# Mutants of Tn*3* resolvase

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This thesis is dedicated with all my love to my family, Mum and Dad, Gran and Aunt Lizzie, Dawn Graeme, Ryan and Connor

## Chapter 4: Mutagenesis of Tn3 resolvase



## Chapter 5: Development of a new expression and purification system for Tn*3* resolvase



## Chapter 6: *In vitro* analysis of altered Tn*3* resolvases

## Chapter 7: Discussion

# Abbreviations.

## Units

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| k | - | $10^{3}$ | m | - | $10^{-3}$ | μ | - | $10^{-6}$ |
| n | - | $10^{-9}$ | p | - | $10^{-12}$ | f | - | $10^{-15}$ |

| | | | | | |
|---|---|---|---|---|---|
| bp | - | base pairs | kb | - | kilo base pairs |
| A | - | Ampères | V | - | Volts |
| W | - | Watts | Ci | - | Curies |
| °C | - | degrees Centigrade | g | - | grammes |
| l | - | litres | m | - | metres |
| M | - | molar | mol | - | moles |
| h | - | hours | min | - | minutes |
| sec | - | seconds | rpm | - | revolutions per minute |
| cps | - | counts per second | cpm | - | counts per minute |

## Chemicals/Reagents.

| | | |
|---|---|---|
| APS | - | ammonium persulphate |
| ATP | - | adenosine triphosphate |
| DNase I | - | deoxyribonuclease I |
| dNTP | - | deoxynucleoside triphosphate |
| DTT | - | dithiothreitol |
| EDTA | - | ethylenediaminetetraacetic acid (disodium salt) |
| EtBr | - | ethidium bromide |
| EtOH | - | ethanol |
| IPTG | - | isopropyl β-D-thiogalactopyranoside |
| RHB | - | resolvase dilution buffer |
| SDS | - | sodium dodecyl sulphate |
| TMED | - | *N,N,N',N'*-tetramethylethylenediamine |
| Tris | - | tris-(hydroxymethyl)aminoethane |

## Other Terms

| | | | | | |
|---|---|---|---|---|---|
| $X^{r}$ | - | resistance to X. | $X^{s}$ | - | sensitivity to X. |
| *ori* | - | origin of replication. | FIS | - | factor for inversion stimulation. |

PAGE - polyacrylamide gel electrophoresis.

| | | | |
|---|---|---|---|
| Ap | - ampicillin | Km | - kanamycin |
| Tc | - tetracycline | Cm | - chloramphenicol |

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## Summary

Prior to this work, no mutants of Tn*3* resolvase had been isolated. To construct a variety of mutants, a suitable plasmid for mutagenesis purposes was created. This plasmid, pAT5, contains unique restriction sites along two thirds of the length of the resolvase open reading frame. This allows double stranded oligonucleotides containing mutations to be cloned into the resolvase gene. pAT5 was also designed to be compatible with the *galK* screen used to investigate the *in vivo* properties of the mutants. The *in vivo* assay was based on change of colony colour on MacConkey agar plates when the cells were unable to utilise galactose, due to the deletion of a plasmid-encoded *galK* gene by resolvase-catalysed recombination.

Two specific mutants of Tn*3* resolvase were made, Y6F and E124Q. Random mutagenesis of the E-helix of resolvase created a library of mutants, which was screened *in vivo* for ability to resolve test plasmids containing either two *res* sites, or two isolated copies of binding site I of *res* (the crossover site) or one site I and one *res*. The mutant resolvase D102Y was isolated, which promoted the reaction between *res* v site I *in vivo*.

In order to purify these and any subsequent mutants, a new purification procedure was developed. This is a denaturing protocol which has been shown to be reproducible for wild-type resolvase and the mutants mentioned above. A new expression plasmid, using a promoter recognised by the phage T7 RNA polymerase was created, resulting in higher yields of resolvase per gram of cells.

*In vitro* analysis of Y6F resolvase showed that the protein was recombinationally inactive on a wild type substrate, but retained binding ability as well as cleavage and religation activities. This supports the theory that resolvase linkage to the DNA during recombination is by a phosphoseryl, and not a phosphotyrosyl linkage as in topoisomerases.

L69F mutant resolvase, inadvertently created during the construction of the mutant selection system, retains recombination activity, but displays topoisomerase activity and aberrations in binding.

The Tn*3* resolvase mutant E124Q was observed to display enhanced cleavage activity, but unlike the γδ E124Q resolvase mutant does not show the ability to resolve substrates containing partial *res* sites.

The mutant isolated using the *in vivo* screen, D102Y resolvase, was shown to display the same phenotype *in vitro* as *in vivo,* and catalysed recombination between *res* and site I. This protein also catalyses intermolecular reactions, and may be described as having a "hyperactive" phenotype. The isolation of a mutant resolvase with this phenotype is a major step towards the isolation of a mutant able to catalyse a recombination reaction between two site I's.

# Chapter 1

Introduction

# Introduction

## 1.1 Features of site-specific recombination

Site specific recombination causes DNA rearrangements in a precise and controlled manner by the breakage and rejoining of the DNA at specific sites. The recombination reactions have no requirement for extensive homology between the DNA segments involved, unlike general recombination, but are instead characterised by the highly specific nature of the DNA sites and their interactions. Some of the essential *in vivo* functions of this method of recombination include the control of gene expression, resolution of intermediates of transposition, the regulation of plasmid copy number, and the integration and excision of bacteriophage DNA from the host chromosome (Matthews, 1992; Stark *et al.*, 1992; Johnson, 1991).

The strand exchange reaction is catalysed by the recombinase protein, specific to the recombination system, although other accessory proteins may be involved. The recombinase binds to the recombination site, and subsequently mediates strand cleavage, exchange and religation, such that there is no loss or synthesis of DNA. The outcome of this event is dependent on the positioning and orientation of the sites involved. In prokaryotic systems, where recombination usually occurs on circular plasmid substrates, an intermolecular reaction (between sites on different DNA molecules) results in the fusion of two molecules, while intramolecular reactions (between sites on the same molecule) can have one of two outcomes (see Figure 1.1). Since recombination between identical sites results in linking of the right half of one site to the left half of the second site, thus preserving the sequence of the sites, recombination sites which exist in inverted repeat result in the inversion of the DNA sequence with respect to the rest of the DNA molecule, while sites in direct repeat result in the resolution of the plasmid DNA.

Most of the known site specific recombination systems can be divided into two families based on sequence homology of the recombinase; the resolvase/invertase family (exemplified by Tn*3* resolvase), and the integrase family (exemplified by the bacteriophage λ integrase). Members of these families also share similarities in their recombination sites and reaction mechanisms.

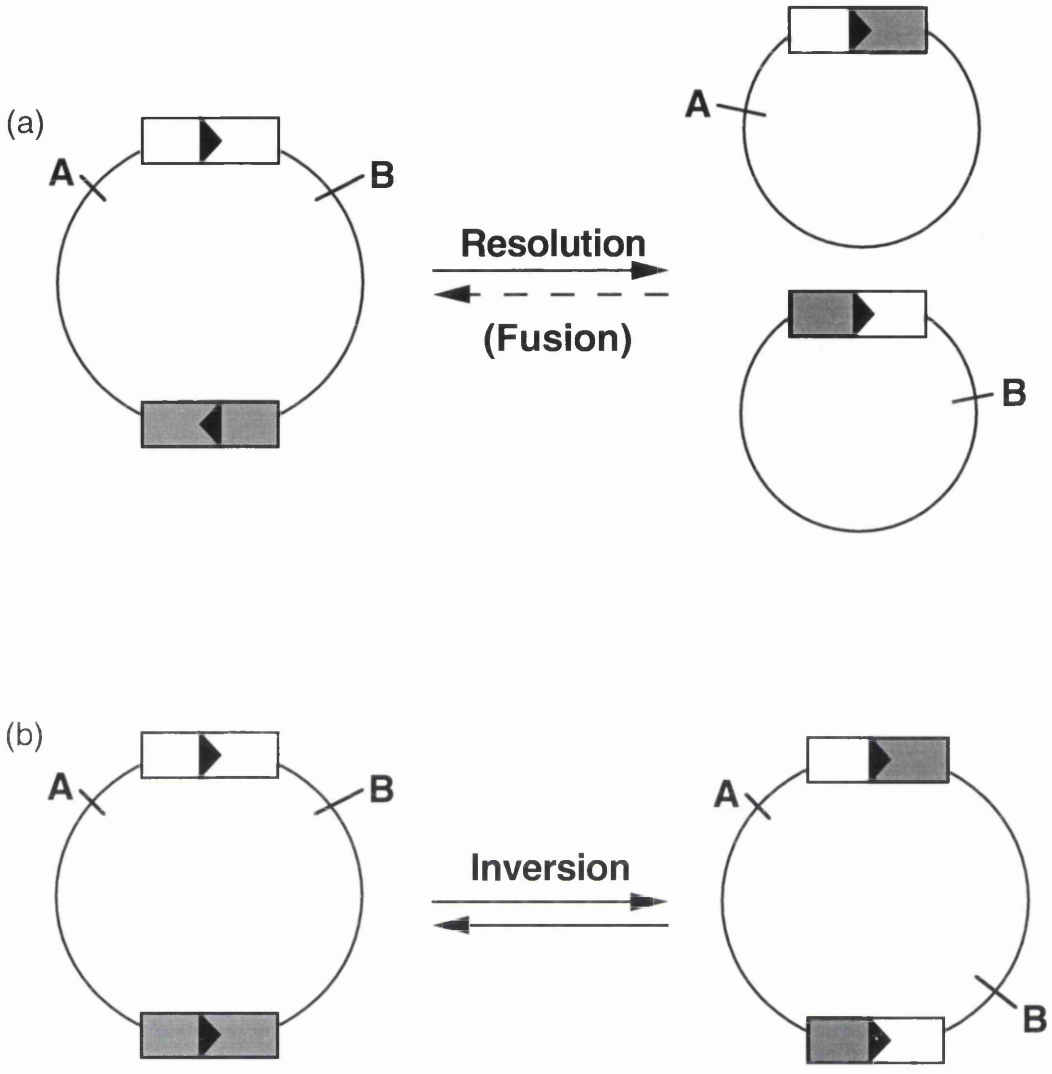



**Figure 1.1. Diagram illustrating resolution and inversion.**

(a) Resolution of a circular substrate containing two directly repeated sites. The reverse reaction is fusion.

(b) Inversion of a circular substrate containing two inverted repeat sites.

Recombination sites are indicated by blank or shaded boxes, with arrow heads to show their orientation, defined by sequence asymmetry. A and B are sequence markers, e.g. restriction endonuclease recognition sites. Figure adapted from Blake, 1993.

## 1.2 The integrase family

Recombination by the integrase family of recombinases is characterised by a two step reaction in which each step involves the cutting and rejoining of two strands of DNA. In the first step, one strand of each DNA double helix in the two recombining sites is nicked, and strand exchange occurs producing a crossed strand Holliday intermediate (equivalent to the Holliday structure of general recombination). The second step in the reaction involves the resolution of the Holliday intermediate by exchange of the second pair of strands (see Figure 1.2).

Members of the integrase family of recombinases are diverse, with molecular weights ranging from 20-65 kDa, and low sequence conservation. The protein-DNA linkage during strand transfer is via a phosphotyrosine bond. This differs from resolvase family recombinases, which become linked to the DNA via a phosphoserine bond.

Several Int-like systems have been studied extensively *in vitro*; particularly λ Int, *lox*/Cre, Xer and FLP (A. Landy, 1989; Hamilton and Abremski, 1984; Sherratt *et al.*, 1993; Cox, 1989).

## 1.3 The resolvase/invertase family

As well as Tn*3* resolvase, this family includes recombinases encoded by many other transposons, such as γδ (also known as Tn*1000* ) and Tn*21*. Tn*3* and γδ resolvases are very similar in sequence, and as such features of the Tn*3* system are generally applicable to the γδ system and *vice versa*. The DNA invertases, such as the phage Mu encoded Gin, while on the surface very different to the resolvase family, also contain many features in common with them which will be discussed throughout this Chapter. Other related recombinases have functions in bacteriophage integration, plasmid monomerisation, and programmed developmental genetic rearrangements.

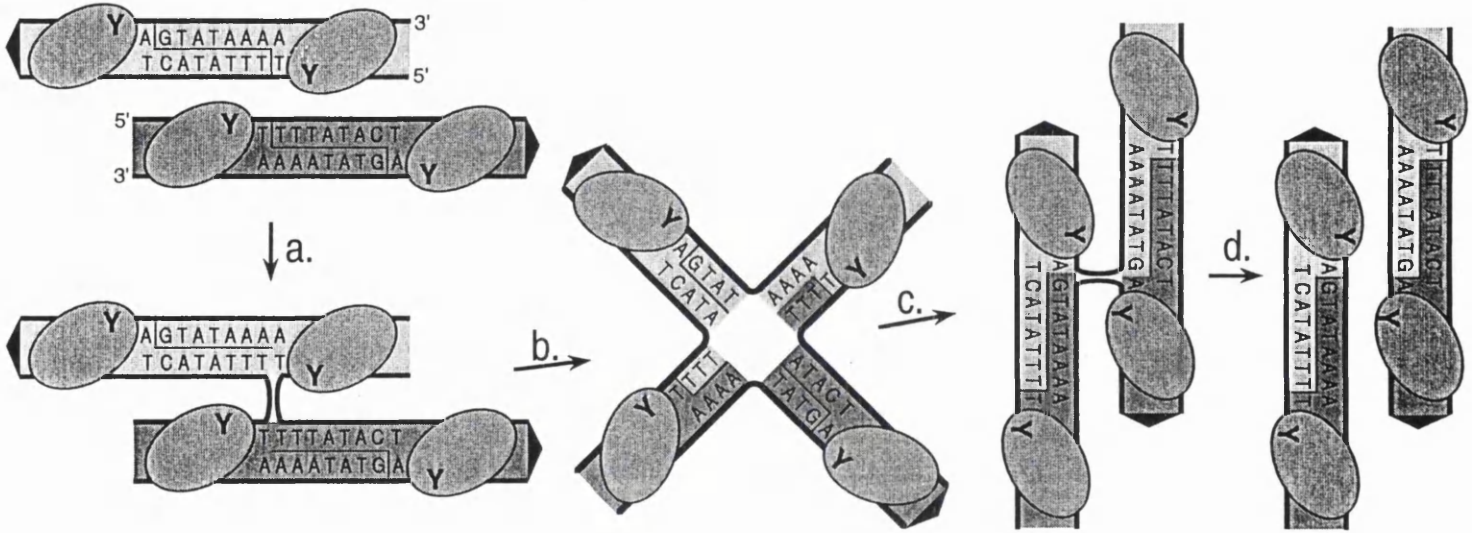



**Figure 1.2 Integrase family strand exchange**

DNA is shown as a flat ribbon, and recombinase as a flat oval. Y is the recombinase's nucleophilic tyrosine residue. The first pair of strand exchanges forms a Holliday junction which is subsequently resolved by branch migration and isomerisation of the Holliday junction. Figure adapted from Stark *et al.*, 1992.

## 1.4 Tn*3* resolvase

Transposition of Tn*3* is a two step process mediated by two distinct proteins. The first of these, transposase, is the product of the *tnpA* gene. Transposase mediates intermolecular replicative transposition, which results in the fusion of donor and target molecules, and the replication of the transposon. Resolvase, the product of the *tnpR* gene, then directs recombination between specific sites, *res*, on each transposon in the intermediate "cointegrate" molecule. This results in discrete donor and recipient molecules, each retaining a copy of the transposon (Figure 1.3a). Subsequent to resolution, the donor and recipient molecules, which exist as a singly linked catenane, are separated *in vivo* by the action of a type II topoisomerase (e.g. DNA gyrase). Tn*3* is 4957 bp long, and as well as the *tnpA* and *tnpR* genes, the transposon confers β-lactam resistance to the host *E. coli* cell by its *Bla* gene (Figure 1.3a). Tn*3* is one of a group of related ampicillin resistance encoding transposons which exist in enteric bacteria. These retain 90% sequence similarity and a high level of functional similarity (reviewed in Sherratt, 1989).

As originally shown by DNase I footprinting, the region of γδ transposon required for recombination (*res*) contains three binding sites for resolvase (Grindley *et al.*,1982), each of which binds a dimer of the protein (reviewed in Hatfull and Grindley, 1988; see Figure 1.3b). In Tn*3*, the very similar *res* site is 114 bp in length and comprises three binding sites of unequal length and uneven spacing, but which retain sequence similarity and imperfect dyad symmetry. Each binding site contains a pair of 9 bp inverted repeats which span a central spacer of 10 bp in site I, 16 bp in site II and 7 bp in site III. The precise spacing between the centre of site I and II (53 bp) is essential for efficient recombination. The insertion of a non-integral number of helical turns in the 22 bp spacer sequence between sites I and II results in very poor recombination, while the addition of 1 or 2 integral turns has less effect on the recombinational activity (Hatfull and Grindley, 1988).

Each site within *res* binds a dimer of resolvase. One noted difference between Tn*3* and γδ resolvases is that, while Tn*3* resolvase binds first as a monomer and dimerises on binding to the *res* site, γδ resolvase has been shown to bind mainly as a dimer in gel shift

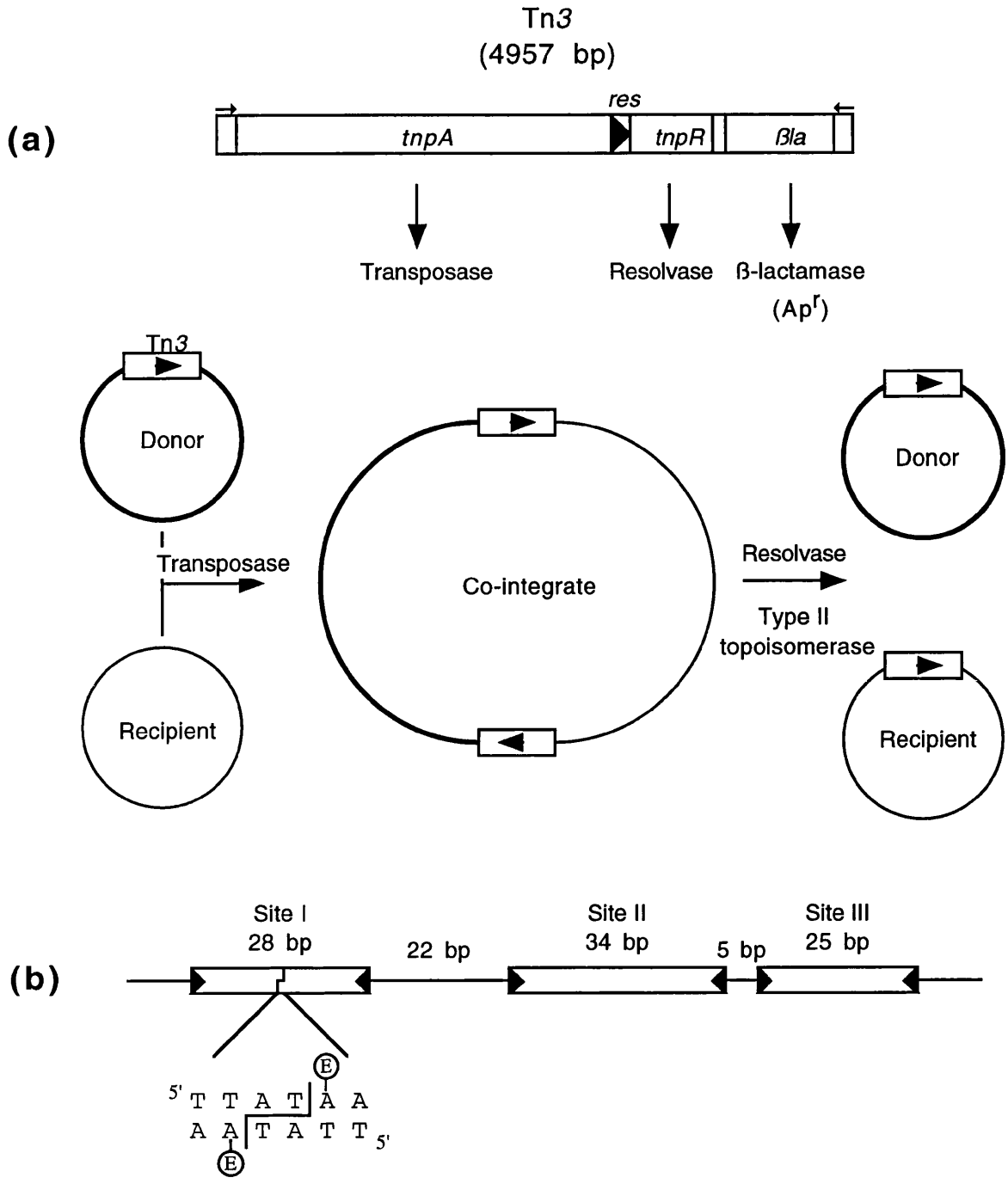



**Figure 1.3 Transposon Tn*3* and *res*.**

(a) Structure of Tn*3*. Terminal inverted repeats are denoted by small arrows. Genes and corresponding gene products are indicated. Replicative transposition of Tn*3* is illustrated. The relative orientations of the Tn*3* elements are indicated by arrowheads. The transposition step requires DNA polymerase for replication of Tn*3*, and the resolution step requires DNA gyrase to decatenate the products of co-integrate resolution.

(b) Structure of Tn*3* *res*. Sites I, II and III are indicated by boxes, and the position of DNA cleavage by resolvase is also shown. The imperfect inverted repeat sequences at the outer arms of each site are represented by arrowheads. The positions at which resolvase becomes covalently linked to the DNA are indicated. Figure adapted from Blake, 1993

assays (Blake *et al.*, 1995). Resolvase binds co-operatively, with the strongest interactions at the outer ends of the consensus sequence of each binding site in the major groove of the DNA, made by the helix-turn-helix binding motif (Yang and Steitz, 1995).

Resolvase has been shown to induce a 2 bp staggered cleavage at the centre of site I within a region of perfect dyad symmetry, the central AT dinucleotide of the sequence TTATAA (Reed and Grindley, 1981; McIlwraith *et al.*, 1995). Cleavage results in 2 bp 3' extensions around which strand exchange and religation take place (Figure 1.3b).

In order for cleavage at site I to take place, sites II and III, the so-called accessory sites, are required to be present (Bednarz *et al.*, 1990). These sites are thought to play an essential role in synapsis, creating the required topology for recombination to take place (Watson *et al.*, 1996).

The Tn*3* family of resolvases are approximately 185 amino acids (20.5 kDa) in length, and display extensive sequence similarity within the subfamily, as well as having similarity to the Cin, Gin, Hin and Pin invertases. The proteins have a classical helix-turn-helix motif which is similar to that found in a variety of other DNA-binding proteins, such as Cro and cI of bacteriophage lambda. Tn*3* and γδ resolvases have been shown to be functionally interchangeable; that is, Tn*3* resolvase will promote recombination at γδ *res* sites, and *vice versa*. Cleavage of these resolvases with chymotrypsin results in two proteolytic fragments, a 5 kDa C-terminal domain and a 15.5 kDa N-terminal domain (Abdel-Meguid *et al.*, 1984). These fragments were shown by footprinting to bind on opposite sides of the DNA helix (Mazzarelli *et al.*, 1993).

The C-terminal fragment comprises 45 amino acids, and contains the helix-turn-helix motif responsible for binding resolvase to the consensus sequences of the inverted repeats of each *res* site. Mutations in γδ resolvase C-terminal region have been shown to impair or abolish binding of the protein to its site (Rimphanitchayakit and Grindley, 1990).

The amino terminal fragment of resolvase, of 140 amino acids, is thought to mediate the protein interactions between the resolvase monomers, as well as containing the residues responsible for forming the catalytic site (Abdel-Meguid *et al.*, 1984; Hughes *et al.*,

1990). Lambda family integrases and topoisomerases cleave the DNA forming a phosphotyrosine linkage with the DNA. However, existing evidence points to the serine at position 10 as providing the nucleophile for the resolvase reaction, resulting in the formation of a phosphoserine bond at the crossover point (Reed and Moser, 1984; Klippel *et al.*, 1988a). The protein is covalently linked to the 5' recessed DNA ends. This serine residue is completely conserved throughout the entire resolvase family. Mutational studies in γδ resolvase and Gin invertase have shown a loss of recombination when serine at position 10 is replaced. The resolvase family do also contain an entirely conserved tyrosine (at position 6 in Tn*3* resolvase). Although this residue has been shown to be important in catalysis (Leschziner *et al.*, 1995; see Chapter 6), it is apparently not the nucleophile required for strand cleavage.

Mutations of a variety of other residues, as well as Tyr-6 and Ser-10, have been shown to abolish recombination, e.g. at Gln-14, Asp-36, Gln-40 and Asp-67 (Hughes *et al.*, 1990; Leschziner *et al.*, 1995; Boocock *et al.*, 1995). These mutant resolvases retain binding activity but are deficient in recombination. These residues are therefore thought to be also important for catalysis.

The initial crystal structure of γδ resolvase, at 2.7 Å resolution determined the structure of the N-terminal but not the C-terminal domain, which remained disordered (Sanderson *et al.*, 1990). This disorder was seen as indicating potential flexibility within the protein, which may account for the comparable binding of resolvase to all three *res* binding sites even though the spacing of the motifs recognised by the C-terminal domain varies between the sites. The structure shows that the first 120 amino acids of the N-terminal domain form a central 5-stranded β-pleated sheet surrounded by five α-helices. The Ser-10 is exposed. The crystal structure (later refined at 2.3 Å; Rice and Steitz, 1994b) has shown that of the 4 dyad related dimers observed, the interface formed by the pairing of the C-terminal helices (the E-helices) of the domain (the 1,2 dimer interface), is the only one formed by all monomers, and the relevant dimer interface in resolvase binding in solution (Hughes *et al.*, 1990, 1993). This interface is hydrophobic in nature and has several conserved hydrophobic residues at its centre.

## 1.5 Resolvase bound to *res*

Resolvase has been shown to induce bending of the DNA when it is bound at each of the three *res* sites. Bending induced at site I of the DNA has been shown to be different from the bending induced at sites II and III; this may be due to the structural distortion of the DNA required at the crossover site. It has been shown that sites I and II of *res* have to be in the correct phase. Cleavage studies with DNase I suggest that the DNA in a fully bound *res* site is looped, possibly through dimer-dimer interactions of resolvase at sites I and III (Hatfull *et al.*, 1987).

Fis-independent Gin mutants have been observed to unwind the DNA at the crossover point (Klippel *et al.*, 1993). This is not the case with wild-type Gin, but is thought to be induced by the interactions of Fis with paired recombination sites. This may aid strand exchange by disruption of base pairing at the crossover site. There has also been evidence to show that γδ resolvase can cause a structural anomaly in the DNA at the centre of site I (Hatfull *et al.*, 1987).

The relationship between the structure of resolvase bound to the *res* site (the resolvosome), and the synaptic structure (the synaptosome) is unclear. Interactions in a single *res* complex may require to be broken before formation of the synaptic complex can occur. But mutants which cannot form a resolvosome are also deficient in recombination, and therefore some of the interactions in the resolvosome are still present in the synaptic complex or as a necessary prelude to or requirement for synapsis.

The co-crystal structure solved at 3.0 Å resolution of a dimer of γδ resolvase bound at *res* site I showed a 1,2 dimer of resolvase bound to a symmetrical site I oligonucleotide (Yang and Steitz, 1995). The entire 28 bp of site I is encircled by the protein. Site I DNA is bent at 60° towards the major groove and away from the N-terminal domains. The DNA is also bent towards protein sequences at the ends of the site bound in the major groove by the helix-turn-helix motif in the C-terminal domain (Yang and Steitz, 1995; Figure 1.4).

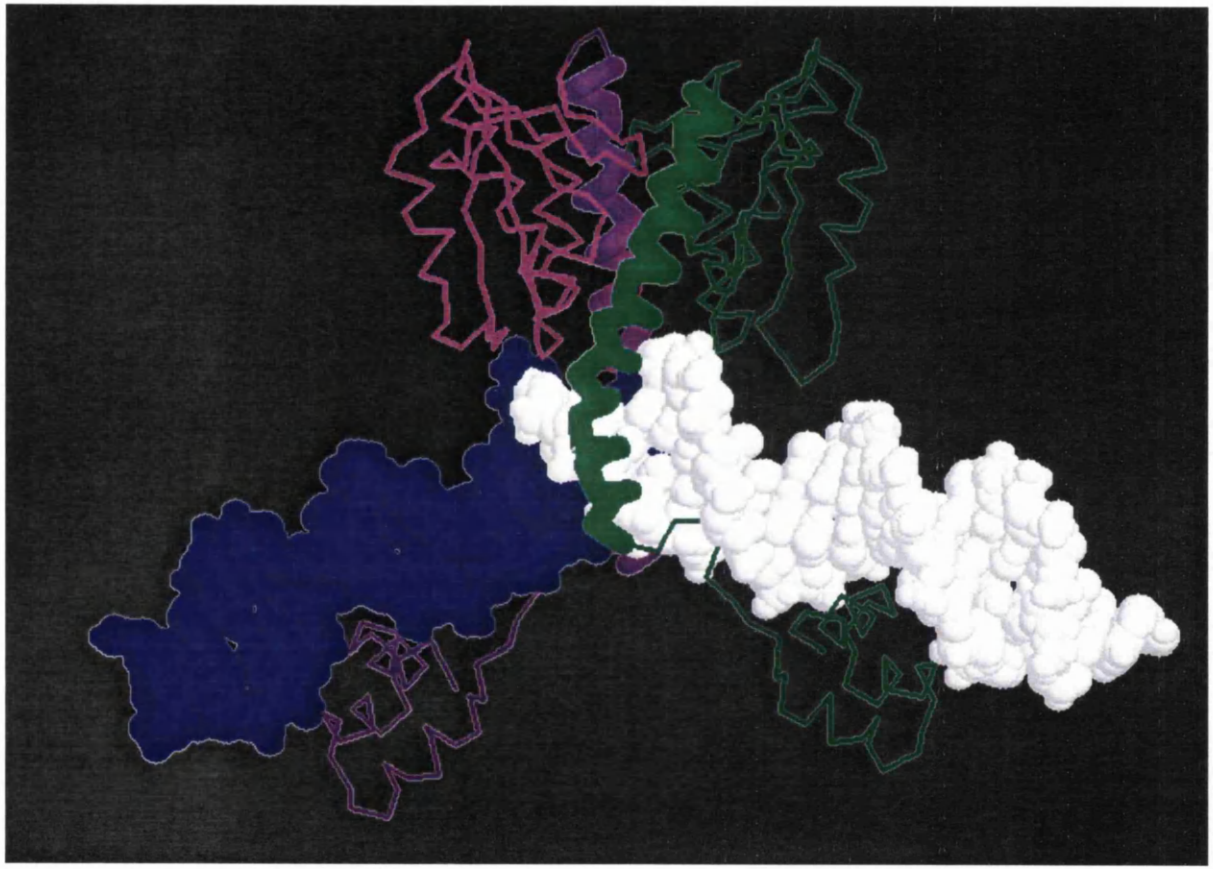


**Figure 1.4 Structure of the γδ resolvase-site I DNA complex**

Illustration of a dimer of γδ resolvase complexed with a 34 bp site I at 3 Å resolution (Yang and Steitz, 1995). The two resolvase monomers are represented in purple and green. The E-helix of each monomer, stretching from residue M103 to G137, is shown in bold. A space-filling model of the site I DNA is represented in blue and white, the crossover point being the boundary of these two colours. Figure adapted from Yang and Steitz, 1995.

The E-helix of one monomer of the resolvase dimer is straight, while the second is bent at 26° into the minor groove. The protein is therefore displaying a degree of asymmetry in the dimer subunits, which may be relevant to the flexibility of the protein in binding to the different sites within *res*.

The active Ser-10 residues are more than 30 Å apart, while the two scissile bonds at the central dinucleotide are only 13 Å apart. Concerted double stranded cleavage is therefore not feasible in this complex. It suggests that either a conformational change, or sequential single stranded cleavage must take place in order to allow the necessary interactions to occur. As yet no dimer-dimer interface has been identified. Therefore, we cannot at present distinguish between two very different models for synapsis of two site I's; the DNA duplexes may be close together, with the N-terminal domains of resolvase on the outside of the synaptic complex, or the N-terminal domains could make a dimer of dimers with the DNA on the outside (Figure 1.5).

Resolvase bound at site II is in a quite different configuration from that bound at site I (Blake *et al.*, 1995). In particular, the two subunits of the dimer interact with the DNA in a different geometry.

## 1.6 *In vitro* resolution

The *in vitro* reaction requires only the presence of a substrate (supercoiled and containing two *res* sites in direct repeat), the protein resolvase and a simple buffer. Cleavage of the DNA and the subsequent strand exchange occur at the centre of site I (Reed and Grindley, 1981). This is thought to be by a double stranded break at the central AT dinucleotide, giving 2 bp 3' extensions. A 5' phosphoserine bond then joins *res* to resolvase. This protein/DNA bond is broken on the religation of the DNA ends in the recombinant configuration (Reed and Grindley, 1981; Reed and Moser, 1984). The product of the *in vitro* recombination reaction is a (-2) catenane. This is also the *in vivo* product, though the linkage of the two circles of the catenane is separated afterwards by the action of DNA gyrase, or another type II topoisomerase (Bliska *et al.*, 1991).

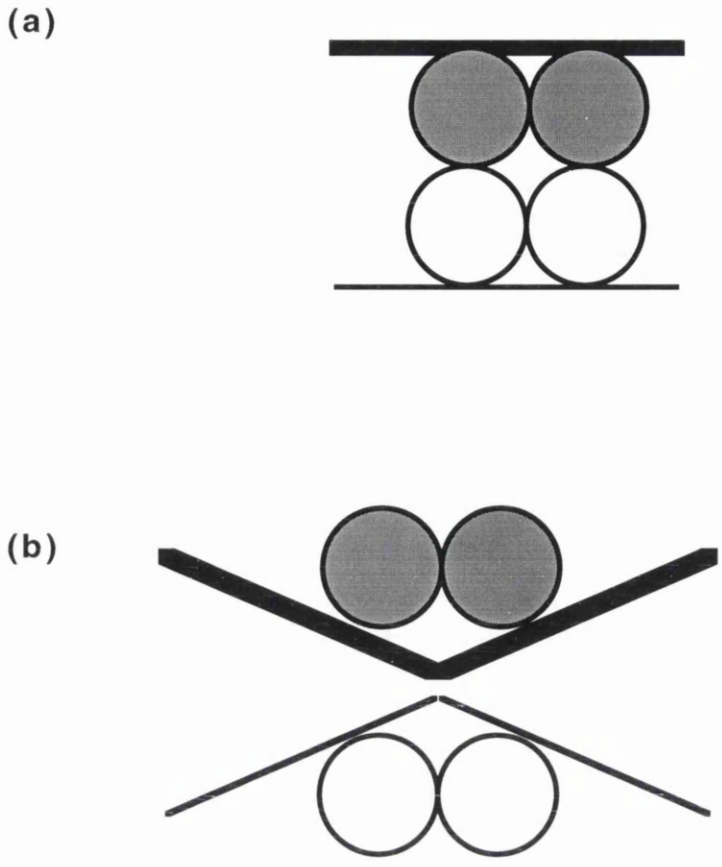



**Figure 1.5 Illustration of two different types of synaptic structure.**

Resolvase monomers are represented by spheres, and site I DNA by thick or thin lines.

(a) Sites come together with the catalytic domains of resolvase at the centre of the synaptic complex, as predicted by the subunit rotation model of strand exchange (see Section 1.8).

(b) Structure in which the DNA is at the centre of the synaptic complex and the resolvase monomers are on the outside, as predicted in the DNA-mediated model of strand exchange (see Section 1.8). Figure adapted from Rice and Steitz, 1994a.

The specificity of this product reflects the topological selectivity of the recombination reaction. Exactly three negative inter-domainal nodes are trapped between the sites in the synaptic complex and strand exchange has a right handed sense. A linkage change of +4 occurs during Tn*3* resolution, and this is reflected in the reverse reaction where a linkage change of -4 is observed (Boocock *et al.*, 1987; Stark *et al.*, 1989b). This change is consistent with the plectonemic wrapping of the DNA/*res* sites and a "simple rotation" mechanism of strand exchange (Stark *et al.*, 1989b). Extensive further studies of the topology of the reaction are also consistent with these notions (Stark and Boocock, 1995a). Topological selectivity has also been observed in the Xer recombination system where 3 negative supercoil nodes are also apparently trapped between the recombining sites (Colloms *et al.*, 1997), and in Gin/Hin inversion.

The reaction is very specific for recombination between *res* sites in direct repeat. No recombination has been found to occur between *res* sites on different molecules or between sites in inverted repeat on supercoiled molecules. This is consistent with the theory of topological constraints which exist within the system.

## 1.7 Synapsis, and proposed models for it

It is as yet unclear exactly how the two *res* sites align themselves to form the synaptic complex while retaining the topological selectivity required for the reaction. A variety of models for the mechanisms leading to synapsis, and the structure of the synaptic complex itself have been proposed (reviewed in Stark and Boocock, 1995a).

The tracking mechanism proposes that resolvase bound at a *res* site diffuses along the adjacent DNA until it aligns itself with a second site. This method explains the strong bias within the system against recombination between inverted sites, and intermolecular reactions. However, the random segregation of "reporter rings" linked to the substrate between the two circles of the resolution product makes it difficult to accept this model of synapsis (Benjamin and Cozzarelli, 1986). Further evidence against the model was its inability to account for reactions showing the occurrence of recombination between a *res* site on a linear molecule and a second *res* site on a supercoiled plasmid, and of

recombination between *res* sites present on separate circles of a catenane (Boocock *et al.*, 1987).

Slithering makes use of the knowledge that supercoiled DNA is plectonemically wound, and that as such it might move so that sites can be brought together in a defined synapse (Benjamin and Cozzarelli, 1986). This type of DNA movement has not been proved experimentally, and slithering does not account for the evidence that resolvase bound to a linear site can react intermolecularly with a supercoiled substrate (Brown, 1986; Boocock *et al.*, 1987). It has also been shown that recombination of a relaxed substrate results in the same (-2) catenane as that produced when recombination occurs within a supercoiled substrate (Boocock *et al.*, 1986). This means that recombination in these two cases would have to occur by drastically different mechanisms and yet result in the same product, which is unlikely.

In the model known as 2 step synapsis, the *res* sites come together by random collision, but require the presence of a topological filter to ensure that strand exchange will only occur in a defined productive synapse (Boocock *et al.*, 1986, 1987; Stark *et al.*, 1989a). The random collision by which it is proposed that the *res* sites come together results in a variety of synaptic structures. However, only one of these structures is energetically favourable such that the next stage in recombination, strand exchange, may take place (Figure 1.6). The requirement for a productive synapse is the trapping of three inter-domainal nodes between the crossover sites (Stark *et al.*, 1989b). Other collisions, such as those occurring between inverted sites, would result in a synapse which is tangled, and energetically unfavourable to the next step in the reaction. These unfavourable synapses would then rapidly dissociate to allow the possibility of forming the more energetically favourable productive synapse. The trapping of the three inter-domainal nodes is thought to be brought about by the wrapping of sites II and III from each site in an antiparallel alignment, with the formation of tetrameric resolvase structures. This is followed by the alignment of site I's (hence the "two"-steps). This model relies heavily on the presence of sites II and III to perform the necessary wrapping to bring about correct alignment, and it has been shown that correct alignment of site I's only takes place in the presence of these accessory sites (Bednarz *et al.*, 1990). Substrates lacking sites II and III were shown to perform recombination (very inefficiently) with both parallel and antiparallel

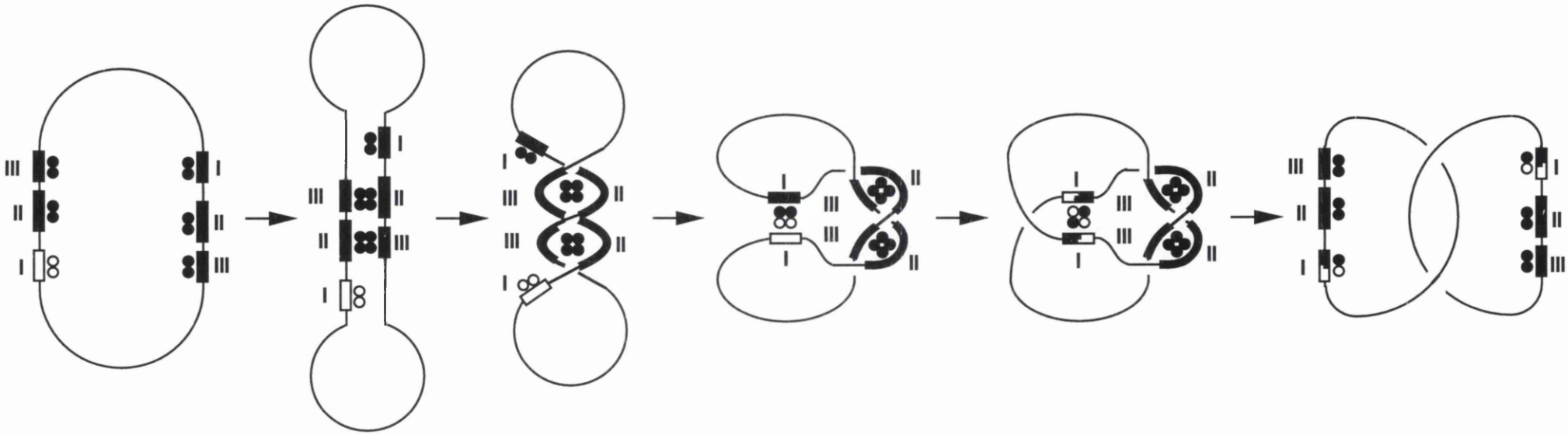



**Figure 1.6 Two-step synapsis model for *res*/resolvase topological selectivity.**

Black and white circles represent resolvase monomers, and the sites of *res* are represented by black or white rectangles. The sites are numbered (I, II, III). Strand exchange is depicted as a rotation of resolvase subunits within the catalytic tetramer at the synapsed site I's. See text for further details. Figure adapted from Stark and Boocock, 1995a.

alignments of the two site I's. It has also been shown that *res* sites form a synapse when present in inverted repeat (Benjamin and Cozzarelli, 1988; Watson, 1994). However the synapse formed is not productive, and does not proceed through the remainder of the reaction. This supports the evidence for a topological filter, since these unproductive synapses can be formed but lack a necessary requirement, such as the correct topology to proceed through strand exchange.

