THE REGULATION AND PROPERTIES OF TN1/3 TRANSPOSASE

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by

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Dedicated to my mother and father
for all their love and support.

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Contents

Abbreviations

Chemicals

APS - ammonium persulphate
ATP - adenosine triphosphate
BSA - bovine serum albumin
CIP - calf intes tinal phosphatase
CsCl - caesium chloride
CTAB - mixed alkyltrimethylammonium bromide
CTP - cytosine triphosphate
dd(NTP) - 2'3'-dideoxy (nucleotide)
DMF - dimethyl formamide
DMS - dimethylsulphate
DNA - deoxyribonucleic acid
d(NTP) - 2'-deoxy (nucleotide)
DMSO - dimethyl sulphoxide
DTT - dithiothreitol
EDTA - ethylenediaminetetra-acetic acid (disodium salt)
EtBr - ethidium bromide
EtOH - ethanol
FSB - final sample buffer
GTP - guanosine triphosphate
IPTG - isopropylthio-B-D-galactoside
NaOAc- sodium acetate
OAc - acetate
ONP - o-nitrophenol
ONPG - o-nitrophenyl-B-D-galactoside
PBS - phosphate buffered saline
PEG - polyethylene glycol 6000
RNA - ribonucleic acid
RNase - ribonuclease
SDS - sodium dodecylsulphate
TEMED - N,N,N'N'-tetramethylethylenediamine
TPEG - phenylethyl-B-D-thiogalactoside
Tris - tris (hydroxymethyl) amino ethane
TTP - thymidine triphosphate
TWEEN - TWEEN 20
Xgal - 5-bromo-4-chloro-3-indoyl-B-galactoside

Antibiotics

Ap - Ampicillin
Cm - Chloramphenicol
Km - Kanamycin
Rf - Rifampicin
Sp - Spectinomycin
Sm - Streptomycin
Tc - Tetracycline
Tp - Trimethoprim

Phenotypes

X^r - resistance to X
X^s - sensitive to X
ori - origin of resolution
res - resolution site
Tra - transfer proficiency

Measurements

bp - base pairs
kbp - kilo base pairs
d - dalton
kd - kilodalton
A - amps
mA - milliamps
V - volts
mV - millivolts
W - Watts
Ci - curies
mCi - millicuries
uCi - microcuries
cpm - counts per minute
^oC - degrees centigrade
g - centrifugal force equivalent to gravitational acceleration
g - grammes
mg - milligrammes

ug - micrograms

ng - nanogrammes

l - litres

ml - millilitres

ul - microlitres

cm - centimetres

mm - millimetres

nm - nanometres

M - molar (moles per litre)

mM - millimolar

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

min - minutes

sec - seconds

hr - hour

Miscellaneous

D&M - Davis and Mingioli

SC - supercoiled

OC - open circular

UV - ultraviolet light

Tn - transposon

WT - wild type

% - percentage

RT - room temperature

Fig - figure

IR(s) - inverted terminal repeat(s)

LIR - left terminal repeat

RIR - right terminal repeat

IS - insertion sequence

RF - replicative form

log - logarithm

aa - amino acid(s)

mRNA - messenger RNA

no - number

OD_X - optical density at wavelength X

HSP - heat shock protein

SUMMARY

Tn*3* transposition is regulated at several levels. It is well established that resolvase, by binding at the *res* site, represses transcription of both *tnpA* and *tnpR*, which encode transposase and resolvase respectively. Binding of resolvase to *res* also leads to resolution of transposition cointegrates by site-specific recombination. In this work, translational fusions of *tnpA* and *tnpR* to *lacZ* have shown that at 30°C, in the presence of resolvase, there are about three times as many resolvase fusion molecules as transposase fusion molecules per cell. This corresponds to approximately 60 and 20 molecules per cell respectively when the transposon is on a high copy number plasmid. If the number of transposase fusion molecules genuinely represents the number of transposase molecules per cell, this will ensure a low frequency of transposition under repressed conditions. In the absence of repressor at 30°C, for example when Tn*3* first enters a cell, resolvase and transposase were shown to be present at higher levels of about 530 and 140 - 280 molecules per cell respectively. This presumably allows a transient burst of transposition, until repression is established.

Experiments with suitable plasmid constructs showed that transposition of Tn*3* is further inhibited by the presence of excess Tn*3* inverted repeat (IR) ends when low levels of transposase are present. This suggests there is non-productive titration of transposase by the excess IR ends which limits the amount of enzyme available for transposition. The regulatory implications of this are discussed.

Transposition immunity, in which a replicon carrying at least one IR end is resistant to further Tn*3* insertions, was also investigated. Evidence was collected which suggested that the left IR was slightly more effective at conferring immunity than the right IR. Models for immunity are discussed.

It has long been known that the transposition of Tn*3* is temperature sensitive: it is at least one hundred-fold lower at 42°C than 30°C. The temperature sensitivity was shown to act at the level of cointegrate formation, rather than resolution: it is resolvase

independent. Additionally, transcriptional and translational fusions to *tnpA* were used to show that the synthesis of transposase is not significantly temperature sensitive. Thus either the transposase itself is temperature sensitive (at the level of activity or breakdown), or there is an undiscovered temperature sensitive host factor involved in Tn*3* transposition. The possible role of the heat-shock protease Lon in breakdown of transposition protein(s) was examined, and was found to play no part in increased breakdown at higher temperatures. Attempts to obtain a mutant transposon which would transpose at 42°C at least as frequently as at 30°C, were not successful. The cause of temperature sensitive transposition remains to be elucidated.

Exonuclease III deletions were used to investigate a plasmid construct in which transposase was expressed from the *lac* promoter at lower levels than expected. An interesting, but so far inexplicable, result was obtained, which may indicate the existence of another regulatory mechanism to prevent high levels of transposase expression.

Finally, a transposase:B-galactosidase fusion protein was purified and used in an attempt to raise polyclonal antibodies to transposase. Both purified transposase and transposase antibodies will play an important future part in understanding the Tn*3* transposition reaction, its regulation, and the evolutionary relationship of Tn*3* to other transposable elements.

CHAPTER 1

INTRODUCTION

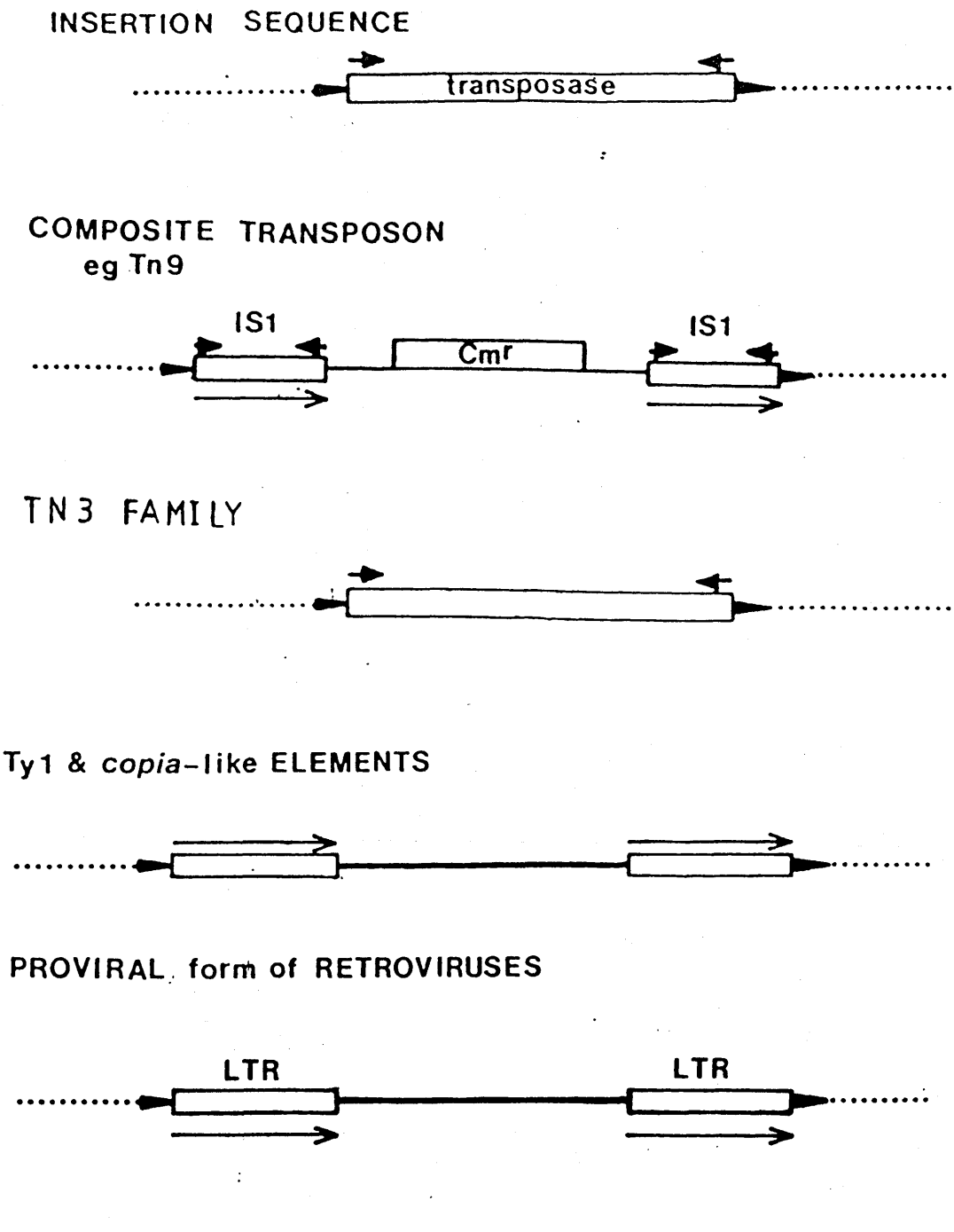


Fig. 1.1 The structural organisation of various transposable elements.

►	Direct repeat of target sequence
→	Short inverted repeats
⟶	Long direct repeats
LTR	Long Terminal repeats
.....	Host DNA

1.1 General features of transposable elements

Transposable elements are defined as segments of DNA with the ability to integrate in a discrete, non-permuted manner into many sites in genomes (Kitts 1982).

Transposition occurs in the absence of any significant DNA homology and does not require a functional homologous recombination system, at least in prokaryotes (Heffron et al. 1977). This is because transposable elements often can make, or have been able to make in the past, their own transposition-promoting enzymes, called transposases. In cases where these have become mutated so that they can no longer function, the transposable element may be complemented in trans by a non-defective element. This is often the case for eukaryotic transposable elements e.g. in Drosophila P and M hybrid dysgenesis (Ch. 3; O'Hare and Rubin 1983) and in maize (Doring and Starlinger 1984). Presumably as a result of the mechanism of action of transposases on the ends of the elements, there is usually a 3 - 13bp directly repeated duplication of target DNA adjacent to the ends of the transposon (Kleckner 1981).

The ends of the elements almost always consist of inverted terminal repeats. The basic organisation of the repetitive sequences in several types of transposable element is shown in Fig. 1.1. Mu is one well known example where true terminal repeats do not exist, but in this case there are regions of homology between the ends.

1.2 The diversity of transposable elements

The existence of transposable elements was first proposed by McClintock (1950) to account for the patterns of pigmentation variation in maize kernels and leaves. Since her discovery they have been found in a wide variety of prokaryotic and eukaryotic organisms: in bacteria, yeast, plants and invertebrates (Calos and Miller, 1980; Kleckner, 1981; Peterson, 1985; Doring 1985).

1.2.1 Eukaryotic transposable elements

The structures of different eukaryotic mobile elements are quite

similar, and their properties are often compared with those of integrated proviruses, leading to speculation that these different classes of genetic elements may share the same evolutionary origin (Temin 1080; Varmus 1982; Baltimore 1985). This hypothesis is supported by experiments demonstrating amino acid sequence homologies predicted from open reading frames located within the internal domains of both these classes of genetic elements, in regions possibly coding for gene products involved in transposition. Additionally the transposition mechanism of some mobile genetic elements, such as that of Ty in yeast, is known to involve an RNA intermediate (Boeke *et al.* 1985). Such elements have been referred to as "retrotransposons".

Mobile genetic elements probably account for a major fraction of the middle-repetitive DNA in the genomes of some organisms, such as *Drosophila melanogaster* (Finnegan *et al.* 1977). The most abundant middle-repetitive sequence families in mammalian DNA may have evolved by processes involving retrotranscription. *Alu*I-repeats, the most highly repetitive family of short, interspersed repeats in human DNA (Houck *et al.* 1979), display a high level of homology with the 7SL RNA sequence and it has been suggested that they may have originated from 7SL RNA by reverse transcription, followed by dispersal of pseudogene-like DNA copies by chromosomal integration, at some stage in evolution preceding mammalian radiation (Ullu and Tschudi 1984). These observations suggest that replicative transposition has probably played a significant role in the evolution of diverse repetitive sequences in eukaryotic genomes (Pearston *et al.* 1985). These repetitive sequences may have been an important feature in the development of eukaryotic regulatory mechanisms (King 1985).

1.2.2 Prokaryotic transposons

Historically, prokaryotic transposons have been divided into three groups. The distinction is based on various structural and functional properties which the elements have in common (Kleckner 1981; see below). The groups are: the IS elements and their composite transposons; the Tn*3*-like transposons; and the transposing bacteriophages. As transposons have become better understood, it is apparent that this division may be too strict. In 1984, Mizuuchi described only two extreme types: those such as Tn*3*-like elements,

which transpose replicatively via a cointegrate molecule in the case of intermolecular transposition; and those such as Tn5 and Tn10 which transpose essentially non-replicatively (1.3). Between these two types there are a number of transposons, including Mu, that produce as the primary transposition product both cointegrates and simple inserts, with different ratios. The evolutionary relationships of these different types of element are unclear.

Examples of insertion sequences are IS1 - IS5, and examples of composite transposons are Tn5, Tn9, Tn903 and Tn10. Each of the latter contain copies of an insertion sequence at the transposon ends which are known as IS50, IS1, IS903 and IS10 respectively. For most composite elements it has been directly confirmed that all of the information relative to transposition is encoded in the insertion modules themselves, and that the IS modules are still capable of independent transposition as individual units. The region bounded by the insertion sequences generally contains resistance to one or several antibiotics.

The IS elements are generally short segments of DNA (750 - 1600bp) that encode only one or two genes involved in transposition. One of these is for a transposase protein which often occupies almost the whole coding capacity. The IS transposases only act efficiently in cis: complementation in trans of a defective element occurs with an efficiency of 1% or less (Grindley and Reed, 1985). This is a significant difference between this type of transposable element and the Tn3-family and the transposing bacteriophages, and may be caused by the transposases, which are all very basic proteins, associating with the DNA immediately after synthesis, and diffusing in one-dimension along the DNA. This would tie in with the fact that the transposition efficiency of composite transposons correlates with length, at least when they are located on plasmids (Way and Kleckner 1985).

Sasakawa et al. (1983) have suggested a possible evolutionary origin for IS elements and composite transposons. They suggested that the transposition-promoting enzymes, the transposases, encoded by the elements may have evolved from various DNA binding proteins similar to today's repressors, helicases or topoisomerases (Kleckner, 1981

supports the latter), which bind, nick and reseal DNA molecules. The recognition sequences could have evolved from operators to which these binding/nicking/resealing molecules bound. At this point there would be no need to suggest that a mobile genetic element existed. The IRs could have arisen from a reverse duplication of the first site, or if the transposase found its binding site by tracking, it might occasionally stop at sequences similar to the first. The element might at this stage shorten, so that the second site is as close as possible to the first. Evolution of the second site might then occur so that it is efficiently recognised. This process could occur at both ends and could lead to overlap between IR sequences and coding sequences. Features of this hypothesis may be applicable to the two other types of elements, the Tn3 family and the transposing bacteriophages.

Tn3 and its relatives form a distinct group of transposons because they encode not only the transposase involved in intramolecular transposition and in forming cointegrates during intermolecular transposition, but also a site-specific recombination enzyme, resolvase, which breaks down the cointegrates by acting at the *res* site, to give simple inserts in the donor and target. The transposase protein is about 120,000 daltons; and the resolvase is about 21,000 daltons. Both gene products are *trans* acting, in contrast to IS transposases which preferentially act in *cis*. The Tn3 family all have terminal inverted repeats of 35 - 40bp and duplicate a 5bp target sequence on transposition (Fig. 1.1).

Over 20 transposons belonging to this group have been isolated from at least 50 different genera (Heffron 1983). Most of these genera are gram-negative, but Tn551 (encoding erythromycin resistance) was isolated from *Staphylococcus aureus* (Kahn and Novick 1980). Other members encode tetracycline resistance, several have multiple resistances, and one carries the gene for lactose metabolism. Tn3 and its immediate relatives, Tn1 and Tn2, carry the most common penicillinase found in gram-negative bacteria, the TEM-type B-lactamase (Heffron *et al.* 1975). This TEM resistance has been divided into two classes (Heffron *et al* 1977): Tn3 was discovered in R1*drd*10 and it encodes a TEM-1 B-lactamase; Tn1 was discovered in RP4 and encodes a TEM-2 B-lactamase. The reason for this difference has been

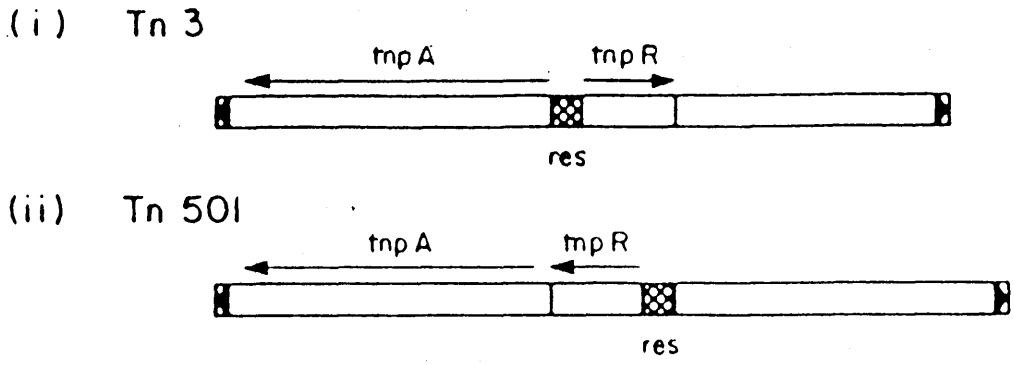


Fig. 1.2 The organisation of the two sub groups of the Tn3 family of transposons. tnpA and tnpR are the genes for transposase and resolvase respectively. res is the site of cointegrate resolution. In the Tn3 subgroup, tnpA and tnpR are divergently expressed and res lies between them. In the Tn501 subgroup the fragment containing tnpR and res has been inverted. The remainder of the transposon may carry additional markers.

elucidated by Chen and Clowes (1987): a base pair difference between Tn*1* and Tn*3* in the *bla* regulatory region means that different promoters are used by the two genes; and another base pair difference within the structural sequence accounts for the isoelectric focusing difference seen between the two proteins. The B-lactamase assays in this thesis (Ch. 4) all involved the Tn*3*-derived TEM-1 protein. The TEM B-lactamases are of medical importance bacause they have been found in disease-causing bacteria such as *H. influenzae* (Elwell *et al.* 1975) and *N. gonorrhoeae* (Fayet *et al.* 1982).

Based on their genetic organisation and on the ability to complement one another, transposons of the Tn*3* family can be divided into two sub-groups (Fig. 1.2) (Grindley and Reed 1985). In one group, represented by Tn*3* and Tn*1000* ($\gamma\delta$), the *tnpA* and *tnpR* genes, encoding the transposase and resolvase respectively, are divergently transcribed from a shared regulatory region. Here the *tnpR* functions, but not the *tnpA*, are interchangeable. The site of cointegrate resolution, *res*, lies within the intergenic region. In the Tn*501* subgroup, the region that contains *res* and *tnpR* is inverted relative to its orientation in Tn*3*.

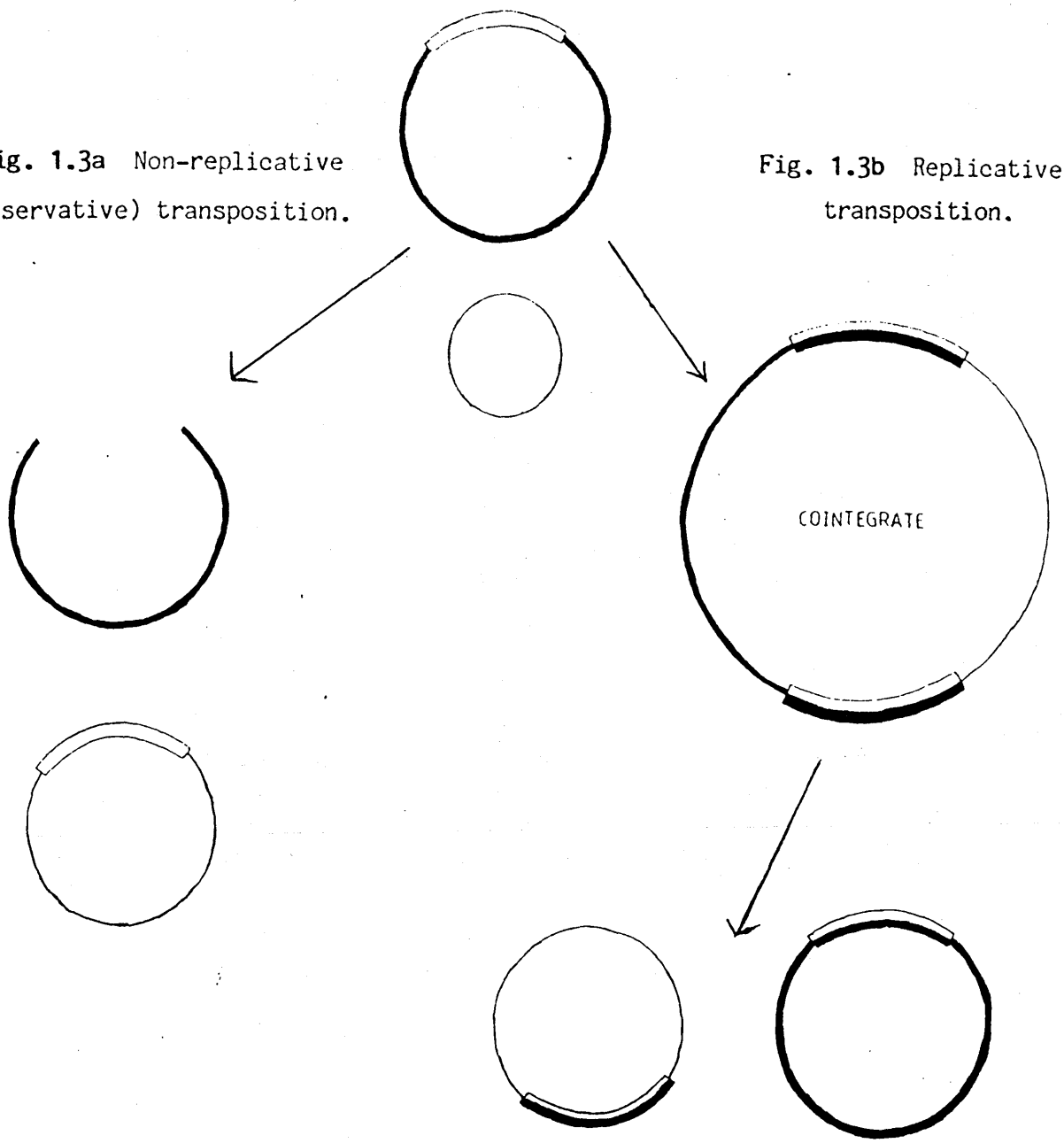
Mu and D108 are examples of transposing bacteriophages (Kleckner 1981). They use replicative transposition for replication of the phage genome during lytic growth, and non-replicative transposition (1.3) for prophage insertion during lysogeny. The two are heteroimmune but have 95% homology and partial cross-reaction (Craigie *et al.* 1984).

During the lytic cycle, Mu transposes replicatively to generate about 100 copies of its genome in almost an hour. This indicates a potential for very efficient transposition relative to the low frequency of other elements.

The two Mu functions directly involved in transposition are Mu A and Mu B. The proteins from these genes have been very well studied (for a recent review, see Mizuuchi and Craigie, 1986). Mu A is absolutely necessary for all transposition related processes, and mutants in Mu B have a markedly decreased frequency of integration. The A product is analogous to the transposases of other elements.

Fig. 1.3a Non-replicative (conservative) transposition.

Fig. 1.3b Replicative transposition.



The transposon is shown as an open box and the donor and target replicons are represented by thick and thin lines respectively.

1.3 Mechanisms of prokaryotic transposition

Prokaryotic transposons may be replicative or non-replicative (conservative) in their transposition mechanism (1.2), although in the latter case limited replication must occur (Mizuuchi 1984). This means that sometimes two copies or more of the element are produced where before only one existed, and sometimes the original element itself transposes, leading to no increase in copy number of the element (Fig. 1.3a and b).

The Tn3 family and Mu, in its lytic cycle, together with at least two IS elements, IS1 and IS903, transpose replicatively, intermolecularly via a cointegrate molecule (Gill *et al.* 1979; Kitts *et al.* 1982; Biel and Berg 1984; Weinert *et al.* 1983). This subsequently may or may not be resolved either by the host's general recombination system, or by a site-specific recombinase encoded by the element itself, to give simple inserts in both the target and donor DNA (Fig. 1.3).

In the case of Mu, biochemical studies have elucidated the structure of an intermediate in the synthesis of cointegrates formed through the action of the Mu transposase (A protein), the B protein, and the *E. coli* histone-like protein HU at the ends of the element. This intermediate has been shown to exist *in vitro* by electron microscopic studies (Miller and Chaconas 1986). Addition of a replication-competent extract to this results in formation of a cointegrate.

Two types of models exist to explain replicative transposition which are compatible with the observed Mu transposition intermediate; these are symmetric and asymmetric models, depending on whether events are initiated at both ends, or at one end of the element respectively. These models have been discussed recently by Grindley and Reed (1985).

The symmetric model is currently favoured for replicative transposition, and this has been found to be the mechanism employed during replicative transposition of Mu (Mizuuchi 1984; Miller and Chaconas 1986). In this model, single strands at both ends of the transposon are cleaved simultaneously, which could involve either the 5' or the 3' ends of the element. Attachment of both ends to the nicked target, at either 3' or 5' protruding staggered cuts,

immediately forms two replication forks. The original proposals (Shapiro 1979; Arthur and Sherratt 1979) then involved assembly of a replisome at each joint and replication of the complete transposon. Final sealing of the replicated DNA to flanking sequences at each end generates a cointegrate. For Mu it has been shown (Mizuuchi 1984) that the 3' ends of the transposon are nicked and joined to protruding 5' ends of the target (Fig. 3.15b).

Asymmetric models propose that transposition is initiated by cutting a strand at one end of the transposon and transferring it to the cleaved target site. The joint then creates a replication fork which travels through the transposon carrying the transposase. When the opposite end of the element is reached, the transposase can terminate the transposition in one of two ways (Grindley and Reed 1985) which can give rise to simple inserts or cointegrates. It is a possibility that one-ended transposition events, which can occur for several transposons (Arthur et al. 1984; Avila et al. 1984; Motsch and Schmitt 1984; Motsch et al. 1985), may proceed by a mechanism akin to the asymmetric one, although Grindley and Reed (1985) believe that these events are equally compatible with symmetric mechanisms.

Conservative transposition is often the sole mechanism for IS elements and composite elements e.g. Tn10 (Bender and Kleckner 1986), and is much more common than cointegrate formation in IS1 and IS903. It also occurs during formation of lysogens in Mu. It is now generally believed that this method of transposition does not proceed via a cointegrate (Grindley and Reed 1985; Derbyshire and Grindley 1986; Harshey 1984).

Two mechanisms exist to account for conservative transposition. The simplest is a cut-and-paste process involving double-stranded cuts by transposase at the two transposon ends. The excised segment is integrated into the target by ligating the extended single strands of the target DNA to the appropriate strands at the transposon ends. This leads to the destruction of the linearised donor. The second mechanism is a modification of the Shapiro/ Arthur and Sherratt model (1979) (Ohtsubo et al. 1981) which involves repair synthesis, before replication of the transposon, that displaces the single strands attaching the transposon to the donor. The polarity of strand

exchange (Fig. 3.15b) could be important in determining whether the repair synthesis displaces the transposon strands attached to the target, breakage of which could destroy the target so no transposition would be detected, or whether it displaces the donor DNA, which could lead to breakage and simple insertion. The polarity of Mu transposition is most likely to lead to the latter, which could explain its ability to transpose both replicatively during lysis, and conservatively during lysogeny (Grindley and Reed 1985).

In the proposal put forward by Ohtsubo et al. (1981), the cointegrate pathway is distinguished from the simple insertion process by its requirement for a complete replisome at the terminal joints. It is possible that it is the efficiency of assembly of the replisome that determines whether a cointegrate process occurs. In the case of Mu, the B protein (Mizuuchi and Craigie 1986), which appears to influence the choice of pathways, may act as a factor that promotes replisome formation. For the ISs the transposase itself may influence the decision: perhaps some transposases, e.g. those of IS1 and IS903, have a weak affinity for some component of the replisome, and others, e.g. that of IS10, have no such affinity. The fact that Tn3 follows the cointegrate pathway could be explained in one of two ways (Grindley and Reed 1985): either there is a strong affinity between its transposase and replication factors, or the polarity of strand exchange (Fig. 3.15b) is reversed (compared to Mu), so that single strand breakage would result in loss of the target molecule.

1.4 The consequences of transposition

As a consequence of their ability to transpose to give simple and/or cointegrate insertions at numerous loci throughout a genome (not entirely at random, Heffron et al. 1975; Kretschmer and Cohen 1977; Picken et al. 1984; Tu and Cohen 1980), transposable elements can exert various effects on any genome in which they are present (Kitts 1982):

Insertion into a gene can inactivate it by disruption of the coding sequence. One example of such a disruption is seen in 3.3.2 where insertion of Tn3571 into the Cm^r gene destroys the Cm^r phenotype. Also, insertion may abolish or diminish the expression of other genes

in a poly-cistronic unit, thus causing a polar effect (Heffron *et al.* 1979).

Although the most commonly observed effect of transposable element insertion is to prevent expression of a gene, they can also activate previously silent transciptional units: genes can be turned on by a promoter located within the element which is directed outwards and can cause transcription of the adjacent target sequences. Thus it is possible for insertion to separate a gene from its normal promoter and to place transcription of that gene under the control of a different regulatory system. This property was exploited in 4.1.2 to construct a transcriptional fusion of the Tn*3* *tnpA* gene to the galactokinase gene, *galK*. It has also been suggested that promoters can be created *de novo* by juxtaposition of sequences at the ends of certain elements with appropriate sequences in the target DNA.

The fusion of replicons by cointegrate formation can have other important consequences: the conjugative plasmid, F, can become integrated into the *E. coli* chromosome by transposition of any one of the insertion sequences which it carries, and can subsequently transfer chromosomal genes to a new host. More frequently, F becomes integrated by homologous recombination between a copy of an insertion sequence on the plasmid and a copy in the *E. coli* chromosome.

Subsequent to the initial insertion event, transposable elements have been found to promote rearrangements of the adjacent DNA including deletions, inversions and translocations:

Deletions and inversions are caused by replicative intramolecular transposition (Bishop and Sherratt 1984). If the second transposon copy inserts in direct repeat to the first, a deletion will occur concurrently with transposition and only one of the products will survive due to the origin of replication. Deletions usually extend from one end of the element into the adjacent DNA, with a copy of the transposable element remaining at the original point of insertion. If the second copy inserts in inverted repeat to the first, then inversion of the intervening section of DNA occurs. This type of event is called a duplicative-inversion and was predicted by transposition models (Shapiro 1979; Arthur and Sherratt 1979) before

the first examples were reported.

Translocation of segments of the host genome can also be caused by transposable elements. If DNA is bounded by two copies of a transposable element, in direct or indirect repeat, a larger element may be formed. These elements are known as composite elements e.g. Tn*9* (1.2.2). Such composite elements may transpose as a discrete unit.

Other rearrangements can be produced by the action of host recombination systems on the inserted sequence: recombination between the direct repeats of target DNA which flank an inserted element may lead to precise excision of the inserted sequence and restoration of the original sequence. Inverted repeats at the end of the element may facilitate this by allowing the formation of a hair-pin loop (Foster *et al.* 1981). Also, if copies of a transposable element are located at several places in a genome, or on multicopy plasmids, then this provides stretches of homology which can be recombined by host homologous recombination systems. This was observed during the initial transposition immunity assays in DS903, a $RecF^-$, $RecA^+$ strain (3.5).

Transposable elements, through all their effects described above are likely to have made a significant contribution to both the frequency of mutation and to the diversity of genetic rearrangements. The resultant genetic variation will probably have been used to further the course of evolution. The possession by some elements of accessory determinants e.g. antibiotic resistance will also undoubtedly have affected the evolution of bacterial populations (Campbell 1981).

1.5 Regulation of transposition

1.5.1 Why regulation is necessary

As previously mentioned (1.3), transposition of prokaryotic elements can be replicative or non-replicative. As far as the survival of the element is concerned, particularly when no accessory determinants are

encoded, the replicative process is the most important, but not necessarily the main, alternative. However, extensive over-replication may be expected to be deleterious to the host, and ultimately to the element. This is because both the element itself and its gene products probably constitute a metabolic burden to the cell, even when they are not outrightly destructive like phages in their lytic cycle (Campbell 1981). Therefore it is reasonable to expect that, for the benefit of both the host and the transposable element, regulation of transposition exists.

1.5.2 The evidence that regulation occurs

It is obvious that transposable elements generally tend to increase their copy number by replication. This is particularly efficient with the Tn*3* family and the transposing bacteriophages. Inasmuch as the number of possible locations is much larger than the number ever occupied in a particular host, some form of regulation must exist.

1.5.3 How regulation of transposon copy number could occur

Three mechanisms could be proposed (Langley *et al.* 1983):

i. The probability of loss of elements could increase disproportionately with copy number, so that under a constant rate of transposition, copy number would increase to some steady-state level. There is no direct evidence for this, but observations in yeast T_y elements and Drosophila (Engels 1983) suggest that loss of elements may occur. Abortive loss of elements has also been reported for Tn*10* (Foster *et al.* 1981).

ii. There could be natural selection of hosts based on the number of transposon copies they contain i.e. those with a relatively high number could have relatively few surviving offspring. This mechanism might stabilise the numbers of transposons, and prevent the establishment of very highly transposing mutants, but in the long term the elements would probably develop a mechanism for regulating their own transposition (iii) which would be more efficient.

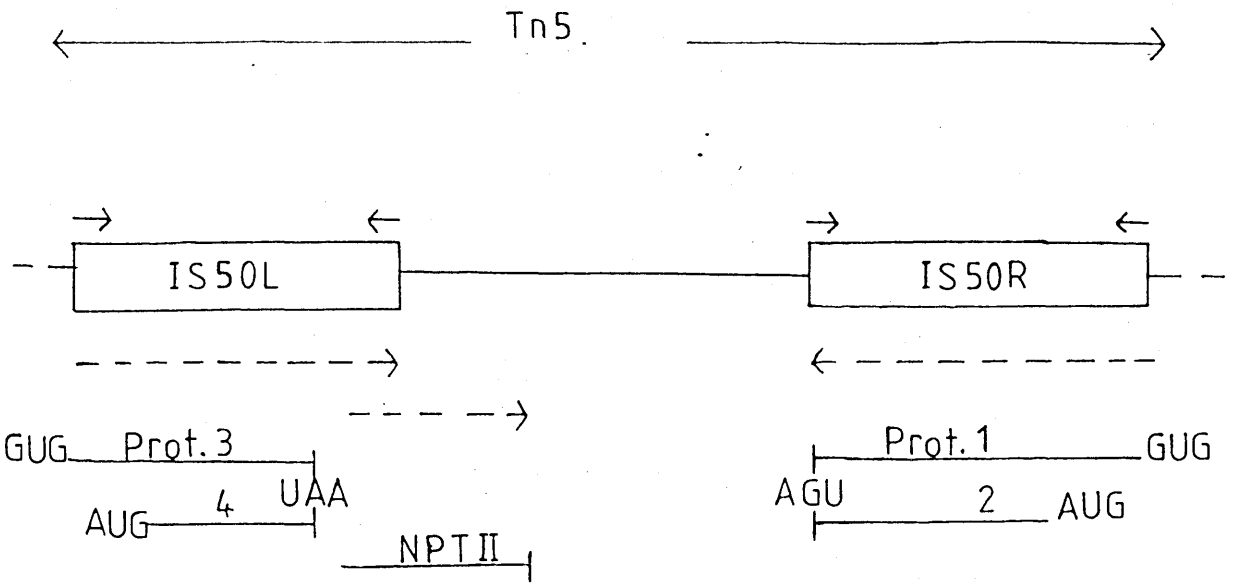


Fig. 1.4 Tn5, showing proteins encoded by IS50R and IS50L. Tn5 and IS50R are transposition +ve. IS50L is transposition -ve. Protein 1 encodes the transposase and protein 2 encodes a -ve regulator of transposition.

---> mRNA

——| protein

→ short inverted repeats

iii. The transposition process itself could be regulated. There is plenty of evidence for this in bacteria (1.5.4 and 1.6.3), and also in Drosphila e.g. when the P factor enters the M cytotype (free of P factor) transposition is highest when copy numbers are low.

1.5.4 Regulation of prokaryotic transposable elements other than Tn3

The following three examples, of Tn5, Tn10 and Mu, serve to illustrate the variety of ways in which prokaryotic transposable elements are known to regulate their own transposition:

Tn5 is a composite element (Fig. 1.1), bounded by two copies of IS50, carrying a gene for aminoglycoside antibiotic resistance (NPT II) e.g. to neomycin and kanamycin (Reznikoff 1982).

Tn5 or IS50R transpose with a high frequency immediately after entry into a cell. Establishment of Tn5 within a cell results in a decrease in this transposition frequency by a factor of 12 - 70 times (Biek and Roth 1981). This behaviour is evidence that Tn5 transposition is under negative control by a factor encoded within the element itself.

Apart from the NPT II gene, Tn5 encodes four proteins, two from each IS50 copy (Fig. 1.4). In IS50L a one base pair change inactivates both proteins by causing an ochre stop codon. In IS50R, protein 1 is 40aa longer than protein 2 at the N-terminus. Protein 1 encodes the transposase which is essential for transposition and acts in cis. Protein 2 is able to inhibit transposition of a Tn5 element freshly introduced into the cell on a λ phage (Isberg et al. 1982), and therefore works efficiently in trans. The difference between the behaviour of the proteins implies that the N-terminal 40aa are crucial for the activity of the transposase. Protein 2 does not act as a repressor of protein 1 synthesis. It therefore either competes for the IR ends with protein 1, interacts with host factors necessary for transposition (but Tn10 and Tn9 can transpose in the same cell and a Tn5-specific host factor is not known to exist), or it might form inactive complexes with protein 1.

Protein 2 is made in approximately four times the amount of the protein in IS50R. The difference is probably due to their translation

initiation signals: AUG for protein 2, GUG for protein 1. The relative amounts of each protein are probably significant in the regulatory process (Reznikoff 1982).

The possible sequence of events on entry of Tn*5* into a host cell may be as follows, bearing in mind that protein 1 acts in *cis*, and protein 2 acts in *trans*:

In some naïve cells, newly introduced Tn*5* DNA will serve as a template for IS*50* mRNA from which protein 1 is translated before protein 2, therefore transposition will occur. In experienced cells, protein 2 will already be present in almost all cells before protein 1, therefore transposition will be very rare. Regulation of Tn*5* transposition therefore appears to be simple but effective.

Tn*10* or IS*10*R transposition is known to be regulated in a more complex manner than that of Tn*5*. Again only one of the IS elements is structurally and functionally intact. IS*10*R contains three promoters (Simons *et al.* 1983). Two are near the outside of the element: pIN is the transposase gene promoter; pOUT is a strong promoter just inside pIN directing transcription outwards. The third promoter is very weak, and its function is not known.

A single chromosomal copy of Tn*10* transposes very infrequently - about once every 10^4 generations. This is primarily because the transposase protein is made in tiny amounts - about 0.15 molecules per cell per generation. This is the result of infrequent transcription (approximately 0.25 transcriptions per element per generation), and inefficient translation (an average of about 0.6 translations per transcript) (Raleigh and Kleckner 1986).

Two separate mechanisms limit IS*10* transposase gene transcription:

i. The promoter for pIN is intrinsically weak (Simons *et al.* 1983): pIN is about one third as active as pOUT.

ii. The opposing promoter, pOUT, interferes with the completion of transcripts initiated at pIN (see "multicopy inhibition of Tn*10* below).

The reason for the poor translation of transposase is not known.

IS10 transposition is also regulated by DNA methylation (Roberts et al. 1985). Two sites for this exist. One is in the pIN promoter, and the other is in the pIN terminator. Lack of full methylation results in increased transcription and termination respectively. IS10 appears to be fully active when it is hemi-methylated, so transposition may be coupled to the occurrence of hemi-methylated DNA after a passage of chromosomal replication.

IS10 also encodes special signals which prevent it from fortuitous activation by insertion into a site within an actively transcribed gene (Davis et al. 1985).

When IS10 is present at high copy number in cells, other regulatory mechanisms come into play. As the number of copies increases, the rate of transposition decreases. This is due to two effects:

i. IS10 transposase acts preferentially in cis

ii. IS10 encodes a trans-acting negative regulator which, when present in high concentration , inhibits expression of transposase protein at the post-transcriptional level. This is an RNA molecule, the product of the pOUT promoter. It pairs with the transposase over a 36bp region of complementarity, and thereby prevents translation of the transposase gene. Kleckner (1985) called this "multi-copy inhibition".

Mu repressor is one of the key elements in the regulation of the Mu life cycle. Its primary mode of action is thought to be negative control, through operator binding, of transcription of the early message for, among other things, the Mu A and Mu B proteins.

Craigie et al. (1984) have found that Mu repressor can bind, at higher concentrations, to the ends of Mu DNA, almost exactly where the Mu A protein does. This raises the possibility of a second level of transposition control by Mu repressor: excess repressor binds to Mu ends and may prevent initiation of transposition by the Mu A protein. A possible role for this mode of regulation might be during lysogenic

Fig. 1.5 The ends of members of the Tn3 family of transposons (Heffron 1983). Tn3, Tn951, Tn1000 (γδ), and IS101 are obviously more closely related to each other than to the Tn501 subgroup.

growth, where the basal level of Mu A protein may occasionally initiate transposition.

1.6 Tn3 and its regulation

1.6.1 The transposition functions of Tn3

The terminal repeated sequences

Tn3 contains identical 38bp inverted repeat sequences (IRs) at each end (Fig. 1.5). These are required intact for normal transposition frequencies (Heffron et al. 1977; Gill et al 1979). The terminal repeated sequences are highly conserved in the Tn3 family (Fig. 1.5). Tn3 and Tn1000 share extensive DNA homology and their IRs have 27/38bp in common, yet these two transposons will not complement each other for transposition (Kitts et al. 1982). Comparison of the sequences of the ends of Tn1000 and Tn3 shows that specific recognition of the IRs by transposase must lie in only 6 non-contiguous bases of the IRs that differ between the two transposons (Heffron 1983). Within the terminal repeated sequences of the Tn3-like transposons Tn1000, Tn951 and IS101, and the bacteriophage Mu, there is a specific heptanucleotide sequence, ACGAAAA, that is conserved. All of these elements generate a 5bp duplication of target DNA on insertion. This may suggest a common evolutionary progenitor for the Tn3-like transposons and MU (Reed et al. 1979).

The transposase

Transcription of tnpA begins at nucleotide 3095 (Heffron 1983). Transcriptional termination does not appear to occur within the gene (Ch. 4), and no transcriptional terminator exists within the LIR (Heffron et al. 1979).

Translation of the transposase transcript starts at the ATG of position 3048 (Ditto et al. 1982), although it is a possibility that an upstream GTG may rarely be used (Heffron 1983). An ochre stop codon exists at nucleotide 36, within the LIR.

The gene is about 3kbp long, and the transposase protein is large

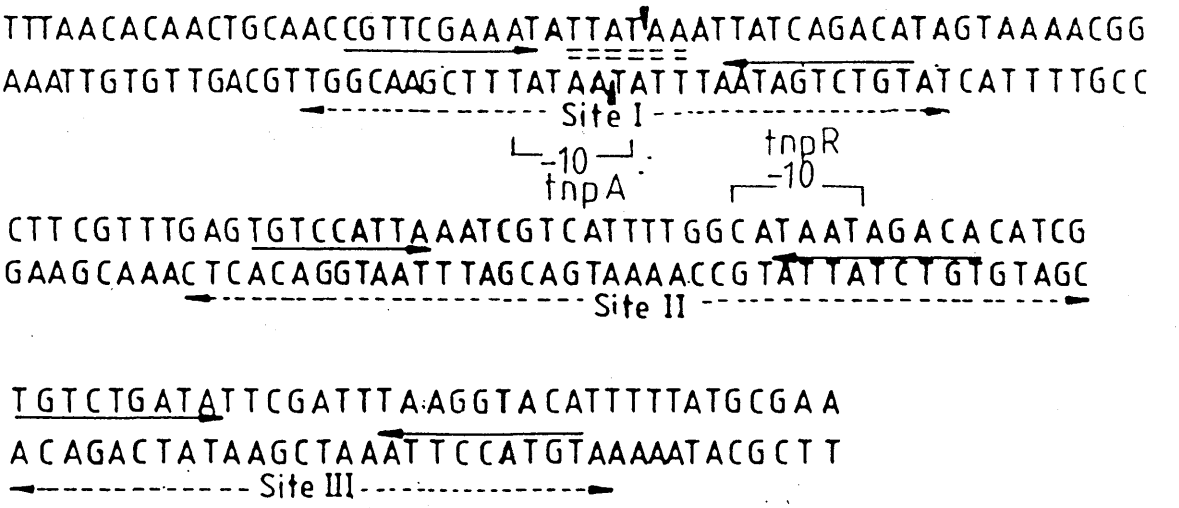


Fig. 1.6 The res site of Tn3. Sites I, II, and III are binding sites for the resolvase protein. The horizontal half arrows show the "binding sequences" that are thought to be recognised by the C-terminal domain of resolvase. The -10 regions of the tnpA and tnpR promoters are marked.

crossover site.

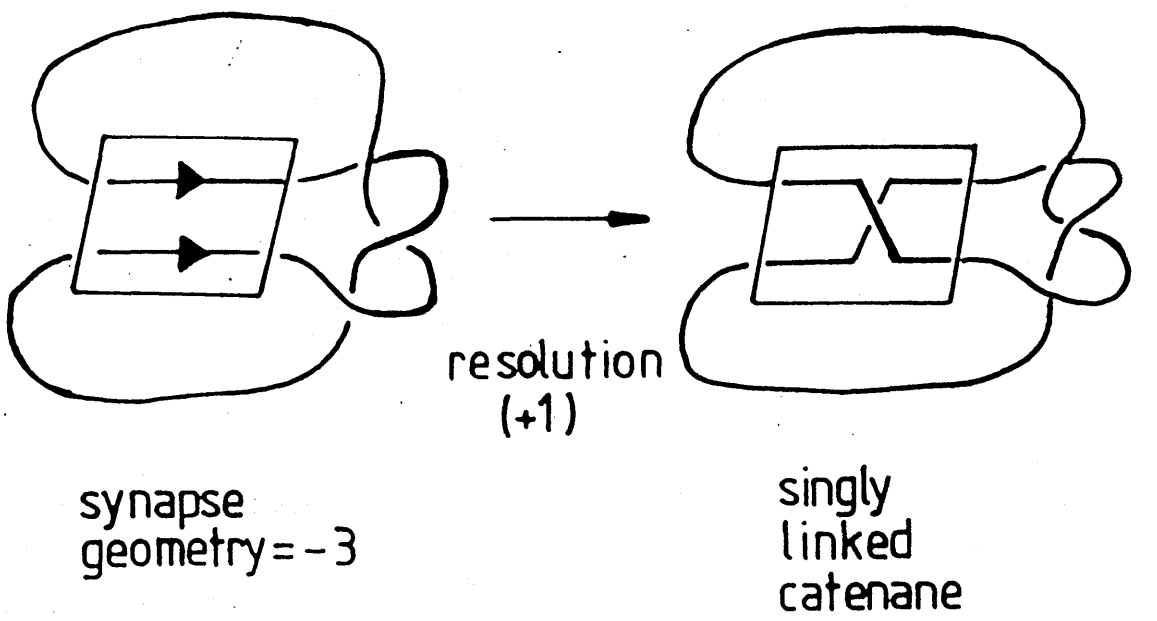


Fig. 1.7 The topology of the resolvase/res synaptic complex and the topological change during resolution (Brown 1985). 3 -ve supercoils are trapped between the two res sites at synapsis. At strand exchange two of these supercoils are converted into the catenane interlock.

(120,000 daltons). It has an isoelectric point near neutrality, but contains several basic regions (Heffron et al. 1979). According to the amino acid sequence there is an extended region between amino acids 887 and 936. This may possibly be an interdomainal region.

The site of action of transposase in the transposon is at the 38bp IRs. According to Evans and Brown (1987), the end binding domain of Tn501 and Tn21 transposases is located between amino acids 28 and 216. Due to the relatedness of these transposons and Tn3 (1.2.2), this may well be the case for Tn3 transposase.

1.6.2 The site-specific recombination function of Tn3

The res site

The location of the res site was defined by Kostriken et al. 1981 and Reed 1981. In Tn1000 and Tn3 these have been extensively mapped and characterised (Kitts et al. 1983), and apparently the res sites of the Tn501 subgroup have the same organisation (Grindley and Reed 1985). They consist of three separate resolvase binding sites, spanning a total of about 120bp (Fig. 1.6). Site I contains the actual cross-over site. Each of the three binding sites consists of a conserved sequence, flanking a variable-sized spacer region. Each site is believed to be bound by a dimer of resolvase. The binding of resolvase to these sites is believed to occur with different binding constants, and the consequence of binding is considerable bending of the DNA. This bending is necessary to align the two directly repeated res sites, such as are found in cointegrates, so that recombination can occur (Fig. 1.7).

The resolvase protein

This is known to consist of two distinct domains (Abdel-Meguid et al. 1984), which can be cleaved by mild proteolysis into a 140aa N-terminal fragment and a 44aa C-terminal portion. The small C-terminal fragment determines the DNA binding specificity, while the large N-terminal domain mediates protein:protein interactions, and carries the enzyme activity necessary for recombination.

The recombination reaction

Cointegrate resolution has been studied *in vitro* (Reed 1981; Symington 1982). Resolvase is the only protein required. The other requirements are for a superhelical cointegrate substrate, a suitable buffer, and Mg^{2+}.

The product of the reaction is a pair of circles that are singly interlinked (Fig. 1.7). This prompted Krasnow and Cozzarelli (1983) to propose that two *res* sites are brought into a synaptic complex by resolvase that is bound to one *res* site sliding along the DNA one-dimensionally, until a second, correctly oriented *res* site is located. This would exclude DNA interwraps that would otherwise cause more complex catenated products to be formed, and could account for the fact that *res* sites must be in direct repeat in a molecule for resolution to occur. However, other hypotheses exist (Boocock, personal communication).

1.6.3 Regulation of Tn*3*

Several features of Tn*1/3* suggest that transposase is normally present in limiting amounts in cells. One is that the transposition frequency from the chromosome to a multicopy plasmid is less than plasmid to plasmid (Kretschmer and Cohen 1977). Another is that increasing the level of transposase within the cell increases transposition (Casadaban *et al.* 1982), although increasing above a certain amount may give no further increase in transposition, implying that another factor has become limiting (Morita *et al.* 1987). The normally limiting amount of transposase in the cell could be brought about by control of synthesis of transposase, or by control of its activity or breakdown, or by all or some of these methods:

TnpA is known to be transcriptionally regulated *in vivo* by resolvase (Grindley and Reed 1985) as a result of its binding to the *res* site (1.6.2). The repression of *tnpA* and *tnpR* occurs concurrently with this binding because two of the three resolvase binding sub-sites overlap with the [illegible] promoter "-10" sequences of *tnpA* and *tnpR* (Fig. 1.6). Translation of transposase is also known to be inefficient, probably due to a poor ribosome binding site (Casadaban

et al. 1982; Morita et al. 1987).

Little is known about the stability or activity of the Tn1/3 transposase protein, but the ability of transposase molecules to catalyse transposition is known to be unstable (Pato and Reich 1983) due to stoichiometric, rather than catalytic usage of the enzyme (Pato and Reich 1984): de novo synthesis of the Mu A protein is necessary for continued replication of Mu. Interestingly, the conclusion that continued protein synthesis is necessary for continued transposition of Tn3 was reached by Kretschmer and Cohen (1979). This raises the possibility that Tn1/3 transposase, like the Mu protein, may also be only used for one round of transposition.

Tn3 transposition is associated with several other phenomena which suggest that more subtle regulatory mechanisms may exist. It was one aim of this project to investigate any such mechanisms underlying some of these phenomena e.g. titration of transposase by the IRs, immunity and temperature sensitivity. During these studies data were obtained on the number of resolvase and transposase molecules present per cell at 30°C in conditions of resolvase repression, and in the absence of this repression at 30°C and 37°C.

Tentative evidence existed from the work of Hettle (1985) that the number of IRs present in a cell might affect Tn3 transposition levels, possibly by acting as a sink to **titrate** out transposase. Such a titration mechanism of regulation may explain the cytotype effect in Drosophila P and M hybrid dysgenesis (Ch. 3 discussion). Therefore one of the aims of chapter 3 was to obtain more data on this with respect to Tn3.

Transposition immunity (Ch. 3) is a property unique to the Tn3 family of transposons (Grindley and Reed 1985). A replicon carrying a copy, or part of a copy of Tn3 acts as a target for additional copies at a substantially reduced frequency (less than 5% of the non-immune). The original observation was made by Robinson et al. (1977). The phenomenon acts only in cis, as expected, since a trans effect would block all transposition. Immunity is found in various replicons, and also apparently, the chromosome. Since the first observation, the property has been noted many times and been studied in detail

(Kretschmer and Cohen 1977; Chiang and Clowes 1980; Wallace et al. 1981a; Wallace et al. 1981b; Chiang et al. 1982; Muster et al. 1983; Lee et al. 1983; Heritage and Bennett 1984; Bishop and Sherratt 1984; Arthur et al. 1984; Huang et al. 1986; Kans and Casadaban 1987). Several of these studies have shown that intramolecular transposition is not subject to immunity. Early attempts to localise the site conferring the property gave conflicting results. Therefore one of the aims of the work described in chapter 3 was to find out more about the role of the 38bp IRs in the phenomenon. Since this work was done, the studies of Huang et al. (1986), and of Kans and Casadaban have given even more detailed information on this (see Ch. 3 discussion), and models to explain the role of transposase and the IRs in immunity have been proposed.

Tn1/3 transposition is **temperature sensitive** (Ch. 4). This observation was first made by Kretschmer and Cohen in 1977. They later found (1979) that the transposition frequency was optimal at 30°C and decreased rapidly at higher temperatures. Work in Glasgow (Ch. 3) showed that the effect occurred during cointegrate formation which is brought about by the tnpA gene product, transposase, without involvement of resolvase. Several possibilities exist to explain the temperature effect. These are:

i. The synthesis of tnpA could be temperature sensitive: the initiation of transcription or translation might decrease with an increase in temperature; the message structure might be altered causing premature transcription termination; or an altered message structure might cause changes in its stability with temperature.

ii. The activity of transposase might be temperature sensitive.

iii. Transposase might be irreversibly denatured more rapidly at higher temperatures, either by more rapid inhibition of its activity, or by more rapid breakdown of the protein.

iv. A temperature-sensitive host protein might be required for cointegrate formation.

The main purpose of the studies in chapter 4 was to examine possibility (i) using transcriptional and translational fusions of galK and lacZ to the tnpA gene, and one of the purposes of the attempt to raise an antibody in chapter 5 was to examine the extent of breakdown of Tn1 transposase at 30°C and 42°C.

Although not studied in this work **regional specificity** of insertion is known to exist (Heffron et al. 1975; Kretschmer and Cohen 1977; Picken et al. 1984; Tu and Cohen 1980). Insertions tend to be clustered in certain regions. Even within a cluster their distribution is non-random. A detailed analysis of hot spots for Tn3 insertion has suggested that many insertions take place preferentially into AT-rich DNA, and near a sequence that is homologous with the end of Tn3. This preference may indicate that local denaturing of the DNA must take place before insertion. The frequent insertion near a sequence related to its end may reflect a preference of the transposase for DNA related to its normal substrate.

In addition to studies on end titration of transposase, Tn3 transposition immunity, and Tn1/3 temperature-sensitive transposition, transposase expression from pSNO15 (Hettle 1985) was investigated. In this plasmid the Tn1 transposase gene was expressed from the lac promoter and RBS, but this construct did not produce the expected amount of transposase. It was thought that elucidation of the reason might show some as yet undescribed regulatory mechanism of the tnpA gene, or would enable the protein to be overproduced sufficiently to allow its simple purification. Purification would enable in vitro studies of transposase end-binding, and might facilitate development of an in vitro system, such as exists for Tn3 resolution (Reed 1981; Symington 1982) and for Mu transposition (Mizuuchi 1983). The development of such a system might elucidate the role that host factors could have in the transposition process and its regulation, as well as clarifying the mechanism of Tn3 transposition.

All these studies would hopefully lead to fuller understanding of the Tn1/3 transposase protein, transposition and its regulation. Ultimately such knowledge could extend ideas on evolution, and might suggest ways to prevent, or control, the spread of antibiotic resistance genes, which is a medical problem of growing concern.

CHAPTER 2

MATERIALS AND METHODS.

Table 2.1 Bacterial strains

Strain	Genotype	Source/Reference
DS903	recF143, thi, leuB6, his^{c}4, thr1, proA2 argE, galK2, supE44, tsx33, rpsL31, ara14	Sherratt
DS903 Sp^{r}	Derived from DS903. Sp^{r}, Sm^{s}.	Jones (1985)
DS902	As DS903 but recA13, ArgE^{+}, RecF^{+}	Sherratt
DS941	As DS903 but lacI^{q}ZΔM15	Sherratt
DS942	As DS903 but Δ(lacpro)	Sherratt
DS947	As DS942 but SupO	Sherratt
DS916	recA56, his, trp, rpoB, lysogen	Sherratt
JM83	ara, Δ(lacproAB), rpsL, ϕ80dlacZΔM15	Vieira and Messing (1983)
JM101	Δ(lacpro), thi, supE, F'traD36, proAB, lacI^{q}ZΔM15	Messing (1983)
CB51	dam-3, ara, thi, Δ(lacpro)	Boyd
CB158	thr1, leuB6, mutD5, hisG4, argE3, thi1, ara14, lacY1, galK2, xyl5, mtl1, rpsL31, tsx-33, supE44.	Boyd
BTA282	rpsL, lac U169, lon, araD139, thi, hflA150 [chr::Tn10], hsdR.	Bone, via Scaife
DS312	AB1157 containing RSF1050	Sherratt

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 Bachmann et al (1976) and Novick et al (1976).

2.2 Plasmids and transposons. The plasmids used and constructed in this study are listed in Table 2.2 and their nomenclature follows that of Novick et al (1976). The transposons used are listed in Table 2.3.

2.3 Chemicals.

CHEMICALS	SOURCE
General chemicals	B.D.H., Hopkins and Williams, Koch-light Laboratories, May and Baker
Media	Difco, Oxoid
General biochemicals	Sigma, Pharmacia
Agarose	BRL
Xgal, IPTG	BRL
Radiochemicals	NEN
10 X core buffer	BRL
Antibiotics	Sigma
Restriction enzymes	BRL, Boehringer Mannheim

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.

