# Subunit interactions in regulation and catalysis of site-specific recombination

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by
Sandra Verena Corinna Tina Wenwieser

Division of Molecular Genetics
Institute of Biomedical and Life Sciences
Anderson College
56 Dumbarton Road
Glasgow

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

This thesis is dedicated with all my love to my family, friends and further animals.
In loving memory of Babette.

"It is a good morning excercise for a research scientist to discard a pet hypothesis every day before breakfast."

Konrad Lorenz in *On Aggression* (1966)

"A new scientific truth does not triumph by convincing its opponents and making them see the light, but rather because its opponenets eventially die, and a new generation grows up that is familiar with it."

Max Planck in *A Scientific Autobiography* (1949)

## Abbreviations

### Units

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

### Chemicals/Reagents

| | |
|---|---|
| AcOH | acetic acid |
| APS | ammonium persulphate |
| ATP | adenosine triphosphate |
| $BM[PEO]_3$ | 1,8-*bis*-maleimidotriethyleneglycol |
| BMPHP | N,N'-*bis*-(3-maleimido-proprionyl)-2-hydroxy-1,3, propane diamine |
| DMF | dimethylformamide |
| DNaseI | deoxyribonuclease I |
| dNTP | deoxynucleoside triphosphate |
| DTNB | 5,5' dithio-bis-2-nitrobenzoic acid |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid (disodium salt) |
| EtBr | ethidium bromide |
| EtOH | ethanol |
| GSSG | gluthathione (oxidised) |
| HBVS | 1,6-hexane-bis-vinylsulfone |
| IPTG | isopropyl ß-D-thiogalactopyranoside |
| KOAc | potassium acetate |
| MeOH | methanol |
| NEM | N-ethylmaleimide |
| PDM(o) | N,N'–o-phenylene-dimaleimide |
| PDM(p) | N,N'-p-phenylene-dimaleimide |
| SDS | sodium dodecyl sulphate |
| TCEP | *tris* -(2-carboxyethyl)-phosphine hydrochloride |
| TEMED | N,N,N',N'-tetramethyl-1,2-diaminoethane |
| Tris | *tris* -(hydroxymethyl)aminoethane |

### Other Terms

| | |
|---|---|
| $X^R$ | resistance to X |
| $X^S$ | sensistivity to X |
| Ap | ampicillin |
| Cm | chloramphenicol |
| Km | kanamycin |
| Tc | tetracycline |
| Str | streptomycin |
| *ori* | origin of replication |
| FIS | factor for inversion stimulation |
| PAGE | polyacrylamide gel electrophoresis |
| DBD | DNA binding domain |
| DSB | double strand break |
| fsd | full scale deflection |
| Tn3R | Tn3 resolvase |
| γδR | γδ resolvase |
| R | Red colonies (*in vivo* assay results listed in the order: pDB34, pDB37, pDB35; e.g. RRR) |
| W | White colonies (*in vivo* assay results listed in the order: pDB34, pDB37, pDB35) |

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# Summary

The role of the 2,3'-interface as an interdimer interface required for synapsis was investigated by creating a double mutant R2A/E56K and combining this with the activating mutations D102Y/E124Q. The effects of multiple 2,3' mutations on regulation of recombination by sites II/III were found to be additive. Mutation of two residues at the 2,3'-interface (R2A/E56K) abolished all detectable activity (except binding), even when the mutations are targeted exclusively to site I. Nevertheless, when targeted to sites II and III, R2A/E56K could fulfil some of the regulatory functions of WT resolvase. Mutation of the same residues in an activated resolvase (R2A/E56K/D102Y/E124Q) did not block catalysis at site I, and had only a partial effect on regulation by sites II and III. Thus, the most critical function of the 2,3'-interface may be to support activation of site I-bound resolvase by sites II and III. While it is clear that the 2,3' interaction contributes to the stability of the synapse, these data imply that it may not be the main architectural interface. This role of the 2,3'-interface supports a new model of the synapse (Sarkis *et al.*, in preparation).

A potential candidate interface for synapsis of resolvase dimers was the region around residue D/E102. Mutations of residues in this region have resulted in activated resolvases, which show reduced dependence on the accessory sites. Residues 96-105 of Tn3 resolvase were replaced with the homologous sequence of Hin, thereby introducing six amino acid substitutions. This resulted in an activated resolvase which showed cleavage and some recombination activity in the absence of sites II and III. This was the first accessory site independent resolvase that did not also carry activating mutations around E124, demonstrating that mutation of the 102 region is sufficient. Furthermore, the rate of *res* x *res* recombination was increased with this mutant. When the corresponding region of γδ resolvase was replaced with Hin sequence, the mutant protein was highly active, recombining crossover sites at a similar rate in the presence or absence of accessory sites. Nevertheless, it was clearly established that both Tn3R (96-105 Hin) and γδR (96-105 Hin) readily synapsed sites II/III, trapping three negative supercoils. Thus, these mutants recombined crossover sites in the absence of sites II/III, yet were synapsis-proficient when sites II/III were present. Both mutants also showed significant supercoil-independent cleavage and recombination activity, but were nevertheless stimulated by negative supercoiling, and by sites II/III. A further mutant, γδR (96-105 Hin) E124Q, generated large amounts of covalent cleavage complexes as predicted. In contrast to the earlier

mutants, it was extremely supercoil and accessory site independent, making it an ideal candidate for crystallisation trials for the cleavage intermediate. Targeting experiments to determine whether mutation in the 102 region disrupted synapsis with WT resolvase were difficult to design and interpret because of the highly activated properties of these (96-105 Hin)-swapped resolvases.

γδR (96-105 Hin) was also used to investigate whether this region of Hin was involved in direct contacts with the Fis/enhancer complex. When assayed with a substrate analogous to the natural Hin substrate (with *res* crossover sites replacing the *hix* sites), a small bias towards inversion over resolution products (approximately 2:1) was observed in the presence of Fis and HU. This bias may be indicative of the formation of an invertasome structure. However, the bias observed was not limited to resolvase carrying the (96-105 Hin) substitution and was also seen with γδR E102Y/E124Q. The analogous experiment to look for possible contact surfaces was performed with a resolvase/Hin hybrid containing a Hin DNA binding domain designed to bind *res* binding sites. A variety of WT and activated resolvase versions of this hybrid were constructed and were found to be catalytically active. Additionally, γδR E102Y/E124Q Hin/Res DBD mutant was active with a site I x site I substrate. However, preliminary results have shown no effect of Fis and HU on the reaction rate or products indicating that the Hin DNA binding domain is not involved in direct contacts with the Fis/enhancer complex.

Models of strand exchange differ radically in their predictions of which resolvase interfaces must be disrupted in order to bring the half-sites together in the recombinant configuration. In order to investigate this, cysteine residues were introduced into resolvase so that three different components of the dimer interface could be disulphide or chemically crosslinked. The mutants initially constructed (D95C/A113C, T73C/A115C (96-105 Hin) and M106C (96-105 Hin)) were fully active when reduced but completely inactive when oxidised (except for binding). These crosslinked derivatives were therefore defective in a step prior to cleavage, thus making it impossible to investigate strand exchange requirements. Nevertheless, resolvases crosslinked in the *trans* and E helix interfaces were found to be proficient in all of the preceding functions including binding to *res*, accessory site synapsis, and activation of resolvase at site I. This rules out a domain-swapping mechanism for synapsis of sites II/III. Use of homobifunctional crosslinking agents, which have greater span lengths, did not

reinstate strand cleavage activity by crosslinked resolvase. Some residues in resolvase are sensitive to NEM modification hence suggesting that modification by bifunctional maleimide crosslinking agents might also be expected to inhibit resolvase activity.

In order to circumvent the problem of the sensitivity of A113C to modification, a different residue was chosen for crosslinking. S112 was selected as it is solvent accessible, and can potentially be crosslinked to T73C (in *cis*) or D95C (in *trans*). Reduced T73C/S112C was similar to WT resolvase in its activity and selectivity. T73C/S112C resolvase with an internal disulphide crosslink was found to be extremely activated for cleavage. In addition, it cuts DNA sequences other than site I. Preliminary mapping of the cleavage points in a linear *res* sites II/III-containing fragment localised the cleavage activity to within the site II region. Such activity has not been observed previously with any activated mutants. This mutant was also the first activated resolvase that does not carry mutations in either the 102 region or around Glu-124. It points to the importance of the *cis* interface in the regulation of catalysis by resolvase. Further characterisation of this mutant, as well as of D95C/S112C, may reveal which interfaces need to dissociate during strand exchange.

# Chapter 1

# Introduction

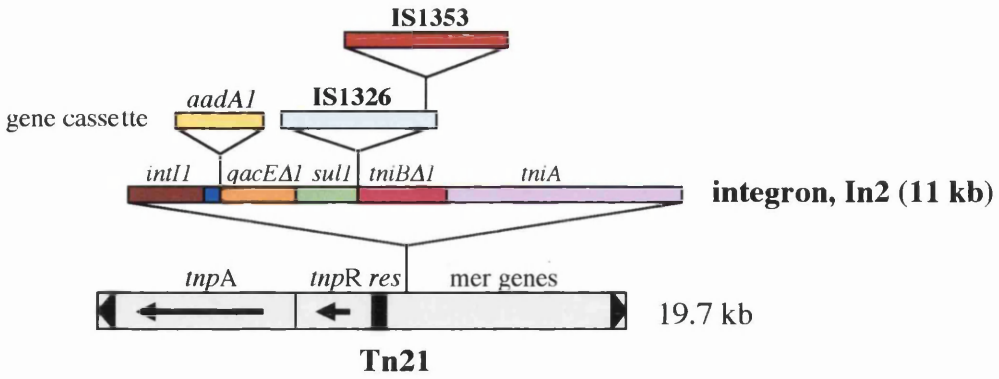
## 1.1 Transposition

As early as 1948, Barbara McClintock had reached the conclusion that the Dissociation locus (Ds) in *Zea mays* could change its location within the genome - a process which was to be termed transposition (Fedoroff, 1992). This work was accepted only reluctantly, resulting in the award of the Nobel Prize 35 years later (1983). By then the existence of 'jumping genes' was firmly established and the total number of transposable DNA elements discovered is rising steadily to this day. The known host range of these elements is also expanding, ranging from higher eukaryotes (e.g. humans) to thermophilic archaebacteria (e.g. *Methanococcus janaschii*). In fact, analysis of the completed human genome has shown that almost half of the human genome is derived from transposable elements, though most of these are inactive (Consortium, 2001).

Importantly, transposable DNA elements were also found in eubacteria such as the gram negative bacterium, *Escherichia coli*, where certain of these elements are responsible for the increased resistance of bacteria to antibiotics. Investigation of this phenomenon revealed that transposable elements encode resistance to specific antibiotics and that due to a combination of transposition and plasmid conjugation these resistance factors could be transferred horizontally between bacteria. One such example is the transposable element Tn3, found in a variety of gram negative bacteria (including *E. coli*), which confers resistance to ampicillin.

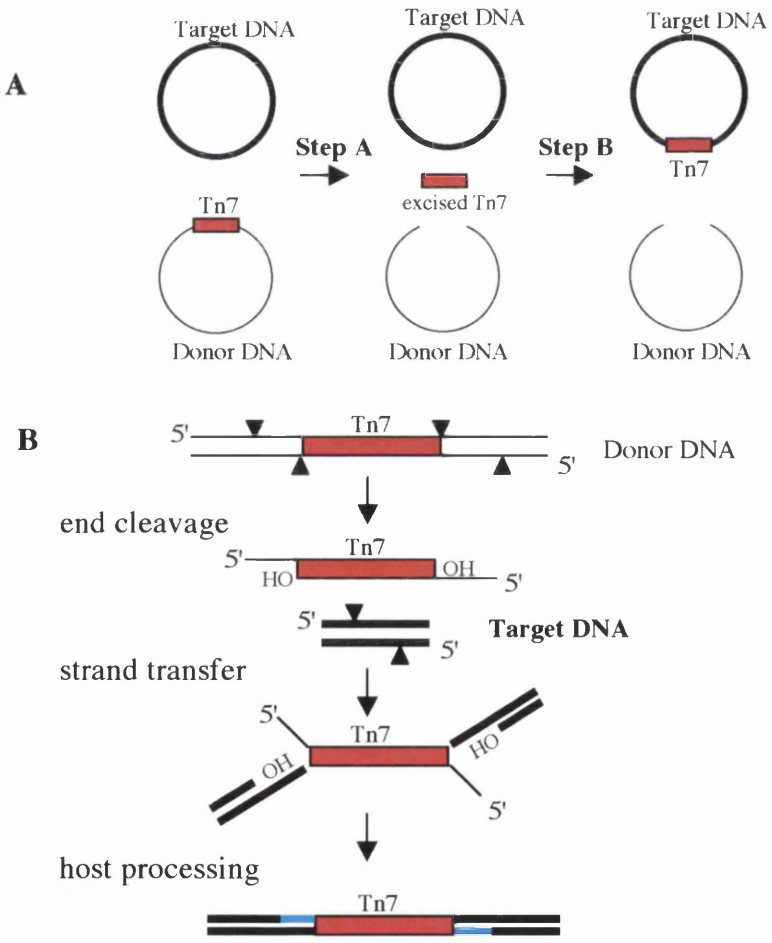
Tn3 and similar antibiotic resistance conferring transposable elements are especially abundant in bacterial populations in hospitals, where they offer their hosts a distinct advantage. Indeed, the horizontal transfer of antibiotic resistance genes is already a serious public health problem as selective pressures are placed upon bacteria to 'collect' resistance genes. Tn21 (19.7 kb), found in *Shigella flexneri*, is an example of a collection of antibiotic resistances (Komano, 1999; Liebert *et al.*, 1999) (Fig. 1.1). In addition to carrying resistance genes to mercury, Tn21 carries an integron, In2 (11 kb). The integron encodes a site-specific integration system that acquires multiple small mobile elements called gene cassettes that encode resistance genes, including quaternary ammonium compound disinfectants and sulfonamide resistance. Inserted into this integron are two further insertion sequences, IS1326 and IS1353.

## Fig. 1.1 The structure of Tn21




The structure of Tn21 (Liebert *et al.* 1999) is shown diagrammatically above (not drawn to scale). The transposition region (containing *tnpA, tnpR* and *res*) and the mercury resistance genes are shown in grey. The integron, In2 (11 kb) is shown directly above, incuding genes encoding resistance to quartenary ammonium compound disinfectants (*qacEΔ1*) and sufonamide resistance (*sul1*) and two insertion sequences, IS1326 and IS1353.

## Fig. 1.2 Tn7 "cut and paste" transposition




The "cut and paste" transposition reaction of Tn7 (red) is shown as an overview (A) and in more detail (B). The entire Tn7 element is excised from the donor DNA (step A) prior to integration into the donor DNA. Arrowheads represent points of cleavage. The newly synthesized DNA (host processing) which results in the target site duplications is shown in blue.

Transposition of Tn3 family transposons is tightly regulated, occurring at frequencies between $10^{-5}$ and $10^{-7}$ per generation. A higher rate of transposition may be harmful enough to the host to cause a decrease in the fitness of the transposon itself. Thus the 'selfishness' of such elements often depends upon a complex relationship with the host and the environment of the host. Another example of transpositional regulation as an adaptation of the transposon is observed for Tn21 transposition. Tn21 transposition is stimulated by the presence of mercury in the environment of its host, thus adapting to environmental changes in order to increase the fitness of Tn21 (Nikiforov *et al.*, 1999).

There is evidence to suggest that some transposable elements increase their chances of spreading within and between hosts by showing preferences for particular target DNA sequences (Wolkow *et al.*, 1996). Transpositional target sequences were often found to be *res* sites (Minakhina *et al.*, 1999). By targeting such sequences transposons presumably increase their chances of transferring themselves into a mobile element, such as a transposon carried on a plasmid. Such re-location would increase the transposons chance of being transferred to another bacterium by conjugation. Re-location of Tn3 into Tn21 by such a mechanism may explain the existence of Tn4, as Tn4 is identical to Tn21 except that it contains a complete copy of Tn3 (thus encoding resistance to ampicillin over and above the numerous resistances encoded by Tn21). Transposition immunity, whereby a transposon will not transpose into DNA already carrying a copy of itself, is also frequently observed (e.g. by Tn3). As this phenomenon depends only on the presence of a single transposon end, this is also likely to be adaptive.

However, not all transposons are beneficial to their host. It is therefore possible that host genomes have fought back at these 'selfish' elements. It has been suggested that invertebrates repress transposition events by DNA methylation (Bird *et al.*, 1979). Another extreme strategy has been adopted by the filamentous fungi *Neurospora crassa*, which detects duplicated sequences and mutates them, thereby destroying any self-replicating elements (this process is named Repeat-Induced Point mutation, RIP) (Bird, 1993). On the other hand, some transposable elements have been able to overcome protective systems of their hosts by locating themselves in a region of the genome which is highly expressed and contains housekeeping genes. An example of this is the R2 non-long-terminal-repeat retrotransposable element located in the highly expressed 28S rRNA genes of arthropods

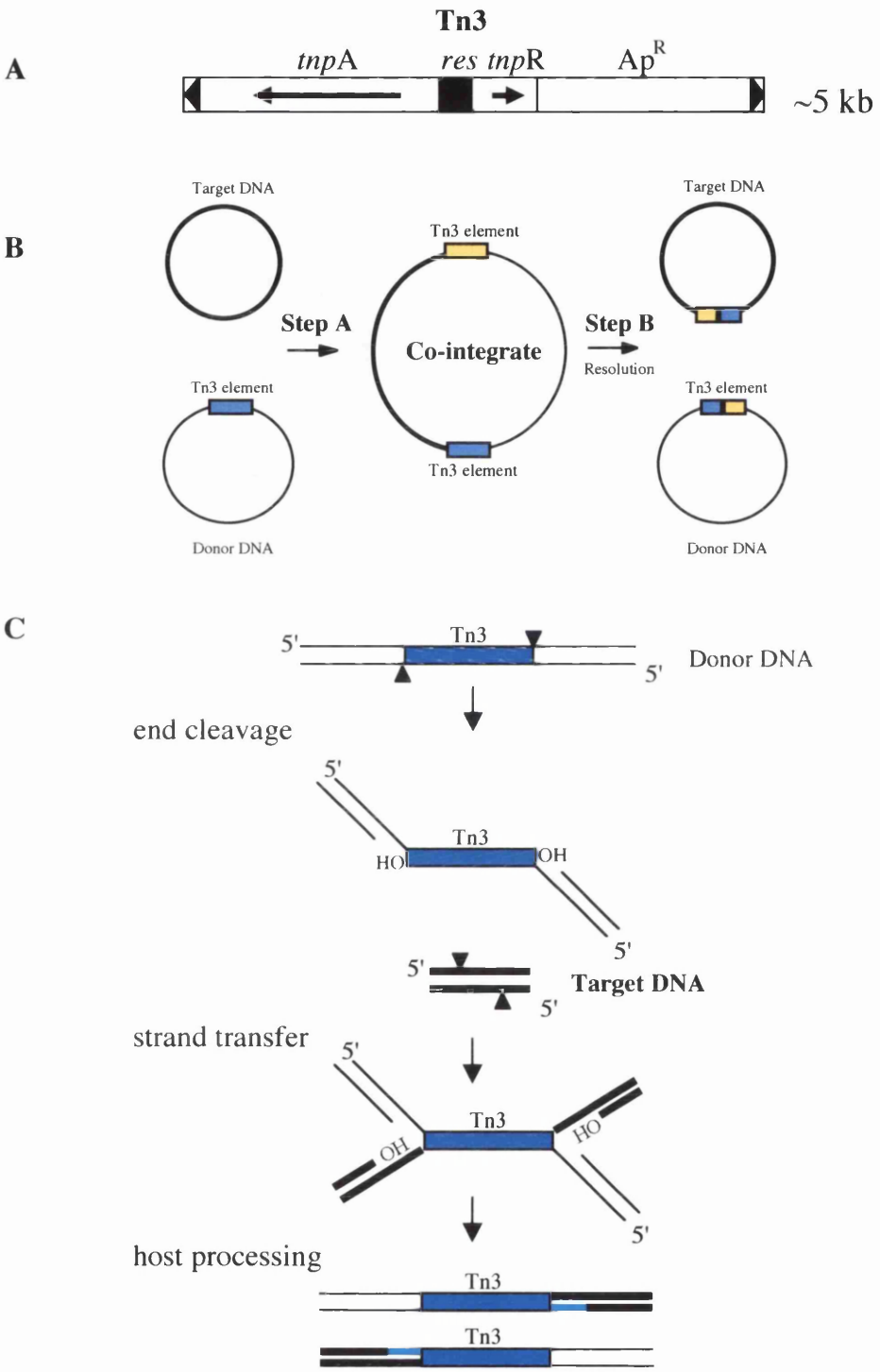
(Eickbush *et al.*, 2000). DNA elements and their hosts can therefore become locked in an arms race of suppression and evasion.

The transposon Tn7 (14 kb) is an example of a non-replicative or "cut and paste" transposon. Tn7 can transpose either to a specific site (*attTn7*) on the bacterial chromosome or to many other sites at a reduced frequency (Craig, 1996). Transposition involves the complete excision of the element followed by insertion into the new locus by the Tn7 encoded enzymes TnsA and TnsB (Fig 1.2). Transposition is initiated by two specific single-strand cleavages, by TnsA, exposing the 3'-OH ends of the element. This is followed by cleavage of the second strand by TnsB, resulting in 5'-overhangs (3 nt) and the excision of the whole element (Sarnovsky *et al.*, 1996). The 3'-OH ends of Tn7 then participate in a concerted strand transfer reaction that joins both ends of the element to staggered phosphates of the target DNA. Host machinery is thought to remove the 3 nt overhangs (5') of the transposed Tn7 element as well as fill in the single-strand gaps created by the staggered insertion (this results in short target duplications in the target DNA).

Tn3 (4957 bp) comprises a transposase gene (*tnpA*), a site called *res*, and a resolvase gene (*tnpR*) (in addition to the previously discussed $Ap^R$ gene) (Gill *et al.*, 1978). It is flanked by inverted repeats of 38 bp (Fig. 1.3, A). Mutants of Tn3 were isolated that were deficient in transposition (Arthur and Sherratt, 1979; Gill *et al.*, 1979). Instead of the normal transposition products, they produced a fused donor and recipient molecule in which the junctions contained copies of Tn3 in direct repeat. This was deduced to be an intermediate of transposition and called a cointegrate. The cointegrate is "resolved" into two functional replicons by a site-specific recombination reaction. This reaction is mediated by the Tn3 resolvase enzyme acting on *res* sites in the cointegrate (Fig. 1.3, B). Thus, transposition by Tn3 involves two separate steps, transposition catalysed by transposase acting on the transposon inverted repeats and site-specific recombination catalysed by resolvase acting on *res*.

The *in vitro* study of the Tn3 transposition reaction has proved difficult (largely due to the insolubility of the transposase enzyme) (Fennewald *et al.*, 1981). The mode of Tn3 transposition has therefore not been studied directly, but it is nonetheless believed to be similar to the replicative transposition pathway of bacteriophage Mu (for Mutator, a linear DNA phage (38 kb) that infects *E. coli*), for which a functional *in vitro* system has been

## Fig. 1.3 Tn3 replicative transposition




**A.** The structure of Tn3 is shown diagrammatically.

**B.** An overview of the two steps of replicative transposition is shown for Tn3. Step A of transposition requires Tn3 transposase and results in the co-integrate structure containing two copies of Tn3. Tn3 resolvase acts upon this substrate, resolving the co-integrate, yielding donor and target DNA, each with a copy of Tn3.

**C.** Step A of the replicative transposition reaction (B) is shown in more detail. Arrowheads represent points of cleavage. The newly synthesized DNA (host processing) which results in the target site duplications is shown in blue.

achieved. The molecular basis of replicative transposition is thought to be as follows: (i) transposase produces single-strand breaks at each 3' end of the transposon (or phage) and staggered cuts on either side of a short target sequence in the recipient DNA, (ii) the free ends of the of the transposon (or phage) and the recipient DNA are joined, forming a Shapiro intermediate, (iii) the remaining free 3' ends of the recipient DNA can then act as primers for DNA synthesis, which will proceed through the transposon or phage (Fig. 1.3, C) (Mizuuchi, 1983; Mizuuchi, 1992).

## 1.2 Site-specific Recombination

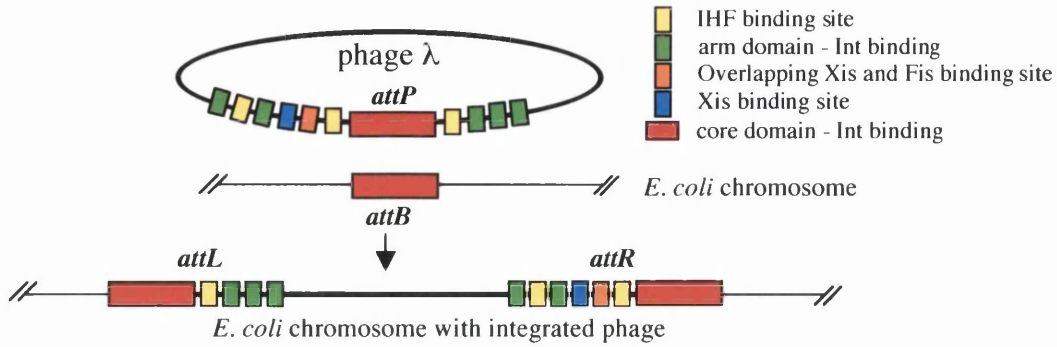
Site-specific recombination is a process whereby a DNA molecule is cut at two specific sites and the ends are rejoined to new partners. Recombination can occur by the sequential exchange of single DNA strands or by exchange of double strand break products. Neither process involves the synthesis or degradation of DNA, and there is no requirement for additional high-energy co-factors, such as ATP. Unlike general recombination, site-specific recombination requires no DNA homology between the sites. Instead, recombinase enzymes recognise and act only at specific DNA sequences.

The resolution of transposition intermediates (e.g. Tn3 resolvase) is not the only function of site-specific recombinases. *In vivo* functions of site-specific recombination are varied, including bacteriophage integration (e.g. by phage λ integrase), the switching of gene expression (e.g. Hin and Gin), and the monomerisation of multimeric plasmids and chromosomes (e.g. XerC and XerD) (Landy, 1989; Sherratt *et al.*, 1995; Summers and Sherratt, 1984; van de Putte and Goosen, 1992). Furthermore, generation of antibody diversity in metazoans is mediated through site-specific recombination reactions to cut and rejoin sets of genes in different combinations. These mechanisms are shown in more detail in Fig. 1.4.

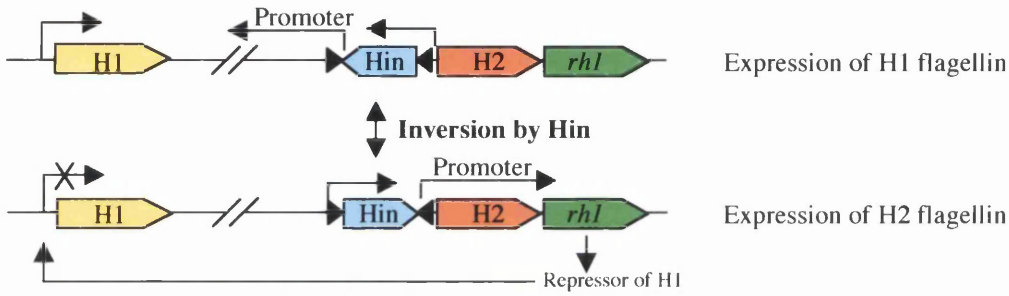
The cutting and joining of two DNA sites can have different outcomes, depending on the position and orientation of the sites in the substrate(s) and the type of reaction performed on them. Many site-specific recombinases are known to perform very specific reactions *in vivo* and *in vitro*. In order to illustrate this, the resolution of the Tn3 cointegrate will be considered in more detail (Fig. 1.5). The *res* site is located in the centre of the transposon, separating the transposase and the resolvase genes. During the resolution reaction the *res*

## Fig. 1.4 Site-specific recombination reactions

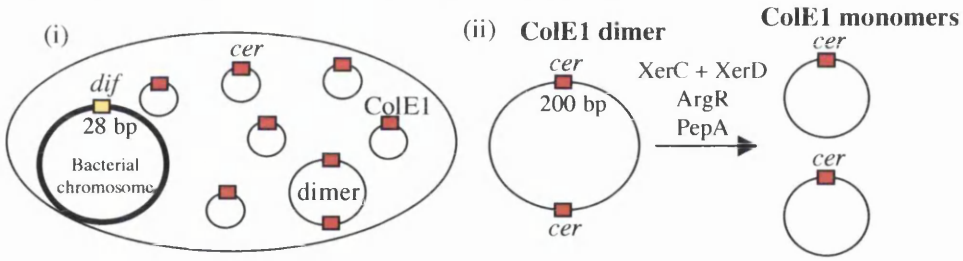
### A Integration of phage λ - λ integrase



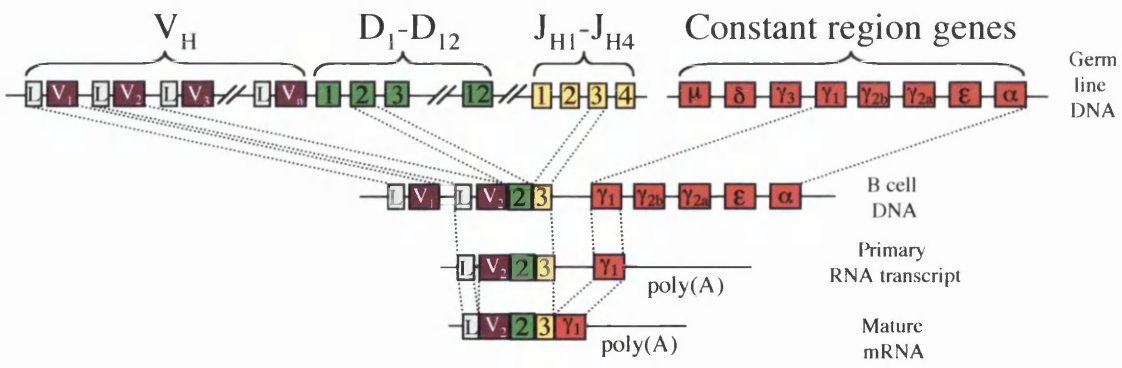

### B Inversion of a promoter - Hin




### C Monomerisation of multimeric plasmids - XerD and XerC




### D Immune system - VDJ recombination




**A.** Integration of phage λ into the *E. coli* chromosome.

**B.** In *Salmonella* the Hin recombinase gene (blue) is flanked by recombination sites, *hix* (shown as black arrowheads). Inversion of the invertible segment (the region between the inverted *hix* sites) by Hin alters the expression of the flagellin gene (H1 or H2) by changing the position and direction of a promoter.

**C.** Monomerisation of a dimer of ColE1 requires XerC and XerD (for catalysis) as well as ArgR and PepA (which are requires for accessory site function). Recombination takes place at the 200 bp *cer* site.

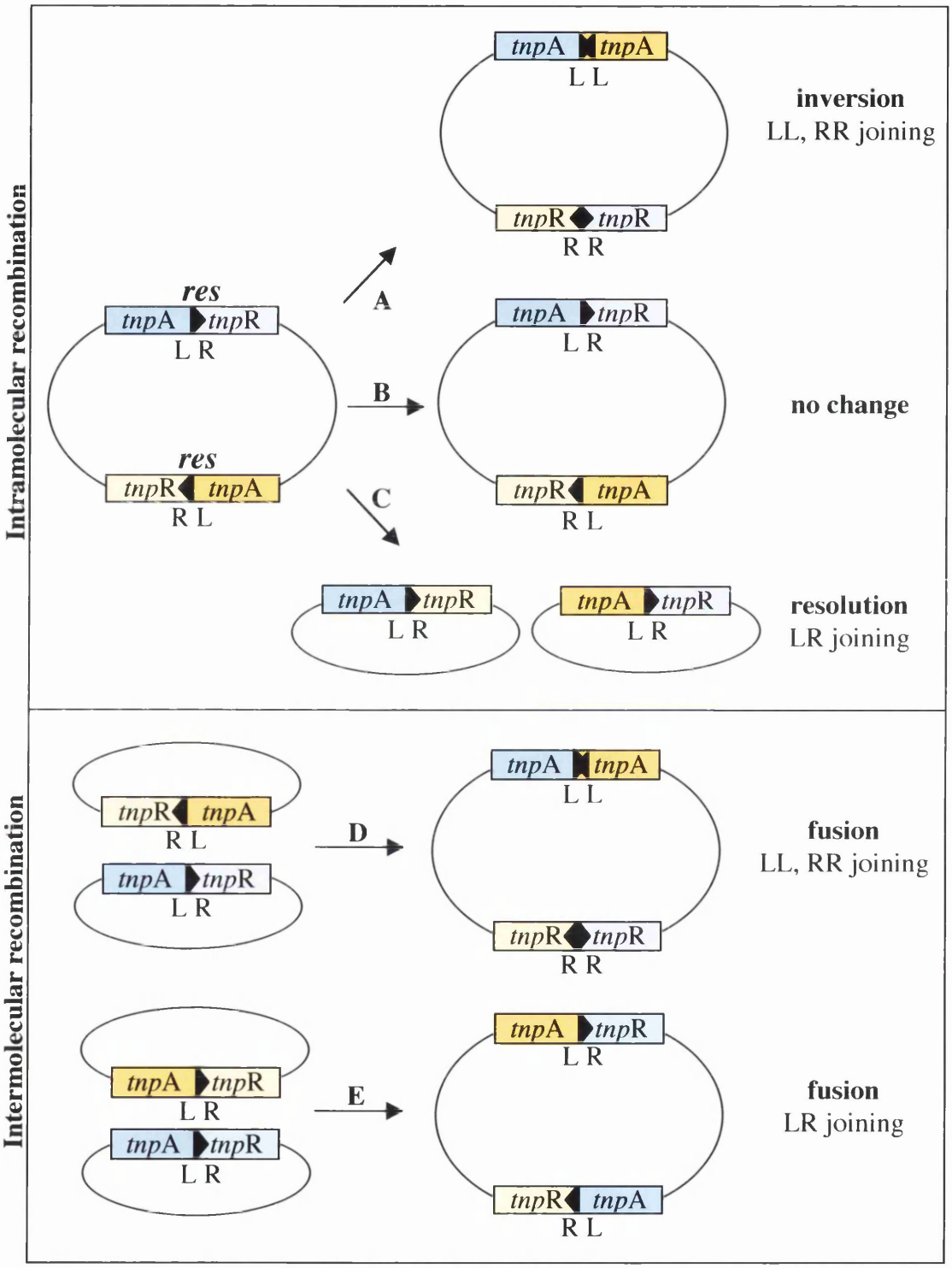
**D.** VDJ recombination to produce a heavy chain protein in mouse is shown. The arrangement represents only one of many possible combinations.

sites of two directly repeated copies of Tn3 are cleaved asymmetrically, generating left (L, *tnp*A-side) and right (R, *tnp*R-side) half-sites. This results in two fragments with two different ends (or four DNA ends). These could be rejoined in three different ways, resulting in either the unaltered cointegrate structure (B), resolution products (as normally occurs) (C) or inversion products (A) (Fig. 1.5). The inversion reaction would result in the rapid "demise" of both copies of Tn3, as they would contain two copies of the transposase encoding end (LL) and two copies of the resolvase encoding end (RR). These hybrid Tn3 sequences would be large inverted repeats and such a plasmid would rapidly be lost from the host bacterium. Note that due to the asymmetric cleavage of *res,* an inversion reaction would also create non-functional hybrid *res* sites (these can also be denoted as LL and RR). Selectivity for the resolution reaction (C) is therefore vital to the replication cycle of Tn3. However, more than one copy of Tn3 may be present in a host bacterium, either due to a replicative transposition event or due to plasmid replication. Intermolecular recombination between two Tn3 *res* sites could lead to reversal of the resolution reaction or other possibly "undesirable" genetic changes within plasmids (such as the multimerisation of plasmids) or within the bacterial chromosome (such as the integration of a plasmid into the host chromosome) (Fig. 1.5). In order to avoid intermolecular recombination resolvase must recognise the connectivity between two *res* sites even if these are separated by many kilobases. Other recombinases are specific for the inversion reaction (DNA invertases) or for fusion of two DNA circles (phage integrases). Because of the potential costs of "incorrect" recombination events, it is not surprising that site-specific recombinases are very specific for their substrates and in their products. The mechanism for this selectivity remained elusive for a long time. However, topological analysis of reaction products of resolvases and invertases with a variety of different substrates suggested how such specificity is achieved. This will be discussed later (section 1.7), following some details on the structure of the *res* site and its interactions with resolvase.

## 1.3 The integrase and resolvase/invertase families

Site-specific recombinases can be classified into two families by amino acid sequence alignments and catalytic mechanism. Nevertheless, the recombinases in each family do not differ in the types of reactions that they catalyse (each family contains enzymes which can perform resolution, inversion and fusion reactions). The integrase group is exemplified by λ integrase (encoded by phage λ) and includes Cre (encoded by bacteriophage P1) and Flp

**Fig. 1.5 Possible outcomes of intra- and intermolecular recombination of Tn3**



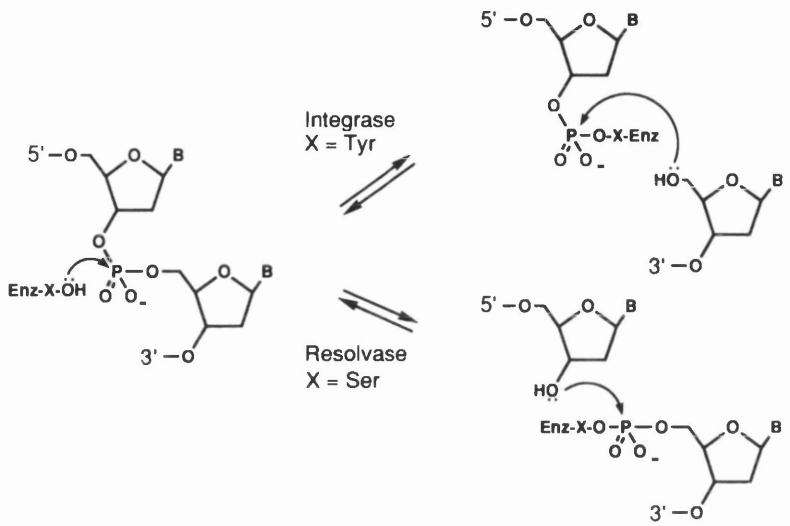

The consequences of intra- and intermolecular recombination of Tn3 are shown. The asymmetry of the recombination site, *res*, is represented as an arrowhead. Cleavage of Tn3 at *res* thus results in two distinct halves, one encoding transposase and the other encoding resolvase. The incorrect joining of the two halves of Tn3 would therefore result in non-functional copies of Tn3 containing aberrant *res* sites (shown as LL, RR joining) with large and unstable palindromic sequences. Intermolecular recombination can also result in multimerisation of plasmids thereby reducing the stability of the plasmid.

(encoded by the yeast 2 μm plasmid). The other group is composed of the resolvases (such as Tn3 resolvase), the DNA invertases (such as Hin from *Salmonella* or Gin encoded by phage Mu), phage integrases, and transposases of certain conjugative transposons (e.g. Tn4451). The λ integrase family is more diverse than the resolvase/invertase family, but all contain four invariant amino acids, RHRY including the catalytic tyrosine residue (Argos *et al.*, 1986). The reactions catalysed by the integrase family of recombinases proceed via a Holliday junction structure. In contrast, the resolvases/invertases cleave the DNA phosphate backbone via a serine residue, and they catalyse a concerted four-strand cleavage reaction (Hatfull and Grindley, 1986; Hatfull and Grindley, 1988; Leschziner *et al.*, 1995). The resulting four half-sites are then ligated in the recombinant configuration. The chemistry of the strand cleavage reactions by the two families are shown in Fig. 1.6.

The mechanism of recombination by the integrase family of site-specific recombinases is understood extremely well as a consequence of a number of co-crystal structures of Cre/*loxP*, corresponding to different stages of the reaction (Gopaul *et al.*, 1998; Guo *et al.*, 1997); the co-crystal structure of Flp/*FRT* (Chen *et al.*, 2000); and the crystal structure of XerD (Subramanya *et al.*, 1997). The Holliday junction/Cre complex (2.7 Å) is shown in Fig. 1.7 (Guo *et al.*, 1997). In this structure the four subunits make very similar contacts with each other, linking all four subunits in a cyclic manner (view A, Fig. 1.7). The crystal structure of Flp/FRT (Chen *et al.*, 2000) shows striking similarities with the Cre/*loxP* structures. However, the active site tyrosine residue is donated in *trans*, in keeping with biochemical data (Chen *et al.*, 1992). The "snap-shots" of the Cre reaction seen in the crystal structures revealed that recombination by Cre does not require large-scale movement of Cre or the DNA, but rather seem to rely on subtle allosteric changes responsible for switching "on" only two subunits at any one time.

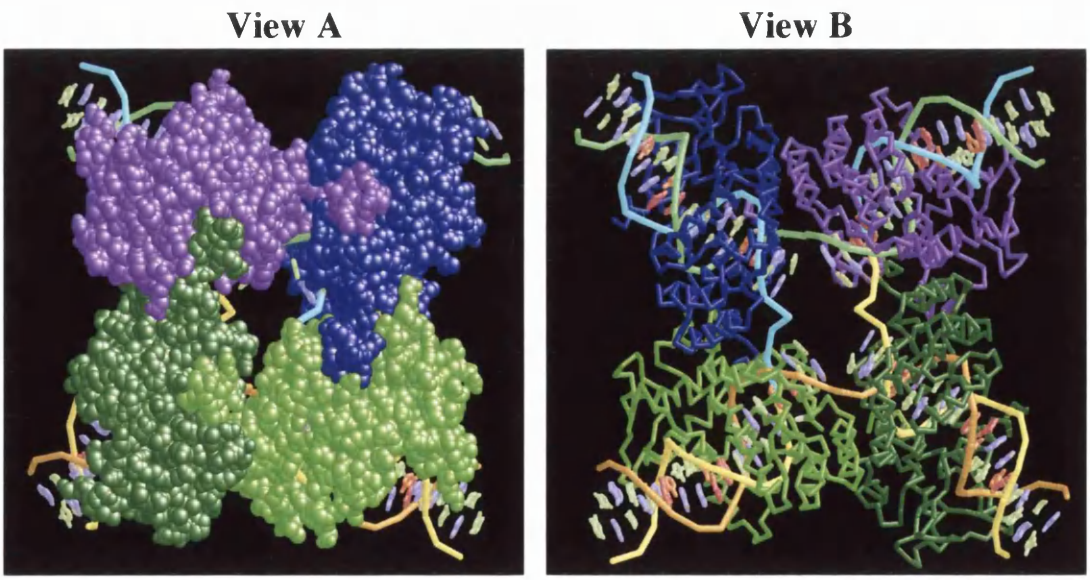
The mechanism of action of the resolvase/invertase family of site-specific recombination enzymes is less well understood. An overview of our knowledge of the resolvase system will be discussed below.

## Fig. 1.6 The chemistry of strand exchange by the integrase and resolvase/ invertase families of site-specific recombinases




The chemistry of strand cleavage is shown for the integrase and resolvase/invertase families of site-specific recombinases. The recombinase subunit is referred to as (Enz), X is the nucleophilic residue (tyrosine or serine) and B stands for base.

## Fig. 1.7 The co-crystal structure of Cre/*loxP* holliday junction (2.7 Å)




Two views of the co-crystal structure of the Cre/*loxP* holliday junction intermediate are shown (Gopaul *et al.* 1998). In view A the four subunits of Cre are shown space-filled, displaying the cyclical contacts made between each monomer and the monomer next to it. In view B the same complex is seen from the opposite side, showing only the backbone of the Cre subunits. The Holliday junction structure of the DNA is produced by the exchange of the cyan and yellow DNA strands.

## 1.4 The Tn3 *res* Site

The Tn3 *res* sequence is important for resolution of the transpositional intermediate, and also for regulation of transposase and resolvase expression. These genes are transcribed from divergent promoters located within the *res* sequence. Binding of resolvase to *res* represses its own expression, thus self-regulating its level in the cell. Transposase expression is also inhibited by resolvase binding, thereby regulating the rate of Tn3 transposition.

The Tn3 *res* site is 114 bp in length. Footprinting of the *res* site revealed three resolvase binding sites (sites I, II and III) (Falvey and Grindley, 1987; Grindley *et al.*, 1982; Kitts *et al.*, 1983). Each site is bound by one resolvase dimer or two resolvase monomers in a highly cooperative manner (Blake *et al.*, 1995) (Fig. 1.8, C). The sites differ in length and are separated by very different spacer lengths (Fig. 1.8, A). Interestingly, the binding sites of most natural *res* sites are separated by an integral number of helical turns of the DNA (centre to centre). This is true for the spacer between sites I and II and is often true for the spacer between sites II and III. The importance of this helical phasing is such that altering the spacing between sites can inhibit recombination (Salvo and Grindley, 1988).

Each resolvase binding site comprises two 12 bp recognition motifs in inverted repeat, separated by a short spacer. The length of the spacer between the binding sequences is different at each site. The most striking difference is seen between sites II and III, which contain spacers of 10 bp and 1 bp, respectively. This indicates that a high degree of flexibility in the resolvase dimer, the DNA, or both is required on binding. Supporting this is the fact that all the individual sites as well as the complete *res* site have been shown to be bent when bound to resolvase (Bednarz *et al.*, 1990; Blake, 1993; Salvo and Grindley, 1987; Yang and Steitz, 1995).

## 1.5 Tn3 and γδ resolvase

Tn3 resolvase is 185 amino acids long and approximately 20 kDa in size. However, non-denaturing gel filtration showed that resolvase has an apparent molecular weight of 40 kDa, suggesting that it is mainly dimeric in solution. Two functional domains were identified by chymotryptic cleavage, which separates the molecule into an N-terminal catalytic domain

(residues 1-140) and a C-terminal DNA-binding domain (residues 141-185). γδ resolvase (from the γδ transposon, or Tn1000) is very closely related to Tn3 resolvase, with approximately 80% sequence identity. They also act on very similar *res* sites and the proteins can bind and resolve each others' binding sites, and complement each other at limiting concentrations.

In the crystal structure of full-length γδ resolvase (Rice and Steitz, 1994), only the N-terminal domain and part of the E helix were visible. The rest of the protein (residues 121-183) was disordered and it seems that these regions only become ordered on binding to DNA. In the large domain crystals, the crystallographic asymmetric unit contained two different dimers called the 1,2 dimer and the 2,3 dimer (Fig. 1.8, B). Higher-order interactions between dimers of resolvase were also seen in these crystals. It was suggested that the higher-order interactions seen in the crystals could represent the interactions in the synaptic complex and a model was proposed by modelling two *res* sites onto the crystal structure (described in chapter 3).

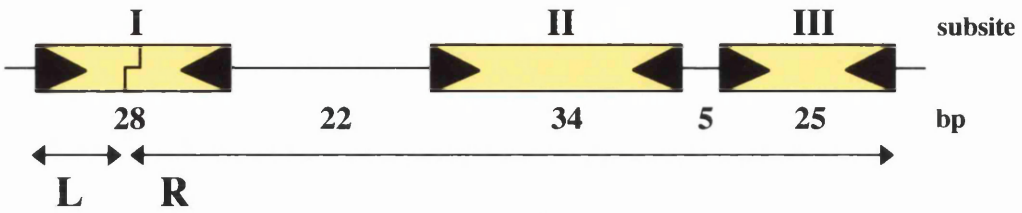
By making cysteine substitutions at residues L50, M106 and I110, which were predicted to crosslink different dimer interfaces, it was shown that the 1,2 dimer (crosslinkable at residues 106 and 110) and not the 2,3 dimer (crosslinkable at residue 50) is the dimer that is present in solution and that binds to *res* DNA (Hughes *et al.*, 1993).

## 1.6 Resolvase/*res* interactions

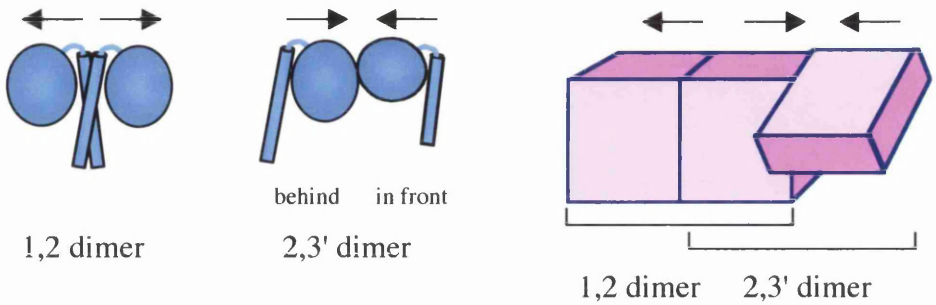
A co-crystal structure of the γδ resolvase dimer bound to a synthetic 34 bp site I has recently been solved at 3.0 Å resolution (Yang and Steitz, 1995) (Fig. 1.9). The co-crystal structure revealed three distinct regions within each monomer. The N-terminal catalytic domain (residues 1-141) is formed by five ß strands surrounded by 5 alpha helices. The C-terminal DNA binding domain (residues 137-183) is a three-helix bundle. Connecting the core of the catalytic domain and the DNA binding domain (DBD) is the E helix (residues 103-136) which contacts the DNA around the minor groove. The DNA in the co-crystal structure is sharply kinked at two points in the central TATA sequence, creating an overall bend of 60°. One of the most striking features of the crystal structure is the asymmetry of the dimer, especially considering the fact that the site I sequence was a perfect palindrome (equivalent

## Fig. 1.8 The structure of the Tn3 *res* site

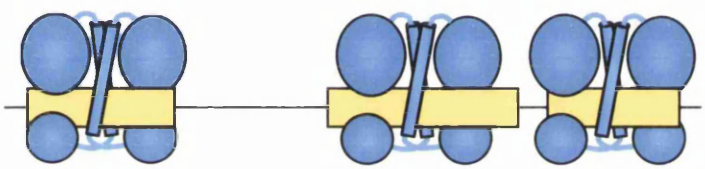
### A The Tn3 *res* site



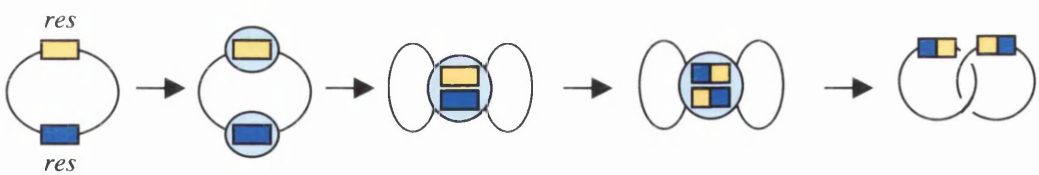

### B Potential structures of resolvase dimers seen in the early crystal structures




### C Binding of resolvase to the Tn3 *res* site



### D The resolvase resolution reaction



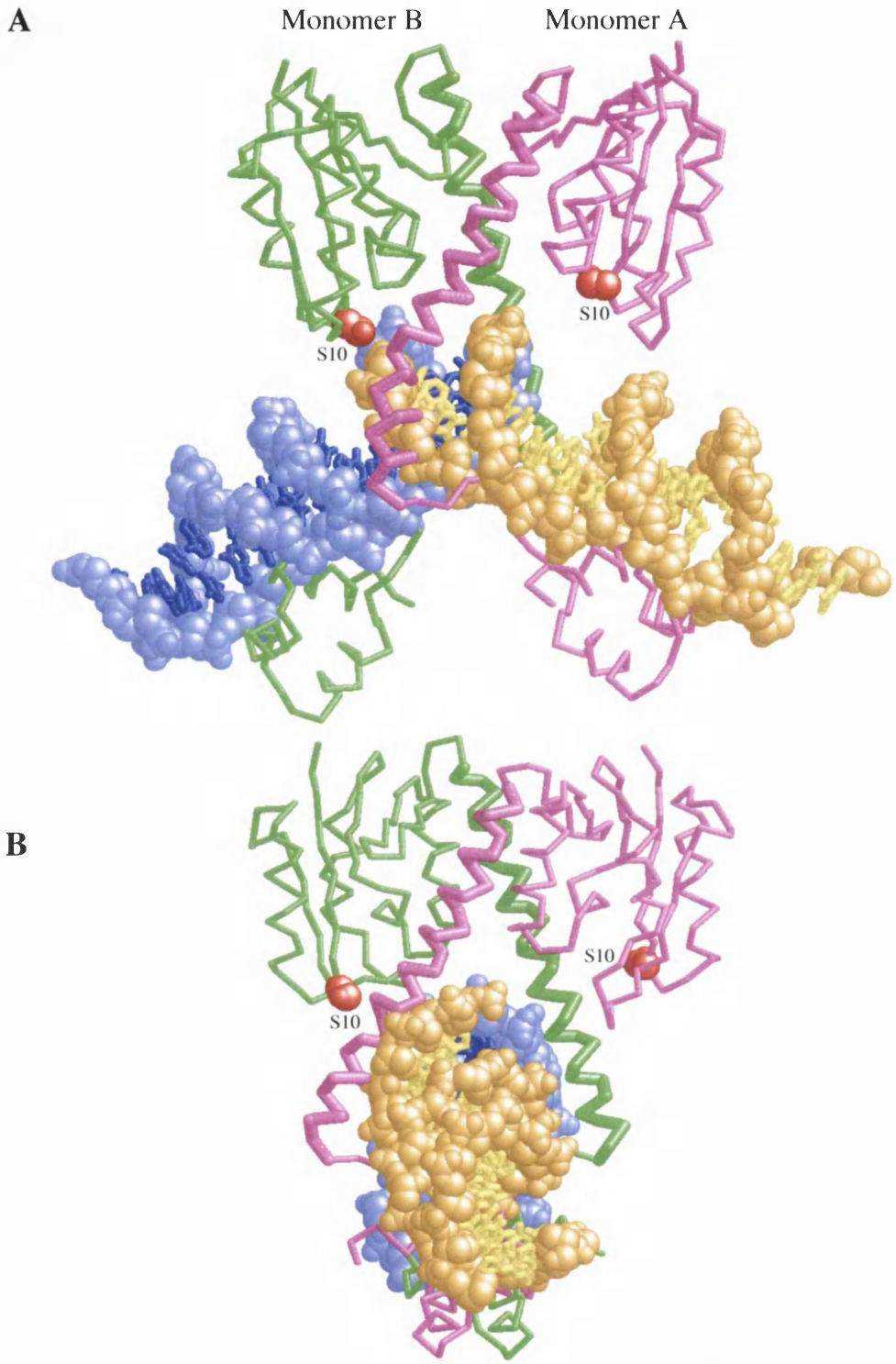

**A.** Tn3 *res* comprises three distinct resolvase binding sites (labelled I, II and III) which differ in length and are separated by different spacer lengths (A). Each subsite contains 12 bp inverted repeats which are bound by the DNA-binding domains of a resolvase dimer (shown as black arrowheads).

**B.** The early crystal structures of resolvase showed monomers interacting across two different important interfaces (Sanderson *et al.* 1990; Rice & Steitz 1994a). These were termed the 1,2 dimer (in which the monomers interacted via extensive hydrophobic alpha helices) and the 2,3' dimer (in which the monomers made contacts via the catalytic domain, forming an interface termed the 2,3'-interface). These represented potential structures of resolvase dimers and are shown diagrammatically above.

**C.** Disulphide crosslinking experiments (Hughes *et al.* 1993) showed that the resolvase dimer which binds to *res* is probably the 1,2-dimer at all subsites. Analysis of resolvase binding (Blake *et al.* 1995) indicated that one resolvase dimer binds each subsite of *res*.

**D.** The complete resolution reaction by Tn3 resolvase can be divided into four steps: (i) binding of resolvase to both *res* sites in a co-integrate, (ii) synapsis of these sites to form a specific nucleoprotein structure termed the synapse, (iii) strand exchange and (iv) the dissociation of the complex. The product was shown to be a singly linked catenane (Reed & Grindley 1981).

**Fig. 1.9 Co-crystal structure of γδ resolvase showing the catalytic residue Ser-10**




Two views of the co-crystal structure of γδ resolvase are depicted above (A and B) (Yang & Steitz, 1995). The monomers are coloured in magenta (monomer A) and green (monomer B). The DNA half-sites are coloured in blue and orange. The catalytic ser-10 residues (red) are shown space-filled (A+B).

to two right ends of site I). The arm region of one monomer contains a straight helix above the DNA minor groove, while the other monomer has its helix kinked into the minor groove. Furthermore, the interactions between the resolvase monomers and the DNA differ, with the centre of the DNA being closer to one of the monomers.

Surprisingly, the structure of the resolvase catalytic domain in the co-crystal did not differ dramatically from the previous crystal structures. In fact, the structure seems to be of an inactive configuration of resolvase; the catalytic Ser-10 residues are 17 Å and 11 Å from the nearest scissile phosphodiester bonds (Yang and Steitz, 1995). The asymmetry of the resolvase-DNA complex, with the nucleophilic Ser-10 of one monomer being closer to the central TATA sequence, suggests the possibility of co-ordinated but sequential single-strand cleavages which may be assisted by sites II and III in the synaptic complex (Yang and Steitz, 1995).

Gel retardation and DNase I cleavage experiments have also shown that γδ resolvase bends the *res* site into a complex termed the resolvosome (Salvo and Grindley, 1988). This complex results from interactions between resolvase subunits bound to the subsites of *res* and induces an intersite bend between sites I and II. The formation of the resolvosome is dependent on the integral phasing of the binding sites. Recombination-deficient mutants of γδ resolvase were identified which bound *res* but did not form the resolvosome structure, suggesting that the ability to form the resolvosome reflects a biologically relevant interaction between resolvase required for recombination (Hughes *et al.*, 1993) (these mutants are discussed in more detail in chapter 3). Nevertheless, resolution requires the formation of a synaptic complex, trapping three negative interdomainal supercoils between the *res* accessory sites (discussed below). Such a structure could presumably only be achieved by the dissociation of the closed loop structure of the resolvosome; therefore the function of the resolvosome in recombination was not obvious.

## 1.7 The Tn3 resolution reaction

An *in vitro* resolution system requires only a simple buffer, magnesium ions, a supercoiled substrate with two *res* sites in direct repeat, and WT resolvase (Reed and Grindley, 1981). The complete reaction involves (i) the binding of resolvase to *res,* (ii) the bringing together

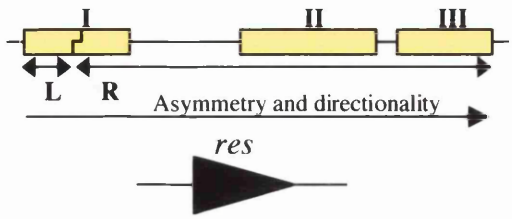
of the two *res* sites such that the crossover sites are aligned correctly (synapsis), (iii) cleavage, strand exchange and ligation, and (iv) release of the products/dissociation of the product synaptic structure (Fig. 1.8, D). *In vitro*, the product of this reaction was not found to be two unlinked circles as anticipated, but instead a singly linked catenane was observed (Fig. 1.8, D) (Krasnow and Cozzarelli, 1983; Reed and Grindley, 1981). It is possible to assign a sign to any crossing of two DNA sequences. Conventionally, *res* is shown as a black arrowhead (representing the asymmetry of this site) and the nodes are assigned as positive or negative (Fig. 1.10, A and B). The product of the resolution reaction catalysed by resolvase was found to be a catenane with two negative nodes, as seen in electron microscopy (Wasserman and Cozzarelli, 1985). The reaction thus proceeds in a topologically organised and specific manner.

Although the major product of resolution is the singly linked catenane with two negative nodes described above, other minor products were also observed. These minor products included a 4-noded knot, a figure-8 catenane, and a 6-noded knot (Fig. 1.10, C). Each product contains one more positive interdomainal node than the previous product (Wasserman *et al.*, 1985). The topologies of these products were indicative of iterative recombination without dissociation of the synaptic complex. The initial synaptic complex therefore must trap three negative interdomainal superhelical nodes with all other substrate supercoils being intradomainal. Furthermore, analysis of the products of an inversion reaction, and of the reverse reaction from a two-noded catenane have shown that the *res* sites wrap around resolvase in a plectonemic rather than toroidal manner (Fig. 1.10, D) (Benjamin and Cozzarelli, 1990; Stark *et al.*, 1994; Stark *et al.*, 1989).

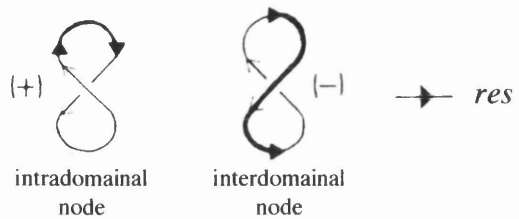
As described previously, the left half of the Tn3 transposon is always ligated to a matching right half (re-creating functional copies of Tn3 carrying the genes for the transposon and resolvase enzymes). When resolvase cleaves the *res* site, the left-half of site I becomes covalently attached to one resolvase monomer and the other monomer becomes covalently attached to the right-half of site I and sites II/III (Fig. 1.11, A). In principle, a recombination site can have an inherent asymmetry in the overlap region created by staggered cleavage; this could ensure correct joining of the left and right ends. However, the overlap region in *res* (2 bp) is symmetrical (Fig. 1.11, A). Instead, the directionality of the site is imposed solely by the presence of the accessory sites. Under 'permissive' reaction conditions, Tn3 resolvase can recombine a *res* x site I plasmid (Bednarz *et al.*, 1990). Although the crossover site is not

## Fig. 1.10 Topology of the Tn3 resolution reaction

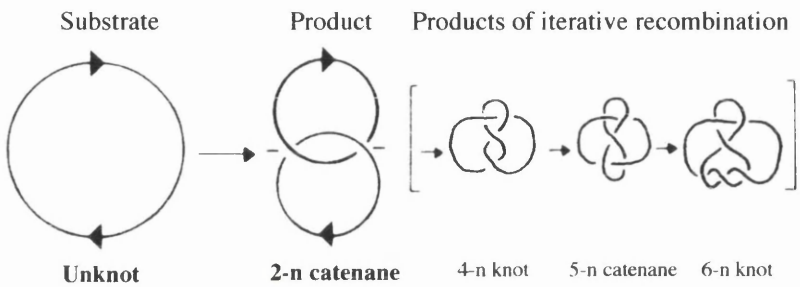
**A** **Asymmetry and directionality of the *res* site can be represented as an arrowhead**



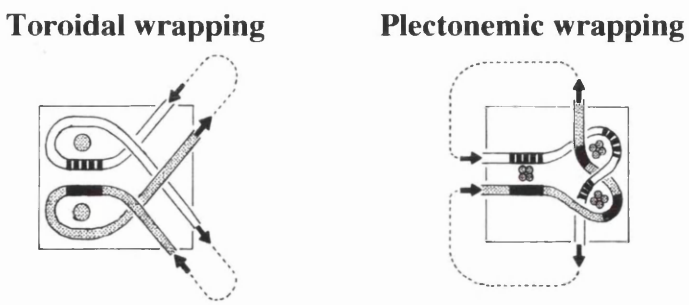

**B** **Crossings of DNA can be assigned as positive or negative nodes which may be intra- or interdomainal**




**C** **Topology of resolution products**




**D** **Reaction product topologies were consistent with two geometries for the productive synaptic complex**




**A.** Cleavage of *res* at site I generates two unequal half-sites which are designated as left (L) and right (R) half-sites as shown. The asymmetry of the *res* site is shown as a black arrowhead (as before).

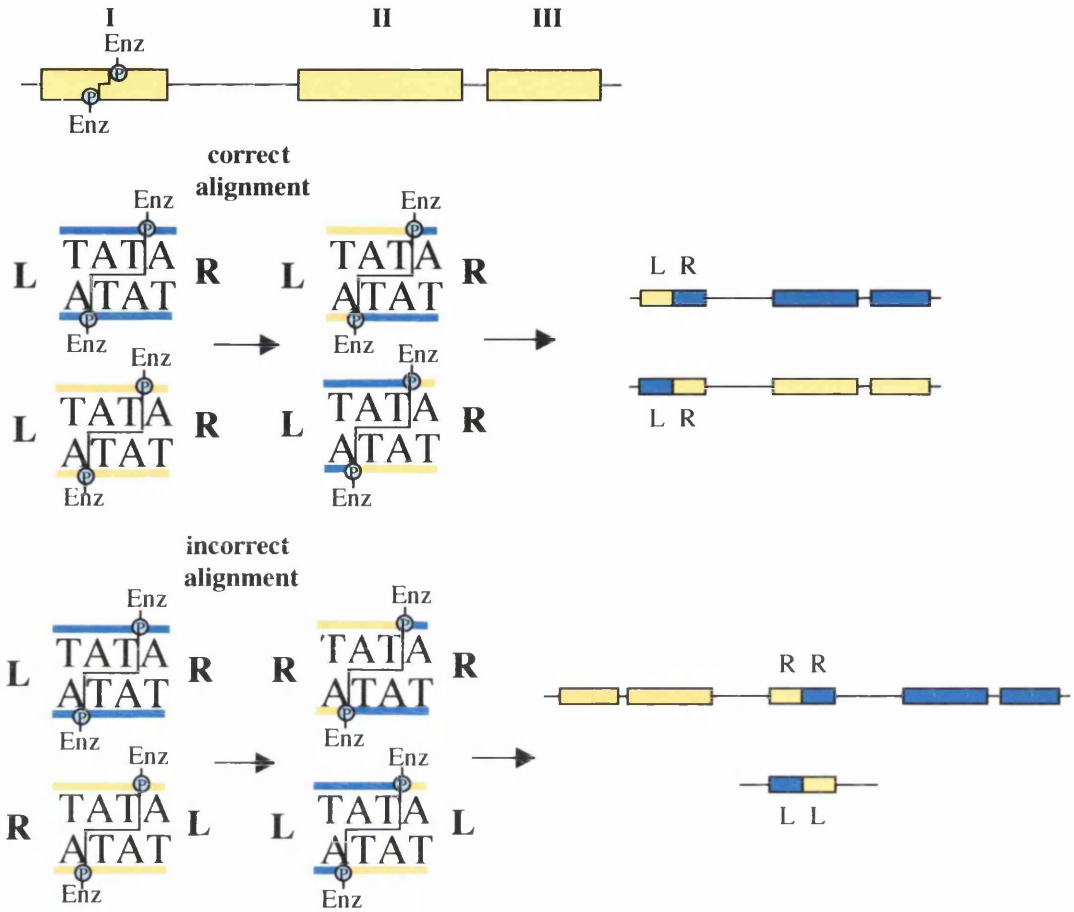
**B.** Nodes created by the crossing of DNA can be described as positive or negative as shown. The two *res* sites divide the plasmid into two domains. Intradomainal nodes are formed by the crossing of DNA within a domain, interdomainal nodes are formed by the crossing of DNA between domains.

**C.** The topology of the Tn3 resolution reaction was studied using electron microscopy by N. Cozzarelli and co-workers and are shown above. The major product of recombination is the 2-noded catenane (Reed & Grindley, 1981). These nodes were found to be negative (Wasserman & Cozzarelli, 1985). Other minor products were also detected (shown in brackets) (Wasserman *et al.* 1985; Stark *et al.* 1991). These were presumed to be products of iterative strand exchange.

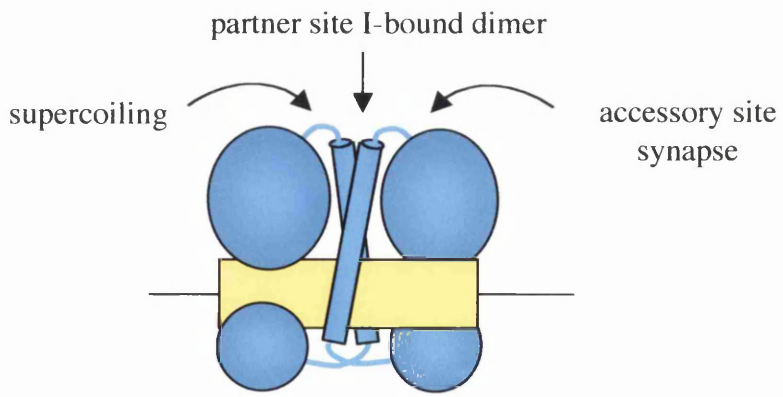
**D.** Presuming that the minor products do indeed represent products of iterative recombination, the initial synapse topology was deduced to contain three negative nodes. The products were consistent with two different geometries for the productive synapse in which the DNA is wrapped in a toroidal or plectonemic fashion.

## Fig. 1.11 Determinants of correct *res* site alignment

**A** **Staggered cleavage of site I creates symmetrical overhangs**




**B** **Activation of resolvase requires a number of signals**




**A.** Strand cleavage by resolvase is staggered and so generates a central 2 bp overlap sequence, AT. The symmetry of this overlap sequence does not prevent the incorrect joining of half-sites, yielding LL and RR junctions.

**B.** Activation of resolvase requires three separate signals; supercoiling, the partner site I-bound dimer and the accessory site synapse.

perfectly symmetrical, resolvase is not sensitive to the orientation of site I. Instead, the topology of the resolvase reaction products suggests that the alignment of the crossover sites is determined by wrapping sites II/III in a specific structure (trapping three negative nodes). Thus, in a *res* x *res* reaction, resolvase always joins a left half-site to a right half-site (creating LR joins) because of the specific structure formed by the synaptic complex, using the directionality imposed by the accessory sites.

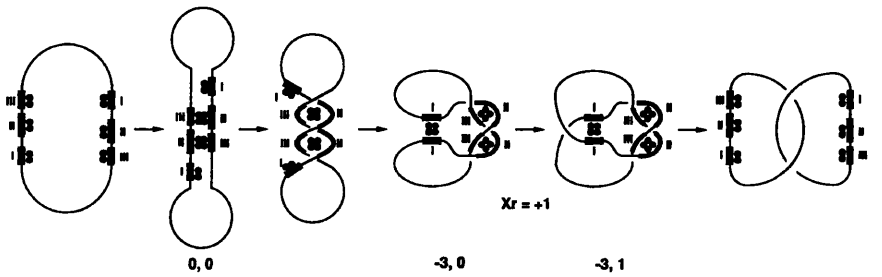
However, any theory tackling resolvase selectivity also has to provide an explanation for resolvase selectivity for sites in *cis*. Resolvase was assayed *in vitro* with a variety of substrates, but found to resolve only substrates with two *res* sites in direct repeat, analogous to its natural substrate (the co-integrate). While it is clear that Tn3 resolvase should not catalyse these reactions (from the point of view of the replicating Tn3) it is not obvious how it can distinguish the orientation of sites which are kilobases apart. One possibility was that resolvase bound at one *res* site could track along the DNA molecule "in search" of another *res* site, thereby preventing intermolecular recombination. However, experimental evidence has refuted this model (Benjamin *et al.*, 1985; Boocock *et al.*, 1986). An alternative model has been suggested to account for resolvase selectivity on topological grounds (Boocock *et al.*, 1987; Boocock *et al.*, 1986) (Fig. 1.12). In this topological filter model the initial synapsis is not regulated. The *res* sites may meet by random collision, however, for a productive synapse to form (namely, one that will lead to recombination) the accessory binding sites II and III must first interwind which results in the alignment of subsites I. It is predicted that this is assisted by negative supercoiling. Incorrect synapse structures involving the entanglement of DNA (such as between sites in inverted orientation) would be energetically costly and would dissociate (Fig. 1.12, B, C and D), allowing the possibility of the formation of a correct synapse. The analysis of synaptic structures formed using various substrates has provided support for this model (Watson *et al.*, 1996).

## 1.8 Synapsis

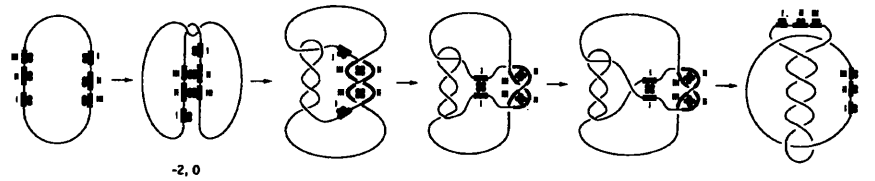
Theoretically the synapse could have one of many possible structures comprising six resolvase dimers and two *res* sites. However, any model of the synaptic complex must fulfil many criteria which constrain both DNA and protein structures. The DNA limitations were set by topological analysis of resolution products and have provided strict limits for any models of the synaptic complex and strand exchange. The accessory sites must wrap

## Fig. 1.12 Topological filter model

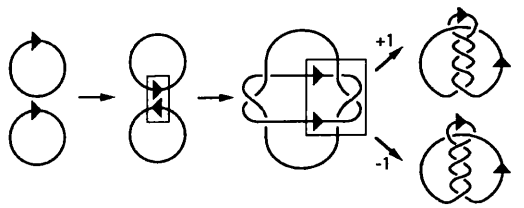
**A. Intramolecular recombination between sites in direct repeat**



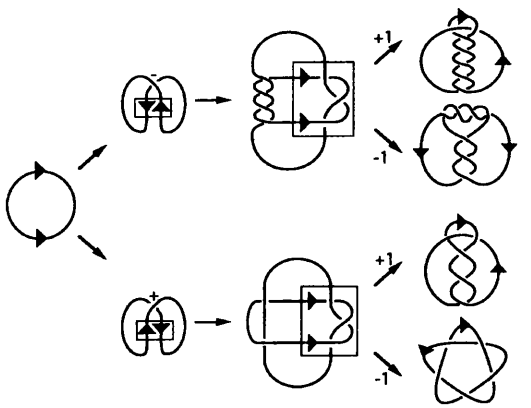

**B. Intramolecular recombination between sites in direct repeat**




**C. Intermolecular recombination**




**D. Intramolecular recombination between sites in inverted repeat**




**A.** The two-step synapsis model is shown for a substrate containing *res* sites in direct repeat. The anti-parallel wrapping of sites II and III traps three negative supercoils and may aid synapsis of the crossover sites in a parallel alignement. Only three interdomainal nodes are trapped and the product topology is as observed.

**B.** The same reaction is shown (as in A) except that additional nodes are trapped (in this case two negative nodes) as would be expected to occur if synapsis occured by random collision events. This would lead to tangling of the DNA in the synapse and yield complex catenated products.

**C.** In order to achieved a local synapse topology (trapping three negative nodes) between sites on different molecules, additional nodes must trapped. The products of right-handed (Xr = -1) and left-handed (Xr = +1) strand exchange are shown.

**D.** As with intermolecular recombination, in order to achieve the correct local synapse topology of synapsis by sites in inverted repeat requires trapping additional interdomainal nodes.

The topological filter model proposes that the initial synapses formed in cases B, C and D would be unproductive and dissociate, allowing another attempt at synapsis to occur (with a possible productive synapse occuring in case B only).

plectonemically and trap three negative supercoils. A more obvious constraint of the DNA is simply the spacing between sites at which the resolvase dimers bind. Any modelling of the DNA around resolvase dimers is thus physically limited. Indeed, the invariability of the spacer sequences between the subsites of *res* presumably reflects the physical constraint of the nucleoprotein complex formed during synapsis.

Studies of the protein interactions within the synaptic complex have been difficult. However, the following points must be considered when modelling resolvase in the synaptic complex.

(i) 6 dimers of resolvase/synaptic complex (inferred) (Blake *et al.*, 1995).

(ii) Resolvase bound at sites II/III traps 3 negative supercoils (Kilbride *et al.*, 1999; Boocock & Bednarz, unpublished).

(iii) Resolvase bound at all subsites of *res* is deduced to be in the form of the 1,2 dimer rather than the 2,3 dimer (Hughes *et al.*, 1993). Disulphide-crosslinked γδ M106C resolvase was proficient at binding to *res*, though it was recombination deficient. Furthermore, targeting experiments with γδR E128K (which fails to bind site III) showed that the inhibitory effect of the disulphide did not operate at site III, indicating that no rearrangement involving the dissociation of the E-helices of the dimer (at site III) is required during synapsis or recombination. Failure of oxidised M106C to function at site I, however, was presumably due to structural perturbations in the 1,2 interface. In fact, the alpha carbons of M106 are 9.5 Å apart and disulphide crosslinking will have caused significant distortion of the dimer.

(iv) The 2,3'-interface is an essential interdimer interface.

Analysis of resolvases which can bind *res* but are defective in resolution have identified an essential interface between resolvase dimers (Hughes *et al.*, 1990). Early targeting experiments supported the notion that the 2,3'-inteface is important only at sites II/III (Grindley, 1993). Recent experiments have greatly refined this picture: in assays of synapsis by sites II and III, and of recombination by complete *res* sites, 2,3'-proficient subunits were found to be needed at specific half-sites within *res* (sites IIL and IIIL for synapsis, sites IR, IIL and IIIR for recombination) (Murley and Grindley, 1998). In fact, "synapsis-up" mutants identified in a random mutagenesis screen, also revealed mutations of residues close to this interface (including V28A, N31T, F34L and M53I/V/L), strengthening the conclusion that the 2,3'-interface is important in synapsis (S. Rowland, unpublished result).

(v) Resolvases with heterologous DNA binding domains retain the functionality of their catalytic domains (Ackroyd *et al.*, 1990; Schneider *et al.*, 2000; N.D.F Grindley, personal communication).

A number of models of the resolvase synaptic complex which take into account the above mentioned structural and topological criteria as well as insights gained from the co-crystal structure and potential higher-order interactions seen in earlier crystal structures have been proposed. These models are discussed in detail in chapter 3.

The kinetics of synapsis by Tn3 and Tn21 resolvases has been studied in detail (Oram *et al.*, 1997). The inital binding to the DNA was found to be complete within 50 msec. Yet recombination required 500 seconds, with synapsis spanning the entire time range. The non-exponential kinetics of synapsis may reflect the association and dissociation of unproductive synaptic structures of random collision events.

## 1.9 Strand Cleavage

Strand cleavage is thought to occur via a nucleophilic attack of the S10 residues on the two strands of the DNA duplex (Hatfull and Grindley, 1986). The transesterification reactions give rise to a covalent resolvase-DNA complex in which the protein is attached to the 5'-recessed ends, releasing a free 3'-OH end. Strand exchange takes place and the 3'-hydroxyls can attack the serine-DNA phosphodiesters, resulting in the ligation of the DNA in the recombinant configuration and the release of resolvase (Fig. 1.6).

Although each binding site is occupied by a dimer of resolvase, it is exclusively at site I that strand cleavage and exchange occurs. This was determined by analysing the cleavage intermediate, which accumulates in the absence of magnesium and in the presence of ethylene glycol (25-40%) (Reed and Grindley, 1981). The cleavage intermediate can be converted to products on addition of magnesium (Reed and Moser, 1984). It thus has the properties of an authentic intermediate in the resolution reaction. A similar experiment performed with Hin led to the same conclusion (Johnson and Bruist, 1989).

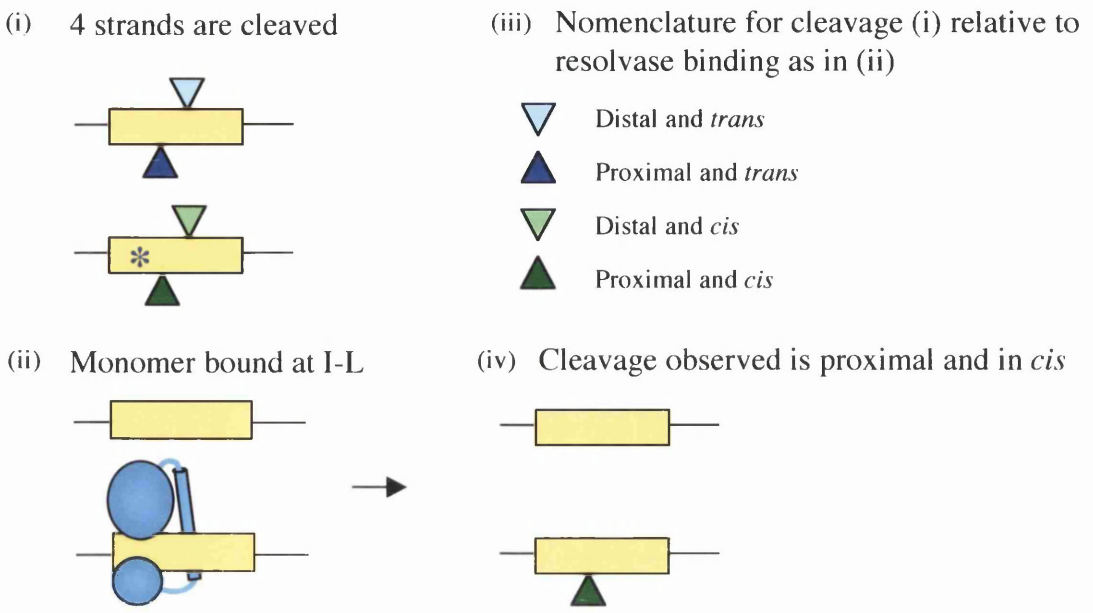
Resolvase is thought to be regulated so that it will only initiate cleavage if a productive synapse has been formed. The cleavage reaction has similar requirements to the resolution

reaction; negative supercoiling and two complete *res* sites (in direct repeat and in *cis*), implying a requirement for a correctly interwound accessory sites and the alignment/synapsis of two crossover sites (Fig. 1.11, B). A picture of the productive synapse emerges in which resolvase bound at site I makes protein-protein contacts with resolvase bound at the opposite crossover site as well as resolvase bound at sites II/III. These contacts may induce allosteric changes in resolvase at site I, resulting in its activation. Certainly, once resolvase becomes committed to initiate cleavage, all four strands are broken in a concerted effort; nicking of one strand is not significantly detected in cleavage reactions with WT resolvase and products in which both sites have been cut are always more abundant than the linear product corresponding to cleavage at only one site. Thus, concertedness of cleavage of DNA strands and of *res* sites is observed.

In order to investigate cleavage at the protein level, mutant resolvases were targeted to specific sites (or half-sites) using a mutant DNA binding domain with an altered DNA specificity (Boocock *et al.*, 1995; Dröge *et al.*, 1990). These assays have shown that all amino acids known to be involved in forming the active site were required in *cis*. In addition, the catalytic Ser-10 residue was found to be provided by the subunit bound proximally to the respective half site (Fig. 1.13). Furthermore, complementation of an active resolvase subunit with a cleavage-deficient resolvase, R68H, bound at the opposite crossover subsite has shown that double-strand cleavage at one subsite can occur independently of cleavage at the partner crossover subsite, but also establishing the importance of interdimer resolvase interactions at site I (Boocock *et al.*, 1995).

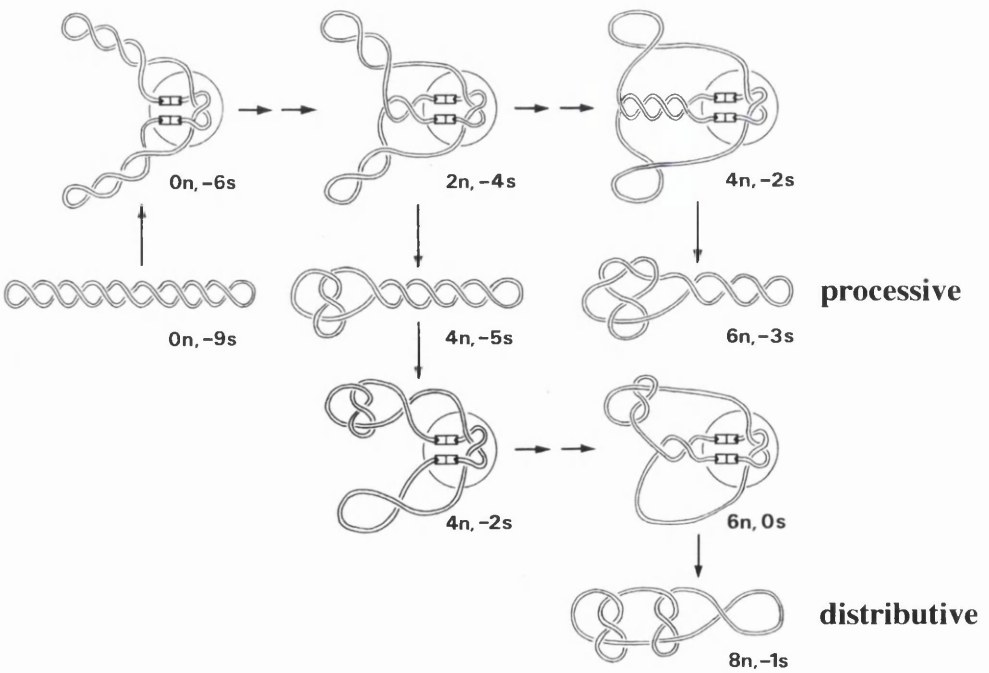
Under certain permissive reaction conditions resolvase has been observed to relax a negatively supercoiled substrate. This activity is similar to that of topoisomerases. Experiments in which catalytically active and inactive subunits were targeted to either half of the crossover site revealed that top-strand cleavage of site I was sufficient for topoisomerase activity by resolvase (Boocock *et al.*, 1995). The relaxation of the substrate was therefore thought to be a consequence of top strand cleavage of the subunit bound to site I-R. The resulting nick could be religated after swivelling of either the enzyme-linked 5' end or the free 3'-OH end around the intact bottom strand.

