the donor plasmid. There was little influence of recipient replicon on this effect (compare pEN300::Tn7 and R388::Tn7 transposition into pMR80 or pEAL1).

3.9 Tranposition of Tn7 is Not Affected by the Orientation of the Transposon in the Donor

Tn7 is known to insert into plasmids in a specfic orientation (Barth and Datta, 1977; Barth and Grinter, 1977; Barth *et al*, 1978). This implies that some orientation specfic aspect of the recipient must be recognised. The availability of Tn7 in both orientations in both pACYC184 and pUC8 allowed the effect of the orientation of the donor

Table 3.11 The orientation of the donor transposon does not affect the transposition frequency.

	DONOR			
RECIPIENT	pACYC184::$Tn7_I$	pACYC184::$Tn7_{II}$	pUC8::$Tn7_I$	pUC8::$Tn7_{II}$
R388	2.7×10^{-5}	2.8×10^{-5}	1.2×10^{-5}	8.9×10^{-6}
pEN300	6.1×10^{-3}	5.1×10^{-3}	1.1×10^{-3}	1.7×10^{-3}

The standard error for the assay between pACYC184::$Tn7_{II}$ to R388 was 1/3 the frequency of transposition. These assays were repeated three times.

Table 3.12 The orientation of the hotsite in the target plasmid does not affect transposition to that target.

Plasmid	Frequency of transposition from DS903
pMR12	2.3×10^{-3}
pMR86	1.2×10^{-3}

The standard errors of these frequencies never exceeded 1/20 of the transposition frequencies. These assays were repeated three times.

on transposition to be examined. All of the Tn7 genomes in these four clones have Tn7 sitting in the same local sequence. Thus any difference observed would be due to the orientation of Tn7. Table 3.11 gives the results of these assays and indicates that the orientation of the donor transposon had no effect on the insertion of Tn7 into R388 or pEN300. The insertion of Tn7 into R388 was independent of the orientation of the donor transposon; all insertions examined occurred in the same orientation (data not shown).

3.10 The Orientation of the Hotsite in a Recipient Plasmids Does Not Affect Transposition to that Site

During the construction of pMR80, pMR12 and pMR86 were made which contain the hotsite cloned into the EcoRI site but in inverted orientation with respect to one another. The transposition of Tn7 into these plasmids was determined (table 3.12) and showed that there was no effect of orientation upon transposition, contradicting the results of Lichtenstein and Brenner (1981). They observed a small (5 fold) difference in the transposition frequency dependent upon the orientation of the hotsite in the target.

This result also confirms than insertion of Tn7 into the hotsite occurs in a sequence directed manner, ie., it is a site-specific event analogous to lambda integration, and highlights a difference in the mechanism of transposition to hotsites and to cold sites. Transposition to the hotsite is sequence specific while transposition to cold sites is not; it occurs in an orientated manner recognising some plasmid determinant, which is probably not sequence, to insert into the plasmid in the same orientation though in a large number of different sites.

3.11 Conclusions

Results presented in this chapter indicate that the transposition of Tn7 is complex. Cointegrates are not observed at a level detectable above the background indicating that the transposition mechanism need not be replicative. Two modes of transposition can be distinguished; transposition to hotsites (cloned on plasmids or chromosomally located) and transposition to plasmids.

Transposition to the hotsite occurs in a sequence dependent manner. The hotsite is specifically recognised and insertions generally occur in identical positions (Lichtenstein and Brenner, 1981, 1982). Deletion analysis of the hotsite reveal it to be complex, extending over about 70bp, with the insertion site lying asymmetrically within this region (C Lichtenstein, pers. comm.; N Craig, pers. comm.)

Transposition to cold sites is not sequence dependent (no obvious preference for specific sites within R388 can be observed) but occurs in a orientated manner. All insertions occur in the same relative orientation with respect to the restriction maps of the transposon and the target plasmid (Barth and Datta, 1977; Barth and Grinter, 1977; Barth et al, 1978). This implies a recognition of some aspect of the plasmid by the transposon, which is orientation specific such as the origin of transfer of a conjugative plasmid.

The context of the donor transposon affects the transposition frequency of Tn7 to the hotsite; transposition from a hotsite is less efficient than transposition from a plasmid. This effect may be influenced by the copy number of the donor though the mechanism underlying it is not understood.

CHAPTER 4

DISSECTION OF THE FUNCTIONS REQUIRED FOR TN7 TRANSPOSITION.

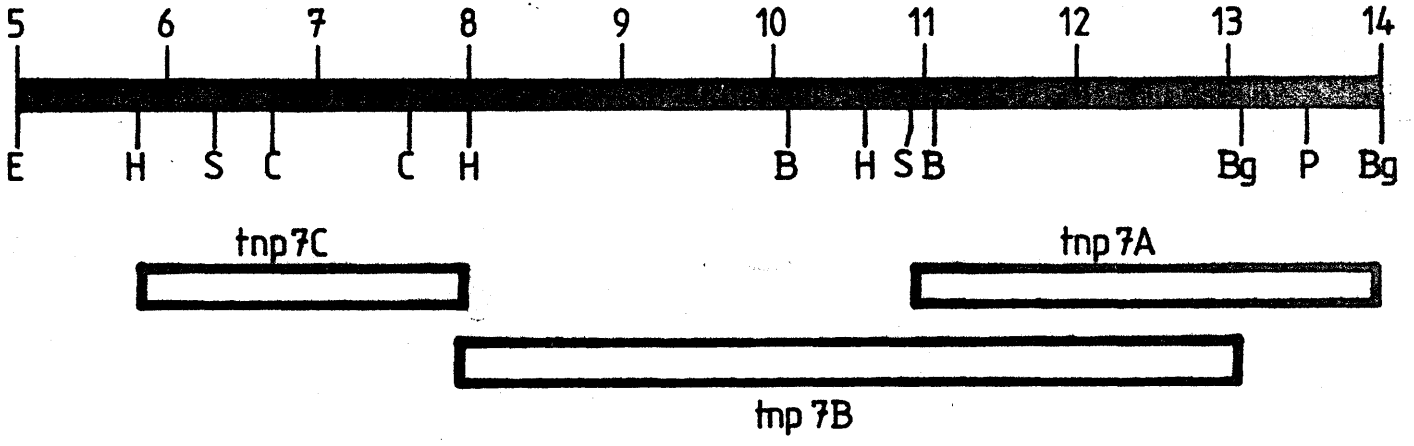


Figure 4.1 Map of Tn7 transposition functions.

Modified from Hauer and Shapiro (1984). Each complementation group is shown by an open box. Tnp 7C is only required for transposition to cold sites.

Abbreviations: B, BamHI; Bg, BglII; C, ClaI; E,EcoRI; H, HindIII; P, PstI; S, SstII

4.1 INTRODUCTION

Though the transpositional capability of Tn7 has been known for several years, little work has been undertaken to determine the genetic organisation of the transpositional functions in Tn7. Several years after the discovery of Tn7, the site specificity of insertion into the chromosome of Escherichia coli was identified, and the site of insertion was cloned and sequenced (Lichtenstein and Brenner, 1981, 1982). This 'hotsite' which has been reduced in size to about 70bp (C Lichtenstein, pers. comm., N Craig, pers. comm.), has been identified recently as the transcriptional terminator of the glmS gene (Gay et al, 1986). The hotsite is functional when cloned into multicopy plasmids which facilitates the study of transposition to this site. In this chapter, trans-acting functions required for transposition to both the hotsite and to plasmids lacking this site have been defined.

During the course of this work, a number of papers were published which partially defined the regions of Tn7 required for transposition (Grinter, 1983; Smith and Jones, 1984; Hauer and Shapiro, 1984; Ouartsi et al, 1985). The approach taken in all of these papers was the complementation of deletions within Tn7 using cloned fragments or other deletion mutants of Tn7. The major results of these studies were;

a) All functions essential for transposition are encoded entirely within an 8.1Kb fragment stretching from the BstEII site at 6.1Kb to the BglII site at 14.0Kb.

b) Three complementation groups were defined, which were each essential for transposition to plasmids, though one was dispensable in transpositions to the hotsite (figure 4.1).

These data indicate that the transposition of Tn7 is complex in terms of the number of functions required and is unlike any transposon studied previously (Kleckner, 1981). They also suggest that the mechanism of transposition to the hotsite (either cloned onto plasmids or in the chromosome) is not the same as that to coldsites (generally plasmids), though this extra complementation group need not be

directly involved in the transposition process but may regulate that process in a manner that permits the transposition of Tn7 to plasmids.

For this thesis, a different approach was taken. The trans-acting functions were analysed by their ability to complement the transposition of a 'mini' Tn7 which did not contain any sequences of Tn7 that might encode a function required for transposition. All sequences encoding trans-acting functions would be supplied on multicopy expression vectors. These vectors encode promoters adjacent to cloning sites which alleviates the requirement of cloning Tn7 promoters with every fragment. This method allows a gross map of Tn7 to be constructed and has the advantage that the different Tn7 functions identified will be cloned and ready for further analysis.

RESULTS

4.2 Defining and establishing a system to map the trans-acting functions required for the transposition of Tn7.

The complementation system, used below to define trans-acting functions, employed Tn7-1, a Tn7 derivative constructed in vitro. It was deleted for all but 168bp of the left end of Tn7 and 535bp of the right end of the transposon. To be useful this system had to fulfill the following criteria.

a) The mini transposon, Tn7-1, should not contain any complete Tn7 open reading frames.

b) Tn7-1 should be incapable of transposition unless complemented in trans, i.e., Tn7-1 should contain all sequences required in cis for transposition.

c) The plasmids used to clone complementing fragments of Tn7 should be compatible and contain controllable promoters which could replace any Tn7 promoters lost during construction.

d) The recipient plasmids should be conjugative, and preferably identical in all respects except for the presence, in one, of a hotsite.

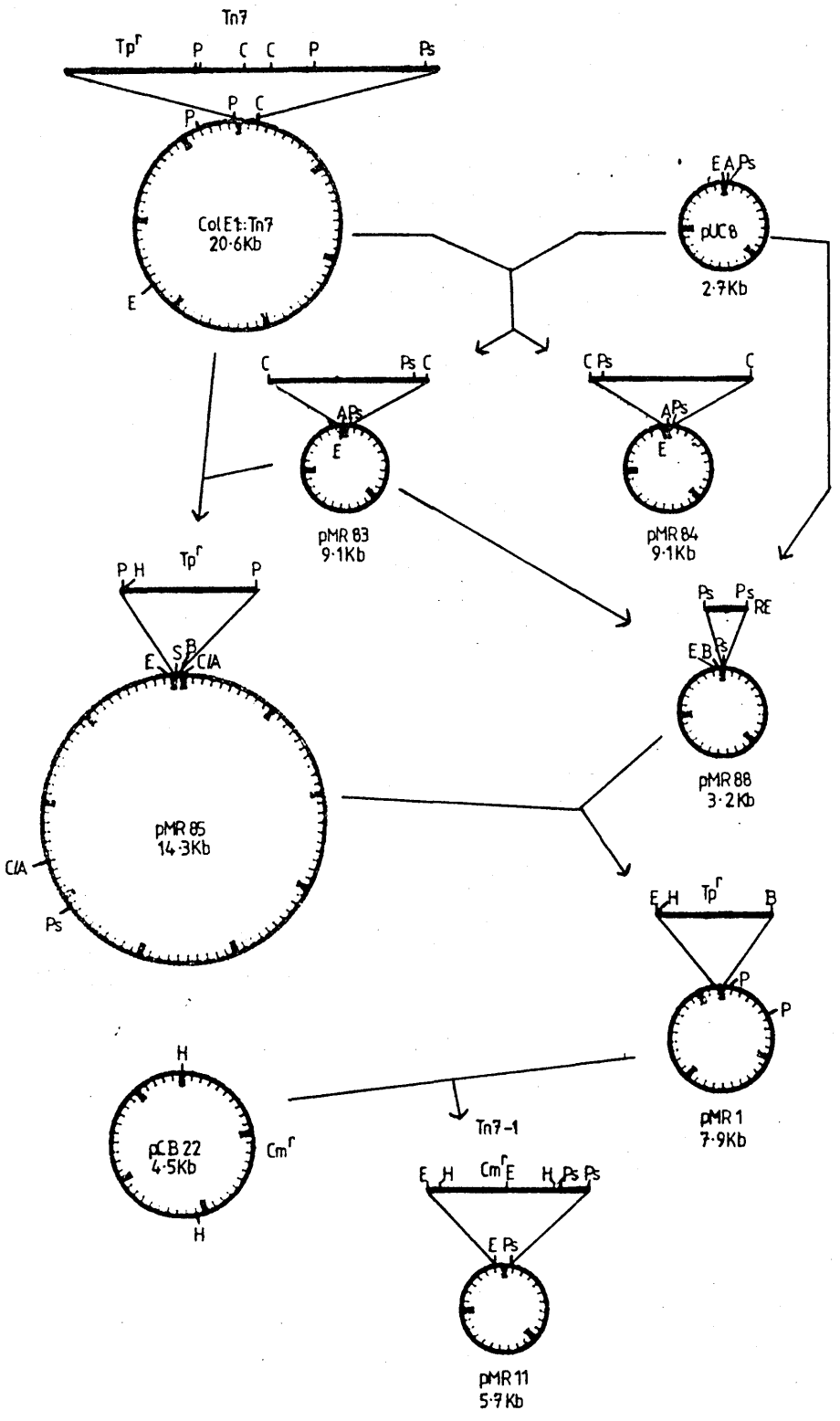


Figure 4.2 Construction of Tn7-1 and Tn7-2.

For an explanation of the strategy, see the text. The newly cloned fragments are represented by the straight lines while the circles represent the vector sequences.

Abbreviations: A, AccI; B, BamHI; C, ClaI; E, EcoRI; H, HincII; Ps, PstI; P, PvuII; RE, Right end.

4.2.1 Construction of Tn7-1 and Tn7-2.

The construction of Tn7-1 was arranged so that all Tn7 sequences present had been determined (Lichtenstein and Brenner, 1982;, Gay et al, 1986). This allowed the sequence of Tn7-1 to be reconstituted and analysed for potential open reading frames.

A second Tn7 derivative, Tn7-2, was also constructed which contained all of Tn7 except for the sequences between the PvuII site at 4.98Kb and the ClaI site at 7.6Kb.

The plan of construction of both these derivatives is detailed in figure 4.2. The plasmid pMR83 was obtained by ligation of a ClaI digest of ColEI::Tn7 to an AccI digest of pUC8. This ligation mix was transformed into ΔM15 and Ap^r colonies selected. Analysis of white colonies (see materials and methods for description of the blue/white selection system in pUC8) on single colony gels identified clones containing the right end of Tn7 up to the ClaI site at 7.6Kb and 8bp of ColEI sequence. Restriction of DNA made from a number of these colonies using PstI identified the plasmids pMR83 and pMR84 which have the alternative orientations of this fragment in pUC8. pMR83 was the required orientation and was used for the construction of pMR88 and pMR85.

The small PstI fragment from pMR83 was purified from a 1% low melting point agarose gel and cloned into the PstI site of pUC8 in a similar manner to that described above resulting in the plasmid pMR88. Only the orientation shown in figure 4.2 was obtained, but this was the correct orientation for the construction of pMR1.

pMR85 was constructed by ligation of PvuII restricted ColEI::Tn7 to SmaI digested pMR83 and subsequent selection of Ap^rTp^r in DS903. Fortunately, the orientation obtained was the required one (figure 4.2). This plasmid was the donor of Tn7-2 which consisted essentially of Tn7 with the region between the PvuII site at 4.98Kb and the ClaI site at 7.6Kb replaced by the BamHI site of the pUC8 polylinker (figure 4.3).

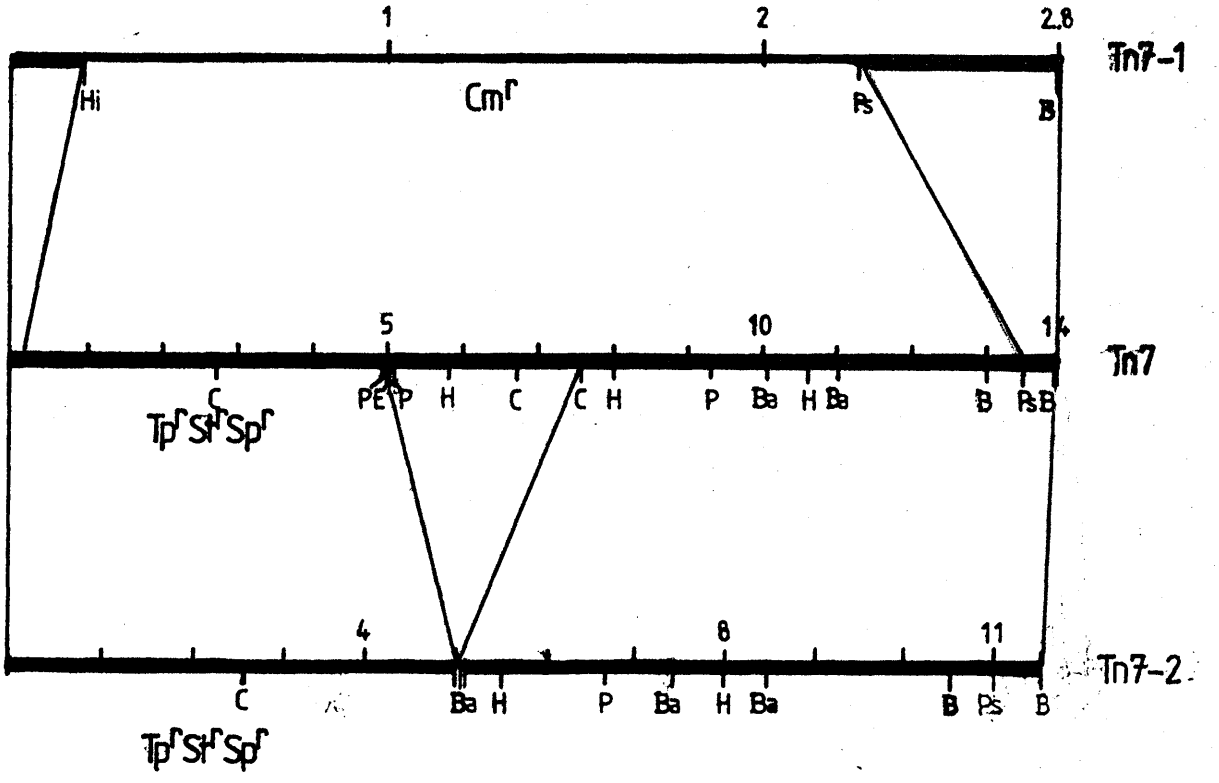


Figure 4.3 Representation of the Tn7 derivatives used in this chapter.

The size of each Tn7 derivative is marked in Kb (Tn7-1, 2.8Kb; Tn7-2, 11.5Kb; Tn7, 14Kb). See text for details of construction and characteristics.

Abbreviations for restriction sites are: Ba, BamHI; B, BglII; C, ClaI; E, EcoRI; H, HindIII; Hi, HincII (only shown for Tn7-1); P, PvuII; Ps, PstI.

For the construction of pMR1, the PvuII fragment cloned in pMR85 was purified from a 1% low melting point agarose gel as a EcoRI/BamHI fragment and subcloned into pMR88 digested with EcoRI and BamHI. pMR1 was digested with HincII and ligated to a HincII digested Cm^r plasmid pCB22. This ligation removed all the Tn7 sequences between the HincII site at 0.168Kb and the HincII site in the pUC8 polylinker and replaced them with a Cm^r gene from pCB22. This plasmid was called pMR11 and was the source of Tn7-1.

Tn7-1 contains 168bp of the left end of Tn7 up to the HincII site and 535bp of the right end up to the PstI site (see figure 4.3). The Cm^r gene is located within a 2.1Kb fragment derived from pCB22 (a lambda-dv vector similar to pCB101 (Boyd and Sherratt, 1986).

4.2.2 Analysis of the reconstituted sequence of Tn7-1.

Using the known sequence of pUC8, pCB22 and the left and right end of Tn7, the entire sequence of Tn7-1 was reconstituted in the university computer and analysed for any complete Tn7 open reading frames. None were found. The start of an open reading frame can be seen in the right end beginning 135bp from that end and reading into the transposon across the PstI site at 13.5Kb. This potential gene is preceded by a possible ribosome binding site and a promoter which has been detected by transcriptional fusion studies (N Ekaterinaka, pers. comm.) and recently by S1 mapping (Gay et al, 1986). The size of the potential polypeptide created in Tn7-1 by the fusion of the right end of Tn7 with the HincII Cm^r fragment will be 137 amino acids, 4 being derived from the Cm^r HincII fragment.

4.2.3 Tn7-1 is incapable of transposition unless complemented in *trans* by Tn7.

pMR11 was transformed into DS903(pEN300) and DS903(R388). R388 and pEN300 were mated out into MR1 in a standard transposition assay. Selection of Tp^r or Tp^rCm^r MR1 allowed the frequency of transposition of Tn7-1 to be estimated. No Tp^rCm^r colonies were found indicating that no transposition of Tn7-1 had occured either to the hot site or to the plasmid R388 (table 4.1). For Tn7-1 to be useful, it had to be capable of transposition when complemented in *trans*. To confirm this,

Table 4.1 Transposition of Tn7-1 from pMR11 to R388 and pEN300.

	Tn7-1	Tn7-1 and pEAL1::Tn7
R388	$<2.3 \times 10^{-9}$	1.3×10^{-5}
pEN300	$<5.8 \times 10^{-9}$	7.2×10^{-4}

These frequencies are the mean of two experiments.

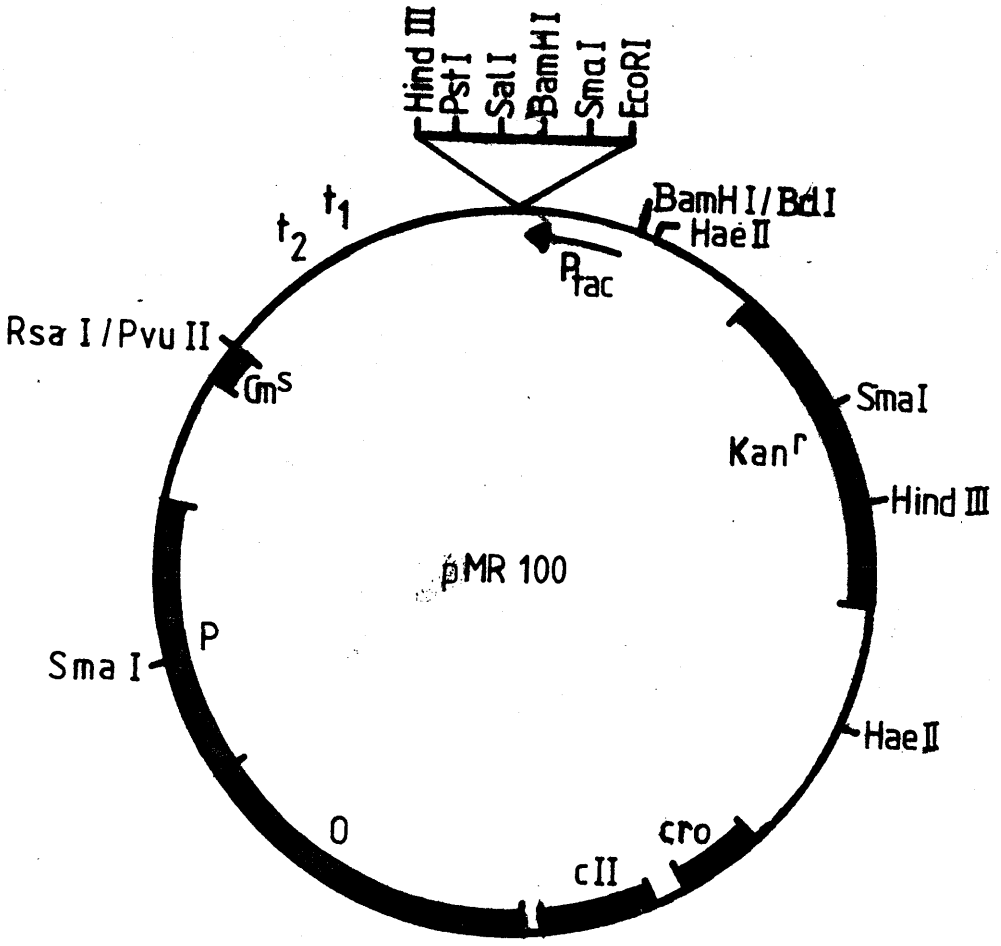


Figure 4.4 Diagram of the expression vector pMR100.