Table 2.2 Plasmids

Plasmid	Marker	Size (in kbp)	Description	Source/reference
R388	Tp^r	33.0	IncW	Datta and Hedges (1972)
pBR322	Tc^r Ap^r	4.36	pMB8-derived	Bolivar *et al.*(1977)
pACYC184	Tc^r Cm^r	4.0	P15A	Chang and Cohen (1978)
pUC	Ap^r	2.67	ColE1-derived	Vieira and Messing (1982)
M13mp9	-	7.23	'phage vector	Yanisch-Perron *et al.*('85)
RSF1050	Ap^r	7.7	pMB1-derived	Heffron *et al.* (1977)
pMA21	Ap^r Tc^r	4.92	2-*res*	Brown (1986)
pGJ28	Km^r		Km^r on 1.43kbp *Hae*II frag.	Warren (1978)
pAA33	Ap^r Tc^r Cm^r	8.5	pACYC184::Tn*1*	Arthur (1981)
pPAK200	Ap^r Cm^r	7.9	pACYC184::Tn*3651*	Kitts (1982)
pPAK316	Cm^r	8.3	$TnpR^+$	Kitts (1982)
pPAK329	Ap^r Tc^r	4.64	pBR322 + *res*-RI*	Kitts (1982)
pDS4153	Ap^r	8.1	$TnpR^+$	Kitts (1982)
pCB101	Cm^r	5.0	λ*dv* vector	Boyd and Sherratt (1986)
λ*dv*Δ25Δ1	Cm^r	3.84	λ*dv* vector	Boyd
pMR18	Cm^r Km^r	5.27	λ*dv* vector	Rogers (1986)
pMR100	Km^r	5.5	λ*dv* vector	Rogers (1986)
pGS301	Tc^r	5.77	λ*dv* vector	Stewart
pKO1	Ap^r	3.9	*galK* vector	McKenney *et al.* (1981)
pKO500	Ap^r	4.2	*galK* vector	Lamond
pKL500	Ap^r	4.2	P_{lac} directs *galK*	Lamond
pJKA	Ap^r	4.26	*tnpA*::*galK* fusion	Kelly (1983)
pJKR	Ap^r	4.26	*tnpR*::*galK* fusion	Kelly (1983)
p253	Cm^r	5.5	pACYC184-based	Burke, Fig. 4.4
pMC1403	Ap^r	9.9	$LacZ^+$, $LacY^+$	Casadaban *et al.* (1980)
pNM480	Ap^r	8.6	*lacZ* vector	Minton *et al.* (1984)
pNM481	Ap^r	8.6	*lacZ* vector	Minton *et al.* (1984)
pNM482	Ap^r	8.6	*lacZ* vector	Minton *et al.* (1984)
pMC1871	Tc^r		*lacZ* vector	Casadaban *et al.* (1980)
pUC8.1	Ap^r	2.91	carries *res*-RI*	Burke, 4.2
pUC18.1	Ap^r	2.91	carries *res*-RI*	Burke, 4.2
pUC9*lacZ*	Ap^r	5.67	P_{lac} & RBS direct *lacZ*	Burke, 4.2
pSNO15	Ap^r	6.7	P_{lac} & RBS direct Tn*1* *tnpA*	Hettle (1985)
pSNO80	Ap^r	9.7	As pSNO15 + *lacZ* at bp984	5.1, Fig. 5.1

pROS1	Ap^r	7.7	RSF1050 perfectly filled-in at *Bam*HI. $TnpR^-$.	3.1
pROS2	Ap^r	7.7	RSF1050 imperfectly filled-in at *Bam*HI. $TnpR^-$.	3.1
pROS3	Ap^r Cm^r	10.5	Tn*3571* transposed into p253. *tnpA* directs transcription of *galK*.	4.1
pROS8	Ap^r Cm^r	10.5	As pROS3, but independent isolate.	4.1
pROS45	Ap^r Cm^r	10.5	As pROS3, but independent isolate.	4.1
pROS46	Ap^r Cm^r	10.5	As pROS3, but independent isolate.	4.1
pROS4	Ap^r Cm^r	10.5	Tn*3571* transposed into p253. *tnpR* directs transcription of *galK*.	4.1
pROS5	Ap^r Cm^r	10.5	As pROS4, but independent isolate.	4.1
pROS47	Ap^r Cm^r	10.5	As pROS4, but independent isolate.	4.1

Table 2.2 cont.

Plasmid	Marker	Size (in kbp)	Description	Ref.
pROS33a	Cm^r	7.0	1.571kbp Tn3 BamHI/ClaI fragment cloned into BamHI/ClaI-cut p253. tnpR directs transcription of galK.	4.1
pROS40	Ap^r Cm^r	11.4	Tn3571 cloned into p253. tnpA directs transcription of galK.	4.1
pROS41	Ap^r Cm^r	11.4	As pROS40, but independent isolate.	4.1
pROS42	Ap^r Cm^r	11.4	As pROS40, but independent isolate.	4.1
pROS43a	Ap^r Cm^r	14.4	As pROS40 with a 3kbp insert at bp984	4.1
pROS44a	Ap^r Cm^r	11.4	Tn3571 cloned into p253. tnpR directs transcription of galK.	4.1
pROS44b	Ap^r Cm^r	11.4	As pROS44a, but independent isolate.	4.1
pROS12	Km^r Cm^r	6.0	pCB101 deleted for small HaeII fragment. Replaced with 1.43kbp Km^r frag. from pGJ28. Km^r reads in same way as Cm^r.	3.3
pROS13	Km^r Ap^r	10.97	Tn3571 transposed into Cm^r gene of pROS12 tnpA reads in same way as Km^r. Insertion point sequenced (Fig. 3.15a).	3.3
pROS14	Km^r Ap^r	10.97	Tn3571 transposed into Cm^r gene of pROS12 tnpR reads in same way as Km^r.	3.3
pROS16	Km^r Ap^r	10.55	Bal31 deletion of pROS13 from Bal1 site. Deletion junction sequenced (Fig. 3.11)	3.3
pROS17	Km^r Ap^r	10.55	Apparently identical to pROS16.	3.3
pROS19	Ap^r	6.87	DraI 4.435kbp frag. from pROS13 into SmaI -cut pUC8 (Fig. 3.12).	3.3
pROS21	Ap^r	7.99	5.326kbp DraI/SmaI frag. from pROS16 into SmaI-cut pUC8 (Fig. 3.16).	3.3
pROS24	Km^r	8.78	4.221kbp BamHI/EcoRI frag. from pROS19 into unmethylated BclI/EcoRI-cut pMR18.	3.3
pROS26	Km^r	9.90	5.336kbp BamHI/EcoRI frag. from pROS21 into unmethylated BclI/EcoRI-cut pMR18.	3.3
pROS31	Ap^r	8.9	tnpA directing translation of lacZ in pNM480.	4.2
pROS32	Ap^r	8.9	tnpR directing translation of lacZ in pNM481.	4.2
pROS35	Ap^r	6.77	4.1kbp BamHI/Sal frag. from pAA33 into BamHI/SalI-cut pUC18.	4.2
pROS36	Ap^r	10.0	pROS35 linearsised by partial PstI digest PstI-bounded lacZ cassette in at 984.	4.2
pROS37	Ap^r	6.46	3.405kbp SalI/EcoRV frag. from pROS35 ligated to SalI/Sma-bounded lacZ cassette from pMC1871.	4.2
pROS38	Ap^r	9.15	6.075kbp SalI/EcoRV frag. from pROS35 ligated to SalI/SmaI-bounded lacZ cassette from pMC1871.	4.2
pROS48	Ap^r	10.3	As pROS36, but with an insert of 335bp at one SmaI site (Fig. 5.3).	5.1
pROS49	Ap^r	8.7	pROS48 cut completely with HinDIII, and partially with PstI. The 8.7kbp frag. ligated (Fig. 5.4).	5.1

Table 2.3 Transposons

Common name or location	Systematic name	Derivation	Phenotype	Reference
Tn*1*	Tn*1*	Naturally occurring on RP4	A^+R^+*res*$^+Ap^r$	Hedges and Jacob 1974
Tn*3*	Tn*3*	Naturally occurring on R1	A^+R^+*res*$^+Ap^r$	Heffron *et al.* 1975
$\gamma\delta$	Tn*1000*	Naturally occurring on F	A^+R^+*res*$^+$	Guyer 1978
Tn*3571*	Tn*3571*	Tn*3* filled-in at *Bam*HI site	A^+R^-*res*$^+Ap^r$	Chapter 3
Tn*365*	Tn*3* 365	*in vitro* deletion of Tn*3*	A^-R^+*res*$^+Ap^r$	Heffron *et al.* 1977
Tn*3651*	Tn*3* 3651	insertion into the *Bam* site of Tn*365*	A^-R^-*res*$^+Ap^r$	Kitts 1982
on pDS4153	Tn*1* *Hae*II53	*Hae*II deletion of Tn*1*	A^-R^+*res*$^+Ap^r$	Sherratt
on pPAK316	Tn*3* *Pst*1inv	*Pst*I deletion and inversion of Tn*3*	A^-R^+*res*$^+Ap^s$	Kitts 1982

2YT broth: 10g bactotryptone, 10g yeast extract, 5g NaCl 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 citrate, 0.1g $MgSO_4.7H_2O$, 17.5g agar, made up to 1 litre in distilled water.

MacConkey agar with galactose: 20g peptone, 1.5g bile salts, 5g NaCl, 0.03g neutral red, 0.001g crystal violet, 10g galactose, 13.5g agar made up to 1 litre with distilled water and adjusted to pH7.1 with NaOH.

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 Medium: 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.

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, 16.4g NaOAc, 3.6g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water, pH 8.2 with acetic acid.

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

10 X Laemmli gel buffer: 144g Glycine, 30g Tris 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 (pH8.0).

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

Formamide dye mix (for sequencing gels): 100ml formamide stirred with 5g "Amberlite" MB1 resin for 30min and then filtered to remove resin. 0.03g xylene cyanol FF, 0.03g bromophenol blue and 0.075g $Na_2EDTA2H_2O$ added and dissolved.

Restriction and Ligation Buffers.

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

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

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

10 X SmaI Buffer: 200mM KCl, 100mM Tris-HCl pH8.0, 100mM $MgCl_2$, 10mM DTT. Stored at 4^oC.

10 X Bal 31 buffer: 120mM$CaCl_2$, 120mM $MgCl_2$, 200mM TrisHCl pH8.0, 10mM EDTA. Stored at 4^oC.

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

10 X Klenow buffer: 100mM Tris-HCl pH8.0, 100mM $MgCl_2$.

10 X T_4 polymerase buffer: 0.33M Tris acetate pH7.9, 0.66M KOAc, 0.1M MgOAc, 5mMDTT, 1mg per ml bovine serum albumin.

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^oC 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^oC.

Miscellaneous buffers.

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

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

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

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

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

Birnboim-Doly III: 5M KOAc pH4.8; mix equal volumes of 3M CH_3COOK and 2M CH_3COOH, pH 4.8.

TELT Buffer: 0.4% Triton X-100, 62.5mM EDTA, 50mM Tris-HCl pH8.0, 2.5M LiCl pH7.5.

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	100ug/ml	10mg/ml (water)
Streptomycin (Sm)	chromosomal	100ug/ml	10mg/ml (water)
Rifampicin (Rf)	chromosomal	50ug/ml	5mg/ml (methanol)
Tetracycline (Tc)	plasmid	10ug/ml	1mg/ml (10mM HCl)
Chloramphenicol (Cm)	plasmid	50ug/ml	5mg/ml (ethanol)
Kanamycin (Km)	plasmid	50ug/ml	5mg/ml (water)
Spectinomycin (Sp)	chromosomal	100ug/ml	10mg/ml (water)
Trimethoprim (Tp)	plasmid	50ug/ml	5mg/ml (50% water/ 50% ethanol)

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

2.8 Indicators. Xgal (5-Bromo-4-chloro-3-indolyl-B-galactoside) was used in conjunction with the host strain JM83 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. Xgal 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. Xgal was also used in conjunction with the lacZ gene, encoding B-galactosidase.

2.9 Growth conditions. Liquid cultures for transformation, DNA preparations or conjugations were routinely grown in L-broth, or when Tp selection was required in isosensitest broth, at 37°C, except where otherwise stated, 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 phage buffer.

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 and M13 RF DNA preparation. Two methods were used to obtain DNA from cells:

Birnboim and Doly (1979) DNA preparation.

a) Plasmid DNA: 200 ml cultures of stationary phase cells were harvested by centrifugation (12000g, 5min at 4°C). The pellet was resuspended in 4ml of Birnboim-Doly I solution and incubated on ice for 5min. 8ml of Birnboim-Doly II solution were added and the solution left on ice for 5min before 6ml of cold Birnboim-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 4.16ml of TE and 1ml of a 3mg/ml EtBr solution was added. 5g of CsCl were dissolved in this 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 against 2 X 500ml 1 X TE. The DNA was then ready for use.

b) M13 RF DNA: the treatment of the overnight culture was as described above for plasmid DNAs. However, the preparation of the overnight culture differed as follows: a single plaque containing the desired 'phage was picked and placed in 1ml of 'phage buffer for 3 to 4hr at 4°C to form a 'phage suspension. A 5ml liquid culture of JM101 cells was grown up to a density of about 1 X 10^8 cells per ml, 0.5ml of the above 'phage suspension added to this and growth continued for a further 2hr. This whole culture was then added to a 100ml culture of JM101 cells (of density about 1 X 10^8 cells per ml) and this was grown overnight at 37°C with vigorous shaking.

Mini plasmid DNA preparation using the modified method of Holmes and Quigley (1981) (Wilimzig 1985). 1.5ml of an overnight culture containing the plasmid of interest was harvested by centrifugation in a 1.5ml eppendorf tube and resuspended in 100ul of TELT buffer. 10ul of lysozyme at a concentration of 10mg/ml in water was added and the tube vortexed briefly. This solution was boiled for 1min and centrifuged in an eppendorf microfuge for 8min at RT. The supernatant was removed and 250ul of cold 100% ethanol was added, followed by microcentrifugation for 15min 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 to 50ul 1 X TE. This DNA was suitable for digestion and other <u>in vitro</u> manipulations.

2.11 Ethanol precipitation of DNA. The DNA solution was made 0.3M in NaOAc and 2.5 volumes of cold ethanol were 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.

2.12 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, except where otherwise stated, 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.

2.13 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.

2.14 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.

2.15 Filling-in recessed 3' ends of DNA. The DNA fragments were resuspended in 2.5ul 10 X nick translation buffer, plus 2.5ul of a 2mM solution of each of the deoxyribonucleoside triphosphates complementary to the single-strand extension, and made up to 25ul with distilled water. The Klenow fragment of DNA polymerase I was added (5units per ug of DNA), and the mixture was incubated at 37°C for 1hr. The reaction was terminated by extraction with an equal volume of phenol (saturated with 1M Tris pH 8.0), followed by three extractions with chloroform. The DNA was precipitated with ethanol.

2.16 Blunting protruding 3' ends. T_4 polymerase will remove unpaired 3' tails from restriction fragments, and will stop when it reaches the first paired base if the complementary deoxyribonucleic acid triphosphate is present. Up to 1ug of DNA was resuspended in a 20ul volume, consisting of 2ul 10 X T_4 polymerase buffer, 1ul of a 2mM solution of all four dNTPs, H_2O up to 16ul, and 2.5 units of T_4 polymerase. The mixture was incubated at 37°C for 5min. Then 1ul of 0.5M EDTA was added before extraction with phenol and chloroform. The DNA was precipitated, washed in 70% ethanol and dried. The DNA was then ready for ligation.

2.17 Bal 31 deletions. Bal 31 is a nuclease which simultaneously degrades the 3' and 5' ends of duplex DNA. It can be used to create deletions of various lengths. 2.5ug restricted DNA was resuspended in up to 39ul distilled H_2O, 5ul of 10 X Bal 31 buffer and 6ul of 5M NaCl. 0.5 units of Bal 31 per ug of DNA was added, and the solution was incubated at 30°C. At various time points 5ul was removed and added to a tube containing 3ul of 0.25M EDTA and 3ul of 0.1M EGTA. At this stage the samples could be kept separate or pooled. To each sample 1ul of 10mg per ml boiled yeast tRNA was added. The enzyme was extracted by phenol and chloroform, and the DNA precipitated with ethanol. The DNA could then be ligated.

2.18 Exonuclease III deletions (Henikoff 1984). 5 to 10ug of DNA was digested to completion with enzymes providing a "start" and "stop" site for exonuclease III (5.1.1). The sample was then treated with RNAse, followed by extraction with phenol and chloroform, and ethanol precipitation. The pellet was dissolved in 66mM TrisHCl pH8.0, 0.66mM $MgCl_2$ to a concentration of about 100ug per ml. A 1/10th volume of exonuclease III was added to give an excess of enzyme over DNA ends of about 20:1. The sample was mixed and incubated immediately at 30°C or 37°C, depending on the desired rate. At various time intervals (35sec) 2.5ul was removed and mixed with 7.5ul of 0.2M NaCl, 5mM EDTA pH8.0. The exonuclease III was inactivated by incubation at 70°C for 10min. The DNA was precipitated by adding 30ul of ethanol and 1ul of 20mg per ml yeast tRNA. The pellet was rinsed with 70% ethanol and dried. Then it was redissolved in 50ul of 0.28M NaCl, 0.05M NaOAc, 4.5mM $ZnSO_4$, containing 0.1 units per ul of S_1 nuclease. This removed any single stranded DNA. The solution was incubated for 30min at RT.

The reaction was terminated by addition of 4M NH_4OAc, 0.1mM EDTA. Portions of each time point were run on an agarose gel, and the rest was extracted with phenol and chloroform before ethanol precipitation, washing and drying. Each pellet was dissolved in 10ul of 20mM TrisHCl pH8.0, 7mM $MgCl_2$, containing 10 units per ml Klenow. The samples were incubated for 2min at 37°C. 1ul of a mixture of the four deoxyribonucleic acids each at 0.125mM was added, and incubation was extended for a further 2min. Each sample was ligated overnight. Before transformation, the DNA was digested to remove non-deletants.

2.19 Transformation with plasmid or M13 RF DNA. This was done in two ways:

Using $CaCl_2$:

a) Plasmids: 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 $MgSO_4$. The cells were pelleted and resuspended in 10ml of 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 at 42°C, or 5min at 37°C) and returned to the ice for a further 15min. An equal volume of L-broth was added and the cells were 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.

b) M13 RF DNA: The recipient strain was always JM101, and the cells were treated as described above for transformation with plasmid DNA up to and including the stage of addition of the transforming DNA. The cells were then left on ice for 1 to 2hr, heat-shocked at 37°C for 5min, and returned to ice for a further 1 to 2hr. The whole transformation mixture was added to molten (45°C) 0.6% agar, along with 200ul of a 20mg per ml Xgal solution and 25ul of a 20mg per ml IPTG solution. All of these components were briefly mixed and the solution poured over the surface of a minimal agar plate containing thiamine and glucose. After allowing the overlay to set, the plates

were incubated at 37°C overnight.

The Hanahan procedure (Hanahan 1985). An individual colony of the strain to be transformed was grown overnight at 37°C in 10ml of 2YT broth. 1ml of the overnight culture was added to 100ml of 2YT and was grown for about 2hr to an OD_{600} of 0.4 to 0.6. The culture was centrifuged in a 250ml centrifuge tube at 2000rpm for 10min at 4°C. The tube was drained well, and the cells were resuspended very gently in 8ml of cold TFB (10mM MES, 100mM RbCl, 45mM $MnCl_2.4H_2O$, 10mM$CaCl_2.2H_2O$, 3mM hexaminecobaltic chloride) and were kept on ice for 15min. 350ul of DMSO was added and the tubes were kept on ice for 5min. 350ul of DTT/KOAc (2.25M DTT, 40mM KOAc pH6.0) was added, and the cells were kept on ice for 10min. 350ul of DMSO were added again, and the cells were kept on ice for a further 5min. The competent cells were kept on ice and used on the day of preparation. This procedure can give up to 100 times the transformants obtained with the $CaCl_2$ method. Substituting the cheaper KCl for RbCl and DMF for DMSO can give a yield about 10 times that of the $CaCl_2$ method. The cells must be treated very gently throughout.

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

2.20 Single colony / single plaque gel analysis. This technique enables the plasmid or M13 RF content of a colony to be observed without the purification of plasmid or M13 DNA.

a) Plasmids: 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 to 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.

b) M13 RF DNA: Cultures were grown up as described above for M13 RF DNA preparations (2.19b) and 1ml of culture removed to a large microfuge tube. The cells were pelleted (12000g, 2min, 4°C), the supernatant discarded and the cells resuspended in 150ul of single colony gel buffer. Subsequent treatment was as described above for

plasmid containing strains.

2.21 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 0.8% 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 to 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.

(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^{o}C and poured between the glass plates. The comb (10 or 13 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.

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).

2.22 Extraction of DNA from agarose gels by the "freeze-squeeze" method of Tautz and Renz (1983). After staining, the gel was placed on a long wave transilluminator (300nM - 360nM) and the band of interest excised. The agarose chip was put in a 1.5ml eppendorf and was equilibrated in the dark at RT with gentle shaking for 15 to 45min with a buffer containing 0.3M NaOAc (pH7.0), 1mM EDTA. At least 10 X the gel slice volume was used. The tube was then drained, and the fragment was frozen to -70°C. Meanwhile, a hole was made in the base of a small eppendorf, and plugged with siliconised glass wool. The frozen fragment was put into this tube, which was placed inside a 1.5ml eppendorf without a lid. The two tubes were centrifuged at 12,000g for 10min. A one hundredth volume of 1M $MgCl_2$, 10% acetic acid was added to the eluted solution in the 1.5ml tube, together with 2.5 volumes of ethanol. After DNA precipitation on ice, the tube was centrifuged, and the pellet was washed twice with 70% ethanol before drying. The DNA was then suitable for ligation.

2.23 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 *recA*$^-$ strains show reduced growth after 30 seconds and do not grow after 60 seconds exposure. *recF*$^-$ strains show an intermediate phenotype. Periodically all strains were checked for their recombination status.

2.24 Protein Gel Electrophoresis. The electrophoresis of proteins followed the procedure of Laemmli (1970). Unless specifically stated all SDS-polyacrylamide gels were 8% running gels with 4.5% stackers. The gel plates were separated by 1mm 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 (8%)	Stacking Gel (4.5%)
Acrylamide/Bis (30 ; 0.8)	10ml	2.25ml (4.5%)
4x Lower Buffer (1.5M Tris pH8.8, 0.4% SDS)	9.37ml	
4x Upper Buffer (0.5M Tris pH6.8, 0.4% SDS)		3.75ml
TEMED	0.013ml	0.060ml
APS (made fresh; 100mg/ml)	0.150ml	0.045ml
dH_2O	18.13ml	8.85ml

The running gel was poured leaving about 1.5 - 2.0cm between the top of the running gel and the bottom of the comb. SDS 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 SDS 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. Residual acrylamide was washed out of the slots using running buffer prior to loading the samples. The gel was run overnight at

about 10mA until the bromophenol blue dye had reached the bottom of the gel.

In order to visualise the protein bands, the gel was stained with 0.1% Coumassie Brilliant Blue-G, 50% methanol, 10% acetic acid, and subsequently destained with 10% methanol, 10% acetic acid. Gels were photographed using illumination from above, and a Pentax 35mm SLR loaded with Ilford HP5 film.

2.25 Conjugation, and transposition assays.

To determine transposition frequencies throughout this work, a system based on the conjugal transfer of small, non-mobilisable plasmids carrying transposons, or transposon derivatives, by the conjugative plasmid R388 was used. R388 contains no known transposable elements and is therefore not immune to transposition, neither does it mobilise non-conjugative plasmids in the absence of transposon sequences. Conjugal transfer of the small plasmids can only occur in this manner if the transpositions occur into a non-essential region of the R388 molecule. A measure of the frequency of transposition into non-essential R388 sequences can be obtained by mating a donor strain, containing R388 and one or more plasmids carrying IRs and encoding tnpA, with a suitable recipient and calculating the fraction of recipients that receive R388 (Tp^r) together with the transposon marker. If the donor plasmid involved is $TnpR^-$, then a stable cointegrate between R388 and the donor plasmid will form and this will be transferred during mating. In this case the fraction of cells containing R388 and the small plasmid marker can give an estimate of the transposition frequency. Obviously this system has limitations e.g. transpositions into essential R388 regions are not detected, and cointegrate molecules tend to use the origin of replication of their small plasmid component and therefore have a much higher copy number than R388, which they tend to out-replicate and displace by incompatibility. However, valid comparisons between transposition frequencies obtained with different donor strains, or under different conditions can be made. For each separate assay, donor strains were freshly constructed, and the R388 and transposon donating plasmids were added in the same sequence.

For transposition assays or strain constructions, the matings were carried out in one of two ways:

Plate matings - 50 to 200 colonies were used to grow up a liquid culture of each donor to about 10^8 cells/ml to get a representative mean figure for transposition. The recipients were grown to stationary phase, then 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 phage buffer and concentrated 10 fold. This was the zero dilution. Serial dilutions were made and plated out on the relevant selective media which selected for recipients with the conjugative plasmid or for recipients which had received the conjugative plasmid and the transposon, and the transposition frequency was calculated. The standard errors of transposition assays were less than 1/5 of the frequency of transposition unless otherwise stated.

Replica plate matings. Donor colonies were patched out in $1cm^2$ patches on selective media and were grown overnight at 30°C or 37°C, Recipients were grown overnight in 2.5ml cultures and 200ul was mixed with 3ml of 0.6% minimal agar, which was poured over L-agar plates containing selection for recipient cells which receive the conjugative plasmid and other mobilised or transposed resistance markers. Replicas of the donor plates were transferred to the recipient plates using velvet. The exconjugates were grown up overnight at 37°C. Any patches growing should have received the conjugative plasmid and the other resistance marker(s) selected for.

2.26 DNA sequencing. In this work the Sanger method of chain termination sequencing was used (Sanger *et al.* 1977). The principal source of reference for this was the "M13 Cloning and Sequencing Handbook" published by Amersham International plc, Amersham U.K. A more detailed account of the theory and practice of sequencing using M13 'phage vectors can be found there.

All the procedures necessary for the generation, isolation and identification of recombinant DNA clones have been described above.

The subsequent procedures needed for the determination of the sequence of the cloned fragment are as follows:

Preparation of single-stranded template DNA. 20ml of 2YT medium was inoculated with 200ul of an overnight culture of JM101 cells, and 1.5ml of this mixture was dispensed into 10ml culture tubes. Each tube was inoculated with a single purified M13 'phage plaque, and grown at 37°C for 5hr with vigorous shaking. The cells (containing all chromosomal, plasmid and M13 RF DNAs) were removed by centrifugation (12000g, 5min, 4°C), the supernatant removed and re-centrifuged to remove all traces of host cells. 1.3ml of this supernatant was added to 200ul of 2.5M NaCl, 20% PEG solution in a large microfuge tube and allowed to stand at RT for 15min to precipitate viral particles. The solution was centrifuged (12000g, 5min, 4°C), the supernatant discarded, the remaining material recentrifuged and all traces of supernatant scrupulously removed, leaving the white viral pellet. The pellet was resuspended in 100ul of TE buffer (pH8.0) and this suspension was phenol extracted three times and chloroform extracted twice to remove viral proteins, and the single-stranded DNA was recovered by ethanol precipitation overnight at -20°C. Finally, the DNA was spun down (12000g, 20min, 4°C) washed twice in 1ml of 85% ethanol in TE buffer, being respun between each wash, and was dried and resuspended in 50ul of TE buffer. This method produced sufficiently pure single-stranded DNA for use in sequencing reactions.

Annealing of primer to template. 5ul of single-stranded template DNA, 1ul (=2ng) M13 universal primer, 1.5ul 10 X Klenow buffer and 2.5ul of distilled H_2O were mixed together, incubated at 60°C for 60 to 90min and then allowed to cool slowly (over a period of 2 to 3hr) to RT. This provided sufficient material for the four sequencing reactions for each clone.

Dideoxy sequencing reactions. All solutions were made up in distilled H_2O and kept frozen at -20°C. Stock solutions at a concentration of 10mM of all four dNTPs were prepared and these were used to make up working solutions at a concentration of 0.5mM. A cold chase solution was prepared which was a uniform mix of all four dNTPs, each at a concentration of 0.5mM. dNTP (N^o) mixes were then prepared from the

working solutions as shown below:

	A^o	C^o	G^o	T^o
0.5mM dCTP	20ul	1ul	20ul	20ul
0.5mM dGTP	20ul	20ul	1ul	20ul
0.5mM dTTP	20ul	20ul	20ul	1ul
1 X TE buffer	20ul	20ul	20ul	20ul

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

ddATP	0.025mM
ddCTP	0.025mM
ddGTP	0.15mM
ddTTP	0.5mM

Equal volumes of each N^o mix and the corresponding ddNTP working solution were mixed to produce the N^o/ddNTP mixes.

The radiolabelled nucleotide used was α-^{35}S-dATP of specific activity 1000 to 1300 Ci per mmole. The concentration of the solution supplied varied from 7.9 to 9.22uM and this was adjusted by the addition of an appropriate volume of a 100uM cold dATP S solution to give a final overall concentration of 16uM dATPαS. Such a volume of this solution was taken (1.5ul) so that each reaction system contained 7.6 to 8.87pmoles (=8.07 to 11.54 uCi) of α-^{35}S-dATPαS.

For the sequencing reactions, 1.5ul of the above labelled nucleotide mix was added to the annealed primer/template mix and 1ul of Klenow enzyme solution (1unit per ul) mixed in carefully by pipetting in and

out. A 2.8ul aliquot was placed into each of four small microfuge tubes labelled A, T, G and C. These each contained 2ul of the appropriate N°/ddNTP reaction mix. Again this was mixed by pipetting in and out. The reaction was allowed to proceed for 20min at RT before 2ul of the chase mix was added. This was mixed and allowed to stand at RT for a further 15min. The reaction was stopped by the addition of 4ul of formamide dye mix to each tube. Samples were then prepared for loading onto a gel or stored at -20°C until required.

High resolution polyacrylamide gel electrophoresis. The gel apparatus used consisted of two 45 X 23cm siliconised glass plates held apart by 0.4mm thick "Plasicard" spacers along each long side and sealed with vinyl tape. Combs were also cut from 0.4mm thick "Plasticard" and had 29 teeth.

For a 50ml 6% gel, the following components were mixed together: 21g "Ultrapure" urea, 5ml 10 X TBE buffer, 7.5ml 38% acrylamide/2% N N' bis-methylene acrylamide (deionised by the addition of 5g of "Amberlite" MB1 resin and stirring at RT for 30min, followed by filtration to remove the resin) and distilled water up to a total volume of 50ml. These were shaken together till the urea was dissolved, 300ul of 10% APS and 50ul of TEMED mixed in, the gel poured, the comb inserted, the plates clamped together firmly and polymerisation allowed to proceed to completion (about 2hr at RT).

The gel was installed in the running apparatus, the comb removed, the reservoirs filled with TBE buffer and the wells washed out with buffer to remove any unpolymerised acrylamide. Just before loading, the wells were again washed out with buffer to remove any urea that may have leached into them, as this would have prevented even loading of the samples.

The samples were heated to 90 to 95°C for at least 3min and 2ul of each loaded immediately onto the gel. The gel was run at a constant power (40 Watts) which maintained it at a sufficiently high temperature (60 to 65°C) to keep the DNA samples denatured. After running, the gel was fixed in a 10% acetic acid (v/v) solution for 20 to 30min at RT, carefully transferred to Whatman 3mm filter paper and dried down under vacuum. Bands were visualised by autoradiography of

a sheet of Kodak "X-Omat" S1 film for 1-4 days at RT.

2.27 Galactokinase assays (McKenney et al. 1981). Assays were performed in duplicate on any one day and assays were repeated twice to get a mean figure for the galactokinase activity of each strain under test.

Overnight cultures of the strains to be assayed were grown up in L-broth without glucose but with antibiotics, at the required temperature. These were diluted 1 in 20 in the same medium and grown up at the desired temperature with shaking till their OD_{650} was in the range 0.2 to 2.35. The OD_{650} of each culture was recorded. 1ml aliquots of each culture were taken, 10ul of mix 3 (100mM $Na_2EDTA.2H_2O$, 100mM DTT, 50mM TrisHCl pH8.0) containing 5mg per ml CTAB added to each and the mixture vortexed for 1min.

Assay reaction mixtures were made up in small microfuge tubes as follows: 5ul of mix 1 (5mM DTT, 16mM NaF)

+ 12.5ul of mix2 (8mM $MgCl_2$, 200mM TrisHCl pH7.9, 3.2mM ATP)
+ 2.5ul of mix4 (240ul 4mM galactose, 10ul D(1-^{14}C)galactose).

The reactions were started by the addition of 5ul of cell lysate. [Blanks were set up by adding 5ul of distilled H_2O rather than cell lysate and additional tubes to act as unwashed "total count" controls were also set up using cell lysates taken at random.] The reactions were incubated at 32°C for 30min and stopped by placing the tubes on ice. The whole reaction mixture was spotted onto a 2.3cm Whatman DE81 filter paper and the filters carrying the unwashed reaction controls dried. The other filters were washed twice (in 1l of distilled water for 10min each time) and then dried. Lastly, the filters were counted in 5ml of "Biofluor" scintillant in a scintillation counter. Each filter was counted at least twice for two minutes each time.

Galactokinase activity was calculated using the following equation:

$$\text{Number of units of galactokinase activity} = \frac{\left[\begin{array}{l}(\text{Cpm from test systems}) \\ -\ (\text{Cpm from blank systems})\end{array}\right] \times 5200}{(\text{Average Cpm from 2 unwashed filters}) \times (\text{Incubation time in min}) \times OD_{650}}$$

Cpm = counts per min.

2.28 B-lactamase assays. The cultures to be assayed were grown overnight at the required temperature, before being diluted 0.5ml into 100ml fresh medium. The cultures were grown at the desired temperature and samples were taken periodically. These were treated with 10ul of 5mg per ml CTAB and were vortexed for 1min. The samples were then either frozen to -20°C for use later, or assayed immediately. The incubation mix consisted of: 2.5ml penicillin solution (= 2.4mg per ml benzyl penicillin in 0.1M phosphate buffer pH7.0) and up to 0.5ml penicillinase i.e. the lysed cell culture. The reaction was allowed to continue for a fixed time (between 10 and 50min), before being terminated by the addition of 5ml of iodine solution (a stock solution of 0.32M iodine, 1.2M KI was diluted 1:20 in 2M NaOAc buffer pH4.2). For each assay tube a control tube was set up consisting of 2.5ml penicillin solution without the penicillinase. This was incubated for the same time as the experimental tube, and had 5ml iodine solution added. At this point, 0.5ml of the lysed cells was added.

After leaving the stopped reactions and controls at 30°C for 10min, the absorbance at 499mu of both was read. The difference between the OD_{499} of the control and the experimental tubes was noted. The B-lactamase activity calculated as follows:

$$\text{Activity} = \frac{\Delta OD_{499} \times (\text{total vol. at end of assay})}{(\text{time of incubation in min}) \times (\text{vol. of cells used})}$$

The total volume varied between 7.5ml and 8.0ml. The volume of cells lysed varied between 0.1 and 0.5ml, depending on how much colour change each volume gave. The problem with this assay was that the OD_{499} is a negative one, and the colour does not develop until the

iodine solution has been added. This means that it is very easy to incubate the cells too long, so that the solution goes almost clear on the addition of the iodine solution. However, when several assays have been done on various strains it is possible to estimate how long the incubation should last.

2.29 B-galactosidase assays. Each strain to be assayed was grown up in duplicate overnight at the required temperature. The following day they were diluted 0.5ml into 100ml of L-broth and were grown shaking at the required temperature. At various time points, 1ml of cells was removed, treated with 10ul of 5mg per ml CTAB and vortexed for one minute. The samples were then either frozen to -20°C for use later, or were assayed immediately in one of two ways:

a) A portion of cells was made to 2.5ml with assay buffer (0.1M NaH_2PO_4 pH7.25, 0.5mM $MgSO_4$). This was incubated at 30°C. After 5min, the reaction was started by the addition of 0.5ml 20mM ONPG (made up in assay buffer). When the reaction mixture started to show a yellow colour, the incubation time was noted and the reaction was terminated by the addition of 2ml of 1M Na_2CO_3. The OD of each solution was measured at 420um and 520um. The B-galactosidase activity was then calculated as described below.

b) A portion of cells was made to 2.5ml with assay buffer prewarmed to 30°C. The mixture was put into a 3ml cuvette, and placed in a Beckman Du-50 spectrophotometer. The machine was programmed using the package "KINDATA" (Beckman) and the reaction was started by the addition of 0.5ml of ONPG as above. The reaction was allowed to continue indefinitely, but the machine was instructed only to print a continuous curve for 5 to 10min. The nM ONPG hydrolysed per min per volume of cells was also printed out. This method showed that the hydrolysis of B-galactosidase was linear over the time of assay.

The activity of B-galactosidase was expressed in two ways for all assays:

a) As Miller units of activity = $1000 \times \left[\frac{OD_{420} - (1.75 \times OD_{550})}{\text{Time} \times (\text{Vol. of cells}) \times OD_{600}} \right]$

(The reference for this is Miller, 1972.)

b) As nM ONPG hydrolysed min^{-1} ml lysed $cells^{-1}$

It was possible to interconvert Miller units to the units in (b) by using a calibration curve, based on the OD_{420} of various concentrations of ONP. It is important to notice that Miller units are proportional to, not equivalent to, the increase in ONP per min per bacterium. Miller units do not take into account the final 5ml volume of the assay, whereas the units in (b) do.

2.30 Western blots. One or two blots could be carried out in the same kit at the same time. For one blot, four pieces of 3mm paper were cut to the size of the gel to be blotted, and one piece of nitrocellulose. A tray was filled with transfer buffer (see below). The 3mm paper and nitrocellulose were thoroughly wetted. A blotting "sandwich" was prepared as follows: the first two layers were 3mm paper, followed by the gel to be blotted. Care was taken to eliminate air bubbles from underneath the gel. The nitrocellulose paper was laid over the gel in one movement, again taking care to avoid trapping air bubbles. The next two layers were 3mm paper again. The "sandwich" was then closed, and the fastening clip marked so that it was clear which side of the "sandwich" the gel and filter were respectively. The "sandwich" was placed into the tank, which was filled with transfer buffer, with the clip at the bottom of the tank. The clip was tilted so that air bubbles would not be trapped. A magnetic stirrer was placed in the tank, together with the cooling tubes, and the lid was replaced on the tank. The current was set at 200mA with the -ve electrode placed behind the gel, and the +ve electrode set in front of the filter. The blot was left running overnight with stirring, and cooling. The following day, the apparatus was dismantled, and the filter cut up as required. The flattened gel was stained and destained as described in 2.24 in order to check that all the protein had been transferred to the filter. The nitrocellulose filter was treated as follows (the buffers are described below):

The blots were washed in TBS-tween to remove any adherent polyacrylamide, one part of the filter was stained to check for

protein transfer (as described below), then the rest of the blots were incubated as required for 1hr with the first antibody, or serum being examined. Various dilutions in HST were tested out during a preliminary run (see text). The blots were then washed 5min in TBS-tween, 5min in fresh TBS-tween, 5min in HST buffer, 5min in TBS-tween, 5min in fresh TBS-tween. Then they were probed with the second antibody diluted 1 in 1000 in HST (Anti-rabbit IgG whole molecule Peroxidase Conjugate - Sigma). 10mls were used per 100cm^2. The blots were then washed 5min in TBS-tween, 5min in TBS-tween, 5min in TBS-tween, 10min in HST, 5min in TBS-tween, 5min in TBS-tween, 5min in TBS-tween, 5min in TBS. The substrate was freshly prepared and used immediately. After bands had developed (about 20min), the filters were stored in the dark, and later photographed on Recordak film.

The buffers required for one or two blots are as follows:

Transfer buffer = 25mM Tris (3g per 1)
(4 litres) 192mM glycine (14.4g per 1)
20% v/v MeOH (200ml per 1)

TBS (pH7.4) = 10mM Tris (1.21g per 1)
(2 litres) 140mM NaCl (8.18g per 1)

TBS-tween = as TBS + 0.1% Tween 20 (1ml per 1)

HST buffer = 10mM Tris pH7.4 (1.21g per 1)
(1 litre) 1M NaCl (58.4g per 1)
0.5% v/v Tween 20 (5ml per 1)

Substrate solution = 18mg 4-chloro-1-napthol in 6ml MeOH.
Add 94ml TBS and 25ul H_2O_2.
Use immediately.

Protein stain = 0.1% amido black in 45% MeOH, 10% acetic acid

Protein destain = 45% MeOH, 10% acetic acid.

CHAPTER 3

THE INTERACTION OF TRANSPOSASE WITH THE INVERTED REPEATS OF TN3 IN VIVO

Introduction

The major aim of these studies was to investigate whether limiting amounts of Tn*3* transposase (1.6.3) *in vivo* are titrated out by site-specific interaction with the 38bp IRs. If this was the case, it might be a subtle way in which the transposition of intact elements may be further limited in a cell where the number of IRs considerably outnumbers the functional *tnpA* genes. This regulation, if it existed, would probably be fortuitous, due to transposase binding available ends to promote transposition, but could be of significance, as it has been proposed to explain the cytotype effect in P-M hybrid dysgenesis of Drosophila (see discussion; Simmons and Bucholz 1985).

A secondary aim was to try and elucidate the role of the IRs in the phenomenon of transposition immunity (1.6.3). Current hypotheses suggest that the binding of transposase to the IRs causes the observed effect. As the Tn*3* transposase gene terminates within the LIR, it was believed that preferential binding of transposase to this end might occur. If this was the case, then it was a possibility that the LIR would confer greater immunity than the RIR.

Intermolecular transposition of Tn*3* proceeds by a two step mechanism involving a cointegrate as an intermediate. The only transposon-encoded requirements for cointegrate formation are the *tnpA* product, transposase, and the 38bp perfect IR ends of Tn*3*. Models for transposition (Shapiro 1979; Arthur and Sherratt 1979) predict that functions which must be performed by the transposase protein are site-specific recognition of the IRs, and nicking and ligating of the donor and target DNA sequences. For this, binding to the target DNA is also necessary.

Site-specific recognition of the IRs of several transposable elements by their transposases has been shown *in vitro* (IS*1* - Zerbib *et al.* 1987; Tn*3* - Wishart *et al.* 1985; Morita *et al.* 1987; Mu - Craigie *et al.* 1984). In the case of Tn*3* the first studies on transposase binding (Fennewald *et al.* 1981) did not show it to be specific for the transposon ends. Later Wishart *et al.* (1985) reported that ATP was required for site-specificity. However that does not now appear to be the case (Morita *et al.* 1987) and the reason for the disagreement and

lack of initial specificity, was probably the differing pH of the reactions caused by the ATP.

In vivo studies on the binding of transposase to the ends of transposable elements have mainly centred on defining the minimum sequences necessary for transposition (IS1 - Gamas et al 1985; Tn10 - Way and Kleckner 1984; Tn3 - Huang et al. 1986). It appears that the entire 38bp IRs of Tn3 are necessary for normal transposition frequencies, although some partial deletants of the IRs can transpose at a reduced frequency. Also one-ended transposition may occur infrequently (Arthur et al. 1984). Almost all IR sequences allowing transposition also confer transposition immunity on their replicons (Huang et al. 1987), suggesting that immunity may be a consequence of transposase binding the ends (see discussion).

In order to study end titration of Tn3 transposase, a set of plasmids were constructed which carry a functional tnpA gene and different numbers of IRs. The effect of these constructs on the transposition of another TnpA$^-$, Tn3-based element in the same cell was examined. The immunity of these constructs to insertions of another Tn3 element was also studied.

Results

3.1 Construction of Tn3571, a TnpR$^-$ derivative of Tn3

Inactivation of the resolvase gene of Tn3, tnpR, was desirable for three reasons. The first was that elimination of the repression of tnpA would increase the levels of transposase in the cells and hence the transposition frequencies would increase (Casadaban et al. 1982). This makes measurement of transposition easier. The second was that elimination of the resolving capacity of the transposon means that cointegrates are the stable end products of intermolecular transposition. This was important in the immunity studies so that transposition could be measured by studying the cotransferral of resistance markers of two plasmids linked in a cointegrate. The third was that eliminating regulation of tnpA by resolvase would enable regulation of tnpA by other factors to be studied (Chapter 4).

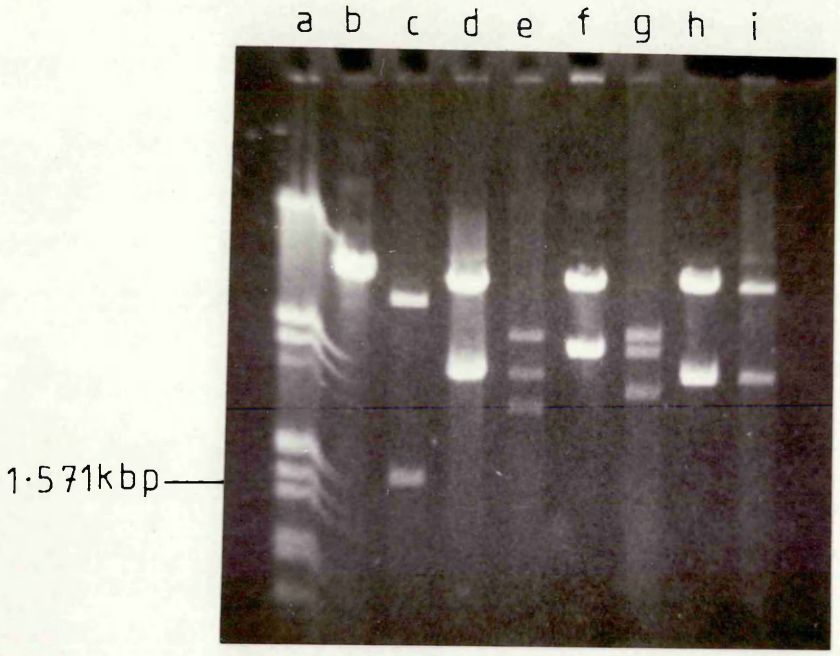


Fig. 3.1

ClaI digestion of DNA from the Dam$^+$ strain DS903 and the Dam$^-$ strain CB51.

a. λHindIII/EcoRI
b. pROS1, methylated
c. pROS1, unmethylated
d. pROS3, methylated
e. pROS3, unmethylated
f. pROS40, methylated
g. pROS40, unmethylated
h. pROS13, methylated
i. pROS13, unmethylated

A 1.571kbp fragment in the unmethylated case indicates perfect filling-in of the BamHI site in pROS1 (c) or pROS2 (e,g,i). pROS3 and pROS40 both contain a DNA methylase sensitive ClaI site in the galK gene.

The source of Tn3 used in these experiments was RSF1050 (pMB8::Tn3, Heffron et al. 1977). This plasmid has a unique BamHI site within the tnpR gene of Tn3, at position 3571 of the transposon. Filling-in of this site should cause a frameshift mutation in resolvase which should truncate and inactivate the protein.

RSF1050 DNA was prepared from DS312. It was cut with BamHI, filled-in, ligated and transformed into DS903. Two single colonies were repurified and DNA was prepared from them. These plasmids were called pROS1 and pROS2. The mutant Tn3 on pROS2 was called Tn3571.

3.2 The properties of Tn3571

3.2.1. The nature of the tnpR mutations on pROS1 and pROS2

DS916 (R388) was transformed to Ap^r with RSF1050, pROS1 and pROS2. About 100 of the resulting colonies from each plate were pooled and examined on single colony gels. In the case of DS916 (R388, pROS1) and DS916 (R388, pROS2) the existence of a high number of cointegrate molecules between R388 and pROS1 or pROS2 could be seen. These were obvious because of their size (larger than an R388 marker), and because of their extremely high copy number compared to the R388::Tn3 plasmids formed by complete transposition in DS916 (R388, RSF1050). It was therefore concluded that pROS1 and pROS2 carried a mutant resolvase gene. pROS2 was subsequently used as the source of Tn3571 for other constructs in which the $TnpR^-$ phenotype was required.

Sometime after pROS1 and pROS2 had been constructed, and pROS2 had been used as the parent of various plasmids, it was learned that perfect filling-in of a BamHI site should give rise to a ClaI site sensitive to Dam methylation. pROS2 derivatives were therefore examined to see whether the BamHI site had been perfectly filled-in or not. The Dam^- strain CB51 was transformed to Ap^r with pROS3 (Chapter 4), pROS13 (this chapter), and pROS40 (Chapter 4), which were all constructed using Tn3571 from pROS2. pROS1 was also introduced. DNA from these strains was digested with ClaI and run on a gel with controls (Fig.3.1). As Tn3 carries a Dam methylase insensitive ClaI site at position 2000, a 1.571kbp fragment would be diagnostic of a DNA methylase sensitive ClaI site at position 3571, and the presence

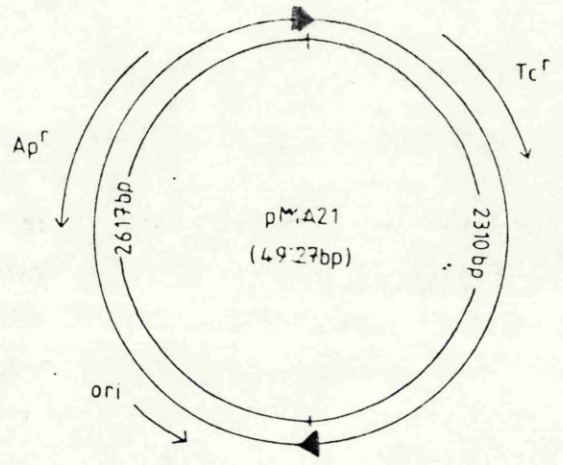


Fig. 3.2 pMA21, showing the two directly repeated res sites across which resolution occurs in the presence of resolvase. Resolution gives two fragments of sizes 2617bp and 2310bp. Only the 2617bp Ap^r fragment has an origin of replication.

▶= res (sites I, II and III)

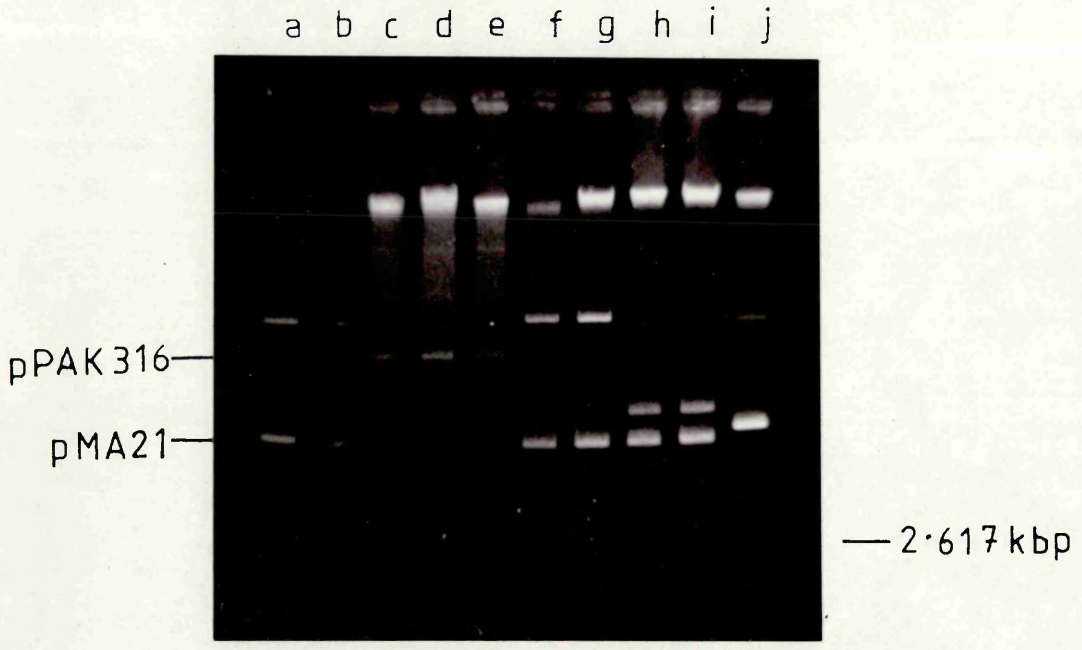


Fig. 3.3 Test for the ability of Tn3571 to resolve pMA21.

a, b. DS902 (pMA21, pROS3)
c, d, e. DS902 (pMA21, pPAK316) +ve control
f, g. DS902 (pMA21, pROS13)
h, i. DS902 (pMA21, pROS12) -ve control
j. DS903 (pUC8 - dimer)

A 2617bp band is an indication that pMA21 has been resolved.

of this fragment would indicate that the BamHI site had been perfectly filled-in.

The results showed that although pROS1 does carry a perfectly filled-in site, the pROS2 derivatives do not - suggesting that pROS2 itself (this DNA was unfortunately no longer available at this time) had not been perfectly filled-in at this site. Thus the nature of the mutation at the BamH1 site of Tn3571 is unknown. This discovery led to the desire to obtain further evidence that the resolving and repressing activities of Tn3571 were completely abolished.

3.2.2 Examination of Tn3571-encoded tnpR for the ability to resolve cointegrates

In order to test whether Tn3571 was capable of resolving a cointegrate-type structure, a plasmid, pMA21 (Brown 1986), bearing two res sites in direct repeat, was used as a substrate (Fig. 3.2).

DS902 (pMA21) was transformed to Cm^r with pROS3, a Tn3571-carrying construct (Chapter 4), and with pPAK316 (Kitts 1982). pPAK316 is a $TnpR^+$ plasmid which is known to be capable of resolving cointegrates, and it was included here as a positive control for resolution. DS902 (pMA21) was also transformed to Km^r with pROS13 (3.3.2) which carries Tn3571, and pROS12 (3.3.1). pROS12 is the parent replicon of pROS13 and does not carry Tn3571. This was included as a negative control for resolution of pMA21. Examination of about 100 pooled colonies of each strain on a single colony gel (Fig. 3.3) showed that two plasmids were present.

As can be seen from Fig. 3.2, resolution between the two res sites of pMA21 divides the plasmid into two smaller circles, only one of which, carrying bla, has an origin of replication. Therefore during overnight growth following transformation, the smaller Tc^r circle will be lost from most cells, whereas the 2.617kbp Ap^r circle will not. If resolution of pMA21 occurs then the 4.927kbp band will not be obvious on the gel, but the 2.617kbp one will be. This was only the case for the positive control, indicating that Tn3571 in pROS3 and pROS13 does not appear to retain the ability to efficiently resolve pMA21.

	30°C	37°C
TnpR$^+$	$2 \cdot 2 \times 10^{-2}$ $\pm 0 \cdot 3 \times 10^{-2}$	$8 \cdot 9 \times 10^{-4}$ $\pm 1 \cdot 7 \times 10^{-4}$
TnpR$^-$	$5 \cdot 2 \times 10^{-1}$ $\pm 3 \cdot 0 \times 10^{-1}$	$3 \cdot 6 \times 10^{-2}$ $\pm 1 \cdot 4 \times 10^{-2}$

Table 3.1 Transposition frequencies of Tn3 (TnpR$^+$) and Tn3571 (TnpR$^-$) from RSF1050 to R388 at 30°C and 37°C. The numbers shown are averages of five replicates.

3.2.3 Examination of Tn3571-encoded tnpR for the ability to repress tnpA expression

In order to discover whether Tn3571 makes repressed or derepressed levels of transposase, the transposition frequency of Tn3571 from pROS2 was compared with that of Tn3 from RSF1050. This was done using plate mating assays performed mainly at 30°C or mainly at 37°C. The reason for doing the assays at both these temperatures is discussed in chapter 4.

DS903 (R388) was transformed with pROS2 or RSF1050, each in duplicate: one transformation with each plasmid was heat-shocked at 30°C, and the other at 37°C, for 5 minutes. After 5 minutes on ice, each transformant was allowed to express for one hour at the same temperature as the heat-shock i.e. 30°C or 37°C. Then the transformants were plated on Sm, Tp and Ap and grown overnight at their respective temperatures of 30°C or 37°C. The following day the strains were grown up at 30°C or 37°C and were mated at 37°C for 2 hours to the recipient, DS916. Exconjugants were selected on plates containing Rf and Tp, or Rf, Tp and Ap. The transposition frequencies were then calculated. These strain constructions and transposition assays were repeated five times to obtain a reliable estimate of transposition frequencies from these donors under these conditions. The results are shown in Table 3.1.

One of the exconjugant R388::pROS2 cointegrate-containing colonies was purified and TnpR$^+$ pPAK316 was transformed in to resolve the cointegrates. Mating of this strain with DS903 gave exconjugants containing R388::Tn3571, one colony of which was purified. This was of use later (3.3.2, 3.5).

The results in Table 3.1 showed that Tn3571 expresses derepressed levels of transposase. The observed 20 - 50 fold level of derepression is consistent with that observed by Gill et al. (1979) when they assayed transposition frequencies of a Tn3 derivative that had also been mutated by filling-in the BamHI site in tnpR.

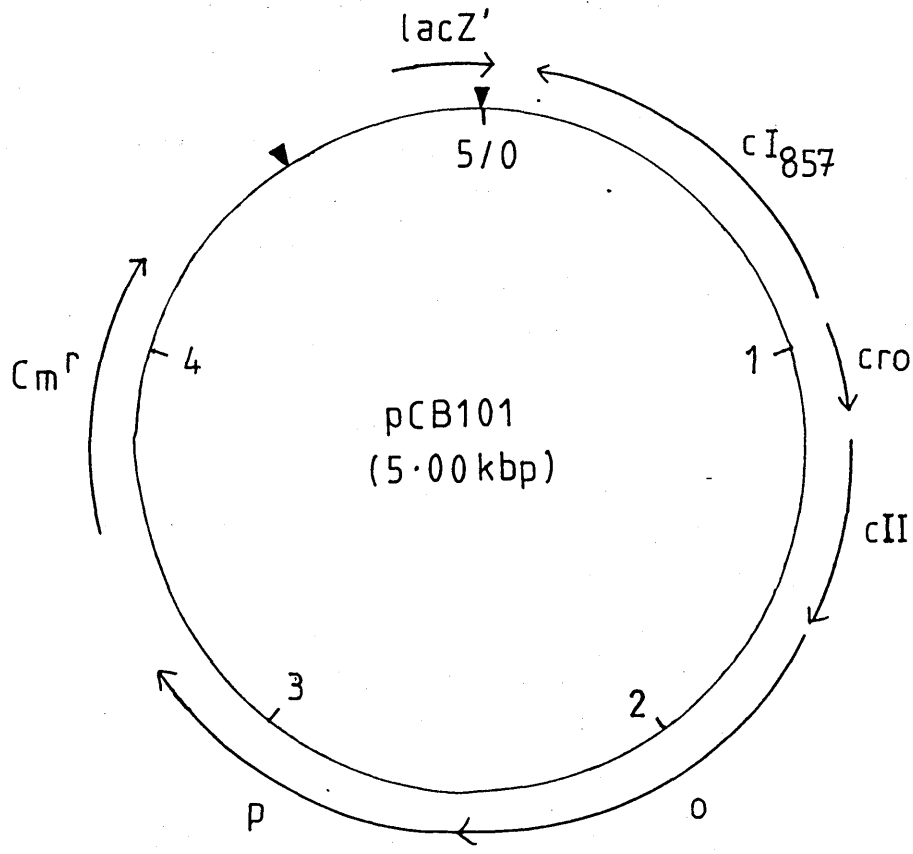


Fig. 3.4 pCB101, a λdv vector.

▼ represents a HaeII site.

3.3 Construction of λdv-based plasmids containing different numbers of IRs.

The plasmid pPAK200 (Kitts 1982) is a pACYC184-based replicon, with Tn3651 transposed into the Tc resistance gene, making the plasmid Tc^S. Tn3651 is a $TnpA^-$, $TnpR^-$ version of Tn3, in which the IR ends are intact and the Ap resistance gene, bla, is functional. pPAK200 is therefore Cm^r Ap^r. Tn3651 can be complemented in trans by tnpA to transpose.