## Fig. 1.13 Cleavage of site I in *cis*




Targeting experiments have determined that cleavage by resolvase at site I occurs proximal and in *cis* (Boocock *et al.* 1995). The four points of cleavage are shown in (i). For a monomer bound at one half-site as in (ii) the nomenclature of cleavage positions relative to resolvase binding as in (ii) is described in (iii). The observed cleavage point (proximal and in *cis*) is shown in (iv).

## Fig. 1.14 The products of Tn3 and γδ resolvase reactions with a knotting substrate




The products of double-round (360° rotations) reactions are shown. The products generated varies depending on whether the synapse tends to remain intact after strand exchange (processive) or whether the synapse tends to dissociate after strand exchange (distributive). The knotting reaction with Tn3 resolvase gives a distributive pattern of products whereas the same reaction (under the same conditions) is more processive for γδ resolvase (Stark *et al.* 1991).

## 1.10 Models of Strand Exchange

Similar to models of the synaptic complex, models for strand exchange are subject to conditions set by previous experimental results. Most importantly, any proposed mechanism for strand exchange must account for the change in linkage number ($\Delta Lk = +4$) measured for the resolution reaction (Stark *et al.*, 1989). Also, knotting reactions and photocrosslinking experiments (described below) have imposed restrictions upon possible strand exchange mechanisms.

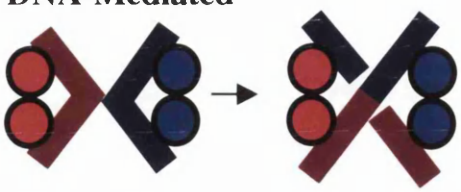
By introducing a single base substitution at the centre of one crossover site (which would result in a mismatch after one round of strand exchange), resolvase can be induced to perform multiple rounds of recombination, producing non-recombinant knotted products. The pattern of knots observed with Tn3 resolvase differed from that observed with γδ resolvase. The patterns were most easily interpreted as being products of iterative strand exchange without dissociation of the accessory site synapse (termed processive, as seen with γδ resolvase) or products of successive rounds of two strand exchange reactions with intermediate accessory site synapse dissociation (termed distributive, as seen with Tn3 resolvase) (Fig. 1.14). These experiments indicated that resolvase can catalyse iterative rounds of strand exchange (Stark and Boocock, 1994; Stark *et al.*, 1991).

Photocrosslinking experiments with resolvase have shown that at least one subunit of the four resolvase monomers bound at the crossover subsites can remain physically attached to the same DNA half-site during recombination (McIlwraith *et al.*, 1996). Furthermore, experiments involving a mismatch in the 2 by overlap region suggest that two rounds of recombination can take place without any rejoining of the DNA strands in an intermediate recombinant form (McIlwraith *et al.*, 1997). Any model of strand exchange therefore cannot involve a reset mechanism after one round, which is necessary for further rounds to occur.

Several models exist for the mechanism of strand cleavage and rotation (Fig. 1.15). They differ in several respects, ranging from a primarily structural and passive role for the protein to a very dynamic one.

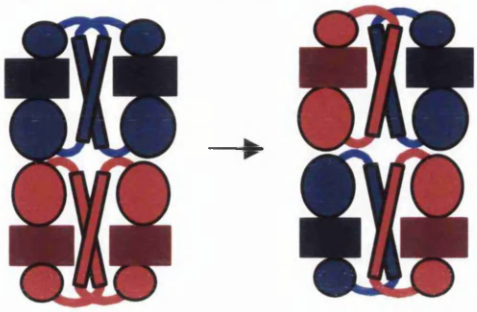
## Fig. 1.15 Models of strand exchange

### DNA-Mediated



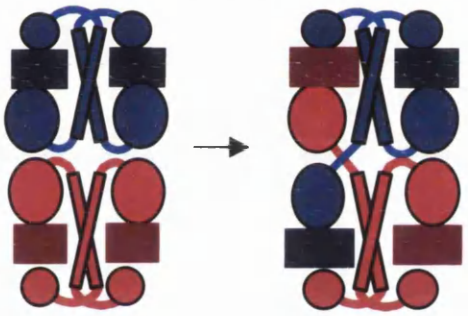
In this model the 1,2 dimers of resolvase bind to site I with the DNA duplexes between them. The crossover points are in close proximity and strand exchange occurs without much protein or DNA movement and without dissociation of the resolvase dimers.

### Subunit Rotation



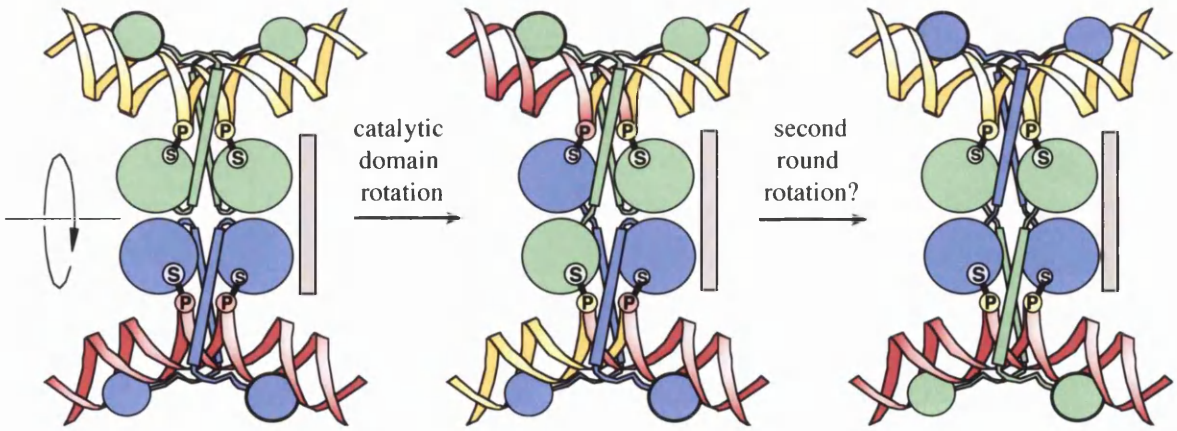
In this model the two duplexes are situated on the outside of the complex and the two resolvase dimers interact via their catalytic domains. Following strand cleavage, two protein monomers (one from each dimer) rotate by 180° in a right-handed sense.

### Domain Swapping



Strand cleavage is followed by only the core catalytic domain rotating by 180°. The E helix dimer interactions remain intact whereas the DNA binding domain of one monomer per dimer needs to dissociate to allow strand exchange.

### Domain Swapping - two rounds of strand exchange without re-setting?




Resolvase is able to catalyse two successive rounds of strand exchange without ligation of the first round intermediate (McIlwraith *et al.* 1997). The diagram above shows that domain-swapping is consistent with two rounds of strand exchange and does not require a re-set mechanism. The rectangle (grey) represents resolvase contacts to the accessory site synapse which are shown to remain intact.

**DNA-mediated strand exchange**

The packing arrangements of earlier crystal structures of γδ resolvase suggested a possible structure for the DNA-mediated synaptic intermediate (Rice and Steitz, 1994), described in more detail in chapter 3. Starting with the six resolvase dimers of the crystallographic unit cell, two *res* sites were added to give a putative synaptic complex (Fig. 1.15). In this model the 1,2 dimers bind to subsite I with the DNA duplexes between them. The crossover points are in close proximity and strand exchange occurs without much protein or DNA movement and without dissociation of the resolvase dimers. No mechanism which would allow for iterative recombination was put forward.

**Subunit Rotation**

The linkage change of each round of recombination is +4 (Stark *et al.*, 1989) which is equivalent to the DNA half-sites rotating by 180° in a right-handed sense. The subunit rotation model postulates that two protein monomers (one from each dimer) covalently attached at site I (presumably to the left half-sites) will rotate by 180° in a right-handed sense. The two DNA duplexes are situated on the outside of the complex and the two resolvase dimers are suggested to interact via their N-terminal domains (Fig. 1.15). The greatest difficulty of this model lies in visualising how the proteins can rotate without allowing the four half-sites to dissociate completely. However, it does provide an obvious scenario for iterative recombination, as seen in the knotting reactions of "mismatch" substrates.

**Domain swapping**

In this model, strand cleavage is followed by the N-terminal core catalytic domain rotating by 180° with the E helix dimer interactions remaining intact (Fig. 1.15). This relative movement of the resolvase domains could be mediated by a potential "hinge" around residue G101. To allow the DNA to "escape", the DNA binding domain of one monomer per dimer would also need to dissociate. An important difference of this model and the subunit rotation model is that the reaction can only proceed through a maximum of two rounds of recombination without dissociation of the synapse unless there exists a reset mechanism after each round that does not require the ligation of the intermediate recombinant form. Importantly, such a mechanism would allow contacts between the four half-sites to be maintained throughout strand exchange. Enzymes which use large scale rotation or swapping of domains during the reactions that they catalyse have been previously identified, with the

number growing steadily. Examples include phage T4 endonuclease VII (Raaijmakers *et al.*, 1999), pyruvate phosphate dikinase (Herzberg *et al.*, 1996), hemolin (an insect immunoglobulin) (Su *et al.*, 1998), diphteria toxin (Bennett *et al.*, 1994), and a calcium pump of the endoplasmic reticulum (Toyoshima *et al.*, 2000). Enzymes using a domain-swap mechanism are also reviewed by (Heringa and Taylor, 1997).

## 1.11 Activation of Resolvase

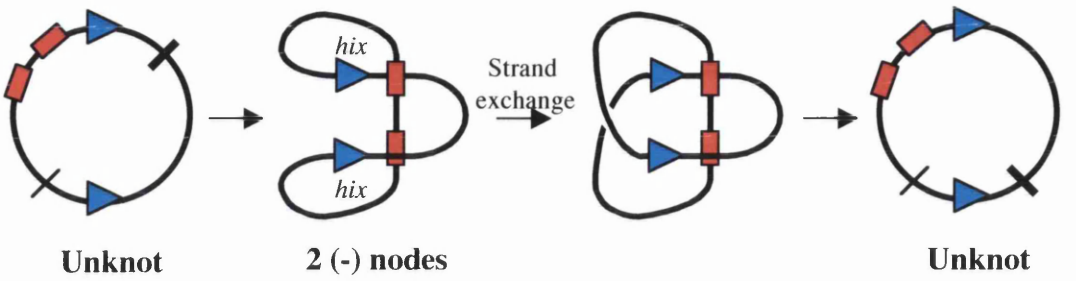
The cleavage intermediate represents a hazardous stage in the Tn3 life-cycle, as DSB's are vulnerable to dissociation, transcription and replication forks. Resolvase therefore only initiates cleavage of the crossover sites when it is fully committed to proceed with the resolution reaction. A resolvase dimer bound at a *res* crossover site is thought to require negative supercoiling, the presence of synapsed accessory sites (trapping three negative supercoils) and a partner dimer bound at the partner crossover site before initiating catalysis (Fig. 1.11, B). The activation of the site-I bound dimers could perhaps be achieved by allosteric interactions between resolvase subunits within the productive synapse, which alter the inactive configuration that resolvase adopts by itself (as seen in the co-crystal structure). "Permissive" reaction conditions allow WT resolvase to recombine a *res* x site I substrate (Bednarz *et al.*, 1990). It was therefore thought that resolvase mutants could be selected that could bypass some or all of the requirements for the activation of wild-type resolvase, and that such mutants may indicate resolvase interfaces that regulate activation.

The first activated mutants of a site-specific recombinase were of the DNA invertase Gin. Site-specific DNA inversion by invertases is greatly stimulated by the presence of an enhancer sequence (*sis*, sequence for inversion stimulation) in *cis* (Huber *et al.*, 1985). Two further host encoded proteins are required to fully stimulate DNA inversion by Hin and its relatives, HU (the histone-like protein of *E. coli*) and Fis (factor for inversion stimulation) which binds to the enhancer sequence, *sis* (Johnson *et al.*, 1986) (Fig. 1.16). Activated invertases which no longer required the enhancer sequence or Fis were selected *in vivo*. Gin S75F/I94V, F104V, H106Y, M108I, M114V; and Cin: R71H, H106Y mutants were isolated and characterised (Klippel *et al.*, 1988; Spaeny-Dekking *et al.*, 1995). *In vitro* analysis of the activated Gin mutants revealed a lack of substrate specificity and supercoil dependence, increased cleavage activity and the absence of topological selectivity (Klippel *et al.*, 1993).

The first resolvase mutant that displayed "activated" properties was substituted in residue E124. Residue E124 interacts with the active site residues S10 and R68 of its partner monomer (in *trans*). Mutation of this residue to glutamine or alanine resulted in an activated γδ resolvase that recombined linear *res* sites (M. Boocock, X. Zhu and N. Grindley, unpublished results). This mutant also gave increased yields of cleavage products, the recombination reaction can be more dissociative, as evidenced by the formation of illegitimate products (Fig. 1.17). This may be a consequence of an altered dimer interface. Such activity can also be enhanced by the addition of 3-12 detergent to the reactions (2 mM) (M. Boocock, unpublished results). The products observed for E124Q under these reaction conditions were consistent with the dissociative cleavage-ligation of half-sites. It was speculated that following the cleavage step, a half-site could escape from the synaptic complex and displace a similar half-site of a different synaptic complex. The displacement of one half site by another was shown to occur in an experiment in which a purified radioactively labelled half-site/resolvase complex was added to a recombination reaction (M. Boocock, unpublished). The resulting radioactively labelled recombinant product confirmed that such a mechanism was feasible.

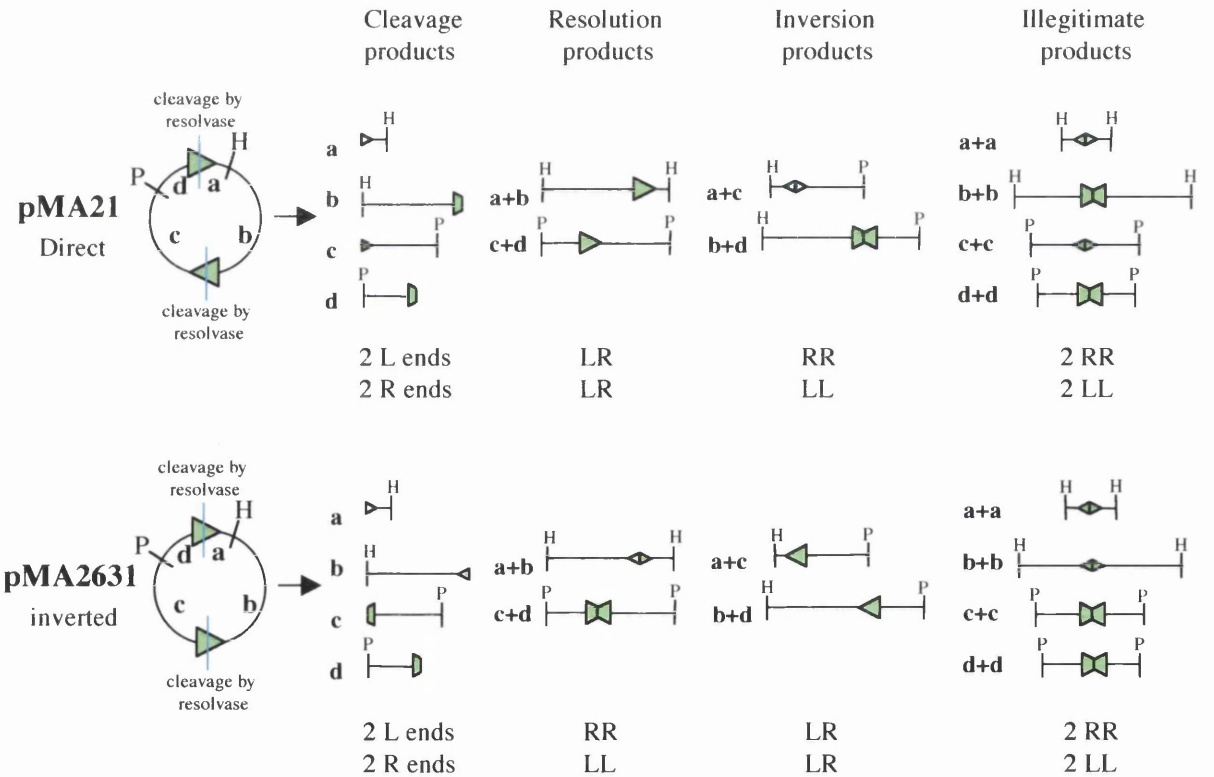
Activated Tn3 resolvase mutants were selected, initially by random mutagenesis of a small region (*Cla*I-*Bam*HI) (Arnold *et al.*, 1999) and then by random mutagenesis of the entire catalytic domain. Selection for a gain-of-function phenotype (as measured by the ability of the mutants to resolve substrates lacking the accessory binding subsites II and III) primarily identified mutations at positions 101 - 103 with the most frequent mutation situated at residue 102 (Arnold P., Ph. D. Thesis 1997; S. Wenwieser and M. Boocock, unpublished results). Mutations in this region activate the protein to recombine without a true synaptic complex being formed. The effects on substrate specificity, reaction rates and reaction topology of activating mutations are discussed in detail in the following Chapters.

## Fig. 1.16 The topology of the Hin invertasome




The reaction catalysed by Hin is shown above. The recombination sites, *hix*, (inverted repeats bound by Hin) are shown as blue arrowheads. The enhancer sequence, *sis*, bound by Fis is shown in red. The synaptosome structure traps two negative nodes (this topology is dependent upon the presence of the enhancer/Fis complex). One round of strand exchange (equivalent to 180° right-handed rotation of the DNA duplexes) yields an unknotted inversion product.

## Fig. 1.17 Restriction analysis of recombination products




Four classes of possible cleavage or recombination products (cleavage, resolution, inversion and illegitimate) are shown for pMA21 and pMA2631. The fragment sizes for these reactions following restriction with *Pst*I and *Hind*III are also listed (for these and further substrates) in Table 6, chapter 2. It should be noted that intermolecular reactions or cleavage-ligation reactions between numerous cleavage products can yield large products.

## 1.12 Conclusion

Despite extensive characterisation of the resolvase reaction, we still do not know how dimers synapse for strand exchange and how resolvase becomes activated for catalysis, nor do we know what structural changes take place within the resolvase dimer during the strand exchange reaction.

A major objective of this work was to gain some experimental insight into resolvase interactions during synapsis and strand exchange and the regulatory features of the reaction. The role of a previously characterised interface, the 2,3'-interface, in synapsis and activation of resolvase was studied in more detail, and is discussed in the light of models of the synaptic complex (Chapter 3). A potential interdimer interface was probed by replacing a region of resolvase (residues 96-105) with sequence from Hin (Chapter 4). This resulted in highly activated resolvase, thus implicating this region of resolvase in regulation. Finally, requirements for the dissociation of specific interfaces within the resolvase dimer during strand exchange were tested by constructing a variety of crosslinkable cysteine-containing mutants (Chapters 5 and 6).

# Chapter 2

# Materials and Methods

## 2.1 Bacterial strains

Two bacterial strains were used, DS941 which is a derivative of *Escherichia coli* K-12 and BL21 which is an *Escherichia coli* B derivative.

| Strain | Genotype | Source |
|---|---|---|
| BL21 (DE3) pLysS | *hsd*, *gal*, (λ *cI ts* 857, *ind*1, *sam*7, *ini*5, $lac_{UV5}$-T7 gene-1), T7 lysozyme expressing plasmid, pLysS | W. Studier |
| AB1157 | *thr*-1, *leu*B6, *his*G4, *thi*-1, *ara*-14, Δ(*gpt-proA*)62, *arg*E3, *gal*K2, *sup*E44, *xyl*-5, *mtl*-1, *tsx*-33, *lac*Y1, *rps*L31 | B.J. Bachmann |
| DS941 | AB1157, but *rec*F143, *lac*ZΔM15, $lacI^q$ | D. J. Sherratt |

## 2.2 Oligonucleotides

Oligonucleotides used during the construction of resolvase mutants are listed in Table 1 and were obtained from Life Technologies (Gibco BRL), MWG or from Marshall Stark.

## 2.3 Plasmids

Plasmids constructed and used are listed in Table 2.

## 2.4 Chemicals

Sources of general chemicals are listed below. All solutions were made using deionised or distilled water. All solutions for enzyme purification were made using distilled 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, Amersham |
| 10x Restriction Enzyme buffers | BRL, New England Biolabs |
| 5x Ligase buffer | BRL |

## 2.5 Solutions

Standard solutions are listed below and will be shown as their abbreviations in the following text. All percentages represent (w/v) measurements throughout unless otherwise stated.

| | |
|---|---|
| TGE | Binding gel running buffer: 50 mM tris, 10 mM glycine, 0.1 mM EDTA (pH ~ 9.4) |
| BRL REact 2 | 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$ |
| C7.5 | 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA |
| C8.2 | 50 mM Tris/HCl (pH 8.2), 10 mM $MgCl_2$, 0.1 mM EDTA |
| C9.4 | 50 mM Tris/HCl (pH 9.4), 10 mM $MgCl_2$, 0.1 mM EDTA |
| DB | Resolvase dilution buffer: 1 M NaCl, 20 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 50% glycerol |
| DissA | Laemmli loading buffer (5x): 50% glycerol, 5% SDS, 200 mM Tris/HCl (pH 6.8), 0.1 mM EDTA (± 2-10% ßME) |
| Doly I | 50 mM glucose, 25 mM Tris/HCl (pH 8.0), 10 mM EDTA |
| Doly II | 0.2 M NaOH, 1% SDS |
| Doly III | 3 M KOAc, 2 M AcOH |
| EGB | 40% ethylene glycol, 50 mM Tris/HCl (pH 8.2), 0.1 mM EDTA |
| Formamide LB | 80% formamide, 100 mM EDTA |
| K mix (5x) | 50% glycerol, 10 mM Tris/HCl (pH 7.5), 0.1 % SDS, 250 µg/ml proteinase K, bromophenol blue (0.01%) |
| Ligation buffer | BRL (1x): 50 mM Tris/HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM DTT, 5% polyethylene glycol-8000, 1 mM ATP) |
| LG buffer | Laemmli gel running buffer (10x): 250 mM tris, 1920 mM glycine, 1% SDS |
| NEB 1 | New England Biolabs restriction enzyme buffer 1: 10 mM bis tris propane/HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7 at 25°C) |
| NEB 2 | New England Biolabs restriction enzyme buffer 2: 50 mM NaCl, 10 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7.9 at 25°C) |
| SDS LB (5x) | Agarose gel loading buffer: 50% glycerol, 10 mM Tris/HCl (pH 7.5), 0.1% SDS, bromophenol blue (0.01%) |
| TAE | 40 mM Tris/AcOH (pH 8.2), 20 mM NaOAc, 1 mM EDTA |
| TBE | 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH ~ 8.3 |

| | |
|---|---|
| TE | 10 mM Tris/HCl pH 8.2, 1 mM EDTA |
| TE/10 | 10 mM Tris/HCl pH 8.2, 0.1 mM EDTA |
| LB | Luria broth: 10 g bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl, made up to 1 litre with deionised water, NaOH (5 M) used to adjust to pH 7.5 |
| 2xYT | 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 |

## 2.6 Bacterial growth media

*E. coli* was grown in liquid L-Broth or on solid L-Agar (LB with 15 g/l Agar). Transformed *E. coli* were allowed to express antibiotic resistance in 2xYT Broth. *In vivo* resolution activity of resolvase was tested using 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. 40 g were made up to 1 litre with deionised water and boiled for 5 minutes. This was cooled to 55°C and supplemented with 20 ml of filter sterilised D-galactose (1% final). Media were sterilised at 121°C, 15 pounds/inch$^2$ for 15 minutes.

## 2.7 Bacterial growth conditions

Bacterial cultures were grown with vigorous shaking (200 rpm) at 37°C. Agar plates were inverted and incubated at 37°C overnight. Bacterial strains stocks were stored at -70°C, following dilution (1:1 v/v) of an overnight liquid culture (LB) in a 40% glycerol, 2% peptone solution.

## 2.8 Antibiotics

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

| Antibiotic | Stock Solution | Selective conditions |
|---|---|---|
| Ampicillin (Ap) | 10 mg/ml in $H_2O$ | 100 µg/ml |
| Chloramphenicol (Cm) | 2.5 mg/ml in ethanol | 25 µg/ml |
| Kanamycin (Km) | 50 mg/ml in $H_2O$ | 50 µg/ml |
| Tetracycline (Tc) | 12.5 mg/ml in ethanol | 12.5 µg/ml |
| Streptomycin (Str) | 5 mg/ml in $H_2O$ | 50 µg/ml |

## 2.9 Transformation of competent cells

### 2.9.1 Competent cells

Competent cells were routinely prepared as follows. 1 ml of an overnight culture was inoculated into 100 ml of L-broth, and grown at 37°C until the $OD_{600}$ was 0.4-0.5. Cells were centrifuged at 3,000 g for five minutes at 4°C using a pre-chilled Beckmann JA-20 rotor. The pellet was resuspended in 50 ml cold (4°C) 50 mM $CaCl_2$ and kept on ice for 30 minutes. Cells were centrifuged as before and resuspended in 1ml of 50 mM $CaCl_2$ and stored at 4°C. Cells made by this procedure remained competent for up to 48 hours.

### 2.9.2 Transformation

Bacteria were transformed by the addition of plasmid DNA or ligation mix (10-100 ng) to 20-50 µl of competent cells. The samples were mixed gently and incubated on ice for 30 minutes. The cells were incubated at 37°C for 5 minutes, and returned to ice for a further 2 minutes. The culture was allowed to express any antibiotic resistance genes carried on the plasmid by incubating with 100 µl of 2xYT broth at 37°C for up to 60 minutes, depending on the expression time required for the antibiotic selection. Aliquots were spread onto selective agar plates.

## 2.10 Preparation of plasmid DNA

### 2.10.1 Large scale preparation of plasmid DNA

Plasmid DNA was prepared by an adoption of the method of Birnboim and Doly (1979). An overnight culture (200 ml in LB) was grown, and the cells were collected by centrifugation (3,000 g, 5 min., 4°C). The pellet was resuspended in 4 ml of Doly I buffer. Cells were lysed by the addition of 8 ml of Doly II and mixed by inverting gently. Lysis and partial DNA denaturation was allowed to proceed for 4 minutes at 4°C before the sample was neutralised and SDS removed by the addition of 6 ml of Doly III (4°C). The samples were mixed gently and centrifuged (39,200 g, 30 minutes, 4°C). The supernatant was removed and the DNA precipitated by adding 12 ml of isopropanol (20°C for 15 min.). The precipitated DNA was recovered by centrifugation (39,200 g, 30 minutes, 20°C). Remaining salts were removed by washing the pellet with 2 ml of 70% ethanol. The pellet was resuspended in 2 ml of TE/10 buffer for 1-2 hours at 37°C and then centrifuged to remove insoluble debris (17,400 g, 5 min, 4°C).

**CsCl Equilibrium Density Gradient**

DNA in TE/10 (2 ml) was mixed with 4.32 ml of a CsCl solution (5 g plus 3 ml $H_2O$) and 0.27 ml of an ethidium bromide solution (10 mg/ml) and the mixture was centrifuged to remove any resulting precipitate (17,400 g, 5 min., 4°C) and the supernatant transferred to a Beckmann quick-seal ultra-centrifuge tube (16 x 76 mm). The tubes were filled with liquid paraffin. The CsCl density gradient was achieved by centrifuging the samples in a Beckman Ti70 fixed angle rotor (200,000 g; 45,000 rpm for 16 hrs. at 15°C); DNA was visualised on a long-wave UV source (365 nm). The supercoiled plasmid DNA was recovered by piercing the tube immediately below the band with a 21G hypodermic needle attached to a 1 ml syringe, removing approximately 0.5 ml of sample. Ethidium bromide was removed by extraction with n-butanol (0.5 ml x 5), and the sample diluted with water (3:1), prior to precipitating DNA with 2 volumes of 100% ethanol (20 min. 4°C). The DNA was recovered by centrifugation in siliconized Corex tubes (17,400 g, 30 minutes, 4°C). The pellet was washed with 70% ethanol (2 ml), and then dissolved in 400 µl TE/10 buffer. The DNA sample was stored at -20°C.

### 2.10.2 Small scale preparation of plasmid DNA

Plasmid DNA was purified from 3 ml of an overnight culture (LB) using the QIAGEN Spin purification kit according to the manufacturer's instructions (Cat. no. 27106). This kit uses silica-gel membrane technology to bind DNA and allow wash steps to be performed. The purified plasmid DNA was eluted with TE buffer (50 µl).

## 2.11 Restriction enzyme digestion of DNA

Restriction digests were carried out in the suppliers' recommended buffer with 2-10 Units of enzyme per µg of DNA, to ensure complete restriction of DNA. The reaction was incubated at 37°C ≥ 30 min. and terminated by the addition of 0.25 vol. of SDS loading buffer, 50 µg/ml proteinase K. For partial restrictions, samples were taken at earlier times.

## 2.12 Filling in DNA 5' overhangs

DNA 3' recessed ends were filled by adding dNTP's (dATP, dCTP, dGTP and dTTP) to a final concentration of 125 µM, with 1 unit of the Klenow fragment of DNA polymerase I in 20 µl of BRL REact 2. The mixture was incubated at 37°C for 30 min. and the reaction stopped by heating for 5 min. at 70°C.

## 2.13 Ligation of DNA fragments

Ligations of foreign DNA (insert) into plasmid DNA (vector) were carried out with a molar ratio of 10:1 insert to vector when double stranded oligonucleotides were ligated into a vector fragment and a 3:1 insert to vector ratio otherwise. DNA ligations were performed in ligation buffer (BRL) with 1 unit of T4 DNA ligase in a volume of 20 µl. The mixture was incubated at room temperature (3-16 hours) and subsequently used to transform competent cells (Section 2.9.2).

## 2.14 Sequencing of plasmid DNA

DNA samples were sequenced using the Sequenase T7 DNA polymerase kit (USB, 1990), based on the method of Sanger *et al.*, 1977. The plasmid template was denatured by incubating the DNA (3 μg) in 200 mM NaOH at 37°C for 15 minutes in a 30 μl volume. This was spun through a G-25 mini-column (Pharmacia), prepared according to the manufacturers instructions. The template was then sequenced as described in the Sequenase protocol.

Alternatively, DNA samples were sequenced by MBSU (Molecular Biology Sequencing Unit, University of Glasgow).

## 2.15 Purification of synthetic oligonucleotides

If necessary, the oligonucleotide was removed from the glass support by the addition of 1 ml of 30% aqueous ammonia. The mixture was incubated at room temperature for 1-2 hours. The glass support was pelleted by centrifugation and the supernatant containing the oligonucleotide was recovered. The oligonucleotide was deprotected by the further addition of 1 ml of 30% aqueous ammonia and the sample was incubated in a 55°C oven overnight (sealed tube). After cooling on ice, ammonium acetate pH 7.5 was added (0.5 M final concentration), and the oligonucleotide was precipitated by the addition of 2 volumes of ethanol, 0°C for 30 minutes. The oligonucleotide was pelleted by centrifugation in an Eppendorf microcentrifuge (14 K, 30 minutes, 4°C) and redissolved in 20 μl of TE/10.

Full-length oligonucleotides were separated from prematurely terminated oligo-nucleotides in a polyacrylamide gel. Formamide loading buffer was added (1 volume) prior to loading on a denaturing polyacrylamide gel (Section 2.17.3). The oligonucleotides were 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 stained in 100 ml of staining solution (20% v/v isopropanol and 10% v/v of 0.1% solution of "Stains all" in formamide). The full-length oligonucleotide was excised and the gel slice crushed in 1 ml of 0.5 M $NH_4OAc$/EDTA buffer in a Nunc tube. The oligonucleotide was incubated at 37°C for 16 hours on a rotating wheel mixer to allow the oligonucleotide to diffuse out of the polyacrylamide. The polyacrylamide was removed by passing the supernatant through a

0.22 μm cellulose acetate filter (Spin-X, supplied by Costar). The oligonucleotide was then ethanol-precipitated, centrifuged, and the pellet washed in 70% ethanol before redissolving in TE/10 buffer.

## 2.16 Annealing oligonucleotides

Two complementary oligonucleotide strands were annealed by mixing equal amounts of purified oligonucleotides (1-10 pmol) in TE/10 buffer, 100 mM NaCl and heating to 75°C for 5 minutes. The oligonucleotides were then slowly cooled to room temperature. Annealed oligonucleotides were stored at -20°C.

## 2.17 Electrophoresis

### 2.17.1 Agarose gel electrophoresis

Agarose gels (0.7% or 1.1% agarose) were prepared by dissolving appropriate amounts of agarose powder in 200 ml of TAE buffer at 100°C. The solution was allowed to cool to 60°C and poured into a gel former (20x30 cm) with an appropriate comb. The gel solution was allowed to set at room temperature. To achieve high resolution, agarose gels were run in gel tanks containing 3.2 l of TAE buffer at 40 V for 16 hours, or 100 V for 4 hours.

### 2.17.2 Non-Denaturing electrophoresis

Resolvase band-shift assays were performed using a non-denaturing PAGE method. An acrylamide gel solution (6% acrylamide:bis-acrylamide 30:0.8, 50 mM tris, 10 mM glycine (pH 9.4), 0.1 mM EDTA) was prepared (30 ml). Polymerisation was initiated by the addition of 18 μl TEMED and 360 μl ammonium persulphate (10%). The gel was pre-run for 30 min. (200 V) and run at a constant voltage (200 V) for 2-4 hours at 4°C or room temperature with TGE.

### 2.17.3 Denaturing polyacrylamide gels

Denaturing polyacrylamide gels were used for plasmid sequencing and purification of synthetic oligonucleotides.

Sequencing reactions of plasmid DNA were electrophoresed using the BRL Sequencing System, Model S2. A standard 6% polyacrylamide sequencing gel was prepared by mixing 80 ml of Sequagel-6 with 20 ml of Sequagel Complete (supplied by Protogel). Polymerisation was initiated by the addition of 600 µl of 10% APS. The gel was allowed to polymerise at room temperature for 20 minutes. The gel was set up and pre-run at 100 W for 45 minutes with TBE buffer (1 l). Following the addition of formamide loading buffer (1 volume) the samples were heated to 80°C for 5 minutes prior to loading. The sequencing gel was run for 1.5-3 hours at 60 W. The gel was dried under vacuum and autoradiographed as described in Section 2.21.

Purification of synthetic oligonucleotides was also carried out on denaturing polyacrylamide gels using CBS Scientific Co. Adjustable Slab gel kits (model # ASG-250). These were set up according to the manufacturer's instructions. A 10% acrylamide/7 M urea gel solution (100 ml) was prepared (10% acrylamide (19:1), 7 M urea, 1 x TBE). Polymerisation was initiated by the addition of 666 µl APS (10%) and 40 µl of TEMED. The gel was pre-run at 15 W. Formamide loading buffer (1 vol.) was added to the samples which were heated to 70°C, 5 min. prior to loading. The gel was run at 15 W, for 2-4 hours.

### 2.17.4 Discontinuous SDS-polyacrylamide gel electrophoresis

Protein samples were analysed using a discontinuous SDS-PAGE system (Laemmli, 1970).

Resolving gels: The resolving gels used were prepared from a solution of the following composition: 15-18% acrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.05% ammonium persulphate, 0.05% (v/v) TEMED. Polymerisation was initiated by addition of freshly prepared 10% ammonium persulphate and TEMED. The gel was overlaid with 0.5 ml of isopropanol and allowed to polymerise for 30-45 minutes.

Stacking gels: Following polymerisation of the resolving gel, the overlay was removed and the surface of the gel rinsed with water to remove any unpolymerised acrylamide. The stacking polyacrylamide gels were prepared from a solution of the following composition: 5% acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 0.1% APS, 0.2% (v/v) TEMED. Stacking acrylamide gel solution was overlaid on the polymerised resolving gel and a comb

was added immediately. After polymerisation of the stacking gel, the wells were rinsed with electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS).

Laemmli loading buffer (DissA) was added to protein samples (0.2 vol.) prior to loading. When required, fresh β-mercaptoethanol (1-5% final) was added. Samples were boiled for 5 minutes to denature proteins and reduce disulphide bonds. Samples were loaded using a Hamilton syringe. Polyacrylamide gels were run at 30-40 mA for 2-3 hours, or at 60 V overnight.

## 2.18 Purification of DNA fragments

Following agarose gel electrophoresis, DNA was visualised on a long wavelength transilluminator (365 nm). The required DNA band was excised. The DNA was purified from the agarose chip by spinning through a Costar filter (0.45 μm) for 10 minutes at 8,000 rpm in an Eppendorf microcentrifuge.

## 2.19 UV spectrophotometry

Concentrations of DNA were estimated by measuring the absorbance at 260 nm in a UV/visible spectrophotometer (Shimadzu). An absorbance at 260 nm of 1.0 was taken to correspond to a 50 μg/ml solution of double-stranded DNA and 20 μg/ml of single-stranded oligonucleotides (Maniatis).

## 2.20 Visualising DNA and proteins

### 2.20.1 Visualising DNA

DNA was visualised in agarose and polyacrylamide gels by staining with an ethidium bromide solution (0.5 μg/ml) in a shaker tray for 30-60 minutes; the gel was destained with deionised water for 60 minutes. DNA was visualised on a 254 nm UV short wavelength transilluminator or, for preparative work, on a long-wave transilluminator at 365 nm.

### 2.20.2 Visualising proteins

Proteins were visualised by incubating Laemmli gels in Coomassie stain (0.1% Coomassie Blue, 50% MeOH, 10% AcOH) for 1 hour at 37°C. Gels were destained at room temperature in 10% MeOH, 10% AcOH.

## 2.21 Photography, autoradiography & phosphor-imagery

Agarose and polyacrylamide gels were photographed using either a Polaroid camera and Polaroid 667 film, or a Pentax SLR camera loaded with Ilford HP5 35 mm film, using a red filter. 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, the gel was dried under vacuum in a Bio-Rad slab gel dryer, and exposed to a sheet of Fuji RX100 film. This was developed in an X-OMAT automated processor (Kodak).

Phosphor-imaging 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.

## 2.22 Expression of resolvase and derived mutants

An overnight culture (prepared from a glycerol stock, -70°C) of the expression strain BL21 DE3 pLysS carrying the appropriate expression plasmid was inoculated (1 ml) into pre-warmed (37°C) L-broth (400 ml) supplemented with Kanamycin (50 μg/ml) and Chloramphenicol (25 μg/ml) in a 2.5 l flask. The cells were grown by vigorously shaking the flasks (200 rpm) at 37°C to an OD ($\lambda$ = 600 nm) of 0.4-0.6. Resolvase expression was induced by the addition of 0.5 mM IPTG and the cells were grown for a further 3-3.5 hours. Cells were harvested by centrifugation in 200 ml buckets (3,000 g, 5 min., 4°C in a pre-cooled JA14 rotor). The supernatant was discarded and the pellet was resuspended on ice in 100 ml of Wash buffer (20 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA). The cells

were pelleted (3,000 g, 5 min., 4°C) and the supernatant was removed. Cell pellets were stored at –70°C.

## 2.23 Purification of Tn3 resolvase and derived mutants

All steps were at 4°C or on ice unless otherwise stated. The frozen cells (see section 2.22) were thawed at room temperature for 5 minutes. The cells were resuspended in 15 ml of resuspension buffer (20 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1.2 mM PMSF, 1% ethanol). Cells were sonicated using a Vibra-cell VC100 sonicator at 40% amplitude with a button probe. Three bursts of 20 seconds were applied, between which the cells were allowed to cool on ice. The crude extract was centrifuged (20,000 rpm, 15 min., 4°C in a pre-cooled JA20 rotor) and the supernatant was collected. The remaining pellet (containing resolvase) was resuspended in 10 ml of 100 mM NaCl buffer (100 mM NaCl, 20 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1.2 mM PMSF, 1% ethanol) using a Dounce homogenizer for 30 minutes. The suspension was centrifuged (20,000 rpm, 15 min., 4°C) and the supernatant was collected. The pellet was resuspended in 10 ml of 2 M NaCl buffer (2 M NaCl, 20 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1.2 mM PMSF, 1% ethanol) using a Dounce homogenizer for 90 minutes. This step solubilizes wild-type resolvase, however, most mutants of resolvase remained insoluble at this stage. The suspension was re-centrifuged as above and the supernatant was retained for potential further purification. The remaining pellet was resuspended in 10 ml of 6 M urea buffer (6 M Urea, 20 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT, 1.2 mM PMSF, 1% ethanol). In the presence of 6 M urea the resolvase mutants were solubilized in a denatured state. Insoluble debris was removed by centrifugation (as before), and the supernatant (containing resolvase) collected for further purification. The resulting final pellet was resuspended in 10 ml of 6 M Urea Buffer, 1% SDS (6 M urea, 20 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT, 1.2 mM PMSF, 1% ethanol, 1% SDS) to provide a sample for SDS PAGE analysis.

The supernatant from the 2M NaCl resuspension step was dialysed against 1 l of low salt dialysis buffer (20 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$,1 mM EDTA, 1 mM DTT, 1.2 mM PMSF). Generally, any resolvase present in this fraction precipitated in the low salt conditions. The supernatant from the 6 M urea buffer resuspension step was dialysed against

one litre of high salt dialysis buffer (1 M NaCl, 20 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 1.2 mM PMSF) for at least 5 hours. The decrease in the concentration of urea allowed resolvase to renature, while the relatively high concentration of salt allowed the resolvase to remain soluble. The dialysed fraction was then transferred to the low salt dialysis buffer and allowed to dialyse overnight. This resulted in the precipitation of resolvase.

After the dialysis steps it had to be decided which fraction contained more and/or purer resolvase (as judged by SDS PAGE). As only mutants of resolvase were purified in this project, the majority (or often all) of the resolvase resuspended in the 6 M urea fraction. Purification therefore continued with this fraction. This dialysed fraction was mixed, transferred to a Nalgene tube and centrifuged (as before). The supernatant was collected and the pellet was resuspended in 10 ml of Chromatography buffer A (6 M urea, 25 mM NaCl, 25 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT). At this stage the fractions still contain a variety of contaminants including DNA, RNA and proteins. A cation exchange column (SP sepharose fast flow) was used to remove these contaminants. As resolvase is slightly positively charged it binds to the resin in low salt (whereas the DNA, RNA and negatively charged proteins elute). A gradient of increasing salt concentration (Chromatography buffer B: 6 M urea, 1 M NaCl, 25 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT) elutes the positively charged proteins (including resolvase). All chromatography steps were performed at room temperature. An SP sepharose column (6 ml, 1 $cm^2$ x 6 cm) was equilibrated with chromatography buffer A. The resuspended resolvase was applied to the column using a peristaltic pump at a rate of 1 ml/minute. Flow-through was collected in a 20 ml universal container on ice for SDS PAGE analysis. The column was connected to a Waters™ 650E advanced protein purification system. Absorbance (260 nm and 280 nm) was read using a Waters 490E programmable multiwavelength detector and recorded on a Millipore chart recorder. A second base-line was obtained and a programmed gradient of increasing salt concentration was initiated.

| | |
|---|---|
| 0-25 min | 100% buffer A to 75% buffer A, 25% buffer B (linear gradient) |
| 25-50 min | 75% buffer A, 25% buffer B to 100% buffer B (linear gradient) |

The flow rate was 1 ml/min throughout the gradient run and 1 ml fractions were collected in Eppendorf tubes and stored on ice. The column was washed and recycled with 20 ml

Chromatography buffer C (6 M urea, 2 M NaCl, 25 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT). Fractions corresponding to absorbance peaks were run on a Laemmli gel to ascertain concentration and purity. Chosen fractions were combined and dialysed (5-6 hours) against high salt dialysis buffer (1 M NaCl, 20 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 1.2 mM PMSF). The samples were then immediately dialysed against a low salt dialysis buffer (20 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1.2 mM PMSF) to allow precipitation of resolvase overnight. The dialysate was transferred to a Nunc tube and precipitated proteins were pelleted by centrifugation in an Eppendorf microcentrifuge (14,000 rpm, 5 min at 4°C). All traces of the supernatant were removed and the pellet was resuspended in 100 - 400 µl resolvase resuspension buffer (2 M NaCl, 20 mM Tris-HCl pH 7.5, 0.1 mM EDTA, ± 2 mM DTT). The suspension was centrifuged as above and the supernatant (containing soluble resolvase) was transferred to a Nunc tube. This procedure was repeated to resuspend any remaining soluble resolvase and the final insoluble pellet was retained. Finally, 50% v/v glycerol was added to the two resuspended resolvase fractions for storage at -20°C. This yields a buffer composition equivalent to resolvase dilution buffer (1 M NaCl, 10 mM Tris-HCl pH 7.5, 0.05 mM EDTA, ± 1 mM DTT, 50% glycerol).

## 2.24 Fractionation of resolvase species using TNB resin

It was possible to remove some reduced cysteine-containing resolvase from a preparation containing a mixture of oxidised and reduced species using a TNB (thio-bis-2-nitrobenzoate) resin (Pierce). The TNB resin (50 µl) was pre-equilibrated with resolvase dilution buffer, pH 8.8 by mixing in an Eppendorf tube and removing the supernatant (5 x 1 ml). Resolvase (in DB) was mixed with the TNB resin in an Eppendorf tube. The binding capacity of the TNB resin is approximately 5 mM; nine volumes (450 µl) of a 25 µM solution of resolvase was added. This slurry was incubated at room temperature for 2 hours and at 37°C for a further 30 min., mixing occasionally. The supernatant, potentially enriched in oxidised species, was removed following centrifugation. Any remaining resolvase that was not covalently linked to the resin (for example, the oxidised monomer of a partially oxidised dimer) was removed with a denaturing solution (8 M urea, 20 mM Tris/HCl pH 8.8). Any remaining resolvase was removed from the TNB resin using the same denaturing solution containing 20 mM

DTT. The removal of the free TNB moiety from the resin produces a yellow colour, and so the reaction of free thiols with the TNB-resin can be monitored.

## 2.25 Fractionation of resolvase species by gel filtration

Gel filtration was used to fractionate only one of the resolvase mutants, namely T73C/S112C. The purpose of this step was to separate the disulphide-linked dimers from other species (which were mostly internally crosslinked). The procedure is therefore described in Chapter 6, section 6.3 and Fig. 6.10.

## 2.26 Determining resolvase concentration

The accurate determination of the absolute resolvase concentration has proved difficult because standard protocols, such as the Bradford assay, have given unreliable measurements. Furthermore, amino acid analysis and $A_{280}$ extinction measurements have generated concentration estimates differing by as much as 3-fold. Resolvase concentrations listed in this thesis are based upon the amino acid analysis performed by D. Blake (Ph. D. Thesis, 1993) on the WT Tn3 resolvase fraction R17 f.47, estimated at 0.4 mg/ml or 20 μM. The relative concentrations of other resolvase fractions were estimated by comparison with this fraction on SDS PAGE gels.

## 2.27 Oxidation and modification of cysteine-containing resolvases

Oxidation of cysteine-containing resolvases occurred on storage at -20°C. The rate of oxidation depended on the mutant. In general, complete oxidation required storage for a minimum of two months. Oxidation was speeded up by dialysing the purified resolvase overnight against resolvase dilution buffer plus 1 mM glutathione (oxidised).

Crosslinking of cysteine residues within resolvase was performed using homobifunctional crosslinking molecules, including PDM (ortho and para), BMPHP, HBVS and $BMPEO_3$. These compounds have the ability to crosslink two sulfhydryl groups. Oxidised resolvase fractions were reduced prior to crosslinking (thereby releasing the reactive free thiol groups) by incubation with 10 mM DTT for 30 min. at 37°C. DTT was removed by passing the

resolvase through a Sephadex G-25 gel filtration spin column (Pharmacia) according to the manufacturer's specifications. The columns were pre-equilibrated with a buffer (25% glycerol, 1.5 M NaCl, 30 mM Tris/HCl pH 7.5, 0.15 mM EDTA), which was found to increase resolvase yields. Similar yields were later obtained by pre-equilibrating the columns with resolvase dilution buffer. Crosslinkers were freshly dissolved in dimethylformamide (10 µM, 100 µM, and 1000 µM final concentrations). Reactions contained resolvase (0.1-1 mg/ml) in 25% glycerol, 1.5 M NaCl, 30 mM Tris/HCl pH 7.5, 0.15 mM EDTA, and 1 mM TCEP, which ensured that resolvase remained reduced without providing reactive sulfhydryl groups. Crosslinking reactions were initiated by the addition of 0.01 vol. of crosslinker/DMF solution, and were incubated for 30 min. at room temperature. The reaction was terminated by adding 10 mM ß-mercaptoethanol.

Resolvase modification at cysteine residues using N-ethylmaleimide (NEM) was achieved in a similar manner. However, NEM was freshly dissolved in ethanol and added to a final concentration of 1 mM only. Either ß-mercaptoethanol or DTT was used to quench the reaction .

## 2.28 *In vitro* recombination by resolvase

Site-specific recombination reactions involving Tn*3* or γδ resolvase and mutants thereof were carried out in several reaction buffers differing only in pH (50 mM Tris-HCl (pH 7.5, 8.2, or 9.4), 10 mM $MgCl_2$, 0.1mM EDTA). Cleavage reactions were performed in the absence of $MgCl_2$ in a buffer containing ethylene glycol (50 mM Tris-HCl (pH 8.2), 0.1 mM EDTA, 40% ethylene glycol). These buffers were sometimes supplemented with DTT (5 mM final) to create reducing conditions and/or spermidine (5 mM final). After addition of resolvase, generally 1/10 volume, (otherwise 1/20 volume) all reactions contained final concentrations of 100 mM NaCl (50 mM) and 5% glycerol (2.5% glycerol).

A typical reaction (20 µl) contained 0.4 µg of plasmid DNA with 0.1 vol. (2 µl) of diluted resolvase. Reactions were carried out at 37°C, typically for 1 hour, though this could range between 30 min-24 hours. The reaction was terminated by heating at 70°C for 5 minutes. In general, 20 µl reactions were carried out and split into two aliquots of 10 µl after incubation. Recombination products were analysed by agarose gel electrophoresis of untreated samples, and of samples digested with restriction enzymes. A *Pst*I/*Hind*III restriction digest was

generally performed by adding these in 0.5 volumes of NEB 2 buffer. Reactions proceeded for 30-40 min. at 37 C. Prior to loading, 5 µl of SDS loading buffer was added to all samples.

Alternatively, the topology of a reaction was investigated by DNase I nicking in the presence of ethidium bromide. The reactions were generally performed in C8.2 buffer with 0.1 volume of resolvase added, as above (yielding a final solution of 100 mM NaCl, 5% glycerol, 50 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 0.1 mM EDTA). This was incubated with 100 µg/ml ethidium bromide and 1 µg/ml DNase I at 37°C for 30 minutes. The samples were treated with SDS loading dye containing 250 µg/ml proteinase K (0.2 vol.). Samples were extracted with phenol/chloroform and centrifuged (16,000 g, 2 min, room temperature) prior to loading on a 0.7% agarose gel.

## 2.29 Accessory site synapsis by resolvase

Assays of accessory site synapsis by resolvase were performed in different reaction conditions (NEB1 buffer, at pH7). Resolvase was added first (1/20 volume) and incubated for 15 min. at room temperature. Cre, dissolved in resolvase dilution buffer was added (1/20 volume-thus the final reaction buffer is NEB1, 100 mM NaCl, 5% glycerol) and incubated for 25 min. at room temperature. The reactions were stopped by heating to 75°C for 5 minutes. This step is important to minimise the formation of Holliday junctions by the Cre protein. The samples were nicked as above, except that the DNase I concentration required for these nicking reactions was only 0.5 µg/ml, as DNase I is more active at the lower pH. A phenol/chloroform extraction was performed prior to loading on a 0.7 % agarose gel.

## 2.30 DNA binding assays (PAGE)

5-10 nM of non-specific carrier DNA (supercoiled plasmid) was combined with the appropriate amount of 3' end-labelled DNA (~200-300 cps) in binding buffer (10 mM Tris-glycine pH 9.4, 0.1 mM EDTA, 10% glycerol) or in a binding/cleavage buffer (25% ethylene glycol, 50 mM Tris/HCl pH 8.2, 1 mM EDTA). Resolvase dilutions were added (1/20 volume). The samples were incubated at room temperature for 15 minutes (or longer for the cleavage reactions). Samples were split if they required different treatments, such as the

addition of SDS (0.1%) and/or proteinase K (50 μg/ml). Samples were loaded on a non-denaturing or denaturing (containing 0.1% SDS) polyacrylamide gel. Electrophoresis was carried out at 200 V for 3-4 hours at 4°C or at room temperature.

## Table 1 Oligonucleotides

Oligonucleotides used in resolvase constructions are shown below.

### T73C Top and Bottom strands

*Age*I T73C *Pac*I *Dra*III

**CCGGTT**AGGCCGCGAC**TGC**GCCGACATGATCCAA**TTAATTAA**AGAGTTTGATG**CACAAGGTG**

**AA**TCCGGCGCTG**ACG**CGGCTGTACTAGGTT**AATTAATT**TCTCAAACTAC**GTGTTCCAC**

TAGCGGTTCGGTTT**AT** 78 nt

ATCGCCAAGCCAAA**TAGC** 76 nt

*Cla*I

### D95C Top and Bottom strands

*Cla*I **D95C** *BstE*II

**CGATTGC**GCGATCAGTACCGACGGTGATATGGGGCAAATGGT**G** 43 nt

**TAACG**CCCTAGTCATGGCTGCCACTATACCCCGTTTACCA**CCAGTG** 46 nt

### A113C Top and Bottom strands

*BstE*II A113C *BamH*I

**GTCACC**ATCCTGTCT**TGC**GTGGCACACGCTGAACGCCGG**AG** 41 nt

**G**TAGGACAGA**ACG**CACCATGTCCGACTTGCGGCC**TCCTAG** 40 nt

### A115 Top and Bottom strands

*BstE*II A115C *BamH*I

**GTCACC**ATCCTGTCTGCAGTG**TGC**CAGGCTGAACGCCGG**AG** 41 nt

**G**TAGGACAGACGTCAC**ACG**GTCCGACTTGCGGCC**TCCTAG** 40 nt

## (96-105 Hin) Top and Bottom strands

*Cla*I *Sac*I *Bst*EII

**CGAT**GAC**TCC**ATC**GAC**AC**GAG**C**TCC**G**C**TATGGGGC**GT**ATGGT**G** 43 nt

**TA**CTG**AGG**TAG**CTG**TG**CTC**G**AGG**C**G**ATACCCCG**CA**TACCA**CCAGTG** 46 nt

## D95C (96-105 Hin) Top and Bottom strands

D95C

*Cla*I *Pvu*I *Sac*I

**CGATTGCTC**GATC**GAC**AC**GAG**C**T** 23 nt

**TAACGAG**CTAG**CTG**TG**C** 17 nt

## M106C (96-105 Hin) Top and Bottom strands

*Sac*I *Nco*I M106C *Bst*EII

**CCG**C**C**ATGGGAC**GTTGC**GT**G** 20 nt

**TCGAGG**C**G**GTACCCTG**CAACG**CA**CCAGTG** 29 nt

## Table 2 Plasmids

Plasmids used and constructed throughout this work are listed below.

| Plasmid | Size (bp) | Anti-biotic Marker | Description/Derivation | Source or Reference |
|---|---|---|---|---|
| pDB34 | 8 371 | Kan | *res* x *res* resolution plasmid (*galK* indicator) | Blake, 1993 |
| pDB37 | 8292 | Kan | *res* x Site I resolution plasmid (*galK* indicator) | Blake, 1993 |
| pDB35 | 8352 | Kan | SiteI x Site I resolution plasmid (*galK* indicator) | Blake, 1993 |
| pMA21 | 4925 | Ap, Tc | pBR322 + 282 bp *Eco*RI fragment from Tn3 in *Eco*RI clockwise, and *Pvu*II 282 bp permutation of *Eco*RI fragment in *Pvu*II site | M. Boocock |
| pMA2631 | 4925 | Ap, Tc | *res* x *res* (inverted repeat) substrate | M. Boocock |
| pNG210 | 4595 | Ap | γδ *res* x *res* (direct repeat) substrate | N. Grindley |
| pAL221*sis* | 5078 | Ap | Tn3 Site I x Site I (inverted repeat) substrate | A. Bednarz |
| pOG5 | 2639 | Ap | Tn3 single *res* substrate | O. Gubbay |
| pSW1233 | 4729 | Ap | pAL221*sis* delta *Cla*I-*Bam*HI (229-581) creating a 111.5 bp site I-*sisN* spacing | Chapter 4 |
| pSW1241 | 4736 | Ap | pAL221*sis* delta *Hind*III - *Bam*HI (235-581) creating a 118.5 bp site I-*sisN* spacing | Chapter 4 |
| pNG345 | 4103 | Ap | *res* x *res* (direct repeat) targeting substrate, G2T mutations in each of the four crossover half-sites | N. Grindley |
| pNG343 | 4798 | Ap | Targeting *res* x *res* (direct repeat) substrate, G2T mutations in both half-sites of one Site I | N. Grindley |
| pGH466 | 5 116 | Ap, Tc | γδ *res* x *res* (direct repeat) substrate, AC x AT mismatch | G. Hatfull |
| pMS34 | 4 925 | Ap, Tc | Tn3 *res* x *res* (direct repeat) substrate, AT x TA mismatch | M. Stark |
| pMS7 | 2 931 | Ap | Single Tn3 *res* substrate | M. Stark |
| pSJH17 | | Ap, Kan | Tn3 *res* x *res* II/III | S. Rowland |
| pAL265 | 4846 | Ap, Tc | Tn3 *res* x site I | A. Bednarz |
| pCO1 | 2 543 | Ap | Single Tn3 site I substrate. *Eco*RI/*Sst*I site I fragment + *Eco*RI/*Sst*I pMTL23 | Corrine Muir |
| pCO2 | 2 543 | Ap | Single Tn3 site I, Tn552 core (4 bp) | Corrine Muir |
| pCO3 | 2 543 | Ap | Single Tn552 site I , Tn3 core (4 bp) | Corrine Muir |
| pMTL23 | 2 505 | Ap | Cloning vector | |
| pEK26 | 4 279 | Ap, Kan | *loxP* x II/III (Tn3) resolution substrate, used for accessory site synapsis assays | E. Kilbride |
| pEK28 | 4 279 | Ap, Kan | *loxP* x II/III (Tn3) inversion substrate, used for accessory site synapsis assays | E. Kilbride |
| pAT5 | 6 252 | Ap, Tc | Tn3R WT ORF: cassetted in pSELECT-1 | P. Arnold |
| pAT6 | 6 252 | Ap, Tc | Tn3R WT ORF: cassetted+ in pSELECT-1 | S. Rowland |
| pAT5Δ | 5 465 | Ap | Cloning vector containing Tn3 resolvase ORF. pAT5 with *Eco*RV-*Nru*I fragment deleted | M. Boocock, Finn Grey |
| pMA59121 | 6 252 | Ap, Tc | pAT5 containing D102Y/E124Q mutations | M. Boocock |
| pMA612 | 6 692 | Kan | pMA61containing D102Y/E124Q mutations | M. Boocock |
| pMA7552 | 5 459 | Ap | Cloning vector containing γδR E102Y ORF in pAT5Δ | M. Boocock |
| pSM22 | 5 459 | Ap | Cloning vector containing Tn3R/γδR (113-183) hybrid ORF; E124Q in pAT5Δ | Sharon Meikle |
| pSA9936.19 | 6 252 | Ap, Tc | pAT6 E56K/D102Y/E124Q | S. Rowland |
| pMA61 | 6 692 | Kan | pSA1101 with Tn3R WT ORF from pAT5; T7 promoter expression vector | M. Boocock |
| pSA1121 | 6 711 | Kan | Tn3R WT- His tagged expression plasmid (not cassetted); T7 promoter expression vector | S. Rowland |
| pSA1112 | 6 688 | Kan | pSA1101 γδR R172L binding domain | S. Rowland |
| pMA82.5 | | Ap | pAT5Δ containing R2A mutation | Jamie Meikleham M. Boocock |
| pMA6240 | 5 461 | Ap | pAT5Δ containing γδ resolvase ORF | M. Boocock |
| pMA7341 | 5 461 | Ap | pAT5Δ containing γδ E102Y/E124Q ORF | M. Boocock |
| D4852 | | Ap | pGEM with synthetic Hin/Res DBD (Baseclear) | Chapter 4 |
| W28 | 6 252 | Ap, Tc | pAT4 L69F S112C | P. Arnold |

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|---|---|---|---|---|
| pAT5 - RMMD | 6 252 | Ap, Tc | pAT5 – RMMD multiple mutations (see Chapter 4) | Juiya He |
| pAT5 - RMMD+ | 6 252 | Ap, Tc | pAT5 - RMMD+ multiple mutations, R2A/E56K (see Chapter 4) | Juiya He |
| pSW1732 | 5 465 | Ap | pAT5Δ containing T73C mutation. T73C top and bottom oligonucleotides were ligated into *ClaI/AgeI* vector fragment from pAT5Δ, introducing T73C mutation and novel *Pac*I and *Dra*III sites | Chapter 3 |
| pSW1952 | 5 465 | Ap | pAT5Δ containing D95C mutation. D95C top and bottom oligonucleotides were ligated into *ClaI/BstE*II vector fragment from pAT5Δ, introducing D95C mutation | Chapter 3 |
| pSW11134 | 5 465 | Ap | pAT5Δ containing A113C mutation. A113C top and bottom oligonucleotides were ligated into *BstE*II/*BamH*I vector fragment from pAT5Δ, introducing A113C mutation and removing a *Pst*I site | Chapter 3 |
| pSW11154 | 5 465 | Ap | pAT5Δ containing A115C mutation. A115C top and bottom oligonucleotides were ligated into *BstE*II/*BamH*I vector fragment from pAT5Δ, introducing A115C mutation | Chapter 3 |
| pSW211 | 5 465 | Ap | pAT5Δ containing T73C/A115C mutations. *PstI/PstI* insert from pSW1732, *PstI/PstI* vector from pSW11154 | Chapter 3 |
| pSW221 | 5 465 | Ap | pAT5Δ containing D95C/A113C mutations. *BsaI/BstE*II insert from pSW1952, *BstE*II/*Bsa*I vector from pSW11134 | Chapter 3 |
| pSW241 | 6 240 | Ap, Tc | pAT5 containing A115C/E124Q mutations. *Hinc*II/*BamH*I insert from pSW11154, *BamHI/Hinc*II vector from pMA59121 | Chapter 3 |
| pSW311 | 6 240 | Ap, Tc | pAT5 containing T73C/E124Q mutations. *PstI/PstI* insert from pSW1731, *PstI/PstI* vector from pMA59121 | Chapter 3 |
| pSW321 | 6 240 | Ap, Tc | pAT5 containing T73C/A115C/E124Q mutations. *PstI/PstI* insert from pSW1732, *PstI/PstI* vector from pSW241 | Chapter 3 |
| pSW411 | 6 692 | Kan | pMA61 containing T73C/E124Q mutations. *NdeI/Asp*718 insert from pSW311, *Asp*718/*NdeI* vector from pMA61 | Chapter 3 |
| pSW421 | 6 692 | Kan | pMA61 containing A115C/E124Q mutations. *NdeI/Asp*718 insert from pSW241, *Asp*718/*NdeI* vector from pMA61 | Chapter 3 |
| pSW431 | 6 692 | Kan | pMA61 containing T73C/A115C/E124Q mutations. *NdeI/Asp*718 insert from pSW321, *Asp*718/*NdeI* vector from pMA61 | Chapter 3 |
| pSW441 | 6 692 | Kan | pMA61 containing D95C mutation. *NdeI/Asp*718 insert from pSW1952, *Asp*718/*NdeI* vector from pMA61 | Chapter 3 |
| pSW451 | 6 692 | Kan | pMA61 containing A113C mutation. *NdeI/Asp*718 insert from pSW11134, *Asp*718/*NdeI* vector from pMA61 | Chapter 3 |
| pSW461 | 6 692 | Kan | pMA61 containing D95C/A113C mutations. *NdeI/Asp*718 insert from pSW221, *Asp*718/*NdeI* vector from pMA61 | Chapter 3 |
| pSW1311 | 5 465 | Ap | pAT5Δ containing S112C mutation. *ClaI/Asp*718 insert from W28, *Asp*718/*ClaI* vector from pAT5Δ | Chapter 6 |
| pSW1312 | 5 465 | Ap | pAT5Δ containing S112C mutation. *ClaI/Asp*718 insert from W28, *Asp*718/*ClaI* vector from pAT5Δ | Chapter 6 |
| pSW1511 | 5 465 | Ap | pAT5Δ containing T73C/S112C mutations. *BstE*II/*Asp*718 insert from pSW1312, *Asp*718/*BstE*II vector from pSW1732 | Chapter 6 |
| pSW1521 | 5 465 | Ap | pAT5Δ containing D95C/S112C mutations. *BstE*II/*Asp*718 insert from pSW1312, *Asp*718/*BstE*II vector from pSW1952 | Chapter 6 |
| pSW1411 | 6 692 | Kan | pMA61 containing S112C mutation. *NdeI/Eag*I insert from pSW1311, *Eag*I/*NdeI* vector from pMA61 | Chapter 6 |
| pSW1621 | 6 692 | Kan | pMA61 containing D95C/S112C mutations. *NdeI/Eag*I insert from pSW1521, *Eag*I/*NdeI* vector from pMA61 | Chapter 6 |
| pSW1611 | 6 692 | Kan | pMA61 containing T73C/S112C mutations. *NdeI/Eag*I insert from pSW1511, *Eag*I/*NdeI* vector from pMA61 | Chapter 6 |
| pSW1Hin1 | 5 465 | Ap | pAT5Δ containing (96-105 Hin) mutations. (96-105 Hin) top and bottom oligonucleotides were ligated into *ClaI/BstE*II vector fragment from pAT5Δ, introducing G96S/S98D/D100S/G101S/D102A/Q105R mutations and a novel *Sac*I site | Chapter 4 |
| pSW231 | 6 240 | Ap, Tc | pAT5 containing (96-105 Hin)/E124Q mutations. *BsaI/BstE*II insert from pSW1Hin1, *BstE*II/*Bsa*I vector from pMA59121 | Chapter 4 |
| pSW471 | 6 692 | Kan | pMA61 containing (96-105 Hin) mutations. *NdeI/Asp*718 insert from pSW1Hin1, *Asp*718/*NdeI* vector from pMA61 | Chapter 4 |

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|---|---|---|---|---|
| pSW712 | 5 465 | Ap | pAT5Δ containing (96-105 Hin) D95C mutations. (96-105 Hin)D95C top and bottom oligonucleotides were ligated into *ClaI*/*Sac*I vector fragment from pSW1Hin1, introducing D95C mutation and a novel *Pvu*I site | Chapter 5 |
| pSW742 | 5 465 | Ap | pAT5Δ containing (96-105 Hin) M106C mutations. (96-105 Hin)M106C top and bottom oligonucleotides were ligated into *SacI*/*BstE*II vector fragment from pSW1Hin1, introducing M106C mutation and a novel *Nco*I site | Chapter 5 |
| pSW611 | 5 459 | Ap | pAT5Δ containing (96-105 Hin) E124Q mutations, (113-183 γδ sequence). *PstI*/*Pst*I insert from pSW1Hin1, *PstI*/*Pst*I vector from pSM22 | Chapter 4 |
| pSW811 | 5 459 | Ap | pAT5Δ - γδ resolvase ORF containing (96-105 Hin) E124Q mutations. *ScaI*/*Cla*I insert from pMA7552, *ClaI*/*Sca*I vector from pSW611 | Chapter 4 |
| pSW911 | 5 459 | Ap | pAT5Δ - γδ resolvase ORF containing (96-105 Hin) mutations. *PstI*/*Pst*I insert from pSW811, *PstI*/*Pst*I vector from pMA7552 | Chapter 4 |
| pSW631 | 5 459 | Ap | pAT5Δ containing (96-105 Hin) A115C mutations. *PstI*/*Pst*I insert from pSW1Hin1, *PstI*/*Pst*I vector from pSW1154 | Chapter 5 |
| pSW831 | 5 459 | Ap | pAT5Δ containing (96-105 Hin) T73C mutations. *Hinc*II/*Cla*I insert from pSW1732, *ClaI*/*Hinc*I vector from pSW1Hin1 | Chapter 5 |
| pSW851 | 5 459 | Ap | pAT5Δ containing (96-105 Hin) T73C/A115C mutations. *Hinc*II/*Cla*I insert from pSW1732, *ClaI*/*Hinc*II vector from pSW631 | Chapter 5 |
| pSW931 | 5 459 | Ap | pAT5Δ containing (96-105 Hin) D95C/A113C mutations. *Hinc*II/*BstE*II insert from pSW711, *BstE*II/*Hinc*I vector from pSW11134 | Chapter 5 |
| pSW1011 | 6 686 | Kan | pMA61 - Tn3 resolvase (96-105 Hin)E124Q mutations ORF. *NdeI*/*Asp*718 insert from pSW231, *Asp*718/*Nde*I vector from pMA61 | Chapter 4 |
| pSW1021 | 6 686 | Kan | pMA61 - γδ resolvase (96-105 Hin) mutations ORF. *NdeI*/*Asp*718 insert from pSW911, *Asp*718/*Nde*I vector from pMA61 | Chapter 4 |
| pSW1031 | 6 686 | Kan | pMA61 - γδ resolvase (96-105 Hin) E124Q ORF. *NdeI*/*Asp*718 insert from pSW811, *Asp*718/*Nde*I vector from pMA61 | Chapter 4 |
| pSW1041 | 6 692 | Kan | pMA61 containing (96-105 Hin) D95C/A113C mutations. *NdeI*/*Asp*718 insert from pSW931, *Asp*718/*Nde*I vector from pMA61 | Chapter 5 |
| pSW1051 | 6 692 | Kan | pMA61 containing (96-105 Hin) T73C mutations. *NdeI*/*Asp*718 insert from pSW831, *Asp*718/*Nde*I vector from pMA61 | Chapter 5 |
| pSW1061 | 6 692 | Kan | pMA61 containing (96-105 Hin) A115C mutations. *NdeI*/*Asp*718 insert from pSW632, *Asp*718/*Nde*I vector from pMA61 | Chapter 5 |
| pSW1071 | 6 692 | Kan | pMA61 containing (96-105 Hin) T73C/A115C mutations. *NdeI*/*Asp*718 insert from pSW851, *Asp*718/*Nde*I vector from pMA61 | Chapter 5 |
| pSW1081 | 6 692 | Kan | pMA61 containing (96-105 Hin) M106C mutations. *NdeI*/*Asp*718 insert from pSW742, *Asp*718/*Nde*I vector from pMA61 | Chapter 5 |
| pSW1151 | 6 240 | Ap, Tc | pAT5 containing R2A/D102Y/E124Q mutations. *Hinc*II/*HinD*III insert from pMA82.5, *HinD*III/*Hinc*II vector from pMA59121 | Chapter 3 |
| pSW1182 | 6 240 | Ap, Tc | pAT5 containing R2A/E56K/D102Y/E124Q mutations. *Hinc*II/ *HinD*III insert from pMA82.5, *HinD*III/*Hinc*II vector from pSA9936.19 | Chapter 3 |
| pSW1841 | 5 465 | Ap | pAT5Δ containing R2A/E56K mutations. *Hinc*II/*Cla*I insert from pSW1182, *ClaI*/*Hinc*II vector from pAT5Δ | Chapter 3 |
| pSW1842 | 5 465 | Ap | pAT5Δ containing R2A/E56K mutations. *Hinc*II/*Cla*I insert from pSW1182, *ClaI*/*Hinc*II vector from pAT5Δ | Chapter 3 |
| pSW1432 | 6 692 | Kan | pMA61 containing R2A/E56K/D102Y/E124Q mutations. *NdeI*/*Eag*I insert from pSA1182, *EagI*/*Nde*I vector from pMA61 | Chapter 3 |
| pSW1811 | 6 692 | Kan | pMA61 containing R2A/E56K mutations. *PinAI*/*AlwN*I insert from pSW1432, and *AlwNI*/*PinA*I vector from pMA61 | Chapter 3 |
| pSW1912 | 5.5 kb | Kan | pSA1112 Tn3 resolvase D102Y/E124Q/R172L (143-183 γδ) ORF. AlwNI/EagI insert from pMA612, EagI/AlwNI vector from pSA1112 | Chapter 3 |
| pSW1921 | 5.5 kb | Kan | pSA1112 Tn3 resolvase R2A/D102Y/E124Q/R172L (143-183 γδ) ORF. AlwNI/EagI insert from pSW1432, EagI/AlwNI vector from pSA1112 | Chapter 3 |
| pSW2011 | 5.5 kb | Ap | pAT5Δ containing Hin/Res DBD. *EagI*/*Asp*718 insert from D4852, *Asp*718/*AlwN*I and *AlwNI*/*Eag*I vector from pAT5Δ | Chapter 4 |
| pSW2021 | 5.5 kb | Ap, Tc | pAT5 containing D102Y/E124Q mutations and Hin/Res DBD. *EagI*/*Asp*718 insert from D4852, *Asp*718/*AlwN*I and *AlwNI*/*Eag*I vector from pMA59121 | Chapter 4 |

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|---|---|---|---|---|
| pSW2031 | 5.5 kb | Ap | pAT5Δ γδ resolvase Hin/Res DBD ORF. *Eag*I/*Asp*718 insert from D4852, *Asp*718/*AlwN*I and *AlwNI*/*Eag*I vector from pMA6240 | Chapter 4 |
| pSW2041 | 5.5 kb | Ap | pAT5Δ γδ resolvase ORF - containing D102Y/E124Q and Hin/Res DBD. *Eag*I/*Asp*718 insert from D4852, *Asp*718/*AlwN*I and *AlwNI*/*Eag*I vector from pMA7341 | Chapter 4 |
| pSW2111 | 5.5 kb | Ap | pAT5Δ containing RMMD mutations and Hin/Res DBD. *Pst*I-*Pst*I insert from pAT5 RMMD, *Pst*I-*Pst*I vector from pSW2011 | Chapter 4 |
| pSW2121 | 5.5 kb | Ap | pAT5Δ Tn3 resolvase RMMD+ Hin/Res DBD ORF. *Pst*I-*Pst*I insert from pAT5 RMMD+, *Pst*I-*Pst*I vector from pSW2011 | Chapter 4 |
| pSW2211 | 5.5 kb | Kan | pMA61 Tn3 resolvase Hin/Res DBD ORF. *Nde*I/*Asp*718 insert from pSW2011, *Asp*718/*Nde*I vector from pMA61 | Chapter 4 |
| pSW2221 | 5.5 kb | Kan | pMA61 containing Tn3 resolvase D102Y/E124Q Hin/Res DBD ORF. *Nde*I/*Asp*718 insert from pSW2021, *Asp*718/*Nde*I vector from pMA61 | Chapter 4 |
| pSW2231 | 5.5 kb | Kan | pMA61 γδ resolvase Hin/Res DBD ORF. *Nde*I/*Asp*718 insert from pSW2031, *Asp*718/*Nde*I vector from pMA61 | Chapter 4 |
| pSW2241 | 5.5 kb | Kan | pMA61 γδ resolvase D102Y/E124Q Hin/Res DBD ORF. *Nde*I/*Asp*718 insert from pSW2041, *Asp*718/*Nde*I vector from pMA61 | Chapter 4 |
| pSW2251 | 5.5 kb | Kan | pMA61 Tn3 resolvase RMMD Hin/Res DBD ORF. *Nde*I/*Asp*718 insert from pSW2111, *Asp*718/*Nde*I vector from pMA61 | Chapter 4 |
| pSW2261 | 5.5 kb | Kan | pMA61 Tn3 resolvase RMMD+ Hin/Res DBD ORF. *Nde*I/*Asp*718 insert from pSW2121, *Asp*718/*Nde*I vector from pMA61 | Chapter 4 |

## Table 3 *In vivo* assays of resolvases

| Plasmid | Resolvase (Tn3 unless otherwise stated) | pDB34 *res* x *res* | pDB37 *res* x Site I | pDB35 Site I x SiteI |
|---|---|---|---|---|
| **pAT5Δ** | Wild-type | W | R | R |
| **pSW1732** | T73C | W | R | R |
| **pSW1952** | D95C | W | R | R |
| **pSW11134** | A113C | W | R | R |
| **pSW11154** | A115C | R | R | R |
| **pSW311** | T73C/E124Q | W | R | R |
| **pSW241** | A115C/E124Q | W | R | R |
| **pSW321** | T73C/A115C/E124Q | R | R | R |
| **pSW221** | D95C/A113C | W | R | R |
| **pSW1Hin1** | (96-105 Hin) | W | R | R |
| **pSW231** | (96-105 Hin) E124Q | 50% W | R | R |
| **pSW911** | γδR (96-105 Hin) | W | W | W |
| **pSW811** | γδR (96-105 Hin) E124Q | W | W | W |
| **pSW742** | (96-105 Hin) M106C | W | R | R |
| **pSW851** | (96-105 Hin) T73C/A115C | R | R | R |
| **pSW851** | (96-105 Hin) T73C/A115C (restreaked) | 40% W | R | R |
| **pSW931** | (96-105 Hin) D95C/A113C | R | R | R |
| **pSW931** | (96-105 Hin) D95C/A113C (restreaked) | 30% W | R | R |
| **pSW1841/2** | R2A/E56K | R | R | R |
| **pSW1182** | R2A/E56K/D102Y/E124Q | W | W | W |
| **pSW1151** | R2A/ D102Y/E124Q | W | W | W |
| **pMA59121** | D102Y/E124Q | W | W | P |

W = white colonies
R = red colonies
P = pink colonies

## Table 4 List of purified protein fractions and estimated concentrations (large-scale purification)

| **Resolvase mutant** (Tn3 unless otherwise stated) | **Concentration (μM)** | **Concentration (mg/ml)** |
|---|---|---|
| D95C | 30 | 0.60 |
| A113C | 40 | 0.80 |
| D95C/A113C | 15 | 0.30 |
| D95C oxidised | 7.5 | 0.15 |
| A113C oxidised | 10 | 0.20 |
| D95C/A113C oxidised | 5 | 0.10 |
| D95C+A113C oxidised | 9 | 0.18 |
| (96-105 Hin) M106C | 60 | 1.2 |
| (96-105 Hin) T73C/A115C | 30 | 0.60 |
| (96-105 Hin) D95C/A113C | 5 | 0.10 |
| (96-105 Hin) M106C oxidised | 30 | 0.60 |
| (96-105 Hin) T73C/A115C oxidised | 30 | 0.60 |
| T73C/S112C | 250 | 5.0 |
| T73C/S112C – TNB purified | 10 | 0.20 |
| T73C/S112C – monomer (GF) | 7.5 | 0.15 |
| T73C/S112C – dimer (GF) | 2.5 | 0.05 |
| Wild-type $-His_6$ | 125 | 2.5 |
| (96-105 Hin) | 5 | 0.10 |
| γδ (96-105 Hin) | 1 | 0.02 |
| γδ (96-105 Hin) E124Q | 0.5 | 0.01 |
| R2A/E56K | 100 | 2.0 |
| R2A/E56K/D102Y/E124Q | 15 | 0.3 |
| D102Y/E124Q/R172L - (γδ BD) | 10 | 0.2 |
| R2A/E56K/D102Y/E124Q/R172L - (γδ BD) | 10 | 0.2 |
| γδ (Hin BD) - 1 M urea resuspension | 7.5 | 0.15 |
| γδ D102Y/E124Q (Hin BD) - 1 M urea resuspension | 2.5 | 0.05 |

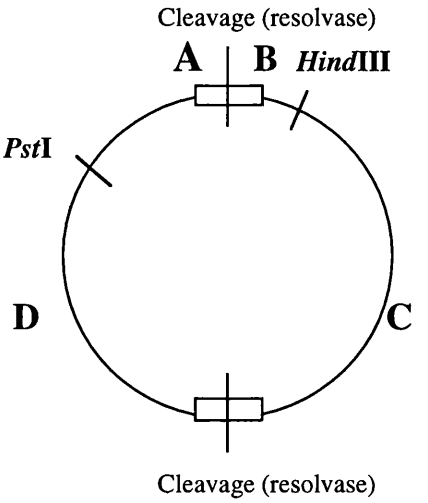
**Table 4 List of purified protein fractions and estimated concentrations (mini-scale purification)**

| Resolvase mutant | Concentration (μM) | Concentration (mg/ml) |
|---|---|---|
| Wild-type | 7.5 | 0.15 |
| Wild-type-$His_6$ | 50 | 1.0 |
| S112C | 10 | 0.2 |
| T73C/S112C | 15 | 0.3 |
| D95C/S112C | 15 | 0.3 |
| Wild-type –Hin/Res DBD | 25 | 0.5 |
| D102Y/E124Q – Hin/Res DBD | 10 | 0.2 |
| γδ Wild-type –Hin/Res DBD | 25 | 0.5 |
| γδ E102Y/E124Q – Hin/Res DBD | 25 | 0.5 |
| G101S/D102Y/M103I/V105L – Hin/Res DBD<br>6 M urea suspension | 7.5 | 0.15 |
| G101S/D102Y/M103I/V105L/E124Q – Hin/Res DBD<br>6 M urea pellet resuspension | 15 | 0.3 |

## Table 5 List of stock protein concentrations of previously purified mutants

| Resolvase mutant | Purified by | Resolvase mutant | Concentration (μM) | Concentration (mg/ml) |
|---|---|---|---|---|
| Wild-type | M. R. Boocock | Wild-type | 7.5 | 0.15 |
| M106C | A. I. MacDonald | M106C | 1 | 0.02 |
| M106C (oxidised) | A. I. MacDonald | M106C (oxidised) | 0.5 | 0.01 |
| D102C | E. Kilbride | D102C | 30 | 0.6 |
| D102Y | P. H. Arnold | D102Y | 10 | 0.2 |
| E124Q | P. H. Arnold | E124Q | 50 | 1.0 |
| D102Y/E124Q | M. R. Boocock | D102Y/E124Q | 3 | 0.06 |
| E56K/D102Y/E124Q | M. R. Boocock | E56K/D102Y/E124Q | 12.5 | 0.25 |
| γδ Wild-type | M. R. Boocock | γδ Wild-type | 9 | 0.18 |
| γδ R2A | R. E. Hughes | γδ R2A | 11 | 0.22 |
| γδ S10L | L. Murley | γδ S10L | 4 | 0.08 |
| γδ R68H/R172L | Xuewei Zhu | γδ R68H/R172L | 30 | 0.6 |
| γδ R68H | Grindley Lab | γδ R68H | 30 | 0.6 |
| γδ R172L | N. D. F. Grindley | γδ R172L | 22.5 | 0.45 |
| γδ M106C | R. E. Hughes | γδ M106C | 22.5 | 0.45 |
| γδ E102C/E124A | Xuewei Zhu | γδ E102C/E124A | 40 | 0.8 |
| γδ E102Y | M. R. Boocock | γδ E102Y | 6 | 0.12 |
| γδ E102Y/E124Q | M. R. Boocock | γδ E102Y/E124Q | 6 | 0.12 |
| γδ E56K/E102Y/E124Q | M. R. Boocock | γδ E56K/E102Y/E124Q | 6 | 0.12 |
| γδ E124Q | Xuewei Zhu | γδ E124Q | 12 | 0.24 |

**Table 6** ***Pst*I/*Hind*III restriction fragment lengths of reaction products of a variety of plasmids**




| | pMA21 | pNG210 | pMA2631 | pAL265 | pAL221 *sis* | pAL221 *sis* Δ *Cla*I-*Bam*HI | pAL221 *sis* Δ *Hind*III-*Bam*HI | pNG345 | pNG343 |
|---|---|---|---|---|---|---|---|---|---|
| Total size (ABCD) | 4925 | 4595 | 4925 | 4846 | 5078 | 4729 | 4736 | 4103 | 4798 |
| Parental AB | 1065 | 899 | 1065 | 986 | 986 | 1719 | 1726 | 1102 | 1102 |
| Parental CD | 3860 | 3696 | 3860 | 3960 | 4092 | 3010 | 3010 | 3001 | 3696 |
| Resolution AD | 2616 | 2220 | 2596 | 2459 | 2596 | 2596 | 2596 | 2423 | 2423 |
| Resolution BC | 2309 | 2375 | 2329 | 2387 | 2482 | 2133 | 2140 | 1680 | 2375 |
| Inversion AC | 3088 | 2628 | 3108 | 2971 | 3340 | 2258 | 2258 | 2136 | 2831 |
| Inversion BD | 1837 | 1967 | 1817 | 1875 | 1738 | 2471 | 2478 | 1967 | 1967 |
| Illegitimate AA | 1844 | 1152 | 1844 | 1570 | 1844 | 1844 | 1844 | 1558 | 1558 |
| Illegitimate BB | 286 | 646 | 286 | 402 | 128 | 1594 | 1608 | 646 | 646 |
| Illegitimate CC | 4332 | 4104 | 4372 | 4372 | 4836 | 2672 | 2672 | 2714 | 4104 |
| Illegitimate DD | 3388 | 3288 | 3348 | 3348 | 3348 | 3348 | 3328 | 3288 | 3288 |
| Cleavage A | 922 | 576 | 922 | 785 | 922 | 922 | 922 | 779 | 779 |
| Cleavage B | 143 | 323 | 143 | 201 | 64 | 797 | 804 | 323 | 323 |
| Cleavage C | 2166 | 2052 | 2186 | 2186 | 2418 | 1336 | 1336 | 1357 | 2052 |
| Cleavage D | 1694 | 1644 | 1674 | 1674 | 1674 | 1674 | 1674 | 1644 | 1644 |

# Chapter 3

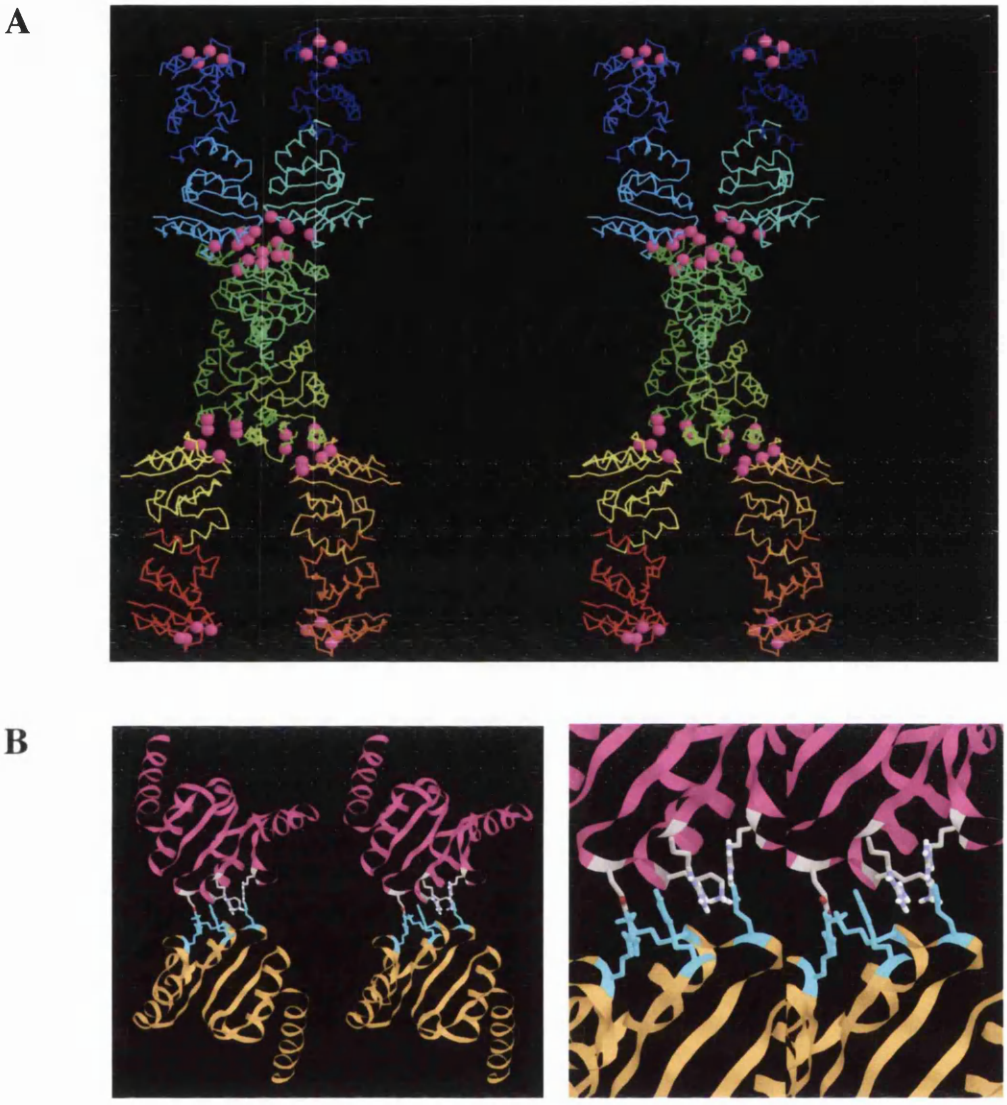
# The 2,3'-interface:
# A new role in the activation of site-I bound resolvase?