The construction is described in the text and figure 4.5. pMR100 is 5.6Kb and encodes resistance to kanamycin. Fragments cloned into the multiple cloning site in the appropriate orientation will have their genes transcribed from the tac promoter which is regulated by the host lacI gene product. Two 5S ribosomal transcriptional terminators prevent excessive transcription from the tac promoter affecting the stability of the replicon. The unique cloning sites in the polylinker are EcoRI, BamHI, SalI and PstI.

Abbreviations: t_1, t_2, 5S ribosomal transcriptional terminators
O, P, cII, cro, Lambda genes essential for replication.

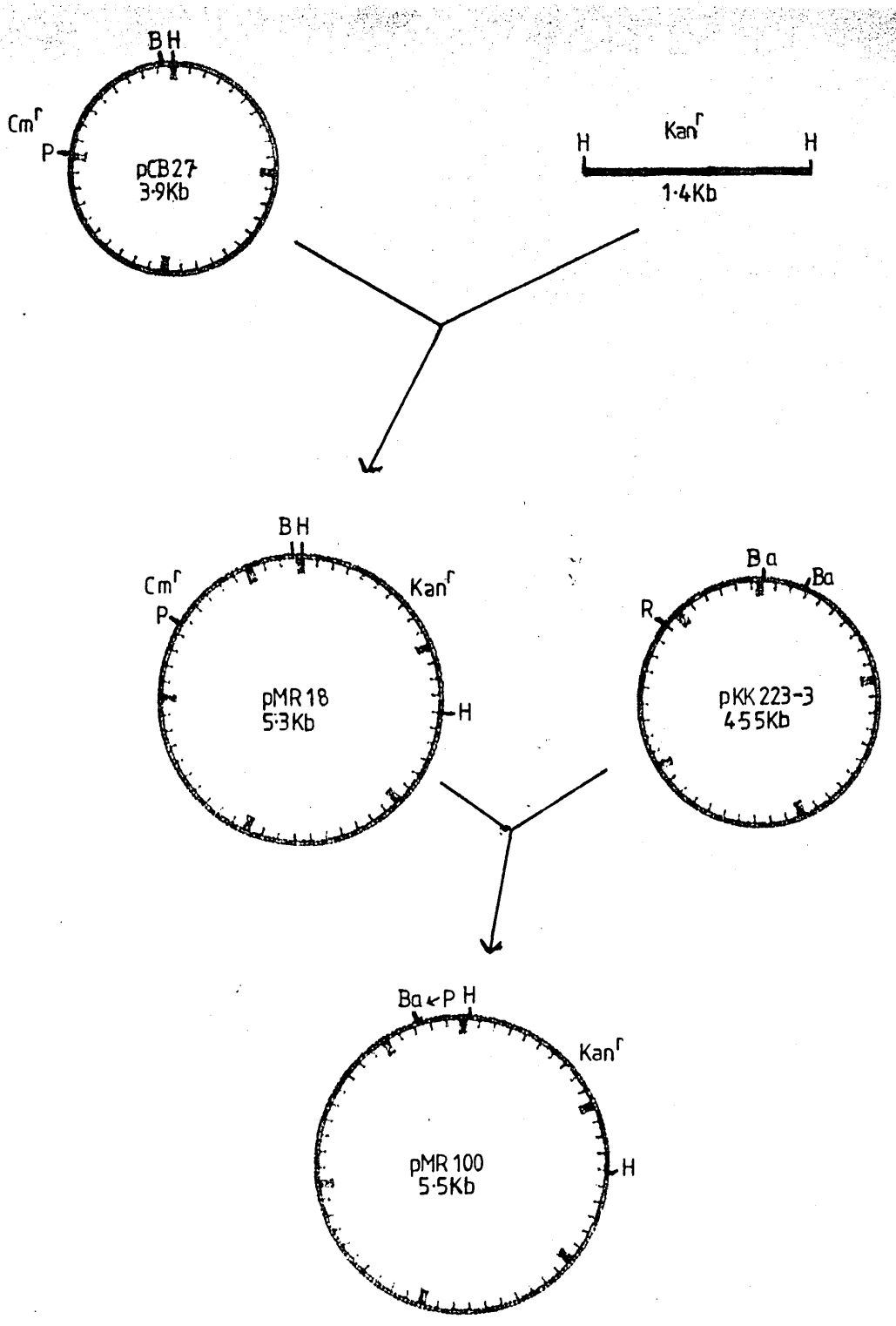


Figure 4.5 Strategy for the construction of pMR100.

The details of the construction are given in the text. All plasmids are drawn to scale and are marked in Kb. The Kan^r fragemnt of Tn903 was a gift from Chris Boyd. pGLW8 is identical to pKK223-3 except for the lack of a BamHI site at 0.28Kb.
Abbreviations: Ba, BamHI; B, BclI; H, ~~HindIII~~ Hae II; P, PvuII; R, RsaI
P--→, tac promoter

pEAL1::Tn7 was transformed into DS903(pEN300, pMR11) and DS903(R388, pMR11) and the mate out assay repeated. Transconjugants were selected on Tp and Cm for plasmids containing Tn7-1 from pMR11 and on Tp for the total number of plasmids transferred. Tn7-1 was complemented by pEAL1::Tn7 to transpose efficiently to both R388 and to pEN300 (table 4.1) and at frequencies comparable to the transposition of Tn7 itself from pEAL1::Tn7 (compare with table 3.9).

4.2.4 Construction of pMR100.

To complement Tn7-1 transposition, multicopy vectors were required which could provide transcription through fragments of Tn7 cloned into them. The expression vector pKK223-3 (Analects) was modified into pGLW8 by the destruction of the BamHI site that is not in the polylinker (Fiona Stuart, pers. comm., 1985) and was used in this form.

However, a second compatible expression vector was required, so the lambda-dv vector pCB27, constructed by Chris Boyd in the laboratory, was converted into pMR100 by the cloning of the tac promoter, polylinker and transcriptional terminators from pKK223-3 into pCB27 (figure 4.4). First, pCB27 was converted to a Cm^rKan^r plasmid pMR18 because pCB27 shares the same resistance gene as Tn7-1. This was acheived by the ligation of a HaeII Kan^r fragment from Tn903 (Oka et al, 1981) into the HaeII site of pCB27. Transformation of the ligation mix and subsequent selection for Cm^rKan^r colonies led to the identification of the plasmid pMR18 which had the structure shown in figure 4.5. pMR18 was transformed into the dam-3 strain CB51 and covalently closed circular DNA prepared. This DNA was sensitive to digestion by BclI.

The Cm^r gene of pMR18 was partially deleted by PvuII and BclI digestion and replaced by a 1.1Kb fragment purified after agarose gel electrophoresis of a RsaI and partial BamHI double digestion of pKK223-3. The resulting plasmid, pMR100, is a lambda-dv based Kan^r expression vector with multiple cloning sites 3' to a controllable tac promoter. The unique sites available in the polylinker are EcoRI, BamHI, PstI and SalI.

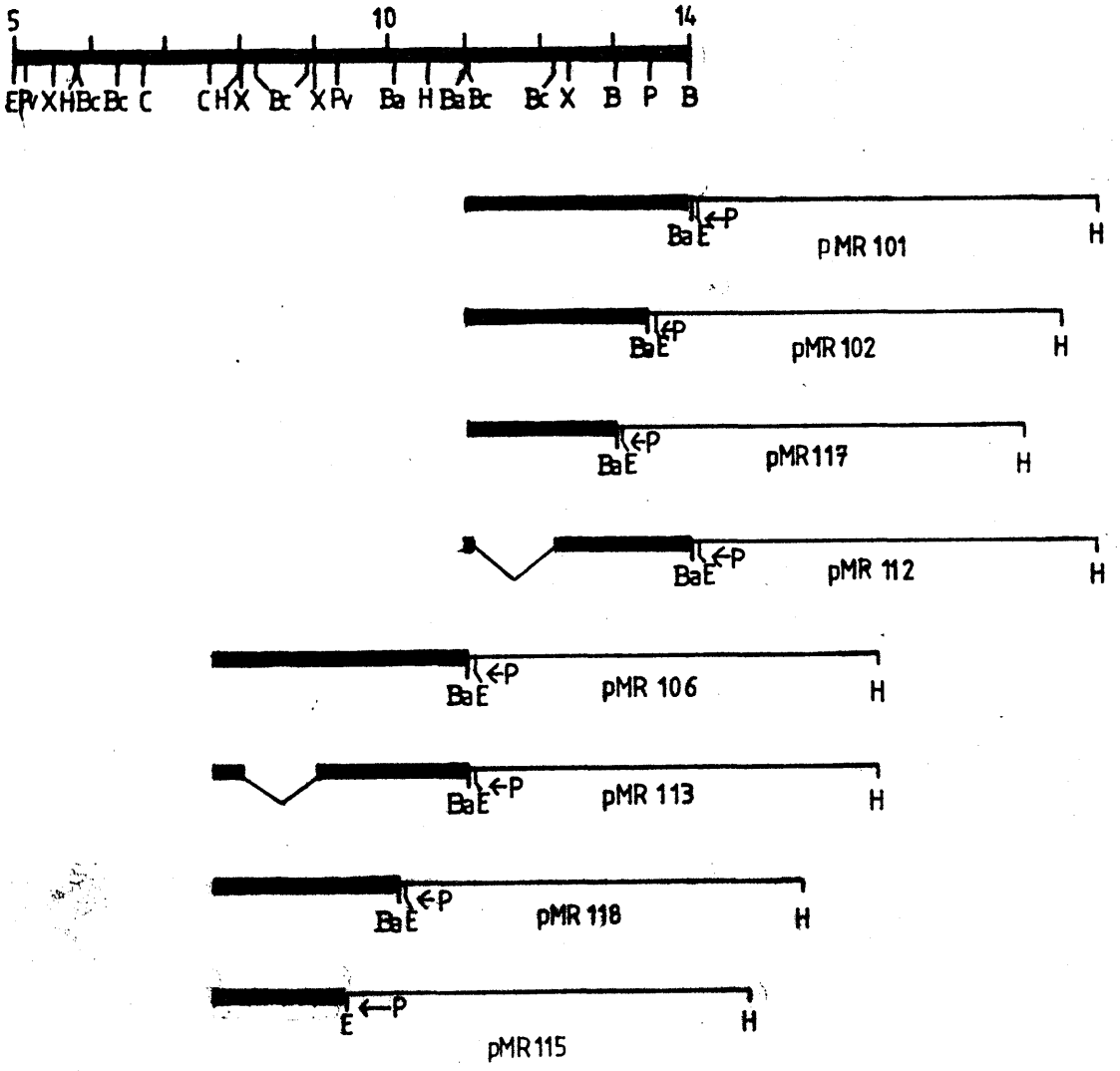


Figure 4.6 Plasmids derived from pMR100 containing Tn7 fragments.

Thick lines represent regions of Tn7. A ⌣ represents a deletion of Tn7 sequences. Thin lines represent pMR100 sequences. Abbreviations: B, BglII; Ba, BamHI; Bc, BclI; C, ClaI; E, EcoRI; H, HindIII; P, PstI; Pv, PvuII; X, XbaI; P→, tac promoter.

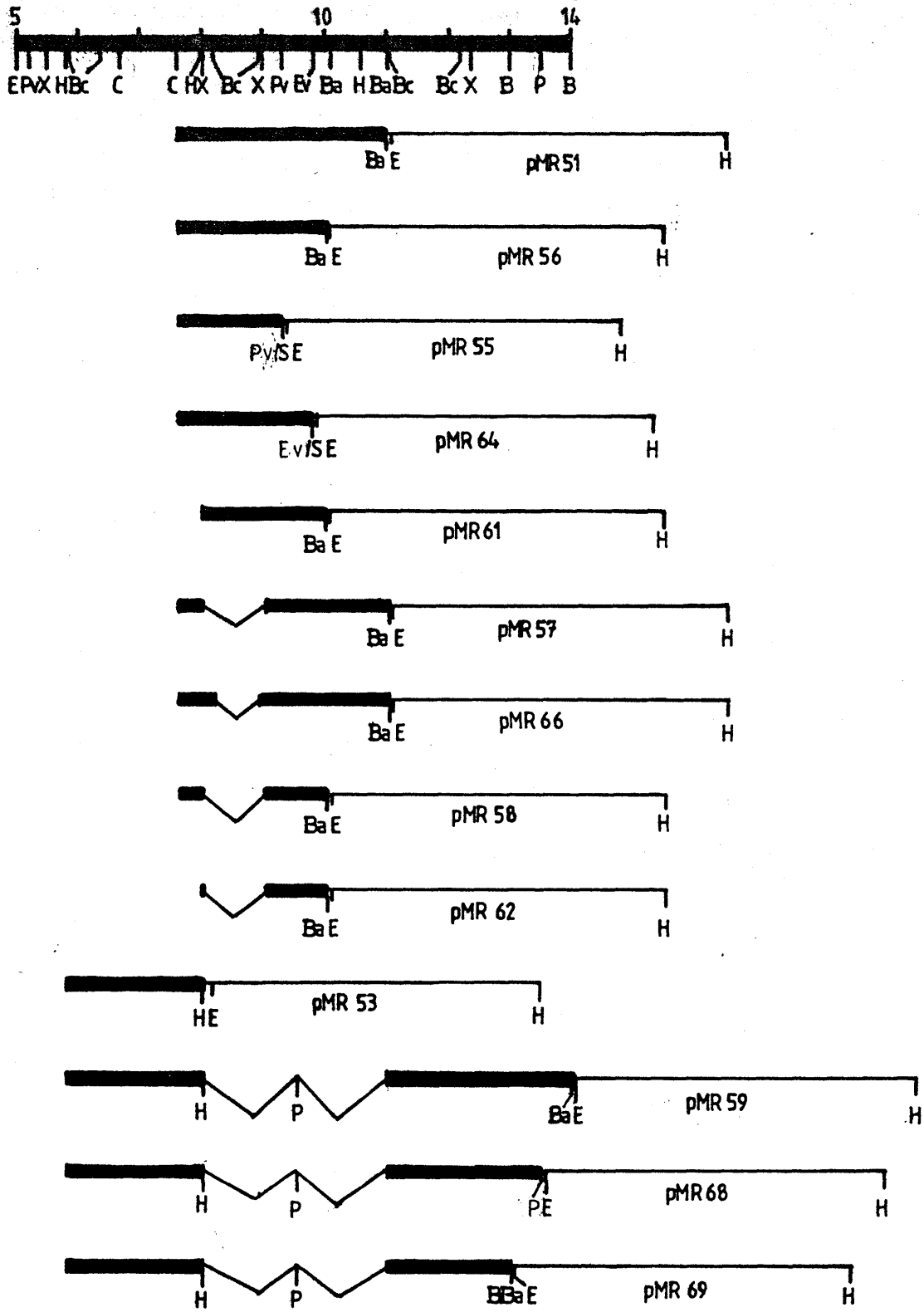


Figure 4.7 Plasmids derived from pGLW8 containing Tn7 fragments.

Thick lines represent regions of Tn7. A ⌣ represents a deletion of Tn7 sequences. Thin lines represent pGLW8 sequences. pMR59, pMR68, pMR69 contain two Tn7 fragments cloned seperately. Abbreviations: B, BglII; Ba, BamHI; Bc, BclI; C, ClaI; E, EcoRI; Ev, EcoRV; H, HindIII; P, PstI; Pv, PvuII; X, XbaI.

4.2.5 Construction of plasmids containing fragments of Tn7.

The fragments used in this study were derived from a number of sources. Clones that were not deletions were obtained by simple digestion of donor and recipient vectors which were mixed in ligation buffer with the insert in excess. T4 DNA ligase was added and the ligation carried out at room temperature for 1 hour prior to transformation into DS941 and selection on the appropriate antibiotics. Figures 4.6, 4.7 and 4.8 show the structures of the plasmids. Three vectors were employed, pACYC184 (P15A origin), pGLW8 (ColE1 like origin) and pMR100 (lambda replication origin). pACYC184 is resistant to chloramphenicol and tetracycline. Any fragment of Tn7 cloned into this vector must be inserted into the Cm gene to allow a positive selection of Tn7-1. The plasmids pMR25, pMR36 and pMR41 have the promoter of the Cm^r gene deleted, so that expression of Tn7 functions can occur only if the fragment has its own promoter. Below, plasmids under construction has been highlighted for clarity.

pMR9 (figure 4.8) was cloned as an EcoRI fragment of pEAL1::Tn7 into EcoRI digested pUC8. **pMR101** (figure 4.6) contains the 4Kb of the right end of Tn7, and was derived by BamHI digestion of pMR84 (section 4.2.1) and ligation to BamHI-restricted pMR100. **pMR102** (figure 4.6) was obtained from pMR101 by subcloning the PstI fragment of pMR101 containing 3.5Kb of Tn7 (11.05Kb 13.5Kb) into the Pst1 site of pMR100. **pMR112** (table 4.6) resulted from religation of BclI digested pMR101. This plasmid is deleted for the Tn7 sequences between the BclI sites at 11.1Kb and 12.2Kb. **pMR106** (figure 4.6) contains the Tn7 sequences between the ClaI site at 7.6Kb and the BamHI site at 11.05. This fragment was cloned as an EcoRI and PstI fragment from pMR23 into pMR100. pMR23 was constructed by partial digestion of pMR84 with BamHI and religation at low DNA concentration. **pMR51** (figure 4.7) contains the same fragment as pMR106 but is based on pGLW8. Digestion of pMR106 and pMR51 with either XbaI or BamHI and religation at low DNA concentration resulted in the plasmids **pMR113** (figure 4.6), **pMR57** (figure 4.7), **pMR118** (figure 4.6) and **pMR56** (figure 4.7) respectively. **pMR117** (figure 4.6) was constructed by digestion of pMR84 with BamHI and BglII and ligation to pMR100 restricted with BamHI.

Digestion of pMR51 with SmaI and PvuII or SmaI and EcoRV followed by

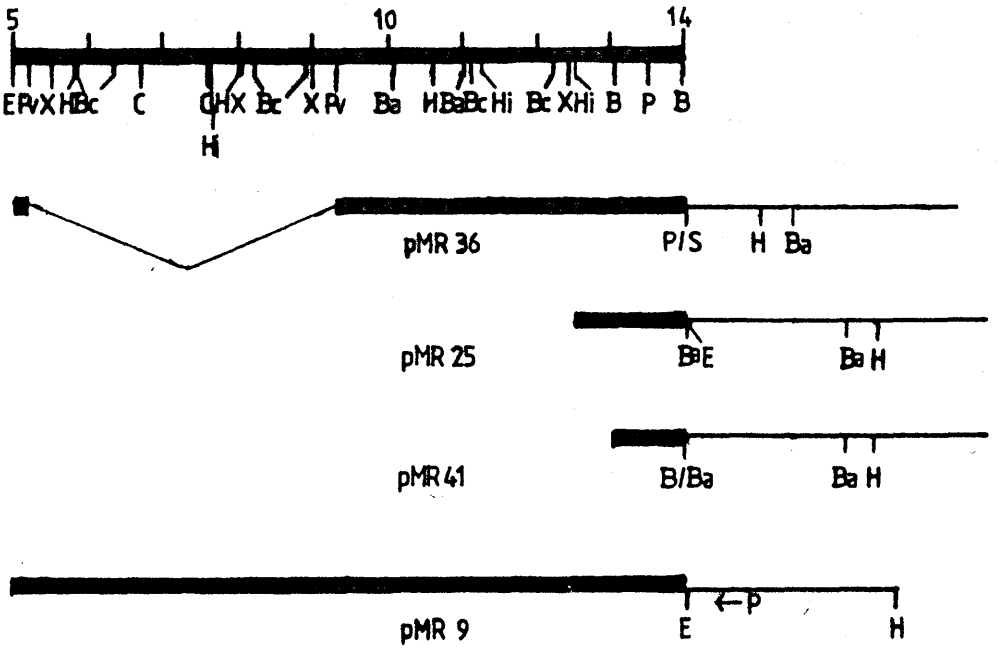


Figure 4.8 Plasmids derived from pACYC184 and pUC8 containing Tn7 fragments.

Thick lines represent regions of Tn7. Thin lines represent pACYC184 sequences or pUC8 sequences (pMR9). The Tn7 sequences in pMR36 contain a deletion of the PvuII fragment between 5.2Kb and 9.3Kb. pMR26, pMR25 and pMR41 have no external promoters and transcription is directed from a Tn7 promoter located in the right end of the transposon between the PstI site at 13.5Kb and the BglII site at 14.0Kb. This transcription reads from right to left as drawn. Abbreviations: B, BglII; Ba, BamHI; Bc, BclI; C, ClaI; E, EcoRI; H, HindIII; Hi, HincII; P, PstI; Pv, PvuII; X, XbaI; P⟶ , lac promoter.

religation at low DNA concentration yielded the plasmids **pMR55** and **pMR64** respectively (figure 4.7). Religation of pMR57 after restriction with BamHI produced the plasmid **pMR58** (figure 4.7). Similarly digestion of pMR56 and pMR58 with HindIII and religation resulted in the plasmids **pMR61** and **pMR62** respectively (figure 4.7).

pMR53 (figure 4.7) was a pGLW8 derivative containing the 2.2Kb HindIII fragment (5.8Kb to 8.0Kb) of Tn7. **pMR59, pMR68** and **pMR69** (figure 4.7) are derived from pMR53 and contain the same fragments of Tn7 as pMR101, pMR102 and pMR117 respectively. All of these clones have the potential to form polypeptide fusions between any open reading frame to the right of the BamHI site at 11.05 and any open reading frame entering the HindIII fragment. However from the limited sequence available it was possible to reconstitute the region of interest of these plasmids. The potential fusions that could be created would extend 111bp in frame 0, 43bp in frame 1 and 5bp in frame 2. Thus the largest fusion would add 37 amino acids from Tn7 to a polypeptide reading out from the BamHI site at 11.05Kb.

pMR25, pMR41 and pMR36 (figure 4.8) were based on the plasmid pACYC184. **pMR25** contains the right end of Tn7 from the HincII site at 12.5Kb cloned as an EcoRI and HincII fragment from pMR84 into pACYC184 restricted with EcoRI and PvuII. **pMR41** had the BglII fragment of Tn7 (13.1Kb to 14.0Kb) cloned as an EcoRI and HincII fragment from a plasmid with this fragment cloned into the BamHI site of its polylinker. **pMR36** was derived from pACYC184::$Tn7_I$ (chapter 3) by sequential digestion and religation first with EcoRI and then with PvuII.

