Catalysis of strand exchange by Tn*3* resolvase

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by

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The research reported in this thesis is my own and original work except where otherwise stated and has not been submitted for any other degree.

Michael J. McIlwraith

CONTENTS

Abbreviations

Units

k	10^3	h	hours
m	10^{-3}	sec	seconds
μ	10^{-6}	cps	counts per second
n	10^{-9}	kb/kbp	kilo base pairs
p	10^{-12}	V	volts
bp	base pairs	Ci	Curies
A	Ampères	g	grammes
W	Watts	m	metres
°C	degrees Celsius	mol	moles
l	litres	min	minutes
M	molar	rpm	revolutions per minute

Chemicals/Reagents

APS	ammonium persulphate
ATP	adenosine triphosphate
Chloroquine	7-chloro-4-[4-diethylamino-1-methyl-butylamino]-quinoline (diphosphate salt)
CIP	calf intestine phosphatase
DNA	deoxyribonucleic acid
DNase I	bovine pancreatic deoxyribonuclease I
dNTP	2'-deoxyribonucleoside 5'-triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
EtBr	ethidium bromide
MOPS	3-[*N*-morpholino]propanesulphonic acid
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
Spermidine	*N*-[3-Aminopropyl]-1,4-butanediamine trihydrochloride
TAE	tris-acetate-EDTA (electrophoresis buffer)
TBE	tris-borate-EDTA (electrophoresis buffer)
TMED	*N,N,N',N'*-tetramethyl-ethylenediamine
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet

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SUMMARY

A novel methodology has been developed to test the mechanism of strand exchange by Tn*3* resolvase. Photocrosslinkable substrate molecules were synthesised by incorporating photoactivatable nucleotide analogues into *res* site I oligonucleotides and then ligating these double-stranded oligonucleotides into supercoiled plasmid molecules *in vitro*. It was then possible to covalently attach a resolvase subunit to a specific half-site of the modified site I by irradiating bound samples with UV laser light, and to assay if the crosslinked complex was catalytically active.

The nucleotide analogues 4-thiothymidine and 6-thiodeoxyguanosine photocrosslinked to resolvase, but were unsuitable because they apparently induced structural anomalies in supercoiled molecules. The use of 5-iodouridine in conjunction with a 325 nm HeCd laser allowed efficient photochemical crosslinking of resolvase to a synthetic site I, and the photoreactivity was retained when the site was incorporated into supercoiled substrate molecules. Using this method, it was shown that fixing a subunit of resolvase to either the left or the right end of site I does not affect its ability to participate in the catalysis of recombination. These results demonstrate for the first time that the resolvase C-terminal domain-DNA interaction is maintained throughout the recombination reaction, supporting a 'subunit rotation' or 'DNA-mediated' mechanism of strand exchange.

A separate series of experiments is also described in which an attempt was made to distinguish between the 'subunit rotation' and 'DNA-mediated' models of strand exchange. The distinction between the models was based on the assumption that there is a requirement for a ligated recombinant intermediate during a 'double round' knotting reaction in a 'DNA-mediated' mechanism but not in a 'subunit rotation' mechanism. Using substrates with mutations at the centre of site I, substrates with closely spaced *res* sites, and substrates containing mismatched base pairs at the centre of site I, it was demonstrated that a fully ligated, mismatched recombinant is not a necessary intermediate in the resolvase-mediated knotting reaction.

To investigate the role of the 'AT' central dinucleotide of site I in DNA cleavage and strand exchange, resolution substrates were constructed with base pairs added or deleted at the centre of site I. It was found that in substrates with an additional base pair at the centre of site I, the resolvase-induced cleavages are not fixed at 2 bp apart (although this is preferred) but can be 3 bp apart. Furthermore, cleavage is not specifically at the AT sequence; rather, the top strand cleavage position seems to be fixed relative to the right end of site I, perhaps due to an interaction between the subunits at the right end of site I and those bound at sites II and III of *res*.

Chapter 1

Introduction

1.1 Site-specific recombination

Site-specific recombination of DNA has been shown to play a major role in many important biological processes in both prokaryotes and eukaryotes. Some examples are the resolution of co-integrate intermediates in DNA transposition, the regulation and switching of alternate gene expression, the amplification and monomerisation of plasmids, and the integration and excision of bacteriophage DNA into and out of host chromosomes (Cox, 1989; Landy, 1989; Stark *et al.*, 1989a; Nash, 1990; Johnson, 1991). The reactions mediate the conservative joining or rearranging of DNA sequences in a precise manner and are distinguished from homologous recombination reactions in that extensive homology within the region of strand exchange is not required.

Each site-specific recombination system encodes a specialised recombinase enzyme that binds to a defined recombination site, cleaves the DNA, exchanges the strands, and ligates the ends in the recombinant configuration. There is no synthesis or degradation of DNA, and no high-energy co-factors are required. Some of the recombination systems have been studied in great detail biochemically, topologically, and genetically, and have become model systems for structural and functional analysis of protein-DNA interactions (Kanaar and Cozzarelli, 1992; Yang and Steitz, 1995).

In prokaryotic recombination systems, the substrates are usually circular plasmid DNA's. The products of the recombination reaction depend upon the orientation of the recombining sites (either in direct or inverted repeat) and also upon whether the sites are on the same DNA molecule (intramolecular recombination) or on different DNA molecules (intermolecular recombination). A single DNA plasmid can be resolved into two circular molecules (for example, in co-integrate resolution); two circular molecules can be fused into one (for example, in phage DNA integration into host chromosome); or the orientation of a region of DNA can be inverted with respect to the rest of the plasmid (for example, to switch gene expression) (Figure 1.1).

Two families of site-specific recombination systems can be identified, based upon sequence similarities in the recombination enzymes and the recombination sites, as well as the similarity in the reaction mechanisms (Stark *et al.*, 1992; Argos *et al.*, 1986). Resolution of co-integrates by Tn*3* resolvase is an example of a system of the resolvase family and integration of bacteriophage λ into host *E.coli* chromosomes is an example of a system of the integrase family.

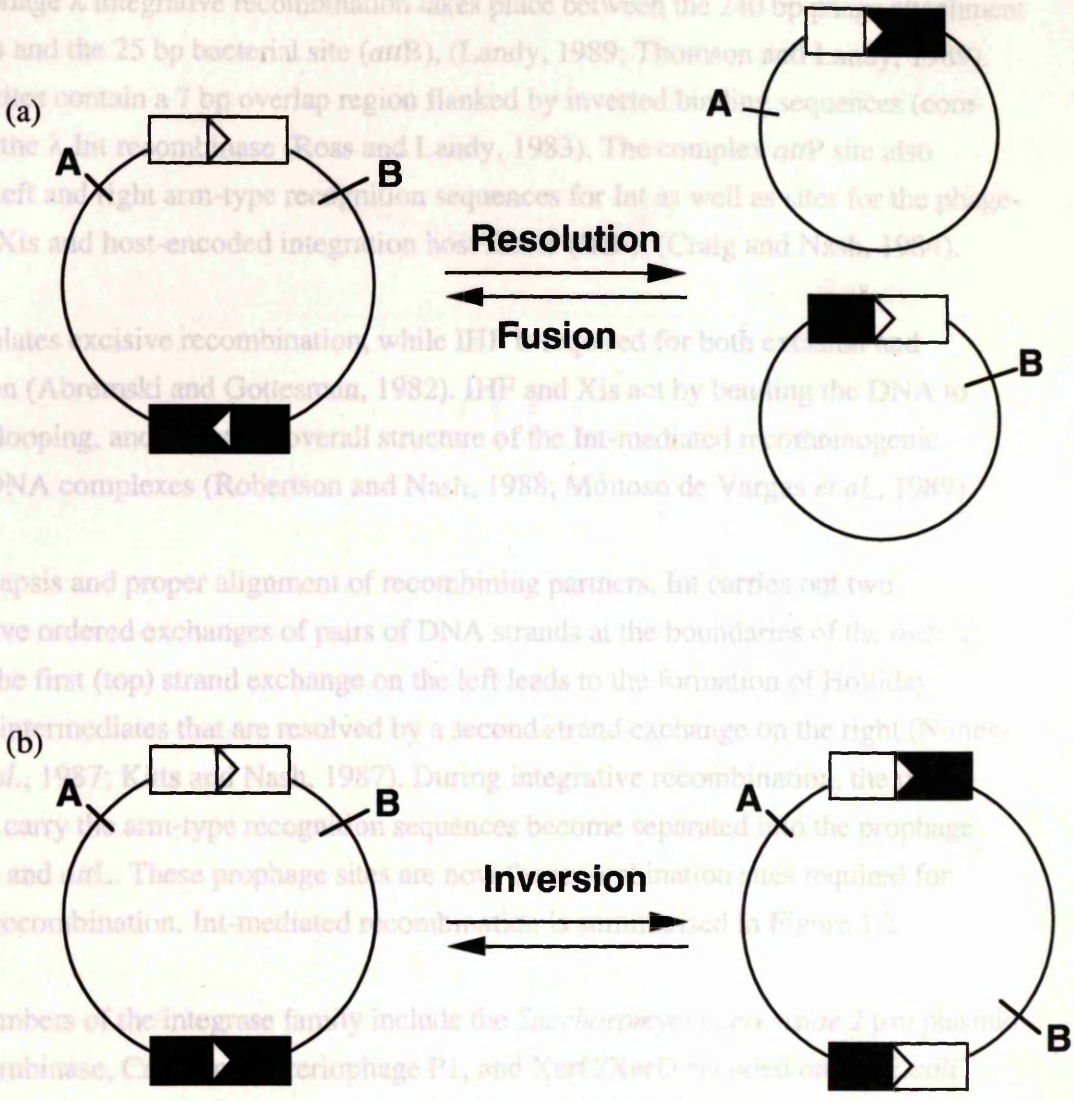


Figure 1.1 The products of resolution and inversion.

(a) Resolution of a circular substrate containing directly repeated sites. The reverse reaction is fusion.
(b) Inversion of a circular substrate containing sites in inverted repeat. Recombination sites are indicated by white or black boxes, with arrow heads to show their orientation. A and B are sequence markers, e.g. restriction endonuclease recognition sites.

1.2 The integrase family of site-specific recombinases

Bacteriophage λ integrative recombination takes place between the 240 bp phage attachment site (*att*P) and the 25 bp bacterial site (*att*B), (Landy, 1989; Thomson and Landy, 1989). Both *att* sites contain a 7 bp overlap region flanked by inverted binding sequences (core-sites) for the λ Int recombinase (Ross and Landy, 1983). The complex *att*P site also contains left and right arm-type recognition sequences for Int as well as sites for the phage-encoded Xis and host-encoded integration host factor (IHF), (Craig and Nash, 1984).

Xis stimulates excisive recombination, while IHF is required for both excision and integration (Abremski and Gottesman, 1982). IHF and Xis act by bending the DNA to facilitate looping, and affect the overall structure of the Int-mediated recombinogenic protein-DNA complexes (Robertson and Nash, 1988; Moitoso de Vargas *et al.*, 1989).

After synapsis and proper alignment of recombining partners, Int carries out two consecutive ordered exchanges of pairs of DNA strands at the boundaries of the overlap region. The first (top) strand exchange on the left leads to the formation of Holliday structure intermediates that are resolved by a second strand exchange on the right (Nunes-Düby *et al.*, 1987; Kitts and Nash, 1987). During integrative recombination, the phage arms that carry the arm-type recognition sequences become separated into the prophage sites *att*R and *att*L. These prophage sites are now the recombination sites required for excisive recombination. Int-mediated recombination is summarised in Figure 1.2.

Other members of the integrase family include the *Saccharomyces cerevisiae* 2 μm plasmid FLP recombinase, Cre from bacteriophage P1, and XerC/XerD encoded on the *E.coli* chromosome. FLP acts at FRT sites to invert a region of DNA and switch between bi-directional replication and 'rolling circle' replication. This results in formation of multiple copies of the plasmid from a single initiation event (Cox, 1989). The bacteriophage P1 Cre recombinase acts to circularise the infecting phage DNA, and to resolve multimers of P1 when it is in the plasmid state, by recombining *lox* recombination sites (Hamilton and Abremski, 1984). XerC and XerD act together to monomerise the bacterial chromosome and naturally occurring plasmids (such as ColE1), to ensure efficient segregation at cell division (Sherratt *et al.*, 1993). Many more members of the Int family of recombinases have been identified by amino acid sequence homology, but have not been studied in any detail. The mechanism of recombination by members of the integrase family of proteins is reviewed in Stark *et al.*, 1992.

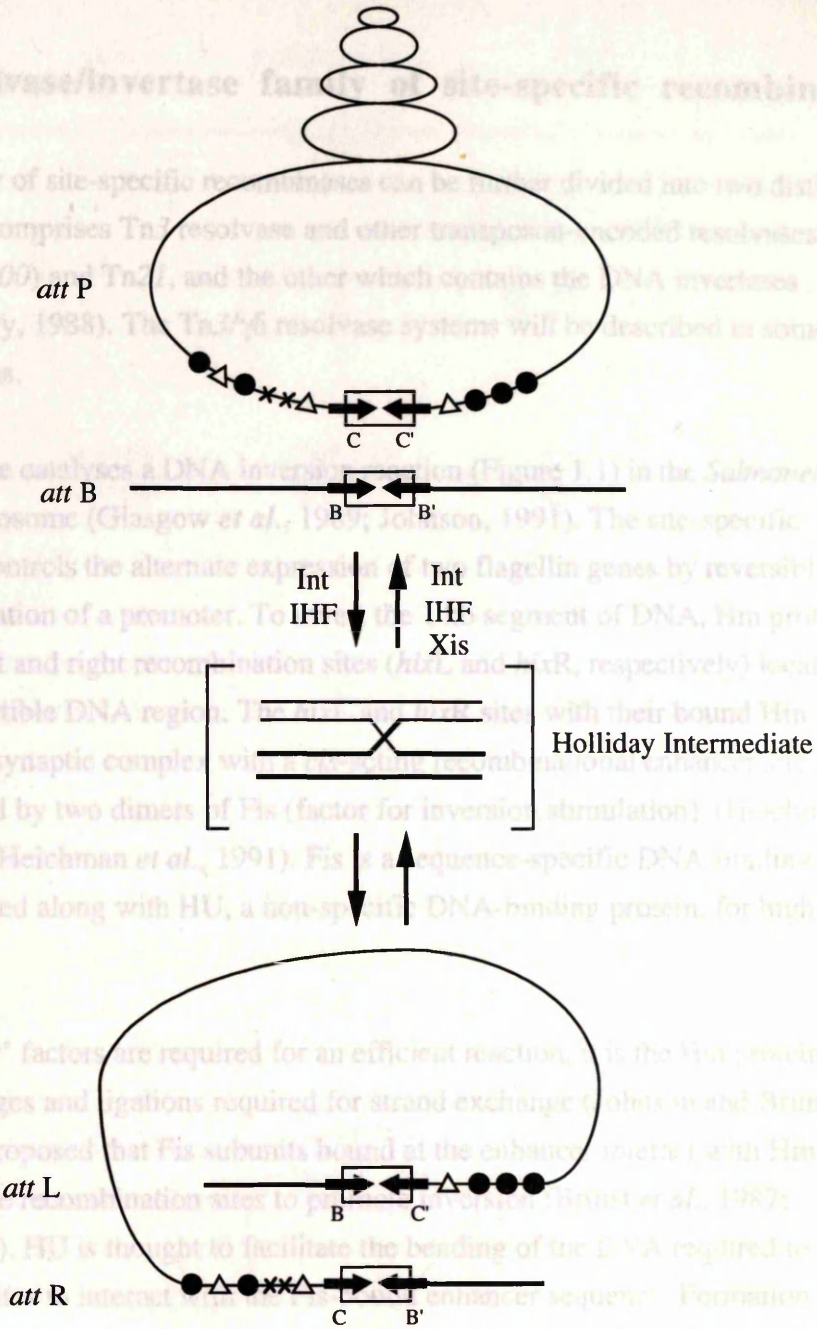


Figure 1.2 The components of integrative and excisive recombination.

Supercoiled *att*P integrates into linear *att*B in the presence of Int and IHF to yield the prophage products *att*L and *att*R; excisive recombination also requires Xis. All four *att* sites have a core region (open rectangles) which is flanked by the inverted core-type Int-binding sites (solid arrows), C, C', B, and B'. The phage *att* sequences on the left and right of core are called P and P' arms, respectively, and carry five arm-type Int-binding sites (filled circles), three IHF-binding sites (open triangles), and two Xis sites (X). Figure adapted from Nunes-Duby *et al.*, 1987.

1.3 The resolvase/invertase family of site-specific recombinases

The resolvase family of site-specific recombinases can be further divided into two distinct groups; one which comprises Tn*3* resolvase and other transposon-encoded resolvases such as those of γδ (Tn*1000*) and Tn*21*, and the other which contains the DNA invertases (Hatfull and Grindley, 1988). The Tn*3*/γδ resolvase systems will be described in some detail in later sections.

The Hin recombinase catalyses a DNA inversion reaction (Figure 1.1) in the *Salmonella typhimurium* chromosome (Glasgow *et al.*, 1989; Johnson, 1991). The site-specific inversion reaction controls the alternate expression of two flagellin genes by reversibly switching the orientation of a promoter. To invert the 1 kb segment of DNA, Hin proteins bind to the 26 bp left and right recombination sites (*hix*L and *hix*R, respectively) located at the ends of the invertible DNA region. The *hix*L and *hix*R sites with their bound Hin protein then form a synaptic complex with a *cis*-acting recombinational enhancer site (*sis*), which itself is bound by two dimers of Fis (factor for inversion stimulation), (Heichman and Johnson, 1990; Heichman *et al.*, 1991). Fis is a sequence-specific DNA-binding protein and is required along with HU, a non-specific DNA-binding protein, for high rates of inversion.

Although 'accessory' factors are required for an efficient reaction, it is the Hin protein that performs the cleavages and ligations required for strand exchange (Johnson and Bruist, 1989). It has been proposed that Fis subunits bound at the enhancer interact with Hin subunits bound at the recombination sites to promote inversion (Bruist *et al.*, 1987; Johnson *et al.*, 1987). HU is thought to facilitate the bending of the DNA required to allow the Hin-bound *hix* sites to interact with the Fis-bound enhancer sequence. Formation of this 'invertasome' complex aligns the two recombination sites correctly and may activate the Hin proteins to initiate the exchange of DNA strands (Figure 1.3).

Hin belongs to a family of bacterial DNA invertases that includes the bacteriophage Mu-encoded Gin and bacteriophage P1-encoded Cin which switch between the expression of alternate forms of a tail fibre protein (thereby increasing host range). In addition to sharing 66 to 80% amino acid sequence identity between pairs of sequences, this family of proteins can substitute functionally for one another in each biological system (Glasgow *et al.*, 1989). Although the invertases only share around 30% amino acid identity with the resolvases and have the additional requirement of an enhancer sequence, the mechanism of strand exchange is believed to be very similar for these two families of proteins (discussed

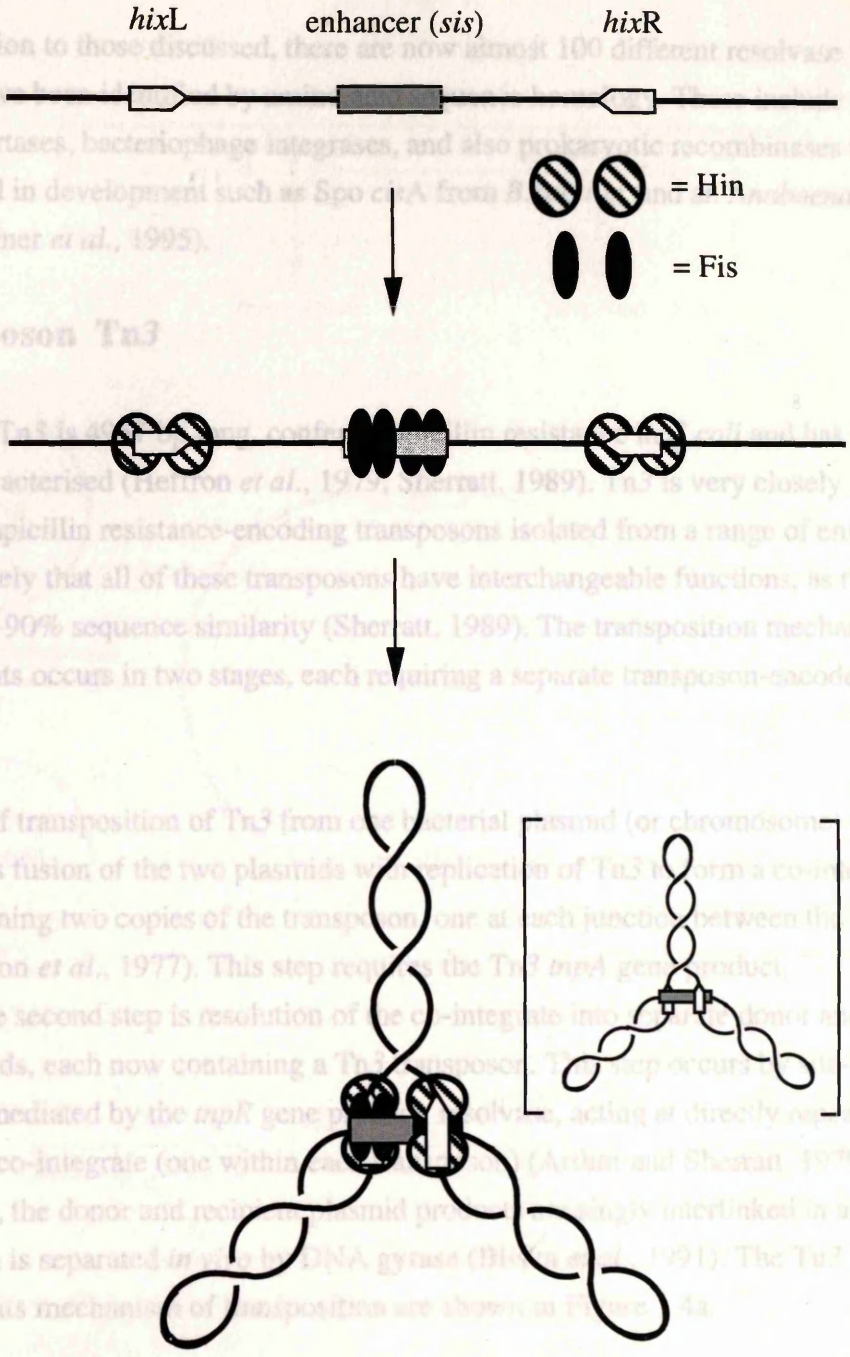


Figure 1.3 The components of the Hin invertase recombination system and possible 'invertasome' structure.

Hin (striped ovals) inverts a region of DNA by binding to the inverted *hix* sites (white boxes). A *cis*-acting enhancer sequence (stippled rectangle) is also required and is bound by two dimers of Fis (black ovals). A proposed structure of the catalytic 'invertasome' complex is shown, although the precise relation of the proteins and DNA sites within the invertasome is not known. Figure adapted from Heichman and Johnson, 1990.

below). In addition to those discussed, there are now almost 100 different resolvase family enzymes that have been identified by amino acid sequence homology. These include resolvases, invertases, bacteriophage integrases, and also prokaryotic recombinases that may be involved in development such as Spo *cis*A from *B.subtilis*, and an *Anabaena* protein (Leschziner *et al.*, 1995).

1.4 Transposon Tn*3*

The transposon Tn*3* is 4957 bp long, confers ampicillin resistance in *E.coli* and has been extensively characterised (Heffron *et al.*, 1979; Sherratt, 1989). Tn*3* is very closely related to a group of ampicillin resistance-encoding transposons isolated from a range of enteric bacteria. It is likely that all of these transposons have interchangeable functions, as they share more than 90% sequence similarity (Sherratt, 1989). The transposition mechanism of Tn*3*-like elements occurs in two stages, each requiring a separate transposon-encoded protein.

The first stage of transposition of Tn*3* from one bacterial plasmid (or chromosome) to another involves fusion of the two plasmids with replication of Tn*3* to form a co-integrate molecule containing two copies of the transposon, one at each junction between the two plasmids (Heffron *et al.*, 1977). This step requires the Tn*3* *tnpA* gene product, transposase. The second step is resolution of the co-integrate into separate donor and recipient plasmids, each now containing a Tn*3* transposon. This step occurs by site-specific recombination mediated by the *tnpR* gene product, resolvase, acting at directly repeated *res* sites within the co-integrate (one within each transposon) (Arthur and Sherratt, 1979). After resolution, the donor and recipient plasmid products are singly interlinked in a DNA catenane, which is separated *in vivo* by DNA gyrase (Bliska *et al.*, 1991). The Tn*3* transposon and its mechanism of transposition are shown in Figure 1.4a.

The *tnpR* gene product (resolvase), as well as being a site-specific recombinase, has also been shown to be a repressor protein that down-regulates the expression of both *tnpA* and *tnpR* genes (Gill *et al.*, 1979). It was shown that the regulatory and resolution roles of resolvase both resulted from the protein binding to a specific sequence (*res*) within the intercistronic region between *tnpA* and *tnpR* (Reed, 1981; Kitts *et al.*, 1983).

The amino acid sequences of the resolvases from many transposons have been determined, and can be used to classify the proteins into several groups, based on their similarity (Hatfull and Grindley, 1988). The Tn*3* and γδ proteins have about 80% amino acid identity

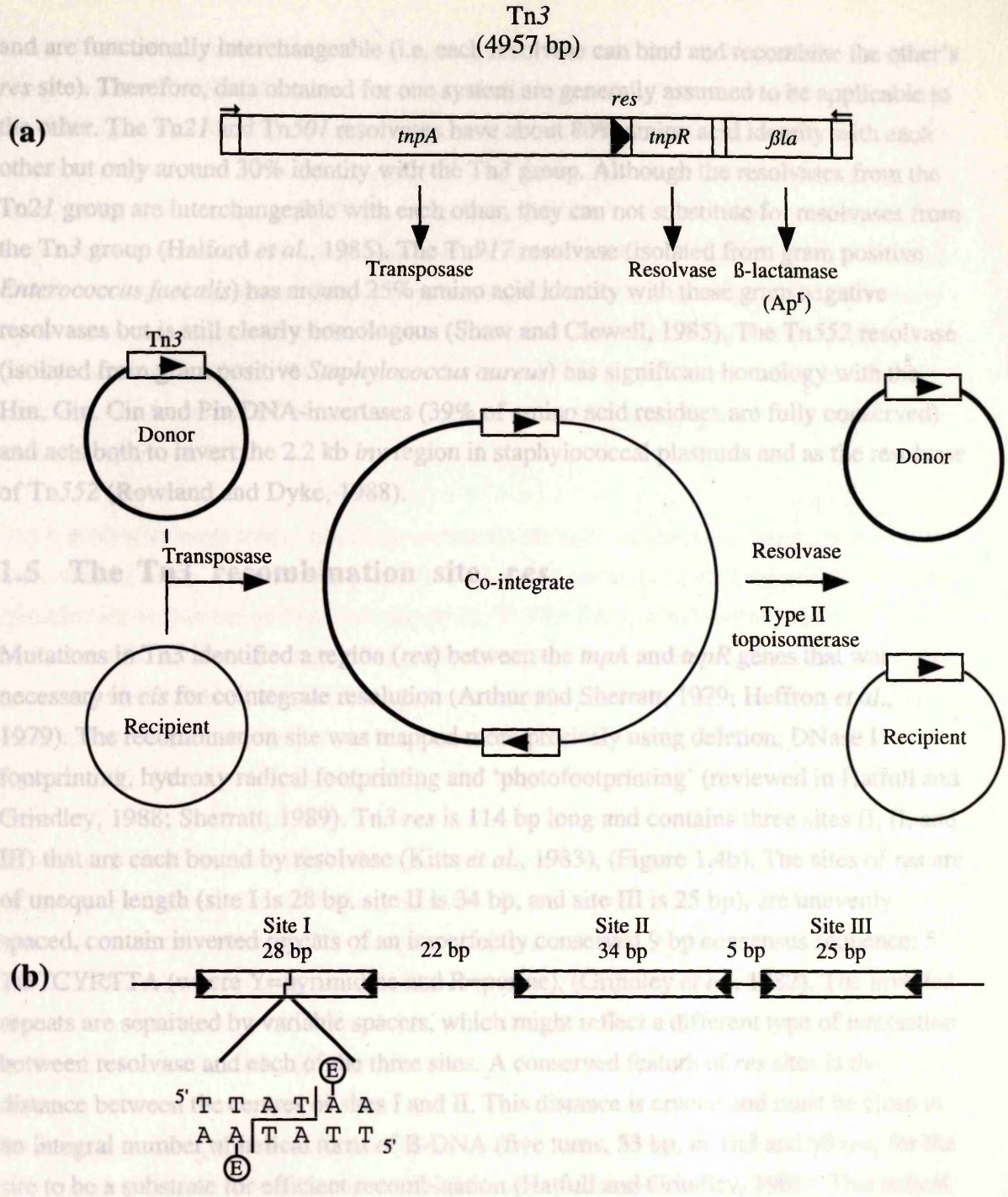


Figure 1.4 Transposon Tn*3* and *res*.

(a) Structure of Tn*3*. Terminal inverted repeats (38 bp each) are denoted by small arrows. Genes and corresponding gene products are indicated. Replicative Transposition of Tn*3*. The relative orientation of the Tn*3* elements is indicated by an arrowhead. The transposition step also requires DNA polymerase for replication of Tn*3*, and the resolution step requires DNA gyrase to decatenate the products of co-integrate resolution.
(b) Structure of Tn*3* *res*. Sites I, II and III are indicated by boxes and the position of DNA cleavage by resolvase is also shown. The imperfect inverted repeat sequences at the outer arms of each site are represented by arrowheads. The positions at which resolvase becomes covalently linked to the DNA are indicated.

and are functionally interchangeable (i.e. each resolvase can bind and recombine the other's *res* site). Therefore, data obtained for one system are generally assumed to be applicable to the other. The Tn*21* and Tn*501* resolvases have about 80% amino acid identity with each other but only around 30% identity with the Tn*3* group. Although the resolvases from the Tn*21* group are interchangeable with each other, they can not substitute for resolvases from the Tn*3* group (Halford *et al.*, 1985). The Tn*917* resolvase (isolated from gram positive *Enterococcus faecalis*) has around 25% amino acid identity with these gram negative resolvases but is still clearly homologous (Shaw and Clewell, 1985). The Tn*552* resolvase (isolated from gram positive *Staphylococcus aureus*) has significant homology with the Hin, Gin, Cin and Pin DNA-invertases (39% of amino acid residues are fully conserved) and acts both to invert the 2.2 kb *inv* region in staphylococcal plasmids and as the resolvase of Tn*552* (Rowland and Dyke, 1988).

1.5 The Tn*3* recombination site, *res*

Mutations in Tn*3* identified a region (*res*) between the *tnpA* and *tnpR* genes that was necessary in *cis* for cointegrate resolution (Arthur and Sherratt, 1979; Heffron *et al.*, 1979). The recombination site was mapped more precisely using deletion, DNase I footprinting, hydroxy radical footprinting and 'photofootprinting' (reviewed in Hatfull and Grindley, 1988; Sherratt, 1989). Tn*3* *res* is 114 bp long and contains three sites (I, II, and III) that are each bound by resolvase (Kitts *et al.*, 1983), (Figure 1.4b). The sites of *res* are of unequal length (site I is 28 bp, site II is 34 bp, and site III is 25 bp), are unevenly spaced, contain inverted repeats of an imperfectly conserved 9 bp consensus sequence: 5' TGTCYRTTA (where Y=pyrimidine and R=purine), (Grindley *et al.*, 1982). The inverted repeats are separated by variable spacers, which might reflect a different type of interaction between resolvase and each of the three sites. A conserved feature of *res* sites is the distance between the centres of sites I and II. This distance is crucial and must be close to an integral number of helical turns of B-DNA (five turns, 53 bp, in Tn*3* and γδ *res*) for the site to be a substrate for efficient recombination (Hatfull and Grindley, 1988). This helical phase effect suggests interactions between the resolvase-bound sites of *res*.

The Tn*3* and γδ resolvases bind to sites I, II and III within *res* to form discrete complexes on non-denaturing PAGE, but while the Tn*3* system forms six complexes, the γδ system forms only three (Hatfull and Grindley, 1986; Bednarz *et al.*, 1990). Recent evidence suggests that Tn*3* resolvase binds to site II of *res* as a monomer to form on-site dimers, whereas γδ resolvase binds to site II mainly as a dimer (Blake *et al.*, 1995). Each site is bound specifically at the outer end of the consensus sequence, in the major groove, by the

C-terminal helix-turn-helix DNA-binding motif of resolvase (Abdel-Meguid *et al.*, 1984; Yang and Steitz, 1995). Further contacts are made by the 'arm' (connecting the C-terminal DNA-binding and N-terminal catalytic domains of resolvase) in the adjacent minor groove closer to the centre of site I (see Section 1.7). In the co-crystal structure of γδ resolvase bound to an artificial site I, the DNA at the centre of the site is bent by 60° towards the major groove and away from the resolvase N-terminal catalytic domains (Yang and Steitz, 1995). Binding of resolvase also induces bends near the centre of sites II and III, as seen by the altered mobility of protein-DNA complexes on non-denaturing PAGE (Hatfull *et al.*, 1987; Blake *et al.*, 1995). The resolvase-mediated bending of the sites within *res* may be important for formation of the nucleoprotein complex (the 'synaptosome') required for the recombination reaction.

The region within *res* where resolvase-induced cleavage and strand exchange occurs is at the centre of site I. The cleavage points were mapped precisely to the central AT dinucleotide within the palindromic sequence: 5' TTATAA, which is cut to leave 2 bp, 3' extensions (Reed and Grindley, 1981), (Figure 1.4b). This AT central dinucleotide of site I, around which double-strand cleavages are made, is well conserved in virtually all *res* sites, and its sequence is important for the recombination reaction (Falvey *et al.*, 1988; see Section 1.19, Chapter 4 and Chapter 5).

Sites II and III of *res* are often called 'accessory sites' and are thought to be involved in bringing *res* sites together (synapsis) with the correct topology for productive recombination (see below). The presence of sites II and III is required for resolvase-induced cleavage to occur at site I (Bednarz *et al.*, 1990; Grindley *et al.*, 1982).

1.6 The recombinase, resolvase

Tn*3* resolvase is a protein of only 185 amino acids (~20.5 kDa), and can act as a transcriptional repressor of the *tnpA* and *tnpR* genes, a site-specific recombinase, or a type I topoisomerase, by binding at multiple sites within *res* and forming highly ordered nucleoprotein complexes. γδ and Tn*3* resolvase are cleaved by chymotrypsin to produce two proteolytic fragments, each containing a functionally distinct domain (Abdel-Meguid *et al.*, 1984; D.Blake and M.Boocock, unpublished results).

The 45-amino acid C-terminal fragment contains a helix-turn-helix DNA major groove-binding motif and binds specifically to the consensus sequences present in the inverted repeats at the outer ends of each site of *res* (Rimphanitchayakit *et al.*, 1989; Yang and

Steitz, 1995), (see above). The arrangement of the helices is very similar to that seen in crystal structures of the Hin invertase DNA-binding domain (Feng *et al.*, 1994) and an *engrailed* homeodomain (Kissinger *et al.*, 1990).

The 140-amino acid N-terminal domain is thought to be responsible for protein-protein interactions which allow multimerisation of resolvase, as well as containing the residues responsible for catalysing strand exchange (Abdel-Meguid *et al.*, 1984; Hughes *et al.*, 1990). Using highly modified conditions (i.e. no Mg^{2+}, +glycerol and using extended incubation times) for the *in vitro* resolution reaction, a cleaved, linear intermediate accumulates in which resolvase is covalently linked to the recessed 5' DNA end at the crossover point via a phosphoserine bond (Reed and Grindley, 1981; Reed and Moser, 1984). Of the 15 serine residues in resolvase, it is the serine at position 10 (Ser-10) that is responsible for the cleavage of DNA during recombination (Reed and Moser, 1984). This serine is conserved throughout the resolvase family of recombinases, and mutations of it in γδ resolvase and Gin invertase result in a loss of catalytic activity (Hatfull and Grindley, 1988; Klippel *et al.*, 1988). This is in contrast to the λ integrase family of site-specific recombinases and many topoisomerases, which cleave DNA and form a phosphotyrosine linkage (Chen *et al.*, 1992). Resolvase does contain an absolutely conserved tyrosine at position 6, which may play an important role in the active site since replacement of this tyrosine (with phenylalanine) also results in a loss of recombination activity, although some topoisomerase activity is seen (Leschziner *et al.*, 1995; P.Arnold and M.Stark, personal communication).

Mutations in the highly conserved residues Gln-14, Asp-36, Gly-40 and Asp-67 also reduce rates of recombination while maintaining topoisomerase activity (Hughes *et al.*, 1990; Leschziner *et al.*, 1995; Boocock *et al.*, 1995). These residues are either important for formation of the active site or are directly involved in catalysis.

A crystal structure of the N-terminal domain of γδ resolvase has been determined at 2.7 Å (Sanderson *et al.*, 1990) and refined at 2.3 Å resolution (Rice and Steitz, 1994b). The C-terminal domain was not seen in the crystal structure of intact resolvase, presumably because it is disordered in the crystals. The first 120 amino acids form a central, five-stranded, β-pleated sheet surrounded by five α-helices (Figure 1.5). The Ser-10 that becomes covalently linked to DNA is located on a loop between β-strand 1 and α-helix A. It is unusual that this residue is not buried in an enzyme active site pocket but instead is exposed on a protruding part of the protein structure. Of the four dyad-related dimers present in the crystal, only one (the so-called 1,2 dimer) was formed by all monomers. The

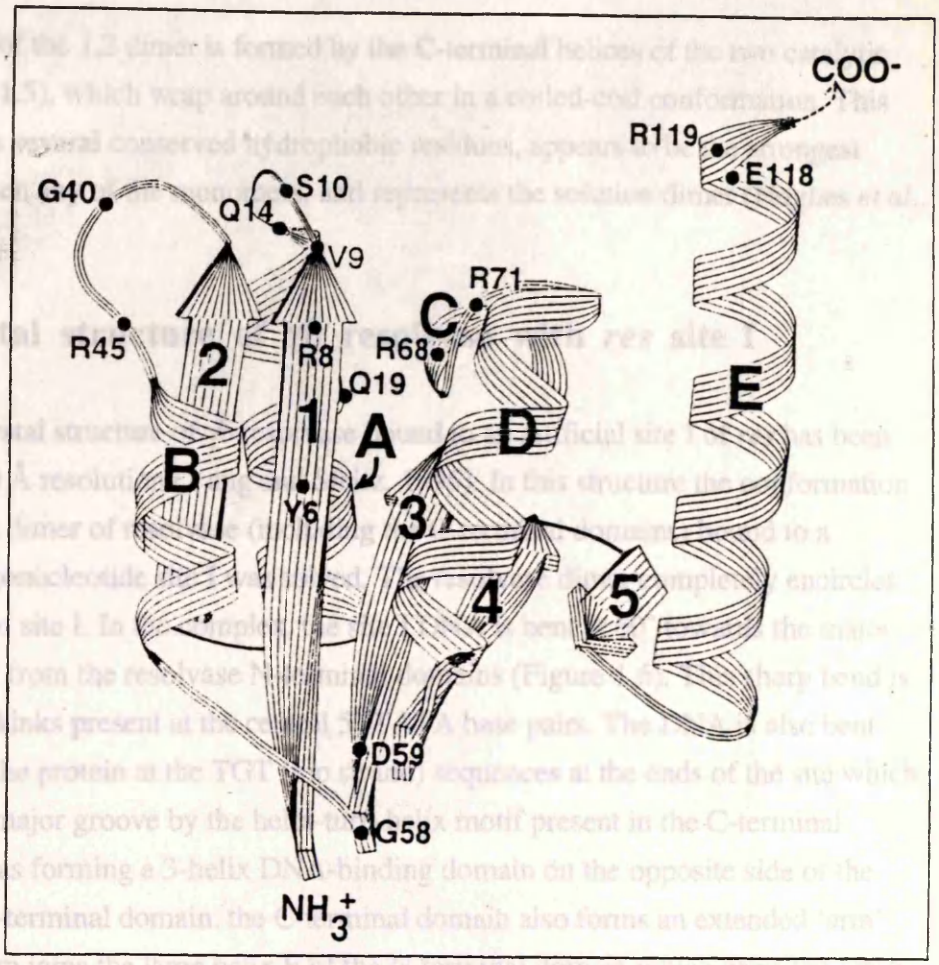


Figure 1.5 α-Carbon ribbon drawing of a resolvase monomer.

Simple representation of the catalytic domain of γδ resolvase refined at 2.3 Å resolution (Rice and Steitz, 1994b). The α-carbon positions of those residues that are absolutely conserved among all recombinases of the Tn*3* type are labelled and marked with circles. The β-strands are numbered 1-5 and the helices A-E. The Ser-10 that becomes covalently attached to the DNA during recombination is located in the loop between β-strand 1 and α-helix A. The C-terminal DNA-binding domain is not resolved. Figure taken from Rice and Steitz,1994b.

subunit interface of the 1,2 dimer is formed by the C-terminal helices of the two catalytic domains (Figure 1.5), which wrap around each other in a coiled-coil conformation. This interface contains several conserved hydrophobic residues, appears to be the strongest interaction between any of the monomers, and represents the solution dimer (Hughes *et al.*, 1990, 1993).

1.7 Co-crystal structure of γδ resolvase with *res* site I

Recently a co-crystal structure of γδ resolvase bound to an artificial site I of *res* has been determined at 3.0 Å resolution (Yang and Steitz, 1995). In this structure the conformation of a complete 1,2 dimer of resolvase (including the C-terminal domains) bound to a symmetrical, oligonucleotide site I was solved. The resolvase dimer completely encircles the entire 28 bp of site I. In the complex, the site I DNA is bent at 60° towards the major groove and away from the resolvase N-terminal domains (Figure 1.6). This sharp bend is achieved by two kinks present at the central 5' TATA base pairs. The DNA is also bent slightly towards the protein at the TGT (top strand) sequences at the ends of the site which are bound in the major groove by the helix-turn-helix motif present in the C-terminal domain. As well as forming a 3-helix DNA-binding domain on the opposite side of the DNA from the N-terminal domain, the C-terminal domain also forms an extended 'arm' structure. This arm joins the large helix E of the N-terminal domain which grips the DNA in the minor groove near the centre of the site (Figure 1.6). Interestingly, helix E of one monomer is straight while helix E' of the other monomer is kinked at 26° into the minor groove, showing some asymmetry of the resolvase dimer which may reflect some conformational flexibility. This structural flexibility may help resolvase bind to the variably sized sites of *res*.

In the crystal structures of γδ resolvase either on its own or bound to site I DNA, the two active site Ser-10 residues in the catalytic 1,2 dimer are more than 30 Å apart, whereas the two scissile bonds at the central dinucleotide are only 13 Å apart. It is difficult to imagine how the resolvase dimer might make concerted double strand cleavages at the centre of the site, but this is what is observed experimentally (Reed and Grindley, 1981; Chapter 4). Either the two active site Ser-10 residues are brought closer to the centre of site I by a conformational change at the catalytic dimer interface, or sequential single-strand cleavages are made (Yang and Steitz, 1995), (see Section 1.15 on strand exchange).

Figure 1.6 The structure of the γδ resolvase-site I DNA complex.

Representation of a complete dimer of γδ resolvase complexed with a 34 bp site I at 3 Å resolution (Yang and Steitz, 1995). The resolvase dimer is shown in ribbon form with one monomer in green and the other in gold. The Ser-10 residues have yellow carbon atoms and red hydroxyl groups. A space-filling model of the site I DNA has the bases in dark blue and the backbone in light blue. The DNA scissile phosphates are highlighted in magenta. Figure taken from Yang and Steitz, 1995.

1.8 The *in vitro* resolution reaction

Detailed biochemical analysis of the Tn*3*/γδ resolvase system followed the establishment of *in vitro* resolution conditions. The only requirements for efficient *in vitro* site-specific recombination are resolvase, negatively supercoiled plasmid DNA containing two *res* sites in direct repeat orientation, and a simple buffer (Reed, 1981). No further host-encoded products, additional DNA sequences or 'high energy' cofactors (e.g. ATP) are required.

The major product of the *in vitro* resolution reaction mediated by Tn*3* resolvase is a singly interlinked catenane with two negative nodes (see Figure 1.7a). The stereostructure of the catenane was determined by partially denaturing the DNA, coating with RecA protein and analysing the products by electron microscopy (Wasserman and Cozzarelli, 1985). *In vivo* the product of the reaction is also the (-2) catenane, but the circles of the catenane are believed to be separated by the action of DNA gyrase (Bliska *et al.*, 1991).

The *res* sites must be in direct repeat; sites in inverted repeat orientation do not lead to any resolution or inversion (Reed, 1981). Furthermore, for efficient recombination the *res* sites must be on the same DNA molecule (intramolecular recombination), (Krasnow and Cozzarelli, 1983). This is in contrast to λ Int site-specific recombination where the reaction is relatively insensitive to the orientation of the sites (Mizuuchi *et al.*, 1980a).

The Hin-mediated DNA inversion reaction has also been reconstituted *in vitro*, with purified components (Johnson *et al.*, 1986). Inversion requires a supercoiled DNA substrate containing two *hix* recombination sites in inverted orientation, a recombinational enhancer sequence and the Hin, Fis, and HU proteins (see Section 1.3). The product of the reaction is an unknotted circle in which the orientation of the region of DNA between the *hix* sites is inverted relative to the remaining plasmid DNA. *In vivo* analysis has shown that when the *hix* sites are in direct repeat orientation, the intervening DNA segment is deleted (Scott and Simon, 1982). The unique products formed by Hin and the other DNA invertases suggest that synapsis of the recombination sites and DNA inversion is not a random process but exhibits topological selectivity, like *res*/resolvase.

The topology of the resolvase reaction products (i.e the -2 catenane) is independent of the degree of supercoiling of the substrate. This suggests that recombination does not result from simple random collision of the sites, which would trap various amounts of supercoils and produce products with different topologies as is seen in λ Int recombination (Mizuuchi *et al.*, 1980b). The specificity for orientation of sites and invariant topology of reaction

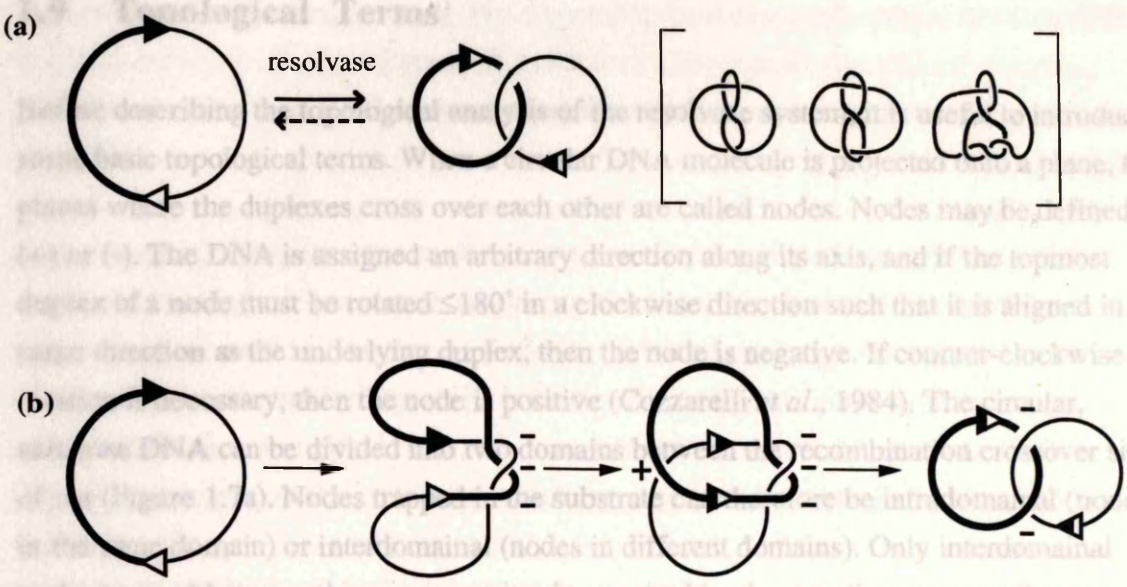


Figure 1.7 Topological features of Tn*3* resolution.

(a) The products of *in vitro* site-specific recombination by Tn*3* resolvase. The two domains of the plasmid DNA substrate are represented by different line thickness. The *res* sites are represented by arrowheads. The major product is a specific (-2) catenane. The products within the square brackets were detected in very small amounts and are the result of multiple strand exchange events (see text for details).
(b) Deduced topology of synapsis and strand exchange. This time, the arrowheads represent site I's (crossover sites). Three negative interdomainal nodes are trapped between the *res* crossover sites at synapsis. Strand exchange has a right-handed sense, generating a positive node. The product of a single strand exchange event is the (-2) catenane. Figure adapted from Stark *et al.*, 1994.

products suggested that synapsis and strand exchange of *res* were also topologically constrained.

1.9 Topological Terms

Before describing the topological analysis of the resolvase system, it is useful to introduce some basic topological terms. When a circular DNA molecule is projected onto a plane, the places where the duplexes cross over each other are called nodes. Nodes may be defined as (+) or (-). The DNA is assigned an arbitrary direction along its axis, and if the topmost duplex of a node must be rotated ≤180° in a clockwise direction such that it is aligned in the same direction as the underlying duplex, then the node is negative. If counter-clockwise rotation is necessary, then the node is positive (Cozzarelli *et al.*, 1984). The circular, substrate DNA can be divided into two domains between the recombination crossover sites of *res* (Figure 1.7a). Nodes trapped in the substrate can therefore be intradomainal (nodes in the same domain) or interdomainal (nodes in different domains). Only interdomainal nodes trapped between the crossover sites in a recombination reaction can contribute to product topology (Benjamin and Cozzarelli, 1986).

1.10 Topology of the resolution reaction

Although virtually all of the products of the *in vitro* recombination reaction catalysed by Tn*3* resolvase are in the form of a (-2) catenane, in some conditions several minor products (1-3% of total product) are also detected (Wasserman and Cozzarelli, 1985). The minor products characterised are a four-noded knot, a five-noded figure-eight catenane, and a six-noded knot (Figure 1.7a), (Krasnow *et al.*, 1983; Wasserman *et al.*, 1985). These products are also topologically unique, so that for example, only one of many possible forms of six-noded knot is observed. The complex knots and catenanes are assumed to occur by rare repetition of the recombination mechanism in which additional rounds of strand exchange occur, before release of the product molecules.

It was proposed that all product forms could be accounted for only if three negative interdomainal DNA nodes were trapped between the recombining *res* sites. If the cleaved DNA strands are then exchanged by rotating the ends 180° in a right-handed sense (looking from the left in Figure 1.7b) introducing a single positive interdomainal node, then a simple (-2) catenane was produced (Figure 1.7b). The minor products were then readily explained by repeating the rotation of the cleaved strands without dissociating the synaptic complex. Thus, the four-noded knot is formed by a 360° rotation of the cleaved DNA ends, the five-

noded catenane by a 540° rotation and the six-noded knot by a 720° rotation. Using the above assumptions, Wasserman and Cozzarelli (1985) predicted that a six-noded knot with only one of eight possible structures should be formed as a product of four rounds of processive strand exchange. Using two-dimensional gel electrophoresis, a six-noded knot was isolated (as 0.1% of total product), coated with RecA and examined by electron microscopy. The six-noded knot was found to have the predicted structure (Wasserman *et al.*, 1985).

These topological experiments verified that processive recombination occurred and that resolvase does indeed introduce a single, positive, interdomainal node during the standard recombination reaction. The driving force for the strand exchange reaction was proposed to be the removal of negative supercoils from the substrate DNA, since negative supercoiling of the substrate was required for an efficient resolution reaction (Stark *et al.*, 1989b). Further topological analysis (of strand exchange) will be discussed in Section 1.18.

It is clear from the topological analysis of the resolution reaction that the interaction of resolvase-bound *res* sites (synapsis) must occur with a specific substrate geometry for a productive recombination reaction to take place. Such topological selectivity must be the product of a specific mechanism of synapsis that brings the *res* sites together in a defined manner to form a productive synapse. Only when the productive synapse is formed, will the strands be exchanged and the recombination products formed. Several models of *res* site synapsis have attempted to explain resolvase topological selectivity, the requirement for *res* sites to be in direct repeat and for the preference for intramolecular recombination.

1.11 'Tracking' and 'Slithering' models of synapsis

An early model called 'tracking' proposed that resolvase bound at one *res* site scans along the adjacent DNA until another *res* site (in the correct orientation) is found (Krasnow and Cozzarelli, 1983). This model was subsequently shown to be inadequate to explain various experimental observations; (i) intermolecular recombination when one *res* site was on a linear molecule and another was on a supercoiled plasmid, (ii) intermolecular recombination between *res* sites on separate circles of a catenane (Boocock *et al.*, 1986, 1987), and (iii) random segregation of 'reporter rings' linked onto the substrate between the two circles of the resolution product (Benjamin *et al.*, 1985).

The slithering model of synapsis suggests that the structure of supercoiled plectonemically wound DNA helps pair the *res* sites using a one-dimensional, conveyer-belt-like motion or

'slithering' of the duplexes (Benjamin and Cozzarelli, 1986). However, slithering has yet to be demonstrated experimentally and several lines of evidence: recombination of non-supercoiled substrates and intermolecular recombination with one *res* site on a linear DNA molecule (Boocock *et al.*, 1986; Brown, 1986) cannot be simply explained using a slithering mechanism for synapsis. Also, the major product of recombination between directly repeated *res* sites on a relaxed, circular substrate is the (-2) catenane, as it is on negatively supercoiled substrates (Boocock *et al.*, 1987; Stark *et al.*, 1994). Thus, negative supercoiling is not necessary for recombination or topological selectivity, but is a requirement for slithering. Tracking and slithering models of *res* site synapsis are discussed further in Stark and Boocock, 1995a.

1.12 Topological filter (two-step synapsis)

The 'two-step synapsis' model proposes that a productive synapse is formed by a 'topological filter' mechanism (Boocock, *et al.*, 1986, 1987; Stark *et al.*, 1989a). Initial synapsis is thought to occur by random collision of the two resolvase-bound *res* sites to give synapsed molecules with different topologies. Strand exchange is thought to occur only within a productive synapse which requires formation of the correct topology i.e. when three negative interdomainal nodes are trapped between the crossover sites (Figure 1.7b). Unproductive synapses do not lead to strand exchange and presumably dissociate, and then the sites may be brought together again with the same or a different topology. A similar model has been proposed for the topological selectivity of the phage Mu transposition system (Craigie and Mizuuchi, 1986).

A possible pathway that would lead to a productive synapse is proposed to occur as follows. Firstly each *res* site binds three dimers of resolvase (one dimer to each site of *res*, as shown in Figure 1.8). There then occurs an antiparallel alignment of site II and III of the two *res* sites; the site II/III interactions might be via a tetrameric interaction of the bound resolvase dimers. Three negative interdomainal nodes are trapped by a right-handed plectonemic wrapping of the antiparallel duplexes around the two hypothetical resolvase tetramers (Figure 1.8); (Stark *et al.*, 1989b). In the second topological step, the two site I's come together in a parallel alignment by interactions of the resolvase dimers bound at each site I. If a productive synapse is formed (with the correct topology) then the catalytic tetramer bound to the site I's can initiate strand exchange.

In support of 'two-step synapsis' it has been shown that the 'accessory' sites II and III of *res* are important for the correct alignment of the two site I's (Bednarz *et al.*, 1990). In this

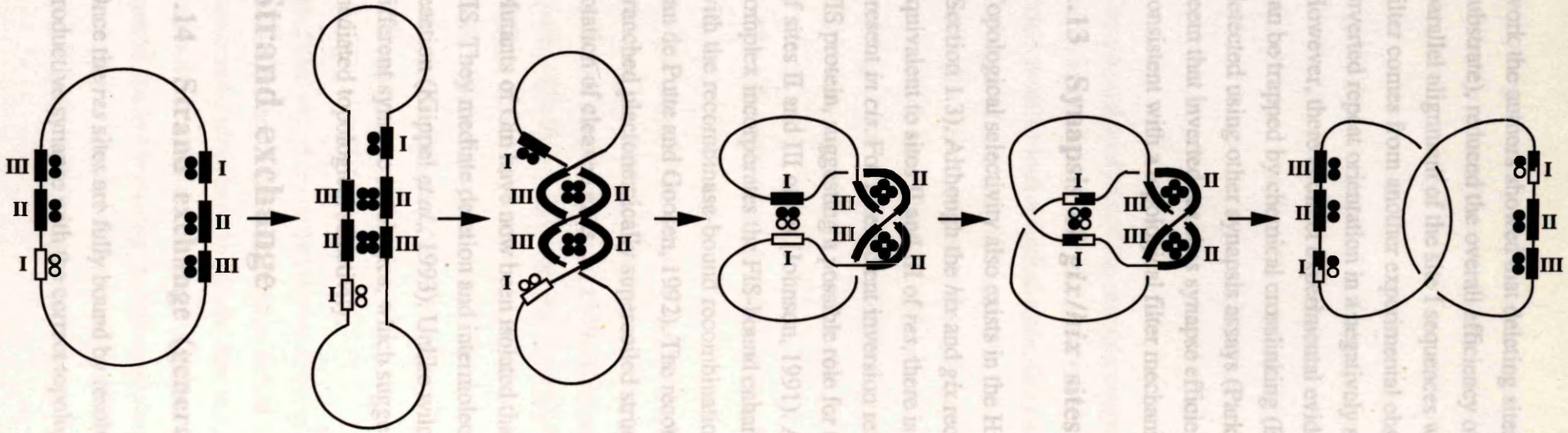


Figure 1.8 Topological filter (two-step synapsis) model for *res*/resolvase topological selectivity.

Resolvase monomers are represented by black or white circles, and the sites of *res* by black or white rectangles. The sites are numbered (I, II, III) as for Figure 1.4b. Strand exchange is depicted as a rotation of resolvase subunits within the catalytic tetramer at the synapsed site I's. See text for further details. Figure adapted from Stark and Boocock, 1995a.

work the authors showed that deleting sites II and III of one *res* site (in a two *res* site substrate), reduced the overall efficiency of recombination, and antiparallel as well as parallel alignment of the site I sequences was observed. Further evidence for a topological filter comes from another experimental observation. It is widely accepted that *res* sites in inverted repeat orientation in a negatively supercoiled plasmid substrate do not recombine. However, there is direct experimental evidence that inverted *res* sites do form synapses that can be trapped by chemical crosslinking (Benjamin and Cozzarelli, 1988; Watson, 1994) or detected using other synapsis assays (Parker and Halford, 1991). On this evidence it would seem that inverted *res* sites synapse efficiently but do not catalyse strand exchange, consistent with a topological filter mechanism.

1.13 Synapsis of *gix/hix* sites

Topological selectivity also exists in the Hin and Gin invertase systems described earlier (Section 1.3). Although the *hix* and *gix* recombination sites do not have accessory sites equivalent to sites II and III of *res*, there is a requirement for an enhancer element to be present *in cis*. For an efficient inversion reaction, the enhancer element must be bound by FIS protein, suggesting a possible role for the enhancer element in selectivity similar to that of sites II and III of *res* (Johnson, 1991). A model proposed for the Hin or Gin synaptic complex incorporates the FIS-bound enhancer site in an 'invertasome' complex together with the recombinase-bound recombination sites as shown in Figure 1.3 (Johnson, 1991; van de Putte and Goosen, 1992). The recombination sites interact with the enhancer in a branched plectonemically supercoiled structure, and strand exchange occurs via a 180° rotation of cleaved ends.

Mutants of Gin have now been isolated that function in the absence of the enhancer and FIS. They mediate deletion and intermolecular fusion as well as the normal inversion reaction (Klippel *et al.*, 1993). Unlike wild-type Gin, mutant Gin can recombine with different synapse topologies, which suggests that there has been a loss of enhancer/FIS-mediated topological selectivity.

Strand exchange

1.14 Strand exchange (general)

Once the *res* sites are fully bound by resolvase and have been brought together to form a productive synapse with the correct topology, the site I DNA duplexes must be cleaved and

then re-ligated in the recombinant configuration. This is the process of strand exchange and will be the main subject of the rest of this introductory chapter and the following results chapters.