In the two-step synapsis model, a highly simplified structural model of the synapse was proposed in which the antiparallel-aligned pair of sites II/III is wrapped around two resolvase tetramers formed by interactions of dimers bound to individual sites, and resolvase dimers bound at site I interact to make another tetramer (see Figure 1.6). Other more elaborate models have been put forward, incorporating some of the structural data (Rice and Steitz, 1994b; Boocock *et al.*, 1995; Yang and Steitz, 1995). However, since no sound relevant biochemical or crystallographic data are as yet available, all such models are at present speculative.

Comparisons have been drawn between resolvase bound at the accessory sites and the Fis protein bound at the enhancer of the inversion systems of Gin and Hin. These inversion systems do not have accessory sites next to the crossover site, but instead require the presence of an intramolecular enhancer sequence bound by Fis for efficient recombination. It has been proposed that synapsis requires Fis-bound enhancer and Gin-bound *gix* sites (or Hin-bound *hix* sites) in an interactive invertasome. A role of the enhancer has been suggested to be in inducing a conformational change in order to unwind the DNA at the crossover site and allow cleavage to take place (Klippel *et al.*, 1993). Gin mutants which can act in the absence of Fis and the enhancer have been shown to mediate normal inversion, as well as displaying the ability to carry out deletion and intermolecular fusion. This suggests a loss of specificity of the topological filter, which may be defined by the Fis-bound enhancer and analogously, the accessory sites II and III of resolvase. The mutants do indeed give topologically complex "random collision" products of recombination. These mutants will be discussed later (in Section 1.9).

## 1.8 Cleavage and strand exchange

The formation of a productive synapse leads to cleavage of the DNA at the centre of site I. Concerted double strand cleavage has been widely accepted as a fact for the resolvase/invertase family (Reed and Grindley, 1981; Boocock *et al.*, 1995; reviewed in Stark and Boocock, 1995b). Evidence to support this has been the total lack of experimental evidence of a Holliday structure, which, if single strand cuts were made in each duplex, would presumably be formed during the strand exchange process. Further evidence for double strand breakage has come from the *in vitro* isolation, using modified conditions, of both γδ resolvase and Tn*3* resolvase-*res* complexes. These complexes show double strand cuts present in the DNA at each *res* site (Reed and Moser, 1986; McIlwraith, 1995). Recent crystallographic and biochemical observations have highlighted that concerted double strand cleavage may not be such an open and shut case. The crystal structure of γδ resolvase bound at site I (Yang and Steitz, 1995) shows the Ser-10 residues, which act as the nucleophile during the reaction, to be too far apart from the cleavage position, the central dinucleotide of site I, for concerted double strand cleavage. This demands that a large conformational change must take place to allow double strand cleavage. When this is taken into account, along with the asymmetry of the protein, which suggests that the monomers comprising the dimer may act independently, there surely exists reasonable doubt. Recent studies using mutant resolvases have shown that single strand breakages can be generated (Boocock *et al.*, 1995). Cleavages may not be truly concerted, but strand exchange might nevertheless have a requirement for two double strand breakages i.e. all four strands to be cleaved, before strand exchange takes place (Yang and Steitz, 1995). It has been shown that the catalytic residues of γδ resolvase act *in cis*, cleaving the DNA strand to which they are bound, and that there is no required order to strand cleavage (Boocock *et al.*, 1995).

Models for strand exchange must be in accord with the current information we possess in order to be considered feasible. The linkage change, the change in the number of helical turns of the DNA double helix, in going from the substrate to the product, helped distinguish between possible mechanisms. The linkage change of Tn*3* resolution was found to be +4 in the forward and -4 in the reverse reactions. This information can be combined with the knowledge that the topology of the product is a (-2) catenane, and that

synapsis creates three negative inter-domainal nodes. This information discounts the possibility of simply breaking and rejoining the paired *res* sites, or recombination through a Holliday junction, since these do not account for the topological changes from the substrate to the product (Stark *et al.*, 1989b).

Changes in DNA topology and the fact that four strand cleavages occur prior to strand exchange are consistent with a model for strand exchange known as subunit rotation (Stark *et al.*, 1992). In this model, the tetramer of resolvase at site I causes double strand cleavage at both crossover sites. Each monomer of resolvase is covalently attached to the half site to which it is bound. One dimer then rotates, along with its attached half site, through 180° in a right handed sense, resulting in the recombinant configuration (Figure 1.7). Religation of the strands and dissociation of the catenane then follows.

This model, as well as being consistent with the topological evidence discussed above, also accounts for minor products formed during the reaction. Further rounds of $180^0$ rotation, in the absence of religation, would account for the 4 noded knot, 5 noded catenane and 6 noded knots observed (Stark and Boocock, 1994; Figure 1.8).

However, as yet no direct biochemical or physical evidence has been shown to support this model, and it must be noted that the model requires a large movement of the DNA/protein complex and the breakage of dimer interactions before rotation can occur. Other models, which do not require subunit rearrangement have been proposed (Rice and Steitz,1994a; Boocock *et al.*, manuscript in preparation). The Rice and Steitz model proposes that the two site I DNA's are bound to resolvase dimers and are brought together such that the crossover points are in close proximity. Strand exchange then occurs with only relatively minor movement of the DNA (see also Chapter 7; see Figure 1.9).

## 1.9 Mutational studies

Recombination between *gix* sites of phage Mu requires the Gin protein bound at these sites and in addition an enhancer sequence bound by the host factor Fis. Mutants have

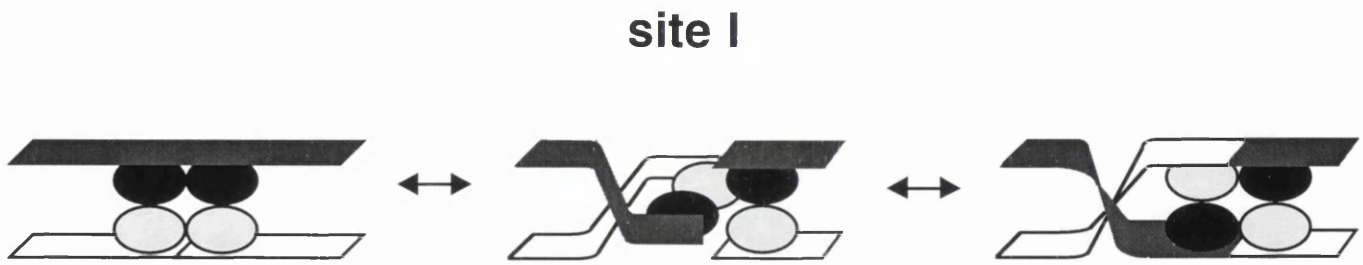



**Figure 1.7 The 'subunit rotation' model of strand exchange.**

The site I DNA is drawn as a ribbon, and resolvase monomers as ovals. The interwrapped sites II/III are to the right as drawn. The two catalytic dimers of resolvase at the synapsed site I sequences promote concerted double-strand cleavage of both crossover sites, and each monomer of resolvase becomes covalently attached to the half-site at which it is bound. One dimer of the catalytic tetramer then rotates (along with its attached half-site) through180° in a right-handed sense to bring about the recombinant configuration. The strands are then re-ligated, and the synapse dissociates to release the recombinant catenane. Figure adapted from Stark *et al.*, 1989a.

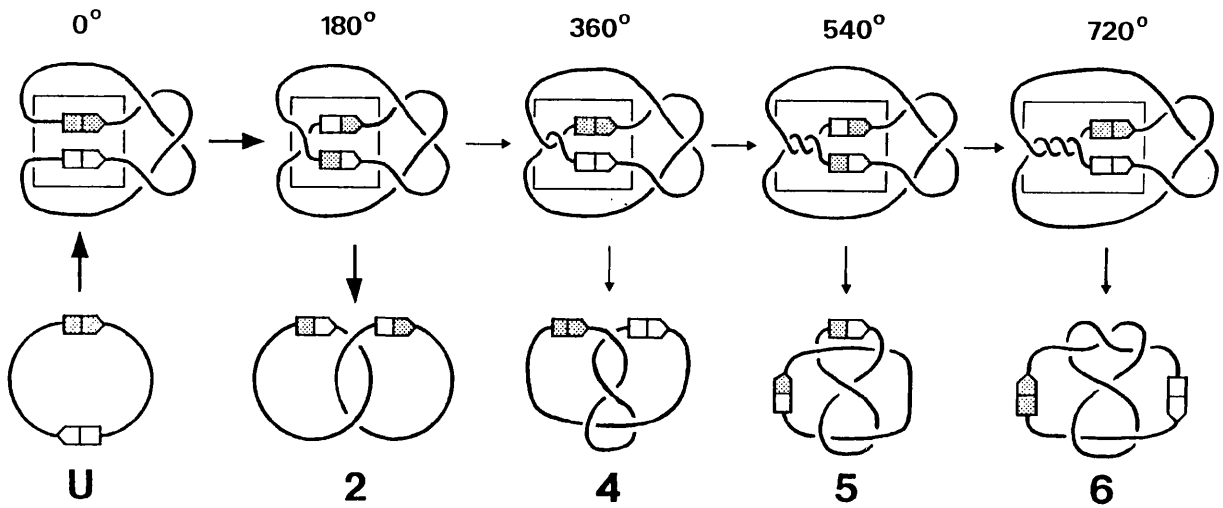



**Figure 1.8 Multiple rounds of strand exchange**

The diagram shows only site I for simplicity. The three intertwinings of the DNA on the right of each picture may be specified by interwrapping of the pair of sites II/III.The topology of multiple rounds of strand exchange and the products formed. The number of nodes present in the product is denoted under each one. Figure adapted from Stark *et al.*, 1989b.

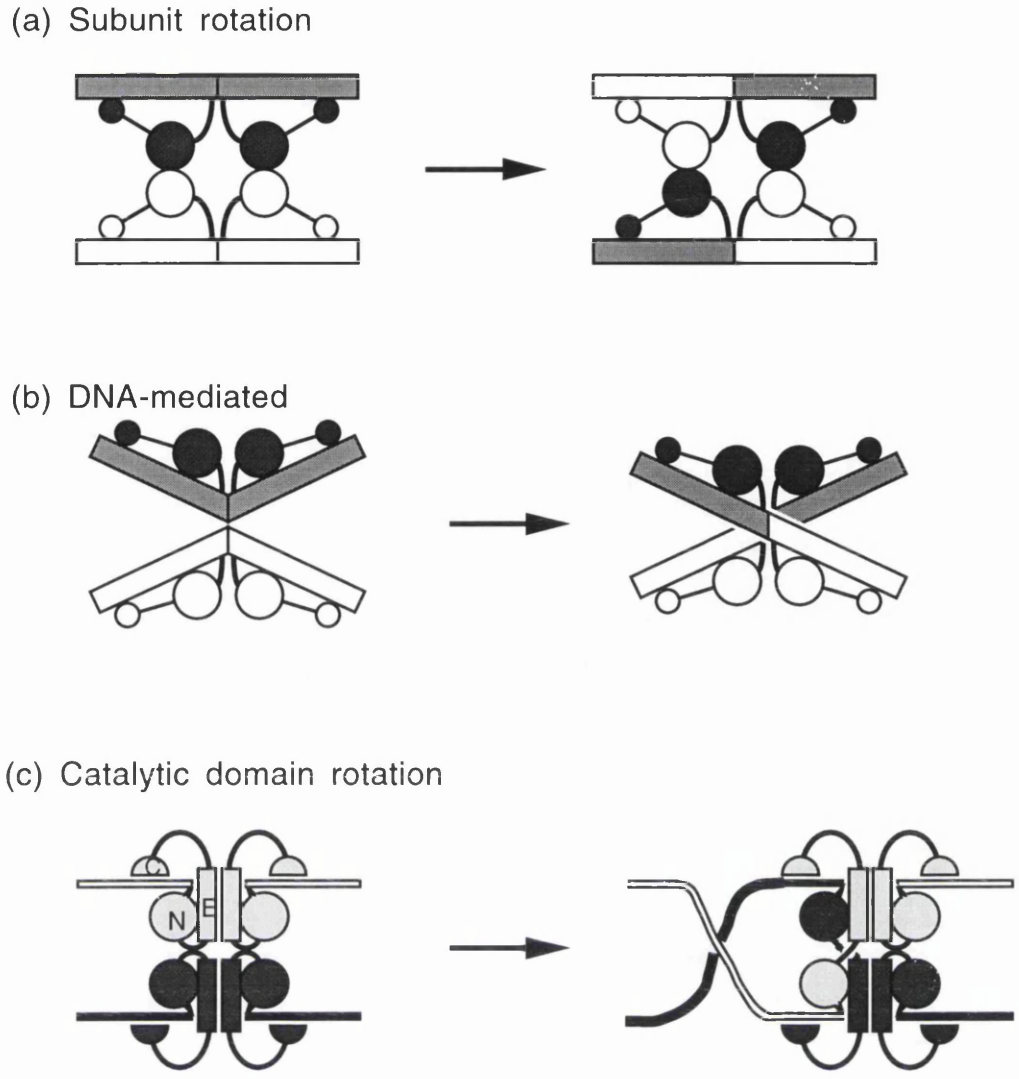



**Figure 1.9 Models of resolvase-mediated strand exchange.**

Only site I DNA is shown for simplicity. Crossover half-sites are shown as white or shaded rectangles. The resolvase N- and C-terminal domains are shown as large and small circles respectively in (a) and (b).

(a) Subunit rotation. Half-sites are exchanged by rotating one pair of subunits (along with its attached half-sites) relative to the other pair.

(b) The DNA-mediated model proposes that strand exchange occurs via movement of the free 3'-hydroxyls, without rearrangement of the bound resolvase dimers (Rice and Steitz, 1994a).

(c)The complexity of this model made it necessary to show the C-terminal and N-terminal domains ("C" and "N" respectively) and the E-helix (denoted by "E"). This model proposes that a flexible hinge exists between the E-helix and the N-terminal domain, which allows rotation of the N-terminal domains without dissociation of the 1,2 dimer (M. R. Boocock *et al.*, manuscript in preparation).

been isolated and investigated *in vitro*, which promote deletion and intermolecular fusion, as well as inversion in the absence of the enhancer-Fis (Klippel *et al.*, 1988b, 1993).

A model for Gin recombination proposes that the *gix* sites bound by Gin come together with the protein Fis bound at the enhancer at a branch point in a negatively supercoiled DNA substrate. This nucleoprotein complex traps two negative supercoils between the *gix* sites. Strand exchange then occurs after staggered double stranded breaks at each *gix* site. One pair of half-sites rotates about the other, and the DNA is religated. This wild-type reaction has a strict specificity for inversion (Figure 1.10).

As well as inversion, relaxation of the substrate is brought about by wild-type Gin, on the formation of the synaptic complex. Gin mutants have been isolated which do not have this pre-requisite and promote relaxation in the presence of a single *gix* site where no synaptic complex could be formed (Klippel *et al.*, 1988b). This was observed not to be by random topoisomerase activity, since substrates lacking any *gix* sites were not relaxed. Therefore, mutant Gin was able to break DNA at a *gix* site, pass a second DNA segment through the break, and rejoin the DNA without the enhancer, Fis or a second *gix* site on the same substrate molecule.

Under specific conditions, recombination by mutant Gin was found to be 20% in the absence and 30% in the presence of Fis. Analysis of the linkage change showed that 60% of the recombinant products had a linkage change of +4, indicating that they were formed through the same pathway as the wild-type reaction, though up to 40% of products had proceeded though a different synaptic complex resulting in a linkage change of 0, indicating that no supercoils had been trapped during the reaction (Klippel *et al.*, 1993).

The Gin mutants were also found to catalyse intermolecular recombination. Analysis of this reaction showed that recombination proceeded through complexes formed by a parallel or antiparallel alignment of the *gix* sites. This seems to indicate that the crossover sequence and the sequence outside the *gix* sites is not important in the determination of the site alignment. Mutant Gin was shown to mediate strand exchange in both the right and left handed sense, probably through the 0-synaptic complex (trapping no topological

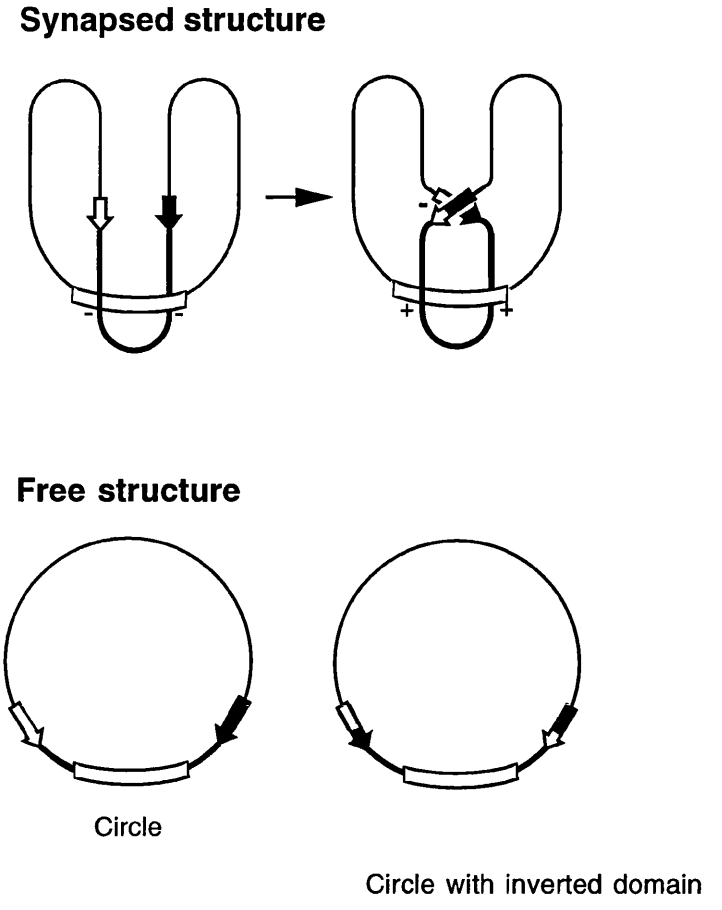



**Figure 1.10 Topological changes during recombination by wild-type Gin.**

The arrows represent the *gix* sites, and the thick and thin lines represent the domains between them. The substrate is shown with only the two essential negative interdomainal nodes. The upper row shows the protein-bound synapsed structures and the lower row shows the free unfolded forms. The enhancer is illustrated by a white box. Figure adapted from Kanaar *et al.*, 1988.

nodes) with parallel or antiparallel sites. It is negative supercoiling which imposes right handed directionality on wild-type strand exchange (Klippel *et al.*, 1993).

Binding studies have shown that mutant Gin protects both *gix* half sites, but in a different manner from the wild type protein. Wild-type Gin protects all but the 2 bp staggered crossover, while mutant Gin leaves an unprotected region of up to 4-5 bp. Mutant Gin altered the DNA structure such that footprinting studies showed an increased accessibility of MPE.Fe(II) (the iron chelate of methidium propyl EDTA, which cleaves the DNA while intercalating via the minor groove) in the minor groove in the region of strand exchange. This was found to be due to a localised unwinding of approximately half a helical turn per *gix* site.

The proposed role of the Fis/enhancer complex is that it induces a conformational change in the Gin/*gix* complex such that cleavage and strand exchange may proceed. This may result in partial unwinding at the *gix* site (Klippel *et al.*, 1993).

These mutants of Gin, H106Y and M114V , have been modelled on the γδ resolvase crystal structure, and are in the E-helix constituting the dimer interface in solution. The changes could change the relative positions of the monomers and thus rotate the half sites bound by them, unwinding the DNA at the centre of *gix*. Unwinding may be required to disrupt base pairing at the crossover region, and may help place the serines into a more favourable position for attacking the DNA.

To learn more about these mutants, secondary mutations which suppress the Fis-independent phenotype were isolated (Merker *et al.*, 1993). Secondary mutations which suppressed the Fis independent phenotype of the Gin mutant M114V were screened for, and 9 single mutations were found to revert the phenotype to wild-type. Separation of these mutants from M114V showed that they all caused the protein to be recombinationally inactive. Most of these mutants were located within 10 amino acids of the amino terminal of the protein. The corresponding region in γδ resolvase has yielded recombinationally inactive γδ resolvase mutants, shown to be defective in dimer-dimer interactions (Hughes *et al.*, 1990).

Mutants of the Cin recombinase have also been isolated which efficiently recombine in the absence of the enhancer, and like Gin M114V have a relaxed phenotype, promoting reaction between *cix* sites on separate molecules and between sites in direct repeat (Haffter and Bickle, 1988). These mutants, R71H and H106Y, unlike the Gin mutants, have a requirement for the presence of Fis. These mutants support the idea that the enhancer defines the topological specificity of the synaptic complex. The loss of enhancer dependence is the reason that these mutants have acquired the ability to recombine sites in direct repeat and sites on different molecules.

Mutants of γδ resolvase have been used to investigate the synaptic structure, and the role and requirements of the accessory sites II and III in comparison to the catalytic site I. Studies using combinations of specific mutants have shown that the requirements for resolvase when bound at site I are different from when the protein is bound at the accessory sites (Grindley, 1993). Resolvase bound at site I has a requirement for Ser-10. The mutant S10L, which is catalytically inactive, when assayed in combination with a mutant (R172L) which binds to a specific synthetic site I used in the reaction, but not the accessory sites, has been shown to recombine. This indicates that S10L can bind and perform the necessary functions of the protein at the accessory sites in the absence of the serine residue which is absolutely required for catalytic activity of the protein bound at site I. Likewise, the mutant γδ resolvase E128K which is unable to bind to site III, but is catalytically active at site I, when used in conjunction with S10L recombines efficiently. It is thought that the mutants complement each other, with S10L binding at the accessory sites while E128K performs the catalytic activity required at site I (Grindley, 1993). The mutant R2A, which cannot perform the "2-3'" interaction thought to occur in the interwrapped accessory sites, does not complement E128K in recombination assays.

Prior to this project, no mutants of Tn*3* resolvase had been isolated.

## 1.10 The role of sites II and III of *res*

Various experiments have been carried out with a view to determining the role of sites II and III in the recombination reaction. A hybrid site (*ges*) was created, consisting of a *gix* site placed next to sites II and III of *res*, with the spacing between the centre of *gix* and

the centre of site II the same as in *res*. These *ges* sites were cloned in direct repeat in a supercoiled plasmid, meaning that the *gix* sites were also in direct repeat. Reactions carried out with Gin but lacking resolvase resulted in no recombination, but the addition of resolvase to the reaction resulted in a recombination event, with the product being a (-2) catenane (M. Boocock and C. Koch, personal communication). This indicates that the presence of sites II and III of *res* can define the topology of the product of Gin-mediated recombination. Sites II and III therefore play a major role in synapsis of wild-type *res* sites.

A second series of experiments, in which site I of *res* was replaced by a *loxP* site, and resulting in the creation of hybrid "*les*" sites, yielded similar observations. The *loxP* site is from bacteriophage P1, and is recombined by the Cre recombinase. Substrates with *les* sites incubated with Cre, resulted in free circle resolution products and unknotted inversion products typical of this system. The addition of resolvase to the reaction resulted in 3 noded inversion products and 4 noded catenane resolution products. Strand exchange in the *lox*/Cre system apparently has a left handed sense, resulting in resolution products of 4 nodes instead of the 2 noded product resulting from the right handed resolvase system. Analysis of these products indicates that sites II and III are regulating the system by bringing the *lox* sites together in a specific geometry and topology (E. Kilbride, personal communication).

The accessory sites II and III have been shown to be important for the alignment of the crossover site in site I (Bednarz, 1989). The left and right arms of the crossover site (site I) differ at 6 of 11 positions: Does resolvase recognise these differences in order to align the sites correctly, or is alignment indirect, via alignment of sites II and III? Correct alignment of the crossover site was shown not to require sequence asymmetry at the crossover site, DNA supercoiling, or the covalent linkage of *res* sites, but was determined by resolvase-mediated interactions of sites II and III of both partners. It was shown that deletion of one of the sets of sites II and III reduced the efficiency of resolution greatly, but that substrates with site I in direct or inverted repeat with the complete *res* were resolved equally well. Therefore, deletion of sites II and III results in the failure of resolvase to recognise the orientation of the site. In a substrate containing *res* sites with a perfectly symmetrical crossover site, the product was shown to be exclusively the

resolution product, a (-2) catenane, indicating that sites II and III are sufficient to determine correct alignment. The above experiments strongly support roles for the accessory sites in promoting the early stages of synapse formation, in correctly aligning the site I's in activating catalysis at site I, and in defining the topology of recombination.

## 1.11 Project aims

The aim of this project was to create and isolate mutants of Tn*3* resolvase, in order to further understand the recombination reaction. In order to do this, a vehicle in which the mutant resolvases could be created had to be developed. This plasmid had also to be compatible with an *in vivo* screen which exists in the laboratory. Analysis of these mutants also demanded the development of a resolvase purification system which was reliable and reproducible for a number of mutants.

Previous work has indicated that sites II and III play an important role in the alignment of the crossover site. A priority was the isolation of a Tn*3* resolvase mutant able to catalyse recombination between two site I's, in the absence of the accessory sites, to understand more about their role in recombination.

# Chapter 2

Materials and methods

## 2.1 Bacterial strains

Bacterial strains used are listed below in table 2.1. The majority of the strains used were derivatives of *Escherichia coli* K-12, with the exception of BL21 which is an *Escherichia coli* B derivative.

| STRAIN | GENOTYPE | SOURCE |
|---|---|---|
| AB1157 | *thr*1, *leu*6, *his*G4, *thi*1, *ara*14, *pro*A2, *arg*E3, *gal*K2, *sup*37, *xyl*15, *mtl*1, *tsx*33, *str*31 | |
| BL21 | *E. coli* B, $F^-$,*dcm*, *omp*T, *hsd*S($r_B$ $m_b^-$) *gal* | W. Studier |
| BL21 DE3 | *hsd*, *gal*, (λ*cI ts* 857, *ind*1, *Sam*7, *ini*5, *lac*$_{UV5}$-T7 gene-1) | W. Studier |
| BMH 71-18 mut S | *thi*, *sup*E, Δ(*lac-pro*AB), [*mut*S::Tn10}, {F', *pro*AB, *lac*ZΔM15, *lac*$I^q$] | Promega |
| DH5α | F',φ80d, *lac*ZΔM15, Δ(*lac*ZYA -argF), U169, *deo*R, *rec*A1, *end*A1, *pho*A, *hsd*R17 ($r_k$-, $m_k$+), *sup*E44, λ$^-$, *thi*-1, *gyr*A96, *rel*A1 | GIBCO |
| DS941 | AB1157, but *rec*F143, *sup*E44, *lac*ZΔM15, *lac*$I^q$ | D. J. Sherrattt |
| EM1 | DS941, but *mut*L | E. Morrell |
| JM109 | *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 ($r_k$-, $m_k$+), *rel*A1, *sup*E44, λ$^-$, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*ZΔM15, *lac*$I^q$] | Promega |

Table 2.1 Bacterial strains

## 2.2 Plasmids

Plasmids used and constructed throughout this work are listed in Table 2.2.

| Plasmid | Size (bp) | Antibiotic Marker | Description/Derivation | Source/ Reference |
|---|---|---|---|---|
| pAL225 | 4.908 | ApTc | Plasmid with 2 x site I in direct repeat | Bednarz *et al.*,1990 |
| pAL265 | 4.848 | ApTc | Plasmid with *res* v site I | Bednarz *et al.*,1990 |
| pAT1 | 6415 | Tc | Resolvase open reading frame in p-SELECT | Chapter 3 |
| pAT2 | 6415 | ApTc | pAT1 + $Ap^r$ Oligonucleotide + *Cla*I - Oligonucleotide | Chapter 3 |
| pAT3 | 6240 | ApTc | pAT2 with resolvase ORF replaced by ORF from pSA1101 | Chapter 3 |
| pAT4 | 6240 | ApTc | pAT3 + Cassette oligonucleotides 1 - 8 | Chapter 3 |
| pAT4-D102Y | 6240 | ApTc | pAT4 + M4 + M8 oligonucleotides | Chapter 3 |
| pAT5 | 6240 | ApTc | pAT4 with L69F change replaced to wild-type | Chapter 3 |
| pAT5-D102Y | 6240 | ApTc | pAT5 containing D102Y mutation | Chapter 4 |
| pAT5-E124Q | 6240 | ApTc | pAT5 containing E124Q mutation | Chapter 4 |
| pATXD102Y | 6692 | Km | pSA1101 with D102Y mutation in ORF | Chapter 5 |
| pATXE124Q | 6692 | Km | pSA1101 with E124Q mutation in ORF | Chapter 5 |
| pATXL69F | 6692 | Km | pSA1101 containing ORF from pAT4 | Chapter 5 |
| pDB34 | 8500 | Km | 3060 bp N/P (Partial) $Kn^r$ pDB703 + 5400 bp N/P pDB4 | Blake, 1993 |
| pDB35 | 8400 | Km | 3060 bp N/P (Partial) $Kn^r$ pDB703 + 5300 bp N/P pDB5 | Blake, 1993 |
| pDB36 | 8400 | Km | 3060 bp N/P (Partial) $Kn^r$ pDB703 + 5300 bp N/P pDB6 | Blake, 1993 |

| | | | | |
|---|---|---|---|---|
| pDB37 | 8400 | Km | 3060 bp N/P (Partial) $Kn^r$ pDB703 + 5300 bp N/P pDB7 | Blake, 1993 |
| pDB6020 | 6410 | Tc | p-SELECT + resolvase ORF | Blake, 1993 |
| pDB6020-Y6F | 6410 | ApTc | pDB6020 + Y6F (T) + (B) oligonucleotides | Chapter 4 |
| pKET-3a | 4952 | Kn | pET3 + Kanamycin gene replacing Ampicillin gene | S.Rowland |
| pKK223 | | Ap | Cloning vector. Accession number M77749 | Brosius and Holy 1981 |
| pMA21 | 4927 | ApTc | 1065 bp P/H pLS139 + 3826 bp P/H pMA44 | Bednarz *et al.*, 1990 |
| pMA6111 | 5900 | Ap | 282 bp P/H pLS139 + 3826 bp PH pMA44 | Blake, 1993 |
| pMTL23 | 2505 | Ap | Cloning vector derived from pUC18 | |
| pOG5 | 2639 | Ap | 130 bp SstI/XbaI RI res + 2505 bp SstI/XbaI pMTL23 | O.Gubbay/ W.M.Stark |
| pSELECT-1 | 5680 | Tc | Cloning vector for directed mutagenesis | Chapter 5 |
| pSA1101 | 6692 | Kn | pTA1 based expression plasmid | S.Rowland/ Chapter 5 |
| pTA1 | 5311 | Kn | Resolvase expression plasmid | Chapter 5 |
| pUC18 | 2686 | Ap | Cloning vector | Yanish-Perron *et al.*, 1989 |
| pUC71K | 3966 | ApKn | Cloning vector | Viera *et al.*, 1982 |

**Table 2.2 Plasmid details**

Antibiotic resistance is detailed under "Marker". The following abbreviations are used in Description/Derivation :-
H = *Hin*dIII, N = NdeI, P = *Pst*I, RI = E*co*RI. ORF denotes the open reading frame

## 2.3 Chemicals

Sources of general chemicals are listed in Table 2.3. All solutions were made using deionised water.

| CHEMICALS | SOURCE |
|---|---|
| General chemicals, Biochemicals, Organic solvents | Aldrich/Sigma, BDH, May & Baker |
| Media | Difco, Oxoid |
| Agarose | BRL, FMC |
| Acrylamide | National Diagnostics, Bio-Rad, Aldrich |
| Radiochemicals | ICN Biochemicals |
| 10x Restriction Enzyme buffers | BRL, Boehringer Mannheim, Promega |
| 10x Ligase buffer | Promega |
| 5x Ligase buffer | BRL |

Table 2.3 Chemicals

## 2.4 Media

*Escherichia coli* was grown in liquid L-Broth and on solid L-Agar. MacConkey galactose agar was used in order to screen mutants. 2xYT broth was used for expression purposes. Growth of cultures for the preparation of competent cells was in φ broth. Details of growth media are listed below.

**L-Broth** : 10 g bacto-tryptone, 5 g bacto-yeast extract, 5 g NaCl, made up to 1 litre with deionised water. NaOH used to adjust to pH 7.5.

**L-Agar** : L- Broth with 15 g/l Agar.

**2xYT Broth** : 16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl, made up to 1 litre with deionised water. pH adjusted to 7.0 with NaOH.

**φ Broth** : 2% w/v tryptone, 0.5% w/v yeast extract, 20 mM $MgSO_4$, 10 mM NaCl, 5 mM KCl, adjusted to pH 7.5 with KOH.

**MacConkey Galactose Agar** : (17 g bacto-peptone, 3 g bactoprotease peptone, 1.5 g bacto bile salts No.3, 5 g NaCl, 13.5 g bacto agar, 0.03 g neutral red, 0.001 g bacto crystal violet) supplied ready made by Difco, 1% D-galactose, made up to 1 litre with deionised water.

## 2.5 Bacterial growth conditions

Bacterial cultures were grown at 37 °C with shaking. Growth on agar plates was at 37 °C with the plates inverted. Antibiotics used for selection are listed in Section 2.7. Bacterial strains were stored long term by diluting an overnight liquid culture, in a 1:1 mixture with 40% glycerol, 2% peptone. These stocks were stored at -70 °C.

## 2.6 Antibiotics

The antibiotics used and their working concentrations in liquid and solid media are listed below, Table 2.4.

| ANTIBIOTIC | STOCK SOLUTION | SELECTIVE CONDITIONS |
|---|---|---|
| Ampicillin (Ap) | 5 mg/ml in $H_2O$ | 50 μg/ml |
| Chloramphenicol (Cm) | 2.5 mg/ml in ethanol | 25 μg/ml |
| Kanamycin (Km) | 5 mg/ml in $H_2O$ | 50 μg/ml |
| Tetracycline (Tc) | 12.5 mg/ml in 70% ethanol | 12.5 μg/ml |

Table 2.4 Antibiotics

## 2.7 Competent cells

Competent cells were prepared by the two methods detailed below. Highly competent cells, DH5α, for the construction of a mutant library were bought in from Gibco BRL.

### 2.7.1 RbCl method

**TFB I** : 30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2.2H_2O$, 50 mM $MnCl_2.4H_2O$, 15% glycerol, pH adjusted to 5.8 with AcOH

**TFB II** : 10 mM MOPS, 75 mM $CaCl_2.2H_2O$, 10 mM RbCl, 15% glycerol, pH adjusted to 6.8 with HCl or KOH

TFB I & II were filter-sterilised.

4 ml of overnight culture, grown from a single colony in φ broth, was used to inoculate 200 ml of pre-warmed φ broth and grown to an $OD_{600}$ of 0.46-0.6. The cells were cooled rapidly in ice and water and pelleted by centrifugation (Beckmann J2-21, 5 000 rpm, 10 minutes, 4 °C) in a pre-chilled rotor. The pellet was resuspended gently in 40 ml of ice-cold TFB I, and the suspension was left on ice for 30 minutes, then spun ( 5 000 rpm, 10 minutes, 4 °C). The pellet was resuspended in 8 ml of cold TFB II and the suspension was left on ice for 15 minutes. Aliquots of 200 µl were dispensed into pre-chilled tubes, and frozen rapidly in liquid nitrogen before storing at -70 °C.

### 2.7.2 $CaCl_2$ method

1 ml of overnight culture was inoculated into 19 ml of fresh L-broth, and grown until the $OD_{600}$ was 0.4-0.5. Cells were spun down, by raising the centrifuge to a speed of 10 K and stopping immediately. The pellet was resuspended in 10 ml pre-chilled 50 mM $CaCl_2$. Cells were respun as above and resuspended in 1ml of 50 mM $CaCl_2$. Cells were then stored on ice where they maintained competence for up to 48 hours.

## 2.8 Transformation

Competent cells prepared by different methods required slightly different transformation procedures. These are detailed below.

### 2.8.1 RbCl prepared cells

DNA (0.1 μg) was added to 50 μl of cells thawed on ice. This was mixed gently and incubated at 0 °C for 30 minutes. Cells were 'heat shocked' by incubation at 37 °C for 5 minutes, and returned briefly to ice where 5 volumes of 2xYT Broth was added. The cells were then allowed to recover by incubation at 37 °C for 90 minutes. Aliquots were spread onto selective agar plates, which were then incubated at 37 °C overnight.

### 2.8.2 $CaCl_2$ prepared cells

DNA (0.1 μg) was added to 200 μl of cells and the samples were mixed gently on ice, then left for 15 minutes on ice. The cells were heat shocked at 37 °C for 5 minutes, and returned to ice for a further 15 minutes. 1ml of L-Broth was added and the cells were expressed at 37 °C for 90 minutes. Aliquots were then spread onto selective plates.

### 2.8.3 DH5α competent cells

0.1 μg of DNA was added to 50 μl of cells thawed on ice. Cells incubated on ice for 30 minutes. Cells were heat shocked for 20 seconds at 37 °C and returned to ice for 2 minutes. 0.95 ml of room temperature 2xYT broth was added and the cells expressed at 37 °C for 1 hour. Cells were then plated onto selective media and incubated overnight at 37 °C.

## 2.9 Preparation of plasmid DNA

### 2.9.1 Large scale preparation of plasmid DNA

The method used here is adapted from Birnboim and Doly (1979).

**Doly I buffer** : 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA

**Doly II buffer** : 200 mM NaOH, 1% SDS, made fresh

**Doly III buffer** : 0.6 vol. 5 M KOAc, 0.115 vol. AcOH, 0.285 vol. $H_2O$

**TE buffer** : 10 mM Tris-HCl (pH 8.2), 1 mM EDTA

200 ml of overnight culture was grown, and the cells were pelleted (12 000 g, 5 minutes, 4 °C). The pellet was resuspended in 4 ml of Doly I buffer. After transferring to 50 ml tubes and chilling on ice for 5 minutes, 8 ml of Doly II buffer was added, and the tubes were mixed by inverting gently . After 4 minutes on ice, 6 ml of ice-cold Doly III was added and the samples were mixed gently before pelleting by centrifugation (39 200 g, 30 minutes, 4 °C). The supernatant was collected into tubes containing 12 ml of isopropanol and the DNA was precipitated at room temperature for 15 minutes, then pelleted by centrifugation (27 200 g, 18 00 rpm, 30 minutes, 20 °C). The pellet was washed with 2 ml of 70% ethanol and allowed to dry. 2 ml of TE buffer was added and the pellet was resuspended at 37 °C.

5 g of CsCl was dissolved in 3 ml of distilled water and the resulting 4.324 ml aqueous CsCl was added to the DNA. 270 µl of 15 mg/ml ethidium bromide was added. The mixture was transferred to a Beckmann ultra-centrifuge tube. The tubes were filled with liquid paraffin and balanced in pairs to within 0.1 g of each other. Tubes were sealed with a heat sealer and placed in a Beckmann Ti70 fixed angle rotor such that the balanced pairs directly opposed each other. They were spun at 200 000 g (49 000 rpm for 16 hours at 15 °C), after which the tubes were removed and observed on a long-wave UV source (365 nm). At this point two bands were observed, the lower of which corresponded to plasmid DNA, the higher being chromosomal DNA. The plasmid DNA was then removed by inserting a needle attached to a 1 ml syringe immediately below the band and through the wall of the tube. 0.5 ml of solution containing DNA was then drawn into the syringe. Four extractions, with 0.5 ml of n-butanol, were carried out to remove the ethidium. The solution was diluted with 3 volumes of water, and the DNA was precipitated with 2 volumes (of the diluted solution) of 100% ethanol at 4 °C for 20 minutes. DNA was pelleted by centrifugation (27 200 g, 30 minutes, 4 °C), the pellet was washed with 70% ethanol and dried, then dissolved in TE (500 µl). The DNA solution was stored at 4 °C.