4.2.6 Construction of MR5(R388) and MR5(pEN300).

For the complementation analyses, a strain containing Tn7-1 in the chromosome was required. This strain also had to be $lacI^q$ to fully control the tac promoter in each expression vector. First, the strain DS903 was co-transformed with the plasmids pMR11 and pMR80::Tn7 (see chapter 3). These plasmids are normally incompatible but can be maintained in the same cell by simultaneous selection for resistance to Cm and Tp. Transformants were grown up overnight to allow pMR80::Tn7 to complement transposition of Tn7-1 from pMR11 to the

Table 4.2 (A) Transposition of Tn7-1 from MR5 to pEN300.

Complementing plasmids	Frequency of transposition	
	- IPTG	+
pMR100, pGLW8, pMR26, pMR101, pMR102, pMR51, (1) pMR25, pUC8, pMR56, pMR53 (2).	$<10^{-8}$	-
pMR84	2.5×10^{-4}	-
pMR9	8.7×10^{-4}	-
pMR51, pMR101	1.5×10^{-4}	2.5×10^{-4}
pMR51, pMR102	$<7.8 \times 10^{-10}$	
pMR51, pMR117	$<1.0 \times 10^{-8}$	
pMR51, pMR102, pMR25	1.3×10^{-4}	1.3×10^{-4}
pMR51, pMR117, pMR25	1.2×10^{-5}	1.4×10^{-5}
pMR51, pMR102, pMR41	1.8×10^{-7}	3.5×10^{-7}
pMR51, pMR117, pMR41	1.6×10^{-7}	1.0×10^{-7}
pMR51, pMR112	$<1.6 \times 10^{-9}$	
pMR56, pMR101	$<6.2 \times 10^{-9}$	
pMR57, pMR101	1.5×10^{-7}	4.5×10^{-7}
pMR66, pMR101	1.5×10^{-8}	2.6×10^{-8}
pMR36	$<4.7 \times 10^{-8}$	
pMR55, pMR36	$<2.6 \times 10^{-8}$	
pMR56, pMR36	3.0×10^{-3}	2.5×10^{-3}
pMR64, pMR36	6.5×10^{-4}	4.9×10^{-4}
pMR58, pMR36	$<4.6 \times 10^{-8}$	1.8×10^{-7}
pMR61, pMR36	$<2.5 \times 10^{-8}$	
pMR61, pMR115, pMR36	$<1.3 \times 10^{-8}$	
pMR62, pMR115, pMR36	1.5×10^{-7}	8.0×10^{-8}
pMR59	$<2.2 \times 10^{-9}$	
pMR59, pMR106	5.8×10^{-4}	1.1×10^{-3}
pMR59, pMR113	8.8×10^{-5}	9.1×10^{-5}
pMR59, pMR118	$<3.6 \times 10^{-9}$	
pMR68, pMR106	$<3.5 \times 10^{-9}$	
pMR69, pMR106	$<2.2 \times 10^{-9}$	
pMR68, pMR106, pMR25	6.5×10^{-5}	5.0×10^{-5}
pMR69, pMR106, pMR25	1.7×10^{-5}	8.3×10^{-7}
pMR53, pMR106, pMR25	$<1.6 \times 10^{-8}$	- (2)

In assays where no transposition was detected the results from the + and - IPTG assays were averaged and only one figure given. Each frequency represents the average of at least three experiments.

(1) These plasmids were all tested individually but none complemented transposition. No $Cm^r Tp^r$ transconjugants were observed amongst at least 10^8 Tp^r transconjugants.

(2) This assay could not be carried out in the presence of IPTG because induction of pMR53 causes cell death.

Angela's strains & plasmids

Glycerols: 1 box at -70°

DS902 *
DS941 *
CB51
MR1 (=DS916 nal^r) *
MR4 *
SRP84
JM109 *
BMH71-18*mutS* *
LA547
DS903::Tn*7* *
DS941::Tn*7*
DS941::Tn*7*K *
DS941::Tn*7*-1 *
LA547::Tn*7*-1
DS902 ColE1::Tn*7* ∞
DS902::Tn*7* pUC18
DS902::Tn*7* pAG085
DS902::Tn*7* pAG086
DS941::Tn*7*-1 pEN300
DS941 pGEX-3X
DS941 pGP1-2 ∞
DS941 pMR78 *
DS941 pMR204 *
DS903 pMR9 *
DS941 pMR100 *
DS903 pEN300 ∞
DS916 pEN300
MR5 pEN300
DS941 pAG011 * ∞
DS941 pAG013 ∞
DS941 pAG051 * ∞
DS941 pAG052 * ∞
DS941 pAG053 * ∞
DS941 pAG077 ∞
DS941 pAG080 ∞
DS941 pAG081 * ∞
DS941 pAG082 * ∞
DS941 pAG083 * ∞
DS941 pAG084 ∞
DS941 pAG085 * ∞
DS941 pAG086 * ∞
DS941 pAG087 * ∞
DS941 pAG088 ∞
DS941 pAG089 ∞
DS941 pAG091 ∞
DS941 pAG092 ∞
DS941 pAG095 ∞
DS941 pAG096 ∞
DS941 pAG101 * ∞
DS941 pAG201 * ∞
DS941 pAG202 * ∞
DS941 pAG203 * ∞
DS941 pAG204 * ∞
DS941 pAG205 * ∞
DS941 pAG206 * ∞
DS941 pAG207 ∞
DS941 pAG208 ∞
DS941 pAG209 ∞
DS941 pAG210 ∞
DS941 pAG211 ∞
DS941 pAG301 *
DS941 pAG302 *
DS941 pAG401 * ∞
DS941 pAG402 * ∞
DS941 pAG403 * ∞
DS941 pAG404 * ∞
DS941 pAG411 * ∞
DS941 pAG412 * ∞
DS941 pAG413 * ∞
DS941 pAG415 * ∞
DS902 pAG416 * ∞
DS941 pAG417 * ∞
DS941 pAG421 ∞
DS941 pAG422 ∞
DS941 pAG423 ∞
DS941 pAG424 ∞
DS941 pAG431
DS941 pAG432
DS941 pAG433
DS941 pAG434
DS941 pAG501 * ∞
DS941 pAG502 * ∞
DS941 pAG503 * ∞

* These are also on slopes,
of varying degrees of desiccation.

∞ CsCl DNA stock; also of:
pSELECT, pT7-7; p253;
pCG8 (Craig Gibbs); pEAL1;
pLA50,pLA77; pNE200;
pMR11
pAG001,002,003,004,005;
pAG012; pAG078,079;
pAG093,094

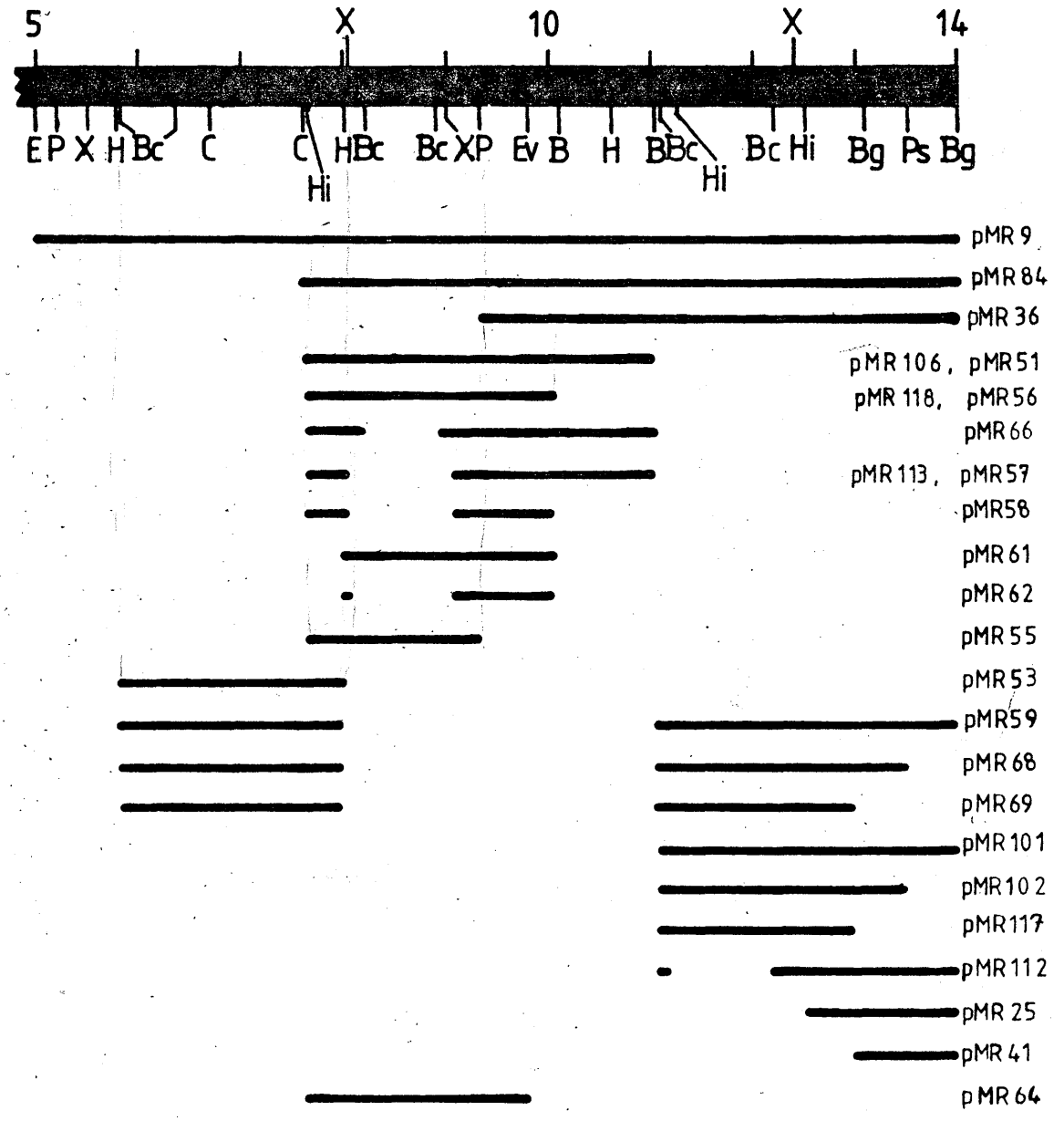


Table 4.2 (B) Illustration of the Tn7 sequences present in the clones used for this study.

The right 9Kb of Tn7 is presented as a thick solid line. The thin solid line represents the region of Tn7 present in the plasmids named alongside. Gaps in this line indicate *in vitro* deletions of Tn7 sequences in these clones.

Abbreviations; B, BamHI; Bc, BclI; Bg, BglII; C, ClaI; E, EcoRI; Ev, EcoRV; H, HindIII; Hi, HincII; P, PvuII; Ps, PstI; X, XbaI

chromosome. Growth in minimal media plus the appropriate nutritional supplements and Cm selected for cells which had lost their resistance to Ap and to Tp, indicating that they had lost both pMR11 and pMR80::Tn7. A number of these clones were analysed on single colony gels to confirm the loss of both plasmids. Chromosomal DNA was prepared from one such colony and analysed by Southern blotting for the presence of Tn7-1 at the chromosomal hotsite (Southern, 1975; Reed and Mann, 1985). The Southern blot confirmed that Tn7-1 had inserted in the chromosomal hotsite in the predicted orientation (data not shown but see figures 6.2 and 6.3). This strain was called MR4. An F'(pro$^+$lacI^{q}ZΔM15) was conjugated into MR4 from BMH71-18 selecting for pro$^+$ colonies. This strain, MR5, was transformed either with R388 or with pEN300 producing the strains MR5(R388) and MR5(pEN300) which were the donor strains in the complementation analysis.

4.3 Complementation of Tn7-1 transposition.

Transposition was measured by the 'mate out' assay described in the materials and methods. The frequency of transposition was defined as the inverse product of the number of Cm^{r}Tp^r exconjugants and the number of Tp^r exconjugants in a mating between the donor strain MR5(R388 or pEN300) containing the complementing plasmids and the recipient MR1.

All the assays which involved derivatives of either pMR100 or pGLW8 were carried out under conditions where the tac promoter was either repressed, or derepressed using IPTG to test if derepression of the tac promoter in these clones affected the transposition frequency. However only two plasmids, pMR69 and pMR58 showed any effect upon IPTG derepression.

4.3.1 Transposition of Tn7-1 to the cloned hotsite.

The constructs tested alone and in combination are shown in figures 4.6, 4.7 and 4.8. The results of these assays are given in table 4.2. Only pMR9 and pMR84 were capable of complementing transposition of Tn7-1 independently to the cloned hotsite. This defined the region of Tn7 required for transposition to a region between the ClaI site at 7.6Kb and the right end and confirmed the results of Hauer and Shapiro

(1984) that the function encoded within the HindIII fragment (5.8Kb and 8.0Kb) is not required for transposition to the hotsite. The Tn7 fragment present in pMR84 was divided at the BamHI site at 11.05Kb to form the plasmids pMR51 and pMR101 which, when together in the donor cell, allowed normal transposition of Tn7-1, indicating no activity was disrupted functionally at this boundary. pMR51 and pMR102 or pMR51 and pMR117 did not support transposition though pMR51, pMR102 and pMR25 together restored transposition to near wildtype frequencies. pMR51, pMR117 and pMR25 also restored transposition. pMR51, pMR102 and pMR41 or pMR51, pMR117 and pMR41 complemented transposition, though at a reduced frequency. No transposition was observed in complementation assays using pMR51 and pMR112 confirming the presence of a trans-acting function, at least partially, within the deleted region in pMR112. Similarly, pMR56 and pMR101 did not complement transposition indicating that a function essential for transposition to the hotsite was, at least partially, encoded within the BamHI fragment between 10.1Kb and 11.05Kb. pMR101 and pMR57, pMR101 and pMR66 or pMR36 and pMR58 (when derepressed) together complemented transposition at a reduced level, which identified another functional region around the XbaI fragment between 8.05Kb and 9.0Kb. Either this region is not essential for transposition or the deletions created in pMR58, pMR57 and pMR66 only partially affect this functional activity. Derepression of pMR58 was required for transposition, even at a reduced level, which supports the arguement that a function essential for transposition but with a reduced activity is present in these clones. This is further supported by the inability of other constructs, probably mutant in this fuction, to complement transposition at all. Productive transposition of Tn7-1 when complemented by pMR36 and pMR56 or pMR36 and pMR64 indicates that the region between the EcoRV site at 9.8Kb and the ClaI site at 7.6Kb contains the function disrupted by the XbaI deletion. Transposition was not observed when Tn7-1 was complemented by pMR36 or pMR36 and pMR55. This gene must cross the HindIII site at 8.0Kb because pMR36 and pMR61 together did not complement Tn7-1 transposition. No evidence was found for more than one gene in the region cloned in pMR64. The frequency of transposition was not enhanced compared to that observed with pMR36 and pMR58 in assays involving either pMR36, pMR115 and pMR62 or pMR36, pMR115 and pMR61.

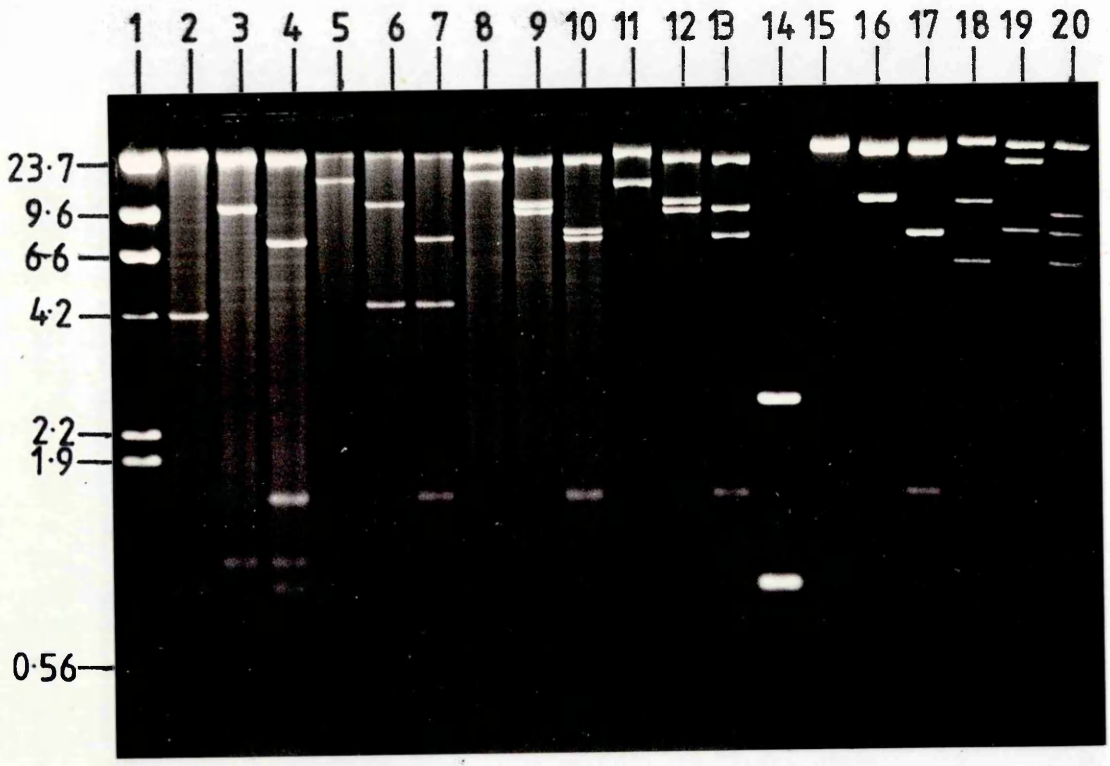


Figure 4.9 Agarose gel of STET prep DNA of four independent insertion of Tn7-1 complemented by pMR59 and pMR113 into pEN300 which have not occurred in the hotsite.

1	Lambda HindIII digested DNA	11	pEN300::Tn7-1_4 (EcoRI)
2	pEN300::Tn7-1_1 (EcoRI)	12	pEN300::Tn7-1_4 (BglII)
3	pEN300::Tn7-1_1 (BglII)	13	pEN300::Tn7-1_4 (EcoRI/BglII)
4	pEN300::Tn7-1_1 (EcoRI/BglII)	14	pMR81 (EcoRI)
5	pEN300::Tn7-1_2 (EcoRI)	15	pEN300 (EcoRI)
6	pEN300::Tn7-1_2 (BglII)	16	pEN300 (BglII)
7	pEN300::Tn7-1_2 (EcoRI/BglII)	17	pEN300 (EcoRI/BglII)
8	pEN300::Tn7-1_3 (EcoRI)	18	pEN300::Tn7 (EcoRI)
9	pEN300::Tn7-1_3 (BglII)	19	pEN300::Tn7 (BglII)
10	pEN300::Tn7-1_3 (EcoRI/BglII)	20	pEN300::Tn7 (EcoRI/BglII)

pEN300::Tn7-1_1, and pEN300::Tn7-1_2 are derived from assays where the complementing plasmids were derepressed while for pEN300::Tn7-1_3 and pEN300::Tn7-1_4 the complementing plasmids were not derepressed. The hotsite remains unoccupied in each of these clone. pMR81 is a pUC8 derivative containing two copies of the 967bp hotsite in direct repeat. All the digests are compatible with Tn7-1 insertions in the A300 region of the molecule and all the Tn7-1 insertions are in the expected orientation except for pEN300Tn7-1_2 which has a Tn7-1 insertion in the opposite orientation. DNA fragments are shown in Kb.

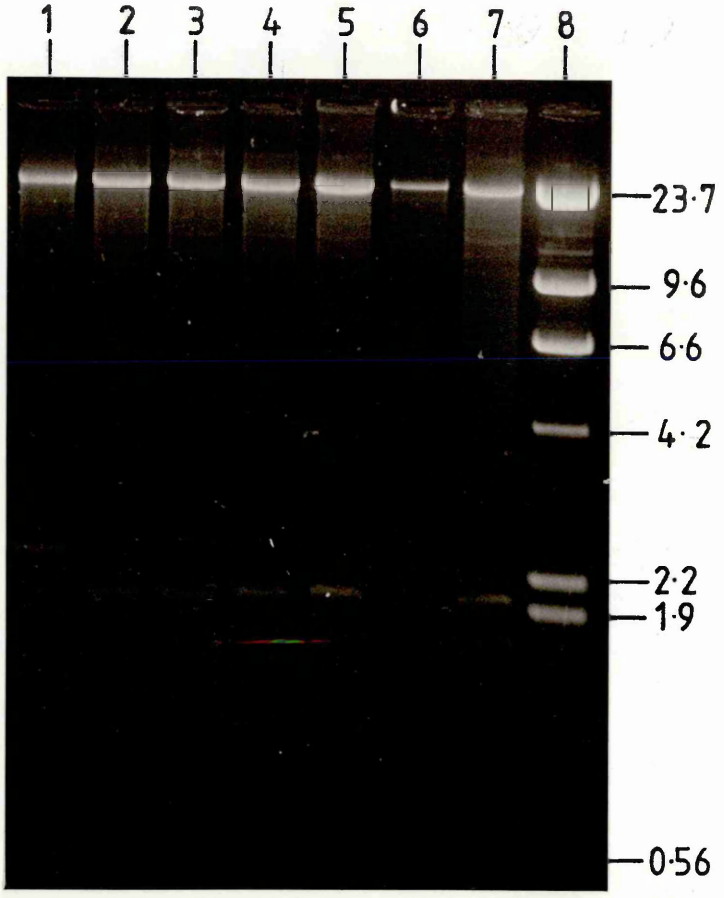


Figure 4.10 Agarose gel of EcoRI restricted pEN300::Tn7-1 derived from the transposition of Tn7-1 to pEN300 complemented by pMR59 and pMR106.

Lanes 1-7 Different clones of pEN300::Tn7-1 digested with EcoRI
Lane 8 Lambda DNA cut with HindIII, used as markers and shown in Kb.

The Tn7-1 insertions have all occurred within the 967bp hotsite fragment. All except that shown in lane 1 have inserted into the hotsite in identical positions. Tn7-1 in lane 1 has inserted within the hotsite fragment but not at the hotsite.

Table 4.3 Transposition of Tn7-2 from pMR85 and DS903::Tn7-2 to pEN300.

Donor	Frequency of transposition
DS903::Tn7-2	2.7×10^{-4}
pMR85	9.6×10^{-4}

These frequencies represent the average of three experiments.

