DISSECTION OF THE Tn3 RESOLUTION SITE

A thesis submitted for
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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.

I dedicate this `book' to
my parents and my sisters,
for everything.

"and what is the use of a book,"
thought Alice, "without pictures
or conversations?"

CONTENTS

ABBREVIATIONS

Chemicals

APS	-	ammonium persulphate
ATP	-	adenosine triphosphate
BSA	-	bovine serum albumin
DTT	-	dithiothreitol
EDTA	-	ethylenediaminetetraaceticacid (disodium salt)
EtBr	-	ethidium bromide
EtOH	-	ethanol
IPTG	-	isopropyl B-D thiogalactopyranoside
SDS	-	sodium dodecyl sulphate
TEMED	-	NNN'N' tetramethyl ethylenediamine
Tris	-	tris (hydroxymethyl) amino ethane

Phenotype

X^r	-	resistance to X
X^s	-	sensitivity to X
ori	-	origin of replication

FIS	-	factor for inversion stimulation
sis	-	sequence for inversion stimulation

Acknowledgements

I thank my supervisor, Dave, for steering me in the right direction with his help and advice. To Martin I am deeply grateful for providing inspiration, enthusiasm and resolvase, and who painstakingly read all my sentences, resolving them into bitable pieces.

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The ladies of the prep. room kept my bugs supplied with food and the glassware clean.

Richard McCulloch constructed some of the plasmids last summer. Other plasmids and FIS protein were sent from Regine Kahmann in Berlin and Pascal Haffter in Basel.

A very special thanks to May, who welcomed me into her home with her two relaxed cats, and to Teresa and Caroline for their friendship throughout.

SUMMARY

Resolvase recognises three different sites within *res*; all three are necessary for recombination *in vivo* and *in vitro*, under standard reaction conditions. However, when resolvase *in vitro* reaction conditions were altered, recombination between a wt-*res* site and an isolated crossover site (i.e. lacking subsites II and III) proceeded at a reduced efficiency. In these substrates, resolvase disregarded the relative orientation of the crossover site, but still selected the resolution event. The resolution products were simply catenated. Resolvase thus recognises the crossover site as functionally symmetrical. Replacing the crossover site within wt-*res* with a perfectly symmetrical subsite I (sym-*res*) resulted in the normal left-to-right alignment of crossover sites for recombination, even for intermolecular recombination between two linear sym-*res* substrates. Therefore, resolvase uses subsites II and III to determine the polarity of *res*. When subsites II and III were removed from both *res* partners, no recombination products were detected.

To investigate the effect of FIS on resolvase-mediated recombination, the enhancer site, *sis*, was cloned into resolvase substrates. Although FIS and *sis* are required to stimulate inversion by the related Gin, Hin and Cin invertases, they did not appear to have any effect on the recombination properties of isolated *res* crossover sites; resolvase reactions with other combinations of wt-*res* and deleted *res* sites were also unaffected by these accessory proteins and sites.

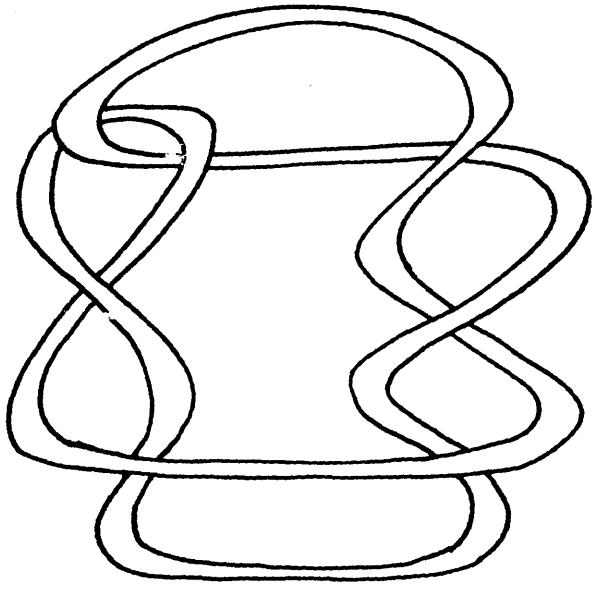
Substrates were made to test whether resolvase acting at subsites II and III can direct a Gin-mediated resolution event between *gix* sites (i.e. '*ges*' site recombination). No recombination between *ges* sites was observed *in vivo* when resolvase and Gin were provided in *trans*. In collaboration with C. Koch, (Berlin) *in vitro*

recombination between *gcs* sites was tested using a FIS-independent mutant Gin protein capable of recombining directly repeated *gix* sites; when resolvase was present recombination between *gcs* sites by the mutant Gin was prevented. This may be interpreted as a consequence of synapsis of subsites II and III by resolvase inhibiting Gin-mediated recombination. Subsites II and III alone were also shown to delay recombination between certain pairs of wt-*res* sites in a multi-*res* site substrate. This result also suggests that subsites II and III are sufficient for synapsis.

Individual *res* subsites, and combinations of *res* subsites, on DNA fragments displayed distinctive retarded complexes in resolvase gel binding assays. By using this assay and a set of circularly permuted DNA fragments, resolvase-induced bending of subsite I was demonstrated. Two complexes per subsite were stabilised in the gel, suggesting that resolvase can occupy a subsite in two steps. No severely retarded complexes were trapped by the gel assay that would be indicative of a higher protein-DNA structure, i.e. a synaptic intermediate. Therefore, intermolecular synapsis of sites by resolvase appears to be difficult to capture in the gel assay.

CHAPTER ONE

INTRODUCTION



Transposition of the Tn3 class of transposons occurs in two stages. Transposase acts at the ends of the transposon and the entire donor molecule is inserted into the target. During this process, the transposon is replicated to form a cointegrate structure (Tn3 transposition reviewed by Sherratt, 1989; figure 1.1). Subsequent resolution between a recombination site, *res*, in each transposon, breaks down the cointegrate structure to give a copy of the transposon in each separate replicon (Arthur and Sherratt, 1979). The resolution event of Tn3 is site-specific (requiring only a limited region of homology), reciprocal and and conservative. Only one transposon-encoded protein (the product of the *tnpR* gene) is involved in resolution of cointegrates.

Several recent reviews cover the resolution systems of Tn3 and related transposons (Hatfull and Grindley, 1988; Hatfull *et al*, 1988; Sherratt, 1989; Stark *et al*, 1989b). Studies on the Tn3 and gamma-delta (Tn1000) resolvases are complementary, as these transposons are virtually identical in their transposition functions, and therefore components of the two systems are interchangeable. The resolvase system is one of several site-specific recombinases that are currently being extensively investigated *in vitro*, following the lead of Nash (1975) with the lambda integration system.

Site-specific recombination systems are found in transposons, phage, plasmids and chromosomes (reviewed by Sadowski, 1986). The systems so far identified appear to belong to one of two categories on the basis of their amino acid sequence homology ie. the resolvase/invertase group and the integrase-like recombinases. Members within each group seem to share common mechanistic features.

Tn3 resolvase is one of several resolvases that are responsible for the resolution of cointegrate structures or are involved in plasmid stability (e.g. the R46 resolution system; Dodd and Bennett, 1986). Related to

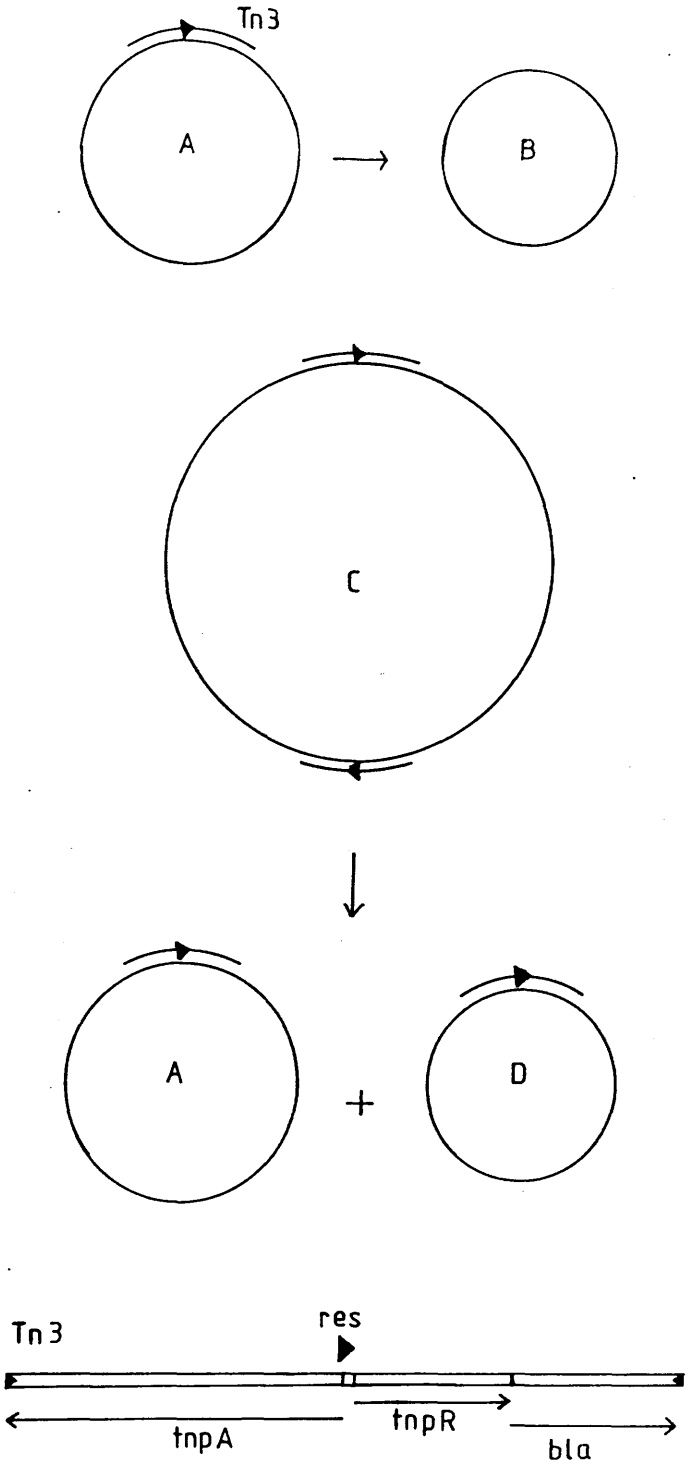


Figure 1.1 Formation and resolution of cointegrate intermediates in Tn3 transposition. Tn3 in a donor molecule (A) transposes into the recipient (B); a cointegrate structure (C) is formed in which the transposon has been replicated. Resolution between *res* sites of each transposon releases two replicons each containing a copy of Tn3 (A and D).

resolvases are the Gin, Hin, Cin and Pin DNA invertases (figure 1.2). In phage Mu, the Gin invertase inverts the G-segment to alter the expression of tail fibre genes and thus change the host-range of the phage. Inversion of tail fibre genes of phage P1 is mediated by the Cin protein. The product of the hin gene of Salmonella typhimurium regulates the expression of flagellin genes by inverting the orientation of their promoter. Pin-mediated inversion in the defective e14 viral element of the E.coli chromosome has an unknown function. These invertases are functionally complementary (Plasterk and van de Putte, 1985).

There is homology in protein domains of the resolvases and invertases. These recombinases can be divided into two domains (Abdel-Meguid et al, 1984) A large amino domain (ca. 140 amino acids) is required for the catalytic function and contacts between protein subunits; a small carboxy-terminal domain contacts the DNA by a putative helix-turn-helix motif (Pabo and Sauer, 1984). Synthetic peptides corresponding to the carboxy-termini of both Hin and gamma-delta resolvase have been shown to contact their respective binding sites (Sluka et al, 1987; Rimphanitchayakit et al, 1988).

The second category of site-specific recombinases can be collectively termed the 'Int' family of recombinases (Argos et al, 1987) Their biological functions are varied. Integration of the lambda phage into the E.coli chromosome is the most extensively studied system. Other phage have related integration systems, i.e. P22, etc. and in some cases their recombinases can be substituted for one another. Another DNA recombination system of bacteriophage P1 requires the action of the Cre protein at lox crossover sites. FLP-mediated inversion of the Saccharomyces cerevisiae 2-micron plasmid provides an intriguing plasmid amplification property (Volkert and Broach, 1986). The Fim invertases of the E.coli chromosome mediate inversion to alter the expression of fimbrial

Figure 1.2 Alignment of predicted amino acid sequences of representative members of the resolvase and DNA invertase family of site-specific recombinases. The putative DNA helix-turn-helix motifs are indicated. The active site serine is also indicated. (Taken from Sherratt, 1989, and Rowland and Dyke, 1989).

(A)

	1		28
consensus	kllvela.st	glrisel.rl	rwsdidld
XerC	RAMLEVMYGA	GLRLSELVGL	DIKHLDLE
Lambda	RLAMELAVVT	GQRVGDLCEM	KWSDIVDG
Phi80	VFLVKFIMLT	GLRTAEIRLS	ERSWFRLD
P2	KKEAELCLST	GARWGEARRL	KAENIIHN
P4	MIAVKLSLLT	FVRSSELRFA	RWDEFDFD
P22	KSVVEFALST	GLRRSNIINL	EWQQIDMQ
Cre	TAGVEKALSL	GVTKLVERWI	SVSGVADD
F_D_Prot	KMLLATLWNT	GARINEALAL	TRGDFSLA
FimB	YCLTLLCFIH	GFRASEICRL	RISDIDLK
FimE	YCLILLAYRH	GMRISELLDL	HYQDLDLN
Tn2603	RLFAQLLYGT	GMRISEGLQL	RVKDLDFD
Tn554a	KLILMLMYEG	GLRIGEVLSL	RLEDIVTW
Tn554b	ATMTMIVQEL	GMRISELCTL	KKGCLLED

(B)

	1			40
consensus	**H**ml**R**hs.at.	lle.g.idir	.iq.llgh..	.is.t.r**Y**th
XerC	**H**KL**R**HSFATH	MLESS.GDLR	GVQELLGHAN	.LSTTQ**I**YTH
Lambda	HEL**R**SLSA.R	LYEKQ.ISDK	FAQHLLGHKS	.DTMASQ**Y**.R
Phi80	**H**DM**R**RTIATN	LSELG.LPPH	VIEKLLGHQM	.VGVMAH**Y**N.
P2	HAL**R**HSFATH	FMING.GSII	TLQRILGHTR	.IEQTMV**Y**AH
P4	**H**GF**R**TMARGA	LGESGLWSDD	AIERQSLHSE	RNNV**R**AA**Y**IH
P22	HDL**R**HTWASW	LVQAG.VPIS	VLQEMGGWES	.IEMV**R**R**Y**AH
Cre	**H**SA**R**VGAARD	MARAG.VSIP	EIMQAGGWTN	.VNIVMN**Y**IR
F_D_Prot	HTF**R**HSYAMH	MLYAG.IPLK	VLQSLMGHKS	.ISSTEV**Y**TK
FimB	**H**ML**R**HSCGFA	LANMG.IDTR	LIQDYLGHRN	.IRHTV**R**YTA
FimE	HML**R**HACGYE	LAERG.ADTR	LIQDYLGHRN	.IRHTV**R**YTA
Tn2603	HTL**R**HSGATA	LLRSG.YDIR	TVQDLLGHSD	.VSTTMIYTH
Tn554a	HML**R**HTHATQ	LIREG.WDVA	FVQKRLGHAH	VQTTLNT**Y**VH
Tn554b	**H**AF**R**HTVGTR	MINNG.MPQH	IVQKFLGHES	.PEMTS**R**YAH
FLP	HIG**R**HLMTSF	LSMKGLTELT	NVVGNWSDKR	ASAVATT**Y**TH

Figure 1.3 Alignment of putative amino acid sequences for members of the integrase family of recombinases. Two domains are conserved (FLP only has the B consensus domain). The positions of three totally conserved amino acids are indicated. (Adapted from Argos et al, 1986.)

genes. A recently identified member of this family, XerC, is a host function, which monomerises the plasmid ColE1 and thus increases the stability of the plasmid within its hosts population (Summers, 1989).

Some amino acid homology exists within two domains of the members of this family of recombinases (figure 1.3). Most importantly, the positions of three specific amino acids are conserved. Of these recombinases so far characterised, some mechanistic properties are shared. However, the range of accessory factors included in some of these systems varies, and some have a substrate selectivity (e.g. lambda integration) but others do not appear to have a strict selection of substrate (e.g. FLP and Cre).

Resolvases and Invertases.

Although resolvases and invertases share many similar characteristics, their functions are different. As their names suggest, resolvases promote resolutions and invertases promote inversions between their respective sites. The functions of these enzymes cannot be reversed; resolvases cannot invert DNA between inverted *res* sites on supercoiled substrates *in vitro* and invertases cannot resolve supercoiled substrates containing directly repeated crossover sites.

Resolvases act at a DNA sequence in Tn3 called the *res* site (approximately 120 bp). Resolvase recognises and binds to three sites (subsites I, II and III) in *res* as determined by footprinting techniques (Grindley *et al*, 1982; Kitts *et al*, 1983) Figure 1.4 shows the arrangement of the subsites in *res*, determined by resolvase contacts or by sequence comparisons with *res* regions of several Tn3 family transposons and related plasmid elements. The *res* sites consist of a crossover site (subsite I) separated by a spacer region from two different accessory sites

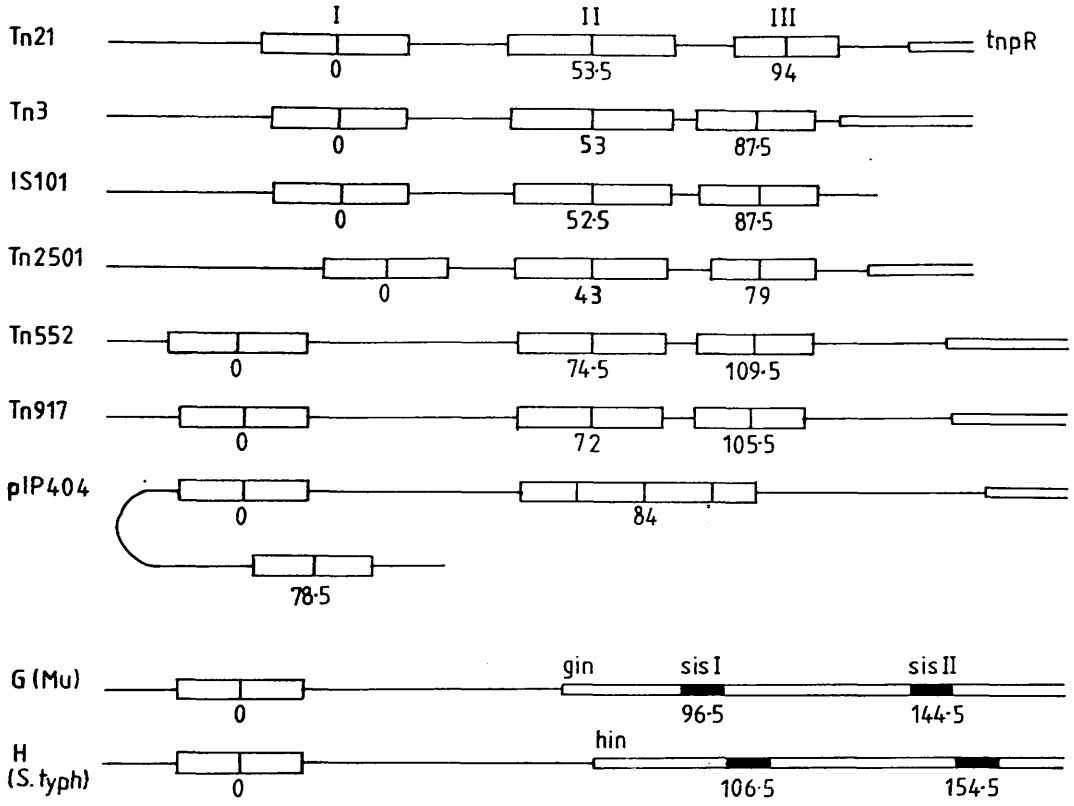


Figure 1.4 The structures of crossover and accessory sites requires for recombination by resolvases and invertases. Three subsites of *res* are arranged as indicated in the diagram. Subsite I is the crossover site of *res*. Each subsite is comprised of an inverted repeat sequence with a central spacer. Two FIS binding sites (*sis*) for the Gin and Hin DNA inversion systems are located within the gene sequences for the invertases. Phosphodiester bond coordinates are defined from the centre of the crossover sites. Note that the *cin* gene is inverted with respect to the crossover sites and therefore the enhancer site is normally located more than 500bp from the *cix*L (Huber *et al*, 1985).

(subsites II and III). The resolvases of Tn3, gamma-delta, R46 and Tn1 can subsitute for one another in resolution reactions as they act at *res* sites containing subsites of identical sizes and spacing (Reed, 1981; Kitts *et al*, 1983; Dodd and Bennett, 1987). However, they cannot recombine *res* sites with different sizes of subsites and spacers e.g. from Tn21 (Halford *et al*, 1985). Resolvases recognise sequence elements in each subsite, arranged with some degree of dyad symmetry, i.e. each subsite of Tn3 *res* contains a sequence similar to a consensus TGT....TA.

The centre of subsite I of Tn3 and gamma-delta *res* contains a sequence TTATAA that is required for strand cleavage and exchange (Wells and Grindley, 1984). Resolvase cleaves the DNA at the centre of subsite I to form 2 bp 3' protruding ends, to which the protein becomes covalently attached by a 5'-phosphoserine link (figure 1.5). The catalytic serine responsible for this link is the conserved Ser-10 of this family of recombinases; a similar link with Ser-9 of the Gin invertase has been found when the *gix* site is cleaved (Klippel *et al*, 1988a).