In order to ensure maintenance of two or more plasmids in a cell it is usual to select for each plasmid by a different antibiotic resistance. Therefore, in order to complement transposition of Tn3651 onto R388, a compatible vector with resistances other than to Tp, Cm, or Ap was necessary. Additionally, for the purposes of the assay described in 3.4, it was desirable that the copy number of the complementing replicon was equal to, or preferably greater than, that of pPAK200 (i.e. pACYC184).

The requirement for a resistance other than to Ap ruled out the use of pUC-based replicons, which are compatible with, and have a higher copy number than, pACYC184 (pACYC184 has 20 copies per chromosome equivalent - Timmis 1981; pUC has 90 copies per chromosome equivalent, of which there are 3.5 per dividing cell - Jones 1985). Also pUC contains the RIR of Tn3 which was undesirable.

The existence within our department of a λdv-based replicon, pCB101 (Boyd and Sherratt 1986), seemed to provide a solution to the problem of compatibility. λdv vectors can be maintained in cells with pACYC184. The cloning sites available made it easy to insert the gene for Km resistance into the vector. The only problem (see 3.4) was that the copy number was apparently less than, not greater than, pPAK200.

3.3.1 Conversion of pCB101 (λdv) to Km resistance.

pCB101 is shown in Fig. 3.4. The presence of the cI_{857} gene is superfluous, as its expression is repressed by the action of cro product. The λ-derived circles can replicate autonomously as plasmids

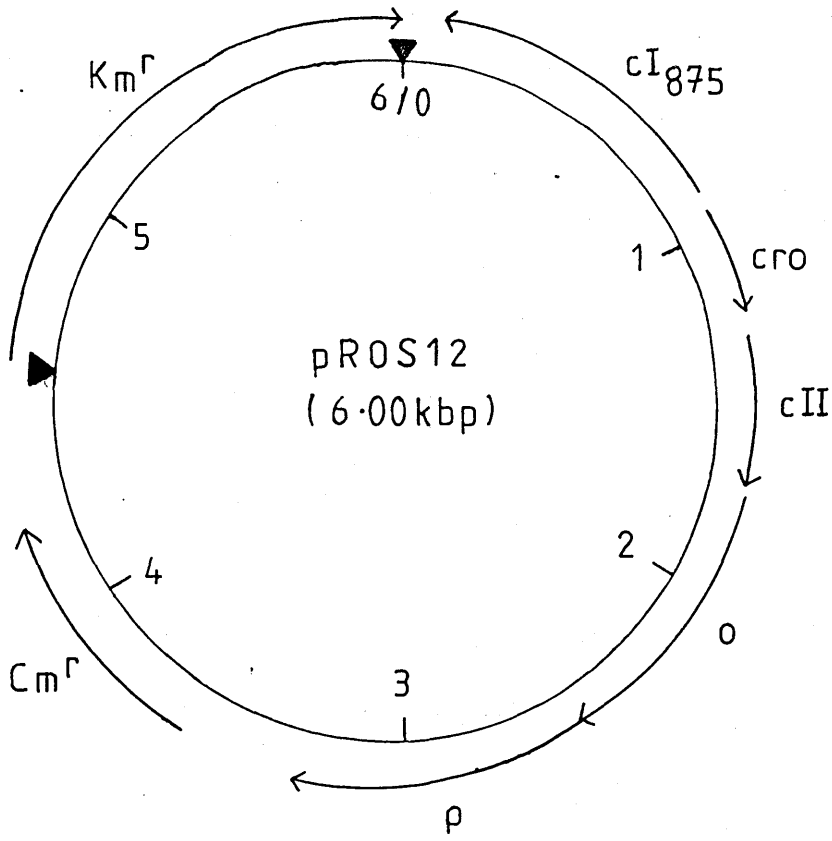


Fig. 3.5 pROS12, carrying the Km^r gene on a 1.43kbp HaeII fragment originally derived from Tn903.

▼represents a HaeII site.

when carrying only the cro, O and P genes.

For subsequent experiments the pUC9-derived HaeII fragment containing the polylinker and lacZ coding region (Vieira and Messing 1982) was not required. Therefore this was replaced by a 1.430kbp HaeII fragment from pGJ28 (Warren 1978) encoding the Km resistance gene, aminoglycoside 3'-phosphotransferase (APH I), derived originally from Tn903 (Oka et al. 1981).

The desired clone was isolated by transforming the ligation mix of HaeII-cut pCB101 and the HaeII APH I-containing fragment into the strain JM83 and plating on L-plates (without glucose) containing Cm, Km, Xgal and IPTG.

In a lacZΔM15 strain, such as JM83 or JM101, plasmids expressing the α-complementing fragment of lacZ, (lacZ'), which is missing from the chromosome, confer a Lac$^+$ phenotype on their host cells. This means that transformant colonies give a blue colour on Xgal-containing plates due to hydrolysis of Xgal by B-galactosidase. The presence of IPTG enhances this blue colour by inducing lac. If the plasmids do not contain the α-complementing fragment, or it is not expressed due to an insertion, then the colonies are colourless on Xgal-containing plates, with or without IPTG. In this construction, colourless, Km^r colonies were sought amongst the blue ones, which would imply that the HaeII fragment containing lacZ had indeed been replaced by the pGJ28-derived fragment.

One colourless, Km^r colony was picked and repurified, and plasmid DNA was prepared from the strain. This construct was called pROS12 (Fig. 3.5). The orientation of the Km^r fragment was determined at a later stage (see Fig. 3.10).

3.3.2 Transposition of Tn3571 onto the λdv vector

DS903 (R388::Tn3571) (3.2.3) was mated with DS916 to give DS916 (R388::Tn3571), which was mated with JM83 (pROS12). The exconjugants were plated on Sm, Km, and Ap. These cells now contained a mixture of plasmids: R388::Tn3571, pROS12, and cointegrates between these two. They were grown up and mated to DS903 and exconjugants were selected

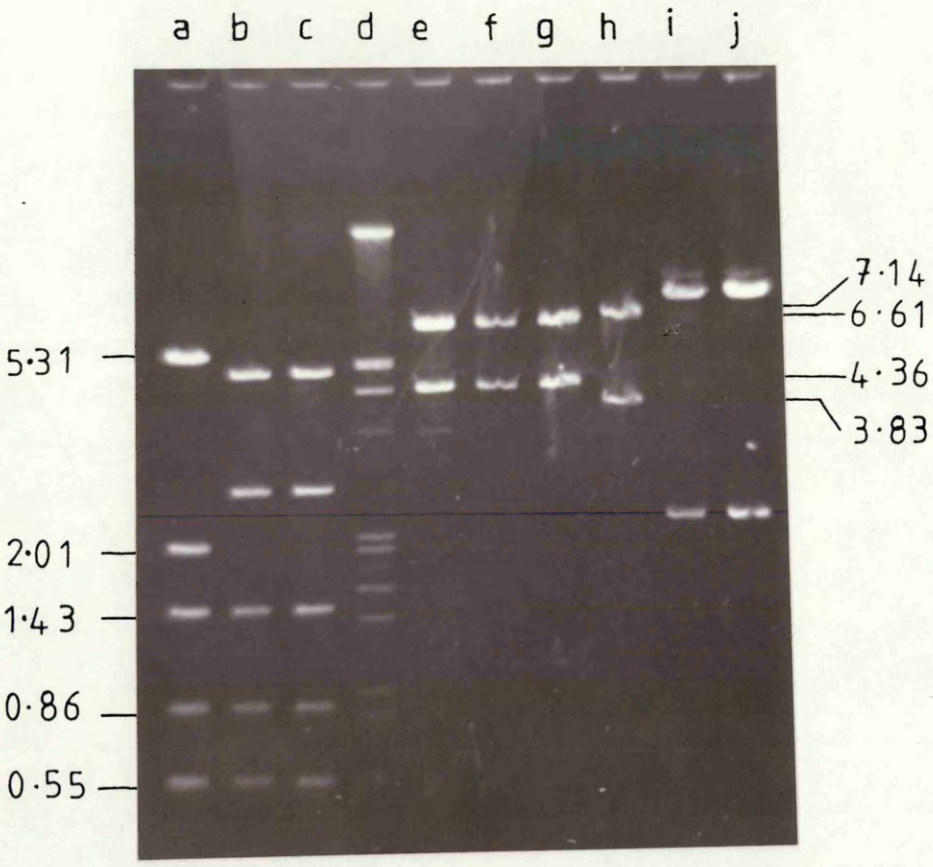


Fig. 3.6a Digests of pROS13, pROS14 and pROS15.

a. pROS13 HaeII
b. pROS14 HaeII
c. pROS15 HaeII
d. λHindIII/EcoRI
e. pROS13 EcoRI
f. pROS14 EcoRI
g. pROS15 EcoRI
h. pROS13 AccI
i. pROS14 AccI
j. pROS15 AccI

Fragment sizes in kbp

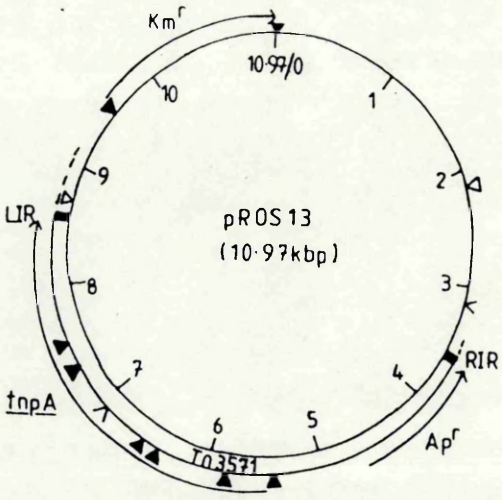


Fig. 3.6b pROS13, showing the position of insertion of Tn3571.

- - - represents the disrupted Cm^r gene
▼ represents a HaeII site
▽ represents an EcoRI site
⋁ represents an AccI site

on agar plates containing Tc, Tp, and Km. The resulting colonies were screened for Cm sensitivity by patching on plates containing Cm. 3 out of 269 of these were Cm^S. This was a much lower frequency than would be expected if insertion of Tn3571 into pROS12 were random: 60% of pROS12 is necessary for replication or was selected for (i.e. Km resistance), and the chloramphenicol resistance gene accounts for 30% of the remaining sequences available for viable transpositions, therefore about 80 Cm^S colonies would be expected out of 269. The reason for the low frequency obtained is not known. Perhaps the chloramphenicol gene is not a favourable region for Tn3571 insertions. Regional preferences are known to exist (Kretschmer and Cohen 1977; Grinsted et al. 1978).

The three Cm^S colonies, containing cointegrates between R388 and pROS12, were grown up and transformed to Cm^r with pPAK316, which is able to resolve cointegrates. Mini DNA preps were carried out on these three strains, and the DNA was serially diluted before transformation of DS903 to lower the chance of double transformants occurring. Transformants were selected on Km and Ap and colonies from the transformation with the lowest concentration of DNA were screened by examination on single colony gels. DNA was prepared from these three strains and mapped with several restriction enzymes (Fig.3.6a).

From the digestions it could be concluded that the transposon in one clone, A, was in a different orientation to that in the other two, B and C. These two appeared to be identical, and either represented two separate insertion events at very similar locations in the same orientation, or they were siblings from one single transposition event.

The transposon in clone A was in a suitable location and orientation for deletion of the LIR from the BalI site of the vector. This clone was therefore used in subsequent constructions. It was called pROS13 (Fig. 3.6b). The actual point of insertion of Tn3571 was later determined by sequencing (3.3.6).

3.3.3 Deletion of the LIR of Tn3571

The location of a unique BalI site on the vector pROS13 about 400bp from the LIR of Tn3571 meant that deletions from this site into the LIR could easily be carried out with Bal31 (Silhavy et al. 1984). Bal31 carries two activities (Lau and Gray 1979): a highly specific, single-stranded endodeoxyribonuclease and exonuclease that catalyses the removal of small oligonucleotides or mononucleotides from both 5' and 3' termini of double-stranded DNA (both strands are degraded at approximately the same rate) ; and a single-strand specific endonuclease similar to nuclease SI. The enzyme is absolutely dependent on calcium ions and can be completely inactivated with EGTA. It can be used to remove nucleotides from the termini of double-stranded DNA with blunt or protruding termini.

About 2ug pROS13 was digested with BalI. Complete digestion was essential to avoid uncut supercoils transforming the cells as well as the deleted plasmids. This was confirmed by agarose gel analysis. The Bal31 digestion was carried out at 37°C in a total reaction volume of 50ul. 2ul samples were removed every 2 minutes and were added to 30ul of stop buffer to give a final volume of 80ul, which was phenol/chloroformed. Ethanol precipitation of the DNA was followed by resuspension in nick-translation buffer. The ragged ends of the DNA were filled-in, the reaction was stopped with EDTA and the solution was phenoled, chloroformed, and the DNA precipitated and resuspended in ligation buffer. The ligation mixture of DNA molecules deleted to different extents was transformed into DS903 (R388, pPAK200). [pPAK200 (Kitts 1982) has been described (3.3).] Transformants were selected on Sm, Tp, Cm, and Km. The latter meant that any molecules deleted into the Km resistance gene would not survive.

The phenotype of the deletants with respect to Tn3571 could be ascertained by mating the individual transformants to DS903 Sp^r. All 89 transformants were patched out and replica-plated onto DS903 Sp^r lawns on plates containing Sp and Tp. The 89 exconjugant patches were then patched out onto plates containing Sp Tp, Sp Cm, and Sp Km. If the colonies were Cm^r Km^r it implied that the deletion in pROS13 had not extended into the the LIR of Tn3571, and both Tn3571 and Tn3651 could transpose into R388 due to the $TnpA^+$ nature of Tn3571. If the

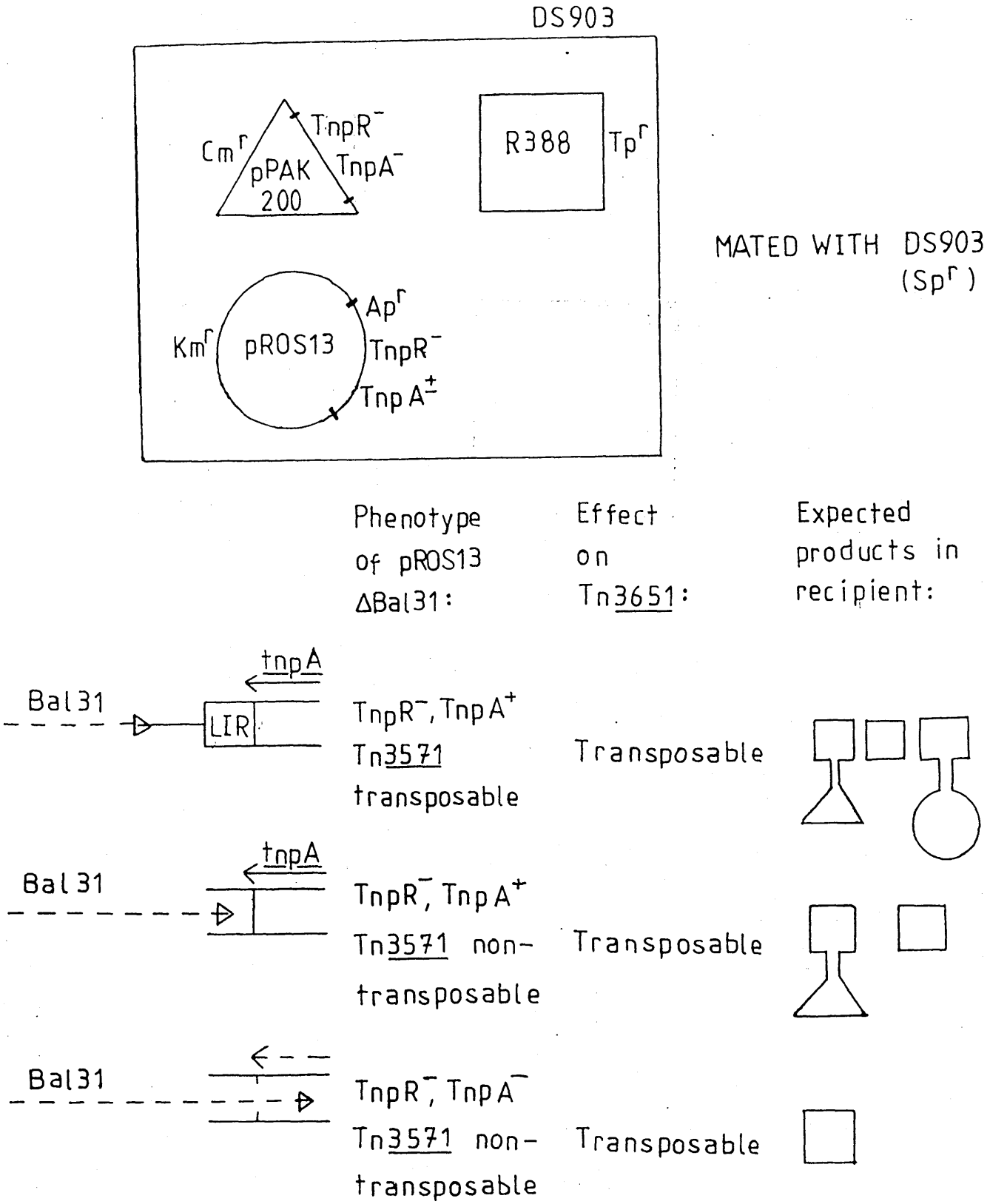


Fig. 3.7 Procedure for ascertaining the Tn3571 phenotypes carried on pROS13 Bal31.

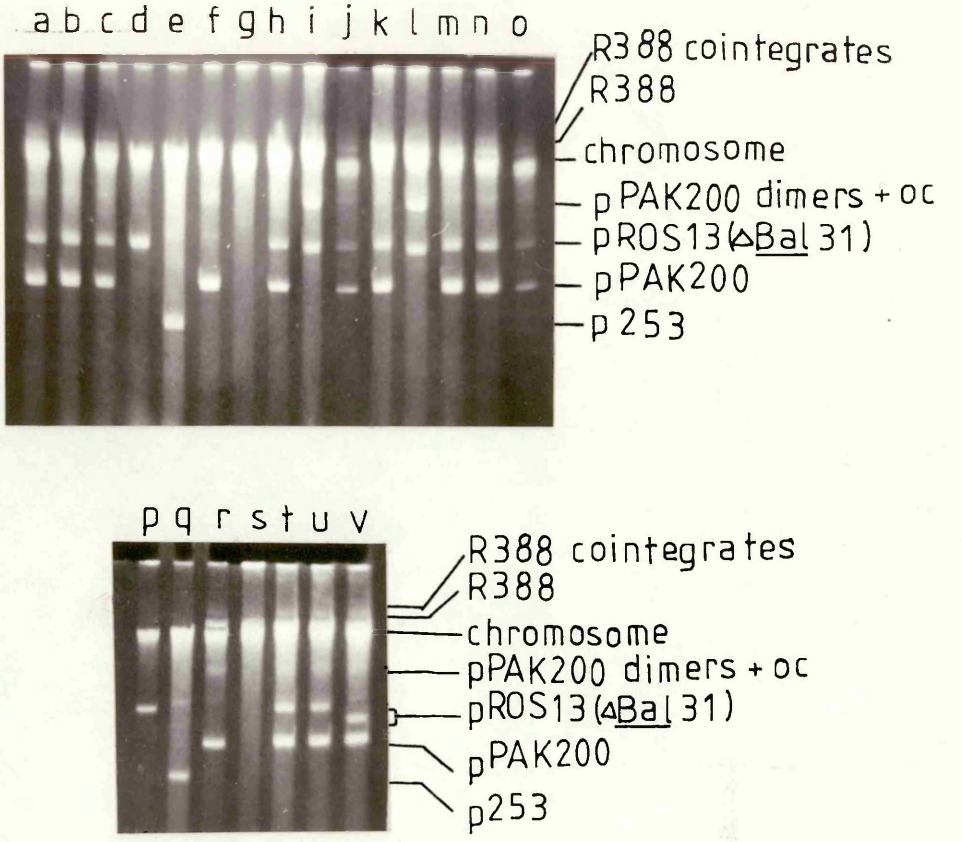


Fig. 3.8 DS903 (R388, pPAK200, pROS13ΔBal31) donors, showing the presence of all three plasmids in the strains.

a - c. Colonies showing a Cm^r Km^r phenotype

d. DS903 (pROS13) marker

e. DS903 (p253) 5.5kbp marker

f. DS903 (R388, pPAK200) marker

g. DS903 (R388) marker

h - o. Colonies showing a Cm^r $Km^{s/r}$ phenotype*

* i and l represent pROS16 and pROS17

p. DS903 (pROS13) marker

q. DS903 (p253) 5.5kbp marker

r. DS903 (R388, pPAK200) marker

s. DS903 (R388) marker

t - v. Colonies showing a Cm^s Km^s phenotype

Colonies mated to DS903 Sp^r	Frequency of Km^r transfer x 10^{-4}	Frequency of Cm^r transfer x 10^{-4}	Frequency of Km^r Cm^r transfer x 10^{-4}	Ratio Cm^r/ Km^r transfer	Ratio Cm^r/ Km^r Cm^r transfer	Ratio Km^r/ Km^r Cm^r transfer
ΔBal31* phenotype:						
Cm^s Km^s						
1	0	0	0	0	0	0
Cm^r Km^r						
2	62	800	2	13	400	41
3	180	1800	8	10	225	23
4	80	680	2	9	486	57
Cm^r $Km^{s/r}$						
5	33	630	2	19	315	16
6	19	540	2	28	360	13
7	40	580	1	15	414	29
8	44	530	2	12	279	23
9	62	960	5	16	218	14
Cm^r Km^s						
10	260	1700	7	7	243	37
11	160	1000	3	6	357	57
12	90	890	4	10	247	25
13	25	340	1	14	395	29
14	80	740	2	9	411	44
15	100	1060	2	11	530	50
16 (pROS16)	0	750	2	-	375	0
17 (pROS17)	0	390	1	-	390	0
Controls:						
DS903 (R388, pPAK200)	0	0	0	0	0	0
DS903 (R388, pPAK200, pROS12)	0	0	0	0	0	0
DS903 (R388, pPAK200, pROS13)	74	1300	5	18	260	15

Table 3.2 Results of plate matings of DS903 (R388, pPAK200, pROS13 ΔBal31) clones, and controls, to DS903 Sp^r. Transfer of Km^r implies Tn*3571* has transposed to R388; transfer of Cm^r implies Tn*3651* has transposed to R388; transfer of both markers implies Tn*3571* and Tn*3651* are present in a large cointegrate with R388 (see text). pROS16 and pROS17 appear to have been deleted into the LIR, leaving *tnpA* intact.
* The ΔBal31 phenotype is that obtained during replica-plate matings (see text).

colonies were Cm^r Km^s, it implied that the deletion in pROS13 had entered the LIR of Tn3571 thus rendering it unable to transpose, but had not destroyed the transposase activity. Therefore Tn3571 was still $TnpA^+$, and was able to complement the transposition of Tn3651 into R388. If the colonies were Cm^s Km^s, it implied that the deletion in pROS13 had removed the LIR of Tn3571 and had entered tnpA. This had abolished transposition of Tn3571 and Tn3651.

Of the 89 exconjugants, 25 were obviously Cm^r Km^r, 19 were Cm^r $Km^{r/s}$ (their phenotype was unclear), 8 appeared Cm^r Km^s, and 38 were obviously Cm^s Km^s. The procedure for ascertaining the Tn3571 phenotypes is outlined in Fig 3.7.

In order to confirm that the DS903 (R388, pPAK200, pROS13) donors had in fact contained all three plasmids, single colony gels were run on several of these (Fig. 3.8).

From the results of the replica-plate matings it seemed possible that either several colonies could have originally been double transformants with two deletants showing different phenotypes, or that another intermediate phenotype was possible, with some of those plasmids deleted into the LIR capable of transposition only at a very low level. Also it was possible that one-ended transposition (Arthur et al. 1984) was causing the intermediate phenotype, but the occurrence of some obviously Cm^r Km^s colonies made this unlikely. It was therefore decided to examine the phenotypes more carefully by repurifying the clones and repeating the matings of DS903 (R388, pPAK200, pROS13 Bal31) with DS903 Sp^r by carrying out ordinary plate matings. DS903 (R388, pPAK200), DS903 (R388, pPAK200, pROS12) and DS903 (R388,pPAK200, pROS13) were included as controls.

The results (Table 3.2) showed that the colonies with the apparently intermediate phenotype Cm^r $Km^{r/s}$ were in fact Cm^r Km^r. Of the 8 which had been marked Cm^r Km^s, only two actually had this phenotype on closer examination. The others were Cm^r Km^r. The two Cm^r Km^s colonies were of special interest, and the plasmids from them were called pROS16 and pROS17. The extent of their deletions has been determined (3.3.4).

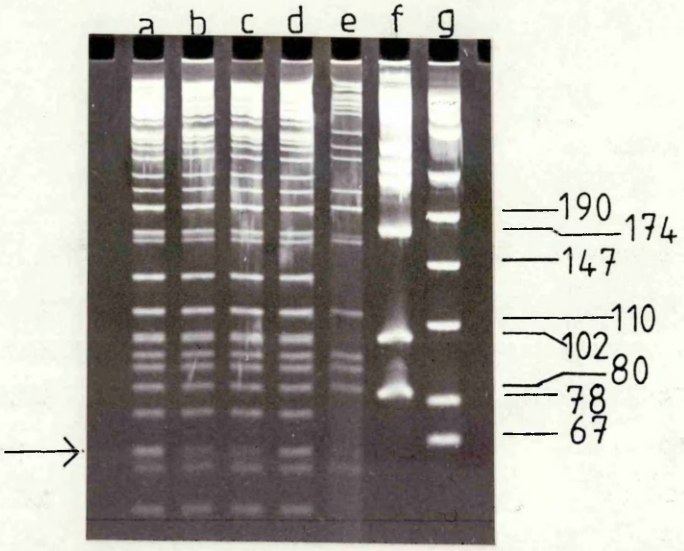


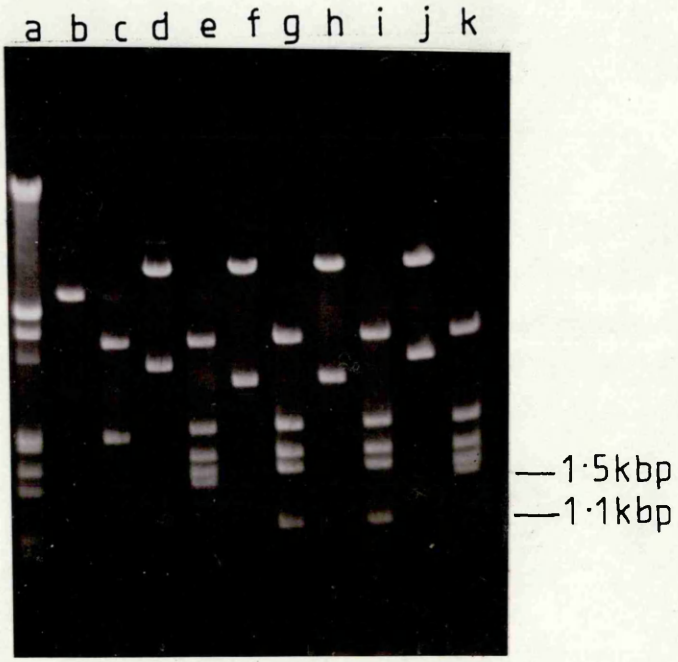
Fig. 3.9 DdeI digests to examine the extent of the deletions in pROS16 and pROS17. The lack of a doublet at the position indicated(→) suggests the deletion is within the LIR, or just within the tnpA gene in these two plasmids.

Fragment sizes are in bp.

a. Clone 5 (see Table 3.2). A doublet is still apparent at the position indicated.
b. pROS16. No doublet is apparent.
c. pROS17. No doublet is apparent.
d. pROS13 control. Doublet is apparent.
e. pROS12.
f. pUC8 HaeIII markers.
g. pUC8 HpaII markers.

Fig. 3.10 BstEII/ClaI digests.

a. λHindIII/EcoRI marker
b. pROS12 ClaI
c. pROS12 ClaI/BstEII
d. pROS13 ClaI
e. pROS13 ClaI/BstEII
f. pROS16 ClaI
g. pROS16 ClaI/BstEII
h. pROS17 ClaI
i. pROS17 ClaI/BstEII
j. Clone 5 (Table 3.2) ClaI
k. Clone 5 ClaI/BstEII



The results also indicated that Tn3651 was transferred on average 3-10 times more frequently than Tn3571 (see 3.4).

The Cm^r Km^r exconjugants were examined further to investigate whether they were growing due to the presence of two different R388 cointegrates within one cell, or to one giant, double cointegrate of R388 with pPAK200 and pROS13 . They were mated to DS903 by streaking on a DS903 lawn and selecting on Sm, Tp, and Cm. Later the colonies were screened for Km resistance. All grew on Km, implying that the Sp^r Tp^r Cm^r Km^r exconjugants from the mating of DS903 (R388, pPAK200, pROS13) with DS903 Sp^r were all due to giant double cointegrates.

DNA was prepared from DS903 (pROS16) and DS903 (pROS17), also from clone 5, and was digested with DdeI in order to see how far the deletions had proceeded towards/into Tn3571. There is a DdeI site starting at position 12 of Tn3571. The digests, together with controls, were run on a 5% polyacrylamide gel (Fig. 3.9). The important feature of this gel was that in the pROS16 and pROS17 tracks only a single band was visible at the point indicated by the arrow, of size 58bp, whereas in the pROS13 control, and in clone 5, the band appeared as a doublet of sizes 62bp and 58bp. The absence of this band in these two tracks strongly suggested the deletion had extended more than 11bp into the LIR.

3.3.4 Sequencing of the deletion end-points in pROS16 and pROS17.

To assist in interpretation of the end-binding and immunity experiments (3.4 and 3.5), knowledge of the precise location of insertion of Tn3571 into pROS12 was required, and the exact nature of the deletions into the LIR. Therefore all three deleted and non-deleted junction sites between Tn3571 DNA and pROS12 DNA were sequenced by the method of Sanger et al. (1977). The sequencing of pROS16 and pROS17 is described below. pROS13 was sequenced later (3.3.6.).

The plasmids pROS12, pROS13, pROS16 and pROS17 were digested with BstEII/ClaI (Fig. 3.10). There is a BstEII site at position 88 of Tn3571, and a cut at this site gives a 5bp 5' overhang. The ClaI site in pROS13 is in the Km resistance gene. On the gel in the case of

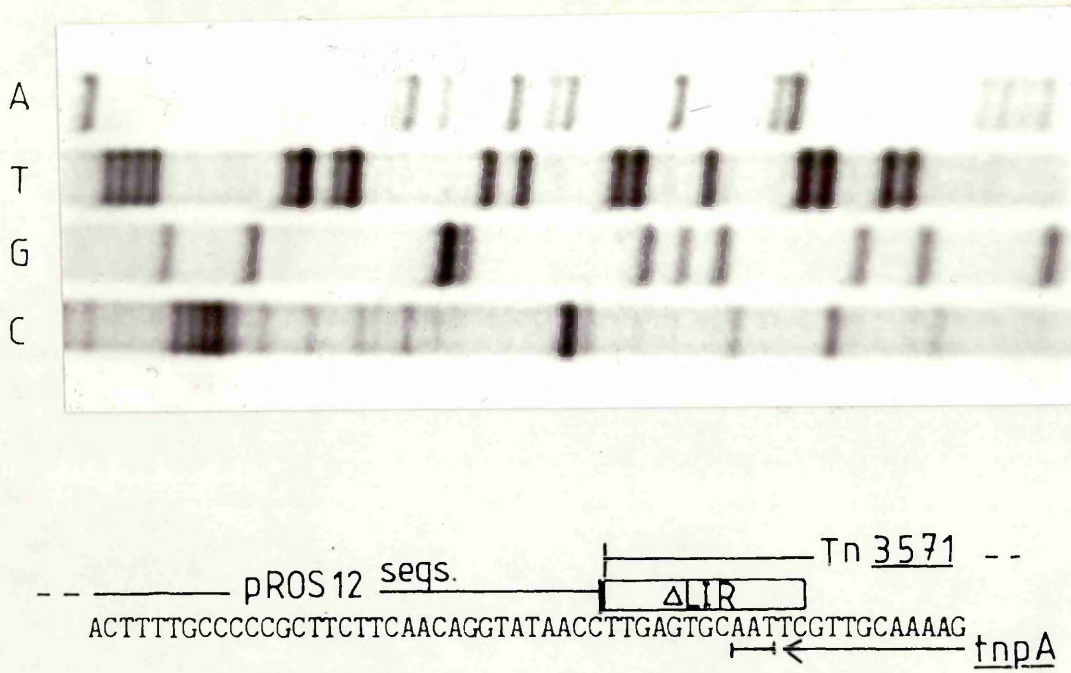


Fig. 3.11 pROS16/pROS17 deletion junction sequence.

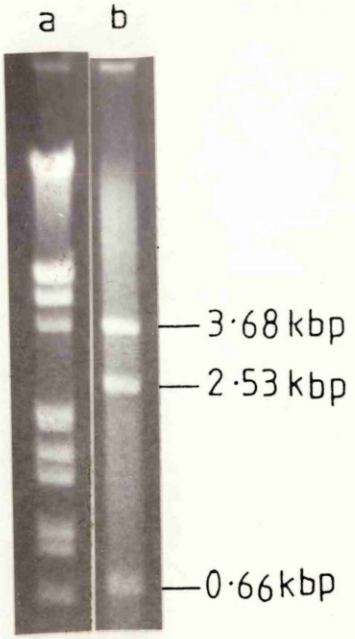


Fig. 3.12a pROS19 PstI digest.

a. λHindIII/EcoRI digest.
b. pROS19 Pst I digest.

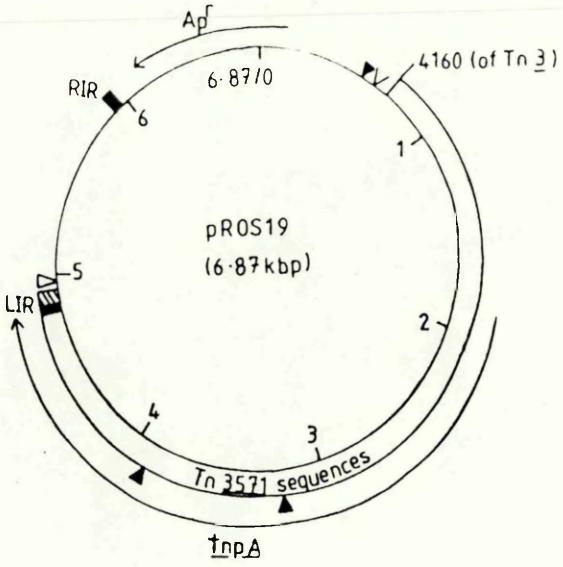


Fig. 3.12b pROS19, showing the Tn3571 sequences present. The construction is described in the text. The presence of the LIR and RIR of Tn3571 in inverted orientation means that part of the plasmid is able to transpose.

▼ PstI sites
▽ EcoRI site
⋁ BamHI site
▨ pROS12 sequences

pROS13 a 1.5bp band was present which had been deleted in pROS16 and pROS17 to a size of about 1.1kbp. The size of the deletion was therefore about 400bp, which meant that the end point was probably within the LIR, or that only a very few tnpA base pairs had been removed. It was decided to sequence the deletion end-points of pROS16 and pROS17 using the 1.1kbp fragment. In order to clone this fragment into SmaI/AccI-cut M13mp19, the 5bp BstEII overhang had to be filled-in. This was done after BstEII digestion, before cutting with ClaI. The purified fragment was then ligated to SmaI/AccI-cut M13mp19 and the DNA was transformed into JM101. Clear plaques were purified and part of the inserts were sequenced.

Both pROS16 and pROS17 were found to be identical in the junction sequence over the deletion end-points (Fig.3.11). The most likely explanation is that they were siblings formed during the expression step in the transformation after Bal31 deleting. This conclusion is supported by the gel in Fig. 3.8, in which pROS16 and pROS17 both occur with pPAK200 as a dimer, in contrast to the other pROS13 plasmids examined.

It was interesting that the Bal31 had only deleted in one direction, towards the LIR of Tn3571, from the BalI site. Asymmetry is often observed during Bal31 digestion (Silhavy et al. 1984). pROS16 was used in further constructions and experiments. The plasmid is deleted for 422bp compared to pROS13 and it lacks 25bp of the Tn3571 LIR. This means that Tn3571 sequences only continue for 8bp after the tnpA stop codon.

3.3.5 Construction of a λdv plasmid carrying Tn3571 lacking the RIR

To remove the right end of Tn3571 from the rest of the transposon, the following strategy was adopted:

pROS13 was digested with DraI and the 4.435bp fragment containing all of the left-hand side of Tn3571 up to position 4160 in bla, was purified. pUC8 was cut with SmaI and treated with CIP, and the 4.435bp fragment was ligated in. The DNA mixture was transformed into JM101 and clear plaques were picked and examined for inserts. DNA from colonies carrying the correct size plasmid was prepared and digested with PstI (Fig. 3.12a) to determine the orientation of

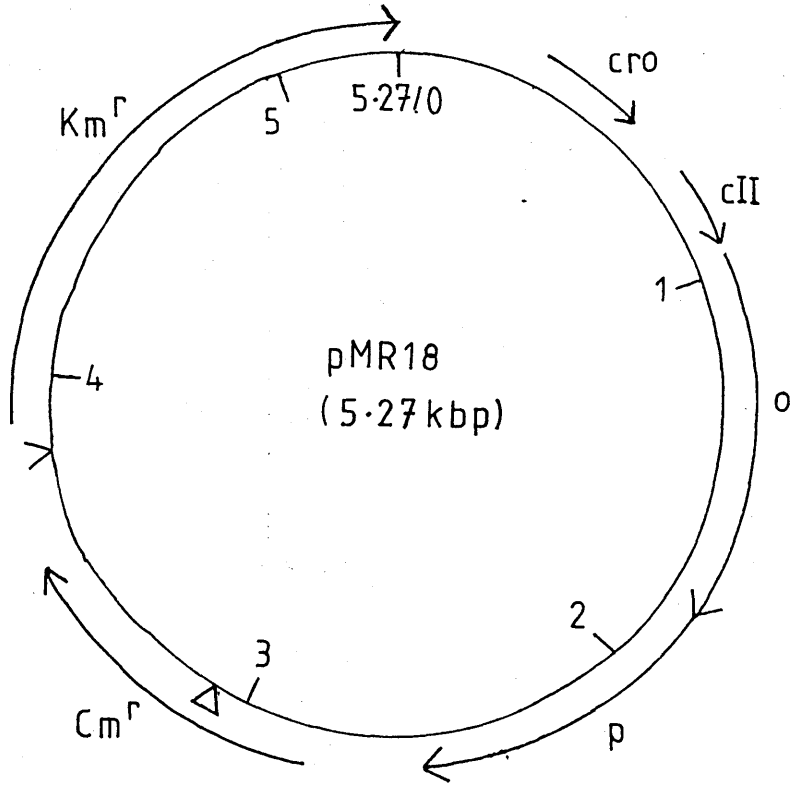


Fig. 3.13 pMR18: λdvΔ25Δ1 (Boyd, Glasgow) with the 1.43kbp HaeII fragment from Tn903 inserted at the HaeII site.

⋁ BclI site
▽ EcoRI site

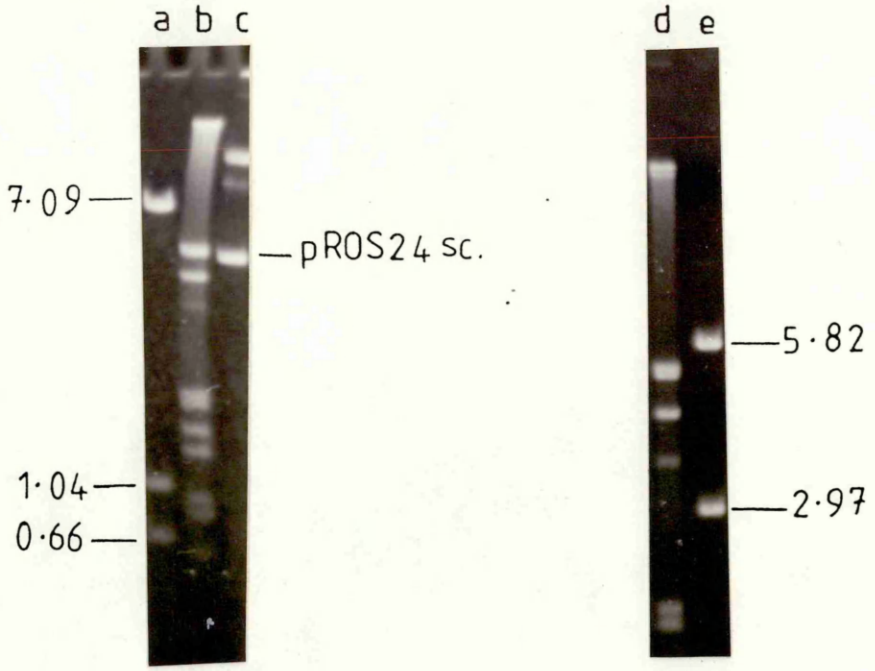


Fig. 3.14a pROS24 digests

Fragment sizes are in kbp

a. pROS24 PstI/EcoRI digest

b. λ HindIII/EcoRI digest

c. pROS24 BalI (no site should be present)

d. λ HindIII/EcoRI digest

e. pROS24 SmaI digest

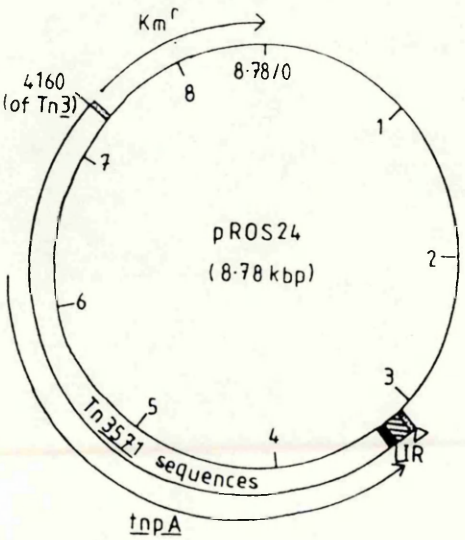


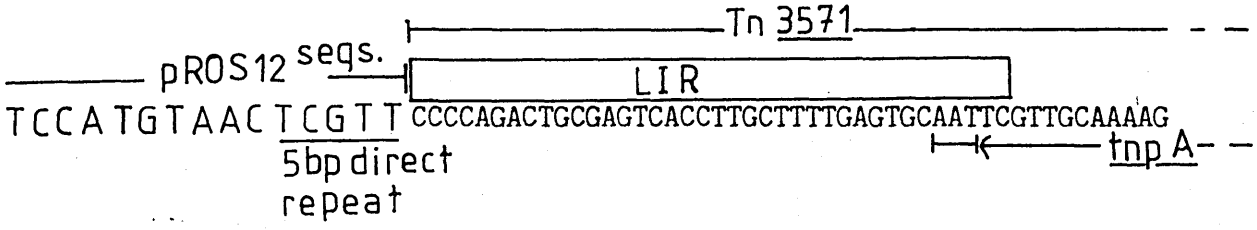
Fig. 3.14b pROS24, showing the Tn3571 sequences present. The construction is described in the text.

▽ EcoRI site

▨ pROS12 sequences

⁘ pUC8 sequences

Fig. 3.15a Sequence of the junction point between Tn3571 DNA and pROS12 DNA in pROS13.



insertion of the fragment. The plasmid was called pROS19 and has the structure shown in Fig. 3.12b.

pROS19 carries Tn3571 sequences from 1-4160. It also carries the Tn3 bla gene and RIR on the pUC8 part of the plasmid. The orientation of the two IRs in this plasmid is such that transposition of the Tn3571 sequences and part of pUC8 can occur. This plasmid was examined for the transposability of this fragment and transposition was found to occur to R388 at 37°C at a frequency of 1.5×10^{-2}. This is the normal level for a derepressed tnpA gene with two IRS at this temperature (see Table 3.1).

In order to separate the Tn3571 sequences, 1-4160, from the pUC8-carried RIR, and to replace them on a λdv construct again, a subcloning was performed: pROS19 was digested with BamHI and EcoRI and the 4.221kbp fragment was purified. pMR18 (Rogers 1986; Fig. 3.13) is a Km^r derivative of λdvΔ25Δ1 which is a λdv construct very similar to pROS12 (Fig. 3.5). pMR18 was introduced to a Dam^- host, CB51, and DNA was made from this. This DNA contains an unmethylated BclI site. Cleavage of unmethylated pMR18 with EcoRI and BclI gives two fragments, one of about 700bp and one of about 4.5kbp. The latter fragment was ligated to the 4.221kbp BamHI/EcoRI fragment from pROS19. The resulting construct, called pROS24, is shown in Fig. 3.14b with confirming restriction patterns (Fig. 3.14a). pROS24 is a Km^r λdv construct containing sequences 1-4160 of Tn3571.

3.3.6 Sequencing of the site of insertion of Tn3571 in pROS13

The plasmid pROS19 was digested with PstI and EcoRI and the approximately 1.05kbp fragment was purified and cloned into PstI/EcoRI-cut M13mp19. The resulting mixture was transformed into JM101 and clear plaques were purified and prepared for sequencing by the method of Sanger (1977). The junction sequence of Tn3571 with pROS12 DNA is shown in Fig. 3.15a. The 5bp target sequence adjacent to the LIR is almost certainly directly repeated adjacent to the RIR.

The generation of short target sequence duplications at the site of insertion is characteristic of IS elements and transposons (Grindley and Sherratt 1979). This has been taken as an indication that a

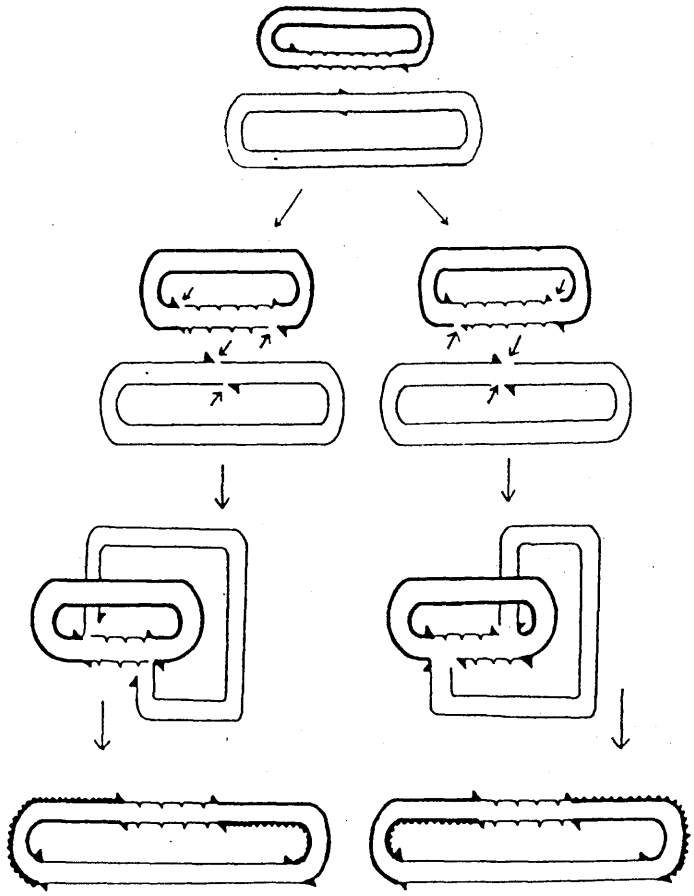


Fig. 3.15b Two possible polarities of the strand transfer reaction (Mizuuchi 1984).

Left: 3' end cutting of the transposon in the donor and 5' protruding staggered cuts of the target lead to a cointegrate in which the upper strand of the left-hand copy of the transposon and the lower strand of the right-hand copy are the newly synthesized strands. For Mu this is the correct polarity.

Right: 5' end cutting of the transposon in the donor and 3' protruding staggered cuts of the target lead to a cointegrate in which the lower strand of the left-hand copy of the transposon and the upper strand of the right-hand copy are the newly synthesized strands.

donor plasmid
transposon DNA
recipient plasmid
newly synthesized DNA strands

Fig. 3.16a pROS21 PstI digest.

a. λ HindIII/EcoRI digest.
b. pROS21 PstI digest.

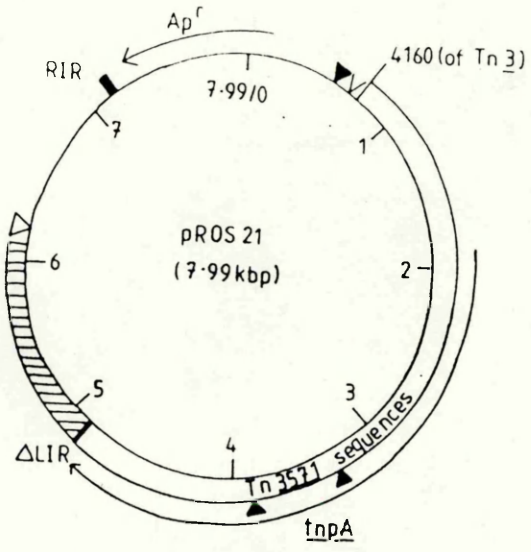


Fig. 3.16b pROS21, showing the Tn3571 sequences present. The construction is described in the text.

▼ PstI sites
▽ EcoRI site
⋁ BamHI site
▨ pROS12 sequences

staggered cut is made in the target DNA and that its protruding ends are joined to the ends of the transposing element during the process of transposition. Models propose that the donor molecule is cut on one strand at each end of the transposable element and the free single-strand terminus at each end of the element is joined to the protruding strand terminus of the target DNA. This generates a set of branched structures with the properties of replication forks from which replication can initiate. A full round of replication produces a cointegrate molecule (1.3 Arthur and Sherratt 1979; Shapiro 1979).

The initial strand transfer event could have one of two possible polarities. But the polarity will be one way or the other for each transposon. For Mu, the polarity is known (Mizuuchi 1984). In the case of Mu, the target site is cut to give 5' overhangs. The transposon sequences are cut at the 3'end. The 5'target overhangs are joined to the 3'ends of the transposons. The two possible polarities giving rise to the insertion of Tn3571 into pROS12 are shown in Fig. 3.15b.

Although a preference for AT-rich sites has been reported to exist for Tn3 (1.6.3; Heffron 1979; Tu and Cohen 1980; Picken et al. 1984), this case of insertion into pROS12 does not seem to show any preference of this type.

3.3.7 Construction of a λdv plasmid carrying Tn3571 deleted for the LIR and lacking the RIR

The plasmid pROS16 (3.3.3 and 3.3.4) was digested with DraI and SmaI and the 5.326kbp fragment containing Tn3571 sequences from 26 - 4160 was purified and ligated to pUC8 cut with SmaI and treated with CIP. The DNA mixture was transformed into JM101 and white colonies were picked and examined for inserts. DNA from colonies carrying the correct size plasmid was prepared and digested with PstI (Fig. 3.16a) to determine the orientation of insertion of the fragment. The plasmid was called pROS21 and has the structure shown in Fig. 3.16b. pROS21 carries the bla gene and RIR of Tn3 as part of the pUC8 sequences.

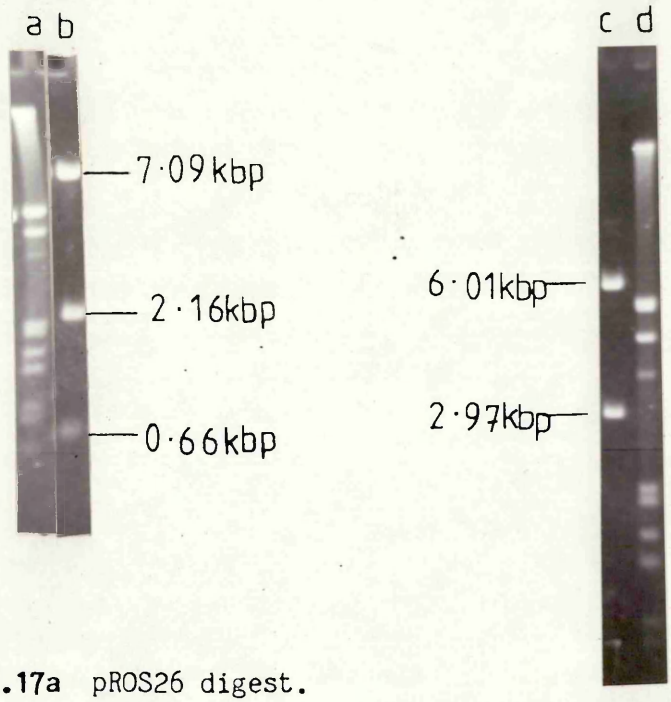


Fig. 3.17a pROS26 digest.

a. λ HindIII/EcoRI digest
b. pROS26 PstI/EcoRI digest

c. pROS26 SmaI digest
d. λ HindIII/EcoRI digest

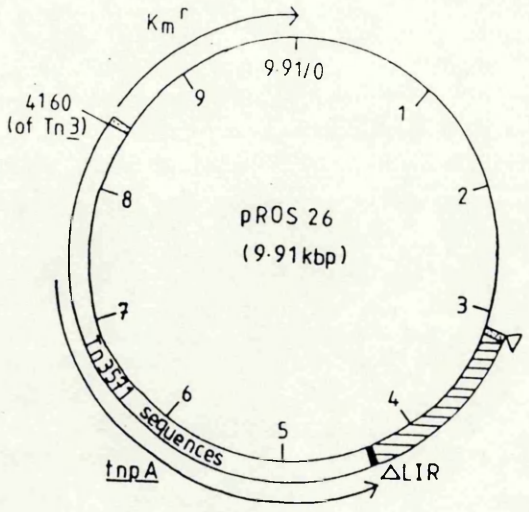


Fig. 3.17b pROS26, showing the Tn3571 sequences present. The construction is described in the text.

▽ EcoRI site
▨ pROS12 sequences
▩ pUC8 sequences

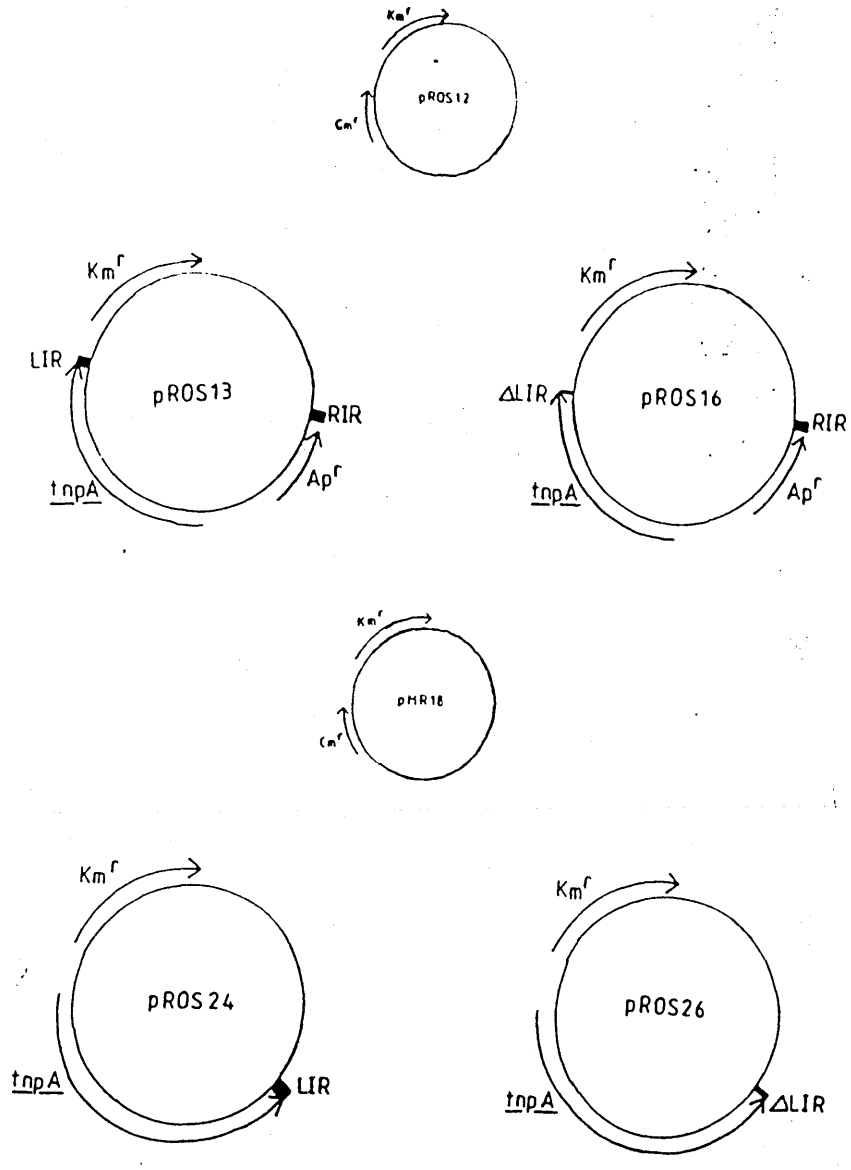


Fig. 3.18 Diagram showing the important features of the set of six plasmids used in 3.4 and 3.5.

Donors	IRs	Frequency of Km^r transfer x 10^{-4}	Frequency of Cm^r transfer x 10^{-4}	Frequency of Km^r Cm^r transfer x 10^{-4}	Ratio Cm^r/ Km^r	Ratio Cm^r/ Km^r Cm^r	Ratio Km^r/ Km^r Cm^r
DS903 (R388, pPAK200)	-	0 0	0 0	0 0	- -	- -	- -
DS903 (R388, pPAK200, pROS12)	-	0 0	0 0	0 0	- -	- -	- -
DS903 (R388, pPAK200, pROS13)	LIR RIR	25 8	140 64	0.4 0.1	6 8	378 638	68 84
DS903 (R388, pPAK200, pROS16)	RIR	0 0	250 91	1.9 0.2	- -	131 505	0 0
DS903 (R388, pPAK200, pMR18)	-	0 0	0 0	0 0	- -	- -	- -
DS903 (R388, pPAK200, pROS24)	LIR	0 0	99 68	0.2 0.1	- -	521 1360	0 0
DS903 (R388, pPAK200, pROS26)	-	0 0	290 110	4.8 4.0	- -	60 275	0 0

Table 3.3. Results of transposition assays 1 and 2 performed at 30°C. DS903 (R388, pPAK200, λdv plasmid) donors were mated to DS903 Sp^r recipients. The transfer of resistance markers has the same implications as in Table 3.2. Only intact IRs are noted.