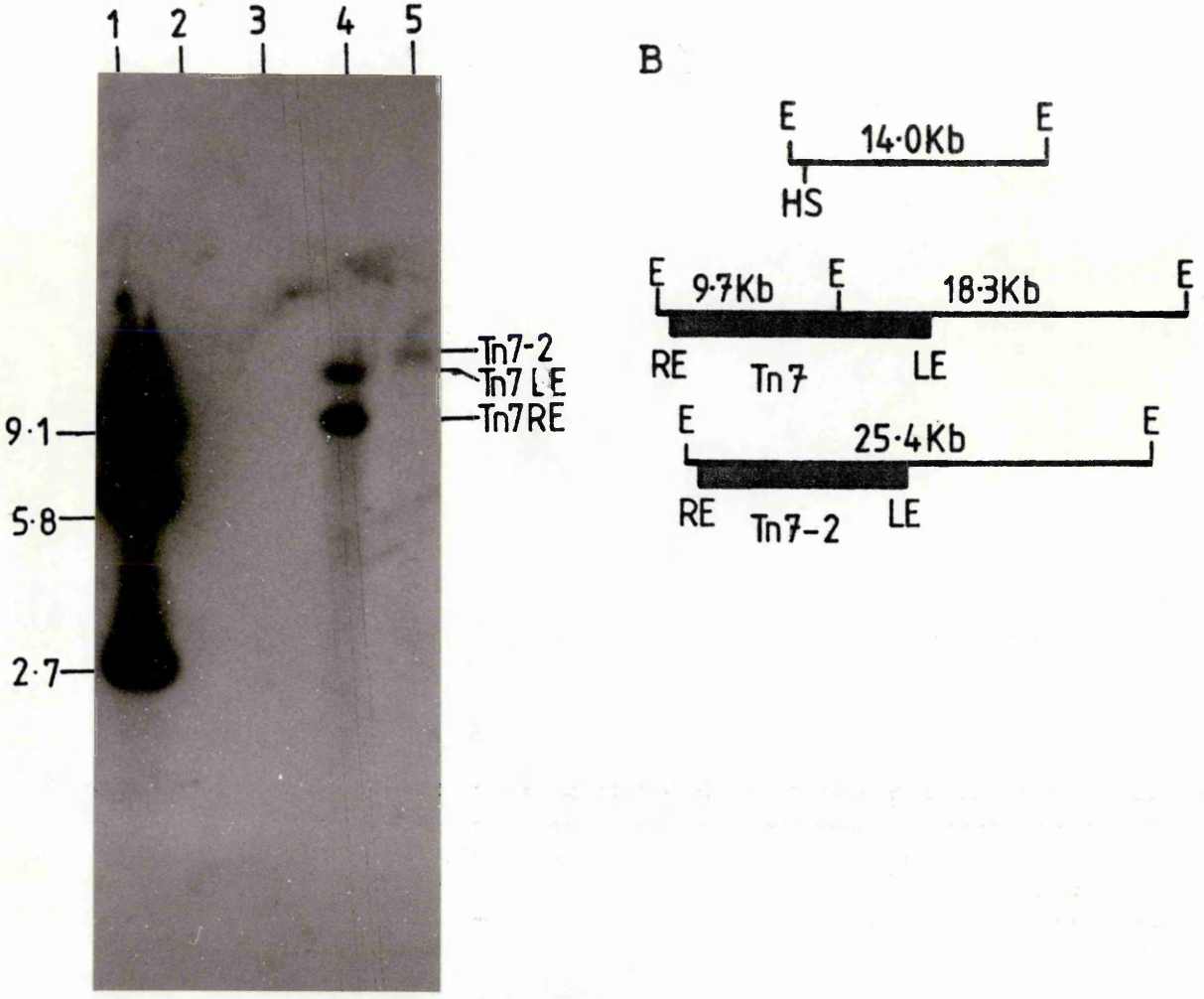


Figure 4.11 Autoradiograph confirming that Tn7-2 has inserted into the chromosomal hotsite in DS903.

A: Southern Blot probed with pUC8::$Tn7_I$ DNA and exposed for six hours. Characteristic bands are shown and agree with the expected sizes of fragments derived from data of Lichtenstein and Brenner (1981).

1 pMR81::Tn7 cut with EcoRI
2 Lambda DNA cut with HindIII, used as size markers (non-homologous to the probe)
3 DS903 chromosomal DNA digested with EcoRI
4 DS903::Tn7 chromosomal DNA digested with EcoRI
5 DS903::Tn7-2 chromosomal DNA digested with EcoRI
DNA markers given in Kb.

B: Map of E.coli chromosome around the Tn7 hotsite derived from Lichtenstein and Brenner (1981) predicting the fragment sizes expected for an insertion of Tn7 or Tn7-2 into the chromosomal hotsite.

Tn7-1 transposition was complemented by pMR59 and pMR113 but analysis of the insertions produced, revealed that these insertions were not in the hotsite (figure 4.9). Insertions into the hotsite occurred when Tn7-1 was complemented by pMR59 and pMR106 (figure 4.10) indicating that the function which lies in this region (9.8Kb - 7.6Kb) is only required for transposition to the hotsite. To ensure this was the only combination of functions which affected the mode of transposition, similar assays were undertaken using plasmids containing the 2.2Kb HindIII fragment. However, no transposition was observed when Tn7-1 was complemented with pMR59 alone, pMR59 and pMR118, pMR68 and pMR106, pMR69 and pMR106 or pMR53, pMR106 and pMR25. To discount the possibility that pMR68 and pMR69 were incapable of complementing transposition under any circumstance, pMR25 was included with pMR106 and either pMR68 or pMR69. These combinations did support transposition. Interestingly, the frequency of transposition of Tn7-1 in the presence of pMR69, but not pMR68, was affected by IPTG. When the tac promoter in this clone was derepressed, the frequency of transposition decreased by about ten fold. The explanation for this result is unclear but will be discussed below.

4.3.2 Transposition of Tn7-2 to the cloned hotsite.

The results from the previous section indicated that a region between the HindIII site at 5.8Kb and the ClaI site at 7.6Kb was not required for transposition to the hotsite. The deletion derivative of Tn7, Tn7-2, was a suitable construct to confirm this result. Two approaches were employed. Firstly, pMR85 was transformed into MR2 and a mate out transposition assay performed (table 4.3).

The second approach was to transpose Tn7-2 into the chromosome of DS903 and use this as the donor in a transposition assay. pMR85 was transformed into DS903 and Ap^rTp^r colonies selected. Growth on supplemented minimal media and Tp, and subsequent screening for Ap^sTp^r colonies identified cells which retained Tn7-2 but had lost pMR85. Analysis on single colony gels and southern analysis of chromosomal DNA of a single Ap^sTp^r colony (figure 4.11) confirmed the absence of pMR85 and the presence of Tn7-2 in the chromosome of DS903 in the predicted position. This strain was called MR6. The conjugative plasmid pEN300 was mated into this strain and a mate out transposition

Table 4.4 (A) Transposition of Tn7-1 from MR5 to R388.

Complementing plasmids	Frequency of transposition - IPTG	Frequency of transposition + IPTG
pMR100, pGLW8, pMR26, pMR101, pMR102, pMR51, (1) pMR25, pUC8, pMR56, pMR53 (2).	$<10^{-8}$	-
pMR84	$<1.7 \times 10^{-7}$	-
pMR9	3.4×10^{-5}	-
pMR51, pMR101	$<3.1 \times 10^{-9}$	
pMR51, pMR102	$<1.0 \times 10^{-9}$	
pMR51, pMR117	$<1.6 \times 10^{-9}$	
pMR51, pMR102, pMR25	$<3.8 \times 10^{-8}$	
pMR51, pMR117, pMR25	$<1.6 \times 10^{-9}$	
pMR51, pMR102, pMR41	$<4.8 \times 10^{-9}$	
pMR51, pMR117, pMR41	$<4.8 \times 10^{-9}$	
pMR51, pMR112	$<2.4 \times 10^{-9}$	
pMR56, pMR101	$<1.7 \times 10^{-9}$	
pMR57, pMR101	$<1.1 \times 10^{-9}$	
pMR66, pMR101	$<6.6 \times 10^{-10}$	
pMR36	$<7.2 \times 10^{-8}$	
pMR55, pMR36	$<4.4 \times 10^{-8}$	
pMR56, pMR36	$<4.3 \times 10^{-8}$	
pMR64, pMR36	$<2.1 \times 10^{-8}$	
pMR58, pMR36	$<6.2 \times 10^{-8}$	
pMR61, pMR36	$<3.5 \times 10^{-8}$	
pMR61, pMR115, pMR36	$<6.4 \times 10^{-9}$	
pMR62, pMR115, pMR36	$<1.3 \times 10^{-9}$	
pMR59	$<1.2 \times 10^{-9}$	
pMR59, pMR106	5.2×10^{-5}	4.3×10^{-5}
pMR59, pMR113	7.6×10^{-5}	8.7×10^{-5}
pMR59, pMR118	$<4.2 \times 10^{-9}$	
pMR68, pMR106	$<2.2 \times 10^{-9}$	
pMR69, pMR106	$<2.2 \times 10^{-9}$	
pMR68, pMR106, pMR25	1.2×10^{-6}	2.5×10^{-6}
pMR69, pMR106, pMR25	3.3×10^{-6}	2.8×10^{-7}
pMR68, pMR106, pMR41	$<5.0 \times 10^{-9}$	
pMR69, pMR106, pMR41	$<3.5 \times 10^{-9}$	
pMR53, pMR106, pMR25	$<4.8 \times 10^{-9}$	- (2)

In assays where no transposition was detected the results from the + and - IPTG assays were averaged and only one figure given. Each frequency represents the average of at least three experiments.

(1) These plasmids were all tested individually but none complemented transposition. The frequency of transposition shown indicates that no $Cm^r Tp^r$ transconjugants were observed in more than 10^8 Tp^r transconjugants examined.

(2) This assay could not be carried out in the presence of IPTG because induction of pMR53 causes cell death.

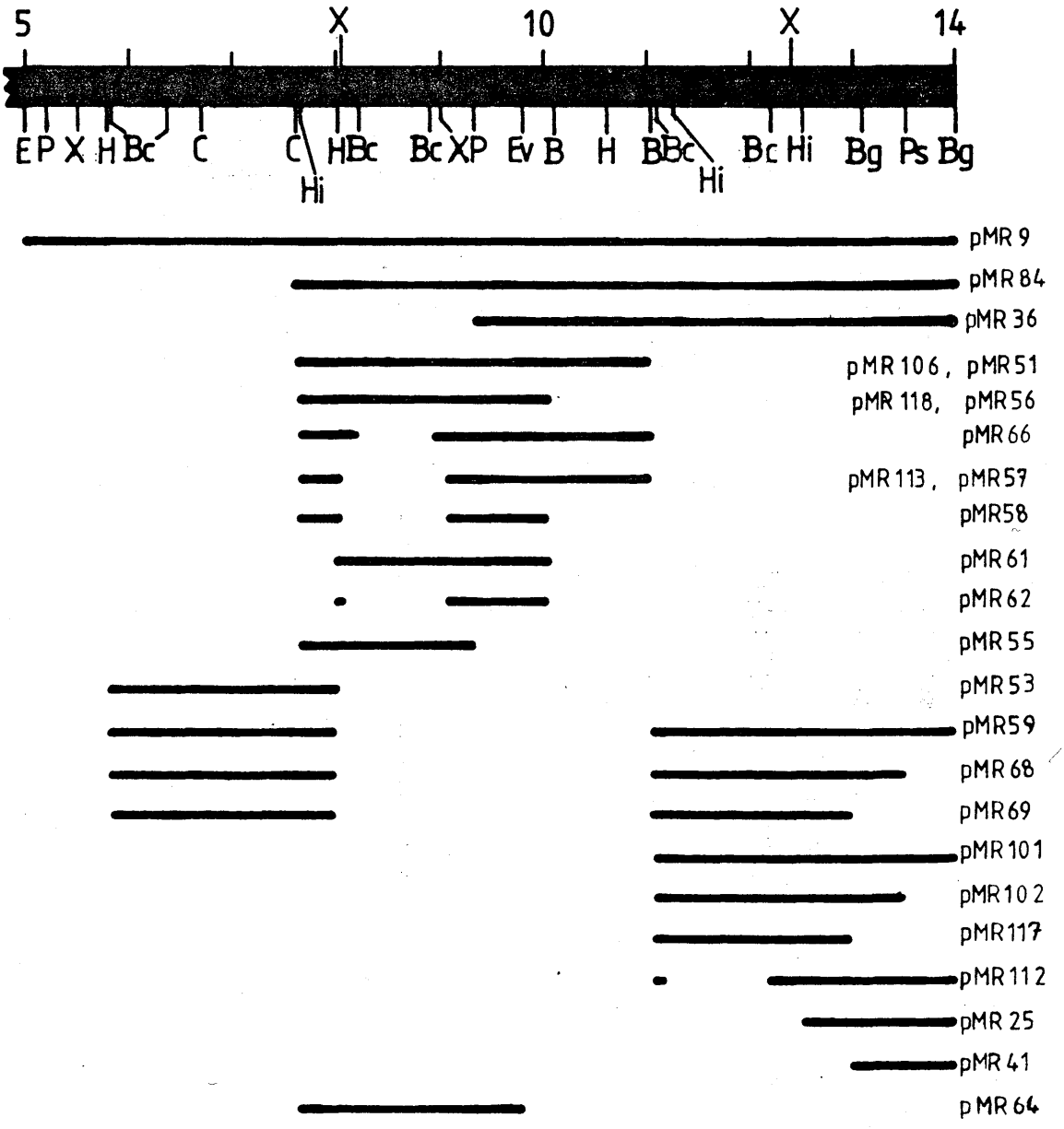


Table 4.4 (B) Illustration of the Tn7 sequences present in the clones used for this study.

The right 9Kb of Tn7 is presented as a thick solid line. The thin solid line represents the region of Tn7 present in the plasmids named alongside. Gaps in this line indicate *in vitro* deletions of Tn7 sequences in these clones.

Abbreviations; B, BamHI; Bc, BclI; Bg, BglII; C, ClaI; E, EcoRI; Ev, EcoRV; H, HindIII; Hi, HincII; P, PvuII; Ps, PstI; X, XbaI

assay performed (table 4.3). In the absence of any selection for pEN300, its presence in MR6 was confirmed by analysis on single colony agarose gels. These results confirmed that transposition to the hotsite does not require a function encoded within the HindIII fragment.

4.3.3 Transposition of Tn7-1 to the plasmid R388.

The results of these assays (table 4.4) confirm the requirement of the region encompassed by the HindIII fragment between 5.8Kb and 8.0Kb for transposition to plasmids lacking the hotsite. The only subclone of Tn7 which was capable of allowing transposition independently was pMR9. pMR84 was not capable of complementing transposition to cold sites. Similarly, transposition of Tn7-1 to coldsites only occurred in the presence of the 2.2Kb HindIII fragment (5.8Kb-8.0Kb). Combinations of plasmids that did not include this region of Tn7 did not complement the transposition of Tn7-1 to R388. pMR59 and pMR106 supported transposition of Tn7-1 at a frequency comparable to that of pMR9. pMR59 and pMR113 also allowed transposition indicating that the function encoded in the region of this deletion is not required for transposition to plasmids, as shown previously in section 4.3.1. pMR59 and pMR118 did not complement transposition. pMR68 and pMR106 or pMR69 and pMR106 did not complement transposition, though the inclusion of pMR25 in these assays allowed transposition to proceed. pMR68, pMR106 and pMR41 or pMR69, pMR106 and pMR41 were not capable of supporting transposition. In assays involving pMR69 a reduction in transposition frequency, similar to that in section 4.3.1, was observed upon the addition of IPTG. Finally pMR25, pMR106 and pMR53 together did not complement Tn7-1 transposition. This assay could not be carried out in the presence of IPTG because the induction of the tac promoter in pMR53 was lethal to the cells.

4.3.4 Transposition of Tn7-2 to R388.

To confirm the requirement for the function encoded within the 2.2Kb HindIII fragment during transposition to plasmids, pMR85 (a Tn7-2 donor) was transformed into DS903 that already contained R388. A mate out transposition assay was performed and the transposition frequency measured (table 4.5). Tn7-2 is capable of transposition from pMR85

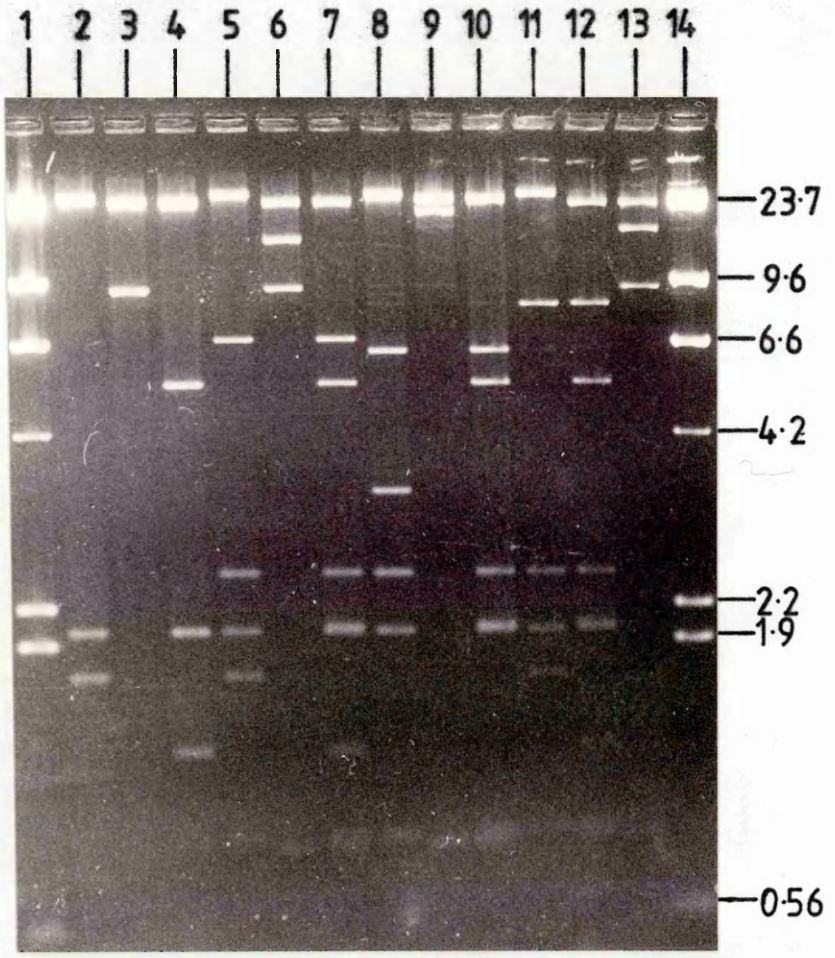


Figure 4.12 Agarose gel of restricted R388::Tn7-2 clones showing insertion of Tn7-2 in both orientations within R388.

1 Lambda (HindIII)
2 R388 (BamHI)
3 R388 (BglII)
4 R388 (BamHI/BglII)
5 R388::Tn7-2_1 (BamHI)
6 R388::Tn7-2_1 (BglII)
7 R388::Tn7-2_1 (BamHI/BglII)
8 R388::Tn7-2_2 (BamHI)
9 R388::Tn7-2_2 (BglII)
10 R388::Tn7-2_2 (BamHI/BglII)
11 R388::Tn7-2_3 (BamHI)
12 R388::Tn7-2_3 (BamHI/BglII)
13 R388::Tn7-2_3 (BglII)
14 Lambda (HindIII)

From these digests a rough estimate of the position of insertion and the orientation may be calculated. The positions are given relative to the R388 map in figure 2.2 using the EcoRI site as the origin. Orientation I is the usual orientation of Tn7 insertion and an example is shown in figure 2.2: Orientation II is the opposite. R388::Tn7-2_1, orientation II, 2.5Kb; R388::Tn7-2_2, orientation I, 1.4Kb; R388::Tn7-2_3, orientation II, 4Kb.
DNA sizes are shown in Kb.

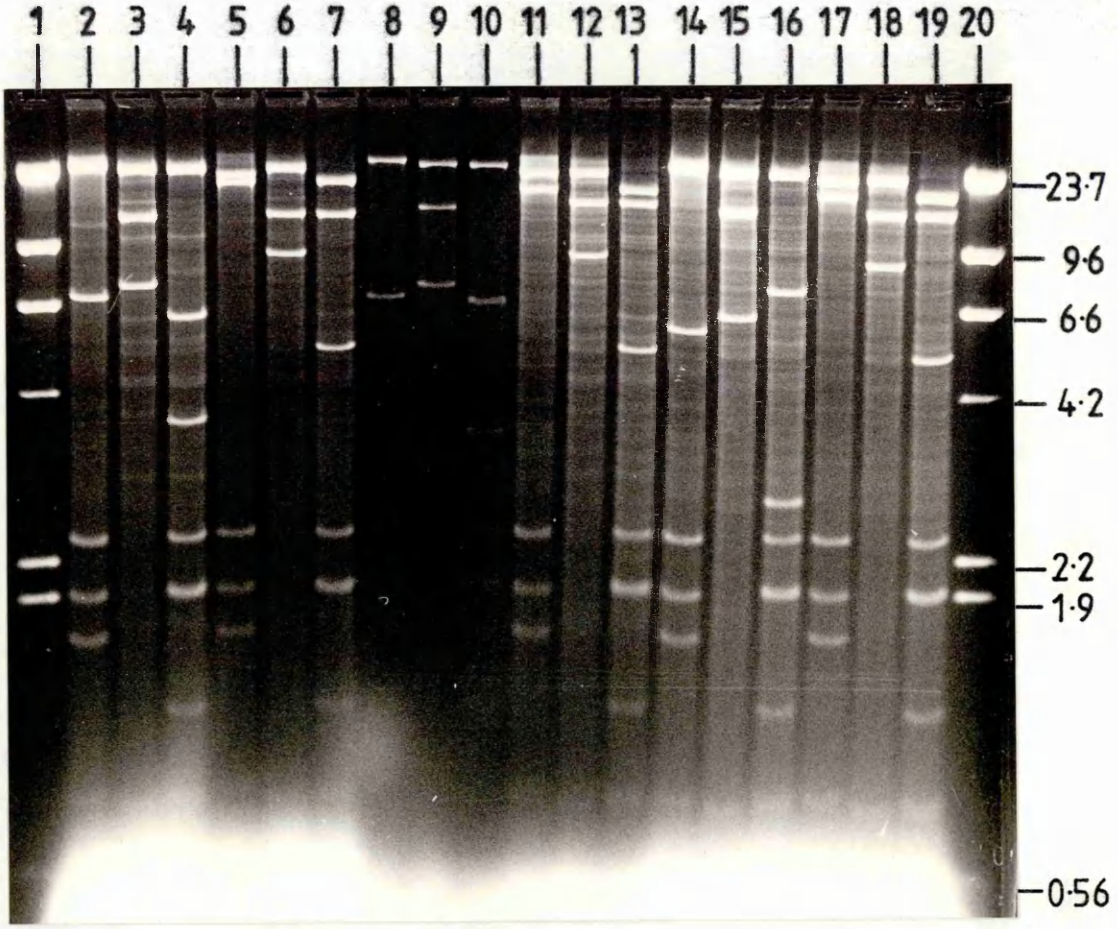


Figure 4.13 Agarose gel of Tn7-2 insertions into R388 showing that Tn7-2 inserts into R388 in both orientations when complemented in *trans* by pNE77.