Invertases act at simple crossover sites arranged in inverted repeat. In addition, a second host protein, FIS, is required to act at an 'enhancer' site, *sis*, on the same substrate molecule (Kahmann *et al*, 1985). The *sis* site can be placed at a distance from the crossover sites and in either orientation. Its natural location overlaps the codon for the catalytic serine within the invertase gene (figure 1.4). As for the resolvases, the invertase genes are located adjacent to their recombination sites, and they can act in *trans*.

A recent addition to this family of recombinases is Bin, which was originally characterised as an invertase of an invertible segment found in a natural plasmid of *Staphylococcus aureus* (Rowland and Dyke, 1988). Since a *bin* gene also resides in the transposon Tn552, it has been suggested that Bin is in fact a resolvase and not an invertase (Rowland and Dyke, 1989). The crossover sites of

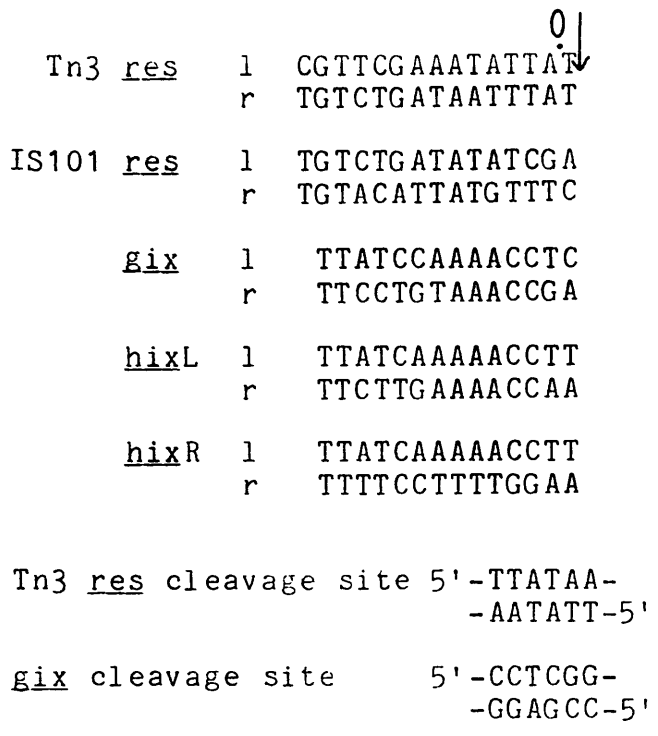


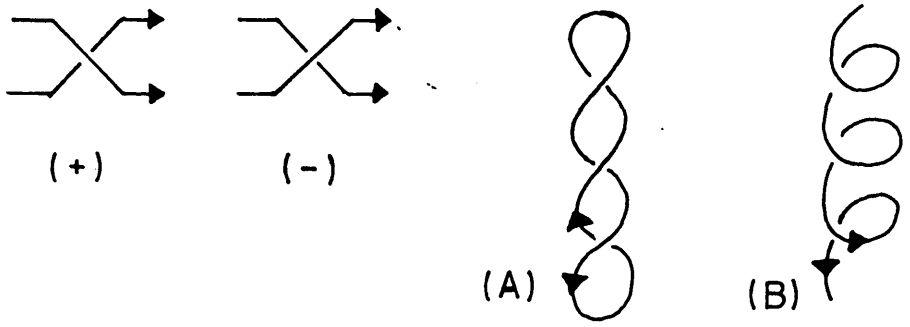
Figure 1.5 Sequences of crossover sites for resolvases and invertases. Each site has dyad symmetry (centred around position 0) and is represented as the 5'-3' sequence of the left and right halves of the site respectively. The sequences of gixL and gixR are identical. The position of cleavage by the recombinase in each case is shown by the arrow.

this system were originally called *bix*, but by comparison with the *res* site of Tn917, two potential accessory sites have been located adjacent to the *bix* crossover site (figure 1.4). Initial *in vivo* experiments have confirmed that Bin can resolve substrates with directly repeated *bix* sites. In addition, the *bin* gene seems to lack any obvious enhancer sequence normally located within invertase genes.

The crossover sites for the invertase systems are similar to those of *res* (figure 1.5). Invertases also cleave at the central dinucleotide of the crossover site to give 2 bp 3' protruding ends. The action of invertases at the crossover site implies a similar mechanism for strand exchange as for resolvases. In both systems, the recombinases cleave both strands of each site and rotate the strands through 180^{o} before religation (Kahmann *et al*, 1987; Stark *et al*, 1989a).

Synapsis of recombination sites by resolvases and invertases.

Both the resolvases and invertases have a strong selection of substrate, such that the crossover sites are in *cis* in a defined order on a supercoiled molecule. By studying the *in vitro* substrate requirements for resolvase and the subsequent product topologies, we can begin to understand the mechanisms of the reaction. The *res* site has been well defined; there are three resolvase binding sites, but only subsite I contains the region for strand exchange (Grindley *et al*, 1982). Resolvase requires two directly repeated *res* sites on a supercoiled substrate; deletion between the *res* sites results in simply catenated products (figure 1.6). When minor products were analysed by electron microscopy, their topological structures indicated that the reaction proceeds via a specific intermediate structure in which three negative interdomainal supercoils are trapped (Wasserman and



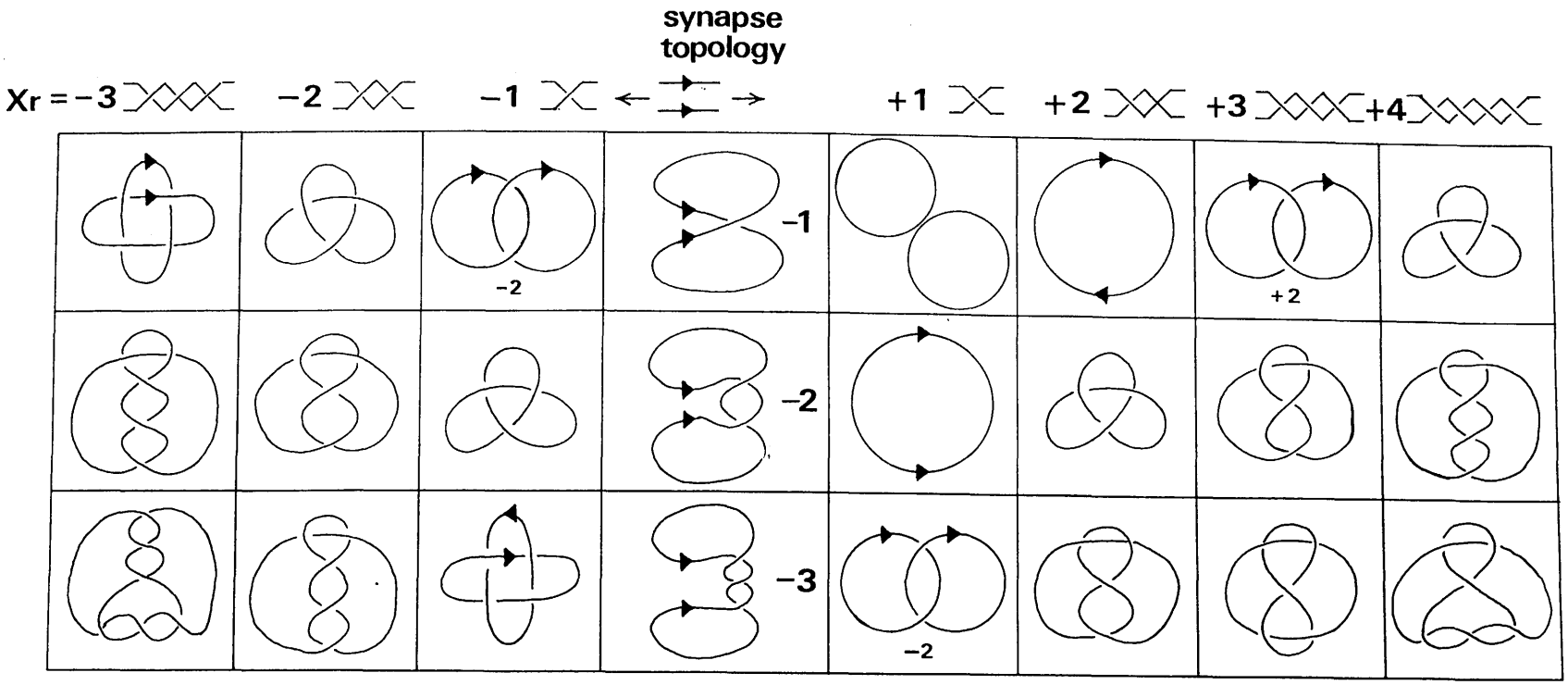
TOPOLOGICAL TERMS

The path of DNA in a molecule crosses in one of two ways, to give a (+) or a (-) node. Strands twisted by rotation (Xr) in the right-handed sense are (+) and those in the left-handed sense are (-).
Supercoils are either (A) plectonemic or (B) solenoidal and are shown as (-) supercoils in the right-handed and left-handed sense respectively.

Figure 1.6 The topology of products expected from recombination intermediates trapping different numbers of interdomainal supercoils and for successive rounds of strand exchange. Tn3 resolution is proposed to occur using a -3 synapse topology and with a right-handed sense of strand exchange (Xr = +1). Iteration of strand exchange for resolvase therefore gives the products shown in the lower right hand corner. Although a -2 catenane can also arise from a -1 synaptic intermediate and a left-hand rotation of strands, the iteration products expected from this intermediate were not detected (Wasserman and Cozzarelli, 1985).

Inversion by Gin and Hin is proposed to proceed using a -2 synaptic intermediate and Xr = +1, to give unknotted products. Complex knots are expected to be generated from an iteration of strand exchange rotation.

The numbers used to define the synapse topology refer to the number and sign of interdomainal nodes trapped (i.e. between the two sites). Two nodes are trapped in the single link of the catenated product rings.


synapse
topology
Xr = −3
−2
−1
+1
+2
+3
+4
−2
−1
+2
−2
−3
−2

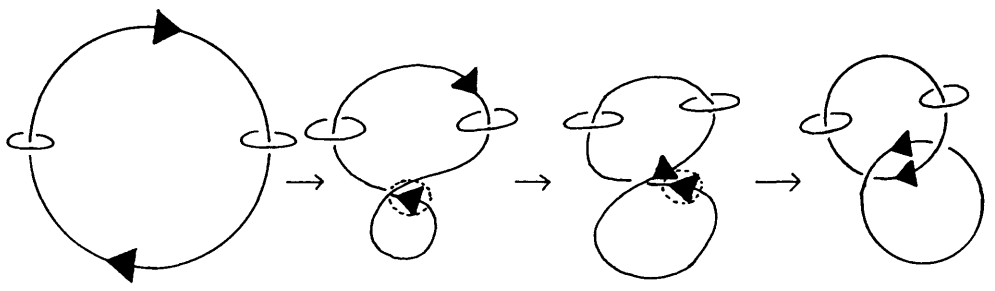


Figure 1.6.1 The reporter ring experiment for the tracking model. Two ØX184 RF molecules were catenated to the resolvase substrate. Both reporter rings would be excluded from the DNA loop formed by resolvase at one res site searching for a second res site, as shown in the diagram. This would result in two rings always being segregated together on one of the circular products. However, some recombination products contained only one reporter ring, suggesting that resolvase does not slide continuously between the two sites. (Benjamin et al, 1985.)

Cozzarelli, 1985). The major product of resolution is a -2 catenane; minor products are a result of an iteration of the strand exchange event before the intermediate structure falls apart (figure 1.6). For the reaction to result in the products found, the strands must be exchanged in the right-handed sense (figure 1.6). A rotation of strands in the right-handed sense is expected to reduce negative supercoiling. Experiments to examine the linkage difference between substrate and product have indicated that the rotation of strands is right-handed, both for resolvase-mediated resolution and Gin-mediated inversion (Boocock et al, 1987; Kahmann et al, 1987).

Several models have been proposed to account for the substrate selectivity of resolvase. Although an adjacent site preference agreed with the idea that resolvase at one site can `track' along the DNA until a second site, in the correct relative orientation, is reached (Krasnow and Cozzarelli, 1983), there has been no further support for the tracking model. Some experimental evidence has discounted tracking. Synapsis of res sites by tracking of resolvase was expected to result in reporter rings on one of the catenated products only, but they were segregated between the resolution products (Benjamin et al, 1985; figure 1.6.1) Not all non-adjacent events are disallowed in substrates containing four or more res sites (J.L.Brown, 1986). Intermolecular recombination between res sites has also been observed (Boocock et al, 1986).

Alternative models incorporate a fixed local alignment of res sites, by resolvase, to give a -3 synapse structure as shown in figures 1.6 and 1.7. In a `slithering' model, the res sites were originally proposed to wrap solenoidally around resolvase (Benjamin and Cozzarelli, 1986). The postulated slithering movements of DNA results in the selection for an alignment of sites with a unique synapse topology. In our `two step synapsis' model, the res sites are aligned by random collision, but a synaptic structure is assembled by the interwrapping of

subsites II and III of *res* around resolvase, with a local structure shown in figure 1.7, in which three negative supercoils are trapped (Boocock *et al*, 1986). Resolvase is proposed to recognise subsites II and III in both sites and align them in an antiparallel sense to form the plectonemically wrapped synapse; the crossover sites (subsite I) are then aligned in a parallel sense for strand exchange. This model, therefore, suggests how the synapse can be formed and how the *res* sites are aligned to maintain their left-to-right polarity upon recombination.

The plectonemic wrap of sites proposed by our model is affected by the topology of the closed circular DNA substrates and the relative orientation of *res* sites. Both inversion and fusion events between *res* sites would be expected to introduce unfavourable tangling and supercoiling of extrasynaptic domains of the substrates if an identical plectonemic wrapping of sites is assembled (figure 1.7). In a random collision of sites, extradomainal supercoils are expected to be trapped. However, if subsites II and III in both sites are used to wrap resolvase plectonemically, then only those collisions which form a synapse without trapping extra supercoils would be accepted for recombination i.e. those that form a 'productive' synaptic structure. Subsites II and III interwrapping with resolvase also excludes inverted *res* sites and fusion of *res* sites from forming productive synaptic complexes when these substrates are supercoiled (figure 1.7). The model incorporates a potential mechanism for resolvase to assess both the topological state of the substrate and the relative orientation of *res* sites, and suggests a function for subsites II and III of *res* in recombination. Differences in the energetics of *res* site synapsis can account for the topological selectivity of the system.

By adaption of *in vitro* reaction conditions for resolvase, non-supercoiled substrates have been shown to recombine (Boocock *et al*, 1986). In supercoiled

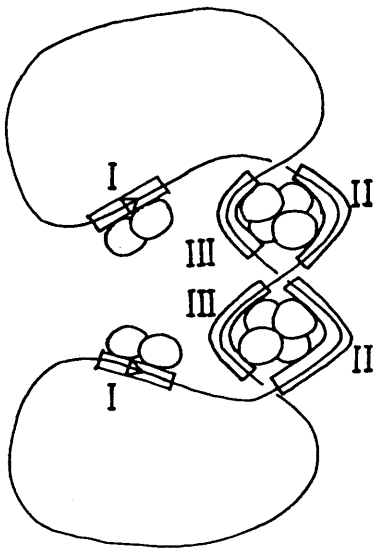
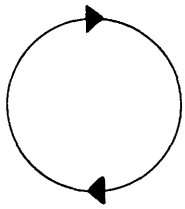
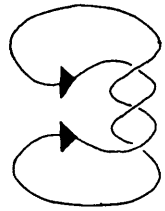
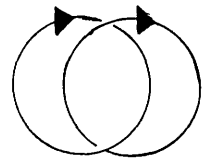
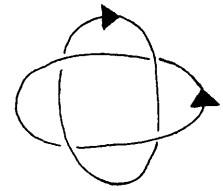
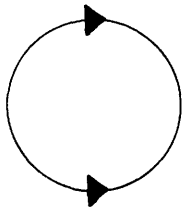
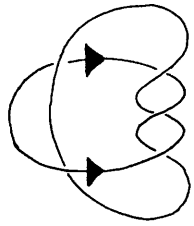
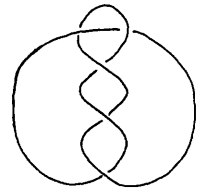
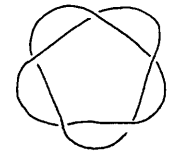
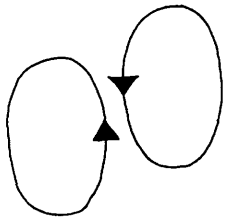
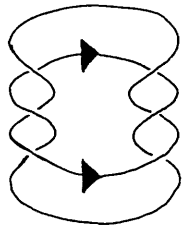
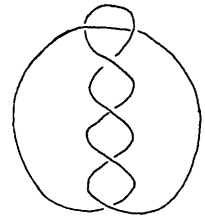
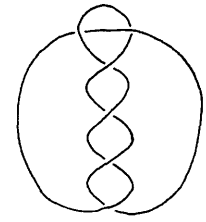
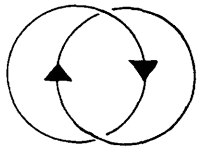
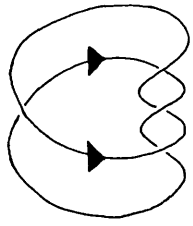
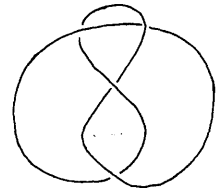
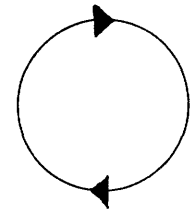
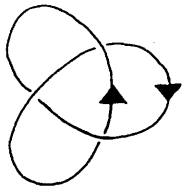
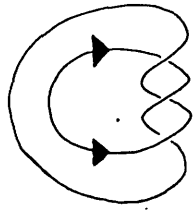
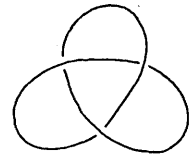
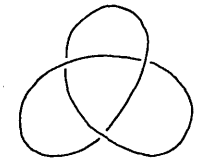


Figure 1.7 Predicted topologies of products of inversion and fusion events in the resolvase system. For resolvase to align the *res* sites for recombination using a -3 synapse, extra supercoils will be introduced into the synaptic intermediates. The product topologies predicted for recombination between inverted sites or a fusion of sites are shown. Catenane fusion has been shown for relaxed substrates (Stark *et al*, 1989a). The product of the fusion of a simple catenane was unknotted and therefore the strands had been exchanged in a left-handed sense (Xr = -1).

reaction	substrate	synapse	predicted product topologies Xr = +1	−1
resolution				
inversion				
circle fusion				
catenane fusion				
trefoil inversion				

substrates, a productive synapse is only expected to form for two *res* sites in direct repeat. Releasing supercoiling by nicking, relaxing or linearising the DNA substrate allows both recombination between inverted *res* sites and intermolecular reactions (providing at least one substrate is not supercoiled) to be observed. Fusion of a relaxed -2 catenane (i.e. the product of a forward recombination reaction which had subsequently been relaxed) was shown to be an efficient reaction *in vitro*, giving an unknotted circular product. Both the topology of this product and the addition of four negative supercoils during the recombination reaction suggested that the catenane fusion was the reverse of the resolution event, i.e. that the -3 synapse had formed, and that the rotation of strand exchange was also reversed (figure 1.7; Stark *et al*, 1989a).

Further predictions from our model have yet to be tested, but some of the experiments presented in this thesis were designed to test the function of subsites II and III in the selection for resolution. Inversion products from nicked substrates were shown to migrate as the expected specific knots, but there structures have not been confirmed by electron microscopy (M. Boocock, this laboratory; figure 1.7). Predictions for the topological states of recombination products of inversion and fusion events are different for a solenoidal and a plectonemic wrapping of *res* sites. The postulated interwrapping of resolvase and subsites II and III would require the protein to sharply bend the DNA within the *res*. site. Resolvase-induced bending of *res* DNA has been shown by gel retardation assays (Brown *et al*, unpublished; see also chapter 3). The *res* sequence has been predicted to preferentially bend when compared to the established data for nucleosome wrapping (Satchwell *et al*, 1986). Although a synaptic intermediate has been trapped by using cross-linking agents, the precise synaptic structure has not been determined (Benjamin and Cozzarelli, 1988).

Recombination between invertase sites can be represented by a similar model to that proposed for resolvase. The requirement for FIS suggested that this protein and the enhancer site are involved in the assembly of the synapse for invertase-mediated recombination. The unique unknotted product and the linkage change of +4 suggested that this synaptic complex traps two negative supercoils in a local structure shown in figure 1.6 (Kahmann *et al*, 1987). A similar model for synapsis has also since been proposed for the synapsis of *hix* sites (Johnson *et al*, 1987). Although there is no direct evidence for this structure, the DNA binding and bending by FIS may be an important feature of the local synaptic structure (Hubner *et al*, 1989; Kanaar *et al*, 1989a). It is unknown how this structure can be assembled and held in place, although supercoiling may contribute to the stabilisation of the synapse. The enhancer site does not have to be on the same molecule as the crossover sites for recombination by Gin and FIS, providing the two supercoiled molecules are multiply catenated. Recombination is also possible when the two *gix* sites are on the separate rings of a supercoiled complex catenane, or if two sites are in direct repeat on a supercoiled knotted molecule (Kanaar *et al*, 1989b). These experiments rule out tracking of the invertase as a mechanism for sensing the orientation of sites. The orientation of the two sites is sensed through the topology of the supercoiled substrates; it is likely that the correct configuration of sites can give a synapse that is stabilised by negative supercoiling. Similar experiments had previously been conducted for Mu transposition *in vitro* (Craigie and Mizuuchi, 1986). Distant Mu ends were brought together by MuA protein when they were on separate molecules of a supercoiled catenane, or when they were in the `incorrect' relative orientation on a supercoiled knotted molecule.