Any mechanism of strand exchange proposed for the resolvase/invertase family of proteins must be able to account for the observed topological changes that occur during the recombination reaction. The model must also be able to explain the major and minor products formed during recombination and the X-ray crystallographic data obtained for resolvase bound to *res*.

Topological analysis of the reaction substrates and products (see Section 1.10) showed that strand exchange by resolvase must have a right-handed rotational sense to introduce a single positive interdomainal node. Only a right-handed strand exchange can satisfactorily explain the formation of the specific (-2) catenane as the major product of recombination, as well as the minor product knots and catenanes (by iteration of the strand exchange mechanism). However, this still leaves various possibilities for how the strands are cut and exchanged; for instance the strands might be cut and exchanged sequentially in pairs, or all four strands might be cut concertedly and exchanged at the same time.

1.15 Single- or double-strand cleavage

If single-strand cuts are made in each duplex, the strands may be exchanged to form a Holliday junction which is then resolved by cleavage and exchange of the other pair of strands (Kikuchi and Nash, 1979; Kitts and Nash, 1988). Lambda Int-like recombination systems (Section 1.2) recombine their DNA using sequential single-strand exchanges, and Holliday structures have been shown to be intermediates in the reaction (Kitts and Nash, 1988; Nunes-Düby *et al.*, 1987). However, no Holliday structures have ever been detected in reactions of the resolvase/invertase family, suggesting a different type of strand exchange mechanism.

Alternatively, if concerted double-strand cuts are made at both recombination sites, strand exchange can be accomplished in one step, by simply rotating the ends 180° and religating the strands. Most experiments to date suggest that the resolvases and invertases cleave all four strands at the crossover sites before any religations are made. γδ resolvase reactions carried out *in vitro* using modified conditions (i.e. no Mg^{2+}, +ethylene glycol or glycerol) produce resolvase-*res* complexes in which the DNA contains double-strand cuts at both *res* sites (Reed and Grindley, 1981; Boocock *et al.*, 1995). This work also established that

staggered cuts are made around the central AT dinucleotide of site I and that resolvase becomes covalently attached to the recessed 5' ends. It has also been demonstrated that in the absence of Mg^{2+} and presence of high concentrations of ethylene glycol (or glycerol), Hin invertase generates double-strand breaks within both *hix* recombination sites (Johnson and Bruist, 1989). These cleaved molecules also contain 2 bp staggered cuts at the *hix* central dinucleotide, and Hin becomes covalently attached to the recessed 5' end.

Although a mechanism involving double-strand cleavage at each crossover point has been widely accepted, and double-strand breaks are observed experimentally, some recent crystallographic and biochemical studies have re-opened the issue. In the crystal structure of a γδ resolvase dimer bound to a site I of *res*, the active site Ser-10 residues are too far away (12 Å and 17 Å) from the proximal cleavage positions at the central dinucleotide (Yang and Steitz, 1995). Concerted double-strand cleavage would require a substantial conformational change of the catalytic dimer bound at the crossover site to bring the Ser-10 nucleophiles closer to the scissile phosphodiester bonds. This, together with the surprising asymmetry of the DNA-resolvase dimer complex, led the authors to suggest that cleavage may occur first on one strand and then on the other (see Section 1.7).

Sequential single-strand cleavage may also be supported by experiments on DNA containing mutations at the central dinucleotide in which single-strand nicking was observed (Falvey *et al.*, 1988). Recent studies on heterodimers of wild-type and mutant resolvases showed that some single-strand cleavages are again generated, and that the top strand is cleaved more frequently (Boocock *et al.*, 1995). Thus, the possibility of ordered, single-strand cleavages still exists, but presumably unlike Int-mediated single-strand cleavage, this does not lead to the formation of Holliday junctions. So, although resolvase might make sequential single-strand cleavages, it is likely that all four strands must be cut before any strand exchanges occur.

1.16 *cis* or *trans* cleavage

Four strand cleavages are required for the complete strand exchange reaction, and so all four recombinase protomers bound at the crossover sites are implicated in catalysis. Each subunit may catalyse a nucleophilic attack on a scissile phosphodiester bond at the crossover site and become attached to one cleaved DNA end either by (i) a 3'-phosphotyrosyl bond (Int family recombinases) or (ii) a 5'-phosphoserine bond (resolvase/invertase recombinases), (Pargellis *et al.*, 1988; Evans *et al.*, 1990; Reed and Moser, 1984; Hatfull and Grindley, 1986). Although it has yet to be proved, it is

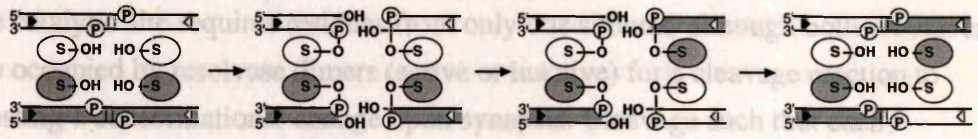
considered likely that four strand cleavages would require four nucleophiles in a 'concerted' mechanism such as that proposed for resolvase (Section 1.17) (Figure 1.9a). This is consistent with the γδ co-crystal structure and the binding stoichiometry of the Tn*3* system which demonstrate that two subunits of resolvase interact at each site I (Yang and Steitz, 1995; Blake, 1993; Blake *et al.*, 1995). Int-family recombinases catalyse sequential single-strand exchanges and would actually only require two nucleophiles, although four subunits are again believed to be involved (Figure 1.9b). In support of this, stoichiometric analysis of the Cre-*lox* system has shown that two subunits of Cre interact with each *lox* recombination site (Mack *et al.*, 1992).

A question that has proved difficult to answer in both systems is whether a recombinase subunit cuts and becomes attached to the half-site to which it is bound (i.e. in *cis*) or to any of the other three half-sites at which it is not bound (i.e. in *trans*), (Figure 1.9c). A recombinase bound at one *res* site and cleaving at the other *res* site could help explain the controlled long-range movement of DNA during strand exchange because the *trans*-covalent attachment may guide the cleaved DNA into the recombinant configuration. *Trans*-cleavage could also explain why the catalytic activities of resolvase require the presence of two *res* sites (Reed and Grindley, 1981; Krasnow and Cozzarelli, 1983).

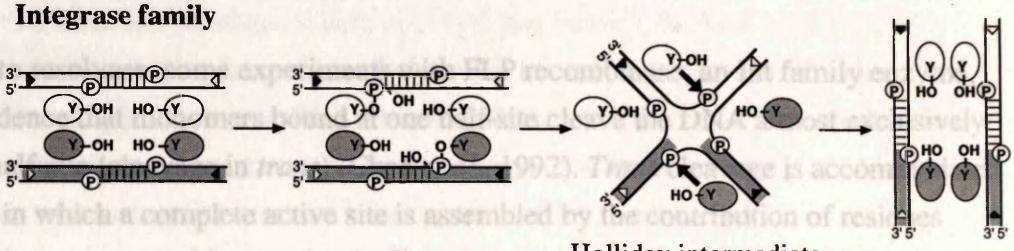
To try to distinguish which *res* site a bound resolvase monomer cleaves, an experiment was carried out where one *res* site was bound by wild-type γδ resolvase and the other site was bound with a mutant that could promote synapsis but not recombination (Dröge *et al.*, 1990). Cleavage of the DNA was then assayed and found to occur mainly (3.5-fold more) at the *res* site bound by the wild-type resolvase. The cleavage seen at the site bound by the mutant is thought to be due to the undesirable exchange of wild-type and mutant resolvase within the synapse, rather than occasional cleavage in *trans*. From this result it was inferred that resolvase subunits cleave the *res* site to which they are bound. However, this experiment does not distinguish which half-site of site I the resolvase cleaves (Figure 1.9c).

Recent experiments utilising γδ resolvase mutants which bind specifically to altered DNA half-sites have addressed this question (Boocock *et al.*, 1995). By using specificity mutants (e.g. R172L resolvase, which binds strongly to half-sites containing TTT instead of TGT at the ends of the site) and catalytic site mutants (e.g. S10C resolvase, which binds but is catalytically inactive) together with modified substrates, it was determined which half-site a specific resolvase subunit cleaves and becomes attached to. It was found that under 'cleavage' conditions (i.e. no Mg^{2+}, +glycerol or ethylene glycol) the Ser-10 nucleophile

a) Resolvase family



b) Integrase family



Holliday intermediate

c)

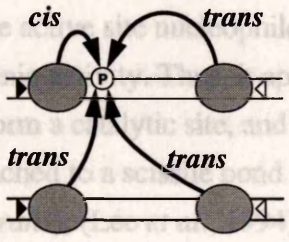


Figure 1.9 Catalysis of recombination by resolvase and integrase family enzymes.

(a) Catalysis of recombination by resolvase family enzymes. The ovals represent recombinase subunits, the circled P's are the phosphates that are the sites of cleavage, the short vertical lines are the 2 bp between the points of cleavage, the arrowheads are the recombinase binding sites, and S-OH is the catalytic Ser residue.
(b) Catalysis of recombination by integrase family enzymes. The symbols are as described in (a). The core sites are aligned in antiparallel to simplify the diagram. Y-OH represents the catalytic Tyr residue. After formation of the Holliday intermediate, a second pair of cleavages and strand exchanges at the phosphates marked by the black arrows gives the recombinant products.
(c) Nomenclature for relationships between subunits and their sites of action. The phosphate (circled P) at the top left of the diagram can be attacked by the nucleophile from the adjacent binding subunit (in *cis*), or by a nucleophile from a subunit bound at any of the other three half-binding sites (in *trans*). Figure adapted from Stark and Boocock, 1995b.

required for strand cleavage is provided by the resolvase subunit bound at the adjacent half-site (i.e. cleavage in *cis*). Furthermore, the catalytic residues Arg-8, Arg-68 and Arg-71 (which are also required for strand cleavage) are also provided by the same adjacent resolvase subunit (Boocock *et al.*, 1995). From these results it appears that formation of the complete catalytic site requires residues from only one subunit, although both crossover sites must be occupied by resolvase dimers (active or inactive) for a cleavage reaction to occur, suggesting a conformational change upon synapsis. Cleavage such that each resolvase subunit becomes covalently attached to its own half-site nicely accommodates a 'subunit rotation' mechanism of strand exchange (discussed in Section 1.17).

In contrast to resolvase, some experiments with FLP recombinase, an Int family enzyme, provide evidence that monomers bound at one half-site cleave the DNA almost exclusively at another half-site (cleavage in *trans*) (Chen *et al.*, 1992). *Trans* cleavage is accommodated by a model in which a complete active site is assembled by the contribution of residues from more than one recombinase subunit. Complementation between mutant FLP proteins suggests that when the 'activating' Arg-His-Arg catalytic triad is present on one bound FLP monomer, the active site nucleophile (Tyr-343) on a second monomer is required for recombinogenic activity. Thus, it appears that residues from more than one subunit are required to form a catalytic site, and that the catalytic Tyr from one half-site cleaves and becomes attached to a scissile bond in the other half-site but within the same full site (i.e. cleavage in *trans*), (Lee *et al.*, 1994).

However, these experiments contradict earlier work on λ Int-catalysed cleavage of one of its recombination sites *att*L (Kim *et al.*, 1990). In this report it was inferred that an Int molecule bound to an arm site of a half-site directly 'bridges' to and cleaves at the core site of the same half-site (i.e. *in cis*). More recent work with cleavage- and activation-deficient mutants of λ Int, targeted to specific half-sites, also strongly indicate that the DNA is cleaved by the Int subunit bound adjacent to the cleavage site (Nunes-Düby *et al.*, 1994). These recent findings for the Int and FLP recombinases are discussed further in Stark and Boocock, 1995b. It will be interesting to see if one of these apparently contradictory results is incorrect or if FLP and Int recombinases do, in fact, utilise different cleavage mechanisms.

1.17 Subunit rotation

A conceptually simple model of resolvase-mediated strand exchange that accounts for most of the experimental data described is called 'subunit rotation' (Sherratt, 1989; Stark *et al.*,

1989a; Stark *et al.*, 1989b). In this model, a catalytic tetramer of resolvase at the synapsed site I sequences promotes a concerted double-strand cleavage of both crossover sites, and each monomer of resolvase becomes covalently attached to the half-site at which it is bound. Now one dimer of the catalytic tetramer rotates (with its attached half-site) through 180° in a right-handed sense to bring about the recombinant configuration (Figure 1.10). The strands are then re-ligated, and the synapse dissociates to release the recombinant catenane.

No direct physical or biochemical evidence exists for subunit rotation, but the model is consistent with all of the topological data obtained (see below). Subunit rotation also explains the minor products formed (the four-noded knot, five-noded catenane and six-noded knot) by simple repetition of the subunit exchange mechanism without re-ligation of the recombinant intermediate. The model also accounts for the introduction of a single, positive interdomainal node introduced during the reaction (Section 1.10).

1.18 Topology of strand exchange

Experimental determination of the topological linkage change, ΔLk (i.e. the change in the number of turns of the double helix) in going from substrate to recombinant product, has proved to be a powerful means of distinguishing between models of strand exchange. Measurement of a ΔLk of +2 during recombination (inversion) of the lambda Int and Cre recombinases provided support for a strand exchange mechanism which proceeds via single-strand cleavages and a Holliday intermediate (Nash and Pollock, 1983; Abremski *et al.*, 1986; Griffith and Nash, 1985).

ΔLk has also been determined for the reaction catalysed by the Gin invertase of bacteriophage Mu (Kahmann *et al.*, 1987; Kanaar *et al.*, 1988). In this work the substrate and product of an inversion reaction were run on an agarose gel alongside a ladder of topoisomers prepared using the substrate DNA. The difference in the linking number between the substrate and product DNA's was measured by comparison with the topoisomer ladder and was +4 for one round of recombination. The second and third rounds of recombination gave products in which ΔLk was +8 and +12 respectively. This result demonstrated that during Gin-mediated recombination, the cleaved DNA ends were not free to rotate and relax the DNA, but that the strands were exchanged in a controlled manner, generating products with precise topologies. The linkage change and specific product formed suggested a model for the Gin synaptic complex in which two negative

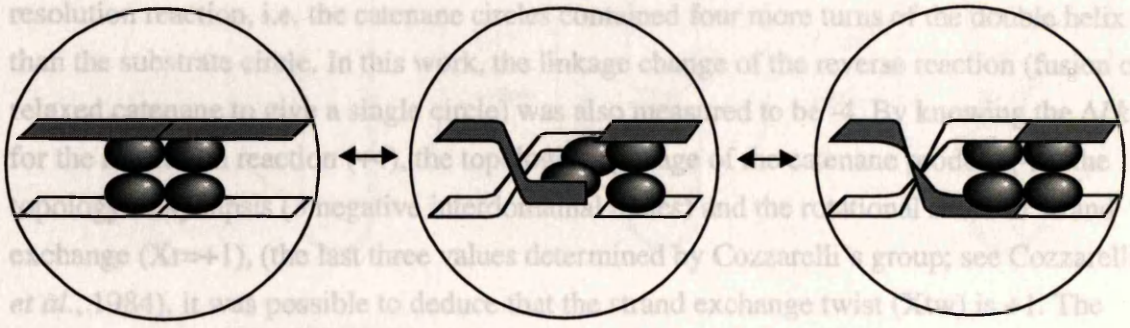


Figure 1.10 The 'subunit rotation' model of strand exchange.

The site I DNA is drawn as a ribbon, and resolvase monomers as shaded ovals. The interwrapped sites II/III are to the right as drawn (and as shown in Figure 1.8). The two catalytic dimers of resolvase at the synapsed site I sequences promote concerted double-strand cleavage of both crossover sites and each monomer of resolvase becomes covalently attached to the half-site at which it is bound. One dimer of the catalytic tetramer then rotates (along with its attached half-site) through 180° in a right-handed sense to bring about the recombinant configuration. The strands are then re-ligated, and the synapse dissociates to release the recombinant catenane. Figure adapted from Stark *et al.*, 1989a.

interdomainal nodes were trapped, and recombination was via a right-handed rotation of the cleaved strands (Kanaar *et al.*, 1988) (Figure 1.11).

The linkage change of the Tn*3* resolution reaction was measured by comparing the circular substrate and the catenane product with reference topoisomers on two-dimensional gels (Boocock *et al.*, 1987; Stark *et al.*, 1989b). ΔLk was measured to be +4 in the forward resolution reaction, i.e. the catenane circles contained four more turns of the double helix than the substrate circle. In this work, the linkage change of the reverse reaction (fusion of relaxed catenane to give a single circle) was also measured to be -4. By knowing the ΔLk for the resolution reaction (+4), the topological linkage of the catenane product (-2), the topology of synapsis (3 negative interdomainal nodes) and the rotational sense of strand exchange (Xr=+1), (the last three values determined by Cozzarelli's group; see Cozzarelli *et al.*, 1984), it was possible to deduce that the strand exchange twist (Xtw) is +1. The strand exchange topology was therefore (Xr=+1, Xtw=+1). The catenane fusion reaction was deduced to be the precise topological reverse of the forward resolution reaction via left-handed 180° strand exchange (Xr=-1, Xtw=-1).

These measured topological changes for the resolution reaction immediately rule out certain models of strand exchange. For example, simply breaking and rejoining the paired sites, or recombination through a Holliday junction intermediate do not account for the deduced changes in helical twist (Xtw), (Stark *et al.*, 1989b). The simplest strand exchange model that is compatible with the determined topological data for resolvase requires a simple 180° rotation of the two half recombination sites around the axis of duplex alignment (Figure 1.12). These measured changes in DNA topology, together with the evidence for concerted four strand cleavage, are entirely consistent with a subunit rotation-like model of strand exchange in which two subunits of resolvase (attached to the cleaved half-sites) bring about the right-handed 180° rotation of ends. Subunit rotation is further supported by the reverse reaction (catenane fusion) which gives the expected product topology if the two resolvase subunits rotate with the opposite, left-handed sense. In a negatively supercoiled plasmid substrate, the reduction in negative supercoiling may help 'drive' the right-handed strand exchange (Stark *et al.*, 1989b).

1.19 The knotting reaction of substrates with altered central dinucleotides

Another approach used to study strand exchange has involved making alterations at the central dinucleotide of site I of *res*. Homology at the central 2 bp of the crossover sites is

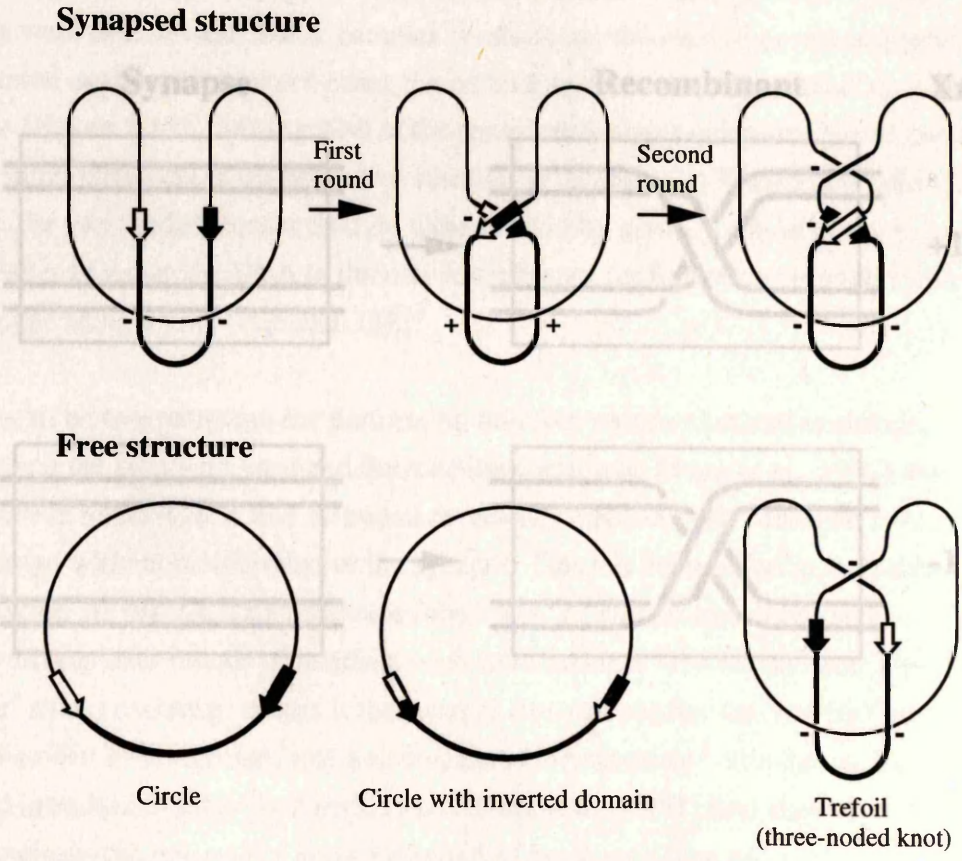


Figure 1.11 Topological changes during two rounds of iterative recombination by Gin.

The arrows represent the *gix* sites, and the thick and thin lines represent the domains between them. The substrate is shown with only the two essential negative interdomainal nodes. The upper row shows the protein-bound synapsed structures and the lower row shows the free unfolded forms. Two processive rounds of recombination are shown in which the protein-DNA complex is not destroyed after the first round. Recombination at the *gix* sites is accompanied by the introduction of a negative interdomainal node. A single crossover inverts the relative orientation of the domains and thereby inverts the sign of the two substrate interdomainal nodes. Two repeated rounds of (iterative) recombination by Gin produce a (-3) trefoil knot. Figure adapted from Kanaar *et al.*, 1988.

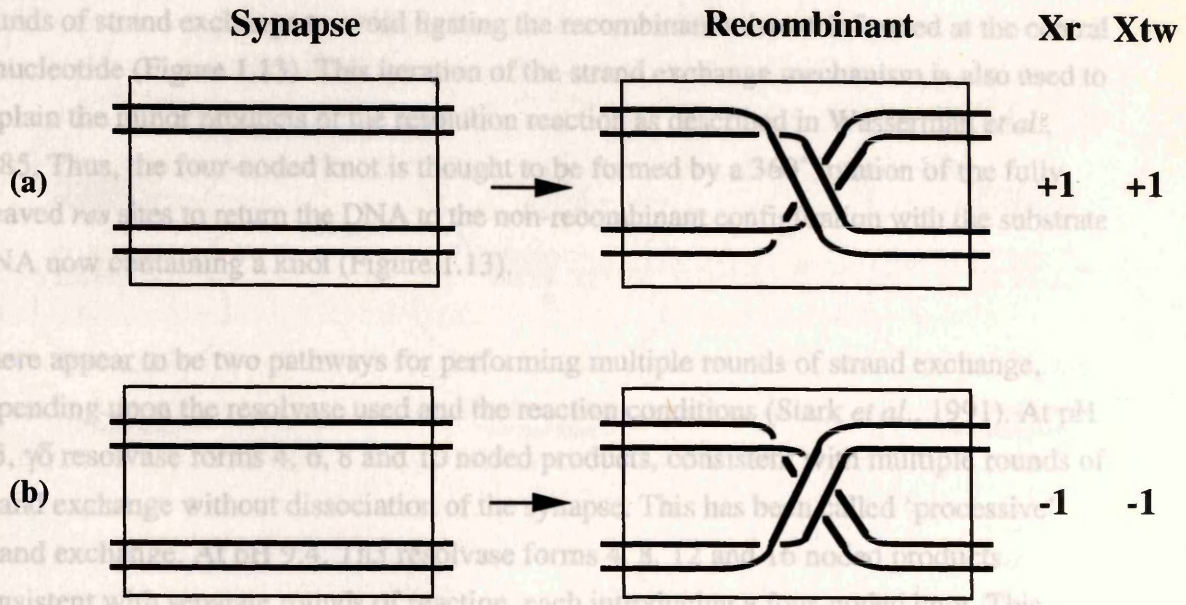


Figure 1.12 The strand exchange topology of Tn*3* resolvase.

The two DNA recombination sites are represented as flat ribbons, held in fixed orientation within a box. The right-hand box shows how the topology differs after the strand exchange reaction has taken place.
(a) Simple right-handed (+180°) rotation of recombining strands.
(b) Simple left-handed (-180°) rotation of recombining strands.
Xr is the strand exchange rotation and Xtw is the strand exchange twist. Figure (a) is consistent with the determined topological data for the resolvase resolution reaction and Figure (b) is consistent with the data for the reverse catenane fusion reaction (see text for details). Figure adapted from Stark *et al.*, 1989b.

important for efficient resolution (Falvey *et al.*, 1988) and mutations of one *res* site to give AC instead of AT (on the top strand) leads to the formation of non-recombinant four-noded knot as the major initial product (Stark *et al.*, 1991). Small amounts of recombinant are also formed, which presumably contain mismatched base-pairs. Six, eight, ten and twelve-noded knots were also formed. These complex products are believed to be due to further rounds of strand exchange to avoid ligating the recombinant mismatch formed at the central dinucleotide (Figure 1.13). This iteration of the strand exchange mechanism is also used to explain the minor products of the resolution reaction as described in Wasserman *et al.*, 1985. Thus, the four-noded knot is thought to be formed by a 360° rotation of the fully cleaved *res* sites to return the DNA to the non-recombinant configuration with the substrate DNA now containing a knot (Figure 1.13).

There appear to be two pathways for performing multiple rounds of strand exchange, depending upon the resolvase used and the reaction conditions (Stark *et al.*, 1991). At pH 7.5, γδ resolvase forms 4, 6, 8 and 10 noded products, consistent with multiple rounds of strand exchange without dissociation of the synapse. This has been called 'processive' strand exchange. At pH 9.4, Tn*3* resolvase forms 4, 8, 12 and 16 noded products consistent with separate rounds of reaction, each introducing a four-noded knot. This 'distributive' strand exchange occurs if the synapse dissociates after the first 360° rotation and the four-noded knot separates into one domain of the substrate while the *res* sites re-synapse and introduce another four-noded knot. Stark *et al.* (1991) also showed that Tn*3* resolvase catalyses the removal of knots from nicked products of the AC x AT substrate to form unknotted circle or four-noded knot. This 'unknotting' reaction is the exact topological reverse of the knotting reaction, since it removes four nodes at a time, and probably proceeds by a left-handed 360° rotation of the *res* half-sites within the synaptic complex.

The highly processive and reversible nature of the strand exchange mechanism is wholly consistent with the subunit rotation model. In fact, the knotting reaction of AC x AT substrates was a prediction of the subunit rotation model of strand exchange (Stark *et al.*, 1991). The lack of homology at the 2 bp overlap between the synapsed site I's is not detected until resolvase is committed to strand exchange, consistent with a model in which the DNA is wrapped around the outside of a catalytic tetramer of resolvase.

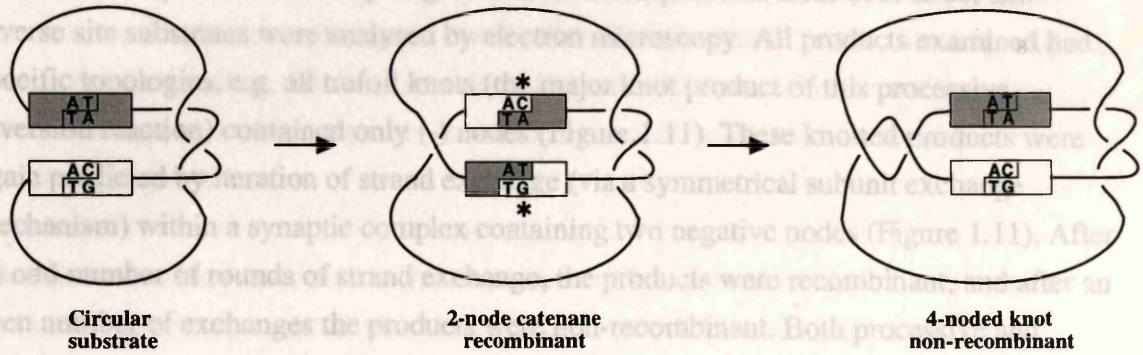


Figure 1.13 Possible reaction of a substrate with an altered central dinucleotide.

Synapsis of the sites traps three interdomainal supercoils. Only the recombining central dinucleotides of site I are shown for clarity. Mismatched basepairs are indicated by an asterisk. The first round of strand exchange generates a catenane containing two mismatched basepairs. A second round of strand exchange restores correct basepairing, and generates a non-recombinant four-noded knot. Figure adapted from Stark *et al.*, 1991.

1.20 Hin/Gin knotting

The mechanism of strand exchange in the Hin and Gin invertase recombination systems has also been studied by analysis of recombinant knots formed by multiple rounds of processive recombination (Kanaar *et al.*, 1990; Heichman *et al.*, 1991). For Gin, manipulation of the reaction conditions caused rapid and efficient processive inversion to create knotted products. The topologies of the knotted products from both direct and inverse site substrates were analysed by electron microscopy. All products examined had specific topologies, e.g. all trefoil knots (the major knot product of this processive inversion reaction) contained only (-) nodes (Figure 1.11). These knotted products were again predicted by iteration of strand exchange (via a symmetrical subunit exchange mechanism) within a synaptic complex containing two negative nodes (Figure 1.11). After an odd number of rounds of strand exchange, the products were recombinant, and after an even number of exchanges the products were non-recombinant. Both processive and distributive recombination products were detected, as with the resolvase system.

Similarly for the Hin invertase, it was found that changing the sequence of the central dinucleotide where the strand exchange occurs, increased the efficiency of the normally rare knotting reaction (Heichman *et al.*, 1991). Again the stereostructure of the knots was analysed by electron microscopy, and a unique pattern of knots was observed. The mechanism proposed to account for the products involves a highly ordered Hin synaptic complex, double-strand cleavages at both recombination sites (covalently attaching Hin to the 5' ends) and finally a 180° rotation of one set of bound subunits. When base-pairing can occur at the central dinucleotide, ligation produces unknotted plasmids containing an inversion. Inability to ligate a mismatched central dinucleotide leads to repetition of the strand exchange mechanism to generate a trefoil knot. Once again, all of the knots examined contained exclusively negative nodes, signifying a right-handed exchange of the DNA strands which is energetically favoured in a negatively supercoiled DNA molecule.

1.21 Topology of the knotting reaction

Although the knotting reaction is well documented for the resolvases and invertases and can be simply explained by a symmetrical subunit rotation mechanism of strand exchange, it remained formally possible that the knots arose by a topoisomerase II activity of resolvase. Under certain conditions resolvase does exhibit a topoisomerase I activity and will relax negatively supercoiled substrates to form a ladder of topoisomers (Castell *et al.*, 1986; Falvey *et al.*, 1988). Rather than knotting substrates via multiple rounds of strand

exchange, resolvase could theoretically form knots by acting as a topoisomerase II on the substrate or the (-2) catenane. However, the subunit rotation model predicts that ΔLk for formation of the four-noded knot should be +4, whereas topoisomerase II activity (or alternative models of strand exchange) would predict different values for ΔLk.

ΔLk for formation of the four-noded knot was determined by comparing circular substrate and knot product with reference topoisomers on two-dimensional agarose gels, and was shown to be +4 (Stark and Boocock, 1994). This result confirms that the local topological change for the knotting reaction is precisely twice that of the resolution reaction. Thus, the linkage change in the knotting reaction is consistent with a 360° right-handed subunit exchange, as predicted.

Clearly the knotting reaction catalysed by resolvase has proved invaluable as a means of analysing the strand exchange mechanism. The kinetics of knot formation and the possibility that knotting involves a ligated recombinant intermediate are examined further in Chapter 4.

1.22 Problems with subunit rotation

Although the subunit rotation model of strand exchange is the simplest explanation available for the products formed by the resolvase/invertase family of site-specific recombinases and elegantly accounts for all of the biochemical and topological data, there is as yet no direct biochemical evidence of such a mechanism, and there are considerable structural difficulties with it. The primary problem is that the two pairs of subunits (within the catalytic tetramer) need to have enough freedom to rotate relative to each other without dissociating completely. The specific topological changes that occur during resolvase/invertase-mediated recombination require that the subunits must maintain some contact with one another. While two flat surfaces may be able to rotate 180° relative to one another and remain in close contact, the interface of the bound resolvase dimer is formed by two extended α-helices (E and E' in Figure 1.6) which are not flat (Yang and Steitz, 1995). It is difficult in structural terms to imagine how this dimer interface could rotate without risking the integrity of the synapse.

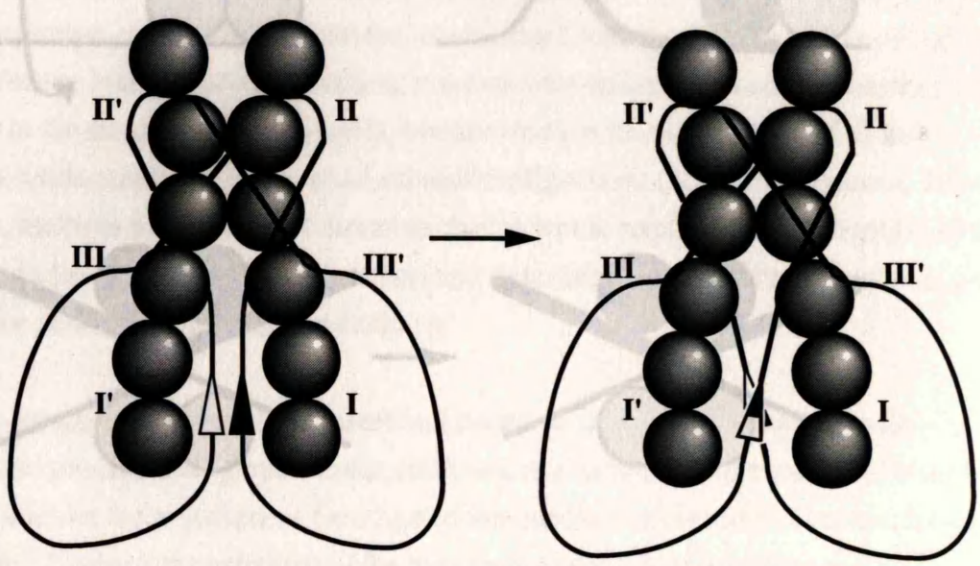
1.23 'DNA-mediated' strand exchange

An alternative model which does not require the rotation of resolvase subunits is the so-called 'DNA-mediated' model of strand exchange (Rice and Steitz, 1994a). This model is

based upon crystallographic studies of the γδ resolvase catalytic subunit together with some of the topological data already discussed. Using the packing arrangement of twelve subunits of γδ resolvase in the unit cell of a hexagonal crystal, Rice and Steitz (1994a) positioned B-form *res* DNA onto the resolvase dimer and tetramer structures seen in the crystal, taking into account *res* footprinting data and the relative positions of Ser-10 and site I. The DNA was positioned around the resolvase tetramers such that three negative interdomainal nodes were trapped between the crossover sites to generate the correct substrate topology (Figure 1.14). The two DNA duplexes are held together in synapsis by interaction between the dimers bound at sites II and III to form tetramers, as in two-step synapsis (Section 1.12). It is also postulated that the dimers bound at the two site I's in the synapse (forming the catalytic tetramer) interact with the resolvase bound at site III, helping to stabilise the whole synaptic structure.

In contrast to subunit rotation, this model places the two recombining duplexes in direct contact with one another (at the points of cleavage/ligation) and proposes that strand exchange occurs via relatively small movements of the cleaved DNA ends. It is suggested that the resolvase catalytic dimers serve only to distort and stabilise the recombining site I's rather than taking part in relatively large rotational movements. Thus, DNA-mediated strand exchange would seem to overcome the problems associated with rotating resolvase subunits relative to each other by proposing a more fixed structural role for the resolvase catalytic tetramer. The resolvase subunits at site I stabilise the DNA in its transition state and cleave the scissile bonds while the actual strand exchange is achieved by the DNA itself. The subunit rotation and DNA-mediated models of strand exchange are compared in Figure 1.15.

While DNA-mediated strand exchange solves the problems associated with exchanging protein subunits, it re-creates the problem of achieving the correct substrate/product topology that the subunit rotation model was originally designed to explain (see Section 1.17). If in DNA-mediated strand exchange the two aligned site I duplexes are simply cut, exchanged and religated, the product formed either contains the wrong helical twist (Xtw≠+1) or contains a node of the wrong sign (Xr=-1), (Stark *et al* ., 1989b). The subunit rotation model predicts the correct topology directly, while the DNA-mediated model can only account for the known topological changes if each site I duplex is distorted. It has been suggested that the correct topology could be achieved by rotating the cleaved, 3' overhanging ends about one another before religation. This rotation could be driven by strain introduced by unwinding the DNA at the centre of each site I by 180° (Rice and Steitz, 1994a). However, the required unwinding at the crossover point of site I predicted



Circular substrate

2-node catenane recombinant

Figure 1.14 Model of 'DNA-mediated' synapsis and strand exchange.

The resolvase monomers are represented as shaded spheres, and the *res* site I's are represented by black or white arrowheads. The arrangement of the twelve resolvase subunits is as seen in a unit cell of a hexagonal crystal of gamma-delta resolvase (Rice and Steitz, 1994a). The substrate DNA is wrapped around the resolvase subunits such that three negative interdomainal nodes are trapped between the crossover sites. In the substrate configuration the site I DNA is bound across a single resolvase dimer while in the product, each recombinant site I is bound across two dimers of resolvase. The approximate positions of sites II and III are also shown. The dimers bound at site I are thought to interact with the protein bound at site III. Figure adapted from Rice and Steitz, 1994a.

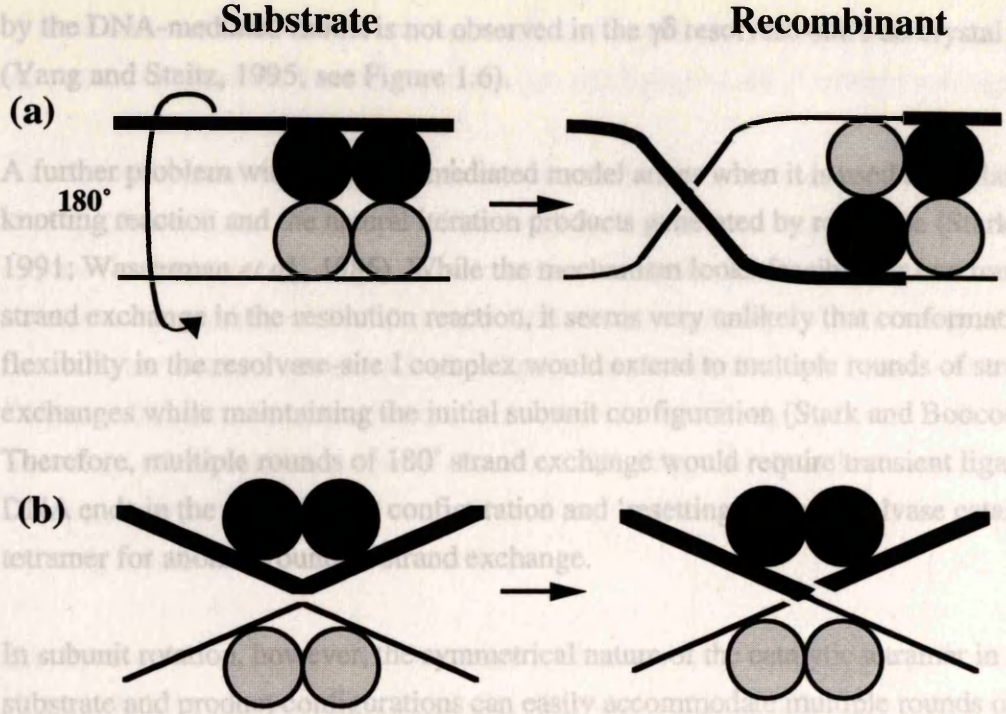


Figure 1.15 The 'subunit rotation' and 'DNA-mediated' models of strand exchange.

Resolvase monomers are represented by stippled spheres and the site I DNA by thick or thin lines.
(a) The subunit rotation model of strand exchange. The site I's are cleaved by a catalytic tetramer of resolvase and the strands are exchanged by rotating one dimer (along with its attached half-sites) relative to the other dimer. The DNA is then religated in the recombinant configuration.
(b) The DNA-mediated model of strand exchange. The site I's are bound by resolvase dimers and are brought together such that the DNA strands are in close proximity. Strand exchange then occurs via movement of the free 3'-hydroxyls, without dissociation of the bound resolvase dimers. Figure adapted from Rice and Steitz, 1994a.

by the DNA-mediated model is not observed in the γδ resolvase-site I co-crystal structure (Yang and Steitz, 1995; see Figure 1.6).

A further problem with the DNA-mediated model arises when it is used to explain the knotting reaction and the natural iteration products generated by resolvase (Stark *et al.*, 1991; Wasserman *et al.*, 1985). While the mechanism looks feasible for one round of strand exchange in the resolution reaction, it seems very unlikely that conformational flexibility in the resolvase-site I complex would extend to multiple rounds of strand exchanges while maintaining the initial subunit configuration (Stark and Boocock, 1994). Therefore, multiple rounds of 180° strand exchange would require transient ligation of the DNA ends in the recombinant configuration and 'resetting' of the resolvase catalytic tetramer for another round of strand exchange.

In subunit rotation, however, the symmetrical nature of the catalytic tetramer in the substrate and product configurations can easily accommodate multiple rounds of strand exchange without the requirement for a ligated intermediate or resetting of the catalytic tetramer. In Chapter 4 the existence of the hypothetical ligated intermediate and the concerted nature of the knotting reaction are investigated further as a means of distinguishing between these two contrasting models of strand exchange.

1.24 Project aims

The main aim of the work described in Chapter 3 was to try and set up a direct biochemical test of the subunit rotation model of strand exchange by Tn*3* resolvase (described in Section 1.17), (Stark *et al.*, 1989a). Although the model accounts for much of the topological and genetic data obtained for the *in vitro* resolution reaction, there is little physical evidence that such a mechanism exists.

To achieve this aim, photoactivatable nucleotide analogues were incorporated into oligonucleotides at specific positions. The oligonucleotides were ligated into plasmid molecules which were then supercoiled *in vitro*. Resolvase was bound to these substrates and covalently crosslinked at the nucleotide analogue by irradiating the mixture with light of the appropriate wavelength. The resolution reaction was then allowed to proceed, and the presence of the covalent complex in the recombinant product was assayed. Using this method, we hoped to test whether a subunit of resolvase (covalently anchored to the site I half-site at which it is bound) was catalytically active, and therefore if the subunit rotation model was valid. A stronger test of subunit rotation would be to determine whether a dimer

of resolvase, with one subunit photocrosslinked to each half-site of an altered crossover site, could catalyse the knotting reaction (i.e. multiple rounds of strand exchange).

An alternative way to distinguish between the 'subunit rotation' and 'DNA-mediated' models of strand exchange is described in Chapter 4. The subunit rotation model does not require the formation of a ligated intermediate during the knotting reaction (Section 1.19), but can accommodate multiple rounds of 180° strand exchange without reorganisation of the catalytic tetramer into an active configuration after each round. In contrast to this, a DNA-mediated knotting mechanism must involve a transiently ligated recombinant to allow 'resetting' of the catalytic tetramer (Section 1.23).Chapter 4 describes experiments in which the requirement for the ligated recombinant intermediate was tested and the properties of the knotting reaction were examined further.

Finally, the role of the *res* site I sequence in cleavage and strand exchange is investigated in Chapter 5. To examine whether changing the length of site I affected resolution, basepairs were added or deleted from the sequence at the centre of the site. At the same time it was investigated whether resolvase-mediated cleavage positions are determined by a specific sequence at the centre of site I or by the relative positions of the resolvase binding sites at the ends of site I.

Chapter 2

Materials and Methods

2.1 Bacterial strains

All bacterial strains used for plasmid DNA amplification were derivatives of *Escherichia coli* K-12.

Strain	Genotype	Source
AB1157	*thr*1, *leu*6, *his*G4, *thi*1, *ara*14, *pro*A2, *arg*E3, *gal*K2, *sup*37, *xyl*15, *mtl*1, *tsx*33, *str*31	
DS941	AB1157, but *rec*F143, *sup*E44, *lac*ZΔM15, *lac*I^q	D.J.Sherratt
DS902 (AB2463)	AB1157, but *rec*A13	D.J.Sherratt

2.2 Plasmids

The plasmids which were used or constructed in this work are listed in Table 2.1, overleaf.

2.3 Oligonucleotide synthesis

All oligonucleotides used in this work (listed in Table 2.2) were synthesised on an Applied Biosystems 391 PCR-Mate oligonucleotide synthesiser using standard cyanoethyl phosphoramidite chemistry. All reagents were supplied by Cruachem Ltd. except 4-thiothymidine and 6-thiodeoxyguanosine protected phosphoramidite derivatives (gifts from B.Connolly and T.Nikiforov) and 5-iododeoxyuridine derivative (purchased from Glen Research).

Oligonucleotides containing 4-thiothymidine were made by introducing a protected base (4-thiothymidine-cyanoethyl-phosphoramidite) which was deprotected post-synthesis by treating with 0.3 M DBU (1,8-Diazabicyclo[5,4,0]undec-7-ene) in anhydrous acetonitrile at 20-25 °C for 3 h to yield 4-thiothymidine. The sample was then concentrated down to an oily residue using a gyro-vap sample concentrator and incubated with fresh 30% aqueous ammonia at 25 °C for 20 h. All other oligonucleotides were deprotected by incubating the glass support in 1 ml of fresh 30% aqueous ammonia at room temperature for 1-2 h. The supernatant was then removed and combined with another 1 ml of fresh 30% aqueous ammonia and incubated at 55 °C overnight in a sealed tube. The deprotected oligonucleotides were precipitated by adding ammonium acetate to 0.5 M, then 2 volumes

Plasmid	Size (bp)	Antibiotic Marker	Description/Derivation	Source or Reference
pUC18	2686	Ap	Standard cloning vector	
pBR322	4363	Ap Tc	Standard cloning vector	
pMTL23	2505	Ap	Cloning vector derived from pUC18	Chambers *et al.*, 1988
pUC71K	3966	Ap Km	Cloning vector derived from pBR322	Vieira *et al.*, 1982
pMA21	4927	Ap Tc	pBR322 derivative with directly repeated *res* sites (see Figure 4.7)	Bednarz *et al.*, 1990
pGC1	4927	Ap Tc	pMA21 derivative (mutagenesis of crossover dinucleotide of one *res* site: AT→TA) see Figure 4.7	G.Calder/W.M.Stark
pGC4	4927	Ap Tc	pMA21 derivative (mutagenesis of crossover dinucleotide of both *res* sites: AT→TA); Figure 4.7	G.Calder/W.M.Stark
pMA2350	3326	Ap	282 bp *Pvu*II (wt *res*) and 358 bp *Hae*III (wt *res*) in *Sma*I and *Hinc*II of pUC18 (see Figure 4.10)	Stark *et al.*, 1989b
pMS8	3326	Ap	pMA2350 derivative (mutagenesis of crossover dinucleotide in one *res* site: AT→AC) Figure 4.10	W.M.Stark
pMS9	3326	Ap	pMA2350 derivative (mutagenesis of crossover dinucleotide in one *res* site: AT→AC) Figure 4.10	W.M.Stark
pMS20	3326	Ap	pMA2350 derivative (mutagenesis of crossover dinucleotide in both *res* sites: AT→AC) Figure 4.10	W.M.Stark
pMM1	4590	Ap Km	1264 bp *Bam*HI/*Bam*HI pUC71K (Km^r) + 3326 bp *Bam*HI pMA2350 (see Figure 4.1)	Section 4.1
pMM2	4590	Ap Km	1264 bp *Bam*HI/*Bam*HI pUC71K (Km^r) + 3326 bp *Bam*HI pMS8 (see Figure 4.1)	Section 4.1
pMM3	4590	Ap Km	1264 bp *Bam*HI/*Bam*HI pUC71K (Km^r) + 3326 bp *Bam*HI pMS9 (see Figure 4.1)	Section 4.1
pMM4	4590	Ap Km	1264 bp *Bam*HI/*Bam*HI pUC71K (Km^r) + 3326 bp *Bam*HI pMS20 (see Figure 4.1)	Section 4.1
pOG5	2639	Ap	130 bp *Sst*I/*Xba*I RI *res* +2505 bp *Sst*I/*Xba*I pMTL23 (see Figure 3.12)	O.Gubbay/W.M.Stark

Table 2.1 Plasmids

Plasmid	Size (bp)	Antibiotic Marker	Description/Derivation	Source or Reference
pOG3	4111	Ap Km	Derivative of pOG5 and pUC71K, with two RI *res* sites in direct repeat (see Figure 5.2)	Section 5.2
pMM5	4903	Ap Tc	2280 bp filled *Eco*RI/*Hin*dIII pMA21 + 2623 bp *Hin*dIII/*Hinc*II pOG5 (see Figure 3.7)	Section 3.2
pMM6	4904	Ap Tc	45 bp *Eco*RI/*Sst*I oligo ('AAT' crossover trinucleotide site I) + 4859 bp *Eco*RI/*Sst*I pMM5 (see Figure 5.2)	Section 5.2
pMM7	4113	Ap Km	Derivative of pMM8 and pUC71K, with two 'AAT' *res* sites in direct repeat (see Figure 5.2)	Section 5.2
pMM8	2640	Ap	45 bp *Eco*RI/*Sst*I oligo ('AAT' crossover trinucleotide site I) + 2595 bp *Eco*RI/*Sst*I pOG5	Chapter 5
pMM12	4904	Ap Tc	45 bp *Eco*RI/*Sst*I oligo ('ATT' crossover trinucleotide site I) + 4859 bp *Eco*RI/*Sst*I pMM5 (see Figure 5.2)	Section 5.2
pMM15	4902	Ap Tc	43 bp *Eco*RI/*Sst*I oligo ('-1 site I') + 4859 bp *Eco*RI/*Sst*I pMM5 (see Figure 5.2)	Section 5.2
pMM18	4901	Ap Tc	42 bp *Eco*RI/*Sst*I oligo ('-2 site I') + 4859 bp *Eco*RI/*Sst*I pMM5 (see Figure 5.2)	Section 5.2
pMM21	2634	Ap	174 bp *Hin*dIII/*Bgl*II pMM12 ('ATT' *res*) + 2460 bp *Hin*dIII/*Bgl*II pMTL23	Chapter 5
pMM22	2632	Ap	172 bp *Hin*dIII/*Bgl*II pMM15 ('-1 *res*') +2460 bp *Hin*dIII/*Bgl*II pMTL23	Chapter 5
pMM23	2631	Ap	171 bp *Hin*dIII/*Bgl*II pMM18 ('-2 *res*') + 2460 bp *Hin*dIII/*Bgl*II pMTL23	Chapter 5
pMM24	4113	Ap Km	Derivative of pMM21 and pUC71K, with two 'ATT' *res* sites in direct repeat (see Figure 5.2)	Section 5.2
pMM25	4109	Ap Km	Derivative of pMM22 and pUC71K, with two '-1 *res*' sites in direct repeat (see Figure 5.2)	Section 5.2
pMM26	4107	Ap Km	Derivative of pMM23 and pUC71K, with two '-2 *res*' sites in direct repeat (see Figure 5.2)	Section 5.2
pMS7	2933	Ap	247 bp *Pvu*II/*Eco*RI (wt *res*) + 2686 bp *Sma*I pUC18 (see Figure 3.12)	W.M.Stark

Table 2.1 Plasmids

Oligonucleotide Description	**Size (nt)**	**Sequence (underlined denotes site I)**	**Reference**
wt site I (top strand)	40	5' CACTAGTCCGTTCGAAATATTATAAATTATCAGACATAGG 3'	Figure 3.2
wt site I (bottom strand)	48	5' AATTCCTATGTCTGATAATTTATAATATTTCGAACGGACTAGTGAGCT 3'	Chapter 3
single 4-S-dT site I (bottom)	48	5' AATTCCTA^{4S}TGTCTGATAATTTATAATATTTCGAACGGACTAGTGAGCT 3'	Figure 3.2
single 4-S-dT site I (bottom)	48	5' AATTCCTATG^{4S}TCTGATAATTTATAATATTTCGAACGGACTAGTGAGCT 3'	Figure 3.2
4-S-dT and 6-S-dG site I (bottom strand)	48	5' AATTCCTA^{4S}T^{6S}G^{4S}TCTGATAATTTATAATATTTCGAACGGACTAGTGAGCT 3'	Figure 3.2
'sym-site I' (top strand)	40	5' CACTAGTCTGTCCGATAATTTATAAATTATCGGACATAGG 3'	Figure 3.2
4-S-dT 'sym-site I' (bottom)	48	5' AATTCCTA^{4S}TG^{4S}TCCGATAATTTATAAATTATCGGACAGACTAGTGAGCT 3'	Figure 3.2
5-I-dU site I (bottom strand)	48	5' AATTCCTA^{5I}UG^{5I}UCTGATAATTTATAATATTTCGAACGGACTAGTGAGCT 3'	Section 3.4
5-I-dU site I (top strand)	40	5' CACTAGTC^{5I}UG^{5I}UTCGAAATATTATAAATTATCAGACATAGG 3'	Section 3.5
'TGT' site I (bottom strand)	48	5' AATTCCTATGTCTGATAATTTATAATATTTCGAACAGACTAGTGAGCT 3'	Section 3.5
5-I-dU 'AC-knotting' site I (top strand)	40	5' CACTAGTC^{5I}UG^{5I}UTCGAAATATTACAAATTATCAGACATAGG 3'	Section 3.6
5-I-dU 'AC-knotting' site I (bottom strand)	48	5' AATTCCTA^{5I}UG^{5I}UCTGATAATTTGTAATATTTCGAACAGACTAGTGAGCT 3'	Section 3.6
'AC-mismatch' site I (top)	40	5' CACTAGTCCGTTCGAAATATTACAAATTATCAGACATAGG 3'	Section 4.5

Table 2.2 Oligonucleotides

Oligonucleotide Description	Size (nt)	Sequence (underlined denotes site I)	Reference
'AAT' site I (top strand)	41	5' CACTAGTCCGTTCGAAATATTAATAAATTATCAGACATAGG 3'	Chapter 5
'AAT' site I (bottom)	49	5' AATTCCTATGTCTGATAATTTATTAATATTTCGAACGGACTAGTGAGCT 3'	Chapter 5
'ATT' site I (top strand)	41	5' CACTAGTCCGTTCGAAATATTATTAAATTATCAGACATAGG 3'	Chapter 5
'ATT' site I (bottom)	49	5' AATTCCTATGTCTGATAATTTAATAATATTTCGAACGGACTAGTGAGCT 3'	Chapter 5
'-1' site I (top strand)	39	5' CACTAGTCCGTTCGAAATATATAAATTATCAGACATAGGG 3'	Chapter 5
'-1' site I (bottom strand)	47	5' AATTCCTATGTCTGATAATTTATATATTTCGAACGGACTAGTGAGCT 3'	Chapter 5
'-2' site I (top strand)	38	5' CACTAGTCCGTTCGAAATATATAATTATCAGACATAGG 3'	Chapter 5
'-2' site I (bottom strand)	46	5' AATTCCTATGTCTGATAATTATATATTTCGAACGGACTAGTGAGCT 3'	Chapter 5

Table 2.2 Oligonucleotides

of cold ethanol. Full length oligonucleotide was then gel-purified using denaturing PAGE (see Section 2.36).

2.4 Chemicals

The sources of most general chemicals are listed below. Specialised chemicals are mentioned in the relevant sections of Chapter 2. Deionised water was used for making all solutions.

Chemicals	Source
General chemicals, biochemicals, organic solvents	Aldrich/Sigma, BDH, May and Baker
Media	Difco, Oxoid
Agarose, acrylamide	BRL, FMC, Bio-Rad, Flowgen
Radiochemicals	ICN Biochemicals
Restriction enzyme buffers	BRL, Boehringer Mannheim, Promega
Ligase buffer	BRL

2.5 Bacterial growth media

Escherichia coli growth was in L-Broth (10 g bacto-tryptone, 5 g bacto-yeast extract, 5 g NaCl made up to 1 litre with deionised water , and adjusted to pH 7.5 with NaOH), or on L-Agar (L-Broth with 15 g/l agar). For antibiotic expression, growth was in 2 x YT Broth (16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl, made up to 1 litre in deionised water, adjusted to pH 7.0 with NaOH). Growth media were sterilised at 120 °C for 15 minutes.

2.6 Preparation of competent *E.coli* cells

20 ml overnight cultures were grown in φ broth (2% w/v tryptone, 0.5% w/v yeast extract, 20 mM $MgSO_4$, 10 mM NaCl, 5 mM KCl, adjusted to pH 7.5 with KOH). 200 ml pre-warmed φ broth was inoculated with 4 ml overnight culture and grown to an OD_{600} of 0.46-0.6 (1-2 h). Cells were cooled and harvested by centrifugation (Beckmann J2-21, 5000 rpm, 4 °C, 10 minutes) resuspended in 40 ml of ice-cold TFBI (30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2 \cdot 2H_2O$, 50 mM $MnCl_2 \cdot 4H_2O$, 15% glycerol, adjusted to pH 5.8 with AcOH) and kept on ice for 30 minutes. The cells were then re-centrifuged (as above) and resuspended in 8 ml of ice-cold TFBII (10 mM MOPS, 75 mM $CaCl_2 \cdot 2H_2O$, 10 mM

RbCl, 15% glycerol, adjusted to pH 6.8 with HCl or KOH) and kept on ice for 15 minutes. 200 µl aliquots were then dispensed into Nunc tubes, frozen in liquid nitrogen and stored at -70 °C.

2.7 Antibiotics

The following concentrations of antibiotics were used in liquid and solid selective growth media:-

Antibiotic	Stock Solution	Selective Conditions
Ampicillin (Ap)	5 mg/ml in H_2O	50 µg/ml
Tetracycline (Tc)	12.5 mg/ml in 70% ethanol	12.5 µg/ml
Chloramphenicol (Cm)	2.5 mg/ml in ethanol	25 µg/ml
Kanamycin (Kn)	5 mg/ml in H_2O	50 µg/ml

2.8 Bacterial cultures

Liquid cultures were grown at 37 °C with vigorous shaking. Growth on agar plates was at 37 °C overnight with plates inverted. Antibiotics were used as required (see Section 2.7). For long-term storage, bacterial strains were grown overnight in L-Broth, diluted 2-fold with 40% glycerol, 2% peptone and then kept at -70 °C.

2.9 Transformation of *E.coli* with plasmid DNA

Aliquots of competent *E.coli* were thawed on ice. 50 µl of cells was added to plasmid DNA on ice and maintained at 0 °C for 30 minutes. The samples were heat shocked at 37 °C for 5 minutes and then left on ice for 15 minutes. 5 volumes (approximately 250 µl) of 2 x YT broth were added and the samples incubated at 37 °C for 2 h. The cells were then plated out on selective media and incubated at 37 °C overnight.

2.10 Large-scale preparation of plasmid DNA

This method was adapted from that used by Birnboim and Doly (1979).

200 ml overnight cultures were grown to stationary phase (approximately 12 h vigorous shaking at 37 °C) and the cells harvested by centrifugation (12 000 g, 4 °C, 5 minutes).

The pellet was resuspended in Doly I buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA), and kept on ice for 5 minutes. 8 ml of fresh Doly II buffer (200 mM NaOH, 1% SDS) was added, the samples mixed gently and maintained at 0 °C for 4 minutes. 6 ml of cold Doly III buffer (0.6 vol. 5 M KOAc, 0.115 vol. AcOH, 0.285 vol. H_2O) was added and the sample mixed and kept on ice. The cell debris was then pelleted by centrifugation (39 200 g, 4 °C, 30 minutes) and the supernatant containing the DNA was mixed with 12 ml of isopropanol to allow precipitation at room temperature for 15 minutes. The DNA was pelleted by centrifugation (27 200 g, 20 °C, 30 minutes) and the pellet was washed with 70% ethanol, dried and resuspended in 2 ml of TE buffer (10 mM Tris-HCl pH 8.2, 1 mM EDTA). Caesium gradient purification was then carried out by adding 4.324 ml aqueous CsCl (equivalent to a solution of 5 g of CsCl with 3 ml of water) to the DNA/TE sample and transferring into a Beckmann ultra-centrifuge tube. 270 µl of 15 mg/ml ethidium bromide was added. The tube was filled with liquid paraffin and heat-sealed. The tube was then centrifuged in a Beckmann Ti70 fixed-angle rotor at 200 000 g for 16 h at 15 °C.