## 3.1 Introduction

### 3.1.1 Initial characterisation of residues constituting the 2,3'-interface

Residues thought to be required for formation of the synapse were first discovered in an extensive *in vivo* screen of recombination-deficient γδ resolvases (Hughes *et al.*, 1990). This screen identified a group of mutants, R32S, K54T and E56K, which formed a spatially distinct cluster far from the active site when mapped onto the crystal structure (Fig. 3.1). These mutants were proficient at binding to *res* (i.e. repressor$^+$) but all revealed two characteristic aberrant phenotypes; a specific defect in cooperative binding to *res* and an inability to form a higher-order, "intra-*res*" structure termed the resolvosome. Formation of the resolvosome structure can be detected in γδ resolvase gel-shift assays, and was shown to derive from interdimer resolvase interactions between binding sites I and binding sites II and/or III. These higher-order interactions result in the segment of DNA between sites I and II being bent or looped out. Regions of DNaseI hypersensitivity in footprints of wild-type γδ resolvase were indicative of resolvosome formation, and these were absent from the footprints of R32S, K54T and E56K. Both of these phenotypes distinguished this group of mutants from R8Q, which carries a mutation close to the active site serine 10. R8Q displayed a similar phenotype *in vivo* (repressor$^+$, resolution$^-$), but otherwise displayed a cooperative, resolvosome-proficient phenotype. Interestingly, the crystal structure revealed that residues R32, K54 and E56 all participate in higher-order interactions between two 2,3 dimers of resolvase within a tetrameric arrangement and between two 1,2 dimers. These interactions occur between dimers that are related by a noncrystallographic two-fold axis and this interdimer interface was termed the 2,3'-interface. At the centre of this cluster lies residue R2, which is also involved in interdimer interactions. By site-directed mutagenesis, the resolvase R2A was made and found to display an identical phenotype to R32S, K54T and E56K, strengthening the conclusion that this interdimer interface is not a crystal packing artefact, but represents a true biologically relevant interaction. Whilst the role of the resolvosome as a potential intermediate of synaptosome formation remains unknown, the evidence supported the hypothesis that the 2,3'-deficient phenotype was a result of disruptions in the higher-order interactions involved in synapsis.

## Fig. 3.1 Hexagonal crystal packing showing 2,3'-contacts



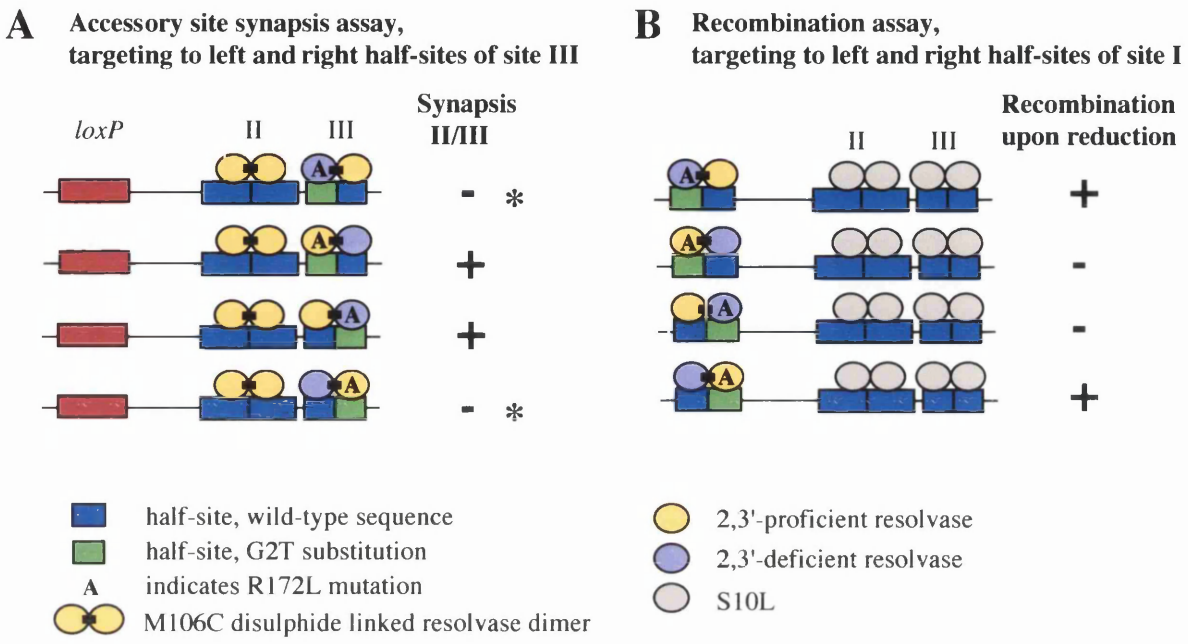

**A**. The packing of γδ resolvase catalytic domains around a 6-fold screw axis in hexagonal crystals (1GDR: Rice and Steitz, 1994). Residues of the 2,3'-interface are highlighted in pink.
**B**. A closer view of the 2,3'-interface. Residues R2, R32, E54 and E56 are highlighted.

### 3.1.2 Targeting 2,3'-defective resolvase mutants to subsites of *res*

Further characterisation of the γδ resolvase mutants, R2A and E56K, involved targeting the defective subunits to either the crossover site or sites II/III (Grindley, 1993). The 2,3'-interface was found to be necessary for accessory site function only; resolvase mutants carrying single mutations of this interface, such as R2A or E56K, were tolerated at the crossover site and could be complemented by the catalytically inactive S10L at sites II and III. As catalysis was necessarily achieved by 2,3'-deficient resolvase, the crystallographic 2,3/2',3' tetramer (whose structure depends upon an intact 2,3'-interface) could be ruled out as the catalytic tetramer. This complementation experiment strengthened the previous conclusion that the 2,3'-interface was involved in the interdimer interactions required to build the accessory site synapse (possibly providing an essential "nucleation" structure from which crossover site synapsis can follow). Whilst initiation of catalysis by resolvase seemed to require a signal from the correctly synapsed accessory sites, it was not clear whether activation by the II/III synapse was purely architectural or whether activation involved direct protein-protein contacts. However, if activation occurs by direct protein-protein contacts it was thought unlikely to involve the 2,3'-interface (the site-I bound resolvase (R2A) was 2,3'-deficient but active in the presence of S10L).

These initial targeting experiments showed that the different roles of the dimers at sites I, II, and III (with respect to catalytic function), were reflected in the different requirements for the 2,3'-interface at site I (no requirement) and sites II/III (absolute requirement). A further study dissected the *res* site into all six half-sites and probed the 2,3'-proficiency requirements of each protomer bound to each half-site (Murley and Grindley, 1998). This was done using an assay for synapsis of sites II/III, and also using a recombination assay. Due to the complexity of the experimental protocol these are illustrated diagrammatically in Fig. 3.2. Accessory site synapsis was found to require 2,3'-proficiency by only half of the total eight bound protomers, at sites II-L and III-L. Resolution was found to require two additional 2,3'-proficient subunits, at site III-R and at site I-R. These results reinforced the previously determined importance of the 2,3'-interface at sites II/III. However, in contrast to the S10L complementation experiment described above, the requirement for 2,3'-proficiency at site I-R reinstated the 2,3'-interface as a potential site I activation signal. Based on the 2,3'-requirements at these specific subsites of *res*, Murley and Grindley presented a model of the synaptic complex. This model will be considered together with other models in section 3.6.

## Fig. 3.2 Targeting of heterodimers of resolvase to subsites of *res* (Murley and Grindley, 1998)




**A**. The requirement of a 2,3'-proficient protomer at all subsites of *res* were examined using the altered-specificity mutant R172L and the weakly binding S173G mutant. To aid targeting, the resolvase subunits were disulphide-linked at position 106 on the E helix. This is displayed as a bold line connecting the monomers of a dimer. The principle of the accessory site synapsis assay is shown above. The substrates contain two identical hybrid *loxP*/*res* recombination sites (see diagram). Cre recombines the substrate at the *loxP* sites yielding simple products (unlinked circles). Upon addition of a synapsis-(II/III)-competent resolvase the product topology is more complex. Importantly, a specific product is observed, namely a 4-noded catenane, as expected if the synapse traps three negative supercoils. Targeting of resolvase was achieved by construction of substrates containing G2T mutations in subsites of *res* (green), which are preferentially bound by binding domains containing an R172L mutation. For simplicity, binding domains are not shown; R172L is denoted by an A (for altered binding specificity). All experiments were performed by targeting "complementary" heterodimers with the relevant substrate in order to reconfirm a result. For example, the requirement of a 2,3'-proficient protomer at site III-L, confirmed for both substrates is indicated with an asterisk.

Recombination assays were similar to the accessory site synapsis assays, except that reduced S173G resolvase was added at a limiting concentration (sufficient to give barely detectable recombination at site I) in the place of Cre.

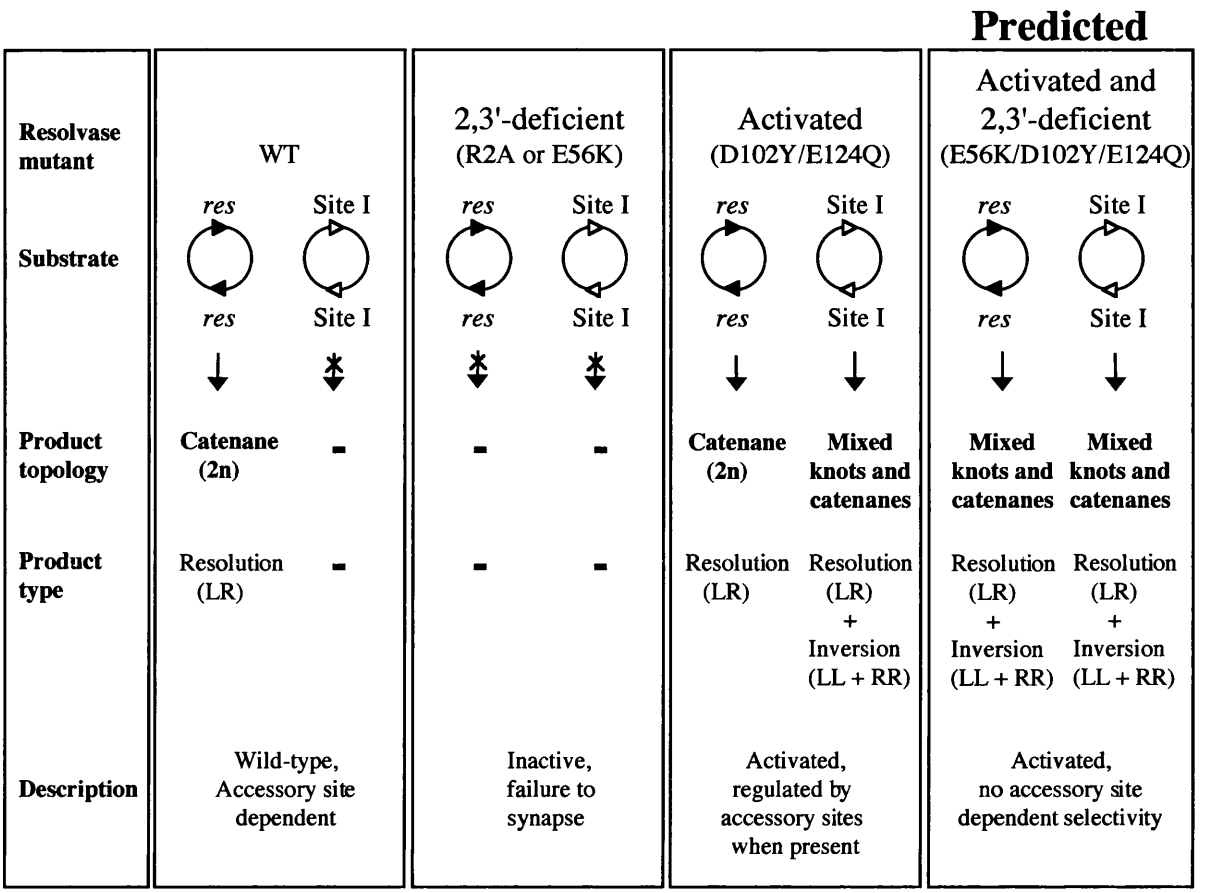
**B**. Strategy used to examine 2,3'-requirements at the crossover site (illustrated above). The catalytic S10L mutant (grey) was used to synapse sites II/III, whereas the various heterodimers were targeted in the desired orientation to the crossover site in their disulphide linked state (purple and yellow). As this disulphide link at M106C inhibits recombination activity, it was necessary to reduce the samples "in situ". The diagram illustrates the finding that a 2,3'-proficient protomer is required at site I-R (Murley and Grindley, 1998).

### 3.1.3 Investigating the effects of 2,3'-mutations on activated resolvase mutants

The generation and characterisation of numerous accessory site independent mutants, such as Tn3 resolvase D102Y/E124Q, enabled new experiments to further test the function(s) of the 2,3'-interface to be performed. Specifically, the use of activating mutations could allow the investigation of the differential effects of 2,3' mutations on different aspects of regulation by sites II and III: activation, alignment and topology. In addition, different combinations of 2,3'-deficient and/or activated mutants could be targeted to site I or sites II/III, to dissect their effects on strand exchange and the stability of the synapse. Importantly, activated 2,3'-deficient resolvase could directly test the hypothesis that the 2,3'-interface is required for accessory site synapsis, as it predicts "random collision" products with any substrate containing two crossover sites regardless of the presence, absence or orientation of accessory sites. Random collision products are predicted with an activated 2,3'-deficient resolvase because it would be unable to synapse the accessory sites (including directly repeated sites). However, recombination would still take place at site I due to the activating D102Y and E124Q mutations following site I synapsis by random collision. An activated 2,3'-defective resolvase is therefore predicted to bypass the topological filter mechanism. The resulting site I synapsis events would trap random numbers of supercoils, yielding a mixture of knotted and catenated products. Furthermore, crossover site alignment would be random, generating equal amounts of LR and LL or RR recombination products. This principle is illustrated for an activated 2,3'-deficient resolvase on a *res* x *res* and a site I x site I substrate in Fig. 3.3.

The triple mutants, E56K/D102Y/E124Q of Tn3 resolvase and E56K/E102Y/E124Q of γδ resolvase were characterised by Martin Boocock (unpublished results). The activated mutant, Tn3 resolvase D102Y/E124Q, was chosen as a background to introduce the 2,3' mutation E56K because it retains the ability to wrap the accessory sites of a *res* x *res* substrate, yielding the topologically specific (-) two-noded catenane. This selectivity fails with a site I x site I substrate, for which the products migrate as a ladder knots and catenanes of increasing complexity (on an agarose gel), consistent with synapsis by random collision. This is reflected in the alignment of the products observed upon restriction enzyme analysis. Exclusively resolution (LR) products are observed with a *res* x *res* substrate but equal amounts of inversion (LL and RR) and resolution (LR) products are observed with a site I x site I substrate. This is expected if the alignment of the crossover sites during recombination is determined solely by the accessory sites. The ability of D102Y/E124Q to be regulated by

## Fig. 3.3 Predicted phenotype of activated 2,3'-deficient resolvase




The selectivity of wild-type resolvase depends upon its ability to wrap the accessory sites into a specific nucleoprotein structure, trapping three negative supercoils, and aligning the crossover sites in a parallel configuration. The product prediction assumes that the R2A and E56K mutations at the 2,3'-interface disrupt the inter-dimer interactions required to build the specific synaptic complex, and thus block activation of resolvase bound to the crossover site. This diagram shows the expected phenotype of an activated 2,3'-deficient resolvase. Previously no products were observed with 2,3' mutants such as γδR R2A and γδR E56K as they were recombination deficient. However, the additional activating mutations of E56K/D102Y/E124Q should reveal the consequences of mutations at the 2,3'-interface on the topology and alignment of recombination reactions. The activated mutant D102Y/E124Q, shows accessory site-independent recombination activity, whilst retaining the ability to be regulated by accessory sites when present. The accessory sites increase the reaction rate and affect the topology of the reaction products. When the accessory sites are present, D102Y/E124Q resolvase generates nearly exclusively resolution products (2-noded catenanes). When the 2,3'-deficient mutations are combined with these activating mutations, it was predicted that the presence or absence of accessory sites would have no effect upon the alignment or topology of the reaction products, or indeed the reaction rate. The recombination reaction was expected to occur following synapsis by random collision, yielding numerous species of knots and catenanes, and a 1:1 ratio of resolution to inversion products, as seen with the activated D102Y/E124Q mutant in the absence of accessory sites.

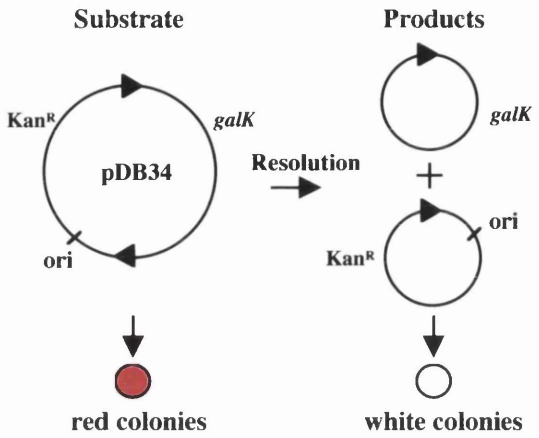
the accessory sites when they are present was essential for the experiments, as this selectivity was predicted to be eliminated by the additional E56K mutation (due to the failure of the topological filter mechanism). Furthermore, the rate of recombination by D102Y/E124Q responds to the presence of appropriately oriented accessory sites, increasing considerably when these are present (as opposed to site x site I reactions). Other "hyperactive" mutants of Tn3 and γδ resolvase, which have improved reaction rates have been isolated and characterised. However, these were less suitable backgrounds (and in some cases completely unsuitable) because they showed less or no regulation by the accessory sites (as judged from the reaction rate as well as the crossover alignment). Furthermore, recombination by these "hyperactive" resolvases was dissociative so that topological analysis would be uninformative (Jiuya He, personal communication, Chapter 4).

Tn3 resolvase and γδ resolvase are not identical in the 2,3'-interface region, although the key residues R2, E56, R32 and K54 are conserved. It was therefore important to establish a phenotype for the E56K mutation in Tn3 resolvase. The activity of E56K resolvase was tested *in vivo* using a complementation assay developed by Blake (1993) (Fig. 3.4). Three test substrates containing a *galK* gene located between two recombination sites (*res* x *res* in pDB34, *res* x site I in pDB37, and site I x site I in pDB35) were available. The *galK* gene encodes galactokinase which phosphorylates galactose. Recombination by resolvase results in two resolution products. The resolution product carrying *galK* does not contain the origin of replication, and is therefore lost. The host strain DS941 does not encode galactokinase (*galK*$^-$) so that cells which have lost *galK* are no longer able to utilize galactose as a source of energy. When plated onto MacConkey agar supplemented with 1% galactose they use amino acids as a nutritional source, releasing ammonia and thereby raising the pH of the media. This turns the Neutral Red indicator in the MacConkey agar white. Thus, in the absence of resolvase activity the colonies are red, whereas resolution of the test substrate yields white colonies. Wild-type resolvase, as encoded by pAT5Δ, produces white colonies with pDB34 (*res* x *res*) and red colonies with pDB37 (*res* x site I) and pDB35 (site I x site I).

Tn3 R2A and E56K resolvases (like γδ R2A and E56K resolvases) were shown to be completely inactive *in vivo*, (RRR, M. Boocock, Fig. 3.4). R2 and E56, therefore, play vital roles in the resolution reaction catalysed by Tn3 resolvase. In contrast, when the 2,3'-mutation E56K was combined with activating mutations, the resulting E56K/D102Y/E124Q derivatives of both γδ and Tn3 resolvase were found to be active on *res* x *res* and site I x site

## Fig. 3.4 Activity of Tn3 and γδ resolvase mutants *in vivo*

A




B

| | Resolvase mutant | | | | *res* x *res* | *res* x Site I | Site I x Site I | Assayed by |
|---|---|---|---|---|---|---|---|---|
| γδ resolvase | + | + | + | + | ○ | ● | ● | M. Boocock |
| | + | + | E102Y | + | ○ | ○ | ● | M. Boocock, S. Meikle |
| | + | + | + | E124Q | ○ | ○ | ● | M. Boocock |
| | + | + | E102Y | E124Q | ○ | ○ | ○ | " |
| | + | E56K | + | + | ● | ● | ● | M. Boocock |
| | + | E56K | E102Y | + | ● | ● | ● | " |
| | + | E56K | + | E124Q | ● | ● | ● | " |
| | + | E56K | E102Y | E124Q | ○ | ○ | ○ | " |
| Tn3 resolvase | + | + | + | + | ○ | ● | ● | P. Arnold |
| | + | + | D102Y | + | ○ | ○ | ● | " |
| | + | + | + | E124Q | ○ | ● | ● | " |
| | + | + | D102Y | E124Q | ○ | ○ | ○ | M. Boocock |
| | + | E56K | + | + | ● | ● | ● | M. Boocock, S. Rowland |
| | + | E56K | D102Y | + | ● | ● | ● | " " |
| | + | E56K | + | E124Q | ● | ● | ● | " " |
| | + | E56K | D102Y | E124Q | ○ | ○ | ○ | " " |
| | R2A | + | + | + | ● | ● | ● | M. Boocock, J. Meikleham |
| | **R2A** | **+** | **D102Y** | **E124Q** | ○ | ○ | ○ | S. Wenwieser: this study |
| | **R2A** | **E56K** | **D102Y** | **E124Q** | ○ | ○ | ○ | " |
| | **R2A** | **E56K** | **+** | **+** | ● | ● | ● | " |

**A**. An *in vivo* resolution assay was developed to screen for activated resolvase mutants (D. Blake, Ph.D. Thesis 1993). pDB34 carries *galK* between two full *res* sites. *E. coli* DS941 (*galK*⁻) carrying unresolved pDB34 is able to ferment galactose, which lowers the pH and yields red colonies on McConkey pH indicator plates. A complementing plasmid, expressing resolvase can be transformed into this strain. *In vivo* recombination by resolvase results in two resolution products. The deletion product carrying the *galK* gene is rapidly lost because the origin of replication (*ori*) is carried on the other resolution product (carrying the *Kan*$^R$ gene). The absence of galactose fermentation leads to an increase in the pH, giving white colonies on McConkey pH indicator plates. pD37 and pDB35 are similar to pBD34 except that they lack one or both accessory sites of *res*, respectively. These plasmids allow accessory site dependence to be assayed *in vivo*.

**B.** *In vivo* results for activated and/or 2,3'-deficient resolvases.

I substrates (M. Boocock, personal communication). As predicted, the activating mutations enabled the E56K/D102Y/E124Q resolvases to bypass the requirement for the 2,3'-interface and for sites II and III. In order to investigate the alignment and topology of these reactions, these resolvases were characterised *in vitro*. Surprisingly, in the presence of the accessory sites (*res* x *res* reactions) the alignment of crossover sites was not random: resolution events predominated over inversion, and the major recombinant product was 2-noded catenane. This implies that recombination proceeds through an interwrapped synapse similar to that formed by wild-type resolvase, trapping three negative supercoils. In contrast, equal amounts of resolution and inversion products were obtained with a site I x site I substrate, and these products were topologically complex, consistent with random collisions of the crossover sites. This confirms that sites II and III are required for the selective alignment of the crossover sites, and that the E56K mutation does not prevent this.

### 3.1.4 Experimental aims

The aim of this chapter was to determine the effect of the 2,3'-interface on the rate, alignment and topology of recombination. The experiments performed with E56K/D102Y/E124Q indicated that the 2,3'-interface is not necessary for selectivity by activated resolvase. However, this conclusion can only be made if the E56K mutation disrupts all 2,3'-contacts between dimers. Although a single mutation at the 2,3'-interface is sufficient to completely block resolution activity, it may not by itself destroy the interface completely. Thus, the retention of topological selectivity (on a *res* x *res* substrate) by the triple mutant E56K/D102Y/E124Q may reflect the formation of a synapse by weakly 2,3'-interacting resolvase dimers. In order to rule out the possibility that resolvase carrying the E56K substitution alone retains the ability to make weak 2,3'-contacts, a second mutation R2A was added. R2 interacts with two residues of the partner monomer (in *trans*), it stacks against R2 and interacts with the negative charge of E56. It also stacks against R32 of the same monomer (in *cis*). The negative charge of E56 makes interactions with R32 and R2 (in *trans*). Mutations of both residues, R2 and E56, were therefore expected to considerably (if not fully) disrupt the 2,3'-interface. Importantly, by combining these mutations the double mutant R2A/E56K could be compared with the single mutant E56K. A functional difference between these mutants would indicate that the E56K mutation alone does not destabilise the 2,3'-interface completely. The role of the 2,3'-interface was further investigated by comparing the effects of E56K and R2A/E56K on the activation, alignment and reaction

topology of activated resolvase. Once again, an additive effect of the R2A and E56K mutations would indicate that the single mutation E56K does not fully disrupt the 2,3'-interface. Alternatively, if no additive effect was observed, one could conclude that a single mutation is sufficient to disrupt the 2,3'-interface and that this interface is not required for the correct alignment and topological selectivity of reactions by activated resolvase.

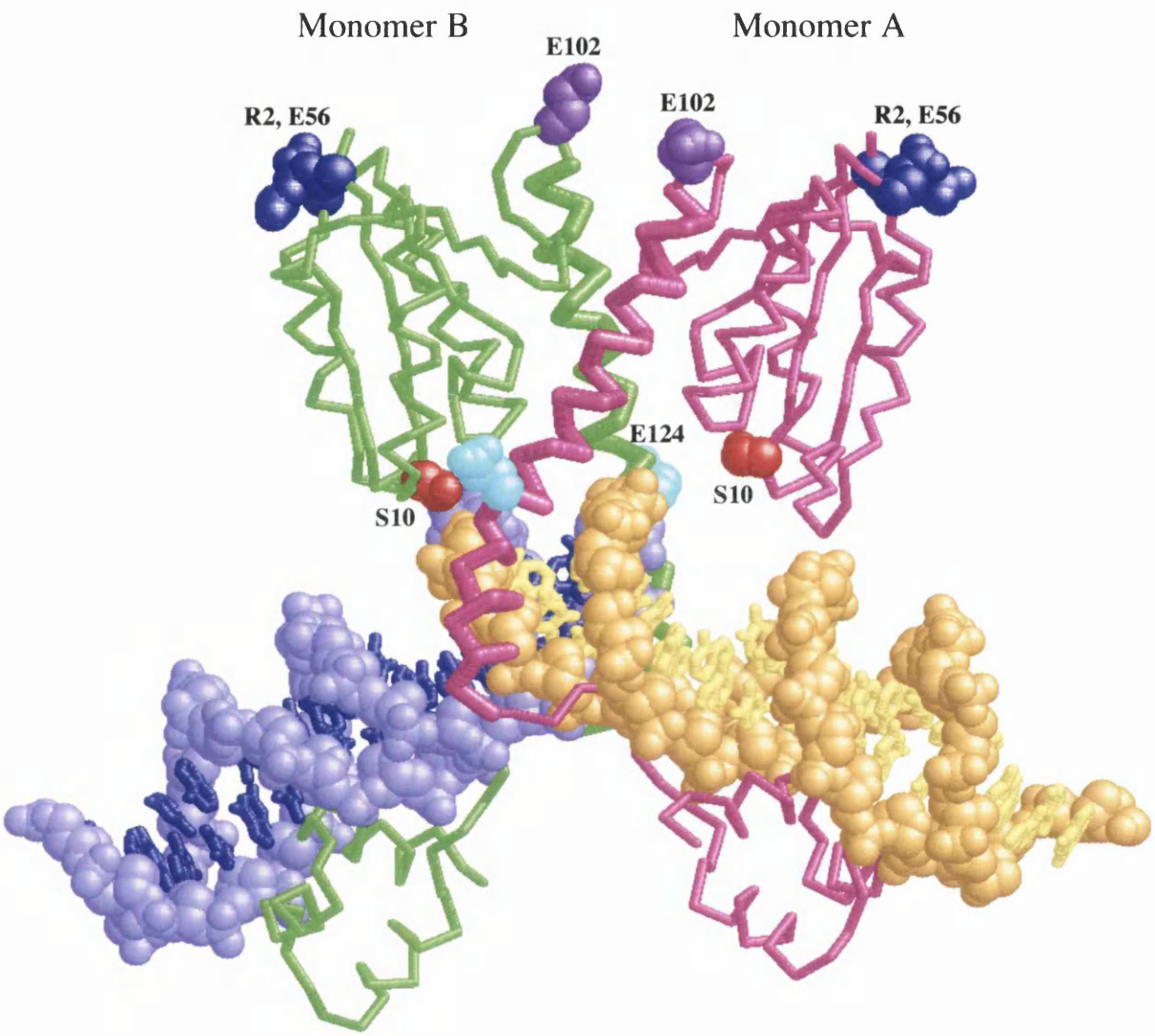
## 3.2 Characterisation of R2A/E56K and R2A/E56K/D102Y/E124Q

The R2A mutation was introduced into the Tn3 resolvase reading frame (pAT5Δ) by J. Meikleham and M. Boocock (Table 2, Chapter 2). Tn3 resolvase was chosen because its reading frame existed with a large number of strategically placed restriction sites (pAT5 and its derivative, P. Arnold, 1997). This enabled the introduction of the R2A mutation using a relatively small double stranded oligonucleotide. The double mutant R2A/E56K, the triple mutant R2A/D102Y/E124Q and the quadruple mutant R2A/E56K/D102Y/E124Q were generated by simple fragment exchanges into pAT5Δ or pMA59121 (pAT5 containing D102Y/E124Q, Table 2, Chapter 2). The positions of these residues are highlighted on the co-crystal structure in Fig. 3.5.

*In vivo,* R2A/E56K resolvase was recombination deficient (RRR, Fig. 3.4). In contrast, R2A/D102Y/E124Q and R2A/E56K/D102Y/E124Q were active on all three test plasmids *in vivo* (WWW). Tn3 resolvase D102Y/E124Q gives pink colonies with the site I x site I test substrate (pDB35) due to incomplete resolution of the substrate. However, the colonies observed with the pDB35 test substrate for both activated 2,3'-defective mutants were white. The triple and quadruple mutants were therefore more active than either parent resolvase. Mutation(s) of the 2,3'-interface thus had a further small activating effect upon D102Y/E124Q resolvase. This activation by 2,3'-interface mutations had been noted previously with the E56K/D102Y/E124Q mutant *in vivo* (M. Boocock, personal communication) and with activating mutations around residue D102 *in vitro* (Jiuya He, personal communication).

R2A/E56K and R2A/E56K/D192Y/E124Q Tn3 resolvases were purified to near homogeneity using the standard resolvase purification protocol described in section 2.23. Their properties during purification did not differ considerably from WT resolvase. *In vitro*

**Fig. 3.5 Co-crystal structure of γδ resolvase displaying residues R2 and E56 of the 2,3' interface**

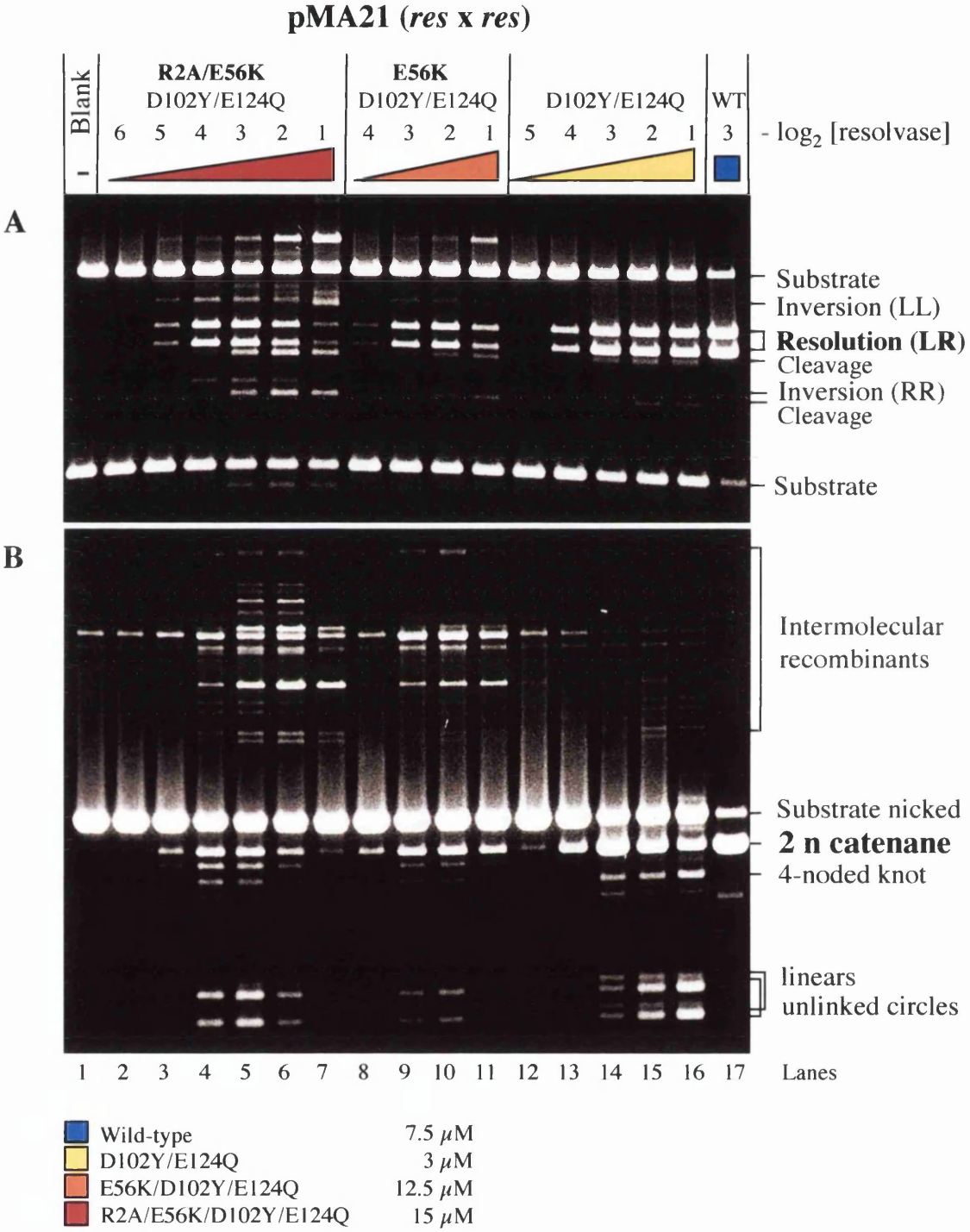



The co-crystal structure of γδ resolvase is depicted as previously. Activating residues E102 (purple) and E124 (cyan), residues R2 and E56 of the 2,3'-interface (blue) and the catalytic serine 10 residues (red) are shown in space-fill.

assays of R2A/E56K resolvase over a wide range of concentrations confirmed its inactivity under standard recombination conditions (C8.2) on a *res* x *res* substrate (pMA21) (data not shown). R2A/E56K/D102Y/E124Q, was found to be an active resolvase, generating resolution products (LR) as the major species (Fig. 3.6). A comparison of the titrations of D102Y/E124Q, E56K/D102Y/E124Q and R2A/E56K/D102Y/E124Q reveals a progressive loss of alignment specificity with increasing numbers of 2,3'-interface mutations. Nevertheless, the amount of "inversion" product produced by R2A/E56K/D102Y/E124Q is significantly less than the "resolution" products, indicating a strong bias towards the "correct" LR alignment. Furthermore, analysis by DNaseI nicking showed that a major product is 2-noded catenane, indicative of the formation of a productive synapse topology as in the wild-type reaction (Fig. 3.6). The rate of the resolution reaction of the quadruple mutant is similar to E56K/D102Y/E124Q, and significantly slower than that of the 2,3'-proficient proteins. This supports the emerging notion that the 2,3'-interface may play a role in the activation of site-I bound resolvase; the mutation of this interface causes the *res* x *res* reaction rate to fall closer to that of a site I x site I reaction (which is considerably slower with D102Y/E124Q). A further notable phenotype associated with the activated 2,3' deficient resolvases is the large amount of linear intermolecular recombinants generated. Nevertheless, restriction enzyme analysis reveals that these intermolecular recombinants mainly contain left-right junctions.

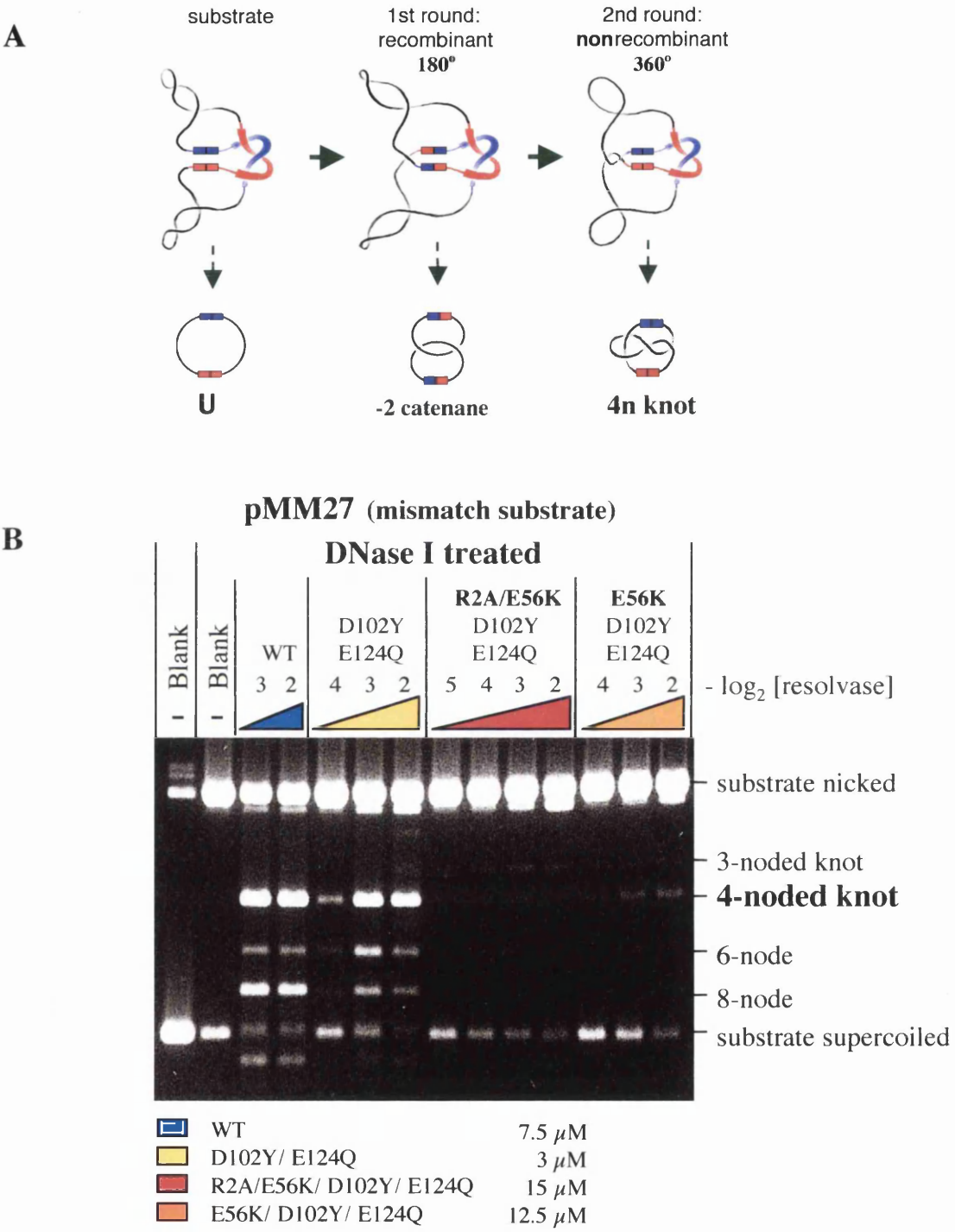
In order to further characterise the topology of the II/III synaptic structure formed by R2A/E56K/D102Y/E124Q, reactions on a "knotting" *res* x *res* substrate (pMM27, AC x AT) were performed (Fig. 3.7). If the accessory sites trap precisely three interdomainal supercoils the predicted product of two processive rounds of strand exchange is a 4-noded knot. This prediction assumes that the topology of the strand exchange mechanism remains fixed. In reactions with pMM27, 4-noded knot as well as products of further 360° DNA double-stranded rotations are generated by WT and activated D102Y/E124Q resolvases (Fig. 3.7). Surprisingly, the activity of E56K/D102Y/E124Q resolvase was considerably reduced; only a trace of the 4-noded knot was visible. In the case of R2A/E56K/D102Y/E124Q the 4-noded product is barely detectable on an agarose gel. Unexpectedly, this assay revealed that activated 2,3'-defective resolvases are defective in recombining a mismatch substrate. Since R2A/E56K/D102Y/E124Q is able to initiate cleavage and strand exchange on a standard *res* x *res* substrate (e.g. pMA21), it is likely that these steps can be performed on the mismatch substrate. However, neither the first round, mismatched recombinant (2n catenane)

**Fig. 3.6 Additive effects of 2,3' mutations on alignment of crossover sites and recombination topology**




Resolution reactions were set up with pMA21 (*res* x *res*) in recombination buffer (C8.2 ) for 2 hours at 37°C. Dilutions of resolvase fractions (stock concentrations listed above) were added (1/10 volume). Reactions were stopped by heating to 75°C for 5 minutes. Aliquots were restricted with *Pst*I and *Hind*III (A) and treated with DNaseI (B).

**Fig. 3.7 Activated, 2,3'-deficient resolvases are defective in knotting reactions of a mismatch substrate**



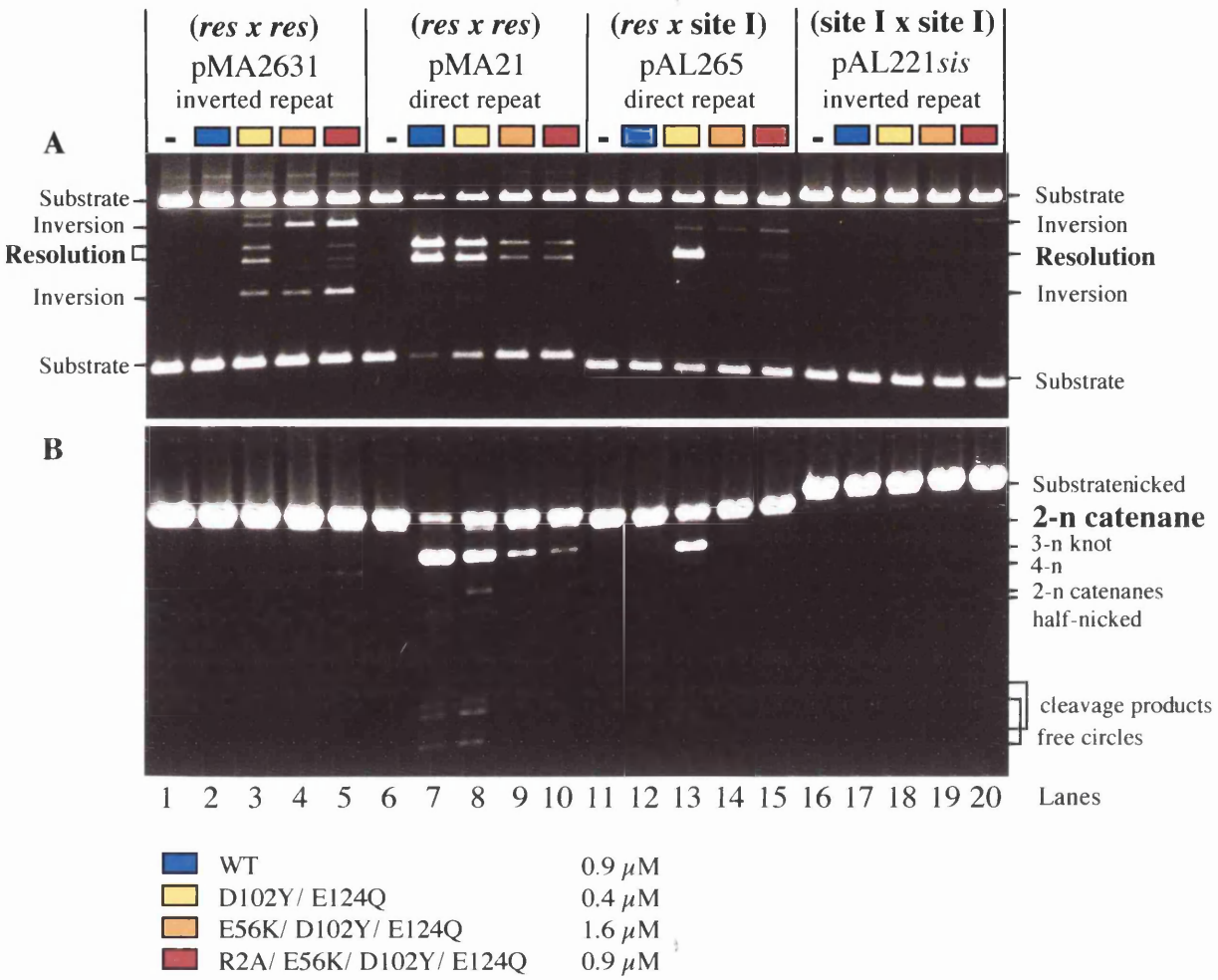

**A.** If the sequences within the dinucleotide overlap of the *res* sites in a substrate differ, the first round recombinant will contain a mismatch. Resolvase catalyses a further round of strand exchange to generate the non-recombinant 4-noded knot species (Stark *et al.* 1991).

**B.** Reactions were performed on the AC x AT mismatch *res* x *res* substrate pMM27 in C8.2 buffer for 2 hours at 37°C. Resolvase dilutions were made from the stock enzyme fractions (stock concentrations listed above). The dilutions of resolvase were added at 1/10 volume. Reactions were stopped by heating to 75°C for 5 min. and analysed by DNaseI treatment.

nor the second round non-recombinant product (4n knot) were detected. One possibility is that R2A/E56K/D102Y/E124Q initiates cleavage and strand exchange, then reverses the reaction and re-ligates in the parental configuration. This may be due to a reduced stability of the first round intermediate in the absence of an intact 2,3'-interface. Alternatively, the activated 2,3'-defective resolvase may be unable to initiate cleavage at the mutant (AC) crossover site.

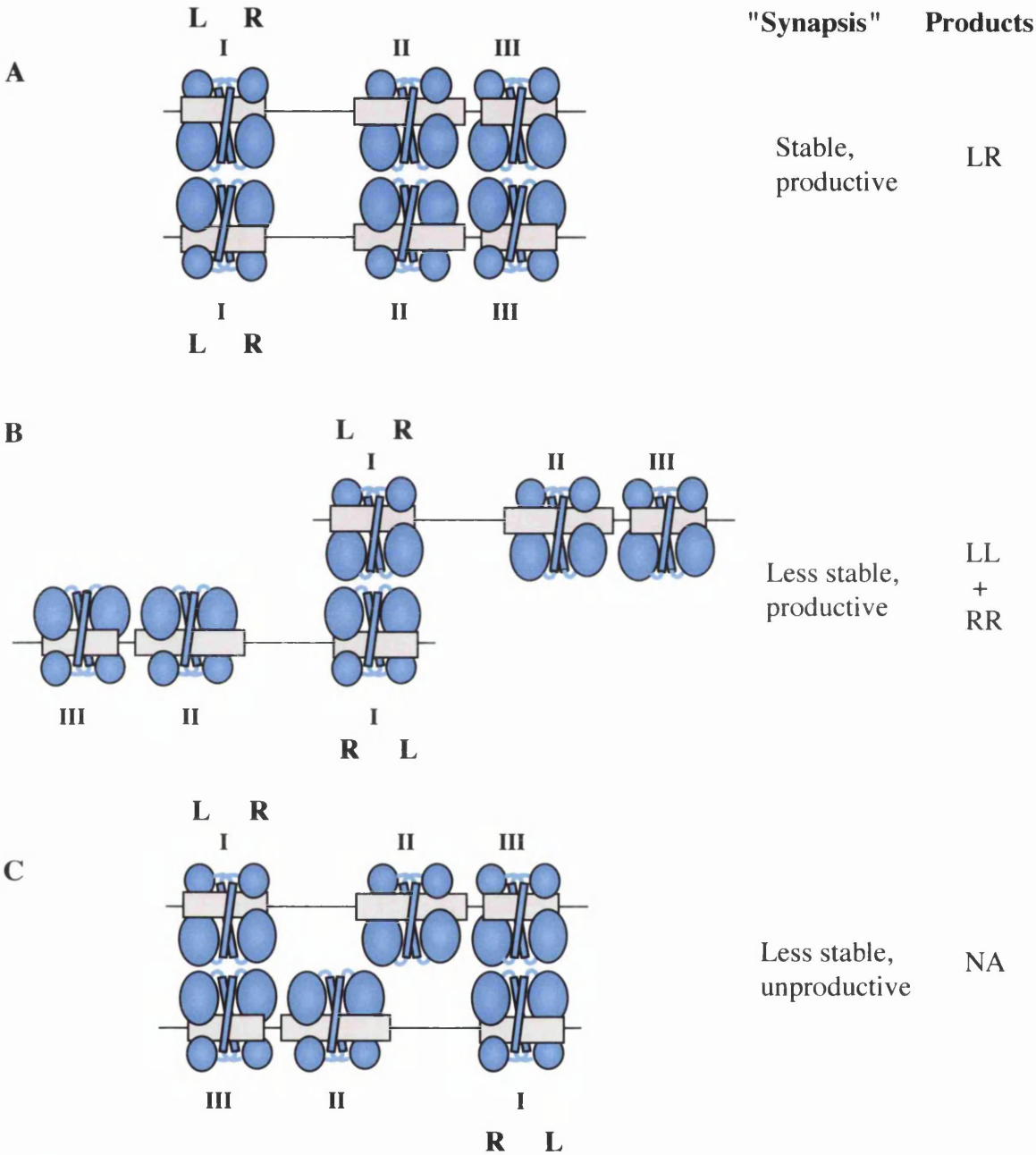
Characterisation of 2,3'-deficient γδ resolvase mutants suggested that the main role of the 2,3'-interface is to enable synapsis of sites II/III. A predicted consequence of disrupting the 2,3'-interface would be that the alignment and topology of reactions with *res* sites in inverted repeat (pMA2631) would not differ from reactions with substrates lacking accessory sites (pAL221*sis*, site I x site I). Comparison of the activities of D102Y/E124Q and R2A/E56K/D102Y/E124Q resolvases on these substrates would reveal any effects of the 2,3'-interface on the reaction products (Fig. 3.8). Restriction enzyme analysis with *Pst*I and *Hind*III showed that activated resolvase D102Y/E124Q was unable to bias the alignment of the crossover sites of pMA2631; equal amounts of inversion (LR) and resolution (LL and RR products) were obtained. At the concentration used, no activity was observed with the site I x site I substrate. However, at higher concentrations D102Y/E124Q has been shown to recombine pAL221*sis*, producing equal amounts of resolution and inversion products (Arnold *et al.*, 1999). Thus, without the possibility/option of forming a -3 synaptic complex (either because of the absence of accessory sites - pAL221*sis*, or because the formation of a -3 synapse with inverted repeat accessory sites faces topological constraints - pMA2631), no bias of the reaction products by D102Y/E124Q resolvase is observed. The same lack of alignment selectivity is observed with R2A/E56K/D102Y/E124Q in the absence of accessory sites (pAL221*sis*). In contrast, the products obtained with pMA2631 with the 2,3'-deficient activated resolvases were mostly of inversion reactions yielding LR joins. A possible explanation of these results is that in the absence of 2,3'-interactions the dimers are free to primarily synapse via the 102 region (Fig. 3.9). Such protein-protein interactions (which may also be important in the formation of the wild-type synapse) would stabilise a parallel alignment of the *res* sites and strand exchange would yield inversion products (LR) with an unknot topology. This may be favoured by slithering of the substrate to bring the *res* sites together in a parallel alignment.

**Fig. 3.8 The 2,3'-interface is essential for activation but not for crossover site alignment in "non-standard" *res* site reactions**




Resolution reactions were set up with various substrates (pMA2631, pMA21, pAL265 and pAL221*sis*) in recombination buffer (C8.2) for 90 min. at 37°C. Dilutions of enzyme fractions were added at 1/10 volume (concentrations of enzyme dilutions prior to addition are listed above). Reactions were stopped by heating to 75°C for 5 min., and divided into aliquots to be analysed by restriction with *Pst*I and *Hind*IIII (A) or by DNaseI treatment (B).

**Fig. 3.9 Suggested mechanism for selective *res* site alignment by 2,3'-deficient resolvase**




Three possible *res* site alignments are shown, in which the bound resolvase dimers interact via a hypothetical interface around residue 102. These would be expected to differ in their stabilities. Their predicted relative stabilities are shown, as are the product alignments upon strand exchange and ligation. If the hypothetical interface plays a determining role in the absence of the 2,3'-interface, then the increased stability of the parallel alignment as in (A) would yield the observed products with an inversion substrate and may be aided by a slithering mechanism to bring the *res* sites together.

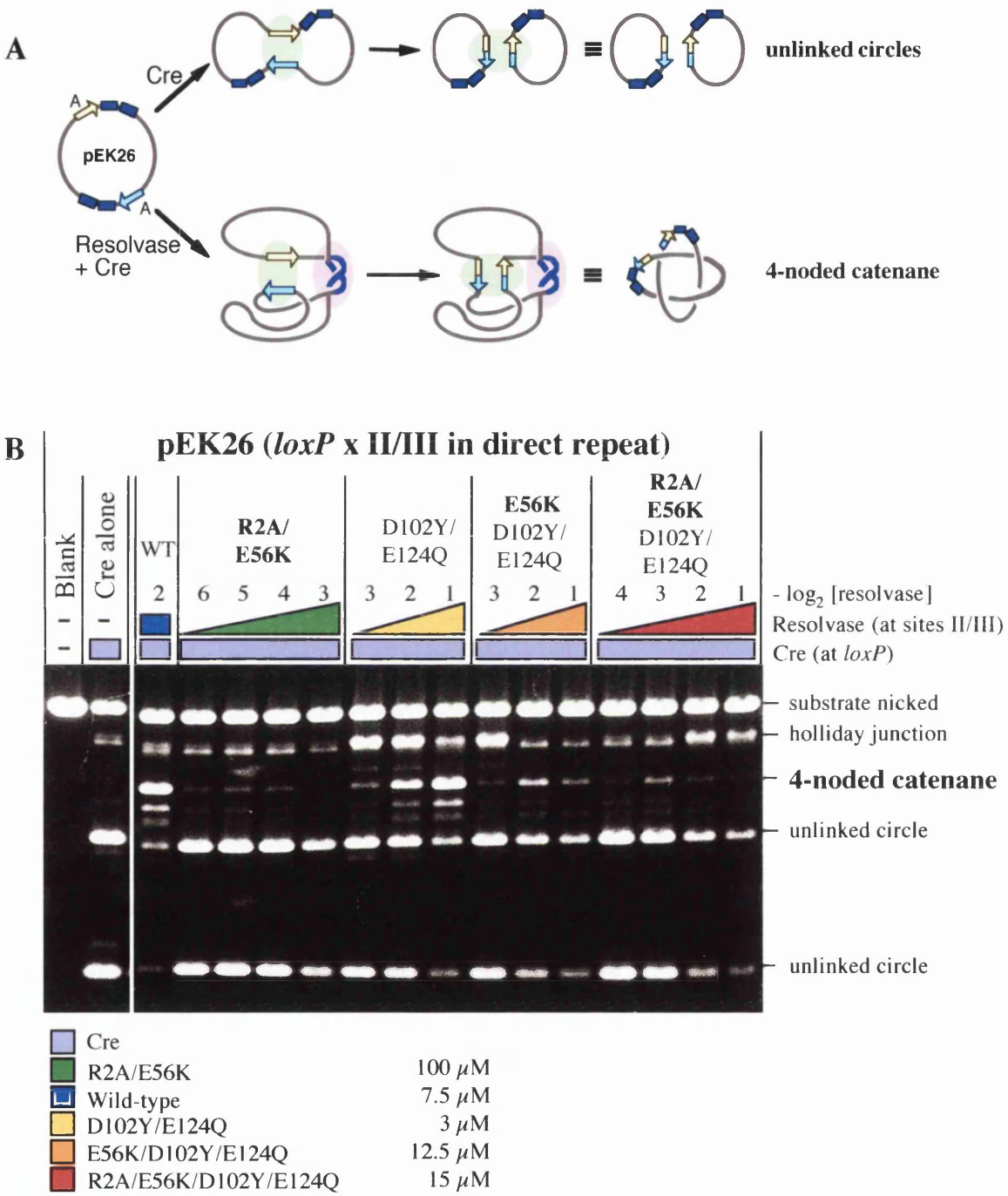
Previous studies have shown that activated resolvases retain the ability to resolve substrates from which one set of accessory sites have been deleted (Arnold *et al.*, 1999). Reactions were performed on pAL265 (*res* x site I) in order to determine whether this reaction is 2,3'-dependent (Fig. 3.8). The reactions of the activated D102Y/E124Q resolvase revealed the ability of this mutant to align the crossover sites despite the presence of only one set of accessory sites. DNaseI nicking of this reaction showed that the main product was 2-noded catenane, indicative of a -3 synapse. Such a structure may be formed by wrapping the single set of accessory sites with DNA adjacent to the isolated crossover site, forming a "pseudo-synapse" which traps three negative supercoils. The reaction rate is slower than a standard *res* x *res* reaction, but much faster than the site I x site I reaction. This is not the case when the resolvase carries additional 2,3'-mutations. The reaction rate of pAL265 by R2A/E56K/D102Y/E124Q drops to the rate observed with isolated crossover sites. In addition, the alignment is random (yielding a 1:1 ratio of LR: LL/RR products) and the topology is consistent with synapsis by random collision. Thus reactions with a *res* x site I substrate revealed a 2,3'-dependency for activation (seen as an increased reaction rate) and for crossover site alignment. These results mirror the *in vivo* data generated in a comparison between resolvase containing activating mutations alone and combined with the E56K mutation (M. Boocock, unpublished results) (Fig. 3.4). The E56K mutation was shown to inactivate the partially hyperactive mutants D102Y and E124Q with a *res* x site I test substrate. Activation of strand exchange by one copy of sites II/III *in vivo* absolutely requires D102Y or E124Q and a 2,3'-proficient catalytic domain. The reliance of this reaction on the 2,3'-interface is only relieved when both activating mutations are present, i.e. when resolvase no longer requires even a single set of accessory sites.

The ability of 2,3'-defective resolvase (R2A/E56K and R2A/E56K/D102Y/E124Q) to wrap sites II/III was investigated using an accessory site synapsis assay. This assay probes for a resolvase-induced topological footprint in the reaction products of the recombinase Cre (Kilbride *et al.*, 1999). The principle of this assay was the same as employed by Murley and Grindley in experiments with 2,3'-defective γδ resolvases (Fig. 3.2) (Murley and Grindley, 1998). A plasmid containing *loxP* sites flanked by sites II and III of Tn3 *res* was used as a substrate for Cre. Cre recombines the *loxP* sites, yielding topologically simple products. The addition of wild type resolvase to these reactions, however, channels the Cre reaction into specific products by trapping an additional three negative supercoils. The products observed in the presence and absence of wild-type resolvase with the substrate pEK26 (4-n catenane

and unlinked circles, respectively) are illustrated in Fig. 3.10. This assay can only determine the topology of the accessory site synapse, and does not address the ability of resolvase bound to the accessory sites to activate site I-bound resolvase. D102Y/E124Q resolvase gives a signal (4-n catenane) approximately equal to that of wild-type resolvase. The activating mutations, therefore, have no discernible effect on the synapsis of sites II and III, as detected in this assay. The signal becomes considerably weaker when the resolvase assayed carries additional mutations at the 2,3'-interface. The synaptic structure detected by this assay is therefore sensitive to 2,3'-interface mutations. Once again the effect of the 2,3'-interface mutations was additive, indicating that the E56K mutation alone was not sufficient to disrupt the 2,3'-interface. 2,3'-defective resolvase carrying no activating mutations (R2A/E56K) also displayed a severe defect in this accessory site synapsis assay, presumably due to defective 2,3'-subunit interactions. However, the signal is not undetectable. This differs from the result reported under similar conditions by Murley and Grindley, in which no residual synapsing activity was detectable with γδ E56K resolvase (Murley and Grindley, 1998). The signal with R2A/E56K is not noticeably weaker than with R2A/E56K/ D102Y/E124Q. The activating mutations therefore do not "rescue" the defect significantly.

The accessory site synapsis assays indicated that the inactivity (on a *res* x *res* substrate) of the 2,3'-deficient R2A/E56K mutant might be due to its reduced ability to synapse sites II/III. γδ resolvase R2A and E56K single mutants were both active when complemented by the 2,3'-proficient γδ resolvase S10L at sites II/III, indicating that these 2,3'-deficient mutants can function at site I. However, when the equivalent complementation assay was performed with Tn3R R2A/E56K and γδR S10L no activity was observed (Fig. 3.11). This indicates that the R2A/E56K resolvase double mutant is more 2,3'-deficient than either single γδ resolvase mutant and therefore implies that the single mutants are partially 2,3'-proficient. Thus, the R2A/E56K mutant is not only defective in function at sites II/III, but is also defective at site I. In contrast, the quadruple mutant R2A/E56K/D102Y/E124Q was found to be an active recombinase. This implied that the 2,3'-interface is not important for site I function by activated resolvase. As anticipated, R2A/E56K/D102Y/E124Q, was complemented by γδR S10L, restoring an approximately wild-type reaction rate, alignment and topological selectivity. Therefore, the 2,3'-defective Tn3 resolvase (R2A/E56K) must carry additional "activating" mutations in order to be complemented by γδR S10L. This supports the hypothesis that the 2,3'-interface normally plays an essential role in activation of the crossover bound resolvase. We infer that targeting 2,3'-deficient resolvase to the crossover

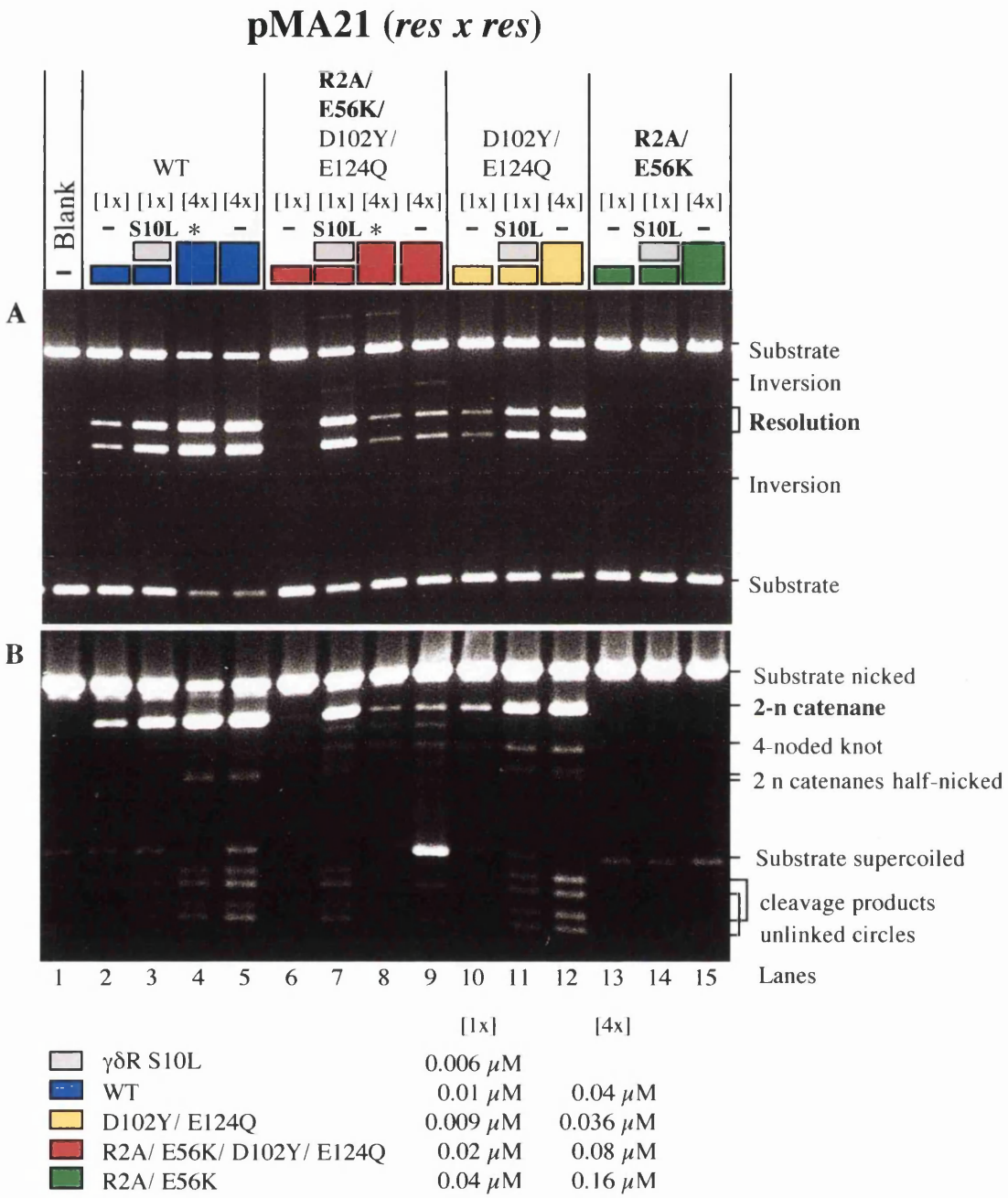
## Fig. 3.10 Assaying synapsis of sites II/III by 2,3'-defective resolvases




**A.** The principle of the accessory site synapsis assay is illustrated for pEK26. Cre acts at the *loxP* sites in pEK26 generating unlinked circles (NEB1 buffer (pH 7.0) at 37°C). However, the prior addition of synapsis-proficient resolvase alters the topology of the Cre reaction, generating 4-noded catenane.

**B.** Synapsis assays were performed with pEK26 in NEB1 buffer. Resolvase (1/20 volume) was added first for 15 min at room temperature. Cre (1/20 volume) was then added and allowed to react for 25 min. at room temperature. Reactions were stopped by heating to 75°C for 5 min. and analysed by DNaseI treatment. The ratio of 4-noded catenane to unlinked circle gives an indication of the amount of synapse formed at sites II and III by the resolvase mutant.

**Fig. 3.11 Activating mutations permit 2,3'-defective resolvase to be complemented by S10L**




Complementation assays were performed with pMA21 in recombination buffer (C8.2) at 37°C for 18 hours, except for reactions labeled with an asterisk which were stopped after 20 min. Reactions were stopped by heating to 75°C for 5 minutes. Aliquots were restricted with *Pst*I and *Hind*III (A) and treated with DNaseI (B). The catalytic serine-10 mutant, γδR S10L, binds sites II/III more tightly than site I and can therefore be used to complement resolvase at the crossover site on a standard *res* site (N. Grindley, 1993). γδR S10L or dilution buffer was added first (1/20 volume), immediately followed by various resolvase mutants (1/20 volume) at the final concentrations listed above.

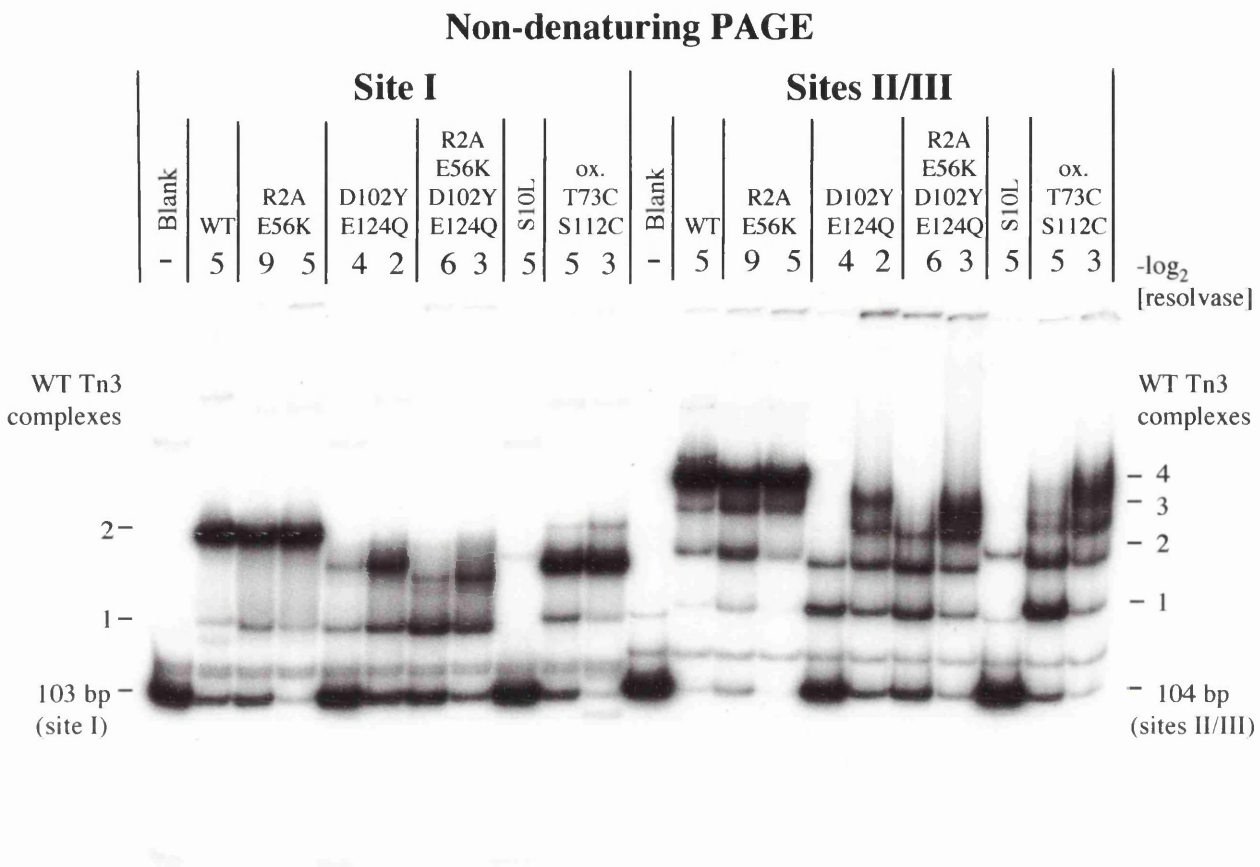
site disrupts contacts between site I- and accessory site-bound resolvase, blocking activation and thereby inhibiting resolution (unless resolvase at the crossover site carries activating mutations).

## 3.3 Binding activity of 2,3'-deficient and/or activated resolvases

In the assays described above, R2A/E56K resolvase showed no activity except for a weak signal in the accessory site synapsis assay. It was therefore crucial to determine its binding activity. Furthermore, it was important to establish whether the 2,3'-deficient Tn3 resolvase mutants would behave in a similar manner to the previously characterised 2,3'-deficient γδ resolvases (Hughes *et al.*, 1990). Binding assays were performed on fragments containing an isolated crossover site, sites II/III and a full *res* site (Figs. 3.12-13). Binding to site I and sites II/III by R2A/E56K gave a pattern of protein-DNA complexes nearly identical to the pattern seen with wild-type resolvase. All binding sites were filled in what appears to be a cooperative manner. The activated mutant D102Y/E124Q, however, displayed an altered binding pattern and this was accentuated by the additional 2,3'-mutations, as seen with R2A/E56K/D102Y/E124Q resolvase. In both cases the monomer complex (for site I and sites II/III) migrated at the wild-type position. However, the dimer complex for site I and all three further complexes with the sites II/III fragment migrated faster than the corresponding wild-type complexes. This altered migration may be due to reduced bending of the DNA. The further decrease in DNA bending of site I observed with the 2,3'-activated mutant (as compared to D102Y/E124Q resolvase) indicated that this phenomenon is not necessarily associated with a lack of DNA bending caused by disrupted interdimer interactions. Thus, the increased mobility of complexes of R2A/E56K/D102Y/E124Q resolvase on sites II/III is not necessarily due to a defect in interdimer interactions.

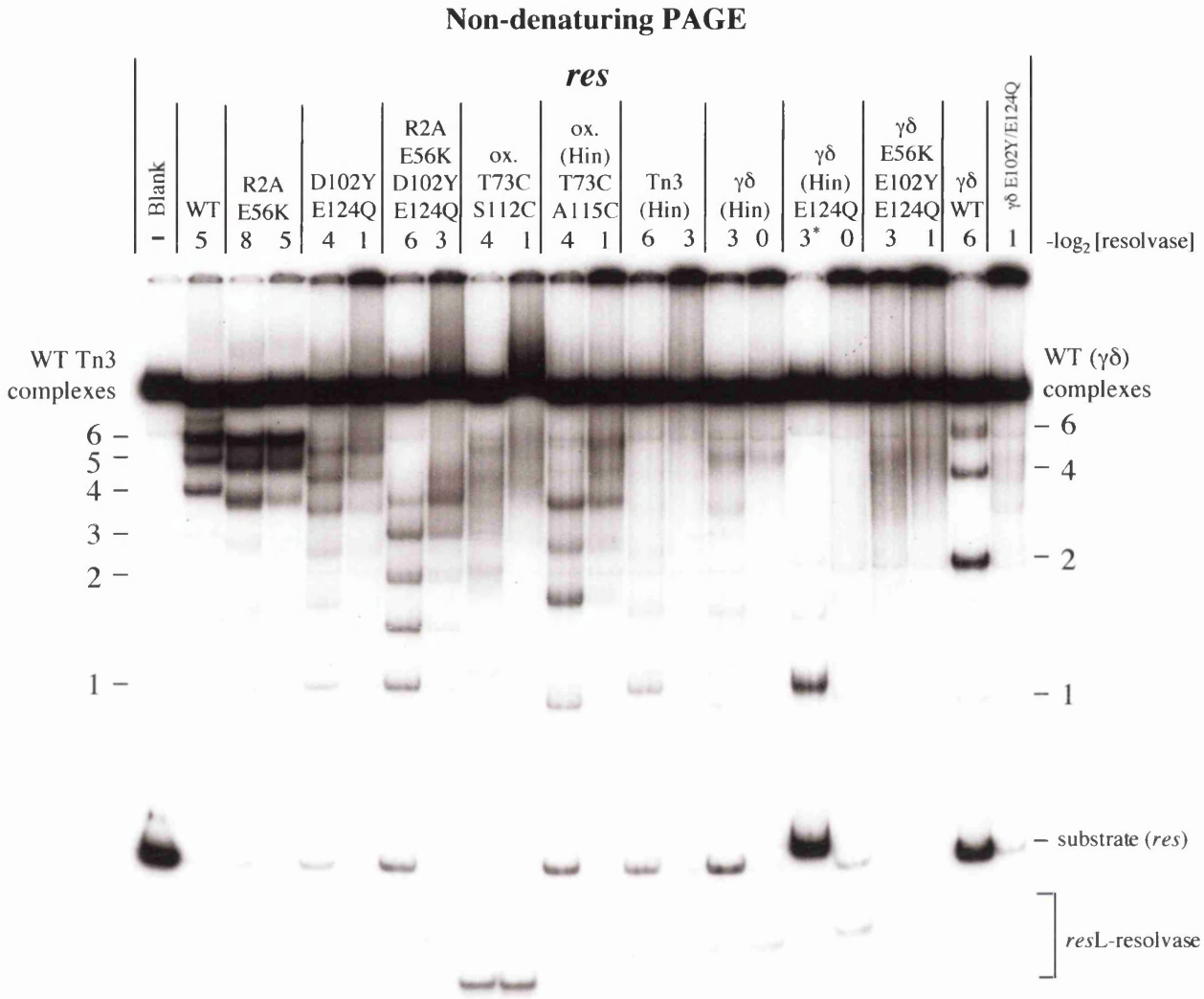
Binding to a full *res* site revealed a similar picture. However, binding by R2A/E56K showed slightly altered binding mobilities of most complexes. The differences in the mobilities of the complexes obtained with Tn3R WT and D102Y/E124Q were exaggerated by additional mutations of the 2,3'-interface (as seen with R2A/E56K/D102Y/E124Q). The complexes were also less well-defined and this smeariness may reflect a decrease in their stability. Furthermore, binding to *res* showed reduced cooperativity of these mutants compared to Tn3R WT (with the notable exception of R2A/E56K). Although this mirrors the results

**Fig. 3.12 Binding to site I and sites II/III by resolvase mutants**




Binding reactions were set up in binding buffer (25% ethylene glycol, 50 mM Tris/HCl pH 8.2, 1 mM EDTA) in the presence of 50-100 nM carrier DNA (supercoiled plasmid). The site I fragment was a 103 bp *Xho*I-*Mlu*I fragment from pCO1, 3' end-labelled at both sites. The sites II/III containing fragment was a 104 bp *Eco*RI-*Xba*I fragment from pOG5, 3' end-labelled at the *Xba*I site. Dilutions of resolvase fractions were added (1/20 volume) and incubated for 10 min. at room temperature. Samples (10 $\mu$l) were loaded on a non-denaturing PAGE Tris/glycine gel and run at 200 V for approximately 4 hours. The concentrations of resolvase fractions prior to dilution were as follows: 7.5 $\mu$M Tn3R WT, 100 $\mu$M R2A/E56K, 3 $\mu$M D102Y/E124Q, 15 $\mu$M R2A/E56K/D102Y/E124Q, 4 $\mu$M γδR S10L, 7.5 $\mu$M T73C/S112C (ox.-intra).

## Fig. 3.13 Binding to a linear *res* fragment by resolvase mutants

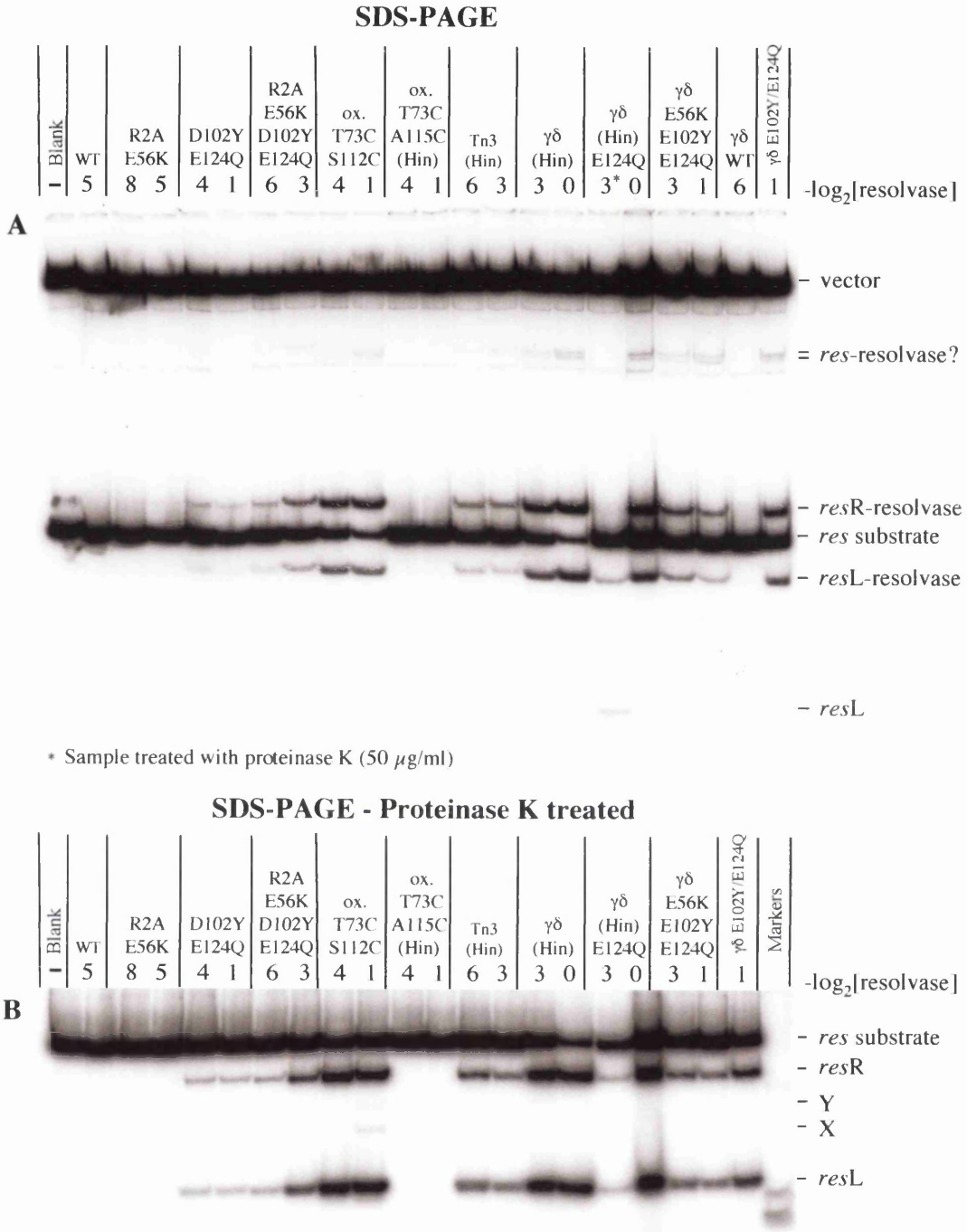



* Sample incubated for 1 minute only

(Hin) : (96-105 Hin) resolvase

Binding reactions were set up with a 199 bp *Xho*I-*Mlu*I fragment from pOG5, 3' end-labelled at both sites in a cleavage/binding buffer (25% ethylene glycol, 50 mM Tris/glycine pH 9.4, 1 mM EDTA) in the presence of 50-100 nM carrier DNA (supercoiled plasmid). Labelled vector is also present (pOG5, *Mlu*I-*Xho*I fragment approximately 2.4 kb). Dilutions of resolvase fractions were added (1/20 volume) and incubated for 1 hour at room temperature. The reactions were split into three aliquots to be analysed in non-denaturing conditions (shown above), in the presence of SDS (Fig. 3.14, A) and in the presence of SDS and proteinase K (Fig. 3.14, B). Samples (10 $\mu$l) were loaded on a non-denaturing PAGE Tris/glycine gel and run at 200 V for approximately 4 hours. The concentrations of resolvase fractions prior to dilution were as follows: 7.5 $\mu$M Tn3R WT, 100 $\mu$M R2A/E56K, 3 $\mu$M D102Y/E124Q, 15 $\mu$M R2A/E56K/D102Y/E124Q, 7.5 $\mu$M T73C/S112C (ox.-intra), 30 $\mu$M Tn3R (96-105 Hin) T73C/A115C (ox.-intra), 5 $\mu$M Tn3R (96-105 Hin), 1 $\mu$M γδR (96-105 Hin), 0.5 $\mu$M γδR (96-105 Hin) E124Q, 9 $\mu$M γδR WT, 6 $\mu$M γδR E102Y/E124Q.