1 Lambda (HindIII)
2 R388::Tn7-2_4 (BamHI)
3 R388::Tn7-2_4 (BglII)
4 R388::Tn7-2_4 (BamHI/BglII)
5 R388::Tn7-2_5 (BamHI)
6 R388::Tn7-2_5 (BglII)
7 R388::Tn7-2_5 (BamHI/BglII)
8 R388::Tn7-2_6 (BamHI)
9 R388::Tn7-2_6 (BglII)
10 R388::Tn7-2_6 (BamHI/BglII)
11 R388::Tn7-2_7 (BamHI)
12 R388::Tn7-2_7 (BglII)
13 R388::Tn7-2_7 (BamHI/BglII)
14 R388::Tn7-2_8 (BamHI)
15 R388::Tn7-2_8 (BglII)
16 R388::Tn7-2_8 (BamHI/BglII)
17 R388::Tn7-2_9 (BamHI)
18 R388::Tn7-2_9 (BglII)
19 R388::Tn7-2_9 (BamHI/BglII)
20 Lambda (HindIII)

From these digests a rough estimate of the position of insertion and the orientation may be calculated. R388::Tn7-2_4 and R388::Tn7-2_6 and R388::Tn7-2_7 and R388::Tn7-2_9 give the same restriction patterns and may not be independent events. The positions are given relative to the R388 map in figure 2.2 using the EcoRI site as the origin. Orientation I is the usual orientation of Tn7 insertion and an example is shown in figure 2.2: Orientation II is the opposite. R388::Tn7-2_4, orientation II, 27Kb; R388::Tn7-2_5, orientation II, 13.5Kb; R388::Tn7-2_6, orientation II, 27Kb; R388::Tn7-2_7, orientation I, 12.5Kb; R388::Tn7-2_8, orientation II, 28Kb; R388::Tn7-2_9, orientation I, 12.5Kb. DNA sizes are given in Kb.

Table 4.5 Transposition of Tn7-2 from pMR85 to R388.

Complementing plasmid	Frequency of transposition
-	4.3×10^{-7}
pNE77	2.8×10^{-5}

These frequencies represent the average of three experiments.

Table 4.6 Transposition of Tn7-2 from DS903::Tn7 to R388.

Complementing plasmid	Frequency of transposition
-	$<2.1 \times 10^{-9}$
pNE77	6.5×10^{-5}

The frequency of transposition represents the average of three experiments.

without complementing plasmids but the frequency of transposition is reduced. DNA was extracted from a number of transconjugants and the nature of the Tn7-2 insertion into R388 determined. Tn7-2 inserts into R388 in both orientations (figure 4.12). The transposition frequency of Tn7-2 was also determined when its transposition was complemented in *trans* by pNE77, a pACYC184 derivative containing the 2.2Kb *Hin*dIII fragment. pMR85 and pNE77 were cotransformed into DS903 which already contained R388 and the transposition frequency determined (table 4.5). pNE77 complemented transposition of Tn7-2 efficiently but analysis of a number of transconjugants on agarose gels revealed that Tn7-2 was inserted into R388 in both orientations. This result is particularly confusing because, in all but one case, Tn7-1 inserted into R388 in a single orientation.

Transposition of Tn7-2 to R388 was also measured using the chromosomal copy of Tn7-2 in MR6 in the presence and absence of the complementing plasmid pNE77 (table 4.6). Tn7-2 could not transpose independently from the chromosome to R388. pMR85 is a pUC8 derived plasmid with a high copy number. It is possible that the presence of Tn7-2 in such a high copy number increased the frequency of transposition to R388 to a level that was detectable by this assay. When complemented in *trans*, Tn7-2 transposed efficiently from the chromosome to R388. Examination of a number of insertion events again showed that integrations occurred in both orientations (figure 4.13).

4.4 DISCUSSION

In this chapter, the construction of a mini Tn7, Tn7-1, deleted for all but 703bp of Tn7 end sequences (168bp of the left end and 535bp of the right end) was described. This transposon was used to map the functions within Tn7 required for transposition by complementation. Fragments of Tn7 were cloned into multicopy plasmids and assessed for their ability, independently or in concert, to promote Tn7-1 transposition.

The transposition of Tn7-1 (when complemented) indicates that all sequences required in *cis* for transposition are located between the left end and the first *Hin*cII site at 0.168Kb and the *Pst*I site at 13.5Kb and the right end. The remainder of the transposon was

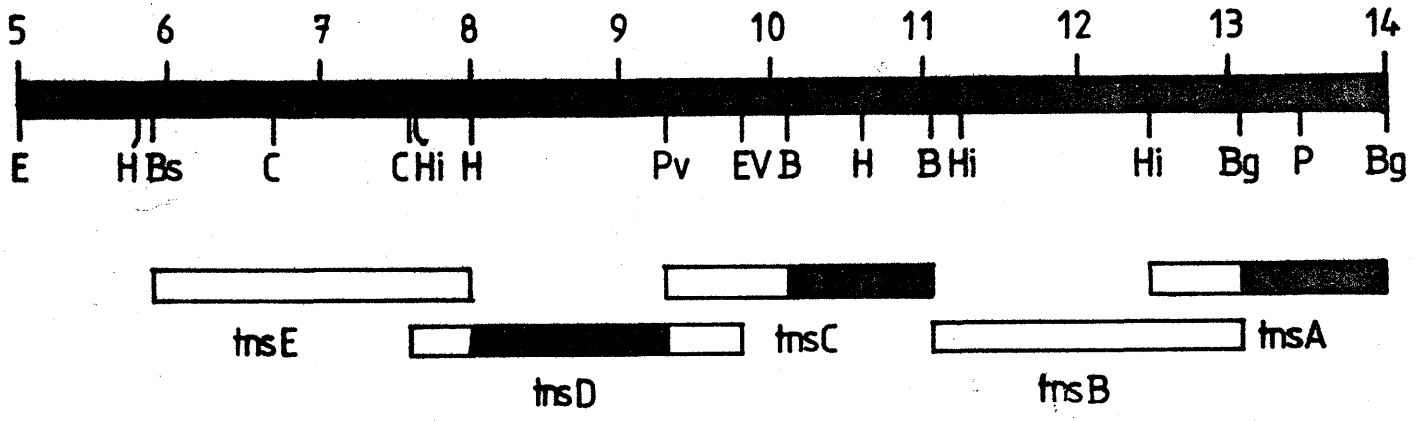


Figure 4.14 Map of transposition functions encoded by Tn7.

The thick lines represent the right 9Kb of Tn7. Solid black boxes indicate regions of Tn7 that must contain Tn7 functions. The open boxes represent regions which must at least partially encode the named function. The sequence of the 2.2Kb HindIII fragment (5.8Kb to 8.0Kb) encoding tnsE suggests that this function requires most of the available coding capacity (Smith and Jones, pers. comm.).

Abbreviations: B, BamHI; Bg, BglII; Bc, BclI; C, ClaI: E, EcoRI; Ev, EcoRV; H, HindIII; Hi, HincII; P, PstI; Pv, PvuII.

replaced by the Cm^r gene from the plasmid pCB27. This result argues against the presence of a site specific recombination system similar to that found in class II elements like Tn3 (Gill et al, 1978, 1979; Heffron et al, 1977; Arthur and Sherratt, 1979). If such a resolvase site does exist in Tn7 it must be located within the sequences present in Tn7-1. No homology was detected between these regions of Tn7 and the res site of Tn3. Secondly, replicon fusions mediated by homologous recombination (see chapter 3) are stable in recA⁻ strains implying that any resolvase system in Tn7 must be very inefficient under the conditions used. All the results are compatible with a resolution system present in one or both ends that acts during the transposition process.

Using Tn7-1, five trans-acting functions were defined (tnsA - tnsE) which were required for the transposition of Tn7-1. Three of these functions, tnsA - tnsC, are required for all transpositions (to hotsites and cold sites). tnsD is required for transposition to the hotsite but is dispensable for transpositions to plasmids lacking this site. tnsE is required for Tn7-1 transposition to plasmids but is not required for efficient transposition to the hotsite (Smith and Jones, 1984; Hauer and Shapiro, 1985; this work). All of these functions are encoded on a 8.1Kb fragment extending from the BstEII site at 5.9Kb to the right end (figure 4.14). Results of transcriptional and translational fusions and limited sequence data suggest that all Tn7 functions are transcribed and translated from right to left as the transposon is drawn in figure 4.14 (N Ekaterinaki, pers. comm.; Smith and Jones, pers. comm.; E Nimmo, pers. comm.).

About 10% of all Tn7-1 insertions into pEN300 did not occur in the usual position within the 969bp hotsite fragment (see figure 4.8). A number of other sites appeared to be targets. All were within the fragment and all insertions analysed were in the same orientation. It is unclear whether this phenomenon is caused by Tn7-1 or by pEN300. A similar insertion of Tn7 into pEN300 has been detected. These insertions cannot be due to the non-stoicheiometric nature of the complementing plasmids because they are found when Tn7-1 is complemented by a full Tn7. Neither can the altered insertion sites be attributed to cold site transpositions because they occur in the absence of the cold site-specific transposition function. Possibly,

they reflect the fact that Tn7-1 was complemented to transpose in *trans*.

tnsA was defined by the inability of pMR102 and pMR117 to complement Tn7-1 transposition to the hotsite in the presence of pMR51 or by the inability of pMR68 and pMR69 to complement transposition to cold sites in the presence of pMR106. The addition of pMR25, or the replacement of pMR102 and pMR117 with pMR101 or pMR68 and pMR69 with pMR59, to these assays restored transposition. However, if pMR41 was used in place of pMR25, transposition to hotsites occurred at a frequency which was reduced by between two and three logs (table 4.2). No transposition to cold sites was detected using pMR41. This suggests that pMR41 produces a partially functional product. Two differences exist between pMR41 and pMR25. Firstly, the 43bp between the right end and the *Bgl*II site at 14Kb are present in pMR25 but absent in pMR41. This does not appear to affect the complementing ability of these plasmids: The presence or absence of this region does not alter the complementation of a mutant Tn7 created by insertion of random DNA into the *Pst*I site at 13.5Kb (E Nimmo, pers. comm.). Secondly, pMR25 has about 600bp extra to the left of the *Bgl*II site at 13.1Kb. This extra sequence may contain the 3' end of *tnsA*.

An open reading frame begining 135bp in from the right end and extending beyond the *Pst*I site at 13.5Kb has been identified and probably encodes *tnsA* (Gay *et al*, 1986).

In these assays, pMR117 complemented the transposition of Tn7-1 to hotsites at about 10% the efficiency of pMR102 in the presence of pMR51 and pMR25. The reason for this remains unclear, though the possibility of a function located at least partially between the the *Pst*I site at 13.5Kb and the *Bgl*II site at 13.1Kb which stimulated transposition, but was not essential, can be excluded. Such a postulated function would have to extend beyond the *Hin*cII site at 12.5Kb, otherwise it would be encoded in pMR25. This would predict that pMR102, pMR41 and pMR51 would be more efficient at complementing Tn7-1 transposition than pMR117, pMR41 and pMR51. This was not observed (table 4.2). However, the formal possibility remains that interactions between the 3' end of *tnsA* (predicted to be missing from pMR41) and this postulated stimulatory polypeptide may be disrupted,

removing the stimulatory effect. This does not seem likely. In chapter 5, evidence is presented which indicates that all the Tn7 sequences in pMR117 could be required to encode the tnsB gene product.

A further observation regarding assays defining tnsA relates to pMR69. When pMR69 was derepressed using IPTG in the presence of pMR25 and pMR106, the transposition frequency to both hotsites (table 4.2) and cold sites (table 4.4) was depressed by 10 to 20 fold (compare pMR69, pMR25 and pMR106 with pMR68, pMR25 and pMR106). pMR68 was not affected by IPTG induction and the cause of this effect remains unclear. pMR53 caused cell death upon derepression with IPTG. This phenotype was not apparent in pMR59, pMR68 or pMR69 which have extra Tn7 sequences inserted between the tac promoter and the 2.2Kb HindIII fragment (5.8Kb to 8.0Kb). Possibly, the extra fragment in pMR69 did not fully protect the host cells from the effects of derepression. This fragment is smaller than those present in either pMR59 or pMR68. The amount or stability of this transcript could reduce the fitness of the assay strain and account for the depressed frequency of transposition.

tnsB is defined by pMR117 and pMR112 for transposition to the hotsite and by pMR69 for transposition to plasmids lacking this site. pMR69 has the same fragment as pMR117 but also includes the 2.2Kb HindIII fragment. Transposition was not detected when Tn7-1 was complemented with pMR53, pMR25 and pMR106 which confirmed that the region between 11.05Kb and 13.1Kb encodes a function or functions essential for transposition to cold sites. This function(s) must extend into the region defined by the BclI fragment (11.1Kb - 12.2Kb) because pMR112 did not complement transposition in the presence of pMR51 while pMR101 did (table 4.2). From the sequence of the BamHI fragment (10.1Kb - 11.05Kb) and the region just to the right of it (Smith and Jones, pers. comm.; E Nimmo pers. comm.), an open reading frame has been identified reading toward the left end which extends some 22 bases beyond the BamHI site at 11.05Kb. This is probably part of the coding region for tnsB. In pMR101, pMR102, pMR117, pMR68, pMR69 and pMR59 the 3' end of this gene has been removed and replaced with polylinker sequences from pMR100 or pGLW8. The C terminal 7 amino acids of this potential polypeptide would be replaced with 17 amino acids derived from the ploylinker of these vectors, which implies that the 7 amino

acids at the 3' end of tnsB are not essential for function.

tnsC is located between the BamHI site at 11.05Kb and the PvuII site at 9.3Kb. pMR36 and pMR64 complemented transposition to the hotsite (table 4.2) while pMR36 and pMR53 complemented transposition to plasmids lacking this site (table 4.4). In the presence of pMR101, pMR51 complemented Tn7-1 transposition to the hotsite while pMR56 did not. Similar results using pMR59 and either pMR106 or pMR118 confirmed that this gene is also required for transposition to cold sites. From the sequence, an open reading frame was identified which began 21bp in from the BamHI site at 11.05Kb and extended leftward beyond the BamHI site at 10.1Kb. The presumptive start of this gene overlaps the presumptive end of tnsB by 4 bases (TCA TGA GT); this is a common event in cases of translational coupling (Nomark et al, 1983).

The function encoded by pMR64 (and other plasmids) identifies tnsD and places it between the EcoRV site at 9.8Kb and the ClaI site at 7.6Kb. pMR36 and pMR64 or pMR56 complemented transposition of Tn7-1 to the hotsite efficiently while pMR36 and pMR61, pMR36 and pMR55 or pMR36 alone did not. This region of Tn7 is not required for transposition to cold sites. pMR59 and pMR113 complemented efficiently transposition of Tn7-1 to plasmids. Similarly pMR59 and pMR113 complemented transposition to pEN300. However, examination of these insertions confirmed that Tn7-1 had not transposed to the hotsite but to alternative cold sites within this plasmid (figure 4.9). One of the insertions examined had occurred in the opposite orientation to all other Tn7-1 insertions examined. The reason or significance of this remains unclear. The complementation experiments place one end of tnsD between the EcoRV site at 9.8Kb and the PvuII site at 9.3Kb. The other end must be located between the ClaI site at 7.6Kb and the HindIII site at 8.0Kb.

Partial complementation was observed using pMR57 and pMR66 in the presence of pMR101 and pMR58 in the presence of pMR36. These results appear to conflict with the inability of pMR61 and pMR36 to complement transposition (though the insensitivity of this assay must be noted) and suggest that this region may encode two functions. However, the results of assays involving pMR36, pMR61 and pMR115 indicate that this

is not the case. The presence of pMR115 did not restore transposition when added to pMR61 and pMR36. Also, the frequency of transposition of pMR36 and pMR58 (under IPTG derepression; see below) or pMR57 and pMR101 was the same as the frequency of Tn7-1 transposition in the presence of pMR36, pMR115 and pMR62. This result suggests that pMR62 was as competent at complementing transposition as pMR57 or pMR58 implying a cis-dominance effect of the XbaI region (8.05Kb - 9.0Kb) over the region between the ClaI site at 7.6Kb and the HindIII site at 8.0Kb. The absence of the XbaI region alleviates the requirement for the ClaI to HindIII region. The presence of the XbaI region on the other hand requires the presence of the ClaI to HindIII region for a functional product. This effect is most likely to be mediated at the level of the polypeptide (or RNA) secondary structure and suggests that these regions interact.

pMR58 in the presence of pMR36 complemented transposition at a reduced level and then only in the derepressed state. A requirement for derepression was not observed for pMR57. Possibly, the Tn7 fragment in pMR57 encodes its own promoter which is not present in pMR58. However, no potential promoters were found within the sequence of the BamHI fragment and both pMR64 and pMR56 were capable of complementing transposition (in the presence of pMR101) in the absence of IPTG. If such a promoter were present it would be within the coding region of tnsC. Alternatively, the stoicheiometry of the Tn7 functions in these assays may be different, which may affect the ability of this partially functional protein (or RNA) to complement transposition. In pMR36, there is no external promoter driving transcription so that the levels of Tn7 functions may be lower in assays using this plasmid.

tnsE is entirely encoded within the 2.2Kb HindIII fragment between 5.8Kb and 8.0Kb (Smith and Jones, 1984; Hauer and Shapiro, 1985; this work). This function is not required for transposition to the hotsite but is essential for Tn7-1 transposition to plasmids which lack this site (compare complementations using pMR101 and pMR51 with assays using pMR59 and pMR106 (tables 4.2 and 4.4)). Tn7-2 transposition to pEN300 occurred at or near wild type levels (table 4.3). All insertions of Tn7-1 into R388 (except one; see figure 4.9) analysed occurred in the orientation expected for wildtype Tn7. This indicates that all cis-acting functions required for normal transposition are

present in Tn7-1. However, the transposition of Tn7-2 to R388 suggests that it is lacking some function required for orientation-specific insertion into R388. Tn7-2 was incapable of independent transposition from the chromosomal hotsite to R388 but could transpose from pMR85 to R388 (tables 4.5 and 4.6) at a reduced frequency. Analysis of some of these insertions identified Tn7-2 in both orientations within R388 (figure 4.11). Possibly the high copy number of Tn7-2 in pMR85 compared with the single copy of Tn7-2 in MR6 allows the low level of transposition of Tn7-2 to be detected in these assays. This would imply a constant probability of Tn7 transposition which was dependent on the number of transposons per cell. This is not consistent with the results in the previous chapter which found that the number of Tn7 molecules in a cell did not affect the frequency of transposition to R388. These events may represent molecules derived from abortive transpositions which could not be completed by the normal mechanism because of the absence of tnsE. The context of the chromosomal donor may prevent recovery of such abortive events. The frequency of transposition was restored to near wildtype levels by complementation with pNE77. This was observed for both chromosomal and plasmid borne Tn7-2 donors (tables 4.5 and 4.6). Again, analysis of some of these events comfirmed that insertion had occured in both orientations (figure 4.12). These data indicate that the loss of the region between the PvuII site at 4.98Kb and the ClaI site at 7.6Kb allows Tn7 to insert in either orientation into plasmid targets. However, this region is also missing in Tn7-1 though insertion is generally in a single orientation (one insertion of Tn7-1 was found in the alternative orientation). Complementation with this region in trans did not restore orientation specific insertion. The absence of cis-acting functions can be ruled out as an explanation because Tn7-1 also lacks all of this region and usually inserts in a specific orientation when complemented. It would require at least two cis-acting regions and some complex transposition 'rules' to explain the results in these terms. The possiblity that the deletion in Tn7-2 does not remove all of tnsE and that the the remaining truncated function in some way mediates these insertions is not supported by the results from the complementation of this mutant. Complemented insertions of Tn7-2 still insert into R388 in both orientations.

In summary, five functions have been identified that are required for

Tn7 transposition. Three of these are essential for all types of insertions. A fourth is required for insertion into the hotsite but not for insertion into plasmids lacking this site. The fifth is involved in transposition to cold sites but is dispensable for transposition to the hotsite. This function may be involved in the orientation specific insertion of Tn7 into plasmids. All sequences required in *cis* for transposition are located within the first 168bp of the left end and the last 535bp of the right end. Recently Brevet *et al* (1985) have analysed the polypeptides produced by Tn7 using maxicells. There results are broadly consistent with those presented here. Further discussion of this paper can be found in chapter 5. Also, Candy Wadell and Nancy Craig have produced a map consistent with that presented in figure 4.14 using insertional mutagenesis of Tn7 by a mini-Mu *in vitro* (N Craig, pers. comm.).

CHAPTER 5

ANALYSIS OF TN7 ENCODED POLYPEPTIDES

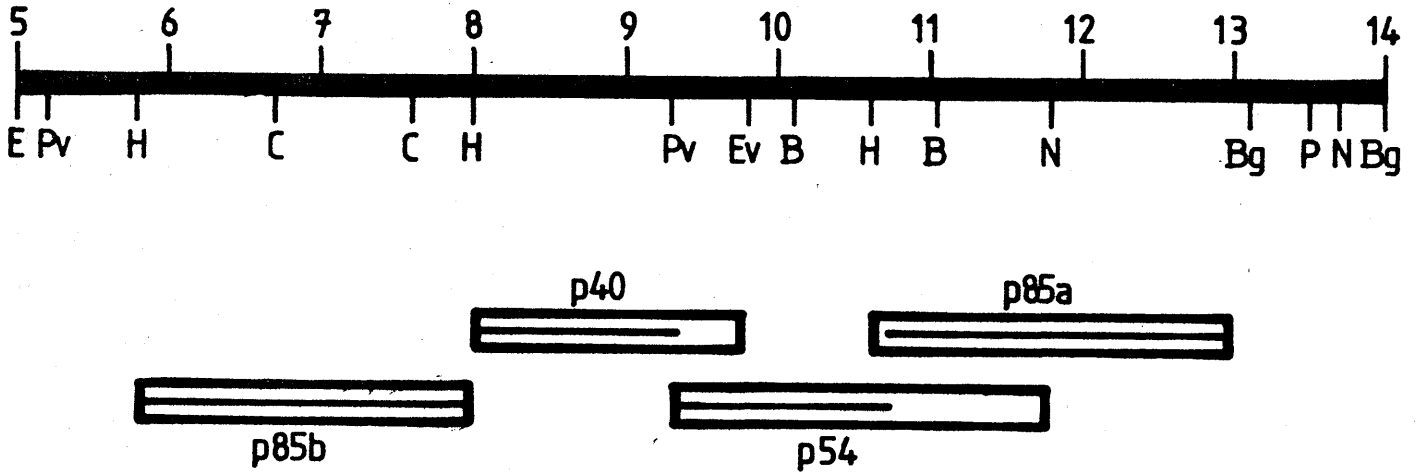


Figure 5.1 Modified diagram of the results of Brevet et al (1985) showing the location of transposition specific functions in Tn7.