Other systems apart from resolvase involve the action

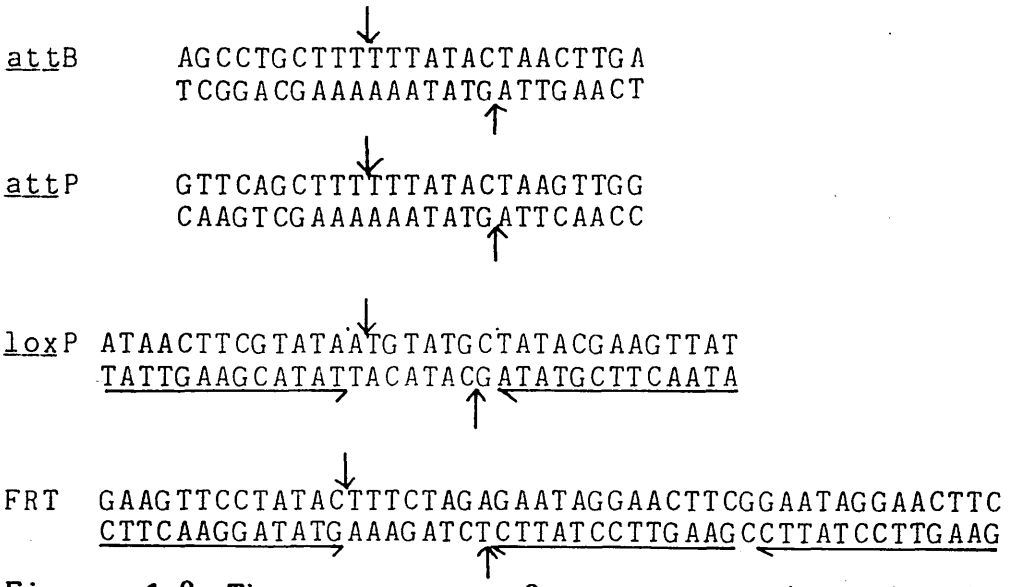


Figure 1.8 The sequences of crossover sites for lambda integrase (att sites), Cre (loxP) and FLP (FRT). Each site is cleaved on either side of a spacer region (core) by their respective recombinase. FRT and loxP contain inverted repeat sequences.

of proteins aligning two distant sites, and models have been developed and tested to show the importance of the topology of the substrate in the selection of a recombination event. For Mu transposition, Gin and resolvase recombination, tracking between sites cannot be the mechanism for bringing sites together. Proteins that act at more than one DNA site influence the regulation of expression of some genes (reviewed by Ptashne, 1986). In some cases, the proteins have been shown to loop the DNA between the bound sites, e.g. lambda, lac and deo repressors (Hochschild and Ptashne, 1986; Kramer et al, 1987; Mortensen et al, 1989).

Integrases

The integrases, in contrast to the resolvase and invertase family, do not select for a deletion or inversion event, as deletion between direct att sites and inversion between inverted att sites occur with similar efficiencies (Pollock and Nash, 1983; Craig and Nash, 1983). Recombination between att sites gives a range of topologically complex products as a result of trapped extra-domainal supercoils prior to strand exchange (Mizuuchi et al, 1980; Spengler et al, 1985). These recombinases cleave their crossover sites to give 5' protruding ends of a variable spacer length, and are attached by a 3'-phosphotyrosine link to the cleaved ends (Pargellis et al, 1988; figure 1.8).

In lambda integration, Int promotes recombination between a complex site, attP, and a simple site, attB. The bacteriophage site, attP, has an asymmetric arrangement of accessory sites flanking both sides of the crossover site (figure 1.9), but attB consists of crossover site only. Recombination between attP and attB requires the host protein IHF in addition to Int and results in two hybrid sites attL and attR. The reverse excision event between

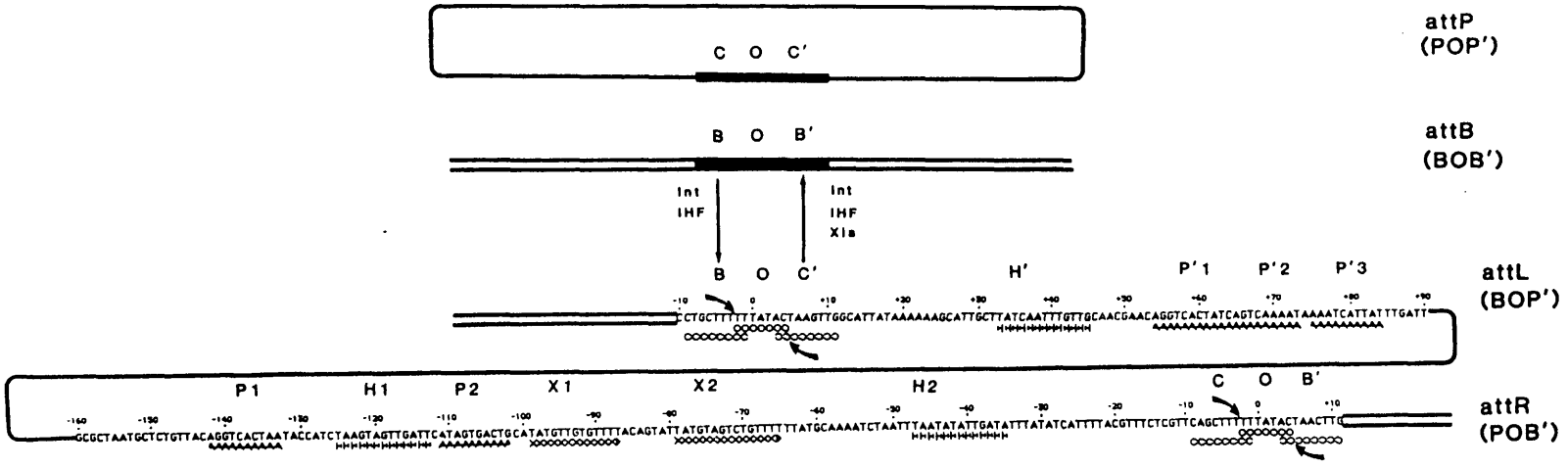


Figure 1.9 Structure of the lambda att site. Recombination between attP and attB generate two hybrid sites attR and attL. The binding sites for IHF (H), Xis (X), arm-type Int (P) and core-type Int (C or B) are indicated. Curved arrows show the sites of strand exchange.

attL and attR requires yet another protein, Xis, which is phage encoded. Intermolecular events are possible with either the integrative or excisive pair of recombination sites. Linear molecules can recombine, with the exception of the substrates containing attP. For integrative events under standard conditions, attP must be on a supercoiled substrate (Mizuuchi et al, 1980). A further exception is an Xis-independent intramolecular excisive event (attR x attL) that requires a supercoiled substrate and sites in direct repeat (Craig and Nash, 1983).

In both the FLP and Cre recombination systems, no accessory sites or accessory proteins are required, although there is a repeat of one half site in the FLP recombination site (FRT) (figure 1.8). There appears to be no selection for a particular arrangement of sites or supercoiling of substrates in either of these systems; the type of intramolecular recombination event is determined by the relative orientation of sites.

Recombination between two directly repeated cer sites of plasmid ColE1 requires at least three host functions. One of these, XerC, has recently been identified as a member of the `Int' family of recombinases from the sequence of its gene (S. Colloms, this laboratory). XerA has now been identified as ArgR, the arginine repressor, and has been shown to bind to sequences within the cer region that are necessary for recombination (Stirling et al, 1988). The third function required for cer recombination is PepA (XerB); the contribution of this amino-peptidase to site-specific recombination is unknown (Stirling et al, 1989).

Both lambda integrase and the FLP recombinase can resolve Holliday structures (Hsu and Landy, 1982; Jayaram et al, 1988). This strongly suggested that integrases have the ability to cleave one strand at a time, first forming a Holliday intermediate and then resolving this structure to give recombinants. The location of the first cleavage step has been determined for lambda integrase, by using

substrates that are either nicked at the cleavage site, or are modified to prevent further strand exchange steps (Nunes-Duby *et al*, 1987; Kitts *et al*, 1988). Int cleaves the top strand of the crossover site at the site adjacent to the P arm.

Lambda integrase recognises sites within the flanking arms of *att*P, but this recognition uses a separate domain of Int (Moitoso de Vargas *et al*, 1988). Models have been proposed that include the ability of an Int subunit to recognise both the crossover site and an arm site within *att*P, and thereby facilitating the wrapping of the site around the enzyme. Accessory proteins are required to bind to *att*P to form an intasome structure that can then attack a naked *att*B site (Richet *et al*, 1988). Supercoiling is a prerequisite for the formation of an intasome structure with *att*P and may facilitate wrapping of the DNA around the accessory proteins.

Objectives

Complex protein-DNA structures are important in several recombination systems e.g. transposon resolution, lambda integration, Mu transposition, etc. and for regulation of transcription. Subsites II and III of *res* are proposed to be a component of a higher order protein-DNA intermediate of recombination. The possible roles of the accessory sites of *res* in the selectivity of the resolvase system are investigated in this thesis.

CHAPTER TWO

MATERIALS AND METHODS

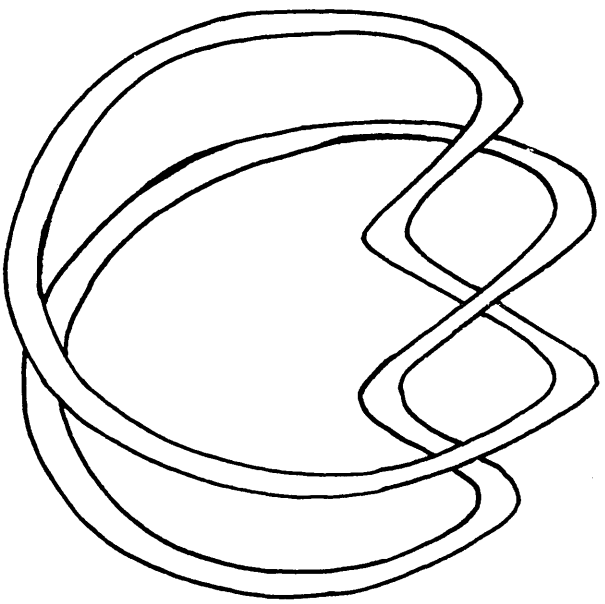


Table 2.1 Bacterial Strains

Strain	Genotype	Source/reference
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	
DS902 (AB2463)	AB1157, but *rec*A13	D. Sherratt
DS941	AB1157, but *rec*F143, *sup*E44, *lac*Z/\M15, *lac*I^q	D. Sherratt
JM101	*sup*E, *thi*, /\(*lac*,*pro*A,B)F', *tra*D36, *pro* A,B, *lac*Z/\M15, *lac*I^q	Yanisch-Perron et al 1985
JC8679 (DS945)	AB1157, but *rec*BC, *sbc*A	D.Sherratt
WA3782 (DS886)	*pin*, *hsd*S, *rec*A (C600 derivative)	T. Bickle
CSH50 (DS887)	*ara*, /*lac*,*pro*, *thi*, *pin*, *rec*$^+$	R. Kahmann
CSH50 fis::Km (DS888)	CSH50, *fis*, *str*, Km^r	R. Kahmann

Table 2.2 **Plasmids**

Plasmid	Size (bp)	Description	Resistance Marker	Source
pBR322	4363	Vector derived from pMB1	ApTc	Sutcliffe 1978
pUC8	2665	Vector derived from pBR322	Ap	Vieri & Messing 1982
pUC18	2686	" " " "	Ap	Yanisch-Perron et al,1985
pMTL23	2505	" " " "	Ap	(CAMR Porton Down)
pMA2350	3326	282 bp *res* PvuII and 358 bp *res* HaeIII in SmaI and HincII pUC18	Ap	M.Boocock
pMA2356	3250	2x 282 bp *res* PvuII in SmaI and HincII pUC18	Ap	M.Boocock
pMA285	2951	resolution product of pMA2356	Ap	M.Boocock
pMA1441	2968	282 bp *res* PvuII pLS138 + SmaI pUC18	Ap	M.Boocock
pMA44	4645	282 bp *res* PvuII pLS138 + PvuII pBR322	ApTc	M.Boocock
pMA21	4927	1065 bp P/H pLS139 + 3862 bp P/H pMA44	ApTc	M.Boocock
pMA2631	4927	2460 bp P/A pMA21 + 2467 bp P/A pMA1961	ApTc	M.Boocock
pMA14	5209	2742 bp P/A pLS138 + 2467 bp P/A pMA44	ApTc	M.Boocock
pMA414	5565	1347 bp P/H pLS138 + 4218 bp P/H pMA11	Ap	M.Boocock
pMA422	5565	1347 bp P/H pLS138 + 4218 bp P/H pMA34	Ap	M.Boocock
pMA19	5283	2816 bp P/A pLS140 + 2467 bp P/A pMA44	Ap	M.Boocock
pMA2615	5565	3098 bp P/A pMA414 + 2467 bp P/A pMA1961	Ap	M.Boocock
pLB30	2947	282 bp *res* RI + RI pUC8	Ap	J.L.Brown
pLB31	2947	as pLB30, but reverse orientation of insert	Ap	J.L.Brown
pBR325:*sis*	(6180)	162 bp *sis* (*gin*) AhaIII + BamHI linkers + BamHI pBR325 (plus filled in site, to make Ap^S)	Cm	R.Kahmann

Table 2.2 continued

pAL30	2958	272 bp *res* SmaI-RsaI pLB30 + HincII pUC18	Ap	Chapter 3
pAL31	2807	121 bp *res* SspI-RsaI pLB31 + HincII pUC18	Ap	Chapter 3
pAL3054	2861	ExoIII deletion of pAL30; subsite I	Ap	Chapter 3
pAL3151	2750	ExoIII deletion of pAL31; subsites II/III	Ap	Chapter 3
pAL3401	2720	34 bp sym-subsite I oligoNT + PstI pUC18	Ap	Chapter 3
pAL214	3092	2 x 203 bp subsite I RI pAL3054 + RI pUC18	Ap	Chapter 3
pAL161	2708	203 bp SspI pAL214 + HincII pMTL23	Ap	Chapter 3
pAL243	2911	406 bp RI partial pAL214 + RI pMTL23	Ap	Chapter 3
pAL195	2579	92 bp subsites II and III RI + RI pMTL23	Ap	Chapter 5
pAL11	4566	203 bp subsite I RI pAL3054 + RI pBR322	ApTc	Chapter 4
pAL15	4566	as pAL11, but reverse orientation of insert	ApTc	Chapter 4
pAL211	4848	2381 bp P/A (I) pAL11 + 2467 bp P/A (wt-*res*) pMA19	ApTc	Chapter 4
pAL215	4848	2381 bp P/A (I) pAL15 + 2467 bp P/A (wt-*res*) pMA19	ApTc	Chapter 4
pAL261	4848	2381 bp P/A (I) pAL11 + 2467 bp P/A (wt-*res*) pMA2615	ApTc	Chapter 4
pAL265	4848	2381 bp P/A (I) pAL15 + 2467 bp P/A (wt-*res*) pMA2615	ApTc	Chapter 4
pAL115	2479	resolution product of pAL215; sym-*res*	Ap	Chapter 4
pAL2115	4958	dimer of pAL115	Ap	Chapter 4
pAL145	2650	145 bp sym-*res* RI pAL115 + RI pMTL23	Ap	Chapter 4
pAL234	4679	34 bp PstI pAL3401 + PstI pMA44	Tc	Chapter 4
pAL12	4705	342 bp subsite I PvuII pAL3054 + PvuII pBR322	ApTc	Chapter 5
pAL13	4705	as pAL12, but reverse orientation of insert	ApTc	Chapter 5
pAL224	4908	2381 bp P/A (I) pAL15 + 2526 bp P/A (I) pAL12	ApTc	Chapter 5
pAL221	4908	2526 bp P/A (I) pAL13 + 2382 bp P/A (I) pAL211	ApTc	Chapter 5
pAL225	4908	2526 bp P/A (I) pAL13 + 2382 bp P/A (I) pAL215	ApTc	Chapter 5
pAL21*sis*	(5107)	1x *sis* (ca. 180 bp) BamHI pBR325*sis* + BamHI pMA21	Ap	Chapter 5
pAL2631*sis*	(5107)	1x *sis* BamHI pBR325*sis* + pMA2631	Ap	Chapter 5
pAL211*sis*	(5208)	2x *sis* BamHI pBR325*sis* + BamHI pAL211	Ap	Chapter 5
pAL261*sis*	(5208)	2x *sis* BamHI pBR325*sis* + BamHI pAL261	Ap	Chapter 5
pAL221*sis*	(5088)	1x *sis* BamHI pBR325*sis* + BamHI pAL221	Ap	Chapter 5
pAL225*sis*	(5088)	1x *sis* BamHI pBR325*sis* + BamHI pAL225	Ap	Chapter 5

Table 2.2 continued

pRM2613	4927	2117 bp RI + 2810 RI partial pMA2631	ApTc	R.McCulloch
pRM313	5234	209 bp (I) PvuII-SphI pAL214 + BamHI (filled in)-SphI pMA14	Ap	R.McCulloch
pRM323	5177	154 bp (II/III) BamHI-SphI pAL195 + BamHI-SphI pMA14	Ap	R.McCulloch
pMS4622	5395	367 bp R46 *res* Hae III and 665 bp R46 *res* RI pMS4611 in PvuII and RI pBR322	ApTc	M.Stark
pCP1420	(3800)	0.8kb Tn21 *res* PstI pEAK9 + PstI pMA1441	Ap	C.Parker
pCIA80	4876	2x 600 bp PvuII-BamHI *res* + 982 bp *cat* BamHI in pUC18	ApCm	P.Haffter
pCIA83	5155	pCIA80 + 372 bp *sis* (cin) PvuII-HindIII	ApCm	P.Haffter
pCIA70	5346	pACYC177 based, p*lac*UV5, Tn3 *tnp*R	Km	P.Haffter
pPAK316	8556	pACYC184 based, Tn3 *res*, *tnp*R	Cm	P.Kitts
pAC:*gin*	(5300)	ca. 1kb *gin* RI + pACYC184 RI	Tc	R.Kahmann
pAL316	(12800)	HindIII pPAK316 + HindIII pAC:*gin*	TcCm	Chapter 5
pAL10	(6200)	RI-H 1375 bp *tnp*R$^+$ pMA6114 + 4.8 kb p*tac*-*lac*I vector	Ap	Chapter 5
pAL3801	2539	34 bp *gix* SstI-RI oligoNT + SstI-RI pMTL23	Ap	Chapter 5
pAL2381	5078	dimer of pAL3801	Ap	Chapter 5
pAL128	2631	92 bp (II/III) RI pAL3151 + RI pAL3801; *ges*	Ap	Chapter 5
pAL2128	5262	dimer of pAL128	Ap	Chapter 5
pAL2195	5500	dimer of pAL3151	Ap	Chapter 5
pAL801	4876	inversion product of pCIA80	ApCm	Chapter 5
pAL831	5155	inversion product of pCIA83	ApCm	Chapter 5
pAL802	4880	NcoI filled in pCIA80	Ap	Chapter 5
pAL832	5159	NcoI filled in pCIA83	Ap	Chapter 5

Abbreviations: P-PstI, H-HindIII, A-AvaI, RI-EcoRI; oligoNT-deoxyoligonucleotide
Sizes in () are approximate.

2.1 **Bacterial strains.** The bacterial strains used were all derivatives of Escherichia coli K-12 and are listed in Table 2.1.

2.2 **Plasmids.** The plasmids used and constructed in this study are listed in Table 2.2.

2.3 **Synthetic oligonucleotides.**

(1) Symmetrical subsite I; a self complementary oligonucleotide (chapter 3):-

5' GTGTCTGATAATTTATAAATTATCAGACACTGCA 3'

(2) gix; two complementing oligonucleotides (chapter 5):-

5' CTTATCCAAAACCTCGGTTTACAGGAAATG 3'

5' AATTCATTTCCTGTAAACCGAGGTTTTGGATAAGAGCT 3'

To hybridise the two DNA strands, up to 50 ug (in 1x TE buffer and 100mM NaCl) of each oligonucleotide were mixed and heated up to 85°C, then allowed to cool slowly.

2.4 **Chemicals.**

CHEMICALS	SOURCE
General chemicals, biochemicals and organic solvents	BDH, May and Baker, Sigma
Media	Difco, Oxoid
Agarose	BRL
X-gal, IPTG	BRL
Radiochemicals	NEN
10x restriction enzyme buffer	BRL, Boehringer Mannheim
Nucleotides	Boehringer Mannheim

2.5 **Proteins.**

Restriction and DNA modification enzymes	BRL, Boehringer Mannheim
Tn3 resolvase	gift from M. Boocock
T4 topoisomerase II	gift from H. Benjamin
FIS	gifts from R. Kahmann and P. Haffter

2.6 **Culture media.**

L-Broth: 10g tryptone, 5g yeast extract, 5g NaCl, made up to 1 litre in distilled water and adjusted to pH 7.5 with NaOH. Supplemented with 2% glucose (from 20% stock solution).

L-Agar: as L-Broth with the addition of 15g/l agar.

4x Davis and Mingioli minimal salts (D & M salts): 28g K_2HPO_4, 8g KH_2PO_4, 1g sodium citrate, 0.4g $MgSO_4.7H_2O$, made up to 1 litre in distilled water.