## Fig. 3.14 Cleavage of a linear *res* fragment by resolvase mutants




(Hin) : (96-105 Hin) resolvase

Binding reactions were set up with a 199 bp *Xho*I-*Mlu*I fragment from pOG5, 3' end-labelled at both sites in a cleavage/binding buffer (25% ethylene glycol, 50 mM Tris/glycine pH 9.4, 1 mM EDTA) in the presence of 50-100 nM carrier DNA (supercoiled plasmid). Labelled vector is also present (pOG5, *Mlu*I-*Xho*I fragment approximately 2.4 kb). Dilutions of resolvase fractions were added (1/20 volume) and incubated for 1 hour at room temperature. The reactions were split into three aliquots to be analysed in non-denaturing conditions (Fig. 3.13), in the presence of SDS (A) and in the presence of SDS and proteinase K (B). Samples (10 μl) were loaded on SDS PAGE Tris/glycine gels and run at 200 V for approximately 2 hours. The concentrations of resolvase fractions prior to dilution were as follows: 7.5 μM Tn3R WT, 100 μM R2A/E56K, 3 μM D102Y/E124Q, 15 μM R2A/E56K/D102Y/E124Q, 7.5 μM T73C/S112C (ox.-intra), 30 μM Tn3R (96-105 Hin) T73C/A115C (ox.-intra), 5 μM Tn3R (96-105 Hin), 1 μM γδR (96-105 Hin), 0.5 μM γδR (96-105 Hin) E124Q, 9 μM γδR WT, 6 μM γδR E102Y/E124Q.

obtained with 2,3'-deficient γδ resolvase mutants, the altered binding phenotype is very weak for R2A/E56K. In fact, the phenotype seems more dependent on the activating mutations than on mutations of the 2,3'-interface. It should, however, be noted that the binding assays were performed using different buffer systems (Tris/glycine instead of TBE). Also, these reactions were performed in conditions, which support cleavage (due to the presence of 25% ethylene glycol). When the identical samples are analysed in the presence of SDS, cleavage products (left and right ends of *res* with a resolvase monomer covalently attached) can be observed (Fig. 3.14). As the substrate is linear, only the hyperactive resolvases D102Y/E124Q and R2A/E56K/D102Y/E124Q yield cleavage products. The 2,3'-interface is therefore not required for supercoil-independent activity by activated resolvase.

## 3.4 Targeting to subsites of *res*

The properties of the quadruple mutant R2A/E56K/D102Y/E124Q and the double mutant R2A/E56K at first seem opposed to the "classical" phenotype associated with mutations of the 2,3'-interface. It has previously been shown that 2,3'-deficient resolvase bound to sites II/III did not complement wild-type resolvase bound at site I. This was suggested to result from a defect in synapsis of sites II/III by the 2,3'-deficient resolvase (see hypothesis I, Fig. 3.15). This interpretation conflicts with the observed product topology of reactions by R2A/E56K/D102Y/E124Q, which indicates that the architecture of the synapse remains largely intact, despite mutations in the 2,3'-interface. We therefore suggested that the 2,3'-interface was not the sole interdimer interface, and was not absolutely required for synapsis. Furthermore, R2A/E56K was not complemented by S10L for activity at site I. This implicated the 2,3'-interface in the activation of site-I bound resolvase. We hypothesised that 2,3'-deficient resolvase is recombination defective because it is unable to activate resolvase bound at the crossover site (see hypothesis II, Fig. 3.15). This new hypothesis II about the role of the 2,3'-interface thus reconciles the previous data with the new data generated with the activated resolvases. Fig. 3.15 displays predictions these hypotheses make about synpasis proficiency, and the alignment and topology of the reaction products for four different combinations of mutants targeted to subsites of *res*.

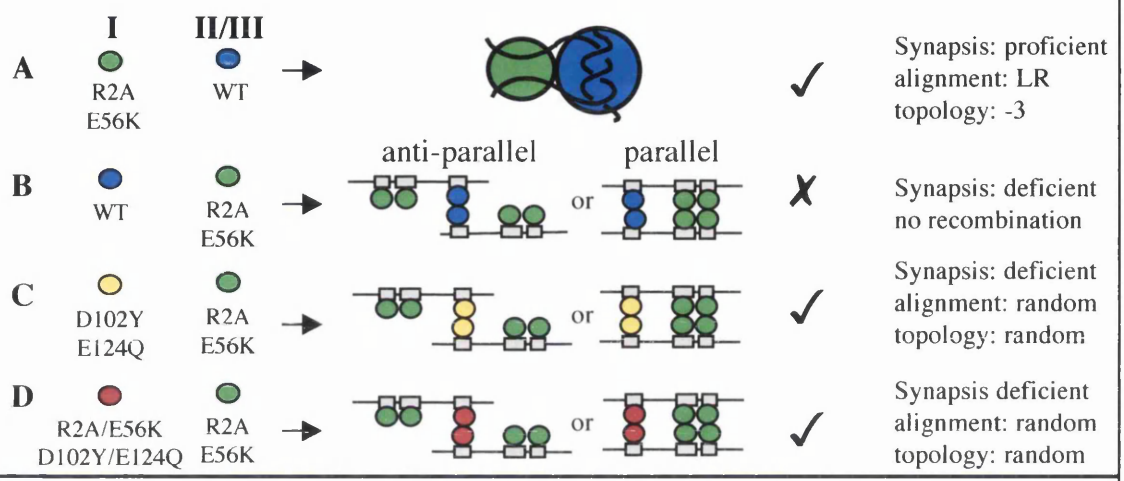
Targeting Tn3 resolvase to subsites of *res* using the altered binding specificity mutation R172L had not been performed previously (Grindley, 1993). It was not clear that the R172L

**Fig. 3.15 Hypotheses about the function(s) of the 2,3'-interface and predictions for targeting experiments**

## Hypothesis I :

**Site I** strand exchange requires activation by sites II/III, but not 2,3'-proficiency at site I **and/or** activating mutations in catalytic domain (D/E102Y, E124Q)

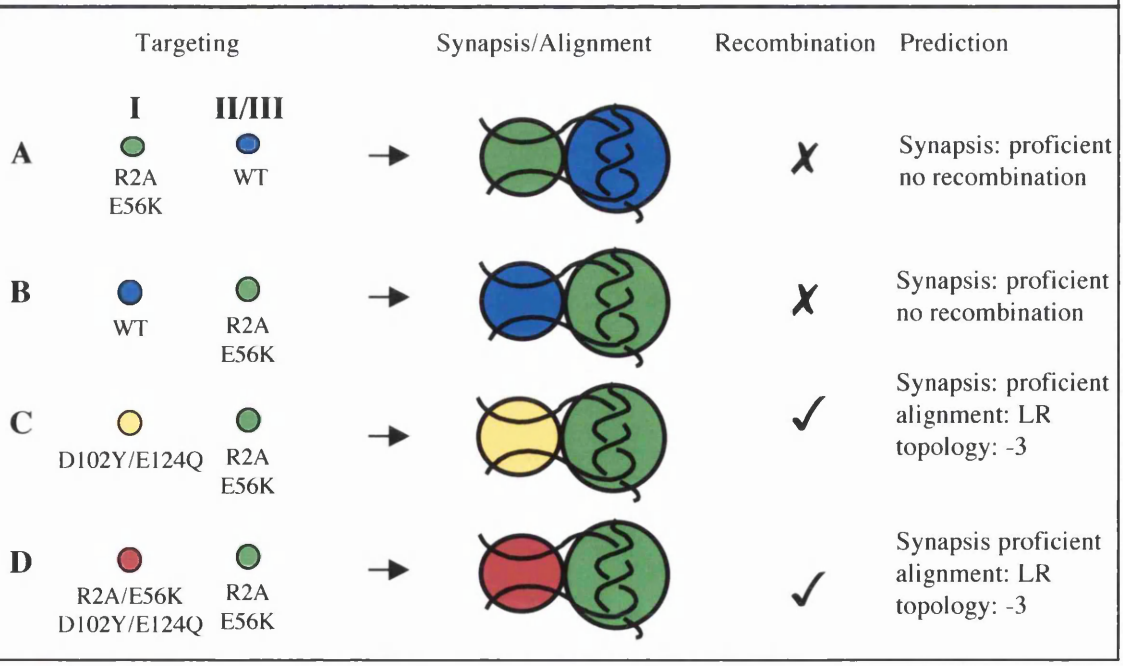
**Sites II/III** synapsis, alignment and -3 topology require the 2,3'-interface




## Hypothesis II :

**Site I** strand exchange requires activation by sites II/III (via 2,3'-interface) **and/or** activating mutations in catalytic domain (D/E102Y, E124Q)

**Sites II/III** synapsis, alignment and -3 topology does not require the 2,3'-interface **however** 2,3'-interface is important for activation of resolvase bound at site I and may stabilise the synapse at sites II/III

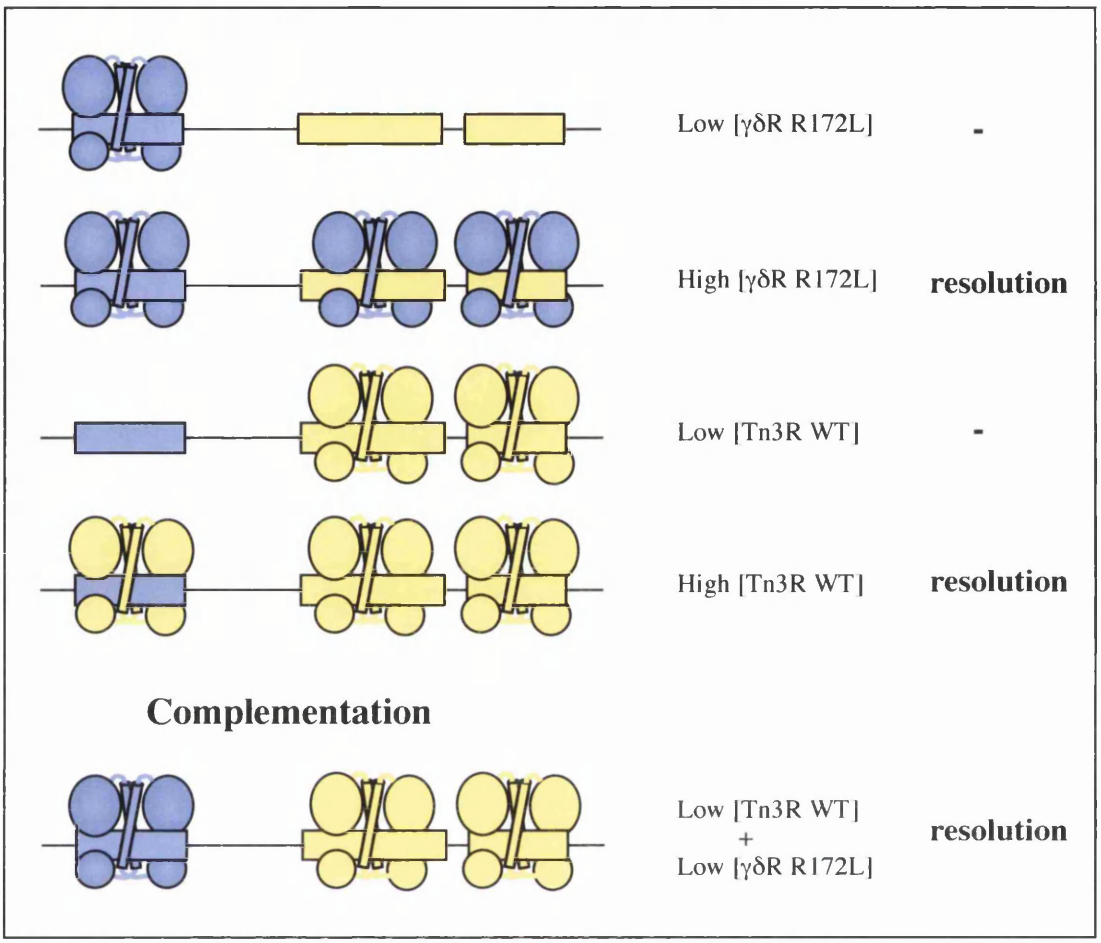


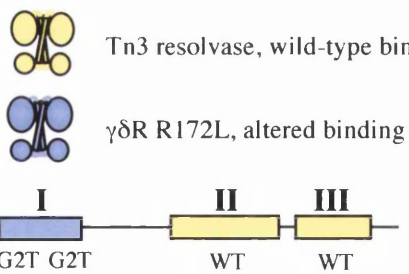
mutation would have the same effect on the recognition specificity of Tn3 and γδ resolvase DNA binding domains. Instead, targetable Tn3 resolvase was made by replacing the Tn3 DBD with the γδ resolvase R172L DBD. This binding domain selectively binds an altered binding sequence containing a G2T substitution. The altered DNA binding specificity mutants D102Y/E124Q/R172L (γδR 143-183) and R2A/E56K/D102Y/E124Q/R172L (γδR 143-183) were constructed. These activated 2,3'-proficient and activated 2,3'-deficient resolvases were designed to be targeted to the crossover sites of pNG345 (*res* x *res* substrate in which all four site I half-sites carry the G2T mutation). Any resolvase with wild-type binding specificity could then be targeted to the accessory sites. If sub-saturating amounts of the resolvase mutants targeted to sites I and sites II/III are used, resolution of the substrate will only occur if the resolvases can fulfil their roles at these sites, thus complementing each other. This principle is illustrated in Fig. 3.16. By targeting different combinations of mutations to site I and to sites II/III, the effects of the 2,3' mutations and activating mutations on strand exchange and the stability of the synapse could be dissected.

## 3.5 Investigating the function of the 2,3'-interface at subsites of *res*

The first targeting experiment was designed to determine whether 2,3'-defective resolvase (R2A/E56K) at the accessory sites could fulfil any accessory site functions (i.e. synapsis and/or activation) (Fig. 3.17). In this experiment, pNG345 was used to target altered binding specificity resolvase mutants to the crossover site. These included γδ resolvase R172L and the activated mutant D102Y/E124Q/R172L (γδR 143-183). Both of these mutants were added at high concentrations in order to saturate all binding sites. At these concentrations, both resolvases were able to resolve pNG345, yielding wild-type products (lane 6 for γδR R172L, and lanes 14 and 15 for D102Y/E124Q/R172L (γδR 143-183). The addition of wild-type resolvase (targeted to sites II/III) complemented both resolvases (lanes 11-13 for γδ resolvase R172L, and lanes 20-22 for D102Y/E124Q/R172L (γδR 143-183). This was especially apparent with the activated mutant, as its activity is reduced (though topologically selective) compared with wild-type resolvase. The addition of R2A/E56K resolvase (targeted to sites II/III) also complemented D102Y/E124Q/R172L (γδR 143-183), lanes 16-18. Moreover, restriction enzyme analysis (A) revealed that the products are mainly resolution products (LR- joins) and treatment with DNaseI (B) shows that the major species is a 2-noded catenane. This indicates that R2A/E56K is able to wrap the accessory sites and form a

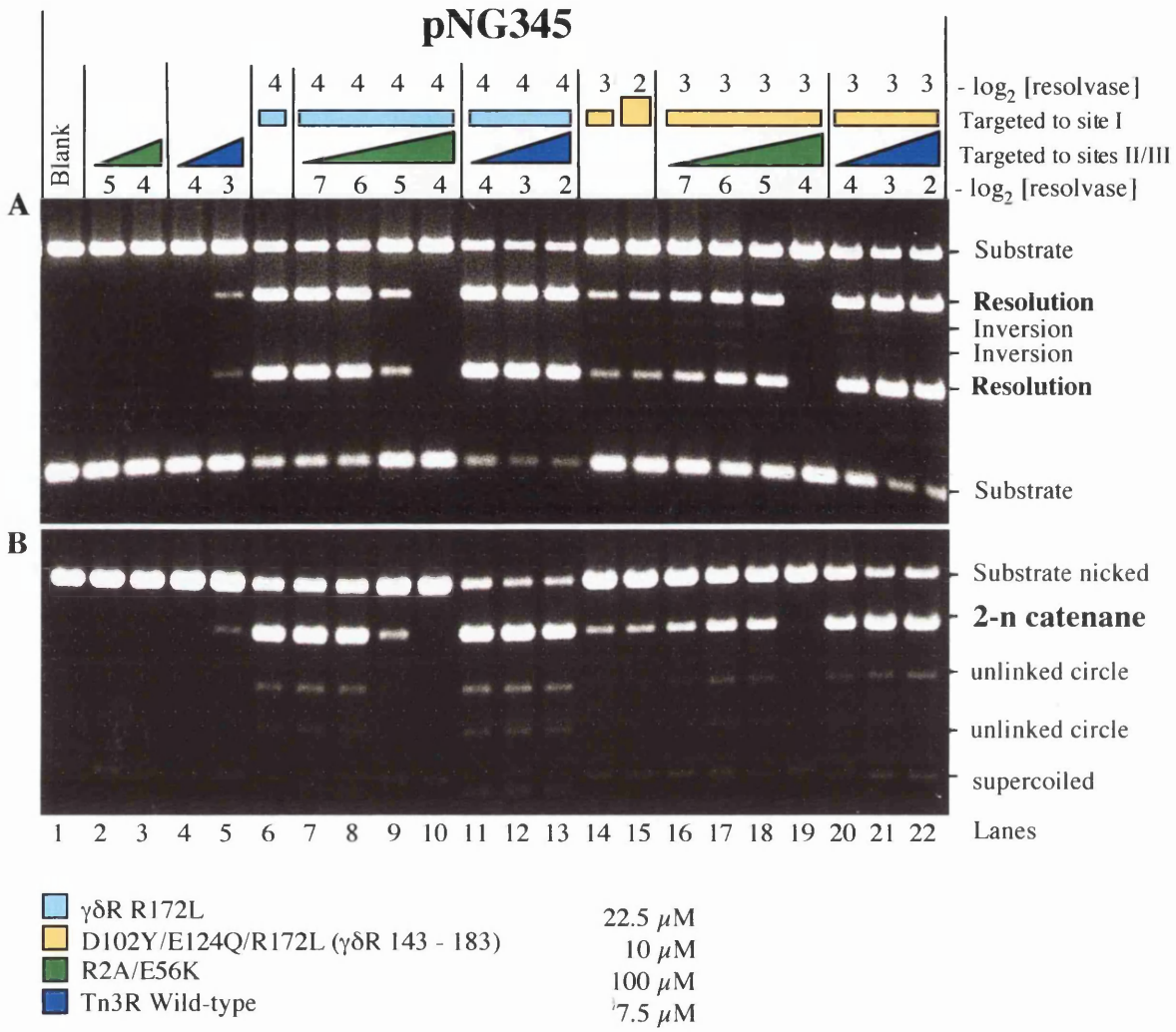
## Fig. 3.16 Complementation by resolvases targeted to subsites of *res*




The pNG345 *res* site with an altered binding sequence at site I (G2T mutation, coloured blue) and wild-type sequence at the accessory sites (coloured yellow) is shown above. The altered binding specificity mutant γδR R172L (coloured blue) preferentially binds to the crossover site of this *res* sequence, whereas wild-type resolvase (coloured yellow) will preferentially bind to the accessory sites. Resolution of this substrate is supported either by complementation of sub-saturating concentrations of both resolvases (final scenario) or by saturation of all binding sites by high concentrations of γδR R172L or wild-type resolvase.

**Fig. 3.17 2,3'-deficient resolvase targeted to sites II/III inhibits R172L but complements activated resolvase bound to site I**

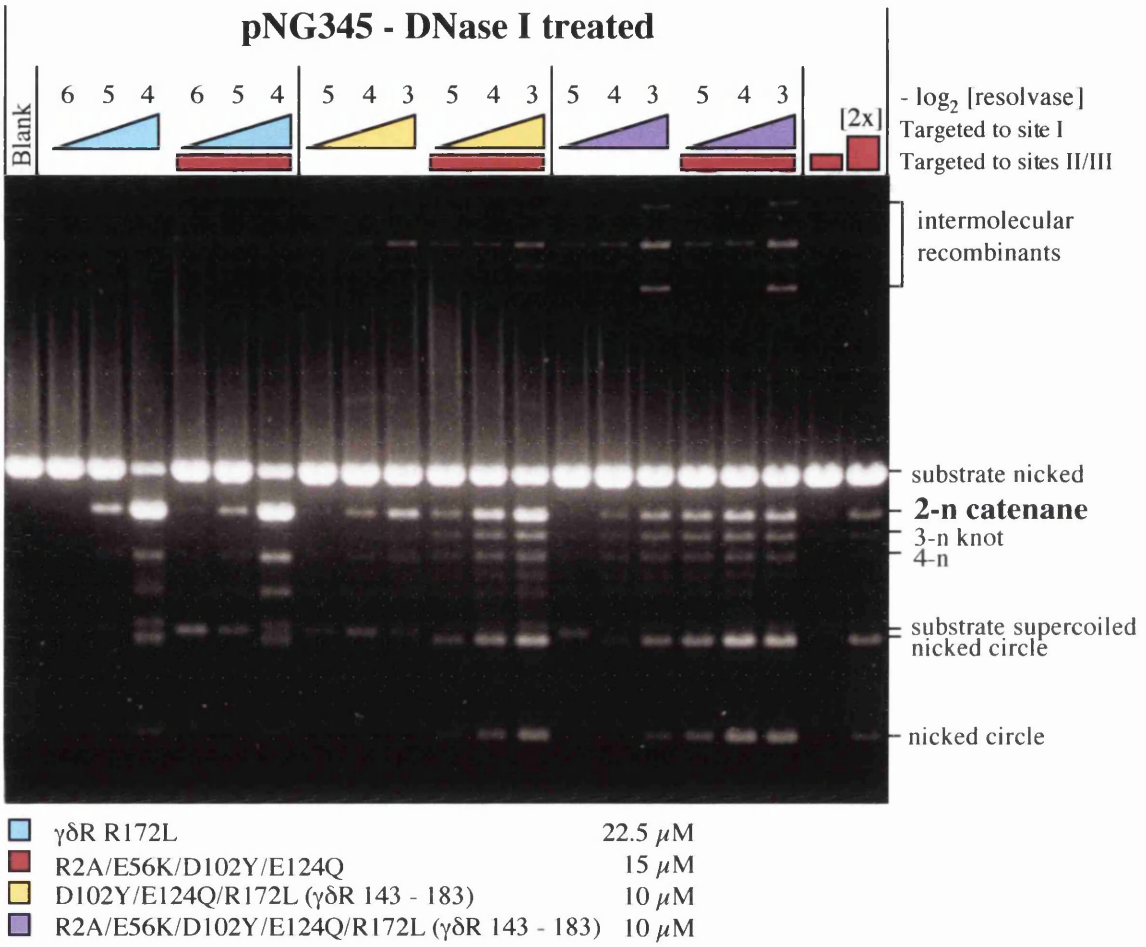



Complementation assays were performed with pNG345 in C8.2 buffer at 37°C for 90 min. The concentrations of resolvase fractions prior to dilution are shown above. The dilutions of the enzymes targeted to sites II/III were added first (1/20 volume). Each sample was then divided into aliquots and resolvase targeted to site I was added (1/20 volume). Reactions were stopped by heating to 75°C for 5 min. and split in order to be analysed by restriction with *Pst*I and *Hind*III (A) and DNaseI treatment (B).

synapse, which traps three negative supercoils. At the highest concentration of R2A/E56K the reaction is blocked (lane 19), presumably due to R2A/E56K binding at site I. It is notable that this complementation reaction does not proceed as far to completion when compared to the complementation reactions with wild-type resolvase (sites II/III). The reaction proceeds faster than in the absence of accessory sites (i.e. D102Y/E124Q/R172L (γδR 143-183) with a site I x site I substrate). Yet the reaction is not as fast as the identical complementation reaction in the presence of the 2,3'-proficient resolvase, S10L, in the place of R2A/E56K at the accessory sites (data not shown). Both of these resolvases are catalytically inactive. The difference in activity by D102Y/E124Q/R172L (γδR 143-183) must therefore occur because of a functional difference at the accessory sites between γδR S10L and R2A/E56K. One difference could be that the synapse formed by S10L is more stable than that of R2A/E56K. Alternatively, γδR S10L may activate resolvase bound to site-I (if this signal depends upon the 2,3'-interface), whereas R2A/E56K may not. If the resolvase bound to site I requires an activation signal and activation depends upon an intact 2,3'-interface (as suggested by hypothesis II) then this is probably due to the reduced activity of D102Y/E124Q/R172L (γδR 143-183) in the absence of an activation signal. If resolvase bound at site I does indeed require an intact 2,3'-interface at sites II/III in order to initiate catalysis, then R2A/E56K would be expected to inhibit γδR R172L at a concentration which complements D102Y/E124Q/R172L (γδR 143-183). This was observed; R2A/E56K inhibits γδR R172L at a dilution of $2^{-5}$ (lane 9) yet stimulates D102Y/E124Q/R172L (γδR 143-183) at the identical concentration (lane 18).

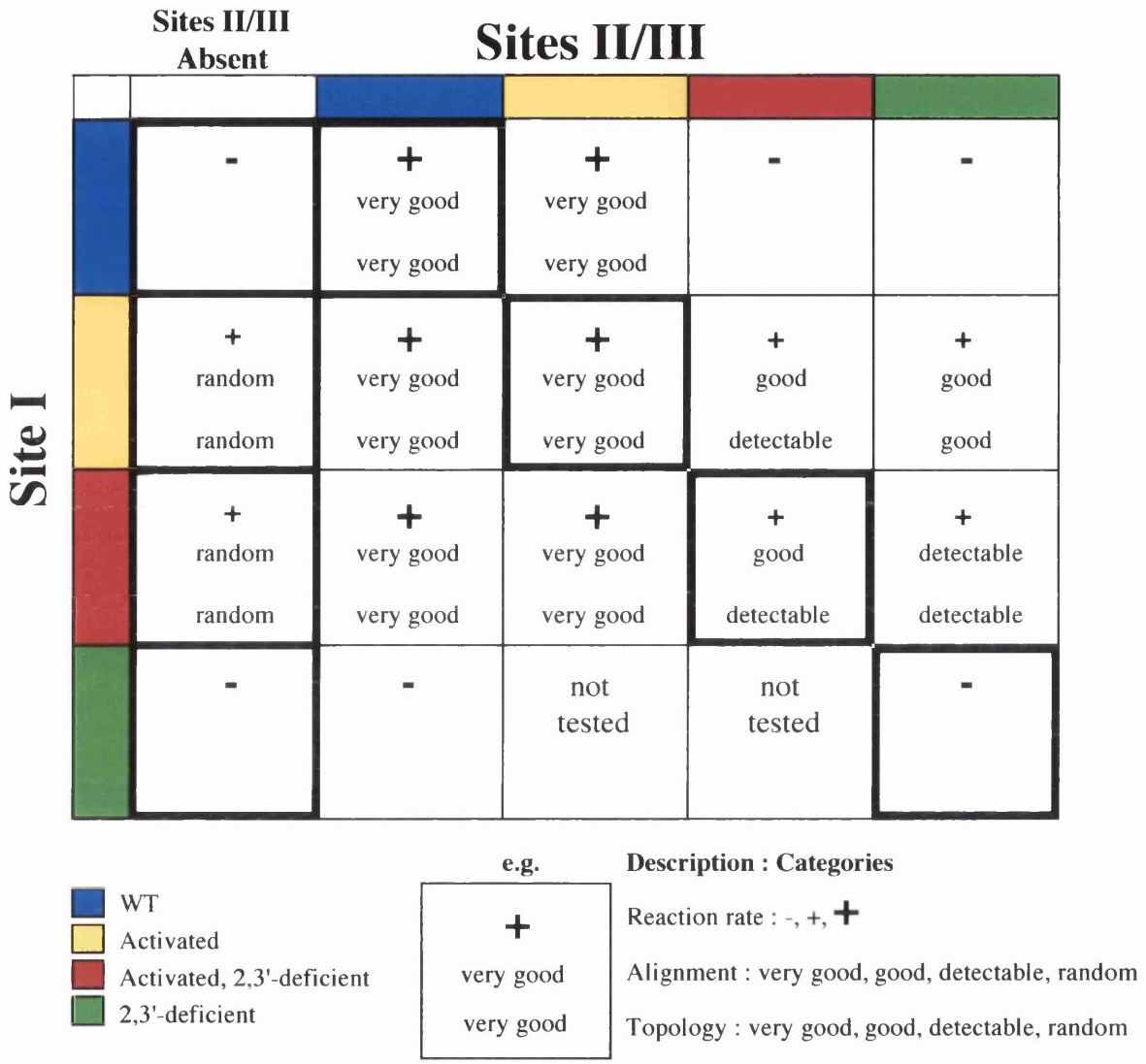
A similar result should be obtained when activated 2,3'-deficient resolvase (R2A/ E56K/D102Y/E124Q) is present at the accessory sites. A fixed (and limiting) concentration of R2A/E56K/D102Y/E124Q was targeted to sites II/III of pNG345 to test for complementation with a variety of mutants (Fig. 3.18). At the concentration used (0.033 μM final) no activity was observed (lane 20) and only a trace of products were observed when this was increased 2-fold (lane 21). This limiting concentration of the R2A/E56K/D102Y/E124Q complemented activated D102Y/E124Q/R172L (γδR 143-183) and R2A/E56K/D102Y/E124Q/R172L (γδR 143-183) targeted to site I. However, γδR R172L was not complemented. As before, a 2,3'-deficient mutant was only able to complement accessory site-independent resolvases (carrying the D102Y/E124Q activating mutations), but not resolvase that has wild-type catalytic function. Topological analysis showed that the reaction products were mainly 2-noded catenanes. However, topological

**Fig. 3.18 Activated, 2,3'-deficient resolvase complements activated resolvase but not WT resolvase targeted to the crossover site**




Complementation assays were set up with pNG345 in C8.2 buffer at 37°C for 75 min. Reactions were stopped by heating to 75°C for 5 min. and analysed by DNaseI treatment. R2A/E56K/D102Y/E124Q, targeted to sites II/III was added first ($2^{-4.5}$ dilution, 1/20 volume, yielding 0.033 $\mu$M final, except control labeled [2x] which is at 0.066 $\mu$M final). The resolvase targeted to site I was added second (1/20 volume). The concentrations of resolvase fractions prior to dilution are shown above.

**Fig. 3.19 Summary of experiments**




The results of experiments with all combinations of wild-type/activated/2,3'-proficient and deficient resolvases are summarised above. Reactions which do not involve targeting to subsites of *res* are shown with a larger border. Column one (coloured white) represents a site I x site I reaction. The diagonal line shows non-targeted *res* x *res* reactions. All other columns show results obtained when resolvase is targeted to subsites of pNG345. Each box displays the products observed from a reaction in which resolvase mutants are targeted to site I (rows) and sites II/III (columns). The class of mutant is colour coded as shown below the table. The reaction rate is displayed as (-) for no activity, (+) for low activity and (+) for full activity. Then the alignment (LR versus LL/RR) is described, followed by the topology (2-noded catenane versus other products).

selectivity indicative of a -3 synapse was more apparent when D102Y/E124Q/R172L (γδR 143-183) was targeted to the crossover site than with its 2,3'-deficient equivalent (e.g. lane 13 versus lane 18). This could be due to residual 2,3'-contacts between a 2,3'-proficient resolvase at site I and 2,3'-deficient resolvase at sites II/III. Alternatively it could be due to D102Y/E124Q/R172L (γδR 143-183) binding at sites II/III. This may be more likely as R2A/E56K/D102Y/E124Q is less cooperative in its binding than wild-type resolvase. In fact, lane 11 corresponds to the complementation of D102Y/E124Q/R172L (γδR 143-183) at the concentration in which no product can be detected by itself (lane 8). The reaction products in this lane are indicative of less topologically selectivity. Intermolecular recombinants were detectable in the reactions containing the activated 2,3'-deficient resolvase at the crossover site (lanes 16 and 19). Such products were also seen in complementation reactions (with pNG345) between R2A/E56K/D102Y/E124Q and S10L. Thus, these products are a site I - 2,3'-deficiency phenomenon.

## 3.6 Models of the Synaptic complex

The data described above, have supported the second hypothesis of the role of the 2,3'-interface in the regulation of recombination. This section looks at three types of models of the synaptic complex (which make different predictions of 2,3'-requirements) in the context of this new data. For clarity, the catalytic domains of the resolvase dimers are shown as dominos in all diagrams of these models, as this is a reasonable approximation of the shape of these domains, (Figs. 3.20 and 3.21).

**Rice & Steitz Model (1994b)**

The packing of resolvase dimers in the crystal structure of resolvase in the absence of DNA suggested a possible structure for the synaptic complex (Rice and Steitz, 1994a; Rice and Steitz, 1994b). The *res* DNA was modelled on to the complex, taking into account topological considerations (Fig. 3.21, A). Sites II/III wrap around the crystallographic tetramer of dimers (section 3.1.1), which are connected via the 2,3'-interface (Fig. 3.20, A (i)), trapping three negative supercoils (Fig. 3.21, A). The resolvase dimers bound to site I are connected by a 2,3-interaction to a dimer bound to site III. Although the DNA wraps around the outside of the resolvase complex at sites II/III it passes through the inside of the dimers bound at site I. The DNA-binding domains were not visible in the earlier crystal

structures, so the model does not make definite predictions of which half-sites require 2,3'-proficiency. Nevertheless, a requirement for 2,3'-proficiency at sites II-R and III-L is in best agreement with the co-crystal structure. No 2,3'-proficiency is required at site I and indeed, modelling a 2,3'-interaction between site I-R and the tetramer of dimers does not yield structures that appear appropriate for recombination.

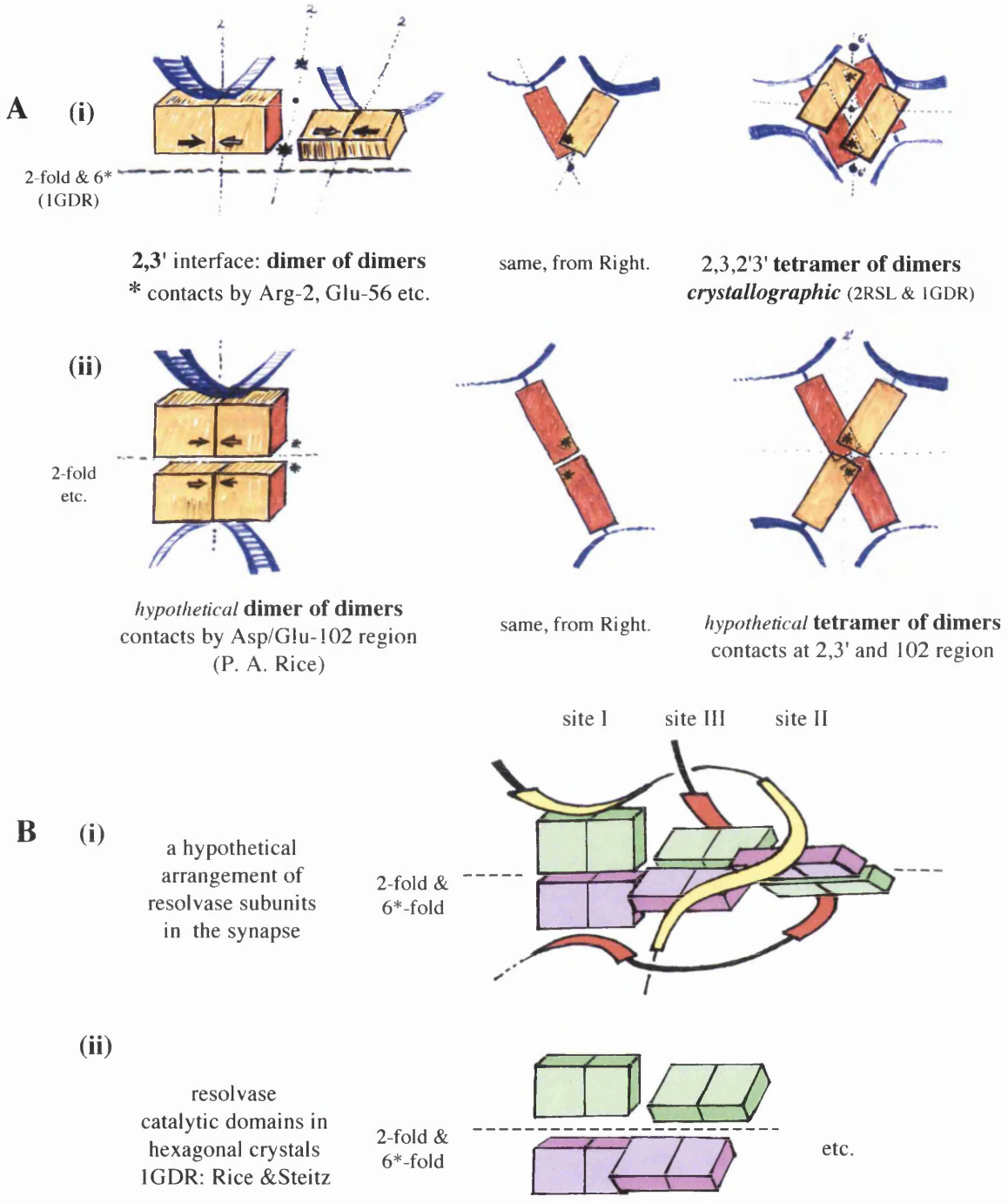
**Murley & Grindley Model (1998)**

The predicted requirements of 2,3'-proficiency of the Rice & Steitz model were not compatible with the experimental 2,3' requirements observed by Murley and Grindley (Murley and Grindley, 1998). The binding data obtained by targeting heterodimers of resolvase to specific subsites of a *res* II/III fragment (section 3.1.2, Fig. 3.2) suggested that the requirement for 2,3' proficiency at sites II-L and III-L in a synapsis assay may represent a single 2,3'-interaction between resolvase dimers bound to adjacent sites (i.e. in *cis*). The additional requirements for 2,3' proficiency at site III-R and I-R (in a recombination assay) were suggested to be due to a direct interaction between these protomers (in *cis* or *trans*) subsequent to synapsis. A different model for the synapsis of sites II/III was proposed, consistent with the data (Murley and Grindley, 1998). Synapsis of two resolvase-bound accessory sites was suggested to be an ordered, DNA-mediated process. The model showed dimers bound at sites II and III interacting in *cis*. This complex then interacts with the identical partner complex to form the same tetramer of dimers as in the accessory site synapse of the Rice & Steitz model (Fig. 3.21, B). However, it should be noted that the DNA is wrapped around this complex in a very different configuration.

**4 + 2 Model (Sarkis *et al.*, in preparation)**

Recently, an alternative type of model has been advocated by M. Boocock and by N. Grindley (Sarkis *et al.*, in preparation). This model requires two interfaces for synapsis; the well-characterised 2,3'-interface and the "hypothetical" interface mediated by contacts by the region including and surrounding residue 102. This model also bases some interactions between resolvase dimers on interactions seen in the crystal structure (Rice and Steitz, 1994a). The 2,3'-interactions seen in the crystal structure are shown diagrammatically, including a view of the tetramer of dimers (Fig. 3.20, A (i)). In the second sequence of diagrams (ii), the dimers are shown interacting via the region including and surrounding residue 102, in a configuration first suggested by P. A. Rice. This type of synaptic configuration is required by the domain swap model for strand exchange. Two of these

## Fig. 3.20 Diagrammatic representations of resolvase interactions

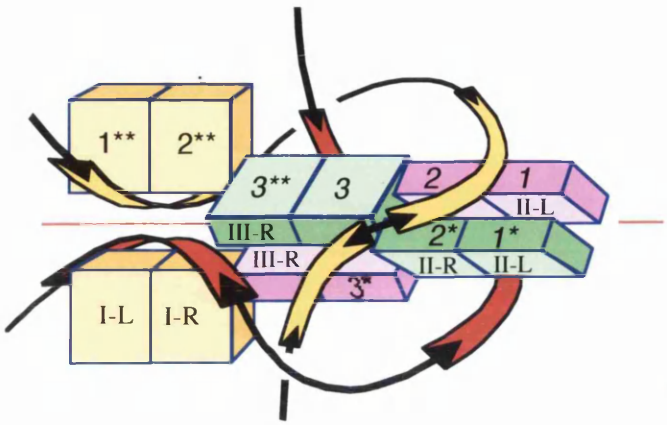



**A**. (i) The catalytic domains of γδ resolvase are represented by flat dominos (yellow) and DNA by a ribbon (blue). The positions of 2,3' residues are marked by asterisks (*). This panel (i) shows views of resolvase dimers interacting via their 2,3'-residues as seen in the crystal structure, including the tetramer of dimers. (ii) This shows resolvase dimers synapsed via their catalytic domains, around residue 102, as suggested by P. A. Rice. Such synapsed dimers could also form a tetramer of dimers by making 2,3'-contacts with another identically synapsed dimer pair. This would create a different, hypothetical, tetramer of dimers which are connected via both the 2,3'-interface as well as the 102 region.

**B**. (i) The hypothetical tetramer of dimers (seen from the side in A (ii)) could be extended to make the same contacts with another (or in principle an infinite number of) synapsed resolvase dimers. This creates a structure around which *res* DNA can be modeled as shown. (ii) This shows four resolvase dimers arranged in a similar manner in the hexagonal crystal structure, except that the resolvase dimers do not make any contacts across the central 2-fold axis.

## Fig. 3.21 Models of the synaptic complexes

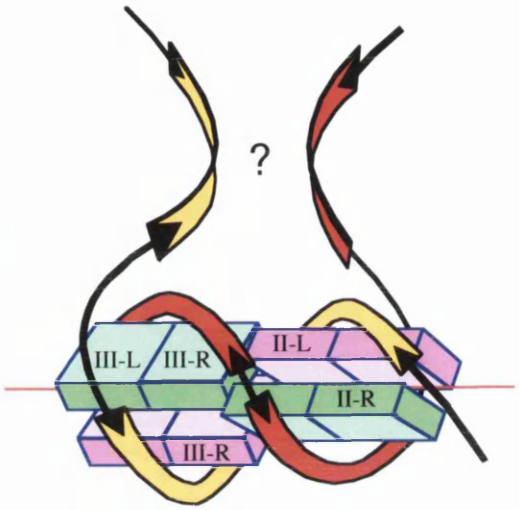
### A Rice & Steitz Model (1994)




**Predicted contacts**

| | | |
|---|---|---|
| 2,3' | II-R + III-L | *(cis)* |
| 2,3 | II-R + III-L | *(trans)* |
| | I-R + III-R | *(trans)* |
| 2,2'/3,3' | II-R + II-R | *(trans)* |
| | III-L + III-L | *(trans)* |

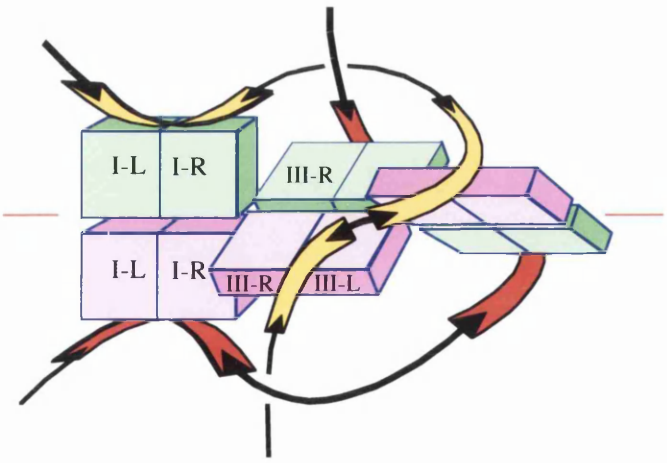
### B Murley & Grindley Model (1998)




**Predicted contacts**

| | | |
|---|---|---|
| 2,3' | II-L + III-R | *(cis)* |
| 2,3 | II-L + III-R | *(trans)* |
| 2,2'/3,3' | III-R + II-L | *(trans)* |
| | III-R + II-L | *(trans)* |

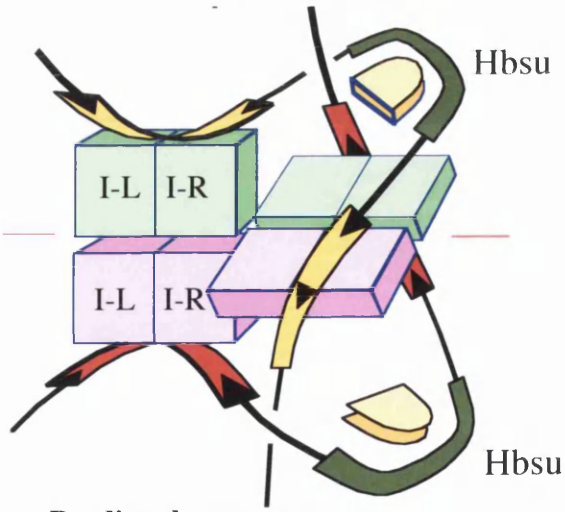
### C 4+2 Model (Sarkis *et al.*, in preparation)




**Predicted contacts**

| | | |
|---|---|---|
| 2,3' | II-R + III-L | *(cis)* |
| | I-R + III-R | *(trans)* |
| "hypothetical" 102 region | I/I | *(trans)* |
| | II/II | *(trans)* |
| | III/III | *(trans)* |

### D Model of Sin/Hbsu synaptic complex (Rowland *et al.*, in preparation)




**Predicted contacts**

| | | |
|---|---|---|
| 2,3' | I-R + II-R | *(trans)* |
| "hypothetical" 102 region | I/I | *(trans)* |
| | II/II | *(trans)* |

hypothetical dimers of dimers (as seen in the first picture, ii) can be positioned to make 2,3'-contacts with each other, producing a hypothetical tetramer of dimers (final picture, ii). In fact a similar symmetric arrangement of dimers was seen in the hexagonal crystal structure (B, ii). However, the resolvase dimers were further apart and did not make contacts via the "hypothetical" interface (A, ii). The hypothetical arrangement of resolvase subunits in the Sarkis *et al.* model of the synapse (B, i) consists of three such dimers of dimers units interacting via the 2,3'-interface. Thus, the hypothetical "tetramer of dimers" (A ii) was extended to make a complex with six resolvase dimers (although in principle such interactions could form an infinite "filament" of "hypothetically-synapsed" resolvase dimers). The *res* DNA can be modelled around the resolvase core, as shown in B, (i).

It is notable that in this structure all resolvase dimers interact in the same manner via the "hypothetical" interface (in *trans*) and via the 2,3'-interface (although the dimers bound to site I and site II make 2,3'-contacts via only one subunit, whereas the dimer bound at site III makes 2,3'-contacts via both its protomers). The 2,3'-interactions would be predicted to occur between site I-R and site III-R (in *trans*) and site III-L and site II-R (in *cis*); the latter prediction is the same as for Rice & Steitz model.

These interactions are not entirely consistent with the data obtained by Murley and Grindley (1998). Although 2,3'-contacts between sites II and III in *cis* are predicted by the model a requirement for 2,3' proficiency at site II-L, as observed by Murley & Grindley, is not readily predicted. In contrast, the additional requirements for recombination (2,3'-proficiency at I-R and III-R) are entirely consistent with the model.

The interactions suggested by these models are summarised below.

| **Interface** | **Rice & Steitz (1994b)** | **Murley & Grindley (1998)** | **4 + 2 Model** |
|---|---|---|---|
| 2,3' | II-R + IIIL (*cis*) | II-L + III-R (*cis*) | II-R + III-L (*cis*) |
| | | | I-R + III-R (*trans*) |
| 2,3 | II-R + III-L (*trans*) | II-L + III-R (*trans*) | - |
| | I-R + III-R (*trans*) | | - |
| 2,2'/3,3' | II-R + II-R (*trans*) | III-R + II-L (*trans*) | - |
| | III-L + III-L (*trans*) | III-R + II-L (*trans*) | - |
| "hypothetical" | - | - | I/I (*trans*) |
| (102 region) | | | II/II (*trans*) |
| | | | III/III (*trans*) |

**How do these models fit the results obtained with 2,3'-deficient and/or activated resolvase described in this Chapter?**

**(i) 2,3'-interface residues are not absolutely required for synapsis of sites II/III.** The accessory site synapsis assay (pEK26) revealed a small amount of product (4-n catenane) indicative of a (-3) synapse, formed upon addition of R2A/E56K (Fig. 3.10). Furthermore, when R2A/E56K was targeted to sites II/III using pNG345, it complemented activated resolvase at site I and 2-n catenane products were observed (Fig. 3.17). This was again indicative of a (-3) synapse formed with 2,3'-deficient resolvase. These data suggested that although the 2,3'-interface was not essential for the synapsis of sites II/III, mutations in residues of this interface do have a deleterious effect upon synapsis.

The Rice & Steitz model and the Murley & Grindley model both place a tetramer of dimers at the accessory sites (although the DNA is wrapped around these very differently). Both models predict 2,3'-interactions between dimers bound at sites II and III in *cis*, whereas interactions in *trans* occur via the 2,3- and the 2,2'/3,3'- interfaces. Neither model therefore places a strong emphasis on the 2,3'-interface in synapsis of the two *res* partners. Indeed, the other interfaces which synapse the dimers in *trans* seem to have a primary role in "holding" the synapse together. A similar argument can be made for the 4+2 model, except that the interface making contacts in *trans* is the "hypothetical" interface. Thus, the evidence that the 2,3'-interface does not play a vital architectural role at sites II/III is compatible with all current models.

**(ii) 2,3'-interactions activate resolvase bound at site I.** The 2,3'-interface appears to be essential for recombination by WT resolvase, but not for "activated" resolvases (Figs. 3.6 and 3.17). This suggests that resolvase bound to site I makes 2,3'-interactions with resolvase bound at the accessory sites, and that this interaction is required for activation. This was also seen in the experiments in which R2A/E56K targeted to the accessory sites could complement activated resolvase (D102Y/E124Q/R172L (γδR 143-183)) but not "non-activated" resolvase (γδR R172L) targeted to site I.

This data is not consistent with the Rice & Steitz model, in which the site-I bound resolvase contacts resolvase at sites II/III only via the 2,3-interface, Thus, this model predicts no requirement for 2,3'-proficiency at site I. The Murley & Grindley model is more difficult to

analyse, as no site-I bound resolvase was modelled in this structure. However, it is difficult to see how resolvase bound at site I could make 2,3'-contacts to the only "free" 2,3'-protomers, II-R or III-L. In contrast, the 4+2 model does make the prediction of 2,3'-contacts between site I-R and III-R. Furthermore, these contacts are in *trans* and are therefore likely to be more useful as an activation signal than an interaction in *cis* (which may occur outwith a productive synapse). The data is therefore consistent with the 4+2 model.

**(iii) 2,3'-proficiency is required for *res* x site I resolution.** 2,3'-proficient activated resolvase, D102Y/E124Q, showed considerable resolution activity on a *res* x site I substrate (Fig. 3.8), generating 2-noded catenane product. In contrast, 2,3'-defective activated resolvase (with the E56K mutation or both mutations, R2A/E56K) was very much reduced in its activity. No bias in the alignment was seen and the reaction rate was no greater than with a site I x site I reaction.

This indicates that the 2,3'-interface is essential for wrapping a "pseudo-synapse" (discussed in section 3.2). Such dependency on the 2,3'-interface suggests that this interface is required for dimer contacts in *trans* that hold the two individual *res* sites in a synaptic complex. The synapse as modelled by Rice & Steitz does not have 2,3'-contacts between resolvase dimers in *trans*. Although the Grindley & Murley synapse could involved 2,3'-contacts between, for example, site I-R and site III-L in *trans*, it is extremely difficult to see how they could then carry out recombination as they would be very far apart. Intriguingly, the 4+2 model of the synapse shows the 2,3'-contacts between protomer I-R and III-R in *trans*. Thus, the dependence upon 2,3'-proficiency for the *res* x site I reaction is fully consistent with this model.

**(iv) 2,3'-deficient resolvase shows a strong bias towards LR joined products with an inverted *res* x *res* substrate.** When assayed with an inverted *res* x *res* substrate, activated resolvase behaved differently depending on whether it was 2,3'-proficient or 2,3'-defective (Fig. 3.8). Whereas 2,3'-proficient activated resolvase showed no bias in the alignment of the crossover site (as deduced from the equal amounts of resolution and inversion products obtained), 2,3'-defective activated resolvase clearly biased the reaction towards LR-joined inversion products. The products were unlikely to occur by dissociative recombination because (i) no significant amount of cleavage product was detected and (ii) this would probably lead to a bias towards LL- and RR-joined resolution products.

A mechanism was suggested to account for the selective *res* site alignment by 2,3'-deficient resolvase (Fig. 3.9). It was speculated that in the absence of the 2,3'-interface and in the presence of topological constraints, resolvase dimers might synapse exclusively via the "hypothetical interface" in *trans*. The increased stability of the parallel alignment (Fig. 3.9, A) would bias the products towards LR-joined inversion products, as observed. The data is therefore consistent with the 4+2 model. However, it does not rule out either the Rice & Steitz model nor the Murley & Grindley model (as the products may derive from dissociative recombination).

## 3.7 Summary and Conclusions

The 2,3'-interface is the only interdimer interface that has been structurally characterised to date. Residues comprising the 2,3'-interface were essential to catalysis. This was thought to be because it contributed to the architecture of the accessory site synapse. However, recent data suggested that the 2,3'-interface may also be involved in contacts between site I-bound resolvase and accessory site bound resolvase. The work described in this Chapter aimed to investigate the role of the 2,3'-interface in the regulation of recombination (rate, alignment and topology) by combining activating mutations with mutations of the 2,3'-interface.

We were concerned that a single mutation at the 2,3'-interface may not be sufficient to completely destabilise the interface. Comparisons of resolvases carrying a single substitution or two substitutions at the 2,3'-interface showed that the effect of the mutations was additive. An accessory site synapsis assay revealed that Tn3R R2A/E56K retained the ability to form a –3 synapse at sites II/III, albeit at a very much reduced efficiency compared to WT resolvase. Thus, while it was clear that the 2,3'-interaction contributes to the stability of the synapse, it was not clear that it is the main architectural interface. Additionally, we had no clear evidence from this assay that activating mutations at residues 102 and 124 have any positive or negative effect on synapsis at sites II and III. This was further confirmed by the retention of topological selectivity and a bias in the alignment of recombination products seen with activated 2,3'-deficient resolvase, Tn3R R2A/E56K/D102Y/E124Q. This suggested the need for a further interface essential for synapsis of *res* sites to generate the –3 synapse topology. The ability of this mutant to function efficiently when targeted to the

crossover site of *res* (Fig. 3.11) and also to recombine a site I x site I substrate, revealed that the 2,3'-interface is not involved in crossover site synapsis or catalysis in site I x site I reactions.

What then is the function of the 2,3'-interface? If a single substitution at this interface inactivates WT resolvase, it presumably has some vital function. Murley and Grindley (1998) observed a requirement for the 2,3'-interface at site I-R and at III-R, in recombination assays, hinting at a potential interaction between resolvase bound at these sites. In support of this interpretation, numerous experiments described in this Chapter suggest that the most critical function of the 2,3'-interface is to support activation of site I-bound resolvase by sites II and III. It was found that if the resolvase is already activated for strand exchange, 2,3'-proficiency is not required (as seen with Tn3R R2A/E56K/D102Y/E124Q); the crossover sites nevertheless respond to the regulatory effect of sites II and III on alignment and topology (as seen in complementation of Tn3R R2A/E56K/D102Y/E124Q with γδR S10L targeted to sites II/III).

This role of the 2,3'-interface supports the recently proposed 4+2 model of the synaptic structure, in which 2,3'-contacts are made between resolvase at site I-R and III-R in *trans*. Activation of strand exchange by the single set of accessory sites of a *res* x site I substrate was found to be absolutely dependent upon an intact 2,3'-interface, supporting the hypothesis that 2,3'-interactions between resolvase at sites II/III and resolvase at site I are in *trans*.

# Chapter 4

# Probing a potential interdimer interface (96-105 Hin)

## 4.1 Introduction

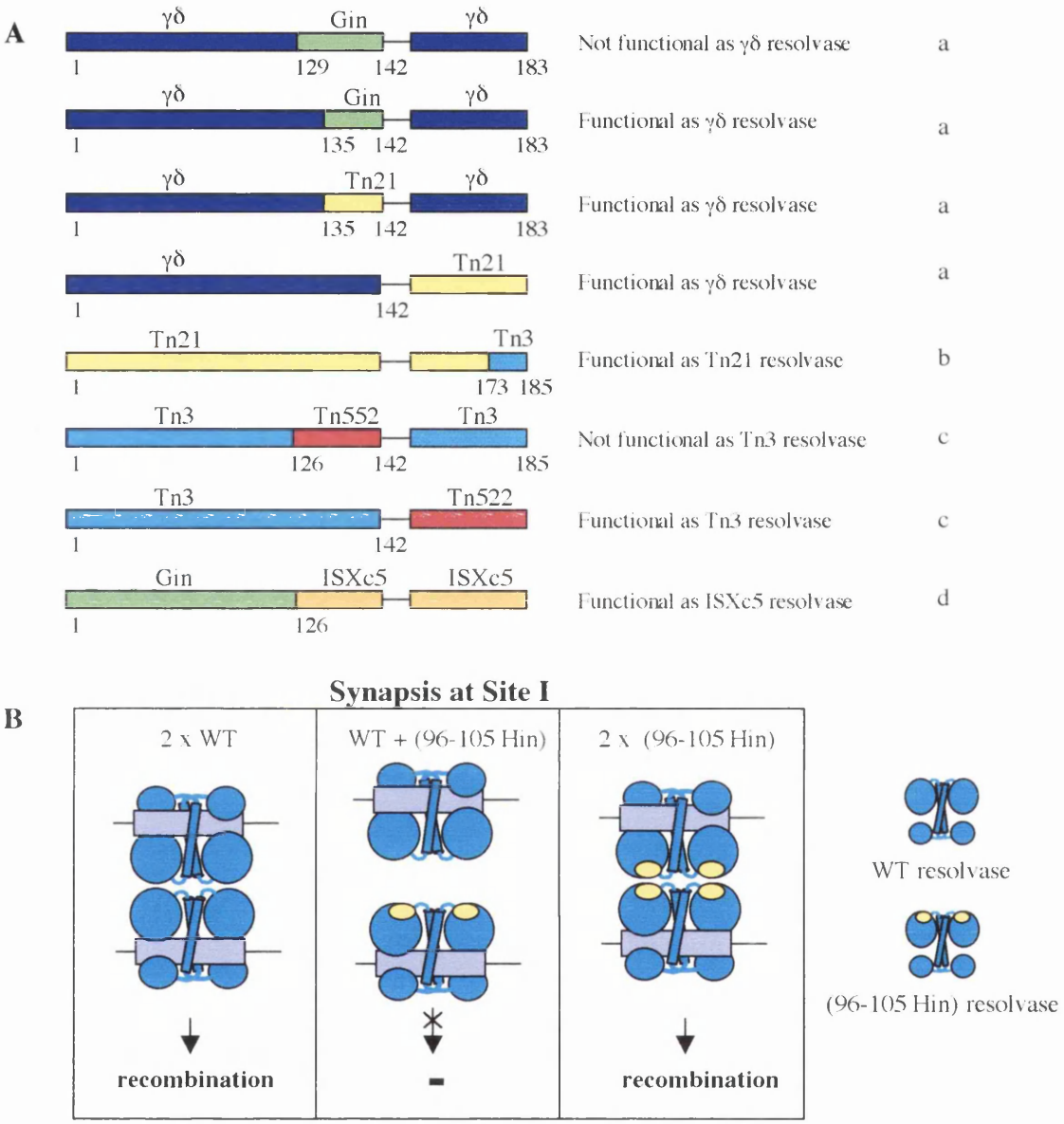
WT Tn3 and γδ resolvases require the presence of two crossover sites, presumably bound by one resolvase dimer each, for catalysis to be initiated. Although synapsis of accessory sites can be detected with sites II/III alone (Kilbride *et al.*, 1999), no cleavage activity can be detected unless site I is present in both partners (e.g. see Fig. 4.9). Furthermore, activated resolvase (such as D102Y/E124Q) generates complex, topologically closed recombination products with a site I x site I substrate, indicative of concerted strand exchange (Arnold *et al.*, 1999). These results are consistent with the hypothesis that the dimers of resolvase bound at each crossover site synapse via an unidentified interface (distinct from the 2,3'-interface) and that this "crossover synapse" is essential for activation of resolvase.

Synapsis models of resolvase and invertases (e.g. Hin) show dimers interacting via either their C-terminal DNA binding domains or their catalytic domains. The possibility that γδ resolvase interacts via its DNA-binding domains was investigated by replacing the entire domain (residues 142-183) with sequence from the Tn21 resolvase (N. Grindley, personal communication), (Fig. 4.1, A). Surprisingly, this hybrid mutant was an active resolvase, which retained the ability to bind and recombine γδ resolvase *res* sites. Further resolvase hybrids have been constructed and all retained the activity associated with that of the resolvase catalytic domain (Fig. 4.1, A). This Chapter describes related experiments in which a small region of the catalytic domain of Tn3 resolvase has been replaced with sequence from the invertase Hin.

Random mutagenesis screens for a gain-of-function phenotype have identified residues in resolvase, including residues G101 to M103, where substitutions enable the resolution of a substrate containing one full *res* site and one crossover site (S. Wenwieser and M. Boocock, unpublished results; Arnold *et al.*, 1999). This region of the protein is not involved in dimer or DNA contacts in any crystal structures, and so it was speculated that it might be involved in interdimer interactions. Furthermore, preliminary crosslinking data from the invertase Hin has suggested that Fis may activate Hin dimers via contacts to the corresponding region.

In order to investigate the hypothesis that the region around residue D102 of Tn3 resolvase is involved in specific interdimer interactions (either at site I, or at all sites of *res*), the region

## Fig. 4.1 Existing hybrid resolvases and rationale for (96-105 Hin) substitution experiment




**A.** The hybrid resolvases constructed to date are shown diagrammatically (a: N.D.F. Grindley, unpublished; b: Ackroyd *et al.* 1990; c: S. Rowland, unpublished; d: Schneider *et al.* 2000). The catalytic domains and the DNA binding domains are shown as coloured rectangles. The flexible linker region is shown as a black line. Residue numbers are shown below the open reading frames. Their function is briefly described.

**B.** Recombination by resolvase is absolutely dependent upon the presence of two crossover sites; cleavage is not observed with a *res* x sites II/III substrate. This suggests that synapsis of the site-I bound resolvase dimers is required to stimulate activity. The region around residue 102 is a potential candidate for a synaptic interface. To test this hypothesis, this region was replaced with sequence from the invertase Hin, introducing 6 amino acid substitutions between residues (96-105, resolvase numbers). This altered region of the resolvase protein is shown diagrammatically in yellow. The diagram illustrates the expected interuption of crossover site synapsis when a wild-type resolvase dimer is targeted to one crossover site of the synapse, and a (96-105 Hin) substituted resolvase dimer is targeted to the other crossover site. Importantly, recombination activity should be reconstituted when two (96-105 Hin) substituted resolvase dimers are bound at the crossover sites.

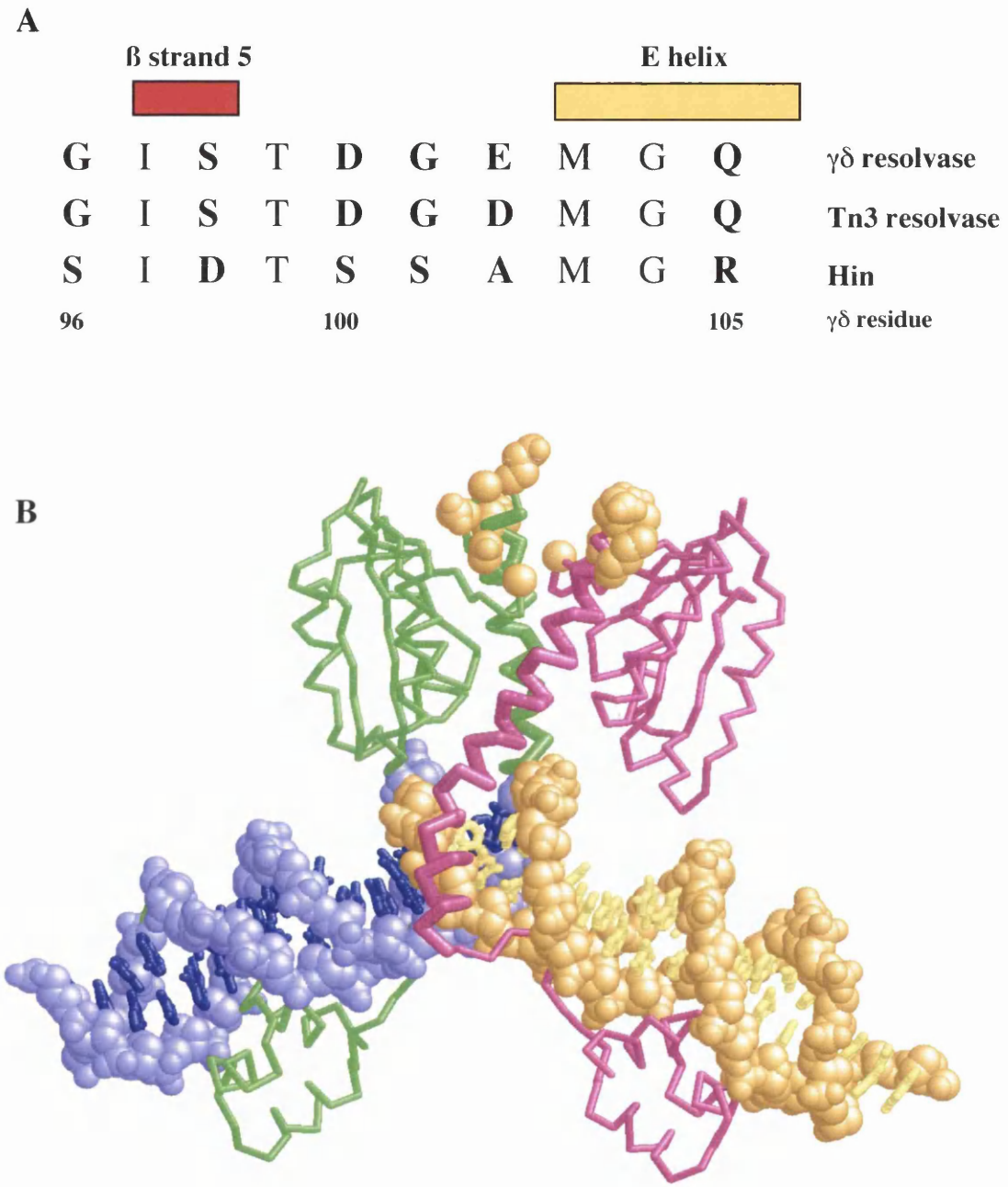
between residues 96-105 (Tn3/γδ resolvase numbering) was replaced with the corresponding amino acid sequence from the invertase Hin. This resulted in six amino acid substitutions, thus creating a hybrid resolvase protein with an altered surface in this region (Fig. 4.2). If a hybrid dimer could interact with a similar dimer, but not with wild-type resolvase, this would indicate that the region is involved in crossover site communications (Fig. 4.1, B). This experiment could be performed by targeting the two different dimers to opposite crossover sites using pNG343, which targets the altered binding specificity mutant, γδ resolvase R172L, to only one crossover site.

Evolutionary trees suggest that invertases have evolved from resolvases. Intriguingly, the region around residue 102 (Tn3 resolvase) is not well conserved between the resolvases (Tn3 and γδ) and the invertases. This region is almost the only surface region of Hin that is conserved in all invertases, yet it is not conserved in different families of resolvases. A potential explanation of this variation is that in resolvase this region is involved in contacts between crossover site and accessory site-bound resolvase, whereas in Hin the corresponding region contacts Fis (which is constant) (Merickel *et al.*, 1998). This region in Hin may have evolved to contact Fis in order to increase its specificity for inversion. Thus, the very different sequence of Hin may have evolved to assume a similar role (i.e. contacting "accessory or enhancer"-bound proteins, which specify alignment). This hypothesis reveals a second use of the Hin-substituted resolvase mutant. By replacing this region of resolvase, it may be possible to test whether the hybrid resolvase would respond to the enhancer/Fis complex (Fig. 4.3). In the presence of a suitable substrate and Fis, the enhancer/Fis complex may substitute for the accessory sites in activation of site-I bound resolvase.

## 4.2 Construction of (96-105 Hin) resolvase

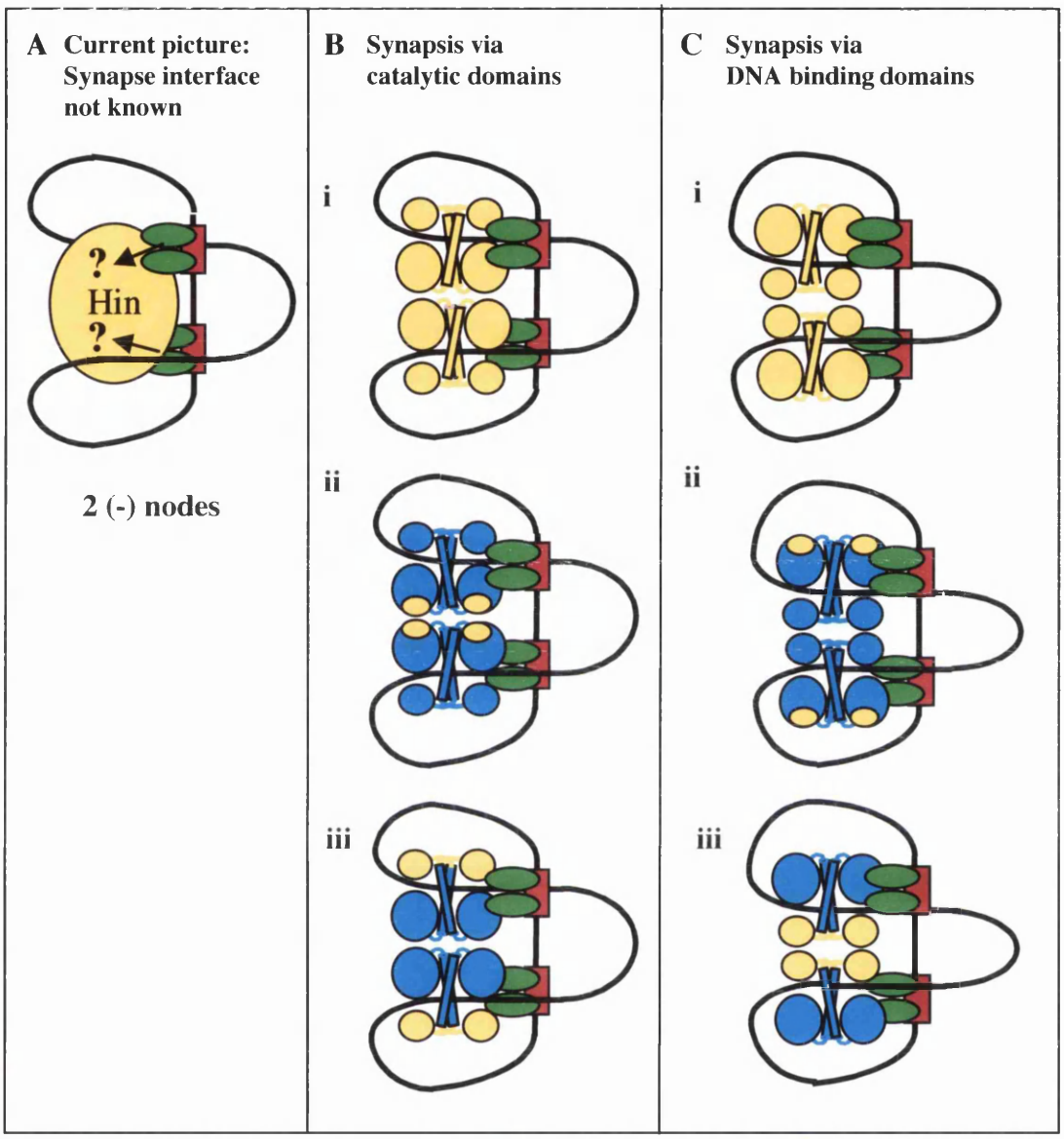
The Tn3 resolvase (96-105 Hin) mutant was constructed by replacing the *Cla*I-*Bst*EII region of the resolvase open reading frame (within pAT5Δ) with synthetic oligonucleotides containing the six codon changes as well as a novel *Sac*I restriction site (Table 1, Chapter 2). Residues 96-105 were replaced, resulting in a total of 6 amino acid substitutions spanning the β strand 5 and the beginning of helix E (Fig. 4.2).

**Fig. 4.2 Alignment and structure of residues (96-105) of γδ resolvase**




**A.** Alignment of γδ and Tn3 resolvase residues (96-105) with homologous residues of the invertase Hin. Amino acids which differ between Tn3 resolvase and Hin are shown in bold.
**B.** The γδ resolvase co-crystal structure is displayed as in chapter 3. Residues G96, S98, D100, G101, E102 and Q105 are shown in orange (spacefill).

**Fig. 4.3 Suggested models for the Hin invertasome and a hypothetical resolvase invertasome**




**A.** The protein contacts and synaptic interfaces within the Hin invertasome are unknown.
**B.** Synapsis of Hin dimers via their catalytic domain (i), and possible structures of invertasomes formed by resolvase with either the (96-105) region (ii) or the C-terminal domain (iii) replaced with Hin sequence.
**C.** Displays synapsis of Hin dimers via their C-terminal domain (Merickel *et al.* 1998) (i), and possible structures of invertasomes formed by resolvase as above.

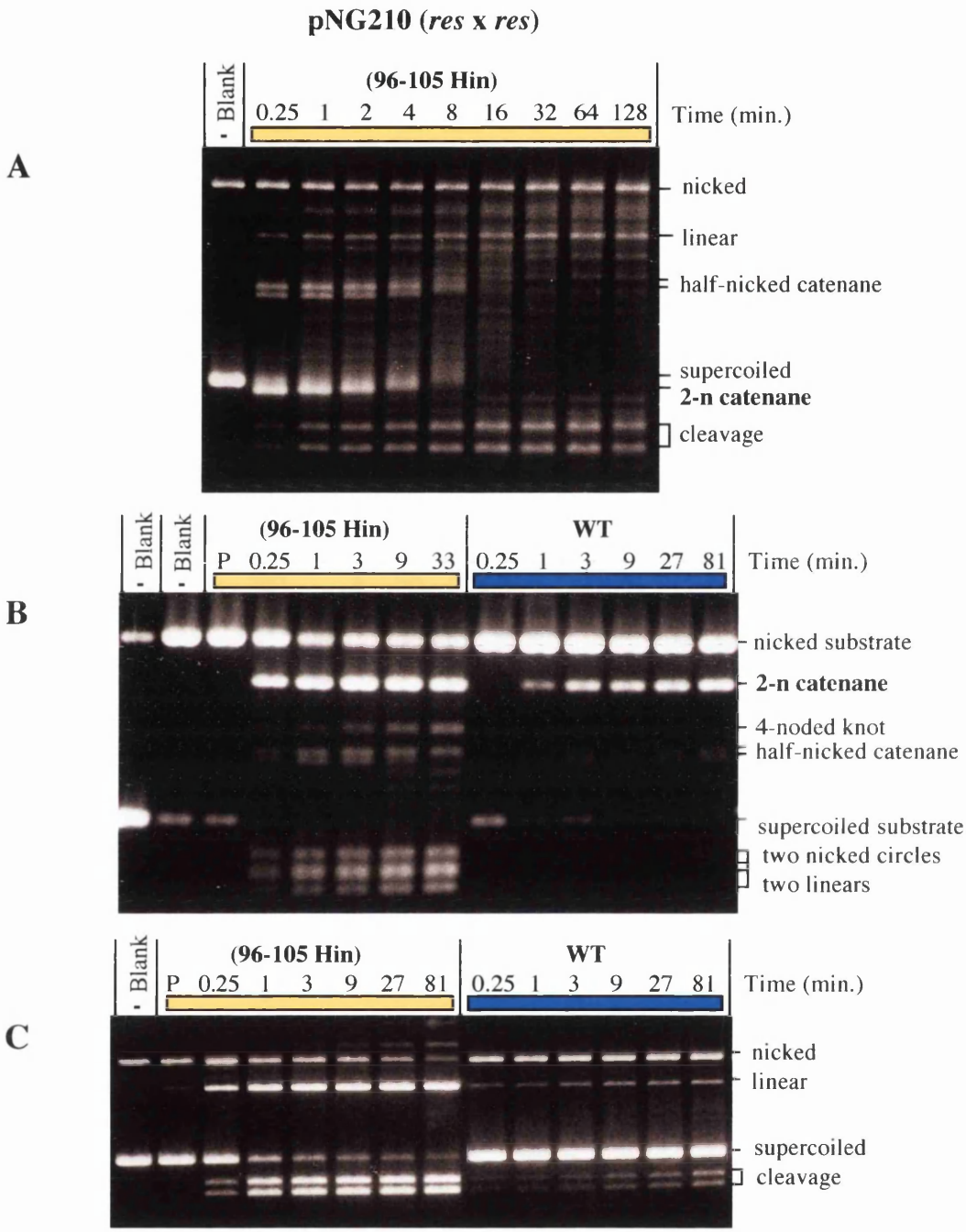
## 4.3 Characterisation of Tn3 resolvase (96-105 Hin)

*In vivo*, Tn3R (96-105 Hin) showed a phenotype surprisingly similar to wild-type (in light of the *in vitro* data reported below), (Table 3, Chapter 2). The protein was successfully purified to near homogeneity by the "standard" protocol (described in Chapter 2).

Initial assays on a *res* x *res* substrate under a variety of recombination conditions (C7.5, C8.2 and C9.4) demonstrated that Tn3R (96-105 Hin) is highly active *in vitro*. Restriction analysis showed that resolution products (LR joined) and some cleavage products were generated. This was further investigated by following the kinetics of the reaction of pNG210 (*res* x *res*) with Tn3R (96-105 Hin) (Fig. 4.4, A). When the reaction products were analysed (without further treatment), the first time point (15 sec.) revealed 2-noded catenane as the major product. This supercoiled 2-noded catenane was depleted over time (becoming undetectable after approximately 8-16 minutes) and a series of slower-migrating bands appeared. Furthermore, a slow accumulation of cleavage products was observed. In a second time-course experiment (Fig. 4.4, B), the samples were DNaseI-treated to reveal their topologies. Again, a species migrating as nicked 2-noded catenane was detected at the first time point (15 sec.). This remained the most prominent species until the final time point (33 min.) indicating that the majority of the slower-migrating bands seen in the uncut samples are probably relaxed forms of the 2-noded catenane. However, a slow accumulation of 4-noded knot, unlinked circles and cleavage products was detected at the later time points. This suggests that Tn3R (96-105 Hin) acts on the 2-noded catenane product (albeit at a slower rate than on the circular substrate) giving further rounds of recombination (in the case of the 4-noded knot) or dissociative products (in the case of the unlinked circles and linear cleavage products). Furthermore, a comparison with wild-type resolvase indicated that the Tn3R (96-105 Hin) catalyses resolution at a faster initial rate (Fig. 4.4, B). A possible reason for the increased reaction rate of Tn3R (96-105 Hin) is that it initiates cleavage faster than wild-type resolvase. A time-course under cleavage conditions was performed, which showed that under these conditions pNG210 was cleaved much faster by Tn3R (96-105 Hin) than WT resolvase (Fig. 4.4, C).

Although mutations in the region surrounding and including D102 are associated with a gain-of-function phenotype (accessory site independent behaviour), the increase in reaction

## Fig. 4.4 Kinetics of recombination by Tn3R WT and Tn3R (96-105 Hin)




**A.** Time course was set up in recombination buffer (C8.2) on a *res* x *res* substrate (pNG210) at 37°C. Aliquots were removed and added to SDS loading dye (1/4 volume) with proteinase K (50 $\mu$g/ml final) to stop the reaction. Samples were analysed uncut.

**B.** Time courses were performed as above with (96-105 Hin) resolvase and wild-type resolvase except that the reaction temperature was 20°C. Aliquots were removed and added to nicking mix containing ethidium bromide and DNase I. Samples were nicked and analysed on a 0.7% agarose gel.

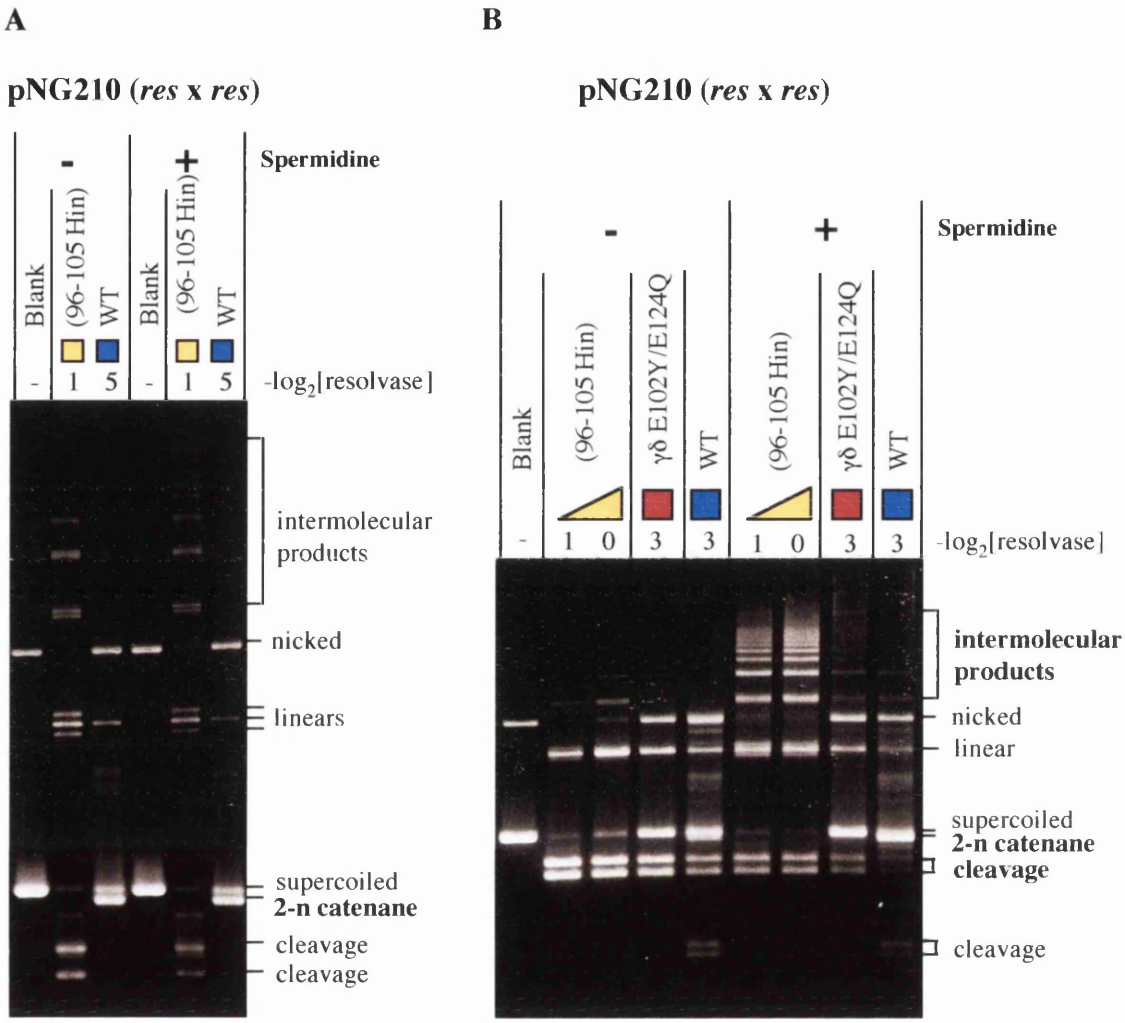
**C.** Time couses were set up in cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA) at 37°C. Aliquots were removed and added to SDS loading dye (1/4 volume) with proteinase K (50 $\mu$g/ml final) to stop the reaction.