DNA bands were visualised using a long-wave UV source (365 nm). Usually two bands were visible, the upper containing chromosomal and nicked plasmid DNA, the lower, stronger band containing supercoiled plasmid DNA. The lower band was removed using a 1 ml syringe and the ethidium bromide was removed by repeated extractions with n-butanol. The DNA was recovered by adding 3 volumes of water and 2 volumes of ethanol, mixing the sample and maintaining at 4 °C for 30 minutes. The precipitated DNA was pelleted by centrifugation (27 200 g, 4 °C, 30 minutes). The pellet was washed with 70% ethanol, dried and resuspended in 500 µl TE buffer. The plasmid DNA was then stored at 4 °C.

2.11 Small-scale preparation of plasmid DNA

This alkaline lysis mini-prep. method was adapted from the above Birnboim Doly prep. (Section 2.10) and uses the same Doly I and Doly II buffers. An Eppendorf microcentrifuge running at 14 000 rpm was used for all following centrifugation steps.

A 10 ml culture was grown to stationary phase (approximately 12 h vigorous shaking at 37 °C) and 1.5 ml of cells was harvested by centrifugation (30 seconds). The cells were resuspended in 200 µl of a Doly I/lysozyme (~4 mg/ml) mixture and left at room temperature for 5 minutes. 400 µl of fresh Doly II was added to the sample which was then

mixed and put on ice for 5 minutes. 300 µl 7.5 M NH_4OAc was added, and the sample was maintained at 0 °C for 10 minutes. The cell debris was pelleted by centrifugation (3 minutes), and the supernatant extracted twice with 0.5 volumes of phenol. The DNA was precipitated by adding 0.75 volumes of isopropanol, maintaining at room temperature for 10 minutes, and then pelleted by centrifugation (30 minutes). The pellet was washed with 70% ethanol, dried and resuspended in 50 µl TE buffer. The plasmid DNA was stored at 4 °C.

2.12 Restriction endonuclease digestion of DNA

Restriction digests were usually done in the suppliers' recommended buffer (generally BRL REact buffer), with between 2-fold and 10-fold excess enzyme to ensure complete cleavage of the DNA. Digests were incubated at 37 °C for ≥ 1 h and were terminated by the addition of an SDS loading buffer (see Section 2.25), or by heating at 70 °C for 5 minutes. Resolvase reactions were usually in C9.4 buffer (see Section 2.40) at 37 °C. After 1 h the reaction was stopped by heating at 70 °C for 5 minutes, the buffer was adjusted appropriately and restriction endonuclease was added for a further 1 h incubation at 37 °C. The reaction was terminated by the addition of SDS loading buffer prior to electrophoresis.

2.13 Ethanol precipitation of DNA

Where necessary, the salt concentration of the DNA solution was adjusted to 0.3 M with sodium acetate. 2 volumes of ethanol were added, followed by thorough mixing of the sample and incubation at -20 or -70 °C for ≥ 15 minutes. The precipitated DNA was pelleted by centrifugation in an Eppendorf microcentrifuge at 14 000 rpm, at 4 °C for 30 minutes. The pellet was then washed in 70% ethanol and re-centrifuged (for 5 minutes). The ethanol was removed, and the pellet was dried and resuspended in TE buffer.

2.14 Purification of DNA by phenol extraction

Organic extraction of proteins was achieved by addition of one volume of phenol (containing 0.1% 8-hydroxyquinoline and equilibrated with 0.5 M Tris-HCl pH 8), vigorous mixing and centrifugation in an Eppendorf microcentrifuge at 14 000 rpm for 3 minutes. The DNA-containing aqueous layer was recovered and re-extracted as necessary. Any remaining phenol was removed by repeating the above extraction with chloroform.

2.15 Calf intestinal phosphatase (CIP) treatment of DNA

1 unit of CIP was added to some restriction digests (to remove 5' phosphate groups from linearised DNA fragments) to prevent ligation, or to allow 5' labelling with γ-[^{32}P]ATP and T4 kinase (see Section 2.16). The digests were then incubated at 37 °C for ≥ 15 minutes. Removal of CIP prior to phosphorylation required two thorough phenol extractions, a chloroform extraction and ethanol precipitation (see above). The dephosphorylated DNA was resuspended in TE buffer.

2.16 5' phosphorylation of DNA

Dephosphorylated linear DNA fragments (see Section 2.15) and synthetic oligonucleotides were 5' phosphorylated with γ-[^{32}P]ATP (or, unlabelled ATP) and T4 kinase. The DNA (~2 pmol) was mixed with 1 x T4 kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) along with either 1 mM ATP or 10 µCi γ-[^{32}P]ATP (8 000 Ci/mmol). 1 unit of T4 kinase was then added and the mixture incubated at 37 °C for 45 minutes. The reaction was stopped by extracting once with phenol, then once with chloroform, and the phosphorylated DNA was recovered by ethanol precipitation. The 5' phosphorylated DNA was resuspended in TE buffer.

2.17 Filling in 3' recessed ends of DNA fragments

This technique was used to blunt 3' recessed ends prior to cloning of a DNA restriction fragment. After restriction enzyme digestion of ~0.5 µg DNA, ~125 µM each of dATP, dCTP, dGTP, and dTTP and 1 unit of the Klenow fragment of DNA polymerase I was added to the reaction, typically in 20 µl of BRL REact 2 buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$). The sample was incubated at 37 °C for 30 minutes and then the reaction was stopped by heating at 70 °C for 5 minutes. The blunt DNA fragment was recovered by either ethanol precipitation or agarose gel-purification.

2.18 3' end labelling of DNA fragments

1-2 µg of DNA was cut typically in BRL REact 2 buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$) to generate at least one 3' recessed end. 10 µCi of an α-[^{32}P]dNTP (3 000 Ci/mmol) and the three remaining non-radiolabelled dNTP's (at 10 µM each) were then added along with 1 unit of the Klenow fragment of DNA polymerase I, and the sample was incubated at room temperature for 20 minutes. All four non-radiolabelled dNTP's were

then added (at ~100 µM each) and the reaction incubated at room temperature for a further 10 minutes. The reaction was stopped by phenol/chloroform extraction, and the 3' labelled DNA was recovered by ethanol precipitation.

2.19 Ligation of DNA restriction fragments

Standard ligations for cloning used 1-2 µg DNA (in a volume of 10-20 µl) in 1 x BRL ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8 000) with 1 unit of T4 DNA ligase (BRL). The molar ratio of vector to insert DNA was generally 1 : 2. Samples were incubated at room temperature overnight, and then used to transform competent *E.coli* cells (see Section 2.9).

2.20 Annealing oligonucleotides

Single-strand oligonucleotides were annealed in 20 µl TE buffer (10 mM Tris-HCl pH 8.2, 1 mM EDTA) with the addition of 50 mM NaCl. The reaction was mixed, heated at 70 °C for 5 minutes and then allowed to cool/anneal at room temperature for ≥15 minutes.

2.21 Ligation of oligonucleotides into plasmid vectors

Double-stranded oligonucleotides (~100 pmol; not phosphorylated) were ligated into plasmid vectors (1-2 µg) with complementary cohesive ends. The reactions were in 1 x BRL ligation buffer (see Section 2.19) with 1 unit of T4 DNA ligase (BRL) and were incubated at room temperature overnight. The samples were then transformed into competent *E.coli* cells (Section 2.9).

2.22 DNase I nicking of plasmid DNA

DNase I (bovine pancreatic deoxyribonuclease I) was purchased from Boehringer Mannheim and diluted in 50 mM Tris-HCl pH 8.2, 150 mM NaCl, 50% (v/v) glycerol. Treatment of DNA with DNase I was carried out in the presence of ethidium bromide to limit the extent of single-strand nicks to about one per plasmid DNA molecule. Resolvase reactions were generally in C9.4 buffer (50 mM Tris-HCl pH 9.4, 10 mM $MgCl_2$, 0.1 mM EDTA) at 37 °C for 1 h. The reaction was then heated at 70 °C for 5 minutes, and the buffer was adjusted by adding 50 mM Tris-HCl pH 7.5, and 300 µg/ml ethidium bromide. 8 µg/ml DNase I was added and the sample was incubated at 37 °C for 2 h. The reaction

was stopped by one phenol extraction followed by addition of SDS gel-loading buffer immediately prior to agarose gel electrophoresis.

2.23 DNase I footprinting

This method was derived from that of Galas and Schmitz (1978). 5' labelled substrate DNA was resuspended in 20 µl of C8 buffer (50 mM Tris-HCl pH 8.2, 10 mM $MgCl_2$, 0.1 mM EDTA). 4 µl aliquots (100-500 cps) of DNA were incubated with 0.2 µl of diluted resolvase (or dilution buffer) at room temperature for 5 minutes. 1 µl of 6.25 µg/ml DNase I was added, the sample was mixed thoroughly, and incubation at room temperature continued for a further 5 minutes. The reaction was stopped by addition of 4 µl of stop mix (3 volumes of formamide loading dye (see Section 2.25): 1 volume 100 mM EDTA). The sample was heated to 80 °C before loading ≤ 4 µl onto a sequencing gel.

2.24 Agarose gel electrophoresis

0.7% or 1.2% agarose gels were made by dissolving an appropriate amount of agarose (BRL, 'Ultrapure') in 250 ml 1 x TAE running buffer (40 mM Tris-acetate pH 8.2, 20 mM sodium acetate, 1 mM Na_2EDTA) by mixing and heating in a microwave oven. The hot agarose solution was allowed to cool to approximately 60 °C before pouring into a gel former and leaving to set at room temperature. 150 x 200 mm horizontal slab gels were generally used, giving 22 adjacent sample wells each with a capacity of around 25 µl. The 'hand-made' electrophoresis tank required about 3 litres of 1 x TAE buffer, and electrophoresis was through the longest dimension of the gel at an applied voltage of between 2 and 5 V/cm. Agarose gels were generally run at room temperature for approximately 15 h.

'Minigels' were commonly run to rapidly analyse restriction fragment sizes or to gel-purify fragments of DNA from low melting-point agarose (see Section 2.31). These gels were made in a similar way but were prepared in smaller kits and required only 30 ml of gel and 400 ml 1 x TAE running buffer. Electrophoresis was at 80-100 V (constant voltage) for 1 h at room temperature.

2.25 Loading buffers

4-5 µl of SDS loading buffer (50% glycerol, 1% SDS, 0.01% bromophenol blue) was added to standard 20 µl resolution reactions, restriction digestions, crosslinking reactions and DNase I nicking reactions prior to loading on an agarose or a polyacrylamide gel.

Non-SDS loading buffer (as above but without SDS) was added to glutaraldehyde synapsis reactions prior to electrophoresis.

1 volume of formamide loading buffer (80% deionised formamide, 10 mM EDTA pH 8.0, 1 mg/ml Xylene Cyanol, 1 mg/ml bromophenol blue) was added to oligonucleotides, sequencing samples and cleavage mapping reactions prior to denaturing polyacrylamide gel electrophoresis. The samples were heated at 80 °C for 5 minutes before being loaded onto the gel.

2.26 DNA molecular weight standards

BRL 1 kilobase ladder was run unlabelled or was 5' labelled with γ-[^{32}P]ATP and T4 kinase (see Section 2.16) and run on both agarose and polyacrylamide gels to help estimate the size of DNA fragments.

2.27 'Single colony' gel analysis

A single bacterial colony of interest was patched onto a fresh L-agar plate (containing appropriate antibiotic selection) to give a patch of ≥ 1 cm^2. After overnight growth at 37 °C a sterilised toothpick was used to transfer cells from the patch into 150 µl of 'single colony lysing' buffer (2.5% Ficoll, 1.25% SDS, 0.05% bromophenol blue, in 1 x TAE). After mixing, the sample was incubated at room temperature for 15 minutes and then centrifuged in an Eppendorf microcentrifuge at 14 000 rpm for 30 minutes. 30-40 µl of supernatant was then loaded directly onto a 1.2% agarose gel and analysed by electrophoresis. This method was used to quickly assay the size of transformed plasmid DNA's.

2.28 Chloroquine gels for analysis of topoisomer distribution

Chloroquine (7-chloro-4-[4-diethylamino-1-methyl-butylamino]-quinoline) is a DNA intercalator, and its presence reduces negative supercoiling in covalently closed circular DNA molecules (see Section 3.3). Chloroquine gels were made by including 7 µg/ml

chloroquine in the agarose gel mixture and the electrophoresis running buffer. Electrophoresis was at room temperature for approximately 15 h at a voltage of 2-5 V/cm. Removal of the chloroquine was necessary for ethidium bromide staining and was achieved by soaking the gel in a 0.3% SDS solution at 37 °C for 1 h.

2.29 Two-dimensional agarose gel electrophoresis

Samples were run on a standard agarose gel (see Section 2.24) and the bands were visualised by long wavelength 365 nm UV illumination following ethidium bromide staining. The entire lane containing the bands of interest was then excised, and at this point could be soaked in a solution of restriction endonuclease or SDS depending on the experiment. The gel slice was then placed in a gel former at one end, replacing the wells, such that the direction of migration in the second dimension of electrophoresis would be at 90 ° to that in the first dimension. The second gel was then prepared by pouring the melted agarose solution into the gel former containing the lane from the first gel. For topoisomer analysis 7 µg/ml chloroquine was included in the first or second gel mixture but not both (see Section 3.3). Chloroquine was removed from the gel run in the first direction, or from the gel run in the second direction, by soaking in a 0.3% SDS solution at 37 °C for 1 h (see Section 2.28). Electrophoresis in the second direction was at 2-5 V/cm for approximately 15 h.

2.30 Ethidium bromide staining of DNA, photography and autoradiography

DNA within agarose and polyacrylamide gels was visualised by staining with a 0.6 µg/ml ethidium bromide solution (made by diluting a 15 mg/ml ethidium bromide stock solution in 1 x TAE electrophoresis buffer or water) for 30-60 minutes. The gel was then rinsed several times and soaked in water for another 60 minutes to remove background ethidium bromide fluorescence.

Visualisation of ethidium stained DNA was by short wavelength 254 nm UV illumination unless bands were to be cut from the gel when a long wavelength 365 nm source was used. Gels were photographed using Polaroid type 667 film. Alternatively a Pentax SLR camera loaded with Ilford HP5 35 mm film was used, and the film was processed according to the manufacturer's instructions with Ilford Microphen developer and Amfix fixative. A Kodak Wratten filter No. 23A was fitted to all cameras.

For autoradiography, the gel (containing labelled DNA) was transferred to 2 layers of 3MM filter paper, dried under vacuum (on a Bio-Rad slab gel dryer at 80 °C for 45 minutes) and then exposed to a sheet of Fuji RX100 film overnight. Exposed film was developed in an X-OMAT automated processor (Kodak).

2.31 Purification of DNA using low melting-point agarose

Low melting-point agarose was purchased from FMC ('Sea Plaque GTG') and was used to prepare agarose gels (as in Section 2.24) from which DNA fragments were to be recovered. Bands of interest were excised using a scalpel from an ethidium bromide-stained low melting-point agarose gel (using long wavelength transillumination) and transferred to a large Eppendorf tube. The gel chip was melted by heating the tube at 65-70 °C for 5 minutes. This was followed by the addition of 1 volume of phenol, vortexing, centrifugation (14 000 rpm for 5 minutes) and then the addition of ~100 µl TE to aid recovery of the aqueous top layer. The DNA-containing aqueous layer was phenol-extracted again, then chloroform-extracted to remove any traces of phenol. The DNA was recovered by ethanol precipitation followed by resuspension in TE buffer.

2.32 Southern blotting

Transfer of DNA from agarose gel to nylon membrane (Amersham Hybond-N) followed the method of Sambrook *et al.*, 1989. Radiolabelled probe was prepared by hexanucleotide random oligo priming (Boehringer Mannheim) according to the manufacturer's instructions. Hybridisation of the probe to DNA fixed on Hybond-N membrane was also according to the manufacturer's instructions. Autoradiography was as described in Section 2.30.

2.33 Polyacrylamide gels

Standard polyacrylamide gels were used for analysis of restriction digests, recombination reactions and for purifying small double-stranded DNA fragments. Similar gels with the addition of SDS (SDS-PAGE) were used for visualising the products of photocrosslinking assays. Varying concentrations of acrylamide and volumes of gel were used, but gels were always constructed in a similar manner.

Glass plates were clamped together with 0.75 mm spacers between them and a length of rubber tubing around the edges of the plates to form a seal. The freshly made acrylamide

gel mixture was poured between the plates. A well forming comb was inserted in the top and clamped in place. The acrylamide was allowed to polymerise for ≥ 1 h. The clamps, tubing and comb were removed and the gel was clamped into the electrophoresis kit. The buffer reservoirs were filled with 1 x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 0.2 mM EDTA pH 8.3) and electrophoresis was at a constant voltage of 200 V at room temperature for 3-4 h. The DNA bands were visualised with ethidium bromide or by autoradiography (see Section 2.30).

A standard 30 ml, X% (w/v), polyacrylamide gel was made by mixing X ml 30% acrylamide : 0.8% bisacrylamide (w/v), 3 ml 10 x TBE buffer, (27 - X) ml H_2O, 360 µl 10% APS (ammonium persulfate,w/v) and 18 µl TMED (N, N, N', N'-tetramethylethylenediamine).

2.34 SDS-polyacrylamide gels

These gels were used for the analysis of photocrosslinked products or other covalent protein-DNA complexes. The most frequently used type of gel was a 10% polyacrylamide, 0.1% SDS, 50 mM Tris-glycine pH 9.4, 0.1 mM EDTA gel which was made by mixing 10 ml 30% acrylamide : 0.8% bisacrylamide (w/v), 3 ml 0.5 M Tris-glycine pH 9.4 (made by dissolving 60.55 g of Tris base and 7.43 g of glycine in 1 litre of deionised water), 15 µl 0.2 M EDTA, 300 µl 10% SDS (w/v), 17 ml H_2O, 360 µl 10% APS (w/v) and 18 µl TMED. The running buffer contained 50 mM Tris-glycine pH 9.4, 0.1 mM EDTA and 0.1% SDS. The gel was cast as in Section 2.33 and electrophoresis was at a constant voltage of 200 V at 4 °C for 4-5 h. In these experiments the DNA was usually radiolabelled, and was visualised by autoradiography (see Section 2.30).

2.35 Non-denaturing polyacrylamide gels

Non-denaturing PAGE was used specifically for resolvase band-shift assays, i.e. the detection of resolvase-DNA non-covalent bound complexes. This is also known as a gel-binding assay. Typically a 6% polyacrylamide, 50 mM Tris-glycine pH 9.4, 0.1 mM EDTA gel was made by mixing 6 ml 30% acrylamide : 0.8% bisacrylamide (w/v), 3 ml 0.5 M Tris-glycine pH 9.4, 15 µl 0.2 M EDTA, 21 ml H_2O, 360 µl 10% APS (w/v) and 18 µl TMED. The running buffer contained 50 mM Tris-glycine pH 9.4 and 0.1 mM EDTA in deionised water. The gel was cast as Section 2.33, but all equipment was kept scrupulously detergent-free. Electrophoresis was as described in Section 2.34.

2.36 Denaturing polyacrylamide gels

These gels were used for plasmid sequencing, purification of synthetic oligonucleotides and the resolvase cleavage point mapping described in Chapter 5. An IBI Base Runner sequencing gel kit was used to analyse plasmid sequence and cleavage mapping products whereas synthetic oligonucleotides were purified using a standard CBS polyacrylamide gel kit as described in Section 2.37. For 100 ml of 6% polyacrylamide/7 M urea gel, a mixture of 46 g of urea, 15 ml of a 40% (w/v) acrylamide solution (19 : 1 ratio of acrylamide to bisacrylamide), 10 ml of 10 x TBE, 666 µl of 10% (w/v) APS and 40 µl of TMED was made up to 100 ml with deionised water. The mixture was poured between clamped sequencing plates or between standard glass plates as Section 2.33. A well-forming comb was inserted, and the gel was allowed to polymerise at room temperature for ≥ 1 h. The gel was pre-run at 45 W (constant power) for 30-60 minutes to heat the gel and prevent renaturation of single-stranded DNA.

Samples containing 1 volume of formamide loading buffer (see Section 2.25) were heated to 80 °C for 5 minutes and then loaded onto the gel. Electrophoresis was at 45 W (constant power) for 2-3 h. The gel was then dried under vacuum, dusted with talcum powder (to prevent sticking) and autoradiographed as described in Section 2.30.

2.37 Purification of oligonucleotides by denaturing PAGE

Synthetic single-strand oligonucleotides were run on 15% polyacrylamide/7 M urea denaturing gels which were made by mixing 15.75 g of urea, 14.75 ml of a 40% (w/v) acrylamide solution (19 :1 ratio of acrylamide to bisacrylamide), 3.75 ml of 10 x TBE, 7.75 ml of H_2O, 225 µl of 10% APS (w/v) and 18 µl TMED. The running buffer was 1 x TBE and the gel was poured between clamped plates as described in Section 2.33. Samples containing 1 volume of formamide loading buffer (see Section 2.25) were heated to 80 °C for 5 minutes and then loaded onto the gel. Electrophoresis was at 400-500 V (constant voltage) for 2-3 h to keep the gel hot and prevent renaturation of single-stranded oligonucleotides.

The gel was stained with 'Stains-all' (1-ethyl-2-[3-(1-ethylnaphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl)naphtho[1,2-d]thiazolium bromide; from Aldrich). 'Stains-all' is a cationic carbocyanine dye which can be used for the staining of DNA, RNA or protein analysed by PAGE.

70 ml of water and 20 ml of isopropanol were mixed with 10 ml of a 0.1% (w/v) solution of 'Stains-all' (in formamide). The oligonucleotide-containing gel was soaked in this solution until sufficient staining was observed (5-10 minutes). The gel was then destained in water by rinsing several times. Single stranded oligonucleotides gave cyan or purple bands depending on their size (smaller DNA's stained cyan, larger DNA's stained purple).

The full size oligonucleotide to be purified (usually the slowest migrating, major product on the gel) was cut out with a scalpel and transferred to a Nunc tube. 1 ml of TE buffer was added to the tube and the acrylamide gel chip was crushed with a glass rod. The acrylamide gel/TE buffer slurry was incubated on a rotary wheel at 37 °C overnight. The gel fragments were pelleted by centrifugation at 14 000 rpm for 1 minute in an Eppendorf microcentrifuge, and the supernatant was transferred to a fresh tube. A further 0.5 ml of TE buffer was added to the pellet of acrylamide; the sample was mixed briefly and re-centrifuged. The clear TE buffer was again removed and the two supernatants were combined. The oligonucleotide was concentrated by ethanol precipitation, and resuspended in TE buffer.

2.38 Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional PAGE was used to examine the binding and crosslinking of resolvase to modified oligonucleotide site I's. Photocrosslinked samples were prepared as in Section 2.45 and run in the first dimension as a standard band-shift assay on non-denaturing PAGE (see Sections 2.35 and 2.42). Electrophoresis in the first dimension was at 200 V (constant voltage) for 3-4 h. The gel was then stopped, the glass plates were separated and the gel was transferred to a solution of 1 x TBE and 0.1% SDS and soaked at room temperature for 15 minutes. The gel was then enclosed between two glass plates and placed in a horizontal electrophoresis tank containing 1 x TBE/0.1% SDS buffer. Electrophoresis in the second dimension was at 40 V (constant voltage) at room temperature for 15 h such that the direction of band migration was at 90 ° to that in the first dimension. The gel was then dried and an autoradiograph made as described in Section 2.30.

2.39 Tn*3* resolvase

Tn*3* resolvase was a gift from M.R.Boocock and M.A.Watson. γδ M106C resolvase was a gift from N.D.F.Grindley (Yale University). Resolvase dilutions were made at 0 °C with 1 x resolvase dilution buffer (50% (v/v) glycerol, 1 M NaCl, 10 mM Tris-HCl pH 7.5, 0.1

mM DTT and 0.05 mM EDTA). In all reactions with resolvase, 50 mM NaCl is introduced by the addition of 0.05 volumes of resolvase diluted in this buffer.

Two-fold serial dilutions of the resolvase stock solution were made, and generally 0.05 volumes of a 2^{-3}, 2^{-4}, or 2^{-5} dilution of the resolvase stock was required for efficient resolution, synapsis, binding or cleavage of 20 µg/ml supercoiled substrate DNA. Dilutions were stored indefinitely at -20 °C. The concentration of the resolvase stock varied between 150 µM and 400 µM depending on the fraction (Blake, 1993; Watson, 1994). A typical resolvase reaction contained 0.05 volumes of a 2^{-4} dilution which is defined as one unit of resolvase. One unit is equivalent to a monomer concentration of approximately 80 nm.

2.40 *In vitro* recombination

Tn*3* resolvase site-specific recombination reactions were performed in C9.4 buffer (50 mM Tris-HCl pH 9.4, 10 mM $MgCl_2$, 0.1 mM EDTA). A typical resolvase reaction contained ~20 µl of C9.4 buffer, 0.4 µg plasmid DNA and to this 0.05 volumes (1 µl) of diluted resolvase was added (~80 nm). After incubation at 37 °C for 1 h, the reaction was stopped by heating at 70 °C for 5 minutes. 60 µl reactions were routinely prepared, and after resolution these were split into three aliquots of 20 µl. The products of recombination could then be visualised by running samples on an agarose gel (i) untreated, (ii) cut with a restriction endonuclease (see Section 2.12), or (iii) nicked with DNase I (see Section 2.22). 5 µl of SDS loading buffer (see section 2.25) was added to all samples prior to electrophoresis. Variations in incubation time and treatment of products were as described in the relevant sections.

2.41 *In vitro* synapsis

The resolvase-mediated synapsis assay was as described in Watson, 1994. Briefly, 0.05 volumes of diluted resolvase was added to 0.4 µg plasmid DNA in a MOPS buffer (50 mM MOPS-NaOH pH 8.0, 100 mM NaCl, 1 mM EDTA) and incubated at 37 °C for 10 minutes. 0.05% glutaraldehyde (Sigma EM grade) was added and the reaction was maintained at 37 °C for a further 5 minutes. Addition of 100 mM sodium borohydride was followed by incubation at room temperature for 10 minutes. The crosslinked reaction mixture was divided into several aliquots which were treated with different restriction endonucleases (Section 2.12). The restriction digests were then divided into two further

aliquots which were treated with loading buffer plus- or minus-SDS (see Section 2.25). The synapsis products were then run on an agarose gel.

2.42 Band-shift analysis

Band-shift analysis was carried out as described in Bednarz, 1989 and Blake, 1993.

Non-specific carrier DNA (pUC18 or pUC71K) at approximately 50 µg/ml was mixed with an appropriate amount of end-labelled *res* DNA (~200-300 cps) in binding buffer (10 mM Tris-glycine pH 9.4, 0.1 mM EDTA, 10% (v/v) glycerol). This binding mix was dispensed to 10 µl aliquots and 0.4 µl of a resolvase dilution was added to each sample. The samples were incubated at 37 °C for 15 minutes and then loaded on non-denaturing PAGE (see Section 2.35) without further treatment. Electrophoresis was at 200 V (constant voltage), at 4 °C for 3-4 h.

2.43 Cleavage assays

Double-strand cleavage of *res* sites by Tn*3* resolvase was assayed by using an ethylene glycol-containing buffer (40% (v/v) ethylene glycol, 50 mM Tris-HCl pH 8.2, 50 mM NaCl, 0.1 mM EDTA). 0.4 µg plasmid DNA was incubated with 1 µl of a resolvase dilution and the reaction volume was made up to 20 µl with ethylene glycol buffer. The reaction was incubated at 37 °C for 16 h and then stopped by the addition of 1 mg/ml proteinase K/0.5% SDS. The samples were then loaded directly onto a 1.2% agarose gel and electrophoresis was as described in Section 2.24.

2.44 Intermolecular recombination

Intermolecular recombination (between *res* sites on different DNA molecules) was assayed by reacting two single *res* site plasmids (one supercoiled and one linearised) in M15M8 buffer (50 mM Tris-HCl pH 8.2, 5 mM spermidine, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% (v/v) glycerol). 1 µg of total DNA (0.5 µg supercoiled DNA and 0.5 µg linear DNA) was incubated with 1 µl of a resolvase dilution in M15M8 buffer at 37 °C for 24-48 h. The reactions were then run (without loading buffer) on an agarose gel and the DNA was visualised by staining with ethidium bromide and/or autoradiography.

2.45 Photocrosslinking Tn*3* resolvase to oligonucleotides

The oligonucleotide photocrosslinking reaction was basically a binding reaction (see Section 2.42) with an additional irradiation step. Non-specific carrier DNA (e.g. pUC71K) at approximately 50 µg/ml was mixed with an appropriate amount of end labelled, double stranded oligonucleotide site I containing appropriate photolabile nucleotide analogues (10-50 cps) in binding buffer (10 mM Tris-glycine pH 9.4, 0.1 mM EDTA, 10% (v/v) glycerol). A small reaction volume (10-20 µl) was preferable. 1 µl of a resolvase dilution was added, and the sample was incubated at 37 °C for 15 minutes. The reaction was then photocrosslinked by irradiation with an appropriate light source.

For oligonucleotides containing 4-thiothymidine or 6-thiodeoxyguanosine modifications, irradiation was by a long wavelength UV source (365 nm) for 10 minutes. For oligonucleotides containing 5-iodouridine modifications, irradiation was by a 325 nm HeCd laser (Liconix) by placing the Eppendorf tube directly in the laser beam for 30-60 minutes. The reaction was split into aliquots to allow plus- and minus-proteinase K (1 mg/ml) treatment before adding 0.2 volumes of SDS loading buffer and running samples on SDS-PAGE (see Section 2.34).

For time-course analysis a 120 µl binding/photocrosslinking reaction was prepared (see above) and incubated at 37 °C for 15 minutes. Before UV or laser irradiation, a 10 µl aliquot was removed to act as the zero time-point sample. The photocrosslinking time-course was initiated by placing the tube containing the reaction mixture on top of the UV light source (365 nm) or directly in the beam of the laser. 10 µl aliquots were removed at various times after initiation of irradiation and stored at 0 °C. An additional aliquot of the final time-point was treated with 1 mg/ml proteinase K. All samples were mixed with 0.2 volumes of SDS loading buffer and run on SDS-PAGE (see Section 2.34).

2.46 Photocrosslinking and recombination of artificial supercoiled substrates

Photoactivatable, artificial supercoiled substrates were prepared as described in Section 2.47. 0.4 µg pMM5 carrier DNA was mixed with an appropriate amount of labelled, artificial supercoiled substrate (20-30 cps) and the reaction volume was made up to 20 µl with 'C9.4 (-Mg^{2+})' buffer (50 mM Tris-HCl pH 9.4, 0.1 mM EDTA). 1 µl of a resolvase dilution was added, and the sample was incubated at 37 °C for 15 minutes. The mixture was then irradiated by placing directly in the beam of a HeCd laser for 30 minutes (or on

top of a UV light source for 10 minutes in the case of the thiothymidine and thiodeoxyguanosine derivatives). After irradiation the buffer was adjusted to 10 mM $MgCl_2$ making the reaction buffer equivalent to C9.4, the standard resolution conditions. The sample was incubated at 37 °C for 1 h, and then stopped by heating at 70 °C for 5 minutes. The reaction buffer was adjusted again and the sample was cut with appropriate restriction endonucleases. An aliquot of the reaction was treated with 1 mg/ml proteinase K, and the samples were mixed with 0.2 volumes of SDS loading buffer, then run on SDS-PAGE (see Section 2.34).

2.47 Construction of artificial supercoiled substrates

Artificial supercoiled substrates were made by ligating natural, modified or mismatched site I, double stranded oligonucleotides into the *Eco*RI and *Sst*I sites of pMM5 (summarised in Figure 3.8). pMM5 (shown in Figure 3.7) was digested with *Eco*RI and *Sst*I, and the large linear fragment was purified by gel electrophoresis using low melting-point agarose (see Section 2.31). This linear fragment was ligated to an annealed, double strand oligonucleotide site I (one strand was labelled at the 5' end with γ-[^{32}P]ATP and T4 kinase, and the other strand's 5' end was fully phosphorylated with unlabelled ATP and T4 kinase). The first ligation was in a volume of 100 µl and contained 1 x ligation buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$; based on Shore *et al.*, 1981), 1 mM ATP, 150 U/ml T4 DNA ligase (BRL); the DNA concentration was about 300 µg/ml. After incubation at room temperature for 15 h, the ligase was inactivated by heating at 70 °C for 5 minutes. The mixture, which now contained concatameric linear DNA, was digested with 800 U/ml *Sst*I by incubation at 37 °C for 1 h, and then the *Sst*I was inactivated by heating at 70 °C for 5 minutes. The mixture was diluted with 1 x ligation buffer to a final DNA concentration of approximately 3 µg/ml, and then 5.1 µg/ml ethidium bromide, 1 mM ATP and 7.5 U/ml T4 DNA ligase were added. After ligation at room temperature for 15 h, the solution was extracted with phenol and the DNA was concentrated by ethanol precipitation. The supercoiled, semisynthetic plasmid was purified by 0.8% low melting-point agarose gel electrophoresis as described in Section 2.31. Natural pMM5 was run adjacent to the ligated products to indicate the position of migration of fully ligated, supercoiled plasmid.

2.48 Topoisomers of supercoiled substrates

To analyse the extrusion of secondary structure from a plasmid substrate containing a single modified *res* site, a set of topoisomers with different superhelical densities was generated using topoisomerase I (see Section 3.3). 1 µg aliquots of plasmid substrate were

mixed with varying concentrations of ethidium bromide in M15M8 buffer. A set of 30 µl samples was prepared which contained 0, 0.5, 1, 2, 3, 4, 6, 8 or 12 µg/ml ethidium bromide. 10 units of topoisomerase I (BRL; calf thymus) was added to each and the samples were incubated at 37 °C for 2 h. The samples were extracted once with phenol and then twice with chloroform. Following addition of 20 mM EDTA and 0.3 M sodium acetate, the topoisomer DNA was recovered by ethanol precipitation. The topoisomers were then either treated individually with S1 nuclease (see Section 2.49) and run on an agarose gel containing 7 µg/ml chloroquine (see Section 2.28), or were combined and run as a single track on a two-dimensional agarose gel (see Section 2.29).

2.49 S1 nuclease digestion

To detect localised regions of base-pair destabilisation and extrusion of secondary structure in a supercoiled substrate, a set of topoisomers with differing superhelical densities (see Section 2.48) was treated with S1 nuclease (Murchie and Lilley, 1989). Aliquots of topoisomer DNA (~1 µg) were mixed with 1 x S1 nuclease buffer (50 mM NaOAc pH 4.6, 50 mM NaCl, 1 mM $ZnCl_2$) and treated with 5 units of S1 nuclease at 16 °C for 30 minutes. The reactions were stopped by addition of 0.2 volumes of SDS loading buffer and then the S1 nuclease-digested topoisomer DNA was run on an agarose gel containing 7 µg/ml chloroquine (see Section 2.28 and Section 3.3).

2.50 Sequencing plasmid DNA

The basic method and buffers used were as described in the Sequenase T7 DNA polymerase technical manual (USB, 1990) which were based on the method of Sanger *et al.*, 1977. The method was modified only in the way in which the plasmid template was prepared. 3 µg plasmid DNA was incubated in 30 µl of 0.2 M NaOH at 37 °C for 10-15 minutes. 10 pmol of sequencing primer was added and the sample was vortexed. 120 µl of ethanol at 0 °C and 12.75 µl 5 M ammonium acetate (pH 7.5) were then added. The sample was mixed and left at -70 °C for ≥ 10 minutes. The template/primer DNA was pelleted by centrifugation (12 000 rpm, 4 °C, 30 minutes), washed in 70% ethanol and then re-pelleted. The DNA was then dried, resuspended and sequenced as described in the Sequenase protocol.

2.51 UV spectrophotometry

DNA concentrations were estimated by measuring the absorbance of diluted DNA at 260 nm on a UV/visible spectrophotometer (Shimadzu). The concentration was then calculated using the approximation that a solution with an absorbance of 1 at 260 nm contains 50 μg/ml double stranded DNA. The absorbance of oligonucleotides was also measured at 260 nm and their concentration calculated using the relationship $A=\varepsilon cl$, (where 'A' is the absorbance at 260 nm, 'ε' is the extinction coefficient (approximately 10 000 l mol^{-1} cm^{-1} per nucleotide), 'c' is the molar concentration and 'l' is the path length of the cell in cm). The absorbance spectrum of modified, photoactivatable oligonucleotides was measured from 200-400 nm using a 'Spectrum' package on the UV/visible spectrophotometer.

2.52 Densitometry

Quantitation of DNA bands on photographic negatives and autoradiographs was done on a Joyce-Loebl scanning microdensitometer. Peaks were cut from the printed spectra and accurately weighed to compare the relative intensities of bands.

Chapter 3

Photocrosslinking resolvase to *res*

Introduction

Site-specific photochemical crosslinking has been widely used in the study of protein-nucleic acid interactions (Blatter *et al.*, 1992; Chen and Ebright, 1993; Nikiforov and Connolly, 1992; Favre, 1990). Usually a modified, photoactivatable base analogue is introduced into an oligonucleotide during synthesis. The DNA-binding protein is then incubated with the modified oligonucleotide binding site and the bound complex is irradiated with strong light of an appropriate wavelength. The resulting protein-DNA complex can be visualised by SDS polyacrylamide gel electrophoresis (SDS/PAGE), either by staining for protein, or using radiolabelled oligonucleotide and exposing to X-ray film.

The photocrosslinking approach has been simply and effectively used to study many different aspects of protein-nucleic acid interaction, e.g. identification of transcription factors bound to promoter/enhancer sites (Kassavetis *et al.*, 1991; Kumar and Sharma, 1993), the movement of replication proteins along DNA (Tinker *et al.*, 1994), and the orientation of DNA-binding proteins relative to their recognition sites (Dumoulin *et al.*, 1993). The majority of work using site-specific photocrosslinking has involved the identification of amino acid-base contacts in the crosslinked protein-DNA complex, which can yield valuable information on how the protein and nucleic acid are juxtaposed (Yang and Nash, 1994; Dong *et al.*, 1994; Chen and Ebright, 1993).

As discussed in Chapter 1, the aim of the work described in this chapter was to try to test the 'subunit rotation' model of strand exchange by Tn*3* resolvase (Stark *et al.*, 1989a). The model proposes that the strand exchange reaction is carried out by a catalytic tetramer of resolvase which cleaves all four DNA strands in a synapsed pair of *res* sites, covalently attaching each resolvase subunit to the site that it has cleaved. One pair of subunits of resolvase then rotates through 180° relative to the other pair of subunits to bring about the recombinant DNA configuration (Figure 1.10). This model is the simplest explanation we have for the DNA topological data and for the 'iterative' recombination products seen in wild type and mutant reactions (Stark *et al.*, 1991; Stark and Boocock, 1994). There is however little biochemical evidence to support subunit rotation.

To test the model directly, photoactivatable nucleotide analogues were incorporated into oligonucleotides which could then be inserted into artificial supercoiled plasmid molecules by ligation *in vitro*. Resolvase could then be covalently crosslinked at these nucleotides in the semi-synthetic plasmid substrate by shining strong UV light of the correct wavelength onto the reaction mixture. This covalently links a single monomer of the catalytic tetramer

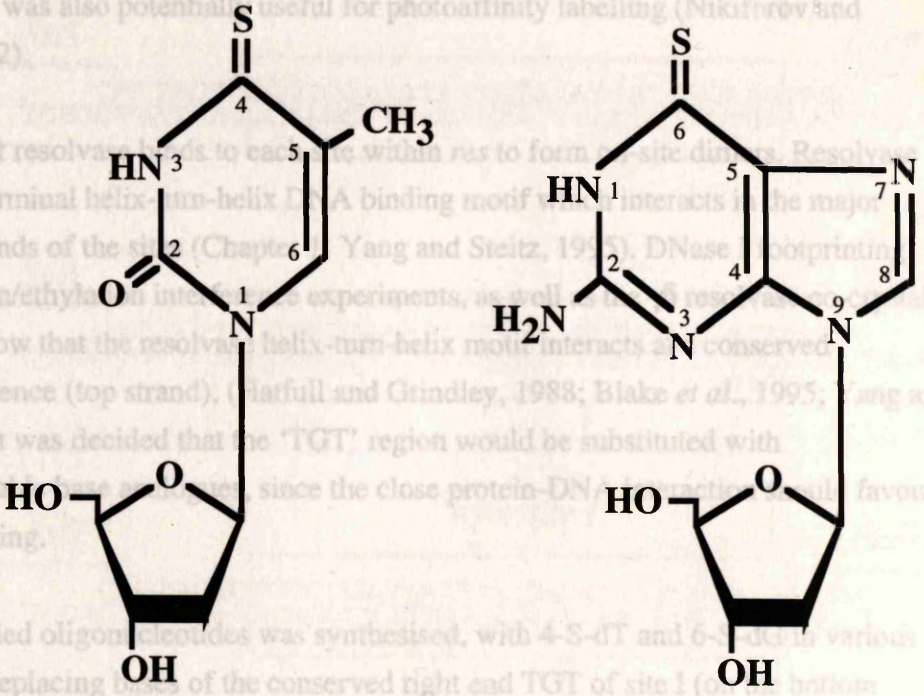
to a specific half-binding site of *res*. If the resolution reaction is then allowed to proceed, recombination products can be assayed by restriction enzyme digestion and SDS/PAGE. By radiolabelling the modified oligonucleotide with γ-[^{32}P] phosphate, it was possible to analyse the crosslinked recombination products by autoradiography, as the photocrosslinked complexes migrate anomalously on SDS/PAGE. This approach was used to ask whether the resolvase covalently attached to the DNA was catalytically active (i.e. gave recombination products), and therefore if the subunit rotation model of strand exchange was valid. Loss of catalytic activity upon crosslinking would suggest that the C-terminal domain of resolvase must dissociate from the *res* DNA during the recombination reaction, and therefore that the subunit rotation model is probably wrong.

The efficiency of photocrosslinking depends upon several factors: (i) the position at which the modified, photoactivatable nucleotide analogue is placed within the DNA; (ii) the nature of the interaction between the DNA-binding motif of the protein and the nucleotide analogue; and (iii) the photoreactivity of the modified nucleotide itself combined with the light source used. There are many photocrosslinkable nucleotides now available, and each has its advantages and disadvantages. The most commonly used analogues include 5-bromouridine, which is readily available and easily incorporated into oligonucleotides (Ebright *et al.*, 1992; Dong *et al.*, 1994), aryl azides such as azidophenacyl derivatives of nucleotides (Yang and Nash, 1994), phenyl-azides (Chen and Ebright, 1993), 4-thiopyrimidines (Nikiforov and Connolly, 1992; Bartholomew *et al.*, 1994; Favre, 1990), 5-mercapto-UTP (He *et al.*, 1995) and 5-iodouridine (Willis *et al.*, 1993).

Results and Discussion

3.1 Photocrosslinking to oligonucleotides containing 4-thiothymidine and 6-thiodeoxyguanosine.

In initial experiments 4-thiothymidine (4-S-dT) and 6-thiodeoxyguanosine (6-S-dG) were incorporated into oligonucleotides by the cyanoethyl phosphoramidite method (Chapter 2). Both of the phosphoramidite nucleotide derivatives were gifts from B. Connolly and T. Nikiforov. The photoreactive sulphur atoms are nucleophilic and would react with reagents used during the synthesis. The modified bases were therefore introduced as protected derivatives and were deblocked post-synthesis to yield 4-S-dT and 6-S-dG (B.Connolly, personal communication). The structures of 4-S-dT and 6-S-dG are shown in Figure 3.1. 4-S-dT was chosen as one photoactivatable base because it is highly photoreactive and introduces only a modest change (oxygen to sulphur) in the natural thymidine base it



4-thiothymidine (4-S-dT) 6-thiodeoxyguanosine (6-S-dG)

Figure 3.1 The structures of 4-thiothymidine and 6-thiodeoxyguanosine.

Replacement of oxygen by sulphur at position 4 of thymidine produces the photoactivatable base analogue 4-thiothymidine. A similar substitution at position 6 of deoxyguanosine produces the photoactivatable base analogue 6-thiodeoxyguanosine. The photochemistry of 4-thiothymidine is reviewed in Favre, 1990.

replaces (reviewed in Favre, 1990). It was hoped that such a slight change would not present a problem in annealing the oligonucleotides or in binding resolvase to the modified site, although the replaced oxygen of the thymidine is known to be involved in base-pair hydrogen bonding. 4-S-dT absorbs at 330 nm (ε = 21 000 $M^{-1}cm^{-1}$), and so the photocrosslinking reaction can be easily effected by exposure to long wavelength UV irradiation, which minimises photodamage to other bases and amino acids. Less data are available for 6-S-dG, but it is also reported to be photoactive, although less reactive than 4-S-dT, and so it was also potentially useful for photoaffinity labelling (Nikiforov and Connolly, 1992).

It is known that resolvase binds to each site within *res* to form on-site dimers. Resolvase contains a C-terminal helix-turn-helix DNA binding motif which interacts in the major groove at the ends of the sites (Chapter 1; Yang and Steitz, 1995). DNase I footprinting and methylation/ethylation interference experiments, as well as the $\gamma\delta$ resolvase co-crystal structure all show that the resolvase helix-turn-helix motif interacts at a conserved 'TGTCT' sequence (top strand), (Hatfull and Grindley, 1988; Blake *et al.*, 1995; Yang and Steitz, 1995). It was decided that the 'TGT' region would be substituted with photocrosslinkable base analogues, since the close protein-DNA interaction should favour photocrosslinking.

A set of modified oligonucleotides was synthesised, with 4-S-dT and 6-S-dG in various combinations replacing bases of the conserved right end TGT of site I (on the bottom strand; Figure 3.2). The left end of site I contains a CGT instead of a TGT, and is thought to bind resolvase less tightly (Rimphanitchayakit and Grindley, 1989). The oligonucleotides were designed such that when annealed to their top strand complementary partners they were equivalent to site I of *res* with *Eco*RI and *Sst*I cohesive ends, that were capable of being ligated into a plasmid at a later stage (Figure 3.8). One set of oligonucleotides was synthesised with an altered sequence to give a symmetrical site I based on the binding site at the right end of site I ('sym-site I'). It was thought that resolvase might bind better to this site, and that this might be advantageous if the modified bases interfered with binding. Incorporation of the thio-analogues was confirmed by UV spectrophotometry. The oligonucleotides containing 4-S-dT had an absorbance at 330 nm as well as at 260 nm, showing that the photocrosslinkable bases were present. The UV spectrum of a modified oligonucleotide is compared with that of a natural equivalent in Figure 3.3.

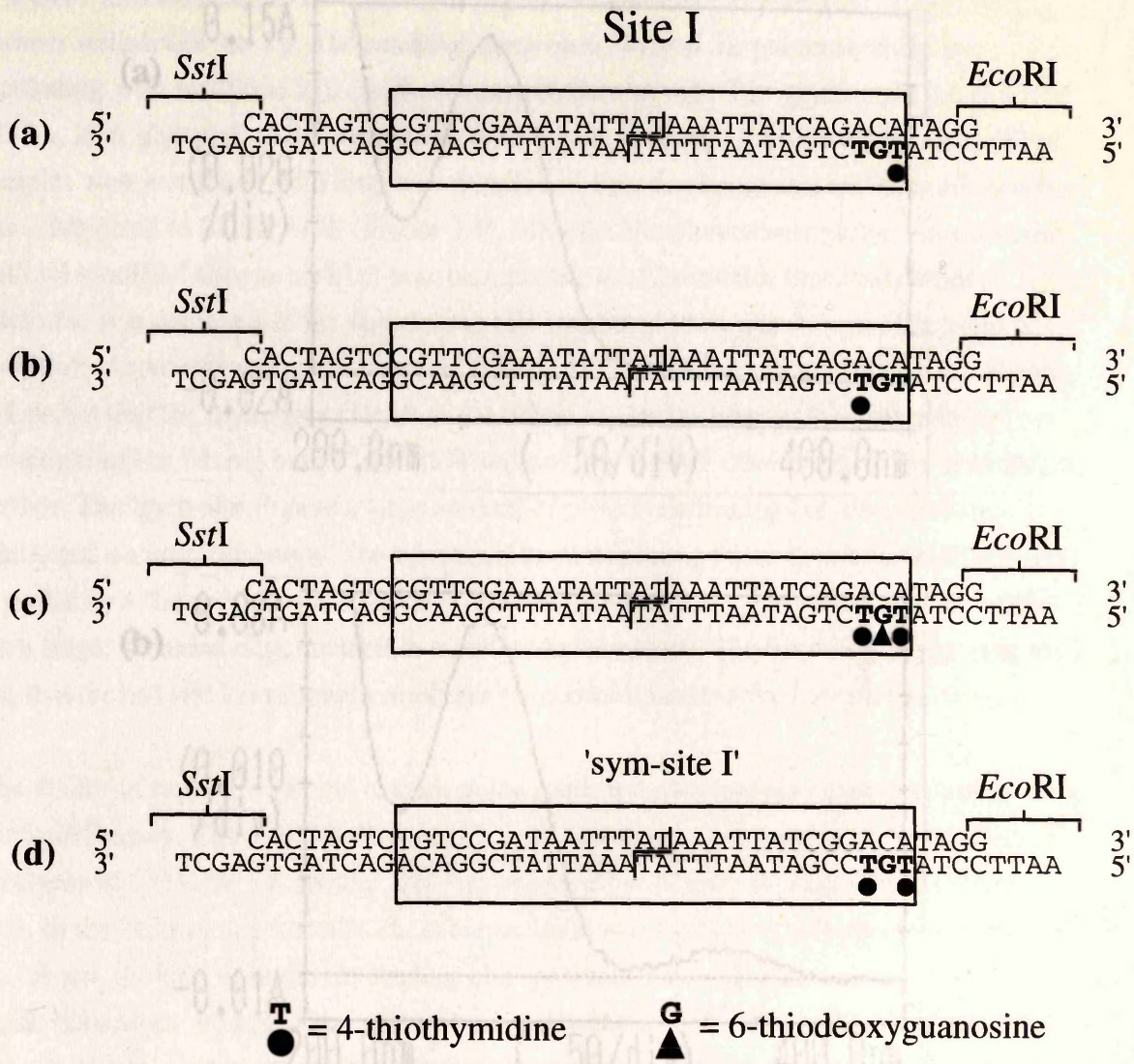


Figure 3.2 Sequence of photoactivatable oligonucleotides containing 4-thiothymidine and 6-thiodeoxyguanosine.

The oligonucleotides shown above were synthesised incorporating 4-S-dT or 6-S-dG at the positions indicated. The oligonucleotides were designed such that when they were annealed in pairs they formed photoactivatable site I's (boxed) with cohesive ends that could be ligated into a plasmid substrate at a later stage. Sites (a), (b), and (c) consist of natural site I sequence (apart from the thio analogue substitutions) but site (d) is a symmetrical site I (called 'sym-site I') which was based on the binding site at the right end of site I.

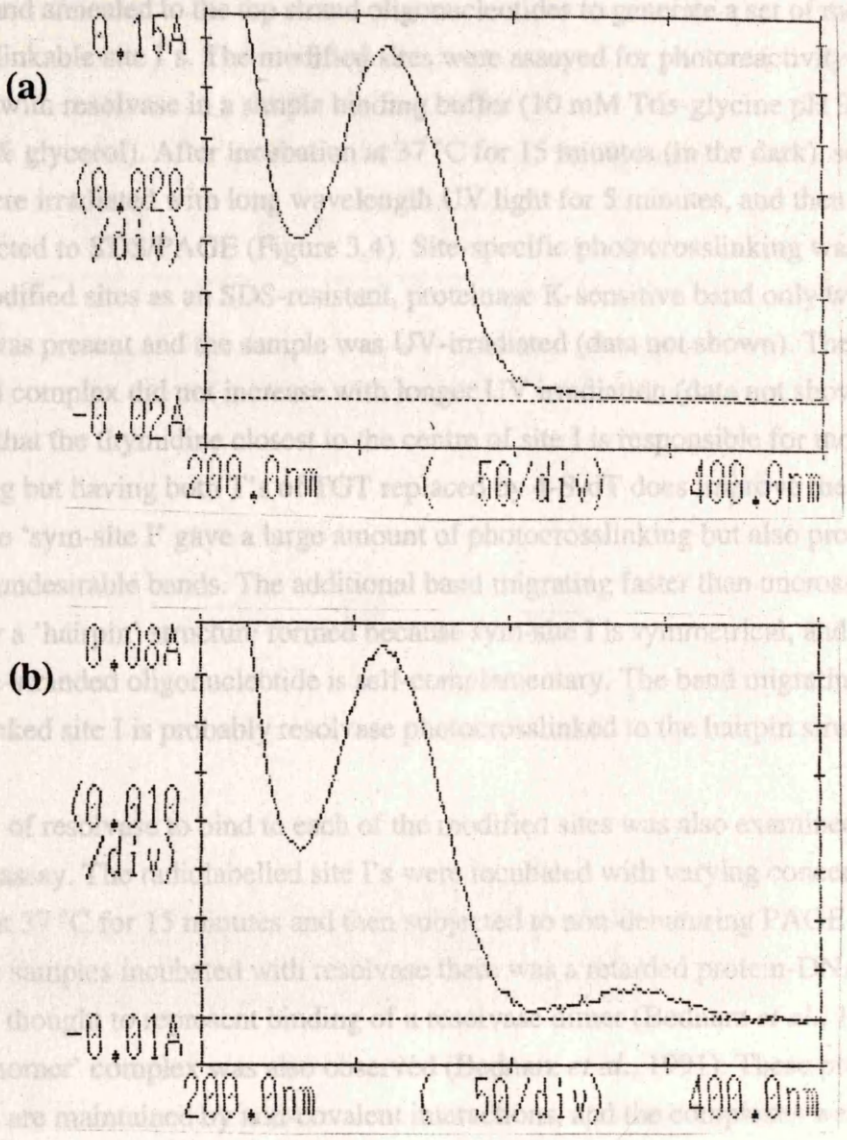


Figure 3.3 The UV absorbance spectrum of an oligonucleotide containing a 4-thiothymidine base analogue.

(a) UV spectrum of a natural site I oligonucleotide. A single peak is detected at ~260 nm.
(b) UV spectrum of a site I oligonucleotide which contains a 4-thiothymidine base analogue. Two peaks are now detected; one at ~260 nm and a second at ~330 nm.

The bottom strand oligonucleotides shown in Figure 3.2 were 5' radiolabelled with γ-[^{32}P]ATP and annealed to the top strand oligonucleotides to generate a set of modified, photocrosslinkable site I's. The modified sites were assayed for photoreactivity by incubating with resolvase in a simple binding buffer (10 mM Tris-glycine pH 9.4, 0.1 mM EDTA, 20% glycerol). After incubation at 37 °C for 15 minutes (in the dark), some of the samples were irradiated with long wavelength UV light for 5 minutes, and then all samples were subjected to SDS/PAGE (Figure 3.4). Site-specific photocrosslinking was observed with all modified sites as an SDS-resistant, proteinase K-sensitive band only when resolvase was present and the sample was UV-irradiated (data not shown). The yield of crosslinked complex did not increase with longer UV irradiation (data not shown). Figure 3.4 shows that the thymidine closest to the centre of site I is responsible for most crosslinking but having both T's of TGT replaced by 4-S-dT does improve the crosslinking further. The 'sym-site I' gave a large amount of photocrosslinking but also produced additional undesirable bands. The additional band migrating faster than uncrosslinked site I is probably a 'hairpin' structure formed because sym-site I is symmetrical, and therefore each single-stranded oligonucleotide is self-complementary. The band migrating faster than the crosslinked site I is probably resolvase photocrosslinked to the hairpin structure.

The ability of resolvase to bind to each of the modified sites was also examined using a band-shift assay. The radiolabelled site I's were incubated with varying concentrations of resolvase at 37 °C for 15 minutes and then subjected to non-denaturing PAGE (Figure 3.5). In the samples incubated with resolvase there was a retarded protein-DNA complex on the gel, thought to represent binding of a resolvase dimer (Bednarz *et al.*, 1991). A very weak 'monomer' complex was also observed (Bednarz *et al.*, 1991). These protein-DNA complexes are maintained by non-covalent interactions, and the complexes were not present on SDS/PAGE (Figure 3.4). Figure 3.5 showed that all of the modified site I's were bound well by resolvase even at the lowest concentrations of protein. The sym-site I was more efficiently bound by resolvase than the natural sequence, but once again there was a substantial amount of hairpin form migrating faster than unbound site I.

For the complete photocrosslinking and recombination experiment to work in a supercoiled, plasmid substrate, it was essential that the photocrosslinked monomer would take part in 'normal' resolvase dimer and tetramer interactions within a synapsed pair of *res* sites. To test if the crosslinked subunit formed part of a normal dimer-DNA complex, two-dimensional gel electrophoresis was used by running crosslinked samples in a standard band-shift assay in the first dimension and then running the same gel, soaked in SDS, at 90° in a second dimension to assay for any photocrosslinked complexes. If the

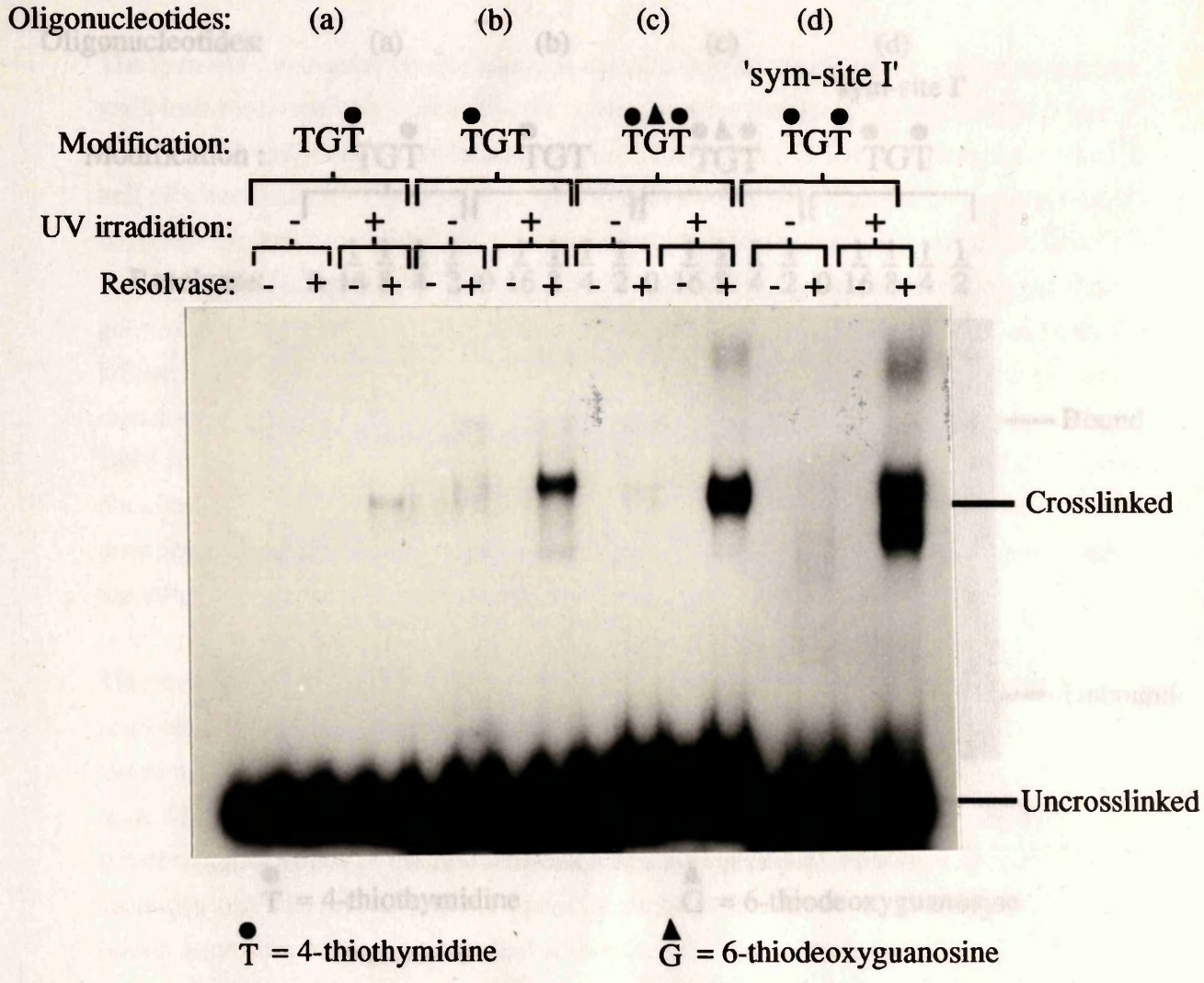


Figure 3.4 UV-crosslinking of resolvase to thio-oligonucleotides on SDS/PAGE.