### 2.9.2 Small scale preparation of plasmid DNA

Several methods were used to isolate plasmid DNA on a smaller scale. All used an Eppendorf microcentrifuge running at 14 000 rpm for the centrifugation steps.

**a) Alkaline lysis**

1.5 ml overnight cultures were grown and pelleted by centrifugation (~30 sec.). The pellet was resuspended in 200 µl of Doly I + 4 mg/ml lysozyme, and left at room temperature for 5 minutes. 400 µl of freshly prepared Doly II was added, and the samples were mixed by inverting several times before leaving on ice for 5 minutes. 300 µl of 7.5 M $NH_4OAc$ was then added, and the samples were kept on ice for 10 minutes. After a 5 minute spin, the supernatant was removed to a fresh tube, whereupon one extraction with 0.5 vol. of phenol was carried out followed by one extraction with 0.5 vol. of chloroform. DNA was precipitated by adding 0.75 vol. of isopropanol, then leaving the mixture at room temperature for 10 minutes. The supernatant was removed, and the pellet was washed with 70% ethanol and dried. DNA was redissolved in 50 µl of TE, and the solutions were stored at 4 °C

**b) Mini CsCl**

This method is adapted from Saunders and Burke, 1990. Unless otherwise stated, all steps in this procedure follow directly on with no incubations.

1.5 ml of overnight culture was spun down and resuspended in 100 µl of Doly I + 4mg/ml lysozyme. 200 µl of Doly II was added and the samples were mixed gently by inversion. 150 µl of Doly III solution was added and the samples were spun for 5 minutes. The supernatant was removed (380 µl) and 720 µl of isopropanol was added. The mixture was vortexed, spun, and the pellet was washed with 70% ethanol. After drying, the pellet was resuspended in 100 µl TE. 100 mg of CsCl was added to the DNA and the samples were shaken until it was dissolved. 15 µl of 15 mg/ml ethidium bromide was added and the samples were spun for 5 minutes. The supernatant was removed and 4 isopropanol extractions (100 µl) were carried out to remove the ethidium. The solution was diluted with 400 µl of TE, and the DNA was precipitated with 50 µl of 3 M sodium acetate and 720 µl isopropanol. The samples were spun for 5 minutes, and the pellet was washed with 70% ethanol before resuspending in 20 µl TE.

**c) Wizard DNA preparation.**

DNA was also prepared using the Wizard DNA purification kit (Wizard Miniprep, DNA Purification System, Promega), according to the manufacturer's instructions.

## 2.10 Restriction enzyme digestion of DNA

Restriction digests were carried out in the suppliers' recommended buffer with an excess of enzyme, between 2-fold and 10-fold, to ensure complete cleavage of the DNA. The digest was maintained at 37 °C ≥ 1 hour. On completion, the addition of 0.25 vol. of SDS loading buffer, (Section 2.23), or heating to 70 °C for 5 minutes, or one phenol extraction (Section 2.12) was carried out to terminate the digest.

Partial restriction digests were carried out using diluted stock enzymes in conjunction with a time course of incubation. Visualising the products on an ethidium bromide-stained agarose gel indicated the conditions required for the desired level of DNA cleavage. DNA was then purified from the gel (Section 2.20).

## 2.11 Phenol extraction of DNA

The removal of unwanted proteins by phenol extraction was carried out by the addition of one volume of phenol (containing 0.1% 8-hydroxyquinoline and equilibrated with 0.5 M Tris-HCl pH 8.0). The mixture was vortexed and subsequently centrifuged in an Eppendorf microcentrifuge (15 000 rpm, 3 minutes). The aqueous layer containing the DNA was removed for further re-extraction if necessary. Residual phenol was removed by repeating the extraction with chloroform.

## 2.12 Ethanol precipitation of DNA

The salt concentration of the DNA solution was adjusted to 0.3 M NaOAc, and 2 volumes of 100% ethanol was added. The sample was mixed well and kept at -20 °C ≥ 15 minutes. The DNA was pelleted by centrifugation in an Eppendorf microcentrifuge (5 000 rpm, 4 °C, 30 minutes). The pellet was washed in 70% ethanol and briefly respun. After removal of the ethanol, the DNA was dried before resuspension in TE buffer.

## 2.13 Filling in DNA ends

In order to blunt 3' recessed ends before cloning a DNA restriction fragment, dATP, dCTP, dGTP and dTTP were added to restriction enzyme-digested DNA to a final concentration of 125 μM, along with 1 unit of the Klenow fragment of DNA polymerase I in 20 μl of BRL REact 2 buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$). The mixture was incubated at 37 °C for 30 minutes, and the reaction was then stopped by heating for 5 minutes at 70 °C. The DNA was then recovered by ethanol precipitation or gel purification.

## 2.14 3' End labelling of DNA

DNA was prepared by restriction. 10 μCi of the appropriate [$\alpha$-$^{32}$P] dNTP was added to the DNA (~1.5 μg), with 1/10 vol. of a mix of the remaining 3 dNTP's (100 μM each), 1 unit of Klenow and $dH_2O$ to the appropriate volume. The mixture was incubated at room temperature for 15-20 minutes. 1 μl of a solution containing containing all 4 dNTP's at 1 mM each was added, and the mixture was left at room temperature for 5 minutes. This was followed by 1 phenol and 1 chloroform extraction, and the DNA was ethanol-precipitated. The labelled DNA was then redissolved in TE buffer.

## 2.15 Ligations

Ligations were carried out with 1-2 μg of vector DNA in a volume of 10 μl. They were carried out between restriction enzyme-digested plasmid DNA fragments, where the molar ratio of the vector to insert was 1:2, or in cases where double stranded oligonucleotides were ligated into a vector fragment, the ratio of vector to insert was 1:1. The buffer used was 1xBRL ligation buffer (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM DTT, 5% (w/v) polyethylene glycol-8 000) or 1xPromega ligation buffer (30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP), with 1 unit of T4 DNA ligase (BRL). The mixture was incubated overnight at room temperature, and subsequently used to transform competent cells.

## 2.16 DNase I nicking of DNA

DNA (20-100 µg) in C8 buffer (50 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 0.1 mM EDTA) with 300 µg/ml ethidium bromide and 1.6 µg/ml DNase I was incubated at 37 °C for 1 hour. DNase I was then removed by extraction with phenol 3 times followed by 1 chloroform extraction. The nicked DNA was ethanol-precipitated and redissolved in TE buffer.

## 2.17 Sequencing of double stranded plasmid DNA

The method used and all buffers were as described in the Sequenase T7 DNA polymerase technical manual (USB, 1990). These were based on the method of Sanger *et al.*,1977.

However, the plasmid template was prepared by an alternative method. 3 µg of DNA was incubated in 200 mM NaOH at 37 °C for 15 minutes. The sequencing primer (~10 pmol) was added, and the sample was mixed vigorously. The DNA was precipitated by adding 120 µl of chilled ethanol and 12.75 µl 5 M ammonium acetate (pH 7.5), and kept at -20 °C for 30 minutes. Pelleting was carried out by centrifugation (12 000 rpm, 30 minutes, 4 °C). The pellet was washed in 70% ethanol, repelleted and dried before resuspension in deionised $H_2O$. The template was then sequenced as described in the Sequenase protocol.

## 2.18 Oligonucleotides

Oligonucleotides used were synthesised on Applied Biosystems 391 or 392 oligonucleotide synthesiser or using standard cyanoethyl phosphoramidite chemistry. All reagents were supplied by Cruachem Ltd.

Randomly mutated oligonucleotides for the purposes of mutagenesis (see Chapter 4) were made by contaminating each of the four phosphoramidite reagents with 1% of each of the other three, resulting in a theoretical rate of mutagenesis of 2.5 base changes per 100 nucleotides.

Oligonucleotides used for mutagenic purposes are detailed where appropriate in the text, oligonucleotides used for sequencing purposes are detailed in Table 2.5.

| | OLIGONUCLEOTIDE SEQUENCE |
|---|---|
| P1 | CGACTCACTATAGGGGC |
| P2 | GGCTGGATTTGTGAGG |
| P3 | TTATTATCATGACATTAACC |
| P4 | GAGTTTGATGCTCAGGGTGTAG |
| P5 | CGACTCACTATAGGGGA |

Table 2.5 Details of sequencing primers

Primer P1 is outside the N-terminal end of the resolvase open reading frame in the p-SELECT-1 vector, priming extension of the top strand, while P2 is used to sequence the reverse strand from the C-terminal end of the open reading frame, again in p-SELECT-1. The primer P3, sits in the resolvase open reading frame, on the 5' strand. P4, also in the open reading frame of resolvase sits on the 5' strand and lies upstream of the *Cla*I restriction site. This primer was used in the sequencing the mutants created during random mutagenesis. P5 was used to sequence the expression plasmids derived from pET plasmids; it is from pET11 T-7 promoter region.

After synthesis, 1 ml of 30% aqueous ammonia was added to the glass support and the mixture was left at room temperature for 1-2 hours. The glass support was pelleted by centrifugation, and the supernatant was removed to a Nunc tube. A further 1 ml of 30% aqueous ammonia was added to the Nunc tube, and the cap was screwed on firmly before incubating in a 55 °C oven overnight. After cooling on ice, ammonium acetate was added to 0.5 M, and the oligonucleotide was precipitated by the addition of 2 volumes of ethanol. The oligonucleotide was then purified on a denaturing PAGE-Gel (see Section 2.21).

## 2.19 5' Phosphorylation of oligonucleotides

Phosphorylation of 100 pmol of oligonucleotide was carried out in 1x T4 kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) with 10 mM ATP. T4 kinase (5 units) was added and the reaction was kept at 37 °C for 30 minutes. The reaction was halted by heating at 70 °C for 10 minutes.

## 2.20 Annealing oligonucleotides

The procedure for annealing oligonucleotides to single stranded DNA can be found in the p-SELECT-1 manual (Promega). This procedure was also used to anneal two complementary oligonucleotide strands. Roughly equal amounts of purified complementary oligonucleotides (1-10 pmol) were mixed in TE buffer and heated at 70 °C for 5 minutes. The oligonucleotides were then cooled to room temperature over a period of 20 minutes. Oligonucleotides were now annealed and could be stored at -20 °C.

## 2.21 Purification of synthetic oligonucleotides

Oligonucleotides were purified in the following manner. 400 µl of the solution of crude oligonucleotide in 30% ammonia (Section 2.3) was precipitated by adding ammonium acetate to a final concentration of 0.5 M, followed by 2 volumes of ethanol. The mixture was left at -20 °C for 30 minutes. The oligonucleotide was pelleted by spinning in an Eppendorf microcentrifuge (15 K, 30 minutes, 4 °C). The supernatant was removed, and the pellet was redissolved in 20 µl of TE. A further 20 µl of Formamide loading buffer (Section 2.23) was added, and the samples were run on a denaturing polyacrylamide gel (Section 2.22). The DNA was then visualised using 'Stains all' (1-ethyl-2-[3-(1-ethylnaphtho[1,2-d]thiazolin-2-ylidene)-2methylpropenyl) naphtho[1,2-d]thiazolium bromide; supplied by Aldrich). The gel was immersed in 70 ml of deionised $H_2O$ + 20 ml isopropanol, and 10 ml of 0.1% (w/v) solution of 'Stains all' in formamide was added. The dish was gently shaken until DNA was observed. The full length oligonucleotide, usually the slowest and most abundant product on the gel, was removed using a scalpel blade, and the gel slice was crushed in 1 ml of TE buffer in a Nunc tube. This was placed on a rotating wheel mixer at 37 °C for 16 hours. The acrylamide was then removed from the TE/oligonucleotide by spinning through a 0.45 µm cellulose

acetate filter (Spin-x, supplied by Costar). The oligonucleotide was ethanol-precipitated, and spun, and the pellet was washed in 70% ethanol before redissolving in TE buffer.

## 2.22 Electrophoresis

### 2.22.1 Agarose gel electrophoresis

**1xTAE buffer** : 40 mM Tris-acetate (pH 8.2), 20 mM sodium acetate, 1 mM $Na_2EDTA$

Gels (0.8%, 1.0%, or 1.2% w/v agarose) were prepared by dissolving the appropriate amounts of agarose powder in 1xTAE buffer by heating in a microwave oven. The solution was allowed to cool to 60 °C and poured into a gel former with the appropriate comb, then left to set at room temperature. When pouring gels made of Low Melting Point agarose (Seaplaque) the gel was allowed to set at 4 °C to ensure the formation of the wells. Two sizes of gels were in general used; 'Large gels' required 250 ml of buffer to form the gel and 3.2 l of buffer to fill the gel tank. Gels were run at 40 V for 16 hours, or 100-150 V for shorter periods of time. 'Mini gels' used 40 ml of buffer + agarose to form the gel and 400 ml in the gel tank to run the gel. These gels were electrophoresed at 80-100 V for the required period of time.

### 2.22.2 Acrylamide gel electrophoresis

The various polyacrylamide gels used were all basically set up in the same manner. 0.75 mm spacers were inserted between glass plates along with a length of rubber tubing in order to form a seal. The plates were clamped together and the gel mixture was poured between them. A comb was inserted to form the wells, and this was also clamped in place. The acrylamide was allowed to polymerise for at least 1 hour before the tubing and comb were removed, and the gel was placed in the gel kit. The required volume of buffer was added to the tank reservoirs, and the gel was run at a constant voltage for the required time.

**a) Non-Denaturing Electrophoresis**

**10xTBE buffer** : 89 mM Tris-base, 89 mM boric acid, 0.2 mM $Na_2EDTA$ (the resulting pH is 8.3).

Standard polyacrylamide gels were used in the analysis of restriction digests and for the purification of small double stranded DNA fragments. For a 30 ml X% polyacrylamide gel, X ml 30% acrylamide : 0.8% bisacrylamide (w/v), 3 ml 10 x TBE buffer, (27-X) ml $H_2O$, 360 µl 10% ammonium persulphate (w/v) (APS) and 18 µl TMED (N, N, N', N'-tetramethylethylenediamine) were mixed, then poured as described above. Reservoirs were filled with 1 x TBE buffer, and the gel was run at a constant voltage of 200 V at room temperature for 3-4 hours. Ethidium bromide was used to stain the gel.

A second non-denaturing PAGE method was used for resolvase band-shift assays. A 6% gel was prepared with 6 ml 30% acrylamide : 0.8% bisacrylamide (w/v), 3 ml 500 mM Tris-glycine (pH 9.4), 15 µl 200 mM EDTA, 21 ml $H_2O$, 360 µl 10% APS (w/v) and 18 µl TMED. The running buffer was 50 mM Tris-glycine (pH 9.4), and 0.1 mM EDTA in deionised water. The gel was poured as previously described, but care was taken to keep all equipment detergent-free. Electrophoresis was carried out at 4 °C at a constant voltage of 200 V.

**b) Denaturing polyacrylamide gels**

Denaturing gels were used for plasmid sequencing and for purification of synthetic oligonucleotides. For a standard 6% polyacrylamide sequencing gel, 80 ml of Sequagel-6 was mixed with 20 ml of Sequagel Complete (supplied by Protogel), then 600 µl of 10% APS (w/v) was added to the mixture. The mixture was poured between the glass plates, a shark's tooth comb was inserted (flat edge inwards) and the gel was left to polymerise at room temperature for 20 minutes. 1 volume of formamide loading buffer was added to samples (see Section 2.23), which were then heated to 80 °C for 5 minutes prior to loading. Two sequencing gel kits were used; an IBI Base Runner kit , which was pre-run at 45 W for 1 hour and run at 45 W for 2-3 hours, and a BRL model S2, which was pre-run at 100 W for 45 minutes before running for 1.5-3 hours at 60 W. The gel was dried under vacuum and autoradiographed as described in Section 2.31.

Purification of oligonucleotides was also carried out on denaturing gels, in a smaller gel kit, but due to variations in length a variety of gel percentages was used. For 100 ml of a 10% polyacrylamide/7 M urea gel, 46 g Urea, 15 ml of 40% (w/v) acrylamide (19 : 1 ratio of acrylamide to bisacrylamide), 10 ml of 10 x TBE, 666 µl of 10% (w/v) APS and 40 µl of TMED was made up to 100 ml with deionised water. This was poured between glass plates, and a comb was inserted to form the wells. The gel was polymerised at room temperature for 1 hour before samples, to which 1 volume of formamide loading buffer had been added, were loaded. The gel was pre-run at 15 W and run, also at 15 W, for 2-4 hours.

Protein denaturing gels were also run; these were of the type described in Laemmli, 1970. They were run in a Biorad "Miniprotean" gel kit. Gels were made and run according to the manufacturers instructions.

## 2.23 Analysis by single colony gel

**Single colony gel lysis buffer** : 2.5% Ficoll, 1.25% SDS, 0.01% Bromophenol Blue in 1xTAE buffer.

This technique was used to confirm the presence and size of plasmids in *E. coli* . Transformants were patched onto fresh selective agar plates and grown at 37 °C for ≥ 8 hours. Bacteria were scraped from the patches with a toothpick, then twiddled into Single colony gel lysis buffer and the samples were incubated at room temperature for 15 minutes, then spun in an Eppendorf microcentrifuge ( 15 K, 30 minutes, 4 °C). Approximately 30 µl was loaded onto a horizontal 1.2% agarose gel, described in Section 2.20.

## 2.24 Purification of DNA from low melting point agarose

The low melting point agarose used was Seaplaque GTG (supplied by FMC Inc.). The gels were run as described in Section 2.20 and stained with ethidium as detailed in Section 2.28. After visualising the DNA on a long wavelength transilluminator (365 nm), the DNA was removed by cutting out a gel chip with a scalpel blade. The chip was

melted at 65 °C for 5 minutes and 100 µl of TE buffer was added to reduce the agarose concentration, which makes it easier to extract and makes the volume more manageable. The agarose was removed by three phenol extractions, followed by one chloroform extraction. The DNA was ethanol precipitated, washed with 70% ethanol and redissolved in the required volume of TE.

## 2.25 Loading buffers

The following loading buffers were used throughout this thesis.

**SDS-Loading buffer** : 50% glycerol, 1% SDS, 0.01% bromophenol blue

**Formamide loading buffer** : 80% deionised formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol, 1mg/ml bromophenol blue

**Laemmli loading buffer** : 50 mM Tris-HCl (pH 6.8), 1% SDS, 10% (w/v) glycerol. 0.01% bromophenol blue.

## 2.26 Size markers

The BRL 1 kb ladder was used as a marker on agarose and acrylamide gels to help determine the size of DNA run on these gels.

Purified resolvase was used as a marker on protein gels.

## 2.27 UV spectrophotometry

Concentrations of DNA were measured by diluting the DNA, then measuring the absorbance at 260 nm in a UV/visible spectrophotometer (Shimadzu).

The concentration of double stranded DNA was calculated by approximation that a solution with an absorbance of 1 at 260 nm in a 1 cm cell contains 50 µg/ml of double stranded DNA.

Synthetic oligonucleotide concentration was estimated using the relationship ;

$$A = \varepsilon c l$$

A = Absorbance at 260 nm

ε = Extinction coefficient, 10 000 l $mol^{-1}cm^{-1}$ per nucleotide

c = Molar concentration

l = Path length of the cell (cm)

## 2.28 Staining of gels

### 2.28.1 Visualising DNA

DNA was visualised in agarose and acrylamide gels by staining with an ethidium bromide solution (0.6 μg/ml) in 1 x TAE buffer. Staining was carried out for 30-60 minutes; the gel was then rinsed and soaked in water for 60 minutes to remove any background staining which had occurred. DNA was visualised on a 254 nm UV short wavelength transilluminator.

### 2.28.2 Visualising proteins

After electrophoresis on a Laemmli poyacrylamide gel (Section 2.20) proteins were visualised using the following reagents;

**Coomassie Stain** : 1% Coomassie Blue, 50% MeOH, 10% AcOH

**Coomassie Destain** : 10% MeOH, 10% AcOH

Proteins electrophoresed on an SDS polyacrylamide gel were stained with Coomassie stain for 30 minutes, then destained as required. Bands were then observed by illumination on a light box.

## 2.29 Photography, autoradiography & phosphorimagery

Agarose and polyacrylamide gels were photographed using a Polaroid camera and Polaroid 667 film, or a Pentax SLR camera loaded with Ilford HP5 35 mm film. Ilford film was processed using Ilford Microphen developer (undiluted, 11 minutes at 20 °C), followed by fixing with Amfix (diluted 1 + 3, 5 minutes at 20 °C).

For autoradiography purposes, the gel was dried under vacuum in a Bio-Rad slab gel dryer, and exposed overnight to a sheet of Fuji RX100 film. This was developed in an X-OMAT automated processor (Kodak).

Phosphorimaging was carried out on gels dried as above, then exposed overnight to phosphor screens. The screens were processed in a Fuji BAS-1000 phosphor-imaging system.

## 2.30 Mutagenesis

### 2.30.1 p-SELECT-1 based directed mutagenesis

The method used was as described in the 'Altered Sites' technical manual (Promega), but the mutagenesis template was prepared by denaturation of double stranded plasmid DNA (Section 2.6) which had previously been nicked by DNase I (Section 2.19).

### 2.30.2 Random mutagenesis

The procedure for random mutagenesis using mutated synthetic oligonucleotides is detailed fully in Chapter 4.

## 2.31 Screening of resolvase mutants

The screening of mutated Tn*3* resolvase was as described in Blake, 1993 and is described fully in Chapter 4.

## 2.32 Expression and purification of Tn*3* resolvase and derived mutants

The expression system used to overproduce Tn*3* resolvase and its mutants is described in Chapter 5. The purification protocol developed is detailed in Chapter 5.

## 2.33 *In vitro* recombination by Tn*3* resolvase

Site-specific recombination reactions involving Tn*3* resolvase and mutants thereof were carried out in several reaction buffers, detailed below.

**C9.4 buffer** : 50 mM Tris-HCl (pH 9.4), 10 mM $MgCl_2$, 0.1mM EDTA
**C8 buffer**: 50 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 0.1 mM EDTA
**M15M8 buffer:** 50 mM Tris-HCl (pH 8.2), 5 mM $MgCl_2$, 5 mM spermidine, 0.05 mM EDTA, 20% v/v glycerol.

A 20 µl reaction in the appropriate buffer contained 0.4 µg of plasmid DNA with 0.05 vol. (1 µl) of diluted resolvase. Incubation at 37 °C was carried out, the duration being dependent on the protein and buffer involved. The reaction was then stopped by heating at 70 °C for 5 minutes. In general, 60 µl reactions were carried out and split into three aliquots of 20 µl after incubation. Recombination products were then analysed by agarose gel electrophoresis of :-

i) Untreated samples
ii) Samples digested with restriction enzymes
iii) Samples nicked with DNase I (Section 2.19)

Prior to loading, 5 µl of SDS loading buffer was added to all 20 µl samples.

## 2.34 Band shift analysis

This was carried out as described in Bednarz, 1989 and Blake, 1993.

50 µg/ml of non-specific carrier DNA (supercoiled pUC71K) was combined with the appropriate amount of end-labelled *res* DNA (~200-300 cps) in Binding buffer (10 mM Tris-glycine pH 9.4, 0.1 mM EDTA, 10% glycerol). The mix was divided into 10 µl aliquots and 0.4 µl of a resolvase dilution was added. The samples were incubated at 37 °C for 15 minutes, then placed on ice until loading on a non-denaturing polyacrylamide gel (Section 2.20). Electrophoresis was carried out at 200 V for 3-4 hours at 4 °C.

# Chapter 3

Construction of a resolvase mutant selection system

# Construction of a resolvase mutant selection system

## 3.1 Introduction

In order to investigate the role of sites II and III of Tn*3* resolvase in the formation of the synapse, it was decided to search for mutants which require only the presence of two site I's for synapsis and recombination. The positions and types of these mutants, and analysis of the products of such a recombination event might help determine the role of sites II and III as well as the actual mechanism of the reaction (e.g. by facilitating crystallisation of an active form of the protein).

The analogy of sites II/III of *res* with the enhancer element required by DNA invertases was noted above (see Introduction). Mutants of the DNA invertase, Gin, have been isolated which can carry out inversion *in vitro* without the presence of the accessory factor, Fis (Klippel *et al.*, 1988a, 1988b; Klippel *et al.*, 1993; Merker *et al.*,1993). Some mutants of Cin have also been reported to carry out recombination in the absence of the enhancer element (Haffter and Bickle, 1988). Hin mutants have also been isolated which are independent of Fis activation *in vitro* (Yuan *et al.*, 1991; Haykinson *et al.*, 1996).

Comparison of the sequences of the resolvases and Gin has shown that the positions of the Fis-independent Gin mutations are in the region which has been shown in γδ resolvase to be involved in protein/protein contacts of the 1-2 dimer (Hughes *et al.*, 1993). In the co-crystal structure of γδ resolvase (Yang and Steitz, 1995) this region is part of the E-helix, a long α-helical segment extending from amino acid residue M103 to G137. Analysis of the Gin mutants supports a model for Gin inversion which is related to models for resolution by Tn*3* resolvase, whereby Fis bound to the enhancer helps in the formation of a synapse of defined topology, allowing the *gix* sites at which Gin is bound to recombine. This model equates site I of *res* with the *gix* sites, and sites II and III (the accessory sites) with the enhancer.

Previous attempts to create Tn*3* resolvase site II/III independent mutants using chemical mutagenesis or mutated oligonucleotides were unsuccessful (Blake, 1993). Since the Gin and Hin mutants found to be independent of Fis were present in the E-helix of the

protein, this seemed the most likely place to target the search for Tn*3* mutants. It was therefore decided to adopt the strategy of specifically mutagenising the sequence corresponding to the region of the E-helix at a high level.

To search for mutants of Tn*3* resolvase which could promote recombination of a substrate containing only two copies of site I, or one complete *res* site and one isolated site I, it was necessary to construct an appropriate expression vector in which the mutants would be created. The vector would be compatible with the *in vivo* screening system which already existed in the lab (Blake, 1993).

## 3.2 Screening of Tn*3* resolvase mutants

The *in vivo* selection system existing in the lab was based on substrates containing the *galK* gene, and was fully described in Blake, 1993.

This system was adapted from resolution substrates such as pMA21 (Bednarz *et al.*, 1990). Resolution of pMA21 results in two smaller circular products, one of which contains the ampicillin resistance gene (*Bla*) and the pBR322 origin of replication. Only this product therefore has the ability to be maintained *in vivo*, while the other product (carrying the tetracycline resistance gene of pBR322) is lost (Figure 3.1). Thus colonies containing only resolved products could be identified by replica plating and identification of $Ap^rTc^s$ colonies against a background of colonies containing $Ap^rTc^r$ unresolved pMA21. By cloning a *galK* cassette (McKenney *et al.*, 1981) into the tetracycline resistance gene region of pMA21, the assay was made more direct. Cloning disrupted the $Tc^r$ gene, and allowed an assay for resolution whereby $Ap^r$ *galK* isolates (containing only the resolved plasmid) could be identified when using $Ap^s$ *galK* cells wild type for the other genes involved in the uptake and metabolism of galactose (e.g. DS941; Miller, 1972). A further refinement was to increase the sensitivity of the assay by reducing the copy number of the substrate plasmid, by replacing the pBR322 origin of replication with that of pSC101 (Blake, 1993). The original system used by Blake (1993) was considered to be non-optimal because the test plasmids had the high copy number pBR322 origin of replication. The high copy number means that very efficient resolution by a mutant is required to delete all *galK* genes from individual cells, thus increasing the

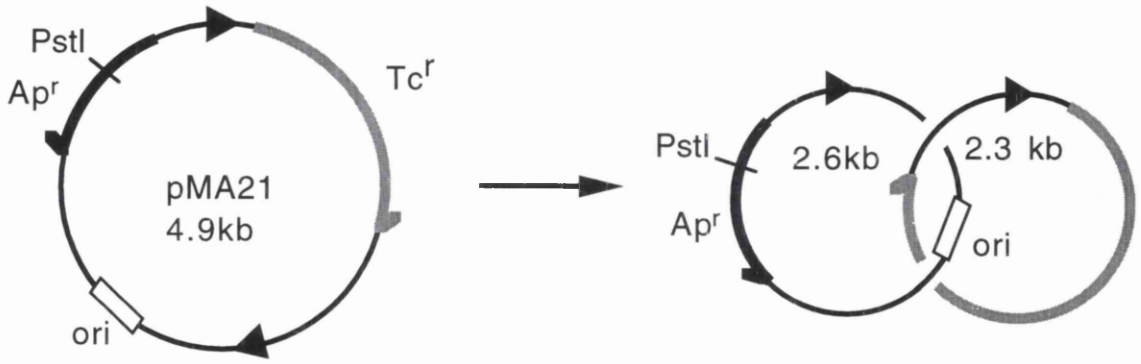



**Figure 3.1 Resolution of pMA21**

Illustration of a standard resolution plasmid, pMA21, containing two copies of *res* in direct repeat, and the products formed by resolution.

difficulty of isolation of mutants. Also, for some earlier strategies for making mutants it was desirable to use the commercially available plasmid p-SELECT-1, which has the pBR322 origin and $Ap^r$ resistance marker. Four new plasmids, pDB34, pDB35, pDB36 and pDB37, were therefore constructed (Blake, 1993) with $Km^r$ marker and the low copy number pSC101 origin of replication. However, they had not been used in resolution assays prior to this work. The plasmids are illustrated in Figure 3.2.

MacConkey galactose plates were used to screen the colonies; on these plates *galK*$^-$ colonies are pale yellow/white, while *galK*$^+$ colonies are red. MacConkey agar contains 2-methyl-3-amino-6-dimethyl-aminophenazine (Neutral red). This indicator is red at pH < 6.8 and yellow at pH > 8.0. Bacterial fermentation of reducing sugars (e.g. galactose) lowers the pH of the medium, giving rise to colonies which are red/pink. If galactose is the only sugar present, *galK*$^-$ cells are forced to metabolise amino acids in the media. This produces ammonia which causes an increase in pH, resulting in yellow/white colonies.

Using this system we were able to screen for mutants with various properties. Not only did we possess a screen for a mutant able to resolve a plasmid containing two site I's, but screens using pDB36 and pDB37 (plasmids containing site I v *res* ) were potentially useful since one round of mutagenesis might not produce a resolvase active on a site I v site I plasmid. A mutant resolving pDB36 or pDB37 might provide a stepping stone to eventually isolating such a mutant.

## 3.3. Development of a mutagenesis plasmid

Construction of a plasmid for the mutagenesis involved the modification of a p-SELECT-1 based plasmid. A suitable plasmid should express a low level of resolvase (to ensure compatibility with the *in vivo* screen), and should contain two unique restriction sites positioned approximately at the ends of the sequence corresponding to the E-helix of resolvase, allowing this region to be removed by restriction digest for the insertion of sequence containing mutations. The open reading frame of resolvase already contains a *Bam*HI site which corresponds to a position near the C-terminal end of the E-helix. To make use of this restriction site, other pre-existing *Bam*HI sites in the p-SELECT-1

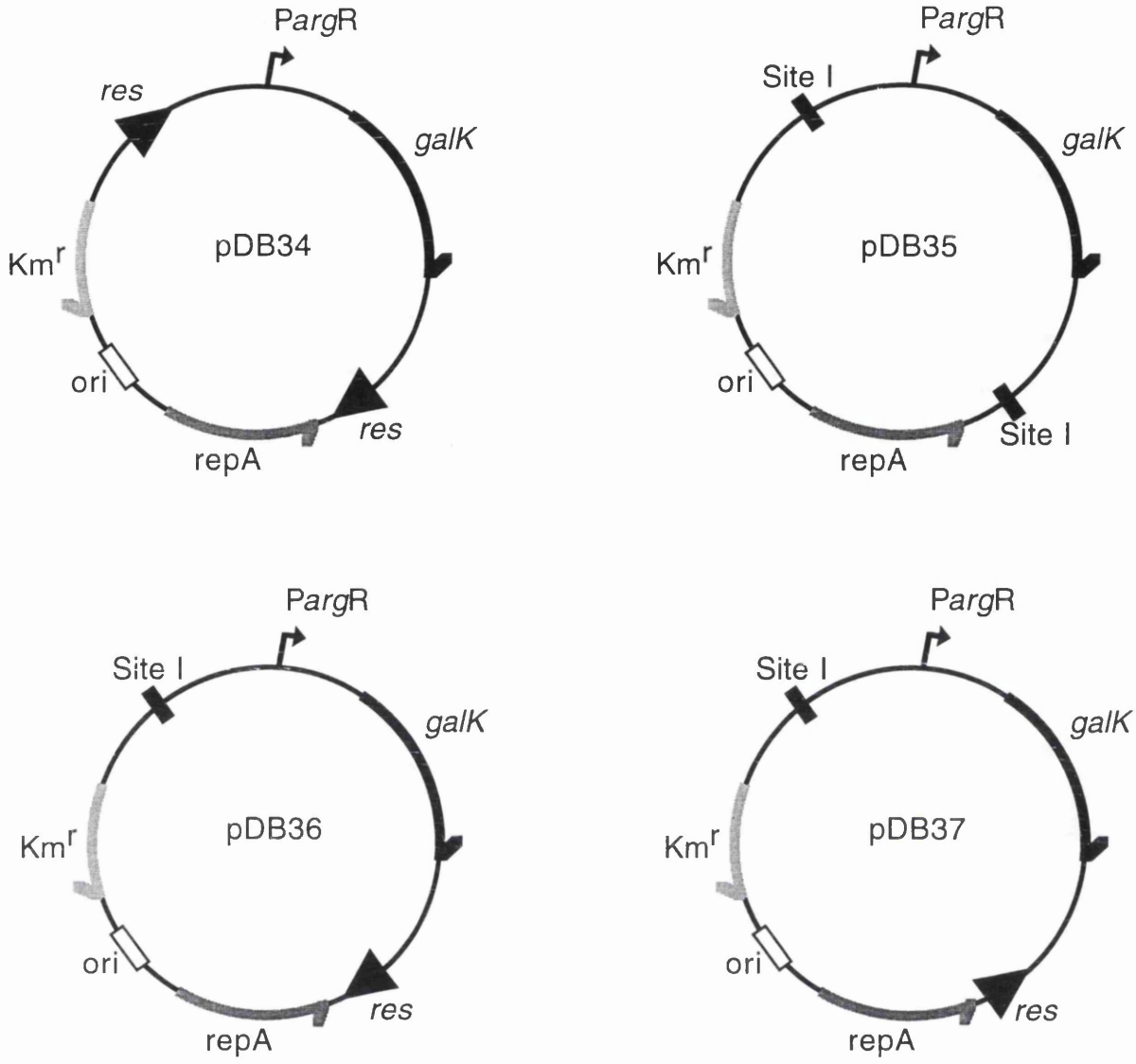



**Figure 3.2 Diagram of resolvase screening plasmids**

The four resolution plasmids used to screen for resolvase mutants. The origin of replication and repA gene for replication initiator protein are from pSC101. *galK* is transcribed from P*arg*R, the promoter of the *E. coli argR* gene.

derived plasmid had to be removed. No restriction site existed at the other end of the E-helix, approximately 80 base pairs upstream, and since the introduction of a new restriction site must not alter the amino acid sequence, its possible type and position were limited. It was found by inspection of the sequence that a *Cla*I site could be introduced in the required region. This again would require the removal of the existing *Cla*I site just outside the tetracycline gene in p-SELECT-1.

### 3.3.1 Site-directed mutagenesis of pSELECT-1

The open reading frame of resolvase was cloned from pMA6111 (Blake, 1993) into p-SELECT-1. pMA6111 was digested completely with *Ssp*I and this was followed by a partial *Bam*HI digest. The desired *tnp*R fragment (742 bp) was gel-purified. A *Sal*I/*Sma*I digest was carried out on p-SELECT-1, and the 5665 bp fragment was also gel-purified. Both fragments were treated with Klenow DNA polymerase to fill in the ends prior to ligation. The ligation mix was transformed into DS941 competent cells, and transformants were investigated to determine the number of *Bam*HI sites present, by restriction digest of DNA prepared by alkaline lysis. Filling in the ends of the insert removed the unwanted *Bam*HI site outside the open reading frame. This plasmid, pAT1, is illustrated in Figure 3.3.

After confirmation that only one *Bam*HI site existed in the plasmid, it was necessary to remove the *Cla*I site near the tetracycline gene. Initially this was attempted by restriction digestion with *Cla*I followed by filling in with Klenow enzyme and religation. However, after transformation and selection with tetracycline, the only transformants observed contained a *Cla*I site. It was observed that the position of the *Cla*I site is close to the promoter of the tetracycline gene on the plasmid. "Filling in" the ends alters the spacing between the -10 and -35 boxes by 2 bp, presumably resulting in successfully filled in clones being tetracycline-sensitive and selected against (Pruss and Drlica, 1986). Tetracycline resistance is the only antibiotic marker present in the plasmid.

It was decided to overcome this problem by an alternative approach. At the same time as annealing the oligonucleotide to create the new *Cla*I site in the open reading frame, and the ampicillin repair oligonucleotide as a means of selection, a third oligonucleotide would

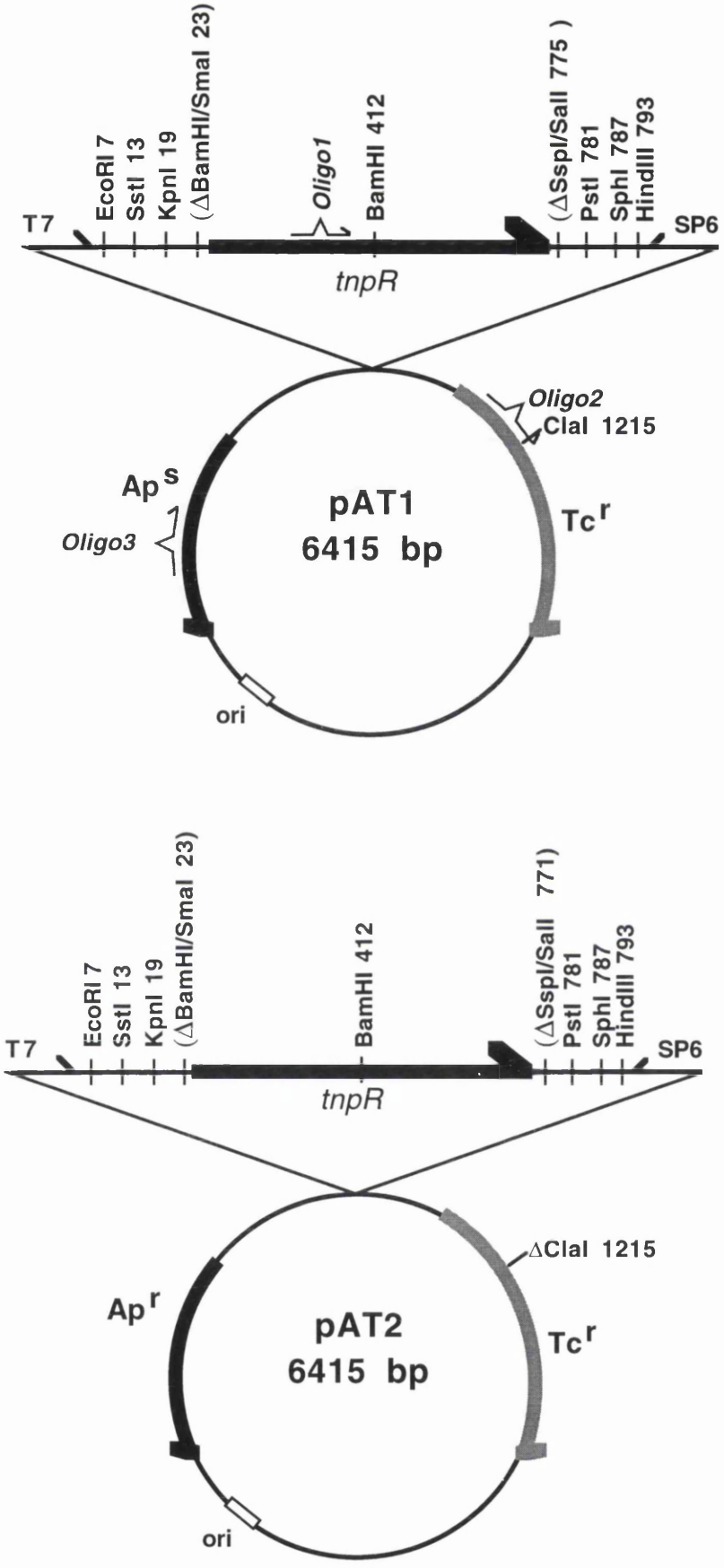



**Figure 3.3 Illustration of pAT1 and pAT2**

Steps involved in constructing pAT2 involved the repair of the Ampicillin gene and the removal of the *Cla*I site in the Tetracycline gene.

be annealed to remove the existing *Cla*I site, by changing one base pair, without altering the length of the promoter sequence. Site-directed mutagenesis was carried out as described in the p-SELECT manual (Promega, 1991) on single stranded DNA of pAT1 (Section 2.30.1). The oligonucleotides used for this are detailed in Figure 3.4.