For each experiment a control sample was prepared in which resolvase was added to the pre-mixed reaction buffer/SDS loading dye with proteinase K, in order to monitor the effectiveness of the stopping procedure (labelled P for Pseudoblank). All experiments were performed with pNG210.

rate with Tn3R (96-105 Hin) was not anticipated. Its behaviour on a *res* x *res* substrate was further investigated under different "permissive" reaction conditions (Fig. 4.5). Reactions with Tn3R (96-105 Hin) yielded substantially more cleavage-ligation products than either wild-type resolvase or γδR E102Y/E124Q. These are indicative of dissociative reactions in which half-sites covalently linked to resolvase are re-ligated giving LL, LR and RR recombinants and intermolecular products. Tn3R (96-105 Hin) was also active on the small proportion of nicked substrate present in the plasmid DNA. Again, neither Tn3R WT nor activated γδR E102Y/E124Q displayed a loss of supercoil dependency under these conditions.

The behaviour of Tn3R (96-105 Hin) showed that the absence of wild-type sequence in this region allows this mutant to bypass some of the normal requirements for recombination (such as supercoiling). Also, the time-course experiments have revealed that this mutant, unlike WT, was active on the product of resolution (2-noded catenane) showing a further loss of the wild-type regulatory mechanisms. The activity of Tn3R (96-105 Hin) was therefore tested on a variety of "non-standard" substrates to investigate its requirement for *res* sites in direct repeat, *res* sites in *cis*, supercoiling, and accessory sites (Figs. 4.6-10). Tn3R (96-105 Hin) was found to be active on all substrates tested. These included pMA2631, which carries two *res* sites in inverted repeat (Fig. 4.6, A and B). Under standard recombination conditions (which largely suppressed dissociative behaviour) activity was low. Nevertheless, it was not limited to cleavage activity, but included recombination products. Approximately equal amounts of resolution (LL + RR) and inversion products (LR) were observed. From this analysis it could not be determined whether these products were concerted (with cleavage and ligation proceeding without dissociation of the synaptic structure) or dissociative. Intermolecular products generated by Tn3R (96-105 Hin) with pMS7, which contains only a single *res* site, indicated that Tn3R (96-105 Hin) no longer requires two *res* sites in *cis* (Fig. 4.6, C and D). Reactions with linear *res* substrates, pMA21 (*res*), under standard recombination conditions again showed products generated by (96-105 Hin) resolvase (and to a lesser extent by the activated γδ resolvase E102Y/E124Q) (Fig. 4.7, A and B). "Resolution" products (LR) exceeded "inversion" products (LL and RR), suggesting that a synaptic structure is formed. This is consistent with the behaviour of previously characterised hyperactive mutants including γδR E102Y/E124Q. Finally, reactions with a substrate containing only crossover sites were performed (Fig. 4.7, C and D). Compared with the previous substrates, the pAL221*sis* reaction by Tn3R (96-105 Hin)

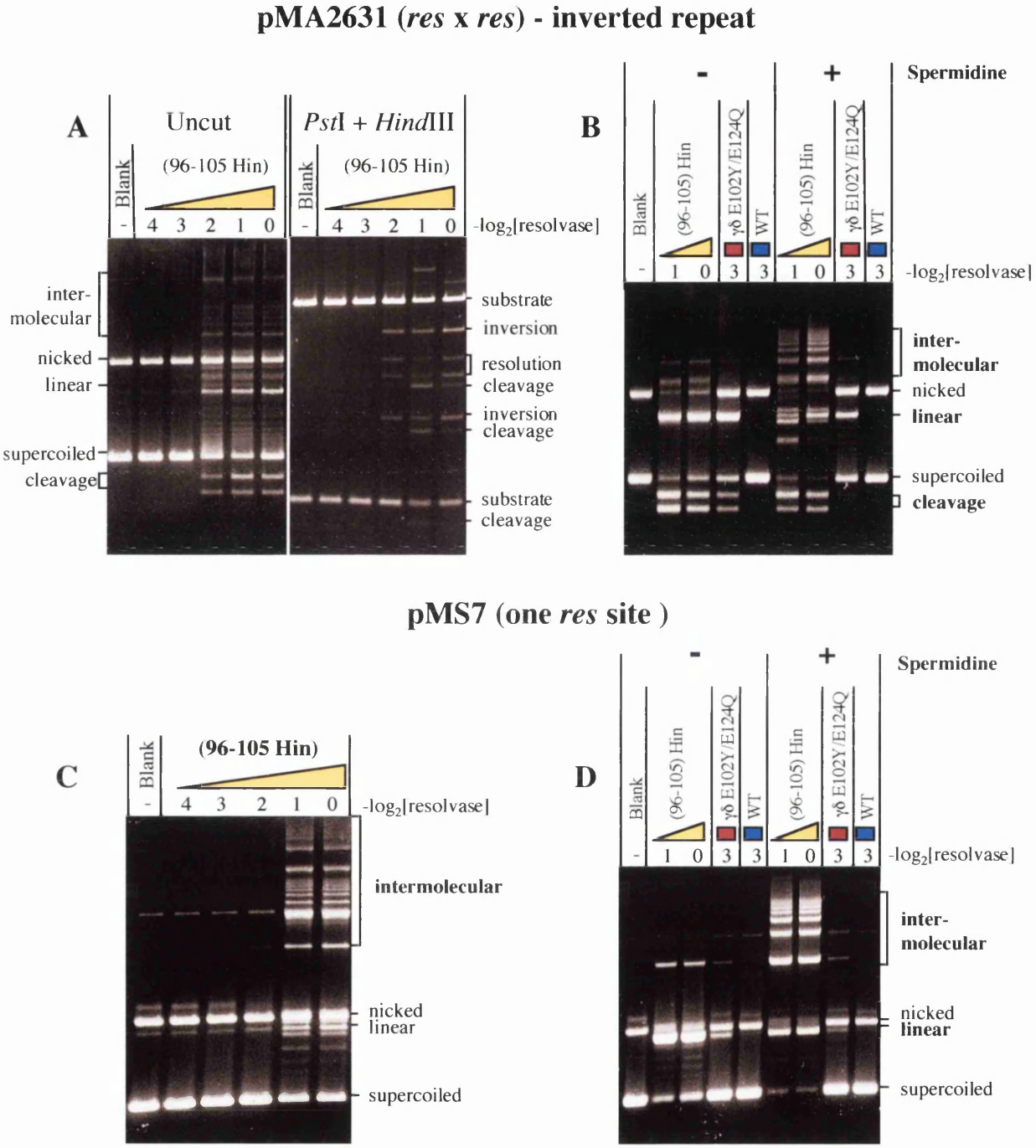
## Fig. 4.5 Dissociative recombination by Tn3 R (96-105 Hin)




**A.** Reactions were set up with pNG210 (*res* x *res*) in cleavage buffer supplemented with magnesium chloride in the presence or absence of spermidine (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 10 mM $MgCl_2$, 0.1 mM EDTA ± 5 mM spermidine). Dilutions were made of fractions at the following concentrations (undiluted); 5 $\mu$M Tn3R (96-105 Hin) and 7.5 $\mu$M Tn3R WT. Reactions were set up with (1/10 volume) additions of resolvase dilutions. Reactions were incubated for 2 hours at 37°C. Samples were treated with proteinase K and run uncut on a 1.1% agarose gel.

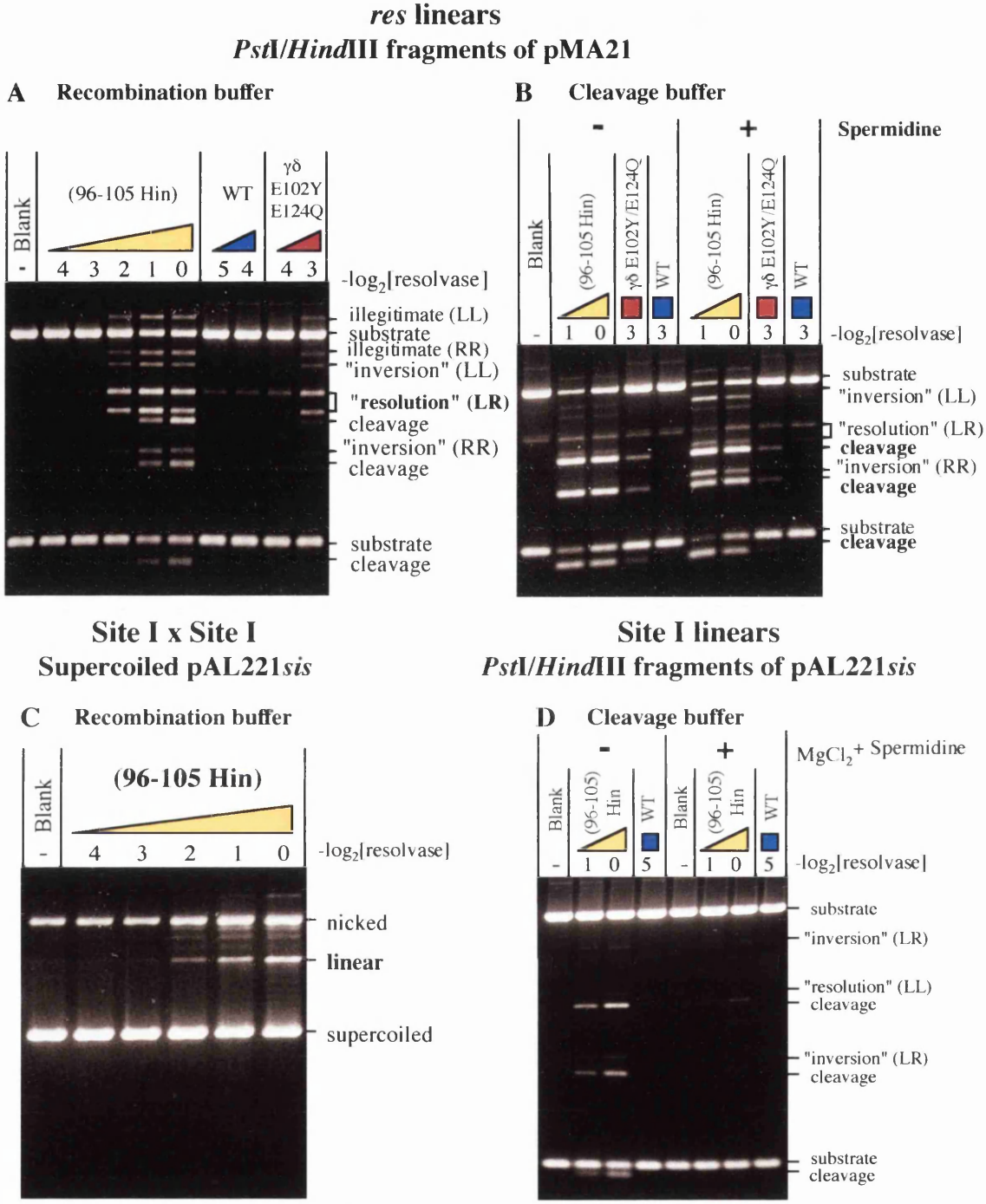
**B.** Reactions were set up with pNG210 (*res* x *res*) in cleavage buffer in the presence or absence of spermidine (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA ± 5 mM spermidine). Dilutions were made of fractions at the following concentrations (undiluted); 5 $\mu$M Tn3R (96-105 Hin), 10 $\mu$M γδR E102Y/E124Q and 7.5 $\mu$M Tn3R WT. Reactions were set up with (1/10 volume) additions of resolvase dilutions. Reactions were incubated for 4 hours at 37°C. Samples were treated with proteinase K and run uncut on a 1.1% agarose gel.

## Fig. 4.6 Tn3R (96-105 Hin) recombines *res* sites in inverted repeat




**A + C.** Reactions were set up in recombination conditions (C8.2) for 24 hours at 37°C with pMA2631 (A) and pMS7 (C). pMA2631 reactions were analysed uncut and restricted with *Pst*I and *Hind*III.
**B + D.** Reactions were set up in cleavage conditions (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 10 mM $MgCl_2$, 0.1 mM EDTA) ± 5 mM spermidine for 4 hours at 37°C with pMA2631 (B) and pMS7 (D). Dilutions were made of fractions at the following concentrations (undiluted); 5 $\mu$M Tn3R (96-105 Hin), 6 $\mu$M γδR E102Y/E124Q and 7.5 $\mu$M Tn3R WT. Dilutions were added at 1/10 volume. All samples were treated with proteinase K (50 $\mu$g/ml) prior to loading on an agarose gel.

## Fig. 4.7 Tn3R (96-105 Hin) shows supercoil- and accessory site independent activities




**A.** Reactions with *Pst*I and *Hind*III linearised pMA21 (*res* x res) were set up in recombination conditions (C8.2) for 24 hours at 37°C.

**B.** Reactions with *Pst*I and *Hind*III linearised pMA21 were performed in cleavage conditions (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA ± 5 mM spermidine) for 4 hours at 37°C.

**C.** Reactions were set up with pAL221*sis* (site I x site I) in recombination conditions (C8.2) for 24 hours at 37°C.

**D.** The substrate, pAL221*sis* (site I x site I), was linearised using *Pst*I and *Hind*III prior to the resolvase reaction. The resolvase reactions were performed in cleavage conditions (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA ± 10 mM $MgCl_2$ and 5 mM spermidine) at 37°C for 4 hours.

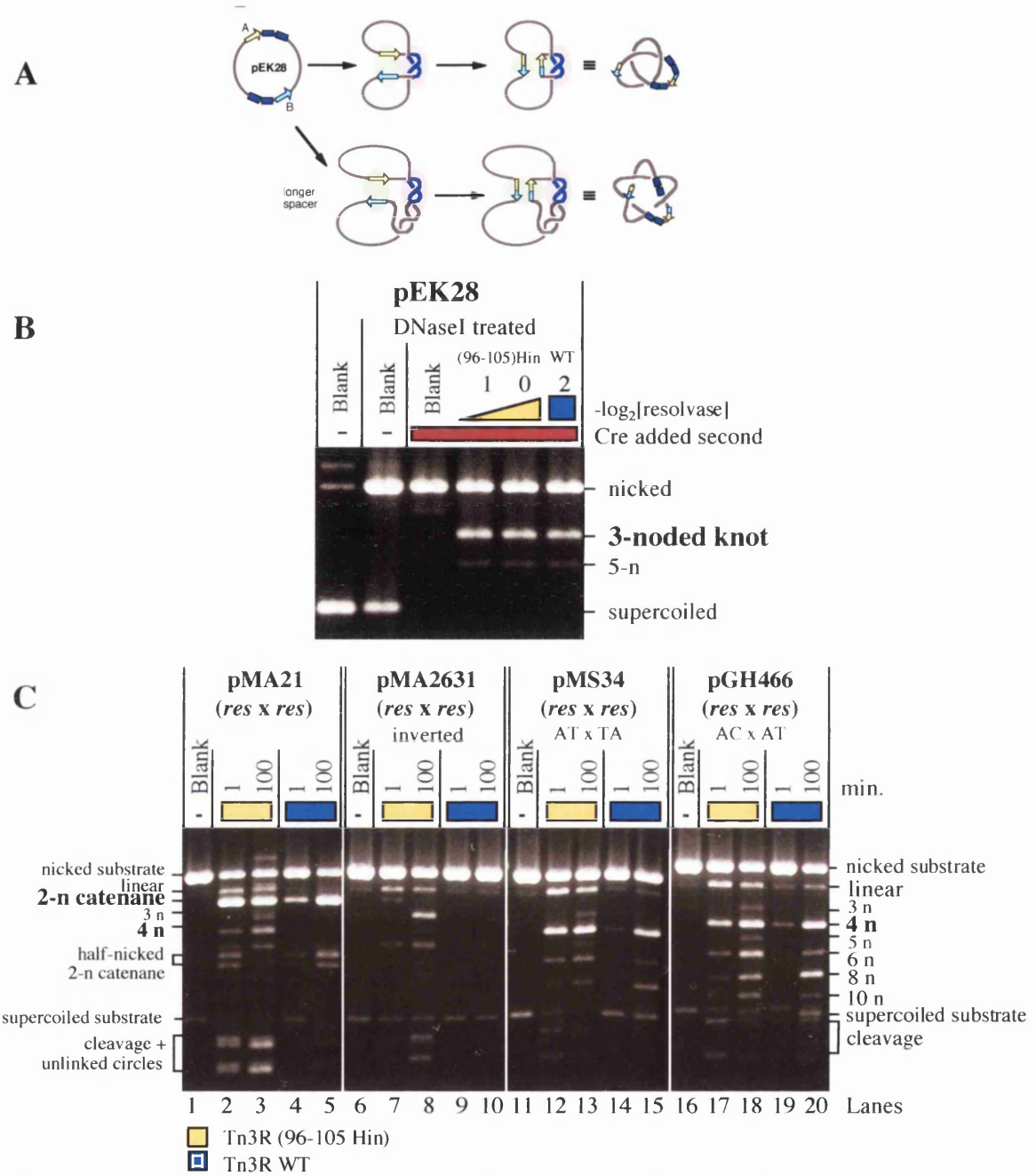
Dilutions were made of fractions at the following concentrations (undiluted); 5 $\mu$M Tn3R (96-105 Hin), 6 $\mu$M γδR E102Y/E124Q and 7.5 $\mu$M Tn3R WT. Dilutions were added at 1/10 volume. All samples were treated with proteinase K (50 $\mu$g/ml).

was the least efficient, generating a small amount of cleavage product over 24 hours under recombination conditions. Recombination products were barely detectable upon restriction enzyme digestion with *Pst*I and *Hind*III (data not shown). Nevertheless, Tn3R (96-105 Hin) was able to cleave linears with a single crossover site under cleavage conditions (Fig. 4.7, D). Thus Tn3R (96-105 Hin) was found to have lost wild-type resolvase selectivities including the requirement for supercoiling, for accessory sites, for sites in *cis* and for sites in direct repeat.

Nevertheless, the time-course experiments showed that Tn3R (96-105 Hin) produces 2-noded catenane product and therefore is necessarily capable of wrapping the accessory sites, if present, and trapping three negative supercoils. Its ability to wrap the accessory sites was also tested using the Cre-resolvase synapsis assay (Fig. 4.8, A and B). Addition of Tn3R (96-105 Hin) yielded as much 3-noded knot product as observed with wild-type resolvase. Its ability to synapse the accessory sites has not been perturbed, neither increasing nor decreasing the synapsis rate/stability as detectable by this method.

The original purpose of this Hin-substituted resolvase mutant was to investigate crossover site synapsis. This synapse was proposed to depend upon the synapsis of resolvase dimers via an interdimer interface involving residues (96-105). The crossover site synapsis requirement of Tn3R (96-105 Hin) was tested using a standard substrate lacking one crossover site (pSJH17, *res* x II/III). Wild-type resolvase is completely inactive on this substrate in both cleavage and recombination conditions (Fig. 4.9). In contrast, this substrate was cleaved by Tn3R (96-105 Hin), revealing that it has lost the wild-type selectivity for the presence of two crossover sites per molecule. A number of other activated Tn3 and γδ resolvase mutants were also assayed with pSJH17 under cleavage conditions (Fig. 4.9). These were also able to linearise the substrate, showing that this property may be a general feature of activated mutants. Notably, both Tn3 and γδ resolvases carrying only the E124Q mutation were active on pSJH17, indicating that this activity does not require mutations in the 102 region of resolvase. Reactions of Tn3R (96-105 Hin) with pMS7, which contains only a single *res* site, generated similar amounts of the linear cleavage product (described previously). Therefore, the reaction of Tn3R (96-105 Hin) with pSJH17 may either be site I synapsis-independent, accessory site synapsis-independent or due to intermolecular synapsis. Nevertheless, the ability of (96-105 Hin) resolvase to cleave one crossover site in the absence of a second crossover site complicated the originally planned targeting experiments.

## Fig. 4.8 Synapsis assay and topological analysis of Tn3R (96-105 Hin) reactions

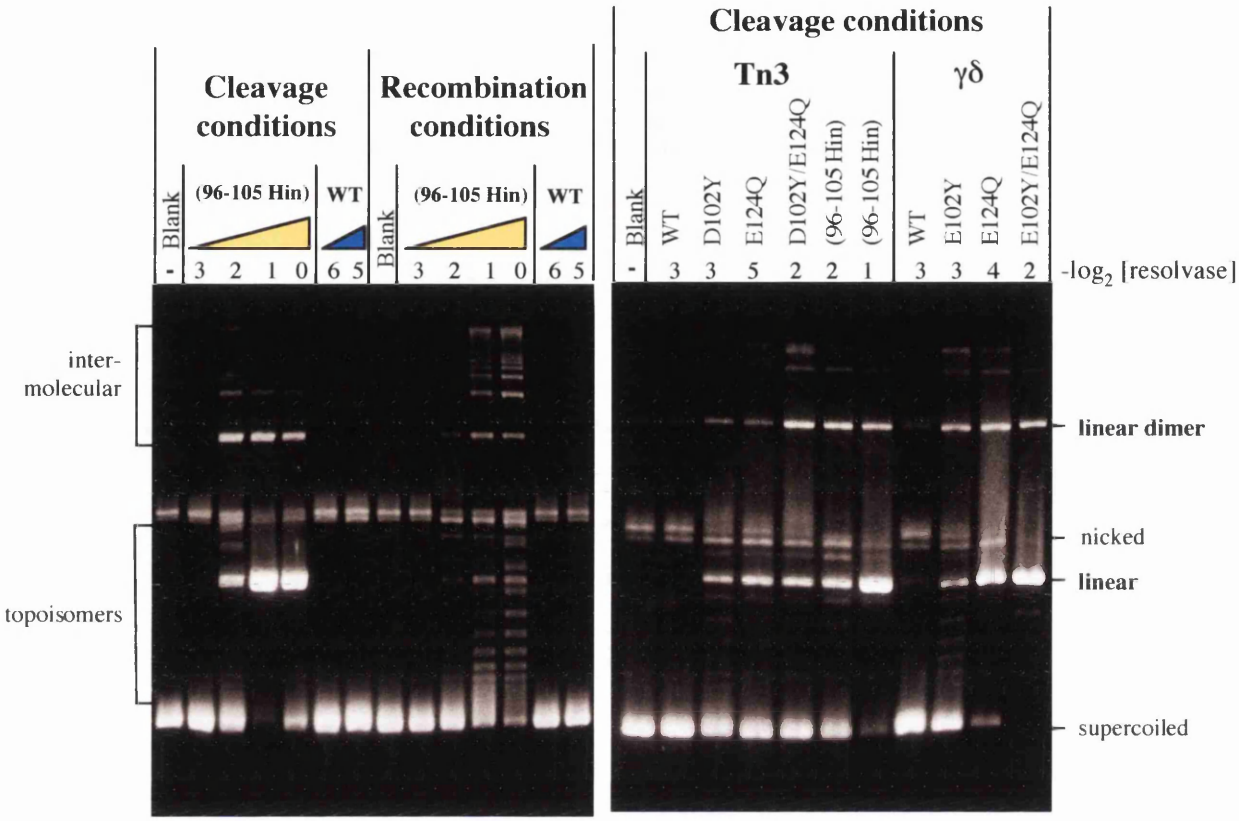



**A.** In the presence of resolvase, Cre recombines pEK28, producing 3-noded and (to a lesser extent) 5-noded knots. In the absence of resolvase, the Cre reaction product is an unknotted circle (not shown).

**B.** Reactions were set up with pEK28 in NEB buffer 1. Resolvase fractions were at the following concentrations; 5 $\mu$M Tn3R (96-105 Hin) and 7.5 $\mu$M Tn3R WT. Dilutions of resolvase fractions (1/20 volume) were added first, allowing resolvase to bind the accessory sites (15 min. at room temperature). Cre, (1/20 volume), was added second and incubated for 25 min. at room temperature. The reactions were stopped by heating to 75°C for 5 min. The samples were nicked using DNaseI and treated with proteinase K.

**C.** Reactions were performed in recombination buffer (C8.2) at room temperature. Time-course reactions were started by adding (1/10 volume) of 1.3 $\mu$M Tn3R (96-105 Hin) and 0.9 $\mu$M Tn3R WT dilutions. Samples were stopped using ethidium bromide, nicked and treated with proteinase K.

## Fig. 4.9 Tn3R (96-105 Hin) does not require two crossover sites in *cis*




Reactions with pSJH17 (*res* x sites II/III) were set up in cleavage conditions (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA) or recombination conditions (C8.2) for 2 hours at 37°C. Concentrations of resolvase fractions (undiluted) were as follows; 7.5 $\mu$M Tn3R WT, 5 $\mu$M Tn3R (96-105 Hin), 10 $\mu$M Tn3R D102Y, 50 $\mu$M Tn3R E124Q, 3 $\mu$M Tn3R D102Y/E124Q, 9 $\mu$M γδR WT, 6 $\mu$M γδR E102Y, 12 $\mu$M γδR E124Q, and 6 $\mu$M γδR E102Y/E124Q. Dilutions were added at 1/10 volume. Reactions were stopped with proteinase K (50$\mu$g/ml).

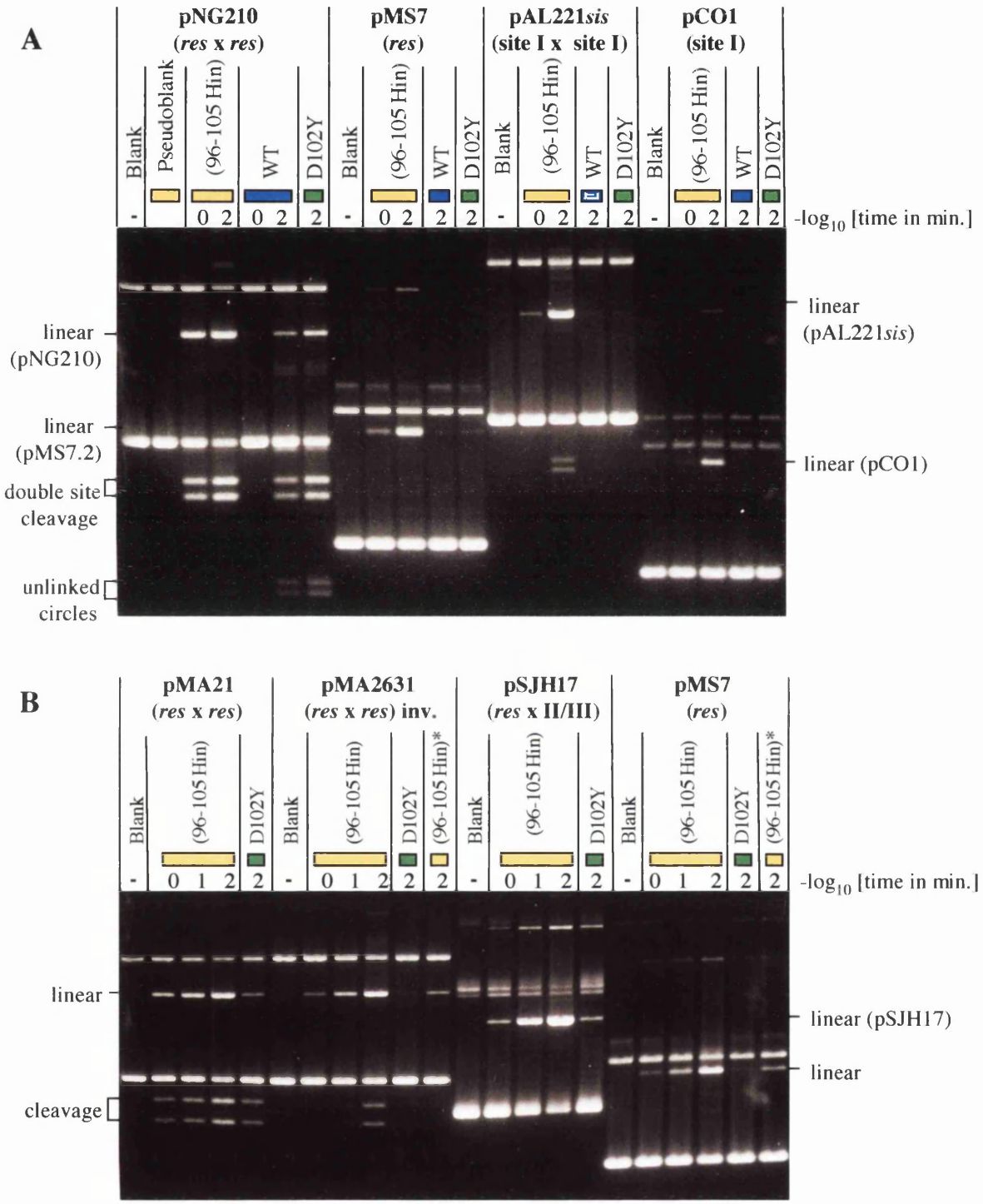
Numerous targeting experiments to address this issue were performed without avail. Thus, the question of compatibility of these surfaces remains.

Nevertheless, this mutant provided the opportunity to directly compare the cleavage activity on substrates carrying two recombination sites in *cis* with substrates carrying only a single copy of a recombination site. This allows the effect of synapsis on the cleavage activity to be investigated. Tn3R (96-105 Hin) activity was tested in cleavage conditions with pNG210 (*res* x *res*), pMS7 (*res*), pAL221sis (site I x site I) and pCO1 (site I) (Fig. 4.10, A). The reaction of (96-105 Hin) resolvase with pNG210 was very efficient, generating more cleavage products within 1 minute than wild-type resolvase generated in 100 minutes. D102Y was also more active than wild-type resolvase; D102Y and WT also gave traces of recombination products (2-noded catenane and unlinked circles). With pMS7 (*res*) as a substrate, the cleavage reaction was much slower for Tn3R (96-105 Hin) and D102Y (which produced only a trace of linear product in 100 minutes), and completely absent for wild-type resolvase. This indicates that intermolecular synapsis is slower or completely inhibited (as compared to synapsis in *cis*). It is not possible to determine whether cleavage of the single *res* site (yielding the linearised full-length product observed) occurs within the context of an intermolecular synapse or in an non-synapsed context. However, it is clear that synapsis activates Tn3R (96-105 Hin) to initiate cleavage (either all observed activity occurs by synapsis-independent cleavage, in which case the rate is much slower than cleavage of pNG210, or none of the observed activity is due to synapse-independent cleavage, making synapsis infinitely activating). The absence of accessory sites also has a profound effect on Tn3R (96-105 Hin). Reactions with pAL221*sis* (site I x site I) reveal that cleavage of this substrate is considerably slower than cleavage of pNG210 (*res* x *res*). Furthermore, the cleavage products generated when both sites within one molecule are cut are far less abundant than the full-length linear product in which only a single site has been cut. The cleavage reaction of pNG210 with WT resolvase shows the pattern of cleavage expected if cleavage of the synapsed sites is concerted, that is, the rate of cleavage at one site increases if its partner site is cleaved. Although cleavage of pNG210 by Tn3R (96-105 Hin) is less concerted than the wild-type reaction it is clearly more concerted than the equivalent reaction by Tn3R (96-105 Hin) on a substrate lacking accessory sites (pAL221*sis*). This non-concerted behaviour could represent synapsis-independent activity. Alternatively, this behaviour could result from an unstable crossover site synapse: dissociation of the synapse following cleavage at one site would release supercoiling and the second site would be less

likely to be cleaved. In fact, approximately 40% of the supercoiled plasmid was linearised (cut at one site), whereas only approximately 15% of this amount was cleaved at both crossover sites. The observation that the activity of Tn3R (96-105 Hin) on pCO1 (site I) was lower than on pAL221*sis* (site I x site I) indicated that site I synapsis increases the reaction rate. This finding further supports the hypothesis that Tn3R (96-105 Hin) is able to synapse isolated crossover sites in *cis*.

A further kinetic experiment was done to look at the effects of the accessory sites (orientation, presence or absence) on the rate and concertedness of the cleavage reaction (Fig. 4.10, B). A comparison of the rate and concertedness of cleavage by Tn3R (96-105 Hin) of the substrates pMA21 (*res* x *res*, direct repeat) and pMA2631 (*res* x *res*, inverted repeat), revealed that the reaction with pMA21 was both faster and more concerted. Intramolecular synapsis of inverted repeat *res* sites is thought to be difficult on topological grounds. The observed differences in reaction rates and concertedness between the direct and inverted repeat substrates therefore indicate that under cleavage conditions, Tn3R (96-105 Hin) is able to synapse a proportion of the substrate. D102Y, however, was almost completely inert (a trace of full-length linear product can be seen) with the inverted repeat substrate, pMA2631. Nevertheless, cleavage activity by this "mildly" activated mutant was clearly detectable with the direct repeat substrate, pMA21. D102Y thus seems more dependent upon synapsis for cleavage than Tn3R (96-105 Hin). In contrast, the reaction with pSJH17 (*res* x II/III) is very efficient, with full-length linear product appearing within 1 minute and the majority of the substrate being cleaved in 100 minutes. In fact, comparison with pMA21 reveals that the absence of one set of crossover sites does not seem to change the reaction rate. D102Y resolvase is also more active with this substrate than with pMA2631. This indicates that for activated mutants, crossover site synapsis only has a stimulatory effect in the absence of accessory site synapsis. In the presence of directly repeated accessory sites, site I synapsis is less important. The activating effect of intramolecular accessory site synapsis is also seen in the comparison of the reaction with pSJH17 (*res* x II/III) and pMS7 (*res*). For both Tn3R (96-105 Hin) and D102Y, the reaction with pMS7 (*res*) is considerably slower. Although a plasmid with a single *res* site is cleaved at a faster rate than a plasmid with a single crossover site, this is possibly entirely due to the cooperative binding of resolvase at a full *res* site (Fig. 4.10, A and B).

## Fig. 4.10 Effects of accessory sites on cleavage activity of Tn3R (96-105 Hin)




The reactions were set up in cleavage conditions (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 10 mM $MgCl_2$, 0.1 mM EDTA) at 18°C (A) and at 20°C (B). Resolvase dilutions were made at the following concentrations; 2.5 $\mu$M Tn3R (96-105 Hin), 7 $\mu$M Tn3R (96-105 Hin)*, 0.9 $\mu$M Tn3R WT, and 1.3 $\mu$M Tn3R D102Y. These dilutions were added at 1/10 volume. Reactions were stopped with proteinase K (50 $\mu$g/ml).

A control sample was prepared (labelled Pseudoblank, A) in which resolvase was added to the pre-mixed reaction buffer/SDS loading dye with proteinase K, in order to monitor the effectiveness of the stopping procedure.

Although the difference between WT and the hyperactive Tn3R (96-105 Hin) is most dramatic under cleavage or "permissive" recombination conditions, it was evident that reactions under standard recombination conditions would be amenable to topological analysis. The activity of Tn3R (96-105 Hin) on a variety of *res* x *res* substrates was studied (Fig. 4.8, C). Once again, its ability to recombine pMA21 (*res* x *res*, direct repeat) considerably faster than WT resolvase could be seen at the 1 minute time point. The slow accumulation of the 4-noded knot (a product which is undetectable in the wild-type reaction) indicates either that strand exchange is processive or that the first round 2-noded catenane product is used in a further round of recombination. Topological analysis of the reaction with pMA2631 (*res* x *res*, inverted repeat), showed a slow (compared with the pMA21 reaction) appearance of 3-noded and 5-noded inversion species (LR) as well as some unlinked circles (resolution products, LL and RR). This mutant is therefore capable of synapsing the *res* sites and catalysing concerted strand exchange. Two mismatch substrates were also tested with Tn3R (96-105 Hin), pMS34 (Tn3 *res*, AT x TA) and pGH466 (γδ *res*, AC x AT). Once again, Tn3R (96-105 Hin) behaved differently from WT resolvase on these substrates. Firstly, some 3-noded and five-noded species were observed (though approximately at an equal ratio to the pMA21 reaction). Secondly, the pattern of iterative products was that of a processive resolvase (such as γδ resolvase, which catalysed multiple double rounds of strand exchange without dissociation of the synapse). Tn3 resolvase, however, gives a dissociative pattern of products (indicative of synapse dissociation between successive double non-recombinant strand exchange reactions) (Stark and Boocock, 1994), Fig. 1.14.

In summary, Tn3R (96-105 Hin) was found to be an activated resolvase mutant. With many substrates (such as supercoiled pMA2631) it was more active than γδR E102Y/E124Q, which was the most hyperactive resolvase mutant characterised at the time. However, its activity on a substrate lacking accessory sites (pAL221*sis* - site I x site I) was very low under recombination conditions (and was, in fact, lower than the activity seen with γδR E102Y/E124Q). Nevertheless, it was the first resolvase carrying mutations exclusively in the 102 region which could catalyse events on a site I x site I substrate (previously all site I x site I recombination required the E124Q mutation too) or indeed a linear site I substrate.

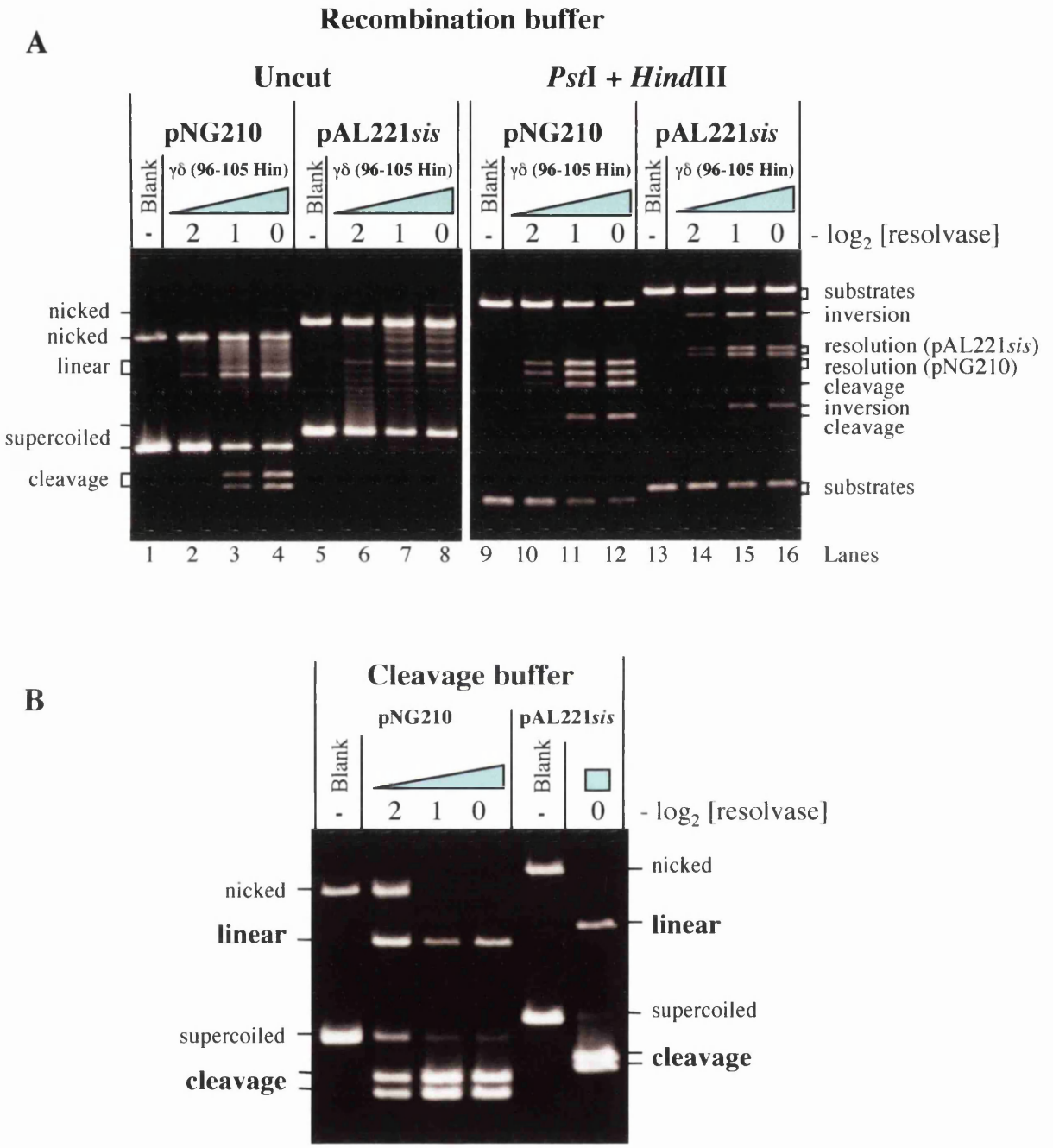
## 4.4 Characterisation of γδ resolvase (96-105 Hin)

It has been found that equivalent activating mutations, such as D/E102Y and E124Q, have a greater deregulating effect in γδ resolvase than in Tn3 resolvase. It was therefore predicted that γδR (96-105 Hin) would be more active than Tn3R (96-105 Hin). The γδR (96-105 Hin) was constructed in two stages (Table 2, Chapter 2). Encouragingly, this mutant was more active than its Tn3 resolvase equivalent in the *in vivo* assay, recombining a site I x site I substrate (Table 3, Chapter 2). It was therefore decided to proceed with the purification and characterisation of this mutant *in vitro*.

γδR (96-105 Hin) was assayed under recombination and cleavage conditions with pNG210 (*res* x *res*) and pAL221*sis* (site I x site I) (Fig. 4.11). Restriction enzyme analysis revealed that in recombination conditions, γδR (96-105 Hin) gave exclusively resolution (LR) products with pNG210 and both resolution and inversion products (in a 1:1 ratio) with pAL221*sis*. Surprisingly, the reaction with pNG210 showed a high level of cleavage products, which were absent from the site I x site I reactions. A possible reason for this could be that the resolution products from the pNG210 reaction are a substrate for cleavage, possibly in a synapsis-independent reaction (thus explaining the lack of ligation products). The absence of inversion products suggests that the *res* x *res* reaction proceeds via a correctly wrapped synaptic structure. Nevertheless, the overall efficiency of recombination did not differ greatly between pNG210 and pAL221*sis*. In this respect γδR (96-105 Hin) differs from Tn3R (96-105 Hin), which barely produced detectable levels of recombination of pAL221*sis* under identical conditions. When reactions were performed with pNG210 and pAL221*sis* in cleavage conditions, both substrates were cleaved close to completion in one hour (Fig. 4.11, B). Again, the reaction efficiencies were similar for the *res* x *res* and the site I x site I substrates. In both reactions the nicked circular substrate was also cleaved, revealing that γδR (96-105 Hin) is also supercoil-independent under these conditions.

To test the ability of γδR (96-105 Hin) to cleave and recombine non-supercoiled substrates, assays were performed on *Pst*I and *Hind*III linearised versions of these substrates (Fig. 4.12). In the absence of supercoiling, the recombination reactions proceeded much slower, especially with pAL221*sis*, for which products were barely detectable. A small amount of "inversion" (LL and RR) product was detectable, though the major recombinants were

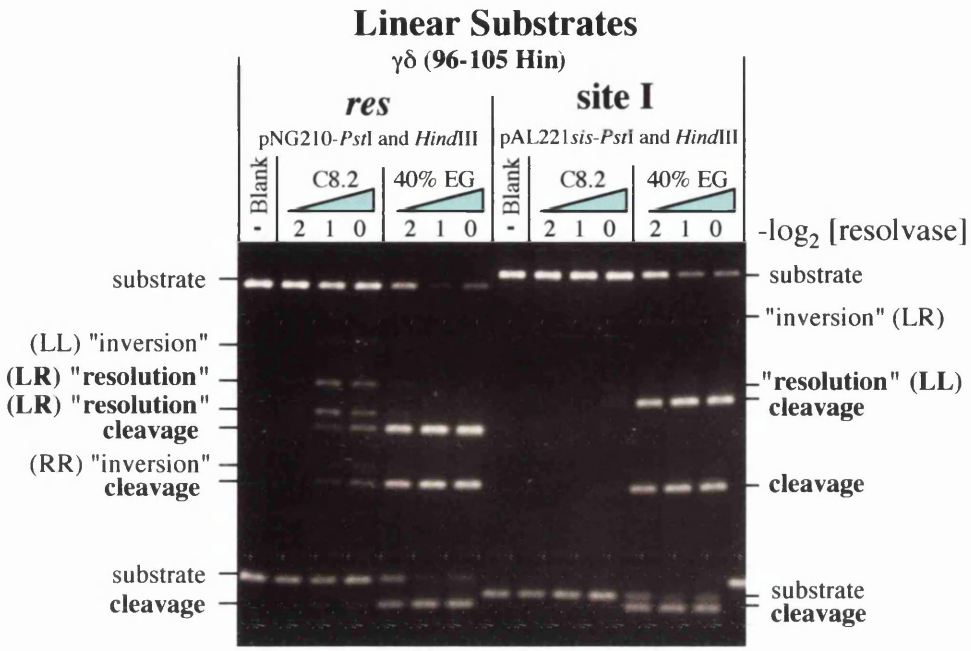
## Fig. 4.11 γδ resolvase (96-105 Hin) cleaves and recombines *res* sites and isolated crossover sites




**A.** Reactions were set up in recombination buffer (C8.2) for 1 hour at 37°C and stopped with proteinase K (50 μg/ml). Dilutions of a 1 μM stock solution of γδR (96-105 Hin) were added as shown (1/10 volume).

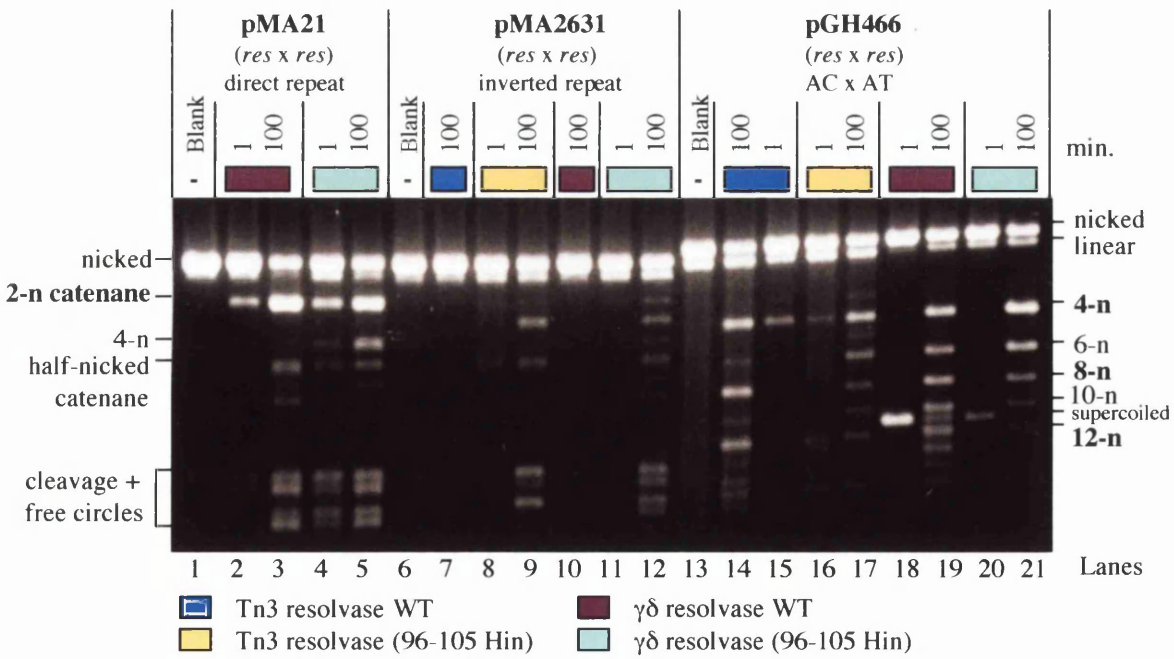
**B.** Reactions were set up in cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA) for 1 hour at 37°C and stopped with proteinase K (50 μg/ml). Dilutions of a 1 μM stock solution of γδR (96-105 Hin) were added as shown (1/10 volume).

## Fig. 4.12 γδ resolvase (96-105 Hin) is active on linear substrates




The substrates were linearised by restriction with *Pst*I and *Hind*III, purified (QIAGEN wizard clean-up resin) and eluted in TE buffer. Reactions were set up in recombination buffer (C8.2) or cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl, 0.1 mM EDTA) for 1 hour at 37°C and stopped with proteinase K (50 μg/ml). Dilutions of a 1 μM fraction of γδR (96-105 Hin) were added at 1/10 volume.

## Fig. 4.13 (96-105 Hin) substituted resolvase mutants - topological analysis




Time course reactions were set up in recombination buffer (C8.2) at room temperature. Resolvase dilutions were added at 1/10 volume. Samples were stopped using ethidium bromide, nicked and treated with proteinase K (50 μg/ml) prior to electrophoresis on a 0.7% agarose gel.

"resolution" products (LR). The products for the linearised pNG210 reaction therefore mostly had the same type of junctions (LR) to those generated when the substrate is supercoiled. γδR (96-105 Hin) could ensure that a left half-site is joined to a right half-site by recombining in an organised "normal" synapse or by dissociative cleavage and ligation (providing only left ends are joined to right ends). It is more likely that the reaction proceeds via a synaptic complex. The cleavage reactions were also efficient with linear substrates, yielding at least 80% cleaved substrate in one hour incubations (37°C). Again, a small difference in rate is now observed between the slightly more efficient *res* (pNG210) reaction and the site I (pAL221*sis*) reaction. This reveals that γδR (96-105 Hin) can still be activated by the accessory sites.

The effect of accessory sites on the topologies of γδR (96-105 Hin) recombination reactions was investigated using pMA21 (*res* x *res*, direct repeat), pMA2631 (*res* x *res*, inverted repeat) and pGH466 (*res* x *res*, AC x AT mismatch) (Fig. 4.13). γδR (96-105 Hin) activity on pMA21 was reminiscent of Tn3R (96-105 Hin). The first time point (1 min.) revealed that formation of 2-noded catenane resolution product is initially more rapid for the Hin-substituted mutant than for γδR WT. Furthermore, the late time point (100 min.) shows the accumulation of 4-noded iteration product. Therefore, like Tn3R (96-105 Hin), γδR (96-105 Hin) was found to have an increased reaction rate and gives increased amounts of processive products. Both versions of the (96-105 Hin-substituted) resolvases were found to be active on supercoiled pMA2631. As observed previously, the Tn3 version of the mutant gave 3- and 5-noded products. γδR (96-105 Hin) also yielded these products, but additionally some 2-noded catenane was observed. The Tn3R (96-105 Hin) reaction with the mismatch substrate, pGH466, was noticeably more processive than Tn3R WT. However, γδR WT is already processive under these conditions (pH 8.2). This is one of the few differences between Tn3 and γδ resolvases. It was therefore anticipated that the γδR (96-105 Hin) reaction would be even more processive, yielding large amounts of complex products (with large numbers of nodes). In fact, the opposite was observed. The reaction was equally efficient as the wild-type γδ resolvase reaction (as judged by the unreacted, nicked substrate). However, although the pattern of products was processive (the intensity of the consecutive products decreases in a regular fashion), the majority of the products had 4-, 6-, or 8-nodes, whereas the reaction with WT γδ resolvase yielded products of further rounds (giving products with greater than 12-nodes). It may be that the processivity observed with WT γδ resolvase already represents the maximum level of processivity attainable.

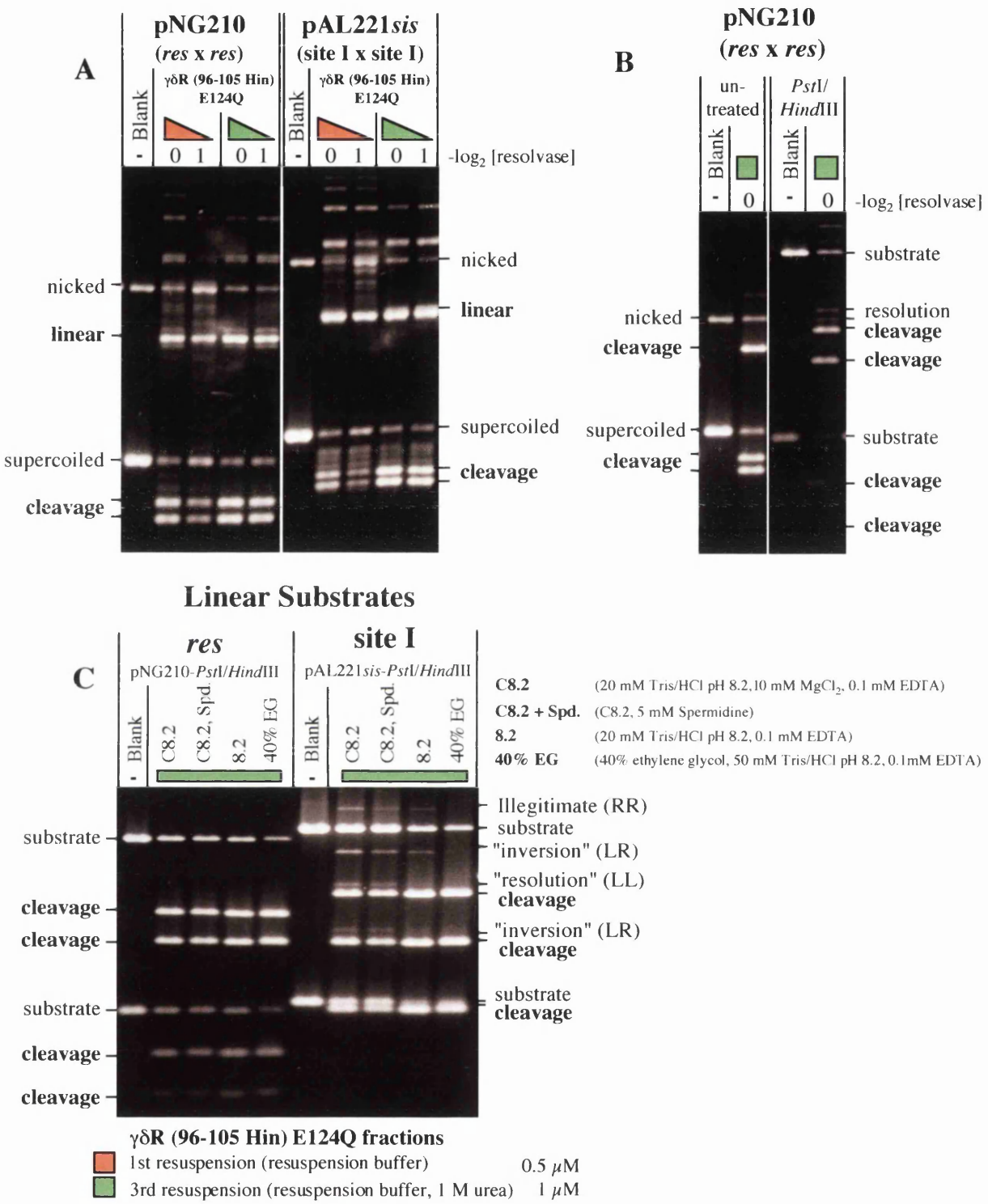
## 4.5 Characterisation of γδ resolvase (96-105 Hin)/E124Q

The E124Q mutation is known to stabilise the covalent intermediate. The remarkable cleavage activity of γδR (96-105 Hin), therefore prompted the construction of γδR (96-105 Hin) E124Q. It was predicted that the introduction of this mutation into the γδR (96-105 Hin) background would further stabilise this intermediate cleavage complex. Such a resolvase mutant would potentially be useful for co-crystallization trials of the cleavage intermediate.

Similar to γδR (96-105 Hin), γδR (96-105 Hin) E124Q showed a fully activated phenotype when assayed *in vivo* (WWW, Table 3, Chapter 2). This mutant fractionated very differently from WT during the standard purification procedures. It has been noted that activated resolvase are not overexpressed as well as wild-type resolvase. This was also the case for γδR (96-105 Hin) E124Q, and may be due to its negative effect on the host *E. coli*. The majority of the purification process proceeded as usual. However, most of the purified γδR (96-105 Hin) E124Q failed to precipitate at low concentrations of salt in the final dialysis (20 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1.2 mM PMSF). The fraction (approximately 20%) which precipitated proved difficult to resuspend in the standard buffer (containing 2 M NaCl). Numerous attempts were made to resuspend the protein, and by increasing the urea concentration the pellet was solubilised to varying degrees. It is possible that most of γδR (96-105 Hin) E124Q re-folded incorrectly when urea was removed in the previous step, and remained soluble in the low salt conditions. All soluble fractions, including the dialysis supernatant which contained the majority of the purified γδR (96-105 Hin) E124Q, were assayed *in vitro* and found to be highly active (Fig. 4.14).

Topological analysis was performed on resolution reactions by γδR (96-105 Hin) E124Q (*res* x *res*) in order to reveal whether the small amount of resolution product observed derives from a wild-type productive synaptic structure (Fig. 4.15, A). As anticipated, more than 80% of the products are cleavage products (in which the substrate has been cut at either one or both *res* sites). However, a small amount of 2-noded catenane recombinant was also detected. This strongly suggests that the resolution products obtained from this reaction were generated from a synapse which trapped three negative supercoils. This was confirmed in the accessory site synapsis assay (with the substrate, pEK28) (Fig. 4.15, B). The addition of γδR

## Fig. 4.14 γδR (96-105 Hin) E124Q activity on supercoiled and linear substrates



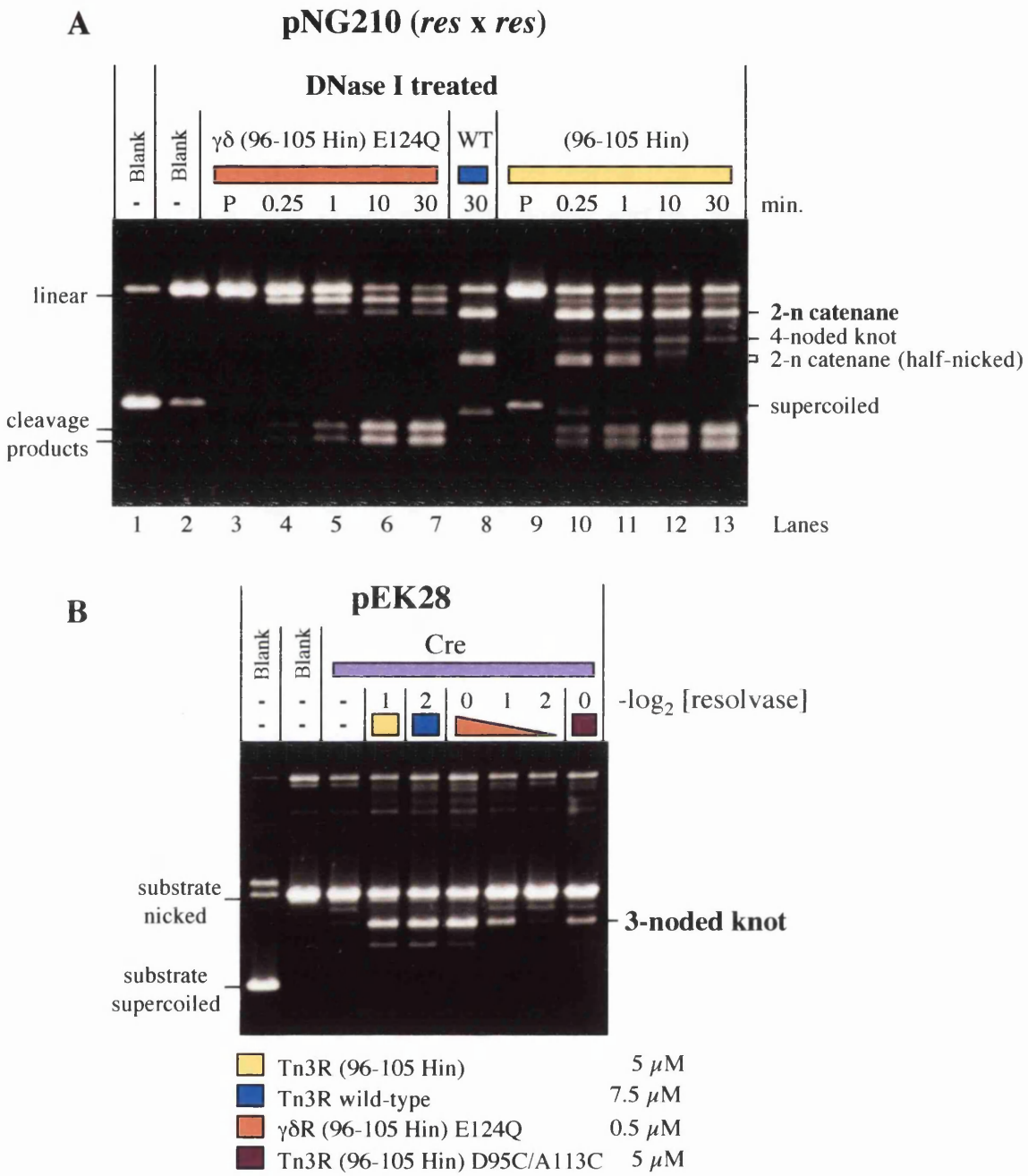

**A.** Reactions were set up with pNG210 (*res* x *res*) and pAL221*sis* (site I x site I) in recombination buffer (C8.2) for 3 hours at 37°C.

**B.** Reactions were set up with pNG210 in recombination buffer (C8.2) for 45 min. at 37°C. Samples were divided into aliquots which were analysed untreated and restriction digested with *Pst*I and *Hind*III.

**C.** Reactions were set up with *Pst*I and *Hind*III linearised pNG210 (*res* x *res*) and pAL221*sis* (site I x site I) in a variety of buffers desribed above for 1 hour at 37°C.

Resolvase dilutions were made from the fractions described above and added at (1/10 volume). All samples were treated with proteinase K (50 μg/ml) prior to electrophoresis.

**Fig. 4.15 γδ resolvase (96-105 Hin) E124Q can wrap accessory sites**




**A.** Topological analysis of time-course reactions. Reactions were set up with pNG210 (*res* x *res*) in recombination buffer (C8.2) at 37°C. Resolvase dilutions of the following concentrations, 0.5 μM γδR (96-105 Hin) E124Q, 0.9 μM Tn3R wild-type, and 1.3 μM Tn3R (96-105 Hin) were added (1/10 volume). Samples were stopped with ethidium bromide, treated with DNaseI and analysed on a 0.7% agarose gel. The pseudoblank (P) was performed by adding resolvase to a combination of the reaction mix and stop mix.
**B.** *lox-res* synapsis assay of (96-105 Hin)-substituted mutants of resolvase. Resolvase (1/20 volume) was added first and allowed to bind the accessory sites for 15 min. at room temperature. Cre (1/20 volume) was added second and incubated for 25 min. at room temperature. The reactions were stopped by heating to 75°C for 5 min. The samples were nicked using DNaseI and treated with proteinase K.

(96-105 Hin) E124Q to the Cre reaction produces 3-noded knot product, as with wild-type resolvase.

In order to facilitate crystallization of the cleavage intermediate using γδR (96-105Hin) E124Q resolvase it would be useful if a wide range of conditions could support cleavage activity. Assays were therefore performed under a variety of conditions with *Pst*I and *Hind*III linearised substrate pNG210 and pAL221*sis* (Fig. 4.14, C). Recombination activity was only completely suppressed in the presence of large amounts of ethylene glycol (40% ethylene glycol, 50 mM Tris/HCl, 0.1 mM EDTA). However, all conditions revealed the exceptional accumulation of cleavage intermediate by γδ resolvase (96-105 Hin) E124Q, thus making this mutant a good candidate for crystallization trials.

## 4.6 Binding of (96-105 Hin) substituted resolvases

Tn3R (96-105 Hin), γδR (96-105 Hin) and γδR (96-105 Hin) E124Q were assayed for their binding/cleavage activities with a radioactively labelled linear *res* fragment (Fig. 3.13). The reactions contained 25% ethylene glycol, identical to the cleavage-inducing conditions used earlier (Chapter 3). Tn3R (96-105 Hin) bound to a significant fraction of the substrate (even at low enzyme concentrations), however, the complexes migrated as diffuse bands. Only three complexes could easily be observed, probably corresponding to 1-3 monomers of resolvase bound. Further complexes may be too diffuse to detect. As seen with the activated and 2,3'-deficient resolvases, the complexes migrated significantly faster than the corresponding Tn3R WT complexes. This may be due to differences in DNA bending. Binding of resolvase monomers also appears to be non-cooperative.

Of the four protein-DNA complexes observed with γδR WT, the weak first complex is believed to represent binding of one monomer, whereas complexes 2, 4 and 6 are thought to correspond to 1, 2 and 3 dimers of resolvase bound. Binding by γδR (96-105 Hin) showed a similar pattern of protein-DNA complexes at the lower enzyme concentration, in which the complexes presumably corresponded to 1, 2, 4 and 6 monomers of resolvase. However, differences to binding by γδR WT were also observed with γδR (96-105 Hin). The complexes migrated faster than γδR WT complexes and were more diffuse. Furthermore, a

probable cleavage product is visible migrating faster than the substrate. This species is thought to be the left half-site of *res* covalently attached to a resolvase monomer.

γδR (96-105 Hin) E124Q was also able to bind the *res* substrate. A 1 minute time point shows two protein-DNA complexes. Once again the migration of the first complex was similar to γδR WT whereas migration of the second complex was aberrant, suggestive of altered bending of the DNA. The complex seen with γδR (96-105 Hin), and presumed to be a cleavage complex, was also observed with γδR (96-105 Hin) E124Q.

When these binding/cleavage samples were analysed following treatment with proteinase K, the left and right *res* half-sites were visible in reactions with all three (96-105 Hin)-substituted resolvase mutants (Fig. 3.14, B).

## 4.7 Attempting to re-constitute an inversion system using (96-105 Hin) substituted resolvases

The invertase Hin acts in conjunction with Fis (factor for inversion stimulation) bound to an enhancer sequence, *sis*, to form an "invertasome" structure (Fig. 1.16). This structure is thought to involve direct protein-protein contacts between Fis and the invertase. The addition of HU to the *in vitro* reaction conditions stimulates the inversion reaction, probably by introducing sharp bends in the DNA which aid the formation of the invertasome (Haykinson, 1993). A recent model of the Hin synaptosome takes into account data on the geometry of Fis bound to the enhancer sequence and a predicted structure of Hin dimers bound to *hix* sites (Merickel *et al.*, 1998). In this model, the Hin dimers are synapsed via their DNA binding domains. There is preliminary evidence that Fis contacts the Hin dimers near the N-terminus of the E helix, around residue 104 (equivalent to 102 in resolvase) (R. Johnson laboratory, unpublished). It has been speculated that the activation of Hin by Fis is similar to the activation of site I-bound resolvase by sites II and III. Resolvase may therefore be directed to form a "synaptosome" in the presence of Fis, if it contains the correct "target" sequence for activation by Fis or if Fis acts indirectly (i.e. without making any direct contact). In the introduction, the possibility that the hybrid resolvase would respond to the enhancer/Fis complex was discussed (Fig. 4.3). If the enhancer/Fis complex could contact the Hin-substituted resolvase, forming a -2 synapse, this should be detectable as a bias

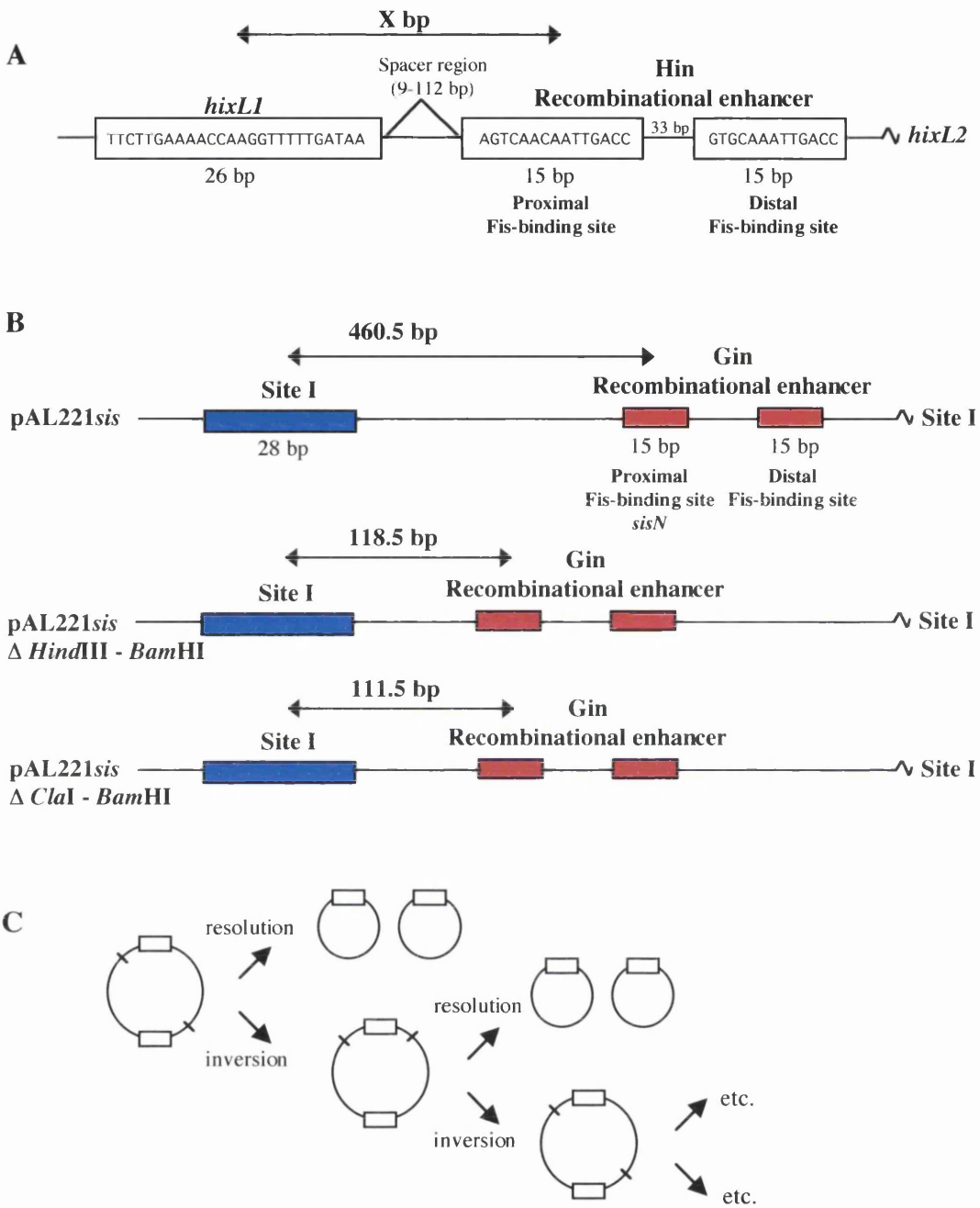
towards inversion products over resolution products. In the absence of Fis, the (96-105 Hin) substituted resolvases give an equal amount of inversion and resolution products with a site I x site I substrate. Unfortunately, the product of an inversion reaction via a –2 synapse does not differ topologically from the substrate (both are unknotted circles), thereby rendering topological analysis uninformative. However, topological analysis could be useful if a substrate with mismatched crossover sites were used. A unique product topology (trefoil knot) would then be expected from a double round of strand exchange (Xr = +2) with a -2 productive synapse topology.

To attempt to create an "invertasome" with resolvase, a substrate with two crossover sites, one flanked by a *sis* site (the enhancer site bound by Fis) at an optimal spacing was required as a substrate. The effect of the enhancer location on Hin invertasome assembly had been studied previously (Haykinson, 1993). It was found that the spacing between the centre of the *hixL1* site and the centre of the proximal Fis-binding site has a large effect on the cleavage reaction of Hin. Low activity was observed up to a spacing of 105.5 bp (distance measured as shown in Fig. 4.16) after which activity oscillated in a manner that correlates with the helical repeat of DNA.

The construct pAL221*sis* (Bednarz, 1990) contains two Tn3 crossover sites (inverted repeat), one of which is flanked by the Gin enhancer sequence. The distance between the centre of site I and the centre of the proximal enhancer binding site, *sisN*, is 460.5 bp (Fig. 4.16, A). In order to try to reduce and optimise this distance, two deletions were made in this substrate (Fig. 4.16, B). This resulted in pAL221sis Δ *Cla*I-*BamH*I (with a site I – *sisN* spacing of 111.5 bp), and pAL221sis Δ *Hind*III-*BamH*I (with a spacing of 118.5 bp). The site I – *sisN* distances in these constructs differ by 7 bp, approximately 2/3 of a helical turn of the DNA. The data from Haykinson and Johnson suggests that a spacing of 111.5 bp would not be geometrically optimal for "invertasome assembly", whereas the 118.5 bp spacing would be near optimal. Although the wild-type spacing of the *hix* crossover and *sisN* sites in the Hin system is 106.5 bp, cleavage activity was found to be slightly improved when the spacing was increased by an extra helical turn to around 118 bp (as in the Gin system).

The three site I x site I x enhancer substrates (pAL221*sis* and its deletion derivatives) were assayed under recombination conditions (C8.2) with all three (96-105 Hin) substituted resolvases, and also with the activated γδR E102Y/E124Q resolvase mutant (Fig. 4.17, A).

## Fig. 4.16 DNA spacing of recombination and enhancer sites

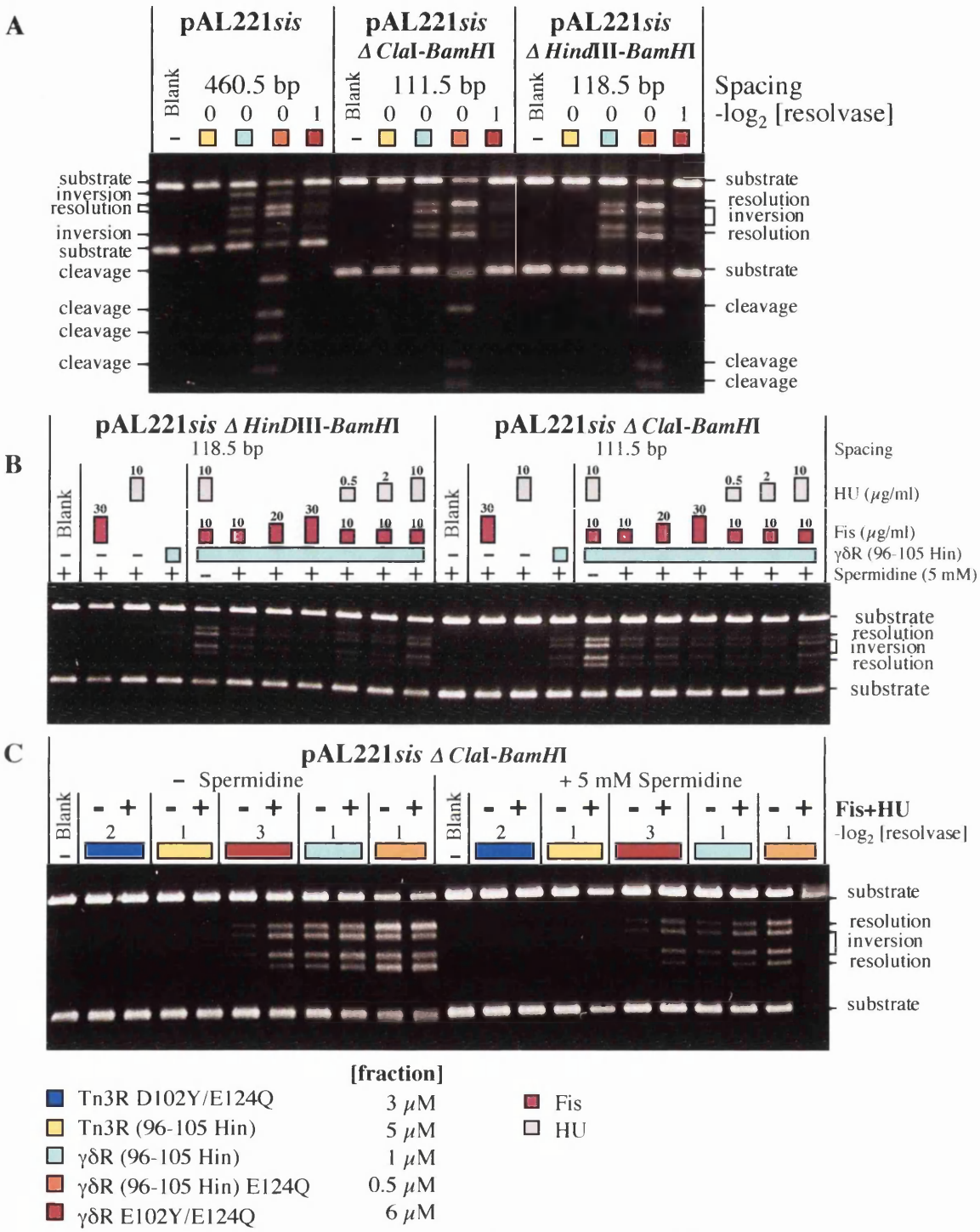



**A.** Haykinson *et al.* 1993 studied the effect of varying the spacer length between the *hixL1* site and the proximal Fis-binding site. Lengths calculated as half *hixL1* site (13 bp) plus the spacer region (9-112 bp) plus 7 bp of the proximal Fis-binding site. The WT spacing for the Hin inversion system is 106 bp.
**B.** The spacing between the centre of site I and the centre of the *sisN* site is shown for the two pAL221*sis* deletion constructs. Note that as the true centre of the proximal Fis-binding site is at position 7.5 bp, these lengths are 0.5 bp larger than the equivalent lengths as calculated by Haykinson & Johnson 1993.
**C.** The initial recombination of a site I x site I substrate with activated resolvase gives a 1:1 ratio of resolution (shown as unlinked circles for simplicity) and inversion products. However, as the inversion product is a substrate for further rounds of recombination, the product ratio will become biased towards resolution products over time.

Note that due to the deletion of the *Hind*III site in the two new substrates, the products were analysed by restriction with *Eag*I and *Pst*I. The relative mobilities of the resolution and inversion products differ between pAL221*sis* and its two deletion products. In the absence of Fis, the site I/enhancer spacing had no discernible effect upon resolvase activity. As noted previously, Tn3R (96-105 Hin) is not very active with site I x site I substrates, and these reactions (which proceeded for only 1 hour) showed no detectable products upon restriction analysis. The reactions with γδR (96-105 Hin) and γδR E102Y/E124Q gave equal amounts of resolution (LL and RR) and inversion (LR) products, consistent with random synapsis of the crossover sites. However, the γδR (96-105 Hin) E124Q reactions with all three substrates yielded considerably more resolution products than inversion products. This bias of γδR (96-105 Hin) E124Q was investigated further by comparing its activity with that of γδR (96-105 Hin) using site I x site I substrates with sites in direct or inverted repeat (data not shown). While γδR (96-105 Hin) showed no bias towards resolution or inversion with either substrate, γδR (96-105 Hin) E124Q gave an excess of resolution products with both substrates. The preference for resolution reaction is therefore independent of the orientation of the crossover sites, and is most likely a consequence of dissociative cleavage and ligation reactions, resulting in unlinked circles or a consequence of multiple rounds of recombination as shown in Fig. 4.16 C. As γδR (96-105 Hin) gave easily detectable recombination products (unlike its Tn3 derivative) with no bias towards resolution or inversion products (unlike the E124Q derivative), this enzyme was used to examine the effects of Fis and/or HU.

The effects of Fis and/or HU (which stimulates the Hin inversion reaction) on γδR (96-105 Hin) activity were assayed under recombination conditions (C8.2 ± 5 mM spermidine) using both pAL221*sis* deletion constructs (Fig. 4.17, B). As increasing amounts of Fis were added, the reaction was slightly inhibited, although a small bias towards inversion products also became apparent. However, in the presence of a low concentration of Fis and an increasing amount of HU, a small stimulatory effect on the overall reaction rate was seen with both substrates. Also, a similar effect on the product ratios could be seen, with the inversion products predominating over the resolution products in the presence of Fis and HU (approximately 2:1). The presence of spermidine (which is known to stimulate inversion by Hin, (Haykinson, 1993)) had the opposite effect on these reactions with γδR (96-105 Hin). However, when these experiments were repeated with activated resolvase mutants that did not contain the (96-105 Hin) substitution, the same effects on the reaction rate and product ratios were observed (Fig. 4.17, C). Thus, no evidence was obtained to support proposed

**Fig. 4.17 Preliminary evidence indicating a small bias in the crossover site alignment by activated resolvases in the presence of Fis**




**A.** Reactions were set up in recombination buffer (C8.2) for 1 hour at 37°C. Reactions were digested with *Pst*I and *Eag*I and treated with proteinase K (50 μg/ml). Dilutions of resolvase were added

**B.** Reactions were set up in recombination buffer (C8.2 ± 5 mM spermidine) for 2.5 hours at 37°C. Fis and/or HU were diluted in 50 mM Tris/HCl pH 8.2 and added first (1/20 volume each, final concentrations shown). 1/20 volume of 0.5 μM γδR (96-105 Hin) was added as indicated. Reactions were digested with *Pst*I and *Eag*I and treated with proteinase K (50 μg/ml).

**C.** Reactions were performed as in (B) for 1 hour at 37°C. However, resolvase dilutions were added (1/10 volume) as indicated. Fis and HU were added (1/20 volume) first to a final concentration of 1 μg/ml final. Reactions were digested with *Pst*I and *Eag*I and treated with proteinase K (50 μg/ml).

direct contacts between the Fis/enhancer and (96-105 Hin). The small change in product ratios observed may therefore be due to contacts of Fis to resolvase in a region other than residues 96-105 or to non-specific contacts of the Fis/enhancer complex with adjacent DNA, which stabilise the -2 configuration. Whether the effect of Fis/HU was dependent upon the presence of the enhancer element was not investigated.

## 4.8 Characterisation of Hin substituted DNA binding domain resolvase

Intriguingly, a variation on the model proposed by Merickel *et al.* (1998) could be constructed in which Fis would contact the Hin DNA binding domain, if synapsis of the Hin dimers occurred via the N-terminal domain (as has been proposed for synapsis of resolvase) (Fig. 4.3, B). This new model (Fig. 4.3, B) is consistent with recent experimental data involving a hybrid Gin/ISXc5 mutant (Schneider *et al.*, 2000). The hybrid Gin/ISXc5 mutant contained the catalytic domain of the invertase Gin (residues 1-123) and a truncated C-terminal, DNA-binding domain from the resolvase ISXc5 (residues 124-205). In contrast to the activities of all previous hybrid recombinases, which retained the activity characteristic of their catalytic domains, this hybrid invertase was shown to function as an active resolvase on an ISXc5 *res* x *res* plasmid *in vivo*. Furthermore, there is evidence that this hybrid has lost the ability to interact with Fis to form an invertasome *in vivo* (although this might simply be due to a reduction in overall activity). Thus, it could be proposed that the residues in Gin or Hin contacted by the Fis/enhancer complex are not within the catalytic domain but are instead in the DNA-binding domain.