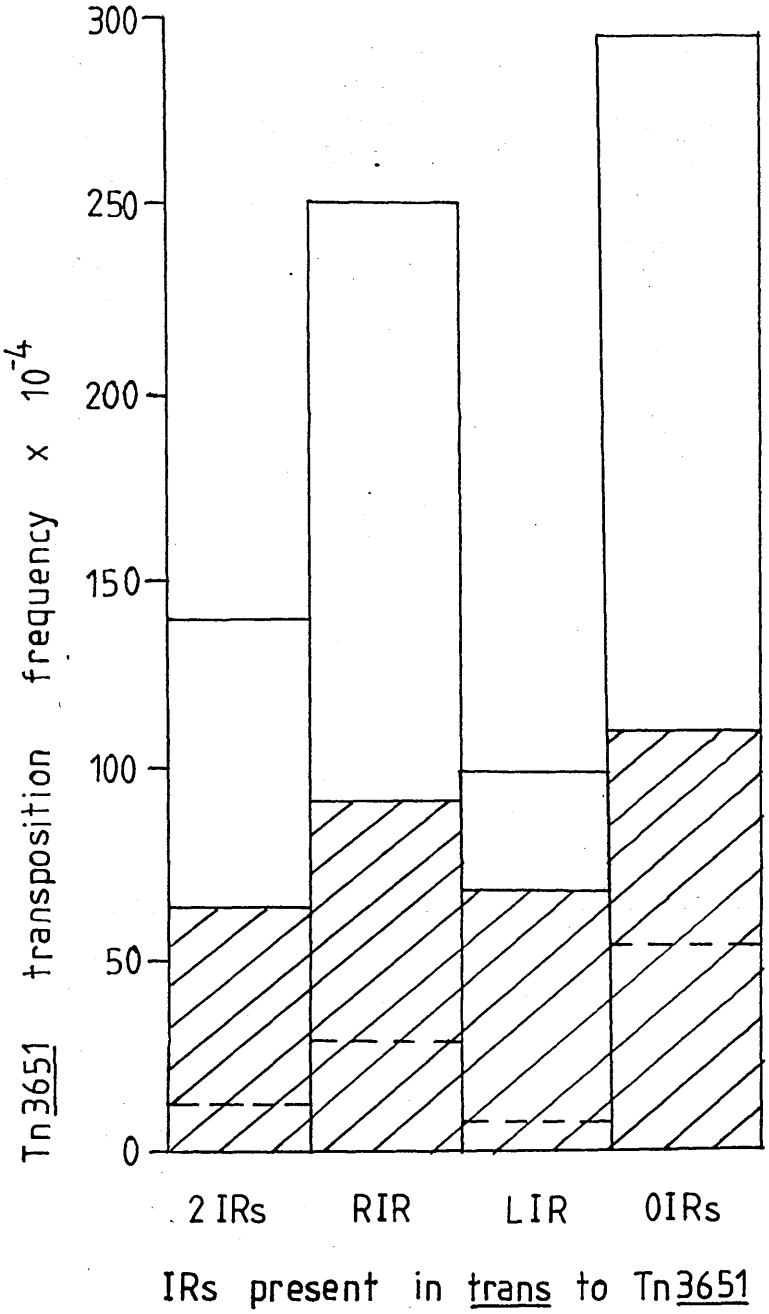


Fig. 3.19 The transposition frequency* (Cm^r transfer) of Tn3651 onto R388 in the presence of λdv plasmids carrying 2IRs, the LIR, the RIR, and no IRs.

Assay 1
Assay 2
--- Assay 3

* In the case of assays 1 and 2 this is per 20 - 30 generations at 30°C. In the case of assay 3, the frequency is per 20 - 30 generations at 42°C, with 1.5 doubling times at 30°C.

In order to separate the Tn3571 sequences, 26-4160, from the pUC8-carried RIR and to replace them on a λdv construct again, a subcloning was performed: pROS21 was digested with BamHI and EcoRI and the 5.336kbp fragment was purified. pMR18 DNA from a Dam⁻ host (3.3.5) was cleaved with EcoRI and BclI and the 4.5bp fragment was ligated to the 5.336kbp BamHI/EcoRI fragment from pROS21. The resulting construct, called pROS26, is shown in Fig. 3.17b, with confirming restriction patterns (Fig. 3.17a). pROS26 is a Km^r λdv construct containing sequences 26-4160 of Tn3571.

3.4 The effect of λdv-based plasmids containing no IRs, the LIR, the RIR, or both IRs of Tn3 on the transposition of Tn3 present in *trans*

A set of six plasmids was used in the following assays to provide different IRs in *trans* to pPAK200. Their construction has been described (3.3). The important features of these plasmids are shown in Fig. 3.18.

In assays 1 and 2, DS903 (R388, pPAK200) was freshly transformed with the six constructs and the transformants were grown overnight at 37°C. The following night 2.5ml cultures of each strain were grown at 30°C from a pool of about 200 transformants. The next morning 200ul of these cultures was used to inoculate 20ml L-broth. Selection for all plasmids was maintained throughout these stages. These cultures were grown at 30°C to an OD_{600} of 0.5 and the DS903 Sp^r recipients were grown at 37°C to an OD_{600} of 1.0. Then donors and recipients were spun down, before being mated at 37°C for 2.5 hours. Exconjugants were plated out on plates containing Sp Tp, Sp Tp Cm, Sp Tp Km, or Sp Tp Cm Km and the plates were incubated at 37°C overnight. The following day transposition frequencies were calculated (Table 3.3 and Fig. 3.19).

The purpose of assay 3 was to see if exposing the cells to 30°C, from 42°C, for approximately one doubling time would give rise to the same frequency of transposition as growing the cells at 30°C for 30 doubling times, as in assays 1 and 2 i.e. to see if the effect of temperature on transposition was immediately reversible, or whether growth into stationary phase was necessary for the level to increase to that obtained by growing the cells for many generations at 30°C.

Donors	IRs	Frequency of Km^r transfer x 10^{-6}	Frequency of Cm^r transfer x 10^{-4}	Frequency of Km^r Cm^r transfer x 10^{-7}	Ratio Cm^r/ Km^r	Ratio Cm^r/ Km^r Cm^r	Ratio Km^r/ Km^r Cm^r
DS903 (R388, pPAK200)	-	0	0	0	-	-	-
		0	0	0	-	-	-
DS903 (R388, pPAK200, pROS12)	-	0	0	0	-	-	-
		0	0	0	-	-	-
DS903 (R388, pPAK200 pROS13)	LIR	260	12	41	5	293	63
	RIR	NA*	NA	NA	NA	NA	NA
DS903 (R388, pPAK200, pROS16)	RIR	5	29	16	644	1812	3
		1	3	4	291	864	3
DS903 (R388, pPAK200, pMR18)	-	0	0	0	-	-	-
		0	0	0	-	-	-
DS903 (R388, pPAK200, pROS24)	LIR	1	8	1	806	6818	8
		0	1	0	-	-	-
DS903 (R388, pPAK200, pROS26)	-	24	53	12	221	4417	20
		2	2	1	112	3800	34

Table 3.4 Result of transposition assay 3. For each strain the top row of numbers refers to the cultures exposed to 30°C, from 42°C, for 1.5 doubling times. The bottom row refers to the cultures kept at 42°C throughout the experiment. DS903 (R388, pPAK200, λdv plasmid) donors were mated to DS903 Sp^r recipients. The transfer of resistance markers has the same implications as in Table 3.2. Only intact IRs are noted. *Not available.

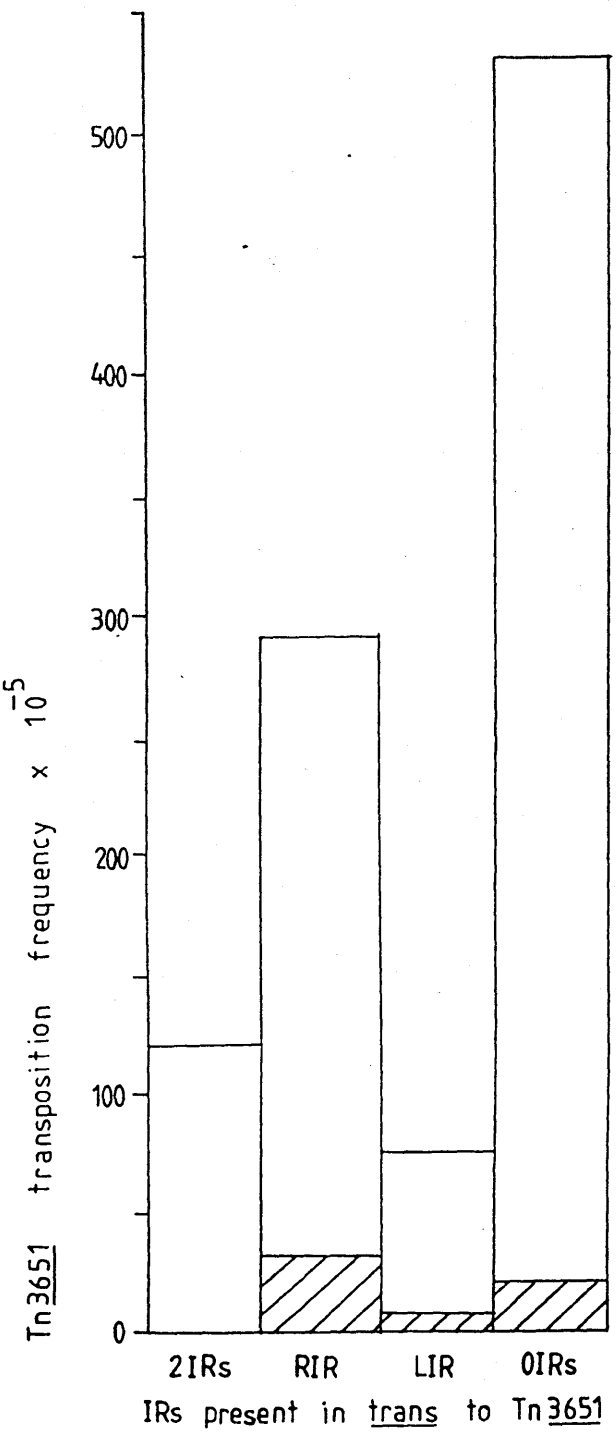


Fig. 3.20 The transposition frequency (Cm^r transfer) of Tn3651 onto R388 in the presence of λdv plasmids carrying 2IRs, the LIR, the RIR, and no IRs.

□ Grown 1.5 doubling times at 30°C, the rest at 42°C.

▨ Grown at 42°C continuously.

OD_{600}	Number of cells/ml	Calculated number of cells/ml at an OD_{600} of 1.0
0.089	6.0×10^7	6.8×10^8
0.187	4.8×10^7	2.6×10^8
0.279	6.0×10^7	2.2×10^8
0.408	3.7×10^8	9.4×10^8
0.430	1.7×10^8	4.0×10^8
0.657	3.9×10^8	5.9×10^8
1.000	5.1×10^8	5.1×10^8

Table 3.5. The data show that the number of cells per ml is directly proportional to OD_{600} in the OD_{600} range 0.1 - 1.0. These results were obtained from viable counts of DS903 at the OD_{600} shown. They may vary slightly for other strains.

The experiments of Kretschmer and Cohen (1979; Ch. 4) had shown the effect to be reversible, but had not determined the minimum time-scale necessary. The effect of temperature on transposition is discussed more fully in chapter 4.

In assay 3, DNA was added to DS903 (R388, pPAK200) competent cells and these were heat-shocked at 42°C for 2 minutes. The cells were put on ice for 20 minutes before adding 1ml of 42°C L-broth and expressing for 1.5 hours at 42°C. Then they were spun down in a rotor pre-warmed to 42°C. They were resuspended in 42°C phage buffer and plated out on pre-warmed plates containing the relevant antibiotics. The transformants were grown overnight at 42°C. The following night, 2.5ml cultures of each strain were grown at 42°C from a pool of about 200 transformants. The next morning 200ul of cells was used to inoculate 20ml L-broth. These cultures were grown at 42°C. Selection for all the plasmids was maintained throughout these stages.

When the cultures were clearly turbid, they were divided into two 10ml lots. The OD_{600} of one of these was read before it was placed into a 30°C shaker. The other was kept at 42°C.

After each of the 30°C cultures had approximately trebled in OD, it was removed from the 30°C shaker and spun down. At the same time the OD_{600} of the other half of each culture at 42°C was noted and it was also spun down. From each culture approximately 1×10^8 donor cells were mated with 5×10^8 recipients at 42°C for 3 hours. The exconjugants were plated out on plates containing Sp Tp, Sp Tp Cm, Sp Tp Km, or Sp Tp Cm Km. The plates were incubated overnight at 37°C and the following day transposition frequencies for the cultures grown either completely at 42°C, or mainly at 42°C with a brief spell at 30°C, were calculated. For results see Table 3.4 and Fig. 3.20.

During the time of the 30°C exposure of about 2 hours, the 30°C cultures roughly trebled in OD_{600}. Therefore the cultures underwent approximately 1.5 doublings. During this same amount of time, the 42°C cultures underwent approximately 2 doublings. This assumes that the number of cells is directly proportional to OD_{600} over the OD_{600} range used (0.035 - 0.35). That this assumption is reasonable is shown by the data in Table 3.5.

The results of assays 1, 2, and 3 showed a pattern (Figs. 3.19 and 3.20). In each case the transposition frequency of Tn3651 onto R388 was greatest in the strain carrying pROS26 (no IRs), and least in the strains carrying pROS24 (LIR) or pROS13 (LIR and RIR). The strain carrying pROS16 (RIR) showed an intermediate level.

It is important to note that the highest and lowest levels of Tn3651 transposition only varied by 2- to 5-fold, although only a small difference was to be expected (see below). This relatively small difference means that any conclusions from these assays must be tentative, although the pattern seen is repeatable (as in assays 1 and 2), and can be observed under different conditions (assay 3).

The simplest explanation for the observed results is that the number of ends present on the λdv plasmids does slightly affect the transposition frequency of Tn3651. The presence of two ends, or the LIR, depresses the frequency, and the absence of any ends alleviates this depression. The presence of only the RIR shows an intermediate effect.

If this explanation is correct, then it is obvious why only a small difference between the highest and lowest levels of transposition was to be expected. The copy number of the λdv plasmids is approximately one third that of pPAK200 (Fig. 3.8). So, removal of both of the IRs from pROS13 to give pROS26 only reduces the number of ends in the cell to which transposase can bind by about one quarter. This then only increases the level of transposase available for end-binding by a third relative to Tn3651. Hence the transposition frequency of Tn3651 should only increase by a third. Similarly, removal of one IR probably only releases about one eighth more transposase for end-binding and hence can only increase the transposition frequency of Tn3651 by about a sixth compared to the case with pROS13.

However, it can be seen that the differences observed, of 2- to 5-fold, are actually greater than would be expected bearing in mind copy number differences. It appears that an approximately 25% reduction in the number of ends leads to an approximately two and a half-fold increase in Tn3651 transposition. A possible reason for this is suggested by the result with pROS24 (LIR), as opposed to with pROS16

(RIR), where it appears that the presence of the LIR represses transposition of Tn3651 more than the RIR. [This differential effect of the LIR has been observed before (Heritage and Bennett 1984).] This could be explained by the transposase binding more rapidly after synthesis to the LIR in cis than to any other IRs in the cell. This is not unlikely as the tnpA gene is much closer to this end, and actually terminates within it. Hence the LIR in cis might be occupied more often than any other end and might sequester more of the limiting transposase than the other ends. Alternatively, some sequences within the tnpA gene could enhance binding to the LIR, and all LIRs in the cell adjacent to these enhancing sequences might be occupied more often than the RIRs. This would mean that removal of the LIR might perhaps release more of the cell's transposase than if that end were equally likely to be bound as all others in the cell. So removal of the LIR, leaving the RIR, or removal of both ends, might lead to a greater increase in transposition than would otherwise be expected.

If this simple explanation is correct, then it appears possible that the ends of Tn3 can act as sinks in vivo for the binding of transposase, with the LIR having a special effect. It also suggests that the transposase is limiting in cells under these conditions, even in the absence of repression by resolvase. If this were not the case then removing two, or one, ends would not have any effect on the transposition level of Tn3651.

In retrospect, the assays would have been better conducted with TnpA$^-$ end constructs being on a higher copy number vector than that carrying a TnpA$^+$ transposon with intact ends. If the hypothesis outlined above is correct then a higher ratio of ends to limiting transposase should result in a more significant effect.

The fact that some Km^r Cm^r exconjugants were detected with not only pROS13, but also pROS16, and pROS24 can be explained by the occurrence of one-ended Tn3571 transposition into R388 (1.3; Arthur et al. 1984), or by the transposition of Tn3651 occurring first into the λdv vector, and then into R388. The latter is probably the explanation for the Km^r Cm^r colonies seen with pROS26. Interestingly, more Km^r Cm^r colonies were found with pROS26 than in the other cases. This may be due to the lack of any immunity to Tn3651 in this plasmid(3.5).

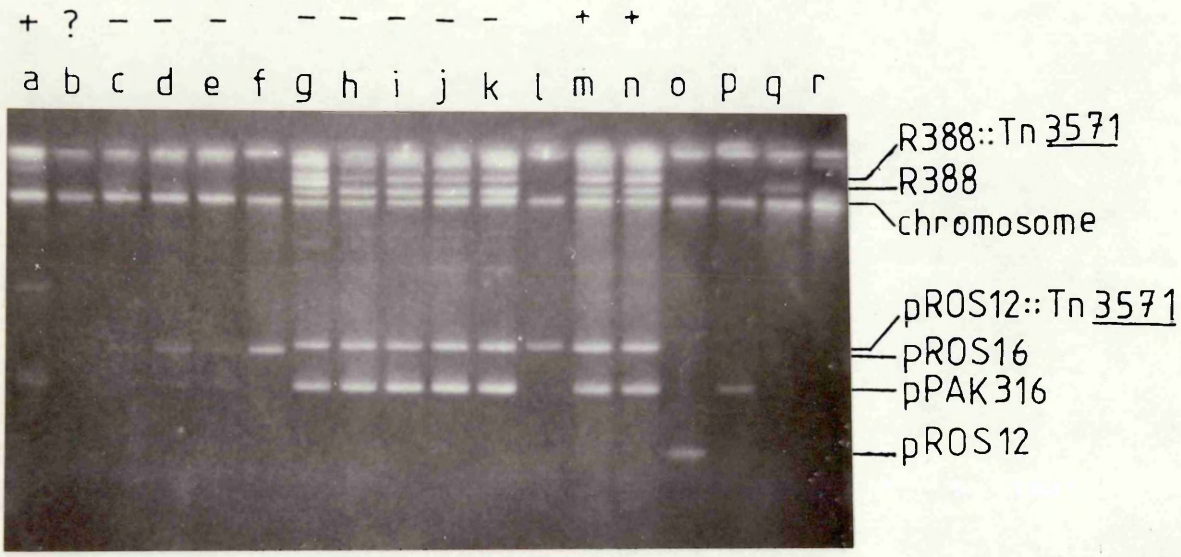
It was noticed again (see 3.3.3) that Tn3651 was transferred about five times more frequently than Tn3571. As the copy number of the λdv plasmids carrying Tn3571 is about one third that of pPAK200 carrying Tn3651, this difference is not surprising, but it may be somewhat on the high side. This could be due to sequence (4.1.5) or environmental (e.g. supercoiling) differences around the two Tn3 derivatives. Therefore, although the numbers of IRs in a cell may affect the transposition frequency, other factors may also play a part.

The third assay showed that the transposition frequency obtained during one and a half doubling times at 30°C was dramatically increased over that seen at 42°C, suggesting that the repressive effect of temperature on transposition can be reversed within only one and a half doubling times. The effect of temperature on transposition is discussed more fully in chapter 4.

3.5 The transposition immunity of λdv-based plasmids containing no IRs, the LIR, the RIR, or both IRs of Tn3

DS916 (R388::Tn3571) was mated with DS903 containing pROS12, pROS13, pROS16, pMR18, pROS24 or pROS26 (Fig. 3.18). The exconjugants were grown up overnight and the following day 50 - 200 colonies of each strain were grown up and mated to DS903 Sp^r. Exconjugants were selected on Sp Tp Ap, or Sp Tp Ap Km and transposition frequencies were calculated.

The observed transposition frequencies of $2 - 6 \times 10^{-2}$ suggested that no immunity was present although pROS13 at least, which contains the whole of Tn3571, was expected to be immune (1.6.3). [A drop in frequency of 10 to 10^6-fold was expected if the plasmids were immune (Heffron 1983; Lee et al. 1983). The extent of immunity varies considerably between experiments (Arthur et al. 1984).] The explanation for the anomaly turned out to be that DS903, which is recF but not recA, allowed a significant level of homologous recombination between the Tn3571 sequences on R388 and on λdv. This meant that the number of Sp^r Tp^r Ap^r Km^r exconjugants did not represent only transpositions of Tn3571 from R388 into the λdv vectors, but also the number of homologous recombinations between them. Therefore the immunity assay had to be modified:



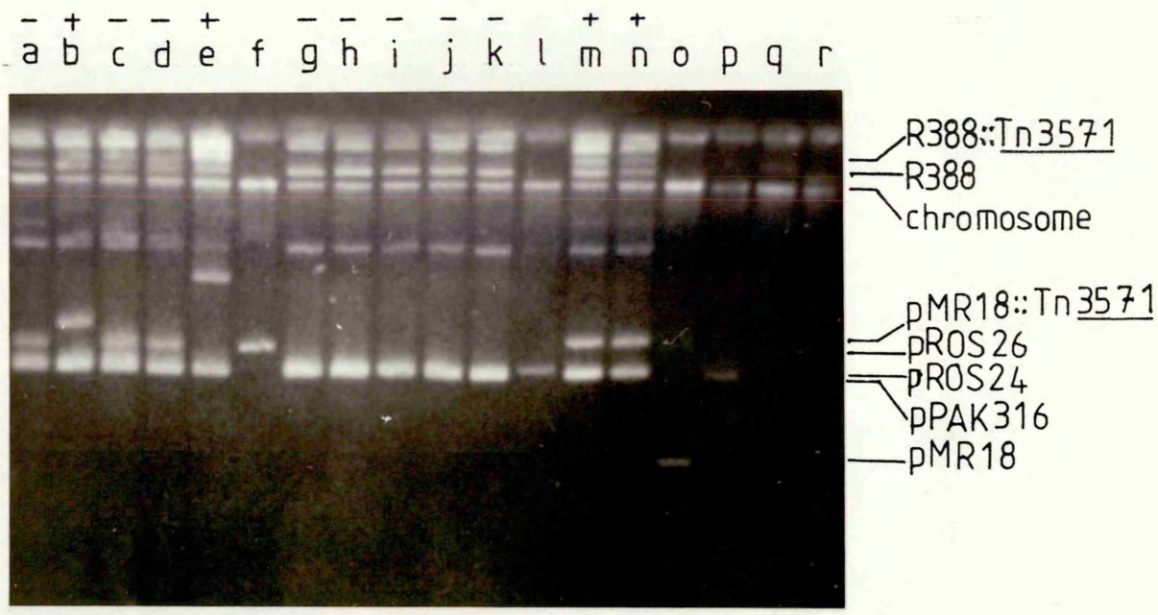
+ ⇒ tranposition

- ⇒ homologous recombination

Fig. 3.21 Sample gels showing the result of the immunity assays described in 3.5.

Gel 1.

a - e. DS903 Sp^r (R388::Tn3571, pPAK316, pROS16). RIR intact only.
f. DS903 Sp^r(pROS16).
g - k. DS903 Sp^r(R388::Tn3571, pPAK316, pROS13). LIR and RIR intact.
l. DS903 Sp^r(pROS13).
m, n. DS903 Sp^r(R388::Tn3571, pPAK316, pROS12). No IRs.
o. DS903 Sp^r(pROS12).
p. DS903 Sp^r(pPAK316).
q. DS903 Sp^r(R388::Tn3571)
r. DS903 Sp^r(R388)



+ ⇒ transposition

– ⇒ homologous recombination

Gel 2.

a - e. DS903 Sp^r (R388::Tn3571, pPAK316, pROS26). No IRs.

f. DS903 Sp^r (pROS26).

g - k. DS903 Sp^r (R388::Tn3571, pPAK316, pROS24). LIR only.

l. DS903 Sp^r (pROS24).

m, n. DS903 Sp^r (R388::Tn3571, pPAK316, pMR18). No IRs.

o. DS903 Sp^r (pMR18).

p. DS903 Sp^r (pPAK316).

q. DS903 Sp^r (R388::Tn3571)

r. DS903 Sp^r (R388).

Plasmid tested	IRs	Number of homologous recombination events		Number of transposition events		Immunity
pROS12	-	0	(0%)	19	(100%)	-
pROS13	LIR RIR	18	(100%)	0	(0%)	+
pROS16	RIR	17	(81%)	4	(19%)	(+)
pMR18	-	0	(0%)	12	(100%)	-
pROS24	LIR	16	(100%)	0	(0%)	+
pROS26	-	10	(59%)	7	(41%)	(-)

Table 3.6. Combined results of two separate assays on the immunity of various plasmids bearing different IR combinations. Only intact IRs are noted.

The six DS903 λdv strains containing R388::Tn3571 were reconstructed by mating from DS916 as before, but this time the exconjugants were mated to DS903 Sp^r (pPAK316). pPAK316 is $TnpR^+$ and is able to resolve cointegrate-type structures. The use of this as a recipient strain enabled true cointegrates to be distinguished from homologous recombinants by examination on single colony gels (Fig. 3.21). Assays were performed on two separate occasions, and the results are summarised in Table 3.6.

Those Km^r exconjugants which were due to homologous recombination were found to contain R388::Tn3571, pPAK316 and the original sized λdv vector after resolution. Those Km^r exconjugants which were due to transposition were found to contain R388::Tn3571, pPAK316 and the λdv vector which had usually increased in size by approximately 5kbp (4.957kbp is the size of Tn3). Occasionally, deleted λdv vector derivatives were seen. These deletants were due to transpositions, and the occurrence of 5kbp insertions or these deletions depended on the orientation of the transposition into the λdv vector with respect to the res site already present on the resident Tn3571. If the res site already present was in inverted repeat to the other two, then an approximately 5kbp increase in size was observed. If the res site already present was in direct repeat to the other two, then resolution between all three sites could occur, giving rise to deletants.

Problems with these assays are that if transpositions occurred into the Km resistance gene, or the cro, O, or P genes they would not be detected. Also not all λdv deletants would be viable, depending on the position of insertion of R388-derived Tn3571 into the λdv plasmid. Therefore the assays tend to underestimate transpositions compared to homologous recombinations. The assays also assume that the levels of homologous recombination are the same for each strain. This is not true because in the case of pROS12 and pMR18 no homologous recombination should occur, and in pROS13, which contains sequences 1-4957 of Tn3571, slightly more should occur than in pROS26, which contains sequences 26-4160. Additionally, the relatively small numbers of Sp^r exconjugants screened means conclusions from the assays must be tentative.

With these criticisms in mind, it can be seen from Table 3.6 that

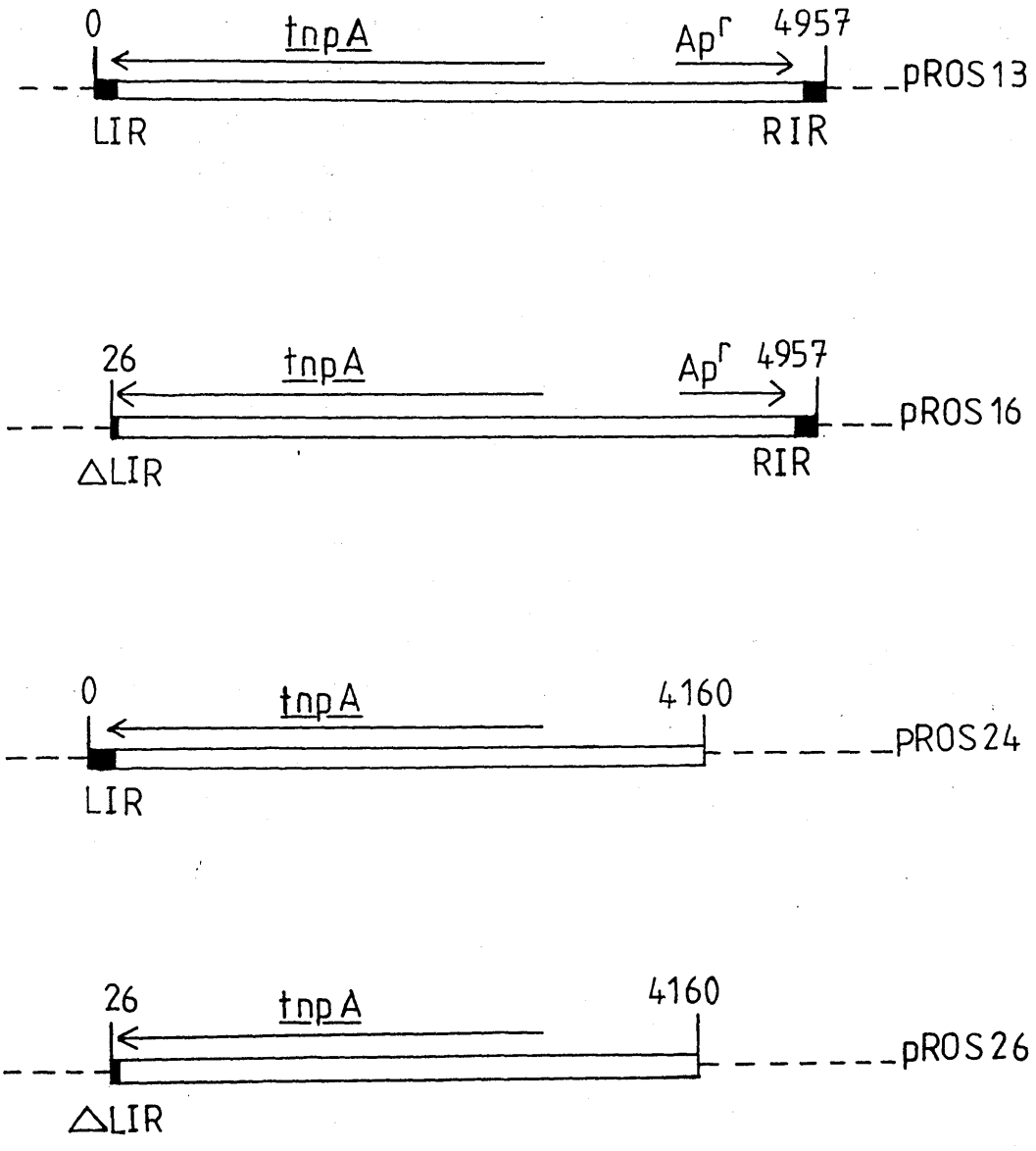


Fig. 3.22 Tn3571 sequences present in pROS13, pROS16, pROS24, and pROS26.

pROS12 and pMR18 are apparently not immune to Tn3571 insertions. pROS13 and pROS24 are apparently totally immune, pROS16 seems to be partially immune, and pROS26 is even less so, perhaps not at all. Fig. 3.22 shows the Tn3571 sequences present in pROS13, pROS16, pROS24 and pROS26. In these assays, sequences 1-4957 confer complete immunity, as do sequences 1-4160. Removal of sequences 1-25 lowers this immunity, indicating that these outer 25bp of Tn3571 are important in the phenomenon. Removal of sequences 1-25 and 4160-4957 lowers the immunity further. This suggests that sequences 4160-4957 do play a part, although the pROS16 result suggests they may not be as important as sequences 1-25.

Previous to these assays being carried out, the immunity-conferring sequences had been localised to Tn3 sequences 1-166 and 4491-4957 (Lee et al. 1983). These include both the LIR and RIR of Tn3. Lee et al. found that either the LH end or the RH end of Tn3 could confer immunity, and the presence of both ends did not confer a significantly greater effect. The results presented here are in agreement with these sequences being responsible for immunity, but they also showed that the LH end is somewhat more effective than the RH end, although the criticism about the length of Tn3571 sequence available for homologous recombination is pertinent here. However, if this result was confirmed, it could be explained in a similar manner to that for the special effect proposed to exist for the LIR in section 3.4. If transposase binding to the ends has a function in causing immunity (see discussion), then it is possible that the LIR has a greater effect than the RIR because the probability of transposase being bound to the LIR present at the immediate 3' end of a functional gene may be greater than that of being bound to the more distal RIR; alternatively, sequences within the tnpA gene may enhance binding to the LIR.

Since these assays were performed it has been confirmed that Tn3 contains no sequence other than either of the IRs which is necessary for transposition immunity (Huang et al. 1986; Kans and Casadaban 1987). These are the same sequences that are necessary for transposition. Huang et al. showed that deletants of the IRs which reduced transposition also reduced immunity, although they did find that an IR deleted for the inner 4bp was not immune, but could

transpose at levels above background. Kans and Casadaban (1987) found that 6bp of the outer 8bp could be changed without significantly altering immunity, but changing 12bp of the outer 17bp eliminated it. In contrast, deletions from the inside altered the immunity phenotype to an intermediate level as soon as base substitutions were made, and changing 8bp of the first inner 9bp eliminated immunity completely. Their results differed slightly from those of Huang *et al.* (1986) in that changing 2bp out of the inner 3bp, or 2bp out of the inner 6bp showed intermediate immunity rather than none. Perhaps the assay used by Kans and Casadaban was more sensitive, or adjacent sequence differences may have had an effect. The correlation between sequences necessary for transposition and immunity is important in models explaining immunity (see discussion).

Analysing the results of the assays presented here in the light of the above workers' results suggests that any immunity conferred by pROS16 is due only to the RIR and not to the remaining LIR, or any other, sequences. Also, it is most likely that no immunity is shown by pROS26.

Discussion

The results presented here show the successful construction of Tn*3571*, a TnpR$^-$ Tn*3* derivative which is unable to resolve cointegrate-type structures and which expresses derepressed levels of transposase. This was used as the source of Tn*3* sequences for several λ*dv*-based constructs containing no IRs, the RIR, the LIR or both IRs of Tn*3*. These constructs were used to examine three questions: does Tn*3* transposase bind the IRs of Tn*3* *in vivo*; do Tn*3* IRs titrate out limiting transposase; and what immunity properties are possessed by constructs with no IRs, the RIR, the LIR or both IRs?

The tentative conclusions from the end titration assays (3.4) were that transposase can bind the IRs *in vivo* and that when transposase is limiting the ends do appear to be able to titrate the protein, and hence lower the amount available for promoting transposition of another transposition-proficient Tn*3* element in the same cell.

Tn*3* transposase has now been purified and shown to bind site-

specifically to the IR ends of Tn3 *in vitro* (Wishart *et al.* 1985; Morita *et al.* 1987). DNA footprinting studies on the binding of transposase to each strand of the inverted repeats, the binding to IR deletion mutations, and to the IRs from transposons with close homology to Tn3 should provide insights into the end-binding action of transposase. The sequence 5'-ACGAAAA-3' is found in the IRs of various transposable elements which generate a 5bp duplication at the site of insertion, such as Mu, D108, Tn3, gamma-delta, Tn551 and Tn501 (Tolias and DuBow 1986). The common occurrence of this sequence suggests an evolutionary relationship between these elements and its conservation suggests this sequence is important in end-binding by the transposases. Additionally, each end of Tn3 contains two interrupted palindromes within the essential part of the immunity-recognition region (Kans and Casadaban 1987). These palindromes may well be important in binding of transposase to the IRs.

The ability of the ends of transposable elements to titrate transposase has been suggested previously for Tn554 (Murphy 1983), Tn3 (Heritage and Bennett 1984), and *Drosophila* (Simmons and Bucholz 1985):

Tn554, a *Staphylococcus aureus* transposon, transposes highly efficiently into a very limited choice of insertion sites. When Tn554 is inserted in the chromosome, transposition is inhibited 100-1000 fold if the cell already has a copy of Tn554 in the chromosome. This suggested to Murphy (1983) that the transposition of Tn554 is negatively regulated, or that an already occupied site is not a substrate for transposition of a second copy. Her experiments showed that 89bp of the "left" end of Tn554, when present on a high copy number plasmid, strongly inhibited the transposition of an incoming transposon to the chromosome, even when the chromosomal site was empty. This suggested that Tn554 transposase could be titrated by the left end of the transposon (Tn554 does not have IR ends), although the occupation of the chromosomal site by another element is probably more responsible for the normally observed inhibition.

In the P-M system of hybrid dysgenesis of *Drosophila melanogaster*, some M strains possess defective, transposase-deficient, chromosomal P elements. These are called pseudo-M strains. In 1985, Simmons and

Bucholz found that in a pseudo-M strain the instability, due to an intact P element, of a P element insertion mutation of the singed bristle locus, was reduced compared to that in an M strain, which contains no P elements. They suggested that the reduction in instability was due to competition for the limited, intact P element-produced transposase between the P elements on the pseudo-M chromosomes and the P elements at the singed locus. P elements carry 31bp IRs at the ends of the elements, and it is here that the transposase is proposed to bind. Simmons and Bucholz also hypothesized that a similar mechanism could regulate the movement of P elements in P strains of D. melanogaster. In the P cytotype, nearly all P element mobility is repressed. They suggested that the P cytotype is a state in which numerous defective P elements bind the transposase and prevent it from causing dysgenesis. They proposed that the majority of these titrating, defective elements are extra-chromosomal. This could explain the manner in which cytotype is inherited. The P cytotype could arise when extra-chromosomal P elements become numerous enough to bind most of the transposase made by intact chromosomal elements. The extra-chromosomal pool was proposed to be derived from chromosomal elements by the action of transposase. In the absence of transposase the pool would not form, leaving the cytotype M. An abundance of defective chromosomal elements would bring about only partial regulation of P element activity, as in pseudo-M strains.

It can be seen therefore that there are several cases where limiting transposase has been proposed to be titrated by the ends of transposable elements. The importance of this property of the ends in regulation of transposition seems to depend on the ratio of ends to the number of functional transposase genes in the cell, i.e., the regulatory effect of large numbers of ends seems only to be apparent when most of the elements are transposase-deficient. The Simmons and Bucholz hypothesis for Drosophila suggests this regulation could be important in eukaryotes. The significance in prokaryotes is not clear because the Tn554 example, and the Tn3 studies reported here, are artificial situations. However, one case where titration of Tn3 transposase may play a role in lowering transposition frequencies within a prokaryotic cell, is when Tn951 is also present (Michiels and Cornelis 1986): Tn951 encodes genes for lactose fermentation, and it

has identical 38bp IRs to Tn3, but has an inactive tnpA gene due to an insertion mutation. Tn951 relies for its transposition on Tn3 transposase being supplied by an intact Tn3 tnpA gene present in trans. Therefore, the presence of Tn951 in a cell with Tn3 could reduce the transposition frequency of Tn3 compared to in a cell without it.

The immunity data presented here are consistent with the 38bp IRs of Tn3 being the regions responsible for the immunity effect (Huang et al. 1986; Kans and Casadaban 1987).

Transposition immunity conferred by a transposon appears to be limited to other closely related transposons (Heffron 1983). Plasmids containing Tn501 are immune to Tn1721 but not to Tn3 insertion. Tn501 and Tn1721 have nearly identical IRs, and they complement one another for transposition functions. Tn501 and Tn3, however, differ at 19 out of 38bp within their IRs, and do not complement each other for transposition. This specificity rules out changes in plasmid structure, such as supercoiling, as an explanation for the phenomenon. It suggests that immunity is the result of highly specific transposases binding the IR ends of each transposon. Thus it is probable that both the transposase-titrating activity and immunity-conferring ability of the IRs are due to transposase binding at these sites as a pre-requisite for transposition.

A model to explain immunity has been proposed by Lee et al. (1983), which is compatible with transposition models proposed by Sherratt (1983). They suggested that integration is the rate-limiting step in cointegration, and a transposition complex, containing transposase and both IRs, moves along a recipient molecule for a time which is long compared to the actual joining step. If the complex encounters a favourable site or region for integration, transposition is initiated in the vicinity. If the complex encounters another copy of Tn3 , or an IR, before inserting into the recipient DNA, the transposition complex dissociates, lowering the frequency of integration. This model assumes an intimate connection between the initiation of transposition and the phenomenon of immunity. This has been shown to exist by Huang et al. (1986), and by Kans and Casadaban (1987). According to Kans and Casadaban, "scanning" of the recipient DNA by

the transposition complex need not be invoked if detection of the immunity site occurs at the protein interaction level prior to strand exchange. Transposase can form multimers *in vitro* (Fennewald *et al.* 1981), and the binding constant of transposase protein-protein interaction might be much greater than that of protein-DNA recognition. So immunity could occur by simple kinetic competition.

Many workers (1.4.5) have found that intramolecular transposition is not subject to immunity. The model proposed by Lee *et al.* (1983) can accomodate this fact. In a plasmid containing one transposon and no other IRs, if the transposition complex contains both IRs (as shown for Mu by Surette *et al.* 1987), then the rest of the molecule will not contain any site-specifically bound transposase which might disrupt transposition. Therefore transposition may occur into these sequences as into other IR-free plasmids. The model predicts that the addition of a third IR might restore immunity. This is in fact the case (Bishop and Sherratt 1984).

In order to form a transposition complex of DNA and two IRs, the IRs must come together. Presumably the transposase binds one IR, perhaps preferentially the LIR (3.4 and 3.5), and diffuses along the molecule to find a second IR. This suggests that the distance between the IRs might affect the transposition frequency. In fact, the distance between the IRs is not at all critical for Tn*3* transposition (Huang *et al.* 1986). So other steps than contact between the IRs limit the transposition frequency. Joining of the transposon sequences to recipient DNA may be a limiting step, as proposed by Lee *et al.* (1983). Interestingly, this length independence of transposition is the opposite of that seen for Tn*5* or Tn*10* (Chandler *et al.* 1982; Morisato *et al.* 1983; Way and Kleckner 1985). These transposons, and Tn*9*, do not show immunity either (Muster *et al.* 1983). So for Tn*5*, Tn*9* and Tn*10*, the formation of a complex including the IRs may be the limiting step of transposition.

The phenomenon of immunity is important in transposition of the Tn*3* family, together with resolution of cointegrates, because it ensures that simple insertions of these transposons occur into as many individual replicons as possible, thus increasing their probability of survival.

CHAPTER 4

THE TEMPERATURE SENSITIVITY OF TN1/3 TRANSPOSITION

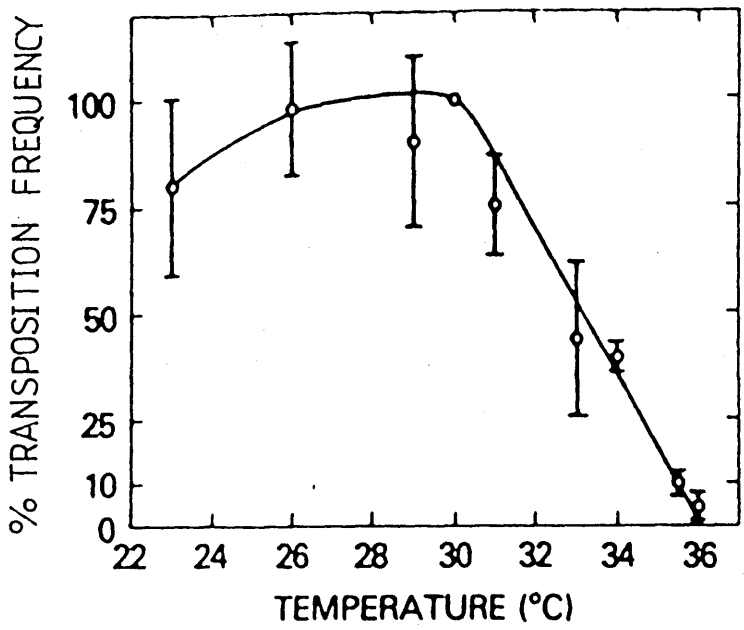


Fig. 4.1 (Kretschmer and Cohen 1979). % transposition frequency of Tn3 with temperature. The 30°C frequency is taken to be 100%.

Introduction

In 1977, Kretschmer and Cohen first made the observation that transposition of Tn*3* was temperature-sensitive, in that transposition frequencies were higher at 32°C than at 45°C by at least one hundred-fold. To measure these frequencies they used a different method from many other workers (Ch. 2), which involved transforming cells already containing donor plasmids with recipient plasmids, and growing the cells at different temperatures immediately after heat-shock for 120 minutes. Antibiotics which selected for transformants were added at this stage and the cultures were grown to stationary phase overnight, before extracting plasmid DNA and transforming recipient cells. The transposition frequencies were estimated by determining the fraction of cells transformed with the recipient plasmid that had also received the transposon marker, and dividing by 8.7 (the size of the recipient plasmid was 8.7kbp) to express the frequency of transposition events per kbp of recipient DNA per at least 25 generations. The results showed that transposition at 32°C was ten times that at 37°C, and at least one hundred times that at 45°C. Additional experiments excluded the possibility that a reduced transformation efficiency at 45°C was responsible for the effect, or that the recipient plasmids were unstable at 45°C when they contained Tn*3*.

Subsequently, Kretschmer and Cohen (1979) confirmed these results by more detailed experiments. The frequency of transposition was studied at temperatures ranging from 23°C to 37°C. The graph in Fig. 4.1 is taken from their paper. Under the conditions they used, the optimum temperature for transposition is in the range from 26°C to 30°C. They also studied the duration of the effect. Their results showed that after introduction of a recipient plasmid into a cell carrying a Tn*3* donor plasmid, the maximum frequency of transposition obtainable at 30°C was reached within about the first 25 generations, and remained constant thereafter on extended incubation for up to 75 generations. Incubation of an identical culture grown at 36°C gave a ten-fold lower frequency, which did not significantly increase after extended incubation for up to 75 generations. These results did not show whether the lower frequency of transposition at 36°C is the result of a slower rate of transposition, or whether it reflects a shift in the apparent transposition equilibrium.

The reversibility of the effect was also shown. The culture grown for 75 generations at 36°C was diluted and grown for 25 more generations at 30°C. The frequency of transposition then obtained was the optimal level obtained within 25 generations of growth at 30°C, demonstrating the complete reversibility of the temperature effect.

Since these studies, there has been only one other report of work on the temperature sensitivity of Tn*3* transposition (Chou *et al.* 1979). They constructed a transcriptional *lacZ* fusion (see below) to the *tnpA* gene, and measured the B-galactosidase activity due to transcription from the *tnpA* promoter. They found 5217, 2895, and 1766 units of B-galactosidase (measured according to Miller, 1972), at 30°C, 37°C and 42°C respectively. However these units were not corrected for plasmid copy number, which is known to decrease with increasing growth rate (Adams and Hatfield 1984; Lin-Chao and Bremer 1986; Klotsky and Schwartz 1987; this chapter), and this result has not been obtained again (this chapter).

The purpose of the studies described in this chapter was to discover the reason for the temperature sensitivity of Tn*3* transposition. Studies in Glasgow (other workers; this thesis - Table 3.1) have shown, using the transposition assay described in Chapter 2, that the observed effect does not depend on the method of assaying transposition, that the temperature effect is independent of the *tnpR* gene, and that the transposition frequencies of $TnpR^+$ and $TnpR^-$ versions of Tn*3* are 10- to 50-fold greater at 30°C than 37°C. Hence attention was focused on the *tnpA* gene (although it was possible that a host factor could be responsible for the effect).

Transcriptional and translational fusions to *tnpA* were constructed, and enzyme assays were carried out together with copy number controls, to determine if transcription or translation of the gene was responsible for the effect. The possible involvement of the heat-shock protease, Lon, in breakdown of transposase at higher temperatures was examined. Also, a mutant Tn*3* was sought, which would be able to transpose as frequently at 42°C as at 30°C. These studies would hopefully give an insight into the nature of the temperature-sensitivity, and elucidate the regions of *tnpA* responsible for the effect.

Results

4.1 Construction and assaying of transcriptional fusions to Tn*3*

The construction of genetic fusions has provided a powerful tool for the analysis of genetic control signals, as well as for other purposes (Silhavy and Beckwith 1985). Strategies for constructing genetic fusions differ as to (i) the generation of transcriptional (operon) versus translational (gene) fusions, (ii) the choice of expressed function e.g. *lacZ* or *galK*, (iii) *in vivo* versus *in vitro* construction, (iv) the generation of multicopy (plasmid) versus single-copy (chromosomal) fusions.

A transcriptional fusion is one in which an exogenous promoter is fused to a reporter gene which lacks its own promoter, but contains its own translation start site. A translational fusion is one in which a reporter gene without its own promoter or translation initiation signals is fused in frame to the coding sequence of a test gene.

4.1.1 Assays using pJKA and pJKR: Tn*3* transcriptional fusions to *galK*

The use of *galK* for transcriptional fusions was developed by McKenney *et al.* (1982). The vectors constructed had all of the following benefits:

(i) Fusions could be made to a gene with a readily assayable product, galactokinase.
(ii) All translation originating in the cloned fragment should be terminated by translational stop codons in all three reading frames, and the mRNA 5' to the assayed product should be long enough (about 170bp) so that changes in the upstream structure should not affect translation of the assayed product.
(iii) The gene function is a readily selectable and scorable marker (on MacConkey plates containing galactose).
(iv) A second, independent marker is present on the vector.
(v) The existence of unique cloning sites allows insertion of the cloned fragment simply and precisely.
(vi) Transcriptional expression of the assayed product depends solely

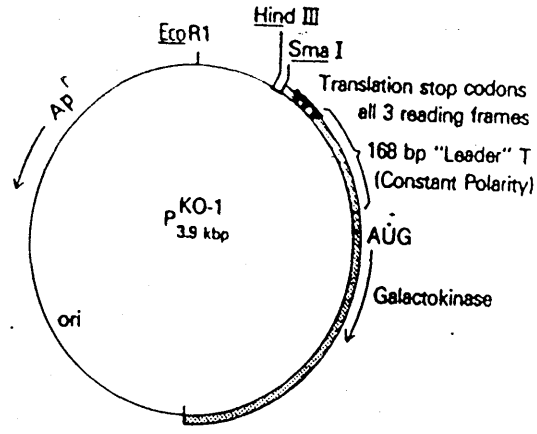


Fig. 4.2 pKO1 (McKenney et al. 1981). The dotted area represents the galactokinase gene (galK) and 168bp of "leader" galT gene sequences preceding the AUG of galK. Translation stop codons in all three reading frames prevent any translation originating in the inserted DNA fragment from reaching the ribosome binding site and AUG of galK. The EcoRI, HindIII and SmaI sites can be used to insert DNA fragments.

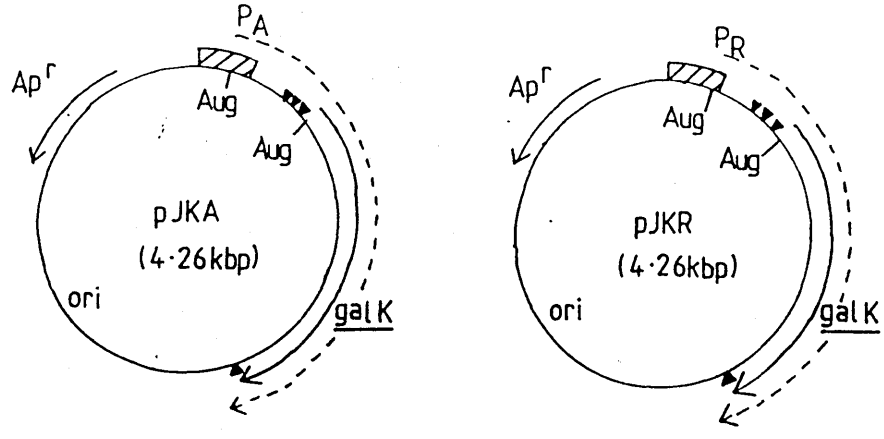


Fig. 4.3 pJKA and pJKR - transcriptional fusions to galK (Kelly, Glasgow). In pJKA, P_A initiates transcription (----→) through galK; in pJKR, P_R initiates transcription through galK.

▨ represents the 282bp EcoRI-bounded Tn3 fragment containing P_A and P_R. The origin of this fragment is described in 4.2.1.

▼ represents relevant translational stop codons.

on the inserted fragment.
(vii) The exact sequence of the region between the cloning site and the assayed product is known.
(viii) The copy number of the vector can be determined easily.

The latter point is especially important in studies where different promoter strengths are being compared, because copy numbers of McKenney vectors are known not only to decrease with increasing growth rate, but also to decrease with the strength of the inserted promoter (Adams and Hatfield 1984). This may be due to strong promoters causing transcription to occur through the origin at the 3' end of galK, thus disturbing normal copy number regulation. It is therefore important to measure the copy number each time the indicator enzyme activity is assayed. Adams and Hatfield (1984) described a simple method for determining the molar amount of galactokinase product produced per mole of plasmid, which involved DNA-DNA dot hybridisation. While this method has the advantage that it can be performed on small samples of the same lysate used for enzyme assays, it has two disadvantages. It requires the use of ^{32}P-labelled probe which has a limited life-time, and must be prepared repeatedly. Second, the procedure involves autoradiography which requires a minimum of several hours of exposure prior to the quantitation of hybridised probe by scintillation counting. An alternative, more rapid measure of copy number is to assay a second enzyme which is expressed by the same plasmid. The levels of the transcriptionally fused galK can then be standardised to the levels of the second enzyme. This method assumes that the second enzyme is constitutively expressed, and that there is a direct relationship between the plasmid-encoded product and the number of plasmids in the cell. That this is the case for B-lactamase at different growth rates has recently been confirmed by Klotsky and Schwartz (1987).

The original McKenney vector, pKO1, is shown in Fig. 4.2. This vector was used in Glasgow by Kelly (1983) to investigate the effect of temperature on the promoter activities of tnpA and tnpR. His two constructs, pJKA and pJKR, are shown in Fig. 4.3. He found that P_A was about two and a half times as strong as P_R (as did Hettle 1985) at 25°C, 30°C, 37°C, and 40°C, and that no significant change was apparent in the activity of either promoter at the different

temperatures. He concluded that the temperature effect on transposition must act at some stage after the initiation of transcription. However, none of his data, or that of Hettle (1985), were corrected for copy number variations, and therefore the observed promoter activity ratios were suspect. Therefore galactokinase assays were repeated on these constructs, together with assays of B-lactamase on the same cultures. Standardisation of the galactokinase levels to B-lactamase levels corrects for copy number at 30°C or 37°C, but does not take into account any variation which may exist in B-lactamase expression with temperature, and although other workers have ignored this possibility (Goransson and Uhlin 1984), the assumption that B-lactamase expression is the same at 30°C and 37°C is not made here. However, B-lactamase assays do enable more accurate comparisons of relative promoter strengths at 30°C or at 37° to be made, than without any standardisation.

The structures of pJKA and pJKR were confirmed by digestion with PvuII/HindIII and SspI (data not shown). DS942 was transformed with the DNA and the resulting strains were grown overnight at 30°C and 37°C without glucose, but with ampicillin, together with pKO500 and pKL500 (Fig. 4.4) as controls. pKO500 and pKL500 are derivatives of pKO1 made by McKenney (personal communication) containing no promoter and P_{lac} respectively. The following day the overnight cultures were diluted 1 in 200 and shaken at 30°C and 37°C. When the OD_{650} was between 0.2 and 0.35, 1ml of cells was removed from each culture and vortexed with 10ul of 5mg/ml of CTAB solution. [It is uncertain whether CTAB - mixed alkyltrimethylammonium bromide - lyses or permeabilises the cells, but in assays where multicopy plasmids are used to carry the gene being examined, this is irrelevant. In cases where the gene being studied is present on the chromosome, or on a unit copy plasmid, the distinction could be important. This is because in the single copy case, if expression is very low and the assayed product is only active as a multimer, some cells may contain only one monomer, and therefore permeabilising the cells thus allowing entry of the substrate into the cells, may give a lower estimation of expression than lysing the cells. The latter would release all the protein from the cells, allowing isolated monomers to multimerise and therefore the estimation of expression would be higher. This difference between permeabilising and lysing cells has been exploited

Plasmid	OD650	ΔOD499 min^{-1} ml^{-1}	ΔOD499 min^{-1} ml^{-1} $OD650^{-1}$	gal K units	gal K units/ ΔOD499 min^{-1} ml^{-1} $OD650^{-1}$	E value - E value for pKO500	F value as % of F value for pKL500
	A	B	C	D	E	F	G
30°C							
pKL500	0.286	0.44	1.54	136	88.3	87.7	100
pKO500	0.272	0.45	1.65	1	0.6	0.0	0
pJKA	0.222	0.34	1.53	26	17.0	16.4	19
pJKR	0.251	0.57	2.27	10	4.4	3.8	4
37°C							
pKL500	0.244	0.41	1.68	193	114.9	113.8	100
pKO500	0.251	0.46	1.83	2	1.1	0.0	0
pJKA	0.300	0.46	1.53	43	28.1	27.0	24
pJKR	0.255	0.57	2.24	19	8.6	7.5	7

Table 4.1. The results of galactokinase and B-lactamase assays on DS942 containing transcriptional fusions of the 5' ends of Tn*3* *tnpA* and *tnpR* to *galK*. Controls are also shown. The method of calculating units of galactokinase and B-lactamase is given in Ch. 2.

by Raleigh and Kleckner (1986) to examine transcription and translation rates of the IS10 transposase gene.] 0.8ml of each culture was frozen at once to -20°C, and the rest was kept for galactokinase assays which were performed immediately. [B-lactamase is stable for weeks at -20°C, whereas galactokinase is not - data not shown.] At a convenient time, within the next two days, the frozen cultures were thawed and assayed at 30°C for B-lactamase activity. The results of both assays are shown in Table 4.1.

The data allow the following conclusions to be made: P_A is approximately 1/5th the strength of P_{lac} (see also Table 4.4); P_R is approximately 1/20th the strength of P_{lac}; and P_R is approximately 1/4th the strength of P_A. These conclusions hold at 30°C and 37°C. Column E shows that the ratio of galactokinase units/B-lactamase units increases by 1- to 2- fold between 30°C and 37°C. This suggests that temperature affects the fusions being studied and B-lactamase differentially: column C shows no significant increase in B-lactamase units at 37°C, whereas column D does show an increase in galactokinase units. The increase in column D does not necessarily mean that mRNA levels of tnpA and tnpR increase with temperature, although this is the most likely explanation. There is a possibility that the levels actually decrease, but this is concealed by an increase in galactokinase translation efficiency at 37°C. However, the fact that the relative strengths of P_{lac}, P_A and P_R remain practically the same at these two temperatures implies that, unless all three behave identically by decreasing with temperature, there is no decrease in the strength of P_A between 30°C and 37°C. Thus it is probable that temperature affects transposition at some stage other than initiation of transcription of tnpA, as originally concluded by Kelly (1983).

4.1.2 *In vivo* construction of *galK* transcriptional fusions to Tn3

Another possibility was that temperature could affect transcriptional termination within the tnpA gene by altering message structure, thus lowering the amount of transposase in the cells at higher temperatures. In order to investigate this, it was desirable to have a transcriptional fusion containing as much as possible of the tnpA gene. According to Heffron *et al.* (1979), there is no transcriptional terminator at the 3' end of tnpA i.e. within the LIR, but there is at

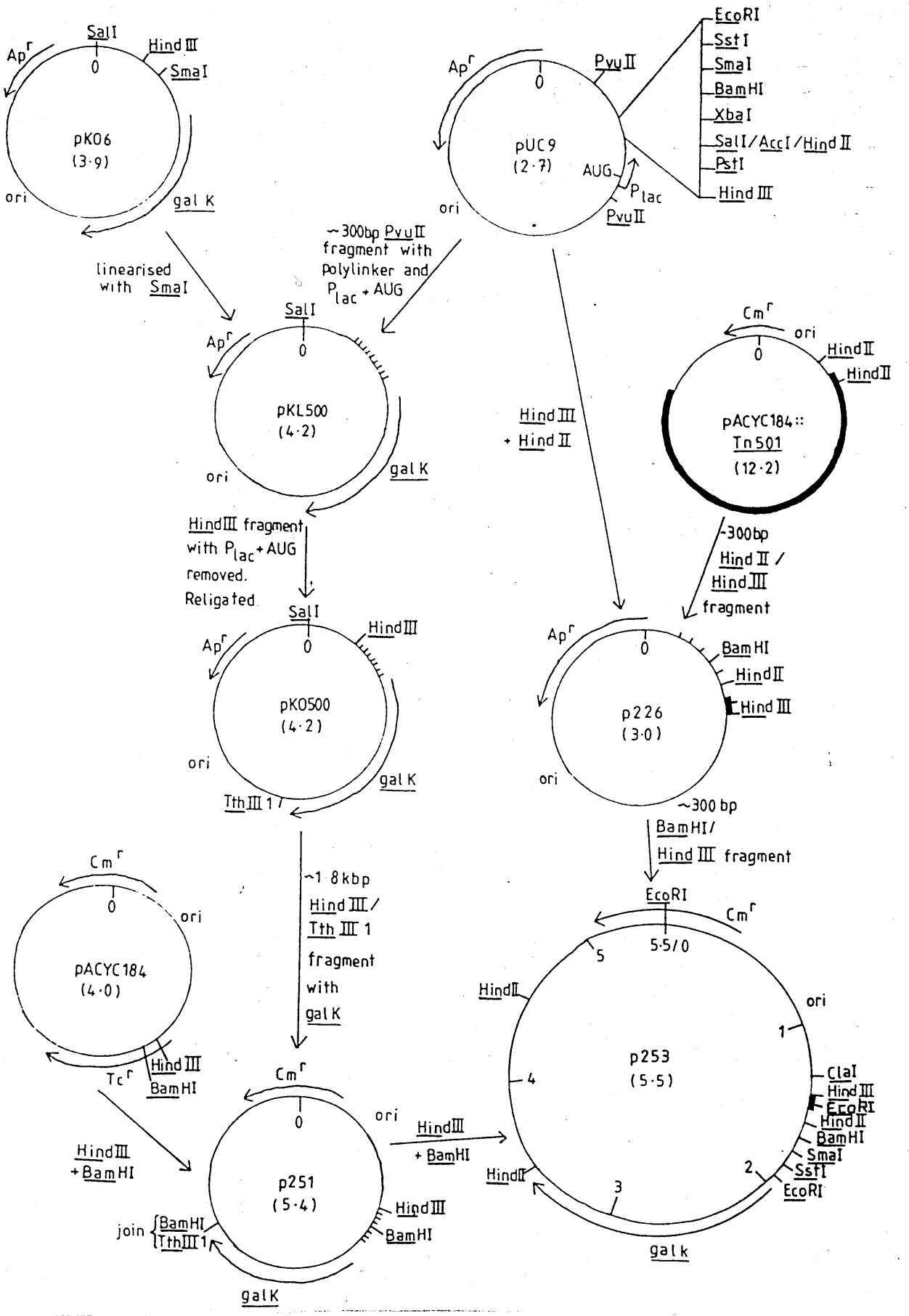


Fig. 4.4 The construction of p253, a Cm^r Ap^s galK transcriptional fusion vector. pKO6 was derived from pKO1 (Fig. 4.2) by replacement of the pKO1 EcoRI site with a SalI linker.