After multiple attempts, this approach proved unsuccessful. The majority of the clones isolated, though having the ampicillin resistance gene and a *Cla*I restriction site, were shown to be approximately 2.5 kb, probably indicating that a deletion had taken place. Further attempts yielded similar results.

By cloning half of the open reading frame stretching between the *Eco*RI site and the *Bam*HI site into p-SELECT, we effectively removed a segment of DNA containing a direct repeat. The construct retained the region of the gene required for mutagenesis. However mutagenesis on this construct , pAT1.1 was also not successful. Similar difficulties in site-directed mutagenesis of *tnp*R have been encountered before, both in this lab and in Nigel Grindley's group (M. Boocock, personal communication).

From the many clones isolated, one was found to have removed the existing *Cla*I site, though it did not contain the desired new *Cla*I site. This clone was the correct size, as determined by Single Colony gel analysis, and conferred resistance to both ampicillin and tetracycline. This plasmid, pAT2, is shown in Figure 3.3.

It was decided to discontinue this approach to introduce the new *Cla*I site in the *Tnp*R, but the plasmid which had been created, pAT2, proved useful in the alternative approach described in Section 3.3.2.

### 3.3.2 The creation of a *TnpR* cassette

Instead of introducing the required restriction sites by site-directed mutagenesis, it was decided to create synthetically two thirds of the resolvase open reading frame by annealing and cloning a series of complementary oligonucleotides, which would maintain the wild type amino acid sequence of resolvase, but which would contain altered nucleotides introducing restriction sites at various points. These oligonucleotides would

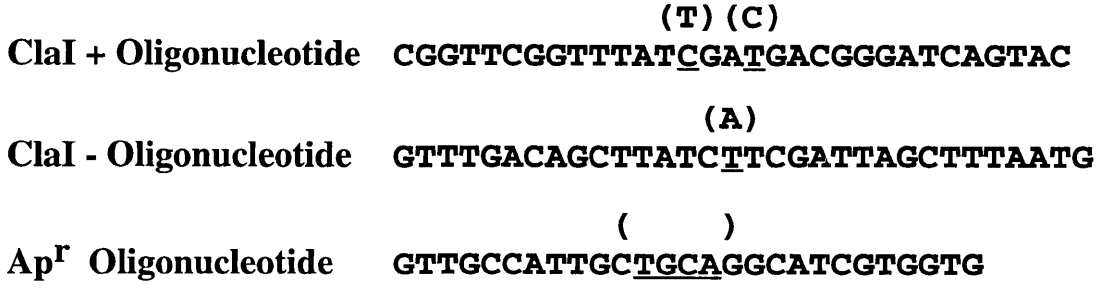



**Figure 3.4 Details of Oligonucleotides for Mutagenesis**

The sequences of the oligonucleotides which introduce the new *Cla*I site in the open reading frame, remove the existing *Cla*I site in the tetracycline gene promoter, and repair the Ampicillin gene in order to allow a means of selection. The original bases are shown in brackets above the oligonucleotides.

utilise the *Mlu*I site near the beginning of the open reading frame present in pSA1101 (S. Rowland, unpublished results; Figure 3.5), and continue to the *Bam*HI site, 341 bp downstream.

In order to carry this out, the resolvase reading frame containing the *Mlu*I site, from pSA1101, had to be cloned into the p-SELECT-1 based vector, pAT2. This was followed by the cloning and piecing together of the required series of oligonucleotides.

pSA1101 was digested with *Xba*I, and the ends were filled in with Klenow enzyme. The 700 bp fragment was gel purified on a mini Seaplaque agarose gel. pAT2 was digested with *Eco*R1 and *Hin*dIII, the ends were filled in with Klenow, and the large 5.639 kb fragment was gel-purified. The two fragments were ligated, and the ligation mixture was used to transform competent DS941 cells. Ligation of these blunt ended fragments recreated both *Xba*I sites and the *Eco*RI site, but removed the *Hin*dIII site originally in the vector. This was useful since a unique *Hin*dIII site could then be incorporated into the cassette. Since a blunt ended ligation was carried out, the insert had the potential to be in either orientation. After the isolation of possible clones, the orientation of the insert was confirmed by analysis of a restriction digest (*Eco*RV to *Mlu*I) . Two clones were isolated which had incorporated the fragment, in different orientations. These clones (pAT3a, pAT3b; Figure 3.6) were checked for resolvase expression *in vivo* using the *galK* system (Section 3.2). Both were shown to express sufficient resolvase to resolve the test plasmid, pDB34 (bearing the *galK* gene) and it was decided to use the clone, pAT3a, in which the reading frame had inserted in the same direction as the T7 promoter (since this was similar to previously constructed plasmids, e.g. pDB6020; Blake 1993). However, it is still uncertain as to whether expression is from the T7 promoter (by *E. coli* RNA polymerase) or an "anonymous" promoter elsewhere.

### 3.3.3 Oligonucleotide cloning and assembly

In designing the oligonucleotides which would comprise the cassette, it was important to make them of a length which was relatively easy to synthesise and manipulate, but this was dependent on the possible positions at which restriction sites could be introduced without changing the encoded amino acid sequence. Figure 3.7 shows a schematic

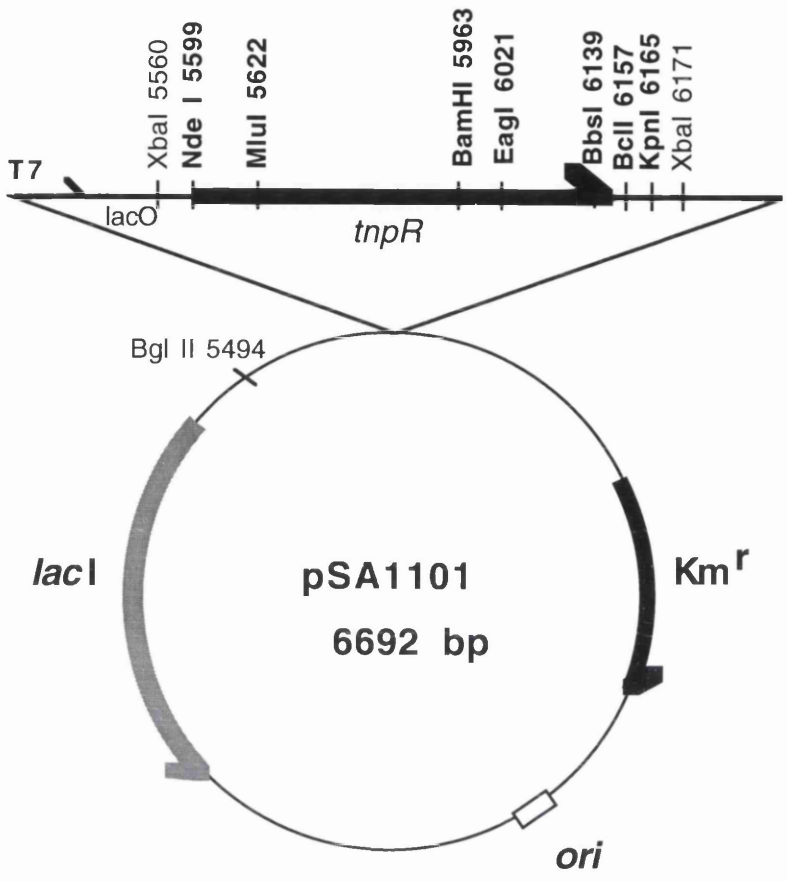



**Figure 3.5 Illustration of pSA1101**

Illustration of the expression plasmid pSA1101. Full details of its construction can be found in chapter 5.

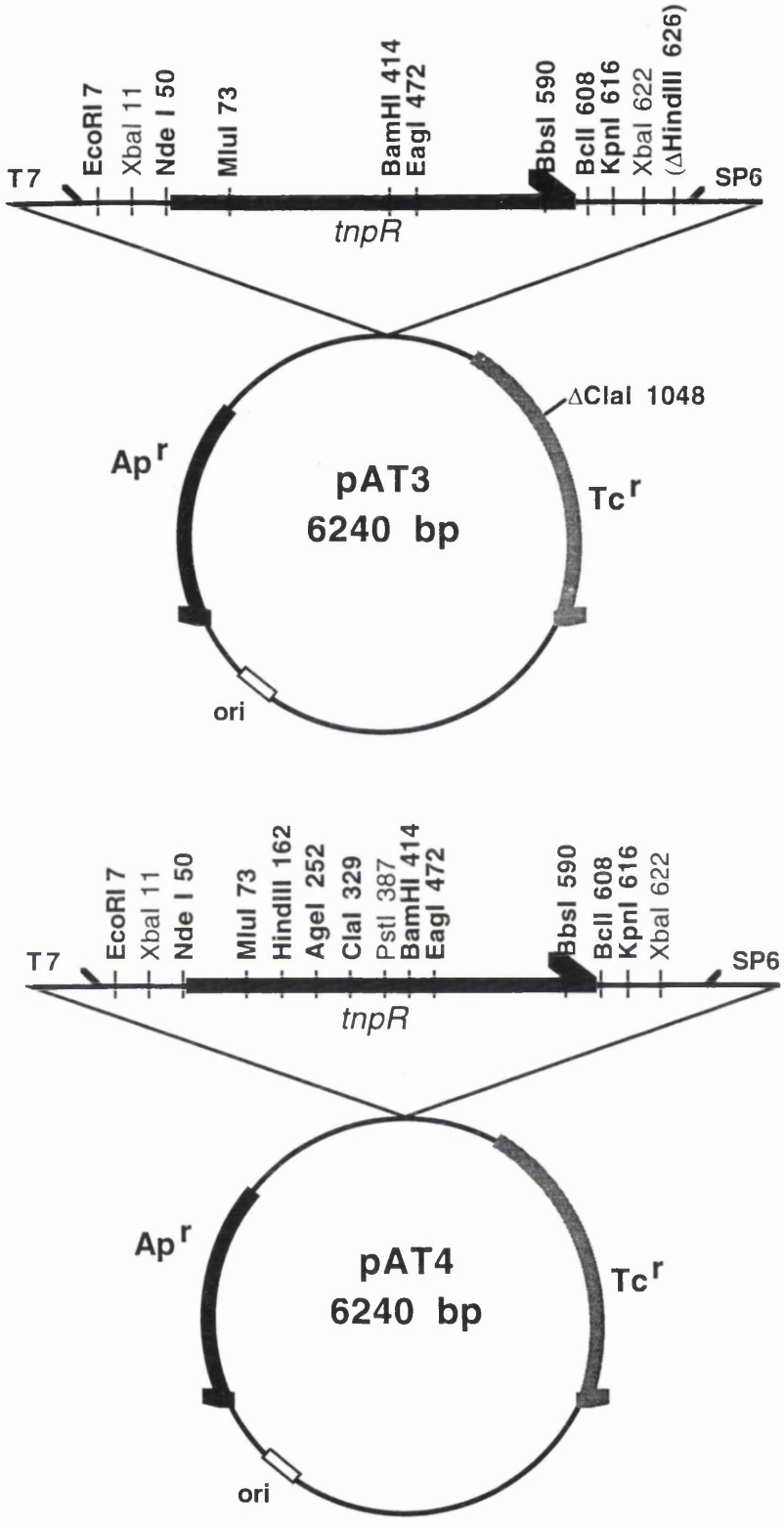



**Figure 3.6 Illustration of pAT3 and pAT4**

This shows the development of the required plasmid for mutagenesis, with the introduction of the new restriction sites in pAT4. pAT3 was created by cloning the open reading frame of Tn*3* resolvase from pSA1101 into pAT2. pAT4 was then constructed by the insertion of oligonucleotides (Figure3.7) into pAT3. The *Pst*I site at position 387 is not unique (see text).

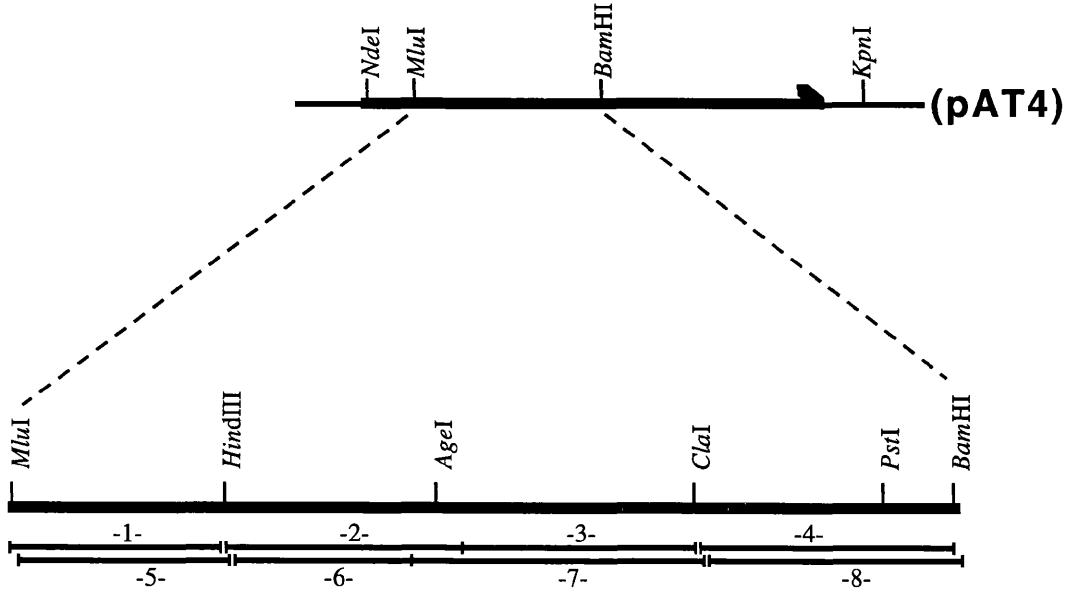



**Figure 3.7 Cassetting of the Tn*3* Resolvase open reading frame**

The diagram shows the open reading frame of Tn*3* resolvase with the *Mlu*I and *Bam*HI sites existing in pSA1101, the positions of the new unique restriction sites to be introduced, and the eight oligonucleotides used in the construction of pAT4.

diagram of the positioning of the chosen new restriction sites. The eight oligonucleotides required were between 76 and 90 bp in length, and their sequences are shown in full in Figure 3.8. In addition to the introduction of unique restriction sites ~ 80 bp apart, a *Pst*I site was introduced near the *Bam*HI site. This site exists in the open reading frame of γδ resolvase, and its incorporation into the Tn*3* open reading frame allows for the future production of γδ/Tn*3* hybrid mutant proteins, which may yield important information.

The oligonucleotides were synthesised by standard methods (Section 2.18) and purified on a 10% polyacrylamide gel (Section 2.22.2b). The oligonucleotides were then annealed (Section 2.20) in the following sets; 1+5, 2+3+6+7, and 4+8. After annealing they were cloned into pMTL23, a standard cloning vector used in this laboratory (Chambers *et al.*, 1988) to create three intermediate plasmids, pAT1/5, pAT23/67 and pAT4/8 (Figure 3.9). The inserts were sequenced to ensure that they carried no mutated bases. Once this had been confirmed, the cloned oligonucleotides were put together in the following manner. The fragment *Mlu*I to *Hin*dIII (1+5) was removed by restriction digest from pAT1/5 and purified. This fragment was then cloned into pAT23/67, containing the oligonucleotides creating the open reading frame from the *Hin*dIII to the *Cla*I site (2+3+6+7), which had been digested with *Mlu*I/*Hin*dIII and the large fragment gel-purified. Confirmation that the six oligonucleotides were now present was obtained by restriction digest analysis, and this plasmid was named pAT123. Unfortunately, due to the positioning of the *Cla*I and *Bam*HI restriction sites in pMTL23, the remaining double stranded oligonucleotide stretching between these sites (4+8), present in pAT4/8, could not be sequentially attached to the others prior to cloning into the vector. To achieve the final construct, a three way ligation was set up. The section corresponding to the cloned oligonucleotides was removed from pAT123 by *Mlu*I/*Cla*I restriction digestion, and the 240 bp fragment was purified. The oligonucleotides 4+8 were removed from pAT4/8 by restriction digestion with *Cla*I/*Bam*HI and the 80 bp fragment was gel-purified. These were ligated with pAT3 which had been digested with *Mlu*I and *Bam*HI and gel-purified. The construction, pAT4, is illustrated in Figure 3.10.

After transformation of the *E. coli* strain DS941, colonies were patched out, and the cloned plasmids were observed on a single colony gel. Alkaline lysis mini-preps (Section 2.9.2a) were prepared from patches showing the presence of the correct sized plasmid.

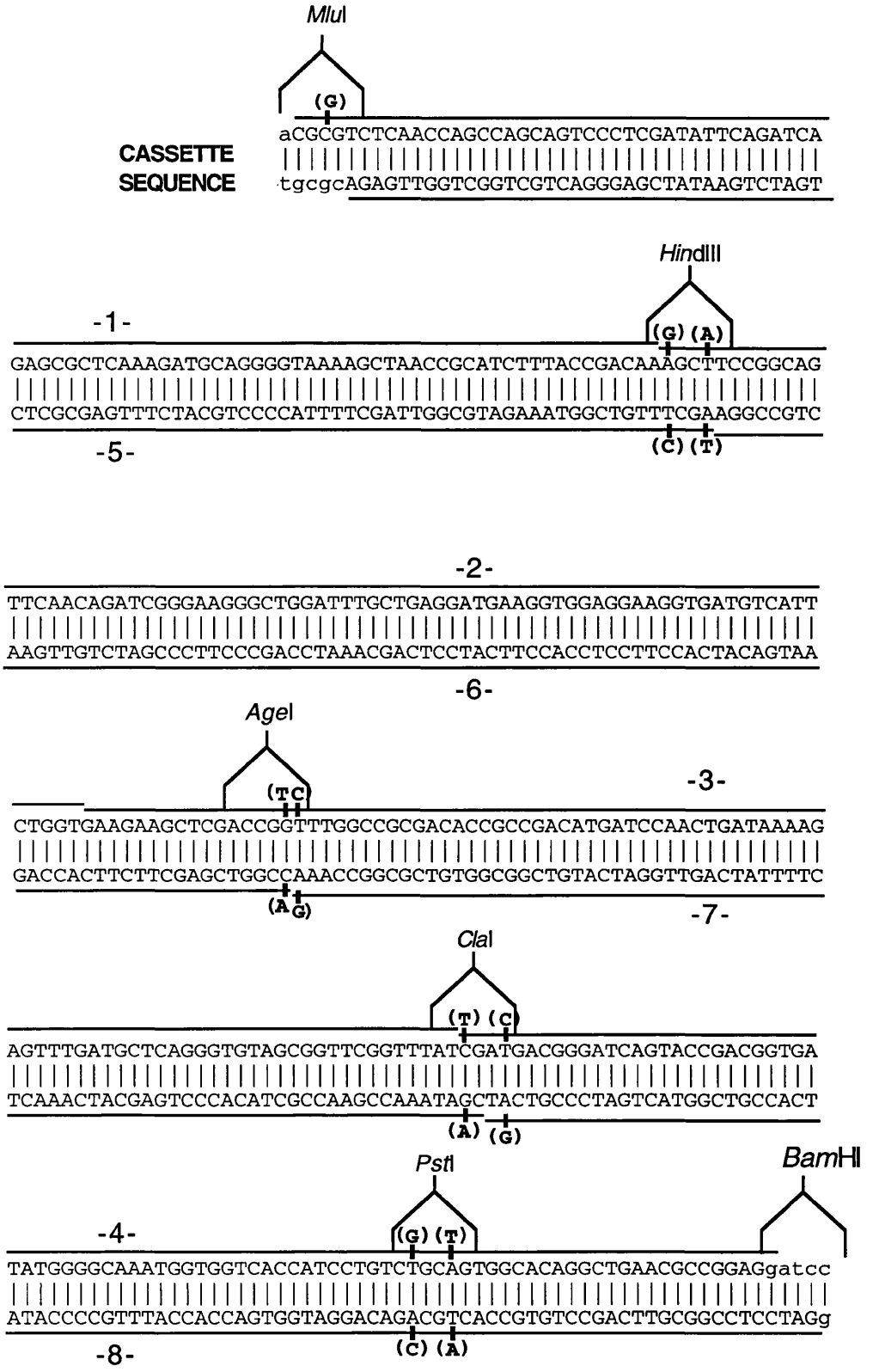



**Figure 3.8 Diagram of Tn*3* resolvase gene cassette in pAT4**

The diagram illustrates the positions of the newly introduced restriction sites relative to each other. Changes introduced by the oligonucleotides are shown, and the wild-type sequence is shown above and below the oligonucleotide sequence in bold. Sequence outwith the oligonucleotide length is denoted by lower case letters.

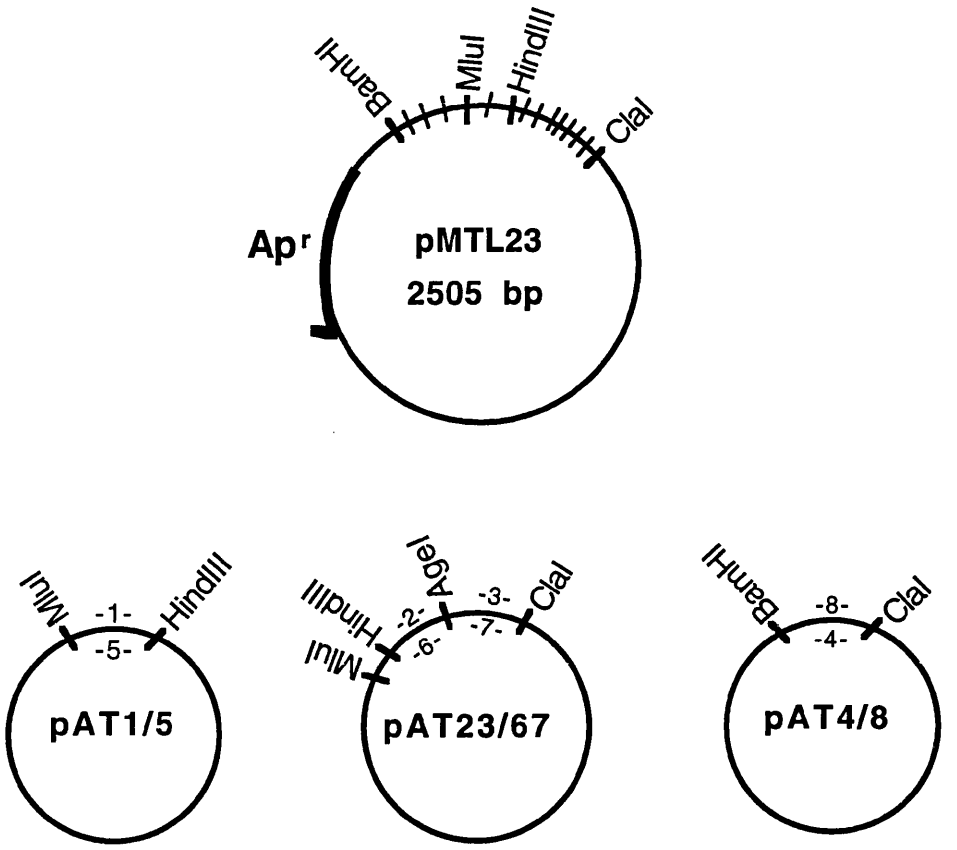
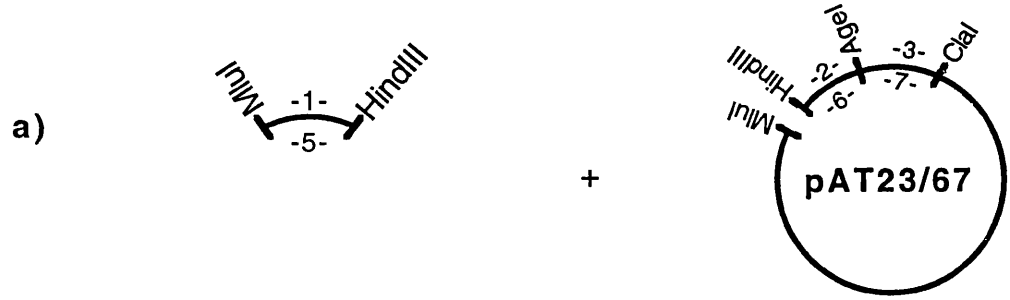
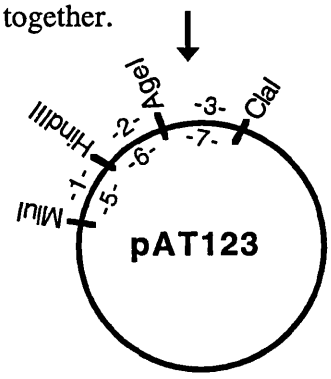


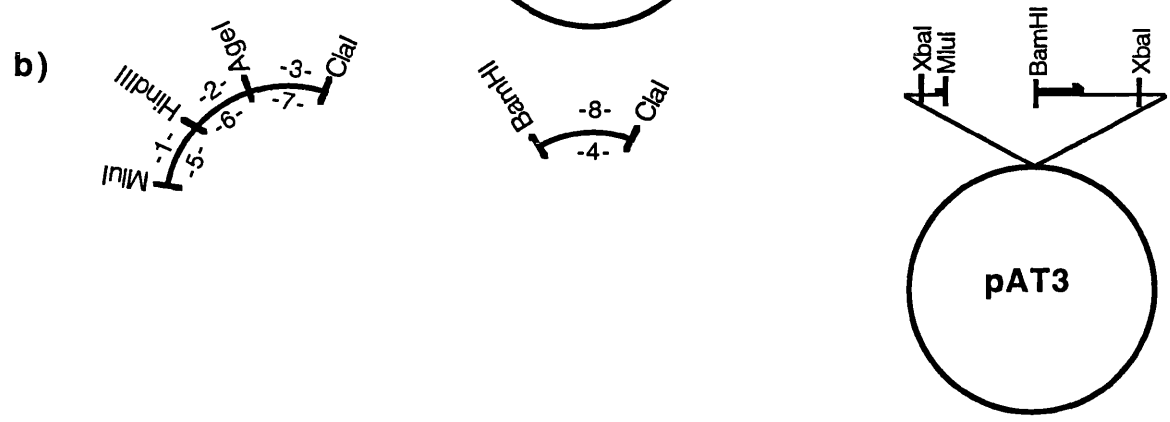

**Figure 3.9 Oligonucleotide Assembly**

This diagram illustrates the cloning vector pMTL23 and the relative positions of the restriction sites flanking the oligonucleotides used to construct the gene cassette. The plasmids resulting from the cloning of the oligonucleotides are also shown, pAT1/5, pAT23/67 and pAT4/8.




Gel purify cloned oligonucleotides 1 and 5 from pAT1/5 on an acrylamide gel. Gel purify pAT23/67 large fragment when restricted *Mlu*I - *Hin*dIII. Ligate these fragments together.




Fragments containing oligonucleotides were purified from pAT4/8 and pAT123 and cloned into pAT3 restricted *Mlu*I - *Bam*HI.

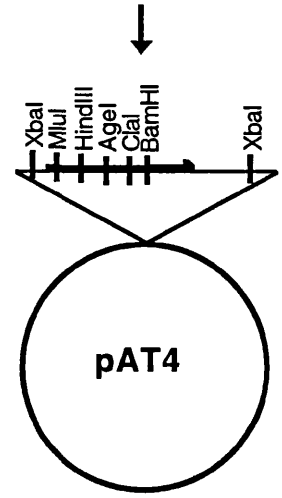



**Figure 3.10 Assembly of pAT4**

Illustration of the cloning steps involved in the assembly of the oligonucleotides comprising the cassette into the p-SELECT-1 based vector, to create pAT4.

Various restriction digests were then carried out to ensure the presence of all the required restriction sites in the correct order (data not shown).

The construct, pAT4 (Figure 3.6) was then checked for resolvase production using the *galK* system (see Section 3.2). The whole open reading frame of the gene was then sequenced on an automatic sequencer (at Strathclyde University). It was confirmed that all oligonucleotides were present in the correct order and that there were no secondary mutations. However, subsequent analysis of this region revealed an error which had been incorporated in the design of the oligonucleotides. While introducing the restriction site *Age*I, the amino acid at position 69, a leucine residue was inadvertently converted to a phenylalanine residue (CTT changed to TTT). This change was later corrected by re-creating the oligonucleotides stretching from the *Age*I site to the *Cla*I site, and amending the TTT to TTA which returns the residue to a leucine. This plasmid was named pAT5.

### 3.3.4. Test screening of pAT4

In order to test the mutagenesis plasmid, pAT4, the following experiment was carried out.

$CaCl_2$ competent cells were prepared from DS941 strains harbouring the resolution screening plasmids, pDB34, pDB35, pDB36 and pDB37 (Figure 3.2). Cells were transformed with pAT4, and after a 90 minute expression step, were spread on MacConkey galactose agar plates containing ampicillin and kanamycin (see Section 2.4). The plates were incubated overnight at 37°C, after which colonies were observed.

As predicted, only pDB34 containing two complete *res* sites in direct repeat was resolved in cells transformed with pAT4, as indicated by the presence of white colonies showing that the *galK* gene had been excised during resolution. The strains containing the other plasmids, pDB35, pDB36 and pDB37, showed only red colonies, indicating the presence of the *galK* gene, and thus a lack of resolution (Figure 3.11).

The products of these *in vivo* tests were then visualised on an agarose gel, by patching out red and white colonies and growing overnight on L-agar plates, then subsequently

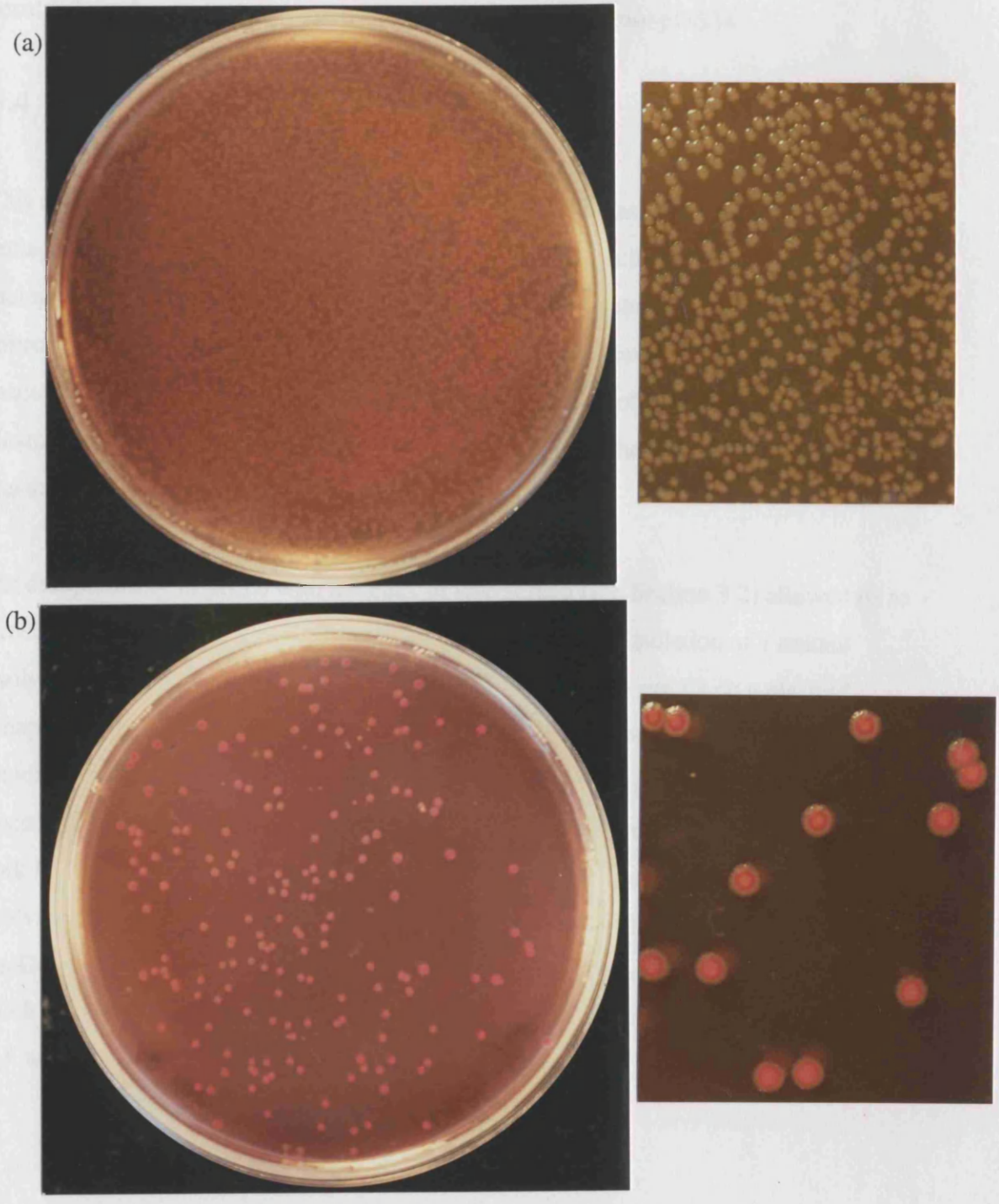



**Figure 3.11 Resolution of pDB34 (*res* v *res*) and pDB35 (site I v site I) by pAT4**

Plates and close ups of (a)white colonies (indicating resolution) of pDB34 and (b) red colonies (no resolution) of pDB35, by pAT4. Even though pAT4 contained the change L69F, it maintained a wild-type activity in this *in vivo galK* assay.

examining the DNA on a Single colony gel (Section 2.23). As illustrated in Figure 3.12, tracks from the white colonies (resolved pDB34) show the presence of the smaller resolved product, which retains the origin of replication, from pDB34.

## 3.4 Summary

This chapter has described the development of a suitable plasmid for the purposes of mutagenesis. Initial attempts to insert the appropriate restriction sites, by site-directed mutagenesis, proved unsuccessful. The problem was overcome by an alternative approach of cassetting the required region of the resolvase open reading frame. The successful construction of this cassette led to the formation of pAT4 (later pAT5), a plasmid with all the requirements for mutagenesis. Using the *galK in vivo* screen, this plasmid was shown to express wild-type resolvase.

The compatibility of pAT5 with the *galK in vivo* screen (see Section 3.2) allowed us to screen the region of the gene (comprising the E-helix) for the isolation of a mutant resolvase able to catalyse recombination *in vitro* between two site I's on a plasmid (Chapter 4). The cassetting of two thirds of the gene also allowed future specific or random mutagenesis to be carried out on a large region of the gene using the simple procedure of inserting mutant oligonucleotides of a relatively manageable size. Indeed, work has already been carried out in order to cassette the remaining one third of the resolvase gene, allowing mutagenesis to be carried out throughout its entire length (A. MacDonald, personal communication). The cassette also inserted the restriction site *Pst*I, which exists in the open reading frame of γδ resolvase, allowing hybrid constructions of Tn*3* and γδ resolvase and mutants thereof to be made.

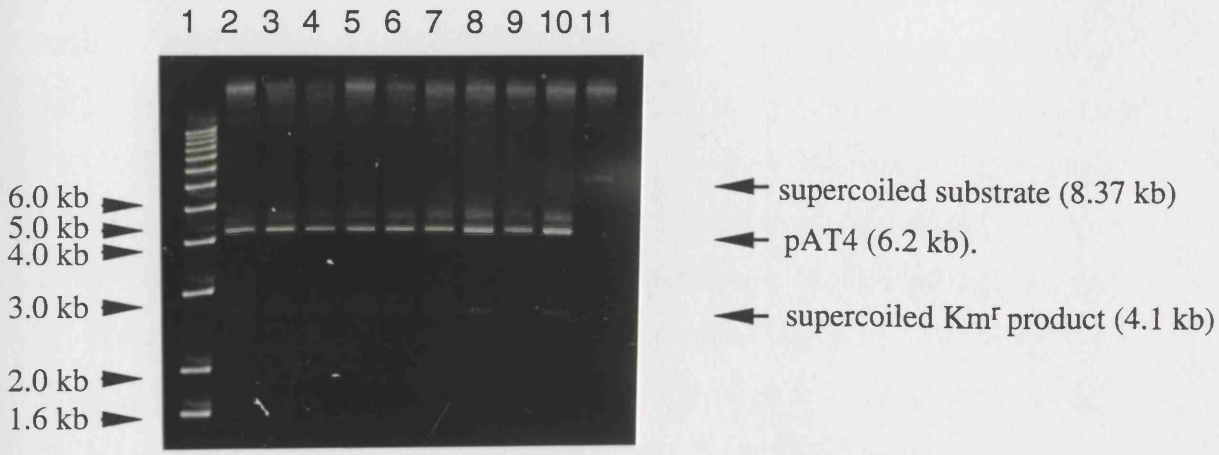



**Figure 3.12 Analysis of Resolution products of pDB34 by Single colony gel**

Resolution of pDB34 by resolvase encoded on pAT4. DNA from white colonies showing resolved Km$^r$ resistant plasmid lacking the *galK* gene.

Lanes 1 - 1 kbp ladder.
2 - 10 - DNA from "white" DS941/pDB34/pAT4 colonies.
11 - DNA from DS941/pDB34 (not transformed with pAT4).

# Chapter 4

Mutagenesis of Tn*3* resolvase

# Mutagenesis of Tn*3* resolvase

## 4.1 Introduction

Once the development of the mutant selection system (see Chapter 3) was complete, it was possible to proceed with the mutagenesis steps to create resolvase mutants. After construction of the mutants, an *in vivo* screen could be used to isolate and characterise them (Blake, 1993; see Chapter 3). This chapter describes the construction and isolation of Tn*3* resolvase mutants. The techniques employed included site-directed mutagenesis by cloning oligonucleotides which had been synthesised with specific altered codons, in order to create specific mutants, and the random mutagenesis of a desired region within the open reading frame to construct a library of mutants for screening, by cloning randomly altered synthetic oligonucleotides. A specific mutant was also constructed by the direct cloning of synthetic oligonucleotides between two restriction sites.

Two specific mutants were made. The first of these was Y6F. The tyrosine-6 residue of Tn*3* is conserved throughout the resolvase family, and is only four residues upstream from the serine residue which has been implicated in the catalysis of DNA strand breakage and rejoining (Hatfull and Grindley, 1988). Examination of the crystal structures of γδ resolvase shows that these residues are close in spatial terms. It was hoped that the construction of the Y6F mutant would clarify this residue's role in catalysis. Changing the tyrosine residue to phenylalanine simply removes the hydroxyl group on the tyrosine, a potential nucleophile. Though evidence supported the serine residue at position 10 as the critical nucleophile (Reed and Moser, 1984; Hatfull and Grindley, 1986; Mazzarelli *et al.*,1993), proof was not conclusive, and the existence of the highly conserved tyrosine-6 residue in such close proximity was potentially significant.

The second specific mutant to be made, E124Q, was deemed desirable after observation of the equivalent mutant's properties in γδ resolvase (M. Boocock, personal communication). This mutant was found to have a hyperactive phenotype in comparison to the wild type protein. The protein was found to be catalytically active *in vitro* on a wild-type substrate, producing the normal 2 noded catenane as the product. However,

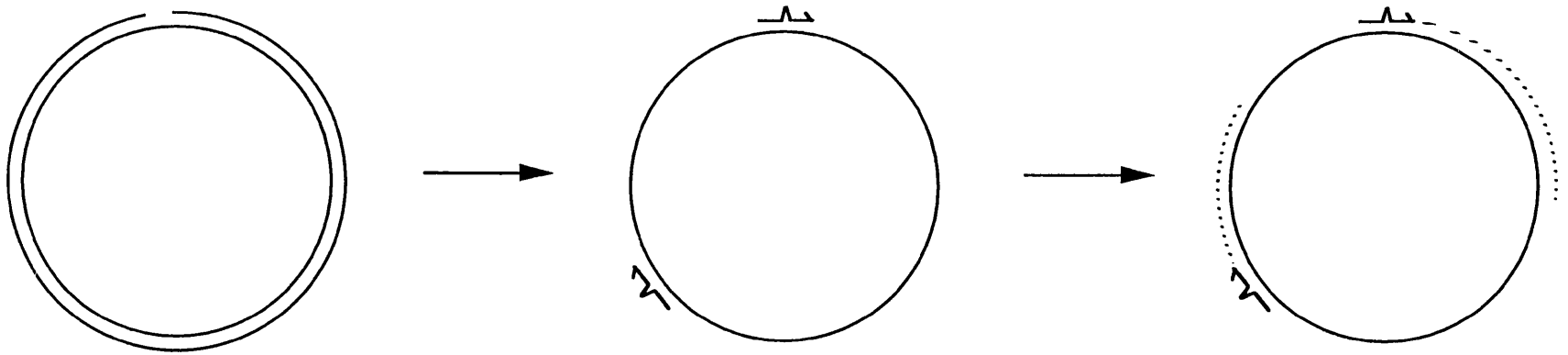
the same reaction was also found to result in free circles and intermolecular products not normally associated with the wild type *in vitro* reaction. Unlike the wild type resolvase, the γδ E124Q protein was also found to be active on linear substrates, and to promote resolution on a substrate plasmid containing a complete *res* site and site I.