To test this hypothesis, a Hin invertase binding domain, containing resolvase residues known to be directly involved in sequence-specific DNA recognition was designed (Fig. 4.18). The altered binding domain begins at residue 141 (Tn3 numbering), although three substitutions (141, 142 and 144) were to residues identical in γδ resolvase and Hin. From residues 144-192, the sequence is that of Hin, with the exception of four residues important for resolvase binding specificity; N169, A171, R172 and K177. In the γδ resolvase co-crystal these residues make specific base contacts with the DNA. The solvent-exposed surface of the modified domain is expected to resemble Hin and therefore should maintain possible contacts with the Fis/sis enhancer complex, but it should also be able to recognise *res* site I. The same experiments (with the same substrates) as were described with the (96-105 Hin)

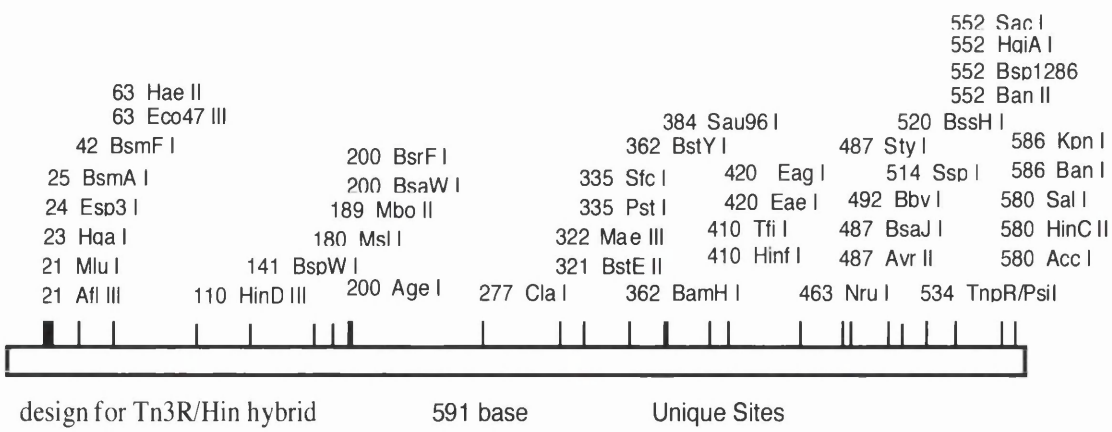
# Fig. 4.18 Design for Tn3R/Hin hybrid

**A** Black: Tn3 sequences
Blue: Hin sequences
Red: changes to Tn3/γδ residues within Hin domain (Hin sequence shown in parenthese)
Green: residues that are identical in Hin and γδ resolvase
restriction sites are underlined; other changes to WT sequences also in lower case.

```
1/1                                                 31/11
atg CGA ATT TTT GGT TAT GCa cgc gtC TCA ACC AGC CAG CAG TCC CTC GAT ATT CAG ATC
M   R   I   F   G   Y   A   R   V   S   T   S   Q   Q   S   L   D   I   Q   I
61/21                                               91/31
AGA GCG CTC AAA GAT GCA GGG GTA AAA GCT AAC CGC ATC TTT ACC GAC Aaa gct tCC GGC
R   A   L   K   D   A   G   V   K   A   N   R   I   F   T   D   K   A   S   G
121/41                                              151/51
AGT TCA ACA GAT CGG GAA GGG CTG GAT TTG CTG AGG ATG AAG GTG GAG GAA GGT GAT GTC
S   S   T   D   R   E   G   L   D   L   L   R   M   K   V   E   E   G   D   V
181/61                                              211/71
ATT CTG GTG AAG AAG CTC Gac cgg tTa GGC CGC GAC ACC GCC GAC ATG ATC CAA CTG ATA
I   L   V   K   K   L   D   R   L   G   R   D   T   A   D   M   I   Q   L   I
241/81                                              271/91
AAA GAG TTT GAT GCT CAG GGT GTA GCG GTT CGG TTT atc gat GAC GGG ATC AGT ACC GAC
K   E   F   D   A   Q   G   V   A   V   R   F   I   D   D   G   I   S   T   D
301/101                                             331/111
GGT GAT ATG GGG CAA ATG GTG GTC ACC ATC CTG Tct gca gTG GCA CAG GCT GAA CGC CGG
G   D   M   G   Q   M   V   V   T   I   L   S   A   V   A   Q   A   E   R   R
361/121                                             391/131
Agg atc cTA GAG CGC ACG AAT GAG GGC CGA CAG GAA GCA AAG CTG AAA GGA ATC AAA TTc
R   I   L   E   R   T   N   E   G   R   Q   E   A   K   L   K   G   I   K   F
421/141                                             451/151
ggc CGt cgt CGt GCG ATC AAC AAA CAT GAA CAG GAA CAG ATT tcg cga CTg TTA GAG AAA
G   R   R   R   A   I   N   K   H   E   Q   E   Q   I   S   R   L   L   E   K
481/161                                             511/171
GGC CAT CCT aGG CAG CAA TTA GCT ATT ATT TTT aat ATT gcg cgc TCC ACC ctt TAt aaa
G   H   P   R   Q   Q   L   A   I   I   F   N(g) I   A(g)R(v)S   T   L   Y   K(r)
541/181                                             571/191
TAC TTT CCG GCg AGC tcg ATc AAg AAA CGA ATG AAT TAA gtc gac ggt acc tct aga
Y   F   P   A   S   S   I   K   K   R   M   N   *   V   D   G   T   S   R
```

```
recognition helix "design": residues close to the DNA:
169 is G in Hin, D in Gin: used N as per γδ
171 is G in Hin, A in Gin, Tn3 and γδ: used A
172 is V in Hin, L in Gin and R172L, R in Tn3 and γδ: used R
177 is R in Hin, Gin and Cin, K in Tn3/γδ: used K
```

**B**




design for Tn3R/Hin hybrid 591 base Unique Sites

**A.** The open reading frame of Tn3R WT Hin/Res DBD is shown above. The four amino acid changes in the Hin DBD are shown in red; the residues at the homologous positions are described below.
**B.** A restriction map of the Tn3R WT Hin/Res DBD open reading frame is shown.

substituted resolvases could be performed with resolvases carrying this altered binding specificity Hin C-terminal domain (referred to as Hin/Res DBD). However, Hin/Res DBD resolvase will test whether the Fis/enhancer complex contacts the C-terminal domain of Hin, which would suggest that these are synapsed via their catalytic domain.

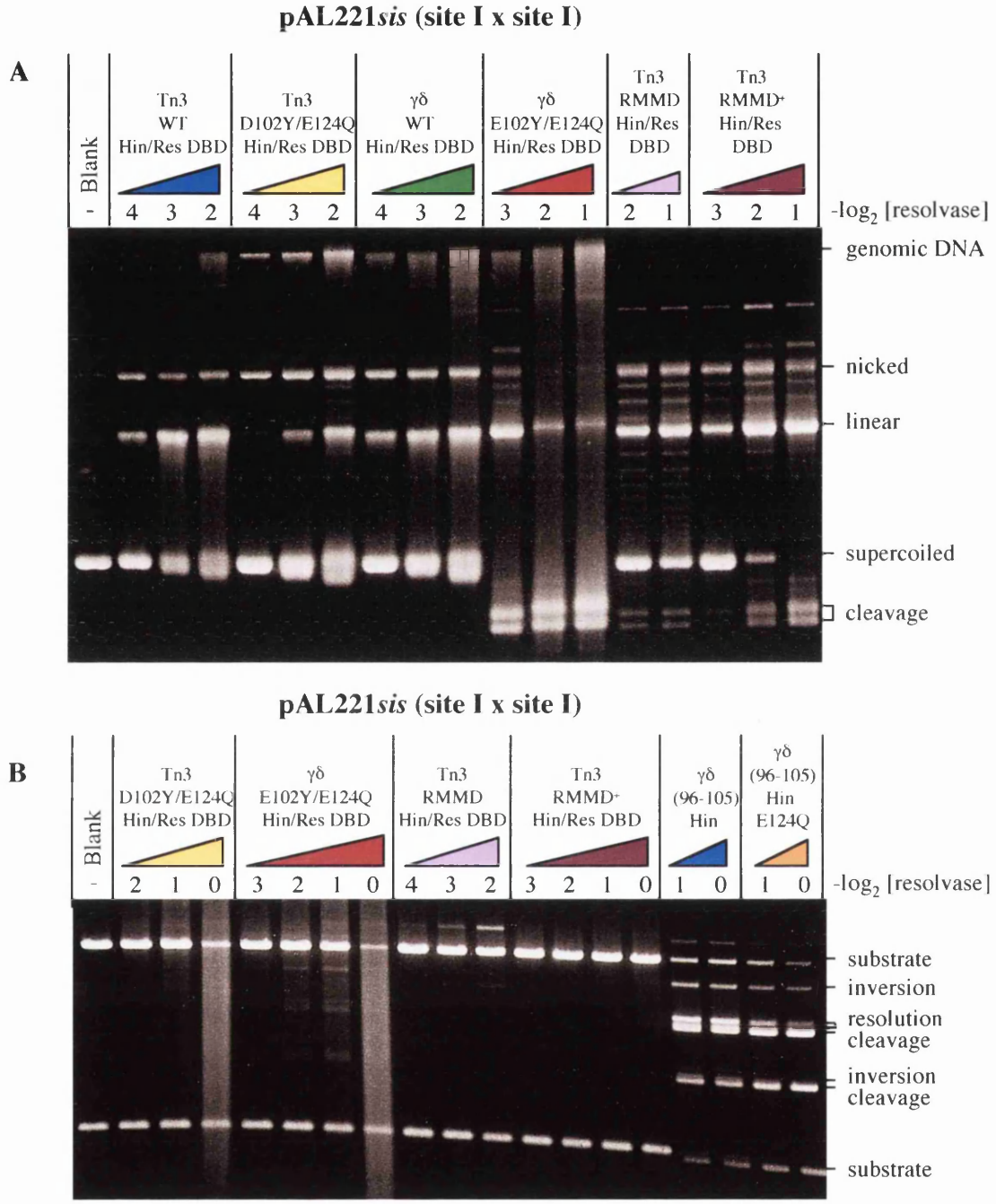
The binding domains of Tn3 and γδ resolvases, and various activated mutants thereof, were replaced with the "redesigned" Hin DNA binding domain. As binding assays have revealed a correlation between activation and un-cooperative and weakened binding, the ability of the hybrids which carry additional activating mutations to bind was a concern. The activated mutants chosen initially were Tn3R D102Y/E124Q and γδR E102Y/E124Q, as these bind site I quite strongly. No activity was detected when the WT and activated resolvase Hin/Res DBD mutants were assayed *in vivo* with a *res* x *res* substrate (Table 3, Chapter 2). Subsequently, two further extremely deregulated Tn3 resolvase mutants were also constructed. These resolvase derivatives contained the mutations G101S/D102Y/M103I/ Q105L (RMMD, Jiuya He) with or without two additional mutations at the 2,3'-interface, R2A/E56K (RMMD+, Jiuya He). The hybrids constructed are listed below.

1. Tn3R WT Hin/Res DBD
2. Tn3R D102Y/E124Q Hin/Res DBD
3. Tn3R G101S/D102Y/M103I/Q105L Hin/Res DBD
4. Tn3R R2A/E56K/G101S/D102Y/M103I/Q105L Hin/Res DBD
5. γδR WT Hin/Res DBD
6. γδR E102Y/E124Q Hin/Res DBD

All six resolvase Hin/Res DBD mutants were purified on a small scale. They had different solubility properties, so different fractions of these mutants had to be used for *in vitro* assays. Initially, assays were performed with a *res* x *res* substrate under recombination conditions (C8.2). All six proteins were found to be active *in vitro* to varying degrees, indicating that they are able to bind to *res* sites (data not shown).

Initially, assays with a site I x site I substrate (pAL221*sis*) were performed under cleavage conditions in order to increase the chances of detecting products (Fig. 4.19, A). Linearisation of the plasmid is visible with all mutants, however, this is almost certainly due to non-specific nicking by contaminating nucleases and thus cannot be interpreted as recombinase

## Fig. 4.19 Assays of Hin/Res DBD substituted resolvases with a site I x site I substrate




**A.** The reactions were set up with pAL221*sis* (site I x site I) in cleavage conditions (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 10 mM $MgCl_2$, 0.1 mM EDTA) at 37°C for 3 hours. The stated dilutions were added at 1/10 volume.

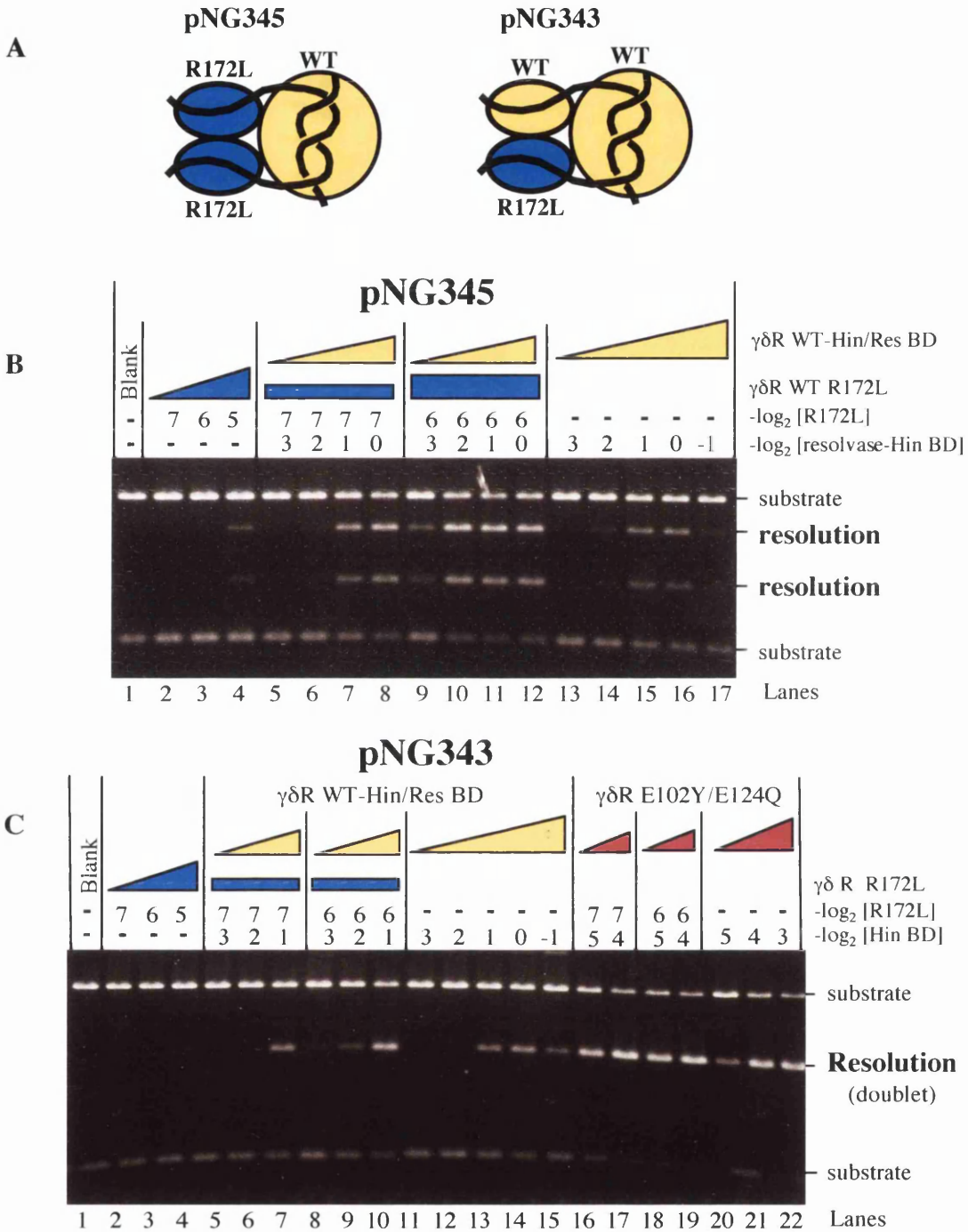
**B.** The reactions were set up with pAL221*sis* (site I x site I) in the absence of magnesium (50 mM Tris/HCl pH 8.2, 0.1 mM EDTA) at 37°C for 3 hours. Resolvase reactions were stopped by heating to 75°C for 5 min. Samples were analysed by restriction digestion with *Pst*I and *Hind*III.

Concentrations of resolvase fractions (undiluted) were as follows; 25 $\mu$M Tn3R WT Hin/Res DBD, 10 $\mu$M Tn3R D102Y/E124Q Hin/Res DBD, 25 $\mu$M γδR WT Hin/Res DBD, 25 $\mu$M γδR E102Y/E124Q Hin/Res DBD, 75 $\mu$M Tn3R RMMD Hin/Res DBD, 15 $\mu$M Tn3R RMMD$^+$ Hin/Res DBD, 1 $\mu$M γδR (96-105 Hin), 0.5 $\mu$M γδR (96-105 Hin) E124Q. Dilutions were added at 1/10 volume. All reactions were treated with proteinase K (50 $\mu$g/ml) and SDS (0.1%) and run uncut.

activity; Tn3 and γδ resolvase derivatives without activating mutations would not be expected to be active on a site I x site I substrate. However, products of the characteristic sizes for cleavage at both crossover sites were observed for a number of the activated resolvases, including Tn3R RMMD Hin/Res DBD, Tn3R RMMD+ Hin/Res DBD and particularly γδR E102Y/E124Q Hin/Res DBD. These products can be interpreted as being recombinase-specific. Thus these activated mutants with an "artificial" DBD nevertheless retain the ability to cleave a substrate in the absence of accessory sites. No activity was detectable with any of these Hin/Res DBD mutants under standard recombination conditions (C8.2), (data not shown). However, in the absence of magnesium, recombination and cleavage by γδR E102Y/E124Q Hin/Res DBD was clearly detectable (Fig. 4.19, B).

γδR WT Hin/Res DBD and γδR E102Y/E124Q Hin/Res DBD were purified on a large-scale. The first stages of purification were surprisingly unremarkable. However, a large amount of these enzymes eluted from the sepharose column in the flow-through fraction. This was unanticipated because the extra positive charges at the C-terminus of the Hin DBD were expected to strengthen binding to the column. Thus, this fraction was also used in further purification steps. Unfortunately, the enzymes proved difficult to resuspend following the final dialysis step, which precipitates resolvase in order to allow resuspension in a small volume. As had been done previously, the final insoluble pellet was resuspended in a small volume of a denaturing urea solution (8 M urea). This was diluted with resuspension buffer (3 vol., 1.33 x concentration) and an equal volume of glycerol added. The final yield of these highly purified enzymes was therefore very low, albeit concentrated enough to assay. The activities of these purified enzymes was similar to the activities observed for the small-scale preparations (data not shown). Due to time limitations these enzymes were not characterised in detail. However, preliminary experiments which addressed effects of Fis and/or HU on these enzymes showed no detectable stimulation (data not shown). Although a firm conclusion cannot be drawn from these preliminary experiments, they suggest that the Hin/Res DNA binding domain does not make specific contacts with the Fis/enhancer complex or HU.

**Fig. 4.20 Complementation between resolvases with different DNA binding domains**




**A.** Illustration of targeting using substrates pNG345 and pNG343 and the altered binding specificity mutant γδR R172L and WT resolvase.

**B.** Reactions were set up with pNG345 in recombination buffer (C8.2) for 1.5 hour at 37°C. γδR WT Hin/Res DBD was added first (1/20 volume, samples kept on ice). γδR R172L was added second (1/20 volume).

**C.** Reactions were performed as for (B) using pNG343 as a substrate.

All reactions were analysed by restriction digest with *Pst*I and *Hind*III and treated with proteinase K (50 μg/ml).

## 4.9 Targeting of Hin/Res DBD substituted resolvases to subsites of *res*

Using γδR WT Hin/Res DBD, the effect of an altered binding domain within a synaptic complex was investigated by targeting to subsites of *res*. Limiting concentrations of γδR R172L could be complemented by γδR WT Hin/Res DBD targeted to the accessory sites (pNG345, Fig. 4.20 A and B) or targeted to the accessory sites and the partner crossover site (pNG343, Fig. 4.20 A and C). The complementation assays therefore suggest that the resolvase with a "Hin" binding domain (which has a completely different surface to a resolvase binding domain) is able to collaborate with WT resolvase to form a functional recombination complex, even when targeted to opposite crossover sites. The residues in the DNA binding domain of resolvase are thus unlikely to be required for essential protein-protein contacts, either for synapsis of the crossover sites, or for activation of site I-bound resolvase by sites II and III.

## 4.10 Summary and Conclusions

The hypothesis that the region around residue D102 of Tn3 resolvase is involved in specific interdimer interactions was investigated by replacing the region between residues 96-105 (Tn3 numbering) with the homologous region of the invertase Hin. Surprisingly, the resulting hybrid enzyme, Tn3R (96-105 Hin) was found to be extremely activated. Kinetic experiments revealed that Tn3R (96-105 Hin) resolved a *res* x *res* substrate faster than Tn3R WT, and DNaseI nicking showed that the reaction remained topologically constrained. Nevertheless, assays with a variety of "non-standard" substrates revealed that Tn3R (96-105 Hin) has lost the strict substrate selectivity observed with wild-type resolvase. Although this mutant was active in the absence of supercoiling and accessory sites, direct comparisons of its activity on different substrates showed that it retained a preference for complete *res* sites in direct repeat.

The identical substitution in γδ resolvase generated a mutant which was more hyperactive than Tn3R (96-105 Hin). The rate of recombination by γδR (96-105 Hin) did not differ greatly in the presence or absence of accessory sites. Nevertheless, accessory site synapsis assays have shown that this mutant retains the ability to wrap the accessory sites, trapping three negative supercoils. This is consistent with the exclusively LR joins seen with a *res* x

*res* substrate and the random alignment seen with a site I x site I substrate (as deduced from the equal ratio of LL/RR:LR joins). Under cleavage conditions the remarkable ability of γδR (96-105 Hin) to cleave supercoiled substrates was observed (whether or not accessory sites are present). However, in the absence of supercoiling and/or accessory sites, the rate of recombination and cleavage by γδR (96-105 Hin) was markedly reduced.

The addition of the E124Q mutation to γδR (96-105 Hin), was anticipated to activate resolvase for cleavage. Such a resolvase would yield large amounts of covalent cleavage-intermediate. This was indeed observed for γδR (96-105 Hin) E124Q, with linearised *res* x *res* and site I x site I substrates under a wide range of reaction conditions, confirming the additive effect of mutations in different regions of resolvase (as also seen with Tn3R D102Y/E124Q and γδR E102Y/E124Q). This mutant may be useful for co-crystallization trials of the cleavage intermediate.

The ability of these (96-105 Hin) substituted resolvase mutants to cleave one crossover site in the absence of a partner crossover site, made the targeting experiments designed to test the compatibilities of the altered surfaces difficult to perform. Nevertheless, the activated phenotype of these mutants was hoped to aid investigations into whether this region was involved in direct protein-protein contacts with the Fis/enhancer complex. Assays were performed with substrates analogous to the natural Hin substrate with *res* crossover sites in place of *hix* sites. Although a bias towards the generation of inversion products was detected with γδR (96-105 Hin) in the presence of Fis and HU (approximately in a ratio of 2:1, inversion to resolution products), this was found to be independent of the Hin-substituted region of resolvase.

The analogous experiment to look for possible Fis contact surfaces was performed with a resolvase/Hin-DBD hybrid designed to bind *res* binding sites. A variety of WT and activated versions of this hybrid were constructed. These were found to be active for recombination on a *res* x *res* substrate. The activated γδR E102Y/E124Q Hin/Res DBD mutant was also found to recombine a site I x site I substrate when assayed in recombination conditions in the absence of magnesium. However, preliminary experiments revealed no detectable effect upon the reaction products in the presence of Fis and/or HU. This suggested that the Hin/Res DNA binding domain does not make specific contacts with the Fis/enhancer complex.

Complementation experiments showed that Tn3R Hin/Res targeted to sites II/III (pNG345) or targeted to sites II/III and a single crossover site (pNG343) was able to complement limiting concentrations of γδR R172L. The resolvase DNA-binding domain of resolvase can thus be replaced by a modified Hin DBD without disrupting any interactions that are essential in the synaptic complex.

# Chapter 5

# Interface dissociation during strand exchange

## 5.1 Introduction

Crystal structures of γδ resolvase, and the co-crystal structure of γδ resolvase bound to the crossover site of *res* (Sanderson *et al.*, 1990; Rice and Steitz, 1994), have not clarified the protein interactions and choreography required for the strand exchange reaction. This is evident from the various models of strand exchange, described in the introduction. These models differ radically in the proposed synaptic interfaces and the relative movements of the resolvase protomers and the DNA. Nevertheless, the structural data provides the opportunity to apply a combination of genetic and biochemical approaches to test these different models.

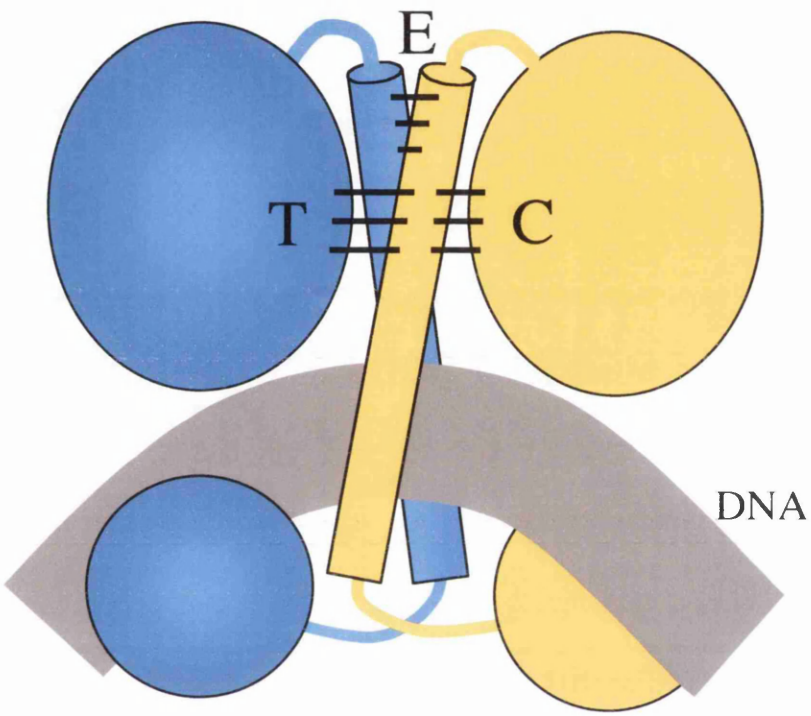
The schematic diagram of the co-crystal structure shows various interfaces within the dimer and the terminology used here to describe them (Fig. 5.1). The three models of strand exchange described earlier make different predictions (shown below) about which interfaces are broken during the reaction.

| | DNA Mediated | Simple Rotation | Domain Swapping |
|---|---|---|---|
| *Trans* Interface | Remains intact | Broken (4/4 subunits) | Half (2/4 subunits) |
| *Cis* Interface | Remains intact | Remains intact | Half (2/4 subunits) |
| E Helix Interface | Remains intact | Broken (4/4 subunits) | Remains intact |
| DNA/DBD Interface | Remains intact | Remains intact | Half (2/4 subunits) |

Interfaces whose dissociation is not a compulsory step in the strand exchange reaction would be predicted to tolerate a physical linkage between residues spanning that particular interface. Wild-type Tn3 resolvase contains no cysteine residues: a physical linkage could therefore be formed by introducing novel cysteine residues at specific locations designed to span the desired interfaces upon formation of a disulphide bond. Experiments with γδ resolvase dimers containing disulphide-linked cysteine residues at positions on the E helix interface (M106C, I110C, and V114C) have been described previously (Hughes *et al.*, 1993).

Each model makes unique predictions about the effect on strand exchange of crosslinking specific interfaces (Fig. 5.2). In the case of simple rotation, disulphide linking of the *cis* interface should not interfere with strand exchange. However, disruption of both the *trans*

**Fig. 5.1 Schematic diagram of the co-crystal structure illustrating various interfaces and their ascribed terminology**




### Descriptions of labelled interfaces

**E** E Helix interface (part of the dimer interface) involves intermolecular zipper interactions between the E helices of each monomer, e.g. residue M106.

**T** *trans* interface involves interactions between the catalytic domain core and the E helix of the partner monomer (e.g. interactions between residues S10, R68H and I97 and the E helix residues around E124).

**C** *cis* interface involves interactions between the catalytic domain core and the E helix of the same monomer (mainly interactions between helix D (A74, I77) and helix E, around E118).

and the E helix interface is absolutely required for the rotation of the subunits proposed by this model. Strand exchange by a domain-swapping mechanism requires dissociation of one of the *cis* and *trans* interfaces per dimer, whereas the E helix interface remains intact. Should linkage of a particular interface block the resolution reaction but not the cleavage step (which immediately precedes strand exchange) then this result would support the hypothesis that disruption of this interface occurs during the strand exchange mechanism. However, it should be noted that a particular model can only be ruled out if an interface, which it requires to be broken, is found to tolerate a crosslink. For example, the simple rotation model could be ruled out if a protein with a crosslink between the E helices of a dimer, or on the *trans* interface, could support resolution. The DNA-mediated model predicts that disulphide linking across all interfaces should, in principle, be tolerated. This model can therefore not be ruled out by this method of investigation. The conclusions that can be drawn if resolution product is observed with *cis*, *trans*, or E helix crosslinked resolvase are shown in Fig. 5.2 for the subunit rotation and domain-swapping models (the DNA-mediated model is not shown, as it is consistent with resolution activity by any crosslinked resolvase). Fig. 5.3 depicts the predicted outcomes of targeting crosslinked resolvase to specific sites using the altered specificity mutant γδR R172L (DBD specificity not shown). However, targeting individual subunits of resolvase containing an intramolecular crosslink (e.g. *cis* crosslinked) is less specific than targeting crosslinked dimers (e.g. *trans* or E helix crosslinked) and may prove difficult to accomplish (site I targeting experiments involved the reduction of oxidised M106C resolvase "in situ", (Murley and Grindley, 1998).

The aim was to determine the requirements for the dissociation of each of these three interfaces (*cis*, *trans* and E helix) in resolvase during the strand exchange mechanism by creating disulphide-linked or chemically crosslinked resolvases. Specifically, the purpose of the experiments was to make active cysteine-substituted mutants appropriate for crosslinking of specific interfaces; to purify and characterise these, to crosslink them, and to characterise the crosslinked species by assaying for activity at different steps in recombination, i.e. binding, synapsis, double-strand cleavage, and recombination.

**Fig. 5.2 Predicted effects of *cis*, *trans*, and E helix crosslinking upon strand exchange**

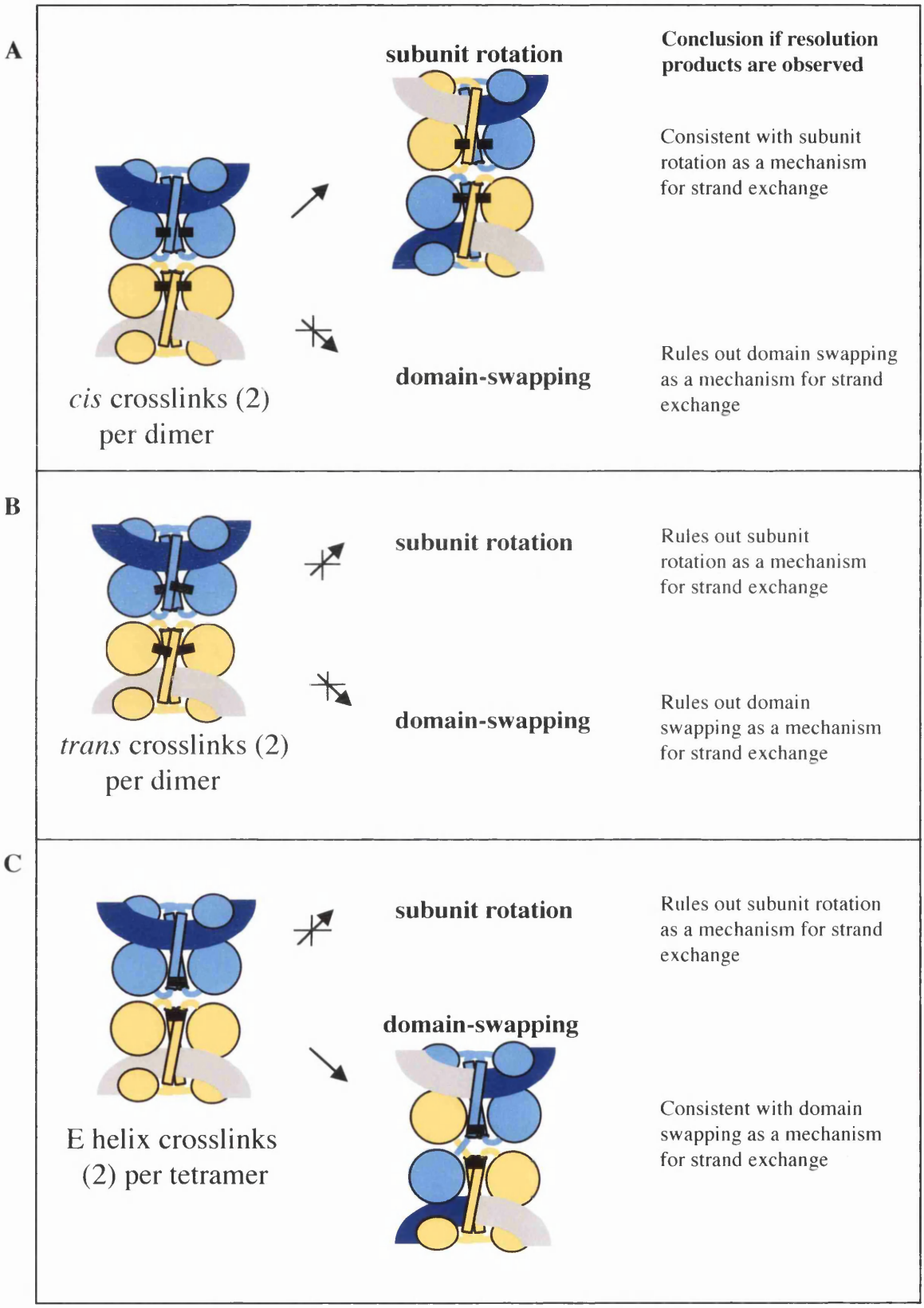
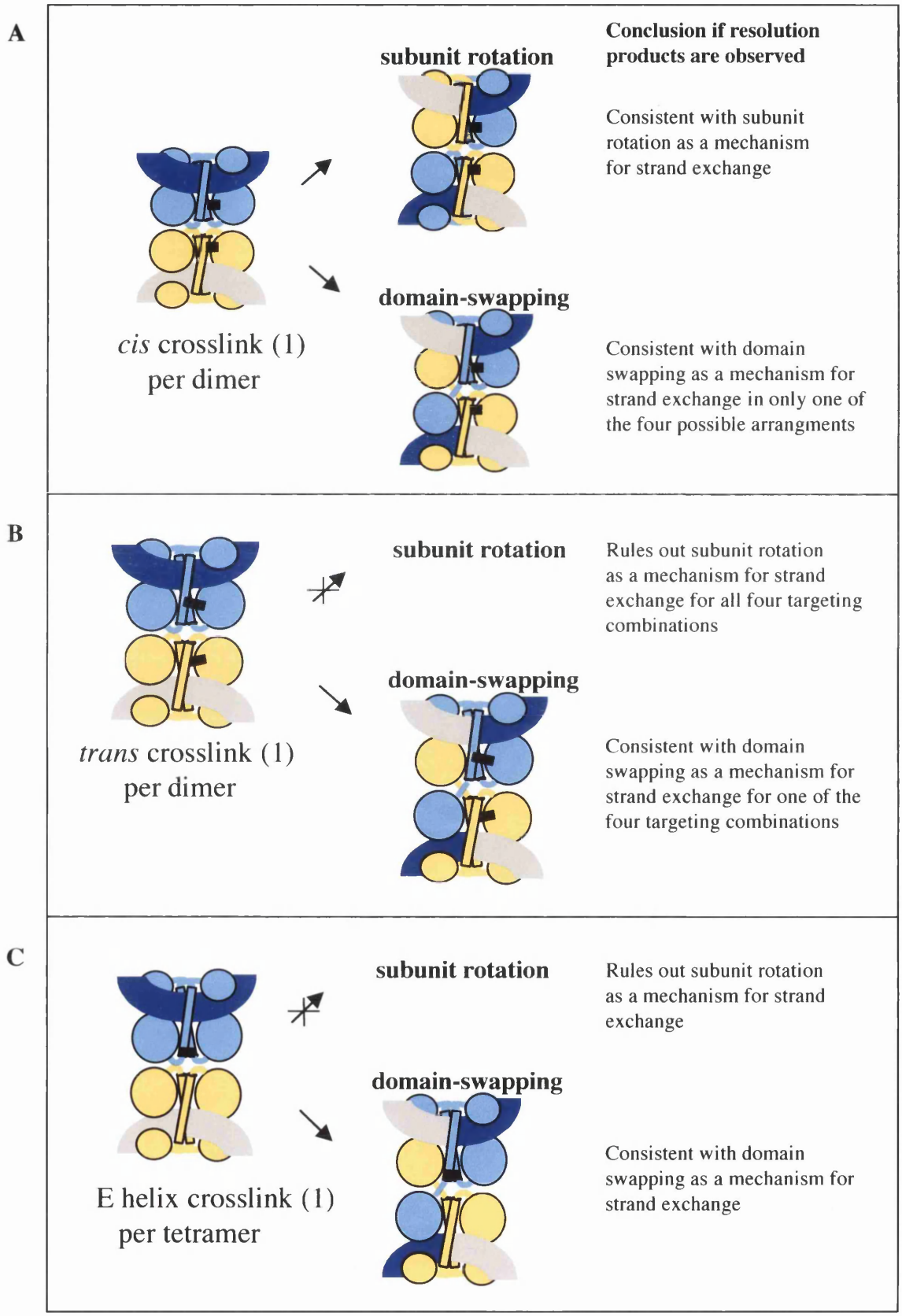


**Fig. 5.3 Predicted effects of targeted crosslinked resolvase upon strand exchange**



## 5.2 Identifying residues to crosslink

Pairs of candidate residues for cysteine substitution were chosen by studying the γδ resolvase co-crystal structure (Table 7). Although the crystal structure is of γδ resolvase, the substitutions were nevertheless made in the wild-type Tn3 sequence, for which an open reading frame with numerous unique restriction sites was available (pAT5 and derivatives, (Arnold, 1997). Using molecular graphics, the distances between the alpha carbons were measured, the aim being to minimise protein deformation upon disulphide bond formation. Only pairs of residues with alpha carbons closer than 7.5 Å were considered (as the typical $C_\alpha$-$C_\alpha$ distance in a cysteine-cysteine crosslink is 6.5 Å). Furthermore, the amino acid pairs were considered such that only residues with suitable geometries ($ß_X$, $\alpha_X$, $\alpha_Y$ angles between 0° and 60°) were chosen. These conditions rapidly eliminated many potential residues. However, the residues T73 and A115 (which span the *cis* interface) and D95 and A113 (which span the *trans* interface) did meet all the requirements (Table 7). Two other residue pairs were chosen as candidate back-ups: S112 and I77 for the *cis* interface and T109 and D95 for the *trans* interface. In addition, two cysteine-substituted mutants, namely D102C and M106C, which would disulphide link across the E helices of the dimer interface, were previously purified and characterised (Tn3 resolvase D102C and M106C, (MacDonald, 1999); γδ resolvase M106C, (Hughes *et al.*, 1993)). The positions of these residues are indicated in the co-crystal structure in Figs. 5.4 and 5.5.

## 5.3 Construction and *in vivo* assays of cysteine-substituted resolvases

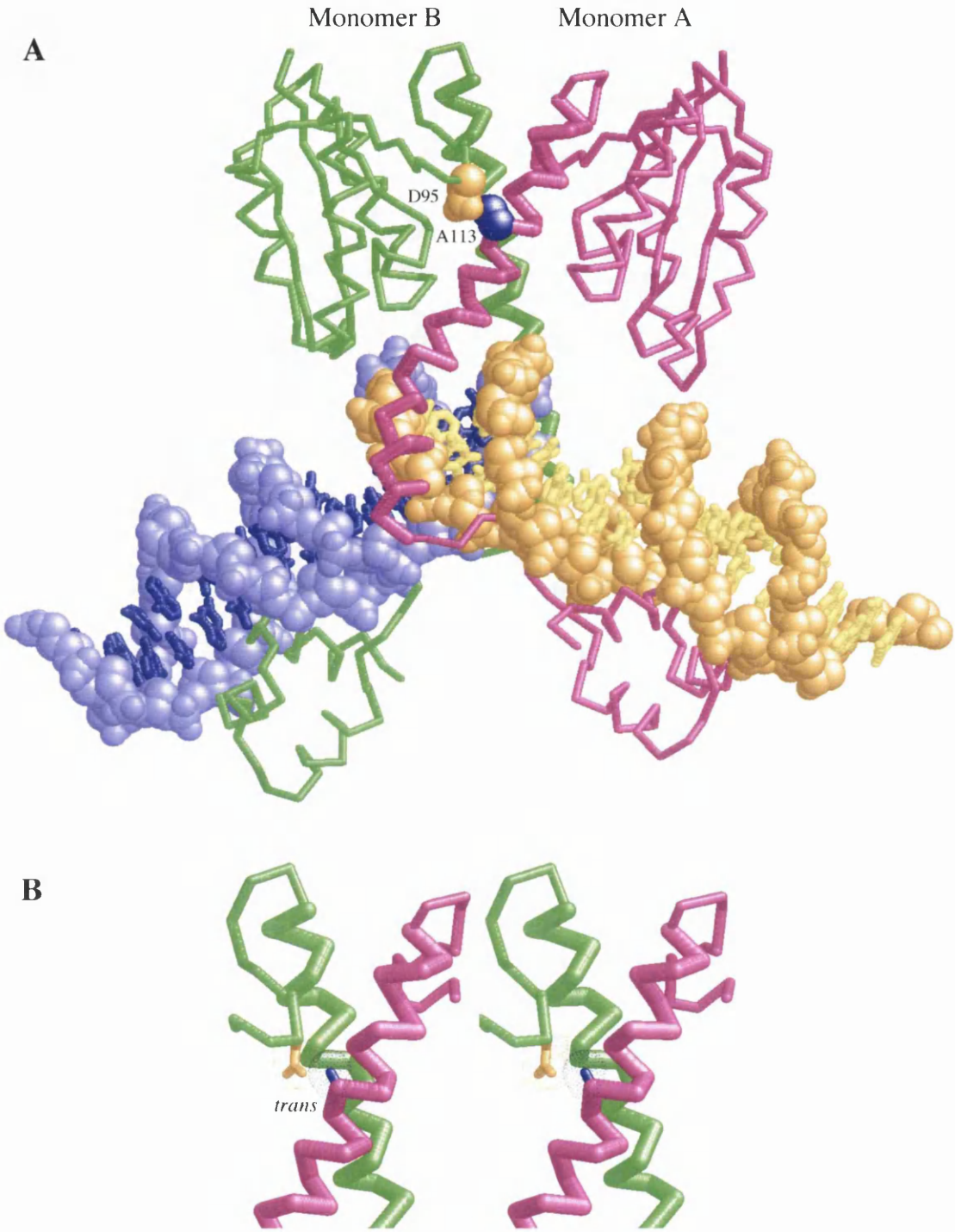
The four single cysteine mutants of Tn3 resolvase, T73C, D95C, A113C and A115C were constructed by replacing regions of the resolvase reading frame of pAT5Δ with synthetic oligonucleotides containing the desired codon substitution (Tables 1 and 2, Chapter 2). The activity of these cysteine-substituted resolvase mutants was tested *in vivo* (Table 3, Chapter 2). T73C, D95C and A113C all showed a wild-type phenotype *in vivo*. However, A115C gave red colonies with all test plasmids, indicating that this substitution reduces or abolishes resolvase activity.

In order to crosslink the *trans* and *cis* interfaces, the double mutants D95C/A113C and T73C/A115C were constructed and further *in vivo* assays were performed (Table 3, Chapter

## Table 7 Distances and bond angles between residues in the co-crystal structure

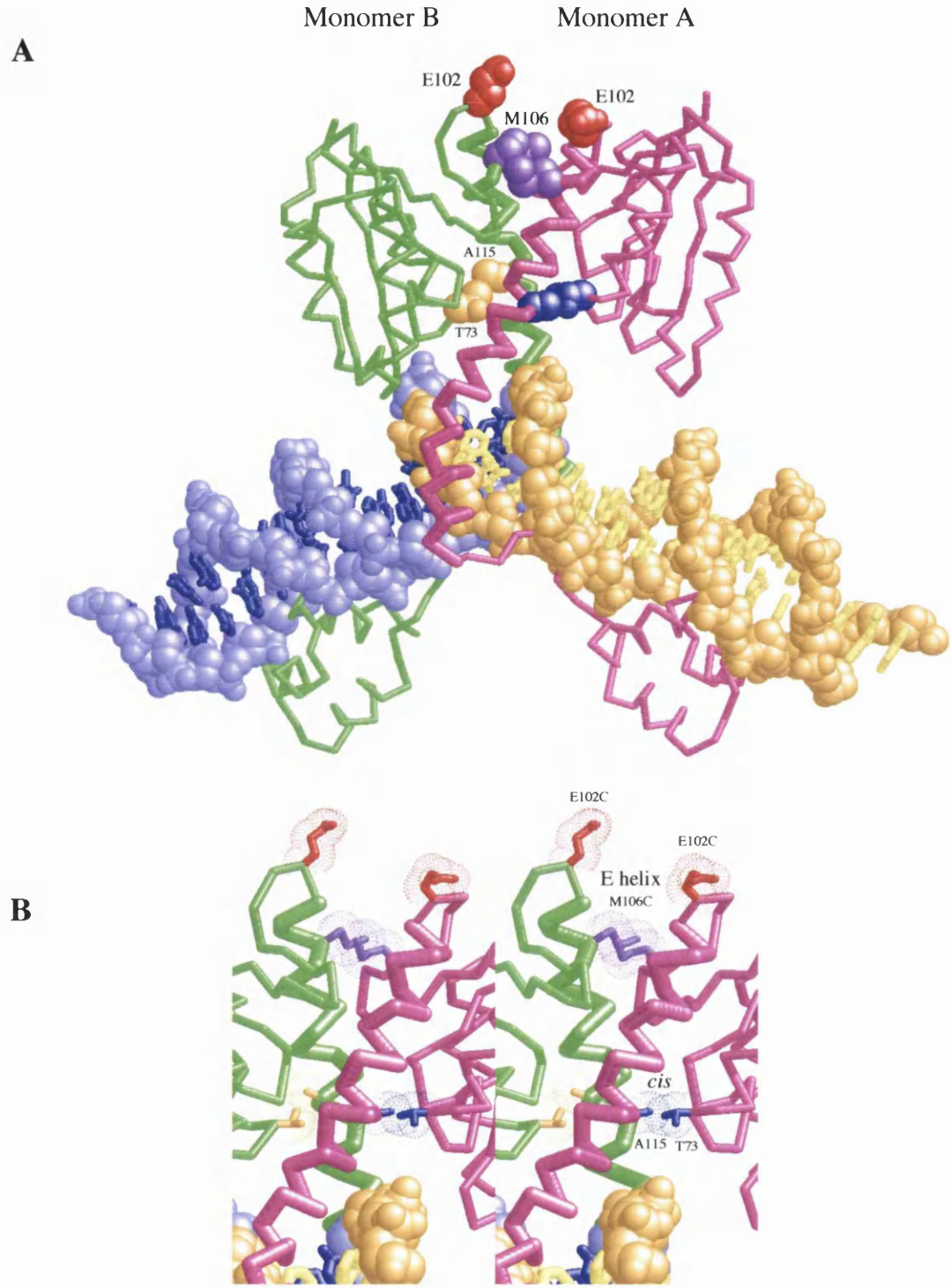
| Inter-face | E Helix Residue X | Residue Y | Distance (Cα-Cα) (Å) | Geometry: ßαα angles (°) | | Comments |
|---|---|---|---|---|---|---|
| | | | | $\beta_X\alpha_X\alpha_Y$ | $\beta_Y\alpha_Y\alpha_X$ | |
| *Cis* | 112 A | 73 A | 7.838 | 114 | 47 | The distance is quite long and the geometry of the side chains is not ideal. |
| *Cis* | 112 B | 73 B | 9.581 | 135 | 70 | |
| *Cis* | 112 A | 74 A | 7.406 | 88 | 106 | The distance is reasonable but the geometry is unfavourable. |
| *Cis* | 112 B | 74 B | 8.745 | 114 | 126 | |
| *Cis* | 112 A | 77 A | 7.077 | 72 | 40 | Ideal distance, however, the S112 side chain is out of the "plane" of the dimer and perpendicular to I77 and may disturb the protein upon crosslinking, considered as a back-up pair. |
| *Cis* | 112 B | 77 B | 6.432 | 100 | 44 | |
| *Cis* | 115 A | 73 A | 7.176 | 41 | 18 | Distance and geometry are good. |
| *Cis* | 115 B | 73 B | 6.876 | 50 | 42 | |
| *Cis* | 115 A | 74 A | 7.895 | 13 | 88 | Distance is reasonable, but A74 points out of the "plane" of the dimer and may create distortion upon linkage. |
| *Cis* | 115 B | 74 B | 7.047 | 41 | 112 | |
| *Trans* | 109 A | 95 B | 7.770 | 59 | 75 | The distance and geometry are not as good as between residue 95 and 113, however, they were considered as a back-up pair. |
| *Trans* | 109 B | 95 A | 7.983 | 59 | 83 | |
| *Trans* | 112 A | 95 B | 9.245 | 85 | 62 | Distance is not ideal. |
| *Trans* | 112 B | 95 A | 9.723 | 69 | 68 | |
| *Trans* | 113 A | 94 B | 9.756 | 67 | 79 | Distance is too long for disulphide linkage. |
| *Trans* | 113 B | 94 A | 10.063 | 50 | 92 | |
| *Trans* | 113 A | 95 B | 6.535 | 54 | 43 | Distance and geometry are good. |
| *Trans* | 113 B | 95 A | 7.161 | 37 | 49 | |
| *Trans* | 113 A | 96 B | 9.422 | - | - | Distances are very long. The geometry could not be determined as the coordinates of the hydrogen (R group) of G96 were not determined. |
| *Trans* | 113 B | 96 A | 9.790 | - | - | |
| *Trans* | 113 A | 97 B | 8.553 | 16 | 35 | Distance is not ideal. |
| *Trans* | 113 B | 97 A | 8.655 | 8 | 37 | |
| E Helix | 102 A | 102 B | 12.527 | 40 | 54 | Distance is extremely long for a disulphide bond. |
| E Helix | 106 B | 106 A | 9.189 | 54 | 33 | Distance is a concern for disulphide linkage. |
| (none) | 95 A | 95 B | 22.247 | | | |
| (none) | 113 A | 113 B | 12.861 | | | Geometry is highly unfavourable |

**Fig. 5.4 Co-crystal structure of γδ resolvase showing residues D95 and A113**




The co-crystal structure of γδ resolvase is depicted as previously. Residues D95 of monomer B (orange) and residue A113 (blue) of monomer A (E helix) are shown space-filled (A). Only one pair of residues spanning the *trans* interface are highlighted for simplicity. The geometry of the side chains is shown in stereo close up (B).

**Fig. 5.5 Co-crystal structure of γδ resolvase showing residues T73 and A115 spanning the *cis* interface and E102 and M106 of the E helix**




The co-crystal structure of γδ resolvase is depicted as previously. Residues T73 and A115 of monomer A (blue) and residues T73 and A115 of monomer B (orange), each spanning the *cis* interface are shown space-filled (A). Residues E102 (red) and M106 (purple) spanning the E helix interface are also shown space-filled (A). The geometry of the side chains is shown in stereo close up (B).

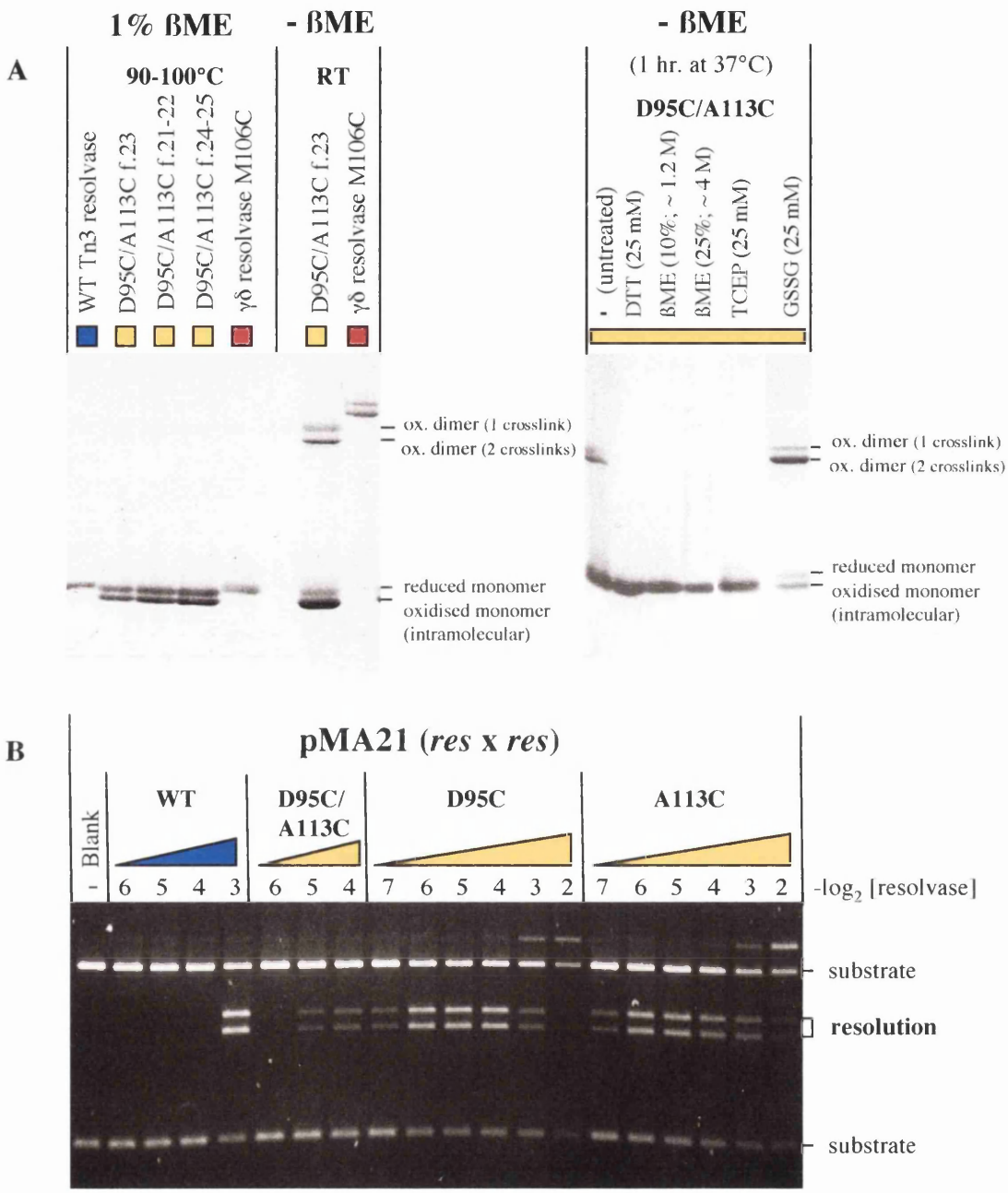
2). The *in vivo* phenotype of D95C/A113C was wild-type whereas T73C/A115C was inactive, like A115C. When the A115C substitution was combined with the activating mutation E124Q, *in vivo* activity with a *res* x *res* substrate (pDB34) was restored. Unfortunately, the triple mutant T73C/A115C/E124Q was still inactive in this assay, and was thus not investigated further.

## 5.4 Purification and characterisation of reduced D95C, A113C and D95C/A113C

The two cysteine-containing Tn3 resolvase mutants, D95C and A113C, and the double cysteine mutant D95C/A113C, were overexpressed and purified. All mutants behaved similarly to wild-type resolvase throughout purification. The concentrations of the purified fractions were estimated by comparison on SDS PAGE gels with a wild-type resolvase fraction, R17 f.47, for which the concentration was determined by amino acid analysis (Blake, 1993), and are listed in Table 5, Chapter 2.

SDS PAGE analysis under standard reducing sample preparation conditions (1% ß-mercaptoethanol (ßME), heated to 100°C for 5 min.) showed that D95C and A113C migrated as single bands at the monomer position, 20 kDa (data not shown). However, the D95C/A113C double mutant migrated as two closely spaced bands near the monomer position (Fig. 5.6, A). The faster-migrating species was thought to be resolvase with an intramolecular crosslink, whereas the slower-migrating species was thought to be reduced protein. More vigorous reduction, including treatment with a final concentration of 25 mM DTT or 2% β-mercaptoethanol, resulted in the complete disappearance of the faster-migrating oxidised species (Fig. 5.6, A). When the D95C/A113C enzyme preparation was analysed in the absence of ßME, two species migrating near the 40 kDa position were seen. These were also seen when D95C/A113C was treated with oxidising agents such as oxidised glutathione, and were thought to be oxidised dimers containing either one or two trans crosslinks. Their conversion to the monomer species upon treatment with reducing agent supported this interpretation. The identification of these species was also supported by comparison with heterodimer crosslinked species described in section 5.5. Upon storage in the -20°C freezer the intramolecular disulphide-linked D95C/A113C species converted to the oxidised dimer over time.

**Fig. 5.6 Oxidation and reduction of D95C/A113C and activities of reduced D95C, A113C and D95C/A113C**




**A.** Laemmli gel analysis (15%) of resolvase fractions, treated with an oxidising agent (GSSG-oxidised glutathione), a variety of reducing agents (DTT, ß-mercaptoethanol, and TCEP) or modifying agents (NEM and DTNB), as labeled. Samples marked 90-100°C were boiled for 15 min. in the presence of 1% ßME final prior to loading. All other samples were run without heating and in the absence of ßME. Some samples were incubated at 37°C for 1 hour prior to loading, as marked.
**B.** D95C, A113C and D95C/A113C assayed for resolution activity on a *res* x *res* substrate (pMA21), in a non-standard reducing recombination buffer (C9.4, 5 mM Spermidine, 2.5 mM DTT; 3 hours 40 min. at 37°C). Dilutions were of fractions of the following concentrations; 7.5 $\mu$M wild-type, 15 $\mu$M D95C/A113C, 30 $\mu$M D95C, and 40 $\mu$M A113C. These were added at 1/10 volume. Samples were analysed by restriction digest with *Pst*I and *Hind*III.

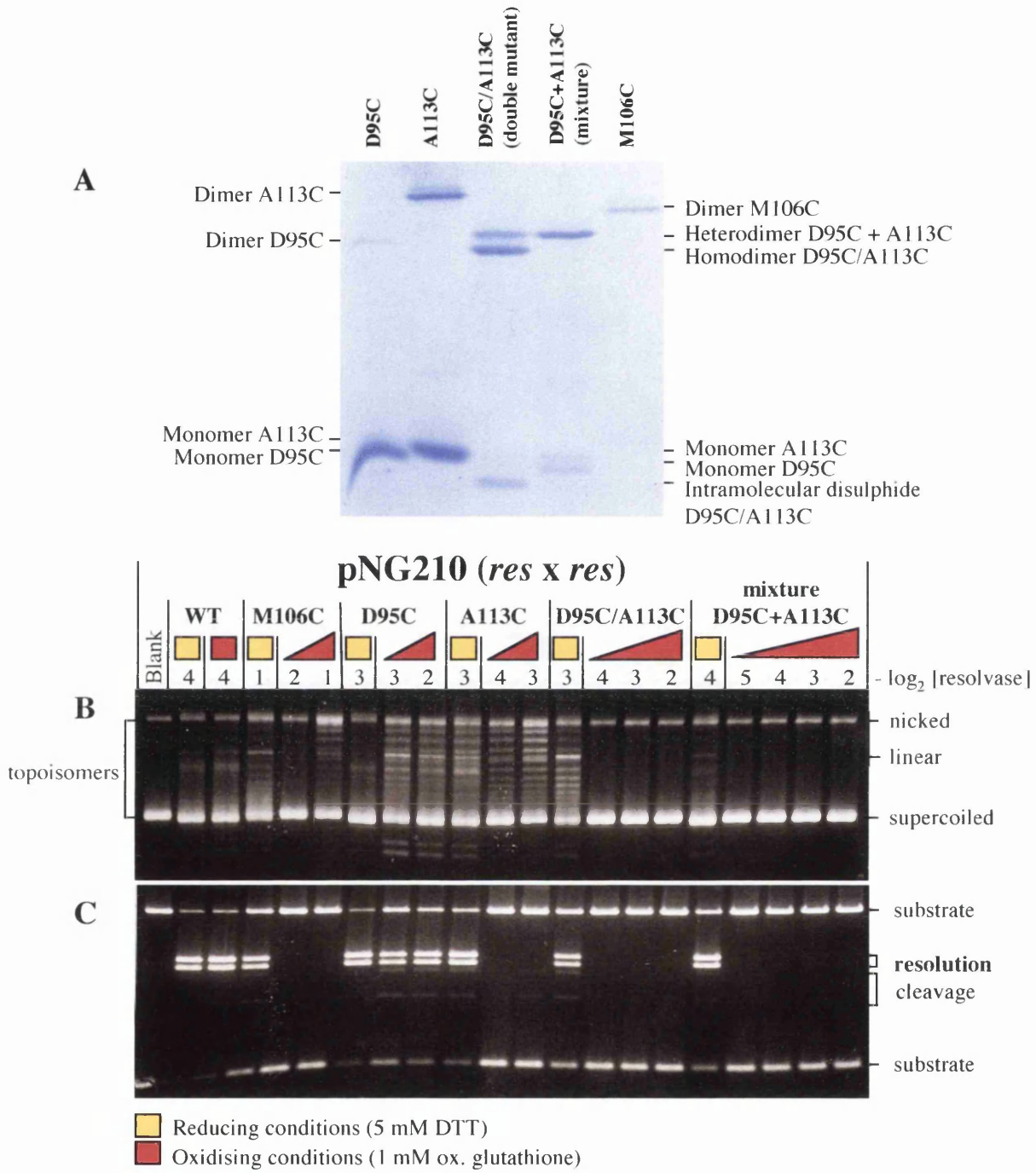
In reducing conditions (C9.4, 5 mM spermidine, 2.5 mM DTT final), D95C and A113C were found to be active recombinases *in vitro*, in agreement with the *in vivo* data. Resolution activities were comparable with wild-type resolvase on a standard *res* x *res* substrate (pMA21). The D95C/A113C double mutant was found to be considerably less active than either single mutant (Fig. 5.6, B).

## 5.5 Oxidation of cysteine-containing mutants

In order to achieve oxidation of D95C, A113C, D95C/A113C, and M106C (provided by A. MacDonald), samples of the purified resolvase stocks were reduced (30 min. at 37°C, 10 mM DTT) and dialysed overnight against a buffer containing 1 mM oxidised glutathione (2 M NaCl, 20 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 1 mM oxidised glutathione). Reducing the resolvases prior to oxidation should remove the intramolecular-disulphide crosslinked species in the D95C/A113C enzyme preparation and allow oxidation of folded resolvase. In a parallel experiment, approximately equal amounts of D95C and A113C resolvase were mixed and dialysed similarly, to obtain heterodimer with only a single *trans* disulphide link.

The extent of oxidation, as observed on SDS PAGE following overnight dialysis, correlated with the distance between the cysteine residues predicted from the crystal structures (Fig. 5.7, Table 7). For example, only a trace of dimer species was observed for D95C (less than 5%), indicating that this was the cysteine-containing mutant most resistant to formation of an interdimer disulphide bond. In contrast, the M106C mutant quantitatively converted to the oxidised dimer species (as detectable on SDS PAGE). The D95C/A113C double mutant was seen to contain a large amount of oxidised dimer species (>90%) which migrated as two closely spaced bands. The slightly slower-migrating species was believed to contain one disulphide bond *in trans*, whereas the faster migrating dimer species was thought to contain two disulphide bonds. The mixture of D95C and A113C also oxidised readily, forming a specific singly-linked heterodimer species (>90%). This migrated at the same position as the slower-migrating oxidised D95C/A113C dimer species, consistent with this species containing only one disulphide bond on the *trans* interface. The different rates of oxidation of D95C, A113C, and D95C/A113C suggest an explanation of the bias towards formation of the oxidised heterodimer (D95C-A113C) as opposed to the oxidised homodimers

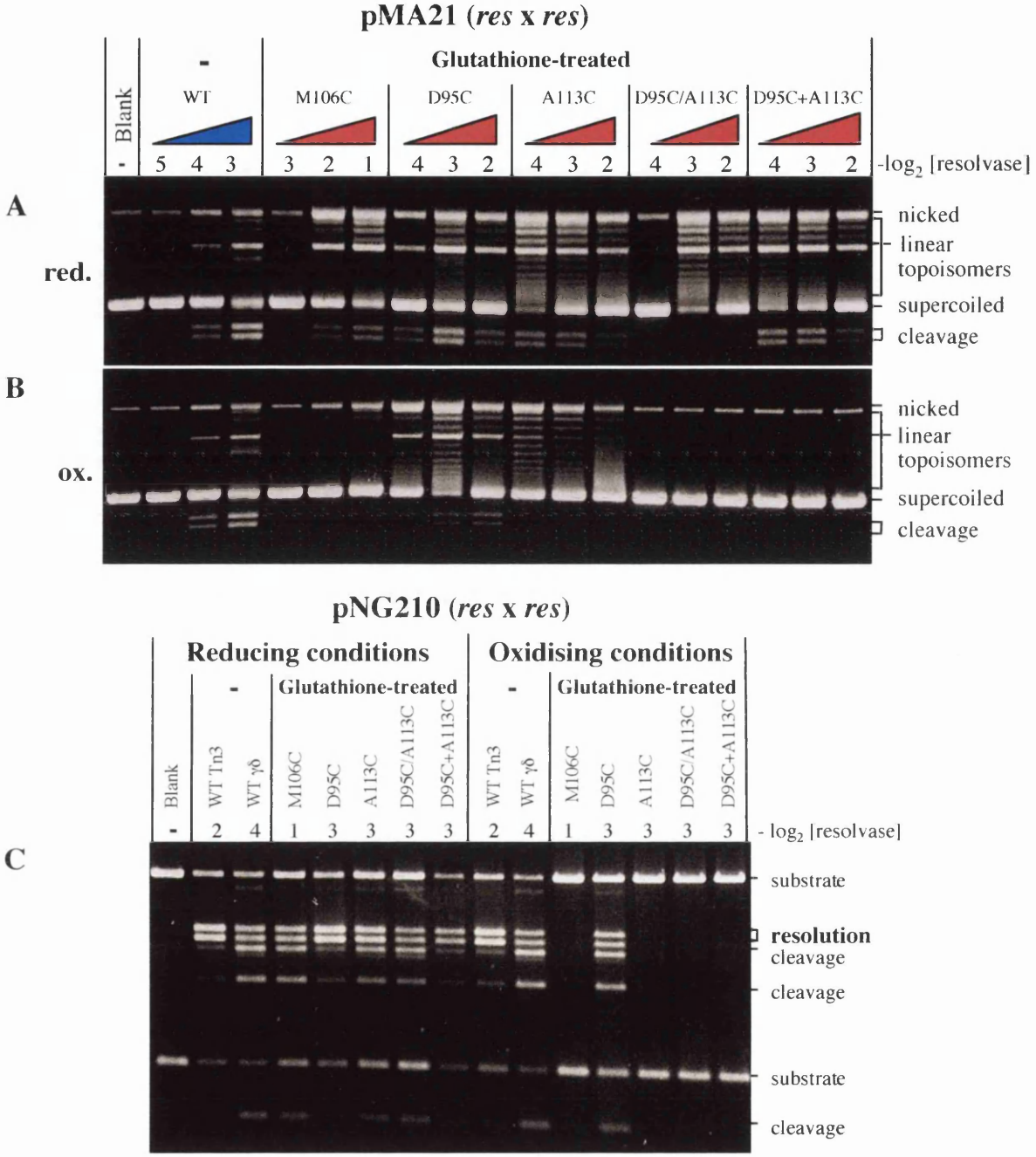
## Fig. 5.7 Oxidation of cysteine-containing mutants inhibits recombination




**A.** Cysteine-containing resolvase mutants were reduced using DTT (10 mM final, 30 min. at 37°C), then diluted 2-4 fold with resolvase dilution buffer. The D95C and A113C single mutants were also combined in equal concentrations. All enzymes (200 $\mu$l) were dialyzed overnight (20 hrs. at 4°C) against buffer (2 M NaCl, 40 mM Tris/HCl pH 7.5, 0.2 mM EDTA) containing 1 mM oxidised glutathione. These were recovered and mixed with an equal volume of glycerol. Samples were analysed on SDS PAGE in the absence of ß-mercaptoethanol.

**B+C.** The glutathione (ox.)-treated fractions (A) were assayed on pNG210 (*res* x res) in recombination buffer (C9.4, 5 mM Spermidine) containing either 5 mM DTT (yellow) or 1 mM oxidised glutathione (red). Dilutions were made from resolvase fractions at the following concentrations; 7.5 $\mu$M Tn3 R WT, 0.5 $\mu$M ox. M106C, 7.5 $\mu$M ox. D95C, 10 $\mu$M ox. A113C, 5 $\mu$M ox. D95C/A113C, and 9 $\mu$M ox. D95C +A113C heterodimer. Dilutions of resolvase were added at 1/10 volume. Reactions were allowed to proceed for 3 hours at 37°C. Aliquots were analysed uncut (B) and restriction digested with *Pst*I and *Hind*III (C).

## Fig. 5.8 Cleavage activities of oxidised preparations of cysteine-containing mutants under oxidising and reducing conditions




**A + B.** Glutathione-treated resolvases were assayed for cleavage activity of pMA21 under reducing (A) and oxidising conditions (B). The cleavage buffer (40% ethylene glycol, 50 mM tris/HCl pH 8.2, 0.1 mM EDTA) was supplemented with 5 mM DTT (for reducing conditions - A) or 1 mM oxidised glutathione (for oxidising conditions - B). Reactions proceeded for 3.5 hours at 37°C and were stopped using proteinase K (50 $\mu$g/ml final).

**C.** Reactions were set up as above but pNG210 (*res* x *res*) was used as a substrate and the buffer was supplemented with 5 mM spermidine. Samples were purified using the Wizard DNA Clean-Up System (Promega, Cat. no. A7280) and restricted with *Pst*I and *Hind*III. Dilutions for both experiments were made from the "oxidised" resolvase fractions. Resolvase stock concentrations were; 7.5 $\mu$M Tn3 R WT, 7.5 $\mu$M γδ R WT, 0.5 $\mu$M ox. M106C, 7.5 $\mu$M ox. D95C, 10 $\mu$M ox. A113C, 5 $\mu$M ox. D95C/A113C, and 9 $\mu$M ox. D95C +A113C heterodimer. Dilutions of resolvase were added at 1/10 volume.

Resolution of the substrate pNG210 requires numerous activities of resolvase. These include binding to all subsites of *res* and the formation a productive synapse, strand cleavage, exchange and re-ligation. The inability of the oxidised species of these various mutants to perform the resolution reaction could be due to a block at any one of these steps. The proposed studies on strand exchange would not be possible if the oxidised resolvases were defective at steps prior to strand cleavage. Therefore, the cleavage activity was assayed in a variety of buffers known to accumulate the covalent intermediate, under reducing and oxidising conditions (Fig. 5.8). These buffers ranged from a standard recombination buffer with magnesium omitted (data not shown) to buffers containing a high concentration of ethylene glycol (Fig. 5.8 A and B) to a buffer containing ethylene glycol as well as spermidine (5 mM), which supports dissociative cleavage/ligation reactions (Fig. 5.8, C). Under reducing conditions all resolvases cleave the substrate pMA21 or pNG210 (*res* x *res*). However, the cleavage reaction by oxidised resolvases (M106C, D95C/A113C, D95C+A113C heterodimer and the partially oxidised A113C) is suppressed. The oxidised proteins are therefore defective in cleavage, or an earlier step.

## 5.7 Targeting oxidised cysteine-containing mutants to subsites of *res*

Wild-type resolvase activity is only initiated when a productive synapse is formed, in which the accessory sites are wrapped trapping three negative supercoils. The oxidised resolvases may be incapable of initiating cleavage of the substrate simply because they are not synapsis-competent. Although oxidised γδ M106C resolvase was known to be able to synapse sites II and III (Hughes *et al.*, 1993; Murley and Grindley, 1998), the possibility remained that accessory site synapsis involved a domain-swapping scenario, which might help to stabilise the productive synapse. If such a synapsing mechanism operates, crosslinking the *cis* or *trans* interface (but not the E-helices, as in oxidised M106C), would block synapsis.

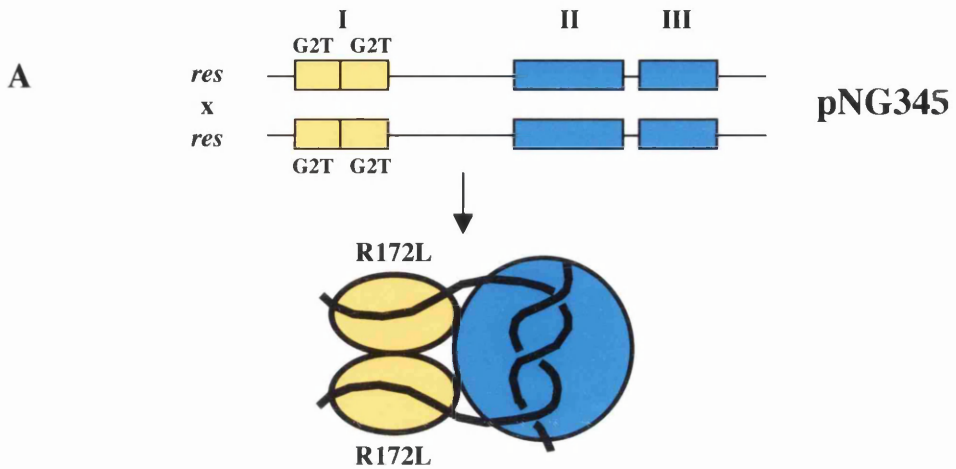
A test of accessory site function can be performed by making use of the specificity mutant, γδ resolvase R172L (Grindley, 1993). By targeting γδ resolvase R172L exclusively to the crossover site of *res*, the pNG345 complementation assay probes whether oxidised mutants bound at the accessory sites retain the ability to stimulate resolvase bound at the crossover sites. Furthermore, using the substrate pNG343, stimulation or inhibition of site I-bound

resolvase in *trans* can be assessed by targeting γδ resolvase R172L to only one of the crossover sites.

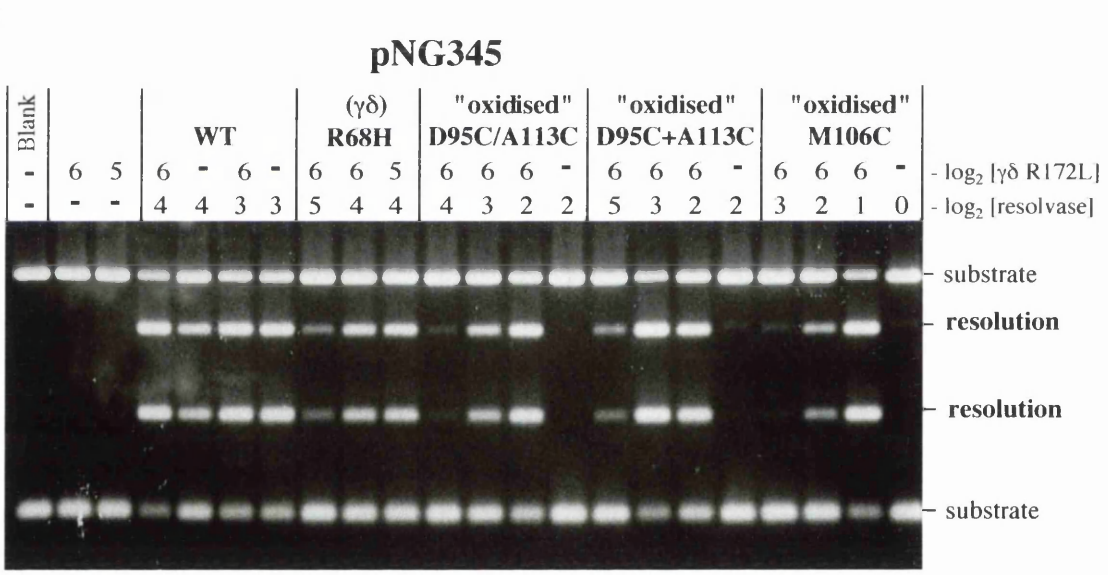
Initially the substrate pNG345 was used, which targets γδ resolvase R172L to both crossover sites. Complementation reactions were performed by adding the disulphide-linked mutants, or control protein with wild-type binding domains, first. This enabled the resolvase to bind and synapse the accessory sites. The specificity mutant γδ resolvase R172L was then added and the reactions were allowed to proceed. γδ resolvase R68H was used as a positive control. This resolvase carries a mutation in an active site residue, R68, and therefore has no catalytic activity at site I. However, when targeted to the accessory sites, γδR R68H is able to complement limiting concentrations of γδR R172L to carry out cleavage and strand exchange of the crossover sites (Boocock *et al.*, 1995). The oxidised resolvase mutants (D95C/A113C, the heterodimer D95C+A113C, and M106C) were also able to complement γδR R172L for recombination of pNG345 (Fig. 5.9, B). These results show that the oxidised mutants bind at sites II/III, are proficient in accessory site synapsis, and complement strand exchange at site I. It was therefore concluded that domain swapping, (or any other major rearrangement of the dimer) is not essential for any of the functions of resolvase at sites II and III, including "activation" of the resolvase bound at the crossover sites.

Having ascertained that oxidised D95C/A113C as well as the heterodimer D95C+A113C are fully functional at sites II and III, it was possible to investigate whether these mutants could stimulate cleavage by a WT resolvase dimer when they occupy the other crossover site. Using the substrate pNG343 it is possible to target γδR R172L to only one crossover site (Fig. 5.10, A) (Boocock *et al.*, 1995). However, when added at a high concentration, γδR R172L can bind all sites of *res* and promote cleavage of both crossover sites of pNG343. When an inactive resolvase, such as γδR R68H, is titrated into the reaction this results in a transition from double *res* site cleavage (at a high [R172L] : [R68H]) to complete inactivity (at a low [R172L] : [R68H]) (Fig. 5.10, B). Importantly, by performing such a titration, one can determine the activity of γδR R172L bound to a single crossover site when all other sites of pNG343 are bound by γδR R68H. The full-length linear cleavage product, in which only the crossover site bound by γδR R172L is cut, indicates that the inactive R68H resolvase bound at the other crossover site is able to stimulate cleavage in *trans* (Boocock *et al.*, 1995). When the oxidised D95C/A113C and the heterodimer D95C+A113C were used in place of

## Fig. 5.9 Accessory site synapsis by oxidised cysteine-containing mutants



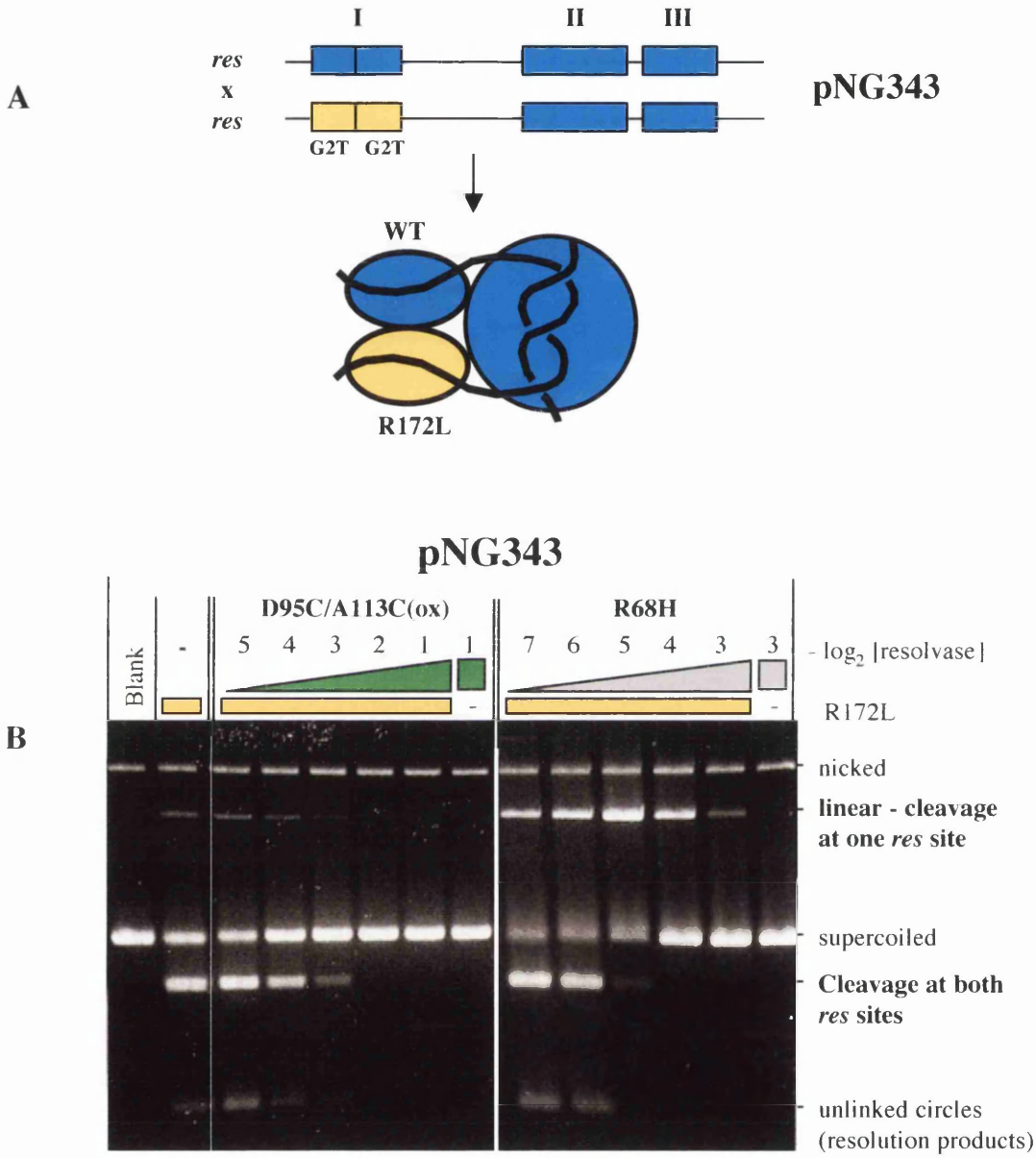

B




**A.** pNG345 targets R172L resolvase to both G2T crossover sites as shown.

**B.** Reactions with pNG345 were set up in resolution buffer (C9.4, 5 mM Spermidine, 1 mM oxidised glutathione). Oxidised resolvase dilutions (or dilution buffer) were added (1/20 volume). An equal volume of a dilution of γδ resolvase R172L (0.7 $\mu$M) was added after approximately 5 minutes. Reactions proceeded for 45 min. at 37°C and were restricted with *Pst*I and *Hind*III. Resolvase stock concentrations were as follows: 7.5 $\mu$M Tn3 R WT, 30 $\mu$M γδ R R68H, 5 $\mu$M Tn3 R "ox." D95C/A113C, 9 $\mu$M Tn3 R "ox." D95C+A113C, and 0.5 $\mu$M Tn3 R "ox." M106C.

**Fig. 5.10 Targeting oxidised D95C/A113C to a single crossover site using pNG343**




**A.** pNG343 targets R172L resolvase to the G2T crossover site, thus targeting of a dimer to only a single crossover site within a synaptic complex can be achieved.

**B.** Reactions with pNG343 were set up in cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1mM EDTA, 1 mM oxidised glutathione). Dilutions of the stock "oxidised" D95C/A113C fraction (5 $\mu$M) or γδ resolvase R68H (30 $\mu$M) were added (1/20 volume) first. γδ resolvase R172L (1.4 $\mu$M) was added (1/20 volume) after approximately 5 minutes. Reactions proceeded for 3.5 hours at 37°C and were stopped by proteinase K treatment (50 $\mu$g/ml final).

R68H this intermediate linear cleavage product was not observed (Fig. 5.10, B). These oxidised dimers were known to complement γδR R172L bound to the crossover site (pNG345 targeting experiment, Fig. 5.9, described above). The inhibition of the reaction must therefore be due to binding at the crossover sites by the oxidised resolvase mutants. The absence of the full-length linear cleavage product in these complementation assays suggests that the crosslinked dimers inhibit cleavage by catalytically active resolvase targeted to the opposite crossover site. This phenotype is unlike that of all other catalytically inactive mutants characterised to date. Furthermore, the "inhibition in *trans*" displayed by these oxidised mutants is a function of their oxidation state. Under reducing conditions, the same mutants could clearly complement limiting concentrations of γδR R172L, yielding the twice-cut cleavage products (data not shown). The failure of the disulphide-linked resolvases to complement R172L in this assay suggests either that they are deficient in crossover site synapsis, or that they block cleavage by an active dimer with which they are synapsed.