Minimal agar: 25ml D&M salts, 75ml 2% agar in distilled water; supplemented with 2% glucose and 20ug/ml thiamine (vitamin B1).

2.7 **Sterilisation.** All growth media were sterilised at 120°C for 15 minutes; supplements and buffer solutions at 108°C for 10 minutes and $CaCl_2$ at 114°C for 10 minutes.

2.8 Buffer solutions.

Electrophoresis

10x E buffer: 242g Tris, 82g sodium acetate, 18.6g $Na_2EDTA.2H_2O$, made up to 5 litres in tap water, adjusted to pH 8.2 with glacial acetic acid.

10x TBE buffer: 109g Tris, 55g boric acid, 9.3g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water; pH is 8.3.

10x TBE buffer (sequencing gels): 121.1g tris, 55g boric acid, 9.3g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water; pH is 8.3.

Single colony gel loading buffer: 2% ficoll, 1% SDS, 0.01% bromophenol blue, 0.01% orange G in buffer E.

Polyacrylamide gel loading buffer: 1% ficoll, 0.1% SDS, 0.02% orange G, 0.01% bromophenol blue in distilled water.

4x Horizontal agarose gel loading buffer (K mix): 25% sucrose, 0.2mg/ml protease K, 0.01% bromophenol blue in distilled water.

Restriction and ligation buffers

10x restriction buffers: used the recommended buffers provided with the restriction enzymes, stored at 4°C or over the long term at -20°C.

Ligation buffer: 660mM Tris/HCl pH 7.5, 66mM $MgCl_2$, 100mM DTT. Stored at -20°C.

4mM ATP: 4mM ATP in 4mM Tris/HCl pH 7.5. Stored at -20°C.

1x TE buffer: 10mM Tris/HCl, 1mM EDTA; pH 8.0

1x TE/10 buffer: 10mM Tris/HCl, 0.1mM EDTA; pH 8.0

2.9 **Antibiotics.** The antibiotic concentrations usually used throughout for both liquid and plate selection were as follows:

Antibiotic	Stock solution	Selective concentration
Ampicillin (Ap)	5mg/ml (water)	50ug/ml
Tetracycline (Tc)	1mg/ml (10mM HCl)	10ug/ml
Chloramphenicol (Cm)	2.5mg/ml (ethanol)	25ug/ml
Kanamycin (Km)	5mg/ml (water)	50ug/ml
Streptomycin (Str)	10mg/ml (water)	100ug/ml

All stock solutions were stored at 4°C.
Antibiotics were added to molten agar which was precooled to 55°C.

2.10 **Indicators.** X-gal (5-bromo-4-chloro-3-indolyl-B-galactoside) was used in conjunction with the host strain DS941 and the pUC vectors, providing a screen for plasmids with inserts in the polylinker region. Clones containing inserts were generally white; clones lacking inserts were blue. X-gal (40mg/ml in DMF) was stored at -20°C and added to L-agar to a final concentration of 20ug/ml.

2.11 **Induction.** A 24mg/ml (100mM) stock solution of IPTG in distilled water (stored at -20°C) was diluted to 6ug/ml in agar plates and 60-120ug/ml in broth, for the induction of expression from ptac and plac promoters in plasmid constructs under lacI control (provided on the plasmid or host chromosome).

2.12 **Growth conditions.** Liquid cultures for transformation, DNA preparations or in vivo recombination assays were routinely grown in L-broth at 37°C with vigorous shaking. Growth on both L-agar and minimal plates were used. Antibiotics were used as required. Plates were generally incubated overnight at 37°C.
Bacterial strains were stored in 50% L-broth, 20% glycerol and 1% peptone at -20°C.

2.13 **Plasmid DNA isolation.**

Large scale DNA preparation (Birnboim and Doly, 1976; as modified in this laboratory).

Solutions:

1. 50mM glucose, 25mM Tris/HCl pH 8.0, 10mM EDTA.
2. 0.2M NaOH, 1% SDS; made fresh.
3. 5M potassium acetate pH 4.8; mix equal volumes of 3M CH_3COOK and 2M CH_3COOH, pH will be 4.8.

200ml cultures of stationary phase plasmid containing cells were harvested by centrifugation (12,400g, 10 min at 4°C). The pellet was resuspended in 4ml of solution 1. 8ml of solution 2 was added and the solution left on ice for a further 5 min. 6ml of solution 3 was then added, gently mixed and the cell debris and chromosomal DNA removed by centrifugation (39,200g, 30 min at 4°C). The plasmid DNA was precipitated from the supernatant (containing 10ug/ml RNase A) with 12ml isopropanol for 15 minutes at room temperature. The DNA was pelleted at 27,200g for 15 min at 20°C and was further purified by banding on a CsCl/EtBr gradient. The DNA was resuspended in 2.09ml of TE buffer and added to 270ul of a 15mg/ml ethidium bromide solution. 5g of CsCl were dissolved in 3ml of water and added to the DNA/EtBr solution. The gradients were centrifuged in a Beckman Ti70 fixed angle rotor at 200,000g for 16 hours at 25°C. Where two bands were visible, a lower supercoiled

plasmid band and an upper nicked DNA band, the lower band was removed using a 1ml syringe. The ethidium bromide was removed by repeated butanol extractions and the DNA was dialysed against 2x1 litre of 1x TE buffer to remove the CsCl. The DNA was then ready for use.

Mini preparation of DNA (modified from from Holmes and Quigley, 1981):

STET buffer: 8% sucrose, 5% Triton X-100, 50mM EDTA, 50mM Tris/HCl pH 8.0

10ml of stationary culture was harvested by centrifugation (12.100g, 30sec), resuspended in 700ul STET buffer and transferred to an Eppendorf tube. 50ul of a 10mg/ml lysozyme solution was then added and after mixing, the suspension was then boiled for 45sec and centrifuged in an Eppendorf microfuge for 15 min at 4°C. The pellet was discarded with a toothpick and the DNA containing supernatant was deproteinised by phenol extraction (subsequent chloroform steps were performed to remove trace phenol before precipitation). The DNA was precipitated with an equal volume of isopropanol for 15 min. After microcentrifugation for 15 min, the pellet was washed in 70% ethanol, dried and resuspended in 50ul TE buffer. 5-10ul of this DNA was suitable for restriction enzyme digests and 1ul of a 1mg/ml RNase A solution was included in the gel loading buffer.

2.14 **Transformation with plasmid DNA:** Genetic transformation introduced plasmid DNA into different host strains. An overnight culture of the recipient strain was diluted 1 in 100 into 20ml L-broth and grown to a density of $2x10^8$ cells/ml (about 90 min). The cells were harvested (12,100g, 1min, 4°C) and resuspended in 10ml of 50mM $CaCl_2$. The cells were pelleted again, resuspended in 0.5ml cold (4°C) 50mM $CaCl_2$ and kept on ice for at least 15 min

before being used. 200ul aliquots of competent cells were added to DNA in TE buffer and, after gentle mixing, were left on ice for 15 min. The cells were then heat shocked (2 min, 42°C or 5 min, 37°C) and returned to ice for 15 min. 200ul of L-broth was added to the cell suspension and incubated at 37°C for 90 min to allow expression of plasmid genes. Transformation to ampicillin resistance was given only 15-30 min expression time. 100ul aliquots of the transformation mixture were spread onto selective plates.

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

2.15 **Single colony gel analysis:** By using this technique, the plasmid content of an isolate can be observed without the need to purify the DNA. A single transformant was patched out (1cm square) on a selective plate and grown overnight. Using a toothpick, a large scrape of cells was collected and resuspended in 150ul of single colony gel buffer. The cells were left to lyse at room temperature for 15 minutes. Cell debris and chromosomal DNA was spun down in an Eppendorf microfuge for 15 min at 4°C). 50ul of the supernatant was loaded onto an agarose gel.

2.16 **Ethanol precipitation of DNA.** The DNA solution was made 0.3M NaOAc and 2 volumes of absolute ethanol were added. After mixing, the DNA was precipitated at -20°C for 20 mins and pelleted for 15 min at 4°C. The pellet was washed with 70% ethanol and dried.

2.17 **Restriction of DNA.** Restriction enzyme digests were usually performed in a total volume of 20 ul, containing 0.3-1.0 ug DNA and 2 ul of 10x restriction buffer. 2-3 units/ug DNA of enzyme was added, mixed and the reactions incubated at 37°C (or at the appropriate temperature) for 1-2 hours. For restrictions of larger quantities of DNA,

the volume was scaled up accordingly. The enzymes were inactivated by the addition of loading buffer, heating to 70°C or by phenol extraction and ethanol precipitation if subsequent manipulations were necessary.

2.18 **Calf intestinal phosphatase (CIP) treatment.** Phosphatase was used to remove 5' phosphate groups from linearised vector to prevent recircularisation of the vector (thus increasing cloning efficiency) or from DNA fragments that were required to be 5' end-labelled by the action of T4 kinase. 1 unit of CIP was added directly to the digest and incubated at 37°C for 15 min (or 45 min for blunt ends).

2.19 **Ligation of DNA fragments.** Restriction fragments were ligated in volumes of 20-50 ul, containing 1x ligation buffer, 0.4 mM ATP, and 1 unit/ug DNA T4 DNA ligase. Generally, a 3:1 insert to vector ratio of fragments was used (10:1 for blunt end ligations). The reactions were incubated at 16°C overnight. Aliquots of the ligation mix were used to transform competent cells.

2.20 **Gel electrophoresis.**

Agarose gels: 0.7-1.2% agarose gels were used.
Agarose powder was dissolved at 100°C in 125 or 200ml buffer E and precooled to 55°C prior to use.
Horizontal gels were used to analyse restriction digests, products of recombination and single colony gel analysis. Gels were usually run for 15-18 hours at 1.5V/cm in gel tanks containing 3 litres buffer E and then stained in 0.6ug/ml ethidium bromide. The DNA was visualised on a 254nm wavelength UV transilluminator.

Polyacrylamide gels: three types were used.
1) Polyacylamide restriction gels. A variety of concentrations were used, depending on the sizes of the

fragments of interest:-

Acrylamide (%)	Range of separation (bp)
5.0	80-500
8.0	60-400
12.0	40-200

Vertical gel kits were used with 1.5mm spacers. The gel apparatus was sealed with 0.6% agarose in H_2O. An appropriate acrylamide gel mix was poured between the plates (with the insertion of a well former) and allowed to set for 60 min.

30ml acrylamide gel consists of:-

30% acrylamide: 0.8% bisacrylamide (w/v) X% gel)	X ml (for
10x TBE	3 ml
H_2O	27-X ml
10% APS (w/v)	360 ul
TEMED	18 ul

The gels were run at room temperature in 1x TBE at a constant current (25-30 mA), for 2-3 hours. DNA bands were visualised under 254nm UV illumination after staining in 0.6 ug/ml ethidium bromide for 10 min.

2) Non-denaturing polyacrylamide gels. These gels were used to separate protein:DNA complexes.
5% 10mM Tris/HCl pH 8.2 (10mM Tris/Glycine pH 9.4), 0.1mM EDTA or 6% 50mM Tris/Glycine pH 9.4, 0.1mM EDTA gels were usually used. Vertical gel kits were sealed with 0.6% agarose in the appropriate TE running buffer.

(A) 30ml of a 5% 10mM TE polyacrylamide gel contained:-

30% acrylamide: 0.8% bisacrylamide (w/v)	5 ml
0.5M Tris/HCl pH 8.2 (Tris/Glycine pH 9.4)	0.6 ml
H_2O	26 ml
0.2M EDTA	15 ul

(B) 30 ml of a 6% 50mM TE (pH 9.4) gel mixture contained:

30% acrylamide: 0.8% bisacrylamide (w/v)	6 ml
0.5M Tris/Glycine pH 9.4	3 ml
H_2O	20.5 ml
0.2M EDTA	15 ul

To each gel, the following reagents were added:-

10% APS (w/v)	150 ul
TEMED	15 ul

The gels were prerun in 10mM Tris/HCl, 10mM Tris/Glycine or 50mM Tris/Glycine accordingly, 0.1mM EDTA at 15V/cm (30-90 min; 4°C). After loading, the gels were run for 2-3 hours at 15V/cm, 4°C. When the gel run was complete, if labelled fragments were used, the gel was transferred to filter paper and dried under vacuum. Bands were visualised by autoradiography of a sheet of Kodak S1 film for 1-3 days. Non-radioactive gels were visualised by ethidium bromide staining, as for polyacrylamide restriction gels.

3) Polyacrylamide sequencing gels. Sequencing reactions electrophoresed on a 6% high resolution polyacrylamide/urea gel as described in the `M13 cloning/dideoxy sequencing instruction manual' published by BRL. Gels were prerun for 30 min and run for 2-3 hours at 40W. Samples were denatured prior to loading (100°C, 3 min). After the gel run was complete, the gel was fixed in 10% acetic acid for 30 min, dried under vacuum and autoradiographed.

2.21 **Photography of gels.** After staining in ethidium bromide, gels visualised by 254nm UV illumination were photographed using Polaroid type 67 Land film or using a Pentax 35mm SRL loaded with Ilford HP5 film. Both cameras were fitted with a Kodak Wratten filter No.23A.

2.22 **Extraction of DNA from agarose gels using GENECLEAN.** After staining, the gel was placed on a long wave transilluminator (300-360nm) and the band of interest was excised. The agarose chip was added to 2-3 volumes of `NaI' solution, then heated to 45-55$^{o}$C for a few minutes, until the agarose had completely dissolved. 3ul of the `Glassmilk' suspension was then added, rapidly mixed and placed on ice for 5 min. After a 5 sec spin in the microfuge, the pellet was washed three times with the `NEW' solution, mixing and spinning for 5 sec each time. All traces of the `NEW' solution were carefully removed from the final wash. The DNA was eluted from the glass beads by adding 20ul TE buffer and incubating at 45-55^{o}C for 5 min. After a 30 sec spin, the DNA containing supernatant was removed. This last step was repeated, adding the supernatants together before respinning to remove further traces of the glassmilk. The DNA was now suitable for subsequent manipulations.

2.23 **Electroelution from polyacrylamide gels.** The band of interest was excised from a gel as from agarose gels. The gel slice was sealed in a short piece of dialysis tubing with 150-200ul of TE buffer and placed across a horizontal gel kit containing E buffer. After electrophoresis for 2 hours or more at 10V/cm, the current was reversed for 0.5-2 min. The DNA/TE solution was removed and the DNA precipitated in ethanol.

2.24 **End-labelling of DNA fragments.**

1) 5' end-labelling by T4 kinase.

Isolated DNA fragments (1-50pmol ends) that had been treated with CIP were end-labelled in a volume of 25-50ul containing 50mM Tris/HCl pH 7.6, 10mM $MgCl_2$, 5mM DTT, 5% glycerol, 0.1mM spermidine, T4 kinase (20units) and 10uCi gamma[^{32}P] ATP (3000 Ci/mmole). The reaction was incubated for 1 hour at 37^{o}C, followed by phenol extraction of the DNA before ethanol precipitation. For blunt and 3'

protruding ends, the reaction buffer contained Tris/HCl pH 9.5.

2) 3' end-labelling by the Klenow fragment of DNA polymerase I.

Appropriately resticted DNA or purified DNA fragment were end-labelled by filling in recessed 3' ends with the Klenow fragment of DNA polymerase I. The reaction contained 1-200ug/ml DNA, 50nM unlabelled nucleotides, 10uCi alpha [^{32}P] NTP, restriction buffer (50mM Tris/HCl pH 8.2, 10mM $MgCl_2$, 50mM NaCl) and 1 unit/ug DNA Klenow enzyme. After incubation at 37°C for 30 min, the reaction was stopped by phenol extraction, and the DNA was ethanol precipitated.

2.25 **Exonuclease III deletions** (adapted from Henikoff, 1984). 5-10ug of DNA were digested with suitable enzymes providing a 5' protruding or blunt end for digestion by exonuclease III and a 3' protruding end for protection of the remainder of the plasmid. After ethanol precipitation, the DNA was resuspended in 50ul of Exo III buffer (66mM Tris/HCl pH 8.0, 0.66mM $MgCl_2$). 5ul (65 units/ul) of exonuclease III enzyme (an excess) was added to the DNA. Incubation of *res*$^+$ constructs with the enzyme proceeded at 12°C. At 1 minute time points, 10ul samples were removed and mixed with 30ul Exo III stop buffer (0.2M NaCl, 5mM EDTA) and heated to 70°C for 10 mins (digestion of 10-15 bp/min). After ethanol precipitation, with the addition of 20ug/ml yeast tRNA, the DNA pellet was resuspended in 40ul of S1 buffer (0.28M NaCl, 0.05M NaOAc pH 4.6, 4.5mM $ZnSO_4$) containing ca. 100 units/ml S1 nuclease and incubated at 37°C for 15 mins. The reaction was terminated by the addition of 6ul S1 stop buffer (4M NH_4OAc, 0.1mM EDTA). Portions from each time point were analysed after restriction on a polyacrylamide gel, and the remainder was extracted by phenol and chloroform before ethanol precipitation. Samples from suitable time points were dissolved in 10ul of 20mM Tris/HCl pH 8.0, 7mM

$MgCl_2$ containing 10 units/ml Klenow fragment and 12.5uM of each of the four deoxynucleotides and incubated at 37°C for 4 mins. The samples were directly diluted for ligation with ligation buffer, ATP and T4 DNA ligase, and ligated overnight. Before transformation, the DNA was digested (to remove non-deletants) with XbaI.

2.26 **Plasmid DNA sequencing.** All DNA sequencing reactions were performed on denatured pUC18 and pMTL23 plasmid derivatives using the dideoxy chain termination technique. All template preparations, solutions and reaction conditions were as described in the `Guidelines for quick and simple plasmid sequencing' published by Boehringer Mannheim, with the following exceptions:
DNA was prepared using CsCl gradients.
Working solutions: 0.5mM deoxynucleotides, 2mM dideoxynucleotides
from which four separate reaction mixtures were made:-

Nucleotide mix	A	C	G	T
	ul	ul	ul	ul
dTTP	40	40	40	2
dCTP	40	4	40	40
dGTP	40	40	2	40
ddGTP	0	0	40	0
ddCTP	0	40	0	0
ddATP	10	0	0	0
ddTTP	0	0	0	75

2ul of 2ng/ul universal or reverse primers were used to anneal to the template. Sequencing and chase reactions were incubated at 37°C. After drying the samples, they were resuspended in 6ul formamide-dye mix and denatured by heating to 100°C for 2-3 min prior to loading 2ul aliquots per gel track.

2.27 **In vitro recombination assays.**

1x resolvase dilution buffer: 1M NaCl
50mM Tris/HCl pH 8.2
1mM EDTA

Resolvase dilutions were at 0°C. 1ul or 0.5ul of diluted resolvase was added to 20ul recombination buffer containing ca. 20ug/ml DNA substrate (final NaCl concentration was 50mM or 25mM respectively). Resolvase concentrations (approximate) of 0-500nM were used.

Recombination buffers:-

(A) 50mM Tris/HCl (pH 9.4), 10mM $MgCl_2$, 0.1mM EDTA

(B) 50mM Tris/HCl (pH 8.2), 10mM $MgCl_2$, 0.1mM EDTA, 5mM spermidine.3HCl, 20% glycerol (v/v).

(C) 50mM Tris/HCl (pH 9.4), 10mM $MgCl_2$, 0.1mM EDTA 5mM spermidine.3HCl, 20% glycerol (v/v).

(D) 50mM Tris/Glycine (pH 9.4), 5mM $MgCl_2$, 0.1mM EDTA, 5mM spermidine.3HCl, 20% glycerol (v/v).

(E) 50mM Tris/HCl (pH 9.4), 10mM $MgCl_2$, 0.1mM EDTA 20% glycerol (v/v).

Buffer A is `standard' buffer; B, C, D and E are `permissive'.

Reactions were carried out at 37°C and stopped by heating to 70°C for 5 min. Samples were then treated with DNase I or restricted, modifying buffer conditions where necessary. Reactions were stopped by the addition of K mix loading buffer.

One unit of resolvase was defined as the minimum activity required for 50% resolution of 1ug of a standard substrate (e.g. pMA21) in buffer A for 30 mins at 37°C, assayed as above. 200nM is approximately 8 units of resolvase.

For assays that included FIS, the FIS protein (0.25-4ug/ml: 10 units/ul, 1 unit is 10ng) was added to reactions as above, prior to the addition of resolvase.

2.28 **DNase I nicking of DNA.** DNase I was diluted in 10mM Tris/HCl pH 8.0, 10mM $MgCl_2$, 50% glycerol (v/v) and stored at -20°C. Recombination reaction samples in recombination buffer B or E were treated with 5-100ng/ml DNase I for 10 min at 0°C. 1ug/ml DNase I was used for samples in recombination buffer A and similarly incubated. Reactions were then mixed with K mix loading buffer containing 0.2% SDS.

2.29 **DNA knotting by T4 topoisomerase II.** Supercoiled DNA (e.g. pMA21) at ca. 20ug/ml in topoisomerase II buffer (50mM Tris/HCl pH 7.5, 60mM KCl, 40ug/ml BSA, 10mM $MgCl_2$, 0.1mM EDTA) was prewarmed to 30-32°C. 0.1ul of T4 topoisomerase II per 20ul reaction mix was added, mixed and incubated at 30-32°C for 3 min. The reaction was stopped on ice, and divided into aliquots for nicking by 0.1ug/ml DNase I (10 min, 0°C). Nicking was stopped by the addition of K mix loading buffer containing 0.2% SDS.