As previously mentioned, it was a priority to isolate a resolvase mutant which could promote recombination between two site I's on a supercoiled plasmid, in the absence of the accessory sites II and III (see Chapter 3). Utilising the resolvase mutant selection system described in Chapter 3, a random mutagenesis protocol was employed to search for a mutant with this phenotype. As described in Section 3.1, the targeted region of the resolvase open reading frame was between amino acid positions 94 and 121, which stretches between the unique *Cla*I and *Bam*HI restriction sites in pAT4 (Figure 3.6), and corresponds to most of the E-helix seen in the γδ resolvase crystal structures.

## 4.2 Construction of the Tn*3* resolvase Y6F mutation

The method employed to make a resolvase containing the mutation from tyrosine at position 6 in the resolvase open reading frame to phenylalanine, was site-directed mutagenesis using the p-SELECT-1 protocol (Altered Sites *in vitro* mutagenesis system technical manual, Promega). This involved annealing an oligonucleotide containing the codon change from tyrosine to phenylalanine (TAT to TTT) along with a second oligonucleotide which repaired the defective ampicillin resistance gene in p-SELECT-1, and allowed for a means of selection for possible mutants. The principle is, that an isolate which has annealed one oligonucleotide has a high chance of annealing the second (Figure 4.1).

Using the plasmid pDB6020 (Blake, 1993) which contains the Tn*3* resolvase gene cloned into the pSELECT-1 polylinker, single stranded DNA was prepared by nicking the DNA with DNase I/ethidium bromide (Section 2.16), then denaturing the singly nicked plasmid molecules with 2 M sodium hydroxide. The standard "Altered Sites" protocol uses bacteriophage to make the single stranded DNA. However, the DNase I method has been shown to produce single stranded DNA which can be used for mutagenesis (M. Boocock, personal communication). Subsequent site-directed mutagenesis was carried



Double stranded DNA nicked with DNase I, denatured and precipitated leaving single stranded circular DNA.

Oligonucleotides annealed to single stranded DNA.

Second strand synthesised with T4 polymerase. Upon transformation daughter plasmids contain changes conferred by both oligonucleotides, or original sequence.

**Figure 4.1 Outline of Site Directed Mutagenesis**

Diagram shows the basic steps involved in site directed mutagenesis as described in the p-SELECT manual (Promega)

out as described (Altered Sites technical manual, Promega). The steps involved in this procedure are outlined in Figure 4.1. The Y6F oligonucleotide also contained changes which introduced a *Mlu*I site, as a further means of detection of possible mutants. The Y6F and Ap repair oligonucleotides are detailed in Figure 4.2.

The reaction mixture (see Section 2.15) was used to transform competent EM1 cells (see Table 2.1) which had been prepared as described in Section 2.7. EM1 is a repair deficient (*mutL*) strain. One tenth of the transformation mixture was plated onto ampicillin plates, and the remainder was grown up overnight in 20 ml L-broth containing ampicillin. Two colonies were observed on the ampicillin plates, suggesting a total of about 20 transformants.

Two alkaline lysis mini preps were carried out (see Section 2.9) on, a) 1.5 ml of overnight culture; b) 1.5 ml overnight culture and picks of the two colonies. Restriction digestion with *Mlu*I was then carried out on the two mini-preps (see Section 2.10). Visualising the DNA on a 1.2% agarose gel (see Section 2.22) showed that a proportion of each DNA sample had been cut with *Mlu*I in both cases. This indicated the presence of a possible Y6F mutant, since the *Mlu*I site is present on the same oligonucleotide as the Y6F mutation.

After transforming DNA into DS941 competent cells, $Ap^r$ transformants were isolated. Twelve transformants were grown up overnight, and alkaline lysis mini prep DNA was prepared from them (see Section 2.9). The DNA was digested with *Mlu*I, and five of the twelve plasmids were found to contain a *Mlu*I site, indicating that they might also contain the Y6F change.

It was important to confirm that isolates containing the *Mlu*I restriction site also contained the change at position 6 to phenylalanine, with no secondary mutations. CsCl DNA was prepared (see Section 2.9) of the five isolates, and the DNA was sequenced (see Section 2.17). Sequencing over the region where the oligonucleotides annealed showed the presence of the Y6F mutation in four of the five isolates. No unexpected secondary mutations were observed throughout the region of the resolvase open reading frame which was sequenced.

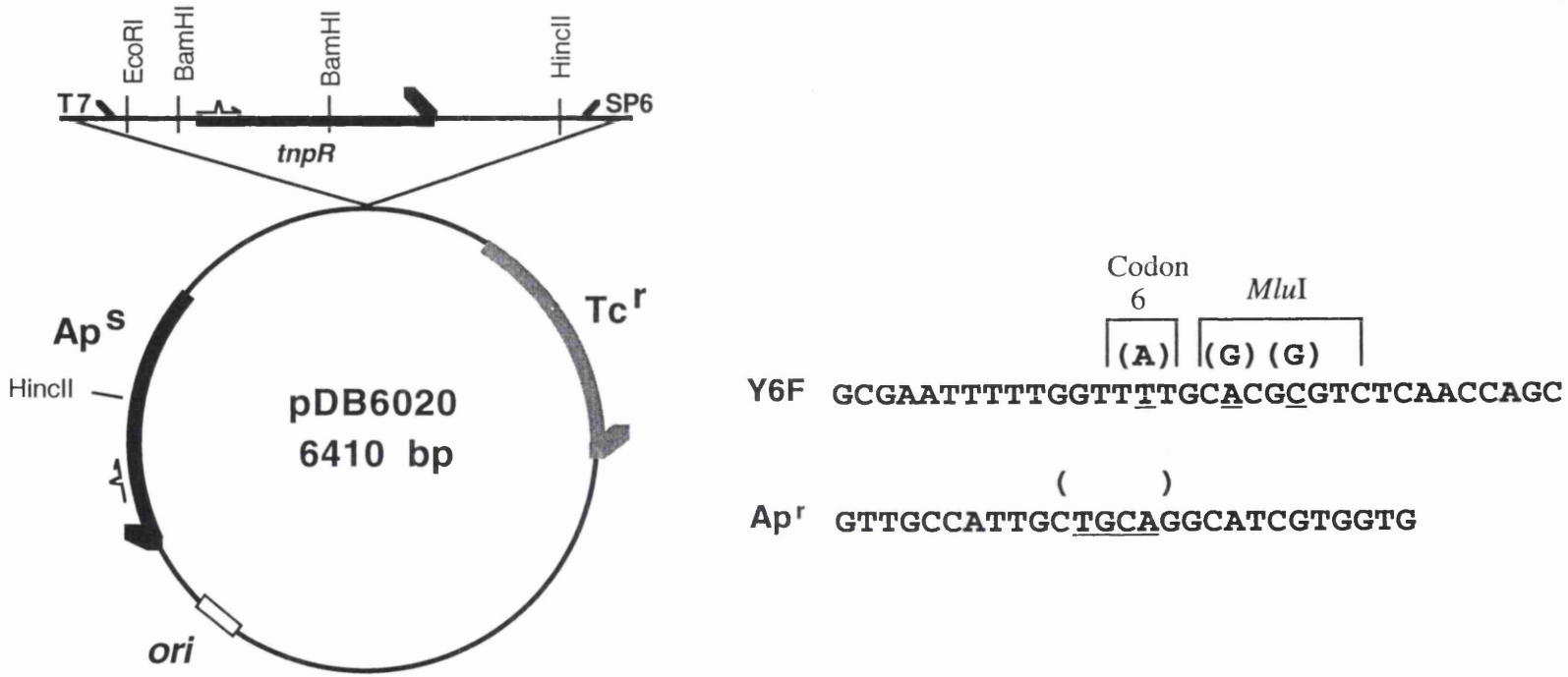



**Figure 4.2 Illustration of the creation of the Y6F resolvase open reading frame**

Diagram shows the sequence of the oligonucleotides used to create the Y6F resolvase open reading frame, and their positions on pDB6020. The sequence changes are shown with the original sequence in brackets above.

The recombinational activity of the Y6F resolvase encoded on the pDB6020-derived plasmid, pATY6F, was then examined *in vivo* using the *galK* screen (see Section 3.2). The results of this screen are shown in Section 4.5. The protein was then purified in order to observe its *in vitro* properties (Chapter 6).

## 4.3 Construction of Tn*3* resolvase E124Q mutation

The construction of the mutant E124Q resolvase was carried out in the expression plasmid pSA1101 (Figure 3.5). This plasmid contained the required unique restriction sites, *Eag*I and *Bam*HI, flanking the E124 codon, which would be essential to the strategy employed.

The strategy for mutagenesis was as follows. E124 lies between the *Bam*HI - *Eag*I restriction sites in pSA1101 (Figure 3.5). It was decided to make two complementary oligonucleotides which are equivalent to the DNA between these restriction sites, but contain the change at position 124 altering the glutamic acid codon (GAG) into a glutamine codon (CAG), along with the introduction of a unique restriction site *Bsi*Wi (see Figure 4.3). These oligonucleotides could then be cloned into pSA1101 (see Section 2.2)) which had been digested with *Bam*HI and *Eag*I (Section 2.15).

Oligonucleotides were synthesised, and purified in the usual manner (Section 2.21). The two oligonucleotides were annealed (10 pmol of each; see Section 2.20).

An overnight ligation was then set up (Section 2.15) comprising annealed oligonucleotide and gel-purified digested pSA1101. Transformation with the ligation mixture resulted in six colonies on L-agar plates containing kanamycin. From overnight cultures of the six transformants, DNA was prepared (Section 2.9.2). Restriction digests with *Bsi*WI showed the presence of the restriction site in five of these six clones. These five clones were sequenced over the length of DNA between *Bam*HI and *Eag*I restriction sites and were all shown to contain the required mutation with no secondary mutations present. This plasmid was named pATXE124Q.

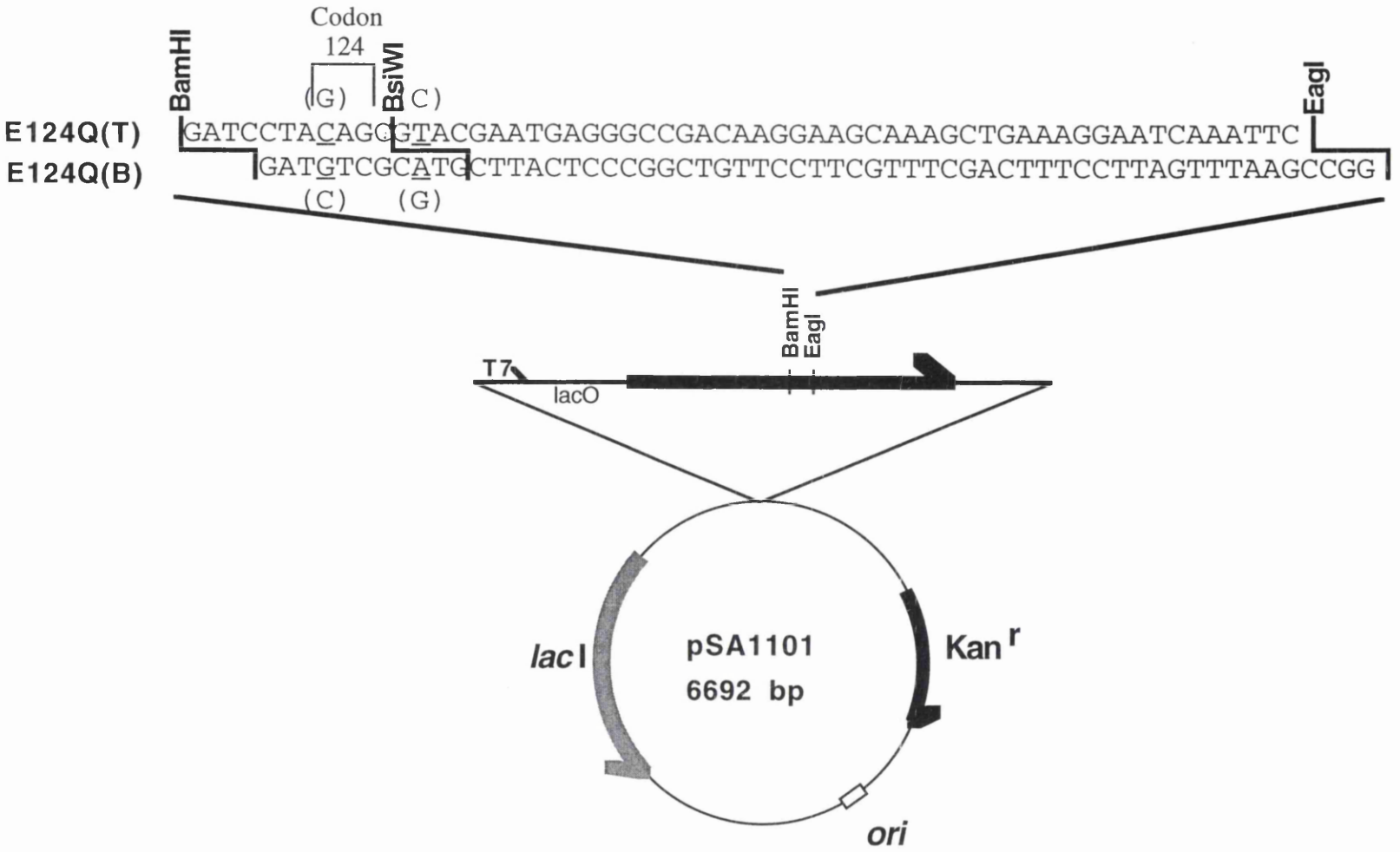



**Figure 4.3 The creation of Tn*3* E124Q resolvase mutant**

Diagram illustrating the changes introduced with the E124Q mutation. The codon change is illustrated, as are the changes creating the *Bsi*WI site.

In order to test the *in vivo* properties of E124Q resolvase, the *Kpn*I - *Bam*HI restriction fragment of pATXE124Q, containing the resolvase open reading frame, was used to replace the equivalent section of pAT5 (Work carried out by Martin Boocock; see also Section 4.5). The protein was also purified for further *in vitro* work (see Chapters 5 and 6).

## 4.4 Random mutagenesis of Tn*3* resolvase

The reasons have been previously described for the mutagenesis of the region contained between the *Bam*HI and *Cla*I restriction sites in the open reading frame of Tn*3* resolvase (see Chapter 3).

In order to ensure, as far as possible, that amino acid substitutions occurred randomly throughout this region, the following method was employed. Oligonucleotides were synthesised which stretched the 80 bp between these two restriction sites, and contained randomly altered bases throughout the length of this region. This was achieved by "spiking" each of the four base reagents used to synthesise the oligonucleotides with the other three, such that (along the length of the oligonucleotide) the "wrong" base would be inserted randomly, resulting in the possibility of an alternative amino acid being encoded. These oligonucleotides were to be cloned into the resolvase open reading frame, and a library of mutants constructed (Figure 4.4), which could then be screened with a variety of test plasmids to isolate potentially interesting mutants.

### 4.4.1 Synthesis of oligonucleotides

In the main, synthesis of the oligonucleotides used was by the usual method (Section 2.18), with the exception of the four base bottles used for the synthesis.

The lengths of the two complementary oligonucleotides stretching between the *Cla*I and *Bam*HI restriction sites are 84 and 86 bp. It was decided to add 1% of each of the "wrong" bases to each phosphoramidite reagent, which should lead to about 2.5 base changes per oligonucleotide. Since not every base change will result in an amino acid substitution, this should result in about 2 amino acid changes per oligonucleotide, on

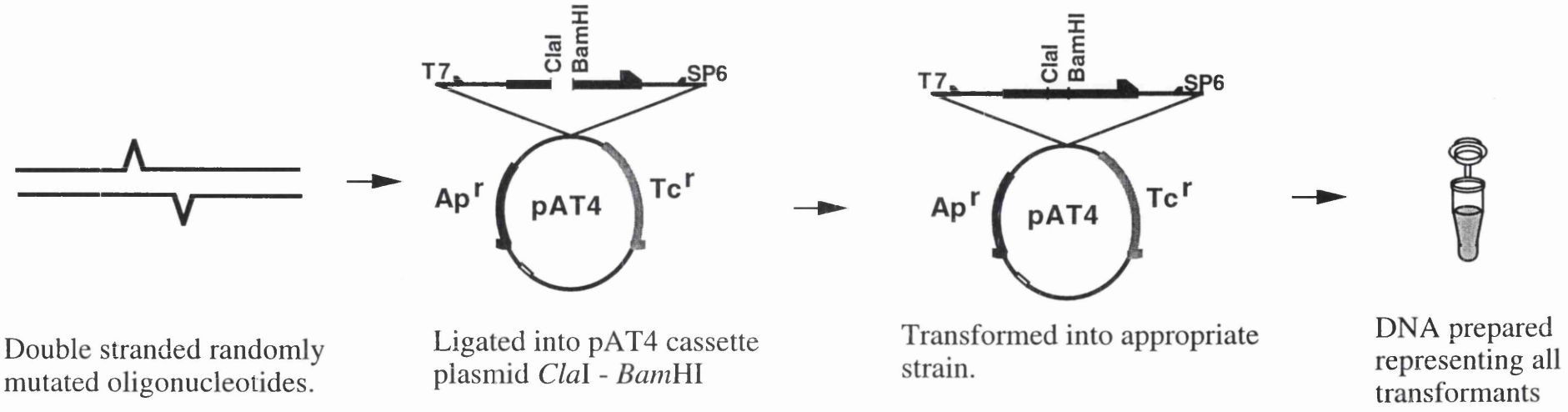



**Figure 4.4 Creation of a mutant library from pAT4**

Figure illustrating the steps involved in the creation of the mutant library.

average . It was decided to spike both oligonucleotides for two reasons. Firstly, all plasmids obtained will have been derived from a mutant strand, resulting in a lower background of "wild-type". Mutating both strands also removes any preferential bias of the repair system towards one strand, thus, for example, giving biased corrections to wild-type.

Equal quantities of solutions of each of the four base phosphoramidites were mixed. This mix was added to fresh bottles of each of the four phosphoramidites, adding sufficient to give 1% of each "wrong" phosphoramidite. These contaminated bases were then used to synthesise the oligonucleotides in the standard manner.

The oligonucleotides made (M4 + M8) were then purified on a 10% polyacrylamide denaturing gel (Section 2.22) and eluted from the gel (Section 2.21). The concentration of the oligonucleotides was determined (Section 2.27). The oligonucleotides, M4 and M8, were then annealed to each other.

### 4.4.2 Construction of a library of mutants

The vector into which M4 + M8 were to be cloned, pAT4 (Figure 3.6) was digested with *Bam*HI and *Cla*I, and the large 6.15 kb fragment was gel-purified on a 1.2% agarose gel (Section 2.22). A ligation was then set up between this DNA fragment and the annealed mutated oligonucleotides. Approximately 0.125 pmol of large DNA fragment, and 0.25 pmol of annealed oligonucleotides were used in the ligation.

Half of the ligation mix was used to transform DS941 competent cells. In order to minimise the number of duplicate transformants resulting from a single cloning event, the expression step, which normally lasts 90 minutes was reduced to 15 minutes. This allowed the cells to recover but prevented subsequent generations. When plated onto ampicillin plates, 500 transformants were observed. In principle, each transformed cell may have two types of plasmid in it, derived from the two strands of the ligated plasmid. However this problem disappears on subsequent transformation of the library DNA into the strain containing the test plasmid. In order to make a DNA library of these mutants, the transformants were pooled by resuspending the colonies in 3 ml of L-broth. The cells

were then spun down, and DNA was prepared by the alkaline lysis method (see Section 2.9). This provided the first library, L1. A control ligation, containing restricted pAT4 DNA but lacking the oligonucleotides, had been carried out to check the efficiency of enzyme cutting. When transformed, this ligation resulted in a very small number of colonies.

The remainder of the ligation mix was transformed into DH5α competent cells (supplied by BRL), which were highly competent. This transformation resulted in 2000 colonies from which a second library was constructed as described in the previous paragraph, and named L2.

These libraries were then screened (Section 4.5.2) for the presence of mutants.

## 4.5 *In vivo* analysis of Tn3 resolvase mutants

Using the *galK in vivo* screen, the specific mutant resolvases encoded by pATY6F and pAT5E124Q, were examined for their recombinational activity. The screen was also used to isolate mutants with a particular phenotype from the libraries created, L1 and L2. pAT4, which contains the L69F mutant resolvase open reading frame inadvertently created during its construction (see Section 3.3.3) was also examined.

The mutation E124Q which had previously only existed in the expression plasmid (based on pSA1101) was cloned into the pSELECT-1 based pAT5 as a *Kpn*I - *Bam*HI fragment swop (M. Boocock, personal communication).

### 4.5.1 Analysis of specific mutants

The three mutant plasmids pATY6F, pAT4 and pAT5E124Q were used to transform DS941 strains containing the screening plasmids pDB34, pDB35, pDB36 and pDB37 (see Chapter 3; Figure 3.2). The strains were also transformed (see Section 2.8) with pDB6020 (see Section 2.2), a plasmid which expresses low levels of wild-type resolvase, to act as a positive control. After plating onto MacConkey galactose plates, resolution of the substrate plasmid results in the observation of white/yellow colonies,

while colonies in which the plasmid is not resolved are red (see Chapter 3). Figure 4.5 depicts the results of these transformations.

The wild-type plasmid pDB6020 resolves only the wild-type substrate, pDB34, containing two complete *res* sites in direct repeat. The pAT5Y6F mutant fails to promote this reaction, and thus Y6F resolvase would seem to be catalytically dead for recombination. The pAT5E124Q mutant behaved as would a wild-type resolvase *in vivo*, i.e. resolving pDB34 (containing two wild-type *res* sites), but with no resolution observed with the plasmids containing partial *res* sites. This was not entirely expected, as the γδ E124Q mutant resolves *res* v Site I *in vivo* using this assay (M. Boocock, personal communication). The mutant pAT4(L69F) also showed recombinational activity typical of the wild-type protein. pAT5, which is identical to pAT4 except for correction of the L69F mutant to wild-type, behaved exactly as pAT4 in this assay (data not shown).

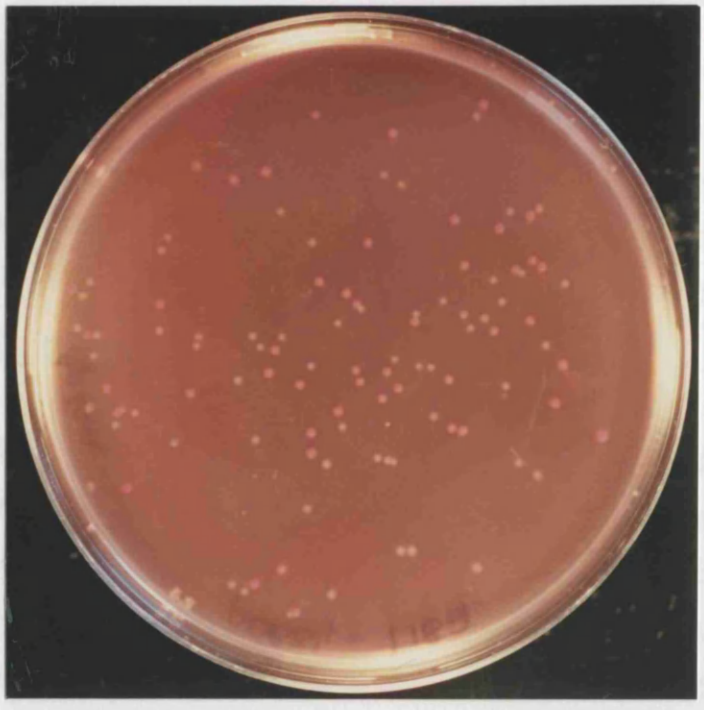
### 4.5.2 *In vivo* screening of the libraries, L1 and L2

DS941 strains containing the four screening plasmids, pDB34, pDB35, pDB36 and pDB37 (Blake, 1993) were then used to isolate potentially interesting resolvase mutants from the libraries made in Section 4.4.

As in Chapter 3, $CaCl_2$ competent cells were prepared (see Section 2.7) from DS941 strains containing these plasmids. 3 μg of library DNA was used to transform the competent cells, and these were then plated onto MacConkey galactose plates containing Ap and Km. Over 2000 colonies were observed for each transformation, to ensure that screening had been complete.

pDB34 (*res* v *res*) transformants showed a roughly 50% split between reds/whites. This indicates a mixture of functional and non-functional resolvase mutants, which was expected since the mutagenesis was aiming for 1-2 substitutions per oligonucleotide length. Some substitutions may result in a conservative change, or a change at a non-essential residue, whereas others may be very damaging to activity.

(a)



(b)

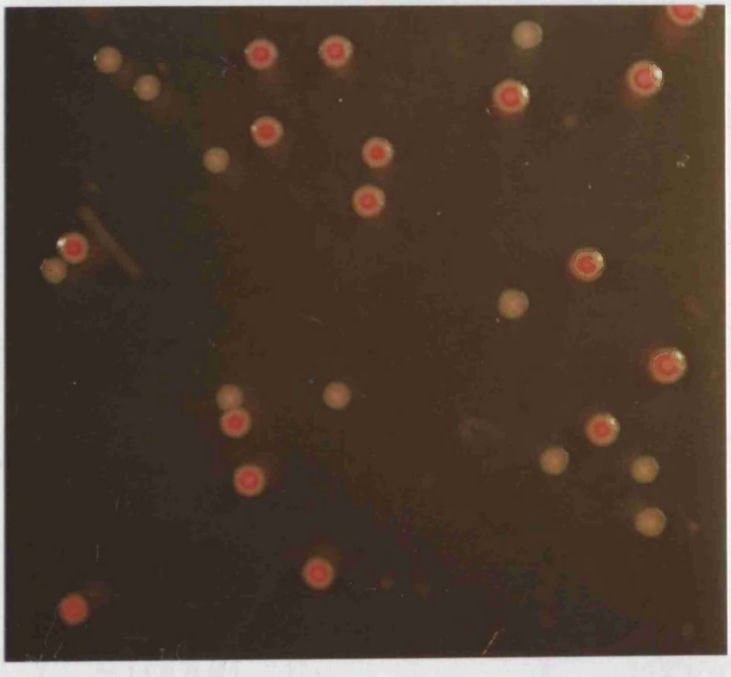


**Figure 4.5** ***In vivo*** **screening of Library I**

Illustration of the red and white colonies observed when the mutant Library-1 was screened with the wild-type substrate pDB34.
(a) Photograph of the whole MacConkey galactose plate
(b) Close up of a section of the plate.

When DS941 containing pDB35 (site I v site I) was transformed, all colonies observed were red, indicating that no mutant had been isolated which could promote resolution between two single site I's. This was initially disappointing. However, this may indicate that the phenotype required to catalyse this reaction may not be conveyed by a single amino acid change, and perhaps a more severe mutagenesis, or combination of mutants was required.

Screening the library with pDB36 and pDB37 (site I v *res*) resulted in a total of 8 colonies which were white (Table 4.1), suggesting that the plasmids containing only one full *res* site and a site I had been resolved. These isolates were then restreaked onto fresh MacConkey galactose plates containing ampicillin and kanamycin to ensure the reproducibility of the test. All eight isolates were found to form white colonies on restreaking, and thus had apparently resolved the plasmid.

| TEST PLASMID | LIBRARY 1 (from 500 transformants) | | LIBRARY 2 (from 2000 transformants) | |
|---|---|---|---|---|
| | **Red colonies** (No resolution) | **White colonies** (Resolution) | **Red Colonies** (No resolution) | **White colonies** (Resolution) |
| pDB34 (*res* v *res*) | ~4800 | ~5200 | ~4900 | ~5100 |
| pDB35 (siteI v siteI) | ~10000 | - | ~10000 | - |
| pDB36 (*res* v siteI) | ~10000 | - | ~10000 | 3 |
| pDB37 (*res* v siteI) | ~10000 | - | ~10000 | 5 |

Table 4.1 : Screening of mutant libraries

The eight isolates were then grown up, and mini CsCl DNA preps were carried out (Section 2.9.2). The DNA of the pAT4 derivatives was then sequenced. In all cases a change was observed at position 102 (GAT - TAT), which resulted in the amino acid

residue being altered from aspartate to tyrosine. Since this mutant displayed a novel phenotype, it was then purified for *in vitro* work (see Chapter 6). Retransformation of pAT4D102Y into DS941/pDB36 and DS941/pDB37 reproduced the previously observed resolution; all colonies were white on MacConkey galactose plates. It was also shown by single colony gel analysis (see Section 2.23) that pDB36 and pDB37 had been resolved. pAT4D102Y retransformed into DS941/pDB34 gave white colonies, showing resolution of the two *res* test plasmid, as expected, while transformation into DS941/pDB35 gave only red colonies, indicating that the D102Y mutation had not conferred activity on a site I v site I substrate (Figure 4.6).

This mutagenesis experiment was repeated subsequently (M. Burke, personal communication) using the same "spiked" oligonucleotides, but cloning into pAT5 (wild-type at position L69). Further mutants that can resolve pDB37 were isolated; all turned out to be mutant at D102 (see Chapter 7).

### 4.5.3 Sequencing of library mutants

In order to investigate the resolvase reading frame (*Cla*I - *Bam*HI) of the mutant pAT4 for the extent and range of mutants contained in the library, it was decided to sequence both red and white colonies obtained by screening the libraries with the wild-type substrate plasmid, pDB34. This would result in the identification of mutants which have lost the ability to carry out resolution by a single amino acid substitution. Also, mutants which contain amino acid substitutions but retain their function could be identified.

The colonies were re-patched onto L-agar plates containing ampicillin, and overnight cultures were set up in L-broth containing ampicillin. DNA was prepared from the overnight cultures by the mini CsCl method (see Section 2.9.2) and sequenced. Figure 4.7 shows the positions of some of the mutants isolated.

Sequencing the library mutants also showed that approximately 80% of the white colonies were in fact wild-type, and contained no codon changes. This indicated that the library was perhaps not as extensive as we would have hoped. This problem may be overcome by creating a library using oligonucleotides which have been synthesised with a higher

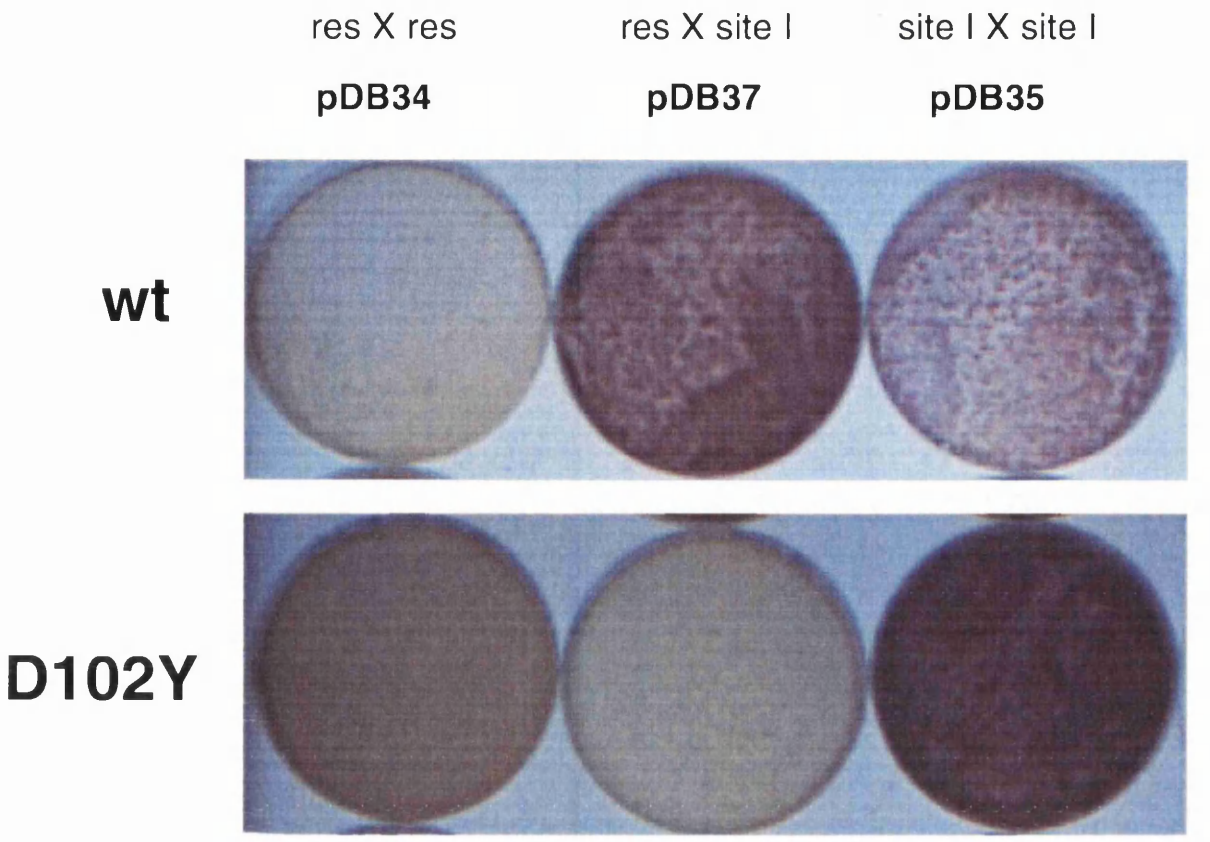



**Figure 4.6** ***In vivo*** **properties of pAT5 and pAT5D102Y**

Figure shows that mutant pAT5D102Y can resolve both pDB34 (*res* v *res*) and pDB37 (*res* v site I) but cannot resolve pDB35 (site I v site I). pAT5, the wild-type control plasmid, can only resolve the wild-type substrate pDB34. In this *in vivo galK* assay, white colonies contain recombinationally active resolvases, while red colonies are recombinationally dead.

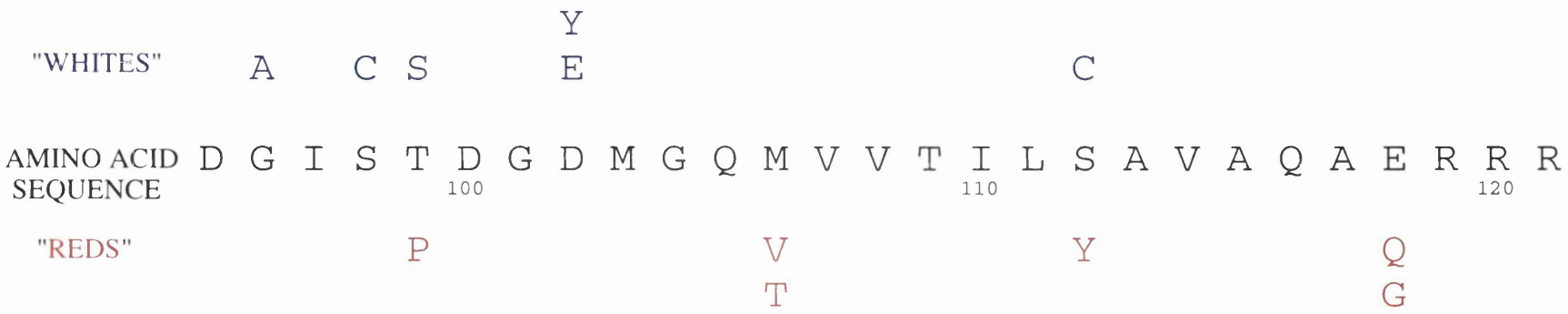



**Figure 4.7 Positions of point mutations**

The diagram shows the amino acids (single letter code) comprising the *Cla*I - *Bam*HI region. In red, are the point mutations which render the protein inactive against the wild type substrate *in vivo*, pDB34. The point mutations in blue do not affect the activity of the protein on the wild type substrate pDB34 *in vivo*.

percentage of altered codons. Also, ensuring that the vector used in the ligation with the oligonucleotides contains no full length linear DNA which may recircularise during the ligation, may reduce the percentage of white colonies containing plasmid with the wild-type sequence. A large proportion of red colonies (no resolution) had frame shifts in the *Cla*I - *Bam*HI sequence. This problem may be due to the method of randomising the mutation using oligonucleotides, and might be strain-dependent..

## 4.6 Analysis of the mutagenesis procedure

It is important to confirm that the mutations introduced by the random mutagenesis procedure were indeed random, and that there was no bias for specific base substitutions, such as transitions over transversions or *vice versa.* In order to do this a few calculations were carried out.

### 4.6.1 Base Replacements

There are twelve base replacements in total (each base can be mutated to three others). However, because mutagenic oligonucleotides were used to replace both strands of the DNA in the *Cla*I - *Bam*HI region, there are only six possibilities to be considered; e.g. a G-C base pair which is mutated to a T-A base pair could have been caused by either a G to T change on one strand or a C to A change on the other DNA strand.

The point mutations in the *Cla*I - *Bam*HI region, detected by sequencing of isolates from the libraries of mutated pAT4, are summarised in Table 4.2.

| | Base Replacement | Number of Occurrences |
|---|---|---|
| **I** | G -> A / C -> T | 7 |
| **I** | A -> G / T -> C | 10 |
| **V** | G -> T / C ->A | 13 |
| **V** | G -> C / C -> G | 14 |
| **V** | A -> C / T -> G | 8 |
| **V** | A -> T / T -> A | 12 |

Table 4.2 : Occurrence of Base replacements

From these data we can see that all possible base changes have been made, and though some have a slightly higher frequency than others, the differences in this relatively small data set are not statistically significant.

From Table 4.2 we can see that there were 17 transitions and 47 transversions (distinguished by the letters "I" and "V" in the table). If mutagenesis were truly random, there should be twice as many transversions as transitions. Again, the excess of transversions observed is probably not significant because of the small sample size.

### 4.6.2 Positions of the mutations

It was interesting to examine the positions of the point mutations, to determine whether mutations in the first and last 10 bp of the targeted region had an effect on the ability of the oligonucleotide to ligate efficiently at the restriction sites. Nine mutations were found in the first 10 bp of the targeted region, and only two in the last 10 bp. The middle section, of 64 bp, contained 53 mutations (Figure 4.8). There is no obvious bias against mutations at the beginning of the targeted region; the small number at the *Bam*HI end of the sequence may indicate a slight bias, or could simply be a statistical aberration due to the small number of isolates sequenced.

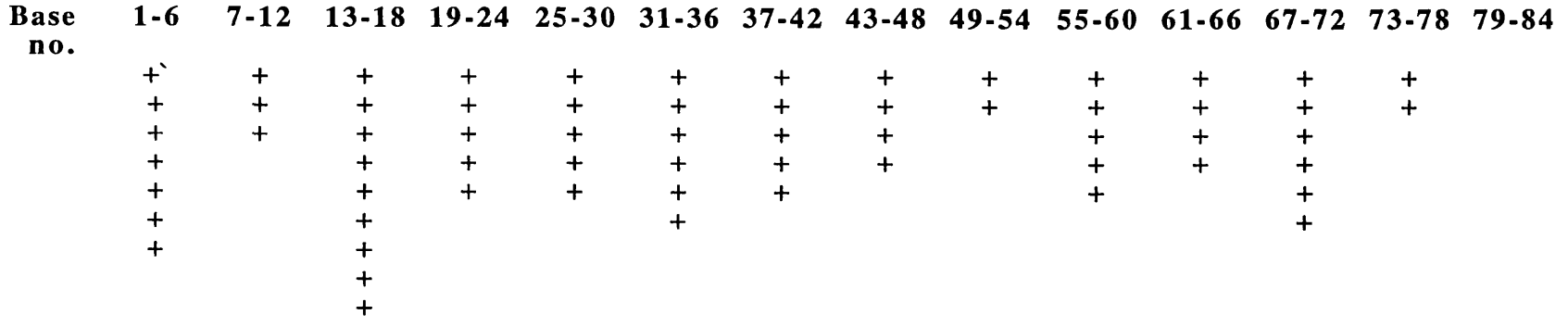



**Figure 4.8 Positions of mutations**

Diagram showing the positions of mutations between the *Cla*I - *Bam*HI sites, observed in the libraries of mutated pAT4. Numbers refer to the bases numbered from the 5'- end of the *Cla*I site to the *Bam*HI site. Each "+" denotes a mutation in the region referred to by the numbers.

From this evidence, it can be deduced that mutagenesis is indeed random, with no bias towards a specific base substitution, or type of substitution, or sequence region. This differs from other methods of mutagenesis such as PCR or chemical mutagenesis, both of which result in a bias towards certain types of alteration.