■ represents Tn501 sequences.

the 3' end of bla. Therefore it was decided that transcriptional fusions to galK could be made using transposition itself. A problem with isolating transpositions into the original McKenney vector (Fig. 4.2), was that it already contains the bla gene so selection for insertion would not be possible. Also, because the bla gene already present was evolutionarily derived from that of Tn3, the RIR of Tn3 is present on the plasmid. This meant that transpositions onto the vector would be very infrequent due to the phenomenon of immunity (Ch. 3). Therefore a different galK-containing vector was constructed by Burke (Glasgow). This vector is a pACYC184-based replicon, unlike pKO1 which is pBR322-based, and it carries the gene encoding chloramphenicol acetyl transferase which confers the Cm^r phenotype on cells containing it. The bla gene and the RIR of Tn3 are no longer present. The construction is outlined in Fig. 4.4.

The procedure by which transcriptional fusions of tnpA to galK were obtained by transposition is described below:

DS903 (R388) was transformed with pROS2 (Ch. 3). Cointegrates formed as the end products of transposition of Tn3571 from pROS2 to R388, were isolated by mating to DS916, and selecting for recipients containg R388::pROS2. pPAK316 was transformed into these cells. pPAK316 encodes tnpR and therefore was able to resolve the cointegrates, giving rise to DS916 (pROS2, R388::Tn3571, pPAK316). These cells were mated with DS903 (p253) made previously, and DS903 (R388::Tn3571, p253) cells were selected on MacConkey agar containing galactose. This agar was used because those cells in which Tn3571 transposes into p253 to form cointegrates such that the galK gene is expressed, grow up as red colonies after 24 hours at 30°C. The background colonies remain white. Any red colonies obtained were picked and mated individually to DS916 (pDS4153). pDS4153 encodes a tnpR gene, and hence was able to resolve cointegrates between R388 and p253 to give DS916 (R388::Tn3571, p253::Tn3571, pDS4153). A TELT plasmid DNA preparation on the recipients from each mating gave total plasmid DNA, which was then transformed into DS903 at various dilutions to reduce the probability of double transformants occurring. p253::Tn3571 DNA was prepared from cultures of single colonies by CsCl centrifugation, and the resulting individual transposition events into p253 were mapped using various restriction enzymes. Five independent

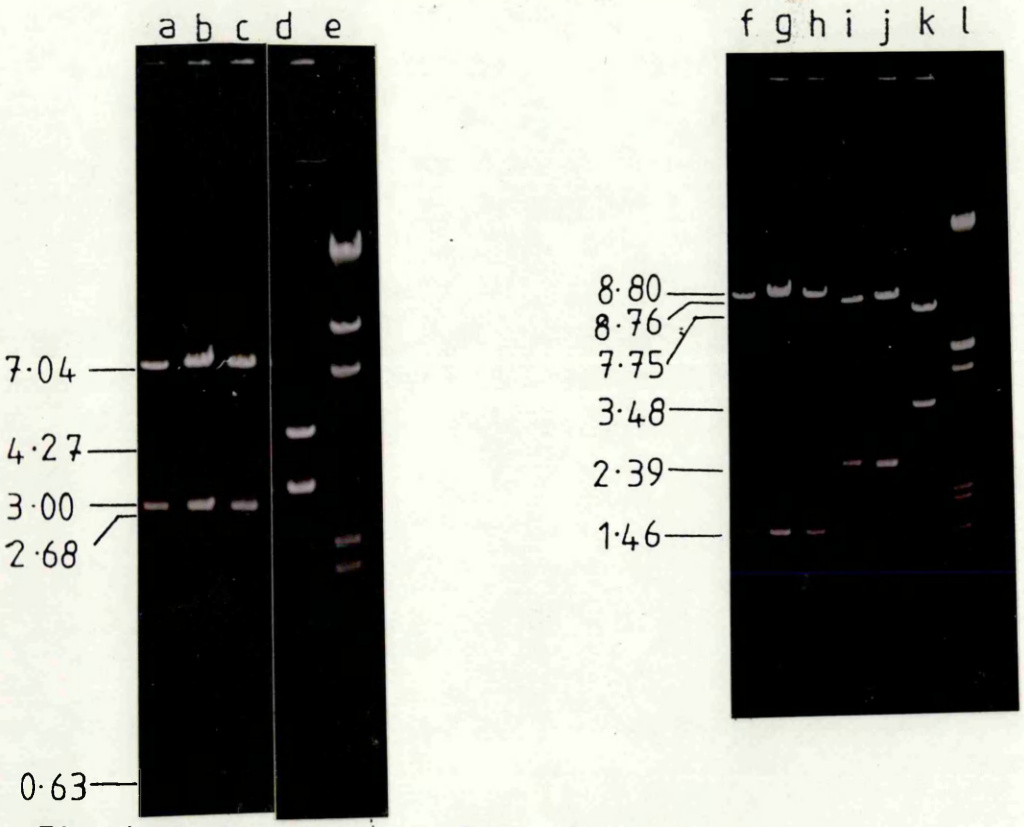


Fig. 4.5a Digestions of pROS3, pROS4 and pROS5.
Fragment sizes are in kbp.

a. pROS5 PstI digest.
b. pROS4 PstI digest.
c. pROS3 PstI digest.
d. pMC1871 PstI digest.
e. λ HindIII.
f. pROS5 EcoRI digest.
g. pROS4 EcoRI digest.
h. pROS3 EcoRI digest.
i. pROS5 ClaI digest.
j. pROS4 ClaI digest.
k. pROS3 ClaI digest.
l. λ HindIII/EcoRI.

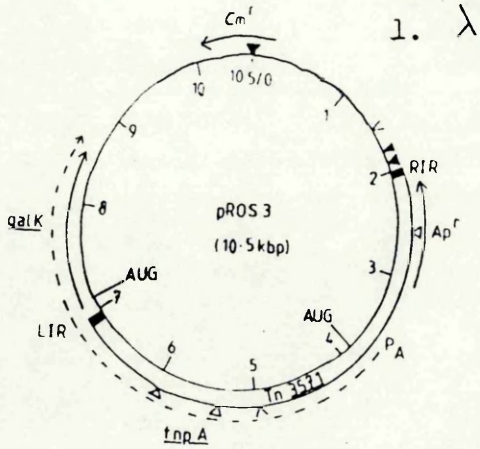


Fig. 4.5b pROS3, showing the transcriptional fusion of Tn3 tnpA to galK. Tn3571 has inserted between the polylinker and galK. In pROS4 and pROS5 Tn3571 is in the same position, but in the opposite orientation.

--- represents transcription from P_A.
▽ PstI sites
▼ EcoRI sites
⋁ ClaI sites.

Plasmid	*tnpA* directing *galK*	Transposition frequency	
		Assay 1 (at 30°C)	Assay 2 (at 37°C)
Tn*3571* transposed into p253:			
Clone 1	Yes	7.3×10^{-2}	-
Clone 2	Yes	3.2×10^{-1}	4.9×10^{-2}
pROS3	Yes	3.5×10^{-1}	4.4×10^{-2}
pROS4	No	7.0×10^{-1}	-
pROS5	No	8.6×10^{-1}	-
Average		4.6×10^{-1}	4.65×10^{-2}
Tn*3571* cloned into p253:			
pROS40	Yes	8.8×10^{-2}	5.2×10^{-3}
pROS44a	No	7.6×10^{-2}	-
pROS44b	No	4.4×10^{-2}	-
Average		6.9×10^{-2}	5.2×10^{-3}

Table 4.2. Results of transposition assays at 30°C and 37°C from various p253-based plasmids into R388. The donor strain was DS903 and the recipient was DS903 Sp^r.

transposition events were detected: three of these were in the correct orientation, with P_A apparently driving synthesis of galactokinase; but two were in the opposite orientation with transcription apparently coming from the bla gene promoter, in spite of the existence of a transcriptional terminator at the 3' end of the gene (Heffron et al. 1979). The transposition frequencies of these clones were assayed (Table 4.2), and were found to be normal for a derepressed transposon at 30°C (Table 3.1), and not to depend on the relative orientation of insertion of the transposon. The restriction analyses and structures of three clones, pROS3, pROS4, and pROS5 are shown in Fig. 4.5.

The mapping data showed that all three insertions in the correct orientation were between the polylinker and the galactokinase gene. This was not very satisfactory because the 170bp mRNA sequence 5' to the galK gene, deliberately maintained in the McKenney vectors to prevent interference with galactokinase expression, was disrupted. However, further attempts to obtain insertions within the polylinker, or to the far side of it, were unsuccessful. A possible reason for this is that selecting in the manner described, for red colonies on MacConkey galactose plates, is not a good way to obtain a variety of transcriptional fusions in different positions. Only the reddest colonies are obvious and others expressing intermediate levels of galactokinase may be overlooked. Another possibility for insertions only being detected downstream of the polylinker is the regional preference of Tn3 which is known to exist (1.6.3; Kretschmer and Cohen 1977). pROS3 and pROS4 were used in galactokinase assays (Table 4.4), but Tn3571 was also cloned into the polylinker, as described below, in order to maintain the 170bp leader sequence.

4.1.3 *In vitro* construction of *galK* transcriptional fusions to Tn3

The plasmid pROS13 (3.3.2) was cut with EcoRI and the linearised plasmid was blunt-ended by filling-in the 5' overhangs. Cleavage with SmaI gave a 5.887kbp fragment containing the whole of Tn3571, with some bounding λdv sequences. This fragment was ligated to SmaI-cut p253, and the resulting plasmids were transformed into DS903 and plated on MacConkey plates containing galactose. 46/93 transformants were very pale pink, and the others were white. 5 of the clones were mapped with various enzymes (Fig. 4.6a) and their structure is shown

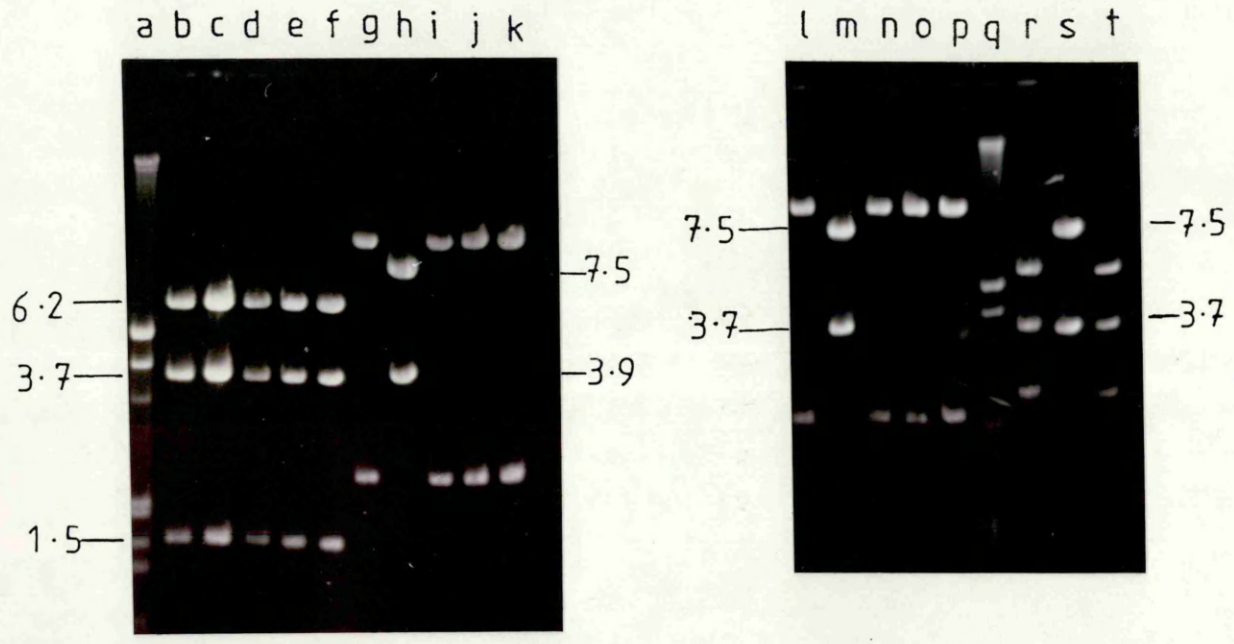


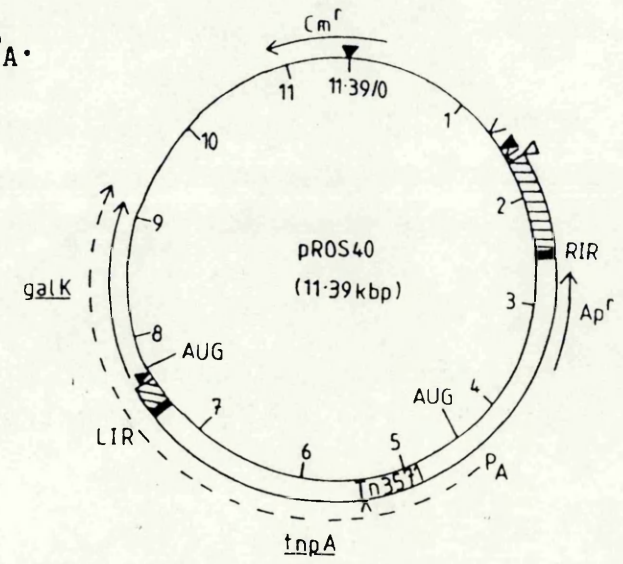
Fig. 4.6a Digests of pROS40 and pROS44a-d.

a. λ HindIII/EcoRI digest.
b, d - f. EcoRI digests of pROS44a-d.
c. EcoRI digest of pROS40.
g, i - k. ClaI digests of pROS44a-d.
h. ClaI digest of pROS40.

l, n - p. BamHI/ClaI digests of pROS44a-d.
m. BamHI/ClaI digest of pROS40
q. λ HindIII/EcoRI digest.
r, t. SmaI/ClaI digest of pROS44d and c.
s. SmaI/ClaI digest of pROS40.

Fig. 4.6b pROS40, showing the transcriptional fusion of Tn3 tnpA to galK. Tn3571 has been cloned into the polylinker. In pROS44a-d Tn3571 is in the same position, but in the opposite orientation.

--> represents transcription from P_A.
▽ SmaI site
▼ EcoRI sites
⋁ ClaI sites
↓ BamHI site.
▨ pROS12 DNA



(Fig. 4.6b). pROS40, pROS44a and pROS44b were assayed for their transposition frequencies by transforming into DS903 (R388) and mating out to DS903 Sp^r. The results are shown in Table 4.2. The data show that the orientation of the transposon in the polylinker does not affect the transposition frequency. The transposition levels are about 5-fold lower than those from the clones derived by transposition of Tn3571 into p253. This difference may be significant bearing in mind the different colours seen on MacConkey plates (pale pink versus red), and also the assay results (4.1.5).

4.1.4 Characterisation of galK transcriptional fusions

In order to confirm that the galactokinase expression in the transcriptional fusions of pROS3 (also pROS8, pROS45 and pROS46) and pROS40 (also pROS41) was under the control of P_A, the ability of the tnpR product to repress galactokinase expression from these constructs was examined. DS903 (R388::Tn3) was transformed with p253 (Fig. 4.4), pROS3 (Fig. 4.5), pROS4 (Fig. 4.5), pROS8 (as pROS3, but independent insertion), pROS33a, pROS40 (Fig. 4.6b), pROS41 (as pROS40, but independent insertion), pROS43a, pROS45 (as pROS3, but independent insertion), pROS46 (as pROS3, but independent insertion), and pROS47 (as pROS4, but independent insertion). [pROS33a is a transcriptional fusion of tnpR to galactokinase, created by cloning the 1.571kbp Tn3 BamHI/ClaI fragment from RSF1050 into BamHI/ClaI-cut p253. Expression of galactokinase in this construct should be repressed by the tnpR product. pROS43a is derived from pROS42 (as pROS40, but independent insertion), and it has a 3kbp insertion into the tnpA gene at position 984 of the transposon. Galactokinase expression in pROS4 and pROS47 should be insensitive to repression by resolvase.]

In order to check if the tnpR product itself, rather than just the presence of R388::Tn3, was responsible for the observed effects, DS942 (R388::Tn3571) was also transformed with the above constructs. R388::Tn3 and R388::Tn3571 only differ with respect to the position of insertion of the transposon, and their TnpR phenotype: Tn3 is $TnpR^+$, and Tn3571 is $TnpR^-$ (3.1). The presence of the two plasmids in each strain was verified by single colony gel analysis (data not shown), and the clones were streaked out on MacConkey galactose plates. The expected and observed phenotypes are shown in Table 4.3, and Fig. 4.7.

Plasmid	*tnpA* directing *galK*	Functional *tnpR* in *trans*		Non-functional *tnpR* in *trans*	
		Expected	Observed	Expected	Observed
Control:					
p253	No	W	W	W	W
Tn*3571* transposed into p253:					
pROS3	Yes	W	W	R	R
pROS8	Yes	W	W	R	NA
pROS45	Yes	W	W	R	R
pROS46	Yes	W	W	R	R
pROS4	No (*bla*?)	R	R	R	R
pROS47	No (*bla*?)	R	R	R	R
Tn*3571* cloned into p253:					
pROS40	Yes	W	W	P	W
pROS41	Yes	W	W	P	W
pROS43a	Yes	W	W	P	W
pROS33a	No (*tnpR*)	W	W	R	R

Table 4.3. Expected and observed phenotypes of DS903 (R388::Tn*3*) or DS942 (R388::Tn*3571*) carrying various plasmids in which *galK* is directed by P_A or P_R. MacConkey galactose plates were used.
Key: W, White; R, Red; P, Pale Pink; NA, not available.

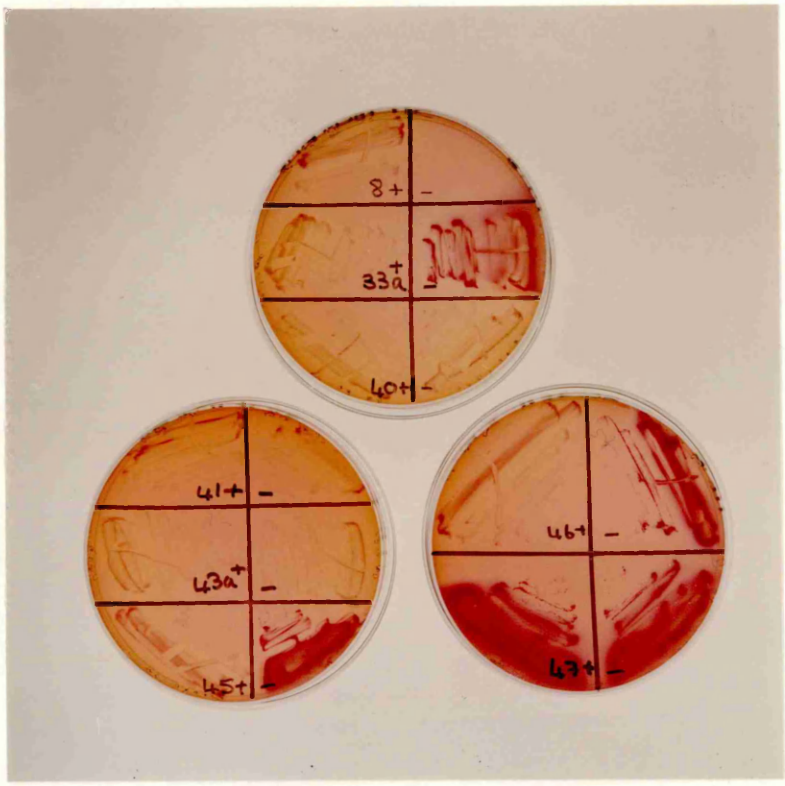


Fig. 4.7 DS903 (R388::Tn3) (resolvase +) or DS942 (R388::Tn3571) (resolvase -) containing pROS8, pROS33a, pROS40, pROS41, pROS43a, pROS45, pROS46 and pROS47 streaked out on MacConkey agar. A red colour is given by 25 - 30 *galK* units (Table 4.4) and indicates that transcription of *galK* is being directed by an inserted promoter. Table 4.3 shows the expected and observed phenotypes of these and other strains.

Plasmid	OD650	ΔOD499 min^{-1} ml^{-1}	ΔOD499 min^{-1} ml^{-1} $OD650^{-1}$	gal K units	gal K units/ ΔOD499 min^{-1} ml^{-1} $OD650^{-1}$	E value - E value for pKO500	F value as % of F value for pKL500
	A	B	C	D	E	F	G
30°C							
Controls:							
pKL500	0.240	0.373	1.55	184	119	102	100
pKO500	0.156	0.301	1.93	32	17	0	0
p253	0.220	-	-	27	-	-	-
Tn3571 transposed into p253:							
pROS3	0.271	0.294	1.08	41	38	21	21
pROS45	0.254	0.293	1.15	32	28	11	11
Tn3571 cloned into p253:							
pROS40	0.270	0.201	0.74	20	27	10	10
pROS43a	0.277	0.210	0.76	17	22	5	5
37°C							
Controls:							
pKL500	0.422	1.123	2.66	290	109	101	100
pKO500	0.326	0.696	2.13	18	8	0	0
p253	0.269	-	-	19	-	-	-
Tn3571 transposed into p253:							
pROS3	0.360	0.553	1.54	37	24	16	16
pROS45	0.393	0.478	1.22	34	28	20	20
Tn3571 cloned into p253:							
pROS40	0.386	0.221	0.57	11	19	11	11
pROS43a	0.314	0.287	0.90	16	18	10	10

Table 4.4. Results of galactokinase and B-lactamase assays on DS903 containing various transcriptional fusions of Tn3 *tnpA* to *galK*. Controls are also shown. The method of calculating galactokinase and B-lactamase units is given in Ch. 2.

The results showed that Tn3 in *trans* repressed galactokinase expression in pROS3, pROS8, pROS45 and pROS46, where P_A is driving transcription of *galK*. It also repressed expression in pROS33a, where P_R is driving *galK*. The repression is due specifically to the *tnpR* product, not to the presence of the second plasmid. As expected, no repression was observed in the cases of p253, which has no promoter, or pROS4 and pROS47, which are both apparently expressed from the B-lactamase promoter. Resolvase was expected to repress synthesis in pROS40, pROS41 and pROS43a, but due to the very low level of galactokinase expressed, even in the TnpR$^-$ case, no repression was observed. It was impossible to distinguish the phenotype of these constructs from that of p253 in this experiment, although the clones pROS40 and pROS41 had originally been identified due to their very pale pink colour.

4.1.5 Assays of *galK* transcriptional fusions

Galactokinase and B-lactamase assays were carried out on most of the plasmids described in 4.1.4. pKL500 and pKO500 were included for comparison. The results are shown in Table 4.4.

A copy number measurement for p253 or pROS33a was not obtained as neither carries *bla*. The values corrected for background levels of galactokinase are shown in column F. Column G shows the F values as a percentage of the F value for pKL500. Column G enables the ratios of promoter activities at 30°C or 37°C to be determined. The pROS3 and pROS45 data again showed (see also Table 4.1) that P_A is 1/5th to 1/6th the strength of P_{lac} at 30°C or 37°C. Therefore having about 3kbp of extra mRNA (compared to pJKA) inserted upstream of *galK* apparently does not affect its expression, even though the 170bp leader sequence has been disrupted.

Although P_A was supposedly directing transcription in pROS40 and pROS43a, the galactokinase assays showed that transcription into *galK* was apparently reduced about 2-fold in these fusions compared to pROS3 and pROS45. This is consistent with the very pale pink colour observed on MacConkey galactose plates (4.1.3). The lower transposition frequencies observed from both pROS40a and pROS44b (Table 4.2) suggest that the lower enzyme assay results in pROS40 and

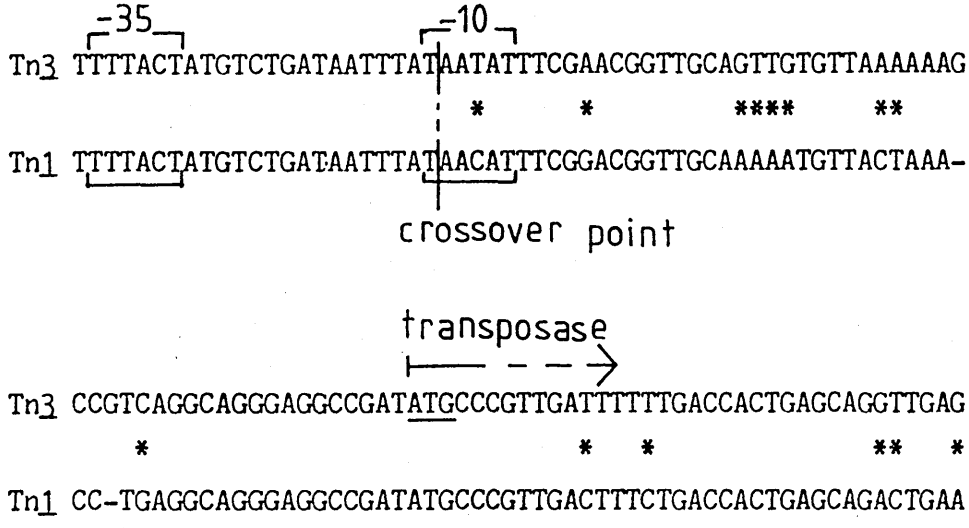


Fig. 4.8 Sequence differences between Tn1 and Tn3 in the region of the tnpA promoters (Burke, Glasgow). The coding strand is shown, and the tnpA -35 and -10 promoter sequences are marked, together with the ATG at which translation (⊢—·→) most often starts.

* marks a known base pair difference between the two transposons.

\- indicates an unknown base pair in Tn1.

pROS43a may be independent of the orientation of the insert in p253, and therefore that the effect may not be due to the increased distance between P_A and galK. Perhaps the sequences bounding Tn3571 from the λdv in these constructs decrease the tnpA message stability (no transcriptional terminator should be present in the region 3' to the tnpA gene, because this sequence is part of the Cm^r gene). That this is a possibility is suggested by the observation (3.4) that transposition from the λdv vector, pROS13, from which pROS40 etc. were derived (4.1.3), is about one third the level that would be expected in that system.

The fact that the P_A:P_{lac} ratios, in the cases of pROS3 and pROS45, are approximately constant between 30°C and 37°C, implies that, unless P_A and P_{lac} both decrease with temperature to the same extent, it is unlikely that temperature affects transposition by causing increased transcriptional termination within the tnpA mRNA at higher temperatures.

4.2 Construction and assaying of lacZ translational fusions to Tn1 and Tn3

It was originally intended that for this thesis all work would be carried out on Tn3 rather than Tn1. Tn3 has been entirely sequenced (Heffron 1979), unlike Tn1, and some restriction site differences are known to exist between the two transposons (Brunton et al. 1981). For the constructions described here, RSF1050 (Heffron et al. 1977) was used as the source of Tn3 where possible. However, for some translational fusions, pAA33 (Arthur 1981) was used as the source of the transposon. According to Arthur (1981) pAA33 is pACYC184 with an insertion of Tn3. After constructions containing this transposon had been made and assayed, it was discovered during digestion with KpnI, that this transposon was actually Tn1. [Tn1 has a KpnI site at about position 750, whereas Tn3 does not.] Although Tn1 is also known to be temperature sensitive in its transposition (Hettle 1985), this mistake was unfortunate because a region of Tn1 extending from the N-terminal end of tnpA to tnpR has been sequenced in this laboratory (Burke, Fig. 4.8), and been found to contain several differences to Tn3. One difference causing particular concern is that within the "-10" promoter sequence of tnpA where there is a change, from a "T" in Tn3

to a "C"in Tn*1*. This is only 3bp from the crossover site where resolvase acts to site-specifically resolve cointegrates. Thus this difference could affect the transposition of the transposons in three ways: it could affect the promoter activity of *tnpA* directly; it could affect binding of resolvase to sub-site I (1.6.2), thus altering the transcriptional repression of *tnpA*; and it could affect the efficiency of cointegrate resolution. In view of the differences, the use of Tn*3* or Tn*1* in the constructions below is clearly noted.

The transcriptional fusions described in 4.1.1 and 4.1.5 seemed to suggest that it is most likely that the temperature effect on transposition takes place at some stage after transcription of the mRNA. Therefore it was considered important to examine the translation of *tnpA* at different temperatures.

The most commonly used gene for translational fusions is *lacZ* (Silhavy and Beckwith 1985). There are several reasons for this: the *lac* operon has been intensively studied, so many genetic and biochemical aspects of the system are known; lactose, the substrate of the system, can be used by *E. coli* as a sole carbon source, so that indicator media available for detecting sugar metabolism can be used; lactose is a disaccharide which provides the opportunity to generate numerous functional analogs of lactose in which the glucose moiety is replaced. The analogue *o*-nitrophenyl-B-D-galactopyranoside (ONPG) is a substrate which, when hydrolysed by B-galactosidase to ONP, gives a yellow colour. It is this analogue that is used as the substrate in the B-galactosidase enzyme assays described in this chapter. This assay is extremely sensitive. The analogue 5-Bromo-4-chloro-3-indoyl-B-D-galactoside (Xgal) is also a substrate of B-galactosidase. It yields a blue colour upon hydrolysis.

The B-galactosidase enzyme itself is a tetramer, and the molecular weight of each monomer is 116kd. From gene fusion studies it is known that up to 26 amino acids can be removed from the N-terminus and be substituted for by other amino acid sequences with very little effect on the specific activity of the enzyme (see Ch. 5). Hybrid proteins with B-galactosidase activity therefore have subunit molecular weights of anywhere from approximately 114kd to 116kd and up. These proteins are among the largest found in *E. coli* which means that, on SDS-

polyacrylamide gels, the hybrid proteins are found in the upper region of the gel in which very few other proteins migrate. Therefore, depending on levels of synthesis, these proteins are easy to localise, and can be excised from the gels with little background contamination, electroeluted, and used to raise antibodies against the fused gene segment (Ch. 5).

In the wild-type lac operon, lacZ coding sequences lie immediately adjacent to the signals for transcription and translation initiation. Casadaban et al. (1980) put a BamHI site at codon 8, which meant that the lacZ gene could be separated from its promoter and translation signals, and they constructed several vectors which contained an easily mobile lacZ "cassette". This could be cloned into other vectors allowing fusions to the gene of interest to be made. This enabled that gene's expression to be analysed.

4.2.1 Construction of lacZ fusions to the 5' ends of Tn3 tnpA and tnpR genes

The plasmid pMC1403 (Casadaban et al. 1980) was used by Minton et al (1984) as the source of a lacZ (and lacY) fragment in the construction of a set of three pUC-based vectors, which differ only in the position of the polylinker relative to lacZ. Cloning a particular fragment into the same sites of pNM480, pNM481 and pNM482 results in lacZ fusions to each of the three reading frames of the fragment. Usually only one of these clonings results in fusion of an open reading frame to lacZ. A blue colour is seen on plates containing Xgal in this case, and the activity of the translational fusion protein can easily be assayed using ONPG.

A partial EcoRI* digestion of Tn3, initially carried on RSF1050, gives rise to a 282bp fragment containing the tnpA and tnpR promoters and translation signals (Kitts 1982). This fragment was originally isolated and cloned via another plasmid into the EcoRI site of pBR322 to make pPAK329. This plasmid was the source of the 282bp fragment for cloning into pUC (Burke, Glasgow): the fragment was excised from pPAK329 with EcoRI, the 5' overhangs were filled-in, and the blunt-ended fragment was ligated to HincII-cut pUC8 and pUC18. The plasmid derivatives carried the fragment in both orientations. In pUC8.1,

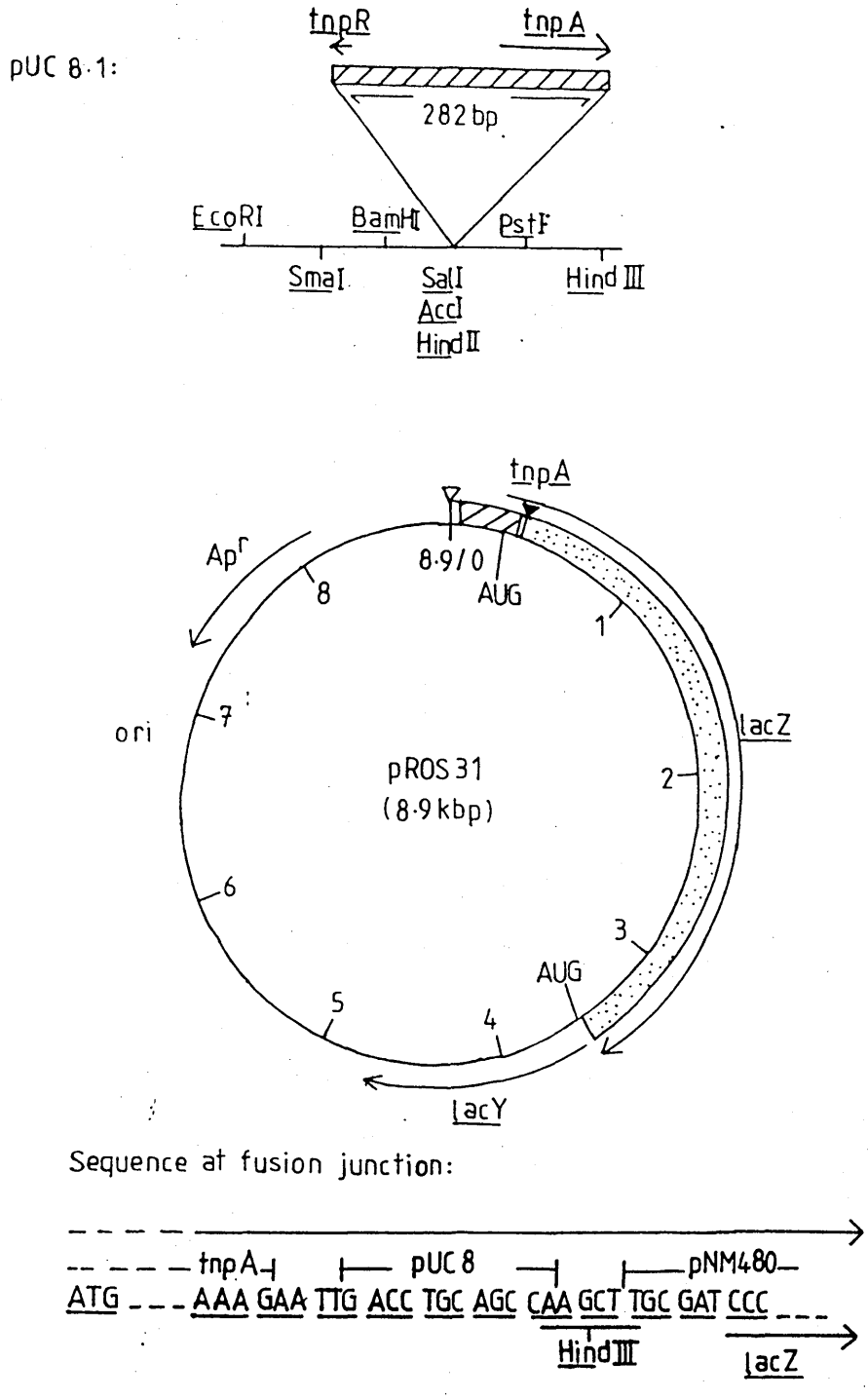
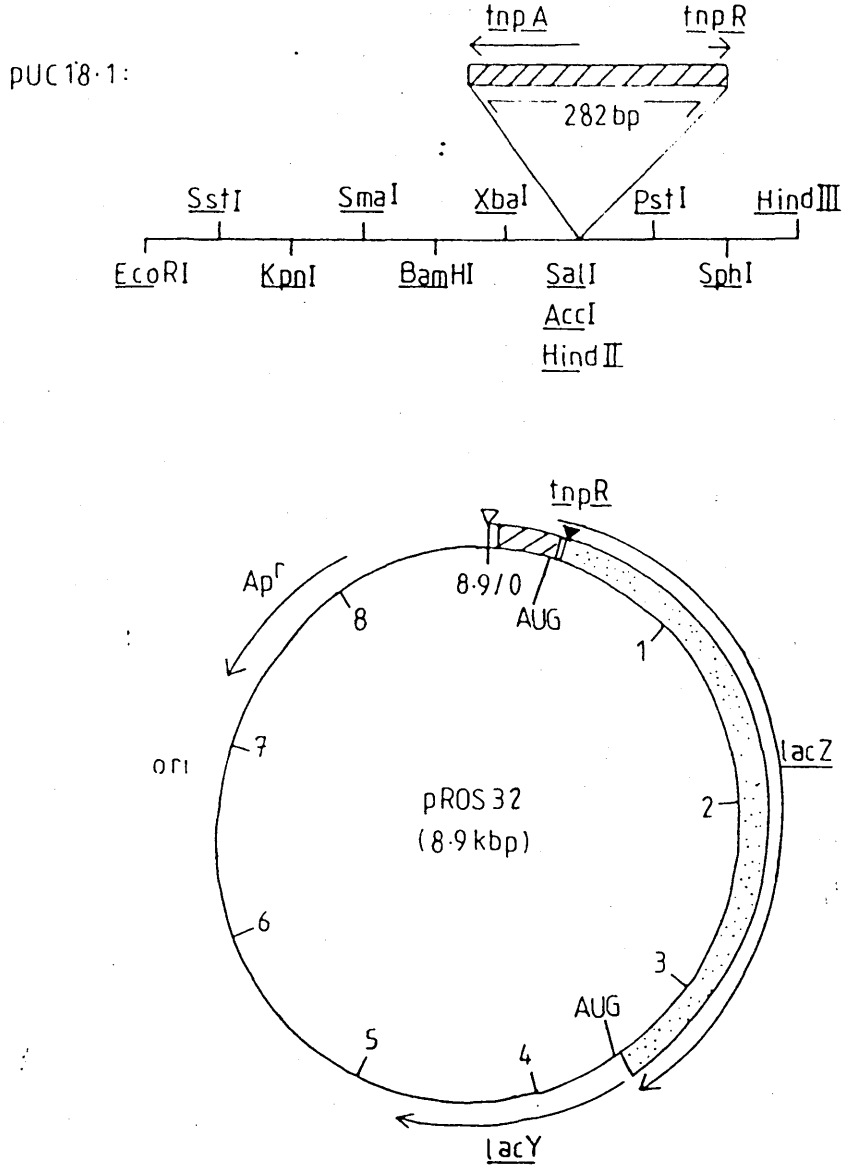


Fig. 4.9a pROS31, showing the translational fusion of Tn3 *tnpA* to *lacZ*.

Tn3-derived 282bp fragment
pUC8 sequences
lacZ sequences
▼ HindIII site
▽ EcoRI site



Sequence at fusion junction:

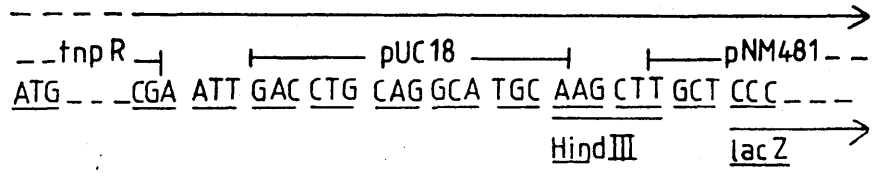


Fig. 4.9b pROS32, showing the translational fusion of Tn3 tnpR to lacZ.

Tn3-derived 282bp fragment
pUC18 sequences
lacZ sequencs
HindIII site
EcoRI site

30°C:

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
A	170	0.008	0.0010	1.470	0.0425	35	125	5.3
	200	0.011	0.0018	2.646	0.0560	47	164	5.1
	240	0.022	0.0029	4.263	0.1006	42	132	4.6
	285	0.045	0.0062	9.114	0.1441	63	138	3.2
	315	0.071	0.0087	12.789	0.1960	65	123	2.8
	345	0.099	0.0127	18.669	0.3097	60	128	3.1
	380	0.137	0.0180	26.460	0.4283	62	131	3.1
	405	0.167	0.0249	36.603	0.4810	76	149	2.9
	430	0.184	0.0329	48.363	0.6090	79	179	3.3
	460	0.209	0.0471	69.237	0.7380	94	225	3.5
	500	0.233	0.0574	84.378	-	-	246	-
B	170	0.008	0.0008	1.176	0.0251	47	100	3.1
	200	0.010	0.0007	1.029	0.0247	42	70	2.5
	240	0.022	0.0025	3.675	0.0889	41	114	4.0
	285	0.048	0.0062	9.114	0.1195	76	129	2.5
	315	0.071	0.0086	12.642	0.0189	67	121	2.7
	345	0.099	0.0120	17.640	0.2851	62	121	2.9
	380	0.140	0.0181	26.607	0.2939	91	129	2.1
	405	0.173	0.0213	31.311	0.4839	65	123	2.8
	430	0.183	0.0370	54.390	0.5260	103	202	2.8
	460	0.204	0.0518	76.146	0.7930	96	254	3.9
	500	0.238	0.0635	93.345	-	-	269	-

Table 4.5a Results of B-galactosidase and B-lactamase assays on DS942 (pROS31) kept still at 30°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

37°C:

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
A	156	0.004	0.0012	1.764	0.0377	47	300	9.4
	220	0.015	0.0021	3.087	0.1124	27	140	7.5
	258	0.029	0.0043	6.321	0.1661	38	148	5.7
	320	0.051	0.0102	14.994	0.3302	45	200	6.5
	343	0.071	0.0181	26.607	0.5863	45	255	8.3
	376	0.093	0.0325	47.775	0.8118	59	349	8.7
	396	0.101	0.0345	50.715	0.7564	67	342	7.5
	443	0.129	0.0541	79.527	1.4044	57	419	10.6
	463	0.146	0.0661	97.167	1.5472	63	453	10.6
	488	0.160	0.0610	89.670	1.6637	54	381	10.4
	513	0.170	0.0713	104.811	1.1461	91	419	6.7
	548	0.176	0.1074	157.878	1.3933	113	610	7.9
B	156	0.010	0.0029	4.263	0.1508	28	290	15.1
	220	0.047	0.0066	9.702	0.2778	35	140	5.9
	258	0.069	0.0109	16.023	0.3476	46	158	5.0
	320	0.089	0.0227	33.369	0.6371	52	255	7.2
	343	0.106	0.0344	50.568	0.9529	53	325	9.0
	376	0.124	0.0469	68.943	1.2023	57	378	9.7
	396	0.138	0.0469	68.061	1.0986	62	336	8.0
	443	0.152	0.0540	79.380	1.7116	66	355	11.3
	463	0.165	0.0478	70.266	1.5168	46	290	9.2
	488	0.181	0.0630	92.610	1.7400	53	348	9.6
	513	0.188	0.0788	115.836	1.9121	61	419	10.2
	548	0.201	0.0744	109.368	1.2791	86	370	6.4

Table 4.5b Results of B-galactosidase and B-lactamase assays on DS942 (pROS31) kept still at 37°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

30°C:

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
A	90	0.002	0.0001	0.147	0.0066	22	50	3.3
	150	0.010	0.0014	2.058	0.0185	111	140	1.9
	195	0.015	0.0027	3.969	0.0226	176	180	1.5
	225	0.018	0.0047	6.909	0.0525	132	261	2.9
	245	0.034	0.0063	9.261	0.0421	220	185	1.2
	267	0.040	0.0095	13.965	0.0418	334	238	1.0
	295	0.053	0.0153	22.491	0.0963	234	289	1.8
	323	0.076	0.024	35.280	0.1534	230	316	2.0
	345	0.097	0.0336	49.392	0.2229	222	346	2.3
	428	0.194	0.1007	148.029	0.3268	453	519	1.7
	458	0.230	0.1279	188.013	0.7426	253	556	3.2
	480	0.251	0.1421	208.887	0.5843	357	566	2.3
B	90	0.004	0.0012	1.764	0.0092	192	300	2.3
	150	0.011	0.0035	5.145	0.0148	348	318	1.3
	195	0.022	0.0059	8.673	0.0331	262	268	1.5
	225	0.031	0.0086	12.642	0.0371	341	277	1.2
	245	0.044	0.0152	41.560	0.0562	740	345	1.3
	267	0.061	0.0206	56.325	0.1111	507	338	1.8
	295	0.082	0.0274	74.917	0.1290	581	334	1.6
	323	0.108	0.0352	96.244	0.1938	497	326	1.8
	345	0.146	0.0490	133.976	0.2638	508	336	1.8
	368	0.171	0.0678	185.379	0.3780	490	396	2.2
	428	0.230	0.1051	287.364	0.4349	661	457	1.9
	458	0.258	0.1051	299.942	0.5287	567	425	2.0
	480	0.261	0.1562	427.082	0.5286	808	598	2.0

Table 4.6a Results of B-galactosidase and B-lactamase assays on DS942 (pROS32) kept still at 30°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

37°C:

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
A	90	0.006	0.0115	16.905	0.0354	478	1917	5.9
	150	0.017	0.0290	42.630	0.0663	643	1706	3.9
	195	0.037	0.0606	89.082	0.1266	704	1638	3.4
	225	0.049	0.0862	126.714	0.1761	720	1759	3.6
	245	0.070	0.1068	156.996	0.2397	655	1526	3.6
	295	0.122	0.1763	259.161	0.4306	602	1445	3.5
	323	0.145	0.2543	373.821	0.6345	589	1754	4.4
	345	0.170	0.2435	357.945	0.8258	433	1432	4.9
	410	0.216	0.5015	737.205	1.3586	543	2322	6.3
	428	0.222	0.5842	858.774	1.4259	602	2632	6.4
	458	0.251	0.6390	939.330	1.5935	589	2546	6.3
	480	0.279	0.9043	1329.321	1.7333	767	3241	6.2
B	90	0.002	0.0103	15.141	0.0329	460	5150	16.5
	150	0.020	0.0285	41.895	0.0612	685	1425	3.1
	195	0.026	0.0526	77.322	0.1001	772	2023	3.9
	225	0.047	0.0705	103.635	0.1700	610	1500	3.6
	245	0.066	0.1114	163.758	0.3065	534	1688	4.6
	267	0.092	0.1120	164.640	0.3437	479	1217	3.7
	295	0.116	0.1560	229.320	0.5008	458	1345	4.3
	323	0.136	0.2244	329.868	0.7079	466	1650	5.2
	345	0.166	0.2084	306.348	0.9520	322	1255	5.7
	410	0.218	0.3514	516.558	1.3667	378	1612	6.3
	428	0.225	0.5459	802.473	1.3678	587	2426	6.1
	458	0.253	0.6606	971.082	1.6599	585	2611	6.6
	480	0.283	0.8113	1192.611	1.7274	690	2867	6.1

Table 4.6b Results of B-galactosidase and B-lactamase assays on DS942 (pROS32) kept still at 37°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

tnpA points towards the pUC8 HindIII site. In pUC18.1, tnpR points towards the pUC18 HindIII site. Excision of the small EcoRI/HindIII fragment from pUC8.1, ligation to EcoRI/HindIII-cut pNM480, and transformation into DS942, gives blue colonies on Xgal plates. pROS31 contains 38 amino acids of the 5' end of tnpA fused to lacZ. The structure of pROS31 is shown in Fig. 4.9a. Excision of the small EcoRI/HindIII fragment from pUC18.1, ligation to EcoRI/HindIII-cut pNM481, and transformation into DS942, gives blue colonies on Xgal plates. pROS32 contains 3 amino acids of the 5' end of tnpR fused to lacZ. The structure of pROS32 is shown in Fig. 4.9b.

pROS31 and pROS32 were examined for repression of B-galactosidase synthesis by putting an intact tnpR gene in trans (4.2.4). In both, levels were found to be repressed, confirming that the B-galactosidase expression in these constructs is under the transcriptional control of tnpA or tnpR.

4.2.2 Assaying of lacZ fusions to the 5' ends of Tn3 tnpA and tnpR

B-galactosidase assays together with B-lactamase assays were performed on pROS31 and pROS32. The cultures were either kept still (low dissolved oxygen), or shaken (high dissolved oxygen), at 30°C and 37°C. This was to see the effect of growth rate and/or dissolved oxygen on tnpA and tnpR expression. 2.5ml duplicates i.e. two transformant colonies of each construct, were grown overnight at the experimental temperature and were subcultured 200ul into 100ml of L-broth without glucose, but with ampicillin. Tests showed that the B-galactosidase and B-lactamase assays were linear over the time of the assays. It would have been ideal to have measured the protein content at each time-point, but this was impracticable due to the small volumes (less than 3ml) removed for assays, which would make sonication awkward, and also to the large number of samples involved. The results of assays on the cultures which were kept still throughout their growth are shown in Tables 4.5 (pROS31) and 4.6 (pROS32). The data in these tables are shown graphically in Figs. 4.10-4.15.

Taking pROS31A as an example (Table 4.5), it can be shown that at 30°C and 37°C, both the B-galactosidase and the B-lactamase units increase in parallel with the OD_{600} until the growth rate slows (Fig. 4.10a and

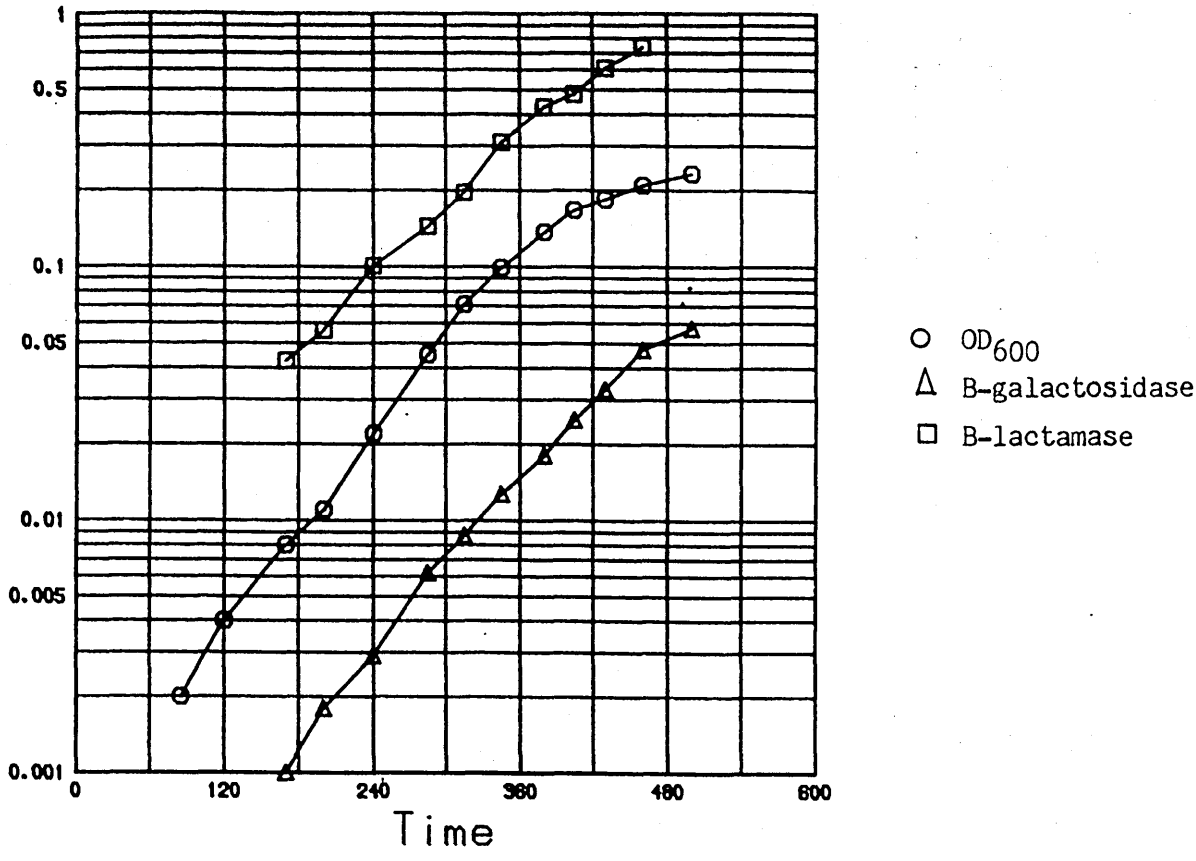


Fig. 4.10a pROS31A, showing OD_{600}, B-galactosidase activity, and B-lactamase activity plotted against time for a culture kept still at 30°C. The raw data are given in Table 4.5.

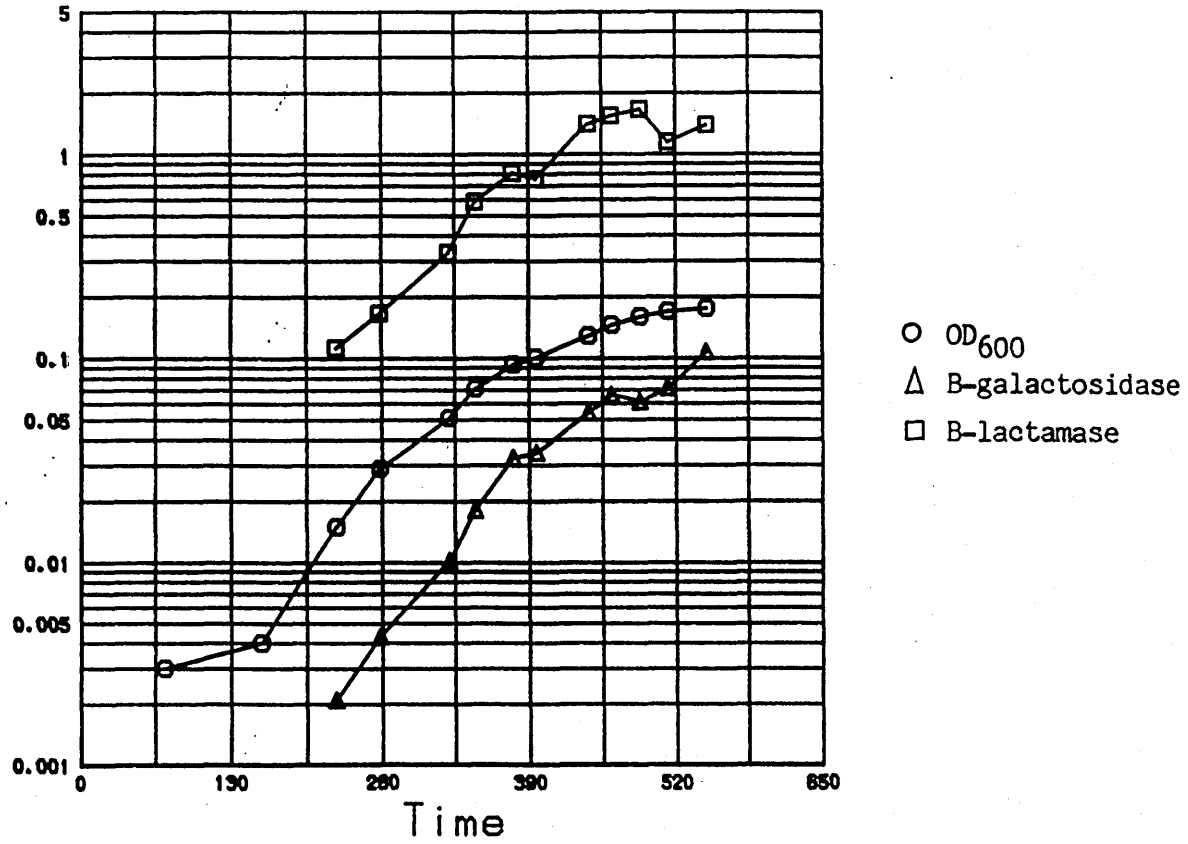


Fig. 4.10b pROS31A, showing OD_{600}, B-galactosidase activity, and B-lactamase activity plotted against time for a culture kept still at 37°C. The raw data are given in Table 4.5.

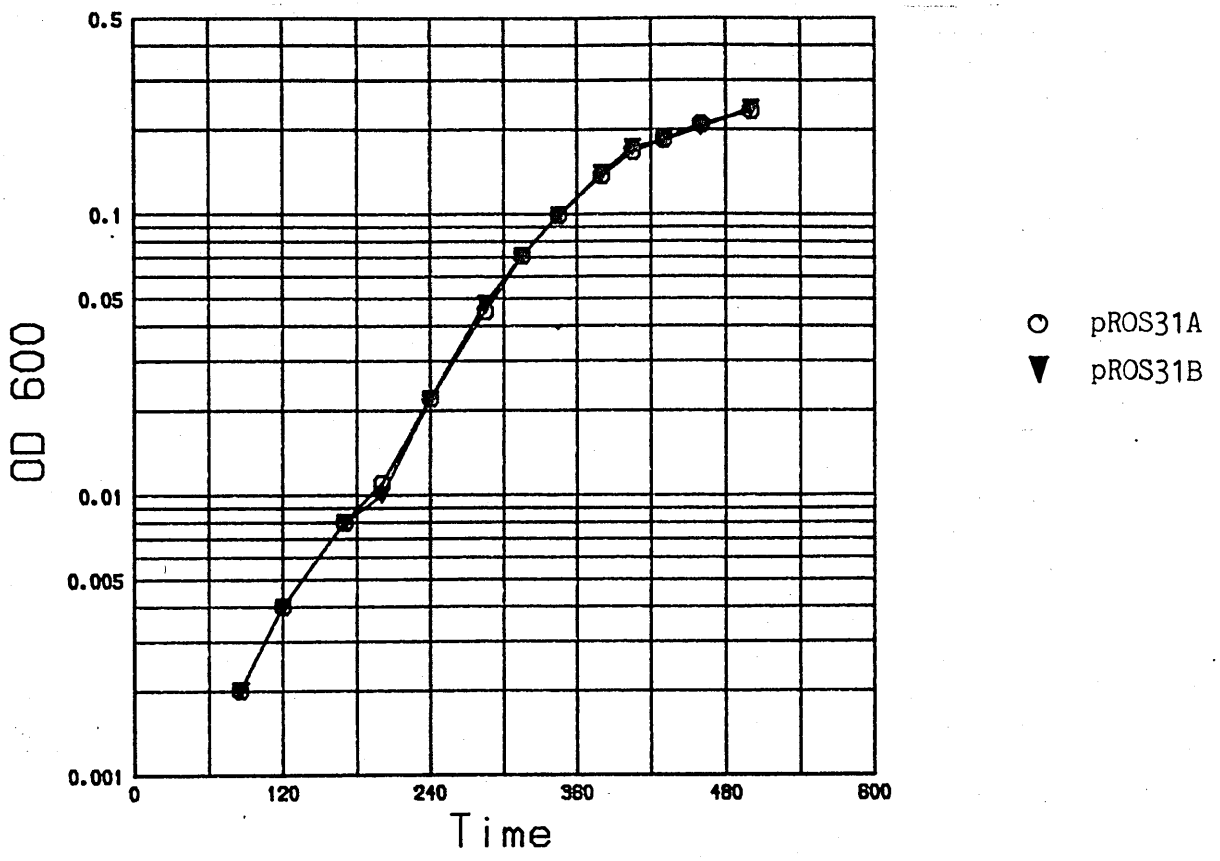


Fig. 4.11a pROS31A and pROS31B, showing OD_{600} plotted against time for still cultures grown at 30°C. The raw data are given in Table 4.5.