The modified oligonucleotides shown from left to right are as shown in Figure 3.2(a), (b), (c) and (d) respectively. The labelled site I's were incubated in binding buffer in the absence or presence of resolvase (2 units) and then irradiated with UV light for 5 minutes. The samples were run on a 12% acrylamide, 0.1% SDS gel.

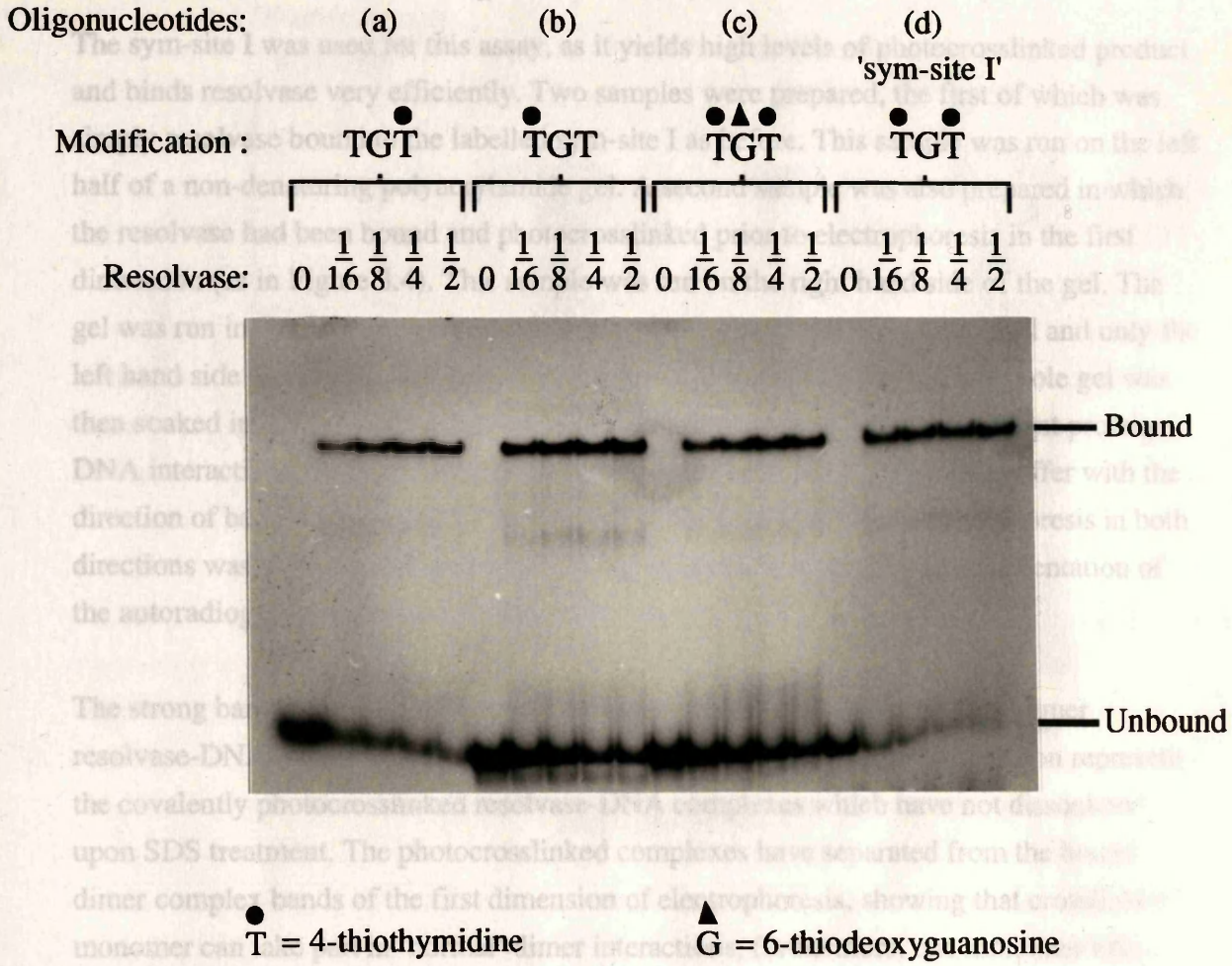


Figure 3.5 Binding of resolvase to thio-oligonucleotides.

The modified oligonucleotides indicated from left to right correspond to those shown in Figure 3.2(a), (b), (c) and (d) respectively. The resolvase concentrations shown are in units where 1 unit is equivalent to a resolvase monomer concentration of approximately 80nM. The labelled site I's were incubated with resolvase in binding buffer (see text) and then run on a 12% acrylamide non-denaturing gel.

photocrosslinked material separated out from the dimer band formed in the first, non-denaturing dimension, then we could assume that the crosslinked monomers had taken part in normal interactions of a resolvase dimer with site I.

The sym-site I was used for this assay, as it yields high levels of photocrosslinked product and binds resolvase very efficiently. Two samples were prepared, the first of which was simply resolvase bound to the labelled sym-site I as before. This sample was run on the left half of a non-denaturing polyacrylamide gel. A second sample was also prepared in which the resolvase had been bound and photocrosslinked prior to electrophoresis in the first dimension (as in Figure 3.4). This sample was run on the right hand side of the gel. The gel was run in the first dimension and then stopped. The plates were separated and only the left hand side of the gel was exposed to UV irradiation for 5 minutes. The whole gel was then soaked in a 0.1% SDS solution for 15 minutes to destroy any non-covalent protein-DNA interactions. The gel was then electrophoresed in an SDS-containing buffer with the direction of band migration at 90° to that in the first direction. After electrophoresis in both directions was complete the gel was dried and exposed to X-ray film; a representation of the autoradiograph is shown in Figure 3.6.

The strong bands retarded in the first dimension represent the monomer and dimer resolvase-DNA complexes. The weaker bands retarded in the second dimension represent the covalently photocrosslinked resolvase-DNA complexes which have not dissociated upon SDS treatment. The photocrosslinked complexes have separated from the bound dimer complex bands of the first dimension of electrophoresis, showing that crosslinked monomer can take part in 'normal' dimer interactions; furthermore, one monomer of a bound dimer can be photocrosslinked within the gel.

This experiment showed that when the C-terminal domain of a resolvase subunit was covalently crosslinked to one end of site I, the subunit was still capable of making normal protein-protein interactions with another subunit. This was important if a photocrosslinked monomer of resolvase was to take part in a recombination reaction within a catalytic tetramer.

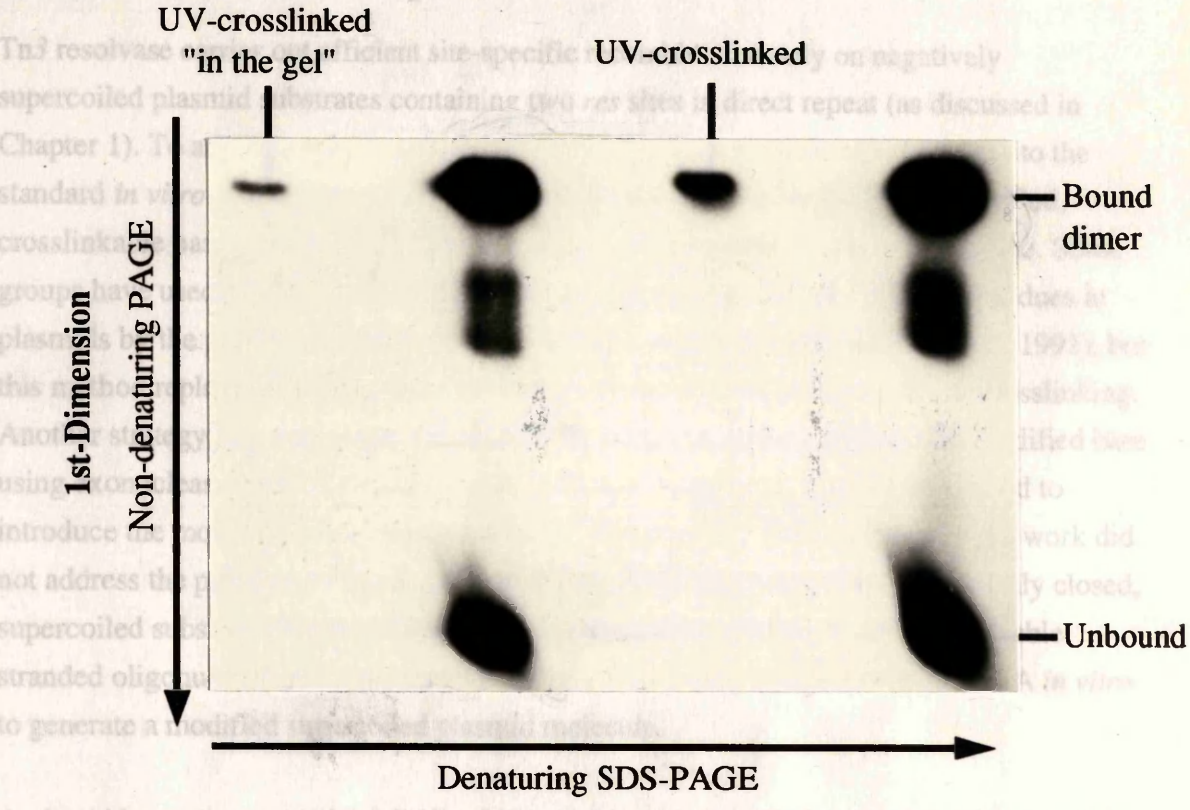


Figure 3.6 Two-dimensional gel electrophoresis of resolvase UV-crosslinked to site I containing 4-thiothymidine.

Labelled 'sym-site I' was used in this assay (see Figure 3.2(d). The sample on the left half of the gel was simply incubated with resolvase (1 unit) in binding buffer. The sample on the right half of the gel was incubated with resolvase (1 unit) in binding buffer and then UV-crosslinked for 5 minutes. Both samples were loaded on a non-denaturing 10% acrylamide gel and electrophoresis in the first dimension was performed as indicated. The left half of the gel was then UV-irradiated for 5 minutes. The whole gel was then soaked in an SDS buffer and electrophoresis was performed in the second dimension using a buffer containing SDS as shown.

3.2 Incorporation of oligonucleotides containing 4-thiothymidine and 6-thiodeoxyguanosine into a supercoiled plasmid substrate.

Tn*3* resolvase carries out efficient site-specific recombination only on negatively supercoiled plasmid substrates containing two *res* sites in direct repeat (as discussed in Chapter 1). To attempt a photocrosslinked recombination reaction, using as close to the standard *in vitro* conditions as possible, it was necessary to incorporate the modified, crosslinkable bases into a site I of *res* in a negatively supercoiled plasmid substrate. Some groups have used modified strains of *E.coli* that can partially replace thymine residues in plasmids by the photocrosslinkable base 5-bromouracil (Katouzian-Safadi *et al.*, 1991), but this method replaces most thymines and does not therefore allow site-specific crosslinking. Another strategy is to use single-stranded DNA templates and incorporate the modified base using exonuclease-free DNA polymerase I (Klenow fragment), which can be used to introduce the modified bases site-specifically (Tinker *et al.*, 1994). However this work did not address the problem of ligating the double stranded DNA molecule to give fully closed, supercoiled substrate. There is no report to date of incorporation of modified, double stranded oligonucleotides into a linearised plasmid and then re-ligation of the DNA *in vitro* to generate a modified supercoiled plasmid molecule.

A plasmid was constructed (pMM5) which contained one natural *res* and another *res* with an engineered *Eco*RI site between sites I and II of *res* (called '*Eco*RI *res*'; Blake *et al.*, 1995) as shown in Figure 3.7. This modified *res* reacts completely normally in resolution assays. pMM5 contains unique *Eco*RI and *Sst*I sites that can be cut to remove site I from one *res* ; it can then be replaced with a modified, photocrosslinkable site I with cohesive *Eco*RI and *Sst*I ends as shown in Figure 3.2.

After considerable manipulation of the reaction conditions, it was found that the most efficient way to incorporate the double stranded oligonucleotides into pMM5 was by a two-step ligation procedure (described in detail in Section 2.47). pMM5 was cut with *Eco*RI and *Sst*I, and the large linear fragment was gel-purified. It was then ligated to double stranded oligonucleotides containing the photoreactive bases. One strand of the oligonucleotide was labelled at the 5' end with γ-[^{32}P]ATP and the other strand was fully phosphorylated with unlabelled ATP. This first ligation step was carried out at high DNA concentrations and resulted in mainly linear, concatameric DNA containing the labelled double strand oligonucleotide. This DNA was then digested with *Sst*I to give linear, monomeric material containing single plasmid molecules ligated to single copies of the

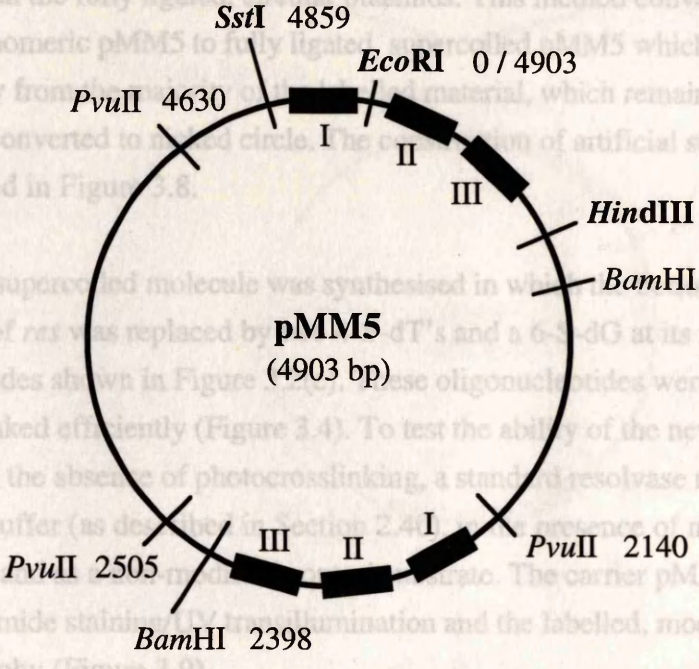


Figure 3.7 A simplified restriction map of pMM5.

pMM5 contains one natural *res* and another *res* with an engineered *Eco*RI site between sites I and II of *res* (see Table 2.1). The unique *Eco*RI and *Sst*I sites can be cut to remove site I from one *res* site, which can then be replaced with a modified oligonucleotide site I with cohesive *Eco*RI and *Sst*I ends such as those shown in Figure 3.2. Unique restriction sites are in bold type. The locations of restriction endonuclease sites that are used in experiments throughout this work are also shown. Site locations are numbered (in bp) relative to the centre of the *Eco*RI site (designated zero).

labelled site I. The *Sst*I was then inactivated at 70 °C for 5 minutes, and a second ligation was carried out at low DNA concentration in the presence of 5 μg ml^{-1} ethidium bromide. The ethidium bromide added intercalates between the base pairs of the DNA and partially unwinds it. Phenol extraction of the ethidium bromide after the ligation results in negative supercoiling in the fully ligated, circular plasmids. This method converted 10-20% of the labelled, monomeric pMM5 to fully ligated, supercoiled pMM5 which could then be gel-purified away from the majority of the labelled material, which remained as linear plasmid or had been converted to nicked circle. The construction of artificial supercoiled molecules is summarised in Figure 3.8.

An artificial supercoiled molecule was synthesised in which the bottom strand 'TGT' of a single site I of *res* was replaced by two 4-S-dT's and a 6-S-dG at its right end, using the oligonucleotides shown in Figure 3.2(c). These oligonucleotides were used because they photocrosslinked efficiently (Figure 3.4). To test the ability of the new substrate to recombine in the absence of photocrosslinking, a standard resolvase reaction was carried out in C9.4 buffer (as described in Section 2.40), in the presence of natural pMM5 to act as carrier DNA and as a non-modified control substrate. The carrier pMM5 was visualised by ethidium bromide staining/UV transillumination and the labelled, modified substrate by autoradiography (Figure 3.9).

The *Hin*dIII restriction analysis showed that the natural pMM5 substrate recombined efficiently to give around 80% recombinant catenane (appearing as 2.3 kbp linear and 2.6 kbp nicked and supercoiled circles). In contrast, the artificial substrate gave almost no recombinant (<5% appearing as a 2.3 kbp linear only, since the label is contained in the smaller domain of pMM5). Attempts were made to purify the new supercoiled substrate further using DEAE-sephacel, and to reduce the superhelical density by titrating with ethidium bromide and relaxing the DNA with topoisomerase I. Neither strategy improved the yield of recombinant products (data not shown).

The lack of efficient recombination of the artificial supercoiled substrate could have been due to several factors: (i) the thio-modifications were somehow blocking recombination; (ii) the synthetic oligonucleotides were somehow adversely affecting the *res* site and resolution; (iii) the method of making the artificial supercoiled substrate was deleterious to recombination, perhaps by giving the wrong negative superhelical density. To address these questions, an artificial substrate was synthesised using 'wild type' oligonucleotides containing no modifications, and was purified using precisely the same method as before. A standard resolution reaction was again carried out on this substrate in the presence of

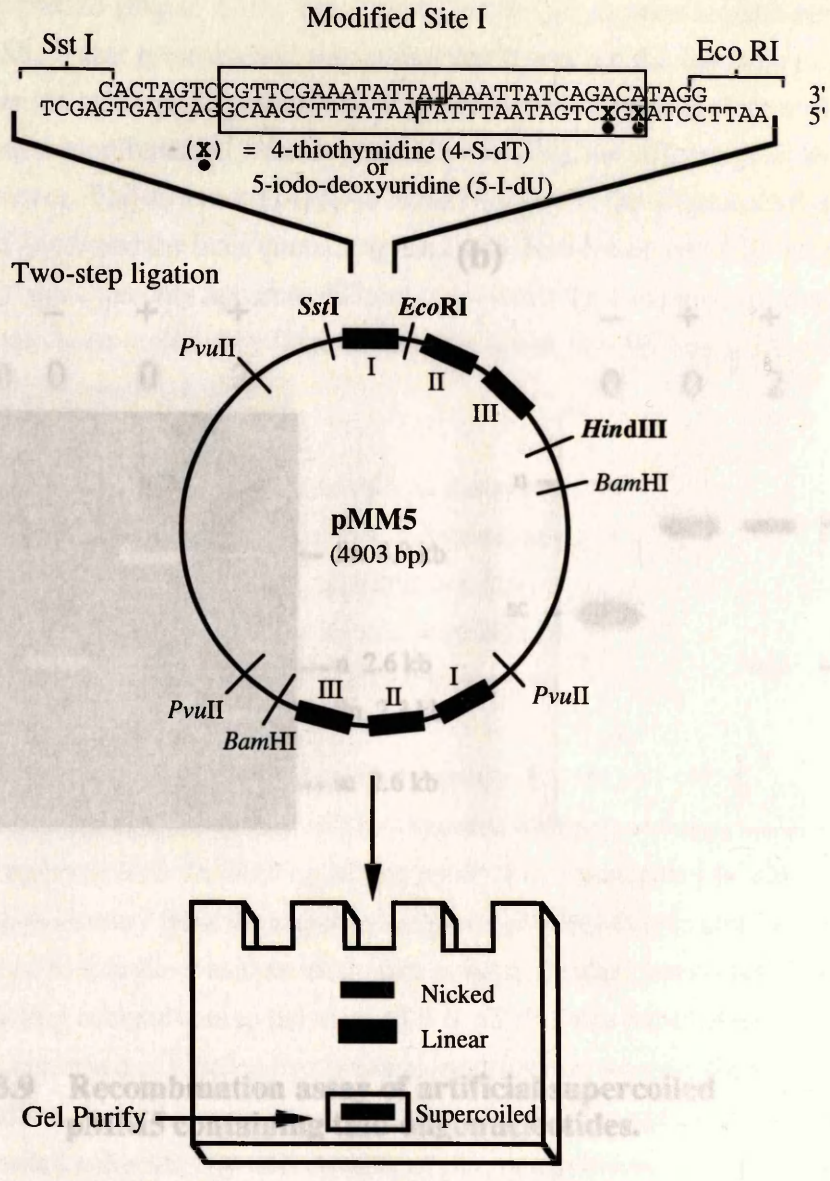


Figure 3.8 The construction of artificial supercoiled molecules containing modified site I oligonucleotides.

Modified site I's were incorporated into artificial supercoiled substrate molecules using the proceedure summarised above. pMM5 was digested with *Eco*RI and *Sst*I (to remove a site I) and the large linear fragment was gel-purified. Labelled, phosphorylated oligonucleotide site I was ligated to the purified pMM5 to give mainly linear, concatameric DNA. This DNA was then digested with *Sst*I to give linear, monomeric pMM5 ligated to a single copy of the labelled site I. A second ligation was then performed at low DNA concentrations in the presence of ethidium bromide. Phenol extraction of the ethidium bromide after the ligation results in negative supercoiling of fully ligated plasmids. The artificial supercoiled pMM5 was then gel-purified away from the linear and nicked pMM5. For full experimental details see Section 2.47.

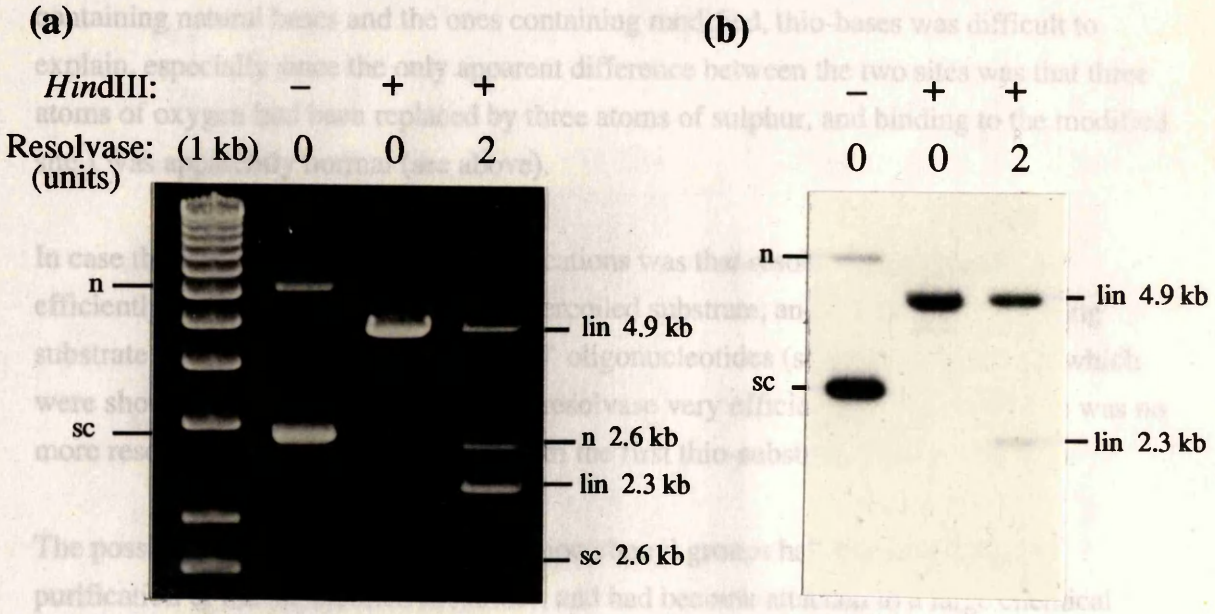


Figure 3.9 Recombination assay of artificial supercoiled pMM5 containing thio-oligonucleotides.

(a) Recombination of natural pMM5 (ethidium staining of gel). The first lane contains a 1 kb size ladder (BRL). Nicked circles are indicated by 'n' and supercoiled circles by 'sc'. Restriction of substrate pMM5 by *Hin*dIII produces a 4.9 kbp non-recombinant linear (lin 4.9 kb). Restriction of resolution products by *Hin*dIII produces a 2.3 kbp recombinant linear and nicked and supercoiled versions of the 2.6 kbp circle as indicated.
(b) Recombination of artificial supercoiled pMM5 which contains the thio-oligonucleotides shown in Figure 3.2(c) (autoradiograph of gel). Labelling is as for (a).

All reactions contained both natural and modified pMM5, were performed in C9.4 buffer and were run on the same 1% agarose gel. (a) and (b) show the same gel (ethidium-stained in (a), autoradiograph in (b)).

unlabelled, carrier pMM5 (Figure 3.10). This time the artificial substrate recombined to give 60-70% 2.3 kbp linear recombinant, suggesting that it was not the insertion of oligonucleotides or the subsequent purification of the supercoiled substrate that was at fault, but that the thio-modifications were dramatically reducing the efficiency of the recombination reaction. The difference between recombination of the oligonucleotides containing natural bases and the ones containing modified, thio-bases was difficult to explain, especially since the only apparent difference between the two sites was that three atoms of oxygen had been replaced by three atoms of sulphur, and binding to the modified site I was apparently normal (see above).

In case the problem with the thio-modifications was that resolvase was bound less efficiently to the modified site in the supercoiled substrate, another 4-S-dT containing substrate was made using the 'sym-site I' oligonucleotides (shown in Figure 3.2) which were shown to bind and photocrosslink resolvase very efficiently. However, there was no more resolution of this substrate than with the first thio-substrate (data not shown).

The possibility that the highly reactive thiocarbonyl groups had reacted during the purification of the supercoiled substrates, and had become attached to a large chemical group that was interfering with the binding of resolvase, was investigated by cutting out the 4-S-dT-containing sym-site I from the supercoiled plasmid using *Eco*RI and *Sst*I. The binding of resolvase to this site was then examined using a standard band-shift assay (Figure 3.11). Binding of resolvase to the excised 4-S-dT site was completely normal, suggesting that interference of binding by chemical modification was not the reason for the lack of recombination. A similar approach was used to check if the site I cut from the artificially supercoiled substrate was still capable of photocrosslinking. Resolvase was incubated with the modified, supercoiled substrate and UV irradiated; the DNA was then cut with *Eco*RI and *Sst*I. The reactions were then run on SDS/PAGE; no photocrosslinking was observed (data not shown).

The lack of photocrosslinking was not unexpected because the oligonucleotides containing 4-S-dT had never photocrosslinked in the presence of 'carrier' DNA (data not shown). The lack of crosslinking in the presence of carrier DNA was never fully understood; in this case the pMM5 vector, in which the 4-S-dT site I was contained, was perhaps acting as 'carrier' and inhibiting photocrosslinking. It was concluded that when oligonucleotides containing 4-thiothymidine and 6-thiodeoxyguanosine were incorporated into artificial supercoiled plasmid substrates, the result was (i) little or no recombination and (ii) no photocrosslinking.

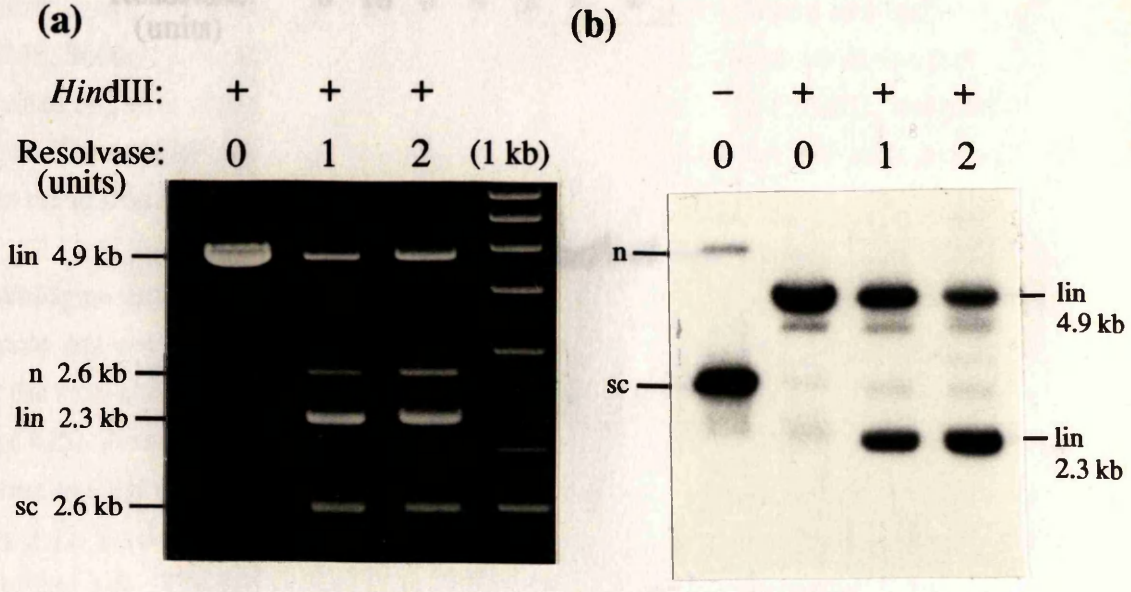


Figure 3.10 Recombination assay of artificial supercoiled pMM5 containing 'wild type' oligonucleotides.

(a) Recombination of natural pMM5 (ethidium-staining of gel). Labelling of the Figure is as for Figure 3.9. Nicked circles are indicated by 'n' and supercoiled circles by 'sc'. Restriction of substrate pMM5 by *Hin*dIII produces a 4.9 kbp non-recombinant linear (lin 4.9 kb). Restriction of resolution products by *Hin*dIII produces a 2.3 kbp recombinant linear and nicked and supercoiled versions of the 2.6 kbp circle as indicated.
(b) Recombination of artificial supercoiled pMM5 which contains 'wild type' site I oligonucleotides (autoradiograph).

All reactions contained both natural and modified pMM5, were performed in C9.4 buffer and were run on the same 1% agarose gel. Both photographs are of the same gel (as Figure 3.9).

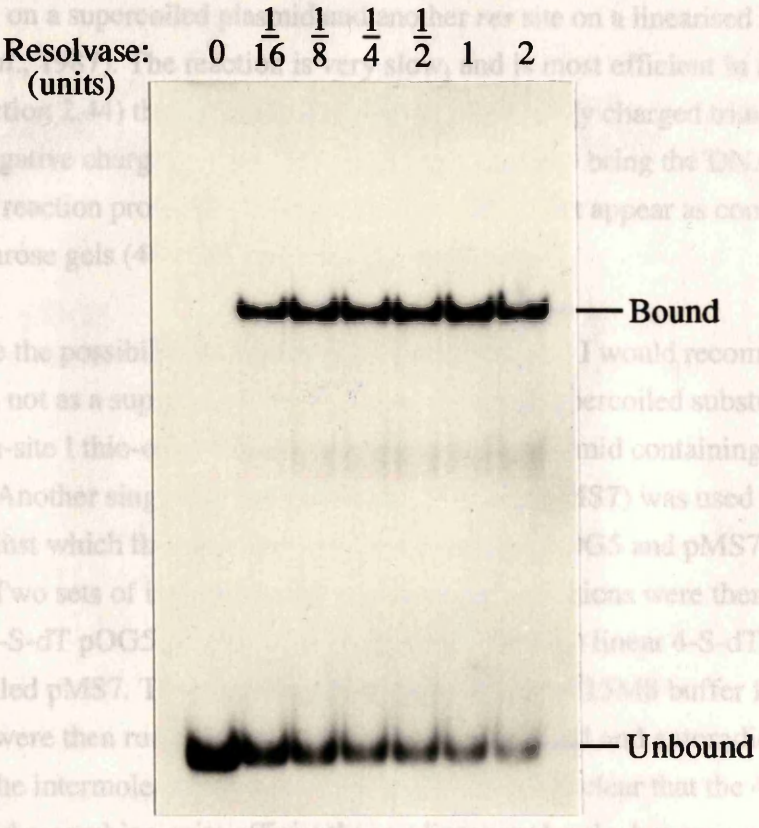


Figure 3.11 Binding of resolvase to a thio-oligonucleotide site I cut from an artificial supercoiled substrate.

The oligonucleotide sym-site I (see Figure 3.2(c)), which contains 4-S-dT base analogues, was cut from an artificial supercoiled pMM5 substrate using *Eco*RI and *Sst*I. This site was incubated with various concentrations of resolvase in binding buffer and then loaded on a 10% acrylamide non-denaturing gel.

3.3 Further investigation of the effect of supercoiling on substrates containing 4-S-dT and 6-S-dG.

Tn*3* resolvase is capable of catalysing intermolecular recombination reactions between a single *res* site on a supercoiled plasmid and another *res* site on a linearised plasmid (Boocock *et al.*, 1987). The reaction is very slow, and is most efficient in a buffer (M15M8, Section 2.44) that contains spermidine, a positively charged triamine that neutralises negative charges on the DNA, making it easier to bring the DNA molecules together. The reaction proceeds to recombinant products that appear as concatamerised linears on agarose gels (40-50% on overnight incubation).

To investigate the possibility that the 4-S-dT containing site I would recombine as a linear molecule, but not as a supercoiled molecule, an artificial supercoiled substrate was made using the sym-site I thio-oligonucleotide inserted into a plasmid containing only a single *res* site (pOG5). Another single *res* site-containing plasmid (pMS7) was used as the wild-type substrate against which the modified pOG5 was reacted. pOG5 and pMS7 are shown in Figure 3.12. Two sets of intermolecular recombination reactions were then carried out: (i) supercoiled 4-S-dT pOG5 reacted with linear pMS7, and (ii) linear 4-S-dT pOG5 reacted with supercoiled pMS7. The reactions were performed in M15M8 buffer for 60 h at 37 °C and samples were then run on agarose gels which were dried and autoradiographed (Figure 3.13). From the intermolecular recombination results it was clear that the 4-S-dT-modified substrate could recombine quite efficiently as a linear molecule, but gave no recombinant products as a supercoiled molecule.

It was possible that the replacements of oxygen by sulphur had affected hydrogen bonding at the modified bases (since the oxygen at position four of thymidine is involved in Watson-Crick hydrogen bonding), and that this combined with supercoiling (which is known to facilitate the melting of base-pairs) was causing localised destabilisation of base pairing. Even a small change in DNA secondary structure might disrupt the binding and protein-protein interactions of resolvase, which could explain the poor yields of recombination products. The problem may have been made worse by the fact that the sites of *res* are imperfect palindromes, and especially that the sym-site I construct contains a perfect palindrome. Perfect and even imperfect palindromic sequences can lead to the possibility of cruciform formation, and these structures can be stabilised in negatively supercoiled DNA (Benham, 1982; Vologodskii and Frank-Kamenetskii, 1982).

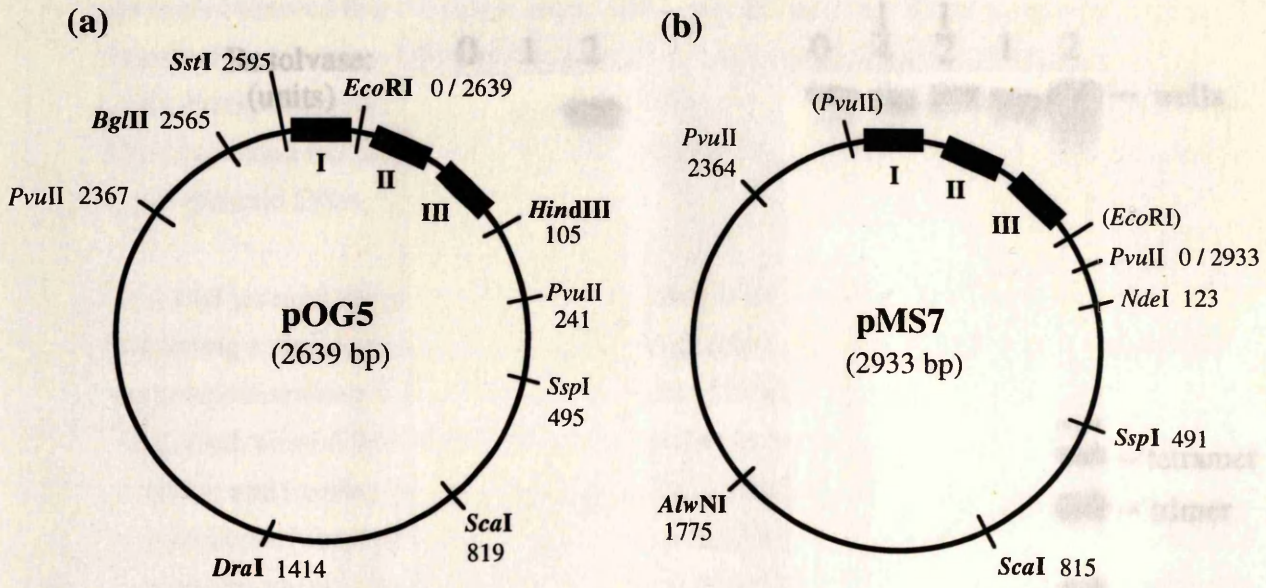


Figure 3.12 Simplified restriction maps of pOG5 and pMS7.

(a) pOG5 contains only one *res* site which has an engineered *Eco*RI site between sites I and II of *res* (see Table 2.1). The unique *Eco*RI and *Sst*I sites can be cut to remove site I from one *res* site, which can then be replaced with a modified oligonucleotide site I with cohesive *Eco*RI and *Sst*I ends such as those shown in Figure 3.2. Unique restriction sites are in bold type. Site locations are numbered (in bp) relative to the centre of the *Eco*RI site (designated zero).
(b) pMS7 contains only one natural *res* site (see Table 2.1). Labelling of restriction sites is as in (a). Site locations are numbered (in bp) relative to the centre of the *Pvu*II site (designated zero).

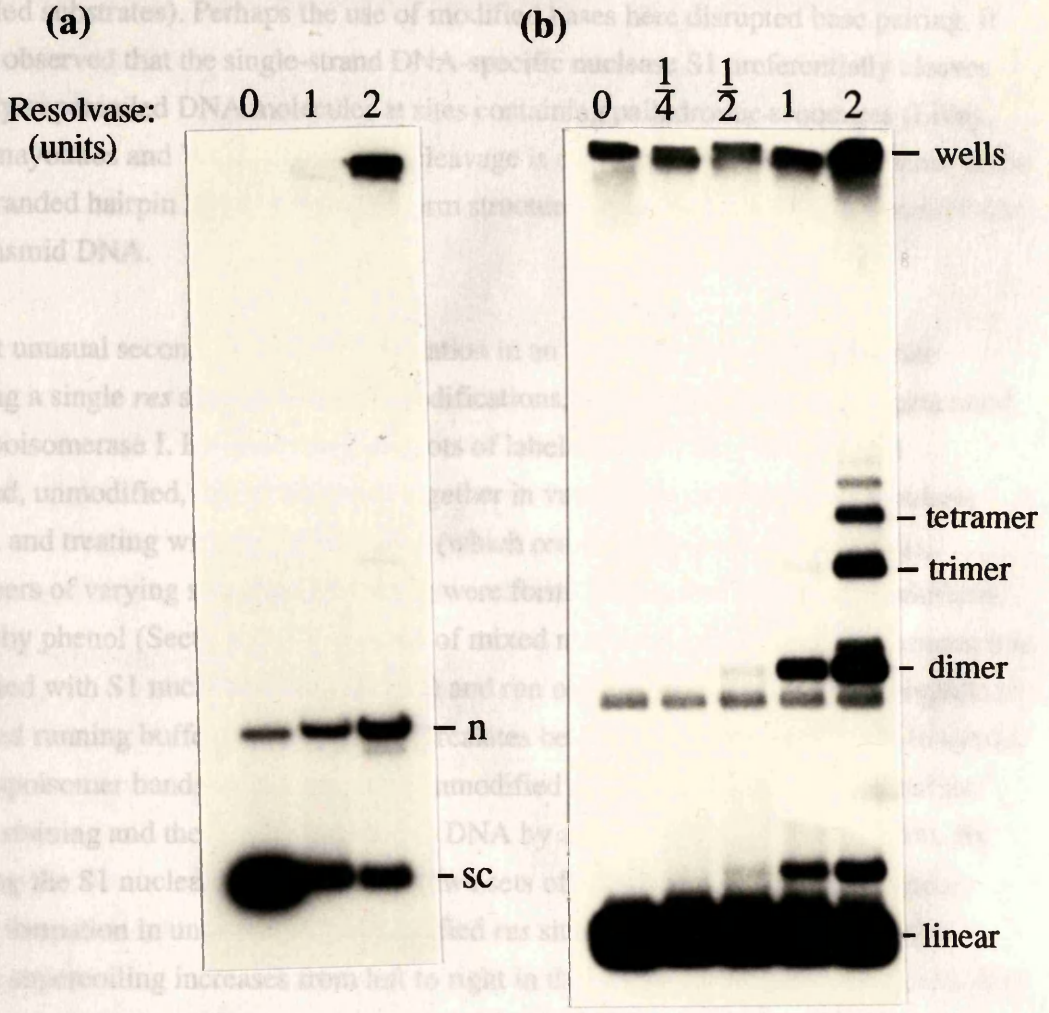


Figure 3.13 Intermolecular recombination between pOG5 and pMS7.

(a) Intermolecular recombination reaction between artificial supercoiled 4-S-dT pOG5 and linear pMS7. Only the supercoiled pOG5 is radiolabelled and is visible on the gel.
(b) Intermolecular recombination reaction between linear 4-S-dT pOG5 and supercoiled pMS7. Only the linear pOG5 is radiolabelled and is visible on the gel. Intermolecular recombination products can be seen as linear concatamers labelled 'dimer', 'trimer', and 'tetramer'.
The reactions shown in (a) and (b) were performed in M15M8 buffer (see Section 2.44) and the products were loaded on a 1% agarose gel.

Cruciform formation is known to be very slow under physiological conditions, but can be greatly enhanced when the DNA is subjected to conditions that destabilise base pairing (Courey and Wang, 1983) such as heating (which is used in the purification of the artificial supercoiled substrates). Perhaps the use of modified bases here disrupted base pairing. It has been observed that the single-strand DNA-specific nuclease S1 preferentially cleaves negatively supercoiled DNA molecules at sites containing palindromic sequences (Lilley, 1980; Panayotatos and Wells, 1981). The cleavage is caused by attack of the nuclease at the single-stranded hairpin loops of the cruciform structure, resulting in nicking or linearisation of the plasmid DNA.

To detect unusual secondary structure formation in an artificial supercoiled substrate containing a single *res* site with 4-S-dT modifications, a set of topoisomers was generated using topoisomerase I. By incubating aliquots of labelled, modified substrate and unlabelled, unmodified, carrier substrate together in varying concentrations of ethidium bromide, and treating with topoisomerase I (which removes negative supercoiling), topoisomers of varying superhelical density were formed upon extraction of the ethidium bromide by phenol (Section 2.48). This set of mixed modified and natural topoisomers was then treated with S1 nuclease (Section 2.49) and run on an agarose gel with chloroquine in the gel and running buffer (chloroquine intercalates between base pairs and helps to spread out the topoisomer bands on the gel). The unmodified DNA was visualised by ethidium bromide staining and the labelled, modified DNA by autoradiography (Figure 3.14). By comparing the S1 nuclease cleavage of the two sets of topoisomers, unusual secondary structure formation in unmodified and modified *res* sites could be compared directly. Negative supercoiling increases from left to right in the samples of Figure 3.14, and at high levels of negative supercoiling even the unmodified DNA was cleaved, presumably due to cruciform extrusion. More importantly, at the higher levels of negative supercoiling (>4 $\mu g\ ml^{-1}$ ethidium bromide), all of the modified topoisomers were completely cleaved by S1 nuclease, but some unmodified DNA had not been cleaved. It was concluded that the *res* containing 4-S-dT was extruding unusual secondary structure to a greater extent than the natural *res* site.

Two-dimensional gel electrophoresis has also been used to detect negative supercoiling-induced cruciform formation (Courey and Wang, 1983; Wang *et al.*, 1982). To look for the presence of cruciforms in the modified, 4-S-dT *res* site, the topoisomers were combined to make a 'ladder', and run in two dimensions on an agarose gel. The topoisomers were run in the first direction on a standard agarose gel; the gel was then soaked in buffer containing chloroquine, and run at 90° to the first direction in chloroquine buffer. Topoisomers

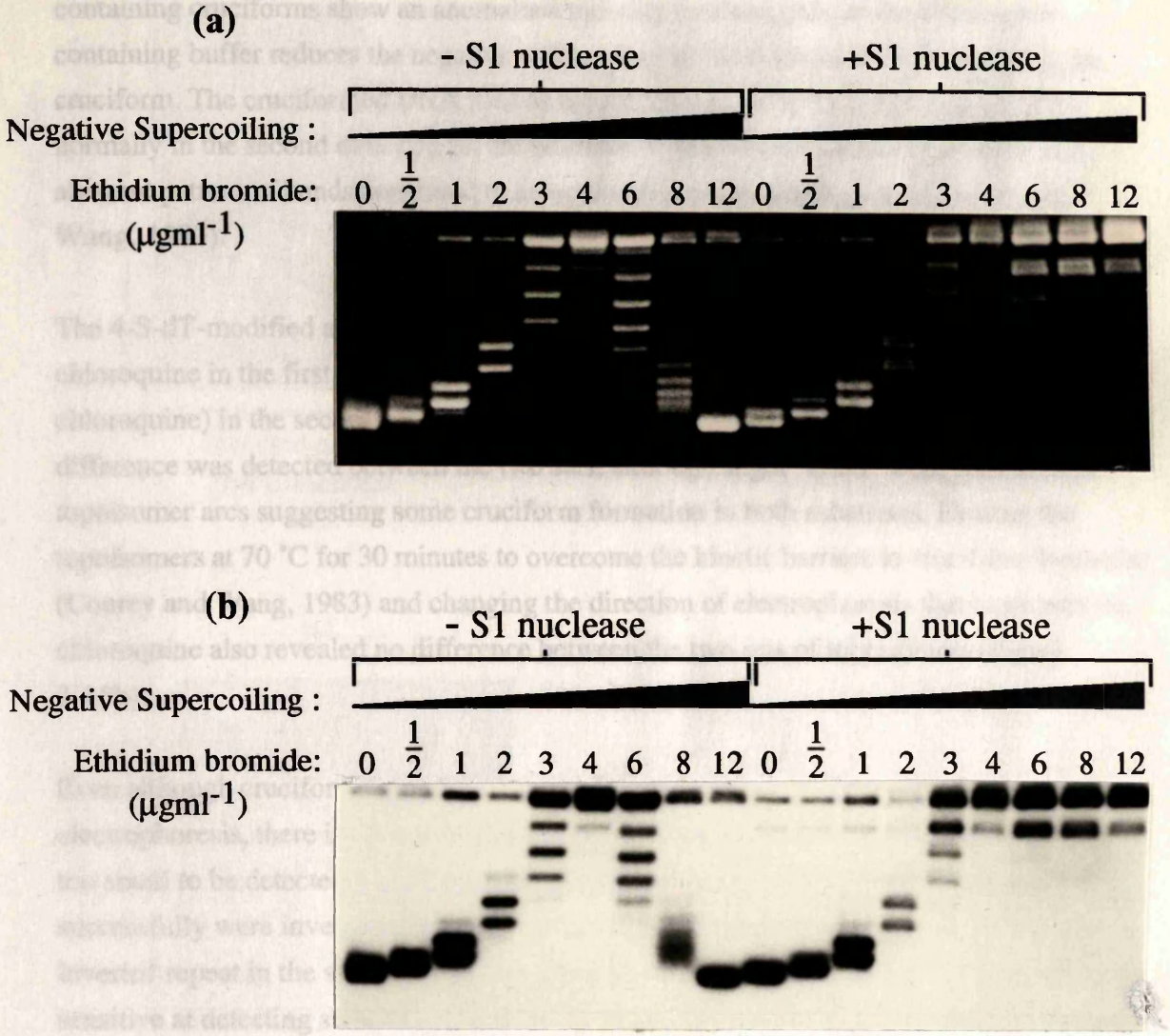


Figure 3.14 S1 nuclease cleavage of topoisomers containing 4-S-dT.

(a) S1 nuclease cleavage assay of topoisomers containing natural *res* sites. The concentration of ethidium bromide used to generate each topoisomer is shown. The resulting increase in negative supercoiling of the topoisomers is also shown.
(b) S1 nuclease cleavage assay of topoisomers containing *res* sites with 4-S-dT modifications. The Figure is labelled as for (a).
Both sets of topoisomers were generated together and were treated together with S1 nuclease in S1 nuclease buffer (see Section 2.49). The products were loaded on the same 1.2% agarose gel containing 7 μg/ml chloroquine.

containing cruciforms show an anomalous mobility on these gels, as the chloroquine-containing buffer reduces the negative supercoiling of the plasmid, thus destabilising the cruciform. The cruciformed DNA runs as retarded bands in the first direction but runs normally in the second direction (in the presence of chloroquine) resulting in an overall aberrant pattern of bands compared to an uncruciformed topoisomer arc (Courey and Wang, 1983).

The 4-S-dT-modified and unmodified topoisomers were run on a gel containing chloroquine in the first dimension, and on a standard agarose gel (containing no chloroquine) in the second dimension and are compared in Figure 3.15a. No definitive difference was detected between the two sets, although slight 'kinks' were seen in both topoisomer arcs suggesting some cruciform formation in both substrates. Heating the topoisomers at 70 °C for 30 minutes to overcome the kinetic barriers to cruciform formation (Courey and Wang, 1983) and changing the direction of electrophoresis that contained the chloroquine also revealed no difference between the two sets of topoisomers (Figure 3.15b).

Even although cruciform formation was not detected using two-dimensional gel electrophoresis, there is a possibility that the cruciform or other secondary structure was too small to be detected using this method, as other groups who have used this method successfully were investigating longer palindromic sequences, typically 70-100 bp. The inverted repeat in the sym-site I is only 28 bp long. The S1 nuclease assay is perhaps more sensitive at detecting smaller cruciforms and loops, and does in this case demonstrate that the 4-S-dT (and 6-S-dG)-modified *res* sites do form unusual secondary structure more readily than their natural equivalent sites. This observation, and the result that the modified *res* site is capable of recombining as a linear molecule, but not as a supercoiled molecule, provide compelling evidence that the lack of recombination with the thio-modified *res* site was due to formation of a small region of abnormal secondary structure within site I in the artificial supercoiled substrate.

The failure of photocrosslinking of resolvase to the 4-S-dT site I after it had been incorporated into a supercoiled plasmid (Section 3.2) was another serious concern if 4-S-dT were to be used in this type of experiment. The phenomenon was not investigated further, although a small amount of photocrosslinking was observed when the site was incorporated into a linear plasmid molecule (data not shown).

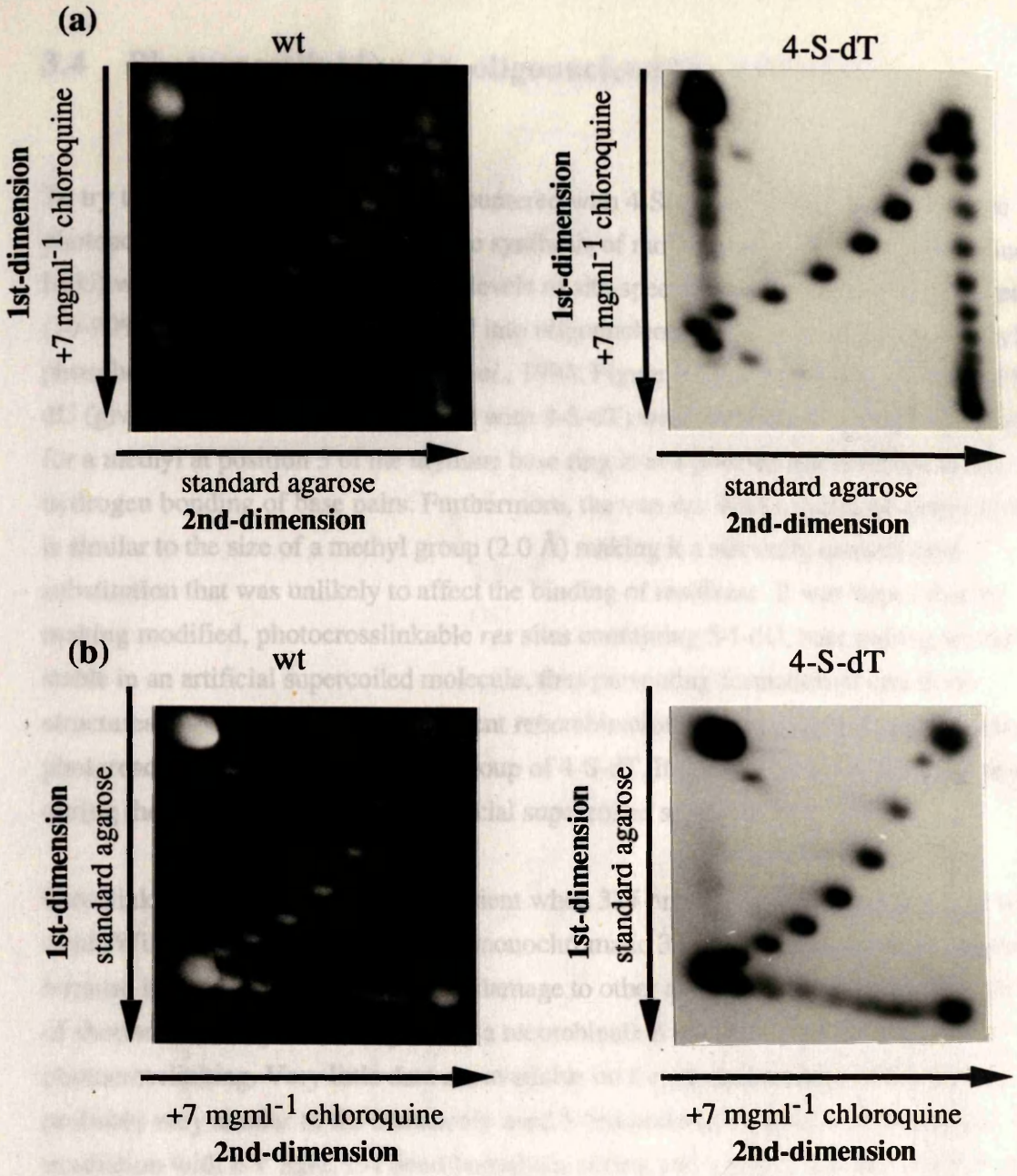


Figure 3.15 Two-dimensional gel electrophoresis of 4-S-dT topoisomers.

(a) Comparison of topoisomers containing natural *res* sites (on the left) and topoisomers containing *res* sites with 4-S-dT modifications (on the right). The modified and unmodified topoisomers were mixed and run on the same 1.2% agarose gel, which contained chloroquine in the first direction of electrophoresis but not in the second (as indicated). (b) As (a) except (i) the topoisomers were heated at 70 °C for 30 minutes before loading onto the gel, and (ii) chloroquine was added in the second direction of electrophoresis, but not in the first.

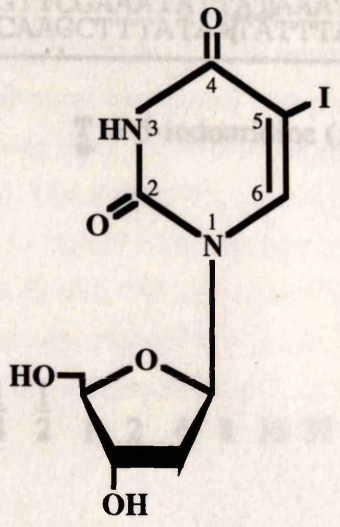
3.4 Photocrosslinking to oligonucleotides containing 5-iodouracil.

To try to overcome the problems encountered with 4-S-dT and 6-S-dG, an alternative photoactivatable base was used for the synthesis of modified sites. 5-iododeoxyuridine (5-I-dU) was reported to give very high levels of site-specific photocrosslinking to proteins (70-90%) and was easily incorporated into oligonucleotides using standard cyanoethyl phosphoramidite chemistry (Willis *et al.*, 1993; Figure 3.16a). A major advantage of 5-I-dU (given the difficulties encountered with 4-S-dT) was that the iodo-group substituting for a methyl at position 5 of the thymine base ring is at a position not involved in the hydrogen bonding of base pairs. Furthermore, the van der Waals radius of iodine (2.15 Å) is similar to the size of a methyl group (2.0 Å) making it a sterically conservative substitution that was unlikely to affect the binding of resolvase. It was hoped that by making modified, photocrosslinkable *res* sites containing 5-I-dU, base pairing would be stable in an artificial supercoiled molecule, thus preventing formation of cruciform structures and thereby allowing efficient recombination. The C-I bond of 5-I-dU is less photoreactive than the thiocarbonyl group of 4-S-dT. It is therefore more likely to be stable during the gel purification of the artificial supercoiled substrate.

Crosslinking to 5-I-dU was most efficient when 325 nm monochromatic laser light was used (Willis *et al.*, 1993). The use of monochromatic 325 nm light is also advantageous because it is less likely to cause photodamage to other amino acids and bases than UV light of shorter wavelength; this might help a recombination reaction to proceed after photocrosslinking. Very little data are available on the photochemistry of 5-I-dU, but it is probably very similar to the commonly used 5-bromodeoxyuridine, such that upon irradiation with UV light, C-I bond homolysis occurs and a highly reactive uracilyl radical is generated (Blatter *et al.*, 1992). If there is organic material in direct van der Waals contact with the radical, covalent bond formation may occur (Figure 3.16b).

A pair of oligonucleotides was synthesised with 5-I-dU bases replacing both thymines of the conserved TGT on the bottom strand, right hand end of site I (Figure 3.17a). The 5-I-dU absorbs at around 308 nm with about an eighth the absorbance of 4-S-dT (ε = 2600 $M^{-1}cm^{-1}$) and so 5-I-dU incorporation could not be detected easily by UV spectrophotometry (data not shown). The oligonucleotides were again synthesised with *Eco*RI and *Sst*I cohesive ends for subsequent ligation into pMM5. The bottom strand oligonucleotide was 5' labelled with γ-[^{32}P]ATP and T4 kinase, and annealed to the top strand to give 5-I-dU site I. The labelled, modified site was incubated with resolvase at

(a)



5-iodouridine (5-I-dU)

(b)

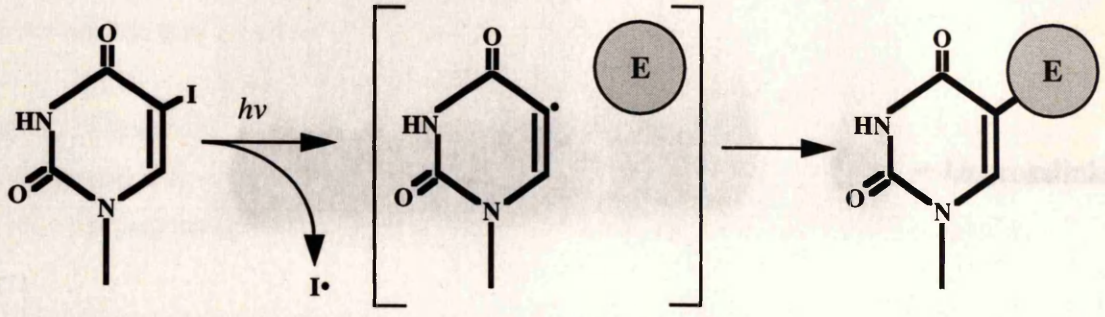


Figure 3.16 The structure and photochemistry of 5-iodouracil (5-I-dU).

(a) The structure of 5-iodouridine. Replacement of methyl by iodine at position 5 of thymidine produces the photoactivatable base analogue 5-iodouridine.
(b) Photochemistry of 5-iodouridine. On irradiation with ultraviolet laser light, bond homolysis occurs and a highly reactive uracilyl radical is generated. If there is organic material (shown as a stippled circle) in direct van der Waals contact with the uracilyl radical, covalent bond formation may occur. Figure adapted from Blatter *et al.*, 1992.