## 4.7 Analysis of mutant libraries

The combined libraries, L1 and L2 contained 2500 individual colonies. However, the number of independent mutants of pAT4 was inevitably less than this. Sequencing of DNA derived from 50 pAT4 "white" colonies (recombinationally active) from the *galK* assay with a wild-type substrate plasmid, pDB34, showed that 13 contained one or more base substitutions ( i.e. 26%). Since 50% of colonies obtained by transformation with libraries were white, about 13% of the total 2500 colonies were recombinationally active on the wild type resolution plasmid but contained base substitutions, i.e about 325 colonies. The remainder of the "white" colonies isolated (harbouring unmutated pAT4) could have arisen by several routes. Firstly, they could be derived from the vector DNA. Some of the pAT4 vector DNA may have only been cut at one of the two restriction sites, and as a result could simply have recircularised on ligation without incorporation of any mutant oligonucleotide. Secondly, some of the oligonucleotides containing substituted bases may have been repaired to the wild type sequence existing on the second strand. Finally, some oligonucleotide may simply have contained no mutations. Since it was estimated that the oligonucleotides would contain 2 base substitutions each on average, it is safe to assume that some may contain no changes.

Sequence analysis of 36 pAT4 mutants with a recombinationally inactive phenotype, giving "red" colonies in the assay with pDB34, the wild-type substrate, showed that 25 of these contained frameshifts (i.e. 69%), with the remaining 11 containing one or more base substitutions. This means that about 31% of the total "red" colonies harboured pAT4 encoding full length mutant resolvase, and this computes to 15.5% of the total isolates within the libraries, i.e. about 387 clonies. It should be noted that the frameshifts were exclusively single base pair insertions or deletions of up to 5 bp. There was no evidence of large insertions or deletions.

In order to avoid a number of isolates resulting from the same transformation event, a very short expression step was carried out during transformation (see Section 4.4.2). Therefore we can be relatively confident that the libraries contain approximately 712 mutants with either single or multiple base substitutions. Considering that of the 82 bp targeted, the number of possible single base substitutions is 246, the libraries contain almost 3 (2.89) times this number of mutants. We can therefore be confident that most of the possible single substitutions were screened. However, any codon can only be changed into 9 other codons by mutation at a single base pair, and some of these may encode the same amino acid. Thus this type of mutagenesis does not create every possible amino acid change at each residue.

Taking this into account, it seems possible that position 102 is the only position in the targeted region of Tn*3* resolvase which can yield a hyperactive point mutant active on a plasmid containing a site I in direct repeat with an entire *res* site, and detectable by this assay. Position 124 (not in the *Cla*I - *Bam*HI fragment) when mutated from glutamate to glutamine in γδ resolvase resulted in a hyperactive mutant of this phenotype. However, the same mutation created in Tn*3* resolvase (see Chapter 6), while obviously displaying hyperactive tendencies in comparison to the wild type protein, is not sufficiently active to promote the site I v *res* reaction *in vivo* or *in vitro,* and would not have been detectable by this assay. This mutant may prove to display a more hyperactive phenotype in conjunction with other mutations (see Chapter 7).

## 4.8 Summary

The specific resolvase mutants, Y6F and E124Q were created and their properties were analysed *in vivo*. As expected, the Y6F protein was recombinationally inactive, but the E124Q protein was shown to behave unlike the γδ E124Q resolvase equivalent. The Tn*3* resolvase mutant, *in vivo*, seemed to behave as the wild-type protein, and displayed none of the hyperactivity of the γδ protein, which resolves substrates containing site I in direct repeat with *res*. However, it was hoped that *in vitro* analysis would yield further information concerning the properties of this protein. L69F resolvase (pAT4) also exhibited wild type behaviour (i.e. the same as pAT5).

Two random mutagenesis libraries were created, and screening revealed a Tn*3* resolvase mutant, D102Y, which resolved a substrate containing site I in direct repeat with *res*. The libraries did not contain a mutant which was able to resolve substrates containing two site I's. Sequencing of DNA from both red and white colonies resulting from screening with the wild type plasmid containing two *res* sites (pDB34) led to the identification of point mutations that caused inactivation of resolvase (red colonies) or did not prevent resolution (white colonies). It was also observed that a the libraries created contained a significant number of frameshifts and unmutated reading frames. Amendment of the procedure to create the libraries may help remove these unwanted isolates.

The *galK* screen used has proved to be very successful in the isolation and characterisation of resolvase mutants. Though the screen requires a relatively low rate of resolution in order to observe the "white colony phenotype" due to the low copy number of the test plasmids, it does require complete resolution of all plasmids in the cell to take place. However the screen has proved to be remarkably accurate in isolating mutants, whose *in vivo* phenotype was similarily displayed after purification, in the *in vitro* assay. It is therefore a very useful tool in the search for mutants which perform specific recombinational activities *in vitro*.

# Chapter 5

Development of a new expression and purification system for Tn*3* resolvase

# Development of a new expression and purification system for Tn*3* resolvase

## 5.1 Introduction

Since the aims of this project required the purification of a range of resolvase proteins containing mutated residues, for *in vitro* work, it was essential that a good system for over-expression of these proteins was available, along with a reliable and reproducible protein purification system.

The expression system in existence used a plasmid based on pKK223 (Brosius and Holy, 1984). In derivatives of this plasmid, expression of the targeted protein is driven by IPTG induction of the *tac* promoter, lying upstream of the gene of interest. For Tn*3* resolvase, induction of *E. coli* cells containing the pKK223-derived plasmid pMA6114 (Blake, 1993) with IPTG resulted in very poor growth in the strain JM101; over-expression of resolvase was observed to a reasonable degree, and JM101(pMA6114) was used for all the Tn*3* resolvase preparations previously carried out in this lab (Blake, 1993; Watson, 1994).

In order to achieve higher expression of resolvase, it was decided to construct a new expression plasmid based on one of the pET plasmids. These employ a promoter recognised by the phage T7 RNA polymerase, but not by the host *E. coli* RNA polymerase. Protein is produced on expression of the T7 RNA polymerase, either by phage infection, or by induction with IPTG of strains (e.g. BL21(DE3)) containing integrated copies of a T7 polymerase gene (Studier *et al.*, 1990).

Since a variety of mutants were to be made, it was desirable that the plasmid in which the mutagenesis was to take place would have certain features in common with the expression plasmid (such as unique compatible restriction sites flanking the resolvase open reading frame), and this was kept in mind when designing the expression plasmid.

A large scale purification procedure was already in existence (see above). The purification of resolvase in this procedure was dependent on the resolvase remaining

soluble after the cells had been lysed by passage through a French Press, and the subsequent precipitation of resolvase (along with the DNA) by the addition of spermine. When the resolvase was redissolved it was found to be relatively pure. However, great care was necessary to ensure that the procedure gave reproducible yields. It was not certain that the procedure would be applicable to all resolvase mutants. One attempt to use it for preparation of the mutant Y6F (Chapter 4) failed because the resolvase was insoluble after lysis of the cells in the French Press. It was decided to use this protocol as a starting point to develop a more reliable procedure.

In a small scale variation of the protocol (Blake, 1993), it had been found that the resolvase, instead of remaining in solution as in the large scale purification, tended to pellet along with the cell debris after lysis of the cells. Getting the resolvase to remain in solution at this point proved problematic, and so an alternative strategy was devised, involving recovery of the denatured protein from the pellet and subsequent refolding. This denaturing procedure had the added advantage of protecting the resolvase against the action of proteases for a large part of the protocol. Refolding was carried out by dialysis to remove the urea denaturant.

## 5.2 Construction of the expression vector, pSA1101

### 5.2.1 Construction of pTA1

The first step in the construction of the expression construct involved inserting the wild-type resolvase open reading frame into the chosen pKET3a vector (Studier *et al.*, 1990; S. Rowland, unpublished). A three way ligation was carried out with the 717 bp *tnp*R fragment created by *Mlu*I/*Hinc*II digest of pDB6020-Y6F (Chapter 4), along with the complementary oligonucleotides, ΔY6FT and ΔY6FB, which stretch from the *Nde*I site just outside the open reading frame to the *Mlu*I site, ~24 bp into the reading frame, were cloned into pKET3a, digested with *Nde*I/*Hinc*II (Fig. 5.1). This step removed the previously created Y6F mutation at the beginning of the open reading frame, restoring the wild type amino acid sequence, but retaining the *Mlu*I site, which was to be used in the creation of the "mutagenesis plasmid" (Section 3.3). The position of the *Nde*I site at the start codon (CATATG) means that the cloning results in the ideal spacing for the open

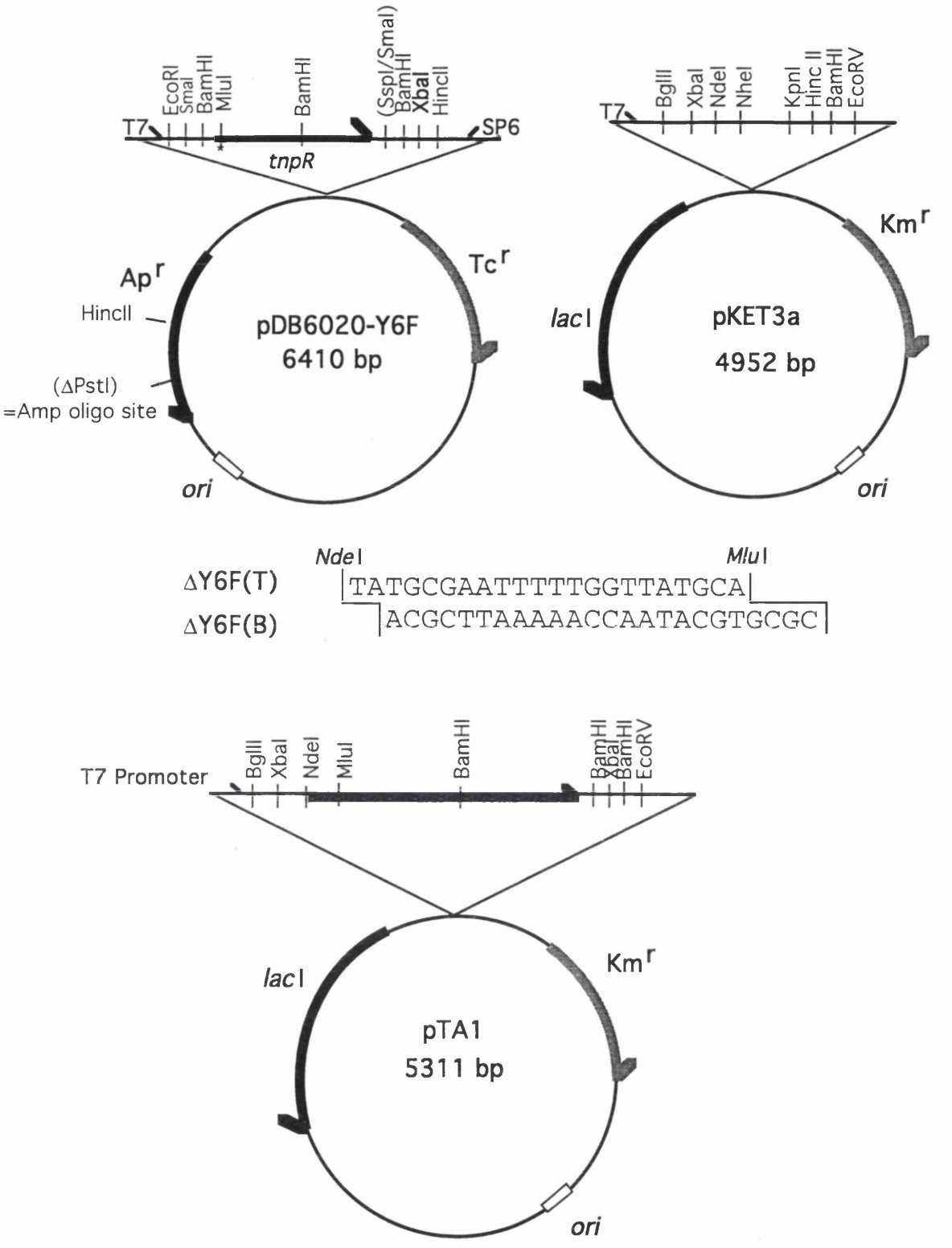



**Figure 5.1 Diagram illustrating the construction of pTA1.**

The ORF of pDB6020-Y6F was removed by restriction digest with *Mlu*I and *Hinc*II and cloned into pKET3a digested *Nde*I to *Hinc*II. The ORF which stretches between *Nde*I and *Mlu*I was reconstructed with the oligonucleotides ΔY6F(T) and ΔY6F(B).

in pET3. The wild type resolvase open reading frame was downstream of the T7 phage promoter in the new vector, pTA1 (Figure 5.1). pTA1 was sequenced over both ligated ends to ensure that the oligonucleotides and open reading frame had inserted as predicted.

### 5.2.2. Induction of pTA1

In order to confirm that pTA1 expressed resolvase, and to determine the level at which expression occurred, small scale inductions were carried out in the four BL21 strains.

pTA1 was transformed into the four BL21 strains (BL21, BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE) (Studier *et al.*,1986). BL21(DE3) has an integrated prophage containing an inducible *lac* UV5 promoter which drives transcription of the T7 RNA polymerase gene. The strains BL21(DE3)pLysS and BL21(DE3)pLysE contain plasmids which reduce the uninduced activity of T7 RNA polymerase by the production of T7 lysozyme. T7 lysozyme acts as an inhibitor of T7 RNA polymerase by forming a complex with it. If the targeted protein is in any way toxic to the cells, reduction of the basal expression can optimise the growth of the culture. These plasmids differ in the expression of T7 lysozyme, with pLysE producing much more. However, this can be counter-productive since large amounts of lysozyme can inhibit cell growth and cause excessive lysis of cultures. It is normal procedure to test expression in the four strains, and choose the strain that gives the best results for further work.

Kanamycin-resistant transformants containing pTA1 were grown up in liquid culture (L-broth) overnight to reach stationary phase. The overnight culture was then diluted 1:100 (200 µl inoculated into 20 ml of fresh broth) and grown until the $OD_{600}$ had reached 0.4 - 0.5. At this point, 10 ml of culture was removed to a separate boiling tube and used as an uninduced control. To the remaining 10 ml, IPTG (1 mM final) was added to induce the cells, and they were grown in a shaking water bath for 3 hours at 37 $^{0}$C.

20 µl samples were then taken, spun down, resuspended in Laemmli Loading Buffer, and run on a Laemmli gel (Section 2.22.2) to observe whether induction had taken place. As the gel in Figure 5.2 shows, the new expression construct over-expressed resolvase, and did so to a higher level than had previously been seen with pMA6114. The protein

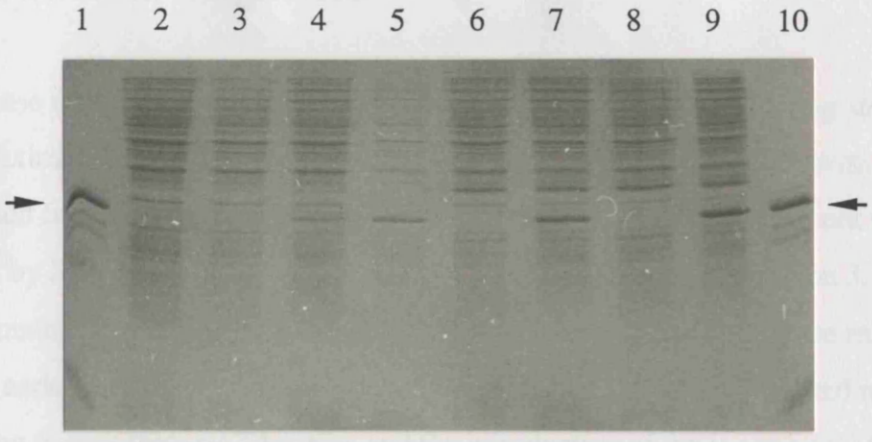



**Figure 5.2 Inductions of BL21 strains containing pTA1**

Laemmli gel showing the relative amounts of resolvase produced on induction of the four BL21 strains containing pTA1with IPTG. The position of resolvase is indicated by an arrow.

Lanes 1 - Resolvase marker
2 - BL21/pTA1
3 - BL21/pTA1 + IPTG
4 - BL21DE3/pTA1
5 - BL21DE3/pTA1 + IPTG
6 - BL21DE3pLysS/pTA1
7 - BL21DE3pLysS/pTA1 + IPTG
8 - BL21DE3pLysE/pTA1
9 - BL21DE3pLysE/pTA1 + IPTG
10 - Resolvase marker

was subsequently purified and shown to be active in the standard resolution assay (see Section 2.33).

### 5.2.3. Construction of pSA1101

The resolvase expression plasmid, pTA1, then underwent a series of cloning steps in order to maximise over-expression of the protein and ensure compatibility with the plasmid used for mutagenesis, pAT4 (see Section 3.3; Figure 3.6). This work was carried out by S. Rowland and resulted in the plasmid pSA1101 (see Section 3.3; Figure 3.5). The main results of these cloning steps were the introduction of unique restriction sites at the ends of the resolvase reading frame to allow the cloning of mutated resolvase open reading frames from pAT4 easily, and the introduction of the *lac* operator from pET1c, allowing a lower basal level of expression by the binding of the *lac* repressor, but at the same time allowing similar levels of induced expression (Figure 5.3).

### 5.2.4. Induction of pSA1101

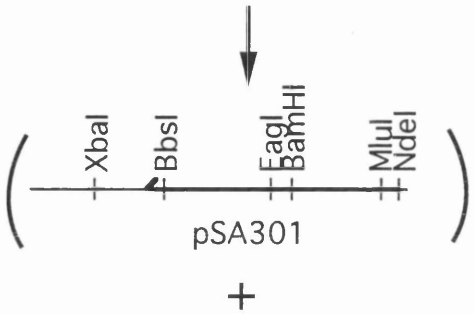
Small scale inductions (as previously described for pAT1) were carried out with pSA1101, and the level of expression was visualised on a Laemmli gel (data not shown), which confirmed that the plasmid was making resolvase to a higher level than previous expression plasmids.

In order to maximise protein production, the effects of time of induction and the length of induction were investigated.

a) Point of induction

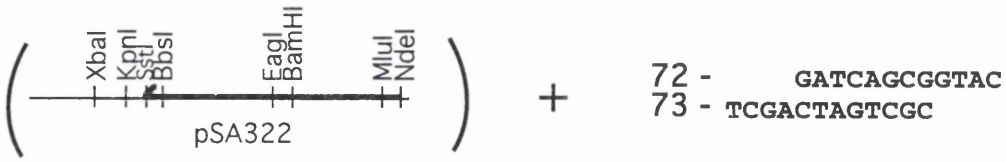
20 ml of L-broth was inoculated with 200 µl of overnight liquid culture of BL21(DE3)pLysS pSA1101. At selected intervals, 1 ml of the culture was removed and induced with 1 mM IPTG for 3 hours. Samples were induced over a range of $OD_{600}$ from 0.1 to 0.9. The $OD_{600}$ at the end of the induction was also recorded and the figures are shown in Table 5.1.


Digest pTA1 *Bam*HI-*Bam*HI; digest pDB6020-63/64 9 (From S. Rowland) *Bam*HI-*Bgl*II and fragment swop, creating pSA301.This introduces the *Eag*I site in the ORF




69 - **AAGGGCGAGCTCTCATCACCATCACCATCACTGATCAGGTACCT**
70 - **CGCTCGAGAGTAGTGGTAGTGGTAGTGACTAGTCCATGGAGATC**

Digest pSA301 *Bbs*I-*Xba*I and insert oligonucleotides (69 + 70), creating pSA322. This introduces a HIS Tag, and new restriction sites.




Digest pSA322 *Sst*I-*Kpn*I, insert oligonucleotides 72 + 73. This removes the HIS Tag and creates the plasmid pSA302. (continued opposite)

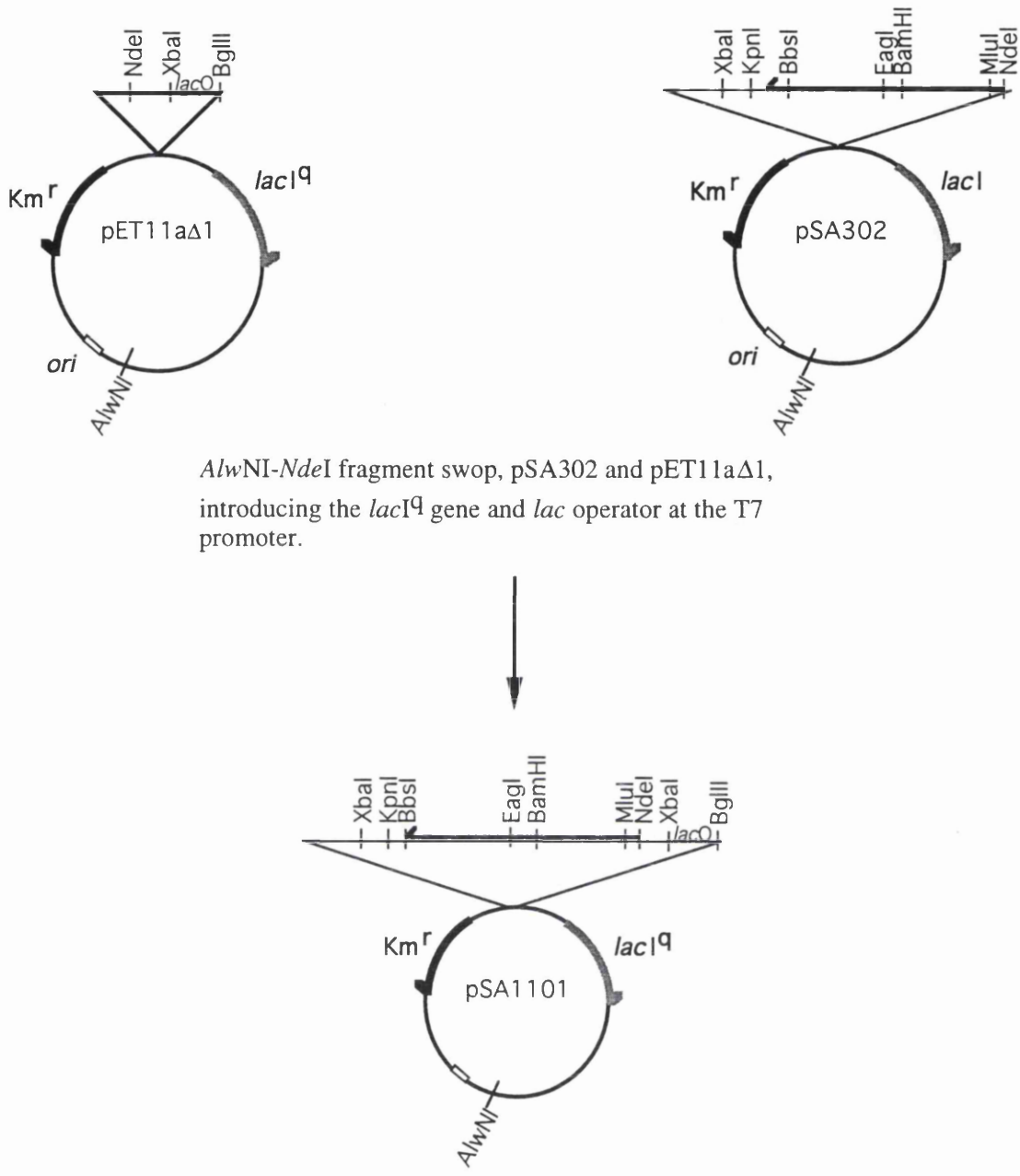



**Fig. 5.3 Illustration of the construction of pSA1101**

Diagram showing the major steps involved in the manufacture of the plasmid for expression purposes pSA1101.

After inducing expression for 3 hours, 20 µl samples were spun down and resuspended in Laemmli buffer before loading on a Laemmli gel (Section 2.22.2) (Figure 5.4). This gel clearly showed that the highest level of induction of resolvase was obtained by inducing the culture while the cells were at $OD_{600}$ of 0.3 - 0.4. However, cells induced at this point failed to reach as high a density as cells which were induced later. It was decided that the optimum point of induction was at $OD_{600}$ 0.4 - 0.5 .

b) Length of Induction

To determine if the length of time that the cells are induced for plays a significant role in the level of resolvase expression, cells were induced for varying times. A 20 ml culture was inoculated with 200 µl of overnight culture of BL21(DE3)pLysS (pSA1101), and grown at 37 °C until the $OD_{600}$ of the cells was about 0.33. 1 ml aliquots of culture were removed and induced with 1 mM IPTG for various lengths of time (Table 5.2).

After the cells had been induced, a 20 µl sample was spun down and resuspended in Laemmli loading buffer before loading on a Laemmli gel (Figure 5.5). Resolvase was obviously present after 1 hour, and the amount increased with time. However, after 3.5 hours, there was little further increase. Based on this experiment, it was decided to induce future batches of cells for ~4 hours.

## 5.3 Purification of resolvase from induced BL21(DE3)pLysS(pSA1101) cells

In previous large scale resolvase purification protocols (Watson, 1994), after the cells had been induced and harvested, they were resuspended and broken by 3 passes through a French Press. However, in this purification, sonication was used.

### Solutions & Buffers

1 x KPM : 25 mM $KH_2PO_4$ (pH 7.0), 5 mM $MgCl_2$, 1 mM EDTA, 0.4 mM DTT, 1 mM benzamidine

RHB : 2 M NaCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 mM DTT

| SAMPLE | $OD_{600}$ AT INDUCTION | AGE OF CULTURE AT INDUCTION | $OD_{600}$ AT END OF INDUCTION |
|---|---|---|---|
| 1 | 0.098 | 1 h 15 min | 0.752 |
| 2 | 0.214 | 1 h 50 min | 0.934 |
| 3 | 0.302 | 2 h 10 min | 1.081 |
| 4 | 0.395 | 2 h 30 min | 1.202 |
| 5 | 0.495 | 3 h | 1.183 |
| 6 | 0.605 | 3 h 25 | 1.523 |
| 7 | 0.890 | 4 h 15 min | 1.577 |

Table 5.1 The effect of time of induction

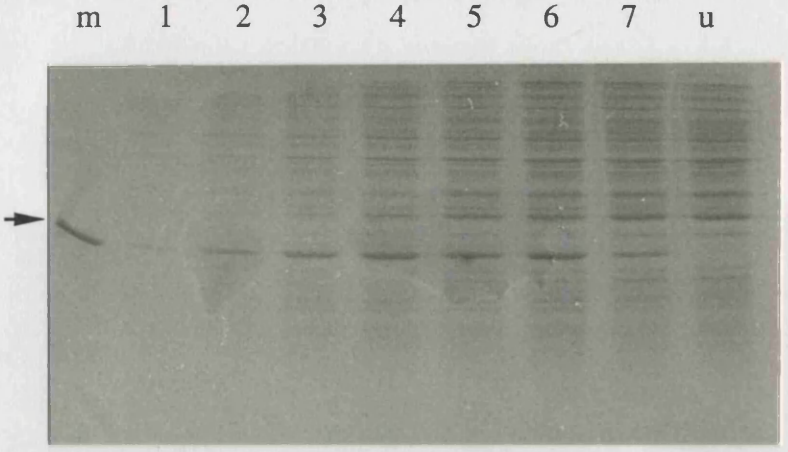



**Figure 5.4 The effect of time of induction.**

Laemmli gel showing the effect of inducing Bl21(DE3)pLysS(pSA1101) cells at various stages in the growth of the culture (i.e.increasing optical density). Details are listed in Table 5.1. Position of resolvase is denoted by an arrow.

Lanes m - Resolvase marker
1 - 7 - As indicated in Table 5.1
u - Uninduced cells

| SAMPLE | LENGTH OF INDUCTION (min) | $OD_{600}$ AT END OF INDUCTION |
|---|---|---|
| 1 | 0 | 0.33 |
| 2 | 30 | 0.427 |
| 3 | 63 | 0.525 |
| 4 | 95 | 0.594 |
| 5 | 120 | 0.678 |
| 6 | 200 | 0.784 |
| 7 | 240 | 0.804 |
| 8 | 420 | 0.875 |

Table 5.2 The effect of varying the length of induction

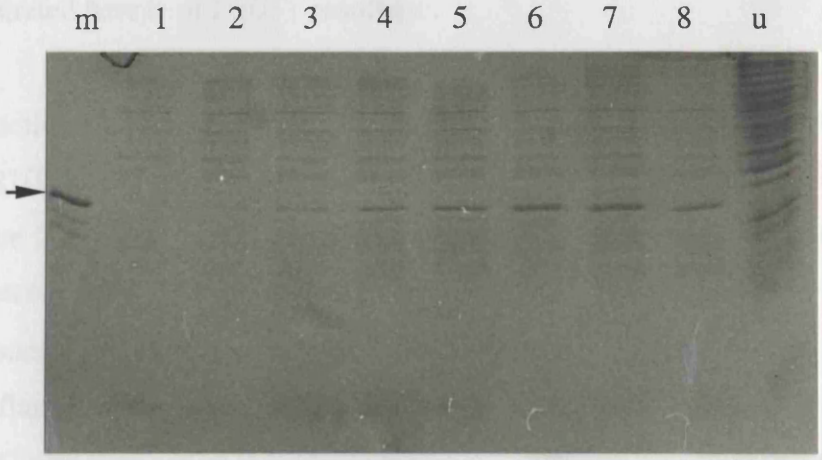



**Figure 5.5 The effect of length of induction.**

Laemmli gel showing the effect of increasing the length of time of induction on BL21(DE3)pLysS(pSA1101) cells. Details are listed in Table 5.2. Position of resolvase is denoted by an arrow.

Lanes m - Resolvase marker
1 - 8 - As indicated in Table 5.1
u - Uninduced cells

RHB : 2 M NaCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 mM DTT

PMSF Solution: 10 mg/ml in ethanol

Tris Buffer-1 : 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.4 mM DTT

Tris Buffer-2 : Tris Buffer 1 + 100 mM NaCl

Tris Buffer -3 : Tris Buffer 1 + 2 M NaCl

Urea Buffer-A : 7 M urea, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.4 mM DTT

Urea Buffer-B : Urea Buffer-A + 1 M NaCl

Urea Buffer-C : Urea Buffer-A + 2 M NaCl

At various stages throughout the purification, ~5 µl samples were taken and run on a Laemmli gel to follow the progress of the protein. These are shown in brackets. Several resolvase mutants were purified by the procedure described; the specific purification illustrated here is of D102Y resolvase.

Induction of the cells was carried out as follows. 5 ml of overnight culture of BL21(DE3)pLysS(pATXD102Y) was inoculated into 500 ml of pre-warmed L-broth in a 2 litre flask with the appropriate antibiotics, chloramphenicol and kanamycin at a concentration of 50 µg/ml (final) each. Two flasks were set up, and cells were grown for 3 hours at 37 °C until the $OD_{600}$ was 0.445. 5 ml of the culture was then removed from the flask and placed in a boiling tube, and grown to act as a control for non-induced cells. IPTG was then added to both the flasks to a final concentration of 1 mM. The cultures were induced for 3 hours at 37 °C with shaking. Cells were then spun down in a pre-chilled rotor at 5000 rpm for 10 minutes, then weighed and stored at -20 °C. The two cultures weighed 2.96 g and 2.87 g.

Ice-cold KPM Buffer (14 ml) + PMSF solution (150 µl) was added to cells thawing on ice, and the cells were resuspended (sample 1S). The mixture was sonicated, with a Vibra cell VC100 sonicator probe, on ice at 40% power, 6 x 20 seconds, with a 10 minute gap between bursts to prevent the cells overheating. The mixture was centrifuged at 18 000 rpm for 10 minutes, 4 °C in a JA-20 rotor, and the supernatant was decanted and kept on ice (samples 2S + 2P). The pellet was resuspended in 10 ml of Tris-Buffer-1, and homogenised (7 ml Dounce Tissue Grinder, supplied by Wheaton) before

centrifuging as above (samples 3S + 3P). The supernatant was removed, and the pellet was resuspended in 10 ml of Tris Buffer 2. After homogenising as before, the mixture was recentrifuged at 18 000 rpm, 10 minutes, 4 °C (samples 4S + 4P).

The pellet was resuspended in 10 ml of Urea buffer A, then homogenised thoroughly and left to resuspend for 1-2 hours. After which, the mixture was spun at 18 000 rpm, 15 minutes at 20 °C. The supernatant and the pellet were kept (5S +5P) and a Laemmli gel was run to check the location of the protein (Figure 5.6).

Once it had been confirmed that the resolvase was present in the supernatant (sample 5S), it was passed down a small SP sepharose Fast Flow cation exchange column ~3 cm high, equilibrated with Urea Buffer A. After loading, the column was washed with 5 column volumes of Urea Buffer A. This removed unbound protein, DNA and RNA from the column. The resolvase was then eluted from the column by washing with Urea Buffer B. 5 x 1.5 ml samples were collected, and these fractions were run on a second Laemmli gel. The resolvase was generally observed to be in the 2nd and 3rd fractions (Figure 5.7).

The appropriate resolvase fractions were then dialysed against Tris-Buffer 1 (1 l). The resolvase precipitated, and after spinning (15 000 rpm, 4 °C, 10 minutes) the pellet was redissolved in Urea Buffer A (400 µl).

The sample was loaded onto a Pharmacia MonoS FPLC column (volume 400 µl), and washed through with Urea Buffer A until a relatively steady base line had returned. A gradient, varying the salt concentration from 0 to 500 mM NaCl over 60 minutes, was then carried out to elute the resolvase from the column at 0.5 ml/minute, and 0.5 ml fractions were taken. The resolvase was, in general, found to elute at between 200 and 300 mM NaCl. Fractions containing resolvase were identified by electrophoresis on a Laemmli gel (Figure 5.8).

Appropriate fractions were then dialysed at 4 °C against 2 litres of Tris-Buffer 3 (FPLC Fractions 6, 7, 8 & 9. These were combined into two dialysis bags, (a) - Fractions 6 & 7 and (b) - Fraction 7 & 8). This allows the resolvase to refold in solution. The two solutions of resolvase (a) and (b) were dialysed for the second time against "low salt"

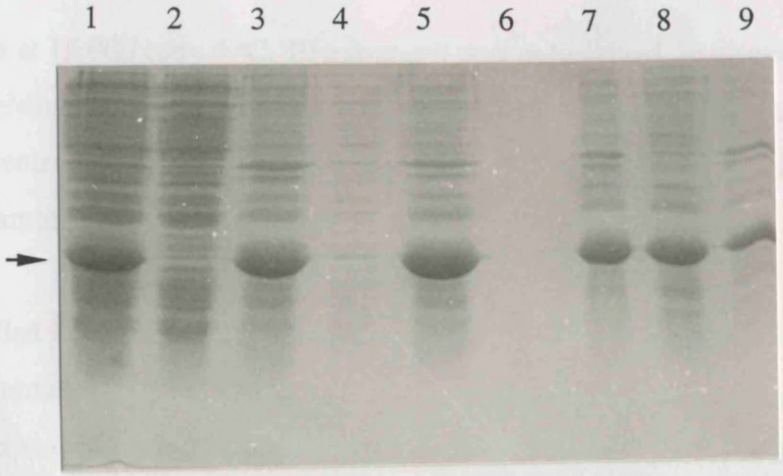



**Figure 5.6 D102Y Resolvase purification - Gel 1**

Illustration of resolvase purification up to the denaturing step. See text for details.

Lanes 1 - Resuspended induced cells (1S)
2 - Supernatant after sonication and precipitation (2S)
3 - Pelleted sonicated cells (2P)
4 - Supernatant from first wash (3S)
5 - Pelleted cell debris (3P)
6 - Supernatant from second wash (4S)
7 - Pelleted cell debris (4P)
8 - Cell debris resuspended in 7 M urea (5S)
9 - Pelleted cell debris (5P)

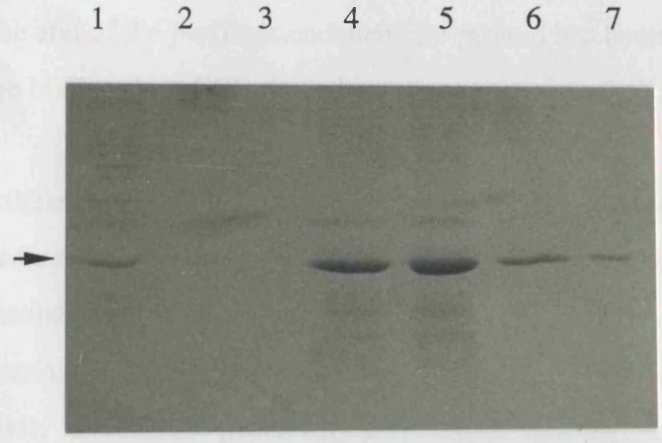



**Figure 5.7 D102Y Resolvase purification - Gel 2**

Continuation of the resolvase purification. Fractionation on the SP sepharose Fast Flow column. See text for details.

Lanes 1 - Column flow-through
2 - Column wash
3 - Fraction1
4 - Fraction 2
5 - Fraction 3
6 - Fraction 4
7 - Fraction 5

these at 15 000 rpm, 4 °C, 10 minutes it was redissolved, in a small volume of RHB (100 µl) giving Sn (a) and (b). These were run on a Laemmli gel to determine their concentration and purity (Figure 5.8). Staining with Coomassie did not reveal any contaminating protein bands.

Purified resolvase was then stored in RHB + 50% v/v glycerol at -20 °C. Considering the dramatic improvement in the over expression of resolvase with the new pET based expression system, the yields from the new purification protocol, though reproducible and consistent, have been relatively low. On examination of the Laemmli gels run concurrent with the purification, it was observed that at several stages the resolvase concentration drops visibly. The first of these is the resuspension of the pellet in Urea Buffer for the first time. Though it is difficult to measure accurately the proportion of the pellet run on the gel, it is obvious that there is a substantial amount of resolvase which remains in the pellet at this time. The second stage where resolvase disappears is during separation of the urea solution down the small SP Sepharose column. On loading, it seems that not all the resolvase is bound to the column, and some may be lost as flow through. However this does not account for the large loss of resolvase. There is little evidence of degradation of the protein at this time. The final stage where resolvase is lost is at the end of the purification where the protein has been dialysed and is redissolved in storage buffer. It is difficult at this stage to resuspend all the resolvase.

Nevertheless, the new procedure seems to be very reproducible, and resolvase has been purified successfully from all mutants tried so far. Wild-type resolvase was purified in this manner from pTA1, a precursor to pSA1101. In standard resolution assays, resolvases produced by this new denaturing method have been shown to be comparable in activity to resolvase previously prepared using a non-denaturing method.

The following mutant resolvases were made by this procedure *in vitro*; L69F, D102Y, and E124Q. The expression plasmids pATXD102Y and PATXL69F were created by removing the (*Asp*718 - *Nde*I) restriction fragment, containing the resolvase reading frame from pAT5D102Y and pAT4 respectively, and replacing the equivalent fragment in pSA1101. The creation of pATXE124Q is described in Chapter 4. Y6F resolvase was made by a similar purification protocol, but using the pKK223-based expression system

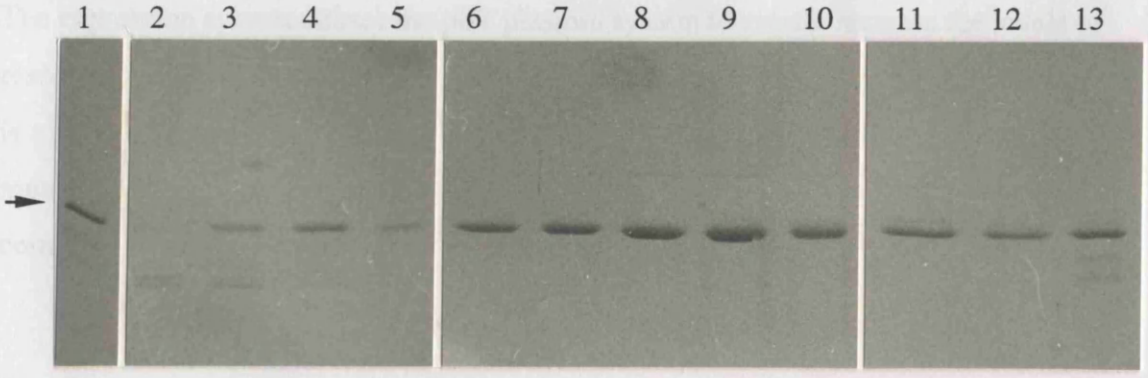



**Figure 5.8 Resolvase purification Gels 3 & 4**

Gels illustrating D102Y resolvase purification by monoS FPLC and showing its final purity after dialysis. Lanes 2-10 comprise fractions taken from the FPLC column. Lanes11-13 show resolvase after the dialysis steps.

Lanes 1 - Resolvase marker
2 - FPLC Fraction 1
3 - FPLC Fraction 2
4 - FPLC Fraction 3
5 - FPLC Fraction 4
6 - FPLC Fraction 5
7 - FPLC Fraction 6
8 - FPLC Fraction 7
9 - FPLC Fraction 8
10 - FPLC Fraction 9
11 - Sn (a) after Dialysis
12 - Sn (b) after Dialysis
13 - Resolvase marker

(see Chapter 3). The *Eco*RI - *Hind*III fragment of pKK223 was replaced by the equivalent fragment of pDB6020-Y6F to give the expression plasmid pATXY6F.

## 5.4 Summary

This chapter has outlined a new expression and purification protocol for Tn*3* resolvase. The expression system utilises the pET plasmid system to greatly increase the levels of resolvase expressed per gram of cells. The purification system which has been developed is a denaturing protocol, and it has been shown to be reproducible for the variety of mutants which have been created (see Chapter 4; A. McDonald, personal communication).