The names of the polypeptides are shown above and below the appropriate box. The open boxes identify the boundaries of the regions encoding these polypeptides. The lines within the open boxes indicate the position assigned to the polypeptide by Brevet et al.
Abbreviations: B, BamHI; Bg, BglII; C, ClaI; E, EcoRI; Ev, EcoRV; H, HindIII; P, PstI; Pv, PvuII

5.1 INTRODUCTION

In the previous chapter, the complementation of Tn7-1 was used to define five functions required for the transposition of Tn7. These assays only defined regions of Tn7 that encoded diffusible gene products and did not demonstrate the nature of these functions, ie, whether they were polypeptides. The available sequence data (Lichtenstein and Brenner, 1982; Gay et al, 1986; Smith and Jones, pers. comm.; E Nimmo, pers. comm.) and transcriptional and translational fusions (N Ekaterinaki, pers. comm.; Rogers et al, 1986) indicate that these regions do encode polypeptides. A correlation of the presence of polypeptides with the results of the complementation experiments would consolidate the mapping data and support the results of the fusion experiments.

Brevet et al (1985) recently published an analysis of the polypeptides produced by Tn7 using maxicells and identified four polypeptides which were encoded in the region required for transposition (figure 5.1). This map agrees well with the complementation data presented in the previous chapter though some discrepancies exist. The complementation groups tnsB, tnsC and tnsE can be related directly to the polypeptides p85a, p54 and p85b respectively in their nomenclature, but they did not observe a polypeptide which correlates with tnsA. Also, the functional analysis from chapter 4 indicated that tnsD extended beyond the HindIII site at 8.0Kb. Brevet et al found that a 40Kd polypeptide, p40, was translated from a fragment of Tn7 with this site as one of its boundaries. This result is not incompatible with the complementation data which relies on the activity of the protein or trans-acting function rather than its size on an SDS-polyacrylamide gel. The p40 polypeptide may be only partially encoded from Tn7 sequences; the C terminal end may be derived from vector sequences adjacent to the boundary HindIII site. Further analysis of this paper will be left to the discussion.

The analysis of Tn7 polypeptides was studied initially using whole cell extracts from strains containing recombinant plasmids with cloned Tn7 fragments. These were examined for differences in the patterns of expression of polypeptides between repressed and derepressed cultures. No differences were observed, suggesting that the levels of the

presumptive polypeptides even in derepressed strains were too low to be detected or that their banding patterns were obscured by background cellular polypeptides.

A minicell analysis of the polypeptides expressed from these plasmids was undertaken. This technique examined the products of translation from plasmids within purified minicells which contain no chromosomal DNA. Hence, any new synthesis must be plasmid-specific and can be detected by ^{35}S-methionine labelling of the nascent polypeptides produced (Adler et al, 1967).

Minicells were first observed among cells selected for increased resistance to ionising radiation, but which remained sensitive to UV radiation (Alder et al, 1967). Strains which produce minicell have an altered cell division process which often causes cell septation at the wrong location in a dividing cell (Khachatourians et al, 1973; Clark-Curtiss and Curtiss, 1983; Zusman and Kroloshi, 1974). The resulting minicells contain no chromosomal DNA but do include plasmid DNA, thought to be segregated randomly with the trapped cytoplasm. The physiological state of minicells is unaffected by this aberrant division and there is no difference in the transcription, translation and replication of the trapped plasmids (Adler et al, 1967). This makes minicells very useful for the analysis of plasmid encoded polypeptides and RNA.

The mutation that causes the minicell phenotype has been mapped to the minB locus at 25.6 minutes on the E.coli chromosome (Schaumberg and Kuempel, 1983; Davie et al, 1984). This mutation does not affect the overall number of cell divisions but only the location at which septation occurs (Teather et al, 1974).

The results of an analysis of the polypeptides produced in DS944 minicells transformed with plasmids containing fragments of Tn7 are presented below. This analysis supports the functional analysis presented in the previous chapter and indicates that the region of Tn7 required for transposition is utilised fully in terms of its available coding capacity.

Table 5.1 Plasmids used in the Analysis of Transposoition Proteins and the functions they encode.

Plasmid	Tn7 Function	Plasmid	Tn7 Function
pMR100	-	pGLW8	-
pMR101	tnsA, tnsB	pMR51	tnsC, tnsD
pMR102	tnsB	pMR56	tnsD
pMR117	tnsB	pMR64	tnsD
pMR112	tnsA	pMR57	tnsC, tnsD[1]
pMR106	tnsC, tnsD	pMR58	tnsD[1]
pMR113	tnsC, tnsD[1]	pMR66	tnsC, tnsD[1]
pMR118	tnsD	pMR61	None
		pMR62	tnsD[1]
pMR26	-	pMR55	None
pMR25	tnsA	pMR53	tnsE
pMR41	tnsA[1]	pMR59	tnsA, tnsB, tnsE
		pMR68	tnsB, tnsE
		pMR69	tnsB, tnsE

1 Partial complementation of this gene resulted from this plasmid. An altered pattern of mobility might be expected for this polypeptide.

5.2 Observations on the purification of minicells from the strain DS944

This section describes some qualitative observations regarding the purification and labelling of minicells from the strain DS944. Clark-Curtiss and Clark (1983) recommended growing minicell strains in minimal media prior to the purification of the minicells. Both M9 and 3XD minimal media produce high yields of minicells from DS944. However, the purification of minicells grown up in these media was difficult and the background level was high due to whole cell contamination. This was probably due to the smaller size of the whole cells which were not separated properly during the purification. Though the yield of minicells was reduced when DS944 was grown up in rich media like L-Broth and Brain Heart Infusion media, the background contamination was also much lower.

Secondly, a greater purity of minicells was achieved by growing the cultures into stationary phase. Under these conditions the ^{35}S-methionine did not incorporate well. The efficency of labelling was increased when the minicells were purified just after the culture had entered stationary phase. In general, autoradiographs were clearer if the minicells were grown well into stationary phase and the consequent lower level of incorporation was accepted. The reduced signal to noise ratio allowed the film to be exposed for extended periods without serious background interference.

5.3 Analysis of polypeptides produced by plasmids containing fragments of Tn7 in DS944 minicells

The plasmids listed in table 5.1 and illustrated in figures 4.6, 4.7 and 4.8 were transformed into DS944. 200ml of L-Broth was innoculated with a single colony and grown up overnight at 37°C. The minicells were purified, labelled with ^{35}S-Methionine and minicell extracts made. The extracts were examined on 10% SDS-polyacrylamide gels following the procedure of Laemmli (1970). The results of these gels are presented in figures 5.2, 5.3, 5.4, and 5.5. Each plasmid was examined independently at least twice. The three vectors (pGLW8,

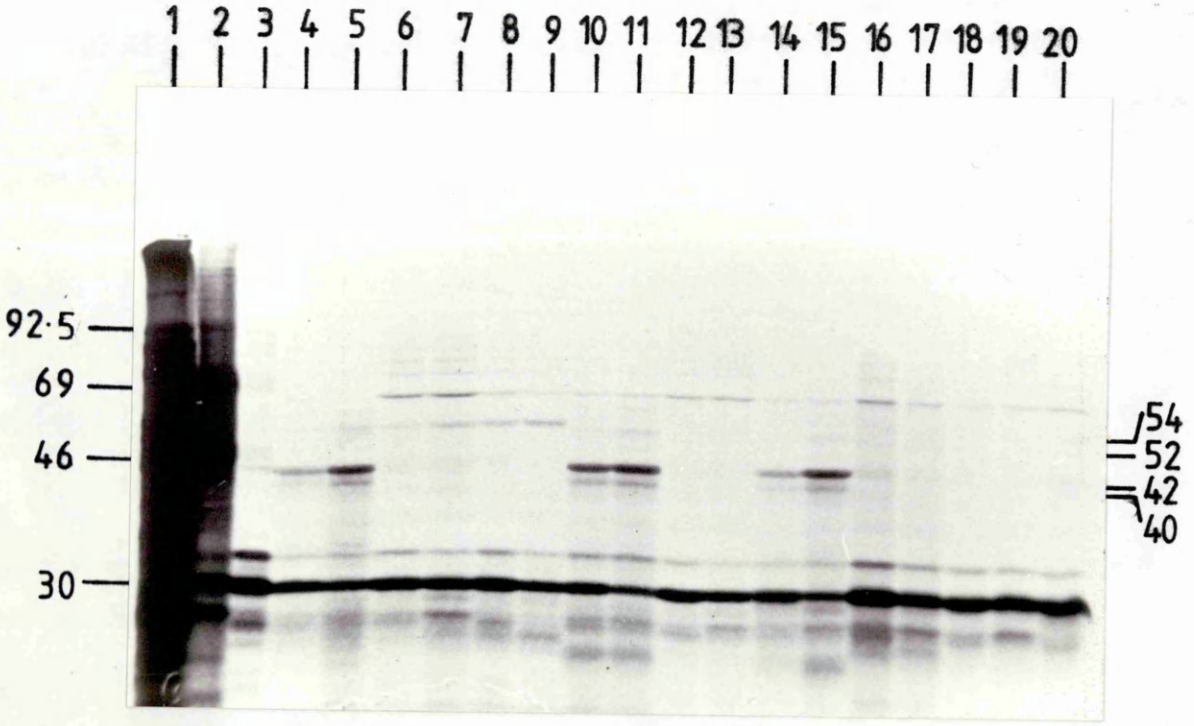


Figure 5.2 Polypeptides produced by pGLW8 and its derivatives containing fragments of Tn7.

1	DS944	11	pMR57 (+)
2	Molecular weight markers	12	pMR53 (-)
3	pGLW8 (+)	13	pMR58 (+)
4	pMR51 (-)	14	pMR66 (-)
5	pMR51 (+)	15	pMR66 (+)
6	pMR56 (-)	16	pMR61 (-)
7	pMR56 (+)	17	pMR61 (+)
8	pMR64 (-)	18	pMR62 (-)
9	pMR64 (+)	19	pMR62 (+)
10	pMR57 (-)	20	pMR55 (+)

The +/- in parentheses idicates the presence or absence of IPTG during the labelling of the minicells. pGLW8 and pMR55 were analysed in the presence and absence of IPTG. Only the +IPTG tracks are shown. The pattern in the - IPTG tracks were identical. The size of bands is given in Kd

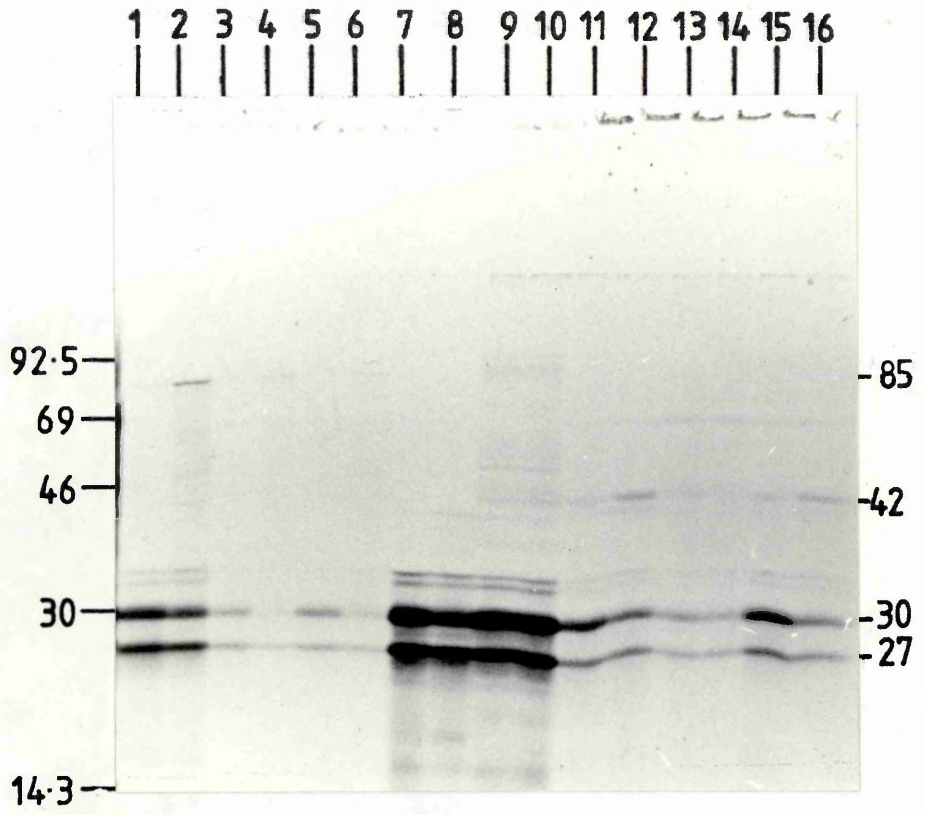


Figure 5.3 Polypeptides produced by pMR100 and its derivatives containing fragments of Tn7.

1	pMR101 (-)	A+B	9	pMR100 (-)	O
2	pMR101 (+)		10	pMR100 (+)	
3	pMR102 (-)	B	11	pMR106 (-)	C+D
4	pMR102 (+)		12	pMR106 (+)	
5	pMR117 (-)	B	13	pMR118 (-)	D
6	pMR117 (+)		14	pMR118 (+)	
7	pMR112 (-)	A	15	pMR113 (-)	C (D)
8	pMR112 (+)		16	pMR113 (+)	

+/- in parentheses indicates the presence or absence of IPTG during the labelling of the minicells.
The size of bands is given in Kd.

pMR100 and a pACYC184 derivative, pMR26) were also analysed as controls for vector encoded polypeptides. pMR26 was deleted for about 300bp between the EcoRI site and the PvuII site, which removed the promoter and 5' end of the chloramphenicol gene and made pMR26 a better control than pACYC184 for vector specific polypeptides. These sequences were also deleted in pMR25 and pMR41.

The use of a plasmid name below refers to the polypeptide profile obtained from the minicells containing that plasmid and the size of any polypeptide is given as its apparent molecular weight.

pGLW8 (figure 5.2) and all its derivatives show two major bands at 30Kd and 33Kd. In some tracks, including pGLW8, there is a further band of 27Kd. These are all different forms of the B-lactamase polypeptide (Dougan et al, 1979). A 68Kd polypeptide was also present in all tracks except pGLW8. The origin of this band is unknown but it is not Tn7 specific. It was not observed at increased levels in the presence of IPTG and was present in lanes derived from plasmids with unrelated Tn7 sequences.

In tracks derived from pMR51, three extra polypeptides were identified with molecular weights of 42Kd, 40Kd and 54Kd. These polypeptides were present in increased amounts when the tac promoter was derepressed using IPTG. Polypeptides of 42Kd and 40Kd were also observed in tracks from pMR57 and pMR66 minicell extracts while pMR56 and pMR64 produced a polypeptide of 54Kd. Again the levels of these polypeptides were enhanced in the presence of IPTG. No extra polypeptides were identified in extracts of pMR55, pMR61, pMR62 or pMR58. A faint band of 52Kd (definitely running below the 54Kd band observed in pMR51, pMR56 and pMR64) was seen in extracts of pMR57 and pMR66. This band was probably due to whole cell contamination of the minicell preparations.

In figure 5.3, minicells containing plasmids derived from pMR100 were analysed. pMR100 encodes five polypeptides, four of which are derived from lambda, the fifth is the kanamycin resistance gene of Tn903. Only three of these polypeptides are observed in minicell extracts of pMR100 and its derivatives. Two bands are observed with molecular weights of 30Kd and 27Kd. These are the products of the O, P and Kan

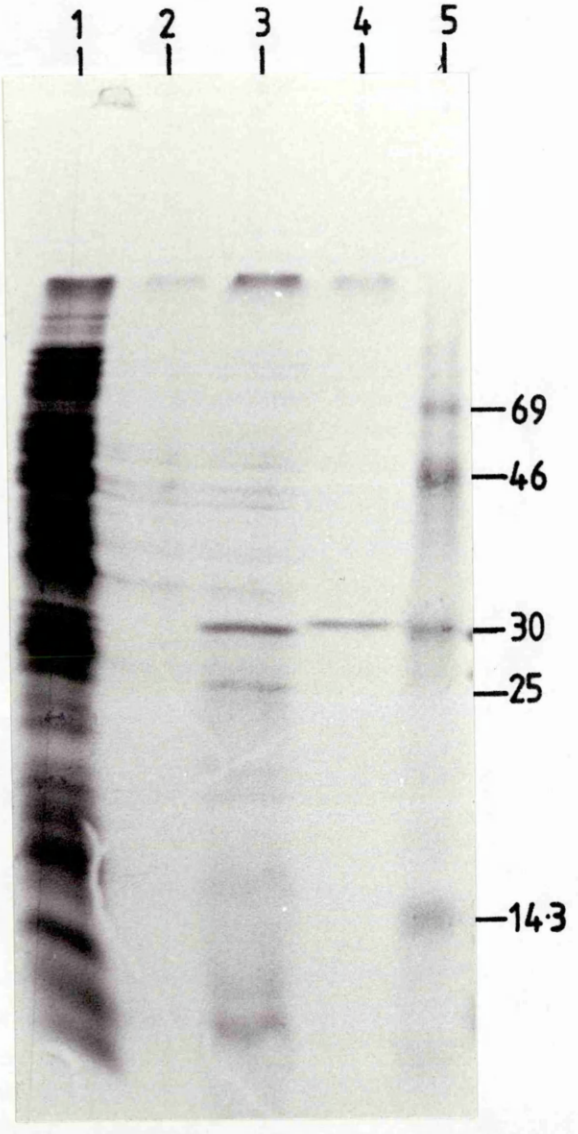


Figure 5.4 Polypeptides produced by pACYC184 plasmids containing fragments of Tn7.

1 DS944
2 pMR26
3 pMR25
4 pMR41
5 Molecular weight markers

The size of bands is given in Kd.

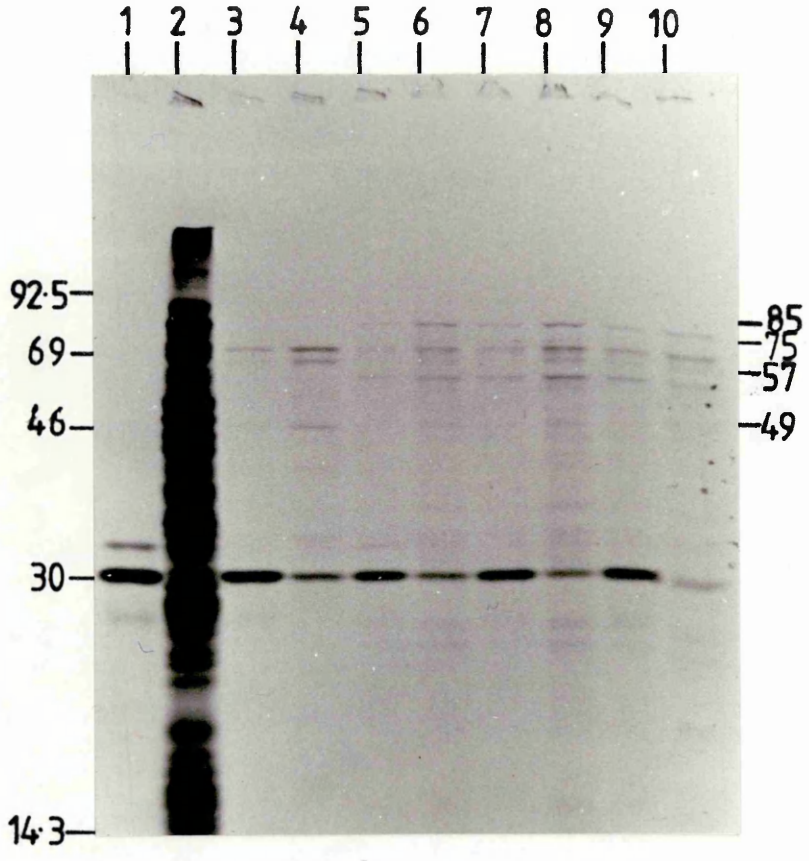


Figure 5.5 Polypeptides produced by pMR53 and its derivatives containing additional Tn7 fragments.

1	pGLW8 (+)	6	pMR59 (+)
2	DS944	7	pMR68 (-)
3	pMR53 (-)	8	pMR68 (+)
4	pMR53 (+)	9	pMR69 (-)
5	pMR59 (-)	10	pMR69 (+)

+/- in parentheses indicates the presence or absence of IPTG during the labelling of the minicells.
The size of bands is given in Kd.

resistance genes respectively (P and Kan run with the same molecular weight).

A polypeptide with an apparent molecular weight of 85Kd was present in tracks derived from pMR101, pMR102 and pMR117 but was absent in all others, including pMR112. This polypeptide was present at increased levels when the tac promoter was derepressed with IPTG. 42Kd and 40Kd polypeptides were observed in lanes derived from pMR106 and pMR113. No polypeptide of 54Kd was present in tracks derived from pMR106 and pMR118. However, in other gels of these plasmids this polypeptide was observed (data not shown). These polypeptides were present at increased levels in the presence of IPTG.

Figure 5.4 shows the polypeptides produced by pMR26 and the other pACYC184 derivatives. Tetracycline resistance is mediated by a polypeptide of 41Kd (Peden, 1983). No polypeptide of this, or any, size was common to pMR25, pMR26 and pMR41. pMR25 encoded two polypeptides of molecular weight 29Kd and 25Kd. Minicells containing pMR41 revealed only a single polypeptide of 30Kd.

The analysis of polypeptides produced by pMR53 and its derivatives is presented in figure 5.5. The vector specific bands described above were present. In addition, a polypeptide of 75Kd was observed in all lanes except pGLW8. The level of this polypeptide was enhanced in the presence of IPTG. Two polypeptides of 49Kd and 70Kd were also present in pMR53 and faintly in pMR59 and pMR68. These may be alternative products of the 75Kd polypeptide.

pMR59, pMR68 and pMR69 encoded a polypeptide of 85Kd which was expected from previous results (figure 5.3). An additional 57Kd molecular weight polypeptide was also present.

5.4 DISCUSSION

In the previous chapter, five functions were identified that were required for transposition (figure 4.14). Table 5.1 indicates which of these complementation groups should be present in each of the plasmids used for the minicell analysis. Table 5.2 summarises the results of the minicell analysis and shows that it is in good

Table 5.2 Summary of the apparent molecular weights of polypeptides observed in figures 5.2, 5.3, 5.4 and 5.5.