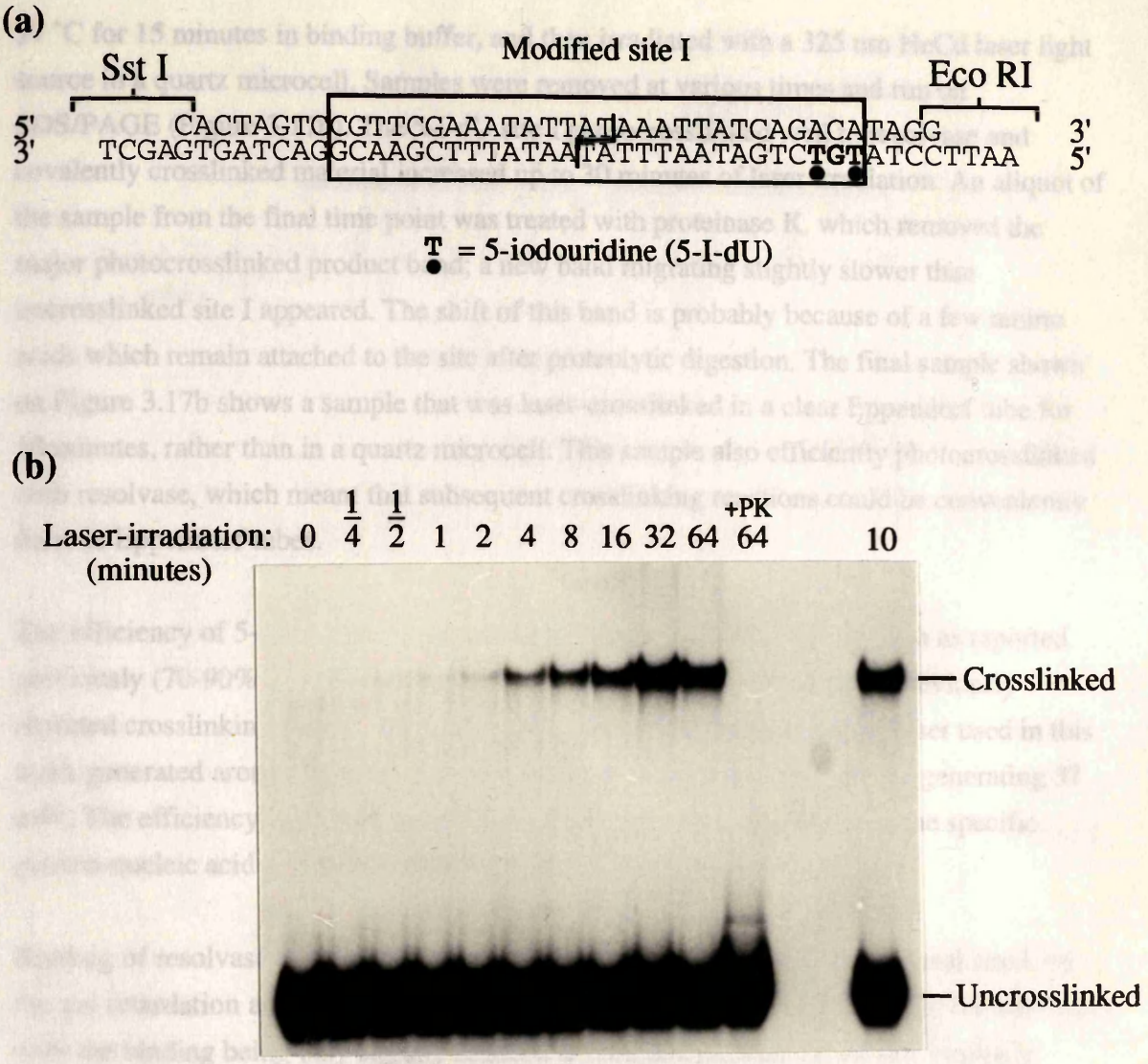


Figure 3.17 Laser-crosslinking of resolvase to 5-I-dU site I.

(a) The sequence of the modified site I oligonucleotides containing two 5-I-dU substitutions at the right hand end. (b) The labelled site I (shown in (a)) was incubated in binding buffer with resolvase (2 units per track) and then laser-irradiated in a quartz microcell for over an hour. Samples were removed at the times shown. One sample was treated with proteinase K (+PK). The final sample was irradiated for 10 minutes in an Eppendorf tube. All samples were then run on a 10% acrylamide, 0.1% SDS gel.

37 °C for 15 minutes in binding buffer, and then irradiated with a 325 nm HeCd laser light source in a quartz microcell. Samples were removed at various times and run on SDS/PAGE (Figure 3.17b). The 5-I-dU site I photocrosslinked well to resolvase and covalently crosslinked material increased up to 30 minutes of laser irradiation. An aliquot of the sample from the final time point was treated with proteinase K, which removed the major photocrosslinked product band; a new band migrating slightly slower than uncrosslinked site I appeared. The shift of this band is probably because of a few amino acids which remain attached to the site after proteolytic digestion. The final sample shown on Figure 3.17b shows a sample that was laser-crosslinked in a clear Eppendorf tube for 10 minutes, rather than in a quartz microcell. This sample also efficiently photocrosslinked with resolvase, which meant that subsequent crosslinking reactions could be conveniently done in Eppendorf tubes.

The efficiency of 5-I-dU photocrosslinking (around 5-10%) was not as high as reported previously (70-90% in Willis *et al.*, 1993) but is still higher than in most previously reported crosslinking studies. This may have been due to the fact that the laser used in this work generated around 11 mW of power, whereas Willis *et al.* used a laser generating 37 mW. The efficiency of photocrosslinking is also very much dependent on the specific protein-nucleic acid interaction being studied.

Binding of resolvase to the labelled 5-I-dU site I was tested, along with natural site I, by the gel retardation assay (Figure 3.18). The 5-I-dU site I was bound strongly by resolvase, with the binding being only slightly reduced in comparison with the natural sequence.

To test if the laser crosslinked monomer could form part of a normal dimer-DNA complex, two-dimensional gel electrophoresis was used by running a crosslinked sample in a standard band-shift assay in the first dimension and then running the same gel, soaked in SDS, at 90° in a second dimension to assay for photocrosslinked complex. A second sample was also prepared in which resolvase was simply bound to the 5-I-dU site I without crosslinking. The samples were electrophoresed in both directions and then the gel was dried and exposed to X-ray film. The autoradiograph is represented in Figure 3.19. As with the sulphur-containing site I, the 5-I-dU photocrosslinked complex separated from the bound dimer complex band of the first dimension of electrophoresis, demonstrating that a laser crosslinked monomer of resolvase can take part in 'normal' dimer interactions. The control sample which was bound by resolvase (without photocrosslinking) produced no additional bands in the second dimension of electrophoresis (Figure 3.19).

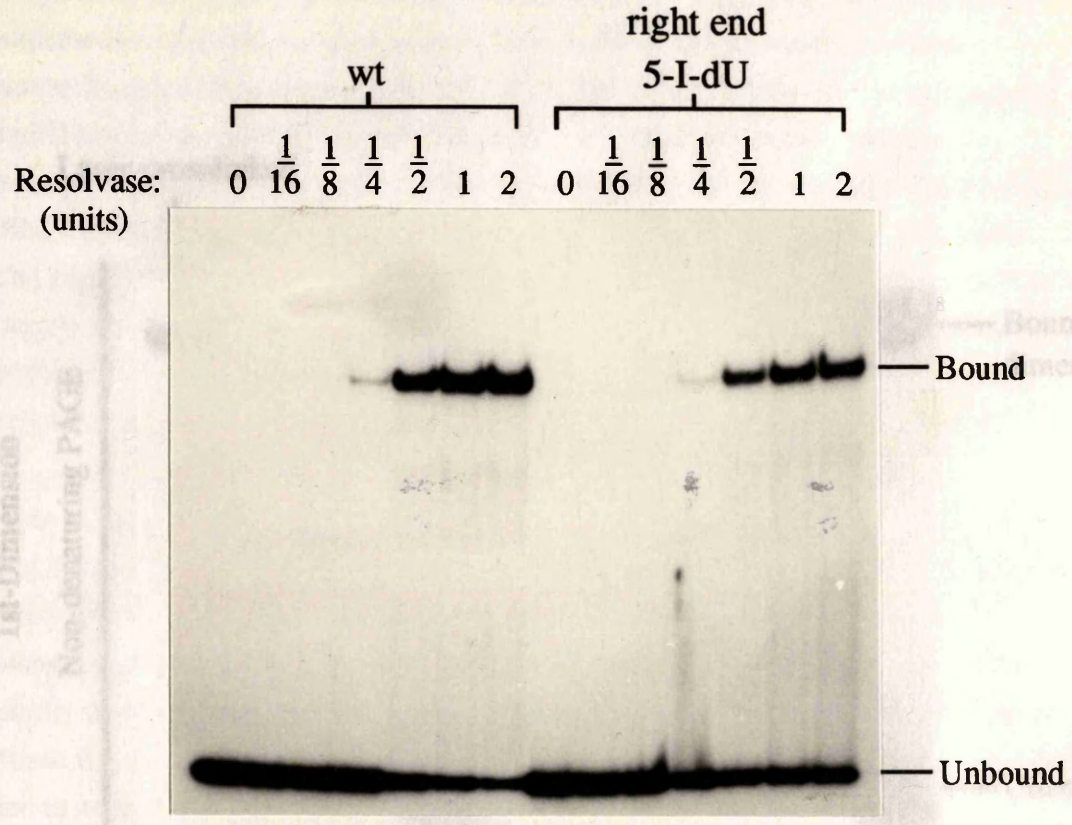


Figure 3.18 Binding of resolvase to a (right end) 5-I-dU site I.

Binding of resolvase to the labelled 5-I-dU site I (shown in Figure 3.17a) was compared to that of a natural site I. Both sites were incubated with various concentrations of resolvase in binding buffer and then run on an 8% acrylamide non-denaturing gel.

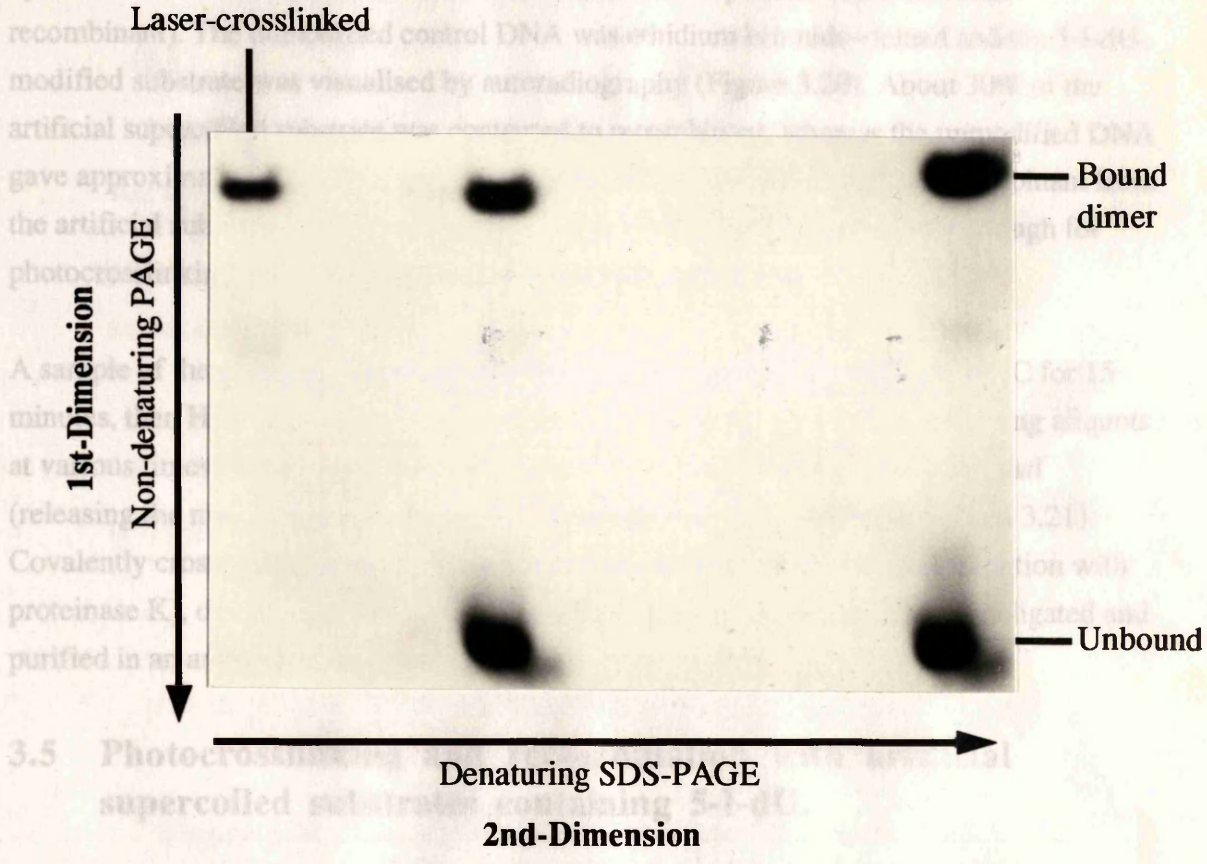


Figure 3.19 Two-dimensional gel electrophoresis of resolvase laser-crosslinked to a site I containing 5-I-dU.

Labelled 5-I-dU site I (shown in Figure 3.17(a)) was incubated with resolvase (0.5 units) in binding buffer and then laser-crosslinked for 60 minutes. The sample was loaded on the left hand side of a 10% acrylamide non-denaturing gel and electrophoresis in the first dimension was performed as indicated. The whole gel was then soaked in SDS buffer and electrophoresis was performed in the second dimension using a buffer containing SDS as shown. The sample on the right hand side of the gel was incubated with resolvase, but was not laser-irradiated.

An artificial supercoiled substrate was then synthesised (as in Section 3.2) by a two-step ligation process, incorporating the labelled 5-I-dU site I into one *res* site within pMM5. This supercoiled substrate was then reacted with resolvase in C9.4 buffer, without photocrosslinking, in the presence of unlabelled pMM5 DNA. The products were assayed by *Hin*dIII restriction (which linearises the smaller 2.3 kbp circle of the catenane recombinant). The unmodified control DNA was ethidium bromide-stained and the 5-I-dU-modified substrate was visualised by autoradiography (Figure 3.20). About 30% of the artificial supercoiled substrate was converted to recombinant, whereas the unmodified DNA gave approximately 40-50% recombinant products. Although the yield of recombinant from the artificial substrate was not as high as from the wild-type reaction, it was enough for photocrosslinking and recombination reactions to be attempted.

A sample of the substrate was incubated with resolvase in binding buffer at 37 °C for 15 minutes, then HeCd laser-irradiated at room temperature for 80 minutes, removing aliquots at various times. Photocrosslinking was assayed by digestion with *Eco*RI and *Sst*I (releasing the modified site I), then running the samples on SDS/PAGE (Figure 3.21). Covalently crosslinked complexes were detected (which disappeared upon digestion with proteinase K), demonstrating that the 5-I-dU site I was still photoreactive when ligated and purified in an artificial supercoiled substrate.

3.5 Photocrosslinking and recombination with artificial supercoiled substrates containing 5-I-dU.

To be able to photocrosslink a monomer of resolvase to site I of *res* and ask whether it is capable of taking part in a full recombination reaction in a supercoiled substrate, it was essential that the monomer of resolvase be covalently joined to the site prior to any recombination taking place. Fortunately for this experiment, resolvase requires the presence of Mg^{2+} ions for fast, efficient recombination over a short time scale. Therefore, resolvase was photocrosslinked to the modified site in the absence of Mg^{2+} ions (giving no recombination); once crosslinking was complete, Mg^{2+} was added, and the resolution reaction was allowed to proceed at 37 °C for 1 hour.

The recombination products were assayed by digestion with *Eco*RI + *Pvu*II, which generates labelled 273 bp non-recombinant and 151 bp recombinant fragments (Figure 3.7). The reactions were run on SDS/PAGE; the autoradiograph is represented in Figure 3.22. In the absence of resolvase no recombinant or crosslinked bands are seen, only 273 bp non-recombinant. The second sample shows that in the presence of resolvase and laser

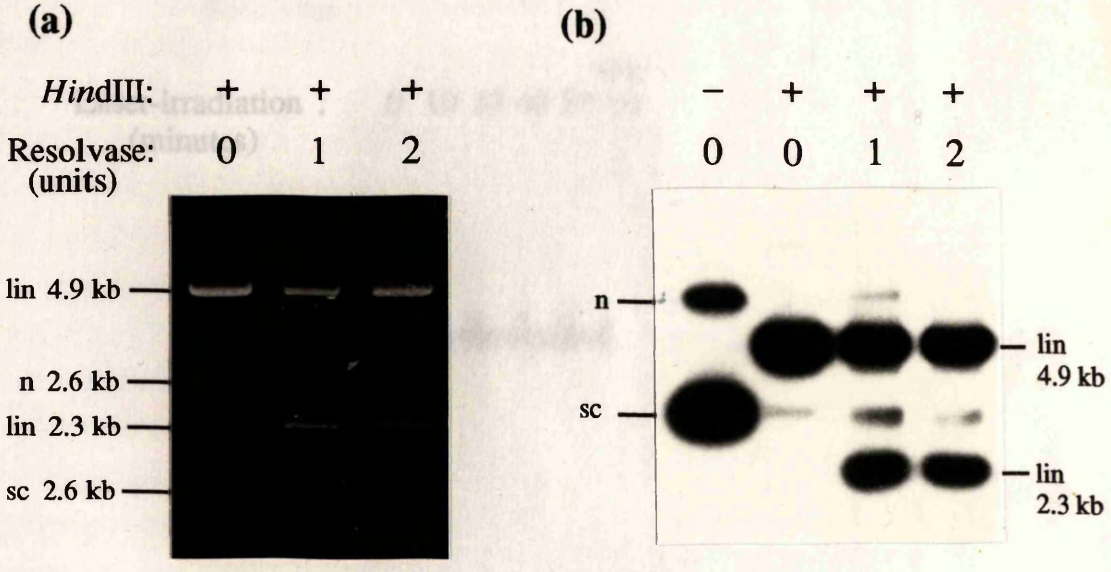


Figure 3.20 Recombination assay of artificial supercoiled pMM5 containing 5-I-dU site I oligonucleotides.

(a) Recombination of natural pMM5 (ethidium-staining of gel). Restriction of substrate pMM5 by *Hin*dIII produces a 4.9 kbp non-recombinant linear (lin 4.9 kb). Restriction of resolution products by *Hin*dIII produces a 2.3 kbp recombinant linear and nicked and supercoiled versions of the 2.6 kbp circle as indicated.
(b) Recombination of artificial supercoiled pMM5 which contains the 5-I-dU site I oligonucleotides shown in Figure 3.17(a) (autoradiograph). Labelling is as for (a). Nicked circles are indicated by 'n' and supercoiled circles by 'sc'.

All reactions contained both natural and modified pMM5, were performed in C9.4 buffer and were run on the same 1% agarose gel. (a) and (b) are photographs of the same gel (EtBr-stained in (a), autoradiograph in (b)).

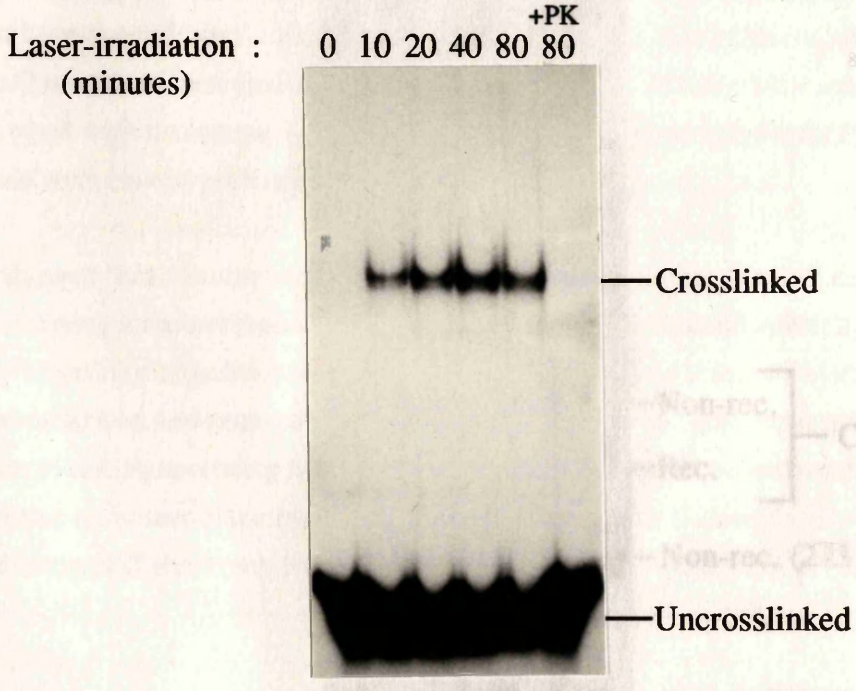


Figure 3.21 Laser-crosslinking of resolvase to a 5-I-dU site I cut from an artificial supercoiled pMM5 substrate.

Artificial supercoiled pMM5 containing 5-I-dU was incubated with resolvase (2 units) in binding buffer and then laser-irradiated for 80 minutes. Samples were removed at the times shown above the lanes. All samples were then digested with *Eco*RI and *Sst*I (releasing the modified site I) and run on a 10% acrylamide, 0.1% SDS gel. The final time-point sample was also treated with proteinase K (+PK).

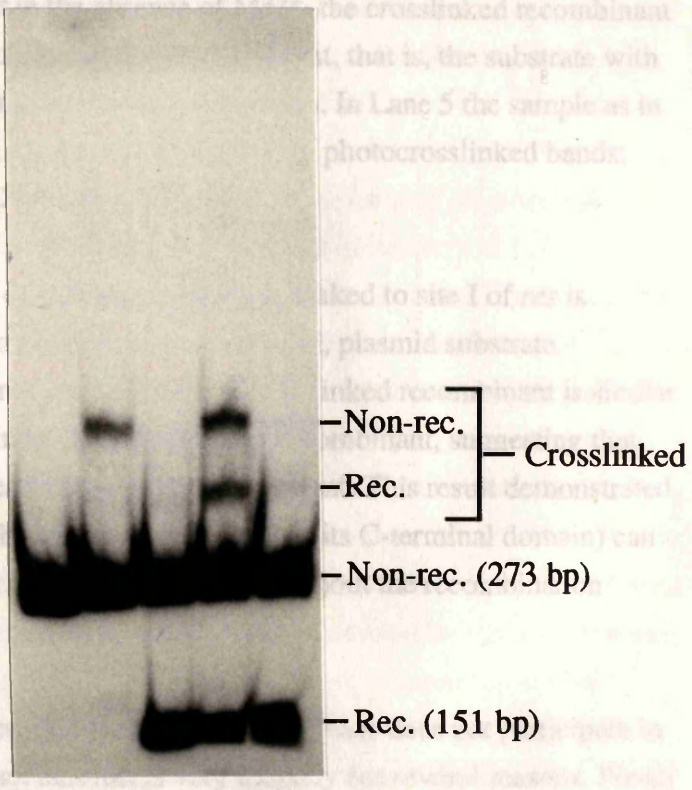


Figure 3.22 Laser-crosslinking to and recombination of an artificial supercoiled substrate containing a right end 5-I-dU site I.

Artificial supercoiled pMM5 containing the 5-I-dU site I shown in Figure 3.17(a) was used in the above experiment. Samples were incubated with or without resolvase (2 units) and certain samples were laser-crosslinked for 30 minutes as indicated. Mg^{2+} was then added back to some samples and the resolution reaction was allowed to proceed. Recombination was assayed by digestion with *Eco*RI and *Pvu*II, which generates labelled 273 bp non-recombinant and 151 bp recombinant fragments (see Figure 3.7). The final crosslinking and recombination reaction was divided and one half was treated with proteinase K. All samples were run on a 6% acrylamide, 0.1% SDS gel.

irradiation, but with no Mg^{2+} added back, only 273 bp non-recombinant and photocrosslinked non-recombinant bands are present. The third sample was not laser-irradiated, but Mg^{2+} was added back, and only the 273 bp non-recombinant and 151 bp recombinant bands appear. The sample in lane 4 was laser-crosslinked with resolvase in the absence of Mg^{2+}, then Mg^{2+} was added back and the sample was allowed to react for 1 hour. A new band appeared, representing the photocrosslinked recombinant product. Since the photocrosslinking was carried out in the absence of Mg^{2+}, the crosslinked recombinant product must have come from the crosslinked non-recombinant, that is, the substrate with the crosslinked monomer took part in a recombination reaction. In Lane 5 the sample as in lane 4 was treated with proteinase K, leading to the loss of the photocrosslinked bands; therefore these were indeed protein-DNA complexes.

Figure 3.22 showed that a monomer of resolvase photocrosslinked to site I of *res* is capable of catalysing a recombination reaction in a supercoiled, plasmid substrate. Furthermore the ratio of crosslinked non-recombinant to crosslinked recombinant is similar to that of uncrosslinked non-recombinant to uncrosslinked recombinant, suggesting that recombination of the photocrosslinked substrate is quite efficient. This result demonstrated that the resolvase monomer at the right end of site I (or at least its C-terminal domain) can stay attached to the half site to which it originally binds throughout the recombination reaction.

There is a possibility that the photocrosslinked subunit of resolvase does not participate in the strand exchange reaction. We think that this is very unlikely for several reasons. Firstly it was shown, by two-dimensional gel electrophoresis, that a crosslinked monomer of resolvase takes part in 'normal' dimer binding interactions (Figures 3.6 and 3.19). We are therefore confident that the crosslinked subunit should also take part in 'normal' catalytic tetramer interactions. Furthermore, the subunit of resolvase was crosslinked to the modified end of site I prior to the recombination reaction; it is therefore very unlikely that another subunit of resolvase could bind at this end of the site. Thus, if the crosslinked monomer did not participate in recombination, then the strand exchange reaction would require a subunit of resolvase not interacting with the modified site I. Since monomers of resolvase cleave and recombine the site to which they are bound (Boocock *et al.*, 1995), we strongly believe that the crosslinked monomer catalyses strand exchange at the site I to which it is covalently attached.

If the subunit rotation model of strand exchange were correct (Chapter 1) then there is a possibility that the monomers of resolvase bound to the right hand side of the synapsed site

I's in the synaptosome remain stationary and it is the monomers in the left hand side of the catalytic tetramer that undergo subunit rotation (Stark *et al.*, 1989; Stark *et al.*, 1992; and Figure 1.10). It has also been suggested that in the synaptosome, the tetramers of resolvase bound at sites II and III contact the dimer to the right hand side of the catalytic tetramer at the synapsed site I's (Rice and Steitz, 1994a). This suggestion would imply that if any subunits of resolvase are to be released from the DNA, then it is more likely to be the subunits to the left of the catalytic tetramer, furthest from the site II/III interaction. It was therefore necessary to repeat the photocrosslinking and recombination reaction for a monomer of resolvase photocrosslinked to the left hand side of site I.

The left end of site I does not contain the 'TGT' contained at the ends of all other half binding sites of *res,* but instead has the sequence CGT. To maximise the yield of photocrosslinking, it was decided to change the top strand, left half of site I to (5-I-dU) G (5-I-dU) and anneal it to a bottom strand oligonucleotide with the complementary ACA sequence (Figure 3.23a). The top strand was synthesised, 5' labelled with γ-[^{32}P]ATP, and annealed to unlabelled bottom strand DNA. Photocrosslinking was assayed by incubating the modified site I with resolvase in binding buffer, and laser irradiating for 1 hour, removing samples at various times, and running the reactions on SDS/PAGE (Figure 3.23b). A crosslinked complex was observed (as in Figure 3.17b) which disappeared upon treatment with proteinase K. Binding of the modified site was assayed by incubating with resolvase in binding buffer and then subjecting the samples to non-denaturing PAGE (Figure 3.24). Efficient binding to the modified site was observed, and changing the left end of site I (top strand) from CGT to (5-I-dU) G (5-I-dU) and bottom strand to ACA improved the binding of the modified site I compared to the right end 5-I-dU site I (see Figure 3.18).

A supercoiled substrate containing the 5-I-dU modifications at the left end of site I was synthesised (Section 3.2) and incubated with resolvase in C9.4 buffer, in a standard recombination assay also containing unlabelled, unmodified pMM5. The reactions were carried out in the absence of photocrosslinking, and were assayed by *Hin*dIII restriction, (*Hin*dIII cuts in the small circle of the catenane). The recombinant can be seen as the nicked and supercoiled forms of the 2.6 kbp circle of the catenane (Figure 3.25). Once again the 5-I-dU supercoiled substrate generated about 30% recombinant catenane.

Photocrosslinking and recombination was then combined, using this synthetic substrate, as described earlier in Section 3.5. Resolvase was photocrosslinked in the absence of Mg^{2+} (therefore without recombination) and then Mg^{2+} was added back and the resolution

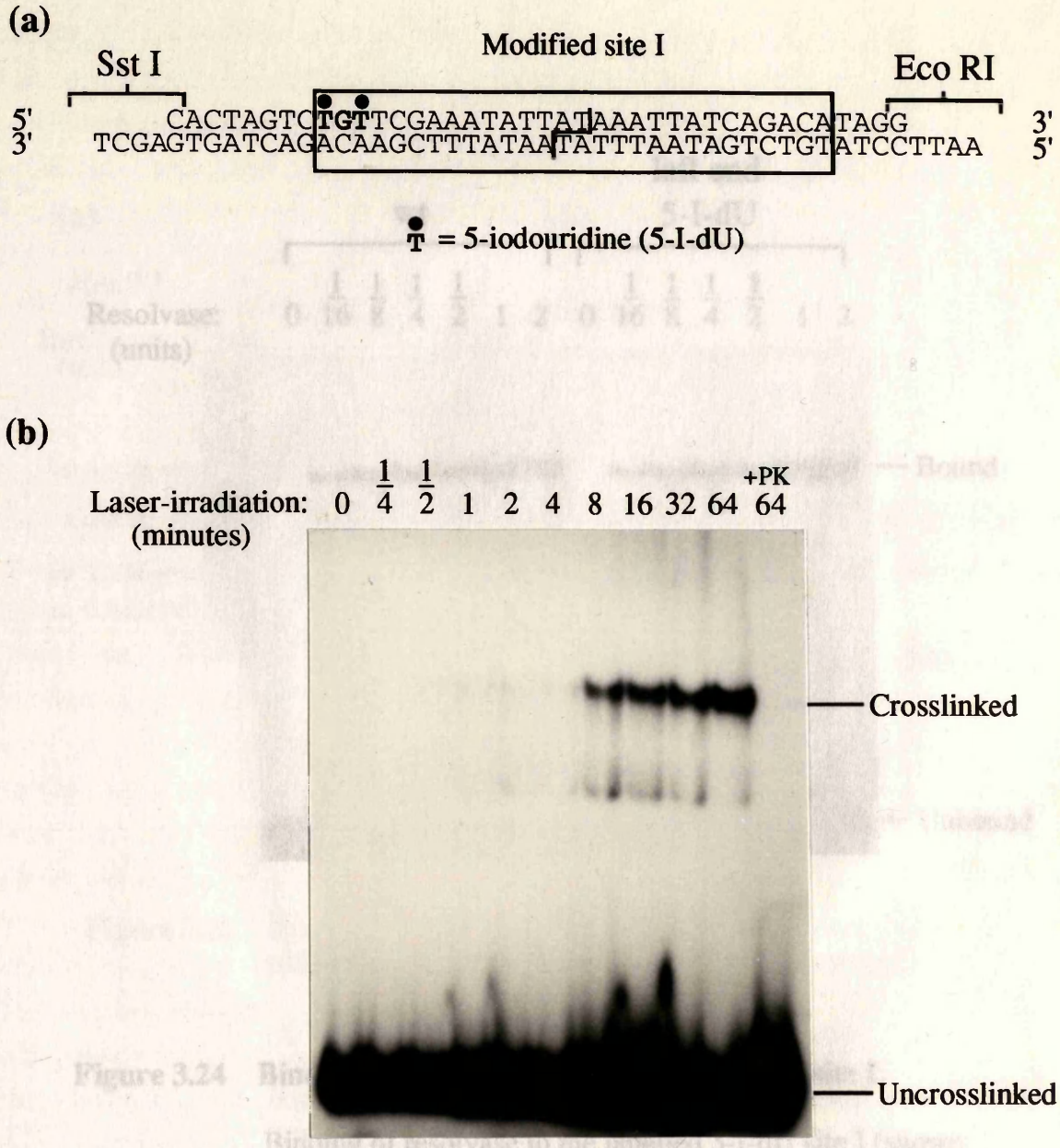


Figure 3.23 Laser-crosslinking of resolvase to 5-I-dU site I.

(a) The sequence of the modified site I oligonucleotides containing two 5-I-dU substitutions at the left hand end. (b) The labelled site I (shown in (a)) was incubated in binding buffer with resolvase (2 units per track) and then laser-irradiated in a quartz microcell for over an hour. Samples were removed at the times shown. One sample was treated with proteinase K (+PK). All samples were then run on a 10% acrylamide, 0.1% SDS gel.

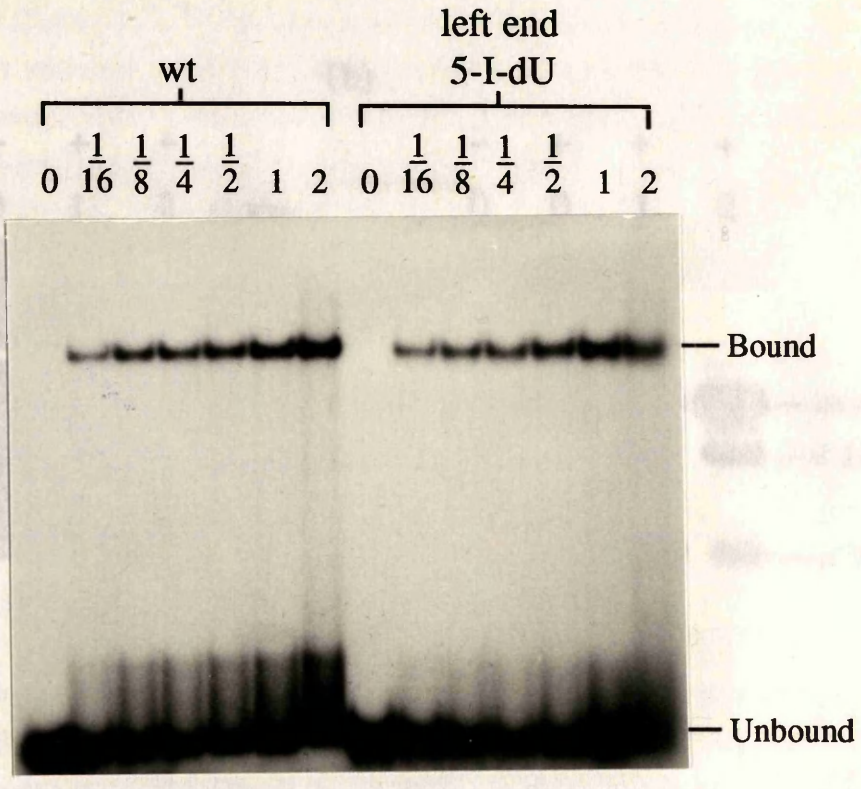


Figure 3.24 Binding of resolvase to a (left end) 5-I-dU site I.

Binding of resolvase to the labelled 5-I-dU site I (shown in Figure 3.23a) was compared to that of a natural site I. Both sites were incubated with various concentrations of resolvase in binding buffer and then run on an 8% acrylamide non-denaturing gel.

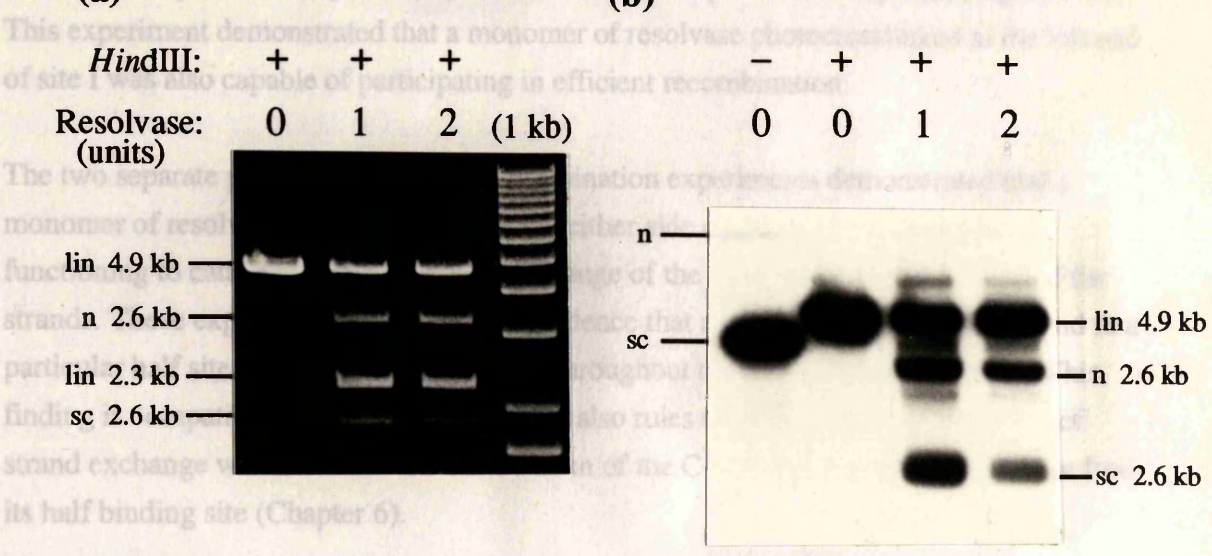


Figure 3.25 Recombination assay of artificial supercoiled pMM5 containing 5-I-dU site I oligonucleotides.

(a) Recombination of natural pMM5 (ethidium-staining of gel). Restriction of substrate pMM5 by *Hin*dIII produces a 4.9 kbp non-recombinant linear (lin 4.9 kb). Restriction of resolution products by *Hin*dIII produces a 2.3 kbp recombinant linear and nicked and supercoiled versions of the 2.6 kbp circle as indicated.
(b) Recombination of artificial supercoiled pMM5 which contains the left end 5-I-dU site I oligonucleotides shown in Figure 3.23(a) (autoradiograph). Labelling is as for (a). Nicked circles are indicated by 'n' and supercoiled circles by 'sc'.

All reactions contained both natural and modified pMM5, were performed in C9.4 buffer together and were run on the same 1% agarose gel. (a) and (b) are photographs of the same gel (EtBr-stained in (a), autoradiograph in (b)).

reaction was allowed to proceed for 1 hour. Because the radiolabel was in the top strand here (at the *Sst*I site), recombination was assayed using different restriction enzymes. *Bam*HI + *Sst*I digestion generated a 495 bp non-recombinant fragment and a 151 bp recombinant fragment (Figure 3.7). The same controls as in Figure 3.22 were used. Reaction samples were separated by SDS/PAGE and exposed to X-ray film (Figure 3.26). This experiment demonstrated that a monomer of resolvase photocrosslinked to the left end of site I was also capable of participating in efficient recombination.

The two separate photocrosslinking + recombination experiments demonstrated that a monomer of resolvase covalently attached at either side of *res* site I was capable of functioning to catalyse strand cleavage, exchange of the DNA ends, and religation of the strands. These experiments provide good evidence that a monomer of resolvase bound to a particular half site stays attached to that site throughout the recombination reaction. This finding is compatible with subunit rotation; it also rules out some alternative models of strand exchange which involve the dissociation of the C-terminal domain of resolvase from its half binding site (Chapter 6).

An important alternative that these results do not rule out is the 'DNA-mediated' model of strand exchange (Rice and Steitz, 1994a) which does not involve any subunit rotation (Chapter 1). In this model, which is based on the X-ray crystallographic studies of resolvase, the synapsed site I's lie in the centre of a catalytic tetramer, and recombination is achieved via direct contact of the DNA duplexes (Figure 1.14). It is proposed that the resolvase catalytic tetramer does not exchange the DNA strands by rotation, but serves only to distort and stabilise the recombining DNA during the recombination reaction. Photocrosslinking a monomer of resolvase to site I would not disrupt 'DNA- mediated' strand exchange because the recombination is achieved by relatively small movements of the cleaved DNA ends at the centres of the site I's. While DNA-mediated strand exchange agrees with the resolvase crystal packing data, it cannot easily explain the precisely measured topological changes of the DNA during recombination without introducing complex unwinding and untwisting of the DNA at site I (Rice and Steitz, 1994a). Furthermore, DNA-mediated strand exchange requires that a 'recombinant intermediate' is formed during a mismatched knotting reaction (described in Chapter 4). Current evidence strongly suggests that the iterative knotting reaction is achieved by multiple rounds of strand exchange without religation of the DNA ends to make a recombinant intermediate (Chapter 4).

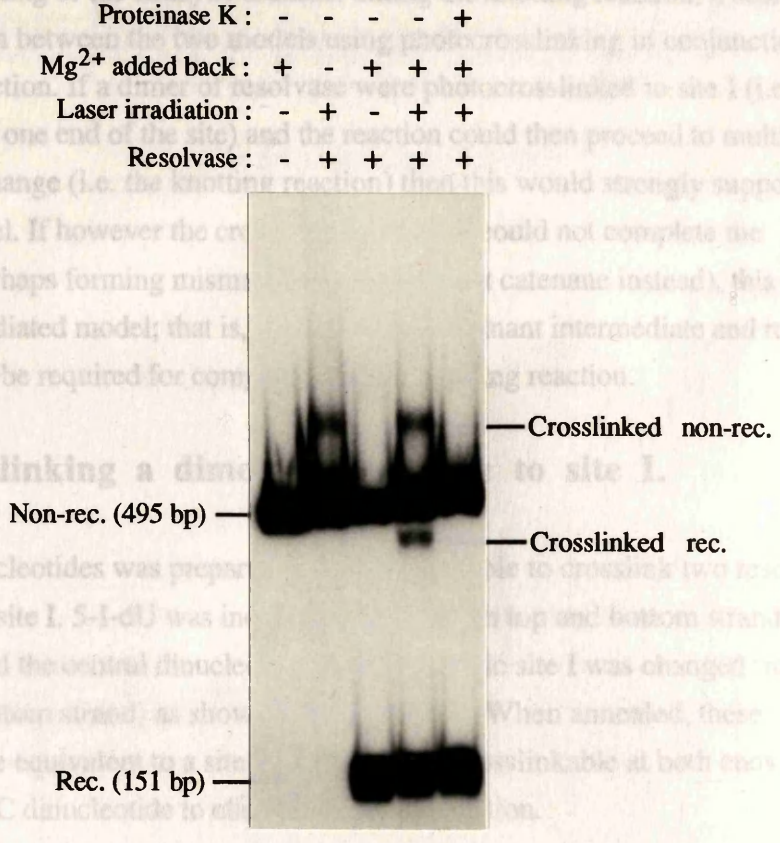


Figure 3.26 Laser-crosslinking to and recombination of an artificial supercoiled substrate containing a left end 5-I-dU site I.

Artificial supercoiled pMM5 containing the 5-I-dU site I shown in Figure 3.23(a) was used in the above experiment. Samples were incubated with or without resolvase (1 unit) and certain samples were laser-crosslinked for 60 minutes as indicated. Mg^{2+} was then added back to some samples and the resolution reaction was allowed to proceed. Recombination was assayed by digestion with *Bam*HI and *Sst*I, which generates labelled 495 bp non-recombinant and 151 bp recombinant fragments (see Figure 3.7). The final crosslinking and recombination reaction was divided and one half was treated with proteinase K. All samples were run on a 6% acrylamide, 0.1% SDS gel.

Since the DNA-mediated model of strand exchange requires formation of a recombinant intermediate and resetting of the catalytic tetramer during the knotting reaction, it seemed possible to distinguish between the two models using photocrosslinking in conjunction with the knotting reaction. If a dimer of resolvase were photocrosslinked to site I (i.e. each C-terminal domain to one end of the site) and the reaction could then proceed to multiple rounds of strand exchange (i.e. the knotting reaction) then this would strongly support the subunit rotation model. If however the crosslinked complex could not complete the knotting reaction (perhaps forming mismatched, recombinant catenane instead), this would support the DNA-mediated model; that is, ligation to recombinant intermediate and resetting of the tetramer might be required for completion of the knotting reaction.

3.6 Photocrosslinking a dimer of resolvase to site I.

A new set of oligonucleotides was prepared to make it possible to crosslink two resolvase subunits to the same site I. 5-I-dU was incorporated into both top and bottom strands at the conserved TGT's, and the central dinucleotide of the synthetic site I was changed from AT to AC (GT on the bottom strand) as shown in Figure 3.27a. When annealed, these oligonucleotides were equivalent to a site I that was photocrosslinkable at both ends and contained a central AC dinucleotide to elicit the knotting reaction.

Photocrosslinking to both ends of site I was attempted by incubating the labelled site with resolvase in binding buffer at 37 °C for 15 minutes and then irradiating the sample with HeCd laser light for over 2 hours. Samples were removed at various time points to analyse the rate of photocrosslinking. As a marker for photocrosslinked dimer the γδ resolvase mutant M106C was used (a gift from N.Grindley). This mutant has a methionine to cysteine mutation (position 106) at the dimer interface. When oxidised it forms disulphide bridges between monomers to yield a high proportion of dimeric γδ resolvase (M106Cox). A sample of M106Cox was photocrosslinked to the modified site I and run along with the Tn*3* resolvase time course on SDS/PAGE (Figure 3.27b). On a long exposure of the gel to X-ray film a small amount of photocrosslinked dimer was detected after 1 hour of laser irradiation. The dimer complex ran in a similar position to the photocrosslinked M106C dimer, as expected. The ratio of uncrosslinked DNA: crosslinked monomer: crosslinked dimer was 92%: 7%: 1% as measured using a phosphorimager. Both monomer and dimer complexes were destroyed upon treatment with proteinase K. It was interesting to note that the crosslinked monomer complex appeared as two bands, probably because photocrosslinked monomer-DNA complexes at the left and right ends of the site have different mobilities. Clearly such a small yield of crosslinked dimer was going to cause

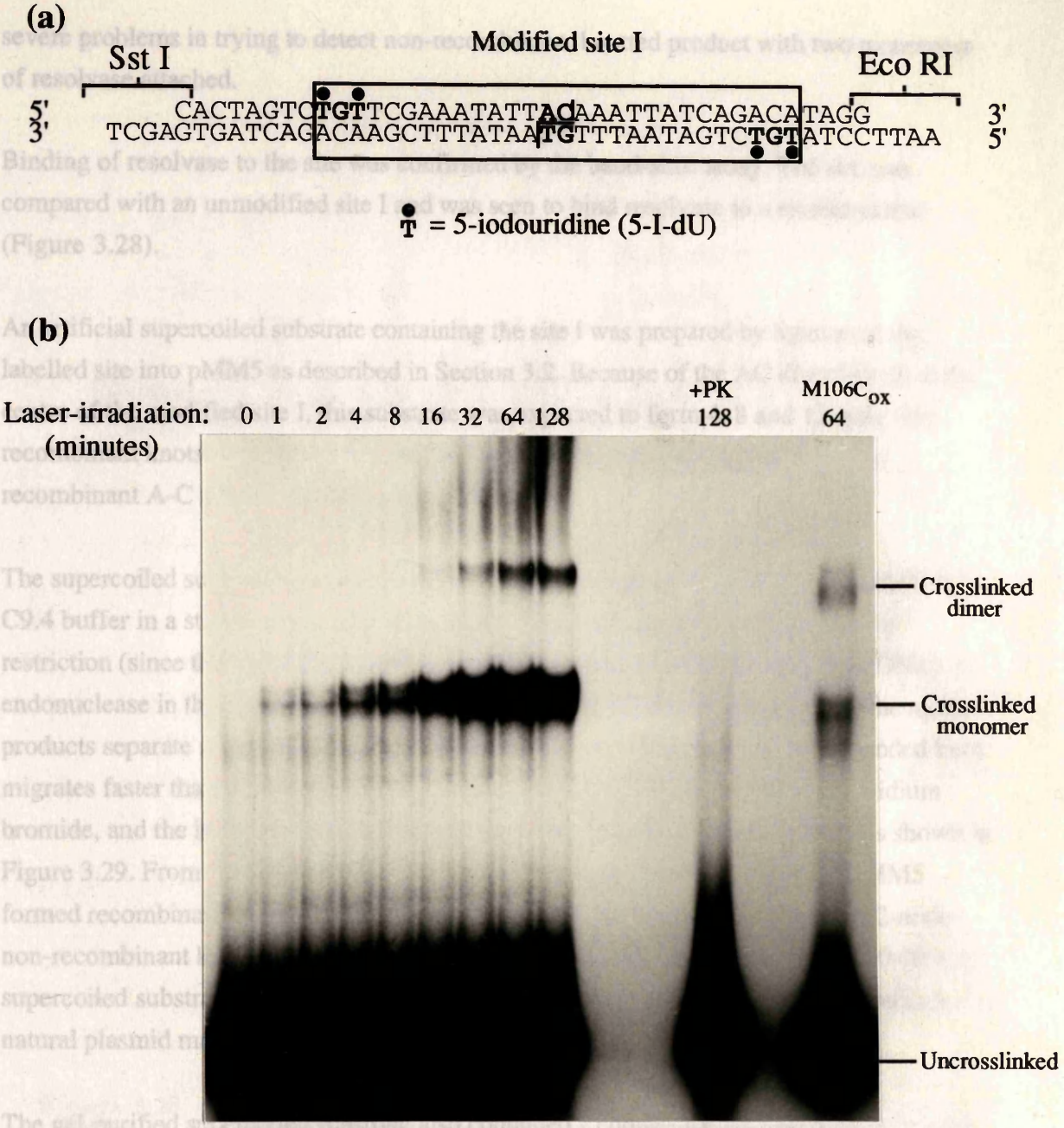


Figure 3.27 Laser-crosslinking a dimer of resolvase to a 5-I-dU site I.

(a) The sequence of the modified site I oligonucleotides containing two 5-I-dU substitutions at each end and an 'AC' central dinucleotide.
(b) The labelled site I (shown in (a)) was incubated in binding buffer with resolvase(1 unit per track) and then laser-irradiated in an Eppendorf tube for over 2 hours. Samples were removed at the times shown. One sample was treated with proteinase K (+PK). The final sample was incubated with the γδ resolvase mutant M106Cox and laser-irradiated for 1 hour. Samples were run on a 10% acrylamide, 0.1% SDS gel.

severe problems in trying to detect non-recombinant, knotted product with two monomers of resolvase attached.

Binding of resolvase to the site was confirmed by the band-shift assay. The site was compared with an unmodified site I and was seen to bind resolvase to a similar extent (Figure 3.28).

An artificial supercoiled substrate containing the site I was prepared by ligation of the labelled site into pMM5 as described in Section 3.2. Because of the AC dinucleotide at the centre of the modified site I, this substrate was expected to form 4, 8 and 12 node non-recombinant knots by performing multiple rounds of strand exchange to avoid the recombinant A-C mismatch (Stark *et al.*, 1991).

The supercoiled substrate was incubated with resolvase (along with unmodified pMM5) in C9.4 buffer in a standard recombination assay. The products were assayed, not by restriction (since the knotted products are non-recombinant) but by nicking with DNase I endonuclease in the presence of ethidium bromide (described in Section 2.21). The nicked products separate according to the number of nodes they contain (therefore a 4-noded knot migrates faster than a 2-node catenane). Unmodified pMM5 was stained with ethidium bromide, and the labelled, modified pMM5 was visualised by autoradiography as shown in Figure 3.29. From the agarose gel it can be seen that although the unmodified pMM5 formed recombinant 2-node catenane, the artificial plasmid generated 4, 8, and 12-node non-recombinant knots in the absence of photocrosslinking. Only an estimated 10-20% of supercoiled substrate was converted to products, whereas the *in vitro* knotting reaction of a natural plasmid may be expected to yield 50-60% product.

The gel-purified supercoiled substrate also contained a contaminating supercoiled molecule (the nicked and supercoiled forms are indicated by '*' in Figure 3.29) whose nature was never fully understood. The contaminant appeared in several purifications and was very difficult to remove. It seemed to contain one *res* site and did not form any products upon treatment with resolvase. Unfortunately the nicked form of the contaminant ran precisely in the region where the 2-node catenane might have been expected to run (Figure 3.29). This band is clearly not 2-node catenane as it appears in the no-resolvase control lane and does not change upon treatment with resolvase.

Because of a combination of factors, including the supercoiled contaminant, the extremely low yield of photocrosslinked dimer, and the relatively low yield of knotted products, the

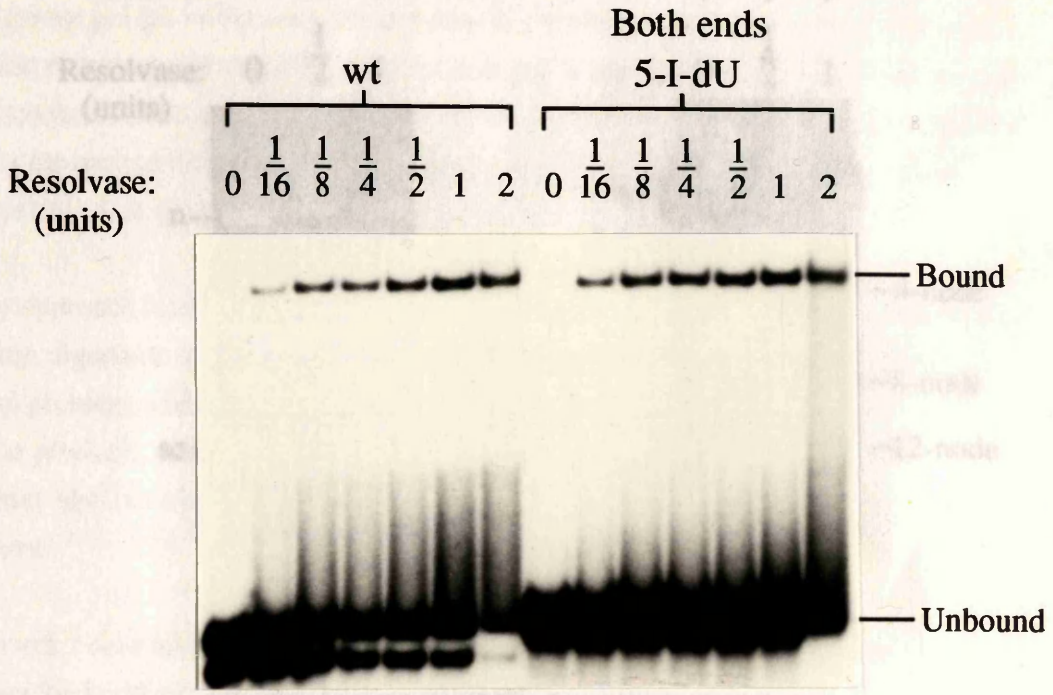


Figure 3.28 Binding of resolvase to a site I with 5-I-dU substitutions at both ends of the site.

Binding of resolvase to the labelled 5-I-dU site I containing 5-I-dU substitutions at both ends and an 'AC' central dinucleotide (shown in Figure 3.27a) was compared to that of a natural site I. Both sites were incubated with various concentrations of resolvase in binding buffer and then run on an 8% acrylamide non-denaturing gel.

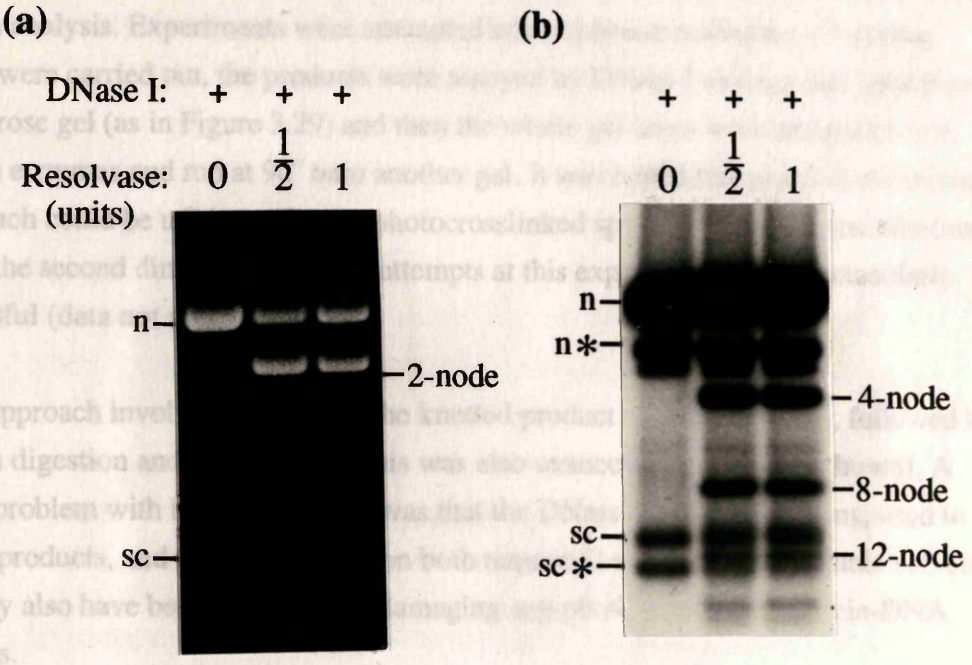


Figure 3.29 The knotting reaction of an artificial supercoiled substrate containing 5-I-dU substitutions and an 'AC' central dinucleotide.

(a) Recombination of natural pMM5 (ethidium-staining of gel). The products were assayed by nicking with DNase I (described in Section 2.21) to separate the different topological species. Nicked circles are indicated by 'n' and supercoiled circles by 'sc'. The position of 2-node catenane is indicated.
(b) Recombination of artificial supercoiled pMM5 which contains the oligonucleotides with 5-I-dU substitutions at both ends of site I and an 'AC' central dinucleotide shown in Figure 3.27(a) (autoradiograph). Labelling is as for (a). The positions of 4-, 8- and 12-node non-recombinant knots are indicated. The nicked and supercoiled forms of a contaminating molecule are indicated by 'n *' and 'sc *' respectively.

All reactions contained both natural and modified pMM5, were performed in C9.4 buffer together and were run on the same 0.7% agarose gel. (a) and (b) are photographs of the same gel (EtBr-stained in (a), autoradiograph in (b)).

knotting experiment with a photocrosslinked dimer of resolvase has not yet been successfully carried out (data not shown). A further complication was caused by the fact that the knotted products are non-recombinant, and therefore cannot easily be analysed by restriction analysis. Experiments were attempted where photocrosslinking + knotting reactions were carried out, the products were assayed by DNase I nicking and separation on an agarose gel (as in Figure 3.29) and then the whole gel lanes were incubated with restriction enzymes and run at 90° onto another gel. It was hoped that a two-dimensional gel approach could be used to visualise photocrosslinked species (of the non-recombinant knots) in the second dimension. Several attempts at this experiment were spectacularly unsuccessful (data not shown).

Another approach involved excision of the knotted product bands from a gel, followed by restriction digestion and SDS/PAGE. This was also unsuccessful (data not shown). A potential problem with both approaches was that the DNase I nicking reactions, used to assay the products, and the gel purification both required extraction with organic solvents, which may also have been extracting or damaging any photocrosslinked protein-DNA complexes.

Future work could involve addressing some of the technical problems indicated above, regarding the knotting reaction with a photocrosslinked dimer of resolvase. This difficult experiment should be able to distinguish between the 'subunit rotation' or 'DNA-mediated' models of strand exchange (see above).

A further use of laser crosslinking might be to photocrosslink resolvase to different binding sites of *res* at various positions, and use proteolytic digestion combined with amino acid sequencing or electro-spray mass spectrometry to identify which residues of resolvase interact with specific bases of *res*. This approach could yield valuable structural information that could complement the X-ray crystallography, and could easily be used to probe the interactions of resolvase at sites II and III, which may be quite different to the interaction at site I (Blake *et al.*, 1995).

Chapter 4

Evidence for a subunit rotation model of strand exchange

Introduction

Two contrasting mechanisms are currently proposed for strand exchange by Tn*3* resolvase class site-specific recombinases (discussed in Chapter 1). In both models the strand exchange is achieved by a catalytic tetramer of resolvase subunits which bring together (synapse) the two recombining site I's of *res* (Stark *et al.*, 1992). All four strands in the two sites are cleaved concertedly, with each monomer of the tetramer becoming covalently joined to the half site at which it is bound (Figure 1.9a). In the 'subunit rotation' model, the DNA strands are then exchanged by 180° rotation of one dimer of the resolvase tetramer in a right-handed sense, and then re-ligation of the attached strands to bring about the recombinant configuration. The reaction is driven forward by the negative supercoiling present in substrate plasmids.