# Chapter 6

*In vitro* analysis of altered Tn*3* resolvases

# *In Vitro* Analysis of altered Tn3 Resolvases

## 6.1 Introduction

After the construction and subsequent purification of a selection of Tn*3* resolvase mutants, (see Chapters 4 and 5) it was now possible to examine their behaviour *in vitro*. Investigation of their *in vivo* properties, using the *galK* screen (see Section 4.5) indicated that the mutant Y6F was catalytically inactive in the wild type reaction, E124Q and L69F showed behaviour typical of the wild type protein, and D102Y in addition to promoting the wild type reaction *in vivo*, could catalyse recombination on a substrate containing one complete *res* site and an incomplete site consisting only of site I (see Chapter 5). Examination of *in vitro* products by agarose gel electrophoresis can provide us with more detailed information as to how the reaction is proceeding as compared to the *in vivo* results. These indicate only whether the recombination reaction has taken place. Analysis *in vitro* may help to determine the relative efficiencies of the reactions of mutants, and if illegitimate reactions, such as intermolecular recombination and cleavage events, are taking place which may not be detected in *in vivo* assays. *In vitro* analysis is also important in determining the topology of the reaction products formed.

The standard resolvase *in vitro* reaction system is described in Section 2.33. A substrate plasmid containing two *res* sites in direct repeat is treated with dilutions of Tn*3* resolvase in an appropriate buffer. Resolution of the substrate by wild type resolvase results in the formation of a singly linked catenane; after restriction digestion with an appropriate enzyme that cuts at only one site in the substrate, a linear piece of DNA and a circle are produced. The reaction is illustrated in Figure 3.1. By using a variety of substrate plasmids containing partial or complete *res* sites, the properties of the mutant proteins can be determined *in vitro*.

The substrates used for analysis of the mutants are detailed in Figure 6.1. These substrates, except for the wild type substrate pMA21 containing two *res* sites in direct repeat, contain a variety of partial and whole *res* sites which would not be resolved by the wild type protein under the standard conditions. The standard conditions for the resolution reaction include incubation at 37 °C for a period of 30 minutes, in the standard

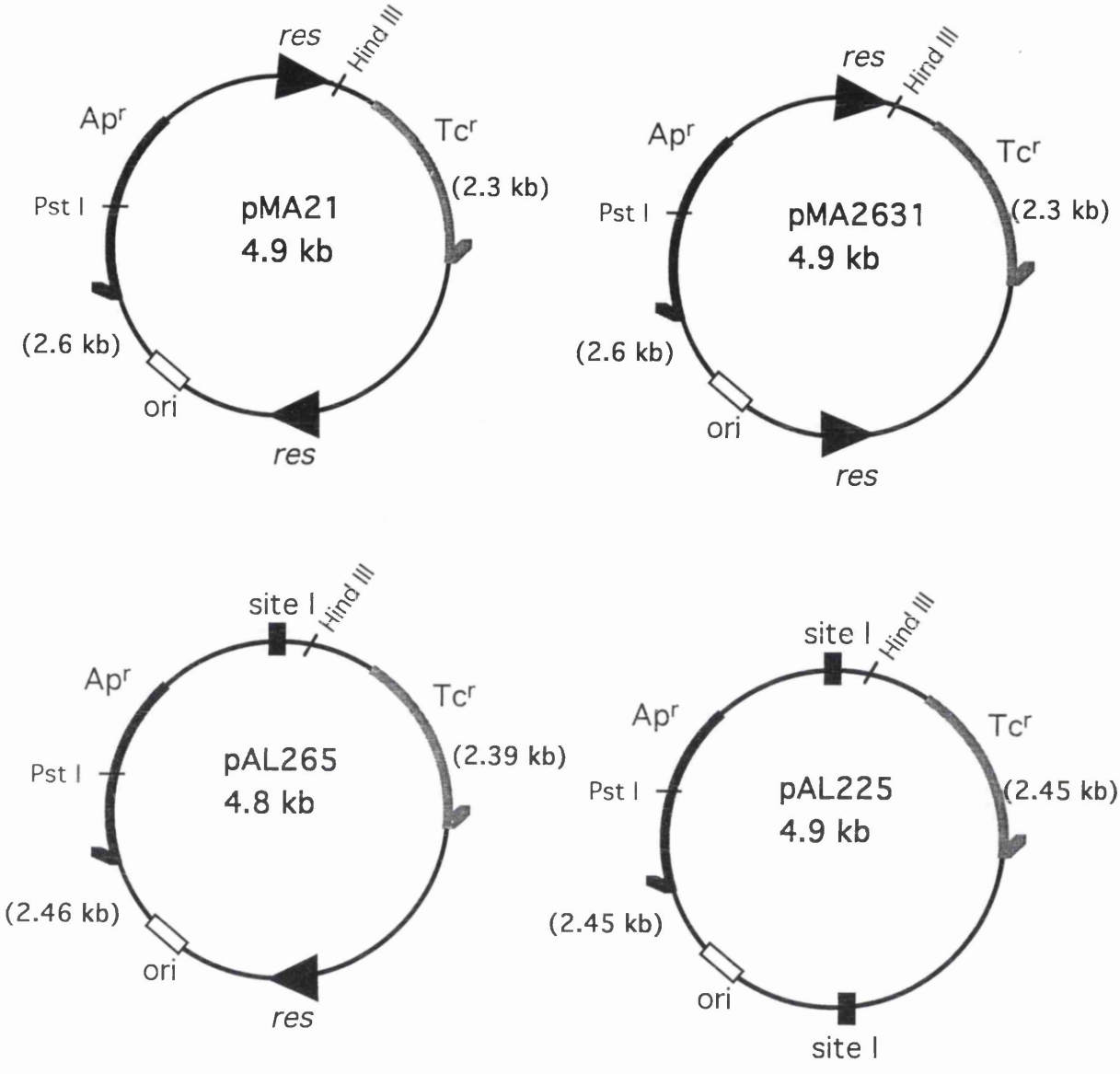



**Figure 6.1** ***In vitro*** **Tn3 resolvase substrates**

Diagrams of the plasmid substrates used for *in vitro* analysis of resolvase recombination. Sizes indicated in brackets refer to the resolution products of the substrate.

reaction buffer, C9.4 (50 mM Tris-HCl (pH 9.4), 10 mM $MgCl_2$, 0.1 mM EDTA) with an aliquot of resolvase (see Section 2.33).

The other technique used in this chapter to analyse the properties of mutant proteins is the band shift assay. This allows us to examine the early steps in the reaction, whether the protein is binding to the *res* site as the wild type protein would, by forming the standard six complexes when bound to a linear full length *res* site. In this assay, the wild-type protein has been shown to bind as a monomer in a co-operative manner (Blake, 1993; Blake *et al.*, 1995). The plasmid used to generate the *res* fragment to which the resolvase is bound in the assay is illustrated in Figure 6.2.

## 6.2 *In vitro* analysis of Y6F Resolvase

After purification of the Y6F mutant, resolvase recombination activity *in vitro* was determined in the standard resolution assay (see Section 2.33).

In order to carry out a titration of the Y6F protein, a series of 2 fold dilutions of the stock solution was carried out. In the case of Y6F, it had been predicted that the protein would have little or no recombinational activity; therefore reactions were carried out for various lengths of time on a wild type substrate, pMA21 (containing 2 directly repeated *res* sites; Figure 6.1). The reaction was carried out in C8 buffer (defined in Section 2.33). 40 µl samples were incubated at 37 °C, after which the resolvase was inactivated by heat (70 °C for 5 minutes). 20 µl was then removed, and a *Hin*dIII restriction digest was carried out. Since there is only one site for *Hin*dIII on the substrate plasmid pMA21, the enzyme cleaves only one of the circles in the catenane product, resulting in a linear piece of DNA and a small free circle; these products can be distinguished from the original substrate by agarose gel electrophoresis.

Agarose gel electrophoresis (Figure 6.3) showed that the Y6F protein was completely inactive, which may be expected since we have mutated a highly conserved residue. However, the Y6F protein did retain some topoisomerase activity, and slowly relaxed the substrate DNA. This suggests that the protein has retained its ability to bind to the substrate, cleave, relax and religate the DNA, but is unable to complete the recombination

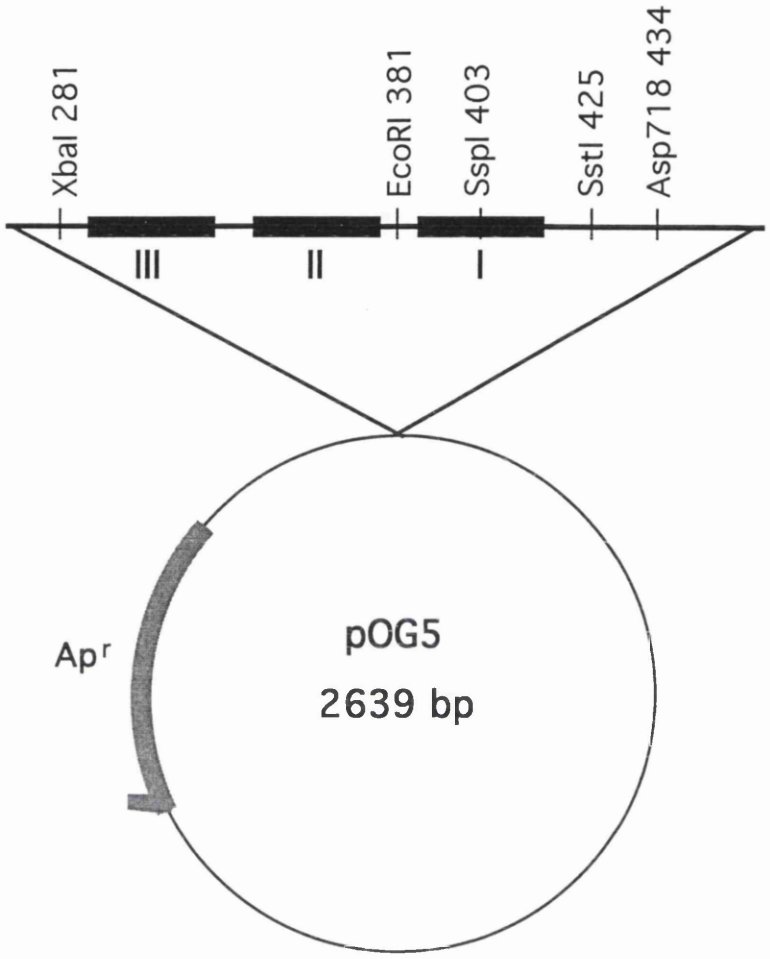



**Figure 6.2 Illustration of pOG5**

The diagram shows the plasmid used in the generation of the fragment used for gel shift assays. The fragment was created by an *Xba*I - *Asp*718 restriction digest. pOG5 is derived from pMTL23. The *res* in pOG5 has been altered from the wild-type at 3 bp between sites I and II, creating an *Eco*RI site (Blake *et al.*, 1995).

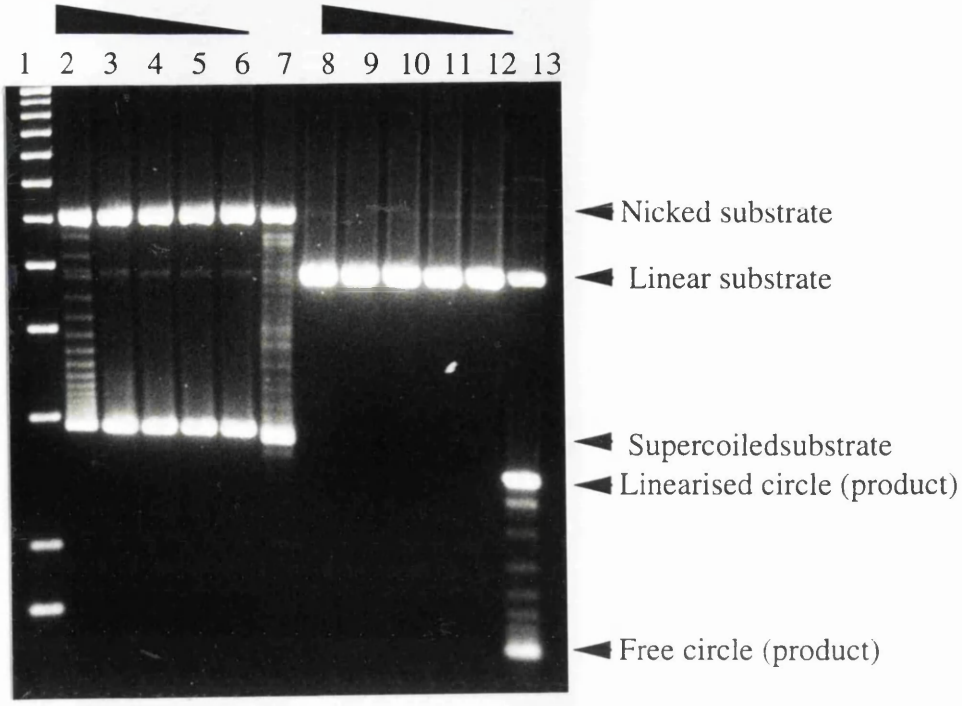



**Figure 6.3 Reaction of pMA21 with the Y6F mutant of Tn*3* resolvase**

Reaction of pMA21 was carried out in C8 buffer for 16 hours at 37 °C. After heat inactivation at 70 °C, a *Hind*III restriction digest was carried out on half the reaction mix (20 µl). Restricted and unrestricted samples were loaded onto a 1.2% agarose gel , which was run at 40 V for 14 h. A series of 2-fold dilutions of Y6F resolvase were used. The approximate stock concentrations of Y6F resolvase and wild-type resolvase are 12 µM and 400 µM respectivley.

Lanes 1 - 1 kbp ladder
2 - undiluted Y6F
3 - 2-fold dilution of Y6F
4 - 4-fold dilution of Y6F
5 - 8-fold dilution of Y6F
6 - No resolvase control
7 - 8-fold dilution of wild-type resolvase
8 - 13 - As samples 2 - 7, but digested with *Hind*III.

recombination reaction. This evidence suggests that the tyrosine-6 is not required as a nucleophile for the cleavage and religation of the DNA to proceed. But, it must have some important role, perhaps as a proton donor/acceptor, or in the structure of the active site. Topoisomerisation is observed but not recombination, perhaps because it requires the cleavage of only one DNA phosphodiester, whereas recombination requires simultaneous cleavage of four phosphodiesters.

Two further experiments were carried out to determine the effect of the Y6F mutation on the recombination reaction. A dilution of wild type resolvase, which promoted recombination at a very low level, was added to a series of Y6F dilutions, and 40 µl reactions were incubated for 30 minutes as above. The reactions were stopped, and 20 µl from each sample was treated with *Hin*d III. As Figure 6.4 shows, the presence of an increasing amount of Y6F protein does not seem to increase the rate or level of recombination taking place, but topoisomerase activity on the substrate is seen at the higher concentrations of Y6F. Note that this topoisomerase activity is much higher than with Y6F alone, as in Figure 6.3 (incubation was for only 30 min rather than 16 h). Equally, an experiment carried out where a Y6F dilution was added to the standard resolvase reaction, carried out in the same way as above (Figure 6.5) showed an enhancement of the topoisomerase activity shown previously by the protein, on the substrate as well as the product. This addition of the wild-type protein resulting in an enhancement of topoisomerase activity, followed by recombination suggests that the replacement of increasing numbers of subunits of the Y6F protein by wild-type resolvase at site I, allows the protein at site I to catalyse the recombination reaction. The possibility has not been ruled out that Y6F resolvase is completely catalytically inactive, and the very weak topoisomerase activity of the Y6F preparation is caused by traces of wild-type resolvase in it (for example, by accidental incorporation of tyrosine instead of phenylalanine during translation of the mRNA). Topoisomerase activity, seemingly induced here by the addition of wild-type resolvase, has been observed in experiments using wild-type resolvase when some defect in the system interferes with normal recombination, such as inappropriately spaced or mutant *res* sites (Stark *et al.*, 1991). This would suggest that the catalytic inactivity of the Y6F protein is not due to the disruption of the synaptosome by the mutation, since the protein may still be bound to the accessory sites, but is due to the modification of the enzyme at the active site. Further

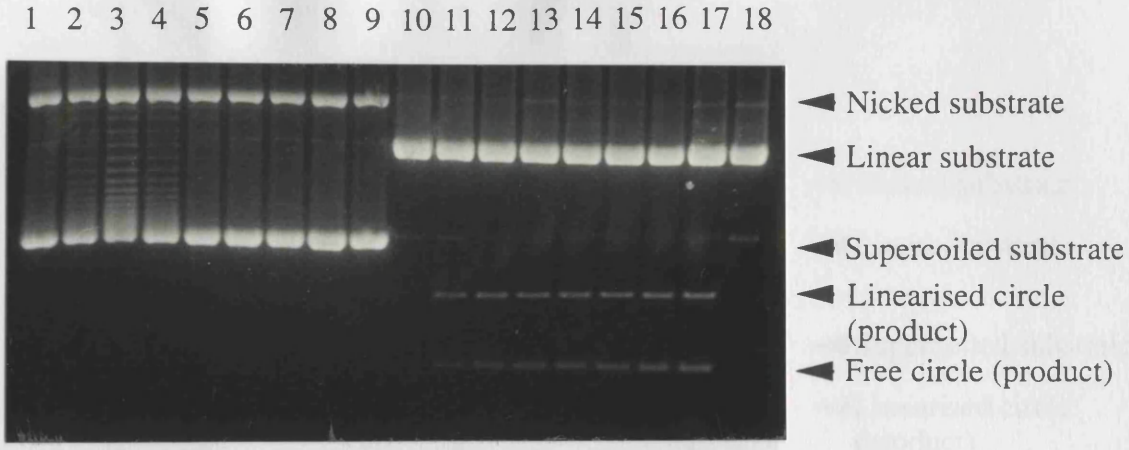



**Figure 6.4 Effect of increasing amounts of Y6F resolvase on the wild-type reaction**

Resolution reactions of pMA21 with wild-type resolvase (diluted to give only a low level of resolution) and increasing amounts of Y6F resolvase. Reactions were incubated for 30 minutes at 37 °C before stopping, and a restriction digest was carried out with *Hin*dIII on half the reaction mixture. Both restricted and unrestricted samples were loaded on a 1.2% agarose gel and run at 40 V overnight. The approximate protein concentration of stock Y6F resolvase is 12 μM, while stock wild-type resolvase is approximatly 400μM.

Lanes 1 - Undiluted Y6F resolvse
2 - Undiluted Y6F resolvase + wild-type resolvase
3 - 2-fold dilution of Y6F resolvase + wild-type resolvase
4 - 4-fold dilution of Y6F resolvase + wild-type resolvase
5 - 8-fold dilution of Y6F resolvase + wild-type resolvase
6 - 16-fold dilution of Y6F resolvase + wild-type resolvase
7 - 32-fold dilution of Y6F resolvase + wild-type resolvase
8 - Wild-type resolvase alone
9 - No resolvase control (dilution buffer)
10 - 18 - Samples from lanes (2) to (9) restricted with *Hin*dIII.

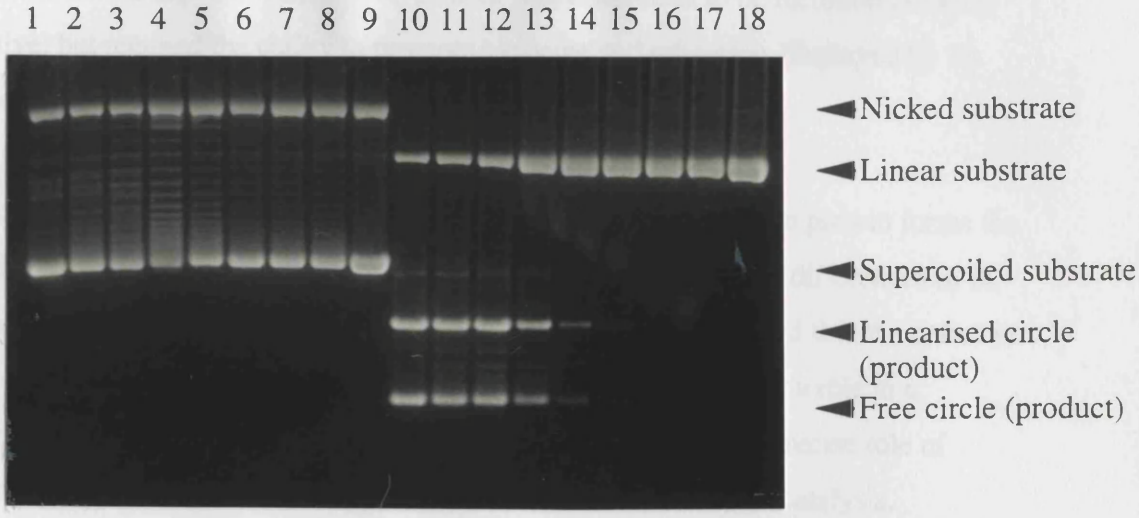



**Figure 6.5 Effect of adding increasing amounts of wild-type resolvase to Y6F reactions**

Reactions with wild-type resolvase and a 4-fold dilution of Y6F resolvase were incubated at 37 °C for 30 minutes then stopped by heat. Half the reaction mix was removed and a *Hin*dIII digest was carried out. The restricted and unrestricted samples were loaded onto a 1.2% agarose gel and run at 40 V. Approximate protein concentrations are as indicated in Figure 6.3.

Lanes 1 - 16 -fold dilution of wild-type resolvase
2 - 16 -fold dilution of wild-type resolvase + Y6F resolvase
3 - 32 -fold dilution of wild-type resolvase + Y6F resolvase
4 - 64 -fold dilution of wild-type resolvase + Y6F resolvase
5 - 128 -fold dilution of wild-type resolvase + Y6F resolvase
6 - 256 -fold dilution of wild-type resolvase + Y6F resolvase
7 - 512 -fold dilution of wild-type resolvase + Y6F resolvase
8 - 4-fold dilution of Y6F resolvase
9 - No resolvase control (dilution buffer)
10 - 18 - As lanes 2 - 10 but restricted with *Hin*dIII

evidence to support this comes from the γδ Y6F protein (Leschziner *et al.*, 1995). Experiments in which γδ Y6F resolvase was targeted to the accessory sites, and using a mutated site I, which bound only a specific mutant able to carry out catalysis, resulted in normal products, indicating that the synaptosome was stable when the Y6F protein was bound at sites II and III. The γδ Y6F protein was also found to be recombinationally inactive, but retained the ability to promote cleavage and religation, displayed by the observation of topoisomerase activity.

Binding studies confirmed that in the presence of a linear *res* site, the protein forms the standard 6 complexes. (Figure 6.6). The protein shows no obvious differences in its binding pattern in comparison to the wild type protein. This indicated that the defect in Y6F does not inhibit the binding of the protein, and so Y6 must play a role in a subsequent step in the reaction. Though it is not clear yet as to the precise role of tyrosine-6 in the reaction, the results have shown it is important for catalysis.

## 6.3 Analysis of E124Q Resolvase

From *in vivo* studies on the E124Q protein (Chapter 5), it was predicted that this protein would retain wild-type recombinational activity, but unlike the γδ E124Q resolvase mutant would have little or no activity on partial *res* sites.

It had been predicted from the co-crystal structure of γδ resolvase bound to site I (Yang and Steitz, 1995) that E124 was important since it formed a hydrogen bond with serine-10 of one of the subunits. This hydrogen bond was thought to be helping to hold the protein in an inactive configuration. E124 mutants may therefore be "activated" in that a mutation may remove the hydrogen bonding and allow the structure more flexibility. This was found to be the case with the γδ E124Q mutant, which was observed to carry out recombination reactions on linear substrates and partial *res* sites, catalyse intermolecular reactions and to sometimes leave cleaved ends. These are all events not normally catalysed by the wild-type protein. The γδ E124Q mutant has what can be described as a hyperactive phenotype in comparison to the wild-type protein.

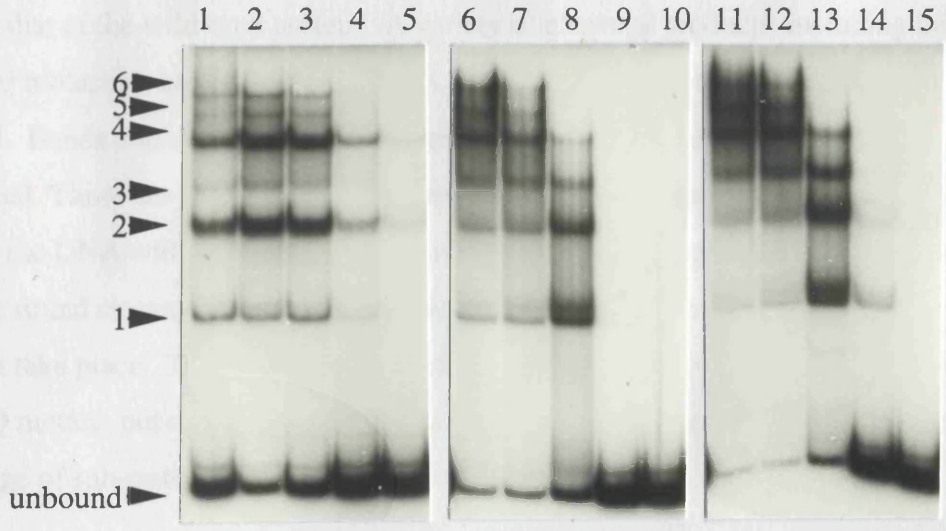



**Figure 6.6 Binding of wild-type, L69F and Y6F resolvases to *res***

2 fold dilutions of each protein were used to carry out a titration on the res fragment. The approximate concentrations of the protein stocks are as follows,

Wild-type resolvase - 400 μM
L69F resolvase - 50 μM
Y6F resolvase - 12 μM

Lanes 1 - 16-fold dilution of wild type resolvase
2 - 32-fold dilution of wild type resolvase
3 - 64-fold dilution of wild type resolvase
4 - 128-fold dilution of wild type resolvase
5 - No resolvase (dilution buffer)
6 - Undiluted L69F resolvase
7 - 2-fold dilution of L69F resolvase
8 - 4-fold dilution of L69F resolvase
9 - 8-fold dilution of L69F resolvase
10 - No resolvase (dilution buffer)
11 - Undiluted Y6F resolvase
12 - 2-fold dilution of Y6F resolvase
13 - 4-fold dilution of Y6F resolvase
14 - 8-fold dilution of Y6F resolvase
15 - No resolvase (dilution buffer).

Initial studies confirmed that the Tn*3* resolvase mutant E124Q was active on the wild-type substrate pMA21 (Figure 6.7). Restriction digests of the reaction with an enzyme which cleaves the substrate DNA once, results in the linear and circular products one would expect from the wild-type reaction; however, the circular product has been relaxed to a relatively high extent. This topoisomerase activity may suggest that the E124Q mutant has enhanced cleavage activity, or has lost the specificity for formation of the synaptosome before cleaving the DNA, possibly due to its "activation". The agarose gel of the undigested resolution reactions indicates the mutant E124Q is displaying behaviour unlike that of the wild-type protein. A variety of cleavage products, including linear and circular molecules are seen on the gel. A large proportion of the circular products are nicked. Bands corresponding to nicked recombinant circles and half-nicked catenane are observed. These are indications that the mutant has an activated phenotype whereby it will cleave the DNA without the full recombination reaction taking place. The presence of double strand cleavage products also indicates that the complete recombination reaction did not take place. This mutant does not seem to have as activated a phenotype as the γδ E124Q mutant, but compared to wild-type Tn*3* resolvase, this mutant gives enhanced cleavage of substrate and products.

No recombinational activity on plasmids containing partial *res* sites (pAL265 and pAL225) by Tn*3* E124Q resolvase was observed. Various conditions were explored, including changing length of incubation and the buffer conditions, but to no avail (data not shown). This is contrary to the γδ E124Q resolvase which had the ability to carry out recombination on partial *res* sites *in vitro* (M. Boocock, personal communication).

In band shift assays (Figure 6.8), E124Q resolvase displayed a slightly different than normal binding pattern. The protein has the ability to form the lower complexes, and seems especially efficient at forming the first complex (corresponding to binding of a resolvase monomer), but seems to find it difficult to form the higher complexes such as the fifth and sixth. This would indicate less efficient binding than normal. This inefficiency in binding may lead to some of the altered behaviour observed in the *in vitro* analysis; for example, the double strand cleavage products might be the result of premature dissociation of resolvase subunits in a recombination intermediate.

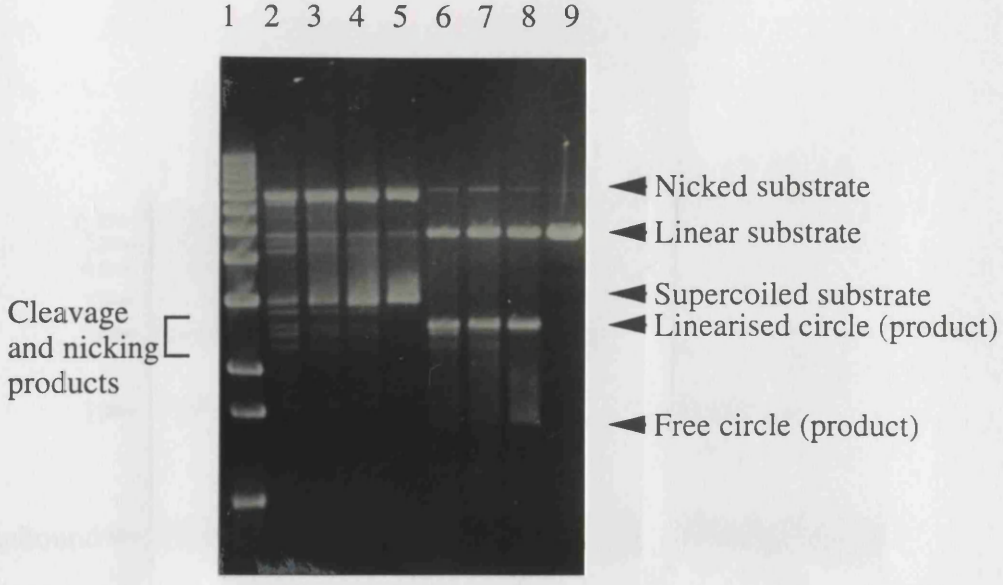



**Figure 6.7 Resolution of pMA21 (*res* v *res*) by E124Q resolvase**

Resolution reactions were incubated with dilutions of E124Q resolvase for 1 h in C8 buffer, then stopped with heat and half the reaction mix was digested with *Pst*I. Samples were run on a 1.2% agarose gel for 16 h at 40 V. Of the four cleavage and nicking product bands, two correspond to nicked products (the top and third bands) and two represent linearised circles cleaved at *res* (the second and fourth bands). The approximate concentration of E124 stock resolvase is 50 μM.

Lanes 1 - 1 kbp ladder
2 - Undiluted E124Q resolvase
3 - 2-fold dilution of E124Q resolvase
4 - 4-fold dilution of E124Q resolvase
5 - No resolvase control (dilution buffer)
6 - Undiluted E124Q resolvase; digested with *Pst*I
7 - 2-fold dilution of E124Q resolvase; digested with *Pst*I
8 - 4-fold dilution of E124Q resolvase; digested with *Pst*I
9 - No resolvase control (dilution buffer); digested with *Pst*I

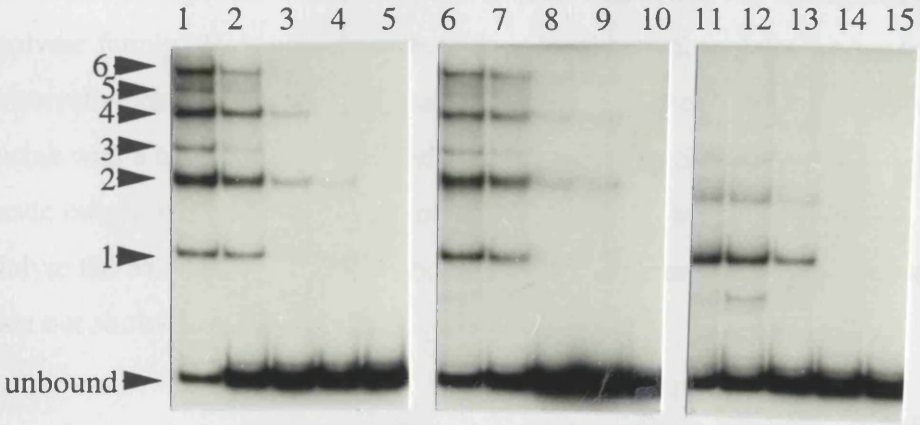



**Figure 6.8 Binding of wild-type, D102Y and E124Q resolvases to *res***

2 fold dilutions of each protein were used to carry out a titration on the *res* fragment.The approximate protein concentrations of resolvase stocks are as follows,

Wild-type resolvase - 400 μM
D102Y resolvase - 100 μM
E124Q resolvase - 50 μM

Lanes 1 - 16-fold dilution of wild-type resolvase
2 - 32-fold dilution of wild-type resolvase
3 - 64-fold dilution of wild-type resolvase
4 - 128-fold dilution of wild-type resolvase
5 - No resolvase (dilution buffer)
6 - Undiluted D102Y resolvase
7 - 2-fold dilution of D102Y resolvase
8 - 4-fold dilution of D102Y resolvase
9 - 8-fold dilution of D102Y resolvase
10 - No resolvase (dilution buffer)
11 - Undiluted E124Q resolvase
12 - 2-fold dilution of E124Q resolvase
13 - 4-fold dilution of E124Q resolvase
14 - 8-fold dilution of E124Q resolvase
15 - No resolvase (dilution buffer)

## 6.4 Analysis of L69F resolvase

Since we had inadvertently made this protein, we observed its *in vitro* properties. Since the first *in vivo* screening experiments were carried out using pAT4 (L69F), it was important to show that its phenotype had not been compromised. L69 is close to the active site of the protein (Chapter 3) and is quite well conserved throughout the Tn*3* resolvase family. However, the change from leucine to phenylalanine is a fairly conservative change, both amino acids being hydrophobic in nature. Replacement of leucine with a hydrophilic or charged residue at position 69 might have resulted in a more drastic outcome. *In vivo* screening of L69F (pAT4) showed that the protein was able to catalyse the wild-type reaction, but had no activity on plasmids containing partial *res* sites (data not shown).

An *in vitro* resolution reaction confirmed the ability of the L69F protein to perform resolution on the wild-type substrate, pMA21 (Figure 6.9). The protein also demonstrated a higher than normal level of topoisomerase activity, indicating that the mutation at L69 was having some effect on the activity of the protein, as previously seen with wild-type resolvase when a defect in the system prevents normal recombination (Stark *et al.*, 1991). Resolution reactions carried out on pAL265 and pAL225 confirmed the inability of the protein to promote resolution between partial *res* sites (data not shown).

The gel shift assay shown in Figure 6.6 demonstrated that this protein is more efficient at forming the higher fifth and sixth complexes in comparison with the wild-type protein. This aberration in binding activity may account for some of the unusual *in vitro* properties of the protein.

## 6.5 Analysis of D102Y resolvase

From the *in vivo* studies, the most likely of the mutants to carry out recombination *in vitro* on partial *res* sites was the "selected" mutant D102Y. This mutant demonstrated the ability to carry out resolution on a substrate plasmid containing one complete *res* site in direct repeat with a single site I, *in vivo*. However, the *in vivo* screen operates on the

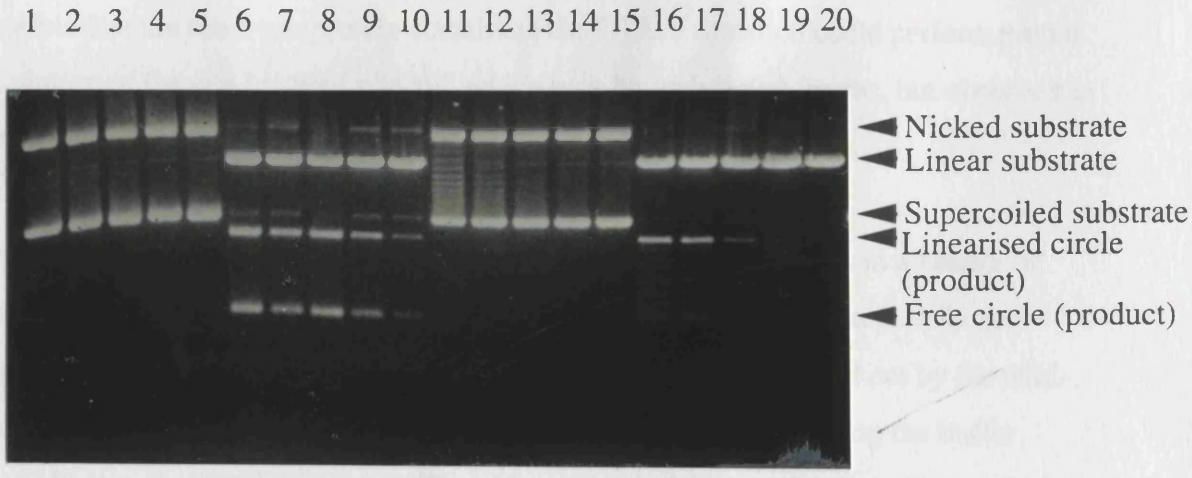



**Figure 6.9 Resolution of pMA21 by L69F resolvase**

Wild-type and L69F resolvase reactions were carried out in C9.4 buffer for 30 minutes before digesting half the reaction mix with *Pst*I. Samples were run on an agarose gel (1.2%) for 14 hours at 40 V. The approximate concentration of L69F stock resolvase is 50 μM, while wild-type resolvase stock concentration is 400 μM.

Lanes 1 - 4-fold dilution of wild-type resolvase
2 - 8-fold dilution of wild-type resolvase
3 - 16-fold dilution of wild-type resolvase
4 - 32-fold dilution of wild-type resolvase
5 - 64-fold dilution of wild-type resolvase
6 -10 - As lanes 1 - 5, but restricted with *Pst*I
11 - Undiluted L69F resolvase
12 - 2-fold dilution of L69F resolvase
13 - 4-fold dilution of L69F resolvase
14 - 8-fold dilution of L69F resolvase
15 - No resolvase control (dilution buffer)
16 - 20 - As lanes 11 - 15, restricted with *Pst*I

basis that very little resolvase is required in order to resolve the low copy number plasmid and result in a *gal*$^{-}$ clone, that is, a white colony on MacConkey galactose medium. This was not necessarily an indication that the protein would promote the reaction sufficiently to demonstrate the same result *in vitro,* or if indeed the reaction did proceed, to give sufficient reaction to allow analysis of the topology of the resulting products. It was also possible that the resolvase protein containing the D102Y mutation could perform partial resolution of the site I v site I plasmid which may be undetected *in vivo*, but observed *in vitro*.

Resolution reactions carried out with the wild type substrate, pMA21, in a variety of buffers showed that D102Y performed the standard resolution reaction as well as a variety of intermolecular reactions not normally observed to be carried out by the wild-type resolvase protein. (Figure 6.11). The products of a reaction using the buffer, M15M8 (see Section 2.33), for an incubation period of 6 h are shown in Figure 6.10. The protein clearly displays topoisomerase activity, relaxing the substrate DNA. Some free circular cleavage products and linear DNA are observed; however, these products are not formed to the same range or extent as the cleavage products from the action of the mutant protein E124Q on the substrate, pMA21, indicating that D102Y is relatively well behaved regarding the rejoining of the DNA ends. The protein clearly demonstrated a relaxed specificity for the substrate since it performed resolution between sites on different plasmids, indicated by the presence on the gel of bands ranging from 6 to 8 kb. Such intermolecular reactions are illustrated in Figure 6.11. These bands correspond to reactions between *res* sites present on different supercoiled substrates where the ends are joined as in the wild-type reaction, the left half of one site to the right half of the second. Bands present also indicate that certain "illegitimate" recombination events are taking place, where the right end of one *res* site is joined to the right end of the second *res* site on the other substrate molecule and left to left. This result is in keeping with the results of resolution reactions carried out on the substrate pAL265 (*res* v site I) (see below) and indicates that the protein is acting, to some degree, independently of the accessory sites II and III, unlike the wild-type protein.