Molecular weight of polypeptide	Figure	Encoded by	IPTG[1]	Tn7 gene
30Kd, 27Kd, 33Kd	5.2, 5.5	pGLW8 and its derivatives	-	-
27Kd, 30Kd	5.3	pMR100 and all its derivatives	-	-
30Kd	5.4	pMR41	NA	*tnsA*[2]
29Kd	5.4	pMR25	NA	*tnsA*
25Kd	5.4	pMR25	NA	*tnsB*[2]
85Kd	5.3, 5.5	pMR101, pMR102, pMR117, pMR59, pMR68, pMR69	+	*tnsB*[2]
42Kd, 40Kd	5.3, 5.2	pMR106, pMR113, pMR51, pMR57, pMR66	+	*tnsC*
54Kd	5.2	pMR106[3], pMR118[3], pMR51, pMR56, pMR64	+	*tnsD*
75Kd	5.5	pMR53, pMR59, pMR68, pMR69	+	*tnsE*
49Kd, 70Kd	5.5	pMR53, pMR59, pMR68 pMR69[3]	+/-	unknown
57Kd	5.5	pMR59, pMR68, pMR69	-	*tnsB*/*tnsE*
52Kd	5.2	pMR57, pMR66	-	unknown

1 A + indicates that the level of the polypeptide was enhanced in the presence of IPTG.

2 These polypeptides may not be entirely encoded by Tn7 sequences.

3 Present in gels not shown.

NA Not applicable

agreement with the complementation data. This analysis also indicates that each complementation group has an associated polypeptide confirming that the trans-acting functions are proteins.

pMR25, pMR112, pMR101 and pMR59 should all encode a functional tnsA. pMR41 partially complemented for this gene. No polypeptide could be attributed to tnsA from pMR101, pMR112 or pMR59 but a 29Kd polypeptide was present in minicells containing pMR25 which was not present in extracts containing pMR26 or pMR41 (figure 5.4). A polypeptide of this molecular weight would be obscured in extracts from pMR101, pMR112 and pMR59 because these plasmids encode 30Kd polypeptides- the O gene product (pMR101 and pMR112) and the B-lactamase gene product (pMR59). Further support for this assignment comes from the presence of a slightly, but distinctly, larger polypeptide in extracts of pMR41. This plasmid did not fully complement a tnsA deficiency and might be expected to encode an altered polypeptide. It is difficult to relate the size of a polypeptide derived from SDS-polyacrylamide gels to the size of the gene which encodes it because of the possible anomalous mobility of polypeptides in these gels. However, a 29Kd polypeptide could be encoded within about 0.8Kb. If the ATG sequence which is 135bp in from the right end represents the translation start codon of tnsA, then this open reading frame should extend (reading into Tn7) beyond the BglII site at 13.1Kb. This BglII site is the boundary of Tn7 sequences in pMR41. Possibly, the polypeptide encoded by pMR41 represents a fusion of N terminal amino acids from Tn7 and C terminal amino acids from the vector. Such a polypeptide might show a reduced activity and could explain the available data.

A second polypeptide of 25Kd was encoded by pMR25 but not by pMR41 or pMR26. This implied that it was, at least in part, encoded by the Tn7 sequences present in pMR25 but absent in pMR41, i.e. the sequences between the BglII site at 13.1Kb and the HincII site at 12.5Kb. Translation of this sequence could produce a polypeptide of about 22Kd. This region probably encodes tnsB (see below) and it is possible that this polypeptide was also a fusion between the N terminal end of tnsB and the C terminal end derived from pACYC184 sequences.

pMR25, pMR26 and pMR41 all exhibit tetracycline resistance that is

mediated by a 41Kd polypeptide (Peden, 1983). This resistance is derived from pSC101 and is shared by pBR322. A 37Kd polypeptide has been observed from maxicells of pBR322 and its presence correlates with tetracycline resistance (Sancar et al, 1979). No polypeptide, common to pMR25, pMR26 and pMR41, of this, or any molecular weight, was observed in this analysis. There are 16 internal methionines in the tetracycline resistance gene so that a lack of potential sites for the incorporation of label does not account for the absence of this polypeptide. The copy number of pACYC184 is lower than pBR322 in normal cells and the maxicell analysis amplifies the copy number of plasmids so that the inability to observe a polypeptide for the tetracycline resistance gene in minicell extracts may not be surprising.

The complementation analysis indicated that tnsB was encoded by pMR59, pMR68, pMR69, pMR101, pMR102 and pMR117. In all minicell extracts containing these plasmids but not in any others, a polypeptide of 85Kd was observed. This band was more intense in the presence of IPTG. IPTG derepresses the tac promoter and enhanced transcription of 3' genes is expected. The presence of any polypeptide in the absence of IPTG (when the promoter is repressed by the lacI gene product) indicates that repression is not complete in minicells or that the insert encodes its own promoter. The lacI gene is on the chromosome in DS944 and is not segregated into minicells. Only repressor which has segregated into the minicells either with the cytoplasm or pre-bound to the operator of the tac promoter can regulate transcription. A higher level of repression might be produced by incorporating the lacI gene in the expression vector. In these circumstances, the repressor would always be present in minicells and should provide tighter control of genes cloned 3' to the tac promoter.

In pMR59, pMR68 and pMR69 a second polypeptide of 57Kd was also observed (figure 5.5). This polypeptide was specific to Tn7 but it was not present in minicell extracts of pMR53 nor in extracts that did not contain the tnsE gene product encoded on pMR53. It seems to require the fragments which define both tnsB and tnsE together indicating that it results from an interaction between these genes or their products. Possibly, this polypeptide represents an alternative product of either the tnsB gene or of the tnsE gene mediated by the

presence of the other gene (tnsE or tnsB), by allowing translation from a second initiation signal or by post-translational processing of the 85Kd (tnsB) or 75Kd (tnsE) polypeptides.

pMR53 and its derivatives encode a 75Kd polypeptide which must be encoded by the 2.2Kb HindIII fragment of Tn7 (5.8Kb to 8.0Kb). This fragment defines tnsE and the polypeptides it encodes were present at enhanced levels in the presence of IPTG. Polypeptides of 70Kd and 49Kd were also encoded by Tn7 sequences in pMR53. These polypeptides were present, though very faint, in pMR59 and pMR68 but were not detected in pMR69. In pMR53 there was a clear enhancement of the levels of these polypeptides when IPTG was added indicating that their transcription was directed, at least in part, from the tac promoter.

The sequence of this fragment (Smith and Jones, pers. comm.) contains a single complete open reading frame which could encode a polypeptide of about 61Kd (which could migrate as a 75Kd polypeptide). Utilisation of a second initiation site within the same frame, detected by a computer search for homology with a concensus ribosome binding site, would translate a polypeptide of about 38.5Kd which could migrate as a 49Kd polypeptide. The sequence beyond this open reading frame extends for 491bp before the HindIII site at 5.8Kb is reached. No extensive open reading frame was found and it is unlikely that either the 49Kd or 70Kd apparent molecular weight polypeptides are encoded in this region. These polypeptides could result from post-translational processing of the 75Kd polypeptide. It is also possible that the 49Kd polypeptide is translated from the second initiation site described above.

tnsC was encoded by pMR51, pMR57, pMR66, pMR106 and pMR113. In minicells containing these plasmids, polypeptides of 42Kd and 40Kd were observed. The levels of these polypeptides were increased in the presence of IPTG. These polypeptides could each be encoded by about an 1100bp region of DNA. The minimum size of a fragment known to encode tnsC was 1.8Kb. One end was defined by the PvuII site at 9.3Kb, the other by the BamHI site at 11.05Kb. Two genes encoding these polypeptides could not be distinguished in complementation assays and unless the open reading frames for these genes overlap, 1.8Kb is insufficient to encode each of these polypeptides separately.

From the sequence of the BamHI fragment (10.1Kb-11.05Kb; Smith and Jones, pers. comm.; E Nimmo, pers. comm.), an open reading frame begining just before the 11.05Kb site and reading out of the 10.1Kb BamHI site was identified. If these polypeptides were encoded separately, pMR56 should have been capable of expressing one but not the other of the polypeptides. This was not observed suggesting that the 40Kd polypeptide is encoded within the same region of DNA as the 42Kd polypeptide. They might represent alternative forms of the same polypeptide or the smaller one might be a degradative product of the larger.

Finally, tnsD was encoded by pMR51, pMR56, pMR64, pMR106 and pMR118. Partial complementation of this fuction was obtained using pMR57, pMR58, pMR66 and pMR62. From figure 5.2, a 54Kd polypeptide could be identified and increased levels of this polypeptide were correlated with the addition of IPTG. A DNA fragment of about 1.5Kb could encode a polypeptide of this size. This function must cross the HindIII site at 8.0Kb (chapter 4). The sequence of the HindIII fragment (5.8Kb-8.0Kb; Smith and Jones, pers. comm.) indicates that an open reading frame entering this fragment can only extend a maximum of 120bp. Thus the other end of the polypeptide must begin at or around 9.4Kb placing it beyond the PvuII site at 9.3Kb, which might explain the absence of a Tn7 specific polypeptide from or any complementation by pMR55. No 54Kd polypeptide was seen in minicell extracts of pMR106 or pMR118 in figure 5.3. This might be explained by the poor incorporation of label into this polypeptide generally and in these samples in particular. Other gels of minicell extracts of these plasmids do indicate a faint band at 54Kd (data not shown).

No polypeptides attributable to tnsD were observed in any of the tracks derived from plasmids which partially complemented for this function. A 52Kd polypeptide was seen in the profile of polypeptides produced from minicells containing pMR57 and pMR66. It was not Tn7 specific because it was not present in lanes derived from pMR58 which should encode a similar polypeptide to pMR57; they both have the same deletion. pMR57 and pMR66 do not have the same deletion and would not be expected to produce an altered polypeptide of the same size.

Table 5.2 summarises all of these results. tnsA - tnsE encode nine

polypeptides of apparent molecular weights 29Kd (tnsA), 85Kd (tnsB), 42Kd and 40Kd (tnsC), 54Kd (tnsD) and 75Kd, 70Kd and 49Kd (tnsE). A 57Kd polypeptide was also observed and was encoded by either tnsB or tnsE. Where more than a single polypeptide is translated from the same region of DNA, the larger polypeptide is likely to be the primary polypeptide and the smaller ones are derived from it or translated, at least partially, from within its coding region. The purification and Cleveland mapping of these polypeptides would confirm whether they are processed products of the same gene(s).

Derepression of the tac promoter did not influence the frequency of transposition but did affect the levels of the polypeptides produced. This implies that the transposition frequency is not limited at the level of expression of Tn7 polypeptides derived from these plasmids. Control of transposition must be mediated at another level, such as activation of the transposon ends.

Brevet et al (1985) have used maxicells to identify the polypeptides expressed by Tn7. They identified four polypeptides which were encoded by regions of Tn7 required for transposition. These polypeptides p85a, p54, p40 and p85b were encoded on fragments that could be related directly to tnsB, tnsC, tnsD and tnsE (figure 5.1 and figure 4.14). The locations of these polypeptides within Tn7 are in good agreement with the complementation data (see below) though there are differences between the apparent molecular weights determined by Brevet et al and those determined in this chapter.

p85a (85Kd) maps to tnsB (which also encodes a polypeptide of 85Kd) and is encoded by pMR117 which has a 2.05Kb fragment of Tn7. This fragment could encode a polypeptide of about 73Kd which is 12Kd smaller than the polypeptide observed. tnsB does cross the BamHI site at 11.05Kb but the frame used (identified by translational fusions; N Ekaterinaki, pers. comm.) extends only 22bp beyond this site. Thus the coding capacity of the fragment does not agree with the apparent molecular weight of the polypeptide encoded by it. Divergence between the actual and observed molecular weight has been reported (Blackshear, 1984). Incomplete binding of SDS to the polypeptide, acidity, basicity, protein glycosylation and membrane assosiation of the protein have all been implicated in causing anomalous migration.

Whether one of these factors is causing the effects observed here is not known.

Allowing for possible anomalies in the way protein gels run, p54 (54Kd) is in reasonable agreement with the size of the polypeptide encoded by tnsC. The difference between p40 (40Kd) and the 54Kd polypeptide encoded by tnsD may also be accounted for by anomalous migration. However, these polypeptides are also running in a different order of apparent molecular weight. This cannot be explained by the running conditions of the gels.

Brevet et al found that p40 (tnsD in this analysis) was encoded between the HindIII site at 8.0Kb and the EcoRV site at 9.8Kb. In the complementation analysis in chapter 4, this HindIII site was found to lie within tnsD. This function must extend beyond the HindIII site toward the ClaI site at 7.6Kb. No evidence was provided that the p40 polypeptide observed by Brevet et al was functional; only its presence on a SDS-polyacrylamide gel was used to map its position within Tn7. Possibly, the C terminal end of p40 was replaced by amino acids derived from the vector pBR322. A similar construct used in this thesis, pMR61, failed to complement for tnsD indicating that the loss of the C terminal end makes the polypeptide assosiated with tnsD inactive. The sequence of the HindIII fragment (5.8Kb to 8.0Kb; Smith and Jones, pers. comm.) indicates that depending on the frame, 1, 14 or 40 amino acids could be encoded by Tn7 DNA between the HindIII site at 8.0Kb and the ClaI site at 7.6Kb. In the construct employed by Brevet et al, these amino acids would be replaced by, at most, 8 amino acids derived from pBR322, while in pMR61, 5, 11 or 14 amino acids would be fused onto the C terminal end of this polypeptide, depending on the reading frame used.

In the minicell analysis, functions which were only complemented partially or not at all could not be correlated with an assosiated polypeptide (ie. pMR57, pMR58, pMR66, pMR55, pMR62 and pMR61). This is not unusual particularly for the completely inactive plasmids like pMR55. The N terminal end of tnsD is postulated to be missing in this construct. The lack of a polypeptide assosiated with pMR61 cannot be explained. It is possible that the stabilities of these altered polypeptides are also different or that they are too small to be

detected by this analysis.

Brevet et al (1985) did not identify a polypeptide encoded in the right end of Tn7 equivalent to the 29Kd polypeptide encoded by tnsA. Clones used in their analysis should have contained this function and the reason for its absence is not clear.

In summary, six polypeptides have been identified which can be mapped to the complementation groups observed in chapter 4. These polypeptides take up nearly all of the available coding capacity of the 8.1Kb required for transposition. No other transposon has been identified which has so many polypeptides assosiated with its transposition. The role of these polypeptides is not understood though it is likely that tnsD encodes a polypeptide which interacts with the hotsite. Similarly, tnsE probably encodes a polypeptide which has a DNA binding function and may be involved in determining the plasmid insertion site.

CHAPTER 6

PRELIMINARY ANALYSIS OF TN7 INSERTIONS INTO THE CHROMOSOME OF *E.COLI* AT SITES OTHER THAN THE HOTSITE.

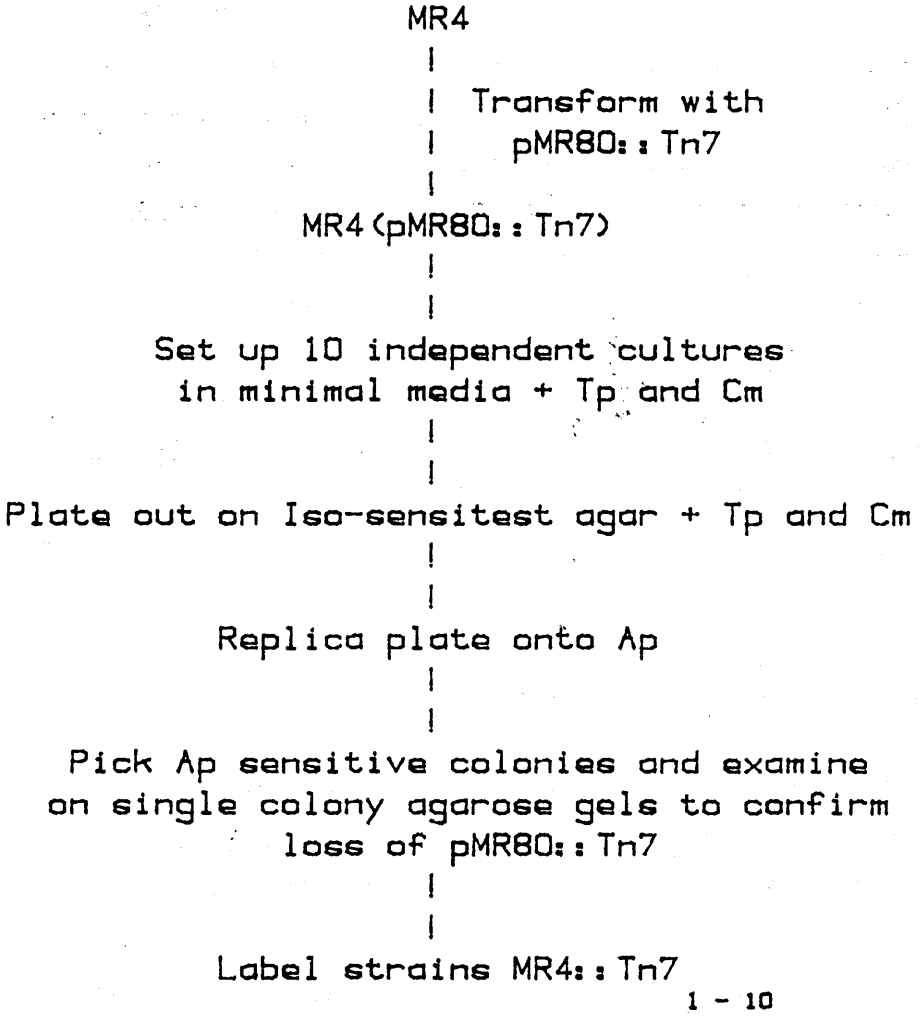
6.1 INTRODUCTION

Insertion of Tn7 into the chromosome of E.coli occurs with high efficiency and specificity (Barth et al, 1977; Lichtenstein and Brenner, 1981). All insertions, examined by conjugal transfer, P1 transduction and Southern blotting, have occurred in a single site, recently identified as the transcriptional terminator of the glmS gene (Barth et al, 1978; Lichtenstein and Brenner, 1981; Gay et al, 1986). This hotsite is superficially analogous to the lambda attachment site (attB) but this analogy does not extend beyond the specificity with which both Tn7 and lambda integrate into the chromosome of E.coli. attB is a relatively simple site while the hotsite is complex in terms of its size and its organisation (Lichtenstein and Brenner, 1982; N Craig, pers. comm.; C Lichtenstein, pers. comm.). The cloning of this region of the chromosome has greatly facilitated the analysis of the hotsite. Deletion mapping defined the hotsite to about 70bp and showed that the information within the hotsite sequence, determining the specificity of insertion, was asymmetric with regard to the point at which integration occured (N Craig, pers. comm.).

Tn7 also inserts into the chromosomes of other genera with a similar specificity (Thomson et al, 1981; Ely, 1982; Turner et al, 1984). Another approach used to analyse this site has been the cloning, sequencing and comparison of a number of hotsites from different genera. The preliminary results of this study identify long regions of homology between the hotsites of different genera which extend beyond the known sequences required to define the hotsite (C Lichtenstein, pers. comm.). The reason for such extensive homology remains obscure.

The lack of evidence for insertion of Tn7 elsewhere in the chromosome of E.coli prompted the investigation outlined below. The identification of secondary sites of insertion (chromosomal insertions not located within the hotsite) in E.coli and the subsequent cloning and sequencing of such sites, should provide further data regarding the factors which define the hotsite. Only preferred secondary insertion sites provide relevant data because insertions may occur by either the hotsite specific or the cold site specific transposition pathway; integrations into preferred sites are more likely to be

Figure 6.1. Strategy for construction of MR4::Tn7.



mediated by the hotsite specific transposition pathway. The results in chapter 4 have suggested a different approach to this problem. Transposition into the chromosome using a tnsE$^-$ Tn7 would avoid any insertions mediated by the cold site transposition pathway (see discussion and concluding remarks).

The highly efficient insertion of Tn7 into the hotsite (probably greater than two logs more frequent than other insertions) would prevent identification of secondary sites unless the hotsite was deleted or occupied by another Tn7 molecule. A similar approach has been used to identify secondary sites of insertion of lambda and comparison of these sites with attB has provided information regarding the important sequences which define that site (Weisberg and Landy, 1983). Using the strain MR4 (chapter 4), a preliminary study of ten independent Tn7 insertions into the chromosome was undertaken by Southern blotting to determine if preferred secondary sites were present.

RESULTS

6.2 Construction of Independent Isolates of MR4::Tn7

The strategy for obtaining Tn7 insertions in the strain MR4 is diagrammed in figure 6.1. MR4 was transformed with pMR80::Tn7. Ten colonies were purified and grown up overnight in minimal media with the appropriate supplements and trimethoprim. Under these conditions plasmid free cells can be isolated. Dilutions were plated onto iso-sensitest agar plates supplemented with trimethoprim and chloramphenicol and incubated overnight at 37°C. These antibiotics maintained a selection for Tn7 and Tn7-1 respectively. Colonies from each culture were replica plated onto iso-sentitest agar plates supplemented with Tp and Ap or Tp. Ap^S colonies derived from each culture were grown up and analysed by single colony agarose gel electrophoresis to confirm the loss of pMR80::Tn7. A colony derived from each of the ten cultures was picked and labelled MR4::Tn7$_1$ to MR4::Tn7$_{10}$.

6.3 Genetic Evidence that MR4::Tn7$_{1-10}$ were Generated by Transposition of Tn7.

The Tp^rAp^s phenotype of MR4::Tn7$_{1-10}$ could have occurred in a number of ways; spontaneous resistance to trimethoprim during the isolation of plasmid free cells, homologous recombination between pMR80::Tn7 and the chromosome or transposition of Tn7. pMR80::Tn7 was derived from pUC8 (see chapter 3) which encodes part of the lacZ gene. This homology is small (about 750bp; Messing et al, 1977) and in pMR80::Tn7 is disrupted by the hotsite containing Tn7. Homology also exists between the hotsite in the plasmid and the chromosome and between Tn7 sequences present in Tn7-1.

Spontaneous resistance to trimethoprim was excluded by screening the Tp^rAp^s colonies for resistance to spectinomycin which is also encoded by Tn7. Resistance to this antibiotic was not selected during the construction, though all ten strains grew in the presence of spectinomycin. Spontaneous resistance to two independent antibiotics, both present in Tn7, would be extremely rare and could only be picked up by selection from a large population of cells.

The sensitivity of all the colonies to Ap ruled out any homologous recombination event involving only a single crossover because this would result in the integration of the plasmid sequences and hence Ap^r as well. The probability of a double crossover within the small region of homology present is low. However, if such a recombination event did occur across the lacZ homology, it could be detected by looking for a $lacZ^-$ phenotype. MR4 is $lacY^-$ so that examining the MR4::Tn7 strains for a lac^- phenotype would be uninformative. MR4 when plated on IPTG and XGAL turns a pale blue, probably due to leakiness of the $lacY^-$ phenotype. This effect was used to test whether any of the Tn7 elements in the strains MR4::Tn7$_{1-10}$ had disrupted the lacZ gene. All these strains and MR4 were plated onto L-agar plates supplemented with IPTG and XGAL and incubated overnight. No difference between the colour of MR4 and of the strains MR4::Tn7$_{1-10}$ was observed which indicated that none of the Tn7 inserts had integrated into the lacZ gene either by homologous recombination or transposition. Homologous recombination between either the hotsites or Tn7 sequences present in both the chromosome and pMR80::Tn7 was

Table 6.1 Transposition of Tn7 and Tn7-1 from MR4::$Tn7_{1-10}$ to pEN300

Strain	Frequency of transposition Tn7	Tn7-1
MR4::$Tn7_1$	2.7×10^{-5}	6.1×10^{-4}
MR4::$Tn7_2$	1.0×10^{-5}	1.2×10^{-3}
MR4::$Tn7_3$	1.5×10^{-5}	1.9×10^{-4}
MR4::$Tn7_4$	3.0×10^{-5}	7.7×10^{-4}
MR4::$Tn7_5$	2.0×10^{-5}	4.8×10^{-4}
MR4::$Tn7_6$	1.8×10^{-5}	7.3×10^{-4}
MR4::$Tn7_7$	1.7×10^{-5}	5.9×10^{-5}
MR4::$Tn7_8$	3.3×10^{-5}	7.0×10^{-4}
MR4::$Tn7_9$	2.4×10^{-5}	5.4×10^{-4}
MR4::$Tn7_{10}$	1.7×10^{-5}	3.0×10^{-4}

This assay was carried out once.

also excluded. Integration of Tn7 under these circumstances would replace Tn7-1 making the cells Cm^S. During construction of these strains selection for Cm^r was maintained. Together these data indicate that neither homologous recombination nor spontaneous resistance to Tp could account for the phenotypes of MR4::$Tn7_{1-10}$. Transposition of Tn7 from pMR80::Tn7 to the chromosome was consistent with the phenotype observed.