'Subunit rotation' is supported indirectly by many biochemical and topological studies (reviewed in Chapter 1), but little effort has been made to produce direct biochemical proof of the mechanism. While rotation of subunits most simply explains the topological changes in the DNA accompanying recombination, the model requires that interactions between two dimers (of the catalytic tetramer) are broken and reformed. After separation of the two dimers, apparently nothing holds the recombining protein-DNA complex together.

An alternative model which does not require subunit rotation is the 'DNA-mediated' model of strand exchange (Rice and Steitz, 1994a). In this model the recombining site I's lie at the centre of the catalytic tetramer, with direct contact of the DNA duplexes (Figure 1.14). Recombination is brought about by exchanging the cleaved DNA ends, with the catalytic tetramer distorting the recombining sites, and holding them together. The catalytic tetramer must presumably undergo some conformational changes to allow cleavage, recombination and re-ligation of the DNA ends. Although 'DNA-mediated' strand exchange requires less structural rearrangement of protein than subunit rotation, it demands much untwisting of the recombining DNA to account for the measured topological changes of the substrate molecules.

Models of strand exchange which involve '*trans*-cleavage', i.e. resolvase cleaving a half-site to which it is not bound (see Chapter 1) are not considered further in this chapter. *Trans*-cleavage has been proposed for the FLP recombinase (Chen *et al.*, 1992; Lee *et al.*, 1994) but recent results strongly suggest that resolvase monomers cleave the site to which they are bound i.e. '*cis*-cleavage' (Boocock *et al.*, 1995).

As a 'tool' to investigate strand exchange (and to possibly distinguish between the 'DNA-mediated' and 'subunit rotation' models) we have been using *res* sites altered at the central dinucleotide ('crossover point') of site I. *In vitro* recombination reactions (of a standard substrate) produce mainly recombinant 2-node catenane, but a small fraction of substrate is converted to non-recombinant knotted products and more complex catenanes (Wasserman *et al.*, 1985). These knots (and catenanes) were thought to be the product of repeated rounds of strand exchange without dissociation of the synaptic complex. After changing the central dinucleotide of one *res* site (in a two site substrate) from AT to AC, reactions with resolvase gave very little 2-node catenane, but instead generated large amounts of 4, 8 and 12-node non-recombinant knots (Stark *et al.*, 1991). Similar observations have been made with the Gin and Hin DNA invertases (Kanaar *et al.*, 1990; Heichmann *et al.*, 1991). The non-recombinant knots are thought to be formed due to detection of the mismatched basepairs in the recombinant, leading to iteration of the strand exchange mechanism (Figure 1.13). Since small amounts of 2-node catenane were detected, correct base pairing is not essential for re-ligation of ends (although re-ligation might be very inefficient in mismatched state) but is probably important for efficient release of products.

This knotting reaction was predicted by the 'subunit rotation' model of strand exchange. The mismatch is recognised only after the first 180° rotation of protein-DNA ends, forcing a second 180° rotation to form the correctly base paired 4-node knot. The 'subunit rotation' mechanism does not require ligation of DNA ends (to form a mismatched recombinant) after the initial 180° rotation, but allows multiple rounds of strand exchange without having to 're-set' the resolvase tetramer into an active configuration. In contrast to this, a 'DNA-mediated' strand exchange mechanism requires that in a knotting reaction, after the first round of strand exchange, the ends must be ligated to form a mismatched recombinant intermediate, to allow re-setting of the catalytic tetramer. Two rounds of strand exchange without ligating the ends and re-setting the catalytic tetramer would require severe distortion of the DNA duplexes and/or resolvase monomers, which is not thought to be feasible (Stark and Boocock, 1994). If the catalytic tetramer is re-set without re-ligation of ends then there is a problem analogous to that with 'subunit rotation'; i.e. the subunits must rearrange while no covalent bonds hold the pieces of the catalytic complex together. It would seem then that a ligated, mismatched recombinant is a necessary intermediate in 'DNA-mediated' strand exchange, but is not required by 'subunit rotation'.

In this chapter experiments are described in which the requirement for the mismatched recombinant was tested, and the properties of substrates with mutated central dinucleotides and mismatched *res* sites were examined further.

Results and Discussion

4.1 Properties of substrates containing AC central dinucleotides at site I of *res*.

Previously published work, using substrates with mutations at the central dinucleotide of site I, used *res* sites from the closely related γδ resolvase system (Stark *et al.*, 1991; Stark and Boocock, 1994). New sites were created using Tn*3* sequences where the 'crossover point' at site I was mutated from AT to AC using site-directed mutagenesis (M.Stark, unpublished results). A new set of substrates was cloned using these altered sites with all four combinations of AT or AC at the centre of site I in *res* (as described in Table 2.1). pMM1 (AT x AT), pMM2 (AT x AC), pMM3 (AC x AT) and pMM4 (AC x AC) are shown in Figure 4.1. The plasmid constructs were confirmed to be as described by sequencing, and are identical except at the mutated *res* sites.

The plasmids were treated with resolvase in C9.4 buffer for 1 hour. The products were assayed by nicking with DNase I, followed by agarose gel electrophoresis (Figure 4.2). As previously reported, AT x AT and AC x AC substrates produced 2-node recombinant catenane, while the AT x AC and AC x AT substrates formed 4, 8, and 12-node knots. Restriction enzyme digestion confirmed that the products were recombinant and non-recombinant respectively (data not shown). Tiny amounts of recombinant catenane (~1%) could also be seen in the reactions of the knotting substrates. This catenane must contain A-C and G-T mismatches at the centre of the site I's. Clearly, in the AT x AC and AC x AT substrates, almost all products are in the form of non-recombinant knots, demonstrating that the mismatched recombinant is strongly selected against. However, the small amount of mismatched recombinant detected could still possibly be a transitory intermediate which is quickly converted to non-recombinant knot.

To determine if the mismatched recombinant is formed at an early stage in the knotting reaction, and is then quickly converted to knot by a second round of strand exchange, time course analysis of the AT x AC reaction was performed. The knotting substrate was incubated with resolvase at 37 °C; samples were removed at various time points and immediately stopped at 70 °C. The products were nicked with DNase I and run on an agarose gel, which was stained with ethidium bromide, and also Southern blotted and probed (Figure 4.3). After 15 seconds of reaction, only 4-node knot was detectable on the ethidium bromide stained gel and on the overexposed blot. No mismatched recombinant (2-node catenane) could be detected until around 4 minutes, by which time a substantial

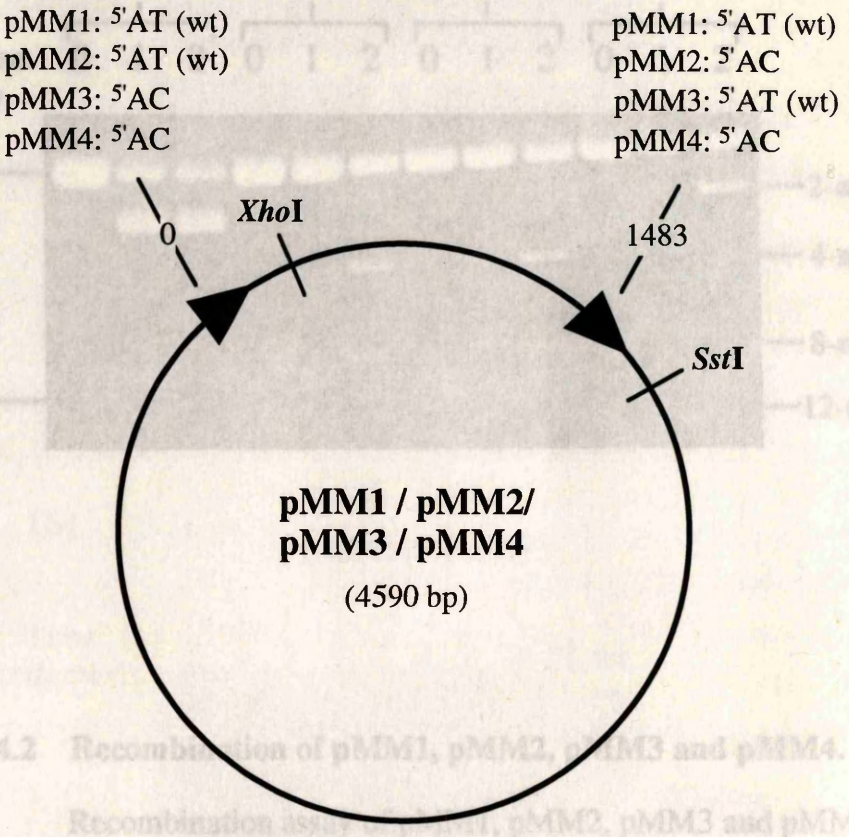


Figure 4.1 Simplified maps of pMM1, pMM2, pMM3 and pMM4.

Simplified maps of plasmids containing copies of the wild-type *res* site and/or mutant *res* sites in which the site I central dinucleotide is altered. The *res* sites are shown as black triangles. pMM1 contains two wild-type *res* sites. pMM2 and pMM3 contain one wild-type *res* site and one *res* site containing an 'AC' central dinucleotide in place of the wild-type 'AT'. pMM4 has the central dinucleotide mutated to 'AC' in both *res* sites. The locations of two unique restriction sites that are used later in this section to linearise the different domains of the substrates are shown in bold type. See Table 2.1 for further details.

Figure 4.2 Recombination of pMM1, pMM2, pMM3 and pMM4.

Recombination assay of pMM1, pMM2, pMM3 and pMM4, which contain wild-type (AT) and/or mutated (AC) site I central dinucleotides as shown above and in Figure 4.1. Nicked substrate is indicated by 'n' and supercoiled substrate by 'sc'. The positions of recombinant 2-node catenane and non-recombinant 4-, 8-, and 12-node knots are also indicated. The plasmids were incubated with resolvase in C9.4 buffer, and the products were assayed by nicking with DNase I (described in Section 2.21) and then running on a 0.7% agarose gel.

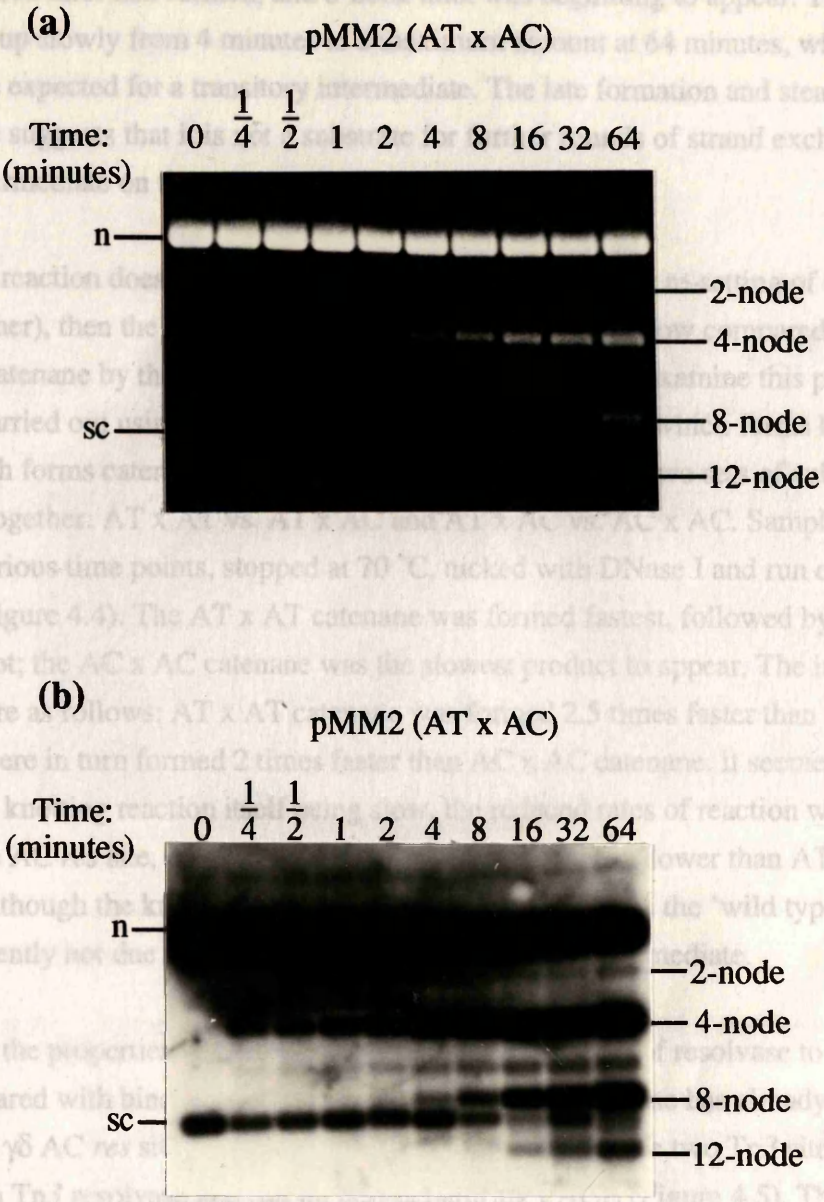


Figure 4.3 Time course analysis of the pMM2 knotting reaction.

(a) Time course analysis of the pMM2 (AT x AC) knotting reaction (ethidium-staining of gel). pMM2 was incubated with resolvase (1 unit per track) in C9.4 buffer and samples were removed at the times indicated and stopped at 70 °C. All samples were then nicked with DNase I and run on a 0.7% agarose gel. Labelling is as for Figure 4.2.
(b) Southern blot of the gel shown in (a) (autoradiograph).

amount of 4-node knot had formed, and 8-node knot was beginning to appear. The catenane built up slowly from 4 minutes to a maximum amount at 64 minutes, which is not what would be expected for a transitory intermediate. The late formation and steady build-up of catenane suggests that it is not a substrate for further rounds of strand exchange, i.e. it is not an intermediate on the pathway to the 4-noded knot.

If the knotting reaction does involve a ligated intermediate (to allow re-setting of the catalytic tetramer), then the formation of knot might be unusually slow compared to the formation of catenane by the AT x AT or AC x AC substrates. To examine this possibility, assays were carried out using mixtures containing both a substrate which forms knot and a substrate which forms catenane to allow direct rate comparisons. Two sets of substrates were reacted together: AT x AT vs. AT x AC and AT x AC vs. AC x AC. Samples were removed at various time points, stopped at 70 °C, nicked with DNase I and run on an agarose gel (Figure 4.4). The AT x AT catenane was formed fastest, followed by the AC x AT 4-node knot; the AC x AC catenane was the slowest product to appear. The initial rates of reaction were as follows: AT x AT catenane was formed 2.5 times faster than AT x AC knots which were in turn formed 2 times faster than AC x AC catenane. It seemed that rather than the knotting reaction itself being slow, the reduced rates of reaction were a property of the AC *res* site, since AC x AC catenane formation is slower than AT x AC knotting. So although the knotting reaction was indeed slower than the 'wild type' reaction, this was apparently not due to the formation of a rate-limiting intermediate.

To investigate the properties of the AC *res* site further, the ability of resolvase to bind the site was compared with binding to a natural AT *res* site. γδ resolvase has already been shown to bind γδ AC *res* sites efficiently (Hatfull *et al.*, 1988). The two Tn*3* sites were incubated with Tn*3* resolvase and run on non-denaturing PAGE (Figure 4.5). The AT and AC *res* sites both formed the expected six complexes (Bednarz *et al.*, 1990) which arise due to the six monomer-binding sites present within the full *res* sites. The AT *res* and AC *res* binding patterns were very similar, demonstrating that Tn*3* resolvase binds both the AT and AC sites efficiently.

Since the reduced rate of AC site reactions was not due to reduced binding of resolvase, it was postulated that the mutant sites might not synapse as efficiently as their natural equivalents. To assay synapsis, the protein crosslinking agent glutaraldehyde was used to trap and visualise the synapsed *res* sites (Watson, 1994 and Section 2.41 for details). The AT x AT, AT x AC and AC x AC substrates were incubated with resolvase (in a MOPS buffer with no Mg^{2+} ions present, to prevent recombination) and then crosslinked with

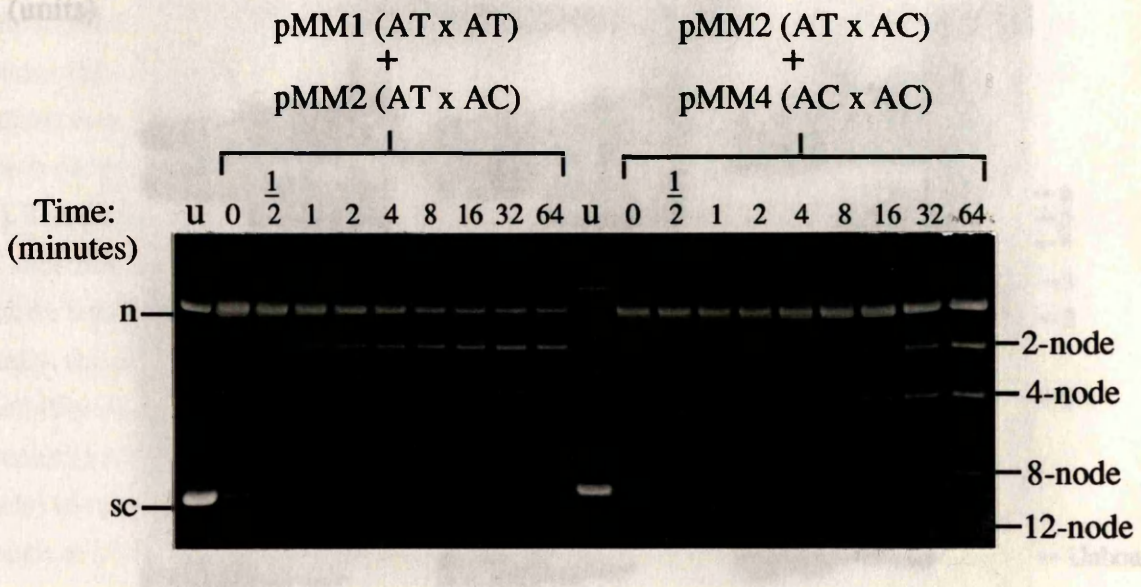


Figure 4.4 Comparison of the rates of catenation and knotting of pMM1-4.

To compare the rates of catenation and knotting, mixtures of equal amounts of plasmids were reacted together with resolvase in time course assays. pMM1 + pMM2 and pMM2 + pMM4 mixtures were incubated with resolvase (1 unit per track) in C9.4 buffer; samples were removed at the times indicated, and stopped at 70 °C. Samples were then nicked with DNase I and run on a 0.7% agarose gel. Unnicked marker pMM2 samples are indicated by 'u'. Products are labelled as Figure 4.2.

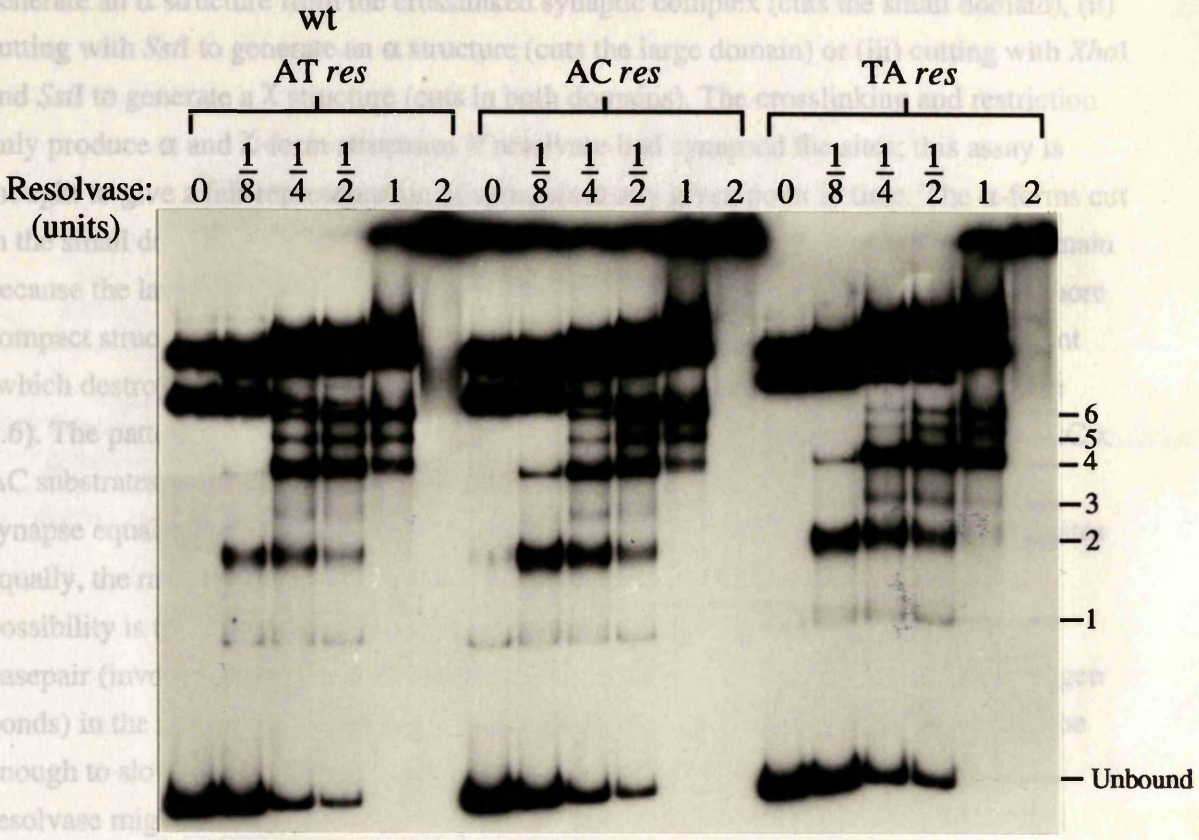


Figure 4.5 Binding of resolvase to AT *res*, AC *res* and TA *res*.

To assay the ability of resolvase to bind to *res* sites with altered site I central dinucleotides, three *res* sites were used in a band-shift assay. Labelled fragments containing a wild-type AT *res* site, an AC *res* site or a TA *res* site were incubated with varying concentrations of resolvase in binding buffer and then run on a 6% acrylamide non-denaturing gel. The six complexes which were formed due to binding by resolvase monomers are indicated. The TA *res* fragment (282 bp) was generated by cutting pGC4 with *Eco*RI (shown in Figure 4.7). The AT and AC *res* fragments (292 bp) were generated by cutting pMS12 and pMS11 (respectively) with *Eco*RI (M.Stark, personal communication).

glutaraldehyde for 5 minutes. The products were assayed by either (i) cutting with *Xho*I to generate an α structure from the crosslinked synaptic complex (cuts the small domain), (ii) cutting with *Sst*I to generate an α structure (cuts the large domain) or (iii) cutting with *Xho*I and *Sst*I to generate a χ structure (cuts in both domains). The crosslinking and restriction only produce α and χ-form structures if resolvase had synapsed the sites; this assay is thought to give a fair representation of synapsis at any given point in time. The α-forms cut in the small domain migrate faster through the gel than the α-forms cut in the large domain because the large, uncut domain remains intact and supercoiled, giving the α-form a more compact structure. Each sample was split in two and run with or without SDS treatment (which destroys any resolvase-induced synapsed structures) on an agarose gel (Figure 4.6). The pattern of α and χ-form structures formed for the AT x AT, AT x AC and AC x AC substrates was identical, strongly supporting the idea that AT *res* and AC *res* sites synapse equally well. Since resolvase seems to bind and synapse the AT and AC *res* sites equally, the rate-limiting step in the AC site reactions remained unclear. A distinct possibility is that after cleavage of the strands, an AC site requires breakage of a G-C basepair (involving three hydrogen bonds) instead of an AT basepair (with two hydrogen bonds) in the natural site. The energy required to break the extra hydrogen bond may be enough to slow the AC site strand exchange. Alternatively, the catalytic domain of resolvase might interact less efficiently at the sites of strand exchange when the central dinucleotide is not AT (see also Section 4.2).

4.2 Properties of substrates containing TA central dinucleotides at site I of *res*.

The A-C and G-T mismatches that are implicated in the reactions described above are quite 'mild'-both are purine-pyrimidine pairs, which might still be able to hydrogen-bond and fit into the stacked DNA double helix. If a transitory, mismatched intermediate must be formed during the knotting reaction, it seemed possible that by making more severe mismatches at the central dinucleotide the knotting reaction might be completely blocked. Alternatively resolvase might cleave the DNA, exchange the strands, and then release a 'cleaved intermediate' because of problems in re-ligating the severely mismatched recombinant site. Either of these possibilities would be informative about the existence of a mismatched recombinant intermediate.

Another set of substrates was made in which the central dinucleotides at site I were mutated from AT to TA (M.Stark and G.Calder, unpublished results). pMA21 (AT x AT), pGC1 (AT x TA) and pGC4 (TA x TA) are shown in Figure 4.7. A recombinant catenane formed

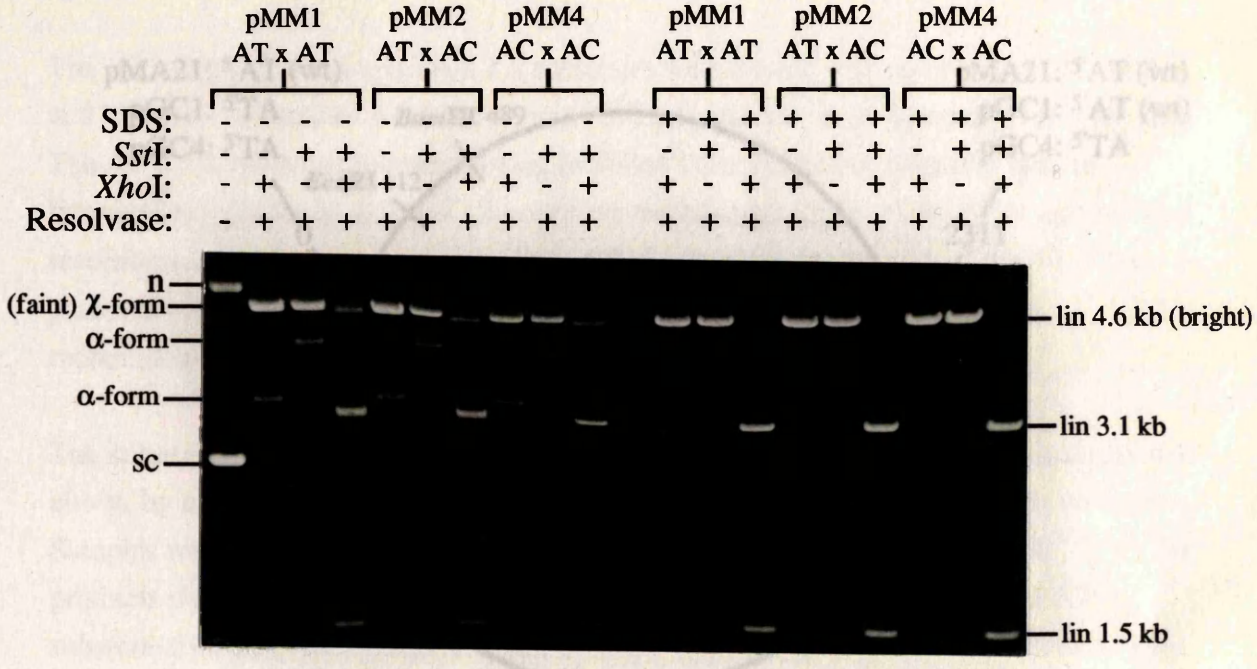


Figure 4.6 Glutaraldehyde-crosslinking of pMM1, pMM2 and pMM4 synapsis reactions.

Chemical crosslinking assays were performed to compare synapsis of substrates containing AT and AC *res* sites. pMM1, pMM2 and pMM4 were incubated with resolvase (1 unit) in a MOPS buffer and then crosslinked with glutaraldehyde for 5 minutes (see Section 2.41 for details). The products were cut with either *Xho*I (generates a fast running α-form), *Sst*I (generates a slow running α-form) or both (generates a very slow χ-form), and run on a 0.8% agarose gel. Split samples were run with and without SDS treatment. Uncrosslinked substrate cut with one restriction enzyme produces a 4.6 kbp linear, and cutting with both restriction enzymes produces a 3.1 kbp linear and a 1.5 kbp linear (see Figure 4.1).

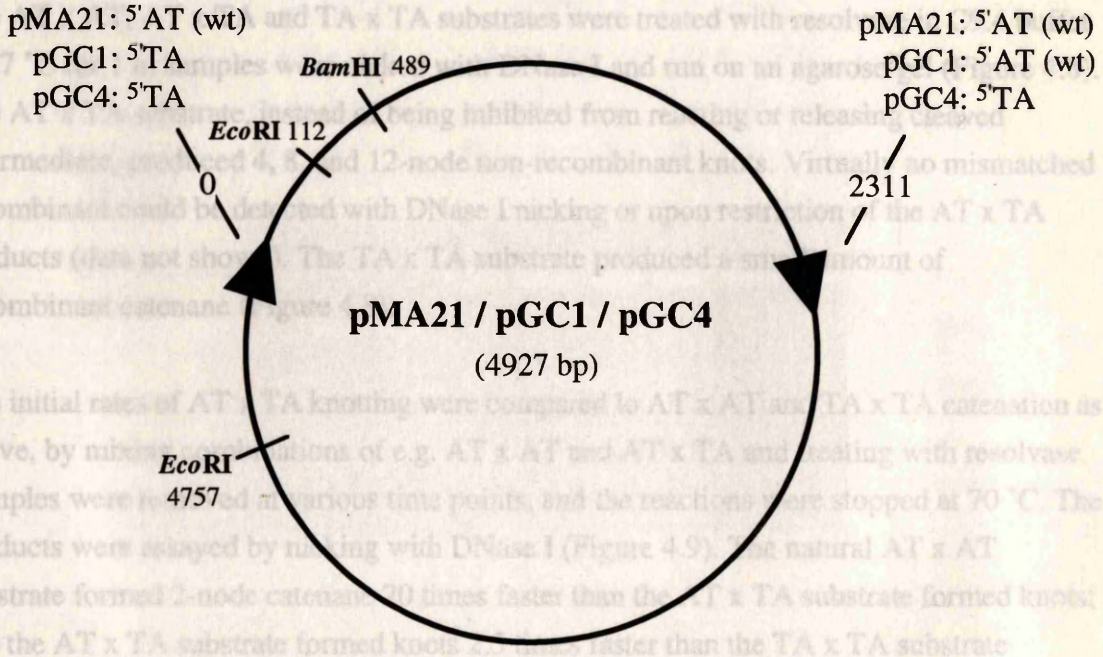


Figure 4.7 Simplified maps of pMA21, pGC1 and pGC4.

Simplified maps of plasmids containing copies of the wild-type *res* site and/or mutant *res* sites in which the site I central dinucleotide is altered. The *res* sites are shown as black triangles. pMA21 contains two wild-type *res* sites. pGC1 contains one wild type *res* site and one mutated *res* site containing a 'TA' central dinucleotide in place of the wild-type 'AT'. pGC4 has the central dinucleotide mutated to 'TA' in both *res* sites. The locations of three restriction sites in different domains of the substrates are shown. See Table 2.1 for further details.

from the AT x TA substrate would contain two T-T and two A-A mismatches; these pyrimidine-pyrimidine and purine-purine pairs are presumably much less stable than the A-C and G-T purine-pyrimidine pairings formed in the putative AT x AC knotting intermediate.

The AT x AT, AT x TA and TA x TA substrates were treated with resolvase in C9.4 buffer at 37 °C for 1 h; samples were nicked with DNase I and run on an agarose gel (Figure 4.8). The AT x TA substrate, instead of being inhibited from reacting or releasing cleaved intermediate, produced 4, 8, and 12-node non-recombinant knots. Virtually no mismatched recombinant could be detected with DNase I nicking or upon restriction of the AT x TA products (data not shown). The TA x TA substrate produced a small amount of recombinant catenane (Figure 4.8).

The initial rates of AT x TA knotting were compared to AT x AT and TA x TA catenation as above, by mixing combinations of e.g. AT x AT and AT x TA and treating with resolvase. Samples were removed at various time points, and the reactions were stopped at 70 °C. The products were assayed by nicking with DNase I (Figure 4.9). The natural AT x AT substrate formed 2-node catenane 20 times faster than the AT x TA substrate formed knots; and the AT x TA substrate formed knots 2.5 times faster than the TA x TA substrate formed 2-node catenane. Just as for the AC *res* substrates, the rates of reaction decreased with increasing number of TA *res* sites. Thus the reduced rate of reaction is dependent on some property of the TA *res* site itself, rather than on homology or non-homology in the partner site.

γδ TA *res* site I is known to be bound inefficiently by γδ resolvase (Hatfull *et al.*, 1988), so the binding of Tn*3* resolvase to Tn*3* TA *res* and AT *res* was compared on non-denaturing PAGE (Figure 4.5). The TA *res* site formed all six complexes, but full binding required a much higher concentration of resolvase than was needed with the natural AT *res* site. Even at the highest concentration of resolvase used, the TA *res* site only formed weak fifth and sixth complexes, suggesting that this mutated site I was bound last and least by resolvase. The poor yields of products and slow rates of reaction of the TA *res* site may be attributable to the weak binding of resolvase to this site. However, despite the severe mismatches that would have been present in the recombinant catenane from the TA x AT substrate, no evidence of enhanced formation of intermediates (e.g. cleavage products) was found.

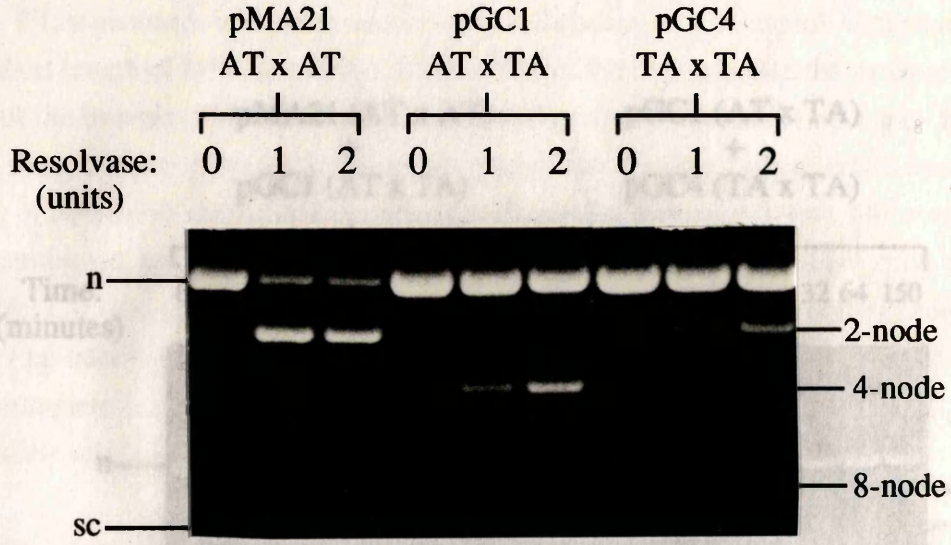


Figure 4.8 Recombination of pMA21, pGC1 and pGC4.

Recombination assay of pMA21, pGC1, and pGC4 which contain wild-type (AT) and/or mutated (TA) site I central dinucleotides as shown above and in Figure 4.7. Nicked substrate is indicated by 'n' and supercoiled substrate by 'sc'. The positions of recombinant 2-node catenane and non-recombinant 4-, and 8-node knots are also indicated. The plasmids were incubated with resolvase in C9.4 buffer, and the products were assayed by nicking with DNase I (described in Section 2.21) and then running on a 0.7% agarose gel.

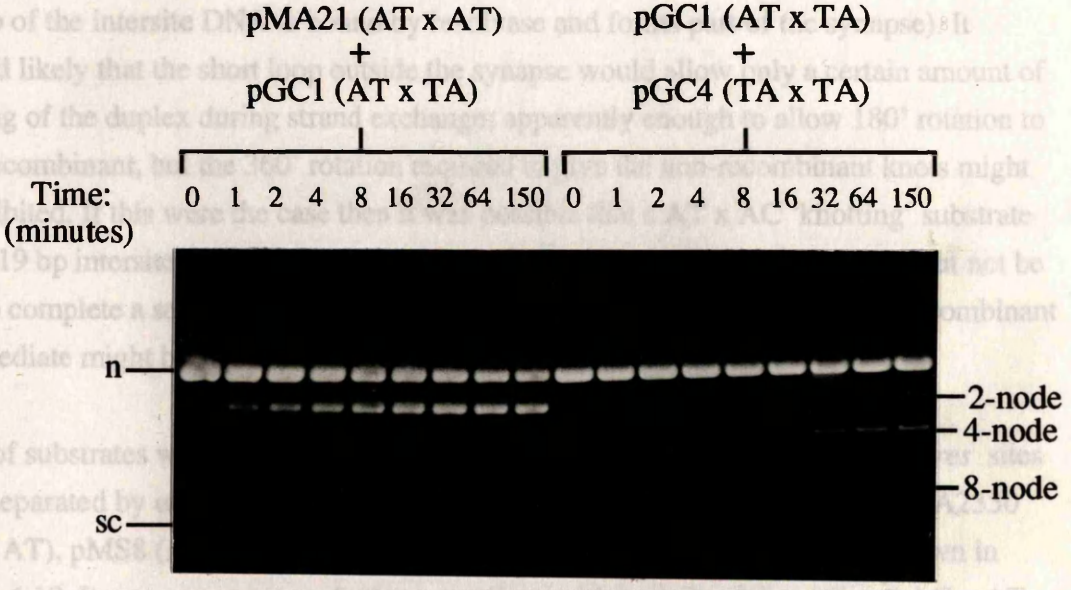


Figure 4.9 Comparison of the rates of catenation and knotting of pMA21, pGC1 and pGC4.

To compare the rates of catenation and knotting, mixtures of equal amounts of plasmids were reacted together with resolvase in time course assays. pMA21 + pGC1 and pGC1 + pGC4 mixtures were incubated with resolvase (1 unit per track) in C9.4 buffer and samples were removed at the times indicated and stopped at 70 °C. Samples were then nicked with DNase I and run on a 0.7% agarose gel. Products are labelled as Figure 4.2.

4.3 Properties of substrates with closely spaced *res* sites.

All of the substrates discussed so far have directly repeated *res* sites that are at least 1 kbp apart to allow efficient recombination. But it is known that resolvase can efficiently recombine *res* sites separated by only 219 bp, as in the substrate pMA2350 (Stark *et al.*, 1989b). When plasmids with such small intersite distances form synapses with resolvase, only a short length of DNA (~100 bp) is left as one of the loops outside the synapse (since 114 bp of the intersite DNA is bound by resolvase and forms part of the synapse). It seemed likely that the short loop outside the synapse would allow only a certain amount of twisting of the duplex during strand exchange; apparently enough to allow 180° rotation to give recombinant, but the 360° rotation required to give the non-recombinant knots might be inhibited. If this were the case then it was possible that a AT x AC 'knotting' substrate with 219 bp intersite spacing would be able to do the first 180° strand exchange but not be able to complete a second round, and therefore ligation to give the mismatched recombinant intermediate might be forced.

A set of substrates was cloned in which the four combinations of AT *res* and AC *res* sites were separated by only 219 bp (M.Stark, unpublished results). The plasmids pMA2350 (AT x AT), pMS8 (AT x AC), pMS9 (AC x AT) and pMS20 (AC x AC) are shown in Figure 4.10. It was necessary to do these reactions with both the AT x AC and AC x AT versions of the substrate because the geometry of the crossover site in the small loop of DNA might be different from the site in the larger loop.

These 'close-site' substrates were treated with resolvase in C9.4 buffer at 37 °C for 1 h; the products were assayed by nicking with DNase I, followed by agarose gel electrophoresis. The AT x AT and AC x AC substrates gave 2-node catenane as expected, but the AT x AC and AC x AT substrates produced no detectable non-recombinant knots and only a tiny amount of mismatched recombinant (Figure 4.11). Clearly the closely spaced substrates were dramatically inhibited from performing two rounds of strand exchange to form knots, and virtually all of the substrate remained as supercoiled non-recombinant plasmid. The AT x AT and AC x AC substrates produced large amounts of recombinant, showing that resolvase can bind, cleave and recombine substrates with closely spaced AT *res* or AC *res* sites. From this it seems likely that in the AT x AC and AC x AT reactions, the closely spaced substrates are also being bound and cleaved, and an attempt is being made at knotting the DNA. The distortion of the small loop of DNA between the sites blocks knotting, but the resolvase still does not ligate more than a few percent of the substrate to form mismatched recombinant. This result suggested that resolvase is highly inefficient at

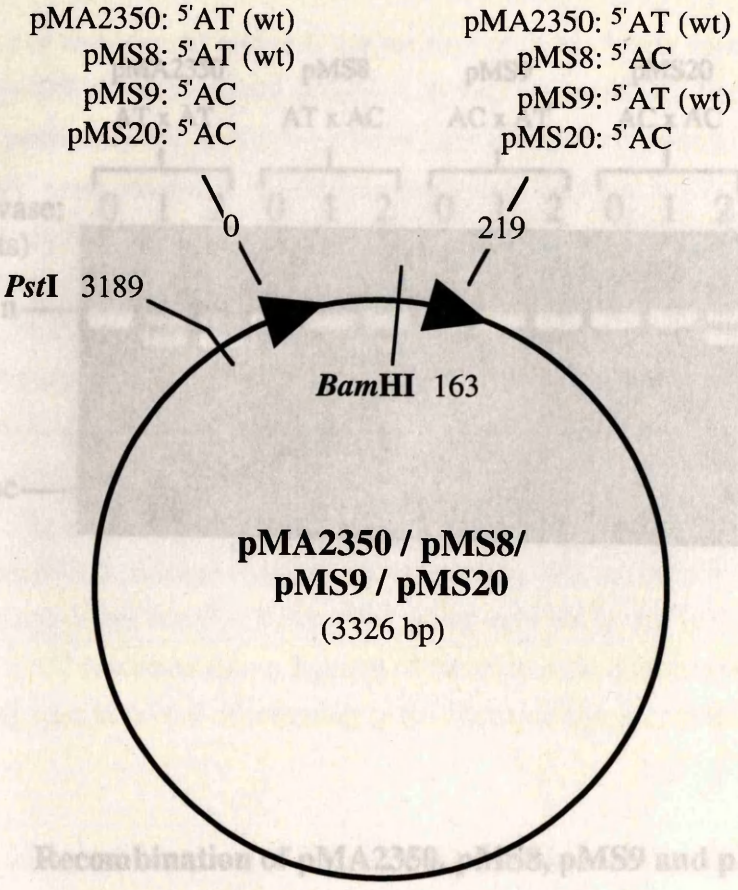


Figure 4.10 Simplified maps of pMA2350, pMS8, pMS9 and pMS20.

Simplified maps of plasmids containing copies of the wild-type *res* site and/or mutant *res* sites in which the site I central dinucleotide is altered. The *res* sites are only 219 bp apart and are shown as black triangles. pMA2350 contains two wild-type *res* sites. pMS8 and pMS9 contain one wild type *res* site and one mutated *res* site containing an 'AC' central dinucleotide in place of the wild-type 'AT'. pMS20 has the central dinucleotide mutated to 'AC' in both *res* sites. The locations of two unique restriction sites that are in different domains of the substrate are shown in bold type. See Table 2.1 for further details.

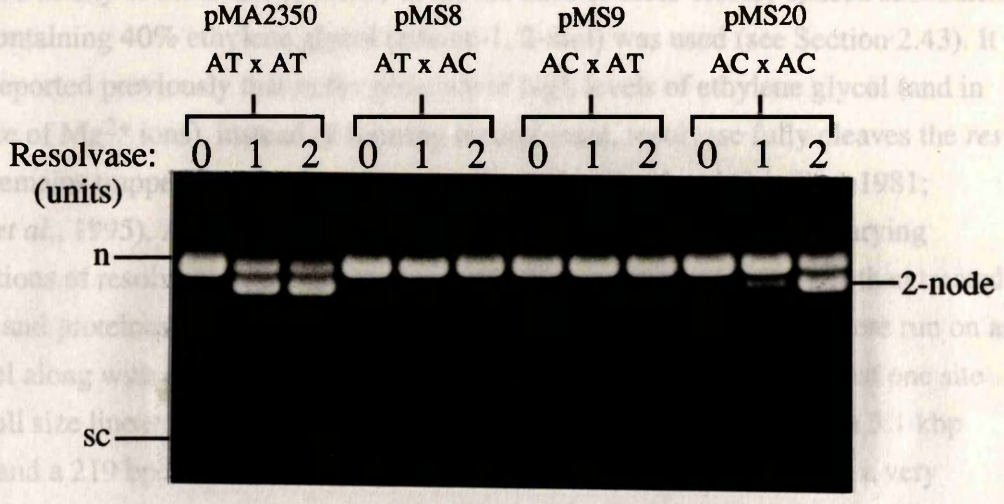


Figure 4.11 Recombination of pMA2350, pMS8, pMS9 and pMS20.

Recombination assay of 'close-site' pMA2350, pMS8, pMS9 and pMS20 which contain wild-type (AT) and/or mutated (AC) site I central dinucleotides as shown above and in Figure 4.10. Nicked substrate is indicated by 'n' and supercoiled substrate by 'sc'. The position of recombinant 2-node catenane is indicated. The plasmids were incubated with resolvase in C9.4 buffer, and the products were assayed by nicking with DNase I (described in Section 2.21) and then running on a 0.7% agarose gel.

ligating the mismatched recombinant, and the intermediate instead prefers to return to the non-recombinant substrate configuration. If ligation to give the mismatched recombinant is so inefficient, then it is unlikely that ligation is essential in the knotting reaction of AT x AC substrates with well-separated *res* sites (e.g. pMM2).

To assay the ability of resolvase to cleave at the *res* sites of these closely spaced substrates, a buffer containing 40% ethylene glycol (ethane-1, 2-diol) was used (see Section 2.43). It has been reported previously that in the presence of high levels of ethylene glycol (and in the absence of Mg^{2+} ions), instead of forming recombinant, resolvase fully cleaves the *res* sites and remains trapped covalently attached to the DNA (Reed and Grindley, 1981; Boocock *et al.*, 1995). All four close-spaced substrates were incubated with varying concentrations of resolvase in the ethylene glycol buffer for 16 h at 37 °C, and then treated with SDS and proteinase K to remove any attached resolvase. The reactions were run on an agarose gel along with a DNA size marker ladder (Figure 4.12). Cleavage at just one site releases full size linear plasmid (3.3 kbp) and cleavage at both sites produces a 3.1 kbp fragment and a 219 bp fragment which runs off the gel. All substrates showed a very similar pattern of resolvase-induced cleavage, demonstrating that resolvase can cleave all of these close-spaced substrates equally. It therefore seems very likely that in the reactions of AC x AT and AT x AC described above, ligation of the mismatched recombinant is strongly selected against in favour of returning to the substrate non-recombinant DNA configuration.

These 'close-site' substrates seem to demonstrate some interesting points about the energetics and reversibility of cleavage and strand exchange. As described in Chapter 1, the recombination and knotting reactions are thought to be driven forward to products (rather than going back to substrate) by an energetically favourable loss of negative supercoiling. Thus in a well-spaced AT x AC substrate reaction, after one round of recombination the reaction is driven forward to perform a second round of strand exchange and form 4-noded knot rather than reverse the direction of strand exchange to re-ligate the non-recombinant substrate configuration. In the close-site AT x AT substrate reaction, there is probably some distortion of the small loop of intersite DNA after 180° rotation of ends, but not enough to make the reaction unfavourable and so the 2-node catenane is formed. However in the close-site AT x AC substrate reaction, after one round of recombination resolvase seems unable to ligate the mismatched recombinant and the direction of strand exchange is reversed to re-form the substrate. This suggests that a double round of recombination in a close-site substrate would create too much distortion of the small DNA loop, and so the energy equilibrium favours the ligated substrate configuration over the 4-noded knot.

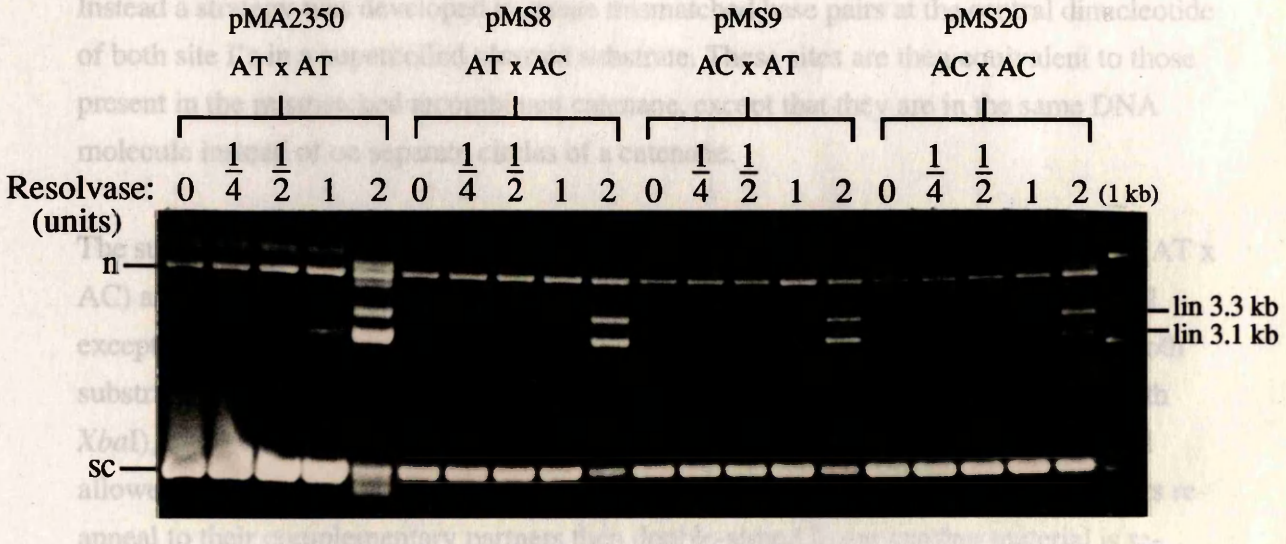


Figure 4.12 Resolvase-induced cleavage of pMA2350, pMS8, pMS9 and pMS20 in ethylene glycol buffer.

Assay of resolvase-induced cleavage at the *res* sites of 'close-site' substrates pMA2350, pMS8, pMS9 and pMS20 (shown in Figure 4.10). The substrates were incubated with resolvase for 16 hours in ethylene glycol buffer (see Section 2.43) and then treated with SDS and proteinase K. The products were run on a 1.2% agarose gel along with a 1 kbp size ladder (1 kb). Cleavage at one *res* site releases full size linear plasmid (3.3 kbp) and cleavage at both *res* sites produces a 3.1 kbp fragment and a 219 bp fragment (runs off the gel).

4.4 A substrate with mismatched base pairs at both site I's of *res*.

As well as trying to detect the formation of the mismatched recombinant, we were also interested in the ability of resolvase to recombine *res* sites with mismatched basepairs at the centre of site I. The mismatched recombinant catenane is present in such small quantities that no attempt was made to purify sufficient amounts to act as a substrate for resolvase. Instead a strategy was developed to create mismatched base pairs at the central dinucleotide of both site I's in a supercoiled plasmid substrate. These sites are then equivalent to those present in the mismatched recombinant catenane, except that they are in the same DNA molecule instead of on separate circles of a catenane.

The substrate containing the mismatches was prepared by using the plasmids pMM2 (AT x AC) and pMM3 (AC x AT) described in Section 4.1. These two plasmids are identical except that they have different *res* site I's mutated to AC at the central dinucleotide. Both substrates were cut with a unique restriction enzyme (pMM2 with *Sst*I and pMM3 with *Xba*I), and then the linear plasmids were mixed, denatured at 85 °C for 6 minutes and allowed to re-anneal at room temperature for a few minutes. If the single-strand linears re-anneal to their complementary partners then double-strand linear starting material is re-formed. However if single-strands from the separate plasmids anneal, then nicked, double-strand circles are formed with an A-C mismatch at one site and a T-G mismatched base pair at the other site. The substrate could then be gel-purified, and supercoiled by ligating the nicks in the presence of ethidium bromide, then extracting the intercalator with phenol. The construction of the mismatched substrate is summarised in Figure 4.13. This method of denaturing and re-annealing plasmids proved to be very inefficient, giving only a small amount of nicked substrate material after considerable refinement of the reaction conditions. The majority of the DNA remained as linear plasmid even after extensive denaturation.

A small amount of annealed pMM2/pMM3, mismatched substrate was purified and supercoiled by ligating in the presence of ethidium bromide. A standard resolvase reaction was then carried out using this substrate along with natural carrier plasmid (pMA2350; shown in Figure 4.10), and the products were nicked with DNase I. The reactions were run on an agarose gel along with uncut samples of the mismatched substrate and the carrier plasmid. The products formed with the 'normal' substrate were visualised by ethidium bromide staining, but such a small amount of the mismatched substrate was used that the gel had to be Southern blotted and probed with a radiolabelled primer (Figure 4.14). From the uncut sample it was clear that less than 50% of the mismatched substrate was

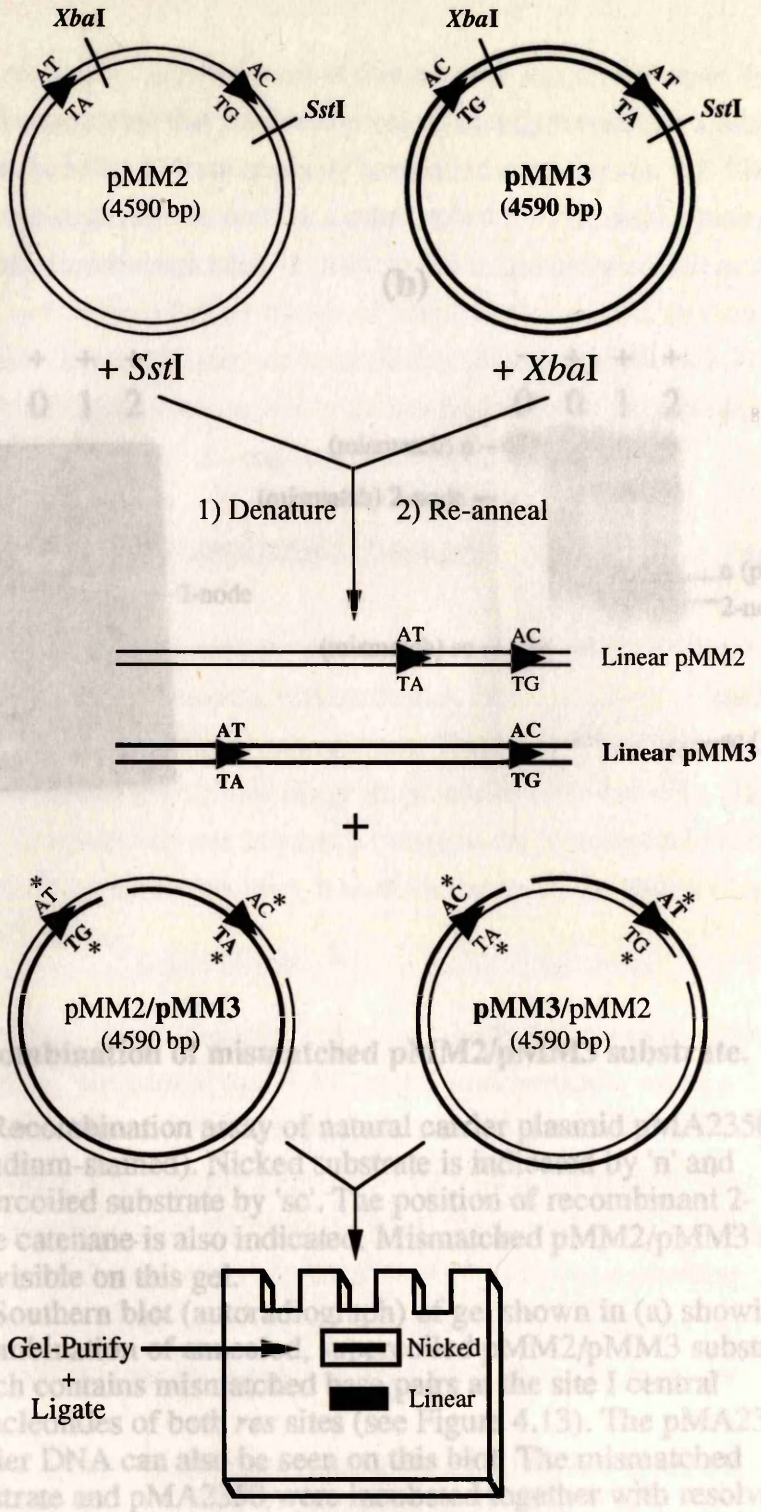


Figure 4.13 Synthesis of mismatched substrates by annealing single-strand plasmids.

Summary of the construction of supercoiled plasmids containing mismatched base pairs (*) at the site I central dinucleotide. The *res* sites are shown as black triangles and the top and bottom strand sequence at the centre of each site I is also shown. pMM2 was cut with *Sst*I and pMM3 was cut with *Xba*I; the linear plasmids were mixed, denatured at 85 °C and allowed to re-anneal at room temperature. The products were run on an agarose gel, and the nicked, mismatched substrate was recovered from the agarose. The nicks were then ligated in the presence of ethidium bromide, and supercoiling was induced by extraction of the intercalator.

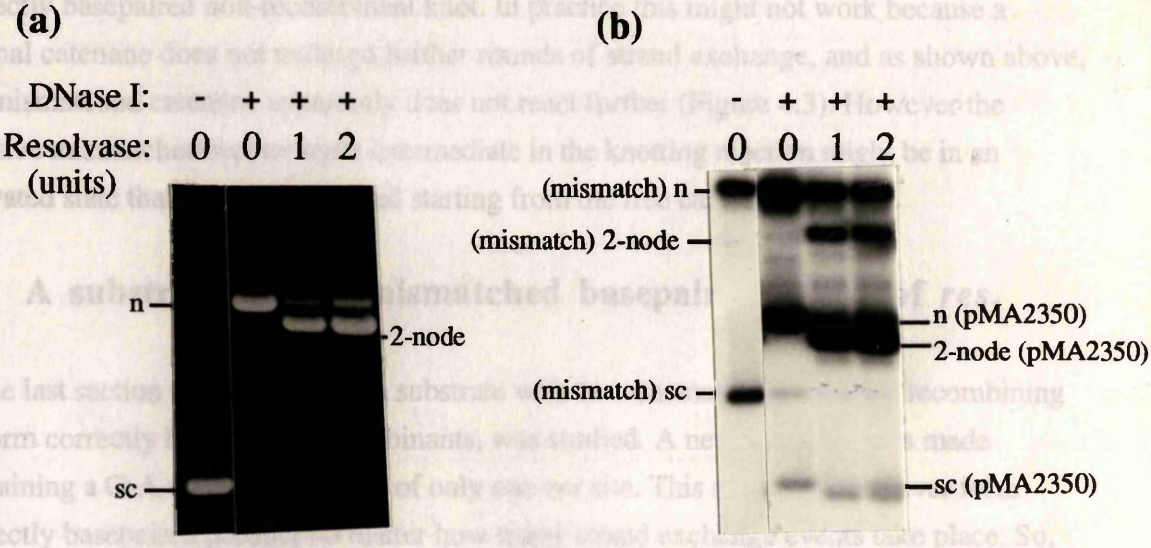


Figure 4.14 Recombination of mismatched pMM2/pMM3 substrate.

(a) Recombination assay of natural carrier plasmid pMA2350 (ethidium-stained). Nicked substrate is indicated by 'n' and supercoiled substrate by 'sc'. The position of recombinant 2-node catenane is also indicated. Mismatched pMM2/pMM3 is not visible on this gel.
(b) Southern blot (autoradiograph) of gel shown in (a) showing recombination of annealed, supercoiled pMM2/pMM3 substrate, which contains mismatched base pairs at the site I central dinucleotides of both *res* sites (see Figure 4.13). The pMA2350 carrier DNA can also be seen on this blot. The mismatched substrate and pMA2350 were incubated together with resolvase in C9.4 buffer, and the products were assayed by nicking with DNase I and running on a 0.7% agarose gel.

supercoiled; however resolvase converted most of this material into recombinant 2-node catenane. This result demonstrated that resolvase could efficiently recombine a substrate containing two mismatched sites to form correctly basepaired recombinant. It is likely then that in principle resolvase could also recombine a mismatched recombinant catenane to form correctly basepaired non-recombinant knot. In practice this might not work because a normal catenane does not undergo further rounds of strand exchange, and as shown above, the mismatched catenane apparently does not react further (Figure 4.3). However the putative mismatched recombinant intermediate in the knotting reaction might be in an activated state that cannot be reached starting from the free catenane.