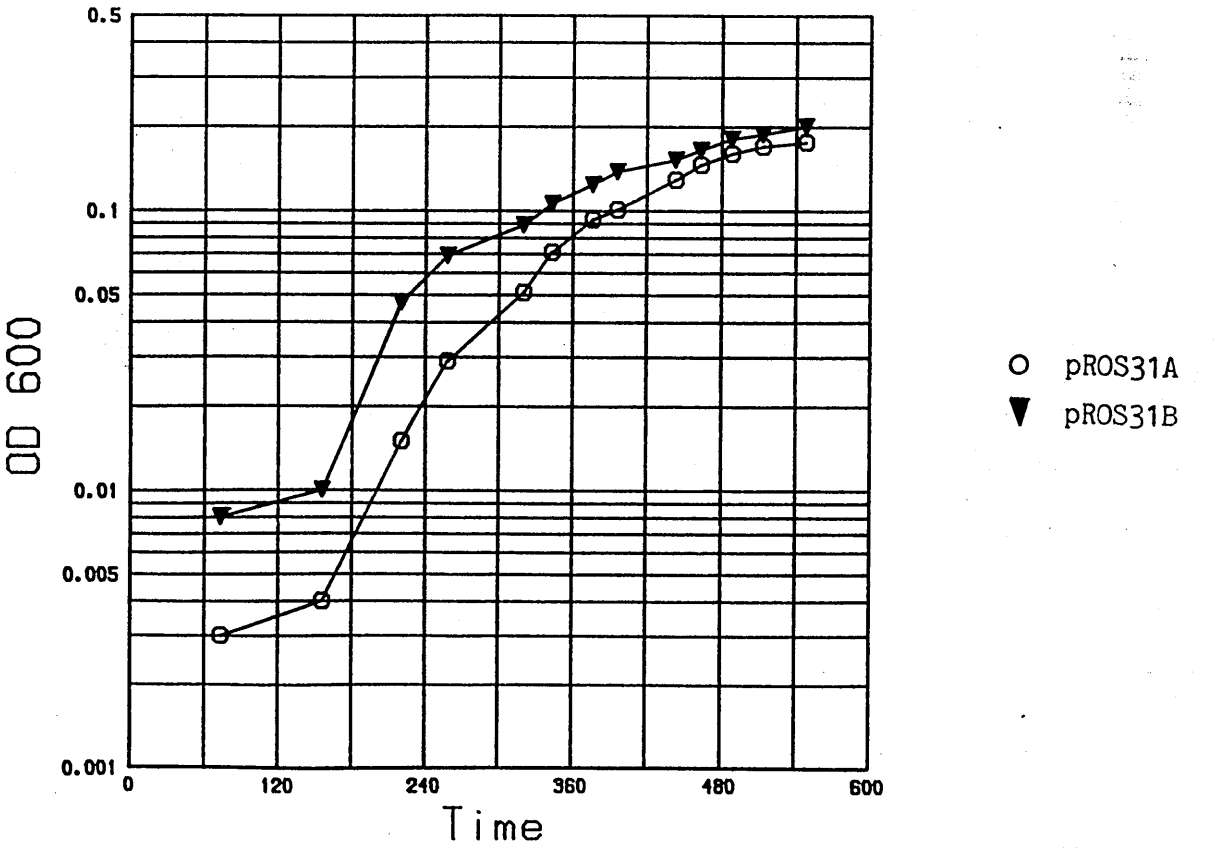


Fig. 4.11b pROS31A and pROS31B, showing OD_{600} plotted against time for still cultures grown at 37°C. The raw data are given in Table 4.5.

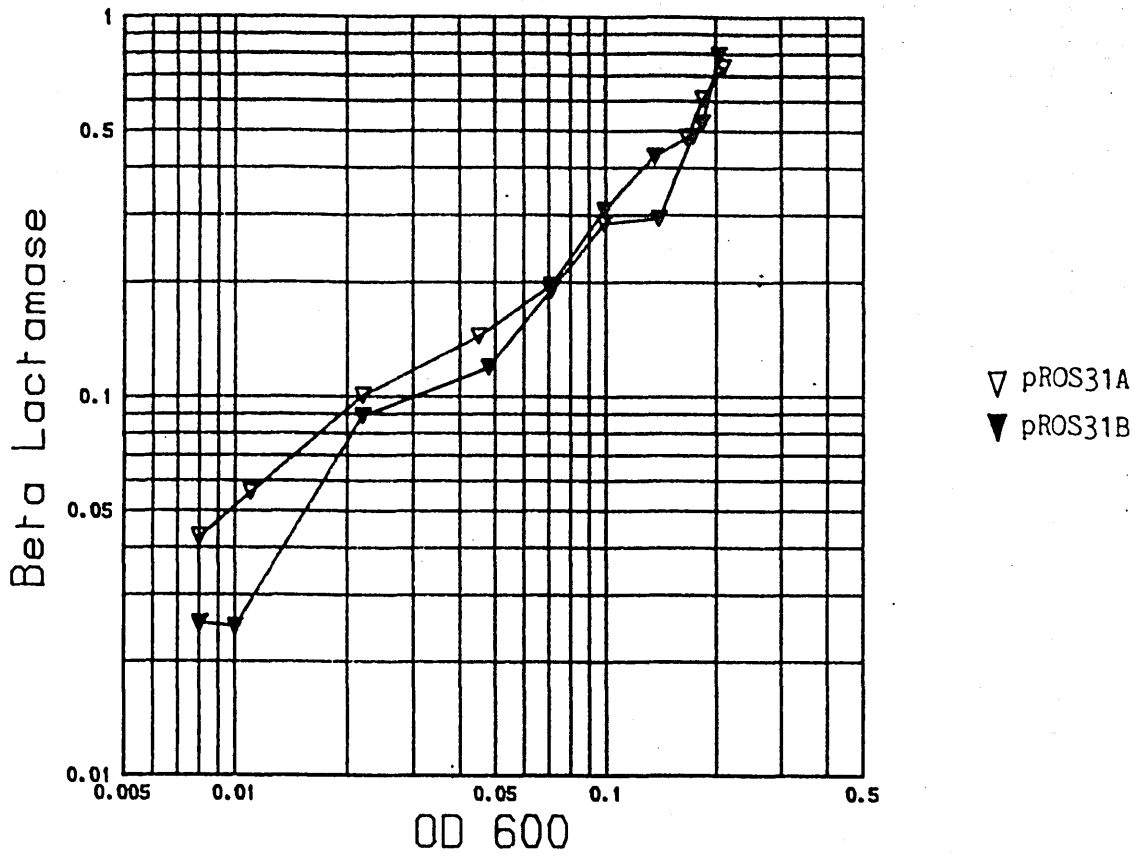


Fig. 4.12a pROS31A and pROS31B, showing B-lactamase plotted against OD_{600} for still cultures grown at 30°C. The raw data are shown in Table 4.5.

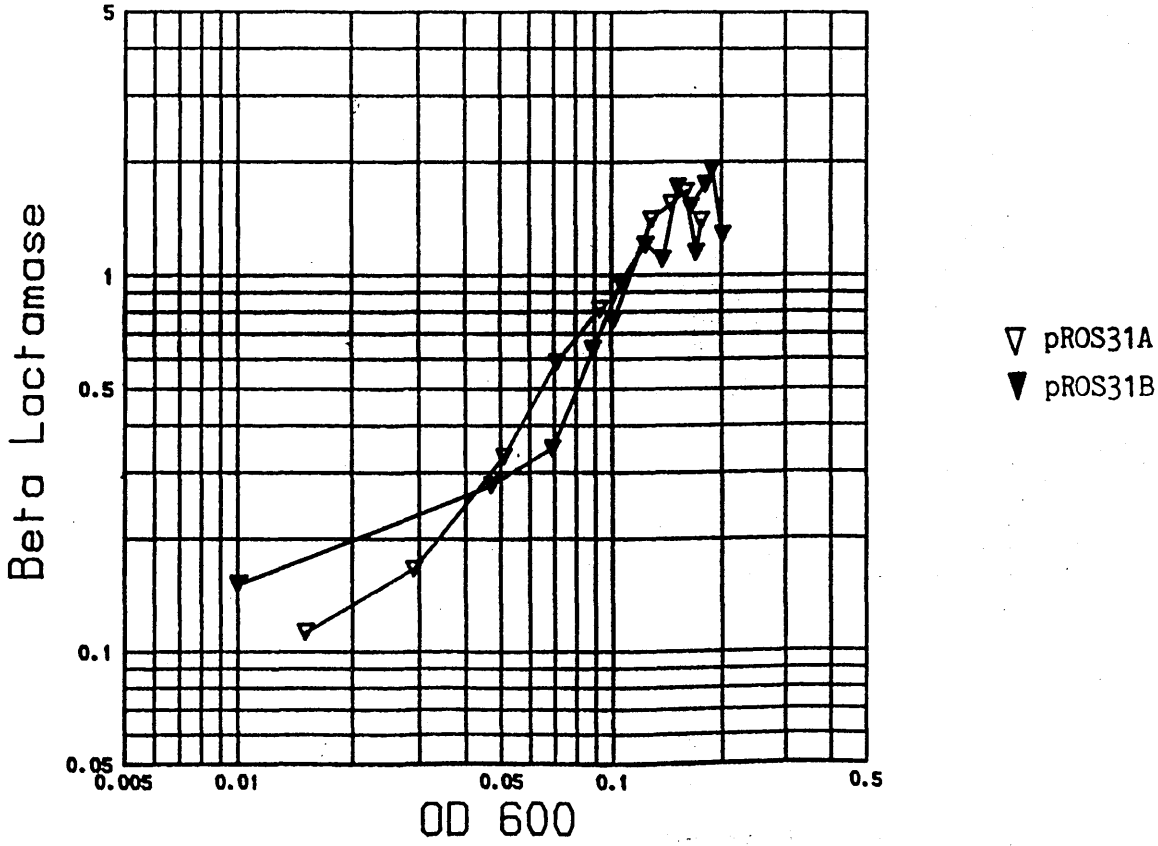


Fig. 4.12b pROS31A and pROS31B, showing B-lactamase plotted against OD_{600} for still cultures grown at 37°C. The raw data are shown in Table 4.5.

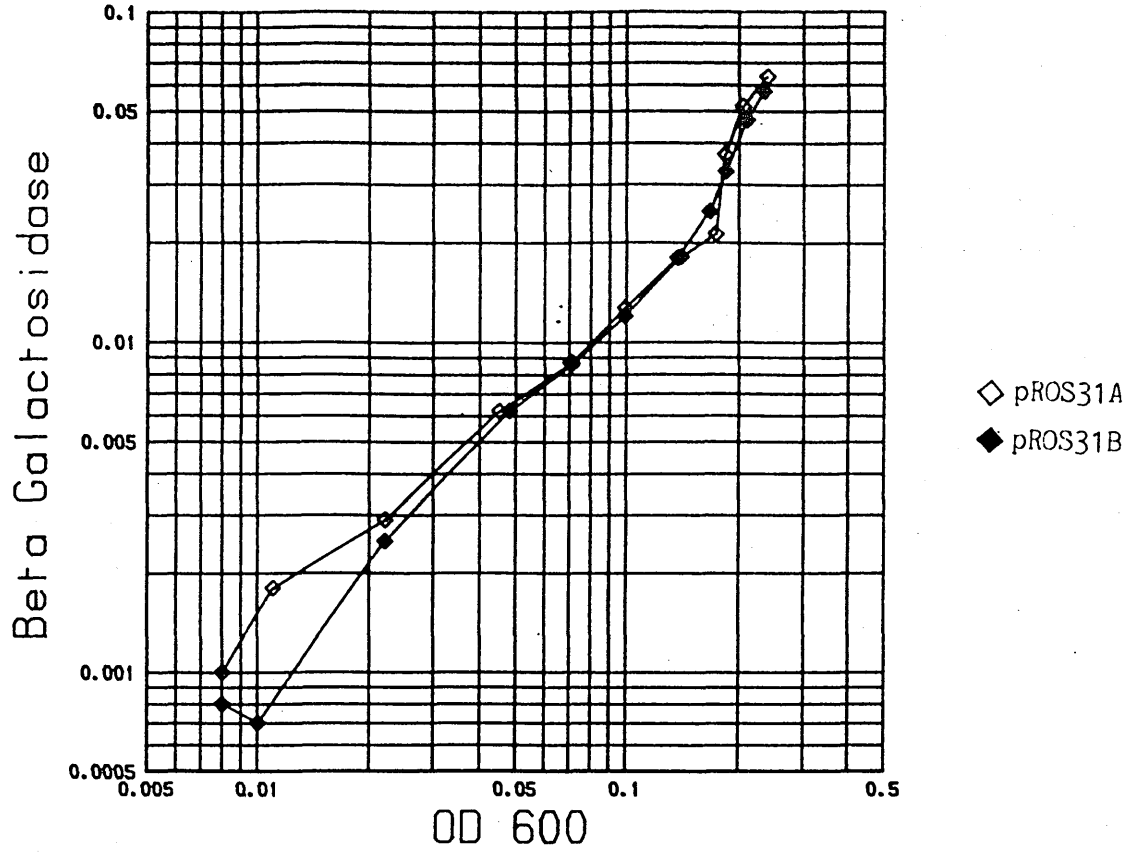


Fig. 4.13a pROS31A and pROS31B, showing B-galactosidase plotted against OD_{600} for still cultures grown at 30°C. The raw data are shown in Table 4.5.

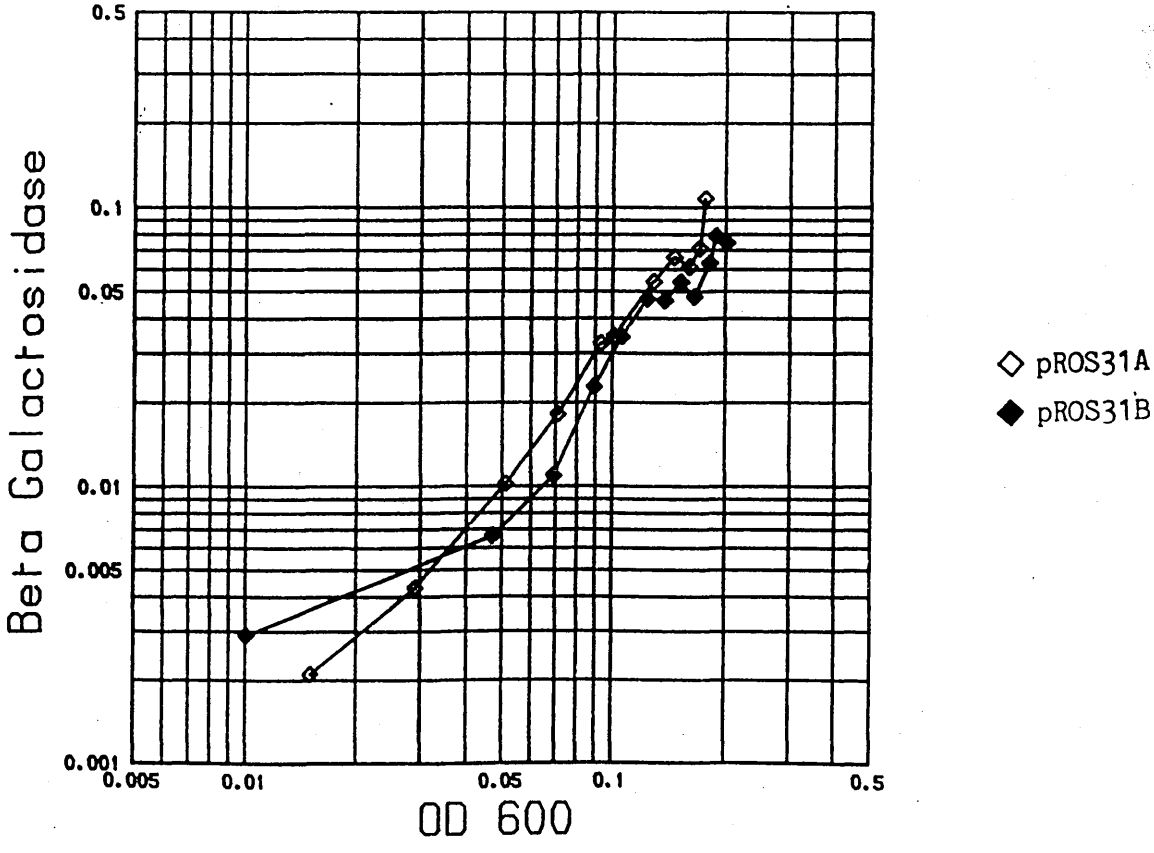


Fig. 4.13b pROS31A and pROS31B, showing B-galactosidase plotted against OD_{600} for still cultures grown at 37°C. The raw data are shown in Table 4.5.

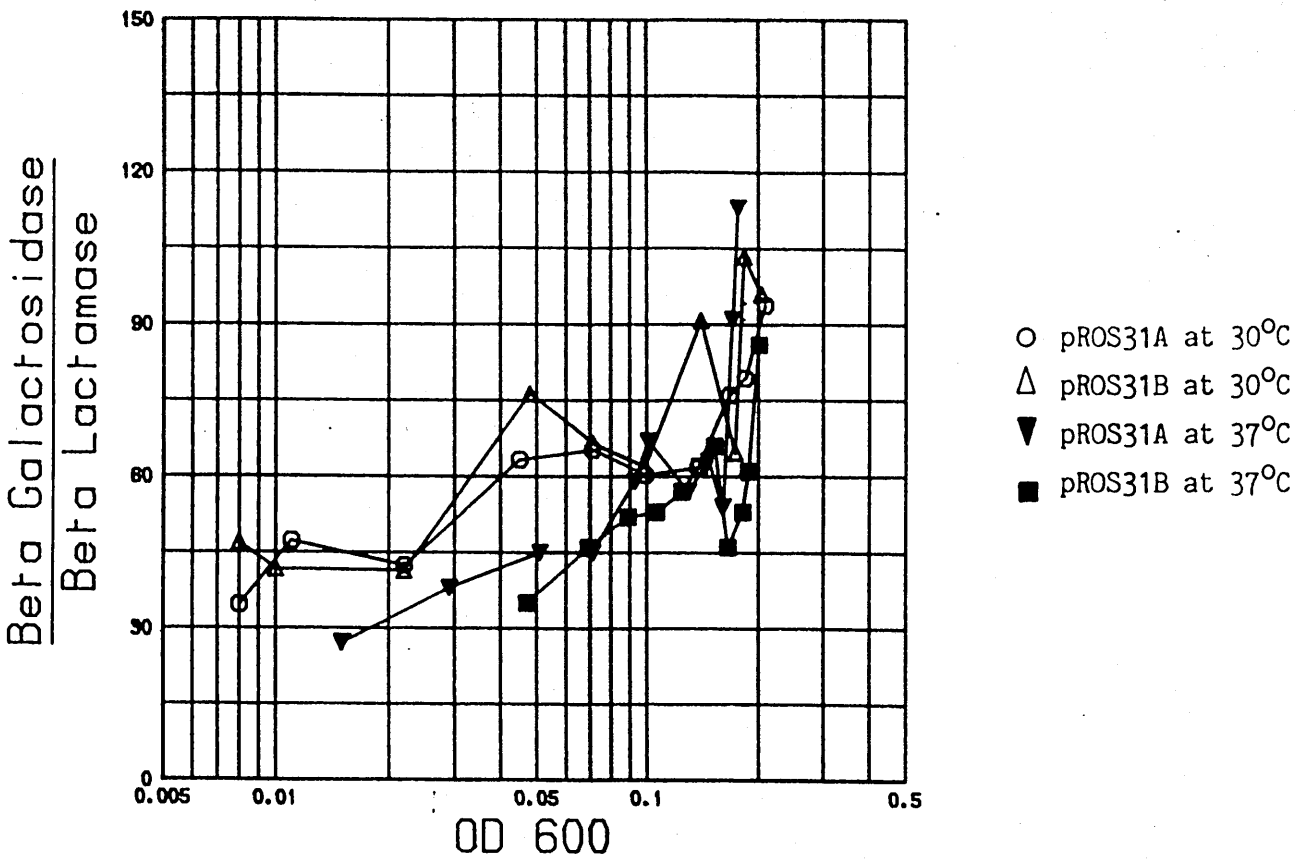


Fig. 4. 14 The ratio of B-galactosidase:B-lactamase for pROS31A and pROS31B for still cultures grown at 30°C and 37°C. The raw data are shown in Table 4.5.

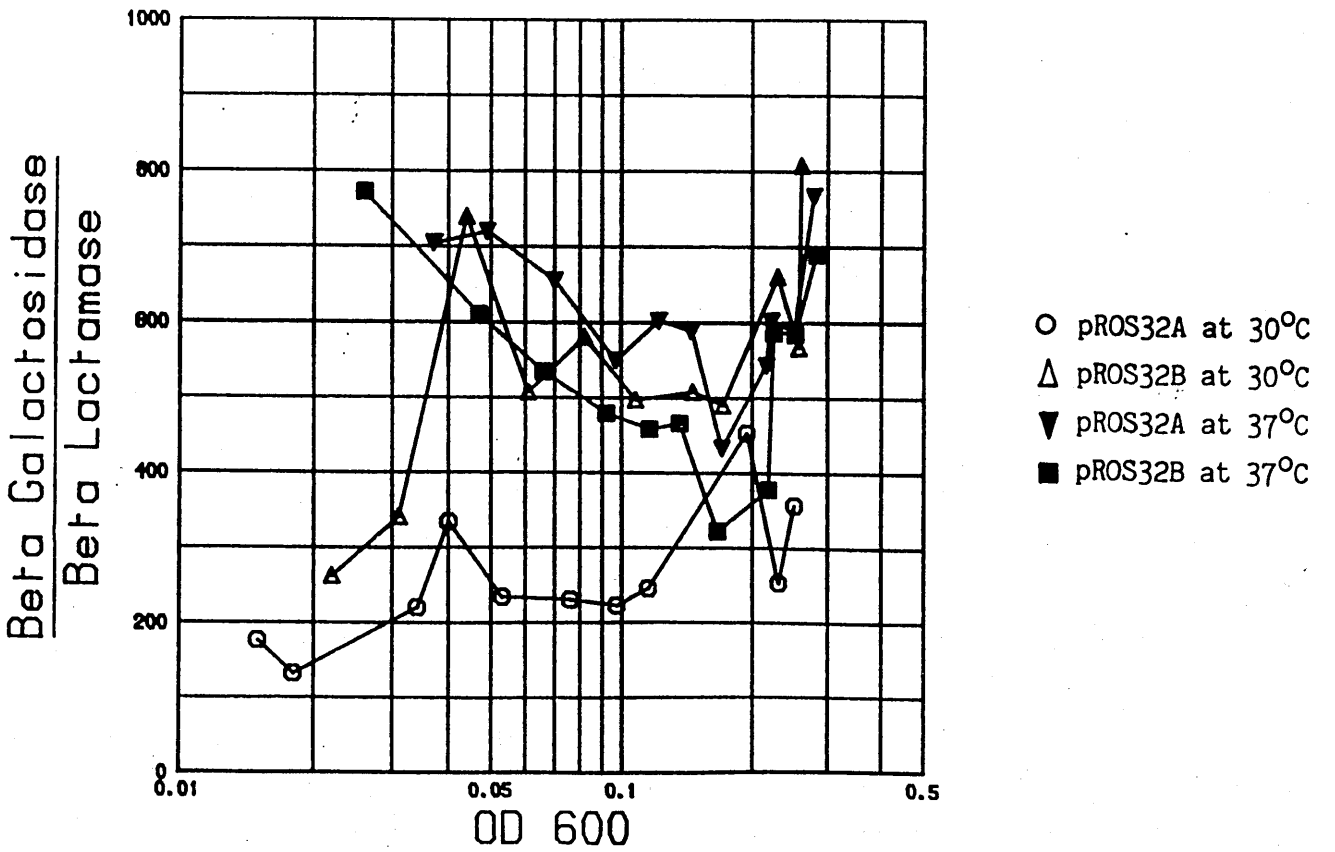


Fig. 4.15 The ratio of B-galactosidase:B-lactamase for pROS32A and pROS32B for still cultures grown at 30°C and 37°C. The raw data are shown in Table 4. 6.

b). Then, particularly obviously in the 30°C case, the enzyme units continue to increase for a time. Presumably this is due to plasmid copy number increasing somewhat in the slowing culture.

The growth curves for pROS31A and B at 30°C and 37°C show that the growth rates of both are the same at 30°C and very similar at 37°C (Fig. 4.11a and b).

Fig. 4.12 shows the logs of B-lactamase and OD_{600} plotted against each other for pROS31A and B at 30°C and 37°C. The graph should show a positive line with a gradient of 1.0 if the increase in the enzyme levels is directly proportional to the increase in OD_{600}. This is so until the growth rate slows. It should also be the case when the log of B-galactosidase is plotted against the log of OD_{600} (Fig. 4.13a and b). However, here the gradient is slightly greater than 1.0, probably reflecting the stability of B-galactosidase. In agreement with this is that when the ratio of B-galactosidase/B-lactamase is plotted against the log of OD_{600} at 30°C or 37°C, it is apparent that the ratio creeps up during growth (Fig. 4.14). This also occurs in fusions to tnpR and to the lac promoter and translation signals (Tables 4.8 and 4.11). The probable reason for this is that B-galactosidase may be more stable than B-lactamase. From this graph it can also be seen that the ratio of B-galactosidase/B-lactamase is very slightly higher at 30°C than 37°C. However, this slight difference is probably not significant with regard to the temperature effect on transposition for two reasons: firstly, the result is not repeatable in other tnpA fusions which are shaken (Tables 4.7, 4.12, 4.13, and 4.14), where a slight increase in the ratio at 37°C is actually observed; secondly, the B-galactosidase units per OD_{600} (Fig. 4.13a and b) actually increase two-fold between 30°C and 37°C, and therefore the number of molecules of B-galactosidase per cell, and by inference the number of transposase molecules, is increased about 2-fold at 37°C.

Fig. 4.15 shows the ratios of B-galactosidase/B-lactamase for pROS32 at 30°C and 37°C while kept still. Here the ratio increases between 30°C and 37°C, which shows that tnpR expression is greater relative to bla expression at 37°C than at 30°C. Numbers of resolvase molecules per cell also apparently increase about 5-fold at 37°C, as suggested

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
30°C:								
A	280	0.156	0.0156	22.93	0.308	74	100	2.0
	350	0.401	0.0701	103.05	1.095	94	175	2.7
B	280	0.185	0.0157	23.08	0.398	58	85	2.2
	350	0.483	0.0727	106.87	1.277	84	151	2.6
37°C:								
A	255	0.178	0.0434	63.80	1.000	64	244	5.6
	305	0.346	0.1388	204.04	2.088	98	401	6.0
B	305	0.180	0.0694	102.02	1.273	80	386	7.1
	360	0.338	0.1642	241.37	2.844	85	486	8.4

Table 4.7 Results of B-galactosidase and B-lactamase assays on DS942 (pROS31) shaking at 30°C and 37°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
30°C:								
A	280	0.160	0.0376	55.27	0.328	169	235	2.1
	350	0.429	0.1857	272.98	1.082	252	433	2.5
B	280	0.163	0.0439	64.53	0.444	145	269	2.7
	350	0.426	0.2172	319.28	1.625	196	510	3.8
37°C:								
A	360	0.110	0.4992	733.82	0.897	818	4538	8.2
B	360	0.120	0.4897	719.86	0.963	748	2449	8.0

Table 4.8 Results of B-galactosidase and B-lactamase assays on DS942 (pROS32) shaking at 30°C and 37°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

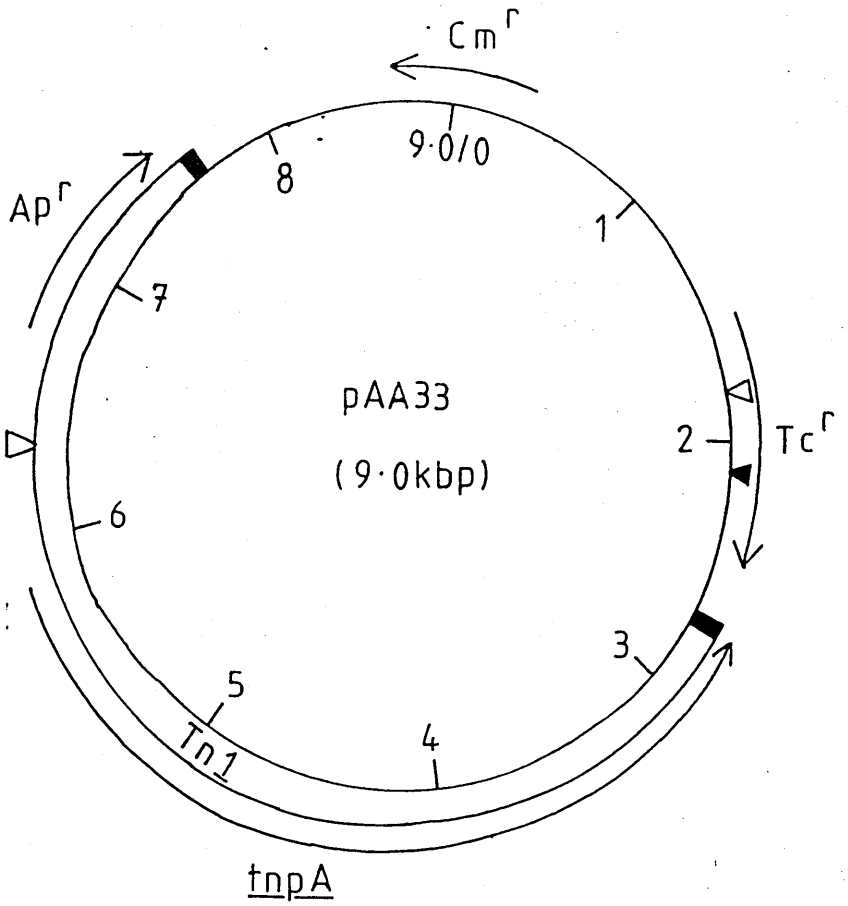


Fig. 4.16 pAA33 (pACYC184::Tn1 Arthur 1981).

▼ SalI site

▽ BamHI sites

by the B-galactosidase units per OD_{600} in Table 4.6.

The results of the assays on pROS31 and pROS32-containing cultures which were shaken at 30°C and 37°C are shown in Tables 4.7 (pROS31) and 4.8 (pROS32). Here the ratio of B-galactosidase/B-lactamase is higher at 37°C than 30°C in both pROS31 and pROS32, again showing the differential effect of temperature on tnpA and tnpR expression relative to bla. The B-galactosidase units per OD_{600}, hence fusion molecules per cell, for pROS31 are greater by 2- to 3-fold at 37°C than 30°C, and those for pROS32 are greater by about 9-fold at 37°C than 30°C.

The results from pROS31, whether shaking or still, suggest that the temperature effect on transposition does not act on the initiation of transcription or translation of tnpA. This is because the fusion assays show no significant effect of temperature on B-galactosidase/B-lactamase ratios which might explain the observed decrease in transposition at 37°C compared to 30°C.

4.2.3 Construction of lacZ fusions at different positions along the Tn1 tnpA gene

It was a possibility that expression of the transposase protein might be affected by temperature due to changes in tnpA message stability with temperature. This was investigated by creating in-frame lacZ fusions at different points along the gene, and assaying levels of B-galactosidase. Assaying fusions at several points could assist in location of the region of the message responsible, if an effect was observed. Attempting to study the problem in this way obviously assumes that the creation of fusions does not alter the normal message stability of transposase.

pAA33 (4.2 and Fig. 4.16) was used as the source of Tn1 because the location of Tn1 in pACYC184 had been mapped approximately (Arthur 1981) and its location was convenient for cloning. Cleavage of pAA33 with BamHI/SalI gives a fragment of size approximately 4.1kbp which contains part of Tn1 including intact tnpA and about 600bp of pACYC184 sequences. This fragment was cloned into pUC18 cut with BamHI/SalI, the ligated plasmids were transformed into JM83, and white colonies

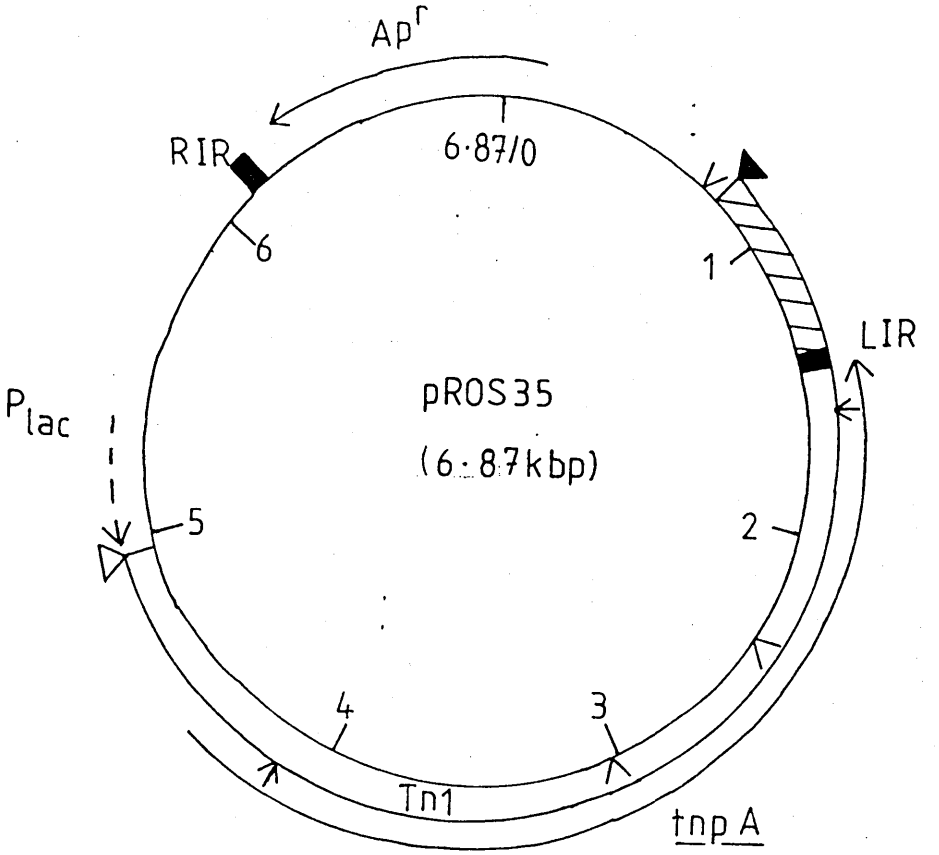


Fig. 4.17 pROS35.

pACYC184 DNA
SalI site
BamHI site
PstI sites
EcoRV sites

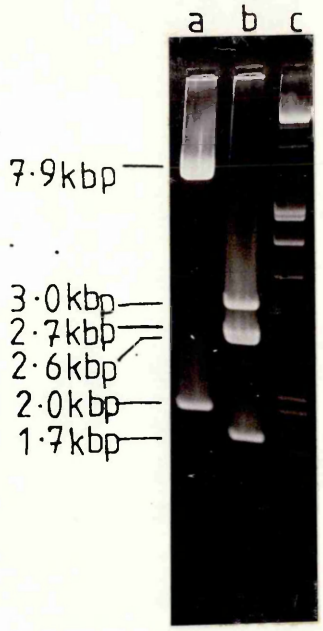


Fig. 4.18a pROS36 digests.

a. ClaI digest
b. BamHI/SalI digest
c. λHindIII/EcoRI digest

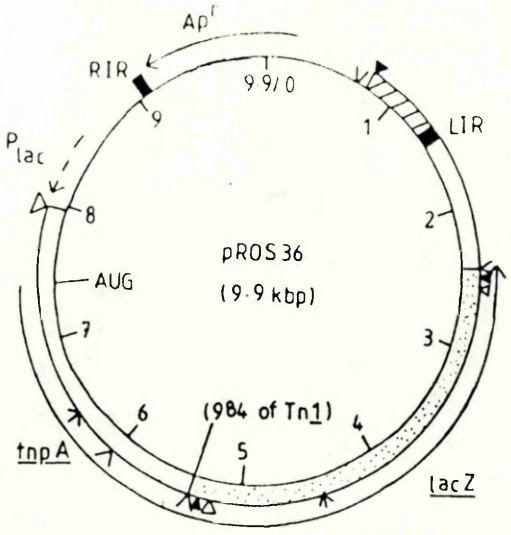


Fig. 4.18b pROS36, showing the translational fusion of Tn1 tnpA to lacZ at position 984 of Tn1.

pACYC184 DNA
Tn1 DNA
lacZ DNA
SalI sites
BamHI sites
PstI sites
ClaI sites

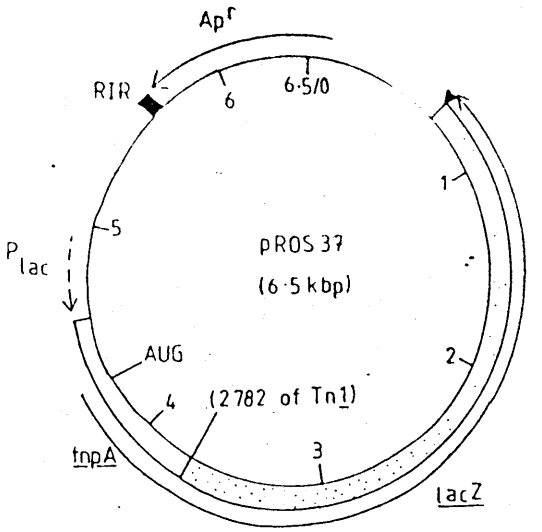


Fig. 4.19 pROS37, showing the translational fusion of Tn1 tnpA to lacZ at position 2782 of Tn1.

Tn1 DNA
lacZ DNA
▼ SalI site

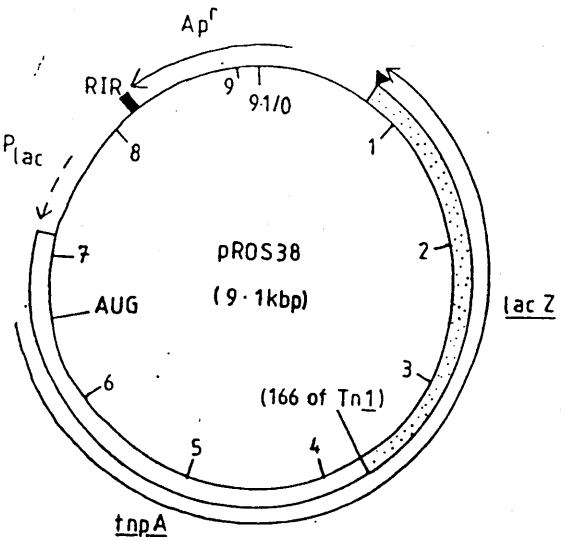


Fig. 4.20 pROS38, showing the translational fusion of Tn1 tnpA to lacZ at position 166 of Tn1.

Tn1 DNA
lacZ DNA
▼ SalI site

were selected on plates containing Xgal. These were examined on single colony gels and found to contain inserts of the correct size. One colony was purified and DNA was prepared from it and mapped. This construct was known as pROS35 (Fig. 4.17).

pROS35 was subsequently digested partially with PstI (in the presence of 30ug/ml EtBr) to give unit length linears which were excised from a gel, purified and ligated to the lacZ-containing PstI fragment from pMC1871. [pMC1871 is a pBR322-based plasmid derived from pMC1403 (Casadaban et al. 1980). It carries an easily portable lacZ cassette.] In the correct orientation, this fragment forms an in-frame fusion to Tn1 tnpA at position 984 in the transposon. Inserts in this orientation can be selected as blue colonies by transformation into DS942 and plating on agar containing Xgal. One of the resulting colonies, pROS36, was purified and mapped (Fig. 4.18a). Its structure is shown (Fig. 4.18b). The ability of tnpR in trans to repress synthesis of the B-galactosidase fusion in pROS36 was tested (4.2.4). The enzyme level was found to be repressed, confirming that the B-galactosidase expression in this construct is under transcriptional control of tnpA. B-galactosidase expression from pROS36 at 30°C and 37°C was examined (4.2.5).

pROS35 was also digested with SalI, then partially with EcoRV. The 3.405kbp and 6.075kbp bands were excised from a gel, purified and ligated individually to the lacZ cassette from pMC1871 bounded by SalI/SmaI ends. The resulting constructs were transformed into DS942 and plated on agar containing Xgal. Blue colonies from each transformation were examined for size, purified and mapped. Two in-frame Tn1 tnpA fusions were obtained: in one lacZ was fused to tnpA at position 2782 of the transposon at the 5' end of the gene; and in the other lacZ was fused at position 166 at the 3' end of the gene. These plasmids were known as pROS37 (Fig. 4.19) and pROS38 (Fig. 4.20) respectively.

By comparison of the structures of pROS37 and pROS38 with that of pROS36 (Fig. 4.18b), it is most likely that in all three constructs B-galactosidase expression is under tnpA transcriptional control (4.2.4). Levels of B-galactosidase expressed from pROS37 and pROS38 at 30°C and 37°C were examined (4.2.5).

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
30°C:								
A	280	0.107	0.196	288.12	0.197	1463	1832	1.8
	340	0.320	0.425	624.75	0.567	1085	1328	1.8
	370	0.473	0.477	657.09	0.960	684	945	2.0
B	280	0.134	0.243	357.21	0.238	1501	1813	1.8
	310	0.231	0.259	380.73	0.361	1055	1121	1.6
	370	0.561	0.542	796.74	1.160	687	966	2.1
37°C:								
A	220	0.124	0.163	239.61	0.387	619	1315	3.1
	255	0.296	0.296	435.12	0.773	563	1080	2.8
	290	0.512	0.535	786.45	1.741	452	1045	3.4
B	220	0.117	0.169	248.43	0.368	675	1444	3.1
	255	0.246	0.309	454.23	0.747	608	1256	3.0
	290	0.460	0.588	864.36	1.815	476	1278	3.9

Table 4.9 Results of B-galactosidase and B-lactamase assays on DS942 (pUC9lacZ) shaking at 30°C and 37°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

4.2.4 Repression of tnpA and tnpR lacZ fusions by tnpR in trans

Repression of the tnpA and tnpR fusions by an intact tnpR gene in the same cell would confirm that B-galactosidase expression was under the transcriptional control of the tnpA and tnpR genes.

As a control for these assays, it was desirable to fuse lacZ to a promoter and translational start that should not be affected by the presence of tnpR in trans. The insertion of the lacZ cassette on a PstI fragment into the PstI site of pUC9 (Burke, Glasgow) provided this control. The resulting construct, pUC9lacZ, fuses lacZ in frame to its original transcriptional and translational control signals. The first 5 codons of lacZ are intact, followed by 15 foreign ones, then the eighth and following codons of the gene. Due presumably to the relatively high expression of this gene, and to the high copy number of pUC (approximately 90 per chromosome equivalent: Jones 1985), cells containing pUC9lacZ are not healthy, and tend to lose the plasmid at high frequencies, even in the presence of ampicillin.

pUC9lacZ was transformed into DS942, and B-galactosidase and B-lactamase assays were initially carried out on this construct in the absence of any other plasmid. This gave information on the effect of temperature on lacZ when it is fused to its own promoter and translation signals in a multicopy plasmid. The results are shown in Table 4.9.

From these data it is apparent that the ratio of B-galactosidase/B-lactamase in pUC9lacZ is less at 37°C than 30°C. The B-galactosidase units per OD_{600} also show that there are fewer B-galactosidase molecules present per cell at 37°C than at 30°C. This effect is also observed with pSNO80 (5.1). The reason for this is not known.

In order to show whether any repression observed was due to the presence of tnpR in trans carried on pACYC184 as pPAK316 (3.2.2; Kitts 1982), rather than to the presence of another plasmid itself, a set of control assays was done at the same time using pACYC184:

The DS942 strains containing pROS31, pROS32, pROS36 and pUC9lacZ were transformed to Cm^r with pPAK316 or pACYC184. 2.5ml cultures of L-

Plasmid	% carrying resistance to		
	Sm Cm	Sm Ap	Sm Cm Ap
pROS31-	86	53	50
pROS31+	93	112	103
pROS32-	92	89	81
pROS32+	97	98	71
pROS36-	110	5	4
pROS36+	89	82	68
pUC9lacZ-	118	108	107
pUC9lacZ+	81	22	13

Table 4.10 Results of viable counts on the DS942 plasmid-containing cultures assayed in 4.2.4 (Table 4.11). Resistance to Cm implied the cells contained either pPACYC184 (-) or pPAK316 (+); reistance to Ap implied the cells contained the pUC-based fusion construct; resistance to Cm and Ap implied both plasmids were present. In all cases most cells (over 81%) contained pACYC184 or pPAK316.

30°C:

Plasmid	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
pROS31-	0.178	0.0100	14.700	0.64	23	56	3.6
	0.657	0.1056	155.200	2.52	62	161	3.8
pROS31+	0.408	0.0055	8.085	1.40	6	13	3.4
pROS32-	0.130	0.0263	38.660	0.42	92	202	3.2
	0.666	0.3567	524.300	3.01	174	536	4.5
pROS32+	0.126	0.0008	1.176	0.30	4	6	2.4
	0.430	0.0231	33.960	1.60	21	54	3.7
pROS36-	0.191	0.0030	4.410	0.11	40	16	0.6
	0.818	0.0219	32.190	0.55	59	27	0.7
pROS36+	0.091	0.0019	2.793	0.50	6	21	5.5
	0.279	0.0075	11.030	1.51	7	27	5.4
pUC9lacZ-	0.042	0.0447	66.000	0.08	825	1064	1.9
pUC9lacZ+	0.033	0.0488	72.000	0.09	800	1479	2.7
	0.089	0.1595	234.500	0.24	977	1792	2.7

Table 4.11 Results of B-galactosidase and B-lactamase assays on pROS31, pROS32, pROS36, and pUC9lacZ at 30°C, in the presence (+) and absence (-) of functional tnpR product. pPAK316 carried tnpR, and pPACYC184 was used as the control. The host strain was DS942.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

broth without glucose, but with ampicillin and chloramphenicol, were grown overnight. Then 200ul was inoculated into 100ml, and the cultures were grown, shaking, at 30°C. [The reason for growing the cells at 30°C was that, at 37°C, in the presence of another pACYC-based or λdv-based plasmid, pUC-based plasmids are very unstable, even in the presence of ampicillin.] It was important that most of the cells containing the pUC-based plasmids also contained the pACYC-based ones at the time of assaying enzyme levels. Therefore, at the time of sampling from each culture, each was diluted and plated out on agar plates containing Sm, Sm Cm, Sm Ap, or Sm Cm Ap. The following day, the number of cells containing one or both plasmids was calculated. The results are shown in Table 4.10. In all cases over 80% of the cells contained the pACYC-based plasmid: in 6 out of 8 cases, over 92% did. Loss of the pUC-based plasmids was variable, and in some cases considerable loss had occurred.

Overall, however, the results did suggest that most cells containing the pUC-based plasmids also contained the pACYC-based ones. Therefore the assay results (Table 4.11) were meaningful.

The control assay of pUC9*lacZ* showed that the *tnpR* product, resolvase, is not capable of repressing synthesis of B-galactosidase when *lacZ* is fused to its own promoter and translation signals. However, the *tnpA* and *tnpR* fusions are specifically repressed by the presence of *tnpR* in *trans*.

In the case of the *tnpA lacZ* fusions of pROS31 (Tn*3*) and pROS36 (Tn*1*), it is clear that an approximately 7-fold decrease in B-galactosidase expression has occurred. This is consistent with the 8-fold increase in Tn*3* *tnpA* levels observed when the *tnpR* gene was interrupted by a nonsense codon (the R49 mutation: Chou *et al.* 1979).

The expression of the *tnpR lacZ* fusion appears to be decreased about 10-fold by the presence of an intact *tnpR* gene in the cell. This is consistent with the 10 to 60-fold increase in truncated Tn*3* *tnpR* observed in the case of the R49 mutation.

The approximate numbers of transposase and resolvase fusion molecules per cell can be calculated from the B-galactosidase units per OD_{600}

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
30°C:								
A	-	0.069	0.0091	13.4	0.186	72	132	2.7
37°C:								
A	230	0.247	0.0867	127.4	1.184	108	351	4.8
B	230	0.236	0.0826	121.4	1.184	103	350	5.0

Table 4.12 Results of B-galactosidase and B-lactamase assays on DS942 (pROS37) shaking at 30° and 37°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
30°C:								
A	310	0.233	0.043	63.21	0.793	80	186	3.4
	340	0.367	0.142	208.74	1.747	119	387	4.8
B	310	0.216	0.040	58.80	0.775	76	185	3.6
	340	0.340	0.122	176.40	1.799	98	359	5.3
37°C:								
A	355	0.293	0.101	148.47	1.354	110	345	4.6
	385	0.430	0.189	277.83	2.295	121	440	5.3
B	385	0.243	0.071	104.37	1.158	90	292	4.8
	420	0.406	0.161	236.67	2.045	116	397	5.0

Table 4.13 Results of B-galactosidase and B-lactamase assays on DS942 (pROS36) shaking at 30°C and 37°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
30°C:								
A	180	0.026	0.0050	7.350	0.071	104	192	2.7
	230	0.060	0.0127	18.669	0.250	75	212	4.2
	265	0.108	0.0180	26.460	0.500	53	167	4.6
	300	0.226	0.0500	73.500	1.258	58	221	5.6
	360	0.588	0.1159	170.373	2.397	71	197	4.1
	400	0.925	0.3009	442.323	4.775	93	325	5.2
	414	1.019	0.3056	449.232	4.962	91	300	4.9
B	180	0.034	0.0085	12.495	0.157	80	250	4.6
	230	0.079	0.0105	15.435	0.368	42	133	4.7
	265	0.154	0.0305	44.835	0.482	93	198	3.1
	300	0.301	0.0677	99.519	0.990	101	225	3.3
	360	0.725	0.1967	289.149	2.079	139	271	2.7
	400	1.075	0.4349	639.303	4.368	146	405	4.1
37°C:								
A	180	0.025	0.0106	15.582	0.218	71	424	8.7
	230	0.073	0.0259	38.073	0.467	82	355	6.4
	265	0.166	0.0451	66.297	0.543	122	272	3.3
	300	0.390	0.1286	189.042	2.023	93	330	5.2
	360	1.020	0.3521	515.587	4.108	126	345	4.0
B	180	0.030	0.0126	18.522	0.199	93	420	6.6
	230	0.081	0.0256	37.632	0.462	81	316	5.7
	265	0.187	0.0566	83.202	0.546	152	303	2.9
	300	0.390	0.1308	192.276	2.118	91	335	5.4
	360	1.029	0.5125	735.375	3.361	224	498	3.3

Table 4.14 Results of B-galactosidase and B-lactamase assays on DS942 (pROS38) shaking at 30°C and 37°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

(4.2.6). It appears that under repression by resolvase at 30°C, there are about 20 transposase fusion molecules and 60 resolvase fusion molecules per cell. This ties in with the fact that transposase is known to be present in very low amounts in cells (1.6.3), which limits the transposition of Tn3.

The results of these resolvase repression assays show that expression of the tnpA and tnpR fusions in pROS31, pROS32, and pROS36 is under transcriptional control of these genes. But it does not rule out the possibility of translational reinitiation occurring in the pROS31 and pROS36 cases, downstream of the most 5' AUG. However, this would have to be in the same reading frame as tnpA, and is not likely to be the main source of translations traversing lacZ, because in pROS38, where lacZ is fused to tnpA at position 166 of the transposon, at the 3' end of the gene, a considerable amount of full length fusion protein is apparent on polyacrylamide SDS gels (Ch.5). Therefore, it is likely that these constructs, and also pROS37 and pROS38, are also under the translational control of tnpA.

4.2.5 Assays of lacZ fusions at different positions along the Tn1 tnpA gene

The strains DS942 (pROS36), DS942 (pROS37) and DS942 (pROS38) were inoculated into 2.5ml of L-broth, without glucose, but with ampicillin, and two of each culture were grown overnight at 30°C or 37°C. The following day, 200ul of culture was inoculated into 100ml of fresh L-broth and the flasks were shaken at 30°C or 37°C. At various points throughout the culture growth, 3ml samples of each were removed and frozen to -20°C for subsequent assays on B-galactosidase and B-lactamase activities. The assay results are shown in Tables 4.12 (pROS37), 4.13 (pROS36) and 4.14 (pROS38).

The ratios of B-galactosidase/B-lactamase are virtually the same as each other in all three constructs, both at 30°C and 37°C, and a small increase is apparent at 37°C compared to 30°C. The B-galactosidase units per OD_{600} for each strain also show an increase with temperature. These data show that the position of the lacZ fusion in the Tn1 gene does not affect the assay results. It is also apparent that under the conditions used here, the number of transposase

Fusion	Condition	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}	Approx. no. of fusion molecules per cell
tnpA::lacZ:				
pROS31	still	66	160	240
pROS31	still	57	308	462
pROS31	shaking	78	128	192
pROS31	shaking	82	379	569
pROS37	shaking	89	132	198
pROS37	shaking	114	346	519
pROS36	shaking	94	279	419
pROS36	shaking	110	369	554
pROS38	shaking	89	239	359
pROS38	shaking	102	360	540
tnpR::lacZ:				
pROS32	still	427	346	519
pROS32	still	573	1876	2814
pROS32	shaking	191	362	543
pROS32	shaking	783	3494	5241
lac::lacZ:				
pUC9lacZ	shaking	1079	1573	2360
pUC9lacZ	shaking	565	1237	1856
pSNO80	shaking	340	588	882
pSNO80	shaking	109	442	663

Table 4.15 Summary of B-galactosidase and B-lactamase assays on lacZ translational fusions. In each case the top row of numbers give the results of assays at 30°C, and the bottom row give the results of assays at 37°C.

molecules per cell is probably about 2-fold greater at 37°C than 30°C. This agrees with the studies on pROS31 (4.2.2), which is a Tn*3* fusion. Therefore it appears that the temperature effect on transposition does not act by altering the message stability of Tn*1/3* transposase.

4.2.6 A summary of the *galk* and *lacZ* fusion results

A condensed summary of the *lacZ* fusion results is shown in Table 4.15. These data, together with the *galK* fusion results (Table 4.4) enable the following conclusions to be made:

I. At 30°C and 37°C derepressed transcription from
a. P_A is about 1/5th of that from P_{lac}
b. P_R is about 1/20th of that from P_{lac}
c. P_R is about 1/4th of that from P_A.

II. At 30°C the combined derepressed transcription and translation of
a. *tnpA* is about 1/15th that of *lacZ*
b. *tnpR* is about 1/5th that of *lacZ*
c. *tnpR* is about 3x that of *tnpA*.

III. At 37°C the combined derepressed transcription and translation of
a. *tnpA* is about 1/7th that of *lacZ*
b. *tnpR* is about 6/5th that of *lacZ*
c. *tnpR* is about 6x that of *tnpA*.

IV. At 37°C compared to 30°C, relative to *bla*, the combined derepressed transcription and translation of
a. *lacZ* decreases about 2-fold
b. *tnpA* increases about 1.4-fold
c. *tnpR* increases about 3-fold.

V. Therefore at 30°C...
a. *tnpA* transcripts are translated with about 1/3rd the efficiency of *lacZ* transcripts
b. *tnpR* transcripts are translated with about 4x the efficiency of *lacZ* transcripts
c. *tnpR* transcripts are translated with about 12x the efficiency of *tnpA* transcripts.

VI. ...and at 37°C...

a. tnpA transcripts are translated with about the same efficiency as lacZ transcripts

b. tnpR transcripts are translated with about 24x the efficiency of lacZ transcripts

c. tnpR transcripts are translated with about 24x the efficiency of tnpA transcripts.

VII. The ratio of derepressed transcripts per gene at 30°C and 37°C, after normalisation to bla, is about
$P_A:P_R:P_{lac}$, 20:5:100

VIII. The ratio of combined derepressed transcriptions and translations per gene at 30°C, after normalisation to bla, taking lacZ as 100, is about
tnpA:tnpR:lacZ, 7:20:100
(Observed B-galactosidase units per OD_{600} are 9:17:100 before copy number correction)

IX. The ratio of combined derepressed transcriptions and translations per gene at 37°C, after normalisation to bla, taking lacZ at 30°C as 100, is about
tnpA:tnpR:lacZ, 10:60:50
(Observed B-galactosidase units per OD_{600} are 17:132:89 before copy number correction)

X. At 30°C, relative to bla, the presence of resolvase in trans represses expression of resolvase fusion proteins about 10 times and expression of transposase fusions about 7 times.

The actual number of transcripts per gene copy is not known, neither is the number of translations per transcript. The B-galactosidase units per OD_{600} shown in Table 4.15 agree with statements (VIII) and (IX) generally, but obviously will not give exactly the expected ratios because of copy number variations.

The B-galactosidase units per OD_{600} can be used to estimate approximate numbers of molecules of transposase, resolvase or B-galactosidase per cell:

B-galactosidase

specific activity = nMONPG hydrolysed min^{-1} mg $protein^{-1}$

$= (nMONPG\ min^{-1})/(OD_{600} \times 0.075^{*})$

$= (OD_{420}\ min^{-1} \times 1470^{**})/(OD_{600} \times 0.075)$

and, disregarding the light scattering correction,

$$\text{Miller units} = (OD_{420}\ min^{-1} \times 1000)/OD_{600}$$

Therefore,

Specific activity = (Miller units x 1470)/(1000 x 0.075)

i.e.

Specific activity = 19.6 Miller units

or

19.6 specific activity units = 1 Miller unit.

* This assumes that a culture with an OD_{600} of 1.0 contains 75ug ml^{-1} of protein (Silhavy et al. 1984)

** This is the conversion factor for OD_{420} min^{-1} ml lysed $cells^{-1}$ to nMONPG hydrolysed min^{-1} ml lysed $cells^{-1}$. This was determined by calibration (data not shown).

If it is assumed that E. coli has 200mg ml^{-1} protein and an internal volume of $1.5(uM)^3$, and that pure B-galactosidase has a specific activity of 450,000 units, then it can be calculated that

1 specific activity unit $\equiv$ 0.078 molecules of B-galactosidase per cell, i.e. 1 Miller unit $\equiv$ 1.5 molecules per cell.

Raleigh and Kleckner (1986) found that 1 Miller unit is equivalent to 6 molecules of B-galactosidase per cell, but they did not show the calculation involved. The ratio will vary depending on what value is taken as the volume of E. coli cells, and how much protein per ml there is believed to be.