2.30 **Relaxation by calf thymus topoisomerase I.** A topoisomer ladder of plasmid DNA was made by relaxing the DNA with topoisomerase I in the presence of varying concentrations of ethidium bromide. 20ug/ml DNA in recombination buffer C containing 0-8ug/ml ethidium bromide was incubated with 2-3 units/ug calf thymus topoisomerase for 1 hour at 37°C. Reactions were stopped by heating to 70°C for 5 min, followed by a phenol extraction and ethanol precipitation. The DNA was redissolved in 1x TE buffer.

2.31 **Two-dimensional gel electrophoresis.** To separate topoisomers containing a cruciform structure from those that do not, a topoisomer ladder was electrophoresed in two dimensions on a 1.2% agarose gel in E buffer. After the first native dimension, the gel was soaked in 25ug/ml chloroquine phosphate for 6 hours, with changes of buffer. The gel was then rotated, with the second dimension

running in the same concentration of chloroquine phosphate. 2D gels were soaked in fresh E buffer for 5-6 hours before staining in 0.6ug/ml ethidium bromide for 45 mins and visualising under UV (254nm) illumination.

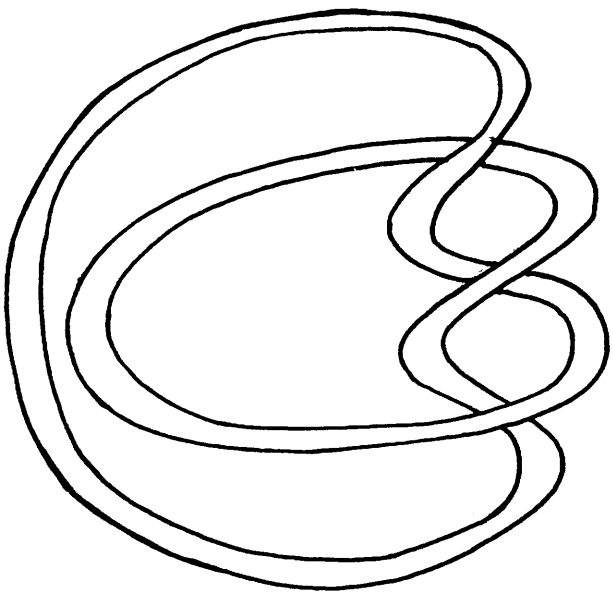
2.32 **Gel binding assays.** Resolvase was diluted as for *in vitro* recombination assays.
Binding buffers:-
(A) 10mM Tris/HCl pH 8.2, 0.1mM EDTA, 10% glycerol (v/v)
(B) 10mM Tris/Glycine pH 9.4, 0.1mM EDTA,
10% glycerol (v/v)

0.4ul of resolvase dilution was added to 10ul containing approximately 1ng end-labelled DNA fragment in binding buffer (with or without 20ug/ml uncut pUC18 or pMTL23 as carrier DNA). Control (blank) reactions contained resolvase dilution buffer only i.e. all binding reactions contained 40mM NaCl, unless indicated. After mixing thoroughly, the reactions were incubated at 37^{o}C for 10 min (or desired time), quenched on ice and loaded onto the gel almost immediately. For reactions with unlabelled DNA, ca. 100ng of fragments were used.

FIS binding assays were carried out in buffer B, as for resolvase. FIS was diluted in 0.6x resolvase dilution buffer; 0.1-50ug/ml FIS protein was added to each reaction.

CHAPTER THREE

GEL RETARDATION OF Tn3 *res*/RESOLVASE COMPLEXES



INTRODUCTION

The binding sites of proteins that interact with DNA can be characterised by examining the effect of nuclease cleavage or chemical modification of the DNA in the presence and absence of the protein. This footprinting technique identified three resolvase recognition sites within an approximately 120 bp region of gamma-delta and Tn3 defined as the functional recombination region *res* (Grindley *et al*, 1982; Kitts *et al*, 1983). Although this has not been done for all the *res* sites found in related transposons and plasmid recombination systems, sequence analysis has indicated three subsites in other *res* regions (figure 1.4). Not all *res* sites have identical spacings between and within subsites, but the general organisation of a crossover site (subsite I) separated from two accessory sites (subsites II and III) is usually conserved, suggesting the functional importance of the three subsites.

The resolvases also share homology at the amino acid level, not only with each other, but also with the related Hin, Gin, Cin and Pin invertases (figure 1.2). All of these related recombinases are believed to recognise their specific DNA sequences by a helix-turn-helix motif in their carboxy-terminal domain (Abdel-Meguid *et al*, 1984).

The invertases, however, act at a simple crossover site, similar to the *res* subsite I (figure 1.5). Resolvase's requirement for subsites II and III is addressed elsewhere in this thesis, but the deletion of these accessory sites severely affects the efficiency of the recombination reaction (P. Kitts, 1982; P. Dyson, 1984; see also chapter 4).

DNA has some degree of structural flexibility. Sequence determinants of bendability have been deduced from studies on nucleosomes (Satchwell *et al*, 1986); AT runs preferentially adopt a compressed minor groove on the inside of a bend, whereas GC runs preferentially become

compressed in the major groove on the inside of a bend. Bendability of the DNA should be distinguished from intrinsic bending, as found in kinetoplast DNA, where phased runs of adenines deform the DNA helix (Wu and Crothers, 1984). Interaction of proteins with DNA can induce a bend, e.g. CAP (Wu and Crothers, 1984), nucleosomes (Drew and Travers, 1985), lambda Xis (Bushman *et al*, 1984), etc. Footprinting techniques can detect changes in DNA as a result of bending, as the narrowing and widening of grooves at a bend alter the accessibility to nuclease and chemical cleavage (Hochschild and Ptashne, 1986; Tullius, 1987). Periodic cleavage between sites was particularly striking when lambda repressor bound two operator sites in the same fragment, where the two sites were separated by 5 or 6 turns of the helix (Hochschild and Ptashne, 1986). This was interpreted as the result of repressor contacting both sites in a loop structure, which was visualised by electron microscopy (Griffith *et al*, 1986). Similar structures have been proposed for the *lac* and *deo* repressors to explain the anomalous retardation of protein:DNA complexes in gel electrophoresis (Kramer *et al*, 1987; Mortensen *et al*,1989). In the case of the *lac* repressor, the loop structure has also been visualised by electron microscopy, and sandwich structures were also suggested to have formed by the repressor tetramers contacting two operator sites on two different molecules.

In non-denaturing polyacrylamide gel electrophoresis, free DNA can be separated from protein bound DNA (Garner and Revzin, 1981; Fried and Crothers, 1981). Bent DNA can be detected by anomalous mobilities of DNA fragments in polyacrylamide gels. The bending locus of protein:DNA complexes can be determined by comparing the gel mobilities of the complexes from a set of circularly permuted fragments which vary only in the position of the bend (Wu and Crothers, 1984). Retardation is most severe when the bend is central to the fragment. Some proteins that bind at specific sites have not been shown to induce

detectable bending by this technique, i.e. lambda repressor (Griffiths *et al*, 1986) and *lac* repressor (Wu and Crothers, 1984; Kramer *et al*, 1987), although recently Zwieb *et al* (1989) have described a gel retardation experiment indicating that *lac* repressor bends a circularly permuted set of *lac* operator fragments.

Our model for synapsis of *res* sites predicts that the DNA wraps around resolvase (figure 1.6). The Tn3 and gamma-delta *res* sites have been extensively footprinted (Grindley *et al*, 1982; Kitts *et al*, 1983). Photofootprinting of *res* on linear and supercoiled molecules suggested no difference in resolvase binding in the different DNA conformations (J.L. Brown, 1986). Subtle changes in the photocleavage pattern suggested that *res* subsites were induced to bend by resolvase, and that probably flexibility in both the DNA and protein dimer allows the enzyme to accomodate the differences in spacing of the subsites.

Other *res* sites which have the same differences in the spacing between and within subsites as for Tn3 *res* i.e. *res* sites of Tn1, gamma-delta, R46 can be recombined by Tn3 resolvase (Reed, 1981a; Kitts *et al*, 1983; Dodd and Bennett, 1987). Tn21 shares the organisation of *res* subsites, but differences in the sizes of subsites and in the crossover sequence appear to block recombination by Tn3 resolvase (Halford *et al*, 1985). However, Tn21 resolvase can footprint subsite II of Tn3 *res*, and gives a somewhat weaker footprint of Tn3 *res* subsite III. Site-directed mutants of Tn21 resolvase have been made to alter the helix motif presumed to contact *res* from the Tn21 to the Tn3 specificity (A.Ackroyd and S.Halford, personal communication). Although the helix swap mutant binds strongly to subsites II and III of Tn3 *res*, still no detectable contacts were made with subsite I. The differences in the sizes of crossover sites and in the central strand cleavage region may be sufficient to prevent the helix swapped Tn21 resolvase from binding

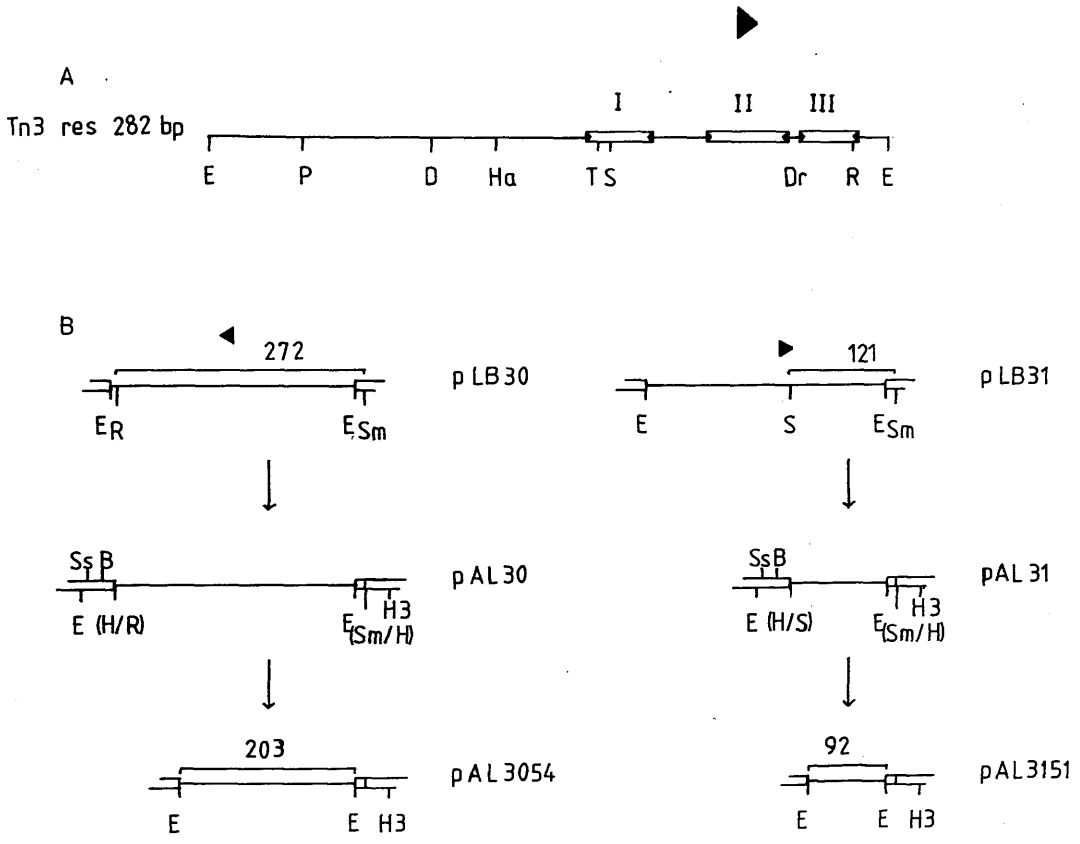
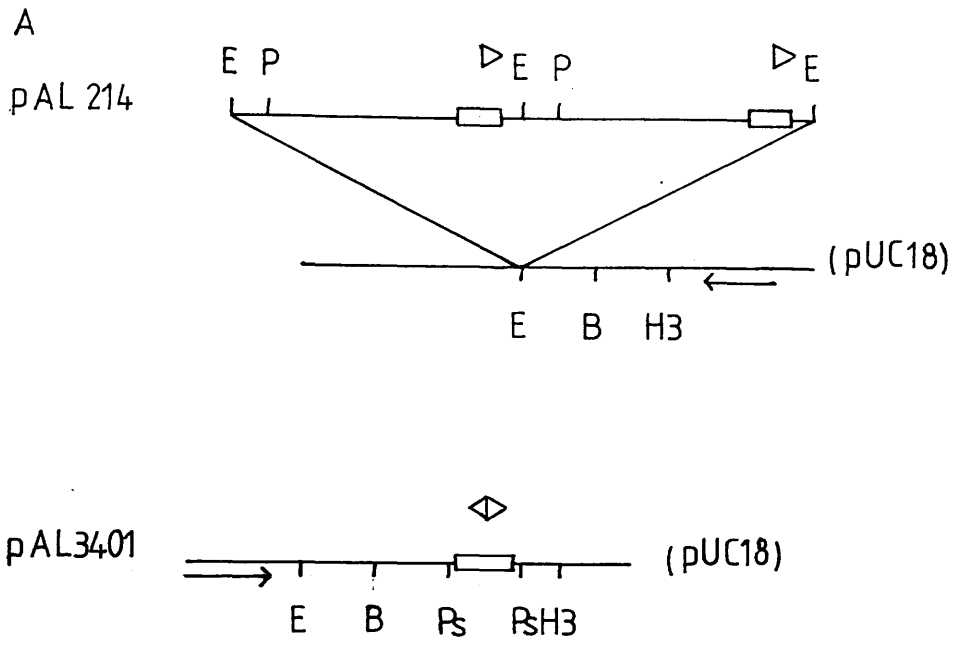


Figure 3.1.
(A) The Tn3 res sequence (282bp EcoRI* fragment). All Tn3 res sequences used in this thesis originated from this EcoRI* fragment: EcoRI sites were formed when the fragment was cloned into pACYC184 (Kitts, 1982).
(B) Dissection of the Tn3 res site.
Schematic diagram showing the strategy for dividing res into subsite I (pAL3054) and subsites II and III (pAL3151). Insertion and orientation of partial res sequences in pUC18 are indicated. Exonuclease III deletion proceeded from BamHI; the remaining plasmid was protected from digestion by cleavage at the SstI site. Non-deletants were removed by XbaI digestion (between BamHI and HincII) prior to transformation of polished and religated deletants.

E=EcoRI, P=PvuII, D=DdeI, Ha=HaeIII, T=TaqI, S=SspI, Dr=DraIII, R=RsaI, Sm=SmaI, B=BamHI, H3=HindIII.



B

(1) Subsite I (pAL214; bottom strand sequenced).

AACACAACTGCAACCGTTCGAAATATTATAAATTATCAGACATAGTAAAACGGCTTCGgaattcgagctc

(2) Subsites II and III (pAL128; top strand sequenced).

II III

gaattcGGCTTCGTTTGAGTGTCCATTAAATCGTCATTTTGGCATAATAGACACATCGTGTCTGATATTCGATTTAAGGTACATTTTTAT

(3) Symmetrical subsite I (pAL3401).

gggatcctctagagtagacctgcagTGTCTGATAATTTATAAATTATCAGACActgcaggcatgcaagc

Figure 3.2.
(A) Constructs of partial *res* sites used for plasmid sequencing. pAL214 is derived from a tandem insertion into pUC18 of the 203bp EcoRI subsite I fragment from pAL3054. pAL3401 contains a symmetrical subsite I (two right halves of Tn3 subsite I) inserted as a synthetic oligonucleotide into the PstI site of pUC18.
(B) DNA sequence of *res* components.
Deletion derivatives containing subsite I (pAL214) and subsites II and III (pAL128; see figure 5.15), were sequenced directly from the plasmid template to determine the deletion end points. Symmetrical subsite I was also plasmid sequenced to confirm the sequence of the synthetic oligonucleotide.

properly to all sites in Tn3 *res*.

The gel binding assay for Tn3 *res*/resolvase interactions was developed as for CAP interactions of Fried and Crothers (1981) i.e. by using TE gel conditions (Brown *et al*, unpublished). Complexes from a set of circular permutation fragments containing the wt-*res* site demonstrated that resolvase can bend a *res* site, which is consistent with our ideas about synapsis. An extension of investigations of *res*/resolvase interactions is presented here, using different components of *res* in the gel retardation assay.

RESULTS AND DISCUSSION

3.1 Dissection of Tn3 *res* into its functional components.

Subsite I of *res* contains the crossover region required for strand exchange. To investigate the functions of the crossover site and the accessory components of *res*, it was necessary to separate subsite I from subsites II and III. Unfortunately, there are no restriction sites between subsites I and II. To dissect *res*, fragments of *res* were cloned into the polylinker of pUC18, whereupon *res* sequence was deleted from the BamHI site by digestion with exonuclease III in both the pAL30 and pAL31 constructs (figure 3.1). Deletions were blocked in the other direction by a 3' protruding end at an SstI site. The deletion products were sized by restriction analysis of EcoRI fragments (figure 3.3; fragments A1 and B1). By sequencing directly from the plasmid template, the deletion end points were determined (figure 3.2).

A second subsite I was made by oligonucleotide synthesis and was composed of two right arms of the Tn3 crossover site. Since the left arm of the wild type crossover site differs from the consensus, we wished to

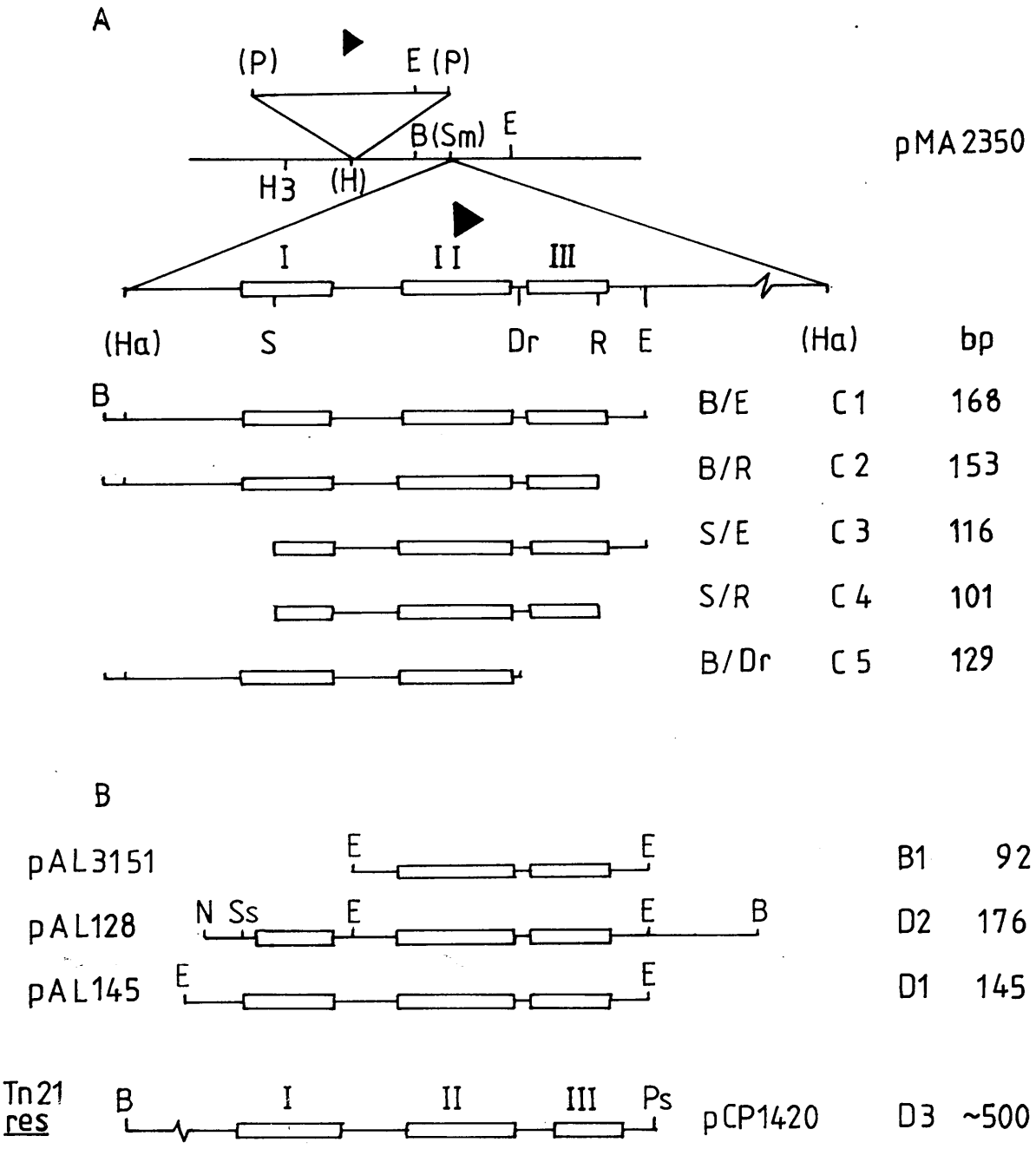
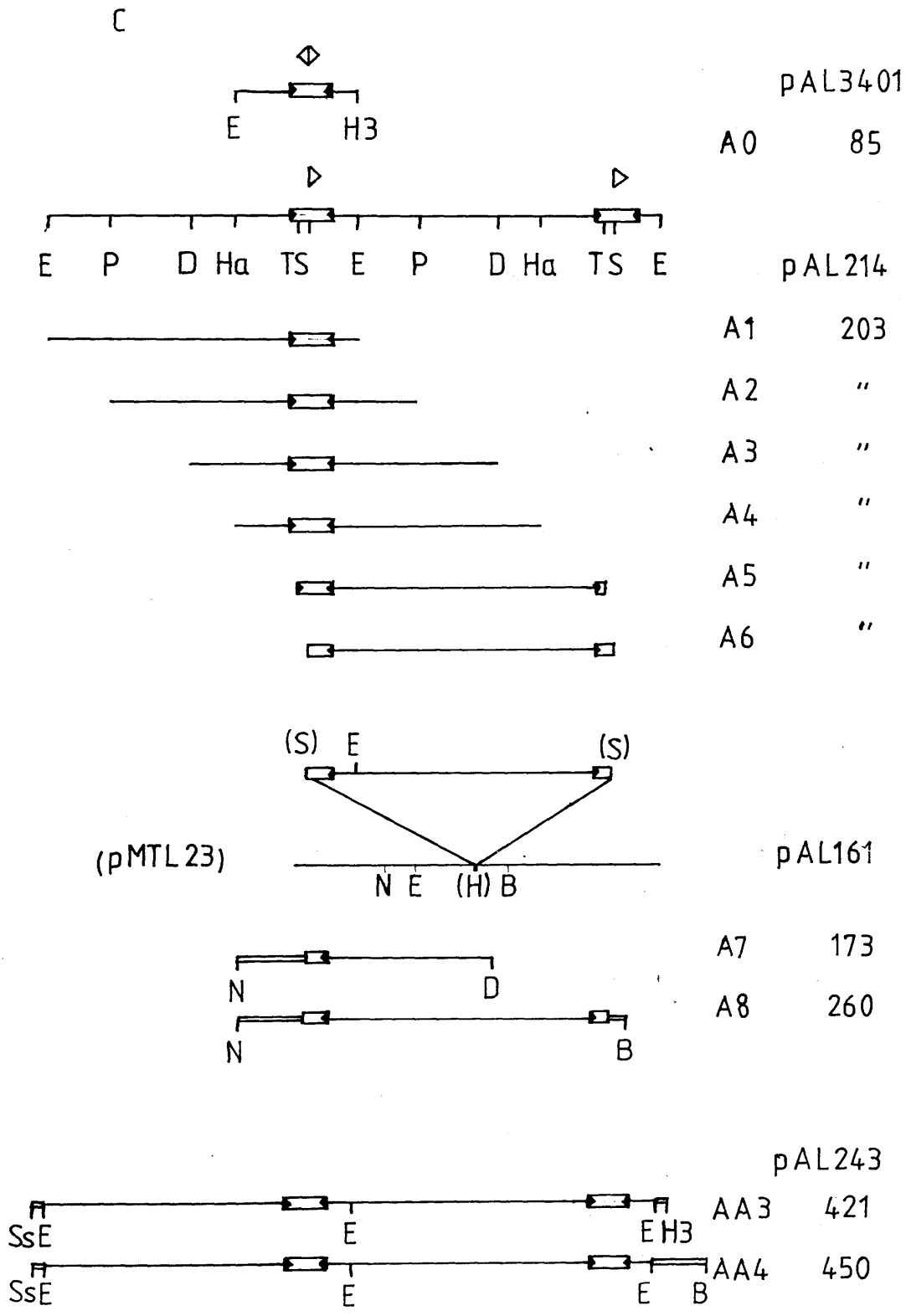