## 5.8 Binding assays with site I of *res*

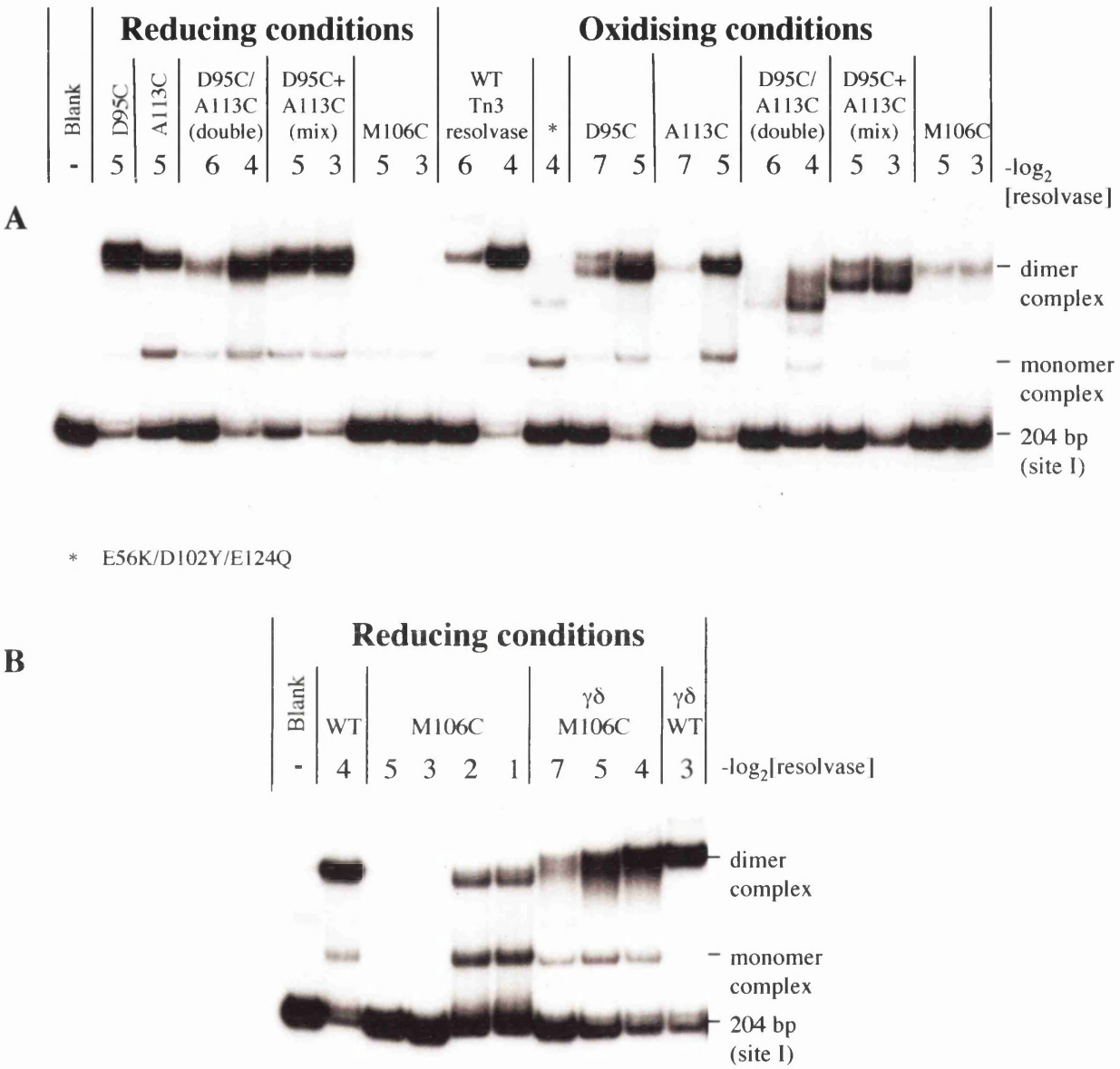
The ability of the oxidised and reduced cysteine-containing mutants to bind to the crossover site was determined using a band-shift assay (Fig. 5.11). Reduced D95C, A113C, the D95C+A113C mixture and D95C/A113C bound the crossover site fragment mostly as dimer complexes. These all migrated similarly to the wild-type resolvase dimer complex (Blake *et al.*, 1995), with the D95C/A113C double mutant migrating only slightly faster. Reduced M106C bound site I in a less cooperative manner, with the monomer and dimer complexes being equally represented (Fig. 5.11 B). This was also noted for γδR M106C, which binds less cooperatively than wild-type γδresolvase, for which no monomer complex was detected. Additionally, the dimer complex was diffuse, indicating that this complex may be less stable than its wild-type equivalent. When the oxidised preparations of these mutants were assayed, some differences were noted. Partially oxidised D95C gave two complexes that migrate near the WT dimer complex. The slower-migrating band is presumed to be the due to the reduced species and the more prominent (especially at higher concentrations) faster-migrating band is thought to be due to binding of the oxidised dimer. This suggests that at lower concentrations the oxidised species binds more tightly (as can be seen for M106C), giving equal amounts of each complex despite the preponderance of reduced D95C (as judged by SDS PAGE, Fig. 5.7, though considerable in-gel oxidation cannot be ruled

out). The ability of oxidised D95C to bind the crossover site (in which the disulphide bond spans residues that are 22 Å apart in the co-crystal structure) may be indicative of extreme flexibility within the dimer. The pattern for A113C binding was the same in reducing or oxidising conditions, presumably because the oxidised species cannot bind the crossover site. Structurally, it is difficult to see how an oxidised A113C dimer would not be severely distorted. The protein-DNA complexes observed may therefore derive solely from the reduced species present in the glutathione-treated A113C fraction.

A striking difference was observed between the binding patterns of both the oxidised D95C+A113 mixture and the D95C/A113C double mutant under oxidising and reducing conditions. While the D95C+A113C mixture gave a single "dimer" band under reducing conditions (presumably because the reduced D95C and reduced A113C dimer complexes migrate at nearly identical positions), the same fraction gave two "dimer" complexes under oxidising conditions. The faster-migrating band probably corresponds to the site I-bound oxidised D95C+A113C heterodimer. The slower-migrating band may represent the binding of the small amounts of reduced D95C and A113C present in the oxidised D95C+A113C fraction. Thus, this band would contain site I-bound reduced D95C, A113C and D95C+A113C dimer species, as observed under reducing conditions. The pattern of binding observed with the oxidised D95C/A113C reveals an even faster-migrating complex. This complex is thought to represent the oxidised D95C/A113C homodimer (containing 2 *trans* crosslinks) bound to site I. Above this dominant band, a diffuse band is observed, which probably contains reduced D95C/A113C and oxidised D95C/A113C heterodimer. At low M106C concentrations and under oxidising conditions, the dimer complex is observed, whereas this complex is absent under reducing conditions. This may be because at these low enzyme concentrations, the M106C mutant does not bind stably as a dimer unless it is covalently linked via a disulphide bond.

Binding assays thus revealed that all the cysteine-containing resolvases, including M106C, D95C, A113C, D95C+A113C heterodimer, and D95C/A113C resolvase, could bind site I in reducing conditions. Furthermore, in oxidising conditions all these mutants (with the exception of A113C) gave protein-DNA complexes with altered electrophoretic mobility, corresponding to crosslinked protein binding to site I. The catalytic inactivity of oxidised M106C, D95C+A113C heterodimer, and D95C/A113C resolvases in recombination is

## Fig. 5.11 Binding of cysteine-containing resolvases to Site I under reducing and oxidising conditions




**A**. Binding reactions were carried out with a 3' end-labelled, 204 bp *Eco*RI fragment from pAL221*sis*, in binding buffer (10 mM Tris/glycine pH 9.4, 10 % glycerol, 0.02 mM EDTA) in the presence of 50-100 nM carrier DNA (supercoiled plasmid). Dilutions of "oxidised" resolvase were added (1/20 volume) and incubated for 10 min. at room temperature. Samples (10 $\mu$l) were loaded on a non-denaturing PAGE Tris/glycine gel and run at 200 V for approximately 4 hours. Reducing reactions contained 10 mM DTT.

**B**. Same as above, except that DTT was added to the polyacrylamide gel (1 mM final) in order to keep the samples reduced throughout electrophoresis. Resolvase stock concentrations were as follows: 7.5 $\mu$M Tn3R WT, 7.5 $\mu$M Tn3R D95C, 10 $\mu$M Tn3R A113C, 5 $\mu$M Tn3R D95C/A113C, 9 $\mu$M Tn3R D95C+A113C, 0.5 $\mu$M Tn3R M106C, 12.5 $\mu$M Tn3R E56K/D102Y/E124Q, 9 $\mu$M γδR WT, 22.5 $\mu$M γδR M106C.

therefore not due to a failure to bind to the crossover site, although there is evidence that the crosslinked protein-DNA complexes may have an altered conformation.

## 5.9 Activation of cysteine-containing mutants through additional (96-105 Hin) substitutions

The failure of the disulphide-linked D95C/A113C resolvase to initiate strand cleavage hindered any further investigation into the mechanism(s) of strand exchange. The hyperactive resolvases with the greatest tendency to form covalent intermediates all contained the (96-105 Hin) substitution. Therefore, in order to make cleavage-competent resolvases which could also be oxidised, the (96-105 Hin) substitution was combined with the cysteine mutations, generating (96-105 Hin) M106C, (96-105 Hin) T73C/A115C and (96-105 Hin) D95C/A113C (Table 2, Chapter 2).

*In vivo*, the (96-105 Hin) M106C multiple mutant exhibited a wild-type phenotype, as expected (although the (96-105 Hin) substitution is activating *in vitro*, the *in vivo* assay does not detect its reduced substrate selectivity) (Table 3, Chapter 2). The (96-105 Hin) T73C/A115C and (96-105 Hin) D95C/A113C mutants, however, were inactive on all three substrates by the standard assay protocol. A number of colonies carrying each test substrate were grown on L-agar plates supplemented with kanamycin and ampicillin. This allowed the cells to grow under non-selective conditions (the medium contains no galactose to ferment) before being re-streaked to single colonies on MacConkey plates. Upon re-streaking, (96-105 Hin) T73C/A115C and (96-105 Hin) D95C/A113C yielded 60% and 30% white colonies with pDB34 (*res* x *res* test substrate), respectively (Table 3, Chapter 2). Therefore, both mutants showed a "slow" resolution activity *in vivo*.

(96-105 Hin) M106C and (96-105 Hin) T73C/A115C were purified on a large scale. Half the resolvase was dialysed into buffer containing oxidised glutathione in order to encourage disulphide bond formation. As with D95C/A113C, the untreated (96-105 Hin) T73C/A115C resolvase migrated as a doublet of monomer-sized bands in SDS PAGE (Fig. 5.12, A). The glutathione-treated (96-105 Hin) T73C/A115C resolvase contained only the faster-migrating species. It was deduced that the faster-migrating species corresponded to the intramolecular disulphide-crosslinked resolvase and that the slower migrating band was reduced monomer

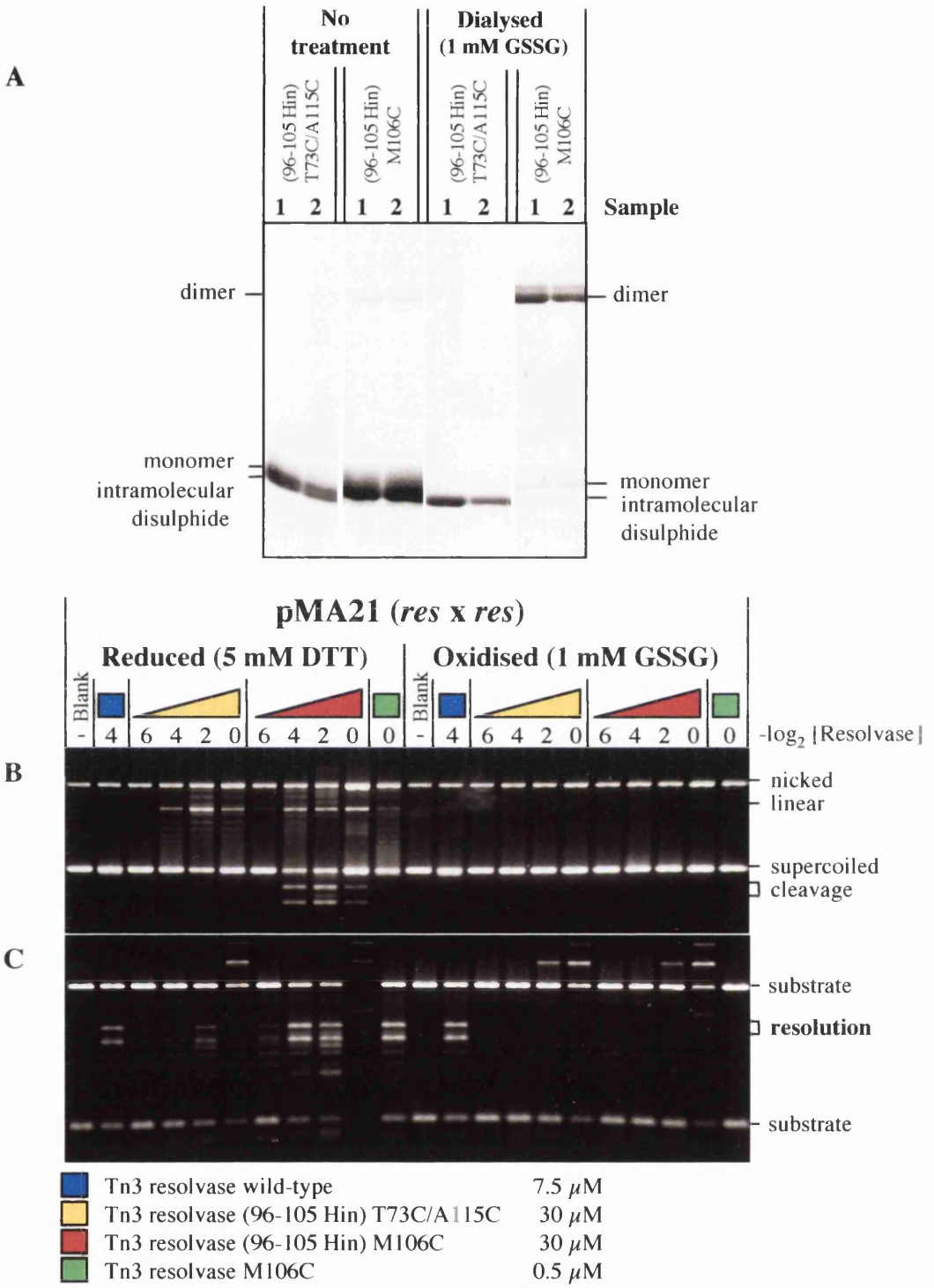
resolvase (as was the case for D95C/A113C). The large proportion (approximately 50%) of oxidised monomer material in the "reduced" samples (initially containing 1 mM DTT) indicates that the formation of an intramolecular disulphide between residues 73C and 115C may be especially favourable. The untreated (96-105 Hin) M106C mutant migrated as a single monomer-sized band, and therefore was fully reduced. In contrast, the glutathione-treated (96-105 Hin) M106C resolvase was found to contain greater than 90% dimer-sized material, corresponding to the oxidised disulphide-linked dimer.

Two attempts to purify (96-105 Hin) D95C/A113C on a large scale resulted in very low final yields, and gave fractions that were too dilute to assay properly. This was largely due to this mutant's remarkable insolubility. Large amounts were lost in the early stages of purification, when approximately half of the resolvase failed to solubilize in the denaturing 6 M urea buffer. (96-105 Hin) D95C/A113C eluted from the SP-sepharose column over a wide range of salt concentrations, as two broad peaks. Furthermore, the majority of the final pellet (following the final dialysis IV - which precipitates re-folded resolvase) did not resuspend in high salt resuspension buffer (2M NaCl, 40 mM Tris/HCl pH 7.5, 0.2 mM EDTA).

The reduced and oxidised stocks of (96-105 Hin) M106C and (96-105 Hin) T73C/A115C were initially assayed for recombination of pMA21 (*res* x *res*) under reducing and oxidising conditions, respectively (Fig. 5.12, B and C). Reduced (96-105 Hin) M106C was remarkably active under recombination conditions, resembling its "parent", (96-105 Hin) resolvase. Reduced (96-105 Hin) T73C/A115C, although clearly less active, nevertheless was able to initiate cleavage (as seen from the full-length linear product in the uncut samples) as well as yielding small amounts of resolution product (as observed upon restriction). In contrast, no activity was observed when the oxidised resolvase mutants were assayed under oxidised conditions (whereas Tn3 WT resolvase was unaffected by the presence of glutathione). The (96-105 Hin) substitution was thus not able to activate these mutants for recombination when crosslinked.

Cleavage of pMA21 (*res* x *res*) by oxidised and reduced (96-105 Hin) M106C and (96-105 Hin) T73C/A115C was also assayed under reducing and oxidising conditions (Fig. 5.13). Reduced (96-105 Hin) M106C, showed cleavage activity similar to that observed with (96-105 Hin). The M106C mutation therefore has little effect on the activity of the reduced form. Reduced (96-105 Hin) T73C/A115C was also active on this substrate. However, the full-

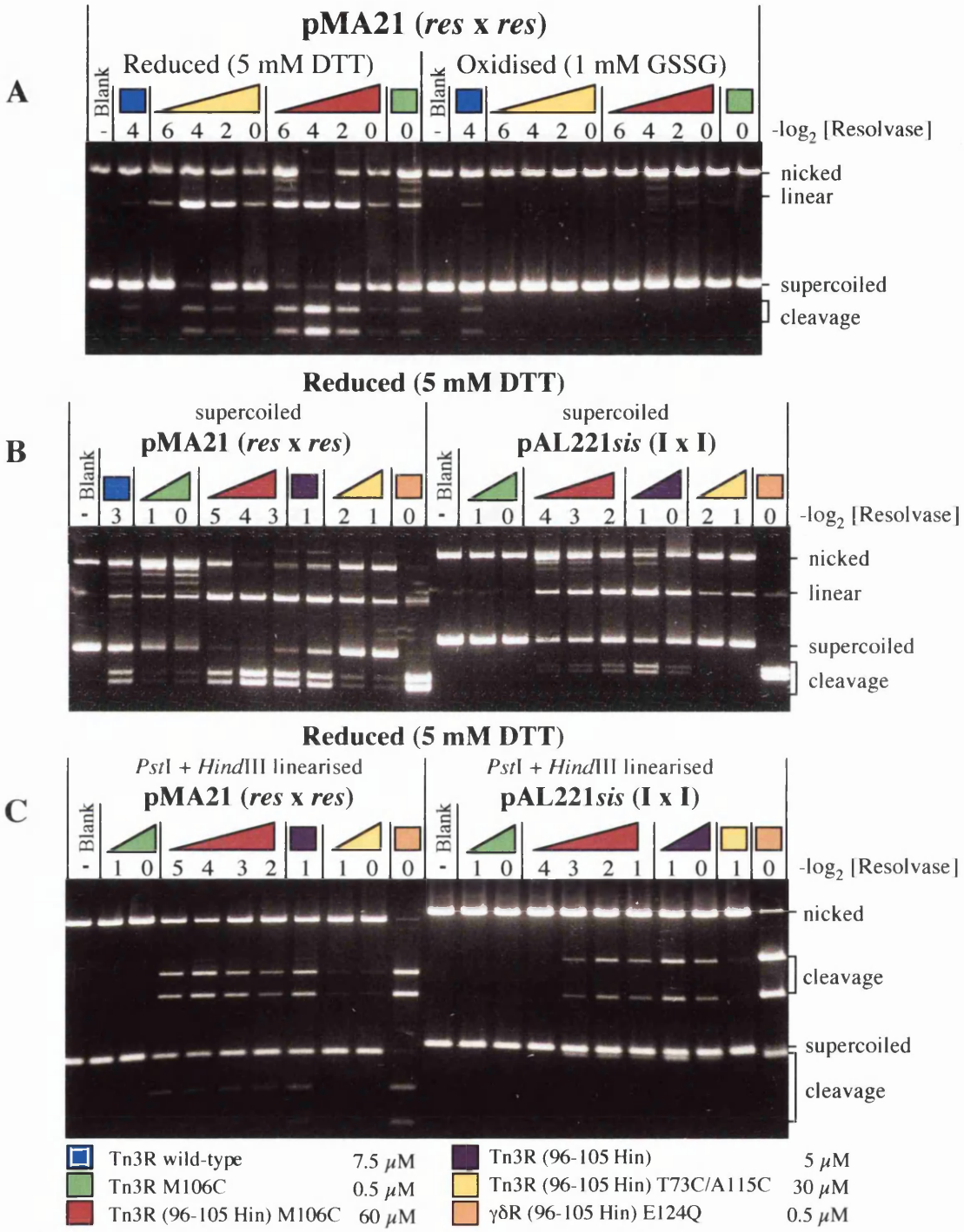
## Fig. 5.12 Characterisation of reduced and oxidised (96-105 Hin) substituted mutants




**A.** Aliquots of untreated and glutathione-treated Tn3R (96-105 Hin) T73C/A115C and Tn3R (96-105 Hin) M106C were analysed using non-reducing SDS PAGE. In each case, samples 1 and 2 represent first and second fractions from resuspension of the final resolvase pellet after purification.

**B+C.** Reactions were set up in recombination buffer (C8.2 plus 5 mM DTT (assays of untreated resolvase) or plus 1 mM ox. glutathione (assays of glutatione-treated resolvase)) and incubated for 3 hours at 37°C. Dilutions of resolvase fractions (see A) were added (1/10 volume). Aliquots were analysed uncut (B) and restricted with *Pst*I and *Hind*III (C). Reactions were stopped by treatment with proteinase K (50 μg/ml).

**Fig. 5.13 Cleavage activity of oxidised and reduced cysteine-containing mutants**




**A.** "Reduced" and "oxidised" fractions of Tn3R (96-105 Hin) T73C/A115C and Tn3R (96-105 Hin) M106C were obtained as described previously (section 5.10). Reactions were performed in cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl, 0.1 mM EDTA; supplemented with 5 mM DTT (reduced resolvase) or 1 mM oxidised glutathione (oxidised resolvase)) with supercoiled pMA21 (*res* x *res*) for 3 hours at 37°C.

**B+C.** "Reduced" fractions of Tn3R (96-105 Hin) T73C/A115C and Tn3R (96-105 Hin) M106C were were assayed under reducing conditions in cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl, 0.1 mM EDTA, 5 mM DTT) with pMA21 or pAL221*sis* and incubated for 22 hours at 37°C. Substrates were supercoiled (B) or linearised with *Pst*I and *Hind*III (C).

Dilutions of resolvase were added at 1/10 volume to all reactions. Reactions were stopped with proteinase K (50 μg/ml).

length linear product accumulates without a concomitant accumulation of the cleavage products resulting from double-strand cleavage at both *res* sites (that is, the ratio of full-length linear to supercoiled substrate is much higher than the ratio of the two "half-length linears" to full-length linear). This indicates that the cleavage reactions at the two *res* sites are not concerted as with wild-type resolvase. This could be due to a reduction in activity caused by the loss of supercoiling following the first double-strand break, and suggests that the reaction may be synapsis-independent or may involve dissociation of the half-sites (or both). No activity that can be ascribed to the oxidised mutants was seen under oxidising conditions. The low level of activity observed with (96-105 Hin) M106C could be due to the small amount of reduced species remaining after oxidation. Oxidised (96-105 Hin) T73C/A115C, was completely inactive when assayed under oxidising conditions. There is evidence that (96-105 Hin) resolvase does not require crossover site synapsis for the cleavage reaction (Chapter 4). Nevertheless, the crosslinked (96-105 Hin) multiple mutants were inactive in cleavage assays. This suggests that their inactivity is not due to a failure to synapse the crossover sites.

The cleavage activity of the reduced forms of these cysteine-containing mutants was then examined with substrates lacking supercoiling and/or accessory sites (Fig. 5.13). Both mutants, (96-105 Hin) M106C and (96-105 Hin) T73C/A115C, were found to be active to varying degrees on all of these substrates. As previously, (96-105 Hin) M106C behaved in an almost identical manner to (96-105 Hin). Interestingly, reduced (96-105 Hin) T73C/A115C displayed a low level of cleavage activity on the supercoiled site I x site I substrate (pAL221*sis*), despite having a lower cleavage activity on a supercoiled *res* x *res* substrate (pMA21) than wild-type resolvase. Hyperactivity, therefore, is not simply a matter of increasing reaction rates; (96-105 Hin) T73C/A115C cleaves a *res* x *res* substrate slower than wild-type resolvase but cleaves a site I x site I substrate faster than wild-type resolvase.

## 5.10 Crosslinking D95C/A113C using homobifunctional crosslinkers

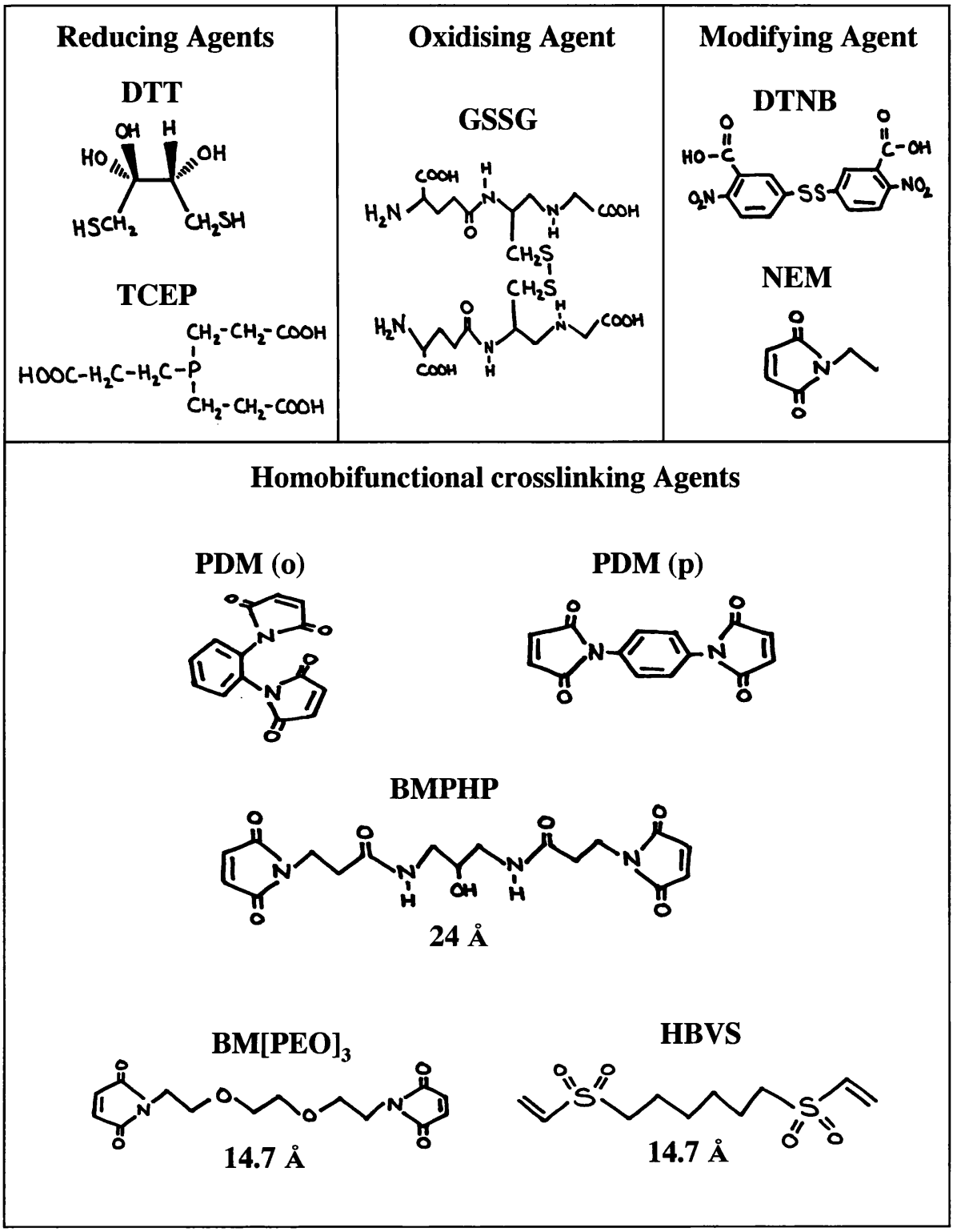
The results described above indicated that the disulphide-linked mutants D95C+A113C (heterodimer), D95C/A113C (homodimer), and M106C were capable of binding to all subsites of *res*, of interwrapping sites II and III, trapping three negative supercoils, and activating catalytically competent resolvase at the crossover site, but were not capable of

initiating cleavage of site I. Furthermore, this was shown to be due to the disulphide link, as the reduced forms of these mutants are all reasonably active resolvases. This was not entirely unanticipated. The distances between the alpha carbons of the crosslinked residues were based on the co-crystal structure, which displays the conformation of an inactive resolvase dimer in which the serine-10 nucleophiles are too far from their DNA target to initiate cleavage. The dimer may require a large degree of flexibility to catalyse strand cleavage. The disulphide bonds introduced in the dimers may reduce the flexibility of the resolvase dimer to the extent that cleavage could not be initiated.

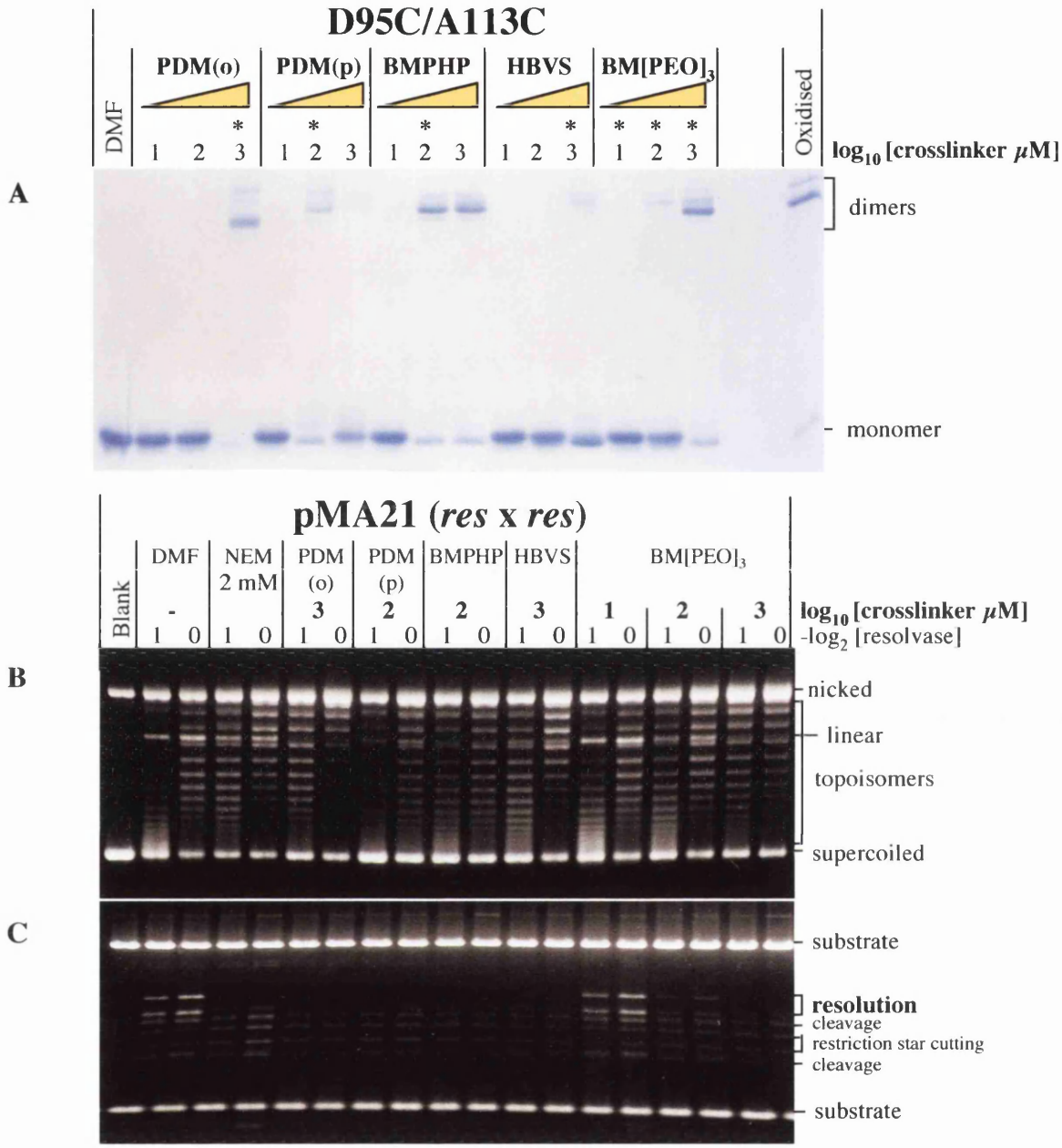
In order to increase the potential flexibility of the crosslinked D95C/A113C, homobifunctional sulfhydryl crosslinking agents were used for crosslinking (Fig. 5.14). The majority of the commercially available crosslinkers contain maleimide groups, which form an irreversible covalent bond with the sulphur of a cysteine side chain. These molecules vary in the distances they can span between two cysteine residues because of differences in the structure of the "arm" of the crosslinker. The crosslinkers of this type used were PDM (ortho and para), BMPHP, and $BM[PEO]_3$ (Fig. 5.14). The maleimide groups are rather "bulky", risking the disruption of the resolvase structure. Additionally, a crosslinking molecule, HBVS, was used. HBVS does not contain maleimides but instead generates a covalent linkage to the sulphur via vinylsulfone groups. As illustrated in Fig. 5.14, the distances that these particular crosslinkers span vary between 14.7 Å and 24 Å. It was hoped that these crosslinkers would enable the dimer to retain enough flexibility to initiate the cleavage step that must precede strand exchange. This increased flexibility of the crosslinked resolvase dimer (as compared with the distance generated by a disulphide link, which maximally spans only 6.5 Å) was nevertheless anticipated to prevent the large-scale rotations required by the subunit rotation and domain-swap models.

The crosslinking reactions were based upon previous crosslinking studies of different enzymes, using the same or similar homobifunctional crosslinking agents (Byrne and Stites, 1995; Franke and Pingoud, 1999; Persechini *et al.*, 1993; Wu *et al.*, 1998; Wu and Kaback, 1997; Yi *et al.*, 1991). The crosslinking efficiency was optimised by titrating the crosslinking molecules (from 10 - 1000 μM final concentrations) and varying reaction times and temperatures. Furthermore, different reaction buffers and different methods for reducing the protein and removing reducing agent before crosslinking were tried. The final optimised procedure is detailed in Chapter 2, section 2.27.

**Fig. 5.14 Structures of reducing and oxidising agents, crosslinkers and cysteine-modifying agents**



## Fig. 5.15 Crosslinking D95C/A113C and assays thereof




**A**. D95C/A113C was reduced by addition of DTT (20 mM final) and incubation at 37°C. DTT was removed using a G-25 column equilibrated with a high salt buffer (25% glycerol, 1.5 M NaCl, 30 mM Tris/HCl pH 7.5, 0.15 mM EDTA). The resolvase was split into 10 μl aliquots to which buffer (1 M NaCl, 25 mM Tris/HCl pH 7.5, 0.05 mM EDTA) was added (1.5 volumes) and 0.25 μl (or V/100) dimethylformamide (DMF) (control), or crosslinker dissolved in dimethylformamide (BMPHP was dissolved in formamide). Reactions proceeded for 30 min. at room temperature and were quenched with DTT (20 mM final). 15 μl samples were run on a 17% laemmli gel (SDS PAGE) under reducing conditions (1% βME), except for the lane containing oxidised D95C/A113C, from which βME was omitted during sample preparation. The crosslinked protein samples were diluted 2-fold with glycerol and stored at -20°C. Samples used in resolution reactions are marked with an asterisk.

**B+C**. Crosslinked or modified D95C/A113C resolvase samples (A) were assayed in C8.2 buffer at 37°C for 2 hours and were analysed directly (B), or treated with *Pst*I and *Hind*III (C). D95C/A113C resolvase was NEM modified as follows; resolvase was reduced (20 mM TCEP), gel filtered into MOPS buffer (25%glycerol, 50 mM MOPS/NaOH pH 7.5, 0.15 mM EDTA, 1.5 M NaCl, 2 mM TCEP/NaOH pH 7.5). NEM was dissolved in ethanol and added at 1/50 volume (2 mM final). The reaction proceeded for 30 min. at room temperature and was quenched using 400 mM βME.

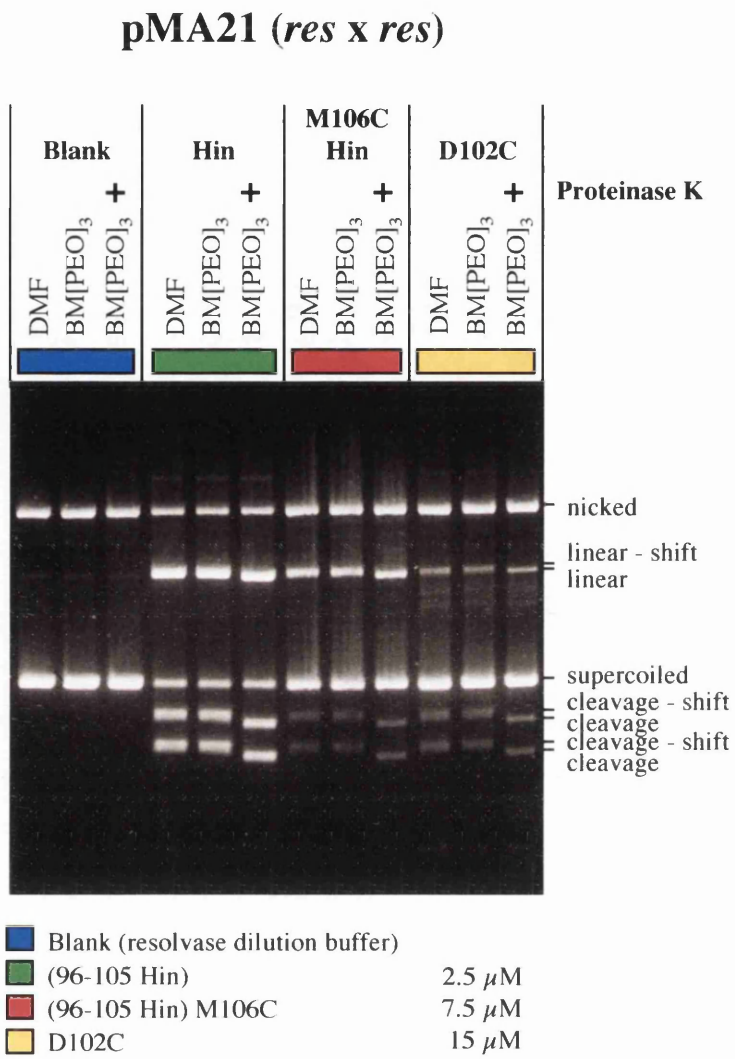
## 5.11 Characterisation of modified and crosslinked species

Reactions with PDM (ortho and para), BMPHP, HBVS and $BM[PEO]_3$ to crosslink D95C/A113C yielded up to 80% crosslinked material (Fig. 5.15, A). The crosslinked protein samples were assayed under recombination conditions. Restriction analysis showed no detectable resolution product. However, D95C/A113C treated with these crosslinkers was found to be able to nick a *res* x *res* substrate and showed substantial topoisomerase activity, suggesting that at least one DNA strand could be cut and rejoined by crosslinked resolvase (Fig. 5.15, B). This contrasts with the complete inactivity of disulphide linked D95C/A113C and implies that the shorter disulphide links physically constrained the resolvase dimer more than the homobifunctional crosslinks, thereby blocking strand cleavage.

NEM modification would mimic the steric effects of the bulky maleimide groups of the crosslinkers, without covalently linking the resolvase dimers, and was therefore used as a control (NEM modification is described in Chapter 2, section 2.27). In fact, when D95C/A113C was modified with NEM, the same nicking activity (but absence of resolution activity) was observed as with the crosslinked species (Fig. 5.20, B and C). This suggests that the lack of resolution activity seen with crosslinked D95C/A113C was not necessarily due to the constraint imposed by the crosslink, but may be entirely due to linkage of the bulky maleimide group. Further experiments revealed that A113C was inactivated by modification with NEM, while D95C (and wild-type) retained activity (Chapter 6, Fig. 6.1).

In summary, the use of the longer crosslinks seems to have alleviated the problem associated with the inflexibility of the resolvase dimer. However, a new problem (inactivation by bulky maleimide groups) was revealed. The sensitivity to NEM modification was found to be specific to the position of the cysteine (and especially deleterious at position A113). Nevertheless, the efficient catalysis of strand breakage and accumulation of the cleaved intermediate complex with the (96-105 Hin) substituted cysteine-containing mutants, indicated a further use for the crosslinking molecules. The proximity of cysteine residues in the cleavage intermediate could be probed by generating large amounts of the cleavage complex under reducing conditions and then crosslinking "in *situ*". In a pilot experiment, shown in Fig. 5.16, the products of cleavage reactions were treated with crosslinkers or mock-treated with formamide and analysed in the presence or absence of proteinase K on an

**Fig. 5.16 Attempting *in situ* crosslinking of cleavage intermediates**




Reactions proceeded in reducing cleavage conditions (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA, 0.5 mM TCEP) for 30 min. at 37°C. The concentrations of enzyme dilutions used are listed above. Dilutions of resolvase were added 1/10 volume. In order to crosslink the cleavage intermediate $BM[PEO]_3$ was added (2 mM final) or DMF as a control and incubated for 15 min. at room temperature. Reactions were treated with 0.2 volumes of loading dye (1% SDS ± proteinase K, 250 μg/ml) for 5 min. at room temperature. The samples were loaded on an agarose gel containing 0.1% SDS in the gel and running buffer.

SDS containing agarose gel. The crosslinkers had no effect on the products observed. As the extent of the difficulties of crosslinking "in *situ*" was unknown, this approach, although potentially very informative, was not investigated further.

## 5.12 Summary and Conclusions

Attempts to investigate the strand exchange mechanism of Tn3 resolvase were made by crosslinking three distinct interfaces of the resolvase dimer. Resolvase mutants containing site-specific cysteine substitutions designed to span each interface were constructed and characterised. These included D95C/A113C (*trans*), D95C+A113C heterodimer (*trans*), (96-105 Hin) T73C/A115C (*cis*), M106C (E helix) and (96-105 Hin) M106C (E helix). All these mutants were found to be active recombinases when reduced, but inactive when oxidised. The disulphide bonds were found to inhibit the strand cleavage step of the resolution reaction. However, oxidised D95C/A113C and M106C were able to complete the preceding steps. The functions which tolerated a disulphide link across either the *trans* interface or the E-helix interface included: binding to all sites of *res*, wrapping of the accessory sites (trapping three negative supercoils), and activation of site-I bound resolvase. In fact, complementation experiments showed that disulphide-crosslinked M106C and D95C/A113C resolvase can perform all activities necessary at the accessory sites in order to support recombination at site I. As these functions do not require the *cis, trans* or E helix interfaces to be disrupted this excludes the possibility of a domain-swapping mechanism (or any other large-scale relative movements across any of these interfaces) at sites II/III.

In the γδ resolvase dimer co-crystal structure the serine 10 residues are far from the relevant scissile phosphates (11 Å and 17 Å). The most likely reason that cleavage of the crossover site is inhibited by oxidative crosslinking of resolvase is that, in order to position the active site close to the phosphate for cleavage, the dimer requires greater flexibility than the disulphide crosslink permits. Homobifunctional crosslinkers were used to increase the distance between crosslinked cysteine residues up to a maximum of 24 Å (BMPHP). D95C/A113C partially crosslinked using PDM(o), PDM (p), BMPHP, HBVS and $BM[PEO]_3$ was found to retain the ability to cleave at least one strand of DNA, thereby relaxing the substrate. Nevertheless, cysteine modification of D95C, A113C and D95C/A113C with NEM revealed that this bulky substituent was tolerated at cysteine 95 but

was not tolerated at other positions, especially 113. It is therefore possible that the homobifunctional crosslinkers have alleviated the problem of inflexibility associated with disulphide crosslinks, while at the same time introducing another problem, the physical obstruction associated with modifying cysteine residues with bulky maleimide reagents. Modification of A113C may in fact be detrimental even if a less "bulky" crosslinking reagent is used such as HBVS (which crosslinks using vinylsulfone groups), as D95C/A113C resolvase crosslinked with HBVS showed identical activities. This introduced a new consideration for the problem of choosing "optimal" residues for cysteine crosslinking, which will be examined in Chapter 6.

# Chapter 6

# The importance of the *cis* interface for strand cleavage

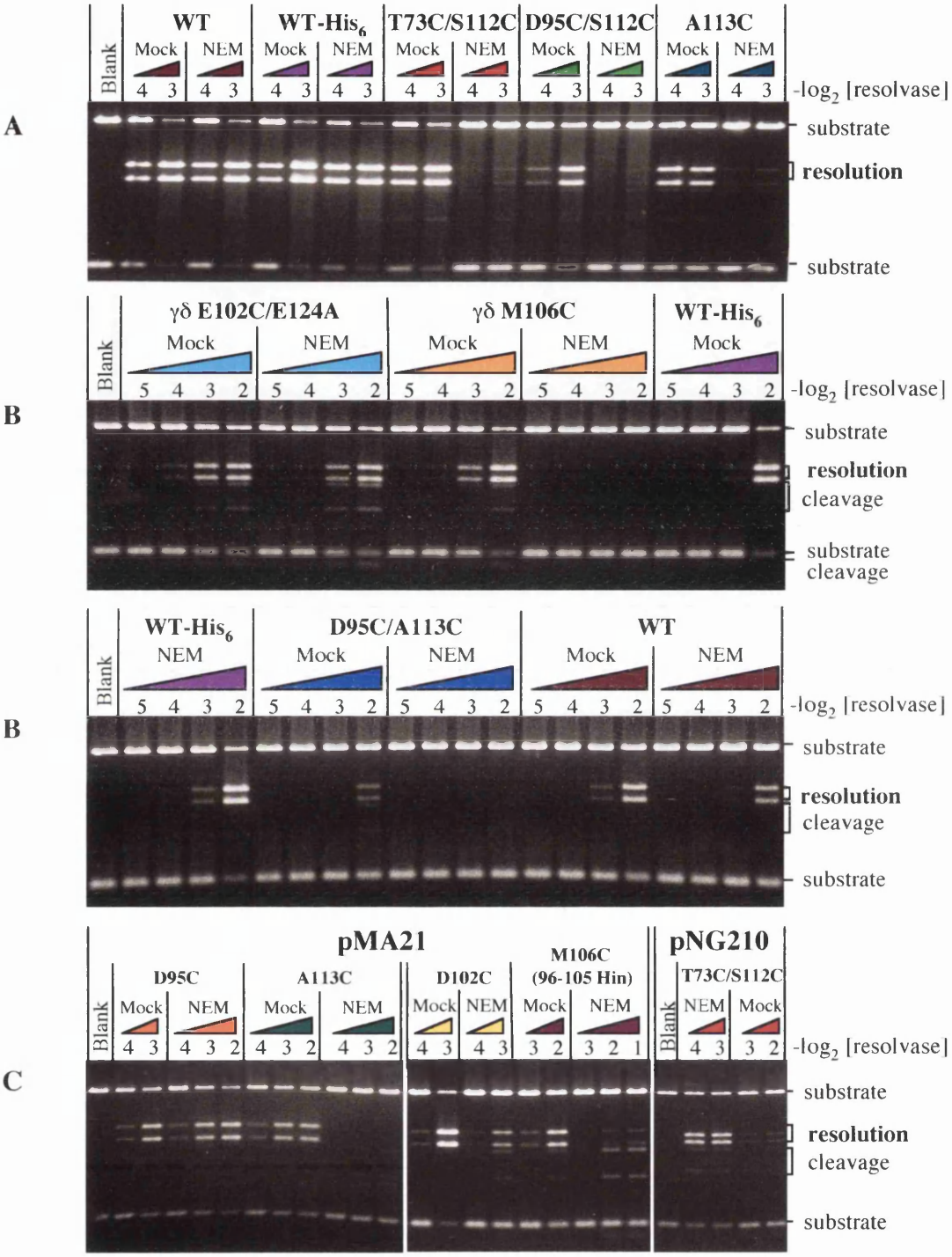
## 6.1 S112C - an alternative to A113C for crosslinking of dimer interfaces?

The results described in Chapter 5 led to the adoption of an alternative strategy to generate cleavage-proficient resolvase crosslinked in the *cis* or *trans* interfaces. The cysteine-substituted resolvase mutants described in Chapter 5 were chosen to fit geometric and spatial conditions to favour oxidative disulphide crosslinking. However, the extra span length of the bifunctional crosslinking molecules (compared to a disulphide bond), meant that more distant residues could be crosslinked. An alternative cysteine substitution at a maleimide-insensitive residue position could be used in place of, for example, A113C, providing it was reasonably close to its prospective crosslinking partner residue. We thus attempted to combine the benefits of bifunctional crosslinkers with the insights gained from NEM modification of cysteines at different positions in the resolvase dimer (Fig. 6.1). NEM modification of resolvases showed a wide range of effects upon recombination and cleavage activities. Resolvases containing no cysteine residues (WT resolvase) were unaffected by the treatment. Certain cysteine-containing resolvases, such as D95C and γδR E102C/E124A, were also unaffected by modification with NEM. In contrast, A113C and γδR M106C were completely inactivated by treatment with NEM. In some cases, such as D102C and M106C (96-105 Hin), NEM modification altered the spectrum of activities: a larger proportion of cleavage products was obtained.

Using the co-crystal structure once more, serine-112 was chosen as a prime candidate residue to substitute with a cysteine (Table 7, Chapter 5). S112 is a surface residue of the E helix, more accessible to solvent than A113 or A115. It was therefore considered less likely that an attached maleimide group would hinder resolvase function. Furthermore, S112 is within 10 angstroms of both T73 (in *cis*) and D95 (in *trans*), so that a cysteine at this position could potentially be used to generate both types of crosslink. These residues are highlighted on the co-crystal structure of γδ resolvase (Fig. 6.2).

S112C, T73C/S112C and D95C/S112C were constructed, overexpressed and purified on a small scale (see plasmid list; Table 2, Chapter 2). All three enzymes efficiently resolved a *res* x *res* substrate under standard conditions (data not shown). However, T73C/S112C and D95C/S112C were partially inactivated by modification with NEM (Fig. 6.1). This may reflect the true activities of NEM-modified resolvases or may represent the activity of a residual fraction of unmodified resolvase.

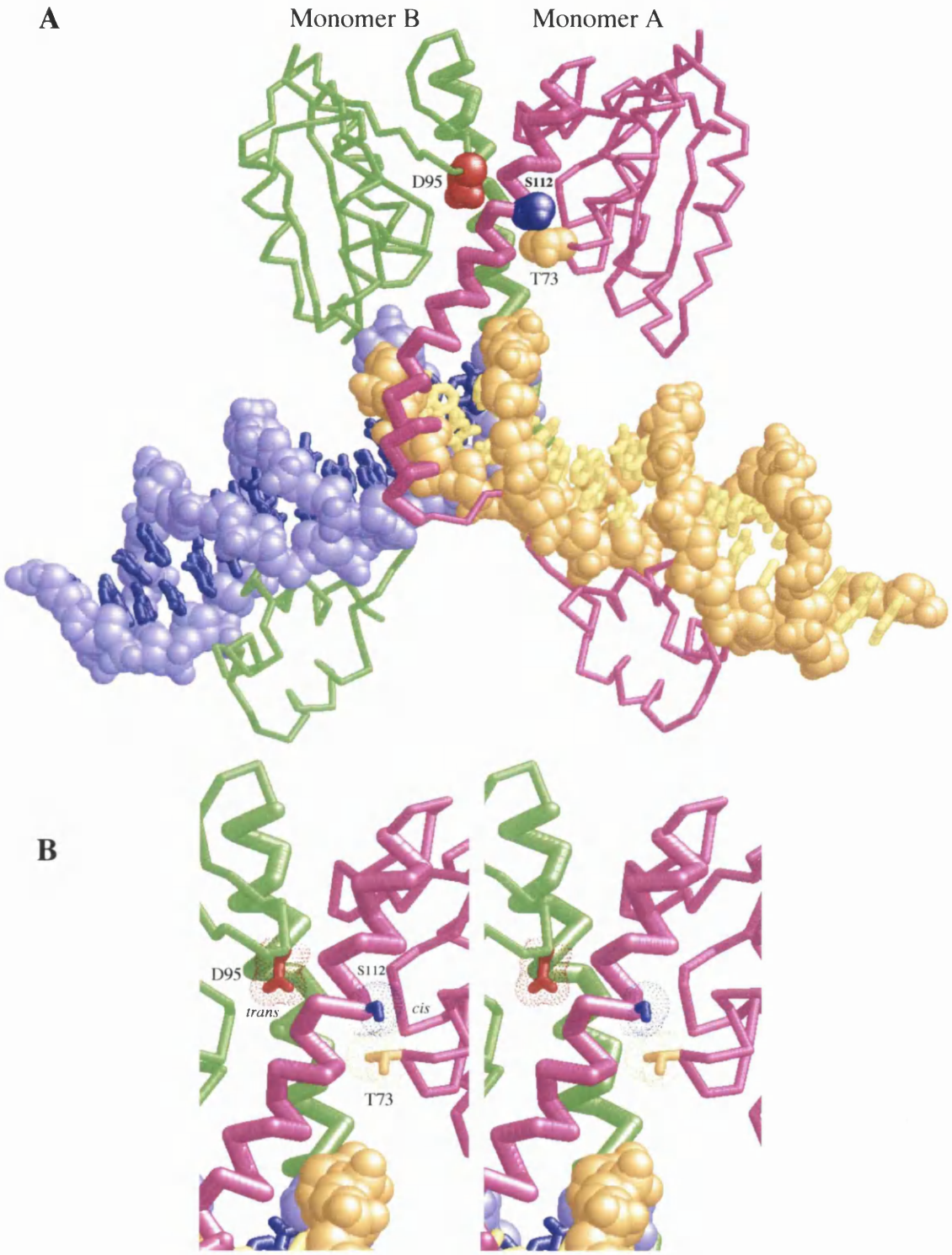
## Fig. 6.1 Recombination activity of cysteine-containing mutants and effects of NEM modification




**A.** NEM modification of resolvases purified on a small scale (contain no DTT), except for A113C which was purified on a large scale (contains 1 mM DTT final, which was removed using a G25 column). NEM (100 mM dissolved in ethanol) or ethanol (mock treatment) was added (1/100 volume). After 20 min. at room temperature, reactions were quenched using 1 M DTT (1/10 volume). Resolution reactions (pMA21) were done in recombination buffer (C8.2, 5 mM DTT) for 40 min. at 37°C and were analysed by restriction with *Pst*I and *Hind*III.

**B+C.** NEM modification of resolvases purified on a large scale. Resolvase was reduced for 1 hour at 37°C (20 mM DTT final, 1/50 volume). DTT was removed using G25 columns. NEM (100 mM dissolved in ethanol) or ethanol (mock treatment) was added (1/100 volume). After 30 min. at room temperature, reactions were quenched using 1 M DTT (1/100 volume). Resolution reactions (pMA21 or pNG210) were set up in recombination buffer (C8.2, 5 mM DTT) for 1 hour at 37°C and were analysed by restriction with *Pst*I and *Hind*III.

**Fig. 6.2 Co-crystal structure of γδ resolvase showing residue S112 and its proximity to T73 (*in cis*) and D95 (*in trans*)**




The co-crystal structure of γδ resolvase is depicted as previously. Residues S112 (blue) and T73 (orange) of monomer A and residue D95 (red) are shown space-filled (A). For simplicity only S112 of one monomer and its interactions with T73(in *cis*) and D95 (in *trans*) are shown. The geometry of the side chains is shown in stereo close up (B).

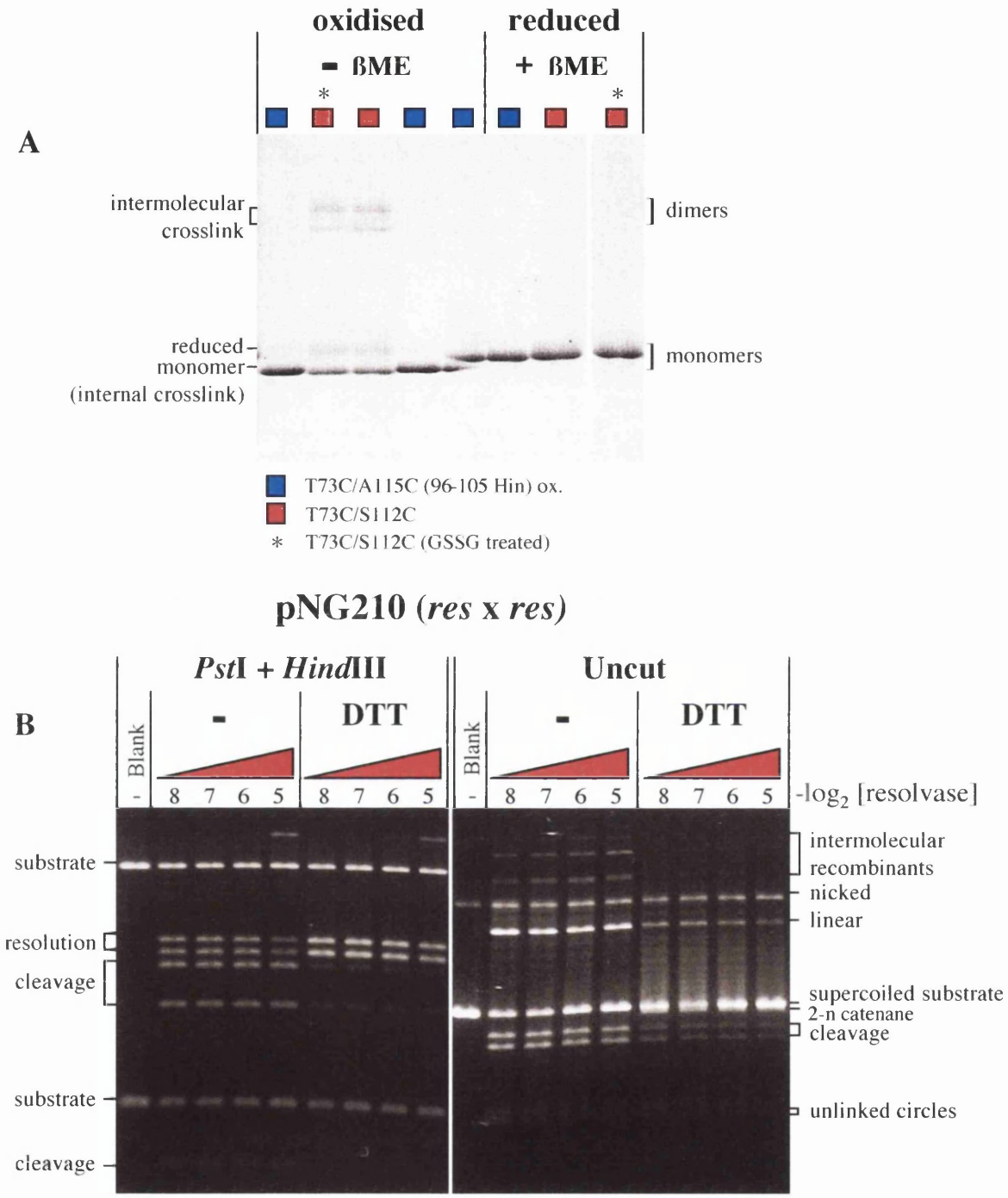
## 6.2 Unexpected activities of T73C/S112C

Initial characterisation of the small-scale preparation of T73C/S112C hinted that this mutant might be slightly "activated"; intermolecular recombination products were observed with a *res* x *res* substrate (data not shown). In order to investigate this further, T73C/S112C resolvase was purified on a large scale. Initial assays revealed an activity very similar to wild-type, including the near-exclusive formation of resolution products with a *res* x *res* substrate, pNG210, under reducing recombination conditions (C8.2, 5 mM DTT, data not shown). No "activated" properties were observed under reducing conditions.

Following storage in the -70°C freezer for nine months, the purified enzyme was subjected to SDS PAGE, in order to reveal its state of oxidation (Fig. 6.3, A). It was previously found that glutathione-treated (96-105 Hin) T73C/A115C resolvase (Chapter 5) formed, exclusively and rapidly, an intramolecular disulphide crosslink across the *cis* interface, inactivating resolvase for strand cleavage. This oxidised fraction of (96-105 Hin) T73C/A115C was used as a marker for the mobility of an internally disulphide-linked resolvase (intramolecular disulphide-linked T73C/S112C resolvase was expected to migrate at a similar position). The T73C/S112C resolvase was found to consist of approximately 20% "reduced monomer" (possibly modified at cysteine residues), 30% "oxidised dimer" (of two different species) and 50% "oxidised monomer". T73C/S112C thus does not oxidise as readily as (96-105 Hin) T73C/A115C, nor does it exclusively form intramolecular disulphide crosslinks. This is in agreement with the distances between the residues (in *cis*) as seen in the co-crystal structure; the distance and geometry for residues 73 and 115 (the candidates originally chosen) appear more suitable for disulphide crosslinking than for residues 73 and 112.

When the mixture of oxidised T73C/S112C isoforms was assayed in reducing and non-reducing recombination buffers (C8.2 ± 10 mM DTT) with pNG210 (*res* x *res*), the products differed, depending on the presence or absence of reducing agent (Fig. 6.3, B). The presence of 10 mM DTT presumably converted the great majority (if not all) of the T73C/S112C preparation into the reduced form. This reduced species behaves similarly to wild-type resolvase, as had been seen with T73C/S112C in the original assays. However, in the absence of reducing agent, products more typical of an activated resolvase were seen, including cleavage and intermolecular recombination products. These reactions are unlikely

**Fig. 6.3 Activity of partially oxidised T73C/S112C species under reducing and non-reducing conditions**




**A.** SDS PAGE analysis of T73C/S112C fractions. These were compared to intramolecular disulphide-linked T73C/A115C (96-105 Hin) resolvase using reducing (ß-mercaptoethanol, 2% final) and non-reducing sample preparation. In track 5, the oxidised isoforms are partially reduced by diffusion of ßME from track 6.

**B.** Resolution reactions were set up in recombination conditions in the presence or absence of reducing agent (C8.2 ± 10 mM DTT) for one hour at 37°C. Dilutions of T73C/S112C (250 $\mu$M enzyme fraction), were added at 1/10 volume. Reactions were stopped by heating to 75°C for 5 min. and analysed untreated and restricted with *Pst*I and *Hind*III.

to be entirely dissociative, as restriction analysis revealed that all the recombination products contain left-right junctions.

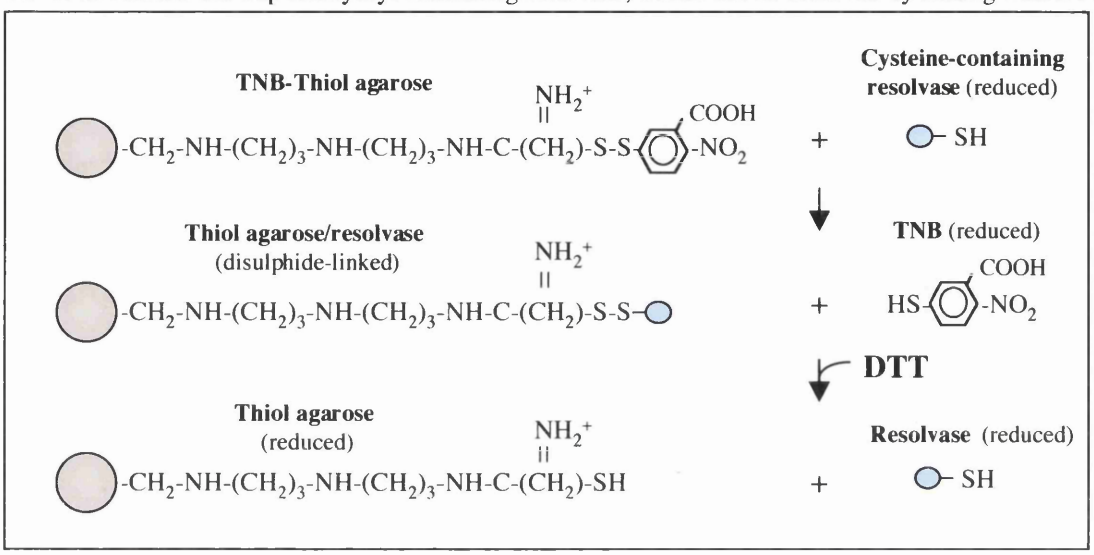
In order to remove the reduced species from the T73C/S112C preparation, small-scale purification on two different resins, SulfoLink and TNB (Pierce), was attempted. The principle and the methodology used is illustrated in Fig. 6.4, see also materials and methods section 2.24. The success of the purification was checked by SDS PAGE analysis (data not shown). No purification of the oxidised T73C/S112C was evident with the SulfoLink resin. However, the TNB-treated sample was depleted for the reduced form, with less than 5% remaining. The final supernatant (8 M urea, 20 mM Tris/HCl pH 8.8, 20 mM DTT), contained reduced species (Fig. 6.5, A).

The various fractions from this small-scale purification experiment were assayed under reducing and non-reducing conditions on a *res* x *res* substrate (pNG210) in cleavage conditions (Fig. 6.5, B). The unfractionated oxidised T73C/S112C preparation, the Sulfo-Link-agarose treated T73C/S112C fraction and the TNB-agarose enriched T73C/S112C (oxidised, fraction 3) fraction were extremely active in non-reducing conditions, cleaving the substrate (both supercoiled and non-supercoiled) almost to completion. In these conditions, the hyperactivity of the oxidised T73C/S112C fractions could be seen more clearly; additional products were observed, which were believed to be generated by cleavage at sites in the DNA other than *res* site I. When the identical protein samples were assayed under reducing conditions, the cleavage reaction was much slower. No "additional" cleavage products were detected, and the non-supercoiled pNG210 substrate was not cut. In fact, the products closely resembled the reaction products with wild-type resolvase, including the presence of some two-noded catenane and unlinked recombinant circles. The reduced resolvase eluting in the final supernatant from the TNB resin (containing 20 mM DTT) also showed activities similar to WT resolvase. This experiment demonstrated that reduced and oxidised T73C/S112C species could be separated, and showed that the hyperactivity was associated with one of the oxidised species, containing an intramolecular or an intermolecular disulphide crosslink.

The hyperactivity of the TNB agarose enriched T73C/S112C (oxidised) was further investigated by assays with substrates lacking one or both pairs of accessory sites (pAL265-*res* x site I and pAL221*sis*-site I x site I) in cleavage conditions (Fig. 6.6). The γδR (96-105

## Fig. 6.4 Use of TNB- and SulfoLink resins to fractionate reduced and oxidised resolvase

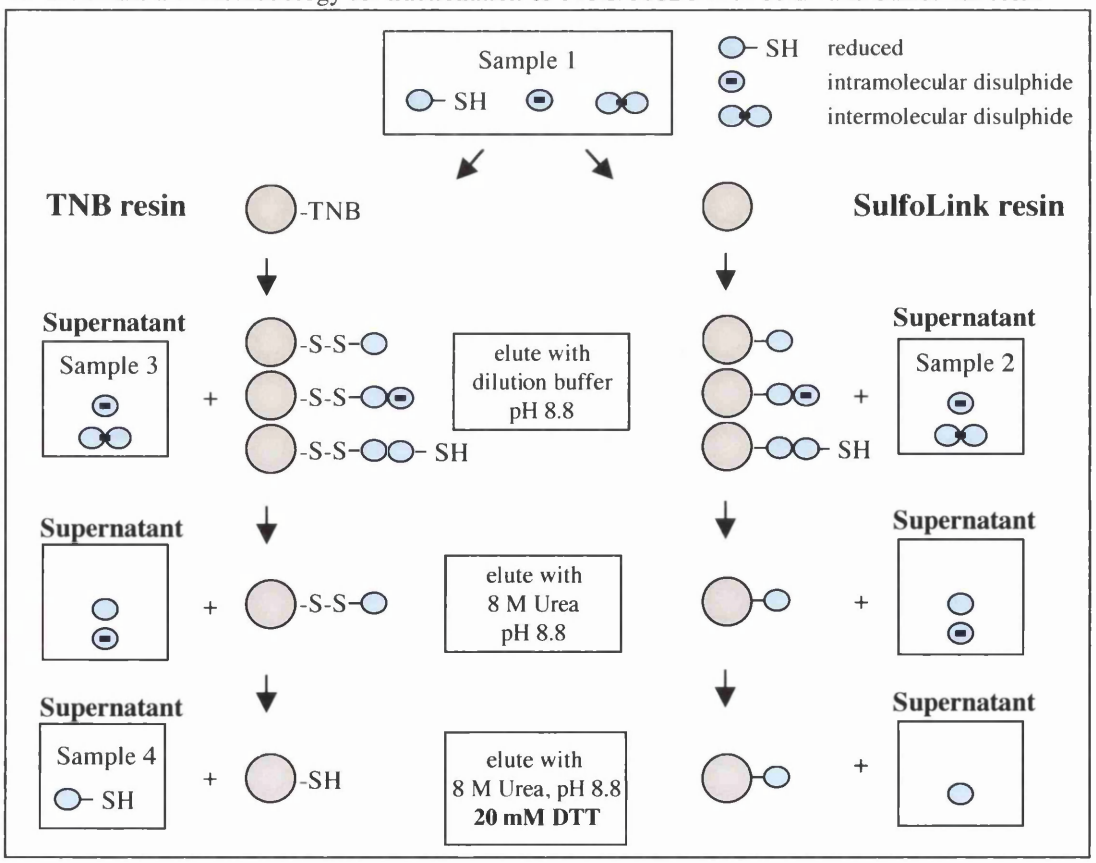
A. TNB column can trap sulfhydryl-containing resolvase, which can be retrieved by eluting with DTT




B. SulfoLink-irreversibly retains sulfhydryl-containing resolvase

- 6% cross-linked beaded agarose with iodoacetyl functional groups
- Specific for free thiols
- The iodoacetyl functional group assures irreversible linkage

C. Rationale and methodology for fractionation of T73C/S112C with TNB- and SulfoLink-resin

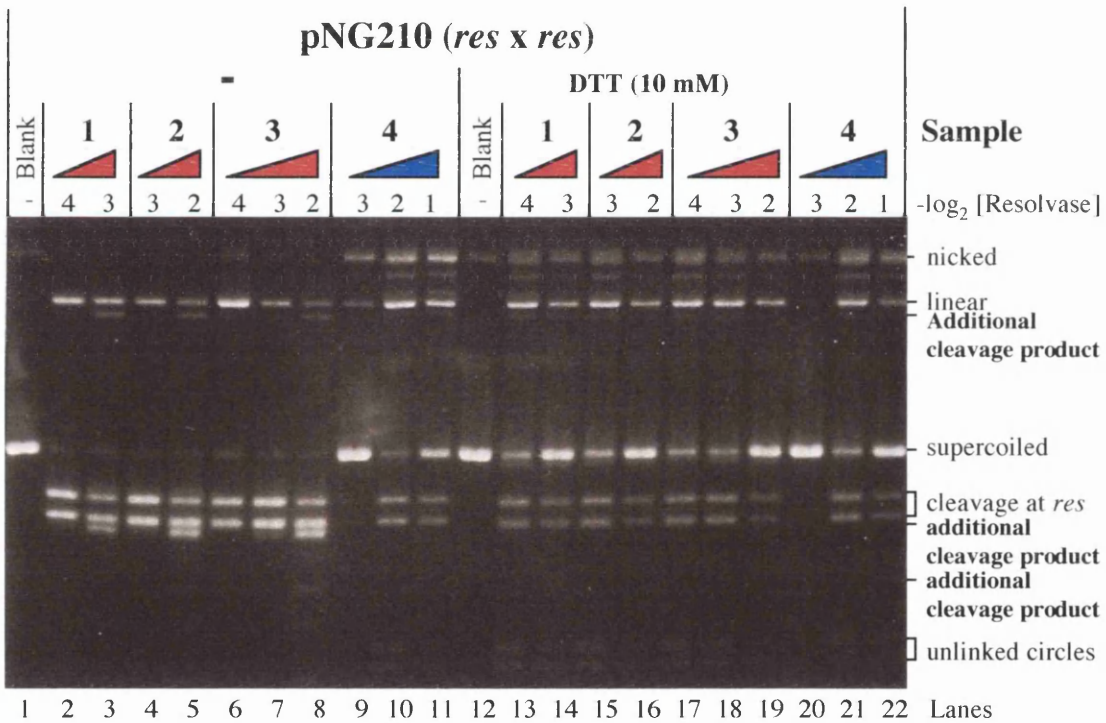


## Fig. 6.5 Fractionation and assay of T73C/S112C species

**A**

| Fraction | Abbreviation | Sample | Reduced | ox.-intra | ox.-inter | "tetramer" |
|---|---|---|---|---|---|---|
| Unfractionated preparation | Pre-purification | Sample 1 | 20% | 50% | 30% | - |
| TNB-agarose enriched (oxidised) | - | Sample 3 | 5% | 60% | 35% | - |
| TNB-agarose Final eluate | reduced | Sample 4 | 100% | - | - | - |
| Sulfo-Link-agarose treated | - | Sample 2 | 20% | 50% | 30% | - |
| Unfractionated column load | Superose load | - | 15% | 50% | 25% | 10% |
| Superose-purified (ox.-intra) | "oxidised monomer" | - | ≤10% | ≥85% | ≤ 5% | - |
| Superose-purified (ox.-inter) | "oxidised dimer" | - | - | 20% | 65% | 15% |

**B**




### Sample

**1** T73C/S112C untreated (1:10 dilution): mixture of reduced and oxidised isoforms (25 $\mu$M)
**2** T73C/S112C eluted from the Sulfo-Link resin: no detectable purification (25 $\mu$M)
**3** T73C/S112C TNB resin supernatant, enriched in oxidised species (20 $\mu$M)
**4** T73C/S112C eluted from the TNB resin with 8 M Urea and 20 mM DTT: reduced (5 $\mu$M)

**A.** Summary of attempts to fractionate T73C/S112C species. Terms used to describe various fractions and estimated percentages of different isoforms.

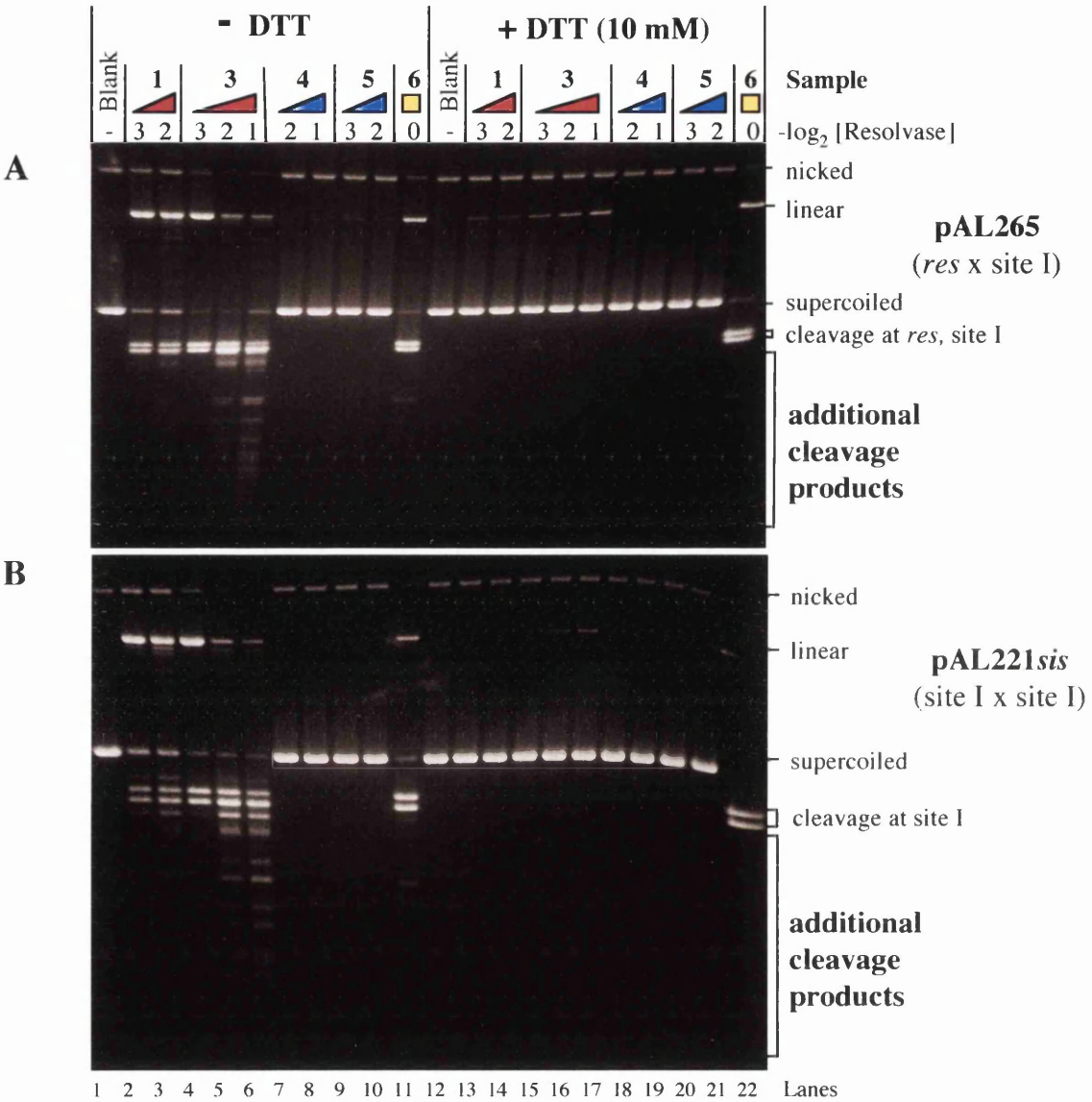
**B.** Assays of fractions of T73C/S112C, differing in their redox status, were prepared as outlined previously. Resolvase dilutions were made in dilution buffer at pH 8.5 (no DTT) and added at 1/10 volume. Reactions were set up in cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA) and incubated for 3 hours at 37° C. The reactions were stopped by treatment with proteinase K (50 $\mu$g/ml) and run uncut.

Hin) E124Q (Chapter 4) was used as a control because it contains no cysteine residues and should not be affected by the redox state of the assay buffer. As expected, γδR (96-105 Hin) E124Q quantitatively cleaved the substrates in the presence or absence of reducing agent (lanes 11 and 22). Astonishingly, in the absence of reducing agent, T73C/S112C (oxidised, fraction 3) was able to cleave both substrates. Furthermore, "additional" cleavage products were observed to an even greater extent with these substrates. At the highest concentration of resolvase the cleavage reaction was almost quantitative; the majority of the substrate (supercoiled or nicked) was cut at both *res* crossover sites (lane 6). A significant proportion appears to be cut at sites other than *res*. In contrast, T73C/S112C pre-treated with DTT, and the reduced TNB resin eluate, were inactive under the same non-reducing conditions (lanes 7-10). When assayed under reducing conditions (10 mM DTT final), all fractions of T73C/S112C resolvase were either completely or almost completely inactive (lanes 12-21). Traces of full-length linear product were seen when T73C/S112C (oxidised, sample 3) was added to the reducing assay mix. This is presumably because the intramolecular and intermolecular oxidised species are rapidly reduced, and the reduced form behaves like WT resolvase (WT resolvase is inactive on all substrates lacking accessory sites). Neither the DTT pre-treated nor the final 10 mM DTT TNB resin eluate (containing reduced resolvase) were active on pAL265 or pAL221*sis*. This confirms that reduced T73C/S112C has no unusual activated properties.

Although extremely hyperactive Tn3 resolvases have been thoroughly characterised, no mutant had previously shown significant cleavage activity at any DNA sequence other than *res* site I, as was seen with "oxidised" T73C/S112C (Jiuya He, personal communication and Chapter 4). The "additional" products observed in the cleavage reactions with T73C/S112C (oxidised, sample 3) migrate faster than the cleavage products obtained from cleavage at both *res* sites. To generate these smaller products, the DNA must be cut at positions other than site I.

In order to obtain a preliminary understanding of which "non-site I" sequences are cut, a variety of substrates were assayed, including a standard supercoiled cloning vector with no *res* sequences, pMTL23 (Fig. 6.7, A). All substrates (including pMTL23) were cleaved by T73C/S112C (oxidised, sample 3), irrespective of the presence or absence of *res* sequences (Fig. 6.7, B). Cleavage of pMTL23 confirmed that oxidised T73C/S112C species can cleave

## Fig. 6.6 Accessory site independent cleavage activity by oxidised T73C/S112C



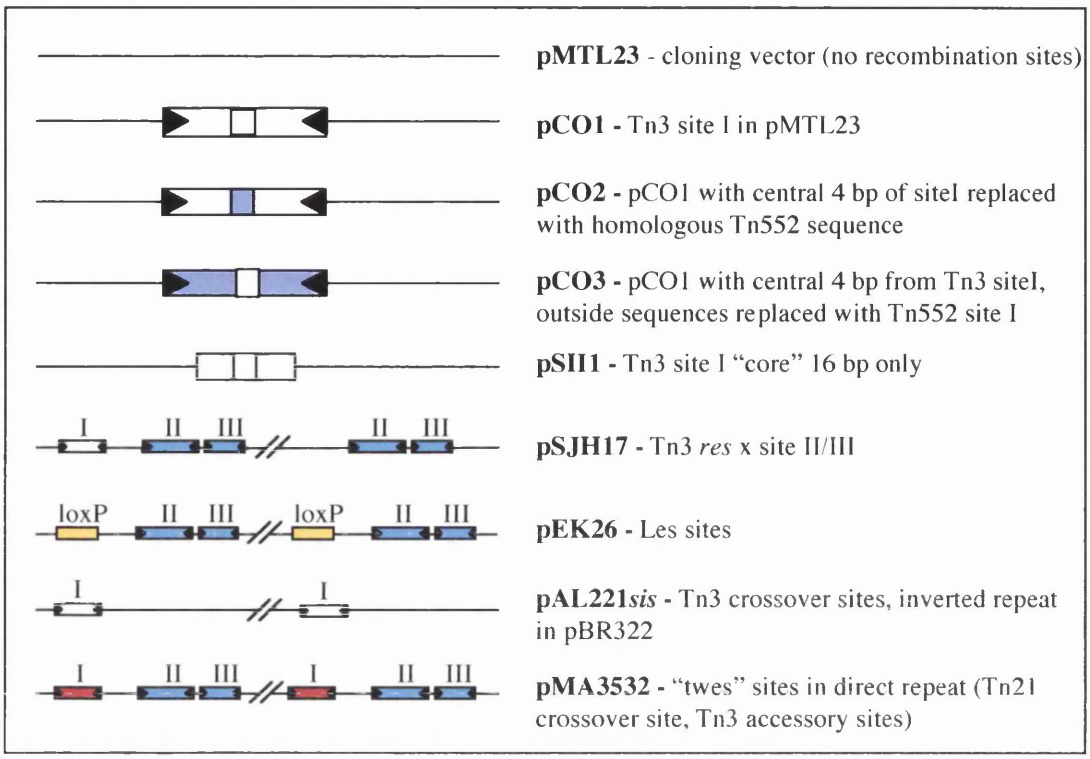

**Sample**

| | | |
|---|---|---|
| **1** | T73C/S112C untreated (1:10 dilution): mixture of reduced and oxidised isoforms | (25 $\mu$M) |
| **3** | T73C/S112C TNB resin supernatant, enriched in oxidised species | (20 $\mu$M) |
| **4** | T73C/S112C eluted from the TNB resin with 8 M Urea and 20 mM DTT: reduced | (5 $\mu$M) |
| **5** | T73C/S112C pre-reduced with 20 mM DTT, 30 min at 37°C (1:10 dilution) | (25 $\mu$M) |
| **6** | γδ resolvase (96-105 Hin) E124Q (fraction containing 1 M urea) | (1 $\mu$M) |

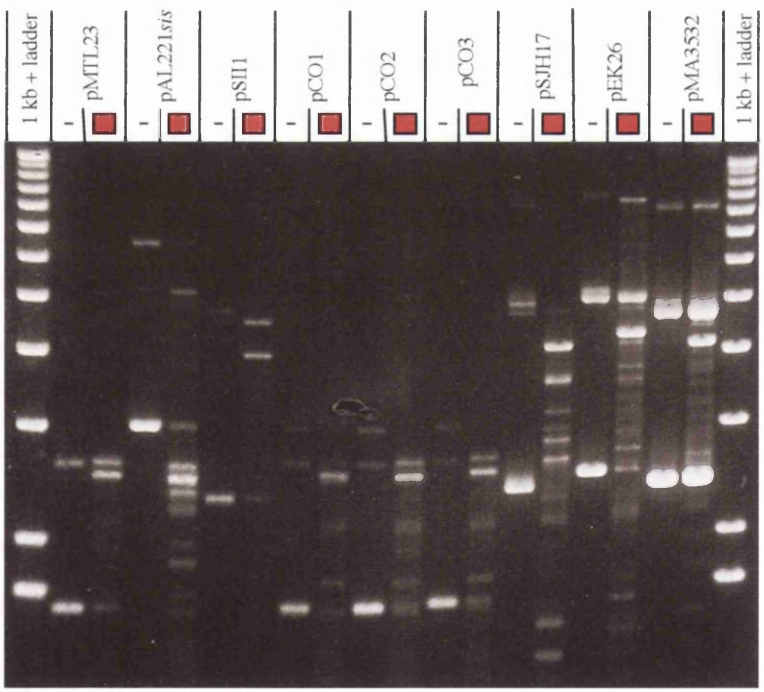
**A+B.** Samples of T73C/S112C, differing in their redox status, were prepared as outlined above. Resolvase dilutions were made with DB (pH 8.5) for samples 1, 3 and 6 and with DB (pH 8.5) plus 20 mM DTT for samples 4 and 5. Reactions were in cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA) for 2.5 hours at 37°C with pAL265 (*res* x site I, A) and pAL221*sis* (site I x site I, B). The samples were stopped by treatment with proteinase K (50 $\mu$g/ml) and run uncut.