4.5 A substrate with a mismatched basepair at site I of *res*.

In the last section the reactivity of a substrate with two mismatched *res* sites, recombining to form correctly basepaired recombinants, was studied. A new substrate was made containing a C-A mismatch at site I of only one *res* site. This substrate can never form correctly basepaired product no matter how many strand exchange events take place. So, although this substrate contains only one mismatch (whereas the hypothetical mismatched recombinant in the knotting reaction has two), it would let us assay the ability of resolvase to ligate a mismatched basepair.

To make this new substrate, oligonucleotides equivalent to site I of *res* were synthesised containing a top strand AC and bottom strand AT central dinucleotide to create a C-A mismatch when annealed (Figure 4.15a). Binding of resolvase to this mismatched site I was compared to binding to a natural site I by incubating both labelled sites with resolvase and then running the samples on non-denaturing PAGE (Figure 4.15b). The site I containing the C-A mismatch was bound remarkably well by resolvase, showing enhanced binding compared to the natural site. Introducing a mismatched basepair may have created a more flexible site I which requires less energy to bend when bound by resolvase.

Labelled, artificial supercoiled substrate was made by ligating this mismatched site I into pMM5 in a two-step ligation process (as described in Chapter 3, Section 3.2 and in Chapter 2, Section 2.47). The purified substrate (pMM5*) was treated with resolvase in C9.4 buffer for 1 h along with natural pMM5 carrier. The reactions were run on an agarose gel (i) uncut, (ii) cut with *Sst*I and (iii) nicked with DNase I (with a sample of each reaction treated with proteinase K). The carrier pMM5 was visualised by ethidium bromide staining and pMM5* by autoradiography (Figure 4.16). The mismatched substrate was recombined by resolvase to form mainly 2-node catenane, with no detected non-recombinant knots.

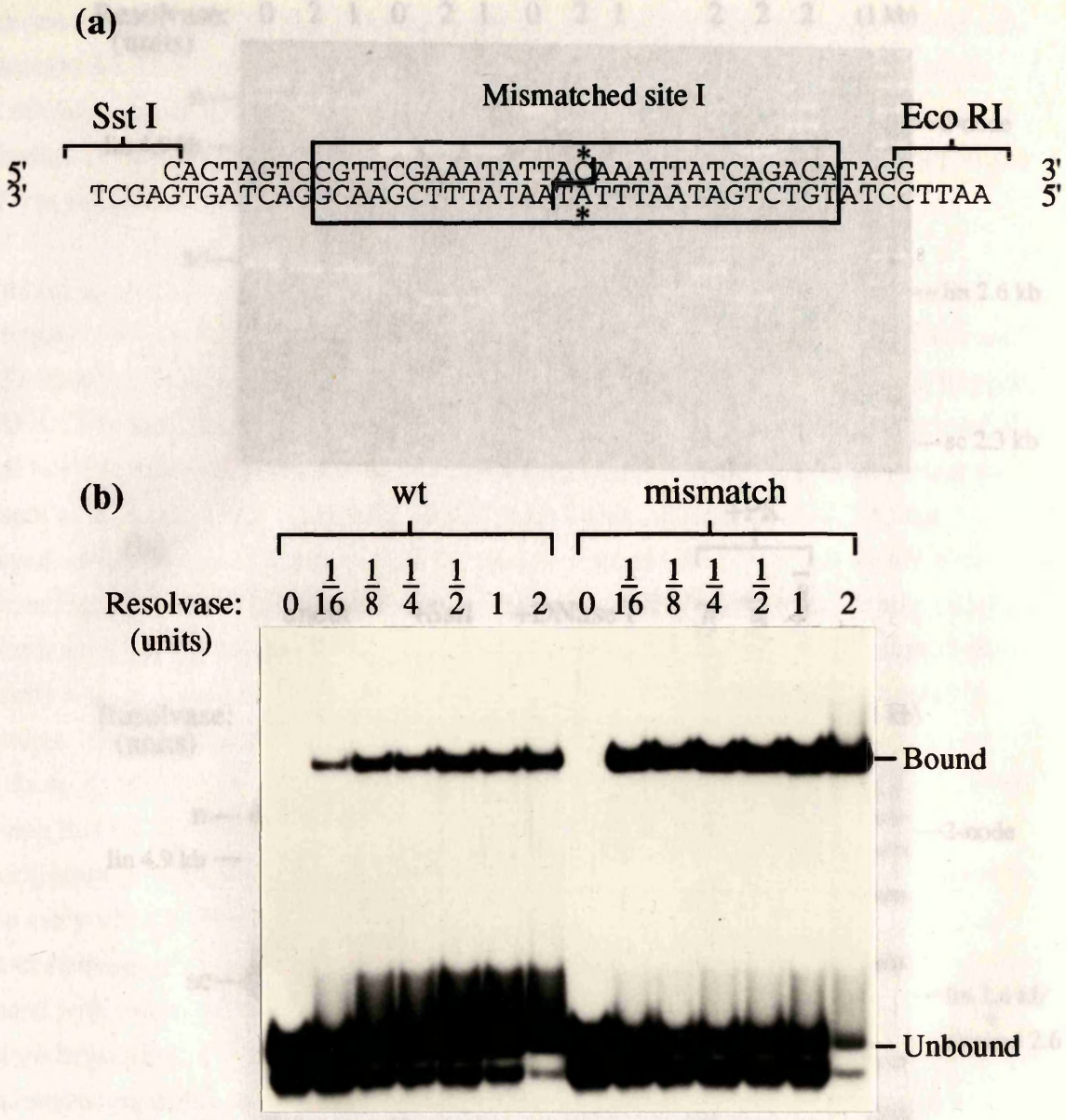


Figure 4.15 Binding of resolvase to a mismatched site I.

(a) The sequence of the site I oligonucleotides that create a C-A mismatch (*) at the central dinucleotide when annealed.

(b) The labelled site I (shown in (a)) and a natural site I were incubated with resolvase in binding buffer and then run on an 8% acrylamide non-denaturing gel.

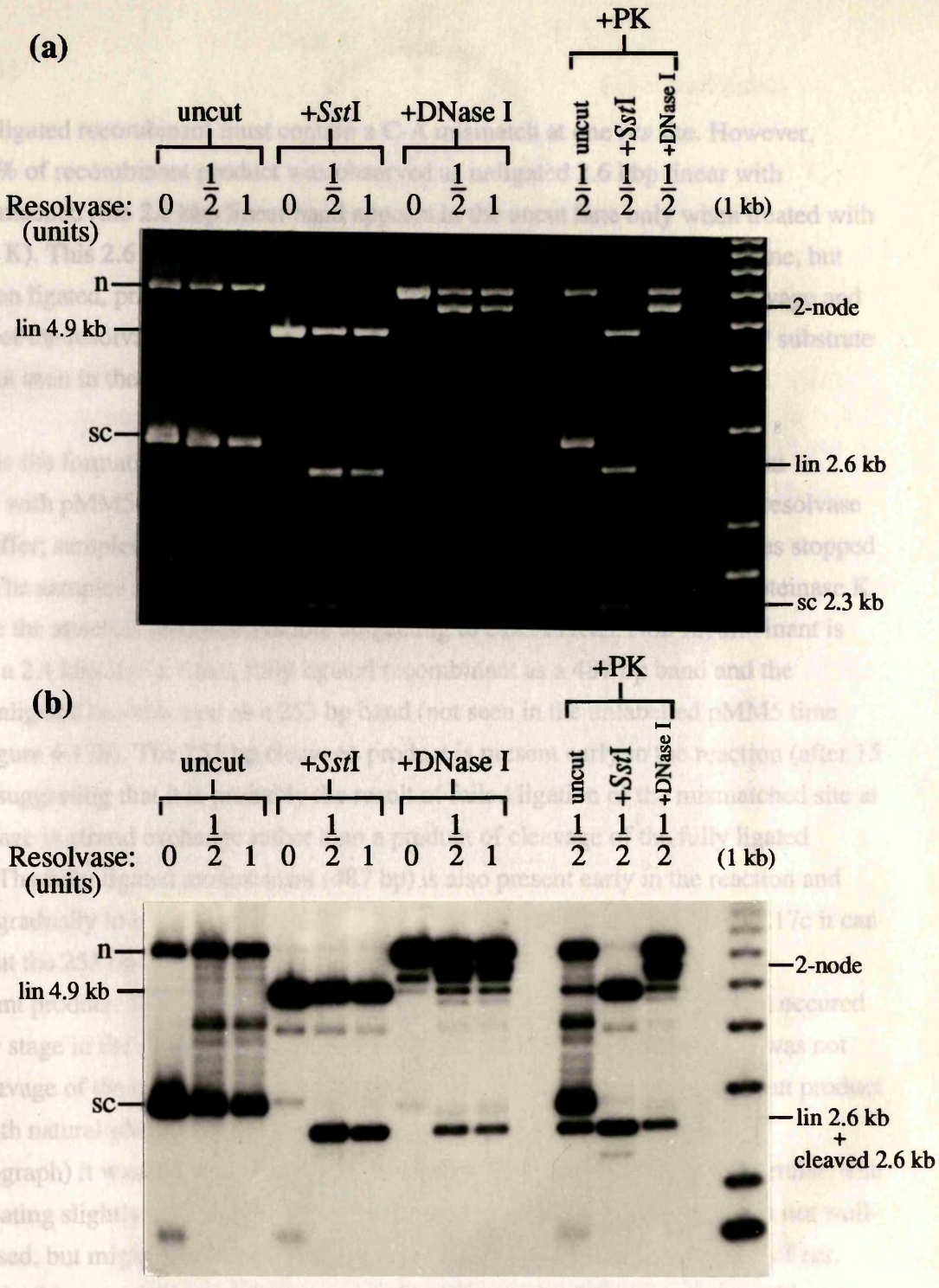


Figure 4.16 Recombination of artificial supercoiled pMM5* containing a mismatched base pair labelled at the *Sst*I site.

(a) Recombination of natural pMM5. Nicked circles are indicated by 'n', supercoiled circles by 'sc' and linear molecules by 'lin'. Restriction of substrate pMM5 by *Sst*I produces a non-recombinant 4.9 kbp linear. Restriction of products produces a recombinant 2.6 kbp linear and a 2.3 kbp circle. Nicking of products by DNase I produces the 2-node catenane.

(b) Recombination of artificial supercoiled substrate (pMM5*) which contains the mismatch site I radiolabelled at the *Sst* I end (shown in Figure 4.15a). All reactions were in C9.4 buffer and products were run on a 1.2% agarose gel (i) uncut, (ii) cut with *Sst*I and (iii) nicked with DNase I. One sample of each was treated with proteinase K (+PK).

This fully ligated recombinant must contain a C-A mismatch at one *res* site. However, around 25% of recombinant product was observed as unligated 2.6 kbp linear with resolvase attached (the 2.6 kbp linear band appears in the uncut lane only when treated with proteinase K). This 2.6 kbp linear would have made the larger circle of the catenane, but has not been ligated, presumably because it contains a mismatched basepair. Cleavage and formation of the resolvase-DNA complex was specific to the mismatched pMM5* substrate and was not seen in the carrier pMM5.

To examine the formation of the cleaved, linear product, a time course reaction was performed with pMM5*. Natural pMM5 and labelled pMM5* were reacted with resolvase in C9.4 buffer; samples were removed at different time points and the reaction was stopped at 70 °C. The samples were all cut with *Pvu*II (Figure 4.17a) and treated with proteinase K (to remove the attached resolvase) before subjecting to SDS/PAGE. Non-recombinant is present as a 2.4 kbp linear band, fully ligated recombinant as a 487 bp band and the cleaved, unligated recombinant as a 253 bp band (not seen in the unlabelled pMM5 time course; Figure 4.17b). The 253 bp cleavage product is present early in the reaction (after 15 seconds), suggesting that it is probably the result of failed ligation of the mismatched site at an early stage in strand exchange rather than a product of cleavage of the fully ligated catenane. The fully ligated recombinant (487 bp) is also present early in the reaction and builds up gradually to eventually become the strongest product band. In Figure 4.17c it can be seen that the 253 bp cleavage product appeared more quickly than the 487 bp recombinant product. This again suggested that formation of the cleavage product occured at an early stage in the strand exchange process as a result of failed ligation, and was not due to cleavage of the catenane. By comparing the 487 bp fully ligated recombinant product formed with natural pMM5 (ethidium-stained gel) and 'mismatched' pMM5* (autoradiograph) it was apparent that the rates of these two reactions were very similar. The band migrating slightly slower than 487 bp recombinant at the early time points is not well-characterised, but might be the 487 bp fragment with a nick at the centre of site I of *res*. This band builds up quickly and then seems to be converted to fully closed recombinant, perhaps as the nick is re-ligated. If this interpretation is correct, it constitutes more evidence that ligation of the mismatched recombinant catenane is indeed inhibited.

The 2.3 kbp recombination product from pMM5* does not contain any mismatched basepairs. Its formation was examined by re-making the supercoiled, mismatched substrate with the radiolabel at the *Eco*RI end of the site I oligonucleotides (Figure 4.15a). The labelled mismatched pMM5* was treated with resolvase as above. The samples were run on an agarose gel (i) uncut, (ii) cut with *Hin*dIII and (iii) nicked with DNase I. The carrier

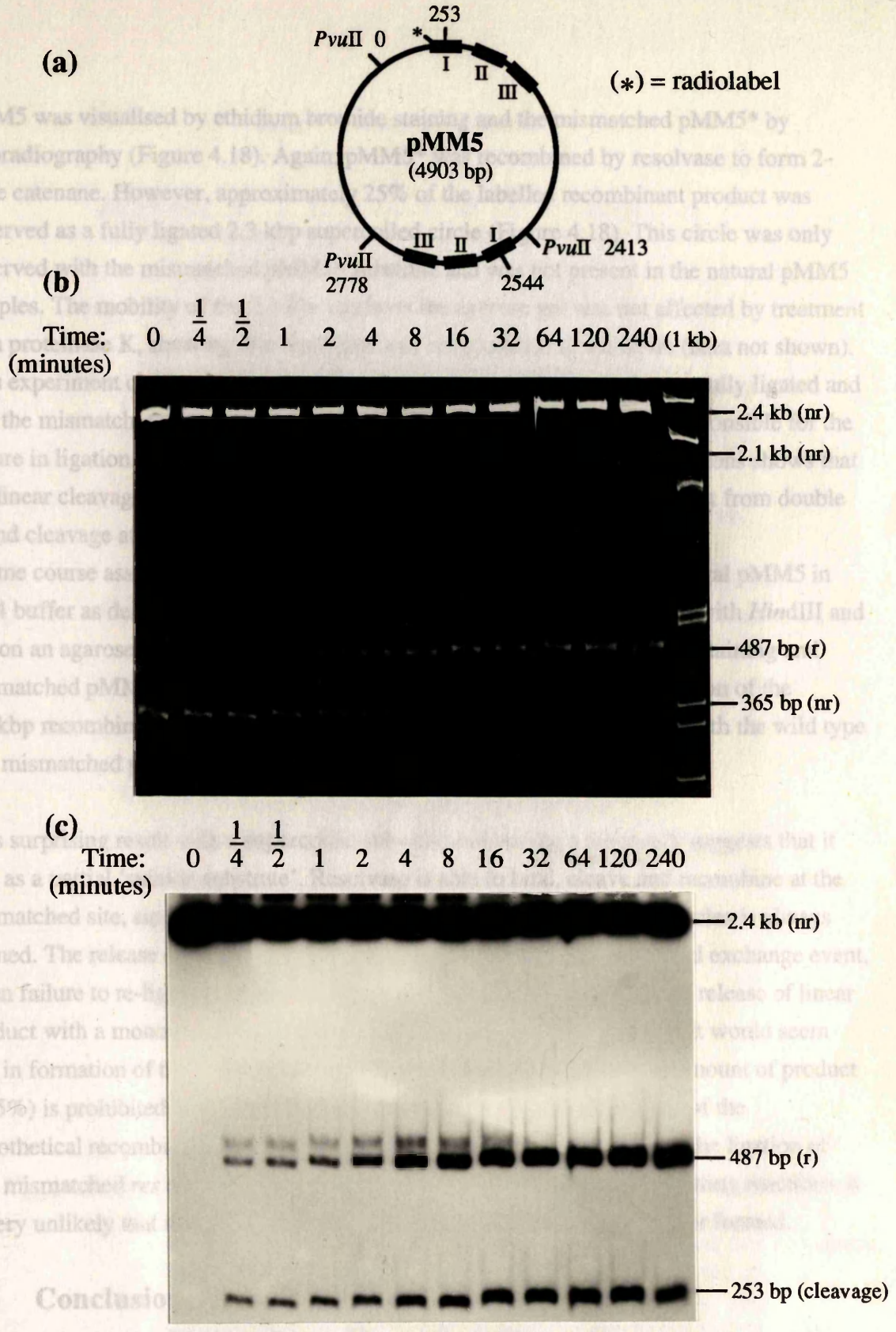


Figure 4.17 Time course of mismatch pMM5* recombination.

(a) Simplified map of pMM5 showing the *Pvu*II sites (in bp).
(b) Time course of natural pMM5 recombination assayed by cutting with *Pvu*II. The sizes of non-recombinant (nr) and recombinant (r) fragments are shown.
(c) Time course of mismatch pMM5* recombination. The additional 253 bp cleavage product is indicated. pMM5* and pMM5 were incubated with resolvase (0.5 units per track) in C9.4 buffer, and the reactions were stopped at the times shown. The samples were cut with *Pvu*II and treated with proteinase K before running on a 6% acrylamide 0.1% SDS gel.

pMM5 was visualised by ethidium bromide staining and the mismatched pMM5* by autoradiography (Figure 4.18). Again, pMM5* was recombined by resolvase to form 2-node catenane. However, approximately 25% of the labelled recombinant product was observed as a fully ligated 2.3 kbp supercoiled circle (Figure 4.18). This circle was only observed with the mismatched pMM5* substrate and was not present in the natural pMM5 samples. The mobility of the 2.3 kbp circle on the agarose gel was not affected by treatment with proteinase K, showing that resolvase was not attatched to the DNA (data not shown). This experiment confirmed that the correctly basepaired 2.3 kbp circle was fully ligated and that the mismatched basepairs in the 2.6 kbp recombinant product were responsible for the failure in ligation. Furthermore, the presence of the free circle in these reactions shows that the linear cleavage product is derived from aborted recombinant, and not just from double strand cleavage at both *res* sites of the substrate molecule.
A time course assay was carried out with this mismatched pMM5* and natural pMM5 in C9.4 buffer as described previously, except this time all samples were cut with *Hin*dIII and run on an agarose gel. Carrier pMM5 was visualised by ethidium bromide staining and mismatched pMM5* by autoradiography (Figure 4.19). The rate of formation of the 2.3 kbp recombinant circle (linearised with *Hin*dIII) was very similar for both the wild type and mismatched pMM5 substrates.

This surprising result with a supercoiled substrate containing a mismatch suggests that it acts as a partial 'suicide substrate'. Resolvase is able to bind, cleave and recombine at the mismatched site, since at least one of the two supercoiled, recombinant circles is always formed. The release of cleavage product presumably comes after the strand exchange event, when failure to re-ligate the mismatched recombinant *res* site results in the release of linear product with a monomer of resolvase attached at each end (Figure 4.20). It would seem that in formation of the mismatched recombinant catenane, a substantial amount of product (~25%) is prohibited from re-ligating the mismatched *res* site. Formation of the hypothetical recombinant in the AC x AT knotting reaction would require the ligation of two mismatched *res* sites. As no cleavage products are detected in the knotting reactions, it is very unlikely that a ligated, mismatched recombinant intermediate is ever formed.

4.6 Conclusions.

It is extremely difficult to prove or disprove theoretical models of strand exchange in one experiment or with a single observation. When taken together, the experiments described in this chapter provide compelling evidence that the mismatched recombinant catenane is not

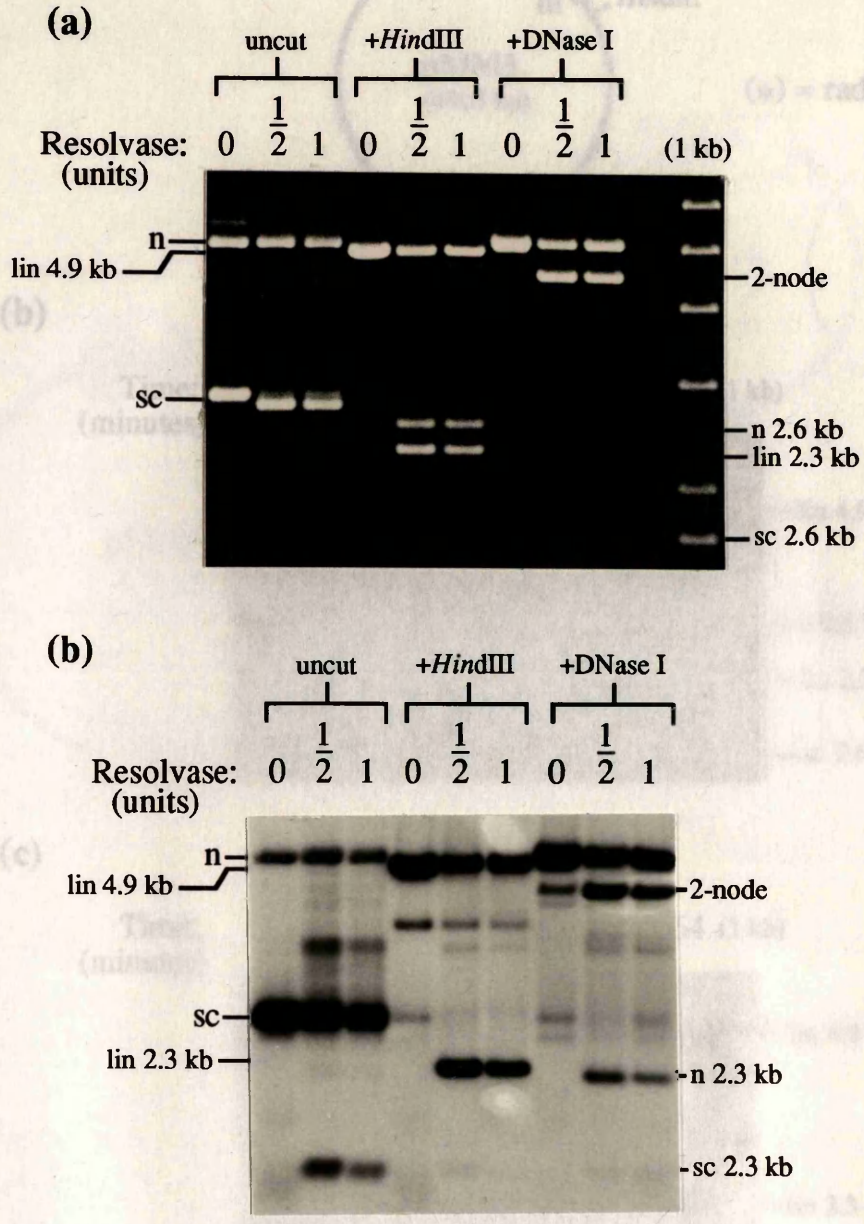


Figure 4.18 Recombination of mismatch pMM5* labelled at the *Eco*RI site.

(a) Recombination of natural pMM5. Nicked circles are indicated by 'n', supercoiled circles by 'sc' and linear molecules by 'lin'. Restriction of substrate pMM5 by *Hin*dIII produces a non-recombinant 4.9 kbp linear. Restriction of products by *Hin*dIII produces a recombinant 2.3 kbp linear and a 2.6 kbp circle. Nicking of products by DNase I produces the 2-node catenane. (b) Recombination of artificial supercoiled substrate (pMM5*) which contains the mismatch site I (shown in Figure 4.15a) radiolabelled at the *Eco*RI site. All reactions were in C9.4 buffer and products were run on a 0.7% agarose gel (i) uncut, (ii) cut with *Hin*dIII and (iii) nicked with DNase I.

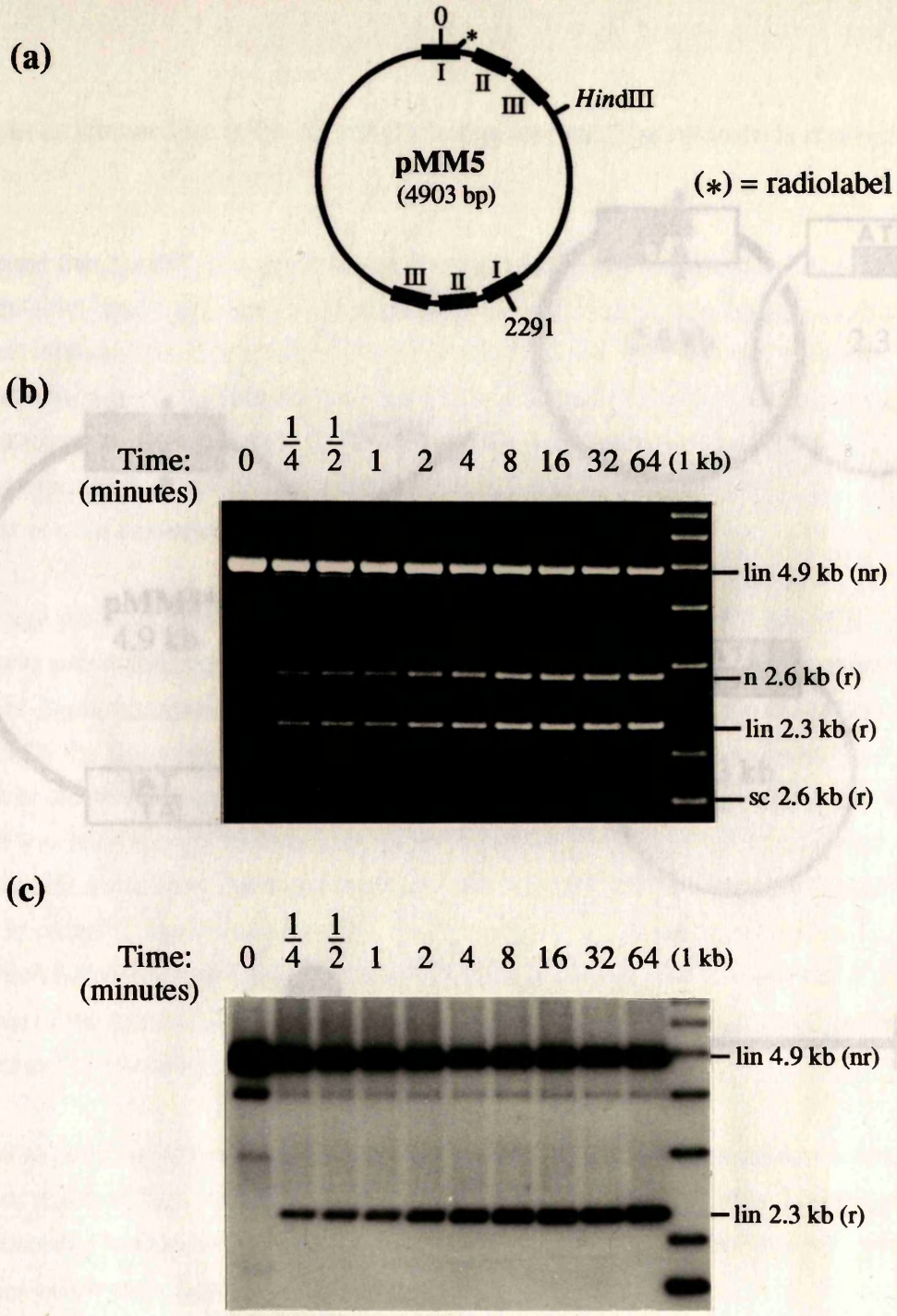


Figure 4.19 Time course of mismatch pMM5* recombination labelled at the *Eco*RI site.

(a) Simplified map of pMM5 showing the *Hin*dIII site.
(b) Time course of natural pMM5 recombination assayed by restriction with *Hin*dIII. Nicked circles are represented by 'n', supercoiled circles by 'sc', linear molecules by 'lin', non-recombinant by 'nr' and recombinant by 'r'.
(c) Time course of mismatch pMM5* recombination (with the radiolabel at the *Eco*RI site). pMM5* and pMM5 were incubated together with resolvase (0.5 units per track) in C9.4 buffer, samples were removed and stopped at the times shown and then cut with *Hin*dIII before running on a 1.2% agarose gel.

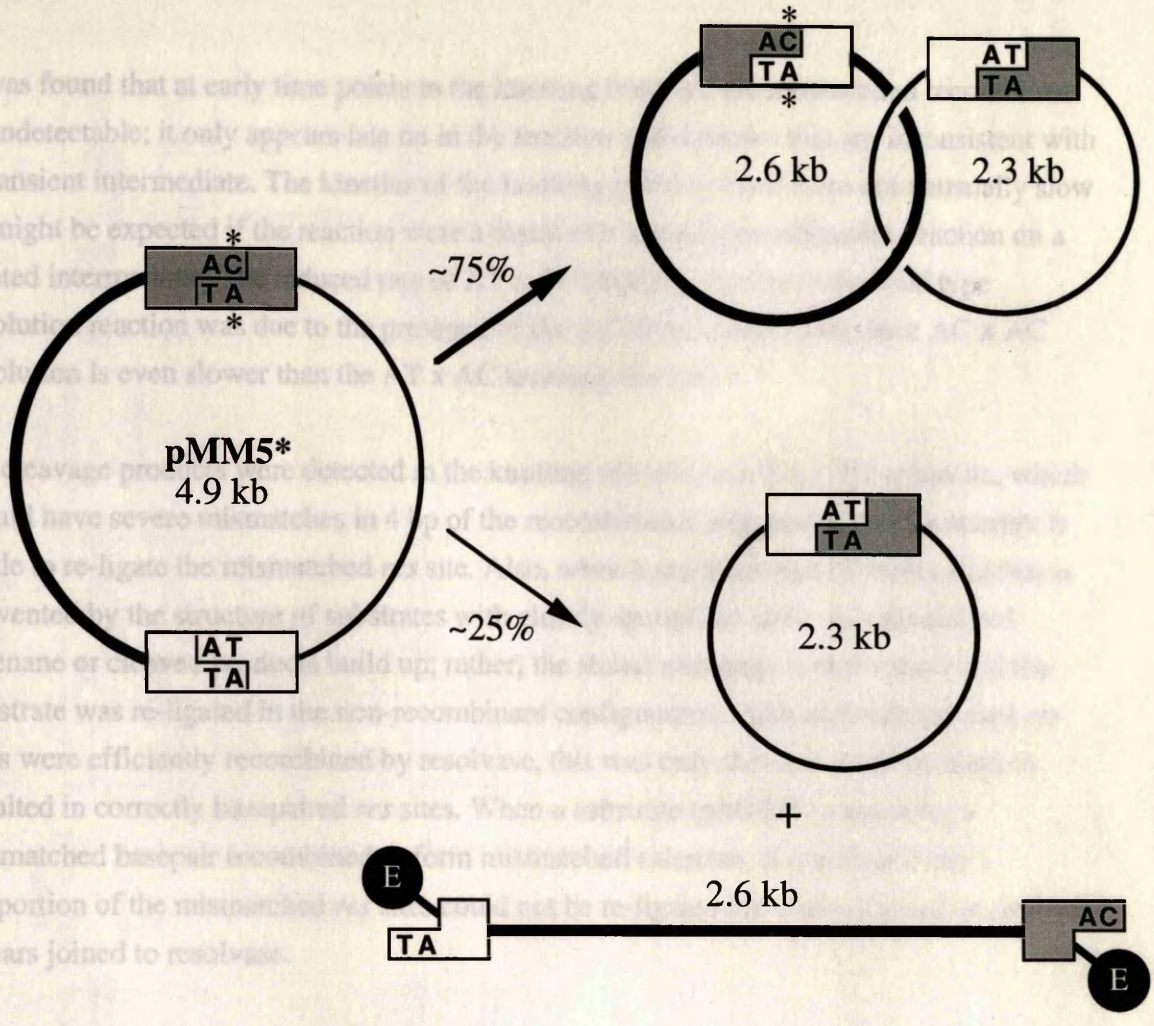


Figure 4.20 Summary of recombination of mismatch pMM5*.

Diagram showing the products formed when an artificial supercoiled substrate (containing a mismatched base pair at the centre of one site I) is treated with resolvase. For simplicity, only the sequence of the recombining site I central dinucleotides is shown. Mismatched base pairs are indicated by '*' and the different domains of the substrate are shown by thick and thin lines. The resolvase monomers that become attached to the cleaved 2.6 kb linear are shown as black circles.

required as an intermediate in the AT x AC knotting reaction. The evidence is summarised below.

It was found that at early time points in the knotting reaction, the mismatched recombinant is undetectable; it only appears late on in the reaction with kinetics that are inconsistent with a transient intermediate. The kinetics of the knotting reaction itself were not unusually slow as might be expected if the reaction were a result of a second recombination reaction on a ligated intermediate. The reduced rate of AT x AC knotting relative to the wild type resolution reaction was due to the presence of the AC dinucleotide itself since AC x AC resolution is even slower than the AT x AC knotting reaction.

No cleavage products were detected in the knotting reaction in a TA x AT substrate, which would have severe mismatches in 4 bp of the recombinants, suggesting that no attempt is made to re-ligate the mismatched *res* site. Also, when a second round of recombination is prevented by the structure of substrates with closely spaced *res* sites, no mismatched catenane or cleaved products build up; rather, the strand exchange was reversed and the substrate was re-ligated in the non-recombinant configuration. Although mismatched *res* sites were efficiently recombined by resolvase, this was only the case when re-ligation resulted in correctly basepaired *res* sites. When a substrate (pMM5*) containing a mismatched basepair recombined to form mismatched catenane, it was found that a proportion of the mismatched *res* sites could not be re-ligated but were released as cleaved linears joined to resolvase.

This final experiment demonstrated that re-ligation of a mismatched *res* site is an extremely inefficient reaction. No cleavage products are detected in the AT x AC reaction, suggesting that the knotting reaction is a concerted 360° strand exchange rather than two 180° strand exchanges involving a ligated, mismatched intermediate.

A possible scheme for the cleavage and strand exchange steps involved in the knotting reaction catalysed by resolvase is represented in Figure 4.21. Only the recombining crossover half-sites are shown for simplicity. Single arrows between different stages represent irreversible reaction steps while double arrows represent reversible reaction steps. The first step requires resolvase-catalysed cleavage of both crossover sites (either individual or concerted cleavages may be involved) to generate two fully cleaved site I's (Figure 4.21). A 180° exchange of DNA half-sites (by any strand exchange mechanism) produces the 'cleaved recombinant', containing the mismatched basepairs, which may or may not be ligated. In a subunit rotation mechanism the 'cleaved recombinant' can undergo

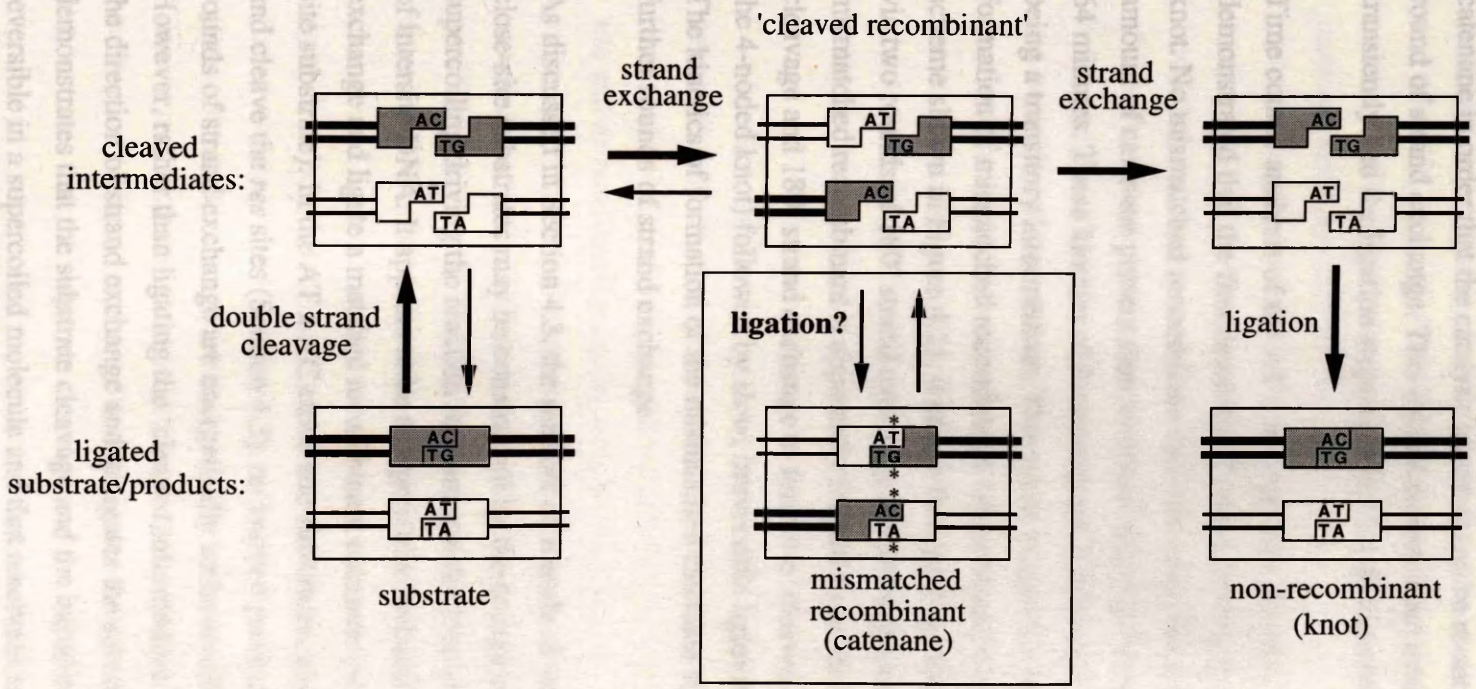


Figure 4.21 Possible scheme for the catalysis of the knotting reaction by resolvase.

Only the recombining crossover half-sites are shown within the boxes for simplicity. The sequences of the recombining central dinucleotides are also shown. Mismatched base pairs are represented by '*'. Double arrows represent reversible reaction steps, and single arrows represent irreversible reaction steps. The top row shows proposed reaction intermediates where both crossover sites contain double-strand cleavages. In a subunit rotation mechanism the 'cleaved recombinant' can undergo the second round of strand exchange, without ligating the mismatched recombinant catenane, to form 4-noded non-recombinant knot. In contrast to this, a DNA-mediated mechanism absolutely requires ligation of the 'cleaved recombinant' (shown boxed) to form mismatched recombinant catenane, so that the catalytic tetramer can be re-set to enable it to perform a second round of strand exchange.

another round of strand exchange, without ligation to recombinant catenane, to form the 4-noded non-recombinant knot. A DNA-mediated mechanism, on the other hand, absolutely requires that the 'cleaved recombinant' be ligated to form the mismatched recombinant catenane in order that the catalytic tetramer can be re-set to enable it to perform a second round of strand exchange. This ligated recombinant intermediate may be formed only transiently and the ligation steps required for its formation might be fast and reversible.

Time course analysis of the AT x AC knotting reaction (Section 4.1, Figure 4.3) demonstrated that the first detectable product formed (after 15 seconds) was the 4-noded knot. No mismatched recombinant catenane was detected until around 4 minutes. The amount of catenane present then increased steadily from 4 minutes to a maximum amount at 64 minutes. These kinetics of formation are inconsistent with the mismatched catenane being a transitory intermediate. This result suggests a branched pathway for the separate formation of mismatched recombinant catenane and 4-noded knot. Using the reaction scheme shown in Figure 4.21, it seems that the 4-noded knot is formed relatively quickly via two rounds of 180° strand exchange without ligation of the 'cleaved recombinant'. The mismatched recombinant is apparently formed more slowly with relatively efficient cleavage and 180° strand exchange to form the 'cleaved recombinant' (as with formation of the 4-noded knot) followed by slow, irreversible ligation to form the mismatched catenane. The kinetics of formation of the mismatched catenane suggest it is not a substrate for further rounds of strand exchange.

As discussed in Section 4.3, the number of rounds of strand exchange that can occur in the close-site substrates may be constrained by the energy equilibrium between substrate supercoiling driving the reaction forward and the level of helical distortion in the small loop of intersite DNA. It appears to be energetically favourable to perform one round of strand exchange and ligate a matched recombinant catenane (with an AT x AT or AC x AC close-site substrate). In the AT x AC close-site substrates, although resolvase can bind, synapse and cleave the *res* sites (Section 4.3), no knotted products were detected indicating that two rounds of strand exchange are energetically unfavourable due to distortion of the DNA. However, rather than ligating the 'cleaved intermediate' (Figure 4.21), resolvase reverses the direction of strand exchange and re-ligates the substrate configuration. This demonstrates that the substrate cleavage and the initial strand exchange reactions are reversible in a supercoiled molecule and that resolvase is extremely inefficient at ligating the mismatched recombinant catenane. This result provides strong evidence that the knotting reaction involves two concerted rounds of strand exchange without ligation of the mismatched recombinant intermediate.

Another assay designed to test the ability of resolvase to ligate a mismatched basepair in a recombinant catenane is described in Section 4.5. An artificial supercoiled plasmid substrate was synthesised containing a C-A mismatch at the centre of site I of only one *res* site (pMM5*). Any products formed using pMM5* would also contain a single C-A mismatched basepair (whereas the presumptive mismatched catenane would contain two mismatched basepairs). When treated with resolvase, pMM5* is mostly converted to fully ligated recombinant catenane which must contain a C-A mismatch at one *res* site. However, a substantial amount of product (~25%) is released as a 'suicide linear' molecule with a resolvase monomer attached at each end. This linear molecule would normally form a circle of the catenane, but has not been ligated, presumably because it contains a mismatched basepair. The other (correctly basepaired) circle of the catenane is fully ligated, supercoiled and has no resolvase attached. The kinetics of formation of the linear molecule are consistent with it being a failed intermediate in the recombination reaction rather than a product of cleavage of the fully ligated catenane. According to the reaction scheme in Figure 4.21, this cleaved linear product is probably released after the first strand exchange event due to the failure of resolvase to ligate the mismatched *res* site.

So why does resolvase not catalyse the knotting reaction with this mismatched substrate or reverse the direction of strand exchange and re-ligate the substrate configuration? Presumably because the 'cleaved recombinant' of pMM5* contains only one mismatched site and so resolvase ligates the correctly basepaired site thus irreversibly committing itself to making full recombinant, and then fails to ligate all of the mismatched sites. Therefore in a well-spaced AC x AT substrate, resolvase may prefer to catalyse the knotting reaction (and ligate a correctly basepaired 4-noded knot) than to ligate the two mismatched crossover sites that would be present in the 'cleaved intermediate'.

A ligated intermediate is necessary during the knotting reaction for the 'DNA-mediated' strand exchange model; our results therefore do not support this model. On the contrary, the results in this chapter indicate that the knotting reaction is catalysed by a concerted, double round of strand exchange without ligation of a recombinant intermediate, consistent with a 'subunit rotation' mechanism.

Chapter 5

The role of *res* site I in recombination

Introduction

Tn*3* and γδ resolvases bind to three sites within *res*; sites I, II, and III (Grindley *et al.*, 1982; Kitts *et al.*, 1983; Chapter 1), each with imperfect dyad symmetry (Figure 1.4b). Cleavage and strand exchange occur at site I, but sites II and III are necessary for efficient recombination (Wells and Grindley, 1984; Bednarz *et al.*, 1990). Each site is bound by two monomers of resolvase to form on-site dimers (Figure 1.6), (Blake, 1993; Blake *et al.*, 1995; Yang and Steitz, 1995). Binding is by insertion of a C-terminal helix-turn-helix motif into the major groove at the end of each site. Some interactions are also made in the minor groove of the DNA towards the centre of each site (Rimphanitchayakit and Grindley, 1990; Yang and Steitz, 1995).

All binding sites contain two copies of a degenerate 12 bp binding sequence. The 12 bp sequences are related by dyad symmetry but separated by varying lengths of DNA at each site (Figure 1.4b). The varying lengths of sites I, II, and III (28 bp, 34 bp, and 25 bp respectively) may be an indication of their differing roles in the recombination reaction. The interaction of resolvase at site I may be quite different to the interaction at sites II and III, giving rise to cleavage and strand exchange exclusively at site I. A co-crystal structure of γδ resolvase bound to a synthetic γδ *res* site I has recently been solved (Yang and Steitz, 1995) and a detailed examination of resolvase binding to *res* site II has also been carried out (Blake *et al.*, 1995); but little is known of the site III complex. Since strand exchange occurs at site I, but sites II and III are required for efficient recombination, it has been proposed that these 'accessory sites' interwrap to form part of the nucleoprotein complex required for catalysis (Grindley, 1993; See also Chapter 1).

Cleavage at site I has been precisely mapped and occurs at the central 'AT' of the palindromic hexanucleotide 5'- TTATAA; the cuts are staggered to leave two-nucleotide 3' single strand extensions. Resolvase becomes attached to the DNA via a phosphoserine linkage at the 5' recessed ends (Figure 1.4b; Reed and Grindley, 1981). The 'AT central dinucleotide' is well conserved in many transposons (although it is 'GT' on the top strand in Tn*2501 res*). Surprisingly resolvase does not appear to contact the 'overlap region' at the centre of site I in footprinting studies, and depurination of any of the central 4 bp does not adversely affect the binding of γδ resolvase (Hatfull and Grindley, 1988). Also, in the γδ resolvase co-crystal the catalytic serine 10 does not interact closely with the central dinucleotide at which it must cleave although the dimer does make interactions at the central 4 bp, creating a bend (Figure 1.6), (Yang and Steitz, 1995). These findings may reflect the

need for a conformational change of the resolvase dimers bound at the site I's before they become catalytically active.

The binding of resolvase induces a bend at each *res* binding site, and is reported to induce a kink at the centre of site I; seen by hydroxyl radical cleavage (Hatfull *et al.*, 1987) and in the γδ resolvase co-crystal (Yang and Steitz, 1995). It has been suggested that this kink allows direct DNA-DNA interactions in the synaptic complex, and introduces strain into the DNA to make strand exchange energetically more favourable (Hatfull and Grindley, 1988).

Synthetic γδ site I's have been constructed containing all possible combinations of the central 2 bp (Hatfull *et al.*, 1988). Although there appears to be no specific interaction between resolvase and the central 2 bp, most base substitutions dramatically reduced the binding of resolvase to site I (by up to 30-fold). For efficient binding it was necessary that at least one of the basepairs was 'wild type' and that neither of the 5' bases was T. In contrast, binding of γδ resolvase to site I was enhanced if any base of the central dinucleotide was removed (by depurination or depyrimidination; Hatfull *et al.*, 1988). A similar enhancement of binding is observed if a mismatched basepair is introduced at the centre of site I (Chapter 4). These results are interpreted as meaning that it requires less energy to bend site I if a base is missing (or mispaired) than if the natural sequence is present. Furthermore, mutant γδ site I's that are bound efficiently by resolvase do not necessarily recombine efficiently when they are part of a full *res* in a substrate plasmid; in fact all mutants were recombined less efficiently than natural *res*. It has been suggested that the failure of substrates with altered central dinucleotides to make recombinants occurs after cleavage and strand exchange and is a result of the failure of resolvase to re-ligate mismatched recombinant sites (Stark *et al.*, 1991; Chapter 4).

The intent of the work described in this chapter was to investigate why cleavage and strand exchange occurs exclusively at site I, rather than at sites II or III of *res*. As described above, it appears not to be the sequence at the centre of the site that makes site I unique (Hatfull *et al.*, 1988). Alternatively, the length of site I may be important for cleavage and may distinguish it from sites II and III since resolvase monomers binding to different sized sites may take up different dimer conformations. Also the position of site I relative to sites II and III within the *res* site may be crucial for formation of a catalytically active synapse (Hatfull *et al.*, 1988).

It was also of interest why the cleavage positions were always two basepairs apart at the centre of site I. This could be due to sequence specific recognition by resolvase or the

position of cleavage could be relative to the binding sites at the ends of site I. If either, or both, of these possibilities were correct, then altering the length or sequence of site I may alter the cleavage positions. Using site I's of different lengths also allowed an investigation of the interaction of resolvase with these altered sites. It has been shown previously that insertion of 5 or 10 basepairs at the centre of site II does not prevent cooperative binding of two Tn*3* resolvase subunits (Blake *et al.*, 1995). Since resolvase can bind cooperatively to sites ranging from 25 bp (site III) to 44 bp (site II+10), it is clearly a remarkably flexible molecule.

Results and Discussion

5.1 Altering the length of site I by adding or deleting bases of the central hexanucleotide.

To determine whether the length of site I could be changed without blocking recombination, and if so how the altered site was now cut, four mutant *res* sites were created with bases added or deleted at the palindromic hexanucleotide 5' TTATAA at the centre of site I. Site I's were made with an extra A or T inserted between the A and T of the central dinucleotide to alter the top strand sequence from 'AT' to 'AAT' (called 'AAT') or 'ATT' (called 'ATT') respectively. By inserting additional A-T (or T-A) base pairs it was possible to conserve the AT-richness at the centre of site I and also preserve the sequence of the 12 bp binding motifs.

Using this strategy we hoped to test (i) if the length of the spacer between the 12 bp binding motifs was crucial for recombination, (ii) whether resolvase recognises and cuts the 'AT' central dinucleotide sequence specifically, or if the cleavage positions are fixed relative to one or both ends of the site. If cleavage by resolvase is relative to the position at which the enzyme binds the site I ends, then the cleavages might be staggered by 3 bp in the 'AAT' and 'ATT' sites. Since resolvase is unlikely to ligate a 3 nt overlap to a 2 nt overlap, cleavages 3 bp apart might give 4, 8, and 12-node non-recombinant knots in a substrate containing an 'AT' *res* and an 'AAT' or 'ATT' *res*.

Two other mutant site I's were also synthesised; one with the 5' T of the central hexanucleotide removed (called '-1 site') and the other with the 5' T and 3' A of the hexanucleotide removed (called '-2 site') to change the top strand sequence at the centre of site I from 5' TTATAA to 5' TATAA and 5' TATA respectively. Shortening site I by 2 bp reduces the length of the site from 28 bp to 26 bp, which is closer to the size of site III (25

bp). If the difference in the interaction of resolvase with site I and site III is due to the length of the site, then resolvase might be expected to bind but not to cleave and recombine the '-2 site'.

5.2 Construction and reactions of substrates containing *res* sites with altered-length site I's.

A set of oligonucleotides was synthesised such that when they were annealed in pairs, they were equivalent to the mutated site I's discussed above with cohesive *Eco*RI and *Sst*I ends (Figure 5.1).

A set of 'single mutant' and 'double mutant' plasmid recombination substrates was cloned by ligating these double-stranded oligonucleotides (shown in Figure 5.1) into plasmids cut with *Eco*RI and *Sst*I to remove the site I of an '*Eco*RI-*res*' (see Table 2.1 for details). The plasmid constructs were confirmed by sequencing and are identical except at the mutated *res* sites; shown in Figure 5.2.

The plasmids were incubated with resolvase in C9.4 buffer for 1 h. The reaction products were run on agarose gels (i) uncut, (ii) cut with a restriction endonuclease (single-mutants were cut with *Sal*I; double-mutants were cut with *Bgl*II), and (iii) nicked with DNase I (to separate catenanes and knots). The results from the single-mutant site substrates are shown in Figure 5.3 and those of the double-mutant site substrates in Figure 5.4. The wild-type AT x AT substrate in Figure 5.3 formed 2-node recombinant catenane as expected, but the AT x AAT and AT x ATT substrates formed mostly 4, 8, and 12-node non-recombinant knots and only a small amount of 2-node catenane. The AT x -1 site substrate formed a small amount of 2-node catenane, but the AT x -2 site substrate formed no detectable products. All 'single-mutant' substrates showed some topoisomerase activity and double strand cleavage when the reactions were run uncut.

In contrast the double-mutant substrates AAT x AAT and ATT x ATT formed mostly 2-node recombinant catenane, with some 4, 8, and 12-node non-recombinant knots also present. The single-mutant and double-mutant substrates containing the ATT *res* sites formed more products than the ones containing the AAT *res* sites. Neither the -1 site x -1 site nor the -2 site x -2 site substrate formed any detectable products, but the -1 site x -1 site substrate showed some topoisomerase activity when run uncut (see Figure 5.4a).

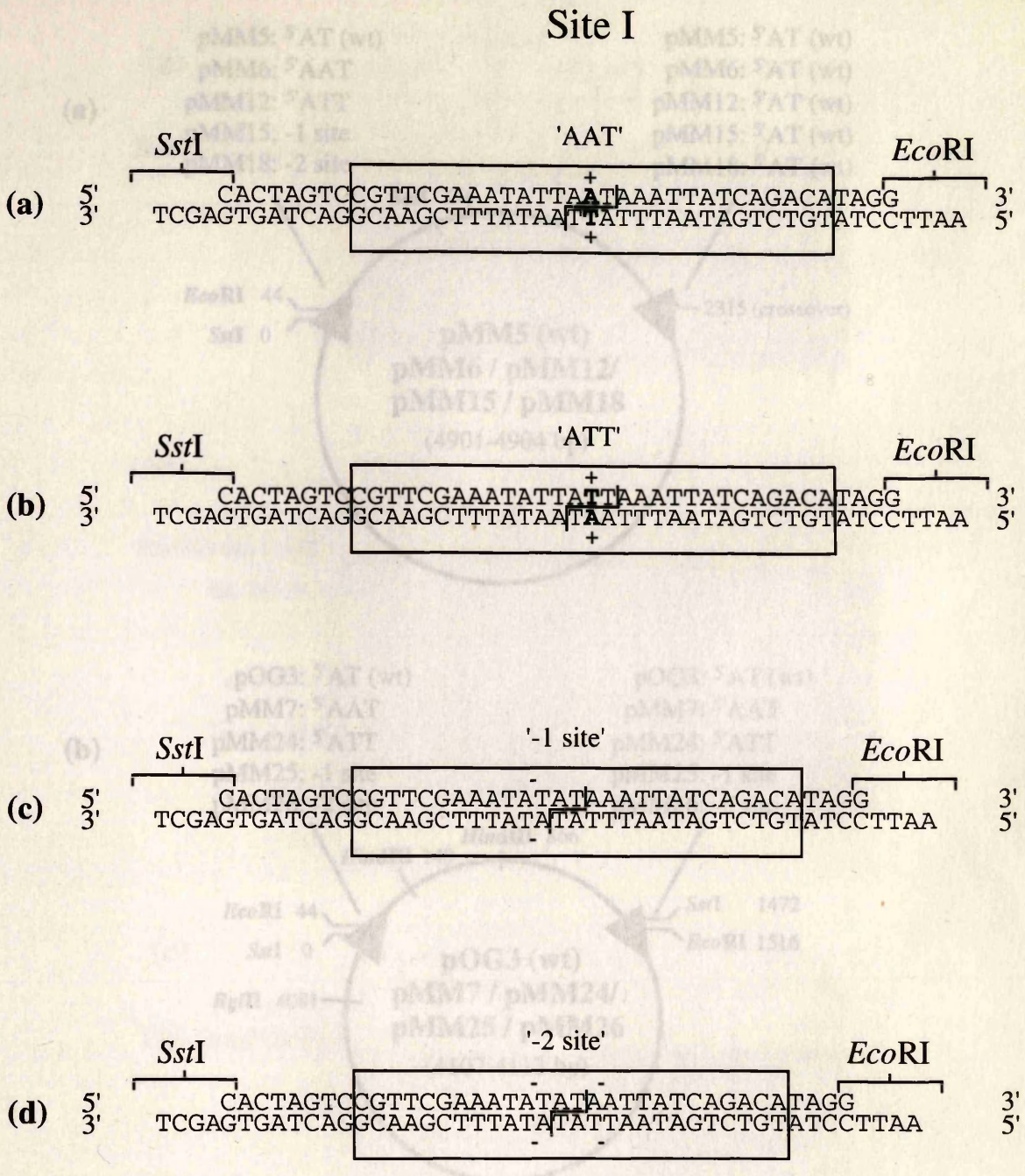


Figure 5.1 Sequence of the 'AAT', 'ATT', '-1 site' and '-2 site' oligonucleotides.

The above oligonucleotides were designed such that when they were annealed in pairs they formed altered site I's (boxed) with cohesive ends that could be cloned into plasmid substrates at a later stage. Added bases are shown in bold type and are marked with a '+'. The positions where bases have been deleted are marked with a '-'. Sites (a), (b), (c) and (d) consist of natural site I sequence except at the positions indicated.

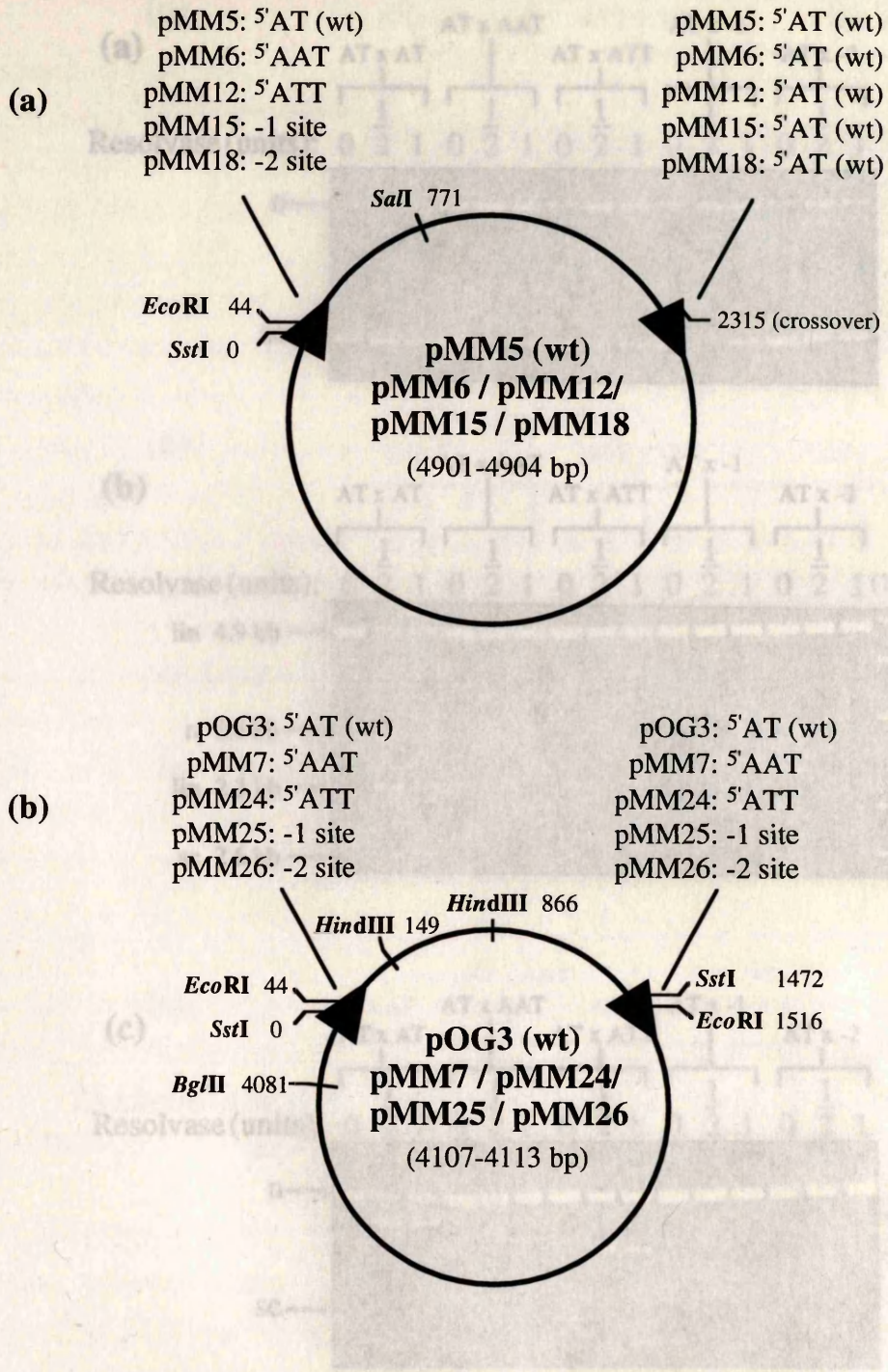


Figure 5.2 Simplified maps of pMM5, pMM6, pMM12, pMM15, pMM18, pOG3, pMM7, pMM24, pMM25 and pMM26.