Figure 3.3. DNA fragments (purified and end-labelled) used in the gel binding assay. Restriction enzyme symbols as for figure 3.1; N=NcoI.
(A) Restriction fragments from a wt-res insert of pMA2350.
(B) Restriction fragments containing subsites II and III alone, with gix site (ges; see figure 5.15) or with a symmetrical subsite I (sym-res; see figure 4.7).
(C) Restriction fragments containing an entire or partial subsite I or symmetrical subsite I. pAL161 is derived by insertion of the SspI fragment from pAL214 (A6) into the HincII site of pMTL23. pAL243 is derived by insertion of a partial EcoRI fragment from pAL214 into the EcoRI site of pMTL23.


C
pAL3401
E
H3
A0
85
E P D Ha TS E P D Ha TS E
pAL214
A1
203
A2
"
A3
"
A4
"
A5
"
A6
"
(S) E
(S)
(pMTL23)
pAL161
N E (H) B
A7
173
N
D
A8
260
N
B
pAL243
AA3
421
SsE
E
EH3
AA4
450
SsE
E
E B

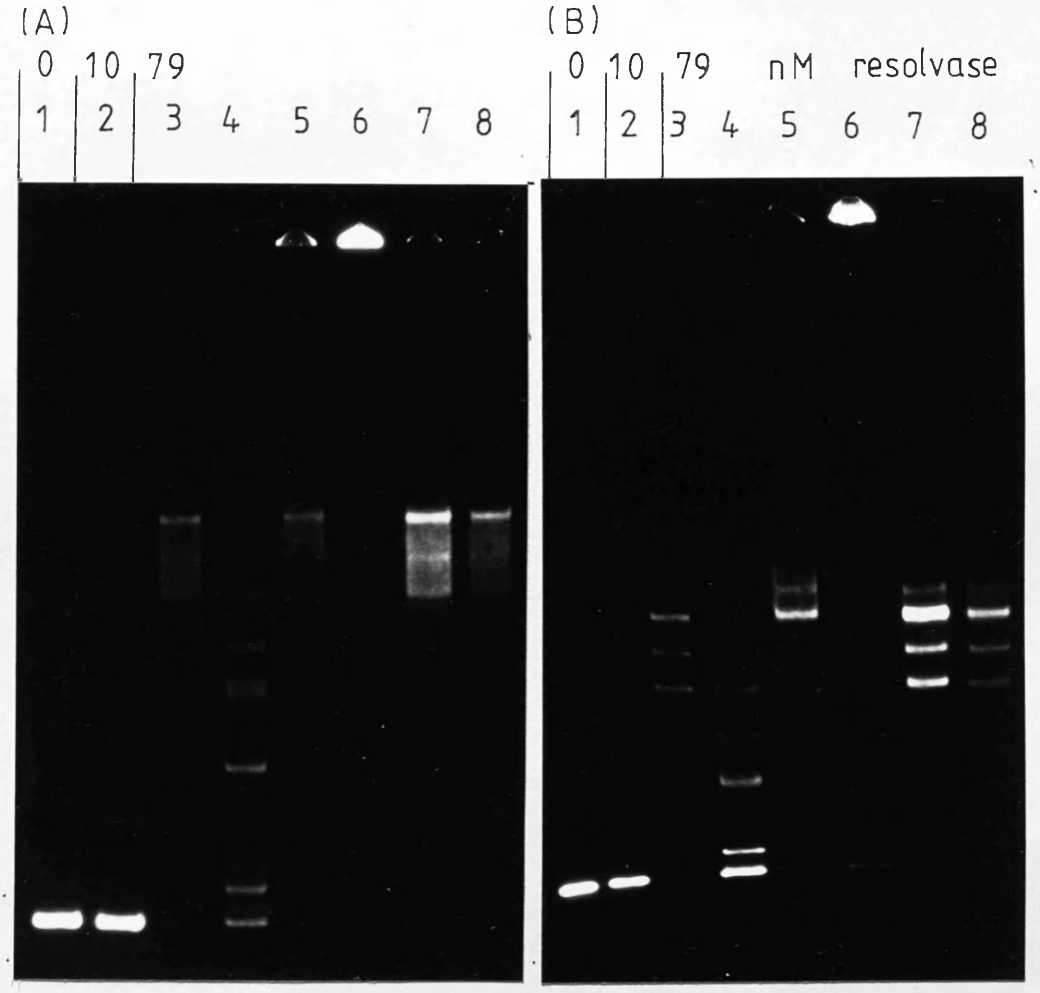


Figure 3.4 The effect of different reaction and gel conditions on resolvase binding to a wt-res fragment (C1). A wt-res fragment (purified and end-labelled) was incubated at 37°C for 10 min, with the indicated concentrations of purified Tn3 resolvase (i.e. 40mM NaCl) under binding conditions B (Tris/Glycine pH 9.4) including carrier DNA, except for the following alterations:-

Lane 4 - incubated at 0°C
5 - + 5mM $MgCl_2$
6 - no carrier DNA
7 - binding buffer A (Tris/HCl pH 8.2)
8 - 20mM NaCl

Gel (A) was 10mM Tris/Glycine, 0.1mM EDTA; gel (B) was 10mM Tris/HCl pH8.2, 0.1mM EDTA. Both gels were 5% polyacrylamide, and electrophoresis was carried out at 4°C.

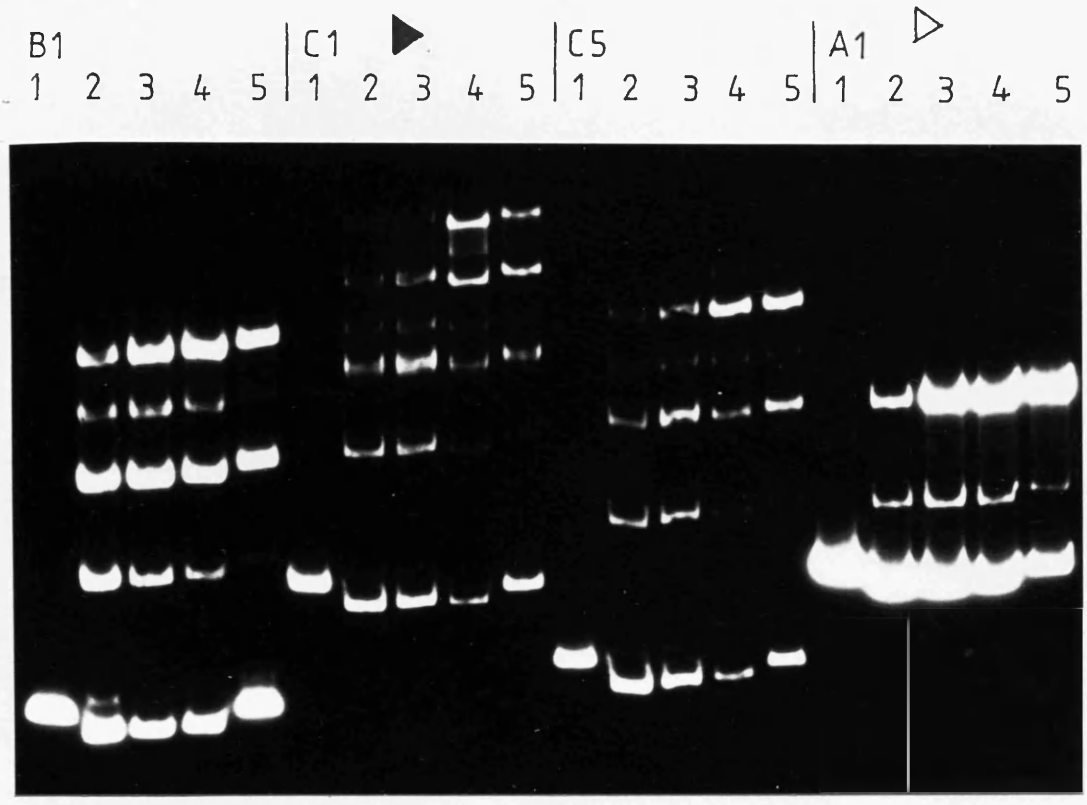


Figure 3.5 Time courses of resolvase binding reactions with <u>res</u> fragments. Purified, end-labelled fragments were incubated at 37°C in buffer B (Tris/Glycine pH 9.4) with 56nM Tn3 resolvase for the times indicated. 6% gel, conditions B (50mM Tris/Glycine pH 9.4).

Lane	Time
1	0 (0nM resolvase)
2	20s
3	1 min 15s
4	4 min
5	18 min

investigate whether the symmetrical subsite I has altered resolvase affinity and recombination properties from the wild type. The self complementary oligonucleotide was cloned into the PstI site of the pUC18 polylinker, from whence its sequence was also confirmed by plasmid sequencing (figure 3.2).

3.2 Resolvase binding to components of *res*.

Figure 3.3 lists a series of restriction fragments from a wt-*res* site and the deleted *res* sites that were isolated and end-labelled for the gel binding assay. When resolvase was bound to the wt-*res* fragment (C1), a pattern of six discrete protein-DNA complexes was obtained, as previously seen (Brown *et al*, unpublished); the sixth complex was the major product (figures 3.4, 3.5 & 3.6). In some of the assays that follow, the binding conditions were identical to previous gel assays. However, by raising the pH from 8.2 to 9.4 (the pH of many of our recombination buffers), the complexes formed with wt-*res* ran as sharper bands (figure 3.4), and were presumably more stable in these conditions. Therefore, many of the gel binding assays shown here were done at the higher pH, as indicated in the figure legends.

Binding resolvase to a fragment containing subsites II and III gave a pattern of four protein-DNA complexes (figure 3.5). An isolated subsite I gave two protein-DNA complexes (figure 3.6). In each case the final complex was the major form. It appears that two separable complexes can be formed for each subsite present in the DNA fragment. If binding to any one site gives one complex, any two sites gives a second complex and all three gives a third complex, then three complexes would be expected for three subsites (figure 3.7A). Why are six complexes seen for wt-*res* and not three? Three complexes were apparently obtained for gamma-delta resolvase binding its

res site (Hatfull and Grindley, 1986). The gamma-delta resolvase was shown to occupy both subsites I and II of partially methylated DNA in the first of the three complexes (Falvey and Grindley, 1988). The interpretation of the footprint pattern of the first complex indicated that the resolvase dimer contacts either subsites I or II, and that subsite III has a lower affinity for resolvase. A possible explanation is a `shuffling' of resolvase between the two subsites to generate a time-average structure captured in the gel as a single retarded complex. Shuffling has also been proposed as an explanation for IHF occupying three binding sites within an IS1 end and its hotsite within pBR322 forming three separable complexes (Prentki *et al*, 1987). It is unlikely that gamma-delta resolvase is transferring between subsites I and II, as the DNA fragment of the first complex still had a partial interference pattern within each subsite. This suggests that a mixture of complexes were isolated in which resolvase occupied one or other of the subsites, but could not transfer between them.

Since more than three complexes were observed for a wt-*res* fragment, the possibility of dimers of resolvase randomly occupying each subsite and shuffling between them, as in figure 3.7A, is ruled out. If no shuffling between subsites occurred (i.e. resolvase was fixed at each site once it had bound there), then seven discrete complexes would be expected (figure 3.7B). Although it is possible that more than one complex would migrate to the same position, any such complexes should be separated when the positions of sites within a fragment are altered, thus changing the bend position and retardation of the complexes. When resolvase bound to circularly permuted fragments of wt-*res*, six complexes were always obtained (Brown et al, unpublished). Random occupation of sites, as in figure 3.7B, would be expected to give three complexes for two subsites, and one complex for a single subsite. In the experiment with one or two subsites, two and four

complexes were obtained respectively. Therefore a non-shuffling random order of resolvase occupying res is also not a suitable explanation of the data.

Alternative possible explanations arise when resolvase occupying each subsite in more than one step is considered. Random occupation of each half-site in wt-res could generate 63 different complexes, which is clearly a vast excess over the number of complexes actually separated. However, if each half-site was randomly occupied, and if all complexes with the same total number of half-sites occupied migrated at the same position (e.g. if resolvase shuffles between sites), the number of complexes expected would be the same as the number of half-sites (figure 3.7C). This model is consistent with resolvase binding to give two complexes per subsite (six for three subsites, four for two, amd two complexes for one subsite).

Resolvase would be expected to bind in two steps per subsite, if the subsites are always occupied in order, giving six complexes for wt-res (figure 3.7D). The bend centres for the circularly permuted fragments of wt-res suggested that subsite II is bound first, then subsite III, and lastly, subsite I. In this explanation for the binding pattern of res, resolvase would not occupy each of the half-sites separately, or the number of complexes would increase to nine for wt-res (all of the complexes drawn in figure 3.7D). This suggests that resolvase either shuffles between half-sites or contacts both half-sites simultaneously in the first step of binding a subsite. Further experimentation with a single subsite to investigate a half-site binding resolvase is outlined below. Ordered binding to two subsites within a fragment should still give four complexes. To check whether each pair of complexes is a result of an independent occupation of a subsite, the individual complexes of modified DNA fragments bound by resolvase should be isolated to reveal which subsites are protected by the protein in which

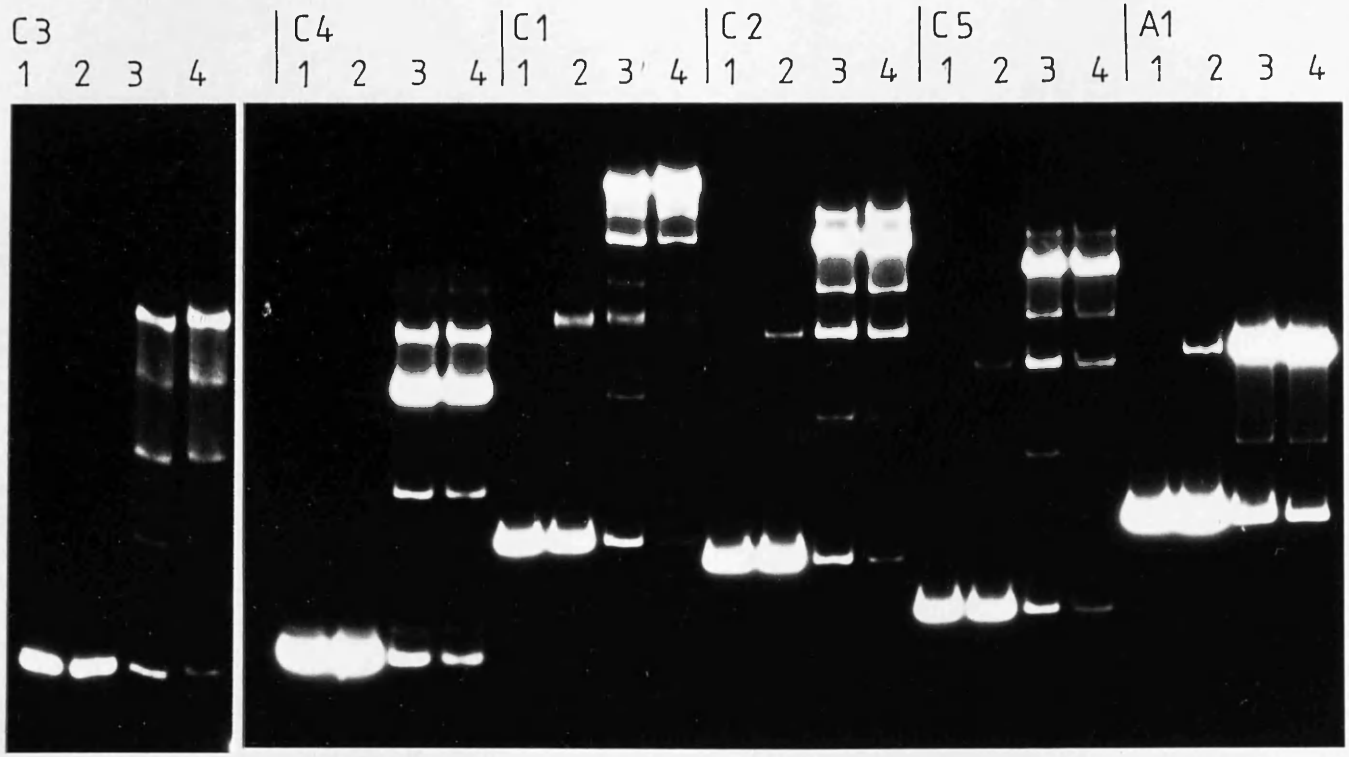


Figure 3.6 Titrations of *res* fragments with Tn3 resolvase. Reactions were incubated at 37°C in binding buffer B with the indicated concentrations of resolvase. Complexes for each fragment are indicated. 6% gel, conditions B.

Lane	nM resolvase
1	0
2	28
3	56
4	112

complex. Any transfer of resolvase between subsites would protect more than one subsite in all the complexes.

Further DNA fragments were made by restriction at sites within *res*, such that subsites I and III were either partially or totally removed (figure 3.3). When only 3 bp were removed from subsite III (fragment C2), five complexes were seen, but the major species was complex 4 (figure 3.6). A similar pattern of complexes was observed when the whole of subsite III was removed (C5). In neither case did the fifth complex become the major bound form, and the complexes observed presumably consisted of protein bound to subsites I and II only. Complex 5 is probably not the result of resolvase binding to the partial subsite III, since a similar extra complex was formed in the absence of any subsite III sequence. When resolvase was bound to a fragment containing only a partial subsite I adjacent to subsites II and III (C3), four complexes were seen, and complex 4 was again the major species. The simplest interpretation of this pattern is that only intact subsites (i.e. subsites II and III) were occupied by resolvase, to give a similar pattern to when a fragment contained subsites II and III alone (B1; figure 3.5). In these partial *res* sequences, subsite II may still be binding resolvase first, as the mobility shift from unbound DNA to complex 1 and 2 was large, and consistent with the idea that subsite II is substantially bent by the protein, as this is the longest of the subsites. Resolvase may be binding subsite II first because of its position within the fragment, preferring to bind a more centrally located site. Also, the gel assay may not be reflecting the distribution of complexes formed in solution, as the different subsites may have altered affinities for the protein during electrophoresis through the gel.