## Fig. 6.7 Oxidised T73C/S112C can cleave many plasmid substrates

A




B




T73C/S112C (TNB-resin purified) 1.25 $\mu$M final

**A.** Structure of plasmids assayed with T73C/S112C resolvase.

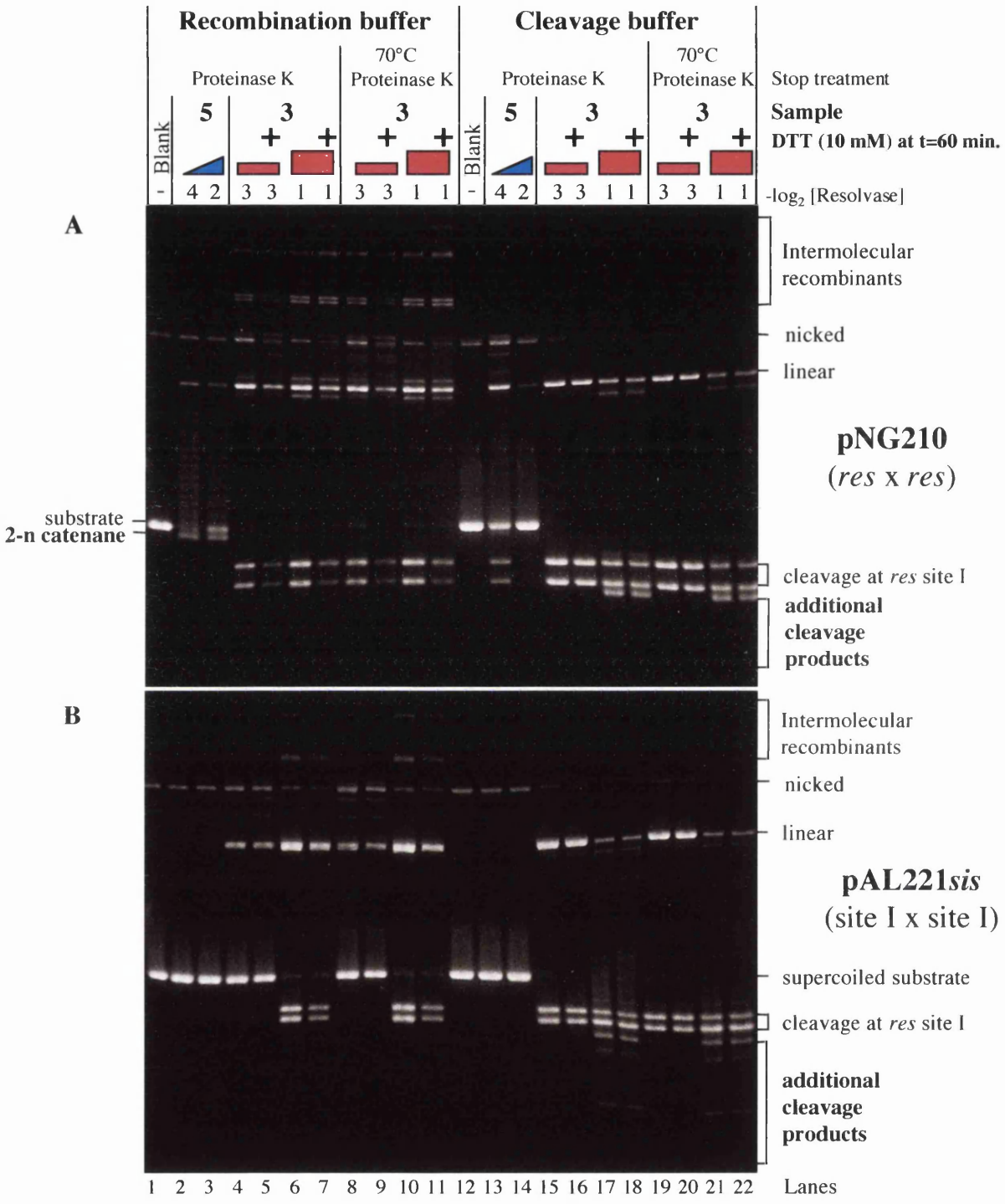
**B.** Reactions were set up in cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA) for 4.5 hours at 37°C. T73C/S112C purified with TNB resin was added (1/10 volume) to the reactions as shown. The reactions were stopped using proteinase K (50 $\mu$g/ml final) and analysed uncut.

non-site I sequences. From this experiment any DNA sequence preference for cleavage could not be established.

As it is possible that the cleavage intermediate is stabilised by the physical constraint of the disulphide bond in oxidised T73C/S112C, we predicted that its reduction may reverse the reaction, pushing the equilibrium back to substrate or through to product. The effect of adding reducing agent after generating the cleavage intermediate was therefore investigated in recombination and cleavage buffers at high and low concentrations of enzyme, using *res* x *res* and site I x site I substrates (Fig. 6.8). The reactions were allowed to proceed for one hour at 37°C, then DTT (10 mM) was added to one aliquot and the reactions were incubated for a further hour (37°C). The addition of reducing agent did result in a decrease in the yield of cleavage product, but it was not clear whether the cleavage product was converted into any other species, such as 2-n catenane. Upon restriction with *Pst*I and *Hind*III, a small increase in pNG210 resolution product (LR) could be seen in the DTT-treated samples (data not shown). Nevertheless, the increase does not account for the marked decrease in cleavage products observed and an unexplained discrepancy remains. This is especially true for the pAL221*sis* reactions, in which no increase in product or substrate can be detected despite a large decrease in cleavage product. As the effect of the standard stop procedure (heating to 70°C) on this extremely activated resolvase was unknown, aliquots were treated with proteinase K (50 μg/ml), or heated to 70°C for 10 min. before treatment with proteinase K (50 μg/ml). Heat treatment had no effect on the products obtained.

Formation of a covalent protein-DNA cleavage complex can be detected on an agarose gel by the shift in mobility of the cleavage products between untreated samples and samples treated with proteinase K (Fig. 6.9). Following a one hour cleavage reaction (37°C), aliquots of the samples were treated with SDS loading mix (2% SDS final) or "K Mix" (0.1% SDS, 50 μg/ml proteinase K final) (0.2 vol.). The reduction in mobility due to covalently bound resolvase can be observed in the SDS-only tracks. Thus, the cleavage reactions catalysed by oxidised T73C/S112C result in a covalent DNA-protein intermediate and the cleavage reaction is not due to hydrolysis.

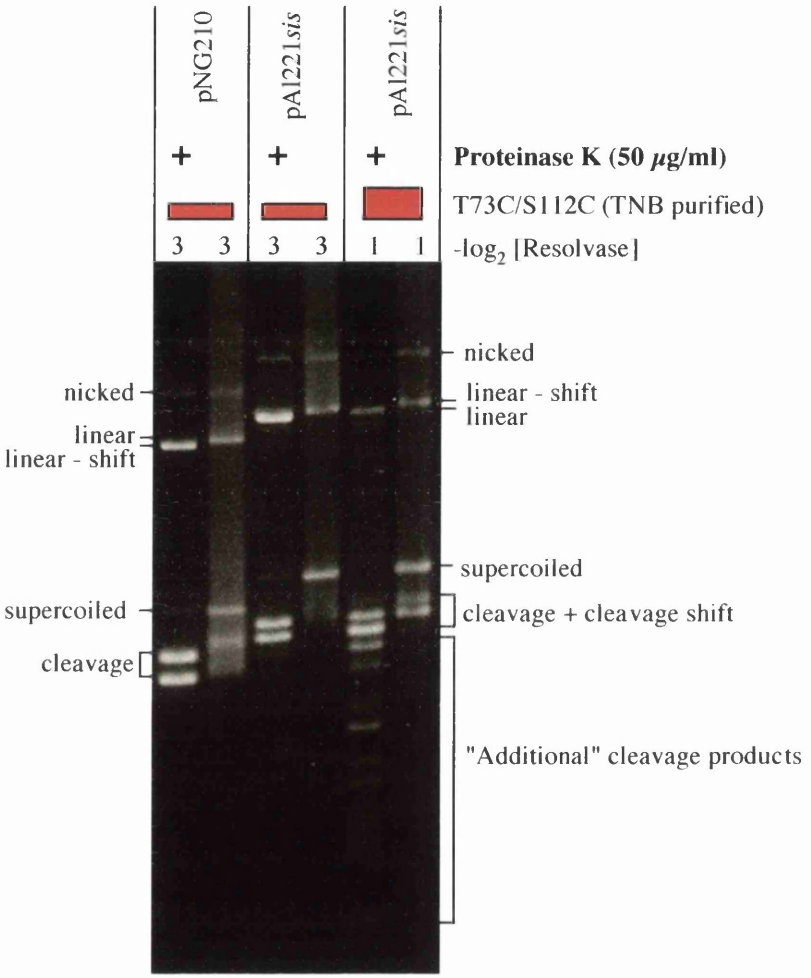
## Fig. 6.8 Oxidised T73C/S112C fails to make normal resolution products




**Sample** **3** T73C/S112C TNB-resin supernatant, enriched in oxidised species (20 $\mu$M)
**5** T73C/S112C pre-reduced with 20 mM DTT, 30 min at 37°C (1:10 dilution) (25 $\mu$M)

**A+B.** Reactions were set up in recombination buffer (C8.2) or cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl, 0.1 mM EDTA) with pNG210 (*res* x *res*, A) or pAL221*sis* (site I x site I, B) for 1 hour at 37°C. Dilutions of resolvase were added at 1/10 volume. Samples were split and DTT (10 mM final) was added to half. These were incubated for a further hour at 37°C before being divided again. One aliquot was treated with proteinase K (50 $\mu$g/ml final) and the other was heated to 70°C for 10 min. prior to proteinase K treatment. Samples were analysed uncut.

**Fig. 6.9 Cleavage by partially oxidised T73C/S112C generates covalent protein-DNA complexes**




T73C/S112C TNB-resin supernatant, enriched in oxidised species (20 μM)

Reactions were set up in cleavage buffer (40% ethylene glycol, 50 mM Tris/HCl, 0.1 mM EDTA) with pNG210 (*res* x *res*) or pAL221*sis* (site I x site I) for 1 hour at 37°C. Dilutions of TNB agarose enriched T73C/S112C (oxidised) resolvase (sample 3) were added at 1/10 volume. Aliquots were treated with SDS loading mix ± proteinase K (50 μg/ml final). Samples were analysed uncut.

## 6.3 Separation of different T73C/S112C species by gel filtration

T73C/S112C is the first "hyperactive" resolvase mutant to be characterised which does not contain activating mutations around the regions of D102 or E124. The co-crystal structure of γδ resolvase gives some insight into why this mutant may be activated for strand cleavage. As previously discussed, in this structure the catalytic serine residues are too far from their phosphate targets to initiate attack. An intramolecular disulphide bond between residues T73C and S112C could alter the structure of the catalytic domain, bringing the serine residues closer to the active configuration required for strand cleavage, and perhaps stabilising the covalent intermediate.

In order to ascertain that the cleavage activity associated with the mixture of oxidised T73C/S112C isoforms was due to the species with an intramolecular crosslink, and not to species with intermolecular crosslinks, separation of these was required. This was thought to be most easily achieved by gel filtration under denaturing conditions, in which non-covalent resolvase dimers would dissociate into monomers.

The gel filtration material chosen was Superose 12 (Pharmacia). This has an optimal separation range of 1-300 kDa (for globular proteins). The final insoluble pellet (approximately 0.2 mg) from the large-scale purification of T73C/S112C was subjected to gel filtration, in order to test whether the monomers and dimers could be resolved in denaturing buffer (6 M urea, 100 mM NaCl, 20 mM Tris/HCl pH 7.5, 0.1 mM EDTA). Four peaks were observed, of which peaks 2 and 3 were presumed to correspond to dimer and monomer, respectively (Fig. 6.10 A). This was supported by SDS PAGE analysis (Fig. 6.10, B).

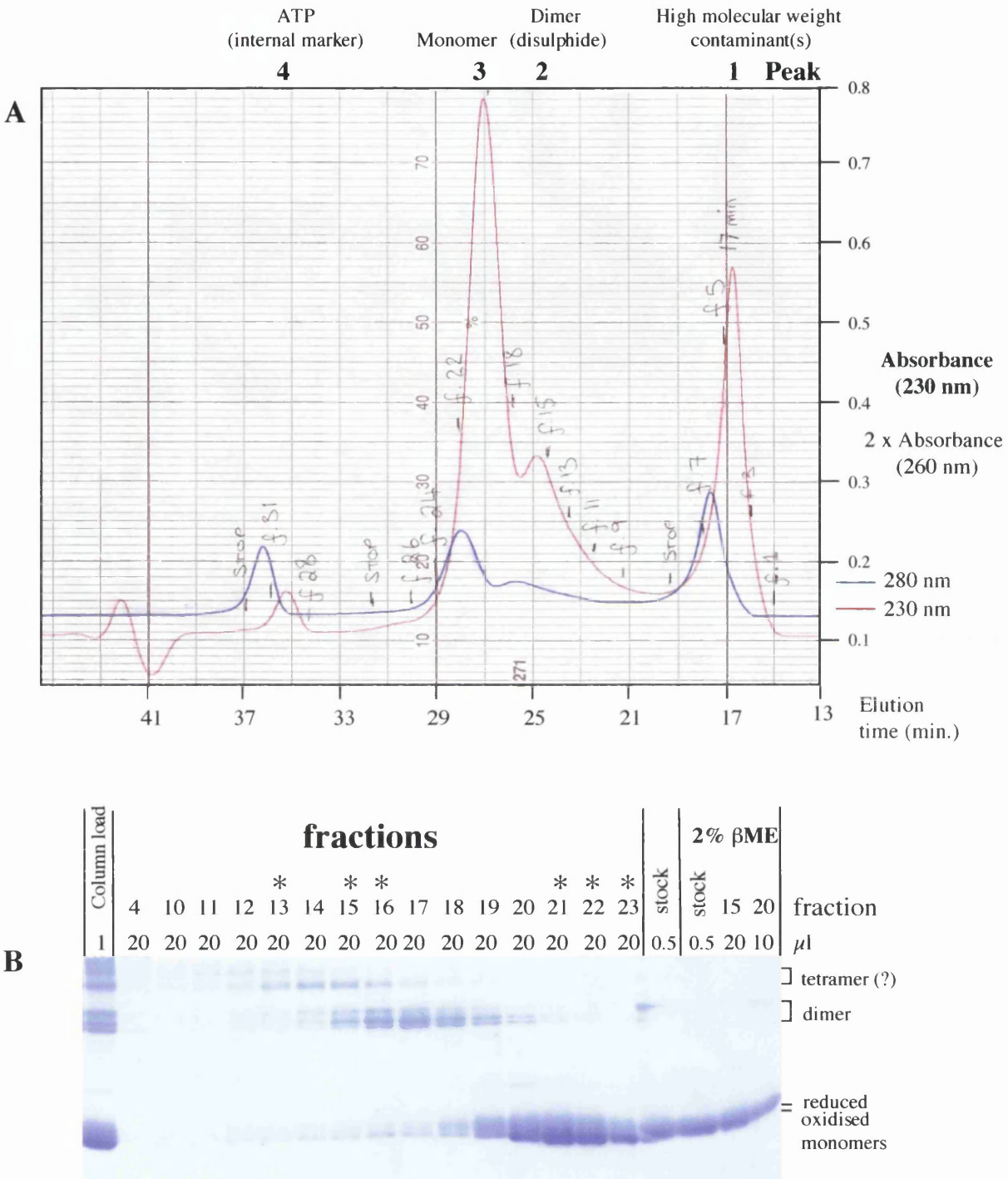
The pooled fractions were initially dialysed against a high salt buffer (2 M NaCl, 20 mM Tris/HCl pH 7.5, 0.1 mM EDTA) and then were concentrated by dialysis against resolvase dilution buffer (50% glycerol, 1 M NaCl, 20 mM Tris/HCl pH 7.5, 0.1 mM EDTA). Samples of the final fractions were analysed by SDS PAGE (Fig. 6.11, A). Under non-reducing conditions, the T73C/S112C pooled dimer and monomer fractions migrated as expected (40 kDa and 20 kDa, respectively). Although the "oxidised dimer" fraction (or superose purified T73C/S112C (ox.-inter)) is enriched when compared with the superose loading sample (lane 5 versus lane 1), this fraction nevertheless contains approximately 20% "oxidised monomer"

species. The "oxidised monomer" fraction (superose-purified T73C/S112C (ox.-intra) (lane 6), on the other hand, is very pure, containing only a trace of "oxidised dimer" species (less than 5%). Importantly, both of these fractions migrate as the reduced species (20 kDa) in the presence of ß-mercaptoethanol (lanes 12 and 13). Thus, gel filtration proved to be a successful technique for separating intramolecular from intermolecular crosslinked resolvase under denaturing conditions (Fig. 6.5, A).

The peak "oxidised dimer" and "oxidised monomer" fractions (in 6 M urea), as well as the original unfractionated sample, were directly assayed for cleavage or recombination activity under reducing and non-reducing conditions (data not shown). Cleavage activity was observed with the internally disulphide-linked species (superose purified T73C/S112C (ox.-intra)). Denatured "oxidised monomer" T73C/S112C (in 6 M Urea) was thus able to re-fold *in vitro* and acquire full cleavage activity. The assays also indicated that the hyperactive phenotype of unfractionated, oxidised T73C/S112C (containing a mixture of species) was due to the internally crosslinked species. Although a trace of cleavage activity was obtained from the "dimer" fraction, the results are consistent with this activity deriving from the small amount of contaminant T73C/S112C (ox.-intra) (visible on SDS PAGE analysis).

The "oxidised dimer" fraction contains a species that migrates considerably slower than dimer (40 kDa) and is labelled as a putative tetramer. The majority of this species eluted from the gel filtration column prior to the dimer species (f.13-16; Fig. 6.10 B). It was thought to be resolvase (and not a contaminating protein) because it migrates at the same position as reduced T73C/S112C in the presence of ß-mercaptoethanol. Thus, it was interpreted to be an oxidised multimer of resolvase. An attempt to purify this species was made (lane 4; Fig. 6.11 A). However, as this potential "oxidised tetramer" fraction was very dilute and contained a trace of internally crosslinked species, it was not assayed for activity. Furthermore, on structural grounds, it is not obvious how T73C/S112C resolvase could form disulphide-linked multimers of any functional relevance (as could in theory be proposed for a domain-swapped *trans*-crosslinked tetramer of resolvase).

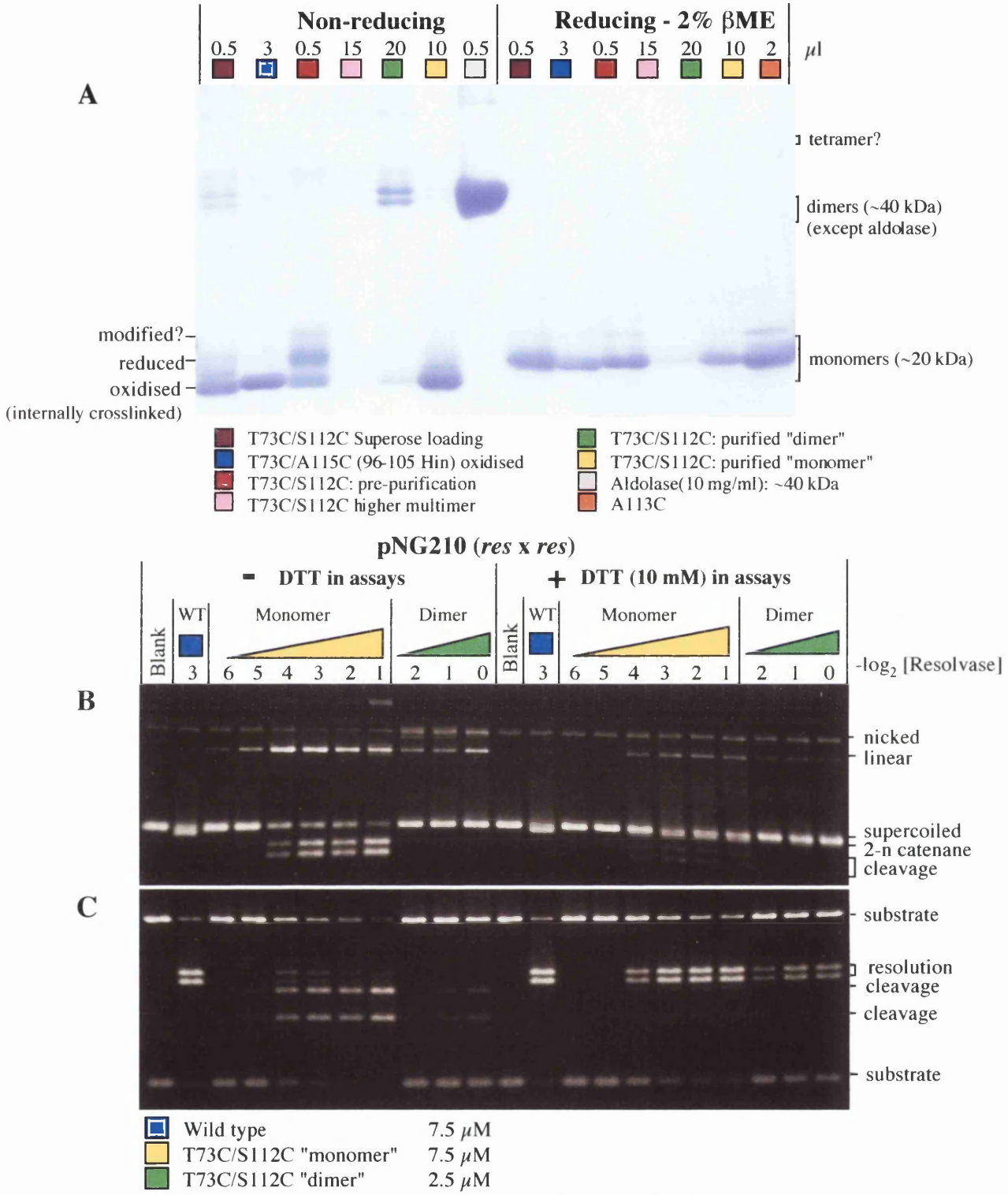
## Fig. 6.10 Separation of oxidised T73C/S112C species by gel filtration




**A.** Superose 12 gel filtration column was equilibrated with denaturing chromatography buffer (6 M urea, 100 mM NaCl, 20 mM Tris/HCl pH 7.5, 0.1 mM EDTA. Oxidised T73C/S112C was resuspended in 66 µl of equilibration buffer. This was loaded and the column was run at 0.5 ml/min. Absorbance was recorded at 280 nm (blue), 0.5 fsd and 230 nm (red), 1.0 fsd at 5 mm/min. ATP (0.2 mM) was added to the sample to provide a low molecular weight marker and elutes as peak four.

**B.** SDS PAGE revealed that peak two corresponds to disulphide linked resolvase dimer and peak three corresponds to resolvase monomers. Fractions marked with an asterisk were pooled and dialysed.

**Fig. 6.11 Characterisation of purified preparations of oxidised T73C/S112C "monomer" and "dimer"**




**A.** SDS PAGE analysis of final pooled fractions of T73C/S112C resolvase following separation by gel filtration, as used for part B. Samples were run under reducing (ß-mercaptoethanol, 2% final) and non-reducing conditions.

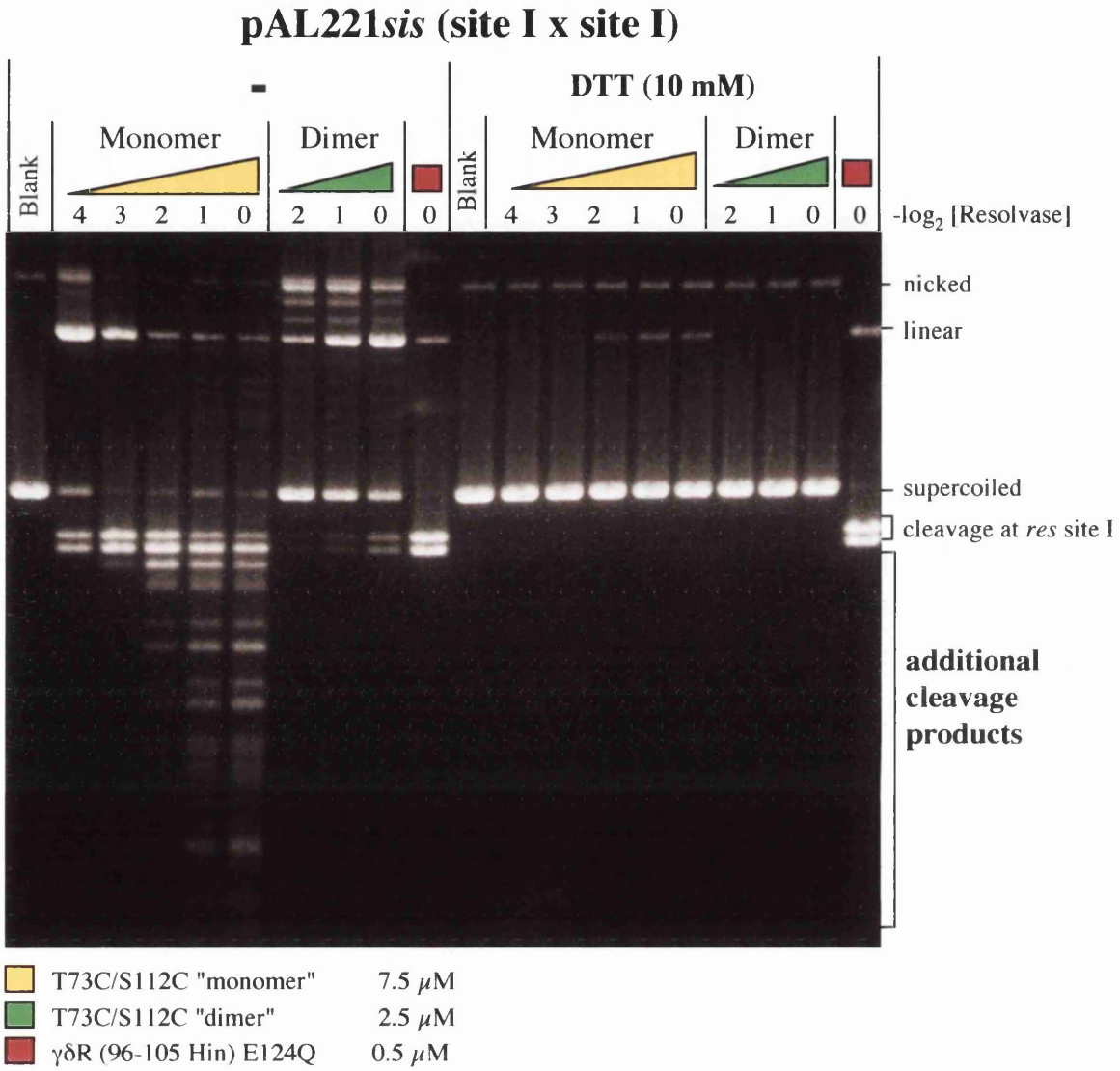
**B+C.** "Monomer" and "dimer" fractions were assayed under recombination conditions (C8.2 ± 10 mM DTT). Pre-reduced resolvase dilutions (15 min. at room temperature, 10 mM DTT) were assayed under reducing conditions and mock-treated resolvase dilutions (15 min. incubation at RT) were assayed in the absence of reducing agent. Dilutions of resolvase were added at 1/10 volume. Samples were analysed uncut (B) or restricted with *Pst*I and *Hind*III (C).

## 6.4 Characterisation of superose purified T73C/S112C (ox.-intra)

Characterisation of the highly purified disulphide-crosslinked T73C/S112C (ox.-intra) has been limited by the time available. Nevertheless, it was firmly established that the intramolecular crosslinked species (and not the intermolecular crosslinked species) was activated for the cleavage reaction of a standard substrate, pNG210 (*res* x *res*), under recombination conditions (Fig. 6.11, B). WT resolvase is unaffected by the presence or absence of DTT, exclusively yielding resolution products. When pre-reduced (10 mM DTT), the sepharose purified T73C/S112C (ox.-intra) and T73C/S112C (ox.-inter) fractions were capable of generating resolution products (exclusively LR joined) under reducing conditions. Two-noded catenanes were visible in the uncut samples, confirming that reduced T73C/S112C can resolve a *res* x *res* substrate via a standard wrapped synaptic complex. However, when these non-treated fractions were assayed in the absence of reducing agent, both behaved differently. T73C/S112C (ox.-intra) produced large amounts of cleavage products, cut at either one or both crossover sites. Small amounts of "resolution" products were observed, indicating that this fraction contains a resolvase species that is proficient at ligation of DNA ends. In contrast, T73C/S112C (ox.-inter) in much less active in the absence of reducing agent. A small amount of cleavage product was detected, which is probably due to the contaminating 20% internally crosslinked material. Thus, T73C/S112C (ox.-inter) is probably inactive, as would be anticipated in view of the structural changes that would be required to make intermolecular crosslinks using residues 73C and 112C.

As anticipated from the previous result with pNG210, cleavage of a site I x site I substrate, pAL221*sis*, was seen only by T73C/S112C (ox.-intra) (under non-reducing conditions) (Fig. 6.12). As before, some cleavage activity was visible with T73C/S112C (ox.-inter), whereas this material is entirely inactive when assayed under reducing conditions. This cleavage activity by the sepharose purified T73C/S112C (ox.-inter) fraction was presumably due to the presence of some internally crosslinked species. Thus the separation and characterisation of intramolecular and intermolecular crosslinked T73C/S112C resolvase species has established that the internally disulphide-crosslinked species is extremely active, whereas the intermolecular crosslinked dimer species is probably inactive.

**Fig. 6.12 The purified T73C/S112C oxidised monomer is associated with activated cleavage at site I, and other sequences**




Purified "monomer" and "dimer" fractions of oxidised T73C/S112C resolvase were assayed under cleavage conditions (40% ethylene glycol, 50 mM Tris/HCl pH 8.2, 0.1 mM EDTA ± 10 mM DTT) with pAL221*sis* (site I x site I) for 2 hours at 37°C. Pre-reduced resolvase dilutions (15 min. at room temperature, 10 mM DTT) were assayed under reducing conditions, and mock-treated resolvase dilutions (15 min. incubation at RT, no DTT) were assayed in the absence of reducing agent.

## 6.5 Binding of T73C/S112C (ox.-intra) to DNA

Binding of purified T73C/S112C (ox.-intra) was assayed with radioactively labelled substrates, containing site I, sites II + III or a full *res* site (Figs. 3.12-13). In the presence of a crossover site, protein-DNA complexes corresponding to monomer and dimer complexes were observed (Fig. 3.12). At a subsaturating enzyme concentration, more dimer complex is observed than monomer complex. Thus, T73C/S112C (ox.-intra) monomers appear to bind to site I. The monomer complexes obtained with T73C/S112C (ox.-intra) and with Tn3R WT had similar mobilities. Nevertheless, the dimer complexes obtained with T73C/S112C (ox.-intra) and D102Y/E124Q (another activated mutant) were found to migrate considerably faster than the dimer complex of Tn3R WT. The altered mobility of the T73C/S112C (ox.-intra) and D102Y/E124Q resolvase dimer complexes may be due to differences in the DNA bend generated upon binding of the second resolvase monomer. Additionally, in the presence of oxidised T73C/S112C (ox.-intra), a minor third complex is observed which migrates slower than the prominent dimer complex. Curiously, this third complex also migrates at the same position as the Tn3R WT dimer-site I complex. The third complex may therefore contain DNA that is bent by the same degree as when bound by wild-type resolvase. This might either be due to the presence of a small amount of reduced T73C/S112C resolvase, or to cleavage activity (nicking one DNA strand) by T73C/S112C (ox.-intra) thereby facilitating the bending/kinking of DNA. A further complex, migrating slightly faster than unbound substrate, is visible exclusively with T73C/S112C (ox.-intra). This probably corresponds to the *res*L-resolvase half-site covalent complex, indicating that T73C/S112C (ox.-intra) can cleave linearised site I DNA.

Binding to a fragment containing the accessory sites II and III revealed a similar picture (Fig. 3.12). Four protein-DNA complexes were obtained in binding assays with purified T73C/S112C (ox.-intra). As observed with binding to site I, the T73C/S112C (ox.-intra) monomer complex migrated at a similar position to the WT resolvase monomer complex. However, all three complexes corresponding to 2-4 bound resolvase monomers migrated faster than the analogous wild-type complexes. Again, this suggested that the DNA in the T73C/S112C (ox.-intra)-DNA complexes is less bent than the DNA in the WT-DNA complexes. The complexes were also noticeably diffuse. In contrast, the complexes formed with wild-type resolvase migrate as discrete bands. This has been observed previously with

activated resolvases, such as D102Y/E124Q. The increased dispersion of the complexes may be due to a decreased binding affinity for the subsites of *res* by these resolvase mutants, especially T73C/S112C (ox.-intra). This may result in unstable complexes, which dissociate during electrophoresis, resulting in diffuse bands.

The patterns of protein-DNA complexes seen with the complete *res* site (Fig. 3.13), are somewhat more complex. Tn3R WT binds to the *res* site giving a characteristic pattern of 6 protein-DNA complexes (lane 2), for which the stoichiometries were determined previously (Blake *et al.*, 1995). Activated mutants of Tn3R such as D102Y/E124Q or R2A/E56K/ D102Y/E124Q gave a similar series of complexes, with some differences. Complexes with the same presumed stoichiometries migrate faster, there appears to be reduced cooperativity in the occupation of the six half-sites and the complexes migrate as more diffuse bands. Even greater differences were seen in binding assays with T73C/S112C (ox.-intra) (lanes 9,10). The input *res* fragment is converted into a diffuse smear of protein-DNA complexes, with some discrete species visible. A prominent fast-migrating species is also seen, which is assigned as the *res*L-resolvase (half-site covalent complex); similar species, with a distinctly slower mobility, are observed in binding reactions with R2A/E56K/D102Y/E124Q and γδ R (96-105 Hin) E124Q, where strand cleavage is also known to take place (Fig. 3.14).

The conditions used in the binding assays supported cleavage activity of activated resolvases on linear substrates. When the reactions with a full *res* site were analysed on an SDS PAGE gel, the covalent resolvase-DNA complexes could be detected (Fig. 3.14, A). The cleaved DNA fragments (left and right *res* half-sites) were seen following treatment with proteinase K (Fig. 3.14, B). When analysed in the absence of proteinase K, the covalent complexes of the left and right half-sites of *res* covalently attached to a single resolvase monomer migrate faster and slower than the free (uncut) *res* substrate, respectively. Small amounts of these species were seen with D102Y/E124Q, and more with the (96-105 Hin) substituted Tn3 and γδ resolvases. T73C/S112C (ox.-intra) also cleaved the *res* substrate, showing a level of activity similar to γδ (96-105 Hin) E124Q resolvase. In the presence of T73C/S112C (ox.-intra) resolvase, small amounts of two additional cleavage products were observed. The characterisation of these products (labelled X and Y, Fig. 3.14, B) will be discussed in section 6.6. These results confirm that the internally crosslinked T73C/S112C resolvase is extremely activated for double-strand cleavage. This strongly contrasts with the complete absence of catalytic activity observed with internally crosslinked T73C/A115C (96-105 Hin)

resolvase, (Fig. 3.14, B), although it is fully proficient at binding (Fig. 3.13). The intramolecular disulphide crosslink in these two mutants only differs in one cysteine partner, S112C or A115C, approximately one full turn of helix E apart. This demonstrates that the consequences of crosslinking the *cis* interface are dependent upon the geometry of the crosslink.

The analysis of the binding/cleavage reactions in the presence of SDS also revealed two further species, migrating as a tight doublet much slower than the substrate and the half-site covalent complexes (Fig. 3.14). These are observed with most activated resolvase mutants, including oxidised T73C/S112C. Preliminary results from analysis by 2-dimensional electrophoresis suggested that the DNA in these complexes co-migrated with the uncut *res* fragment after treatment with proteinase K (P. Mitchell and M. Boocock, personal communication). The two species therefore may represent the *res* substrate covalently attached to a single resolvase monomer, with either the top or bottom strands remaining intact. In each sample these bands are roughly equal in intensity. If these species are indeed the single-strand cleaved complexes described above, one can deduce that resolvase has no preference for top or bottom strand cleavage at site I in these assay conditions.

## 6.6 Mapping the cleavage site in a fragment containing sites II/III by superose-purified T73C/S112C (ox.-intra)

Cleavage reactions by intramolecular disulphide-crosslinked T73C/S112C resolvase revealed additional cleavage products that could not be accounted for by cleavage at the crossover sites. It was suspected that this resolvase mutant might be able to cut other sites in the substrate whose sequence was similar to site I. As the sequences of sites II/III are similar to site I (differing mostly in the length of the spacer regions) these were predicted to be substrates for cleavage by T73C/S112C (ox.-intra) resolvase. A similar experiment to the SDS PAGE binding/cleavage experiment described above was performed with a fragment containing only sites II/III. This fragment was 104 bp in length and was 3'-end labelled at the *Xba*I site located at the right (site III) end of the fragment (Fig. 6.14, B), (it also contains a small amount of a contaminant species, migrating slower than the sites II/III fragment, and thought to contain a full *res* site radioactively labelled at both ends). Reactions were set up with superose purified T73C/S112C (ox.-intra) and γδR (96-105 Hin) E124Q resolvase,

which had been seen to generate some additional cleavage products of the same length as T73C/S112C (as judged by analysis on an agarose gel, Fig. 6.6). The binding/cleavage reactions with sites II/III were divided, and half was treated with proteinase K. Both sets of reactions were analysed in the presence of SDS on a polyacrylamide gel (Fig. 6.13). The most abundant product visible in the reactions with T73C/S112C (ox.-intra) migrates slower than the input II/III substrate (and very close to the position of the contaminant *res* DNA band). This was thought to be a double strand cleavage product of the site II/III substrate, with resolvase covalently attached to the bottom strand (as only the "right-end" carried a radioactive label). Supporting this is the comparison with the proteinase-K-treated samples, in which a novel species with a similar abundance was visible. This species migrates faster than the substrate, indicating that it must contain a double strand break. The apparent size of this fragment was estimated to be 86 bp, by comparison with DNA fragments of known lengths (Fig. 6.13). Assuming the fragment is derived from the R-end (which carries the radioactive label), the DSB can be mapped within site II, as shown in Fig. 6.14, B. A product of the same apparent length (though of a lower intensity) was also observed for the reactions with γδR (96-105 Hin) E124Q and was also assumed to be due to a DSB within site II.

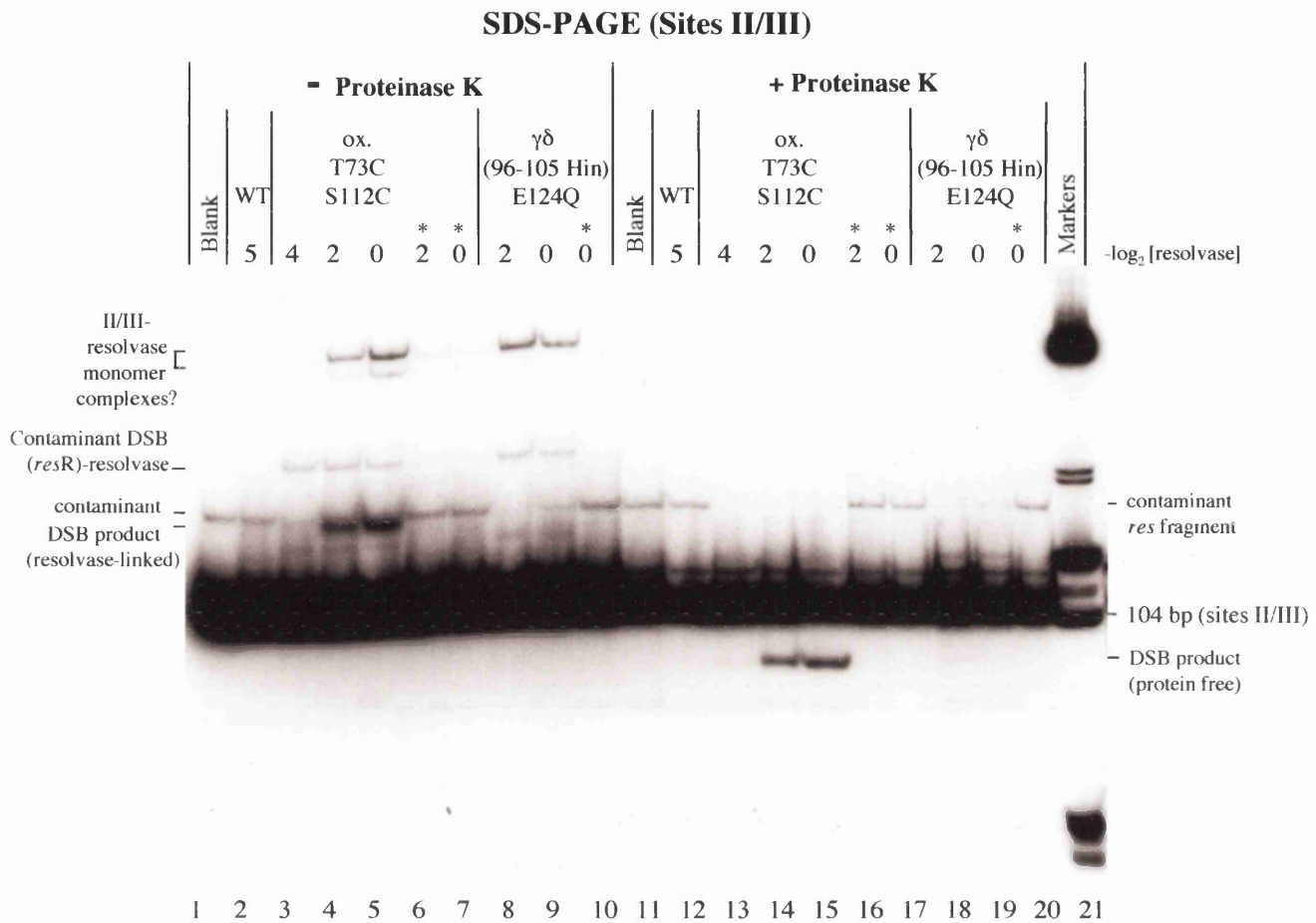
Further slow-migrating species were detected in the presence of the activated resolvase mutants, especially in the presence of carrier DNA (Fig. 6.13). These are sensitive to proteinase K, indicating that these are covalent protein-DNA complexes. The two slowest-migrating species were reminiscent of the two extra covalent complexes observed with the full *res* site previously (Fig. 3.14). It was suggested that these two species migrate as a tight doublet because they represent top and bottom strand cleavage of the *res* substrate and that these were equally represented because T73C/S112C (ox.-intra) resolvase showed no preference of *res* site I strand cleavage (section 6.5). The species observed with the sites II/III substrate may be similar in origin and may represent covalent complexes of sites II/III and a single monomer of resolvase. If this is the case, then the higher abundance of one of the two species might indicate a preference for cleavage of a particular strand of the sites II/III fragment, especially for γδR (96-105 Hin) E124Q resolvase (Fig. 6.13). Two further proteinase K-sensitive novel species are observed for both mutants, migrating faster and slower than the contaminant fragment. These are probably covalent complexes of resolvase and the contaminating *res* DNA (which is anticipated to be cleaved efficiently by these activated resolvases if it carries a *res* site); the pattern observed was similar to that previously seen with a *res* site fragment (Fig. 3.14). Also the contaminant fragment is

depleted in the tracks containing the novel species, (as would be expected if these are complexes of the contaminant DNA and resolvase), Fig. 3.14. Therefore, these two species probably represent the left and right *res* half-site resolvase complexes.

Previously, a similar experiment was described in which two "additional" cleavage products (i.e. non-site I cleavages) were observed for a 199 bp full *res* site labelled at both ends (section 6.5, Fig. 3.14). These were mapped in the identical manner and found to migrate at positions expected for DNA fragments 88 and 107 bp in length (Fig. 6.15). The sum of the estimated sizes of these fragments is 195 bp, which is in good agreement with the size of the original 199 bp fragment. The cleavages could either be mapped within site II or within the spacer between site I and site II, Fig. 6.15 A (the two possibilities are shown in blue and black). However, only the cleavage sites within site II (black) are consistent with the cleavage experiment of sites II/III. The sites II/III fragment is identical to the *res* fragment in the region between *Eco*RI and *Xba*I in which both cleavage possibilities map, nevertheless cleavage clearly maps within site II. This is also consistent with a further mapping experiment described below. Therefore, the simplest explanation of these data is that cleavage occurred at site I, and at a position within site II, independently. The less abundant 107 bp fragment is the product of cleavage at site II alone whereas the more abundant 88 bp fragment is the product of cleavage at site II alone or of site II and site I. The observed ratios of the fragments support this hypothesis.

Finally, a more accurate determination of the cleavage position(s) on the bottom strand of *res* site II was tried by running the reaction products on a sequencing gel. The substrate used was the 104 bp fragment containing sites II/III (Fig. 6.13). A specific cleavage product was observed in the presence of T73C/S112C (ox.-intra) and to a lesser extent with γδR (96-105 Hin) E124Q; these products were of identical apparent sizes. Six markers were used to estimate that this cleavage product migrates at the position of a 76 nt. single-stranded DNA fragment. This firmly established the cleavage position within site II of this fragment, Fig. 6.15 B and C. In order to avoid the uncertainty associated with a small peptide remaining linked to the DNA after proteinase K digestion of the covalently attached resolvase, the experiment would need to be performed with 5' end-labelled DNA.

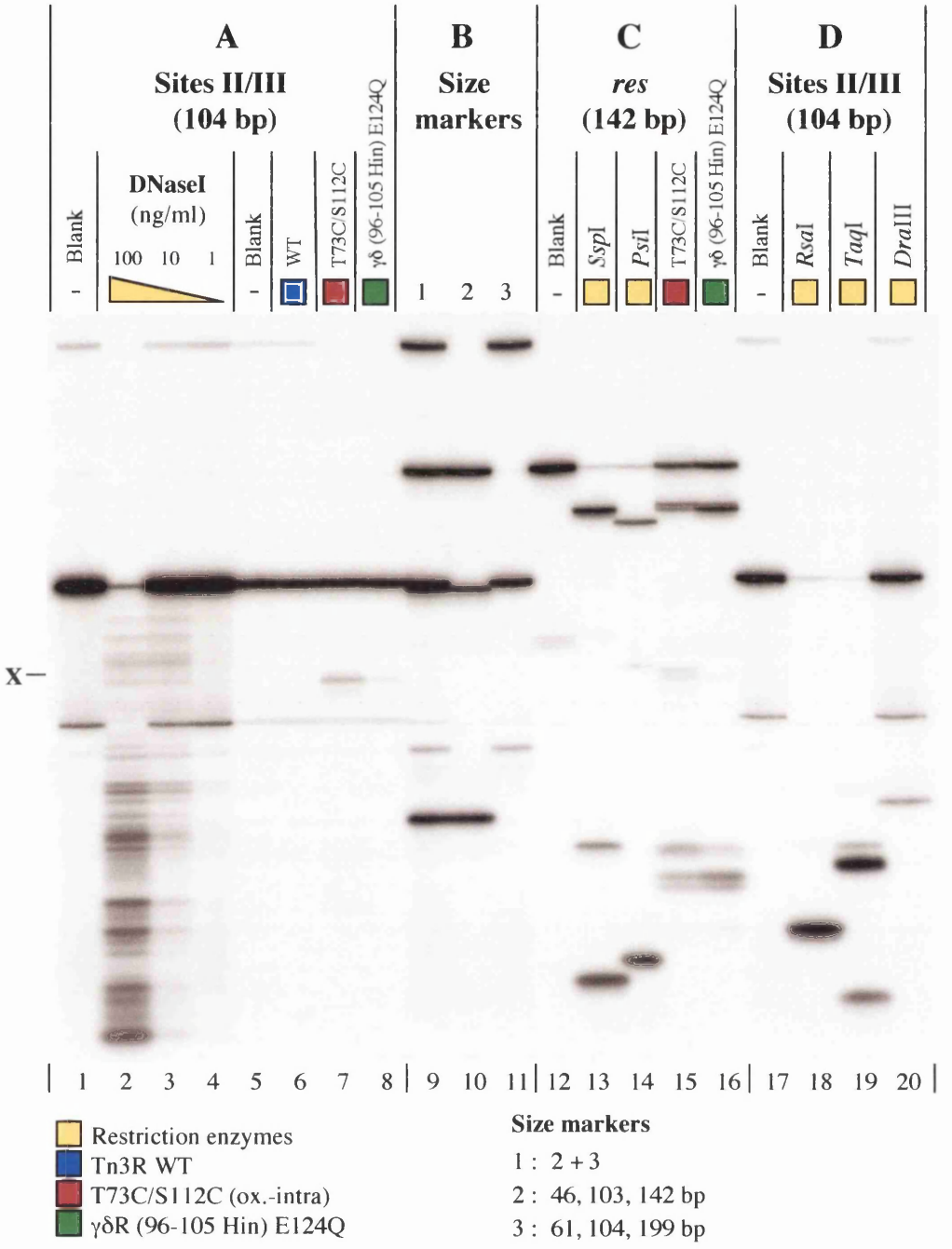
## Fig. 6.13 Cleavage of a linear sites II/III fragment by resolvase mutants




* No carrier DNA present in the samples labeled with an asterisk

Binding reactions were carried out with a 104 bp *Eco*RI-*Xba*I pOG5 fragment, 3' end-labelled at the *Xba*I site in binding buffer (25% ethylene glycol, 50 mM Tris/glycine pH 8.2, 0.1 mM EDTA) in the presence or absence (*) of 50-100 nM carrier DNA (1/240 volume supercoiled plasmid, *gix* x *gix*). Resolvase was added (1/20 volume) at room temperature (8 min.) and reactions were incubated for 75 min. at 37°C. Samples were split (2 x 10 $\mu$l), and 5 $\mu$l loading buffer (SDS % final ± proteinase K) was added. Samples were loaded on a SDS Tris/glycine gel, run at 200 V for approximately 2 hours. Resolvase stock concentrations were as follows: Tn3R WT (7.5 $\mu$M), T73C/S112C (ox.-intra) (7.5 $\mu$M), γδR (96-105 Hin) E124Q (0.5 $\mu$M).

## Fig. 6.14 Preliminary mapping of DSB's induced by T73C/S112C at site II



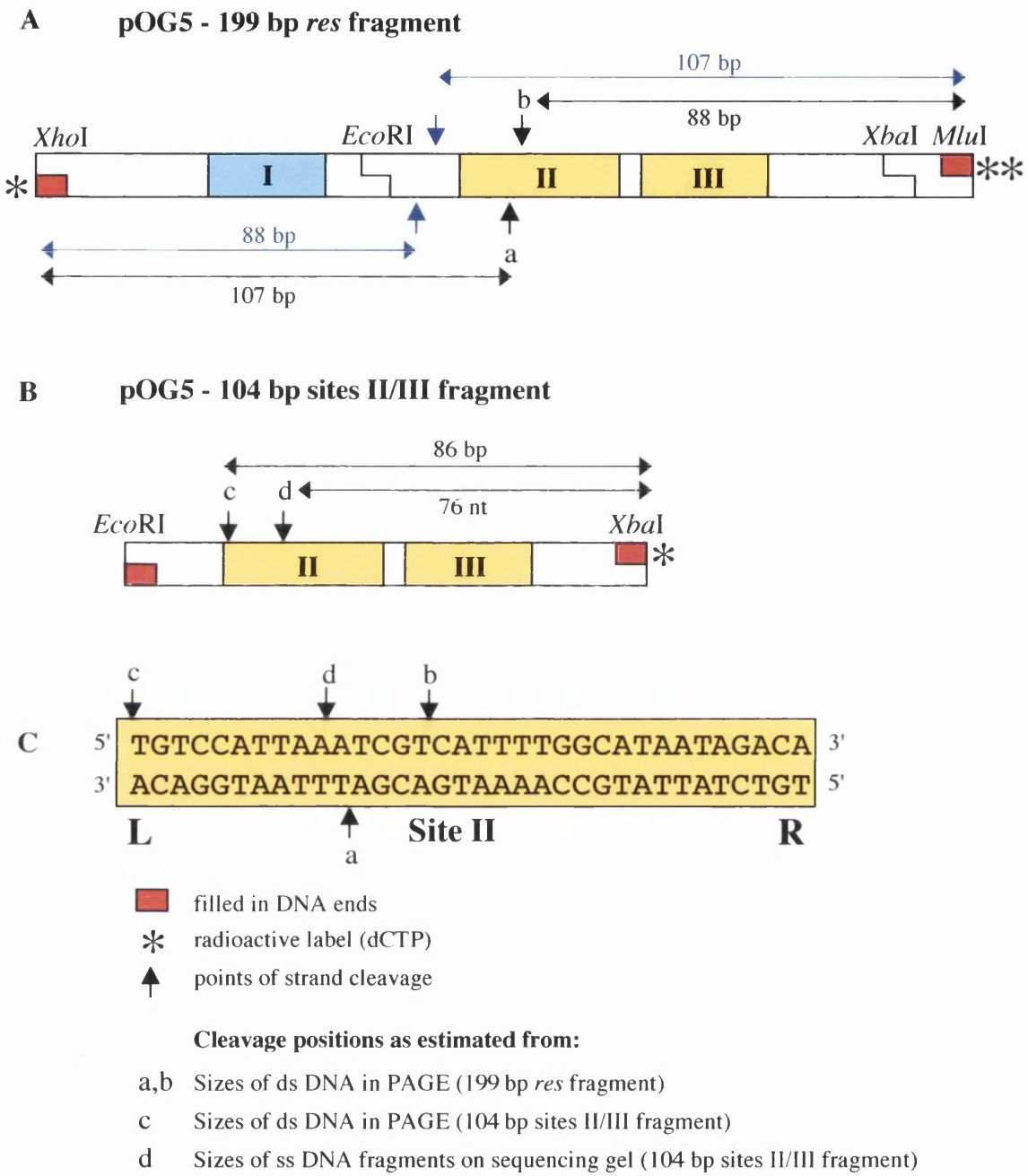

**A.** The substrate was a 104 bp *Eco*RI-*Xba*I fragment containing sites II/III, 3' end-labeled at the *Xba*I site only (see also D). Aliquots of previous assays (Fig. 6.13, samples of lanes 11, 12, 15, 19)) were analysed on a sequencing gel in the presence of formamide (lanes 5-8). The same DNA fragment was treated with DNaseI to generate a ladder of ssDNA fragments (lanes 2-4).

**B.** Fragments of known size were used as markers to calculate the sizes of fragment X (lanes 9-11).

**C.** A 142 bp *Spe*I-*Xba*I *res* fragment, 3' end-labeled at both ends was assayed with restriction enzymes *Ssp*I and *Psi*I (45 min. at 37°C) and with resolvases (75 min. at 37°C, otherwise identical resolvase concentrations and reaction conditions as in (A) were used). Reactions were treated with proteinase K (50 $\mu$g/ml).

**D.** The 104 bp sites II/III containing fragment (used in A) was restricted with a variety of restriction enzymes in order to determine that it was 3' end-labeled exclusively at the *Xba*I site.

## Fig. 6.15 Preliminary mapping of DSB's induced by T73C/S112C at site II




**A.** The radioactively labelled 199 bp *Xho*I-*Mlu*I fragment from pOG5 is displayed diagrammatically. The two possible cleavage points generating radioactively labelled fragments of 88 bp and 107 bp are shown in black and blue.

**B.** The radioactively labelled 104 bp *Eco*RI-*Xba*I fragment from pOG5 is displayed diagrammatically. The cleavage point at 88 bp from the *Xba*I site, as estimated from the PAGE experiment (Fig. 6.12), is shown. The cleavage point of 76 nt from the *Xba*I site is also shown, as estimated from the sequencing gel experiment (Fig. 6.13).

**C.** The sequence of site II is shown as well as the estimated cleavage points for all experiments.

## 6.7 Further work

As mentioned previously, this work is at a preliminary stage. Further characterisation of purified T73C/S112C (ox.-intra) should include attempts to investigate the topology of reactions with various substrates, particularly a standard directly repeated *res* x *res* substrate. This analysis is especially important because the formation of 2-noded catenane product by resolvase crosslinked in the *cis* interface would be consistent with the DNA-mediated and simple rotation strand exchange models but not with the domain-swapping model. In fact, this experiment was performed with the original preparation of T73C/S112C (prior to further fractionation). Although a trace of 2-noded catenane was obtained when this material was assayed under non-reducing conditions, a much larger amount was seen when the assays were repeated in the presence of reducing agent (10 mM DTT), (data not shown). Thus, the trace of 2-noded catenane observed in the absence of DTT may be due to the presence of reduced T73C/S112C present in this fraction (approximately 20%). If the domain-swapping model were not ruled out by this experiment, targeting experiments could be performed. Targeting of T73C/S112C (ox.-intra) to either half-site of the crossover site would further test the domain-swapping model, as this model predicts that positioning the *cis*-crosslinked monomer at a specific half-site of site I would be compatible with strand exchange. However, such half-site targeting experiments may prove difficult to perform. Half-site targeting at site I was achieved previously by targeting M106C disulphide-linked heterodimers and reducing these *in situ* (Murley & Grindley, 1998). As reduction of disulphide crosslinks is not specific, reduction of M106C would also result in the reduction of the internal disulphide between T73C and S112C, unless the *cis* crosslink were irreversible (as with the crosslinking agents described in Chapter 5). The success of gel filtration in separating intramolecular from intermolecular crosslinked species under denaturing conditions may enable the generation of appropriate crosslinked species. Prior to performing targeting experiments it may therefore be essential to establish whether such a crosslinked species would be active for cleavage. Finally, the characterisation of oxidised T73C/S112C should include mapping of numerous "non-site I" cleavage positions to determine sequence preferences. This may be achieved most easily by primer extension "footprinting".

The objective of the crosslinking experiments was to attempt to rule out strand exchange models by determining which interfaces do not need to dissociate during strand exchange. In order to fulfil this objective, the effect(s) of crosslinking the *trans* interface on strand exchange need to be investigated. Crosslinking this interface using D95C/A113C resolvase was not informative (Chapter 5). Time limitations have prevented the construction and characterisation of D95C/S112C (which is expected to form a disulphide bond in *trans*). It should be noted that disulphide bond formation across the *trans* interface may also perturb the *cis* interface. Since disruption of the *cis* interface may reduce resolvase activity (as was observed for oxidised T73C/A115C (96-105 Hin) resolvase, Chapter 5), homobifunctional crosslinking agents may be required. However, in the light of the NEM modification data, Fig. 6.1, (which established that modification of D95C/112C inhibited resolvase activity), it may be prudent to attempt to make a singly crosslinked heterodimer of D95C and S112C subunits. Upon crosslinking, this heterodimer would contain only a single disulphide bond or chemical crosslink (and consequently would involve modification of only 2 rather than 4 cysteine residues). For purification purposes the addition of a histidine tag onto one mutant (e.g. S112C) should prove useful as the heterodimer could be purified using an immobilised nickel column (as shown by Murley & Grindley, 1998). Furthermore, targeting of this heterodimer could be achieved using the γδR R172L DNA binding domain (as in Chapter 3).

## 6.8 Summary and Conclusions

The preliminary characterisation of T73C/S112C resolvase has been very informative. In its fully reduced state T73C/S112C was found to be an active recombinase, not differing substantially from wild-type resolvase. Under non-reducing conditions the cysteine residues became disulphide-crosslinked, primarily (though not exclusively) in an intramolecular configuration. The intramolecular crosslinked species was successfully separated from the other crosslinked species by gel filtration, and this species was found to be active in cleavage reactions at *res*. The cleavage reaction therefore does not necessitate dissociation of the *cis* interface. A small amount of resolution product was observed (LR joined) indicating that T73C/S112C (ox.-intra) is proficient at least in dissociative recombination. Furthermore, T73C/S112C (ox.-intra) was found to display a hyperactive phenotype, including the loss of supercoil and accessory site dependence. This was evidenced by the ability of T73C/S112C (ox.-intra) to quantitatively cleave isolated crossover sites on linear substrates. Astonishingly, cleavage by T73C/S112C (ox.-intra) was not limited to *res* site I. Instead this

mutant was found to cleave DNA sequences other than site I, including plasmids containing no related recombination sites. Analysis in the presence and absence of proteinase K, revealed that the linearisation of the plasmid substrates was due to resolvase-induced DSBs with covalent linkage of resolvase at specific sites in the DNA and not to hydrolysis. This was confirmed in binding/cleavage reactions. Preliminary mapping experiments have indicated that a specific site within *res* site II appears to be cut by T73C/S112C (ox.-intra). Furthermore, these experiments involved linearised substrates, thereby establishing that cleavage at site II by T73C/112C (ox.-intra) and γδR (96-105 Hin) E124Q does not require supercoiling.

T73C/S112C (ox.-intra) thus represents a new type of activated resolvase mutant, as it has no mutations in the 102 or 124 regions, unlike all previously characterised activated mutants. T73C/S112C (ox.-intra) appears to be trapped in an activated configuration, which might be similar to the configuration of wild-type resolvase when it is activated for strand cleavage by an as-yet unidentified signal. This may be a consequence of the disulphide bond, which might be expected to "pull" the serine 10 nucleophile closer to the cleavage site in the DNA backbone. However, a similar mutant, (96-105 Hin) T73C/A115C, which already carries activating mutations, was found to be inactive when disulphide crosslinked across the *cis* interface. Regulation of catalysis by resolvase is thus extremely sensitive to perturbation of the *cis* interface by crosslinking.

# Chapter 7

# Discussion

## 7.1 Introduction

The work described in this thesis has concentrated on two general themes, (i) the protein-protein contacts within the synaptic complex and their role in the regulation of the resolution reaction and (ii) the requirements for the dissociation of protein interfaces (*cis, trans,* and E helix) during strand exchange, with the aim of testing the domain-swapping and other models. These studies have influenced each other. This is not surprising, since hypotheses describing the relative protein movements required for strand exchange were linked to hypotheses about the elusive synaptic interface between resolvase dimers bound at site I (and maybe sites II/III as well). Thus, the proposed domain-swapping model requires synapsis of resolvase dimers via the catalytic domain. Furthermore, these issues tied in with hypotheses about the structure of the invertasome of Hin. We tried to determine how Hin dimers are activated by the Fis/enhancer complex and ask whether these mechanisms are analogous to the mechanism of resolvase activation by the accessory site synapse. This Chapter discusses the findings described in Chapters 3-6, in the context of other work and current thinking on these issues.

## 7.2 Synapsis

Numerous experiments have shown that resolvase bound to site I is largely in an inactive configuration, requiring a number of signals (specific to the productive synapse) to activate resolvase at the crossover sites. An inactive configuration of an isolated resolvase dimer bound to DNA was seen directly in the co-crystal structure of γδ resolvase bound to site I (Yang and Steitz, 1995). Although several regions of the resolvase dimer make dimer-dimer contacts in resolvase crystals (Rice and Steitz, 1994; Sanderson *et al.*, 1990), the only interdimer interface which is supported by functional evidence is the 2,3'-interface (Hughes *et al.*, 1990). Numerous experiments suggested that the 2,3'-interface was required to build the structure of the synaptic complex; it was initially thought to play an architectural role at sites II/III and was thought not to be required for activation of resolvase at site I (Grindley, 1993). However, a more recent study indicated that this interface might also function as a signal between resolvase bound at the crossover site (I-R) and resolvase at sites II/III (Murley and Grindley, 1998). Studies described in Chapter 3 support this role in the activation of resolvase at site I via contacts between residues constituting the 2,3'-interface.

The experiments described in Chapter 3 attempted to address the role of the 2,3'-interface in the context of activated resolvase. Based on previous data, it was hypothesised that the 2,3'-interface is required to wrap the accessory sites of *res*, trapping three negative supercoils and thereby constraining the alignment of the crossover sites. In order to test this, mutations in the 2,3'-interface were combined with activating mutations. A resolvase carrying activating mutations and mutations of the 2,3'-interface would not be expected to respond to the alignment and topological constraints imposed by accessory sites. In the absence of the 2,3'-interface, recombination sites would be expected to meet by random collision and because any initial synapse could be used for recombination, the alignment of the sites and the topology of the products would not be constrained. Thus, the topological filter mechanism would be side-stepped. It should be noted that if synapsis were to occur exclusively by a slithering mechanism, the crossover sites might align in a parallel configuration, generating an excess of unlinked circular resolution products upon strand exchange.

Comparison of Tn3R E56K/D102Y/E124Q and Tn3R R2A/E56K/D102Y/E124Q showed that the effects of mutations at the 2,3'-interface are additive (e.g. Fig. 3.6). This suggested that a single mutation in the 2,3'-interface is not sufficient to completely disrupt this interface. However, because Tn3R R2A/E56K (unlike γδR R2A) was not complemented by γδR S10L, the combined effect of R2A and E56K was considered to be effective in disrupting the 2,3'-interface (as measured by the failure to respond to activation by sites II/III, Fig. 3.11). Nevertheless, Tn3R R2A/E56K/D102Y/E124Q retained topological selectivity when assayed with a *res* x *res* substrate (Fig. 3.6). The architecture of the accessory site synapse had therefore not been disrupted, despite the disruption of the 2,3'-interface. Although we considered it unlikely that the activating mutations could restore the stability of the synapse, compensating for the 2,3'-interface, recent data has demonstrated a "synapsis-up" phenotype for proteins that are multiply mutant in the 102 region (G. Sarkis *et al.*, in preparation). Whether the D102Y mutation alone is sufficient to confer a synapsis-up phenotype remains undetermined. Nevertheless, the bias towards inversion product (LR joining) seen with 2,3'-defective activated resolvases with pMA2631 (*res* x *res*, inverted repeat), is consistent with another interface (such as the "top" surface of the catalytic domain) being responsible for synapsis in the absence of the 2,3'-interface (Figs. 3.8 and 3.9). Certainly, the residual topological selectivity observed with R2A/E56K/D102Y/E124Q

suggests that the 2,3'-interface is not the only interdimer interaction required to build a synaptic structure that traps three negative supercoils.

The evidence presented in Chapter 3 suggests that an intact 2,3'-interface is required for the activation of resolvase bound at site I by resolvase at sites II/III. This requirement can be partially removed by the presence of "activating" mutations (D102Y/E124Q), although a decrease in the reaction rate is observed. The "activating" mutations were found to be required only at the crossover sites; in this context, 2,3'-defective resolvase R2A/E56K is functional at sites II/III (the product topology observed, 2-n catenane, indicated that a –3 accessory site synapse is formed, Fig. 3.17). From this data it could be concluded that the 2,3'-interface is not essential for the architecture of the accessory site synapse (although it does contribute to the stability of this structure, Fig. 3.10). However, the 2,3'-interface does play a vital role in the activation, by sites II/III, of resolvase catalysis (at site I). This role (summarised as hypothesis II, Fig. 3.15) is consistent with a model (4+2) of the synapse proposed recently (Sarkis *et al.*, in preparation, see section 3.6).

The 4+2 model of the synaptic complex is furthermore similar to a model of the Sin recombinase synaptic complex (S. Rowland *et al.*, in preparation). The structure of the Sin recombination site is considerably different from *res* - it has only one accessory site (site II) with binding sites arranged as a direct repeat. Furthermore, between the crossover site and site II lies a binding site for Hbsu. Despite the considerably different structure of the Sin and resolvase recombination sites, the topologies of the recombination reactions are identical. In the model of the Sin synaptic complex proposed by S. Rowland *et al.*, two Sin "dimers of dimers" are arranged as in the 4+2 model of the resolvase synapse (Fig. 3.21, C and D). The DNA between site I and II is looped out and bent sharply by Hbsu binding, thus trapping three negative supercoils.

These models postulate the existence of another as yet uncharacterised interface (i.e. other than the 2,3'-interface), which is required for synapsis. This interface has been called the "hypothetical" interface. The identification of accessory site-independent resolvases (such as Tn3R D102Y/E124Q and γδR E102Y/E124Q), revealed that concerted recombination could occur between resolvase dimers at site I in the absence of sites II/III (Arnold *et al.*, 1999). This and other data indicated that the dimers at site I could form stable contacts via an interface other than the 2,3'-interface. Random mutagenesis of the entire catalytic domain

showed that activating mutations were clustered at the loop around residues 101-103 (S. Wenwieser and M. Boocock, this laboratory). Several different substitutions at residue 102 yielded activated resolvase (L. Tyrell, unpublished results). This suggested that the wild-type sequence may inhibit resolvase function (thus the replacement with almost any other residue relieves this inhibition - resulting in an activating effect on resolvase). It was therefore speculated that:

(i) The surface of the catalytic domain, particularly the loop between alpha helix E and ß strand 5, was involved in interdimer contacts required for synapsis. Such synapsis would be required if strand exchange proceeded by a domain-swapping mechanism. However, it should be noted that there is no evidence of a synapsis phenotype associated with resolvases carrying single activating mutations in the 102 region.

(ii) Mutations in the 102 region may alter a property of the loop, thereby activating strand exchange, consistent with the domain-swapping mechanism for strand exchange.

(iii) Alternatively, the 102 region may be directly contacted by resolvase bound at sites II/III. Mutations in the 102 region may thus mimic the activation signal received in this region of resolvase. This model would be especially attractive if crossover site synapsis occurs via the C-terminal domains (Fig. 4.2, C) leaving the 102 region accessible to solvent.

In order to test the possibility that the 102 region of resolvase was involved in interdimer interactions during synapsis (as speculated in (i) and (iii) above), this region (Tn3R residues 96-105) was replaced by the homologous region of the invertase Hin (Chapter 4). Surprisingly, this yielded activated resolvases, which are discussed in the section on activation below.

## 7.3 Activation

The catalytic activity of resolvase is clearly regulated by various classes of protein-protein contacts within and between dimers. As discussed above, the properties of activated resolvase mutants suggested the existence of another synaptic interface, distinct from the 2,3'-interface. This was probed by substituting residues 96-105 of resolvase with the homologous residues of Hin, thereby generating six amino acid substitutions (Chapter 4). The activating effect of these substitutions confirmed the importance of this region in the regulation of resolvase activity. Tn3R (96-105 Hin) did not require supercoiling or accessory

sites. However, in the presence of a supercoiled *res* x *res* substrate and standard recombination conditions, the activity seen with this mutant did not differ substantially from WT resolvase (yielding mostly 2-n catenane products, Fig. 4.3). Nevertheless, kinetic experiments revealed that Tn3R (96-105 Hin) was a faster resolvase than Tn3R WT. The identical substitution of residues (96-105) of Hin in the context of γδ resolvase produced an even more active resolvase. Why activating mutations often have a greater effect when introduced into γδ rather than Tn3 resolvase is not known. γδR (96-105 Hin) efficiently recombined a substrate with two crossover sites. The products of this reaction were consistent with concerted recombination; no cleavage products and no illegitimate products were observed. For the first time, this revealed that activation of resolvase for accessory site independent activity can be achieved by mutations in the 102 region of resolvase alone. The addition of the E124Q mutation generated a resolvase which was extremely activated for the cleavage reaction. Thus, the effect of these mutations (in very different regions of the resolvase structure) were found to be additive.

A Tn3 resolvase mutant, carrying known activating substitutions only in this region, G101S/D102Y/M103I/Q105L, was subsequently characterised, and confirmed that mutations in this surface region of resolvase alone were sufficient to generate accessory site independent resolvase (Jiuya He *et al.*, in preparation). Curiously, the presence of accessory sites had an inhibitory effect on this mutant in contrast to the (96-105 Hin)-substituted resolvases. Intriguingly, a similar mutant that carried additional mutations in the 2,3'-interface (R2A/E56K/G101S/D102Y/M103I/Q105L) was even more active than the 2,3'-proficient version. The small activating effect conferred by mutations in residues contributing to the 2,3'-interface was also noticeable in a comparison of the *in vivo* phenotypes of D102Y/E124Q and R2A/E56K/D102Y/E124Q (Chapter 3, Fig. 3.4, B). This small activating effect of the R2A/E56K mutations on D102Y/E124Q resolvase was only seen for site I x site I activity and not for *res* x *res* activity (for which 2,3'-proficiency is required for maximal activity). The presence of the single substitution E56K was also seen to increase the site I x site I reaction rate of D102Y/E124Q *in vitro* (M. Boocock, unpublished results). Why mutations of the 2,3'-interface have an activating effect (or indeed any effect) on site I x site I reactions is unknown. A potential explanation is that the mutations in residues of the 2,3'-interface of R2A/E56K/D102Y/E124Q alter the conformation of the enzyme, increasing its ability to synapse with another site I-bound resolvase dimer (possibly via the "hypothetical" interface). An alternative explanation may be that the interaction of

two site I bound dimers of resolvase via their 2,3'-interface places the crossover sites in a configuration unsuitable for recombination. Thus, in the absence of 2,3'-proficiency these unsuitable (though potentially quite stable) interactions are not made, resulting in a more active recombinase.

A further resolvase mutant, internally disulphide crosslinked T73C/S112C, was activated for the cleavage reaction (Chapter 6). This mutant was very different from any previously characterised mutant of resolvase; the locations of the mutations were not in the previously identified regions of resolvase (around residues 102 or 124) known to activate resolvase and the hyperactive property was completely dependent upon the disulphide crosslink. This was the first definitive evidence of the importance of the *cis* interface in the regulation of resolvase activity. It was postulated that T73C/S112C (ox.-intra) was activated because the disulphide bond brings the active site of resolvase closer to the cleavage site in the DNA backbone. T73C/S112C (ox.-intra) may therefore be trapped in an activated configuration. However, it was also shown that activation was not simply a matter of crosslinking this interface; (96-105 Hin) T73C/A115C resolvase was inactivated by formation of an internal disulphide crosslink across the *cis* interface. The residues involved in crosslinking differ only in approximately one turn of the alpha helix (112 versus 115). Thus, perturbation of the *cis* interface by crosslinking can have radical effects upon resolvase function.

In summary, activation of resolvase can occur in a number of different ways:

(i) mutations of residue 102 and surrounding residues: The resolvases of this type which have been characterised are D102Y (and a large number of other single substitutions at this residue) (Arnold *et al.*, 1999). A random mutagenesis screen of the entire catalytic domain in combination with E124Q also revealed G101S, D102V, M103I (S. Wenwieser and M. Boocock, this laboratory). A combination of activating mutations in this region - G101S/D102Y/M103I/Q105L (Jiuya He *et al.*, in preparation), and (96-105 Hin) substituted Tn3 and γδ resolvases (Chapter 4) were also found to be extremely activated.

(ii) mutations of residue 124 and surrounding residues (close to the active site of the partner monomer): The resolvases of this type characterised include Tn3R and γδR E124Q and γδR E124A (Boocock, Zhu and Grindley, in preparation), Tn3R R121K and A117V (P. Arnold, unpublished results). However, Tn3R A117V/R121K/E124Q was severely

impaired in its resolution activity *in vivo*, indicating that multiple mutations in this region (unlike region 102) are not tolerated by resolvase.

(iii) mutations in the 2,3'-interface (when combined with activating mutations of type (i) or (ii): R2A/E56K/G101S/D102Y/M103I/Q105L (Jiuya He *et al.*, in preparation), and R2A/E56K/D102Y/E124Q (Chapter 3).

(iv) disulphide crosslinking of the *cis* interface between residues 73 and 112: T73C/S112C (ox.-intra), (Chapter 6).

The properties of activated resolvase mutants differ dramatically. Activated resolvases differ in the amounts and types of products that they make and also in their dependence upon supercoiling and accessory sites. They cannot be organised on a "linear scale of activation". While resolvase mutants such as Tn3R D102Y or γδR E124Q display relatively "mild" activated properties (such as reduced dependence on supercoiling), the highly activated γδR (96-105 Hin) shows greater dependence upon supercoiling than upon *res* sites II/III (Figs. 4.11 and 4.12). Also, (96-105 Hin)-substituted resolvases show a greater tendency to cleave the DNA without re-ligating the ends, compared with another similarly active resolvase, γδR E102Y/E124Q (Fig. 4.7). Hyperactivity is not simply a matter of increasing the reaction rate; reduced (96-105 Hin) T73C/A115C cleaves a *res* x *res* substrate slower than WT resolvase, but is nevertheless active on a substrate lacking accessory sites, on which WT resolvase shows no activity (Fig. 5.13). Also the Tn3R (96-105 Hin) was the only mutant found to resolve a *res* x *res* substrate faster than wild-type resolvase under standard recombination conditions (Fig. 4.4). Finally, while T73C/S112C (ox.-intra) in some ways represents the most active mutant characterised to date (as it is able to cleave non-site I DNA sequences and also shows little selectivity for any of the usual features required by WT resolvase), it does not efficiently perform a complete resolution reaction (not even with a *res* x *res* substrate, Fig. 6.11), indeed it may completely lack this activity.

The Fis/enhancer complex has been compared to the accessory site synapse of resolvase. Certainly, their roles are analogous: both are required for the activation of a recombinase bound to the crossover sites and the alignment of the recombination sites (by imposing a topologically distinct synaptic structure) and both requirements can be overcome by mutation of the dimer interface. To what extent are their roles structurally similar? Are direct protein-protein contacts involved? Do they target similar regions of the "catalytic" recombinase dimers, and are these synapsed in a similar manner? Does activation require

both "catalytic" recombinase dimers to be activatable (i.e. make interactions with the Fis/enhancer or accessory site complexes)? Does activation involve the entire tetramer or can monomers within the tetramer act independently (i.e. is activation of the four individual monomers an "all or nothing" event)?

A series of experiments using mutants of Fis which are unable to activate Hin (Q21C ox. and D20K, (Safo *et al.*, 1997)) have revealed that Hin activation requires only one functional ß-hairpin activating arm of Fis at each enhancer binding site. Similar experiments with Hin dimers disulphide crosslinked at position M101C (considered as "unactivatable" (Haykinson *et al.*, 1996)) and the active site mutant S10G (Merickel *et al.*, 1998) showed that all four Hin subunits must be "activatable" by Fis or contain a mutation conferring Fis independence (e.g. H107Y (Haykinson *et al.*, 1996)) though they must not necessarily be proficient in catalysis (Merickel *et al.*, 1998). Merickel *et al.* thus speculated that a single Fis ß-arm contacts Hin, thereby triggering simultaneous conformational change in both subunits of a dimer (and resulting in coordinate attack of both DNA strands of *hix*).

Targeting of crosslinked D95C/A113C or crosslinked M106C resolvase to one crossover site of pNG343 was observed to inhibit γδR R172L bound at the opposite crossover site (Chapter 5, Fig. 5.10). This may be due to a failure of the crosslinked resolvase dimer to synapse with the partner resolvase dimer (targeted to the other site I) or to a problem at a later step in resolvase activation. The lack of activity of γδR R172L in this experiment suggests that resolvase becomes activated by quaternary structural changes in the entire catalytic "tetramer". When a similar complementation experiment was performed with oxidised (96-105 Hin) M106C and γδR R172L, it was found that the activating (96-105 Hin) substitution did not abolish the inhibitory effect of the oxidised dimer on R172L resolvase (data not shown).

A similar type of experiment was attempted using 2,3'-defective resolvase R2A/E56K (data not shown). It was thought that this resolvase may be analogous to the "unactivatable" Hin mutant M101C (ox.). It was speculated that the contacts between 2,3'-residues of resolvase at site I and sites II/III (possibly III-R) would be equivalent in function to the contacts between Hin dimers and the Fis/enhancer complex. If cleavage/recombination by resolvase requires all four subunits of resolvase at site I to be activatable by the accessory site synapse it would then be predicted that targeting R2A/E56K to one crossover site would also "inhibit" the

resolvase dimer bound at the partner crossover site. Testing this prediction requires complementation by three different resolvase mutants; 2,3'-proficient but catalytically inert resolvase (such as γδR S10L) targeted to sites II/III, 2,3'-deficient resolvase (R2A/E56K) targeted to site I and γδR R172L targeted to the other crossover site. Alternatively, complementation experiments between γδR R172L targeted to one crossover site and the activated mutant D102Y/E124Q targeted to sites II/III and the other crossover site would address the same question and may prove easier to perform. A preliminary attempt at both of these experiments was made, however, no conclusions could be drawn. Nevertheless, the predictions are in principle testable and should prove interesting.

An attempt to define the region of Hin contacted by the Fis/enhancer complex was made (described in Chapter 4). Although these experiments did not pinpoint the region required, it was found that hybrid resolvases containing large segments of Hin sequence could be purified and were catalytically active. This bias towards inversion seen in some site I x site I recombination reactions with added Fis is also very intriguing. Thus further work to generate a resolvase "invertasome" should be conducted.

## 7.4 Strand exchange

The relative movements of resolvase domains and/or subunits during the strand exchange mechanism are unknown. This crucial part of the resolution reaction requires four covalent resolvase/DNA half-sites to move relative to one another in order to be rejoined in a recombinant configuration (LR joined). The change in the linkage number associated with each round of strand exchange suggested that this occurs by a 180° right-handed rotation of two half-sites relative to the partner half-sites. It is not clear how resolvase maintains connectivity between the cleaved ends, avoiding the "release" or "escape" of half-sites that would result in double strand breaks.

The domain-swap model offers an elegant solution to this problem; only the core catalytic domain of resolvase is involved in the rotational movement (with the covalently joined DNA half-site) allowing the E helix to maintain contacts with the partner monomer. This model requires the loop between the "top" of the E helix and ß strand 5 to act as a flexible "hinge" between the core catalytic domain and the E helix. The loop region may thus be an important

regulatory feature of resolvase. Importantly, this model postulates a change in the protein conformation during strand exchange (particularly of the potential "hinge" region). In this respect it is intriguing that the (96-105 Hin)-substituted Tn3 resolvase showed increased processivity with a *res* x *res* substrate. This is consistent with a regulatory role of the "hinge" region, acting to inhibit further rounds of recombination following strand exchange. Mutations in this region may thus alter the ability of resolvase to stop after one round of strand exchange. Alternatively, it could stabilise the synapse at sites II/III.

Strand exchange models were tested by constructing numerous cysteine-containing resolvases (Chapters 5 and 6). These were designed to allow crosslinking of the *cis*, *trans*, and E helix interfaces (as defined in section 5.1, see also Fig. 5.1), to determine which interfaces do *not* require to be broken during the strand exchange reaction. Unfortunately, the disulphide crosslinks were found to inhibit the cleavage step preceding strand exchange. Thus, no conclusions about the effect of crosslinking at these interfaces on strand exchange could be drawn (except that this step probably requires considerable flexibility by the resolvase dimer). However, a domain-swapping mechanism for synapsis at sites II/III could be ruled out because the *trans* crosslinked mutant D95C/A113C retained wild-type resolvase function at sites II and III (Fig. 5.9).

Unfortunately, the use of homobifunctional crosslinking agents to chemically crosslink the *trans* interface of D95C/A113C did not yield a catalytically proficient crosslinked dimer (Fig. 5.15). Instead, a different approach to this experiment was taken (described in Chapter 6, section 6.1). Residue 112 was chosen as a candidate residue to crosslink with residue 73 (in *cis*) and residue 95 (in *trans*). To date only T73C/S112C resolvase has been characterised. The internally crosslinked T73C/S112C species was found to be extremely active for DNA cleavage. Intriguingly, a preliminary experiment suggested that the disulphide crosslink in the *cis* interface inhibits concerted strand exchange. This does not support the subunit rotation model of strand exchange (but importantly also cannot rule out this model). Further work with this mutant, and characterisation of D95C/S112C resolvase may be very informative (see section 7.6).

## 7.5 Methodologies trialed

This work required the construction, purification and characterisation of a large number of resolvase mutants, some of which carried many independent mutations. Whereas an approximate correlation between the number of mutations and difficulty of purification was observed, the majority of these enzymes were purified without problems and without altering the purification protocol. Nevertheless, a number of mutants were relatively insoluble at the last resuspension step. It was found that resuspension in a small volume of urea (8 M) and subsequent dilution into a high salt buffer and glycerol proved effective in these cases. Resolvase seems to re-fold efficiently after dilution of the urea solution, as no noticeable decrease in activity was observed between these fractions and those resuspended in the usual high salt buffer.

A method to covalently modify cysteine-containing resolvase mutants with NEM or homobifunctional crosslinking agents in high yields was also achieved. This may prove to be useful to generate further crosslinked species (described below, section 7.6). In this context, the successful fractionation of different species of T73C/S112C using gel filtration in denaturing conditions provides another technique that may be very useful in generating pure preparations of specific crosslinked resolvase species.

## 7.6 Further Work

There are a number of investigations that could benefit from further work:

(i) The exceptional cleavage activity displayed by activated mutants, γδR (96-105 Hin) and T73C/S112C (ox.-intra), may be useful for crystallization trials of the covalent protein-DNA complex.

(ii) The attempt to construct a resolvase/Fis/enhancer "invertasome" also requires more work. No convincing bias in the alignment was observed specific for Hin-substituted resolvase. This may be because the region of Hin that is contacted by Fis has not yet been substituted into the resolvase reading frame. The data seem to rule out regions (96-105) and (142-189) as possible regions of contact between Hin and the Fis/enhancer

complex. Experiments using a mismatch site I x site I substrate, as described in Chapter 4, should be attempted.

(iii) Further experiments with the fractionated T73C/S112C (ox.-intra) species that should be performed are outlined in section 6.7. It also outlines possible strategies to generate *trans* crosslinked D95C/S112C (or heterodimer D95C+S112C) resolvase active for strand cleavage. Such experiments would allow the subunit rotation and domain-swapping model to be tested and may give valuable insights into the workings of this intricate protein machine.

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