The estimated number of molecules of the fusion proteins per cell in the derepressed state is shown in Table 4.15. At 37°C there are about 4000 resolvase fusion molecules per cell, and about 500 transposase fusion molecules. At 30°C there are about 530 resolvase fusion molecules and about 280 transposase fusion molecules. It is believed that a single chromosomal copy of *lacZ* gives about 1000 Miller units at 37°C when assayed by the method used here (Miller 1972). This is equivalent to about 1500 molecules of B-galactosidase per cell. If it is true that resolvase is expressed at 37°C at 6/5ths the level of *lacZ* (III), and there are many copies of the gene in the cell (up to 315 - Jones 1985), then it is apparent that the number of resolvase molecules per cell is not nearly as high as expected; similarly for transposase. One reason for this could be that many cells have lost the plasmid (pUC9*lacZ* is known to be unstable), and/or that some factor necessary for gene expression has become limiting at this number of plasmid copies. Additionally, it must be remembered that pUC9*lacZ* is itself a fusion construct, and may not behave in the same way as the wild-type gene.

In summary, under the conditions used, none of the data shown are consistent with the hypothesis that increasing temperature decreases transposase synthesis. It is unlikely that growth into stationary phase would change these conclusions, because the experiment on transposition described in 3.4 shows that placing the cultures at 30°C for only 1.5 generations gives an increase in the frequency of transposition over the frequency at 42°C. Therefore it is probable that the temperature effect acts after translation of transposase, possibly on its activity or breakdown, unless the use of fusions has masked an effect on synthesis.

4.3 The effect of the heat-shock induced protease, Lon, on transposition of Tn*3*

E. coli cells contain at least eight distinct, soluble enzymes capable of degrading proteins to acid-soluble material (Swarmy and Goldberg 1981). One of these, the product of the *lon* gene, Lon (or La), is an ATP-dependent serine protease of size 94kd, which is present in the cytoplasm (Swarmy and Goldberg 1982). Lon is believed to play a major role in the degradation of abnormal proteins and in regulating

turnover rates of certain normal cellular proteins (Lindquist 1986). Lon mutants are pleiotropic: among other things they overproduce capsular polysaccharide, resulting in mucoid colonies; they have an abnormal SOS response, with concomitant filamentation; and they have a decreased ability to lysogenise phage λ (Gottesman and Gottesman 1981). The latter phenotype is due to the effect of Lon on the stability of many of the proteins synthesized by the phage. In the case of early control proteins, this, in conjunction with transcriptional regulation, may provide a way to finely tune the levels of the regulatory proteins.

The Lon protease is a heat-shock protein (Lindquist 1986). A heat-shock protein (HSP) is generally defined as one whose synthesis is sharply and dramatically induced at high temperatures during the heat-shock response. These proteins are also induced by a wide variety of other stresses and seem to have very general protective functions. They are probably produced by all organisms including Drosophila, Soybean, and yeast. The heat-shock response appears to be transient in some organisms, and sustained in others. In E. coli the response is transient when cells raised at 30°C-37°C are shifted to 42°C, but sustained when they are shifted to 45°C-50°C. However, transient responses are not completely transient. As HSPs accumulate, their synthesis declines but plateaus at a higher level (Lindquist 1986). Therefore cells grown at 42°C are likely to produce higher levels of Lon than cells grown at 30°C. In view of this, and the known effect of Lon on λ proteins, it was decided to examine transposition frequencies in a Lon strain, in case Lon is responsible for an increased rate of transposase breakdown at higher temperatures.

The Lon strain, BTA282, was mated with DS916 (R388) to give BTA282 (R388), which was transformed with pROS13 (3.3.2). After heat-shock at 37°C for 5 minutes, the culture was put on ice for 5 minutes, and then split into three. One lot of cells were expressed at 30°C, one at 37°C and one at 42°C for 1.5 hours. Then the transformations were plated out on plates containing Sm Tp Km and were grown overnight at their respective temperatures. The following day, about 200 transformants from each temperature were grown up at the relevant temperatures and mated to DS903 Sp^r. The exconjugants were selected on plates containing Sp Tp, or Sp Tp Km.

Temperature	Transposition frequency
30°C	8×10^{-2}
37°C	2×10^{-4}
42°C	6×10^{-7}

Table 4.16 Results of transposition assays of Tn3571 from pROS13 to R388 in the *lon* strain BTA282. The recipient was DS903 Sp^r (pPAK316).

The results (Table 4.16) showed that a deletion in the lon gene does not alter the effect of temperature on transposition frequencies. Therefore breakdown of transposase by the Lon protease does not seem to be the explanation for the temperature effect. Investigation of the possible role of protein degradation in the effect was one of the reasons for trying to obtain a transposase antibody (Ch. 5). This could then be used to quantitate transposase proteolysis at 30°C and 42°C.

4.4 A procedure for the isolation of a mutant Tn3 which would transpose as frequently at 42°C as at 30°C

As the studies on transposase synthesis had not provided an explanation for the temperature sensitive phenomenon of transposition, it was decided that the isolation of a mutant Tn3, that could transpose at the same frequency at 42°C as at 30°C, would contribute to solving the problem. Therefore an enrichment procedure for possible transposon-encoded mutants was devised:

The spontaneous mutation rate of E. coli has been found to be approximately 1 x 10^{-9} per base pair per generation. In order to make the numbers of cells that had to be screened reasonable, a mutD strain, CB158, was selected as the host in which the mutation was expected to occur. This strain is known to give rise to spontaneous Rf^r mutations at 1000 times the rate of other non-mutD strains (C. Boyd, personal communication). Therefore only 1 x 10^6 cells needed to be screened. A thymine-rich medium is required for this high mutation rate.

DS916 (R388::Tn3571) was mated with CB158, and CB158 (R388::Tn3571) colonies were selected on plates containing Sm Tp Ap. This strain was grown up overnight at 37°C in 20ml of L-broth containing glucose, antibiotics and 20ug per ml thymine. The strain DS902 (pGS301) was also grown up overnight in 20ml of L-broth, with antibiotics, at 37°C. pGS301 (Stewart, Glasgow) is a 5.77kbp λdv-based plasmid carrying resistance to tetracycline. The next day, 0.5ml of the overnight culture of CB158 (R388::Tn3571) was inoculated into 20ml of fresh medium and was grown at 37°C for 5 hours. Then the whole culture was spun down and mated, at 42°C, to all 20ml of the DS902 (pGS301)

culture. Immediately after the mating, dilutions were plated out on agar containing Sm Tp Ap Tc to check the number of exconjugants. There were about 5 x 10^8 recipient cells altogether. The rest of the cells from the mating were grown at 42^oC for 24 hours in 100mls of L-broth containing Sm Tp Ap Tc. This killed off the donors, but did not allow much amplification of the culture because of the high density of the cells. During this time, transposition of Tn3571 could occur from R388 into the Tc^r λdv plasmid, to form cointegrates. If a mutant temperature-insensitive transposon was present, it should transpose at a higher frequency than the wild-type Tn3571. It was possible all the time while the culture was at 42^oC, that the desired mutation could arise. However, at this stage, a mutant might be recessive, and therefore it might not be detected unless it was isolated during one of the mating steps. Also the probability of mutations arising was greatly reduced compared to in the mutD strain.

In order to resolve cointegrates that should have formed due to transposition, DS902 (R388::Tn3571, pGS301) cells were mated at 42^oC to DS903 Sp^r (pPAK316). pPAK316 carries an intact resolvase gene and should be immune to Tn3571 insertions (Ch. 3). Immediately after mating, dilutions were plated out onto plates containing Sp Tp Ap Cm and Sp Tp Ap Cm Tc. The total number of exconjugants was 5 x 10^7, and the total number of transpositions was 1.25 x 10^4, therefore the transposition frequency was 2.5 x 10^{-4}. This was the usual low frequency expected at 42^oC. The rest of the cells from the mating were plated on a total of 10 plates containing Sp Tp Ap Cm Tc and were grown overnight at 42^oC. This was an amplification step during which cells potentially carrying a mutant could multiply. Performing this step on plates was necessary because a previous attempt at the experiment had shown that putting the cultures into 1l of L-broth did not allow amplification due to the cell density.

The cells were scraped off the plates and were resuspended in 1l of L-broth with the relevant antibiotics. Some single colonies were isolated, patched out , and examined on single colony gels for the presence of pPAK316. This plasmid was present in all cells, but unresolved cointegrates, as well as resolved ones, were also present. 20ml of this culture were spun down and mated to 20ml of an overnight culture of DS902 (pMR100). pMR100 is a 5.58kbp λdv-based

plasmid which is Km^r (Rogers 1986). The reason for using a λdv plasmid with a different resistance at this stage, rather than pGS301 again, was to prevent res mutants, which might have arisen, from being amplified more than the required mutants: without the requirement for Tc resistance to be "picked up" at this stage, res mutants could pass round the cycle becoming more and more abundant; with this requirement, the transposons at the greatest advantage were those which could transpose at the highest frequency. Immediately after mating, the culture was diluted and plated out on agar containing Sm Tp Ap Km and Sm Tp Ap Km Tc. The latter was to see whether resolution of cointegrates between R388::Tn3571 and pGS301 by pPAK316 had been complete. The number of exconjugants was 2.2×10^7, and of these one third still carried Tc resistance. This meant that pPAK316 had not been able to irreversibly break down all the cointegrates. At this stage it was apparent that this approach would not be successful, but one final mating of DS902 (pMR100), containing both cointegrates and resolved R388::Tn3571, was carried out to DS903 (pPAK316). Immediately after mating the culture was diluted and plated out on agar containing Sp Tp Cm, Sp Tp Cm Km or Sp Tp Cm Km Tc. The number of exconjugants was 1.1×10^9; the number of transpositions into pMR100 was 3.4×10^5; and therefore the frequency of transposition was 3×10^{-4}. This was not significantly higher than the previous frequency of transposition into pGS301, but further amplification might have been necessary before a mutant could spread through the population to a sufficient extent to alter this frequency. The plates also showed that approximately the same number of cells were resistant to Tc and Km.

A repeat of the entire procedure came up against the same problem.

There could have been several reasons for the apparent failure of complete resolution. The most obvious of these were that:

a. pPAK316 was being lost from some cells during liquid culture, but these cells were not dying. This was investigated by growing a culture of DS903 (pPAK316) overnight at 42^oC in L-broth containing chloramphenicol, then plating out to see how many of the cells had lost the plasmid. The results showed that the plasmid was stable in this situation.

b. A mutation could have occurred in the tnpR gene of pPAK316, or tnpR might not work efficiently at 42°C. This was investigated by transforming pPAK316 into DS902 (pMA21) (3.2.2; Brown 1986), growing the transformants at 30°C or 42°C immediately after heat-shock, and examining them for breakdown of the 2-res plasmid, pMA21, by the tnpR gene. Breakdown appeared to be complete at both temperatures, showing that tnpR was not mutated for its resolving power, and that resolution is as efficient at 42°C as at 30°C.

Other reasons for the problem include the possibility that unresolved cointegrates are not common, but that they mate out more efficiently than resolved ones, or are incompatible with their resolution products. This has not been generally observed in transposition assays. Another reason might be that pPAK316 does resolve the cointegrates efficiently, but that homologous recombination occurs continuously between the Tn3571 sequences to recombine the products. The strain DS903 Sp^r is recF but not recA. This is supposed to lower inter-plasmid recombination, but as was shown in 3.5, recombination still occurs at an appreciable frequency in this strain.

In view of the problems obtaining complete resolution in this system, an alternative protocol was developed:

CB158 (R388) was transformed with pROS13 (3.3.2) and the cells were expressed at 42°C after being placed on ice for 5 minutes following heat-shock. The transformants were grown overnight at this temperature in 20ml 2-YT, containing Sp Tp Ap Km and 20ug per ml thymine. Hopefully, a temperature-insensitive mutant of tnpA would arise at this stage. 5ml of the culture was mated at 42°C with 15ml of a 20ml overnight culture of JM101. [JM101 was used because it contains an F' (tra^-), which carries Tn1000 ($\gamma\delta$). Tn1000 encodes a tnpR gene which is able to complement the mutated tnpR gene of Tn3571, and can therefore resolve cointegrates which form between pROS13 and R388.] Immediately after mating, dilutions were plated out on agar containing Tp, or Tp Ap Km. The number of exconjugants was 1×10^9, and the number of transpositions into pROS13 was 1.1×10^5, therefore the transposition frequency was 1.1×10^{-4}. The rest of the culture was grown up overnight at 42°C in 100ml liquid minimal media containing glucose, B1 and Tp Ap Km. The following day, CsCl was used

to prepare plasmid DNA from the cells. This contained a mixture of F', R388::Tn3571, pROS13::Tn3571, and possibly some unresolved cointegrates. This was to be transformed into DS903 (R388) competent cells, and grown at 42°C. Due to its comparatively small size (11kbp), pROS13 would transform the cells with the greatest efficiency, and then transposition could occur again into R388 to form cointegrates, which could then be mated out again to JM101, and the transposition frequency noted. After several rounds of this cycle, any temperature-insensitive mutant should have spread through the population, and should become apparent by an increased transposition frequency.

If a temperature-insensitive mutant arose and was recessive, then the entry of two pROS13 molecules into the same cell during the transformation step might mask this. Therefore it was important to obtain conditions in which only one molecule should enter each cell. It was also important to transform as many recipient cells as possible, because at least 1×10^6 transformants were necessary, to give a reasonable chance of detecting the required mutation. So it was desirable to use the minimum amount of DNA to transform the maximum number of cells in order to obtain at least 1×10^6 transformants. The advantage of this procedure over the preceding one, was that complete resolution of cointegrates was not essential, as the efficiency of transformation of recipients with cointegrates would be so low. This should also decrease the possibility of *res* mutants being detected.

Using the $CaCl_2$ method of plasmid transformation, observed levels of competence if the cells are transformed on the same day that they are prepared, are around 1 in 5×10^3 cells. If the cells are kept in $CaCl_2$ overnight on ice, this level increases about 5-fold to 1 in 10^3 cells (Stirling, personal communication). According to these estimates, growing 100ml of culture to an OD_{600} of 0.5, should give 1 to 5×10^{10} cells, of which, on the same day as the cells were prepared, about 5×10^6 should be competent, and on the following day, about 2.5×10^7 should be competent.

The results of several experiments carried out on 20ml of cells are shown in Table 4.17. In these preliminary transformation studies,

OD_{600}	Total no. of cells in 20ml	No. of cells ml^{-1}	No. cells transformed	No. that would be transformed in 100ml	Comments
0.217	1.6×10^9	7.8×10^7	4.2 in 10^5	3.3×10^5	Same day. 1.5ul + antibiotics
0.217	2.0×10^9	9.9×10^7	6.9 in 10^6	7.0×10^4	Same day. 0.15ul + antibiotics
0.210	2.3×10^8	1.2×10^7	1.5 in 10^4	1.7×10^5	Same day. 1.5ul - antibiotics
0.210	1.9×10^8	9.5×10^6	5.2 in 10^5	5.0×10^4	Same day. 0.15ul - antibiotics
0.210	1.3×10^8	6.5×10^6	4.0 in 10^4	2.7×10^5	2nd day. 1.5ul - antibiotics
0.210	1.0×10^8	5.2×10^6	2.0 in 10^4	1.1×10^5	2nd day. 0.15ul - antibiotics
0.282	2.9×10^8	1.4×10^7	2.0 in 10^5	2.8×10^4	Same day. 0.15ul - antibiotics + glucose
0.282	1.1×10^8	5.6×10^6	1.8 in 10^4	1.0×10^5	2nd day. 0.15ul - antibiotics + glucose
0.500	5.9×10^8	2.9×10^7	2.4 in 10^5	7.0×10^4	Same day. 0.15ul - antibiotics + glucose
0.500	3.4×10^8	1.7×10^7	5.9 in 10^5	1.0×10^5	2nd day. 0.15ul - antibiotics + glucose

Table 4.17 Results of transforming DS903 (R388) with pROS14 under various conditions.

pROS14 DNA (clone B, 3.3.2) was used instead of the hopefully mutated pROS13 DNA, in order to conserve this. The amount of DNA used in the transformations was estimated by examination on a gel. 1.5ul of pROS14 contained about 100ng of DNA. pROS14 has a size of approximately 11kbp, which is about 7.3MDa. Therefore in 1.5ul of pROS14 there are about 8×10^9 molecules, and 0.15ul contains about 8×10^8 molecules. Parallel transformations were done using these two dilutions in order to check that the DNA was limiting when 0.15ul was used. If this was the case, the two transformations should show a difference in transformation frequency. If no difference was observed, this would imply that the cells were saturated with DNA, and that there was a risk of double transformants occurring.

The data in Table 4.17 show that diluting the DNA did affect the transposition frequency about 5-fold, suggesting that the DNA was not saturating when 0.15ul was used. The competence of the cells did increase about 3-fold when left overnight in $CaCl_2$ on ice, but during this period about half of the cells apparently died, so the result of leaving the cultures until the following day before transformation was only a 1 to 2-fold increase in transformation efficiency. Overall the transformation efficiencies were less than had been expected. Same-day transformations gave a frequency of only about 1 in 10^4 cells (2-fold less than expected), and following-day transformations gave a frequency of 1 in 5×10^3 (5-fold less than expected). This low level of efficiency was not high enough, even if 100ml cultures were used, to give the minimum required 1×10^6 transformants. Increasing the OD_{600} of the culture above 0.5 lead to a decrease in the transformation efficiency (data not shown). The same low efficiency of transformation was obtained with pAA33, which was used as a control to see if there was an inhibitory substance in the pROS14 DNA preparation. Another reason for the low efficiency could have been the presence of antibiotics in the cultures before transformation, while growing the cells. But their presence or absence made no difference, neither did that of glucose (Table 4.17). The efficiency of transformation into DS903 without R388 was also of the same order as that observed above, suggesting it was not the presence of R388 that lowered the frequency. Different rotor speeds while spinning down the cells also made no difference.

An attempt was made to freeze competent cells in glycerol, so that batches could be prepared on different days until sufficient cells were available. However, thawing and testing several lots showed that at least half the cells in a batch died when frozen. So this was also impracticable.

A final attempt to increase the transformation efficiency was made by using a different protocol (Hanahan 1985). The results did not show any improvement at all over those in Table 4.17. Due to this problem of transformation efficiency, the enrichment procedure for a temperature-insensitive mutant could not proceed. The attempt to obtain a mutant was therefore abandoned. If the transposition frequencies after mating with JM101, at some point had indicated that a mutant was present, several individual colonies would have been purified , mini-DNA preps would have been made and retransformed into DS903 to isolate the mutant pROS13. Large-scale DNA preps using CsCl would then have been carried out, followed by retransformation into DS903 (R388), and subsequent re-assaying to confirm the phenotype. The purified mutant would then have been studied by *in vitro* methods to localise the mutation, by replacing parts of the *tnpA* gene with a non-mutated gene, and assaying the temperature phenotype.

Discussion

The purpose of the studies in this chapter was to elucidate the reason for the temperature sensitivity of Tn*1/3* transposition. Work done by Kretschmer and Cohen (1977) and in Glasgow, has shown that transposition frequencies are at least 100-fold greater at 30°C than 42°C. This phenomenon is independent of the method of assaying transposition, and of the plasmids used as donor and recipient. Growth of the cultures into stationary phase is not necessary for the effect to be observed, as 1 to 2 generations at 30°C is enough to increase the transposition frequency considerably over that of a culture kept at 42°C (3.4). This agrees with the observations of Kretschmer and Cohen (1977), that the effect is reversible. It is not clear whether the temperature effect is due to a slower rate of transposition or to a shift in the position of equilibrium.

The temperature effect has been shown to be independent of *tnpR* (Table

3.1), the product of which is known to regulate the transposition frequency by repressing transposase synthesis, hence attention was focused on the tnpA gene. Various transcriptional and translational fusions to the tnpA genes of Tn1 and Tn3 were created, and in spite of the sequence differences between these two transposons (4.2), the assays on translational fusions to both showed no significant difference (Table 4.15).

In the studies of transcription and translation of tnpA, fusions to tnpR and to the lacZ promoter and translational signals were also assayed for comparison. The levels of expression of galK or lacZ were then standardized to bla expression, which is carried on the same plasmid, is constitutively expressed, and correlates with the plasmid copy number at different growth rates (Klotsky and Schwartz 1987). In retrospect, bla expression is not a good copy number control for experiments at different temperatures, because of the possibility that the expression varies with temperature. A recent report (Kuriki 1987) suggests that bla expression may be repressed by heat-shock, showing the opposite effect to other genes such as Lon (4.3), which are induced. However, the significance of this in assays at 37 ^{o}C in cultures which have been grown previously for over 20 generations at this temperature, is uncertain. A better, more direct approach to copy number control, would have been the method of Adams and Hatfield (1984; 4.1.1). This would have enabled more direct statements on the changes in gene expression at 30^{o}C compared to 37^{o}C. However, even using bla expression as a standard, a number of observations was possible (4.2.6). In particular it was apparent that either tnpR has very efficient translation signals, or those of lacZ in pUC9lacZ and those of tnpA are very inefficient. The evidence obtained by selecting for overproducing mutants of transposase (Casadaban et al. 1982; Morita et al. 1987) suggests that the latter is true because mutations in the RBS of tnpA can give a considerable increase in expression.

Overall, the B-galactosidase units per OD_{600} showed that the number of transposase molecules per cell, assuming that the fusions give a true representation of this, was always greater at 37^{o}C than 30^{o}C, whether the cultures grew slowly or fast (4.2.2), and that the number of molecules per cell was low, confirming the observations that

transposase is limiting for transposition (Ch. 3). The assays seemed to suggest that the temperature effect acts at some other stage than the synthesis of transposase, possibly on its activity or breakdown. The results shown here, together with those of Kretschmer and Cohen (1977), which showed that protein synthesis is necessary during the transposition process, suggest that perhaps the transposase protein is partially, irreversibly inactivated at higher temperatures; its activity is blocked; or it is actually broken down to a greater extent. If breakdown is the explanation, it has been shown to be independent of the heat-shock induced protease, Lon (4.3). Breakdown could be further studied using transposase antibodies (Ch. 5).

It was unfortunate that the search for a temperature-insensitive mutant of tnpA had to be terminated. However the procedure was very time-consuming, and success was uncertain anyway, due to the fact that the protocol would not allow detection of host-encoded mutants. It is a possibility that a temperature-sensitive host protein may be required for binding to the transposon ends, and a mutation arising to increase the production of this would not be detected by the procedures used.

The reason for the temperature sensitivity of Tn1/3 transposition remains to be discovered.

CHAPTER 5

PURIFICATION OF A TN*1* *TNPA* *LACZ* FUSION PROTEIN, AND AN ATTEMPT TO RAISE ANTIBODIES

Introduction

There were several reasons for desiring an antibody to the transposase protein of Tn*1/3*: to assist in monitoring the purification of transposase on SDS polyacrylamide gels; to compare the extent of breakdown of the transposase protein at 30°C and 42°C; to enable the cellular localisation of transposase; to examine cross-reactivity with other transposable elements; and to determine if any (host) proteins, or end-labelled DNA containing the IR sequence, would co-precipitate with the transposase protein.

Purification of transposase was desirable, so that various studies could be carried out on its activity *in vitro* e.g. on end-binding, using gel retardation and footprinting; and so that an *in vitro* system for transposition could be developed like that available for Mu (Mizuuchi 1983). Problems had been encountered with purification in Glasgow, although other workers had achieved this (Fennewald *et al.* 1981; Wishart *et al.* 1985; and recently, Morita *et al.* 1987). The difficulties were that (i) transposase was not obvious on SDS-polyacrylamide gels of whole cell extracts, due to its position being masked by other bands, or on gels of minicells, presumably due to its low expression, and (ii) placing the transposase gene under the control of P_{lac} and *lacZ* translation signals in pSNO15 (see below), had not given the expected increase in levels of the protein (Hettle 1985).

The data presented in this chapter show the results of an investigation of the reason for the poor expression in pSNO15, and the successful purification of a *tnpA lacZ* fusion protein produced by pROS38 (4.2.3). This fusion protein was used to try and raise polyclonal antibodies to transposase.

Results

5.1 An investigation of Tn*1* *tnpA* expression in pSNO15

To simplify the purification of proteins, an attempt is usually made to maximize their expression. The three successful purification procedures published for Tn*3* transposase show different ways of

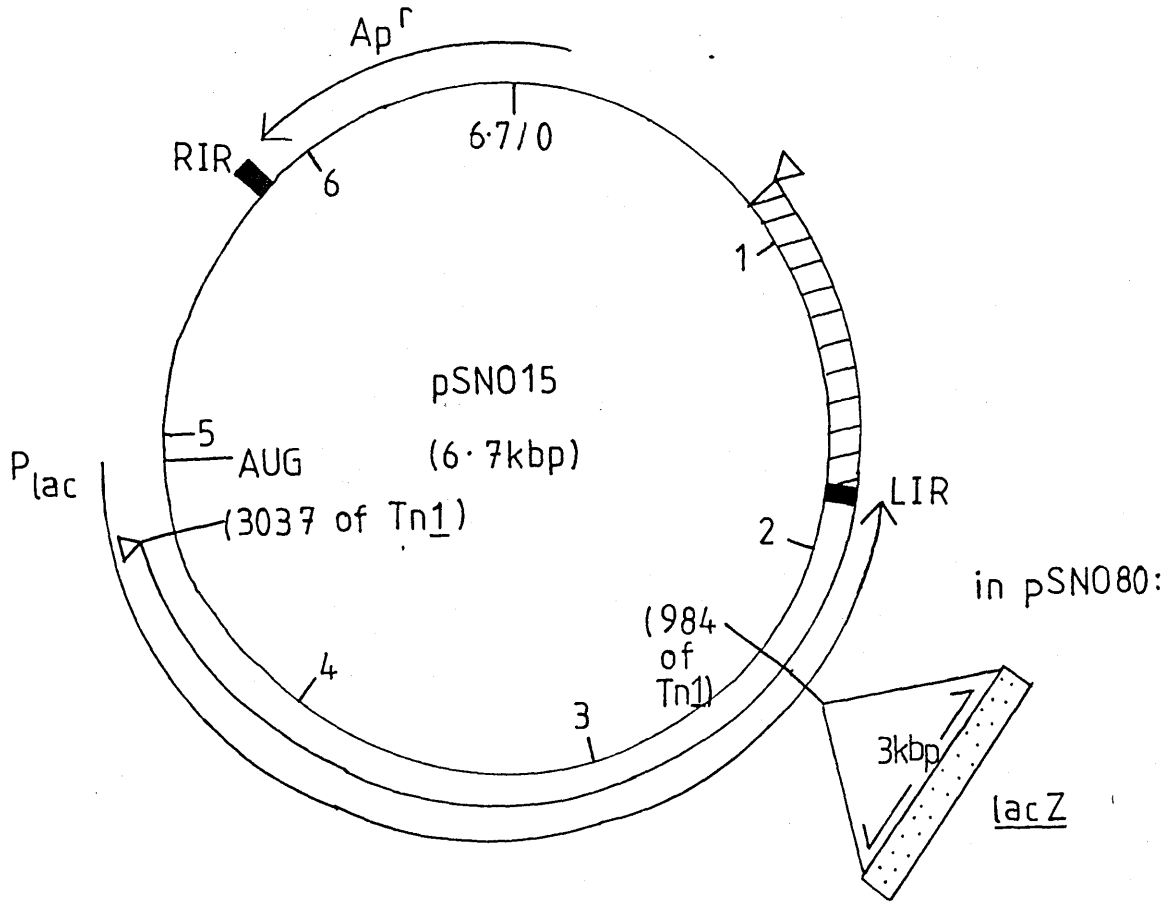


Fig. 5.1 pSNO15 (Hettle 1985), showing the translational fusion of P_{lac} and AUG to tnpA.

pACYC184 DNA

Tn1 DNA

HindII site

In pSNO80 lacZ is inserted at the PstI site at 984 of Tn1, creating a translational fusion: P_{lac} and AUG:tnpA:lacZ.

	Time in mins	OD600	OD420* min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}	(B-lact) ΔOD499 min^{-1} ml^{-1}	nM ONPG min^{-1} ml^{-1}/ ΔOD499 min^{-1} ml^{-1}	(Miller units) OD420 min^{-1} ml^{-1}/ OD600	ΔOD499 min^{-1} ml^{-1}/ OD600
30°C:								
A	280	0.061	0.0346	50.862	0.1989	256	567	3.3
B	280	0.078	0.0463	68.061	0.2228	514	594	2.9
37°C:								
A	290	0.226	0.1005	147.735	1.6173	91	445	7.2
	345	0.376	0.1940	285.180	2.2336	128	516	5.9
	525	0.661	0.3114	457.758	3.3067	138	471	5.0
B	290	0.258	0.1092	160.524	1.6324	98	423	6.3
	345	0.399	0.1925	282.975	2.2854	124	482	5.7
	525	0.665	0.2080	305.76	4.2376	72	313	6.4

Table 5.1 Results of B-galactosidase and B-lactamase assays on DS942 (pSNO80) shaking at 30°C and 37°C.

* The OD420 min^{-1} ml $cells^{-1}$ does not take into account the total assay volume, whereas the nMONPG min^{-1} ml $cells^{-1}$ and the ΔOD499 min^{-1} ml $cells^{-1}$ do.

approaching this: Casadaban *et al.* (1982) combined a chemically-induced *tnpA* ribosome binding site mutation with an inactivated *tnpR* gene on a high copy number plasmid. After the mutation step, a total of five transposase overproducing mutants were sequenced. All were due to single base changes; three within the ribosome binding site, the other two close by. This showed that poor expression of transposase (4.2.6) is due, at least in part, to inefficient initiation of translation. Wishart *et al.* (1985) used a high copy number plasmid, carrying a transposon with an inactivated *tnpR* gene, in the minicell strain DS410. In this case, transposase accumulated to about 1.5% of cell protein. Morita *et al.* (1987) also used a TnpR$^-$ transposon carried on a high copy number plasmid, with a *tnpA* ribosome binding site mutation, in DS410. In the latter case, transposase accumulated to about 4% of total cell protein at 30^{o}C.

pSNO15 (Fig. 5.1) was constructed by Hettle (1985). It is a pUC-based construct with the *lac* promoter and translation signals fused to the fourth codon of the Tn*1* *tnpA* gene. It was reasonable to hope that in this construct the transposase fusion protein would accumulate to as much as 8% of the cell protein at 37^{o}C (providing that a high level of the protein was not toxic) due to a combined effect of the high copy number of the plasmid, the absence of resolvase, and the efficiency of the *lac* promoter and translation signals (4.2.6). At 30^{o}C the level might be between 10% and 20% of the cell protein.

However, the transposition frequency promoted by pSNO15 was no higher than that by just a TnpR$^-$ transposon on a high copy number plasmid (Hettle 1985). Reasons for this could have been the method of assaying transposition, a host protein being limiting, or a difference in the specific activity of the fusion protein, but minicell studies (Hettle, Glasgow) did not show the expected increase in protein levels. It appeared that expression of transposase was being limited in some way in this construct. This was confirmed by fusing the *Pst*I-bounded *lacZ* cassette to the *Pst*I site at position 984 of the *tnpA* gene in pSNO15, a construct known as pSNO80 (Burke, Glasgow). B-galactosidase and B-lactamase assays carried out on this construct (Table 5.1) showed that at 30^{o}C the expression of the fusion was about four times that of derepressed transposase using its own translation signals, but one third that from pUC9*lacZ* (Table 4.15). The sequence

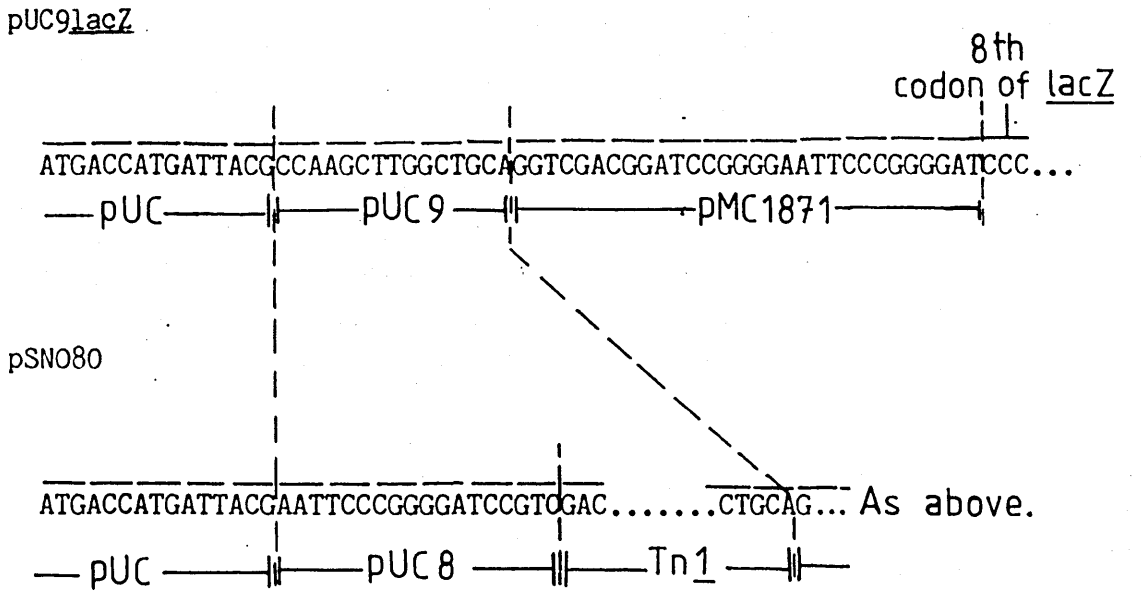
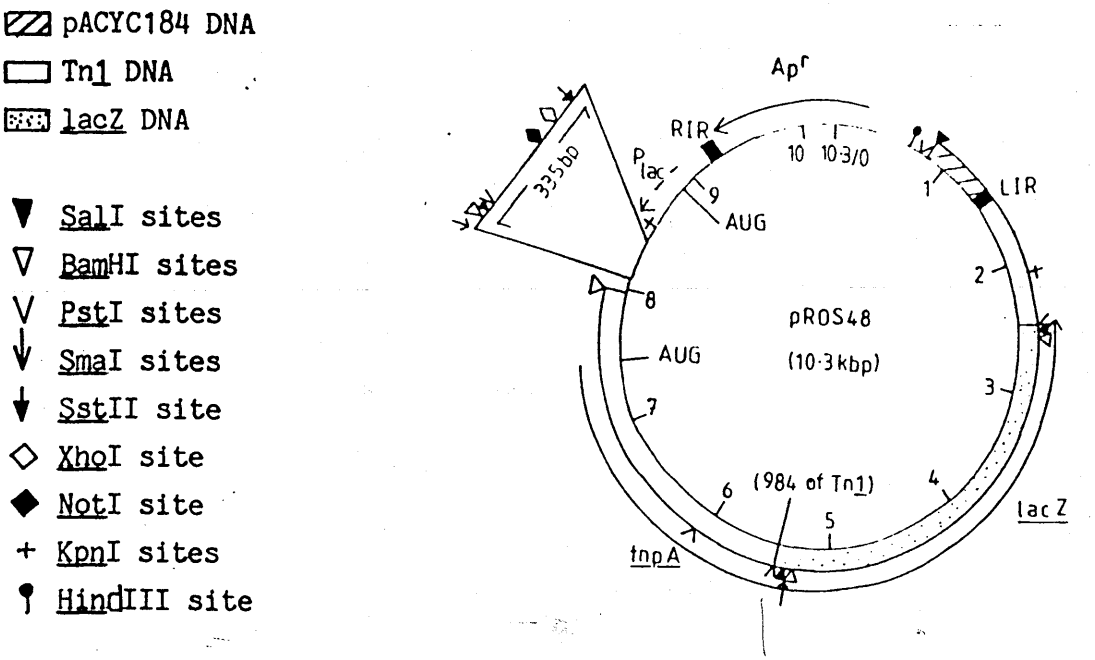


Fig. 5.2 Sequence differences between pUC9lacZ and pSNO80.

Fig. 5.3 pROS48, showing the insert of a 335bp fragment from pCS301 into pROS36 (Fig. 4.18b). The construction is described in the text.



differences of the fusions in pUC9lacZ and pSNO80 are shown in Fig. 5.2.

The decrease in fusion expression from pSNO80, relative to bla expression, at 37°C compared to 30°C, was expected from the pUC9lacZ results (Table 4.15). This has also been observed by Burke (Glasgow). The decrease probably has nothing to do with the temperature-sensitive behaviour of Tn1/3 transposition (Ch. 4), but concerns the translation efficiency of lacZ in the multicopy fusion system used. The assay results implied that some feature of the Tn1 tnpA gene between positions 3039 and 984, or in the 18bp sequence derived from pUC8 (Fig. 5.2), was causing a 3-fold (at 30°C) or 5-fold (at 37°C) decrease in expression of B-galactosidase from pSNO15.

A way in which to localise the inhibitory region was devised. Firstly, this involved the construction of pROS49, which contained useful sites for Exonuclease III deletions into the 5' end of tnpA.

5.1.1 The construction of pROS49

pROS36 (4.2.3) was digested partially with SmaI and the approximately 10kbp linear fragment was purified by excision from a gel. pCS301 (Stirling, personal communication) was digested with BalI/SmaI and the 335bp fragment, consisting of 313bp of Tn5 from the BalI site to the PstI site, and 22bp of pUC19 from the PstI site to the SmaI site, was purified and ligated to the linearised pROS36. One of the resulting constructs, pROS48, is shown in Fig. 5.3. The reason for inserting this fragment was to introduce a unique "start" site for Exonuclease III deletion. The KpnI site adjacent to this insertion was to have been the unique "stop" site, but it was discovered at this point that the transposon sequences present in pROS48 were Tn1, and not Tn3 derived (4.2), and therefore had an additional KpnI site within the tnpA gene. [A start site for Exonuclease III is one which has 5' protruding ends since the enzyme digests double-stranded DNA by removing nucleotides from each recessed 3' end, leaving extensive single-stranded 5' protrusions. When followed by digestion with S1 nuclease, exonuclease III can be used to shorten DNA fragments for the construction of deletions. The enzyme is inefficient at digesting 3' protruding ends (Henikoff 1984), hence a unique restriction site with

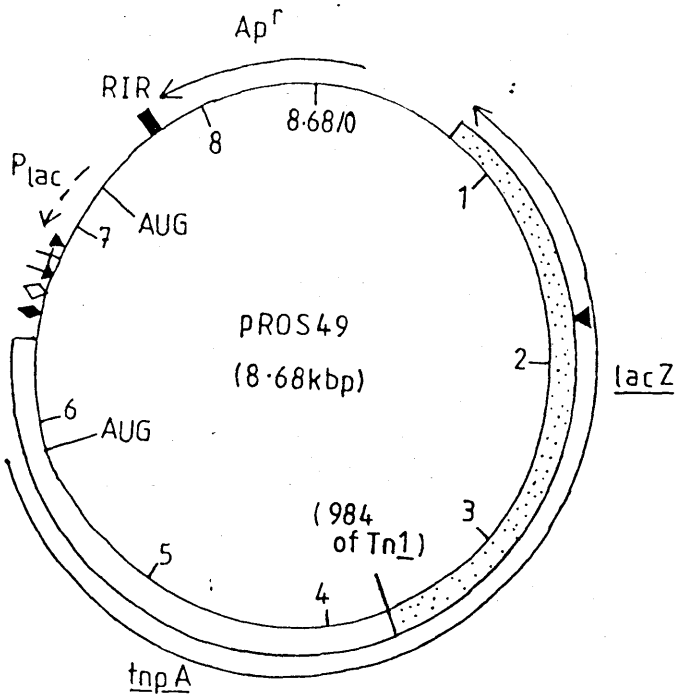


Fig. 5.4 pROS49, a HindIII/partial PstI deletant of pROS48, lacking the Tn1 sequences downstream of lacZ.

▼ SstI sites
↓ SstII site
◇ XhoI site
◆ NotI site
\+ KpnI site

3' overhangs can be used as a "stop" site.]

In view of the problem with the two KpnI sites, it was initially considered that the SstII site within the inserted 335bp fragment might work as a stop site, although it only had a 2bp 3' overhang. pROS48 was therefore cut with SstII, digested with Exonuclease III, treated with S1 nuclease and examined on a gel. pROS48 was treated the same way after digestion with NotI, which has 5' overhangs and therefore should act as a positive control substrate for Exonuclease III.

The results (data not shown) demonstrated that Exonuclease III digested pROS48 from the NotI site at about 60bp per minute at 30°C, and the presence of discrete bands decreasing in size suggested that all molecules were attacked at about the same rate. However, pROS48 was also digested from the 2bp 3' overhangs, giving a smear which moved progressively down the gel with time. This showed that the 2bp 3' overhang of the SstII site was not sufficient to act as an effective stop site, and a 4bp 3' overhang unique site was apparently necessary. Therefore the KpnI site in the tnpA gene downstream of lacZ in pROS48 was removed: pROS48 was cut with HindIII and partially with PstI, and the 8.7kb fragment was purified, blunt-ended with T4 DNA polymerase, and religated to give pROS49 (Fig. 5.4). This now contained two possible unique start sites, NotI or XhoI, and a unique stop site, KpnI.

5.1.2 Exonuclease III deletions of pROS49

As pROS49 was derived from pROS36 (4.2.3), it contained the Tn1 tnpA gene fused at position 984 to lacZ, which was the same location as the fusion in pSNO80. It also carried the lac promoter and ribosome binding site directing translation in the same orientation as, but upstream of, tnpA. The idea of the Exonuclease III digestions was to create progressive deletions, starting from within the 335bp fragment and ending at various points within tnpA, which could then be fused back to the lac signals. Depending on the end-point of the deletions, the constructs would initially resemble pSNO80, with the lac signals being fused to the very 5' end of the tnpA gene, and finally pUClacZ, where the lac signals are fused just upstream of lacZ. There would be

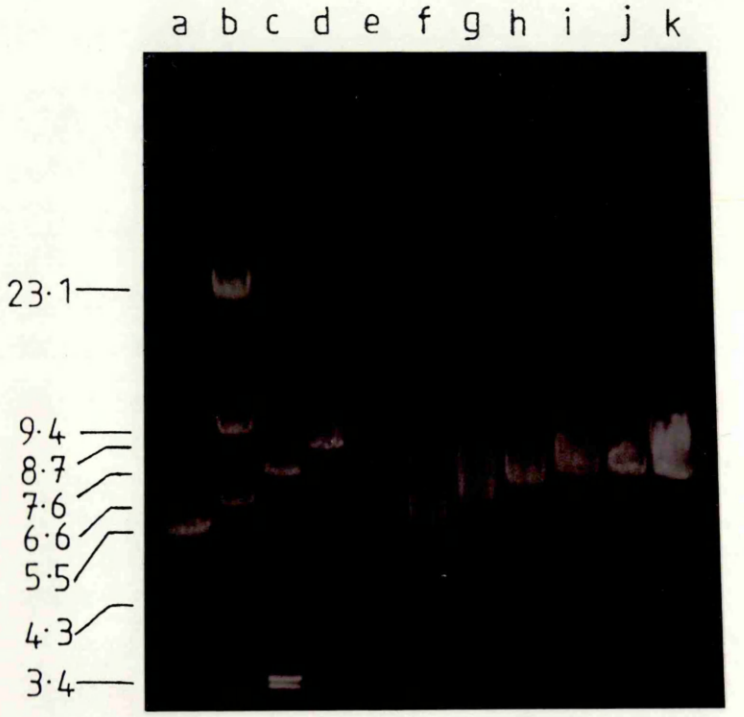


Fig. 5.5 Exonuclease III deletion of pROS49.

a. p253 HindIII 5.5kbp marker
b. λ HindIII digest
c. pROS13 ClaI
d. pROS49 XhoI
e. pROS49 Exonuclease III 35 min
f. pROS49 Exonuclease III 28 min
g. pROS49 Exonuclease III 21 min
h. pROS49 Exonuclease III 14 min
i. pROS49 Exonuclease III 7 min
j. pROS49 XhoI/KpnI
k. pROS49 KpnI

Time	No.of blue colonies	No. of white colonies	Total	% Blue colonies
7	61	29	90	68
14	54	28	82	66
21	22	4	26	85
28	7	16	23	30
35	15	81	96	16

Table 5.2 Results of Exonuclease III deletions on pROS49.

a whole range of deletions lacking various amounts of tnpA. The extent of the deletions would be examined, and assays carried out on the fusions to see where the transition point lay between the pSNO80 level and the pUClacZ level of B-galactosidase expression.

About 10ug of pROS49 was digested overnight to completion in a 20ul volume with XhoI and KpnI. It was important that complete digestion with both enzymes occurred, otherwise Exonuclease III might not work, or might delete past the stop site giving undesired transformants after the final ligation. The next day, 1.5ul was removed from the tube as a zero time point sample, and the rest was treated with RNAse. This step was found to be necessary because any contaminating RNA interferes with digestion of the DNA substrate by Exonuclease III. The DNA was subsequently treated with Exonuclease III, then S1 nuclease. The samples were divided in two, and one aliquot was run on an agarose gel with markers (Fig. 5.5).

The gel showed that Exonuclease III had processively deleted pROS49, although some processivity had been lost with increasing incubation time. The rate was estimated to be about 120bp per minute at 30°C.

The other half of the samples were treated with Klenow to neaten the overhanging ends of the deletions, and the DNA was religated. Before transformation into DS942, the religated plasmids were redigested with XhoI to prevent non-deleted ones from transforming the cells. Transformants were selected on plates containing Sm Ap and Xgal.

The number of blue and white colonies from each transformation is shown in Table 5.2. It was to be expected that the first time point would give a high percentage of blue colonies, because many of the DNA molecules would not have been deleted as far as tnpA, so that B-galactosidase would be expressed from the transposase signals. After 14 minutes and 21 minutes, it was expected that the ratio of blues:whites would decrease because only one in three fusions would be in frame with translation initiated by the lac signals. However, this decrease was not observed (see 5.3.1 for explanation). After 28 minutes and 35 minutes, it was expected that the ratio of blues:whites would decrease even further because many of the deletions would have extended into the lacZ cassette. This is what was observed.

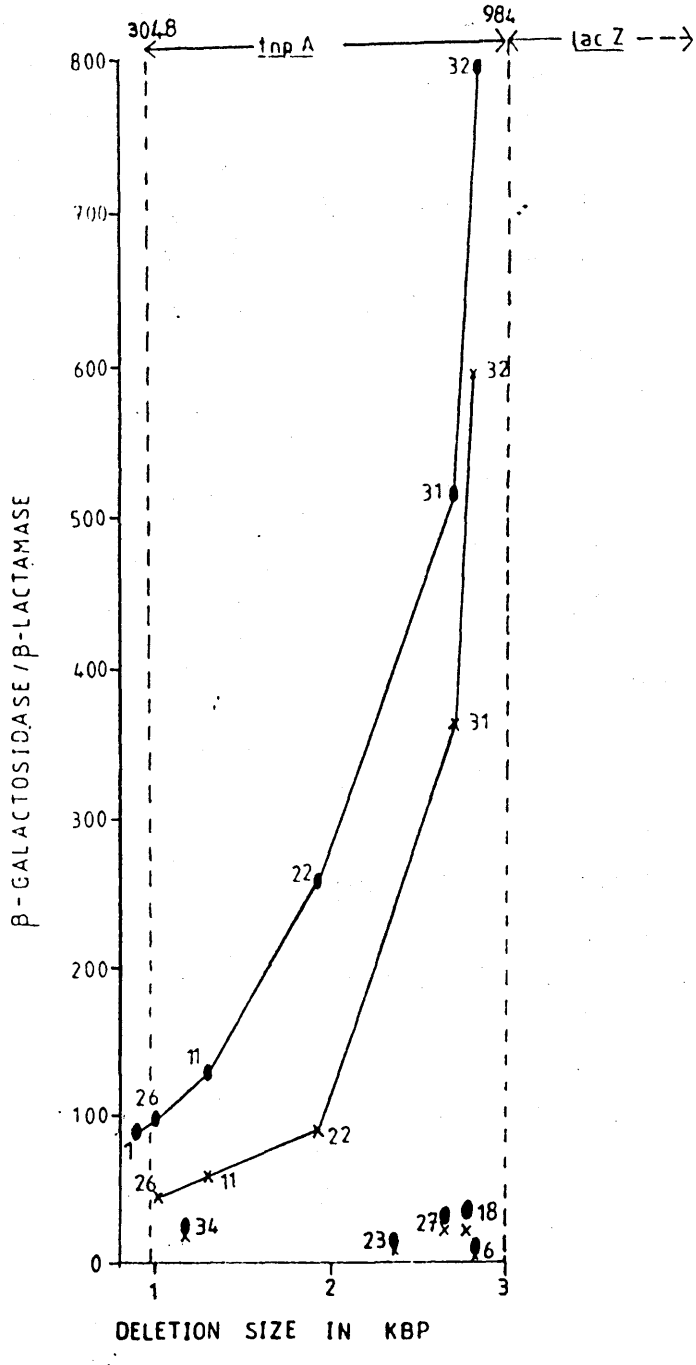


Fig. 5.6 B-galactosidase/B-lactamase ratio plotted against pROS49 Exonuclease III deletion size. B-galactosidase units are in nMONPG min^{-1} ml $cells^{-1}$. B-lactamase units are in ΔOD_{499} min^{-1} ml $cells^{-1}$.

● Samples taken between OD_{600} 0.073 and 0.205.
X Samples taken between OD_{600} 0.205 and 0.408.

This delineates the 2kbp of *tnpA* sequences within which the end-points of all the deletions lie, except that of clone I.

The expression of all deletants except clone 1 is controlled by *lac* signals. Clone 1 is controlled by *tnpA* signals.

The blue and white colonies from each time point were patched out and examined on single colony gels. It had been noted that three of the blue colonies also had a blue "halo" around the outside. This phenotype had previously been observed with pUC9lacZ. The gels showed that in these colonies the plasmids had been deleted for almost all the tnpA sequences 5' to lacZ. The "halo" is probably due to the high amount of B-galactosidase produced by the plasmids (5.1.3) diffusing into the medium after lysis of some cells in the colony.

TELT plasmid preparations of various deletants were carried out and these were retransformed into DS942. Those cells transformed with plasmid from colonies with a blue "halo" gained this phenotype, indicating that it was plasmid-linked. The plasmid DNA from all the retransformed cells was digested with SstI overnight before being run on a gel. This enabled more accurate estimates of the extents of the deletions to be made.

5.1.3 Assays of pROS49 Exonuclease III deletants

Several of the deleted plasmids were assayed for B-galactosidase and B-lactamase expression at 37°C. The B-galactosidase/B-lactamase ratios plotted against the deletion sizes are shown in Fig. 5.6.

It was a surprise to find that there was apparently a gradual increase in B-galactosidase/B-lactamase which was related to the deletion size: the greater the amount deleted, the higher the ratio. Plasmid number 1, deleted only a short way so that tnpA was still intact, showed a comparable level of expression to the tnpA-directed B-galactosidase synthesis in fusions at various points along tnpA (Table 4.15). Plasmids 31, 32 and 33 (all had a blue "halo"), deleted for almost all of tnpA, showed a comparable level of expression to B-galactosidase from pUC9lacZ at 37°C. Intermediate deletants showed intermediate levels, except for a few which showed a comparable low level. The latter fusions were probably out of frame to B-galactosidase. This was concluded because 0 out of 25 white colonies examined had deletion end-points upstream of lacZ i.e. ending within the tnpA gene; all 25 had extended into the cassette. The explanation for this low level of expression might be that all out-of-frame fusions are reinitiating

translation only just 5' to lacZ. In the Tn3 sequence, the other two reading frames are open across position 984. This might also be the case in Tn1. Alternatively, a low level of ribosome slippage might be occurring. Both of these explanations have been invoked for similar results observed by Zabeau and Stanley (1982) and by Stanley (1983).

The purpose of these Exonuclease III deletions had been to elucidate the reason for the low expression of B-galactosidase from pSNO80 compared to that from pUC9lacZ. The results seemed to show that there was a correlation between the amount of upstream sequence and the levels of expression. This has also been observed by Zabeau and Stanley (1982) and Stanley (1983). However, this was not the case in assays on lacZ fused to tnpA at different positions (4.2.3; Table 4.15). Sequence analysis of Tn3 tnpA (Russell and Boyd, Glasgow, personal communication) suggests poor codon usage is not the explanation for the effect observed here, and although Tn1 has not been entirely sequenced, the 5' end (250bp) is 88% homologous at the DNA level and codon usage in this part does not seem to be poor either. Another possible explanation involves the specific activity of the fusions. It could be that the 5' end of the tnpA protein plays an important part in folding. If this were present and correctly folded, and the lacZ part were too, then a fragile "hinge" might exist between the two moieties (this is the case in pROS38 and pGRO577, 5.2.1). If cleavage occurs at this point in vivo, then the B-galactosidase will be free to act with the specific activity of the native protein. However, if the 5' end of the tnpA protein is absent, being replaced by the end of the lacZ gene, then the resulting protein fusion might not consist of two correctly folded portions, with a vulnerable "hinge", but might contain a relatively disordered N-terminal end which could inhibit the specific activity of the B-galactosidase portion, depending on its length. For the fusions constructed here, this explanation is probably not the correct one, because 5 to 10-fold differences in amounts of polypeptide were observed on SDS-polyacrylamide gels in the cases of pSNO80 and pUClacZ (Russell, personal communication). It is also possible that disruption of the 5' end of the tnpA mRNA, giving rise to different structures, might cause attenuation, or stability differences, but this would have to be dependent on length.

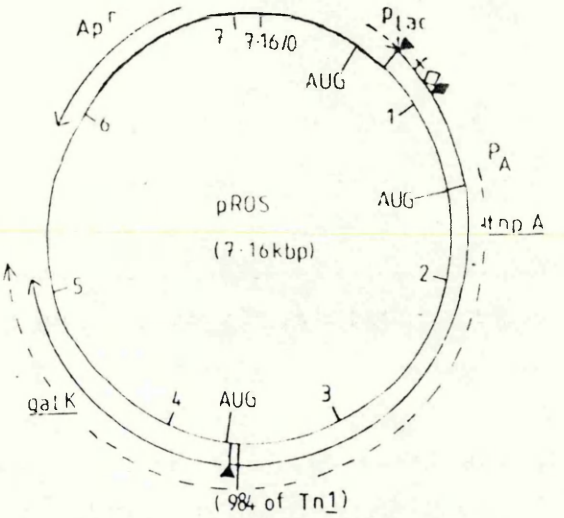


Fig. 5.7 Showing desired transcriptional fusion of Tn1 *tnpA* to *galK* at position 984 of Tn1. This clone would be suitable for Exonuclease III deletions from the unique start sites ◆(*Not*I) or ◇(*Xho*I), using the unique *Kpn*I site as a stop site. Deletions into the 5' end of *tnpA* could be joined back to the *lac* signals in an analogous manner to the Exonuclease III deletions of pROS49 (5.1.2).

▭ 2.96kbp *Eco*RI (▼) fragment from pROS49 (Fig. 5.4).
\+ *Kpn*I site.

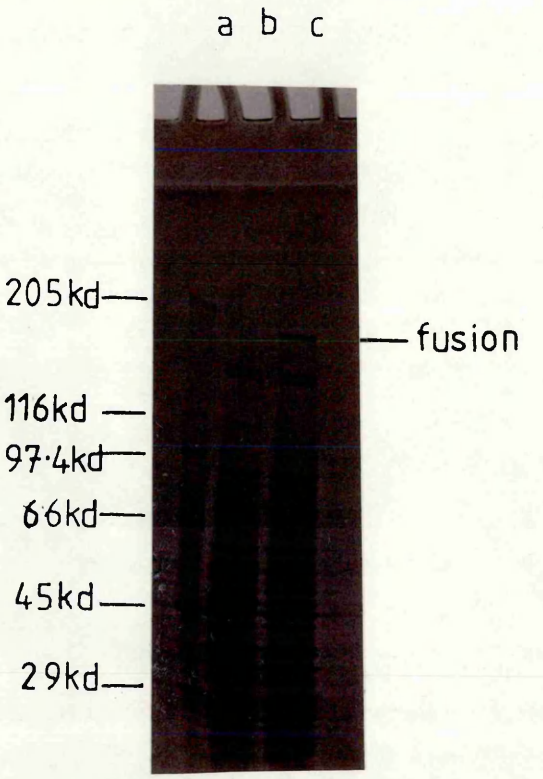


Fig. 5.8 8% SDS/polyacrylamide gel, showing the *tnpA lacZ* fusion protein produced by DS942 (pROS38).

a. Markers
b. DS942
c. DS942 (pROS38)

An attempt was made to see whether the effect would be mirrored in Exonuclease III deletions of a tnpA transcriptional fusion to galK. If it had been, it would have ruled out the possibility that the effect was due to specific activity differences between the fusions, or that codon usage was responsible. It would have implicated mRNA structure and/or stability.

The required construct for these deletions is shown in Fig. 5.7. The 2.963kbp EcoRI/EcoRI fragment from pROS49 was purified and cloned into EcoRI-cut pKL500 treated with alkaline phosphatase. The resulting ligation mix was transformed into DS942 and plated out on agar plates containing Sm Ap. Unfortunately, none of the 7 colonies examined had the EcoRI fragment in the correct orientation, with tnpA pointing towards galK. Due to lack of time, this cloning was not repeated, so the reason for the low expression of B-galactosidase from pSNO80 compared to in pUC9lacZ remains to be elucidated.

5.2 Purification of a Tn1 tnpA lacZ fusion protein

As the attempt to overproduce transposase by fusion to the lac signals in pSNO15 had been largely unsuccessful, it was decided to use a derepressed tnpA fusion to lacZ on a high copy number, pUC-based plasmid as the source of protein. This was expected to accumulate to 1 - 2% of the cell protein according to Wishart et al. (1985) and supported by the fusion assays (Table 4.15 and Section 4.2.6: at 37°C the tnpA lacZ fusion gives 360 Miller units of B-galactosidase, which is equivalent to a specific activity of 7056. If pure B-galactosidase has a specific activity of 450,000 (Fowler 1972), then 1.6% of the cell protein will be synthesized as the fusion.). For the purpose of raising antibodies, it was desirable to have as much as possible of the tnpA sequences in the fusion, and therefore pROS38 was chosen as the source of protein. Here lacZ is fused at position 166 of the transposon, i.e. 133bp from the 3' end of the tnpA gene. The protein is clearly visible on SDS-polyacrylamide gels (Fig. 5.8).

Two ways of purifying the fusion protein were considered: by use of affinity chromatography; or by excision and electrophoresis from SDS-polyacrylamide gels. Due to the apparent simplicity of affinity chromatography this was the method initially chosen.

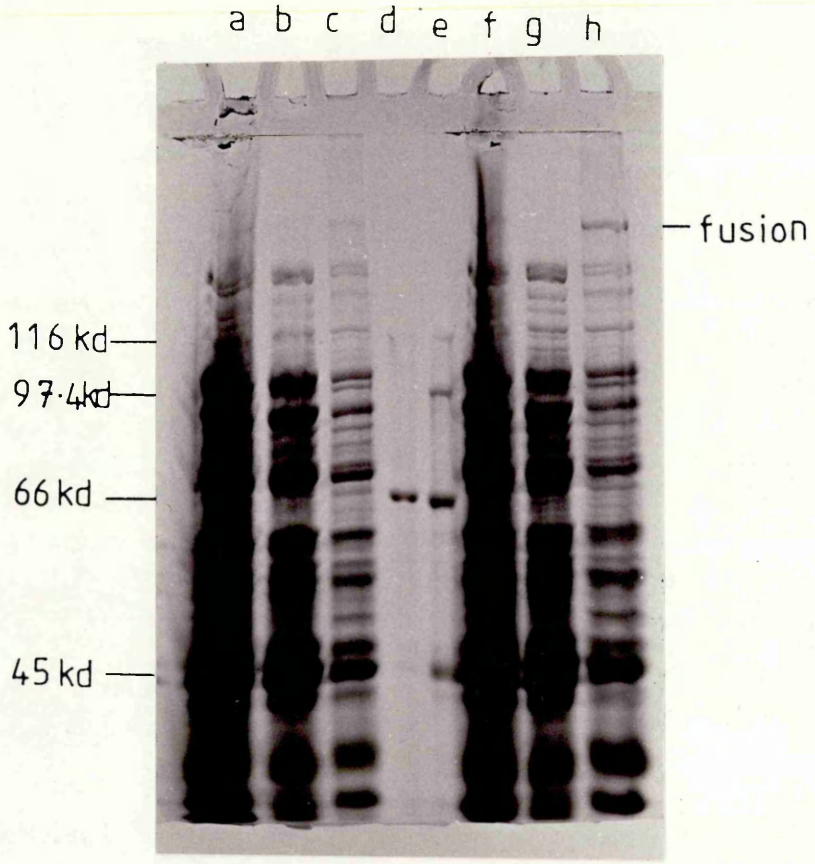


Fig. 5.9 8% SDS/polyacrylamide gel, showing that the tnpA lacZ fusion protein made by DS942 (pROS38) is mainly in the pellet after sonication or treatment with lysozyme in a NaCl-free buffer and centrifugation.

a. DS942 (pROS38) whole cells
b. DS942 (pROS38) sonicate supernatant
c. DS942 (pROS38) sonicate pellet
d. Pure B-galactosidase protein
e. Markers
f. DS942 (pROS38) whole cells
g. DS942 (pROS38) supernatant after lysozyme treatment
h. DS942 (pROS38) pellet after lysozyme treatment

5.1.2 Attempted purification of a Tn*1* *tnpA*-*lacZ* fusion protein by affinity chromatography

The purification of B-galactosidase by this procedure was first described by Steers *et al.* (1971). They fractionated the supernatant of a B-galactosidase-containing sonicate with $(NH_4)_2SO_4$ before running the resuspended, dialysed precipitate onto a column consisting of *p*-aminophenyl-B-D-thiogalactopyranoside (TPEG) covalently bound to derivatives of agarose and polyacrylamide. The enzyme adsorbed tightly at neutral pH to the columns. Elution of the enzyme was brought about by 0.1M NaBorate, pH10.