6.4 Transposition of Tn7 and Tn7-1 from MR4::$Tn7_{I-10}$ to pEN300

To confirm that each of the strains contained a functional Tn7, pEN300 was mated from MR1(pEN300) into MR4::$Tn7_{1-10}$. MR4::Tn7 was resistant to Tp so no selection for pEN300 was available. Transfer of pEN300 to most of the recipients was assured by using an excess of donor. The presence of pEN300 in each of the MR4::Tn7 strains was confirmed by single colony gel electrophoresis.

A mate out transposition assay was carried out selecting for both Tn7 and Tn7-1 transposition. The results of these matings confirmed that in each strain a functional Tn7 was present (table 6.1).

The frequency of transposition of Tn7 was low in all of these strains compared to the frequencies in table 3.9. However, comparisons are difficult because the conditions under which the assays were performed were different. Tn7-1 transposed between 12 and 120 times more frequently than Tn7 in these assays possibily refecting differences in the ability of Tn7, located in different regions of the chromosome, to complement Tn7-1 transposition. The greater efficiency of Tn7-1 transposition generally compared to Tn7 is indicative of a size dependence of transposition. However, these assays were only performed once so that it is not reasonable to draw any conclusions except that there was a functional Tn7 and Tn7-1 present in the chromosome of each of the ten strains.

6.5 Southern Analysis of the Strains MR4::$Tn7_{1-10}$

To determine if any of the Tn7 insertions had integrated into the same position (within the sensitivity of the technique), chromosomal DNA of each strain was prepared, digested with either EcoRI or HindIII and run out on a 0.8% agarose gel. The DNA was transferred to Pall membrane and hybridised against pUC8::$Tn7_I$ (Southern, 1975). The results of these blots are shown in figures 6.2 and 6.3. pUC8::$Tn7_I$ shares homology to Tn7-1 and the pattern of hybridisation includes bands derived from the presence of Tn7-1 in the chromosomal hotsite.

6.6 DISCUSSION

This analysis was a preliminary step to identify if any secondary sites within the chromosome of E.coli were preferred by Tn7. Before the analysis of the sites of insertion was undertaken, evidence that these insertions were due to transposition had to be obtained. Formally, the presence of Tp^r cells could be explained by spontaneous mutation, homologous recombination between the plasmid pMR80::Tn7 and the chromosome and by transposition. Spontaneous mutation was ruled out because the Tp^r strains were also resistant to Sp. Resistance to spectinomycin was not selected during construction. Homologous recombination was ruled out by examining the phenotype of the strains obtained and comparing them with the phenotype predicted if homologous recombination had occurred. No difference was observed between the parent and its derivatives. Finally, The transposition of Tn7 from each of these strains confirmed the presence of a functional transposon in the chromosome.

The site of insertion was defined by Southern blotting using two different restriction enzymes. Analysis with a single enzyme is liable to error, particularly if (as in this case) the diagnostic bands are relatively large. The sensitivity of the analysis relies on the resolution of the gel. Large fragments resolve poorly in 0.8% agarose so that the use of a single enzyme would not distinguish Tn7 insertions into different fragments of approximately the same size. Analysis with a second enzyme should prevent this type of error as the probability of an insertion of Tn7 into independent chromosomal fragments which produce the same pattern upon digestion with EcoRI and

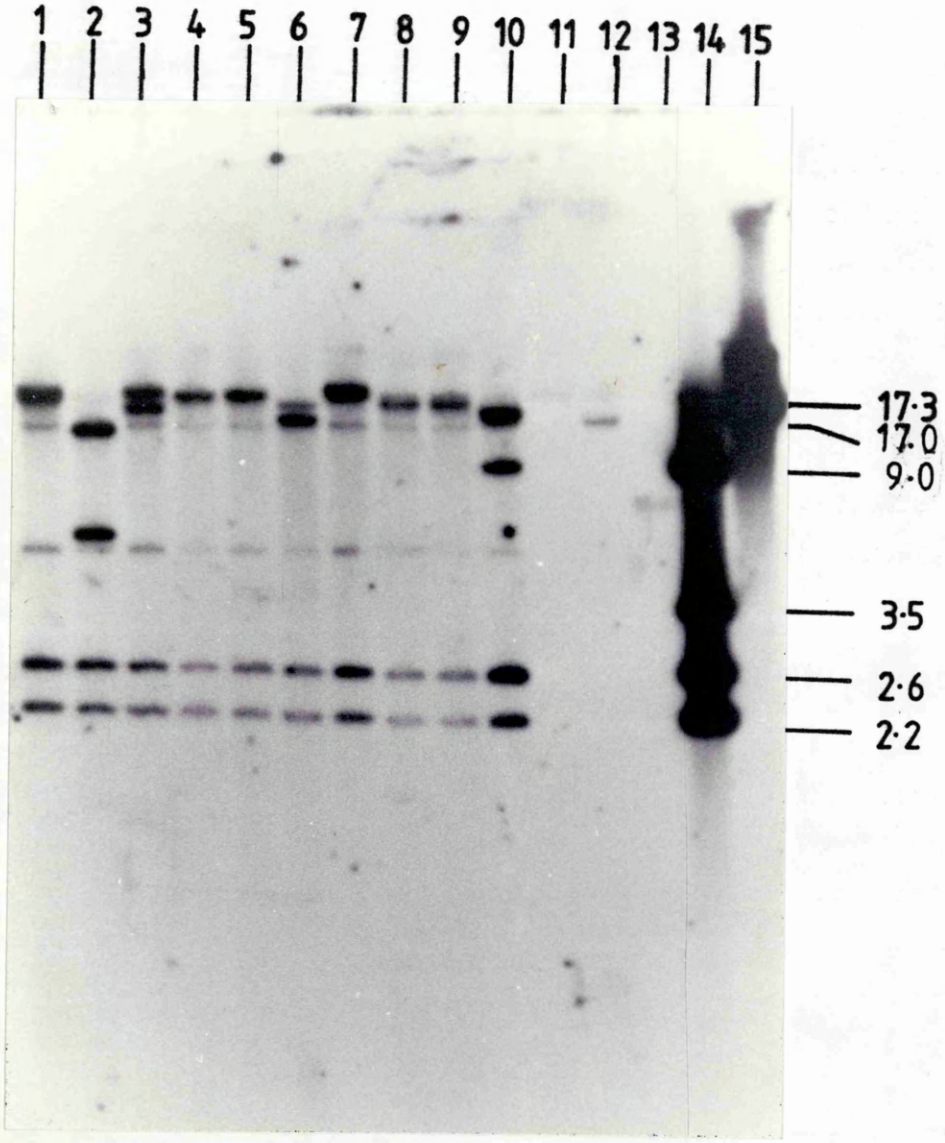


Figure 6.2. Southern blot of pUC8::Tn7$_I$ against HindIII digested MR4::Tn7$_{1-10}$.

1 MR4::Tn7$_1$ (HindIII)
2 MR4::Tn7$_2$ (HindIII)
3 MR4::Tn7$_3$ (HindIII)
4 MR4::Tn7$_4$ (HindIII)
5 MR4::Tn7$_5$ (HindIII)
6 MR4::Tn7$_6$ (HindIII)
7 MR4::Tn7$_6$ (HindIII)
8 MR4::Tn7$_7$ (HindIII)
9 MR4::Tn7$_9$ (HindIII)
10 MR4::Tn7$_{10}$ (HindIII)
11 DS903 (HindIII)
12 MR4 (HindIII)
13 Lambda (HindIII)
14 pUC8::Tn7$_I$ (HindIII)
15 pUC8::Tn7$_I$ (SalI)

Size markers are in Kb.

HindIII is low. The occurrence of identical patterns in different strains in both blots indicates that the insertions have occurred within the same fragment and in the same region within that fragment. Only sequencing of the insertion sites would confirm whether such clones had Tn7 insertions in identical positions. No time was available to initiate the cloning of these insertions, and results from previous chapters indicated a more rigorous approach to the analysis. This will be discussed below and in the concluding remarks.

The blot in figure 6.2 was a hybridisation of pUC8::$Tn7_I$ (figure 3.4) against HindIII restricted chromosomal DNA of MR4::$Tn7_{1-10}$, MR4 and DS903. In every lane containing chromosomal DNA except DS903 a band corresponding to the chromosomal hotsite containing Tn7-1 could be observed. This was the only band present in the MR4 DNA track. In all lanes containing Tn7, two bands diagnostic of the transposon were present. The 2.2Kb and 2.6Kb fragments are internal Tn7 fragments. The remaining major bands represent the junction fragments between Tn7 and the chromosome. For HindIII these bands cannot be smaller than 3.4Kb (the length of Tn7 between the HindIII site at 10.6Kb and the right end of the transposon). A faint band of about 4.8Kb is also visible in all tracks containing Tn7 but not in any other. This may be a partial digestion product of the 2.2Kb and 2.6Kb internal HindIII fragments. A number of tracks appear to have only a single boundary fragment band. It is probable that these bands are doubletons which the electrophoreis has failed to resolve.

Tracks 1, 4 and 5 show identical banding patterns in figure 6.2. Similarly lane 8 and 9 appear to be identical. The pattern obtained using pUC8::$Tn7_I$ as a probe against EcoRI digested chromosomal DNA (figure 6.3) shows that tracks 1, 4 and 7 are the same and that tracks 8 and 9 are different. Taken together these data indicate that only MR4::$Tn7_1$ and MR4::$Tn7_4$ have Tn7 insertions in the same DNA fragment which are indistinguishable. Cloning and sequencing of these insertions would determine if the sites of integration were identical in both cases.

In figure 6.3 a faint band is present in all lanes containing chromosomal DNA except DS903. The size of this band (2.3Kb) agrees well with that predicted for the right boundary between Tn7-1 and the

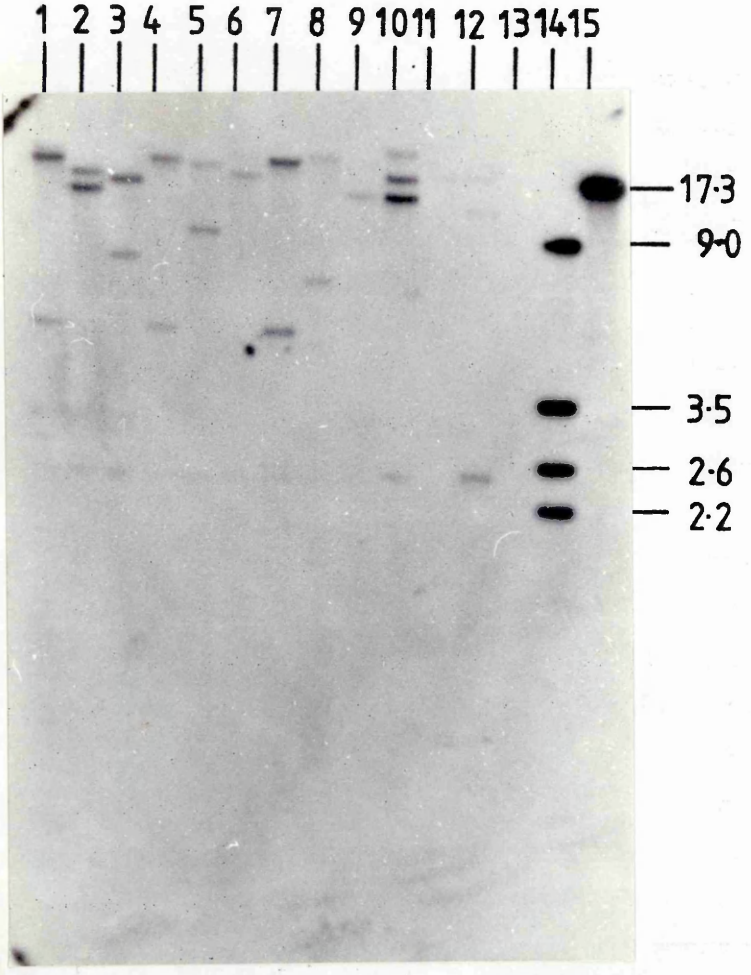


Figure 6.3. Southern blot of pUC8::$Tn7_I$ against EcoRI digested MR4::$Tn7_{1-10}$.

1 MR4::$Tn7_1$ (EcoRI)
2 MR4::$Tn7_2$ (EcoRI)
3 MR4::$Tn7_3$ (EcoRI)
4 MR4::$Tn7_4$ (EcoRI)
5 MR4::$Tn7_5$ (EcoRI)
6 MR4::$Tn7_6$ (EcoRI)
7 MR4::$Tn7_6$ (EcoRI)
8 MR4::$Tn7_7$ (EcoRI)
9 MR4::$Tn7_9$ (EcoRI)
10 MR4::$Tn7_{10}$ (EcoRI)
11 DS903 (EcoRI)
12 MR4 (EcoRI)
13 Lambda (HindIII)
14 pUC8::$Tn7_I$ (HindIII)
15 pUC8::$Tn7_I$ (SalI)

Size markers are in Kb.

chromosomal hotsite. In the lane containing MR4 DNA a second faint band running at about 14Kb is of a size consistent with that predicted for the left boundary fragment of Tn7-1 and the chromosomal hotsite. This band cannot be seen in other lanes derived from MR4 though it should be present. A higher concentration of MR4 DNA was loaded onto these gels compared to the MR4::$Tn7_{1-10}$ tracks. The extent of the homology between this band and pUC8::$Tn7_I$ is small (0.168Kb) and this may account for its absence.

Two bands were expected for each Tn7 insert in these strains which had to be greater than 5Kb (the length between the EcoRI site at 5Kb and the left end of the transposon). Generally this prediction was confirmed though only single bands (probably doubletons) were seen in tracks 6 and 9. Another faint band was present in lane 6 but was not in a stoicheiometric ratio with the first suggesting that it was due to a partial digestion of the DNA. Three bands (apart from the Tn7-1 band) were observed in lane 10 but again one was not of equivalent intensity and was probably the result of a partial digestion.

This analysis was not taken any further due to a lack of time though the development of a system for simplified cloning of subsequent insertions was begun. The principle of this system will be discussed in the next chapter. Another reason for not examining these insertions further arises from the results of chapter 4. Two related mechanisms of transposition are used by Tn7, one for transposition to plasmids which requires the tnsE gene product, the other for transposition to hotsites which requires the tnsD gene product. It is impossible to determine if a particular insertion into the E.coli chromosome was mediated by the tnsD pathway or the tnsE pathway though it is likely that insertions which occur in the same site preferentially are mediated by the tnsD pathway. Only insertions mediated by this pathway are true secondary hotsite insertions. Cold site insertions are background (in terms of this study) and make the analysis attempted here more difficult. Analysis of insertions into the chromosome using a tnsE$^-$ Tn7 would remove this background and allow a simpler identification of preferred secondary sites. The construction of the relevant plasmids and transposon is underway.

The preliminary results presented here indicate that Tn7 is capable of

insertion into the chromosome of E.coli even in the presence of Tn7-1 in cis. Two of the ten independent insertions obtained have probably inserted into the same region of the chromosome and appear to have integrated into the same site within the resolution of the gels employed. N Craig (pers. comm.) has made a similar though more extensive study of secondary insertions of Tn7 and has found no evidence for preferred secondary insertion sites. Her work employed a wild type Tn7 and was also liable to a high background because of insertions mediated by the cold site transposition pathway.

CHAPTER 7

CONCLUDING REMARKS AND FUTURE AIMS

The major result of this analysis of Tn7 was the dissection of the functions required in *trans* for Tn7 transposition (chapter 4). Five functions have been localised within the right 9Kb of Tn7 and their activities correlated with the presence of polypeptides (chapter 5), confirming that Tn7 transposition is mediated by proteins.

Some of these proteins will probably bind to DNA, either specifically to the ends of the transposon or to the hotsite, or non-specifically to cold site targets. The polypeptide encoded by *tnsD* is a good candidate for specific binding to the hotsite. It is only required for transposition to hotsites and is likely to mediate the recognition and integration of the transposon to that site. Similarly, the *tnsE* gene product probably plays a role in the identification and integration of Tn7 into plasmid targets. Preliminary work studying the effect of this protein on plasmid DNA *in vivo* indicates that it is capable of altering the electrophoretic mobility of supercoiled plasmids in agarose gels. The nature of this effect is unclear but it may involve the relaxation of supercoiled plasmids suggesting that a nicking activity may be assosiated with the protein.

tnsA - *tnsC* are involved in both modes of Tn7 transposition, ie. transposition to hotsites and cold sites. No role for these gene products has been identified but they are probably involved in interactions with the transposon. Perphaps they also interact with the *tnsD* and *tnsE* gene products bringing the ends of Tn7 into contact with the target. Studies using transcriptional and translational fusions have shown that the presence of a plasmid containing the *tnsB* gene acts to repress the promoter located in the right end of Tn7 which is believed to initiate transcription of *tnsA*, *tnsB* and *tnsC* (Rogers *et al*, 1986). This promoter is located in the repeats of the right end of the transposon. Whether the effect is caused by the binding of the *tnsB* gene product to the promoter directly or to the sequences in the right end of the transposon is unclear. The purification of these proteins and their subsequent analysis *in vitro* should provide data on their role in transposition and its regulation.

Binding assays both *in vitro* on purified or partially purified proteins and *in vivo* using photofootprinting, methylation protection and alteration in electrophoretic mobility could be used to determine

the sites and proteins which interact (Siebenlist and Gilbert, 1980; Becker and Wang, 1984; Wu and Crothers, 1984). These analyses would also allow the binding constants and order which sites are occupied to be determined.

Transcriptional fusions where the galK gene is placed 3' to Tn7 sequences have been used to locate promoters within Tn7. Two have been localised to the right end, a strong promoter, P_1, is repressed in the presence of plasmids capable of expressing the tnsB gene product and a weaker promoter, P_2, which probably also initiates transcription of tnsB and tnsC (Rogers et al, 1986). These fusions could be used to study the control of transcription in Tn7 by placing plasmids expressing Tn7 fuctions in trans and analysing the effect on transcription. Similarly, competition experiments using multicopy plasmids containing regions of Tn7 could be used to look for the effect of ends or other sequences in trans. Observations suggest that the presence of the right end in high copy number inhibits the transposition of Tn7 from plasmids in trans (N Craig pers. comm.). However, care must be taken in any fusion study to allow for copy number effects and it may be necessary to integrate the fusions into the chromosome to alleviate this problem.

Deletions into Tn7 sequences could be generated usig Bal31 or ExoIII to further trim back the sequences required in cis and in trans for transposition.

The sequence of the right 9Kb of Tn7 is being determined and will provide considerable information on the organisation and the nature of Tn7 and its genes. Comparisons with other DNA and polypeptide sequences may help provide insight into the functions of the Tn7 proteins.

In chapter 3 evidence was presented which indicated that Tn7 did not transpose to produce cointegrates. This implied that Tn7 might use a nonreplicative mechanism to transpose. A more rigorous analysis of the DNA rearrangements mediated by Tn7 would provide further data on the type of mechanism used. Such an analysis would have to study both modes of Tn7 transposition because it is clear that these mechanisms differ in a number of respects which have been discussed. Evidence

has been published that the transposition of both Tn10 and Tn5 is conservative (Hirschel et al, 1982; Harayama et al, 1984; Kleckner and Ross, 1984; Morisato and Kleckner, 1984; Bender and Kleckner, 1986). Neither of these elements promote replicative inversions, adjacent deletions or cointegrates which are the intramolecular and intermolecular equivalents of transposition via a replicative cointegrate pathway. Tn10 does promote inversion deletions but these can be explained by intramolecular transposition of an inverse Tn10 into itself via a simple transposition pathway (Kleckner and Ross, 1980; Hirschel et al, 1982; Berg, 1983; Weinert et al, 1983, 1984; Way and Kleckner, 1984; Isberg and Syvanen, 1985). Further understanding of the mechanism employed could be derived by experiments similar to those carried out by Bender and Kleckner (1986) where heteroduplex Tn10 molecules were used to follow the seperate strands of DNA in the transposition process.

In chapter 6, preliminary evidence was presented that Tn7 can insert into the chromosome of E.coli at sites other than the hotsite. Two out of ten independent insertions may have integrated into the same site. This in conjunction with the results of a similar, and more extensive, analysis by Nancy Craig suggest that no preferred secondary sites of insertion are present in the chromosome of E.coli. These analyses employed a Tn7 capable of inserting into both hotsites and cold sites. In order to determine if any secondary hotsites exist within the E.coli chromosome, a transposon incapable of transposition to cold sites (tnsE$^-$) would be a preferable donor. Any inserts observed would have to be generated by transposition via the hotsite specific pathway and analysis of these insertions would provide a more rigorous demonstration of the presence or the absence of secondary hotsites.

If any such sites were identified they would have to be cloned and sequenced to confirm that independent insertions had integrated into the same sequence. The cloning of these insertions would be greatly simplified if the transposon contained its own origin of replication. Cloning would then only involve intramolecular ligation of chromosomal DNA restricted by an enzyme that did not cut within the tnsE$^-$origin$^+$ Tn7.

Preliminary experiments using a Tn7 containing pMR100 at the EcoRI site confirm that this element is capable of transposition (though about 10% less frequently than a wild type Tn7 control). pMR100 is a lambda-dv based expression vector and its replication is blocked by the presence of the cI gene product. The loss of pMR100 in the presence of a cI overproducing plasmid is very rapid. Further work on the construction of a Tn7 molecule with the correct properties is underway, in preparation for its insertion into the E.coli chromosome.

One of the most interesting and unusual properties of Tn7 transposition is the nature of its transposition into plasmids. Nearly all insertions occur in a specific orientation relative to the restriction map of the plasmid and the transposon. The mechanism by which Tn7 is capable of determining the orientation of the recipient plasmid is unclear. It cannot be the replication of the recipient because insertions into plasmids with bidirectional origins of replication are in a single orientation. It is unlikely to be a specific sequence which is recognised because the sequence would have to be complex enough to be present only once in a large conjugative plasmid like RP4 but simple enough to be present in small plasmids like ColE1.

In chapter 4 it was shown that Tn7-2 inserts at a low frequency into cold sites under some conditions and that these insertions occurred in both orientations. This suggests that tnsE may be involved in this process though the presence of this gene in trans did not restore the orientation specific insertion phenotype.

It is clear that the transposition of Tn7 is complex and involves more proteins than any other transposon studied to date. The elucidation of the function of the proteins involved and the mechanism of transposition used, should provide novel information on the process of site-specific recombination in E.coli.

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