Resolution reactions carried out on pAL265 (*res* v site I) at 37 °C for 16 hours in C9.4 buffer, showed that *in vitro* D102Y resolvase was able to resolve this plasmid. On

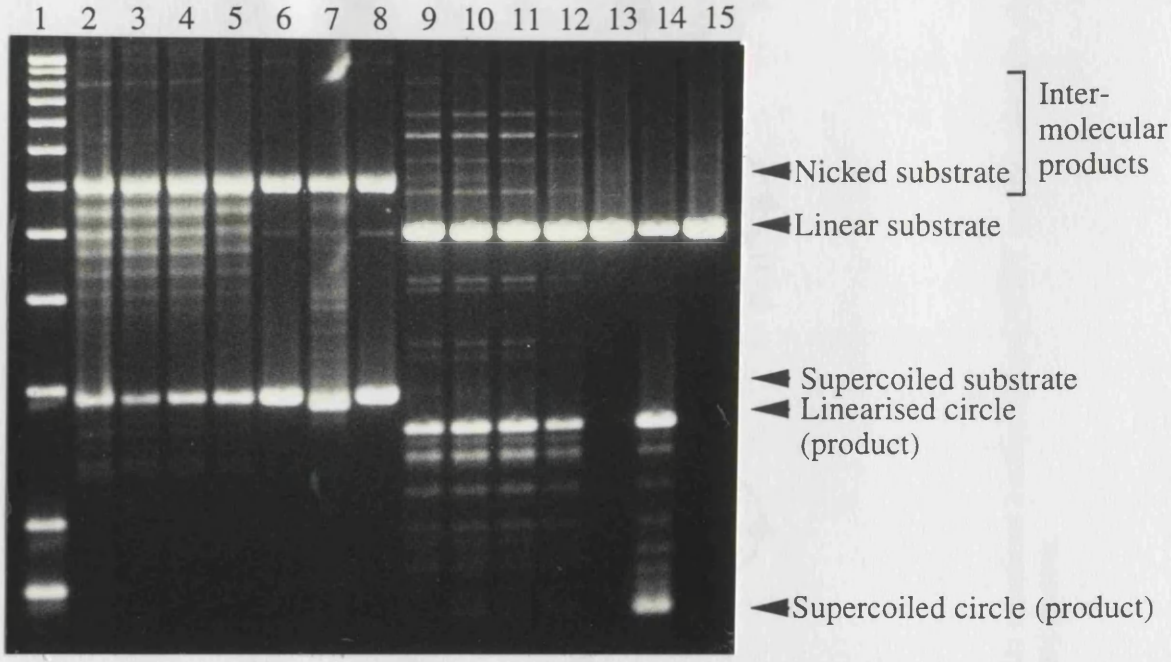



**Figure 6.10 Resolution of pMA21 (*res* v *res*) by D102Y resolvase**

Resolution reactions were carried out in M15M8 buffer for 6 h at 37 °C, and subsequently half the reaction mix was digested with *Pst*I. Samples were run on an agarose gel at 40 V for 16 hours. The gel clearly shows many products which have been accounted for as products of cleavage reactions, and the intermolecular reactions illustrated in Figure 6.11. The approximate concentration of stock D102Y resolvase is 100 μM, while the approximate concentration of wild-type resolvase stock is 400 μM.

Lanes 1 - 1 kbp ladder
2 - Undiluted D102Y resolvase
3 - 2-fold dilution of D102Y resolvase
4 - 4-fold dilution of D102Y resolvase
5 - 8-fold dilution of D102Y resolvase
6 - No resolvase control (dilution buffer)
7 - 16-fold dilution of wild-type resolvase
8 - No resolvase control (dilution buffer)
9 - 15 - As lanes 2 - 8, digested with *Pst*I

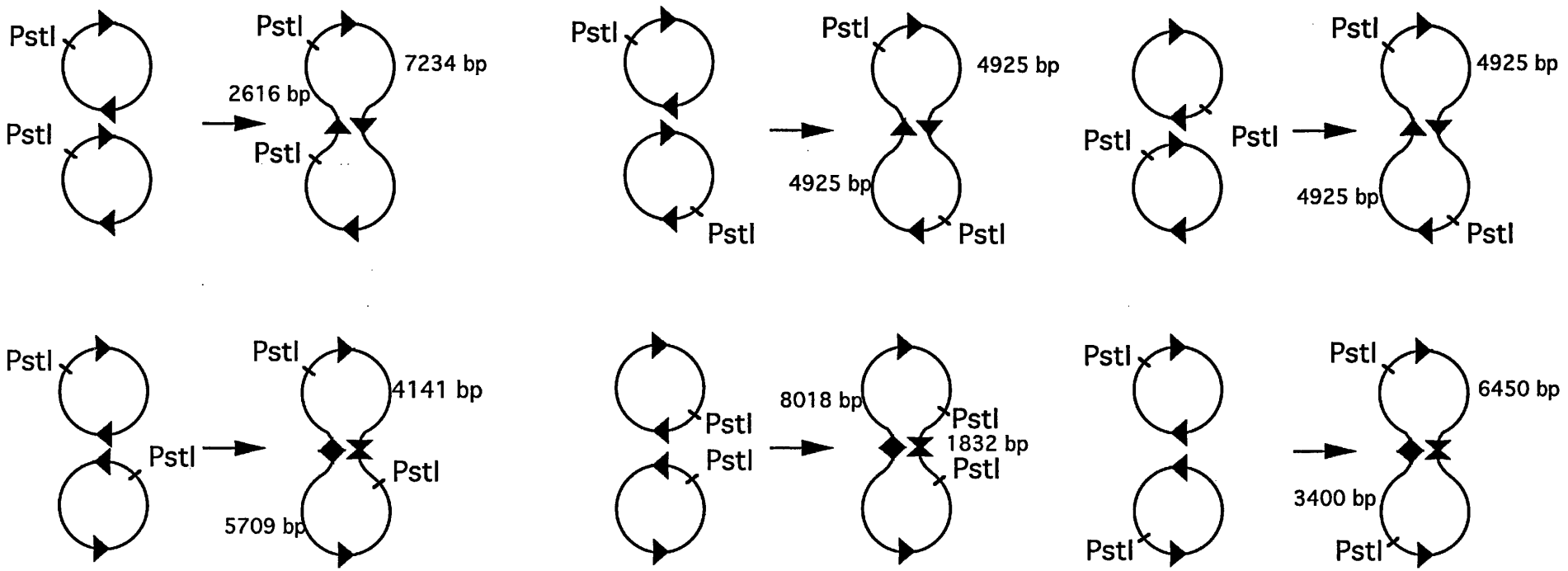



**Figure 6.11 Intermolecular Reactions of pMA21**

Figure showing the possible legitimate and illegitimate intermolecular reactions catalysed by D102Y on the substrate pMA21. The sizes of *Pst*I restriction fragments generated from the products are shown.

examination of the agarose gel (Figure 6.12), the *PstI* digest showed that the products were as expected, a free circle and a linear length of DNA. The protein is relatively poor at promoting the reaction, which proceeds at about 20%; but this is a dramatic improvement on the ability of the wild-type protein to promote this reaction under these conditions. This shows the ability of the protein to carry out the reaction in the absence of the accessory sites II and III on at least one of the *res* sites present in the substrate. The reaction is however proceeding through the standard pathway resulting in the catenane product (Figure 6.13), showing that even in the absence of these accessory sites the protein is bringing the sites together to form the normal synapse. However, D102Y resolvase was completely unable to perform any observable recombination on the site I v site I substrate, pAL225. It also failed to show any recombinational activity on the plasmid pAL2631, which contains inverted *res* sites. These results show that although D102Y resolvase has lost some of its dependency on the accessory sites, it is not totally independent of their presence, and still prefers to carry out the wild-type reaction, recombining two *res* sites in direct repeat.

Binding studies (Figure 6.8) showed that the protein displayed a normal binding pattern, indicating that its enhanced activity was not due to any greater affinity of the protein for the DNA.

## Summary

The *in vivo* results on the four mutant resolvases investigated have been upheld by the *in vitro* analysis, which has also expanded our knowledge of the reactions taking place. Y6F resolvase has been confirmed as being recombinationally inactive, but has been shown to retain its binding ability as well as cleavage and religation activity. Though studies of topoisomerases and strand transferases have shown the catalytic breakage and rejoining of DNA via transesterifications using phosphotyrosyl linkages, the evidence here supports the linkage, in the case of Tn*3* resolvase as being a phosphoseryl (serine-10). L69F resolvase has been shown to resolve the wild type substrate pMA21, but has also demonstrated some topoisomerase activity as well as aberrations in binding. Unlike the γδ E124Q resolvase mutant, the equivalent Tn*3* resolvase E124Q mutant has not shown the ability to resolve partial *res* sites, but can perform intermolecular reactions.

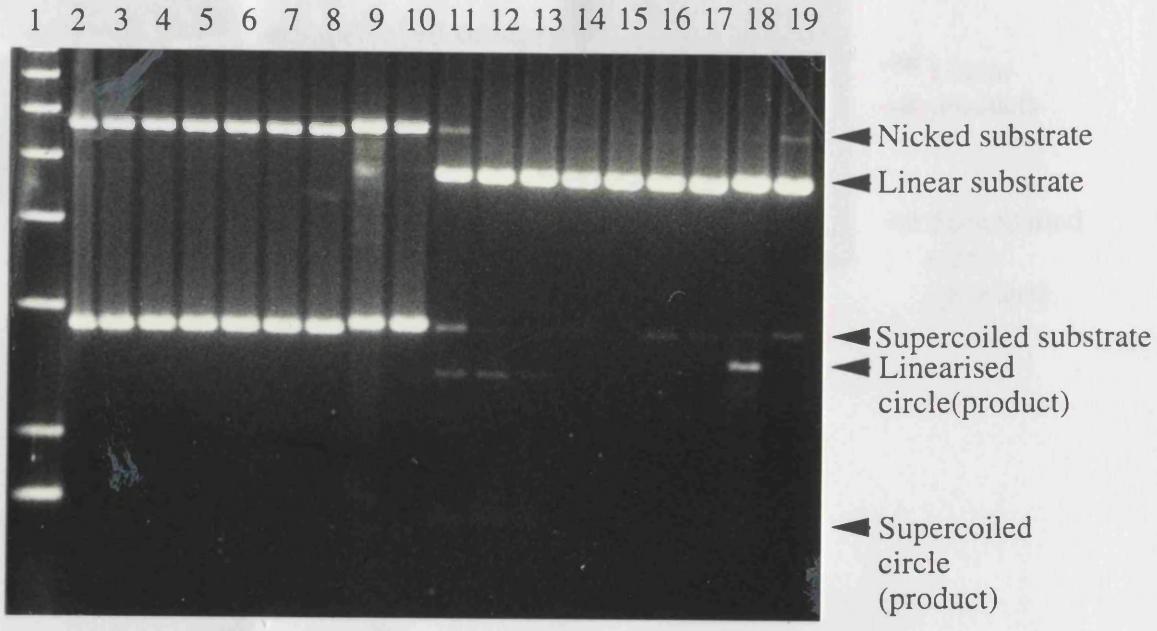



**Figure 6.12 Resolution of pAL265 (*res* v site I) by D102Y resolvase**

Resolution reactions were carried out in C9.4 buffer for 30 minutes, and half the mixture was digested with *Pst*I. Samples were run on a 1.2% agarose gel at 40 V for 16 hours. The approximate concentrations of D102Y resolvase and wild-type resolvase stocks are as indicated in Figure 6.10.

Lanes 1 - 1 kbp ladder
2 - Undiluted D102Y resolvase
3 - 2-fold dilution of D102Y resolvase
4 - 4-fold dilution of D102Y resolvase
5 - 8-fold dilution of D102Y resolvase
6 - No resolvase control (dilution buffer)
7 - 2-fold dilution of wild-type resolvase
8 - No resolvase control (dilution buffer)
9 - Undiluted D102Y resolvase on pMA21 (wild-type control substrate)
10 - No resolvase control, with pMA21 (dilution buffer)
11 - 19 - As lanes 1 - 9, digested with *Pst*I

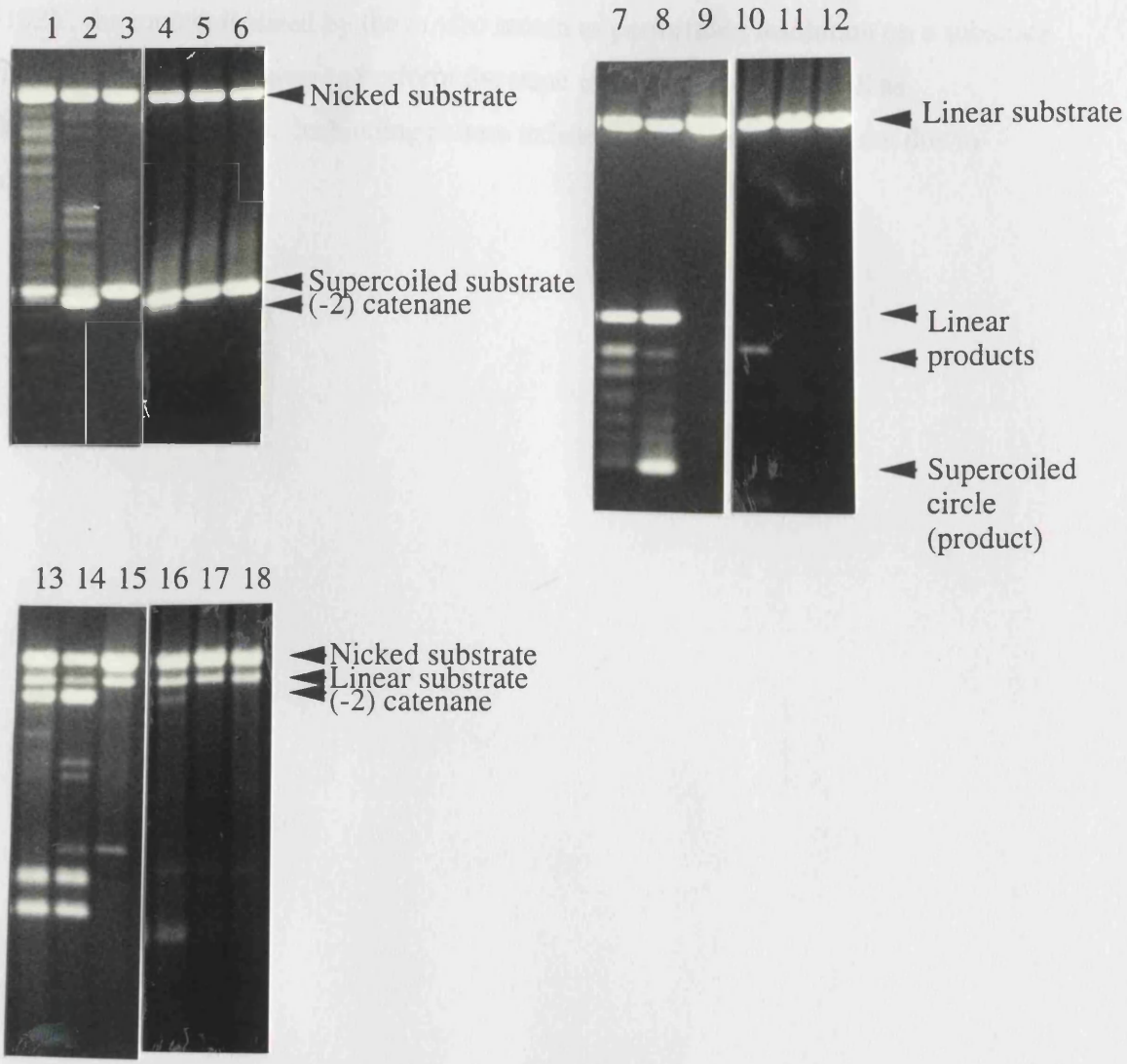



**Figure 6.13 Comparison of activity of wild-type and D102Y resolvases**

The figure compares the DNase I nicking reactions of wild-type resolvase and D102Y resolvase on the wild-type substrate pMA21. Also shown is the nicking reaction of D102Y on the plasmid pAL265 (*res* v site I) indicating the presence of the (-2) catenane as the major product of the reaction. 60 µl resolution reactions were carried out for 1 h at 37 °C then stopped with heat. One third of each mixture was removed and digested with *Pst*I and one third was nicked with DNaseI. All samples were run on a 1.0 % agarose gel for 16 h at 40 V. The approximate concentrations of the stock resolvase solutions are as indicated in Figure 6.10.

Lanes 1 - Undiluted D102Y resolvase / pMA21
2 - 16-fold dilution of wild-type resolvase / pMA21
3 - No resolvase control (dilution buffer) / pMA21
4 - Undiluted D102Y resolvase / pAL265
5 - 6-fold dilution of wild-type resolvase / pAL265
6 - No resolvase control (dilution buffer) / pAL265
7 - 9 - As 1 - 3, digested with *Pst*I
10 - 12 - As 4 - 6 digested with *Pst*I
13 - 15 - As 1 - 3. treated with DNase I
16 - 18 - As 4 - 6 treated with DNase I.

D102Y, the mutant isolated by the *in vivo* screen as performing resolution on a substrate with site I v *res*, was shown to perform the same reaction *in vitro,* as well as intermolecular reactions. Its binding pattern indicates that this activity is not due to enhanced binding.

# Chapter 7

Discussion

# Discussion

## 7.1 General remarks

The first Tn*3* resolvase mutants have been isolated, using the plasmids pAT4 and pAT5, developed for the purposes of mutagenesis and compatible with an *in vivo galK* screen (see Section 3.1). Two specific mutants were created by site-directed mutagenesis, Y6F and E124Q; a third, L69F, was made inadvertently (Figure 7.1). All the mutants were tested for their activities *in vivo.* Random mutagenesis of the E-helix of Tn*3* resolvase did not produce the long awaited mutant able to catalyse recombination between two site I's. However a mutant, D102Y, was isolated with the ability to catalyse resolution between *res* v site I (*in vivo*). In order to analyse these proteins *in vitro,* a new denaturing purification system was developed, now shown to be reproducible for a range of Tn*3* resolvase mutants. Greater yields of resolvase were achieved by employing a new expression vector, with resolvase transcription from a T7 RNA polymerase promoter. Subsequent analysis has determined the *in vitro* properties of the mutant resolvases.

## 7.2 Analysis of resolvase point mutants

A number of catalytically active and inactive mutants were isolated during screening of the mutant libraries created (Chapter 4; Figure 4.6). On closer examination, a number of observations can be made. These mutations are highlighted in Figure 7.2.

Catalytically active Tn*3* resolvase mutants ("white" colonies) in general are relatively conserved changes of the surface residues e.g. G96A, S98C, T99S, D102E and S112C. The minor changes apparently do not disrupt the E-helix, leaving the catalytic activity of resolvase unaffected.

Figure 7.3 shows sequence alignment of some related proteins. These include the Gin, Cin and Hin invertases and Tn*21.* The alignment shows the most conservative residues in this region, the E-helix, as E118, R119 and E124.

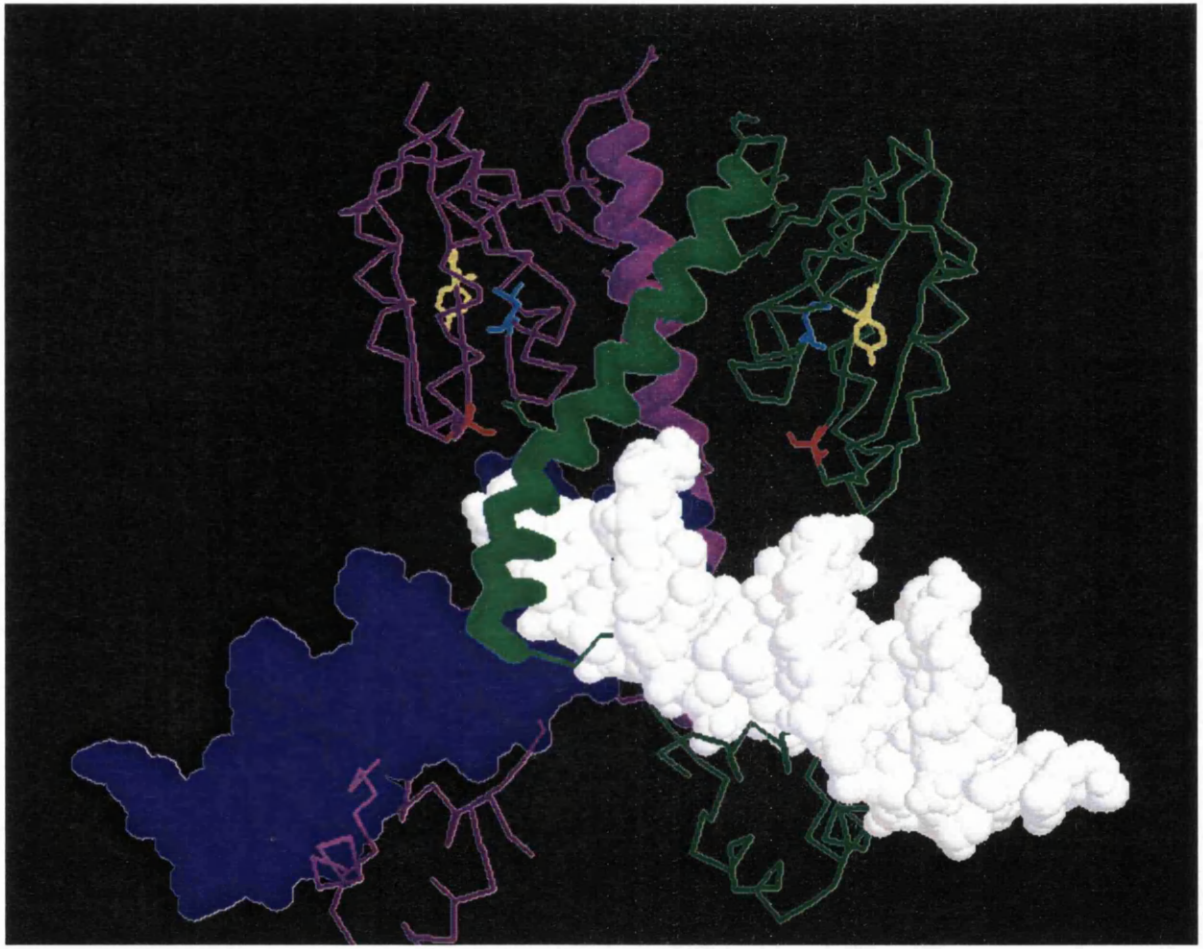


**Figure 7.1 Illustration of Y6F and L69F mutations**

Diagram of γδ resolvase bound at site I (Yang and Steitz, 1995). Serine-10 is highlighted in red, while L69 and Y6 are in blue and yellow respectively. Note how the hydroxyl of the tyrosine is close to the serine-10 residue, and may be contributing to the catalytic site.

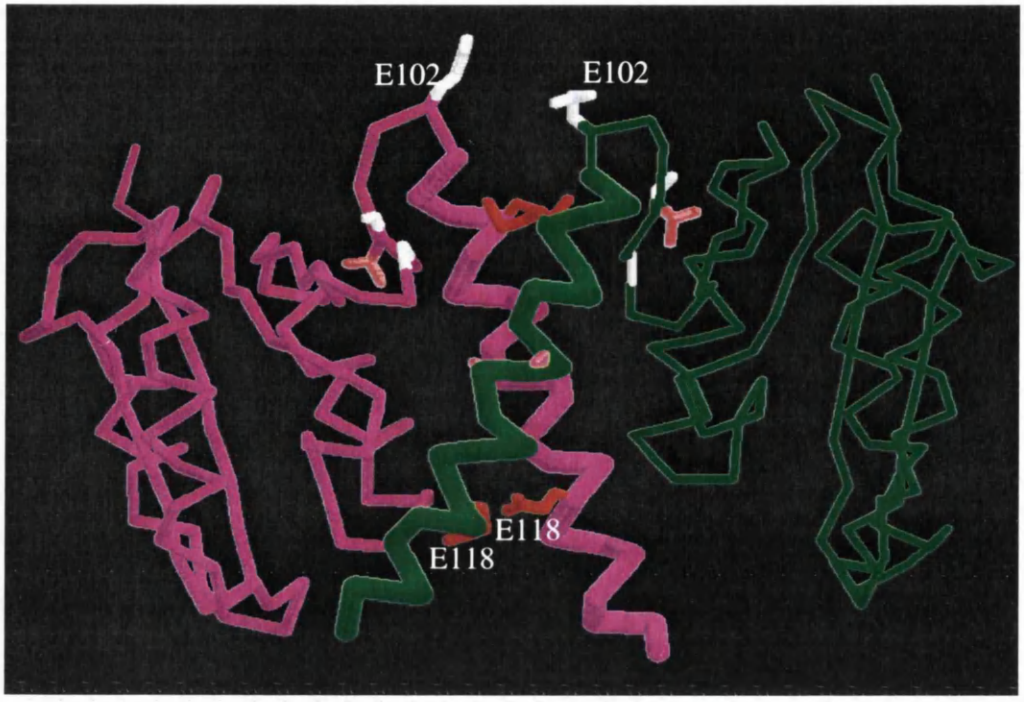



**Figure 7.2 Point Mutations**

Diagram illustrating the positions of the point mutations isolated from the mutant libraries. These mutants are also represented in Figure 4.7. The positions where catalytically inactive mutants were found are shown in red, while catalytically active mutants are displayed in white. The positions at which both inactive and active mutants were isolated are illustrated in pink.


**Figure 7.3 Alignment of amino acid sequences of representative members of the resolvase and DNA invertase family of recombinases**

Position numbering is of the Tn*3* resolvase sequence. Every tenth position is underlined and where possible numbers are given. Identical residues are denoted by an asterisk. Serine-10 is denoted by an arrow. The helix-turn-helix motif is also indicated. Figure adapted from Blake 1993.

Analysis of the catalytically inactive mutants ("red" colonies) is perhaps more complex. The methionine at position 106 has a relatively long slender hydrophobic side-chain, pointing inwards to the 1,2 dimer interface. Mutation of this residue to valine introduces a short bulky side-chain, as does the threonine mutation (also red/dead) which in addition is less hydrophobic. It is not surprising since these mutants disrupt the packing of the E-helix and are recombinationally inactive.

Mutation of threonine at position 99 to proline yields a catalytically dead mutant. Proline does not fold into sheets and so may disrupt the configuration of the loop between the D and E-helices (the D-E loop).

At first glance, the mutation at position 118 from glutamate to glutamine seems very mild and it is surprising that this renders resolvase inactive. However, position 118 is almost 100% conserved throughout the family (Figure 7.3). This conservation indicates that even mild changes at this position cannot be tolerated. The inactivity of the mutant containing glycine at this position is more readily explained since the introduction of a short side-chain may disrupt and possibly break the helix.

Both "red" and "white" mutants have been found at position 112. Mutating this serine to a cysteine results in a catalytically active protein. However mutation to a tyrosine kills the recombinational activity. Serine is a hydrophilic residue, its mutation to a hydrophobic tyrosine, may disrupt the helix, being counter to the amphipathicity of the E-helix.

## 7.3 Analysis of D102Y resolvase

Though the random mutagenesis carried out did not produce a Tn*3* resolvase mutant with the ability to catalyse *in vitro* reaction between two site I's, the isolation of the mutant D102Y, which had the ability to catalyse recombination *in vitro* and *in vivo* on a plasmid containing only site I v *res*, is definitely informative, and a step towards the eventual isolation of a mutant recombinationally independent of sites II and III.

So, how does the change from aspartate to tyrosine at this position influence the ability of the protein to carry out this reaction?

Position 102 is not strictly part of the E-helix observed in the crystal structures of γδ resolvase; it is at the very top end of the E-helix, and the residue of resolvase which is furthest from the DNA in the co-crystal structure. It is not present on any known interface between subunits, either in the crystals or in solution. The co-crystal structure of γδ resolvase bound at site I (Yang and Steitz, 1995) shows residue 102 on one subunit pointing outward into space, while the second points inward to the helix (Figure 7.4). In γδ resolvase residue 102 is a glutamate, and on the residue pointing inward is making an ionic interaction with the lysine at position 105 (Figure 7.5). This residue is glutamine in Tn*3* resolvase, and so it seems unlikely that there will be an equivalent aspartate-glutamine interaction. D102 is not a well conserved residue, being glutamate in γδ resolvase and Tn*21*, leucine in Bin3, and alanine in Tn*4451* resolvase (Figure 7.3) The nature of the sidechain at position 102 could be important for some control feature of the reaction, regulating recombination.

Substitution of tyrosine for aspartate at position 102 in the Tn*3* mutant resolvase may have caused a conformational change, by altering the secondary structure of this region of the protein. A model for strand exchange proposed after analysis of the recent co-crystal structure and taking into account features of this structure, has suggested the possibility that the loop around this position between the bottom of the E-helix and the N-terminal domain, is flexible. This model has been named "catalytic domain rotation" and is illustrated in Figure 1.9. It is proposed that the loop acts as a flexible hinge, allowing the illustrated movement of the catalytic N-terminal domain. As in the subunit rotation model (see Figure 1.9) the model proposes that the DNA is on the outside of the synaptic structure, but this model requires that only part of the protein rotates with the DNA via the hinge mechanism, and thus the 1,2 dimer interface created by the paired E-helices is maintained. A reduction of the H-bonding in this area by the D102Y mutant resolvase may allow this hinge a greater degree of flexibility.

A second possibility is that the protein is displaying an enhanced level of synapsis by forming a more stable synaptic complex. In assays for synapsis, substrates with an isolated site I and a full *res* give only traces of synaptic complex (Watson, 1994). A mutant resolvase with enhanced synapsis capability might increase the recombination

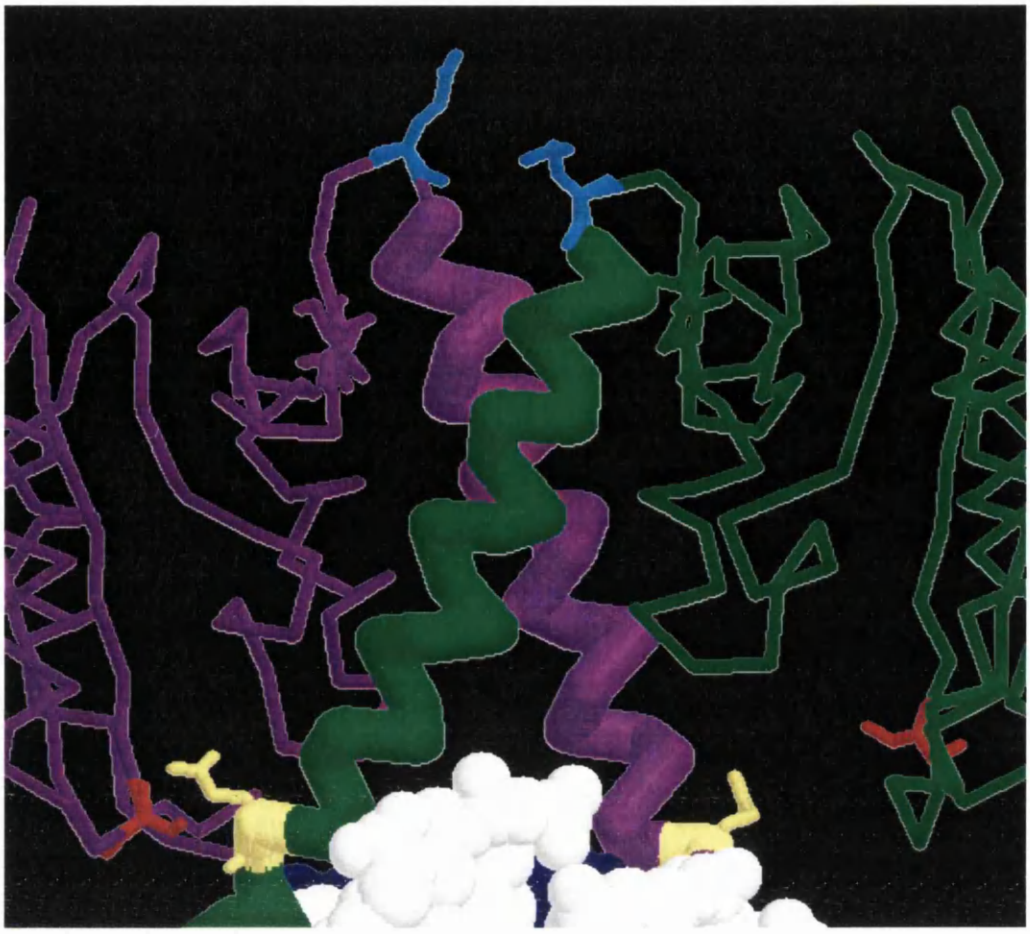


**Figure 7.4 Illustration of the positions of D102 and E124**

Serine-10 on each monomer is coloured red, while E102 is blue and E124 is yellow. E124 of the green subunit lies in close proximity to S10 of the purple subunit, while E102 is the residue furthest from the DNA and points into space on one monomer.

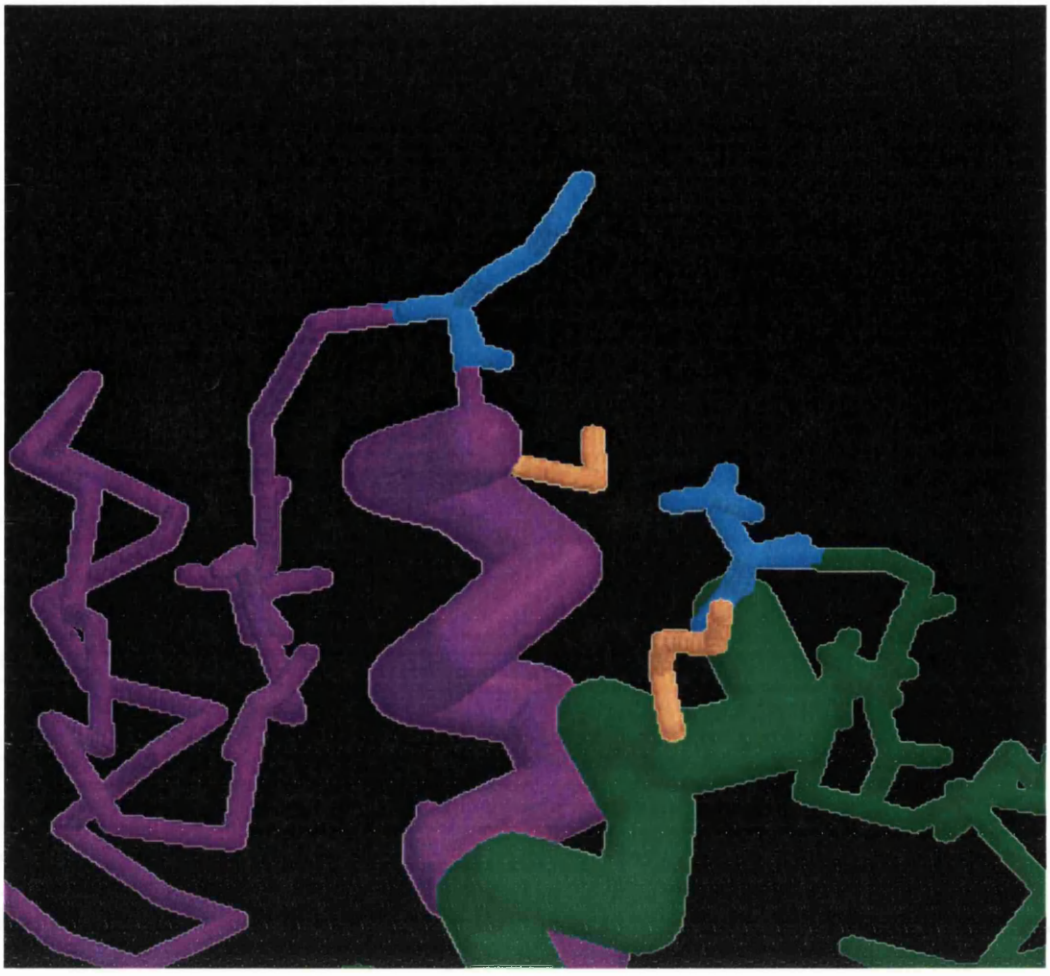


**Figure 7.5 Diagram of the interaction of E102 and K105**

Position 102 is highlighted in blue while position 105 is highlighted in orange. On one γδ resolvase monomer (coloured in green) these seem to be making an ionic interaction.

activity on an isolated site I. This may be due to dimer-dimer interactions making the protein "stickier". This suggests the possibility that position 102 exists on the as yet unlocated dimer-dimer interface, predicted by both the subunit and catalytic domain rotation models of strand exchange, and that the mutation to the more hydrophobic tyrosine side-chain increases the stability of the interface.

A third possibility is that the D102Y mutant resolvase has an increased ability to utilise non-specific DNA in the plasmid to perform the necessary wrapping of sites II and III in order to form the productive synapse. It has been shown that wild-type resolvase can, to a certain extent, utilise non-specific DNA in order to wrap sites II and III in the necessary conformation (Bednarz *et al.*, 1990). In order to carry this out, sites II and III must be present on at least one of the two recombining sites. Since D102Y resolvase has been shown to produce a (-2) catenane as the product *in vitro* from a plasmid containing *res* v site I, it is probable that it is utilising non-specific DNA to wrap together with sites II and III of the full *res*, to create the required 3 interdomainal supercoils. This suggests that D102Y resolvase may have enhanced binding activity on non-specific DNA. Binding studies to a full *res* site have indicated binding comparable to that of the wild type protein. A "stickier" dimer-dimer interface as suggested above, might also stabilise a synapse containing non-specific DNA by helping to hold all the pieces of the structure together.

## 7.3 Developments of this project

Because of the potential significance of resolvase mutations which confer a "hyperactive" phenotype, a considerable amount of further related work has been carried out since the completion of this project. Further *in vivo* screenings of a new mutant library (constructed in a similar manner to L1 and L2) have led to the isolation of 12 mutants with the ability to catalyse the reaction between a site I and *res* (M. Burke, personal communication). All these resolvase mutants contain a mutation at position 102, though some are in conjunction with additional amino acid substitutions. The closest aspartate to position 102, D100, has also been mutated to a tyrosine (M Burke, personal communication). It is interesting to note that this mutant, D100Y is *in vivo* less recombinationally active than the wild-type protein, and is inactive on a *res* v site I

substrate. Thus the equivalent mutation of the other aspartate on this surface of the protein does not produce the hyperactive phenotype.

Saturation mutagenesis at position 102 has indicated that substitution of aspartate by isoleucine, tyrosine, phenylalanine and to some extent valine and threonine causes hyperactive behaviour (M. Burke, personal communtcation). The mutants catalyse the reaction between a site I and *res in vivo*. All other amino acid changes (with the exception of methionine, tryptophan, proline and asparagine mutants which have not yet been isolated) give resolvases which are as recombinationally active as the wild-type protein in the *in vivo* assay. It seems unusual that a position which can yield a number of catalytically enhanced mutants can accommodate a wide range of amino acids which only enhance or at the very worst retain the function of the protein,and do not seem to have a detrimental effect on its catalytic ability. From the range of hyperactive mutants isolated, is seems that neither the charge nor the hydrophobic nature of the amino acid is responsible for its behaviour. Although the most hyperactive mutants have bulky rather hydrophobic side-chains, the D102L mutant is not hyperactive.

D102Y resolvase has been shown to have activity on a site I v site I substrate in a seperate *in vivo* assay (Martin Boocock, personal communication). The substrate in this assay contains a *StrA* gene, causing naturally streptomycin resistant cells to be streptomycin sensitive. The presence of a second plasmid expressing "active" resolvase excises this gene, in the same manner as previously described for the *galK in vivo* assay. The loss of this gene returns the cell to a streptomycin resistant phenotype which can be detected by patching colonies onto agar plates containing streptomycin. Using a substrate containing two site I's and *StrA* gene, this assay has shown D102Y resolvase to carry out the site I v site I reaction at a very low level. Analysis of the products of this reaction by agarose gel electrophoresis has shown the reaction to proceed at approximately 5 - 10%.

A double mutant of Tn*3* resolvase, containing D102Y and E124Q mutations has been constructed (M. Boocock, personal communication). Using the *galK in vivo* assay, the double mutant has been shown to resolve a substrate plasmid containing two site I's (pDB35). After purification of the mutant resolvase, studies have shown that it retains this phenotype *in vitro*, promoting recombination between two site I's on a plasmid.

Agarose gel electrophoresis has shown both resolution and inversion products from this reaction, though the topology of these products has not yet been analysed. This would seem to suggest that the removal of sites II and III on both the recombining sites has effectively removed the previous strict specificity of this system for resolution.

This double mutant is also active on site I oligonucleotides; this will hopefully prove useful for mechanism studies, where chemically modified oligonucleotides can be used.

## 7.4 Future considerations

Overall, there still remains the question of the function of the accessory sites II and III. Do they perhaps perform a regulatory function controlling recombination at site I? If so, then mutation at D102Y may have removed such a control mechanism. More specifically, in what way have the changes we introduced altered or removed this mechanism, and how do we find this out?

If we consider that the mutation of aspartate to tyrosine at position 102 is resulting in a "sitckier" interface, possibly on a dimer-dimer interface not yet discovered, then this may be causing the protein to form a tetramer in solution. Gel filtration proved pivotal in determining that resolvase existed as a dimer in solution. With the use of suitable size markers, gel filtration may determine the existance of a tetramer of D102Y resolvase in solution.

If D102Y resolvase contains a more flexible hinge mechanism allowing the N-terminal of resolvase a greater freedom of movement then cysteine mutants may prove useful. Strategically positioned cysteine mutants, forming disulphide bridges which would essentially "fix" the position of the N-terminal domain would be useful in determining the validity of this proposal, and of the catalytic domain rotation model of strand exchange.

If however, the mutant is displaying enhanced levels of synapsis, then synaptic studies using the method previously used in this laboratory (Watson, 1994), may be useful.

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