When both subsites I and III were partially deleted (C4), an unusual pattern of resolvase-mediated complexes resulted (figure 3.6). Although the major form was the second complex, as expected for one intact site, a third

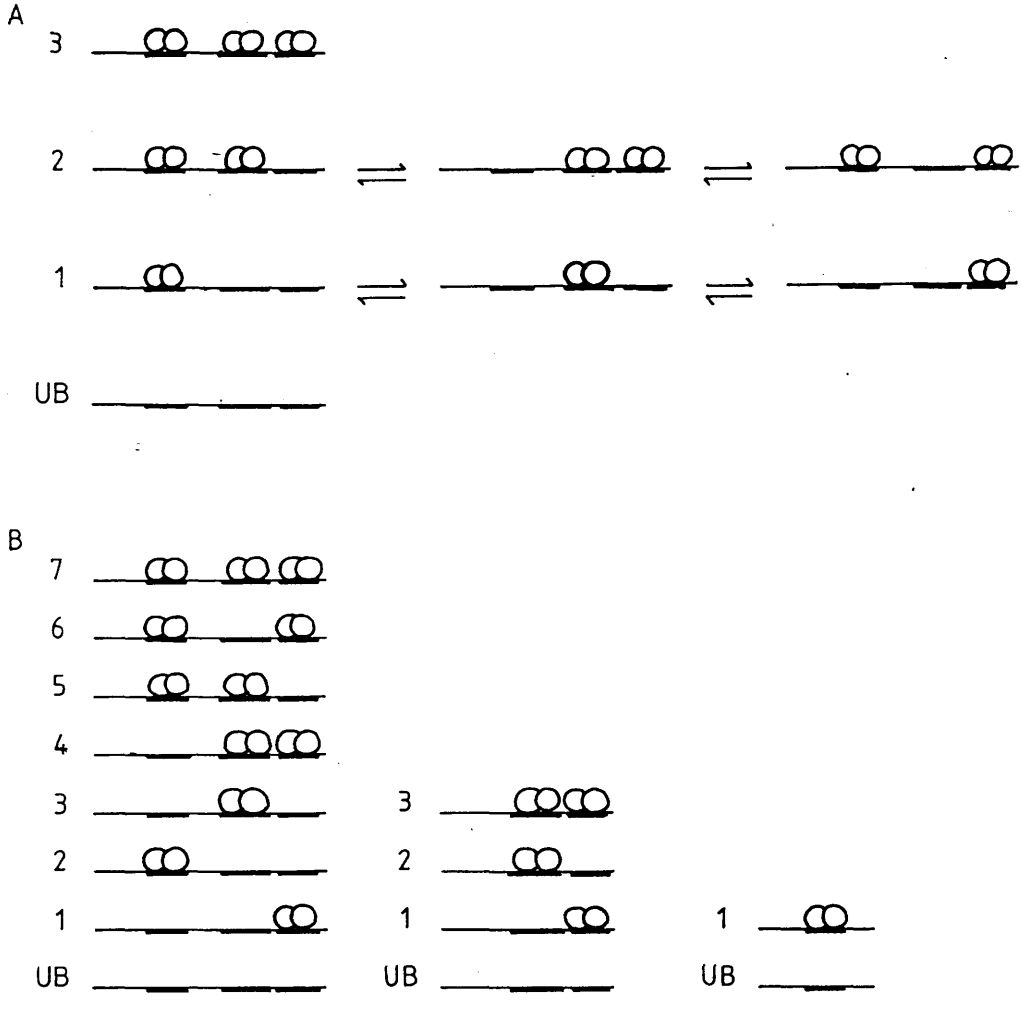
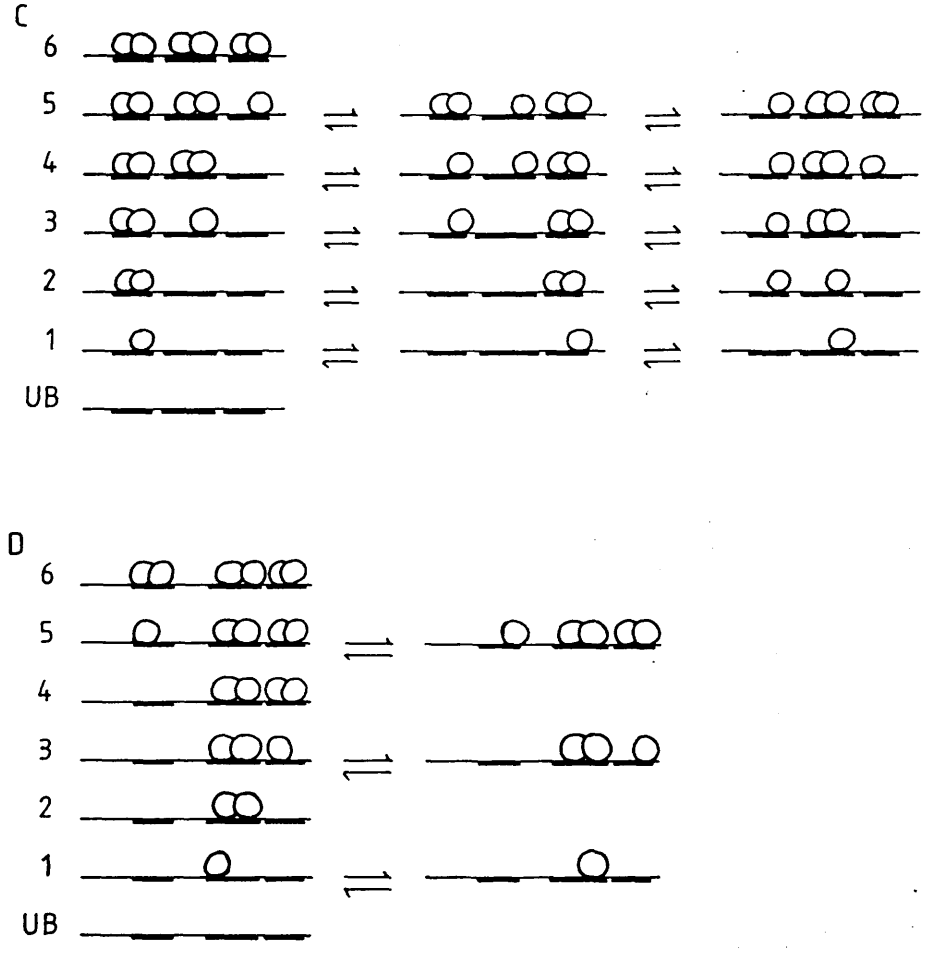


Figure 3.7 Schematic representation of different possibilities for *res* site occupation by resolvase.
(A) Random occupation, one step per subsite, with shuffling between subsites. Separation of 3 complexes from wt-*res* would be expected.
(B) Random occupation, one step per subsite, but no shuffling. 7 complexes would be expected for wt-*res*; 3 for two subsites and only one complex for a single subsite.



(C) Random occupation, two steps per subsite, with shuffling between subsites.
(D) Ordered occupation in two steps per subsites.
In possibilities (C) and (D), half-site occupation is generalised. Suggestions for resolvase occupying a half-site is are shown in figure 3.9.

complex was prominent and two further faint complexes were observed. The third complex suggests that once the only intact subsite is occupied (subsite II), one of the other partial subsites also binds resolvase. It is possible that resolvase cannot bind a single partial subsite to form a stable complex, but when a second partial subsite is present, this may allow a dimer to contact both partial subsites to stabilise a complex. This could be tested by constructing a fragment carrying two partial subsites. Fragments from the circular permutation of subsite I (figure 3.3; and see below) were not suitable substrates for this experiment, as the left half of the site contained only 5 or 9 bp in the TaqI and SspI fragments respectively.

The fragment in which 3 bp of subsite III was removed by RsaI restriction gave a fourth major complex. Previous data for the RsaI circularly permuted fragment of wt-*res* also revealed a four-complex pattern, but the major species was complex 2 and not 4. Since the binding and gel conditions were altered by raising the pH, sharper, and presumably more stable, complexes were observed. Removing 3 bp from the end of subsite III now resulted in complex 4 being the major species, suggesting that binding at subsite I is stable. The results indicate that resolvase will bind any combination of subsites within a fragment until all intact sites are bound, but do not provide any support for the idea that resolvase must bind subsites in a particular order.

3.3 Resolvase binds and bends an individual *res* subsite.

The affinity of and the rate of resolvase binding to subsite I, as determined by a resolvase `titration' and a time-course experiment, was not reduced when subsite I was separated from subsites II and III (figures 3.5 & 3.6). Therefore, binding of resolvase at subsite I in the wt-*res*

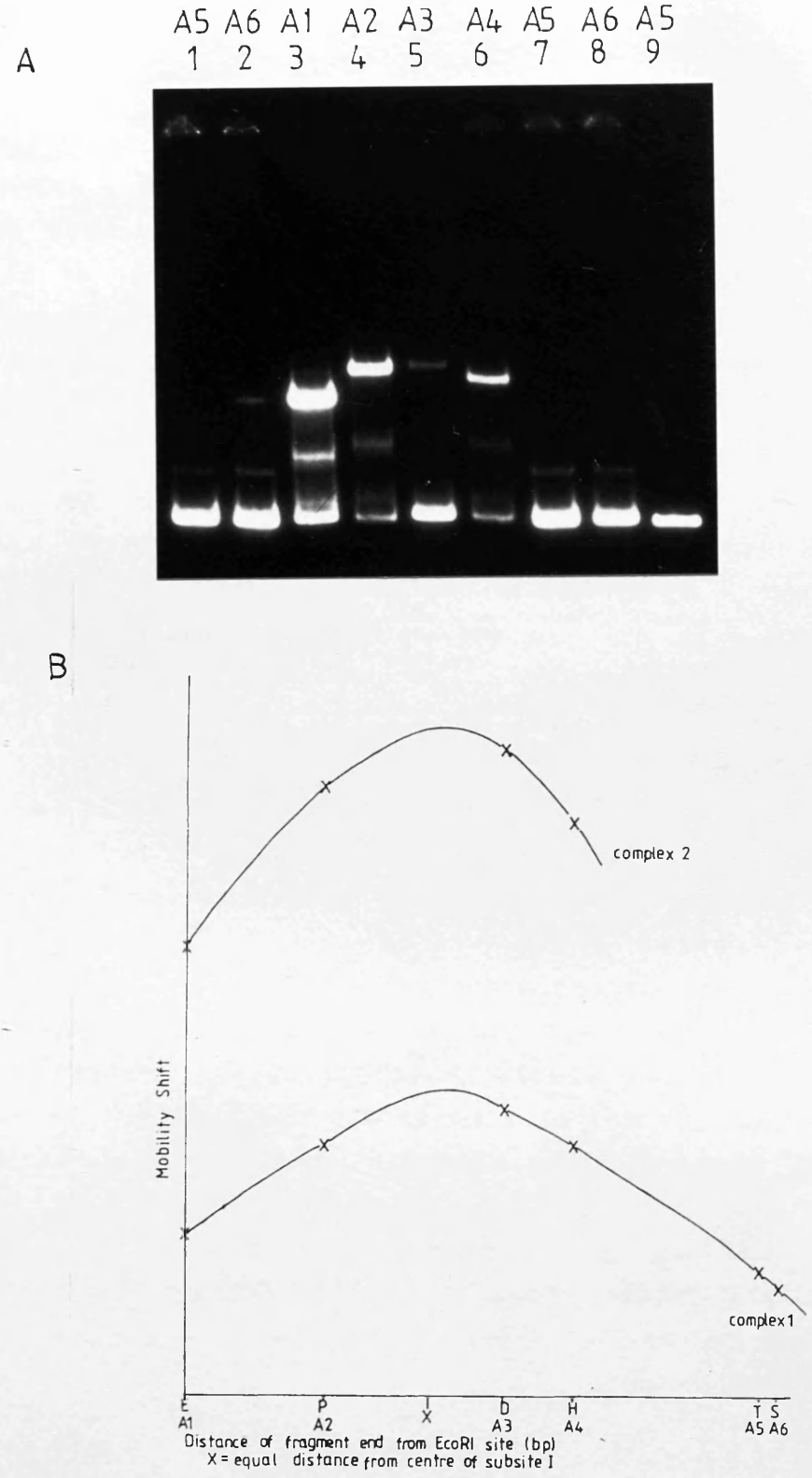


Figure 3.8 (A) Circular permutation of the wt-<u>res</u> subsite I. End-labelled fragments (see figure 3.3) were incubated in buffer A with 39nM purified resolvase (except lane 9, OnM resolvase) and electrophoresed on a 5% polyacrylamide gel in conditions A.
(B) Mobilities of protein-DNA complexes are plotted against the restriction site position of each fragment.

fragment was not a result of cooperative binding by the three *res* subsites. If the stepwise binding has a defined or preferred order, there should be some difference between the *res* subsites. A competition experiment of resolvase binding to fragments of the three different isolated subsites may show the same preferential order of appearance of the protein-DNA complexes for each subsite as in the wt-*res* fragment. This would test the idea that resolvase can distinguish between the three subsites in the gel assay, and would help determine if the location of subsite II within *res* is important.

Resolvase-induced bending at an isolated subsite I was investigated by using a set of 203 bp circularly permuted fragments (figure 3.3), identical in sequence composition and differing only in the location of subsite I, made from a tandem repeat of the subsite I fragment (pAL214). The degree of retardation of complexes formed in the gel assay depended on the position of the subsite (figure 3.8A). Fragments containing intact subsite I were incorporated into two distinct complexes, as previously seen for this isolated subsite. However, when the circular permutation of the 203 bp fragment disrupted the binding site, binding of resolvase gave only one retarded complex. By plotting the gel mobility of both complexes versus the position of the subsite within the fragment, it was found that the lowest mobility occured when the subsite was approximately at the centre of the fragment. The inferred bend centre for the second complex was near to the centre of the subsite I, lying between the centre and one or two base pairs to the right of the centre. For the first complex, the bend centre was further towards the right end of the subsite (figure 3.8B). Retardation of a partial subsite I complex suggested that resolvase binding to an isolated half-site can induce a similar bend to a complex 1 of an intact subsite and thus resolvase contact with both ends of a subsite is not required to induce a bend at a half-site.

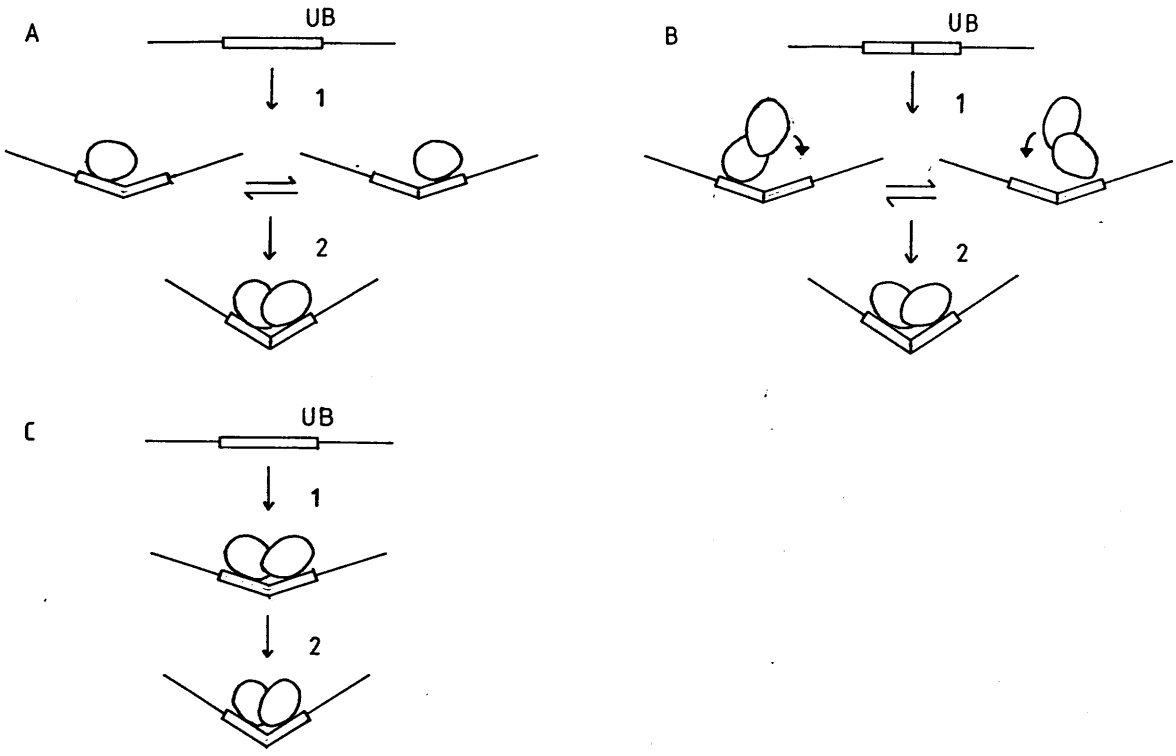


Figure 3.9 Schematic representation of resolvase binding a single subsite of <u>res</u> in two steps.
(A) Binding to each half-site as a monomer, with shuffling between half-sites.
(B) Binding to each half-site as a dimer and shuffling.
(C) Binding to the whole subsite as a dimer, but bending the DNA in two steps.

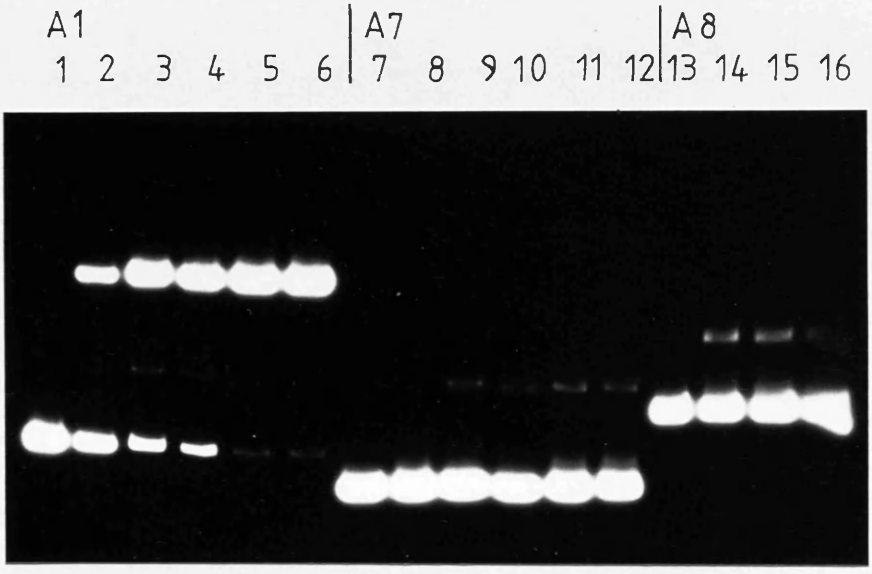


Figure 3.10 Titrations of intact and partial subsite I fragments with resolvase. Purified, end-labelled fragments were incubated in binding buffer B with resolvase for 10 min at 37°C. 6% gel, conditions B.

Lanes	nM resolvase
1, 7 + 13	0
2 + 8	28
3 + 9	56
4, 10 + 14	112
5, 11 + 15	224
6, 12 + 16	448

Application of the gel binding assay to a fragment containing an isolated subsite I of Tn3 *res* showed that Tn3 resolvase is capable of bending this individual subsite. This is in agreement with data for gamma-delta *res* subsites, each of which has been shown to have a resolvase-induced bend (Salvo and Grindley, 1988). Gamma-delta resolvase is proposed to kink the DNA within subsite I (Hatfull *et al*, 1987). In the related Gin invertase system, Gin has also been shown to bend the *gix* crossover site (Mertens *et al*, 1988).

3.4 Characterisation of complexes with subsite I.

The titration of *res* fragments with resolvase (figure 3.6) revealed a steep sigmoidal increase in the occupation of the binding sites over only a four-fold increase in resolvase concentration. Also, binding to form complexes was rapid; stable complexes were captured after incubation for one minute and the major species for each fragment predominated after four minutes (figure 3.5). Assuming that each pair of complexes is a result of resolvase occupying a single site in order, the initial complex for each pair (i.e. complexes 1,3 and 5 for wt-*res*) did not build up at subsaturating resolvase. At higher resolvase concentrations, or later time points, the levels of these complexes went down (figures 3.5 & 3.6). In some cases, this may have been due to a dissociation to the unbound form. Alternatively, the initial complex may be converted to the final bound form.

It is conceivable that dimers of resolvase may contact each half of the subsite independently, and that the initial complex represents binding to one half-site and the final complex represents binding to both halves (figure 3.9B). The observation that only a single complex is formed from the partial subsite I fragments (A5 and A6) is consistent with this idea. Incubating a partial subsite

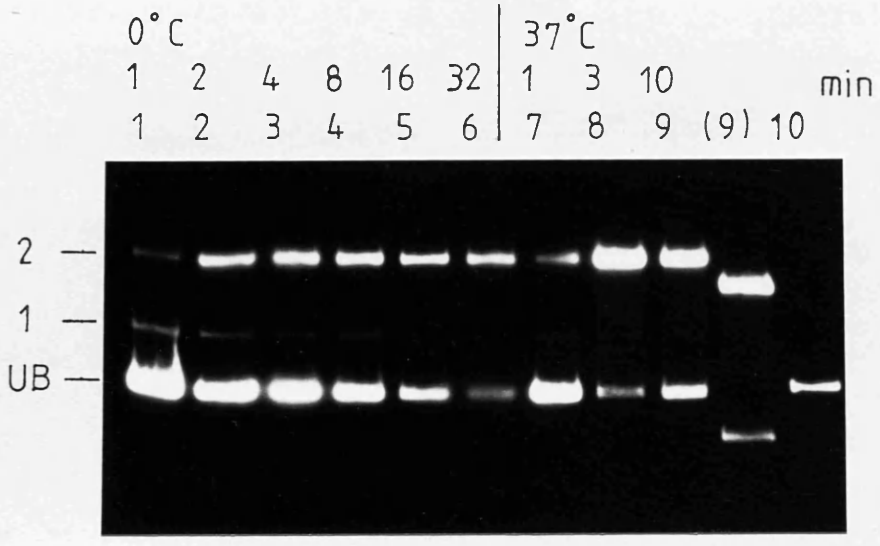


Figure 3.11 Temperature effect on binding subsite I. Incubations of a subsite I end-labelled fragment (A3) with 20nM resolvase (except lane 10, 0nM resolvase) in binding buffer A at 37°C or 0°C for the times indicated. 5% gel, conditions A.