In 1984, Ullman published a one-step purification of *lacZ* fusion proteins which have B-galactosidase activity. This omitted the $(NH_4)_2SO_4$ step used by Steers *et al.*, and was designed to minimize proteolytic degradation of large hybrid proteins, which can be a problem. According to Ullman, a high salt concentration (1.6M) is an important part of the binding procedure. This is supposed to increase the capacity of the column by decreasing the nonspecific adsorption of foreign proteins. Ullman studied five different protein fusions whose size varied from just over 116MDa to 175MDa. The cells were sonicated in a NaCl-free buffer, and the supernatant after centrifugation was adjusted to 1.6M NaCl before running on the column. In all cases, highly purified (33 to 100-fold) fusion proteins were eluted from the columns with 0.1M NaBorate, pH10. This procedure seemed very simple, so the column matrix, TPEG-agarose, was obtained from Sigma.

A trial 1ml column was prepared, and 2gm wet weight of DS942 (pROS38) cells grown at 30°C were sonicated in an NaCl-free buffer as described by Ullman (1984). After centrifugation of the sonicate, the supernatant and pellet were assayed for protein and B-galactosidase, and samples were run on a gel overnight together with a whole cell sample (Fig. 5.9). The assays suggested that about 10% of the B-galactosidase activity was in the supernatant, and the rest was in the pellet. Thus the fusion was mainly in the pellet and was less than 2% of the cell protein. The reason for this became apparent later.

Neither Steers (1971) nor Ullman (1984) had reported problems with solubilising B-galactosidase fusion proteins. However, the necessity

for solubilisation of cro-lacZ fusions in 4% SDS had been reported by Zabeau and Stanley (1982) and Stanley (1983). Their studies showed that precipitation is a function of the structure of the hybrid protein rather than the concentration of the hybrid protein in the cytoplasm.

Attempts were made to resolubilise the pellet in various solvents, at various pHs. None were effective until SDS was tried. 66% of the pellet B-galactosidase activity was soluble in 2% SDS, and the rest in 5% SDS. Surprisingly B-galactosidase was still active in 5% SDS, but it would not bind the column under these conditions, or in 0.1% SDS. Dialysis failed to remove the SDS which was apparently tightly complexed to the protein. Therefore efforts were concentrated on that percentage of the protein which was soluble.

The supernatant buffer was adjusted to 1.6M NaCl, as described by Ullman, and some of it was run onto the trial column, which had been prewashed with the same buffer. Fractions were collected immediately. The column was rinsed with 50ml of buffer before elution was attempted with NaBorate, as described by Ullman. The fractions and eluate were assayed for B-galactosidase activity.

It was apparent that the initial few fractions contained all of the B-galactosidase activity which had been loaded, and none had been retained by the column to be eluted by NaBorate. At least five possibilities for this failure to bind existed. These were:

1. The protein concentration loaded might have been too great.
2. The salt concentration might have been too high (the sonicate itself will contain some salts).
3. The flow rate of 1ml per minute might have been too high.
4. The tnpA lacZ fusion might not bind the column due to its structure.
5. The TPEG-agarose beads might be defective.

The sonicate supernatant was diluted 16-fold and the salt concentration was adjusted to 0.25M. This was loaded onto the pre-washed column as before. Again the activity loaded flowed straight through without binding. This suggested that the protein and salt

Comments	Buffer	Rate in nM ONPG min^{-1} ml^{-1}	Amt. emerging as % of that loaded
(Loaded	A	2632	-)
Old beads:			
Supernatant 1	A	1545	59
Supernatant 2	A	504	19
Borate wash 1		415	16
Borate wash 2		38	1
New beads:			
Supernatant 1	A	1034	39
Supernatant 2	A	502	19
Borate wash 1		963	37
Borate wash 2		86	3
(Loaded	B	2125	-)
Old beads:			
Supernatant 1	B	1605	76
Supernatant 2	B	229	11
Borate wash 1		347	16
Borate wash 2		32	2
New beads:			
Supernatant 1	B	290	13
Supernatant 2	B	35	2
Borate wash 1		908	43
Borate wash 2		67	3
(Loaded	C	2431	-)
Old beads:			
Supernatant 1	C	1684	69
Supernatant 2	C	168	7
Borate wash 1		294	12
Borate wash 2		24	1
New beads:			
Supernatant 1	C	40	2
Supernatant 2	C	22	1
Borate wash 1		1267	52
Borate wash 2		77	3

Table 5.3 Results of "batch method" tests on binding of pure B-galactosidase to old and new batches of TPEG-agarose beads under different NaCl conditions. The buffers were as follows:

	NaCl	TrisHCl pH7.4	$MgCl_2$	B-mercaptoethanol
Buffer A	1.6M	20mM	10mM	10mM
Buffer B	0.25M	20mM	10mM	10mM
Buffer C	0.05M	20mM	10mM	10mM

concentrations were not the reason for the lack of adsorption.

The experiment was repeated in 0.25M NaCl with a flow rate of 1ml per hour. Again no activity bound the column. This ruled out the possibility that flow rate was a problem.

Pure B-galactosidase protein was then used in the tests. The loaded activity again emerged straight through before elution. At this point it was concluded that the TPEG-agarose beads were at fault. This turned out to be the case, as a different batch supplied by Sigma was shown to bind pure B-galactosidase well (see below).

In order to get the conditions right for maximal binding to, and elution from, the column, a "batch method" test was carried out on the old and new batches of beads. This involved equilibrating 50ul of beads with one of three buffers (Table 5.3), then adding 950ul containing a known activity of B-galactosidase. After allowing the tubes to stand on ice for 30 minutes, with periodic vortexing, the supernatant was removed and assayed for its activity. The beads were washed once in the appropriate buffer, and the activity of this supernatant was again assayed. Then the beads were treated with two washes of NaBorate buffer, pH10, and the activities of these eluates were assayed (Table 5.3).

The data showed that the old beads were ineffective at binding at any salt concentration. The new beads retained, and released, most activity (52% of that loaded) at the lowest concentration of NaCl (50mM). This result was in contradiction to that obtained by Ullman (1984), but she used a column containing non-commercial TPEG-sepharose and only presented data on binding of adenylate cyclase-B-galactosidase fusion with respect to NaCl concentration. Her conclusion does not seem to be applicable to pure B-galactosidase, and may well not be generally true for other fusion proteins.

It was obvious from the results of the batch test that in cases where binding and release did occur, not all of the activity loaded could be accounted for after the borate washes. This suggested that either the specific activity of B-galactosidase was less in NaBorate, or that some of the activity loaded might be retained permanently by the

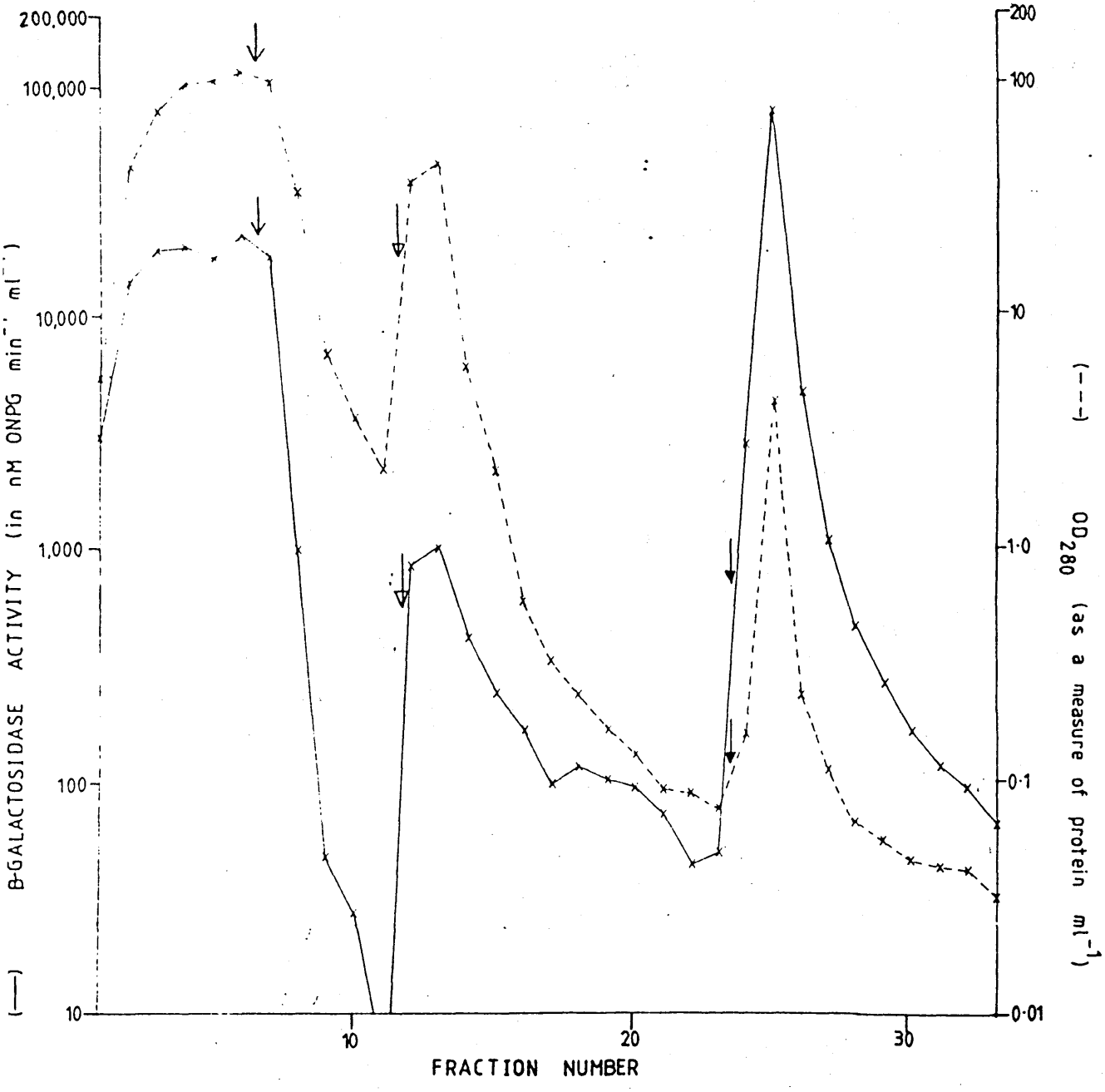


Fig. 5.10 Chromatographic pattern of DS942 (pROS38) sonicate supernatant. Protein was measured spectrophotometrically at 280mu, (---) and enzymatic activity was measured spectrophotometrically at 420mu (——). Each fraction volume was 1ml. Fractions 1-6 were taken while the protein was being loaded on the 0.7ml column in buffer C (Table 5.3).

↓ indicates commencing of wash stage using buffer C.

⇓ shows the point at which the flow rate was trebled from 4 bed volumes/hour to 12 bed volumes/hour, and at which the buffer was changed to buffer B (Table 5.3).

↓ indicates replacement of buffer B with 0.1M NaBorate pH10.

Fractions 24-27 contained one third of the total activity loaded.

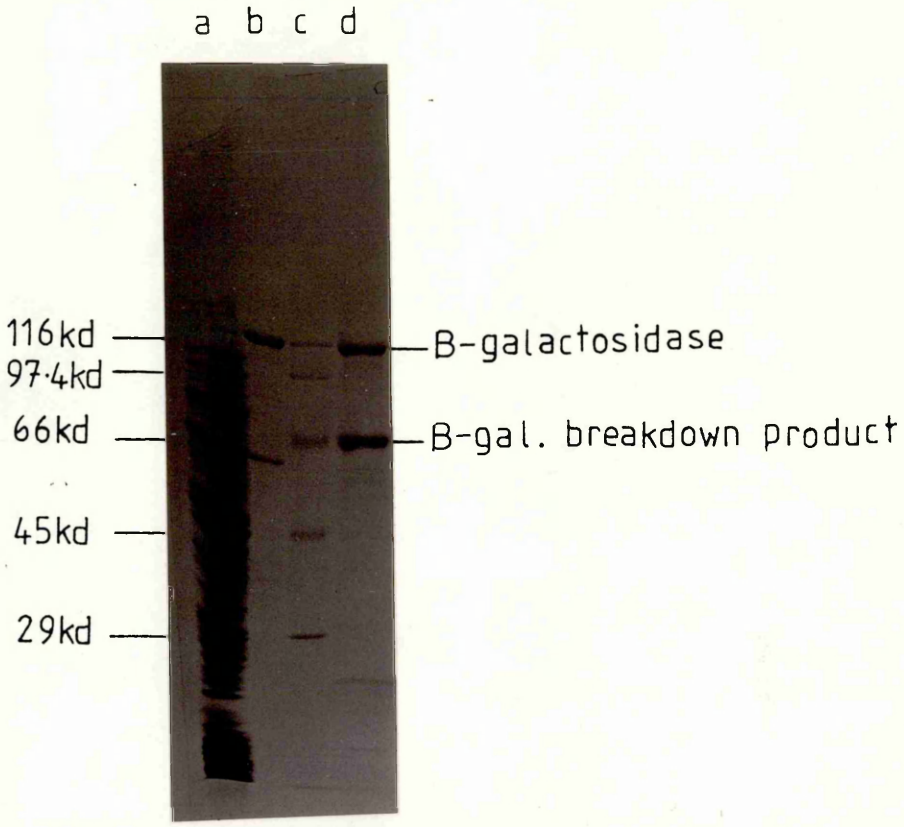


Fig. 5.11 8% SDS/polyacrylamide gel showing the protein eluted from the TPEG-agarose column with NaBorate pH10.

a. DS942 (pROS38) total sonicate
b. Protein eluted from column
c. Markers
d. Pure B-galactosidase

beads. Tests showed that the former was not the case. Buffer C (50mM NaCl) was used as the binding buffer in subsequent column tests.

A 0.7ml column was set up with the new batch of Sigma TPEG-agarose beads. Ideally, a much longer one of about 3ml would have been used, but unfortunately Sigma could not supply more than 1ml of the new batch for several weeks. Some of the fusion protein sonicate supernatant was loaded on the prewashed column as before, in buffer C (Table 5.3). The OD_{280} was used to monitor the protein being washed through the column and B-galactosidase assays were carried out on the fractions (Fig. 5.10). After about 10ml had flowed through, the washing buffer was changed to buffer B (Table 5.3). This contained 0.25M NaCl and was supposed to decrease non-specific binding of proteins to the column, although a significant amount of activity was also released (Fig. 5.10). After 23ml had been collected, NaBorate was added to the top of the column and the eluate was collected. Fractions 24 to 27, containing one third of the activity initially loaded, were pooled and precipitated overnight on ice with 5ml of saturated $(NH_4)_2SO_4$ solution. The pellet was resuspended in 1ml of buffer C and was dialysed against TE buffer. Later a sample of the eluate was examined on an SDS-polyacrylamide gel (Fig. 5.11).

Surprisingly, the gel showed that the purified protein had the size of B-galactosidase. No intact fusion was obvious. The B-galactosidase fragment was also apparently broken down. This was true in the purified "fusion" tracks, and in the pure B-galactosidase track. Apparently, B-galactosidase has an internal site at which cleavage readily occurs. The smaller fragment in the "fusion" and pure B-galactosidase tracks had a slightly different size on the gel. Perhaps this represents a difference in the position of the cleavage site in the two parental molecules due to slightly different folding.

The B-galactosidase protein purified from the strain containing pROS38 could only be a breakdown product of the intact fusion, as the strain was *lac*. As breakdown gave a protein of the same size as B-galactosidase, it appeared that cleavage had occurred at the region just 5' to *lacZ*. This region probably represents a weak "hinge" between the transposase and B-galactosidase parts of the protein. The breakage at this point did not appear to occur during boiling, because

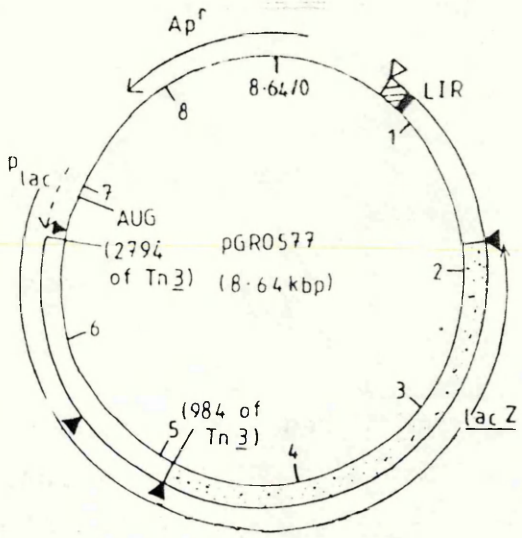


Fig. 5.12 pGRO77, showing the translational fusion of the lac signals at the pUC19 SmaI site to Tn3 tnpA, with the insertion of the lacZ cassette at position 984 of the transposon.

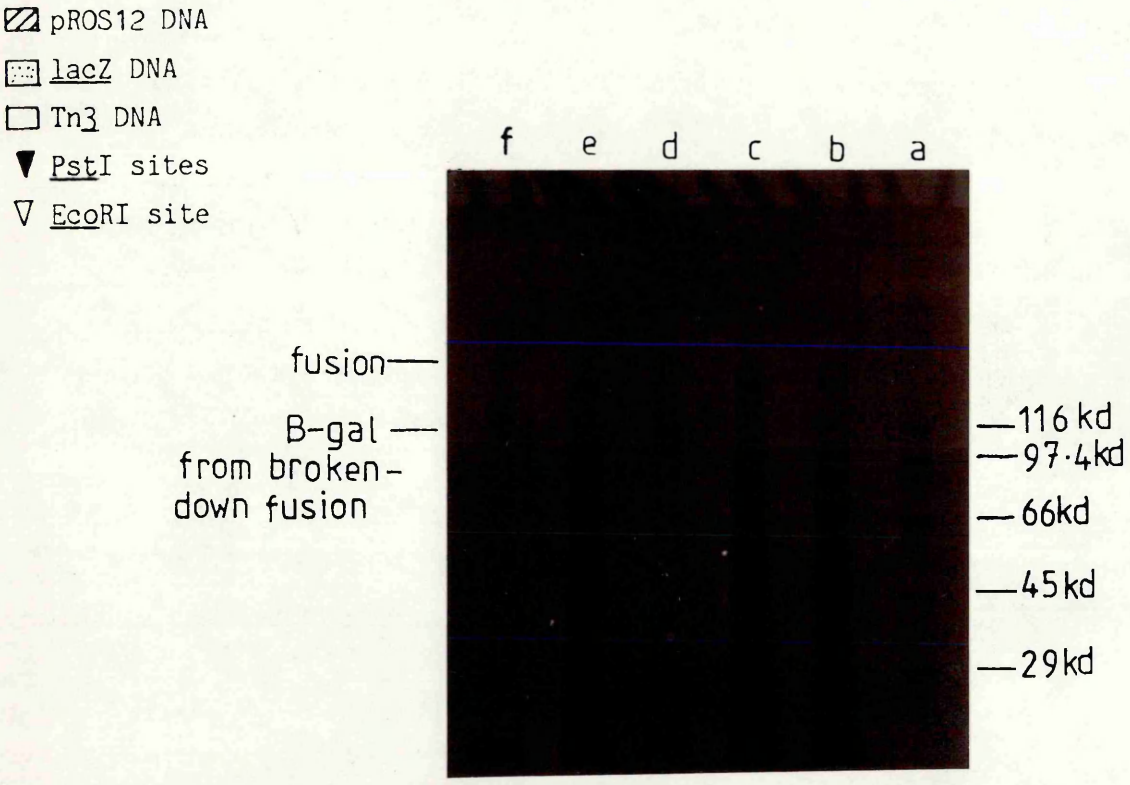


Fig. 5.13 SDS/polyacrylamide gel showing binding to the column of B-galactosidase from broken-down fusion in preference to intact fusion.

a. Markers
b. Total sonicate loaded on column
c. Protein emerging straight through column
d. First eluate with NaBorate
e. Protein in (c.) was reloaded. (e.) shows the protein flowing straight through.
f. Second eluate with NaBorate

the tnpA part of the fusion was not apparent. Therefore it had probably occurred either in vivo due to proteolysis, or in vitro during sonication or freezing and thawing. The results of the Western blot in 5.3 suggested that at least some of the degradation was due to mechanical cleavage.

The sonicate supernatant which had been loaded on the gel had originally contained a little of the protein as fusion (Fig. 5.9). The lack of binding of this to the column suggested that either all the fusion had degraded by being cleaved to B-galactosidase since the gel had been run, or the B-galactosidase bound the column preferentially. It was important to determine which of these reasons was true if the columns were going to be of use. At this point Russell (Glasgow) ran a sonicate supernatant from a strain containing pGRO577 (Fig. 5.12) on a column. The extract was loaded on the column and the flowthrough was collected. The bound protein was eluted with NaBorate and was purified. The flowthrough was put back onto the rewashed column and the second flow-through was collected. The bound protein was again eluted with NaBorate and purified. An SDS-polyacrylamide gel on the purified protein is shown in Fig. 5.13. It showed that the first time through the column, only B-galactosidase was retained and eluted. This accounted for about three quarters of the total activity loaded. The fact that this fusion protein, to position 984 in the transposon, broke down as well as the fusion at 166 encoded by pROS38, confirmed that there must be a weak hinge just 5' of the lacZ portion, probably in the polylinker region of pMC1871. The second time through the column, some purified fusion was retained, together with a little B-galactosidase. This implied that the column had a higher affinity for B-galactosidase than for the fusion protein. This observation suggested that running two columns in series might have the desired result of purifying the fusion protein rather than B-galactosidase. However, in order to obtain enough fusion to raise antibodies, longer columns would be necessary. Therefore, at the time this was impossible due to the lack of functional column beads, so the second method of purification was attempted.

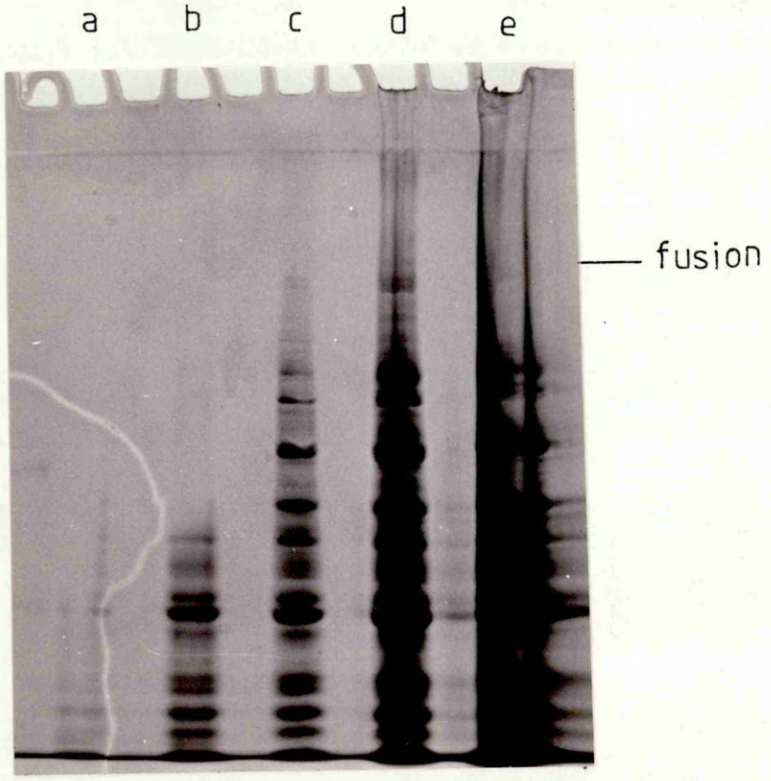


Fig. 5.14 Calibration of amount of DS942 (pROS38) total protein that can be loaded and resolved to show the *tnpA lacZ* fusion protein.

a. 15ug protein (1×10^8 cells) per 0.7cm width of gel
b. 75ug protein (5×10^8 cells) per 0.7cm width of gel
c. 150ug protein (1×10^9 cells) per 0.7cm width of gel
d. 750ug protein (5×10^9 cells) per 0.7cm width of gel
e. 1500ug protein (1×10^{10} cells) per 0.7cm width of gel

5.2.2 Purification of a Tn1 *tnpA-lacZ* fusion protein by excision and electroelution from an SDS-polyacrylamide gel

This method had an advantage over the previous one in that the 5% SDS-soluble protein could be run on the gel, and therefore was not wasted.

Initially a calibration was carried out to determine the maximum amount of protein that could be loaded and resolved to show the fusion protein (Fig. 5.14). The results indicated that about 1mg of protein could be loaded per cm width of gel, i.e. on a gel 0.15cm thick and 17cm wide, the protein from 250ml of a culture with an OD_{600} of 1.0 could be loaded and resolved sufficiently to show the fusion.

The procedure for purification was as follows:

The cells from 1l of DS942 (pROS38) grown to an OD_{600} of 2.0 at 30°C were spun down in 250ml lots (equivalent to 2gm wet weight of cells each), and were frozen to -70°C. Each lot was enough for two gels. When required, the cells were thawed and kept on ice. They were washed in a buffer containing 0.2M Tris HCl pH8.0, 0.25M NaCl, 0.01M MgAcetate, 10mM B-mercaptoethanol, 5% glycerol (Germino *et al.* 1983). Then 250ml-worth of cells were resuspended in 5ml of this buffer before sonication at 0°C, at a microtip limit of 7, for a total of 1 minute (5 seconds per minute). Alternatively, lysozyme could be used as described by Germino *et al.*. It was found that protease inhibitors were not required during this procedure.

After sonication, the extracts were spun at 20K for 30 minutes. This step was probably not essential, but it did remove some of the background protein and this possibly assisted in resolution. The supernatant was discarded, and the pellet was resuspended to form a slurry in 5ml of a buffer containing SDS: 0.2M Tris HCl pH8.0, 0.01M MgAcetate, 10mM B-mercaptoethanol, 5% glycerol, 1mM EDTA, 5% SDS. [This buffer does not contain NaCl because this precipitates out the SDS-protein complexes. MgAcetae is probably not necessary because biologically active B-galactosidase is not required.]

2.5ml of the slurry, together with 2.5ml of 2 x loading buffer was boiled and loaded onto an 8% gel with a 4.5% stacker, without wells.

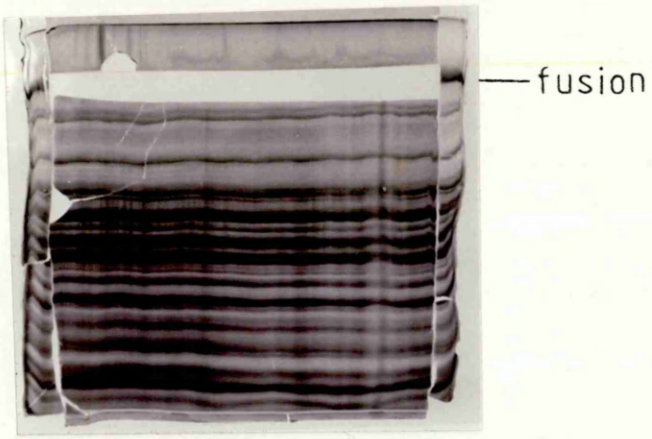


Fig. 5.15 TnpA lacZ fusion protein in sonicate pellet excised from an SDS/polyacrylamide gel for subsequent electroelution.

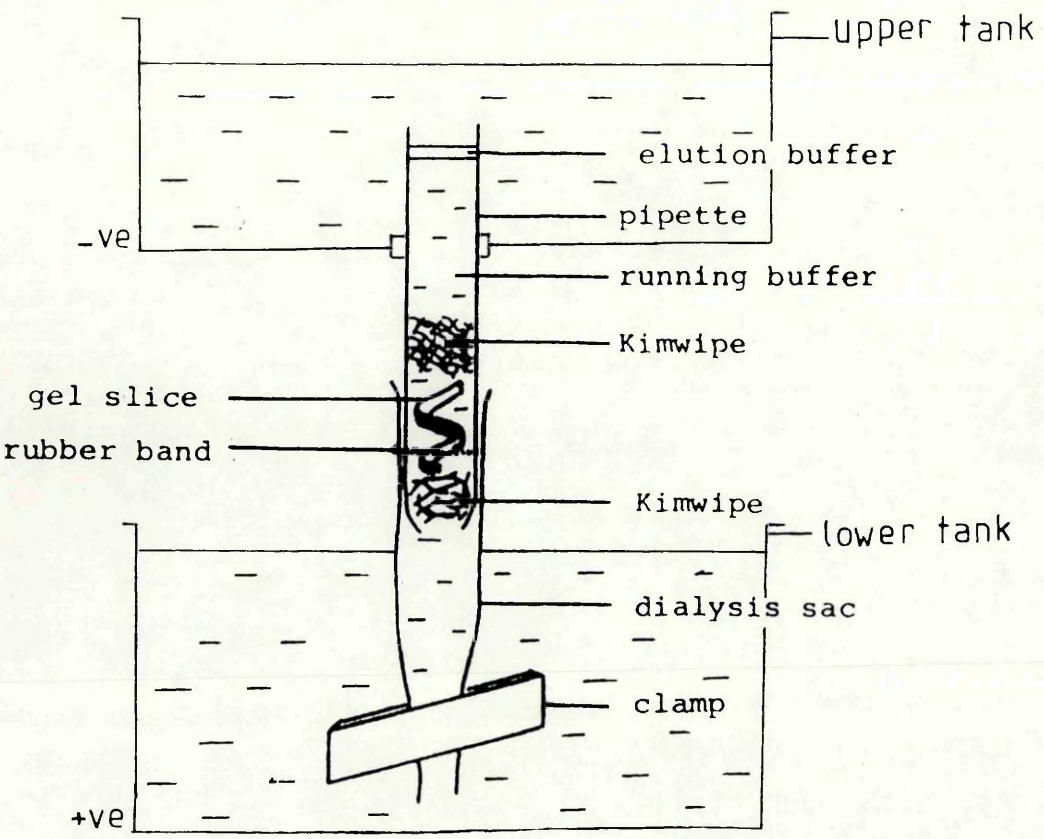


Fig. 5.16 Apparatus for electroelution of protein bands from SDS/polyacrylamide gels (see Materials and Methods).

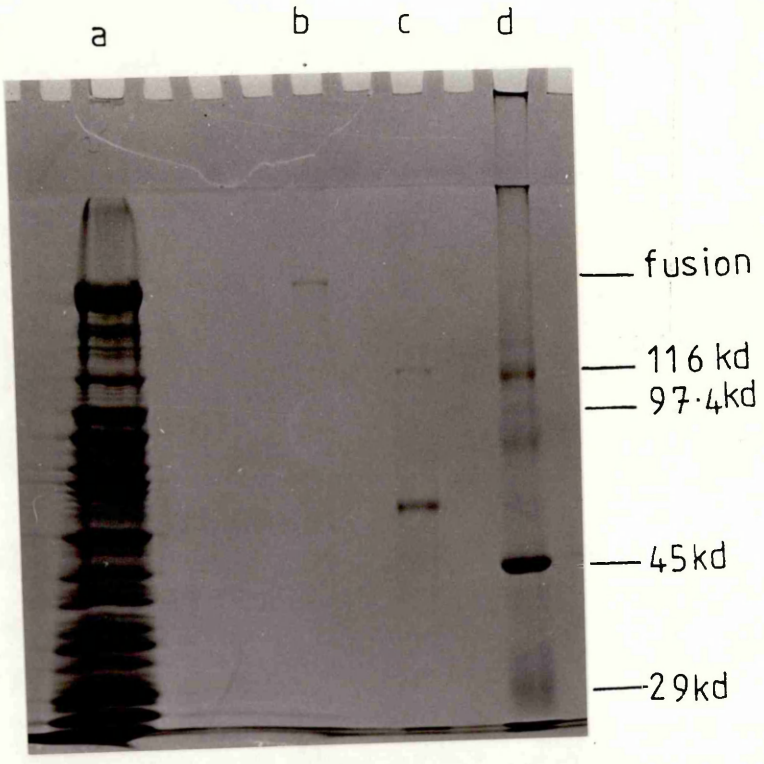


Fig 5.17 8% SDS/polyacrylamide gel showing DS942 (pROS38) tnpA lacZ fusion protein purified by electroelution.

a. Sonicate pellet
b. Purified fusion protein
c. Pure B-galactosidase
d. Markers

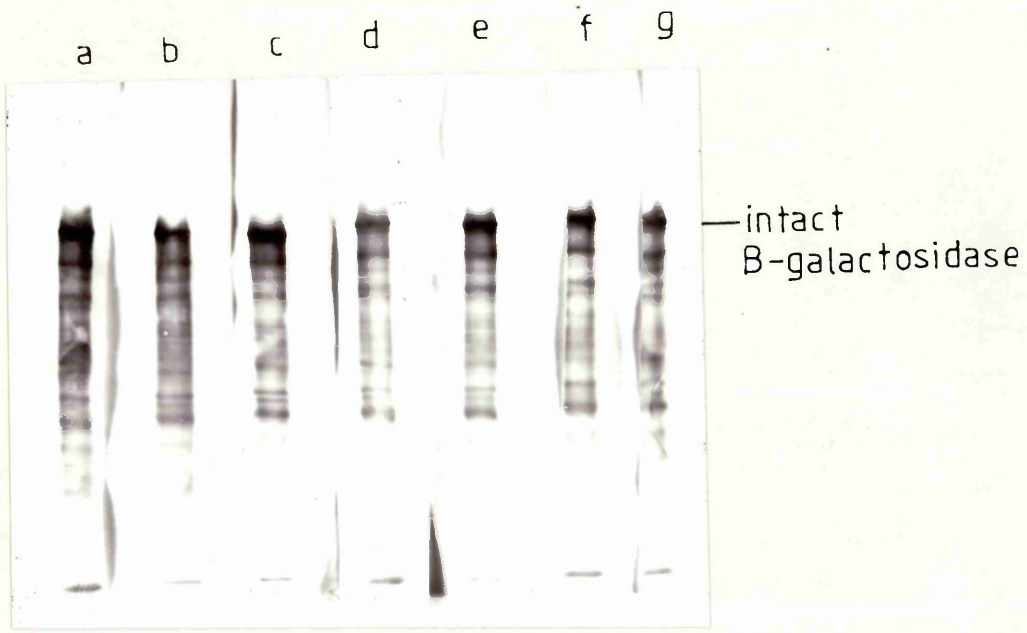


Fig. 5.18 Western blot showing calibration of the concentration of anti-B-galactosidase polyclonal antibody necessary for subsequent blots.

a. 1 in 200 dilution
b. 1 in 300 dilution
c. 1 in 400 dilution
d. 1 in 500 dilution
e. 1 in 600 dilution
f. 1 in 800 dilution
g. 1 in 1000 dilution

The gel was run at a constant current of 12mA overnight until the dye reached the bottom of the gel.

Two 1cm wide strips were cut from each vertical side of the gel. These were stained for protein and destained. Then the fragments were lined up with the rest of the gel and the fusion band position could be identified. A 1cm wide strip containing the protein was excised. The remainder of the gel was stained for protein and destained to confirm that the correct fragment had been cut out (Fig. 5.15). The protein-containing strip was cut into smaller strips and packed into an EDTA-treated tube as shown in Fig. 5.16. The tube was set up in a vertical tube gel tank, and protein running buffer was used for electroelution overnight.

The sample was collected as a 2ml volume in a dialysis bag. It was dried down in a Speed Vac, and subsequently acetone precipitated to remove SDS and salts. The white powdered protein was then resuspended in PBS. The protein from one gel, when resuspended in a volume of 100ul, gave a concentration of about 1ug per ul. Examination on a gel showed that the procedure had been successful (Fig. 5.17).

5.3 Western blots using anti-B-galactosidase antibody

The Western blotting technique was used to show unequivocally that the purified protein was the tnpA lacZ fusion:

Anti-B-galactosidase polyconal antibody and preimmune serum was obtained from Marsden (Virology, Glasgow). An initial calibration was carried out to test what concentration of the antibody was the lowest that could be used. The result is shown in Fig. 5.18.

The antibody worked very well at a dilution of 1 in 1000. This was therefore the concentration used for the experimental blot. The B-galactosidase used in this test was 10 years old, and several breakdown products were observed.

The experimental blot was run on two 8% SDS-poly acrylamide gels with 4.5% stackers. On the first 20-track gel, tracks 1 to 4, 6 and 7, were repeated three times. On the second gel, they were repeated

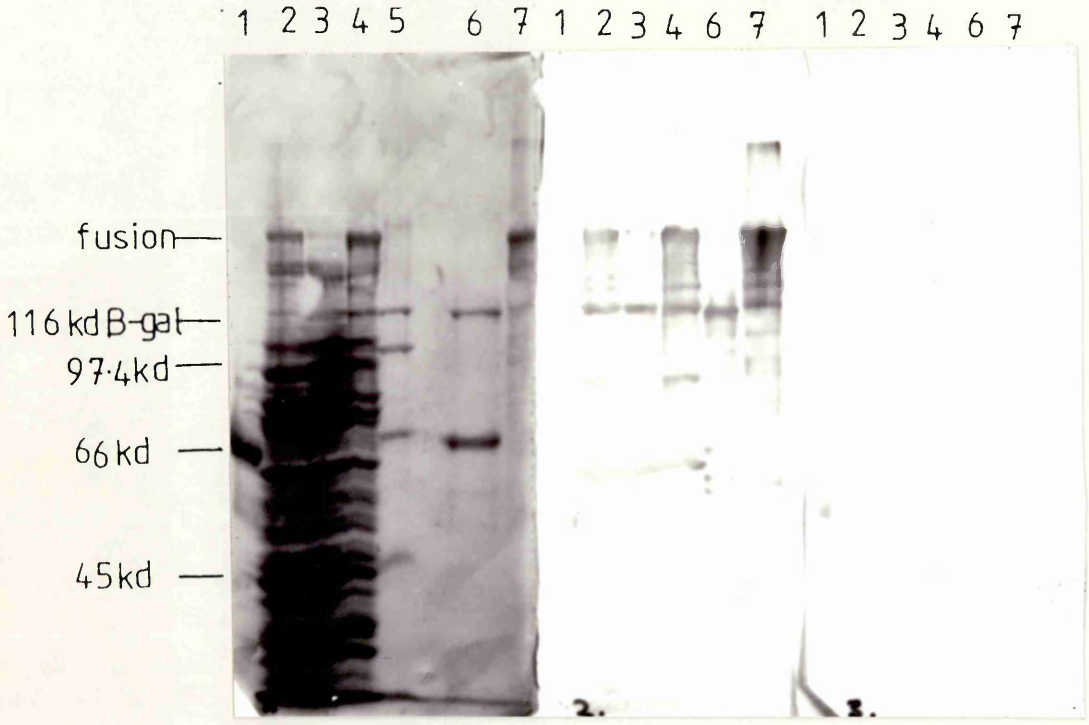


Fig. 5.19 Western blot using anti-B-galactosidase polyclonal antibodies at 1 in 1000 dilution.

Block 1: Filter stained for protein to check transfer and to act as markers.

1. 5ug rabbit serum
2. whole cells
3. sonicate supernatant
4. sonicate pellet
5. Markers
6. pure B-galactosidase
7. Purified fusion protein

Block 2: Filter treated with anti-B-galactosidase antibody (antibody 1) and with anti-rabbit IgG peroxidase conjugate (antibody 2) both at 1 in 1000 dilution before staining.

1- 4, 6, 7 as for Block 1.

Block 3: Filter treated with pre-immune serum and with antibody 2 both at 1 in 1000 dilution before staining.

1-4, 6, 7 as for Block 1.

twice. After running the gel overnight, the five blocks of tracks were treated as follows: [Antibody 1 = anti-B-galactosidase antibody, 1 in 1000 used. Antibody 2 = anti-rabbit IgG (whole molecule) peroxidase conjugate, 1 in 1000 used.]

Block 1: The nitrocellulose filter was stained for protein, to check transfer and to act as markers for blocks 2 and 3.

Block2: The filter was treated with antibody 1, then antibody 2, then with the substrate solution.

Block3: The filter was treated with preimmune serum (1 in 1000 dilution), then antibody 2, then with the substrate solution.

Block 4: The filter was stained for protein to check transfer, and to act as markers for block 5.

Block5: The filter was treated with antibody 2, then with the substrate solution.

The results of blocks 1, 2 and 3 are shown in Fig. 5.19.

Blocks 1 and 4 showed that transfer had worked well. The blotted gel was stained for protein and destained. This showed that transfer of small and intermediate-sized bands had been complete, but a few very high molecular weight bands (including the fusion, the B and B' RNA polymerase subunits, and myosin) had not completely transferred.

In block 3 one major band was apparent in track 1. This was due to the anti-rabbit antibody (antibody 2) binding IgG in the rabbit serum. Fainter bands lit up in tracks 2, 3 and 4, suggesting that the rabbit from which the serum was taken had had antibodies cross-reacting with some *E. coli* proteins before immunization. This faint banding was also obvious in block 2. Block 5 showed one band only, in track 1. Again this was antibody 2 reacting with the rabbit serum.

Block 2 showed one band in track 1, as in blocks 3 and 5. Tracks 2, 3 and 4 consisted of the same volume of whole cells containing pROS38, sonication supernatant, and sonication pellet respectively. In all

three, and track 7, intact fusion protein was apparent, although in track 3 the amount was at least five times less than in track 4. This showed that the fusion was mainly in the 5% SDS-soluble pellet. Several bands could be seen in track 6, the largest of which was intact B-galactosidase. The others were breakdown products (Fig. 5.18) due to the age of the protein stock (10 years). The B-galactosidase band was also clearly visible in tracks 2, 3, 4 and 7. This confirmed the affinity column results that the fusion protein has a weak hinge just 5' to B-galactosidase. That this can be the site of mechanical cleavage is shown by track 7.

The conclusion from the blots was that the protein purified by excision from a gel was indeed the B-galactosidase fusion. This protein was used to immunize rabbits.

5.4 Immunization of New Zealand White rabbits with the Tn*1* *tnpA*-*lacZ* fusion protein

The source of information on immunization procedures was Hurn and Chantler (1980).

Two New Zealand White rabbits, numbers 214 and 215, were obtained. Ideally, preimmune serum should be taken before immunization, but here this was not done. They were injected intramuscularly into both hind quarters with a total per rabbit of approximately 175ug of fusion protein, resuspended in 300ul of PBS, mixed with an equal volume of Freunds Complete Adjuvant. Seven weeks later the rabbits were given a booster of approximately 25ug each, in PBS mixed with Freunds Incomplete Adjuvant. This time the injection was sub-cutaneous. 13 days later, 5ml of blood was taken from each rabbit. This was kept at 4°C overnight to allow clotting to reach completion, before removing the serum and freezing it at -20°C. One week after this, 50ml of blood was taken from each rabbit, allowed to clot, and the serum was frozen at -20°C in 5ml aliquots.

Further booster injections are advisable to increase the titre and avidity of any antibodies. However, the first blood samples should give information on whether the immunization is working or not.

5.5 A preliminary check on the rabbit antiserum

The strains DS947 (pROS35) and DS947 (pROS21) were constructed. pROS35 (Fig. 4.17) carries the Tn*1* *tnpA* gene, and was to be a positive control for antibodies to Tn*1* transposase. pROS21 (Fig. 3.16b) carries the Tn *3* *tnpA* gene, and this was to be a positive control for antibodies to Tn*3* transposase. DS942 (pUC9*lacZ*) (4.2.4) was to be a positive control for antibodies to B-galactosidase, and purified fusion was to be a positive control for antibodies to transposase or B-galactosidase. DS947 alone was to be a negative control.

A Western blot was carried out using the first blood samples from 214 and 215: a 20-track 8% SDS-polyacrylamide gel was poured. Tracks 1 to 10 were for 214 and tracks 11 to 20 were for 215. After running, the gel was blotted overnight. The following day, tracks 7 and 17 were stained for protein to check transfer and to provide markers. Tracks 1, 2, 8, 9, 10, 11, 12, 18, 19,and 20 were probed with the first antibody (1 in 100), then with the second antibody (1 in 1000) before being treated with substrate. Tracks 3, 4, 13, and 14 were probed with the first antibody (1 in 500), then with the second antibody, before being treated with substrate. Tracks 5, 6, 15, and 16 were probed with the first antibody (1 in 1000), then with the second antibody, before being treated with substrate. The results are shown in Fig. 5.20.

Unfortunately, the first antibody was too dilute at 1 in 100 to show any specific binding. Quite a high background was apparent at this dilution. Probing at a higher concentration would be expected to increase this, and therefore might mask any specific reaction. However, one final blot was attempted.

The first antibody was diluted 0 times, 1 in 4, and 1 in 20 to give some indication of whether an antibody to the fusion was present; unfortunately, the peroxidase activity of the second antibody had been destroyed by repeated freezing and thawing, and nothing lit up on the blots during incubation with the substrate. The filters were washed and reprobed with a new batch of second antibody, and a colour did develop, but this was all over the blot due to substrate being still present on the surface. Due to lack of time, the blot was not repeated.

More work needs to be carried out to optimize conditions so that any specific reaction can be seen. Firstly, more purified protein must be made. This is required for further booster injections, and for blots as a positive control. Secondly, the rabbit serum should be absorbed before use with an *E. coli* extract containing no transposase or B-galactosidase protein. This should reduce the background in the blots. Thirdly, the first antibody should be incubated with the filters in the presence of a non-rabbit protein to minimize non-specific, protein:protein interactions. This should also reduce the background.

Discussion

This chapter has been concerned with the attempted overproduction of Tn*1* transposase, and the successful purification of a Tn*1* transposase:B-galactosidase fusion protein. An attempt to raise antibodies to the latter has also been described.

Purification of Tn*1/3* transposase is the logical step, after *in vivo* studies, in attempting to understand the mechanism of action of the protein. It is reasonable to expect that *in vitro* studies would give a wealth of information on the transposition mechanism of Tn*1/3*, as has been the case with Mu: based on *in vitro* studies using Mu transposase, a very detailed model of the process of Mu transposition and its requirements now exists (Surette *et al.* 1987).

An antibody to transposase would be useful, amongst other things (see Ch. 5 introduction and Ch. 6), for monitoring transposase purification, and for studying breakdown of the protein *in vivo* under various temperature conditions. This would hopefully show whether breakdown occurs to a greater extent in cells grown at 42°C or 37°C than at 30°C, and thus might explain the temperature effect on transposition (Ch. 4). Obviously, the success of this approach would depend on the ability of the polyclonal antibodies to recognise degraded fragments of transposase, and also would require that mechanical cleavage of the protein did not occur during preparation of the samples for gel electrophoresis.

Recently, Nakayama et al. (1987) have used purified Mu transposase in conjunction with antibodies specific to various regions of the protein, to study structural domains in the protein. This has enabled identification of three domains, two of which bind DNA, with the N-terminal one being specific for Mu ends. Such studies on Tn1/3 would back up in vivo experiments which have shown that for the transposons Tn501 and Tn21 the endbinding specificity determinant lies within the N-terminal region between amino acids 28 and 216 (Evans and Brown 1987), and also might show how many extra domains are encoded in the greater length of the Tn3 family transposases (1066aa, compared to 662aa in Mu).

This chapter has illustrated the fact that properties of B-galactosidase gene fusions are variable, and unpredictable: it might be concluded that the lack of solubility of the Tn1 tnpA-lacZ fusion product used here was due to the insolubility of the transposase part, which would explain why Ullman (1984) did not have the same problem using various other fusions. However, the three published procedures for the purification of Tn3 transposase vary in their accounts of transposase solubility. According to Wishart et al. (1985), transposase was extracted from the membrane fraction, but no details of the buffers used were given. The most recent report by Morita et al. (1987), found that 70% to 80% of the transposase (which was 4% of the cell protein) was found in the sonicate supernatant. Apparently the solubility of transposase depends on the buffer used for extraction, and also the concentration in the cell. According to Zabeau and Stanley (1982), the solubility of B-galactosidase fusions depends on their structure, not on the cellular concentration. Overall therefore, it is probably a feature of the fusion size and structure that caused over 90% of the fusion studied here to be extractable only in 5% SDS.

The ineffectiveness of affinity chromatography in purifying the fusion due to cleavage of the protein, mechanical or otherwise, has not been reported in the literature (Steers et al. 1971; Germino et al. 1983; Ullman 1984; Silhavy and Beckwith 1985). This is surprising because both pROS38 and pGRO577-encoded fusions were cleaved just at the fusion joint, possibly within the pMC1871 polylinker region. Perhaps other workers treated their proteins more gently, although using

lysozyme rather than sonication to break the cells had just the same result (data not shown) and Ullman (1984) reported that the fusions could be stored at -20°C for several weeks without loss of activity (although this does not necessarily imply that no breakdown occurred). The greater affinity of the column for B-galactsidase rather than the fusion proteins was not surprising, although it was an added inconvenience in trying to purify these proteins.

Overall, electroelution of the fusion protein proved to be the simplest method for purification. This was due to the large size of the fusion causing separation of this band from other E. coli proteins on SDS gels. A significant amount of protein could be prepared quickly in this manner. Another, supposedly similarly efficient, purification method is that of Shumann et al. (1980), which involves sizing columns that can be run in the presence of detergent.

The raising of antibodies to lacZ fusions should not be a problem (Silhavy and Beckwith 1985). If the protein is excised from a gel, of course, those antibodies raised will be to the denatured protein. In cases where the antibody is required for immunoblots of SDS-polyacrylamide gels this is not a problem, indeed it is a benefit. The presence of B-galactosidase antibodies in serum from immunized animals acts as a control for the success of the procedure, although it is possible that the protein fused to B-galactosidase might only provoke a very weak response, due to an inherent lack of immunogenicity. Unfortunately, the data here do not show whether a response to either transposase or B-galactosidase has been provoked in 214 or 215. More work is necessary to optimize the blotting sensitivity before this can be determined. Frustratingly, this could not be pursued due to lack of time.

CHAPTER 6

CONCLUDING REMARKS

Today the therapeutic use of antibiotics in human and veterinary science, and subtherapeutic use in animal feeds provides a tremendous selective pressure for the spread and maintainance of bacterial transposable elements such as the Tn3 family, which carry antibiotic resistance genes.

Considering the present day wide host range of the Tn3 family (1.2.2), the probable common ancestry of these elements, and economy of use of genetic information, it is obvious that these transposons are very successful in transposition. Reasons for this success probably include: their plasmid location, which can lead to their rapid dispersion across species boundaries; their replicative mechanism of transposition (1.3), which increases their copy number within a cell so that simultaneous deletion of all copies is unlikely to occur; and their various regulatory mechanisms, which are involved in keeping transposition levels low enough to ensure that the host survives. This thesis mainly concerns the regulation of Tn1/3 transposition and the tnpA gene, which encodes the transposase.

The primary method of regulation of Tn1/3 transposition is that by the resolvase protein, which binds at sub-sites I, II and III of the res site and consequently represses transcription of both tnpA and tnpR. Data obtained in chapter 4 while using lacZ translational fusions to both of these genes to study the effect of temperature on transposition, gave information on the approximate amounts of transposase and resolvase fusion proteins present in cells in the repressed and derepressed states. Apparently, at 30°C resolvase represses its own synthesis about 10 times and that of transposase about 7 times. This observation ties in with the known fact that resolvase binds more tightly to sub-site II than sub-site I. Approximate calculations (4.2.6) show the number of transposase fusion molecules present per cell at 30°C in the derepressed state is about 140 (Table 4.11) - 280 (Table 4.15). Repression by resolvase decreases this to about 20. Similarly, the number of resolvase fusion molecules present per cell at 30°C in the derepressed state is about 530, which repression decreases to about 60. Therefore in the repressed condition at 30°C, each cell contains about three times as much resolvase fusion protein as transposase fusion protein, and contains both in very low amounts. If the number of transposase

fusion molecules is a true indication of what the transposase levels would be, then it suggests that these low levels are responsible for the observed low transposition frequency. Data on repression at 37°C were not available due to the instability of the pUC fusion constructs in the presence of another plasmid.

The decrease in transposase levels due to repression by resolvase is 7-fold, and yet the decrease in transposition due to repression is 10 - 50 times (Table 3.1). This suggests that in the normal repressed condition, the amount of transposase present per cell is too low in most of the cells to bring about any transposition at all. But a 7-fold increase may be sufficient to raise the levels of transposase per cell high enough to ensure some transposition in most of them. This observation has implications for the study of the effect of temperature on transposition as described in chapter 4: it is obvious that in order to explain the difference in transposition frequencies at 30°C compared to 42°C of at least a 100-fold, a 100-fold difference in levels of the transposase protein need not be required; a 5 to 10-fold difference may be all that is necessary to give the observed effect. However, even this was not observed, showing that the synthesis of transposase does not appear to be temperature sensitive in a way that could explain the observed transposition frequency.

It is possible that transposition has evolved to occur less frequently at 37°C than at 30°C because the former is almost the normal body temperature of organisms harbouring the host *E. coli* cells, and a high level of transposition, and therefore mutation (1.4), might harm the *E. coli* cells and the host organism. However, it is equally possible that temperature sensitivity is a necessary out-come of either the structure of transposase, or of some host protein involved in the transposition process, and it might be due to the activity or breakdown of either of these.

From the data in chapter 3, it can be concluded that in addition to their role in the transposition process as binding sites for transposase and possibly for host proteins (IHF is known to bind at the ends of IS1, Gamas *et al.* 1987; and Hu is known to be involved in Mu transposition, Craigie *et al.* 1985), the IRs of Tn3 may play a (probably fortuitous) part in limiting transposition in two ways:

firstly, in the presence of a limited amount of transposase a large number of ends may titrate out the available protein thus lowering the probability of any one element transposing; and secondly, the presence of transposase bound to IRs of an element in a genome may prevent a productive interaction of another element with that genome. The competition of IRs for limited transposase and immunity both lead to a lowering of the transposition frequency within a cell, and immunity favours transposition of elements to as many individual replicons as possible.

In order to define the topology of the DNA substrates, intermediates and products in the Tn3 transposition process, it would be useful to have an *in vitro* system. Such a system already exists for Mu transposition (Craigie *et al.* 1985; Surette *et al.* 1987), and for the Tn3 cointegrate resolution reaction (Symington 1982). To construct such a system, a primary requirement is purification of the transposase protein. This has now been achieved by three sets of workers (Fennewald *et al.* 1981; Wishart *et al.* 1985; Morita *et al.* 1987). However, an attempt in Glasgow to overproduce the protein to the expected degree by placing it under the *lac* promoter and translation signals failed (Hettle 1985). Although the reason for this was investigated (Ch.5), and it was shown that the expression of a *lactnpAlacZ* fusion seemed to depend on the length of the *tnpA* fragment present, the explanation for this effect was not apparent.

In addition to its use in an *in vitro* system, purified transposase could be used in DNA footprinting on wild-type and altered IRs. This should show the exact sequences protected by transposase, and thus whether the protein binds to one face of the DNA or wraps around it. Potentially, binding of transposase could alter the conformation of the DNA at the IRs leading to bending, or exposure of the sequences at the end of the transposon so that nicking can be facilitated. If bending occurs this must be incorporated into mechanistic models for the transposition reaction, provided it can also be corroborated by *in vivo* data.

One important feature of the ends of elements which generate a 5bp duplication at the site of insertion such as Mu, D108, Tn3, Tn1000, Tn551 and Tn501 is that they have at least one copy of the

heptanucleotide sequence 5' ACGAAAA 3' near both ends of the element, typically located between 12 and 21bp from the ends of these elements. This suggests an evolutionary relationship between these transposons with respect to the DNA sequences present at the ends of the elements which their transposase proteins may recognise and bind. The purified protein could be used in footprinting to the ends of these transposons, because although they do not all complement each other for the transposition reaction, the transposases may be able to bind these related ends. If they bind but fail to orient the DNA correctly for subsequent IR complex formation, nicking, and binding to recipient DNA, information may be obtained on the structure of the protein:DNA complexes required for transposition to occur.

Purified transposase could also be treated with proteolytic enzymes and the breakdown products analysed with antibodies to various parts of the protein. This could assist in identifying those domains of transposase responsible for the end-binding specificity, non-specific DNA binding, and nicking. This approach has been successful for the MU A protein (Nakayama et al. 1987).

Ultimately, the purified protein could be analysed by X-ray crystallography to determine the structure of its active site.

Many uses also exist for transposase polyclonal antibodies: they could assist in monitoring purification of transposase on SDS-polyacrylamide gels, or could be linked to a sepharose column to aid purification; they could be of use in examining the extent of breakdown of transposase from cells grown at 30°C and 42°C, and to identify protease-cleaved fragments; they could be used to localise transposase within cells in electron-microscopic studies of the transposition process.

Purified Drosophila transposase:B-galactosidase proteins have been employed to generate antigens to different regions of the transposase protein (Rio et al. 1986) and these were used in immuno-precipitation studies to see what ^{35}S-methionine-labelled proteins were precipitated by the antibody. They found two major Drosophila heat-shock proteins coprecipitated with the transposase, presumably fortuitously trapped in the complex, and also another smaller protein. This was possibly a

host protein involved in the transposition reaction, or could have been a stable breakdown product of the actual transposase. This approach for Tn*3* using transposase:B-galactosidase antibodies might show which, if any, host proteins are involved in the transposition complex. Similar precipitation studies could be performed using labelled DNA containing the IRs of Tn*3* and other elements to look for specific DNA binding and cross-reactivity. Transposase:B-galactosidase fusion antibodies to different parts of the protein might enable studies on the inter-relatedness of the domains of transposases from various sources.

It is obvious that there is no shortage of questions to be answered once the transposase protein has been purified and antibodies prepared. The data in chapters 4 and 5 showed that the best yield of transposase will probably come from a ribosome binding site mutant such as those used by Fennewald *et al.* (1981) and Morita *et al.* (1987), because transposase transcripts are translated at about 24 times lower efficiency than the resolvase transcripts (4.2.6), which implies the ribosome binding site is inefficient. These mutants in conjunction with the protocol of Morita *et al.* should provide a supply of purified protein sufficient for the experiments described above. Raising antibodies to this or to fusions should also be straightforward, provided enough time is available to persist with the inoculation and monitoring in order to obtain the maximum titre and avidity.

The future prospects for a detailed understanding of the Tn*3* transposition process, including cointegrate resolution, are good. Comparisons of the process with that of Mu (Craigie *et al.* 1985) may elucidate the evolutionary relationship between the Tn*3* family and the transposing bacteriophages.

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