(a) Simplified maps of single mutant plasmids containing one copy of the wild-type *res* site and one copy of a mutated *res* site (containing either the AAT, ATT, -1 or -2 site I's) as shown above. Mutant plasmids were constructed by cloning the appropriate altered site I (shown in Figure 5.1) into the *Eco*RI and *Sst*I sites of wild-type pMM5. Restriction sites are numbered correctly for the wild-type plasmid (pMM5).
(b) Simplified maps of double mutant plasmids which contain two copies of the mutant *res* sites shown above and in Figure 5.1. The wild-type version of these plasmids (pOG3) which contains two natural *res* sites is also shown. Restriction sites are numbered correctly for pOG3.

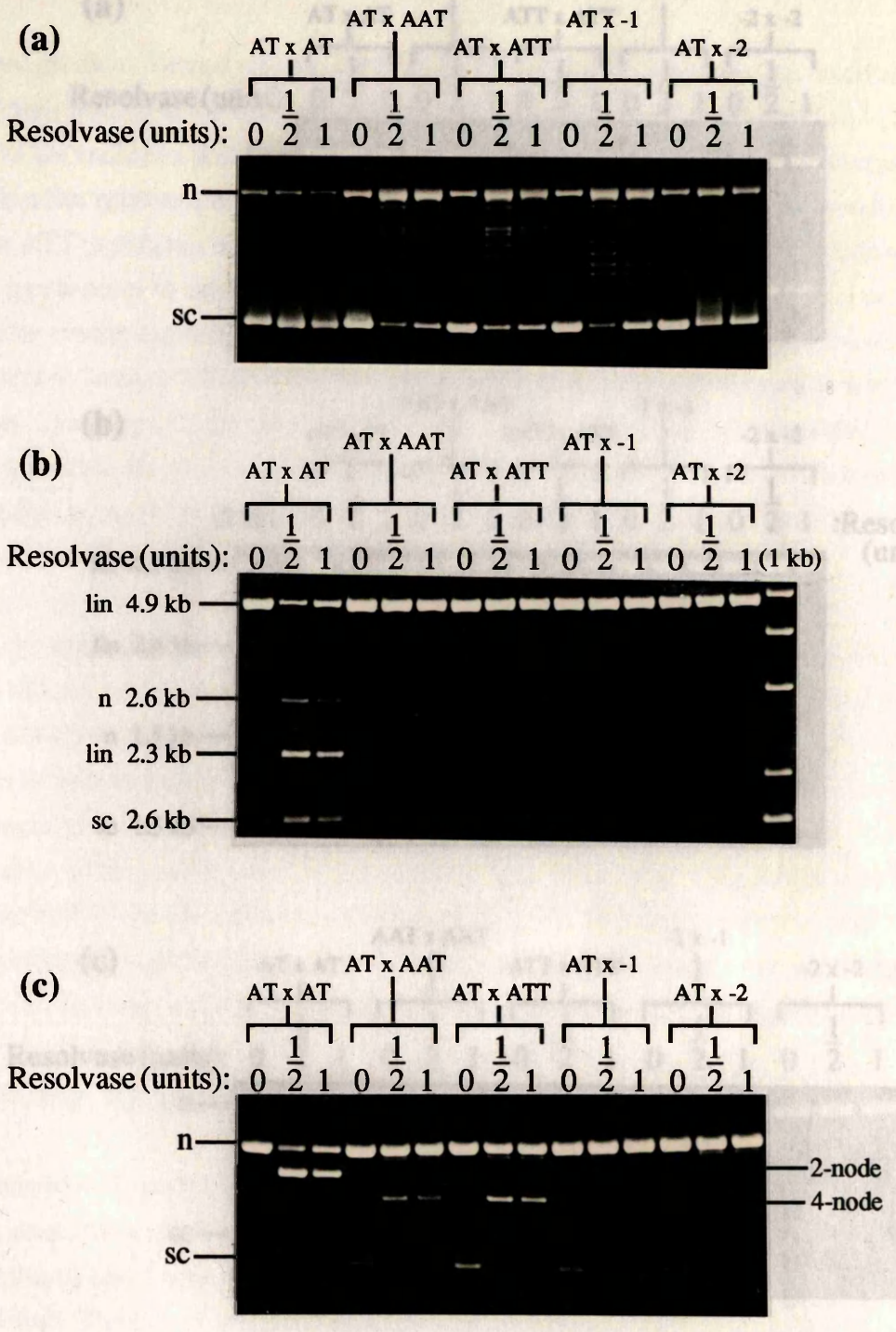


Figure 5.3 Recombination of AAT, ATT, -1 site and -2 site single mutants.

(a) The single mutant site substrates shown in Figure 5.2a were reacted with resolvase and run (uncut) on a 1.2% agarose gel. Each substrate is identified by the sequence at the centre of its two crossover sites. Nicked circles are shown by 'n' and supercoiled circles by 'sc'.
(b) As above except that the products were assayed by cutting with *Sal*I (see Figure 5.2a) which cuts in the small circle of the catenane.
(c) As (a) except that the products were assayed by nicking with DNase I and running on a 0.7% agarose gel. The positions of recombinant 2-node catenane and non-recombinant 4-node knot are indicated.

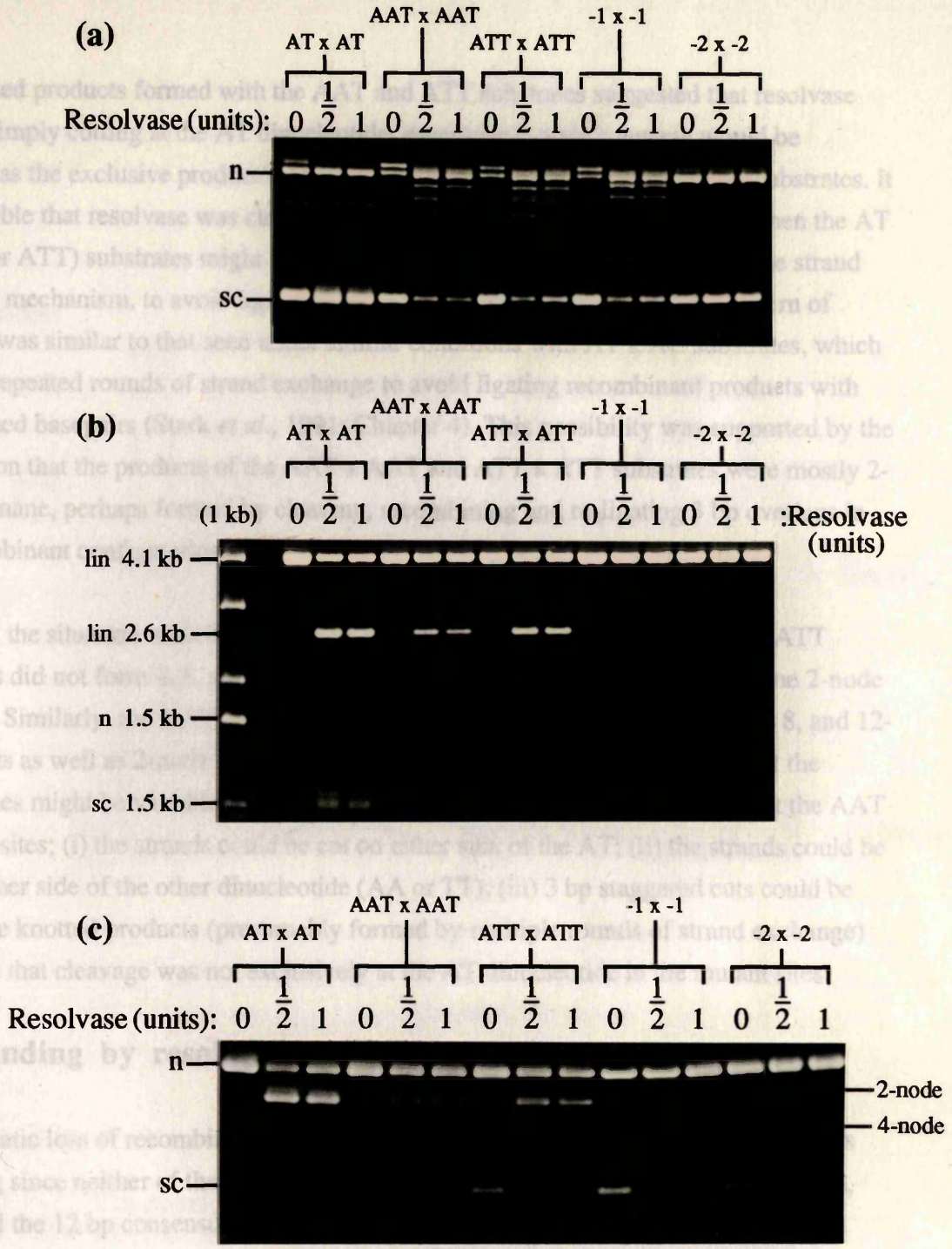


Figure 5.4 Recombination of AAT, ATT, -1 site and -2 site double mutants.

(a) The double mutant site substrates shown in Figure 5.2b were reacted with resolvase and run (uncut) on a 1.2% agarose gel. Each substrate is identified by the sequence at the centre of its two crossover sites. Nicked circles are shown by 'n' and supercoiled circles by 'sc'.
(b) As above except that the products were assayed by cutting with *Bgl*II (see Figure 5.2b) which cuts in the large circle of the catenane.
(c) As (a) except that the products were assayed by nicking with DNase I and running on a 0.7% agarose gel. The positions of recombinant 2-node catenane and non-recombinant 4-node knot are indicated.

The knotted products formed with the AAT and ATT substrates suggested that resolvase was not simply cutting at the AT dinucleotide, otherwise 2-node catenane would be expected as the exclusive product in both the single-mutant and double-mutant substrates. It was possible that resolvase was cleaving the top and bottom strands 3 bp apart; then the AT x (AAT or ATT) substrates might form non-recombinant knots, by iteration of the strand exchange mechanism, to avoid ligating a 3 nt overlap to a 2 nt overlap. This pattern of products was similar to that seen under similar conditions with AT x AC substrates, which undergo repeated rounds of strand exchange to avoid ligating recombinant products with mismatched basepairs (Stark *et al.*, 1991; Chapter 4). This possibility was supported by the observation that the products of the AAT x AAT and ATT x ATT substrates were mostly 2-node catenane, perhaps formed by cleaving, recombining and re-ligating 3 bp overlaps in the recombinant configuration.

However, the situation was complicated by the fact that the AT x AAT and AT x ATT substrates did not form 4, 8, and 12-node knots exclusively, but also formed some 2-node catenane. Similarly, the AAT x AAT and ATT x ATT substrates formed some 4, 8, and 12-node knots as well as 2-node catenane, suggesting that the position of cleavage at the mutant sites might be variable. Three types of cleavage appeared to be plausible at the AAT and ATT sites; (i) the strands could be cut on either side of the AT; (ii) the strands could be cut on either side of the other dinucleotide (AA or TT); (iii) 3 bp staggered cuts could be made. The knotted products (presumably formed by multiple rounds of strand exchange) suggested that cleavage was not exclusively at the AT dinucleotide in the mutant sites.

5.3 Binding by resolvase at the altered-length site I's.

The dramatic loss of recombinant products with the -1 site and -2 site *res* substrates was surprising since neither of the missing bases is contacted by resolvase (Yang and Steitz, 1995) and the 12 bp consensus binding sequence, as well as the central dinucleotide, remained intact. The lack of products could be explained by a change in the interaction of resolvase with the site (perhaps now resembling the interaction with site III) or simply by a reduced efficiency of resolvase binding to the site.

Binding of resolvase to the mutant site I's was assayed by incubating the labelled oligonucleotides (shown in Figure 5.1) with resolvase in binding buffer for 15 minutes and then separating the samples by non-denaturing PAGE (Figure 5.5). The natural site I was bound efficiently by resolvase to form a dimer complex. The AAT and ATT sites were bound much less efficiently than the natural sequence, and the -1 site showed extremely

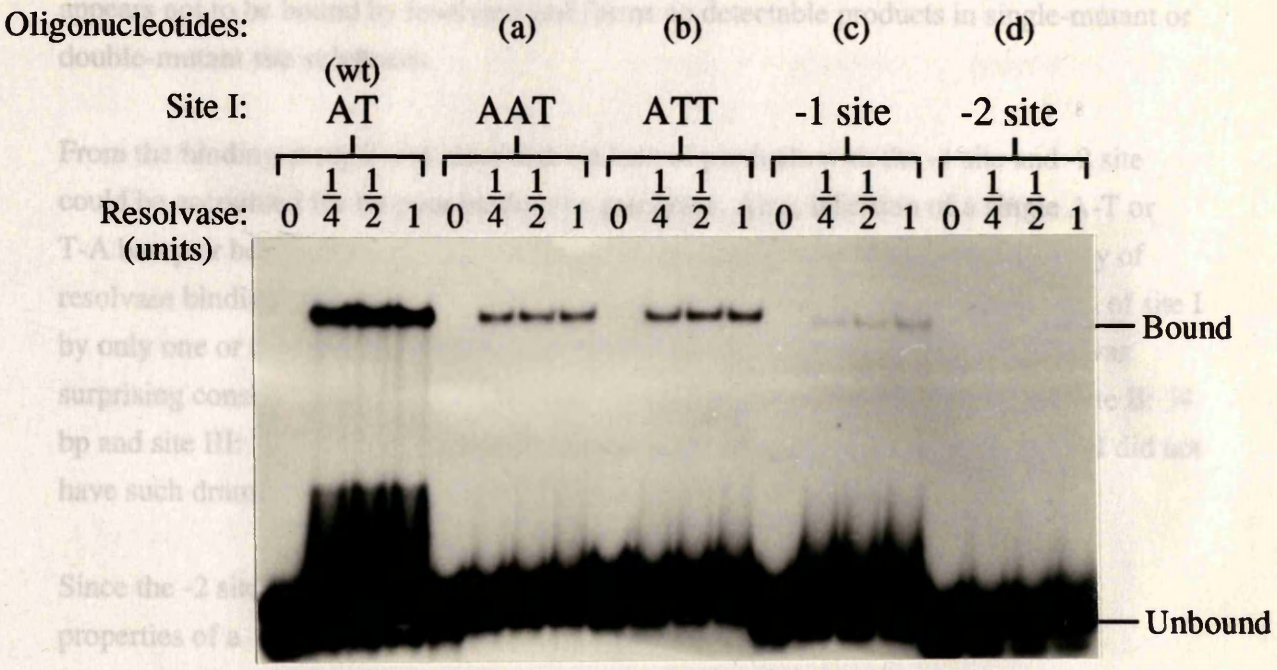


Figure 5.5 Binding of resolvase to AT, AAT, ATT, -1 and -2 site I.

The altered oligonucleotide site I's shown above correspond to those shown in Figure 5.1. The site labelled AT (wt) is made of oligonucleotides that form a natural site I when annealed together. The radiolabelled site I's shown above were incubated with resolvase in binding buffer and then run on an 8% acrylamide non-denaturing gel.

weak binding. No complexes at all were detected for the -2 site. The extent of binding seen in Figure 5.5 correlates with the yield of products formed with the substrates containing these sites. The AAT and ATT sites are bound more weakly than natural site I and also give less products; the -1 site is bound very weakly by resolvase and forms only very small amounts of 2-node catenane in a substrate with one mutant and one natural *res*; the -2 site appears not to be bound by resolvase and forms no detectable products in single-mutant or double-mutant site substrates.

From the binding assay it was clear that the lack of products with the -1 site and -2 site could be accounted for by poor binding by resolvase. Also, insertion of a single A-T or T-A basepair between the AT (top strand) central dinucleotide reduces the efficiency of resolvase binding to the site. It would seem that increasing or decreasing the length of site I by only one or two basepairs drastically reduces the efficiency of binding, which was surprising considering the variation in the length of the sites of *res* (site I: 28 bp, site II: 34 bp and site III: 25 bp). It has also been observed that changes in the length of site II did not have such dramatic effects on binding efficiency (Blake *et al.*, 1995).

Since the -2 site I is 26 bp long and binds resolvase very inefficiently, the binding properties of a -3 site (which would be the same length as natural site III) might be interesting. Poor binding of a -3 site might suggest that there is more to the difference between sites I and III than just the length of the spacer between the recognition motifs for resolvase's C-terminal domain. Perhaps the sequence of site III is adapted for a different type of resolvase-DNA interaction altogether from that at site I, just as the interaction at site II seems to be quite different (Blake *et al.*, 1995).

5.4 Cleavage by resolvase at the altered-length site I's.

To assay the ability of resolvase to cleave these mutant sites, a buffer containing ethylene glycol was used (described in Section 2.43). It has been reported previously that in the presence of ethylene glycol or other solvents (and in the absence of Mg^{2+}), resolvase fully cleaves the *res* sites and remains covalently attached to the DNA instead of forming recombinant (Reed and Grindley, 1981; Boocock *et al.*, 1995). The AT x AT, ATT x ATT, -1 site x -1 site and -2 site x -2 site substrates were incubated with different concentrations of resolvase, in the ethylene glycol buffer at 37 °C for 24 h. The samples were treated with SDS and proteinase K (to remove the attached resolvase) and then separated on an agarose gel (Figure 5.6). Cleavage at only one *res* site releases full size linear plasmid (4.1 kbp)

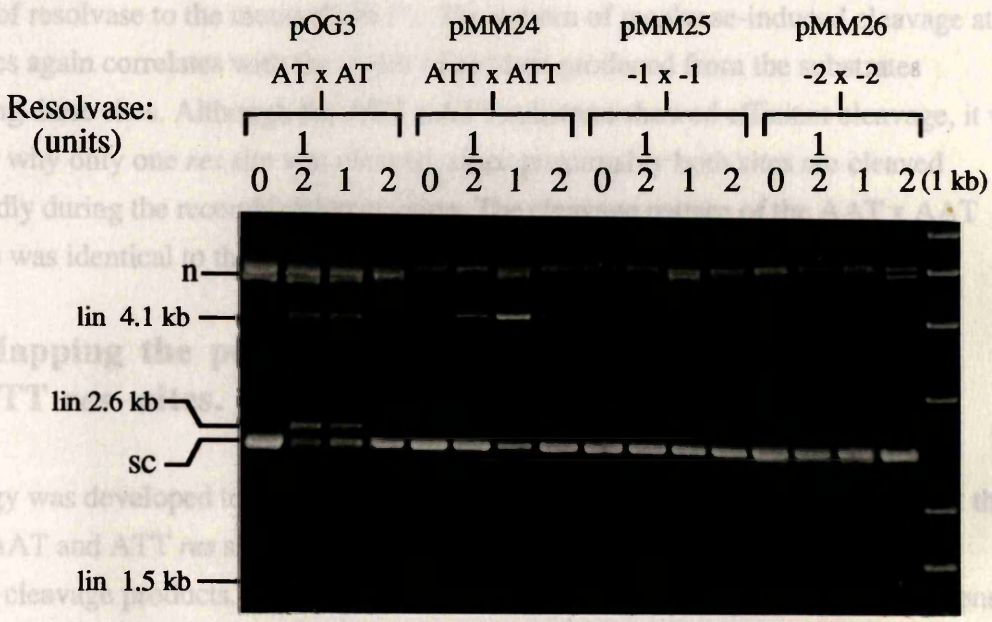


Figure 5.6 Resolvase-induced cleavage of substrates containing AT, ATT, -1 and -2 *res* sites.

Assay of resolvase-induced cleavage of site I spacing double mutants pOG3 (wt), pMM24 (ATT), pMM25 (-1 site) and pMM26 (-2 site) as shown in Figure 5.2b. The substrates were incubated with resolvase for 24 h in ethylene glycol buffer (see Section 2.43) and then treated with SDS and proteinase K. The products were run on a 1.2% agarose gel along with a 1 kbp size ladder (1 kb). Cleavage at one *res* site releases full size linear plasmid (4.1 kbp); cleavage at both *res* sites produces a 2.6 kbp fragment and a 1.5 kbp fragment.

and cleavage at both sites produces a 2.6 kbp fragment and a 1.5 kbp fragment (see Figure 5.2b).

The AT x AT (wild-type) substrate was cleaved efficiently at both *res* sites, but the ATT x ATT substrate was cleaved at only one *res* site. The -1 site x -1 site and -2 site x -2 site substrates produced no cleavage products, presumably because of the very inefficient binding of resolvase to the mutated site I's. The pattern of resolvase-induced cleavage at these sites again correlates with the yields of product produced from the substrates containing these sites. Although the ATT x ATT substrate showed efficient cleavage, it was not clear why only one *res* site was cleaved, since presumably both sites are cleaved concertedly during the recombination reaction. The cleavage pattern of the AAT x AAT substrate was identical to that of the ATT x ATT substrate (data not shown).

5.5 Mapping the positions of cleavage at the AAT and ATT *res* sites.

A strategy was developed to 'map' the positions of the resolvase-induced cleavages at the mutant AAT and ATT *res* sites, by comparing the sizes of natural and mutant single-stranded cleavage products. The method relies on making two experimental assumptions; firstly that Tn*3* resolvase cleaves the natural site I at the AT central dinucleotide, as has been reported for the γδ system (Reed and Grindley, 1981), and secondly that the ethylene glycol cleavage reaction reflects 'normal' cleavage in a standard resolution reaction. We believe both of these assumptions to be correct (see also below).

To map the cleavage positions at the ATT *res*, the AT x AT and ATT x ATT substrates were incubated in ethylene glycol buffer as before, and the full size linear cleavage products were purified from low melting point agarose (see Chapter 2 for details). The linears were then cut with either *Bgl*II (to examine the top strand cleavage) or *Hin*dIII (to examine the bottom strand cleavage), (Figure 5.7a). The cleaved linears were then dephosphorylated by treatment with calf intestinal phosphatase and 5' labelled with γ-[^{32}P] ATP and T4 kinase. Size markers were generated by cutting the same substrates with *Bgl*II or *Hin*dIII, 5' labelling with kinase and then treating the samples with *Ssp*I which cuts site I five bases to the left of the crossover region (Figure 5.7a).

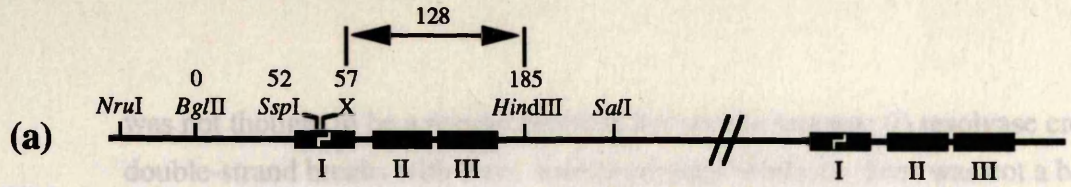
For the ethylene glycol-induced cleavage reaction to work it was necessary to use substrates containing two *res* sites. A potential problem with this was the possibility of single-strand nicking at the second *res* site being a source of interference in the results. This

Figure 5.7 Mapping the positions of cleavage at the ATT *res* site.

(a) A simplified map of the pOG3 (AT x AT) *res* site showing the position of the crossover site (X) relative to the restriction sites used in the cleavage mapping experiment. The numbers above the restriction sites (and crossover site) show the size (in nucleotides) of the single stranded radiolabelled fragment generated by cutting at that site.

(b) Mapping the positions of resolvase-induced cleavage at the AT and ATT *res* sites. pOG3 (AT x AT) and pMM24 (ATT x ATT) cleavage products (full-length linears) were gel-purified and then cut with either *Bgl*II (to examine the top strand cleavage) or *Hin*dIII (to examine the bottom strand cleavage). Size marker samples were cut with *Ssp*I and then either *Bgl*II or *Hin*dIII (see Figure 5.7a). All fragments were then radiolabelled at the 5' end and run on an 8% denaturing-sequencing gel with DNase I cleavage ladders at each side. Sizes were assigned using overexposures of the DNase I-generated reference ladders (not shown). The approximate positions of the sites of *res* are indicated as well as the sizes of the relevant cleavage fragments (in nucleotides).

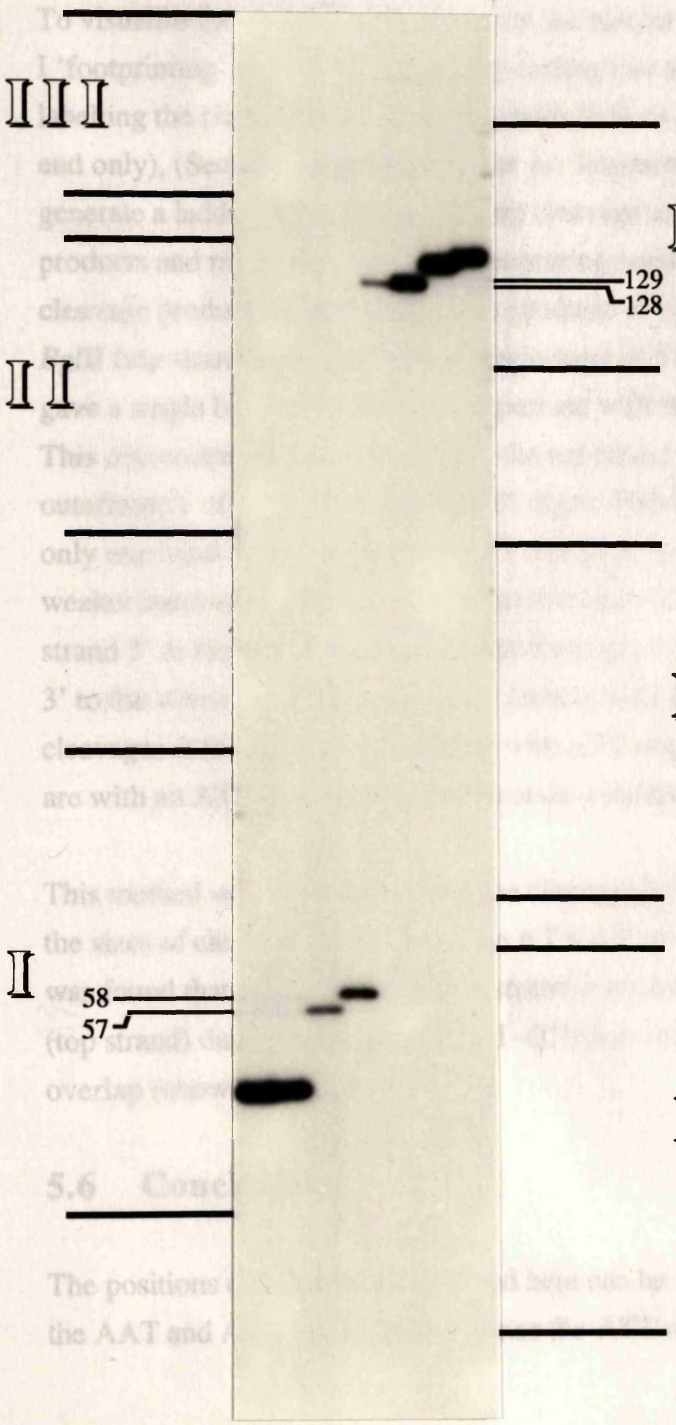
(c) An enlargement of the *Hin*dIII cleavage fragments to show the single 128 nt band for the wild-type (AT x AT) substrate and the 128 and 129 nt bands for the mutant (ATT x ATT) substrate.



(a)

(b)

*Ssp*I:	+	+	-	-	-	-	+	+
*Hin*dIII:	-	-	-	-	+	+	+	+
*Bgl*II:	+	+	+	+	-	-	-	-
Resolvase:	-	-	+	+	+	+	-	-
ATT x ATT:	-	+	-	+	-	+	-	+
AT x AT:	+	-	+	-	+	-	+	-



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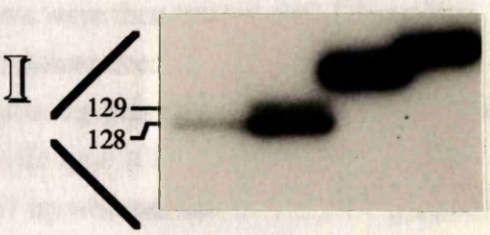


was not thought to be a serious problem for several reasons; (i) resolvase created mostly double-strand breaks with these double-mutant substrates; there was not a big increase in the amount of nicked DNA (see uncut samples, Figure 5.4a), (ii) resolvase cleaved the double-mutant substrates at one or other of the *res* sites, but not both, in the ethylene glycol reaction (although the reason for this is unclear since AT x AT wild-type substrates are cleaved at both *res* sites), and (iii) any single-strand cleavages that were made are resolvase-induced and would presumably be in the same position as where the double-strand cuts would have been made.

To visualise the size difference between the natural and mutant cleavage products, a DNase I 'footprinting' ladder was created by cutting one substrate with *Bgl*II or *Hin*dIII, 5' labelling the plasmid and then cutting with *Nru*I or *Sal*I (to generate *res* sites labelled at one end only), (Section 2.23). These linear *res* fragments were then treated with DNase I to generate a ladder of bands representing cleavage at almost every base of the *res* site. All products and markers were run on denaturing-sequencing gels to separate the single-strand cleavage products (Figure 5.7b). The products of wild-type AT x AT cleavage cut with *Bgl*II (top strand cleavage) gave a single band of 57 bp whereas the ATT x ATT products gave a single band of 58 bp (by comparison with the size markers and DNase I ladder). This demonstrated that resolvase cut the top strand of the mutant site exclusively 3' to the outermost T of ATT. With the *Hin*dIII digest (bottom strand cleavage), the natural *res* gave only one band of 128 bp, but the ATT site gave two bands; a strong band of 128 bp and a weaker band of 129 bp. This demonstrated that ~83% of the time resolvase cut the bottom strand 3' to the AA of the bottom strand trinucleotide AAT, but about 17% of the time cut 3' to the whole AAT. By combining these results it seems that ~83% of the double-strand cleavages at the ATT mutant site are with a TT (top strand) dinucleotide overlap and ~17% are with an ATT (top strand) trinucleotide overlap (shown in Figure 5.9).

This method was also used to map the cleavage positions of the AAT *res* site by comparing the sizes of cleavage linears from the AT x AT and AAT x AAT substrates (Figure 5.8). It was found that ~78% of the double-strand cleavages at the AAT mutant site are with an AT (top strand) dinucleotide overlap and ~22% are with an AAT (top strand) trinucleotide overlap (shown in Figure 5.9).

5.6 Conclusions.

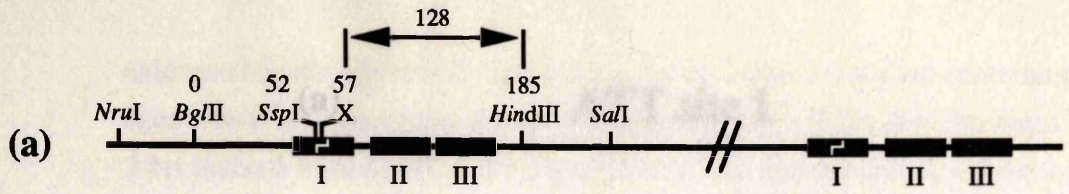
The positions of cleavage determined here can be used to explain the products formed by the AAT and ATT *res* substrates. Since the ATT site is cut mostly with a TT dinucleotide 3'

Figure 5.8 Mapping the positions of cleavage at the AAT *res* site.

(a) A simplified map of the pOG3 (AT x AT) *res* site showing the position of the crossover site (X) relative to the restriction sites used in the cleavage mapping experiment. The numbers above the restriction sites (and crossover site) show the size (in nucleotides) of the single stranded radiolabelled fragment generated by cutting at that site.

(b) Mapping the positions of resolvase-induced cleavage at the AT and AAT *res* sites. pOG3 (AT x AT) and pMM7 (AAT x AAT) cleavage products (full-length linears) were gel-purified and then cut with either *Bgl*II (to examine the top strand cleavage) or *Hin*dIII (to examine the bottom strand cleavage). Size marker samples were cut with *Ssp*I and then either *Bgl*II or *Hin*dIII (see Figure 5.8a). All fragments were then radiolabelled at the 5' end and run on an 8% denaturing-sequencing gel with a 1 kbp ladder down one side (1 kb). The sizes of the relevant cleavage fragments are shown (in nucleotides).

(c) An enlargement of the *Hin*dIII cleavage fragments to show the single 128 nt band for the wild-type (AT x AT) substrate and the 128 and 129 nt bands for the mutant (AAT x AAT) substrate.

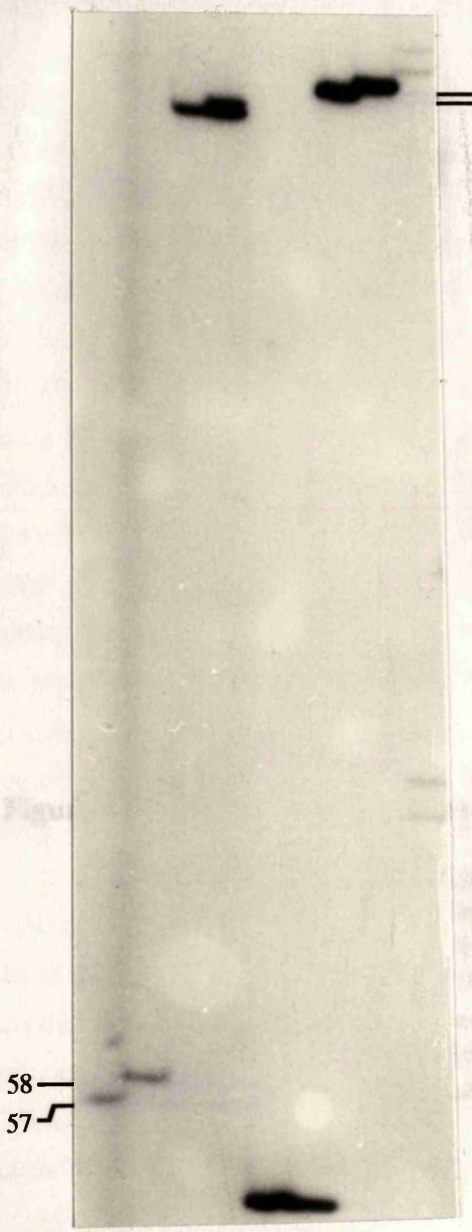


(a)

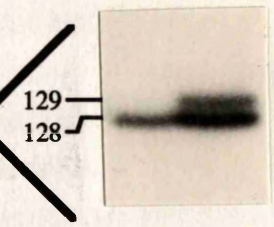
(b)

*Ssp*I:	-	-	-	-	+	+	+	+
*Hin*dIII:	-	-	+	+	-	-	+	+
*Bgl*II:	+	+	-	-	+	+	-	-
Resolvase:	+	+	+	+	-	-	-	-
AAT x AAT:	-	+	-	+	-	+	-	+
AT x AT:	+	-	+	-	+	-	+	-

(1 kb)



(c)



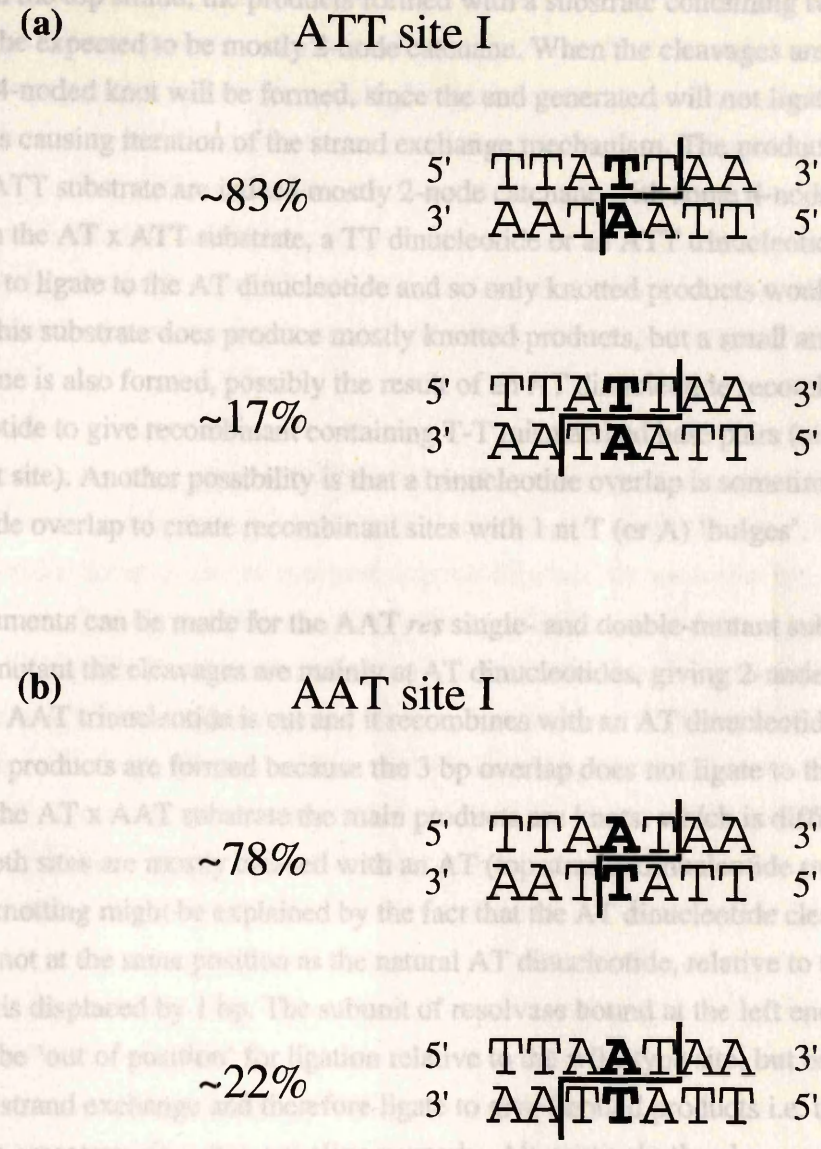


Figure 5.9 Summary of resolvase-induced cleavage of ATT and AAT *res*.

(a) Summary of the cleavage mapping data for the ATT *res* site shown in Figure 5.7. Only the 7 bp at the centre of the site I are shown for simplicity (the complete site I is shown in Figure 5.1). The bases that were added to the centre of this site are shown in bold type. The estimated levels of cleavage are also shown.
(b) Summary of the cleavage mapping data for the AAT *res* site shown in Figure 5.8. Labelled as (a).

extension on the top strand, the products formed with a substrate containing two of these sites would be expected to be mostly 2-node catenane. When the cleavages are staggered by 3 bp, some 4-noded knot will be formed, since the end generated will not ligate to a 2 nt overlap, thus causing iteration of the strand exchange mechanism. The products seen with the ATT x ATT substrate are indeed mostly 2-node catenane with some 4-node knot. However, in the AT x ATT substrate, a TT dinucleotide or an ATT trinucleotide would not be expected to ligate to the AT dinucleotide and so only knotted products would be expected. This substrate does produce mostly knotted products, but a small amount of 2-node catenane is also formed, possibly the result of an AT dinucleotide recombining with a TT dinucleotide to give recombinant containing T-T mismatched base pairs (one in each recombinant site). Another possibility is that a trinucleotide overlap is sometimes ligated to a dinucleotide overlap to create recombinant sites with 1 nt T (or A) 'bulges'.

Similar arguments can be made for the AAT *res* single- and double-mutant substrates. In the double-mutant the cleavages are mainly at AT dinucleotides, giving 2-node catenane, but when an AAT trinucleotide is cut and it recombines with an AT dinucleotide-cleaved site, knotted products are formed because the 3 bp overlap does not ligate to the 2 bp overlap. In the AT x AAT substrate the main products are knots, which is difficult to explain if both sites are mostly cleaved with an AT (top strand) dinucleotide overlap. The increase in knotting might be explained by the fact that the AT dinucleotide cleaved with the AAT site is not at the same position as the natural AT dinucleotide, relative to the ends of the site, but is displaced by 1 bp. The subunit of resolvase bound at the left end of the AAT site I might be 'out of position' for ligation relative to the wild-type site, but be 'in position' after a 360° strand exchange and therefore ligate to give knotted products i.e. the recombining crossover sites may not align properly. Alternatively the cleavage site biases in ethylene glycol buffer might not be exactly the same as in C9.4 buffer. In principle these problems could be sorted out by sequencing the actual products, but in practice the results would be difficult to interpret because of similarities of sequence and the problems of associating together the top and bottom strand sequences of individual recombinants.

Clearly the cleavage of the mutant AAT and ATT *res* sites is complicated and variable. The AAT site is cleaved mostly with an AT dinucleotide, which if taken on its own might be interpreted as resolvase cleaving sequence-specifically. However, if resolvase did selectively cut AT dinucleotides then we would expect the ATT sites also to be cleaved at the AT dinucleotide. The fact that the ATT sites are never cleaved at AT (but mostly at the TT dinucleotide) shows that cleavage at the crossover site by resolvase is not AT sequence specific. Also, resolvase-induced cleavages are not fixed at 2 bp apart (although this is

preferred) but can be 3 bp apart, and show some variability. The 'top strand' cleavage position to the right of the centre of site I is fixed relative to the right end. The left (bottom strand) cleavage position is variable, although it also seems to prefer being a fixed distance from the right end of site I. It has been proposed that dimers of resolvase bound at the 'accessory sites' (II and III) contact the resolvase at the synapsed site I's by protein-protein interactions with the right half of the catalytic tetramer (Rice and Steitz, 1994a). The cleavage data for the AAT and ATT *res* sites are consistent with this proposal, because the interactions would tend to constrain most of the resolvase subunits bound at the right end of site I.

A model has recently been proposed where rather than making a 'concerted' double strand cleavage, resolvase catalyses sequential, individual strand cleavages at site I of *res* (Yang and Steitz, 1995). Some evidence that may support this model shows that the top strand cleavage is made first (Boocock *et al.*, 1995). The results presented here are consistent with this model. A 'concerted' double strand cleavage mechanism would most likely require a fixed geometry at catalysis, leading to fixed cleavage positions (2 bp apart). The pattern of cleavage seen with the AAT and ATT mutant *res* sites suggests that the top and bottom strands are cut separately, with perhaps the fixed right end (top strand) cleavage made first, followed by the more variable left end (bottom strand).

Chapter 6

Conclusions

Conclusions

In Chapter 3, a novel method was developed to test the mechanism of strand exchange by Tn*3* resolvase. By incorporating photoactivatable nucleotide analogues into site I oligonucleotides and then ligating these double-stranded oligonucleotides into supercoiled plasmid molecules *in vitro*, photocrosslinkable substrate molecules were synthesised. It was then possible to covalently attach a resolvase subunit to a specific half-site of the modified site I by irradiating the samples with UV light (prior to recombination) and to assay if the crosslinked complex was catalytically active.

To overcome the problems encountered with the sulphur-containing nucleotides, the photocrosslinkable nucleotide analogue 5-iodouridine was used in conjunction with a 325 nm HeCd laser. 5-iodouridine allowed efficient crosslinking of resolvase to oligonucleotide site I, and the photoreactivity was retained when the site was incorporated into supercoiled substrate molecules. An assay was then developed to detect catalytic activity in covalently photocrosslinked resolvase-*res* complexes using restriction analysis and SDS-PAGE.

Using the above technology, it was shown that fixing a subunit of resolvase to either the left or the right end of site I does not affect its ability to participate in the catalysis of recombination. These results demonstrate for the first time that the resolvase C-terminal domain-DNA interaction is maintained throughout the recombination reaction. Taking this into account, only certain models of strand exchange are still feasible (see below).

In a series of experiments described in Chapter 4, an attempt was made to distinguish between the 'subunit rotation' and 'DNA-mediated' models of strand exchange. The distinction between the models was based on the assumption that there is a requirement for a ligated recombinant intermediate during the knotting reaction of 'mismatch' substrates in a 'DNA-mediated' mechanism but not in a 'subunit rotation' mechanism (discussed in Chapter 1 and Chapter 4). It was found that although a tiny amount of mismatched recombinant is made, its reaction kinetics are not consistent with it being a transitory intermediate. Instead, it seems to be a 'dead-end' product of a side-reaction. Experiments with 'close-site' substrates (where the geometry of the synapse does not allow the knotting reaction, but does allow the recombination reaction), showed that resolvase seems to prefer to reverse the direction of strand exchange (to regenerate the substrate) rather than to ligate the mismatched recombinant.

Experiments with artificial supercoiled substrates containing mismatched basepairs led to the formation of linear molecules joined to resolvase (as well as recombinant catenane), demonstrating that resolvase-mediated ligation at mismatched basepairs is an inefficient reaction. If the mismatched recombinant was a necessary intermediate in the knotting reaction, then it would require resolvase-mediated ligation of half-site ends with (at least) two mismatched basepairs (one in each recombinant joint). Since no resolvase-cleaved linear molecules are detected in the knotting reaction, it seems highly unlikely that resolvase ligates mismatched basepairs as part of the knotting mechanism. Taking all of the evidence presented in Chapter 4 together, it was concluded that a fully ligated, mismatched recombinant is not a necessary intermediate in the knotting reaction.

The results presented in Chapter 3 and Chapter 4, when taken together, let us rule out some previously plausible mechanisms of strand exchange. Two models which can now be discounted are the 'end-swapping' mechanisms. The simplest 'end-swapping' model involves a tetramer of resolvase subunits, in which each subunit cleaves the crossover half-site to which it is bound (Figure 6.1a). The resolvase tetramer remains in approximately the same conformation while two of the half-sites swap places, with each half-site releasing the C-terminal domain to which it was bound and reassociating with the other one. This model is somewhat similar to the 'DNA-mediated' model of strand exchange (Figure 6.1c) in that the recombinant protein configuration must be different from the substrate configuration, and catalysis of a knotting reaction would also require ligation of a mismatched intermediate and re-setting of the catalytic tetramer.

A more complicated 'end-swapping' mechanism might again involve a 'static' resolvase tetramer with each subunit cleaving and becoming joined to the half-site to which it is bound. However, the catalytic Ser-10 nucleophile of one subunit then 'passes' its DNA end to a second nucleophilic residue of resolvase, for example to the Tyr-6 of another subunit, (and vice versa), exchanging the positions of the two DNA half-sites. In one scenario, the second nucleophilic residue might deliver the DNA end back to the Ser-10 residue of the same resolvase subunit before re-ligation of the DNA strands (Figure 6.1b). This second type of mechanism is compatible with multiple rounds of strand exchange without re-ligation of a mismatched intermediate only if the half-site-C-terminal domain interaction is broken. Alternatively, this mechanism could occur without breaking the DNA-C-terminal interaction, but it would then require resetting of the catalytic dimers (and ligation of a recombinant intermediate) in order to catalyse multiple rounds of strand exchange.

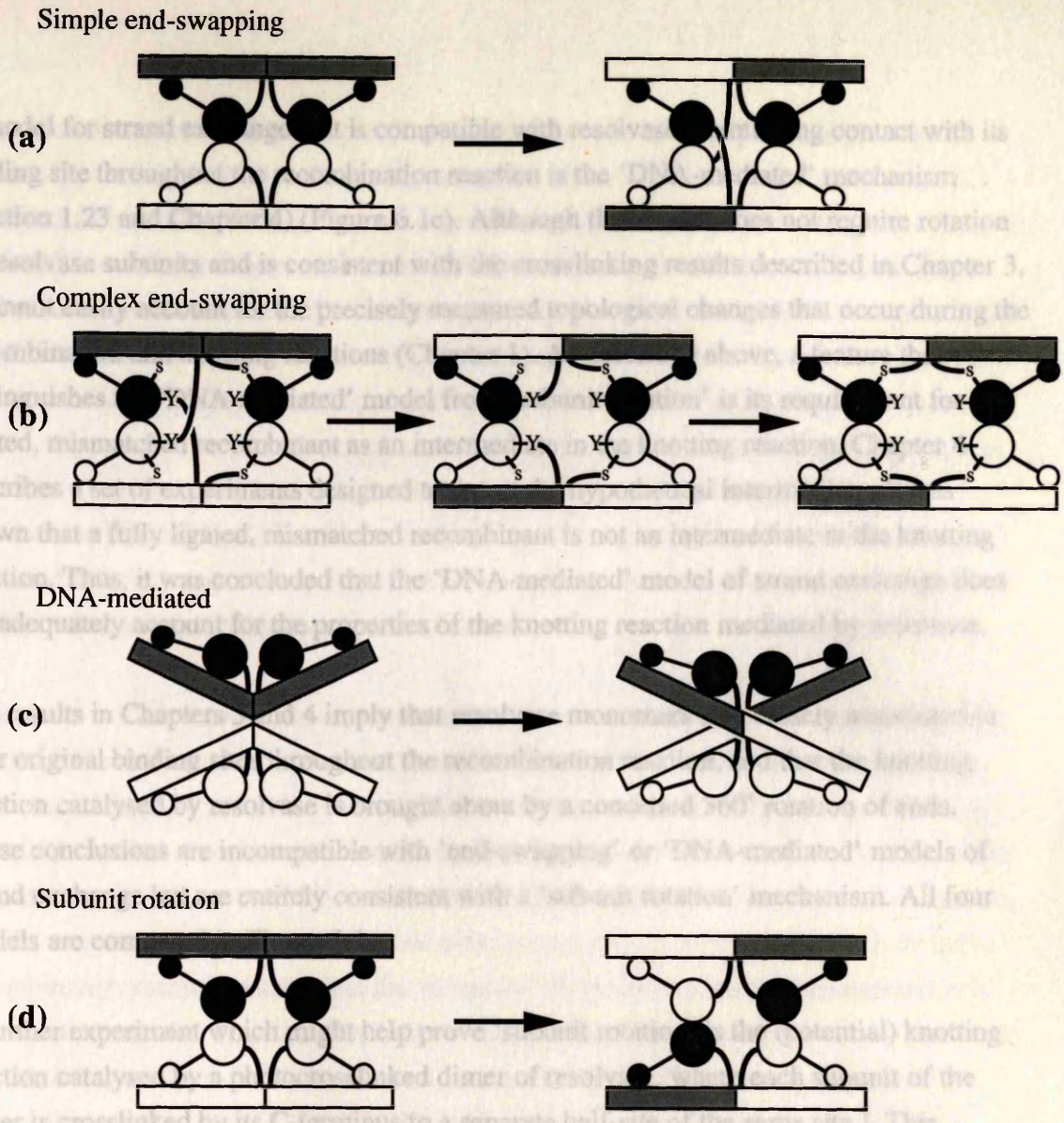


Figure 6.1 Models of resolvase-mediated strand exchange mechanisms.

Only site I DNA is shown for simplicity. Crossover half-sites are shown as white or shaded rectangles. The resolvase N- and C-terminal domains are shown as large and small circles respectively. Serine 10 residues are indicated by 'S' and Tyrosine 6 residues by 'Y'.

(a) Simple end-swapping. Each resolvase subunit cleaves the half-site to which it is bound. Two half-sites then swap places, with each half-site releasing the C-terminal domain to which it was bound and reassociating with the other one.

(b) Complex end-swapping. One possible scheme is shown where again each subunit cleaves and becomes joined (via Ser-10) to the half-site to which it is bound. However, the Ser-10 of one subunit then 'passes' its DNA end to a second nucleophilic residue of resolvase (e.g. Tyr-6) of another subunit (and vice versa) exchanging the positions of the two DNA half-sites. The Tyr-6 might then deliver the DNA end back to the Ser-10 residue of the same subunit before re-ligation of the DNA strands.

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

(d) Subunit rotation. Half-sites are exchanged by rotating one dimer (along with its attached half-sites) relative to the other dimer.

A model for strand exchange that is compatible with resolvase maintaining contact with its binding site throughout the recombination reaction is the 'DNA-mediated' mechanism (Section 1.23 and Chapter 4) (Figure 6.1c). Although this model does not require rotation of resolvase subunits and is consistent with the crosslinking results described in Chapter 3, it cannot easily account for the precisely measured topological changes that occur during the recombination and knotting reactions (Chapter 1). As described above, a feature that distinguishes the 'DNA-mediated' model from 'subunit rotation' is its requirement for a ligated, mismatched recombinant as an intermediate in the knotting reaction. Chapter 4 describes a set of experiments designed to detect the hypothetical intermediate. It was shown that a fully ligated, mismatched recombinant is not an intermediate in the knotting reaction. Thus, it was concluded that the 'DNA-mediated' model of strand exchange does not adequately account for the properties of the knotting reaction mediated by resolvase.

The results in Chapters 3 and 4 imply that resolvase monomers stay closely associated to their original binding sites throughout the recombination reaction, and that the knotting reaction catalysed by resolvase is brought about by a concerted 360° rotation of ends. These conclusions are incompatible with 'end-swapping' or 'DNA-mediated' models of strand exchange but are entirely consistent with a 'subunit rotation' mechanism. All four models are compared in Figure 6.1.

A further experiment which might help prove 'subunit rotation' is the (potential) knotting reaction catalysed by a photocrosslinked dimer of resolvase, where each subunit of the dimer is crosslinked by its C-terminus to a separate half-site of the same site I. This experiment (discussed in Chapter 3) could not be completed for technical reasons. A positive result from this experiment would demonstrate that subunits of resolvase covalently attached to each end of site I could catalyse multiple rounds of strand exchange (i.e. the knotting reaction); this would strongly support the 'subunit rotation' model. If however the crosslinked complex could not complete the knotting reaction (perhaps forming mismatched, recombinant catenane instead), this would support the 'DNA-mediated' model; that is, ligation to recombinant intermediate and resetting of the tetramer might be required for completion of the knotting reaction.

If 'subunit rotation' (Figure 6.1d) is indeed the most accurate description for how resolvase-mediated strand exchange occurs, how can the structural difficulties involved in rotating two dimers of resolvase relative to each other be reconciled with the co-crystal structure? It has been argued that as the resolvase subunits rotate (while still attached to the cleaved DNA ends), there might not be enough surface interaction between the two dimers

to maintain the contact that is required to stop the synapse falling apart (Chapter 1). It should also be noted that the dimer that is formed when resolvase binds to site I (the 1,2 dimer) must be broken during the rotation mechanism, and a new dimer interaction must be formed. The surface of the resolvase subunit that might be involved in these new dimer interactions has yet to be identified unambiguously. However, it should be recognised that the γδ resolvase co-crystal structure might not represent a 'catalytic dimer' bound to site I. The Ser-10 nucleophiles are still too far away from the scissile bonds which they must cleave. There must therefore be some sort of conformational change that occurs within a productive synapse (perhaps upon formation of a tetramer at site I) that brings resolvase into the catalytic configuration. Once resolvase is in this catalytic state the active sites may be closer to the phosphodiester bonds which they must cleave, and the dimers may be in a configuration more suitable for subunit rotation.

It may also be the case that we are being rather 'Newtonian' in our treatment of the strand exchange mechanism. Perhaps rather than thinking of subunit rotation as a kind of 'one-step' mechanism (Figure 6.2a), we should consider it as a series of 'quantum' diffusion steps that together constitute an overall rotation of two dimers relative to each other, while maintaining some contact within the tetramer at all times (Figure 6.2b). Such a mechanism may be a more realistic representation of what is occurring in solution, during a dynamic recombination reaction, rather than the 'snapshot' observations that are represented in X-ray crystal structures. Another possibility is that the ends are cleaved, the two halves come apart and then undergo 'one-dimensional rotational diffusion' (driven by negative supercoiling) with a very high probability of contacting each other in the first available 'specific' configuration, i.e. recombinant. This idea is similar to the proposed one-dimensional diffusion of DNA binding proteins along non-specific DNA scanning every possible sequence until they find their specific sites.

Although site-specific recombination by resolvase has been studied in some detail over the past fifteen years using genetic, biochemical, and more recently structural techniques, several key issues have yet to be resolved. Even after crystallisation of a solution dimer of resolvase bound at the recombination site, it is still not completely clear what role sites II and III play in the reaction, or how the mechanism of synapsis brings the crossover sites together, not to mention how the strands are eventually exchanged. It will be interesting to see what further light is shed on these extremely complex protein-DNA interactions by an ever increasing range of molecular biological techniques.

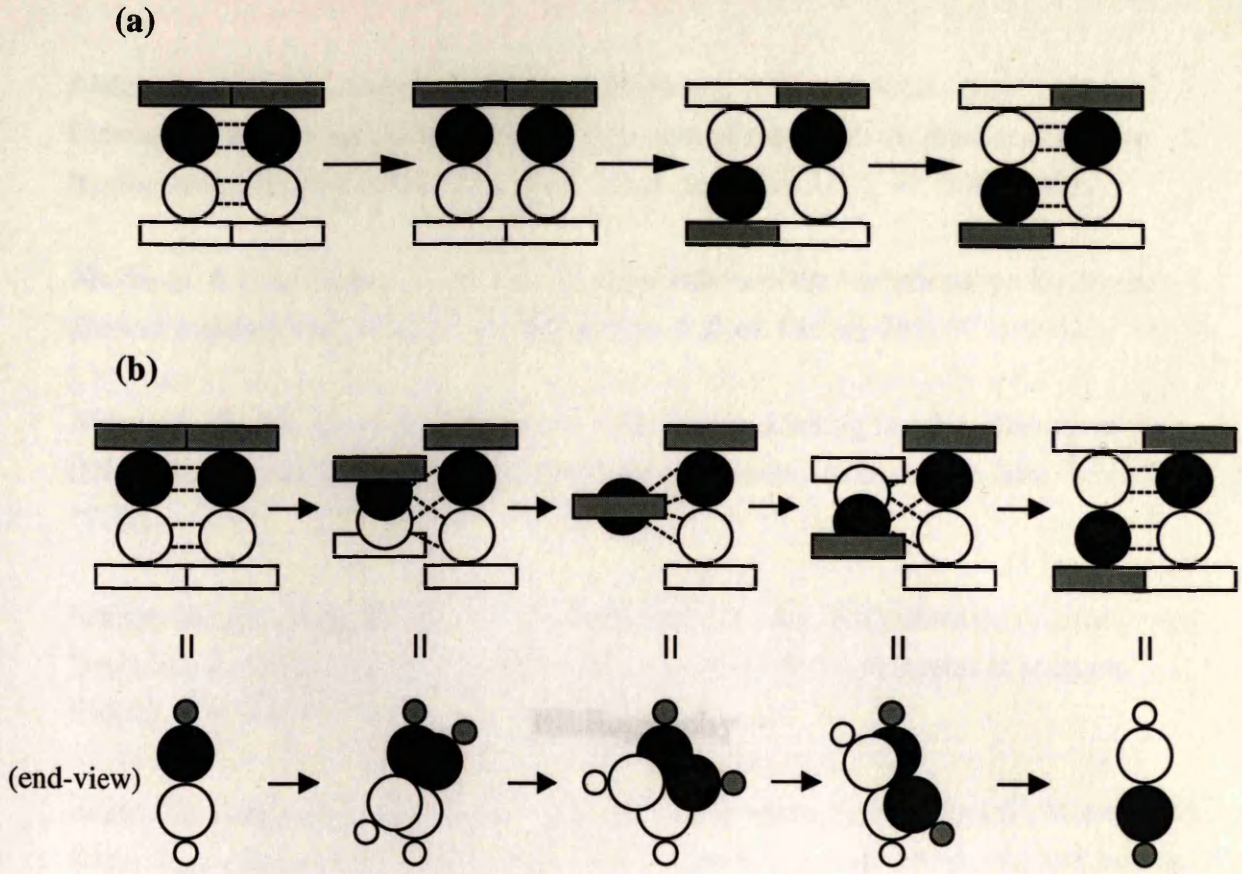


Figure 6.2 Interaction of resolvase dimers during subunit rotation.

Crossover half-sites are shown as white or shaded cylinders and resolvase monomers are shown as white or stippled circles. Protein-protein interactions are indicated by dashed lines.
(a) 'Classical' subunit rotation. Resolvase subunits cleave and become attached at the half-sites to which they are bound. The protein-protein interactions of the 1,2 dimers are broken and then one pair of subunits rotates (with its attached half-sites) relative to the other. Once in the recombinant configuration, new 1,2 dimer interactions are formed and the recombinant strands are ligated.
(b) An alternative mechanism for subunit rotation. Rather than a one-step rotation mechanism, subunits may be exchanged by a series of smaller diffusion steps that together constitute an overall rotation of two dimers relative to each other. This time some contact is maintained within the catalytic tetramer at all times thus preventing dissociation of the synaptic complex. Below Figure (b) is an 'end-view' of the mechanism looking from the left along the rotational axis of Figure (b).

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