I fragment with increasing resolvase did not result in an increase in observable levels of the protein-DNA complex (figure 3.10). If resolvase is contacting each half of a subsite as a dimer, the close proximity of the remaining free subunit of the dimer might allow rapid contact with the second half of the subsite (figure 3.9B). To test this hypothesis, the circularly permuted SspI fragment from pAL214 was subcloned into the pMTL23 vector to substitute the left end of subsite I with non-res sequence (figure 3.3). The availability of extra DNA extending the partial subsite I might be expected to induce the second half of a resolvase dimer to contact this non-res sequence and alter the mobility of the complex. However, no extra complexes were observed for such fragments (figure 3.10). The formation of a single complex for the partial subsite in the circular permutation experiment was not an effect of the site being located at the end of a fragment, as the same site internal to a fragment also showed a single complex. It is possible that any complex formed by resolvase contacting non-res sequence adjacent to the partial subsite I would be unstable in the gel assay. Resolvase monomer binding provides an alternative explanation for the single complex of a partial res subsite (figure 3.9A).

Retention of intermediate complexes for a resolvase bound wt-res fragment is observed when binding reactions are incubated at 0°C (Brown et al, unpublished). Slightly increased levels of the initial complex of an isolated subsite I fragment were also observed when the reactions were incubated on ice for a short period (figure 3.11). Even then, this complex was rapidly converted to some other form.

A more extensive investigation of complexes of subsite I was conducted to follow their behaviour with a combination of different resolvase concentrations and incubation times (figure 3.12). Again, at 0°C, the conversion to fully bound form was slow. At the higher

(A)

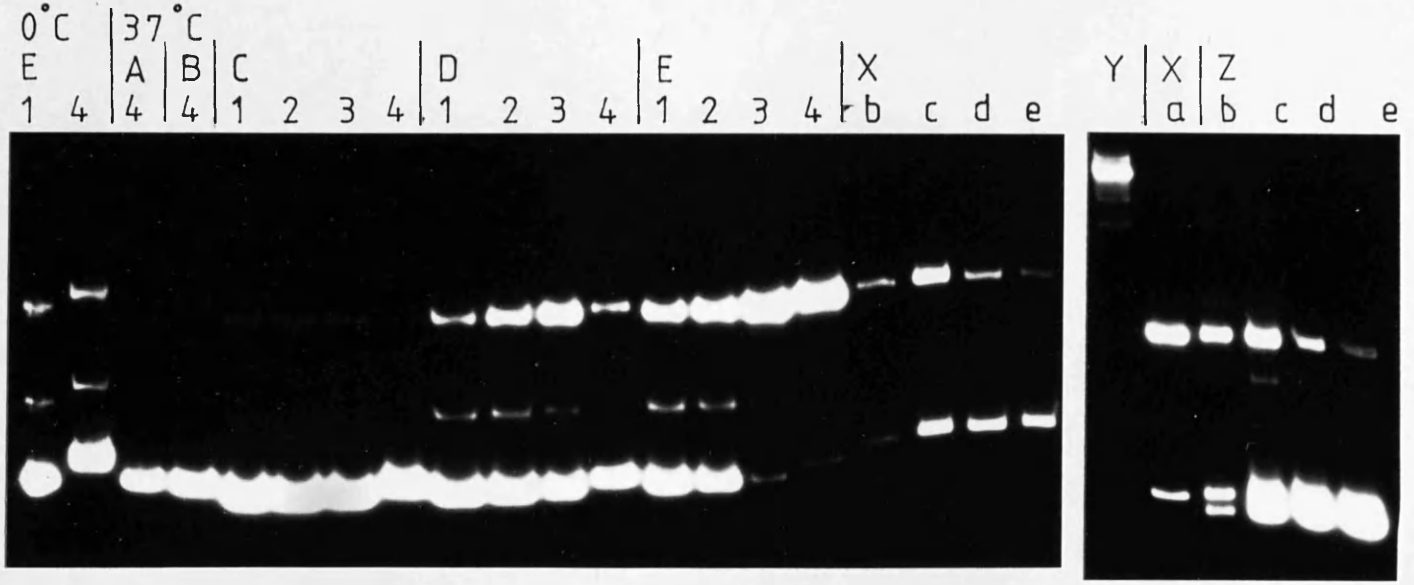
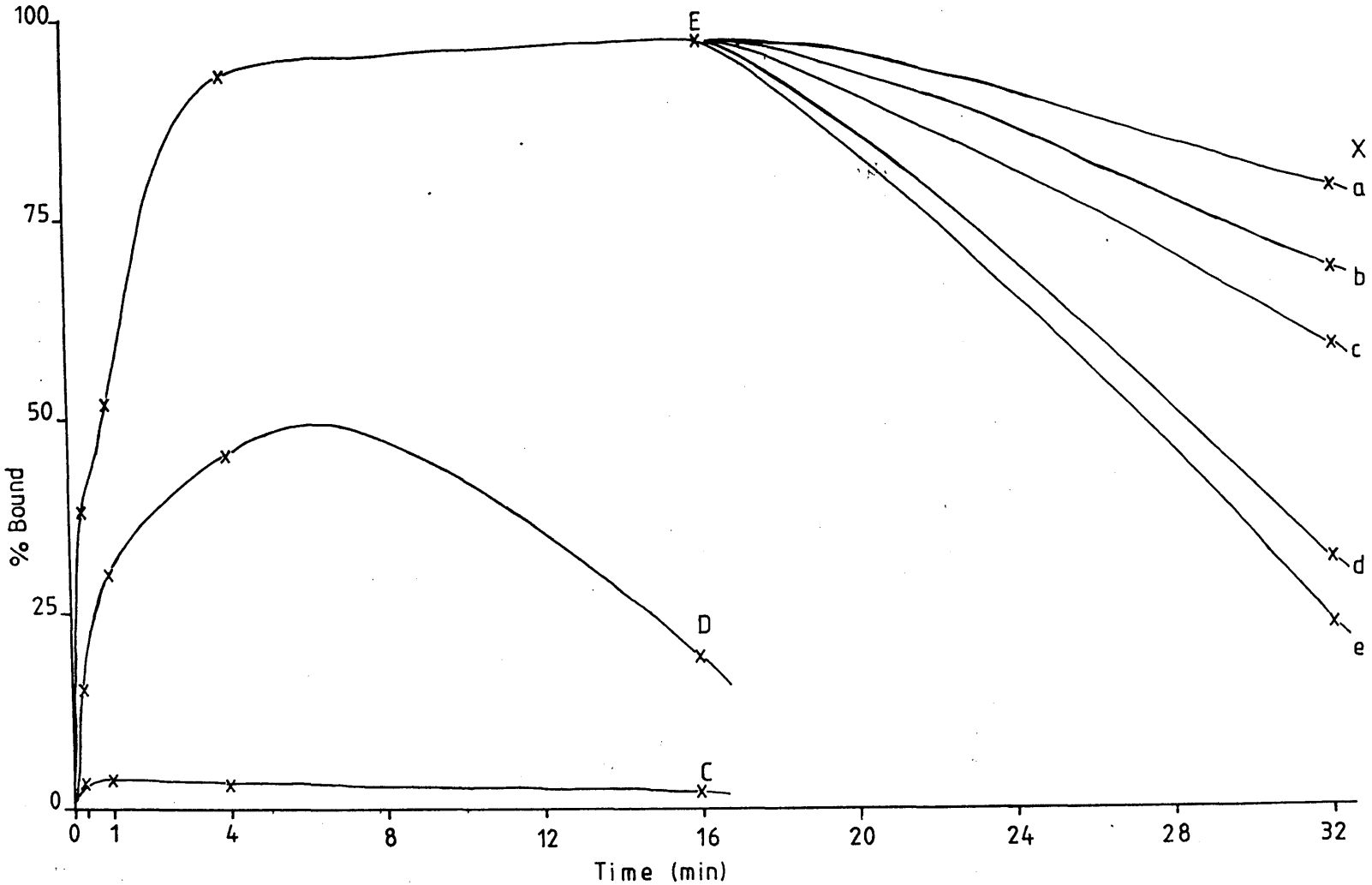


Figure 3.12. Different time points in a titration of a subsite I fragment.
(A) Incubations of a subsite I fragment (A1) in buffer B with resolvase for 20s, 1 min, 4min or 16 min (lanes 1-4 respectively) at 0^{o} or 37^{o}C. Samples A-E contained 0, 14, 28, 56, 112 nM resolvase respectively. The final time point for the highest concentration of resolvase used was diluted 0, 2, 4, 8 and 16-fold (lanes a-e) in prewarmed resolvase dilution buffer containing 40mM NaCl (with (Z) or without (X) the addition of a labelled wt-<u>res</u> fragment) and incubated for a further 16 min at 37^{o}C. The wt-res fragment was incubated alone with 112nM resolvase (for 10 min) in the same conditions as above



(B) Plot of percentage of final complex bound by resolvase at different time points for the different concentrations of resolvase.

incubation temperature (37°C), a higher yield of both complexes was seen with increasing resolvase concentrations. At the later time points, the initial complex was gradually converted to some other form at all concentrations of the enzyme. This was also true for the final time point for the second complex, at all concentrations except the highest. The increase of unbound fragment at these time points suggests that these complexes had dissociated. (There was no time course for the lowest resolvase concentration used.) At the highest concentration , a high percentage of bound complex 2 was maintained even after an additional 15 minutes incubation on the last time point (figure 3.12B).

Dissociation of complexes was investigated by diluting the highest resolvase concentration sample after the binding reaction was incubated for 16 minutes. The sample was diluted 2, 4, 8 and 16-fold in prewarmed binding buffer with or without an additional different end-labelled fragment (wt-*res*, C1) and after mixing, was further incubated for 16 minutes prior to loading into the gel. The extent of the apparent dissociation upon dilution of the resolvase-DNA complexes was slow unless the reaction sample was diluted more than 8-fold, when resolvase was presumably diluted sufficiently to prevent reassociation (figure 3.12B). An approximate association constant of 1.7×10^5 $M^{-1}s^{-1}$ was calculated for the 112nM resolvase reaction; dilution of the reaction 16-fold gave an estimated off rate of 1.5×10^{-3} s^{-1} and a half-life of the complex of 462s. A value for the dissociation constant of a resolvase/subsite I interaction, calculated by using the gel binding assay, is approximately 9nM, which agrees with the sort of concentrations of resolvase required to form a complex. The half-life of the undiluted 112nM resolvase reaction mix, calculated from the percentage of bound subsite I remaining 16 min after the 95% bound reaction was remixed, was approximately 55 min. Incubation in the presence of a second fragment in the dilution mix

checked that the concentration of active resolvase remaining in the reaction, or dissociated from subsite I was sufficient to bind res (figure 3.12A). As already indicated, dissociation was not rapid and thus little resolvase became free, although the reaction mix contained sufficient resolvase to give the six complex pattern of the protein bound to the wt-res fragment. Dissociation of complex 2 did not result in a build up of complex 1. This suggests that the initial complex of a subsite I is both rapid in its association and in its dissociation with resolvase.

The two complexes for subsite I displayed different properties. Complex 1 did not form quantitatively, and was seen at early times and low temperatures. Complex 2 did form quantitatively and was stable at longer times, although less was formed at low temperatures. The result obtained for resolvase binding to a partial subsite suggests that the first complex represents the protein binding and bending a half-site. In an intact site, the unstable complex 1 formed early may be rapidly converted to a form in which both half-sites are occupied. No second complex was observed from resolvase binding to the partial site.

When the DNA binding domain of gamma-delta resolvase (the carboxy-terminal domain) was used to footprint res, some differences in affinities for the half-sites of subsite II were observed (Abdel-Meguid et al, 1984). This suggested that the half-sites can be contacted independently. In the related Gin invertase system, footprinting one half of a gix site by the intact Gin protein has also been observed (Mertens et al, 1988).

Because complex 1 is not formed quantitatively with a half-site, this suggests some limiting factor in the assay. A half-site may bind monomer stably, but not dimer (figure 3.9A). However, if monomer and dimer are in equilibrium, the concentration of monomer should increase with increasing resolvase concentration. The possibility

that monomer is limiting could still arise if the monomer aggregates or is converted to some form other than dimer. Alternatively, the protein-half-site complex could be unstable in the gel assay and consistently fail to be trapped (e.g. 90% of the complex may collapse when it enters the gel).

Resolvase binding a whole subsite did form large amounts of complex 1. It may bind to the second half-site better if one half-site is already occupied. During dissociation, the levels of complex 1 may not build up if resolvase drops off faster once one half of the subsite has become unoccupied.

There are two possible explanations for the finding that each subsite is occupied in two steps. A half-site might be occupied by resolvase independently, in a fixed order. Alternatively, the protein can transfer between the half-sites by the previously proposed shuffling method. In either case, the contact with the half-sites could be made with either monomers or dimers, as depicted in figure 3.9A and B. Resolvase exists as dimers in 1M NaCl and these are probably present in the reaction. Whether monomers or dimers are binding to sites can be resolved by assessing the stoichiometry of the complexes using radiolabelled protein and DNA fragments. It has been shown that two gamma-delta resolvase subunits bind one <u>res</u> subsite (Salvo and Grindley, personal communication). Treating the complexes with footprinting agents before their isolation from the gel should reveal if only one or both half-sites of a particular complex can be contacted by the protein. Although shuffling may provide an explanation for both halves being protected, the same result would be seen for a dimer contacting both ends of a subsite and bending the subsite in two steps (figure 3.9C).

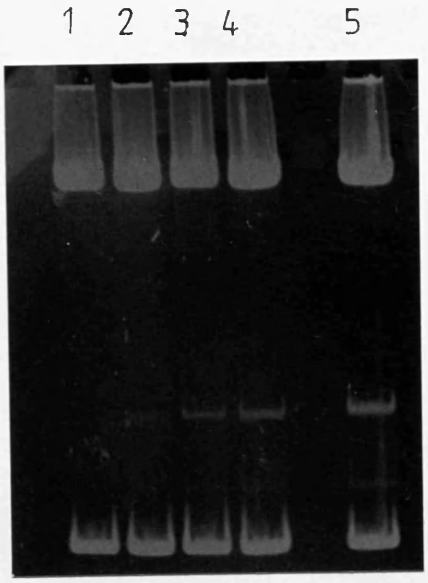


Figure 3.13 Titration of an unlabelled fragment of subsite I. Each reaction contained 100ng of a subsite I (A1) fragment (not isolated from the vector fragment) and was incubated under binding buffer A conditions, without additional supercoiled carrier DNA, and with the resolvase, for 10 min, 37°C. Lanes 1-5 respectively:- 0, 10, 20, 39 and 79 nM resolvase. 5% gel, conditions A (pH 8.2). Ethidium bromide stained.

3.5 Gel retardation of unlabelled *res* DNA fragments.

The amount of a *res* subsite required to bind the available resolvase can be estimated by increasing the concentration of the resolvase binding site, as resolvase becomes limiting in the binding reaction. By using an unlabelled EcoRI restriction digest of pAL3054, the amount of fragment containing subsite I was increased from 1ng, of labelled assays, to >100ng in a 10ul reaction sample. At this concentration of DNA fragment, the complexes formed upon the addition of resolvase were visualised by ethidium staining (figure 3.13). Two complexes were observed, as for a subsite I fragment in figures 3.5 and 3.6. The concentration of resolvase required to form complexes remained unaltered, but the percentage of bound complex was reduced to 10%, compared to 95% at similar resolvase concentrations for limiting DNA conditions. Since the concentration of available subsite I had increased 100-fold, with only 10% bound, the concentration of bound complexes had increased 10-fold. Therefore, the likelihood of synapsis between two resolvase-occupied fragments was expected to increase 100-fold. No additional complexes were observed in the assay using unlabelled fragments at a high concentration compared to an assay with a lower concentration of fragment. This suggests that any interactions between resolvases bound at a site are not strong enough to be stable in the gel assay. However, the presence of a vector fragment may have obscured any severely retarded complexes that could be interpreted as a synapsis of fragments, but an addition of tracer labelled fragment to the reaction should enable the visualisation of any unusual complexes.

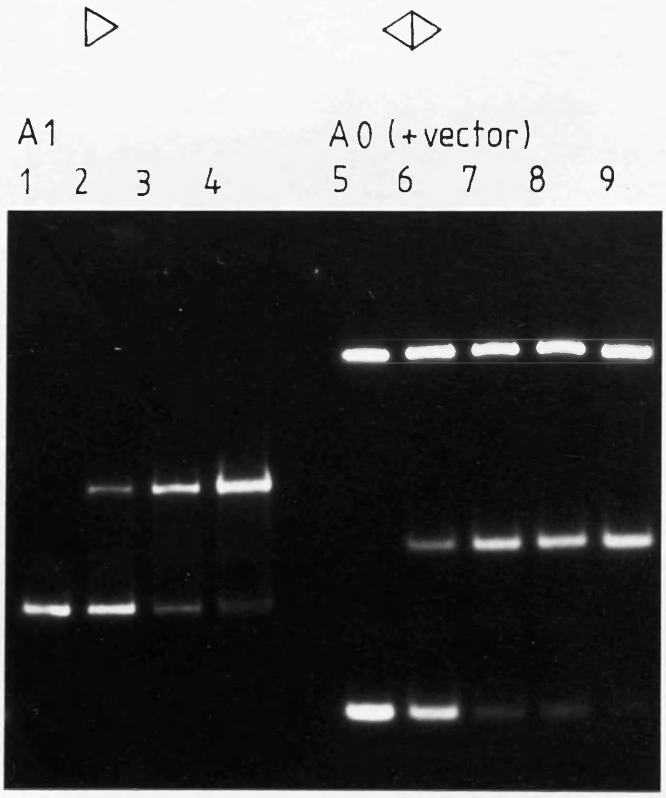


Figure 3.14. Titration of a symmetrical subsite I fragment. The effect of resolvase binding to an isolated wild type subsite I compared to a symmetrical subsite I was determined by incubating either fragment A0 or A1 under binding conditions A for 10 min at 37°C. 5% gel; conditions A (pH 8.2).

Lanes	nM resolvase
1 + 5	0
2 + 6	5
3 + 7	10
4 + 8	20
9	39

3.6 Binding of resolvase to symmetrical res subsites

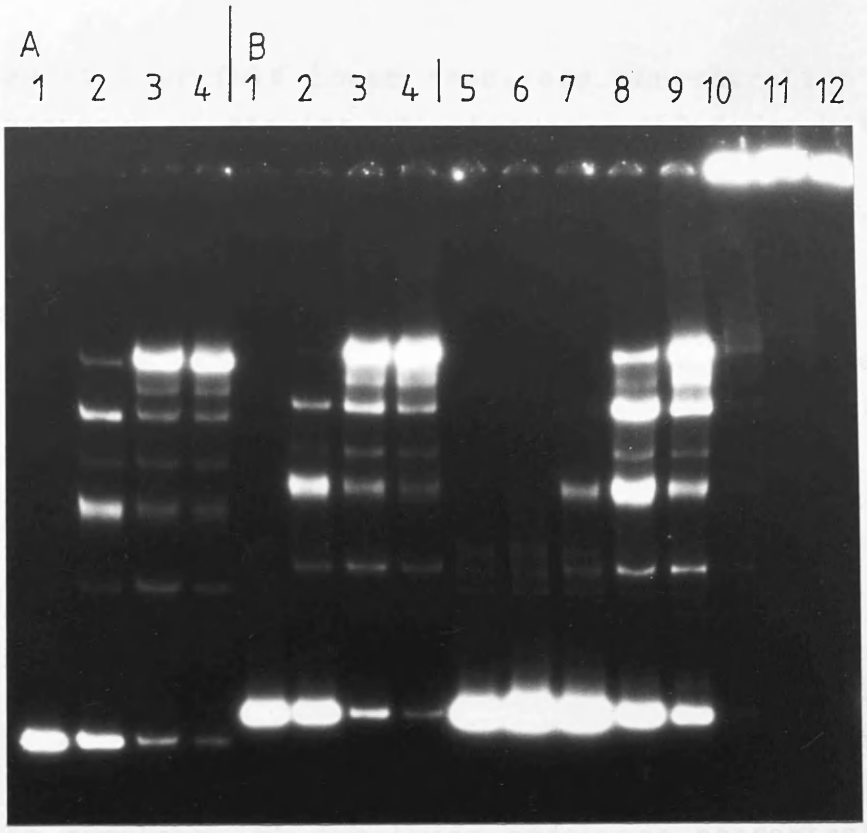
The left end of subsite I is more different from the consensus than the right end and we might suspect that the left end would have a lower affinity for resolvase than the right end. A perfectly symmetrical subsite I (figure 3.1), composed of two right halves of subsite I, might be expected to generate complexes at a lower resolvase concentration than a wt-subsite I. The results are incompatible with this possibility, as both the symmetrical and the wild-type subsite I exhibited similar binding at the same resolvase concentrations (figure 3.14). A fragment containing the same symmetrical subsite I, but located adjacent to subsites II and III as in a wt-res (sym-res; see chapter 4), gave a similar six complex pattern as to the wt-res fragment and at the same resolvase concentrations (figure 3.15A). Since the two arms of subsite I have no apparent difference in affinity for resolvase, the two complexes seen for a wild type subsite I can be interpreted as resolvase failing to bind either the left or right half separately, which might be expected to give two different initial complexes, if they are positioned at different distances from the end of the fragment.

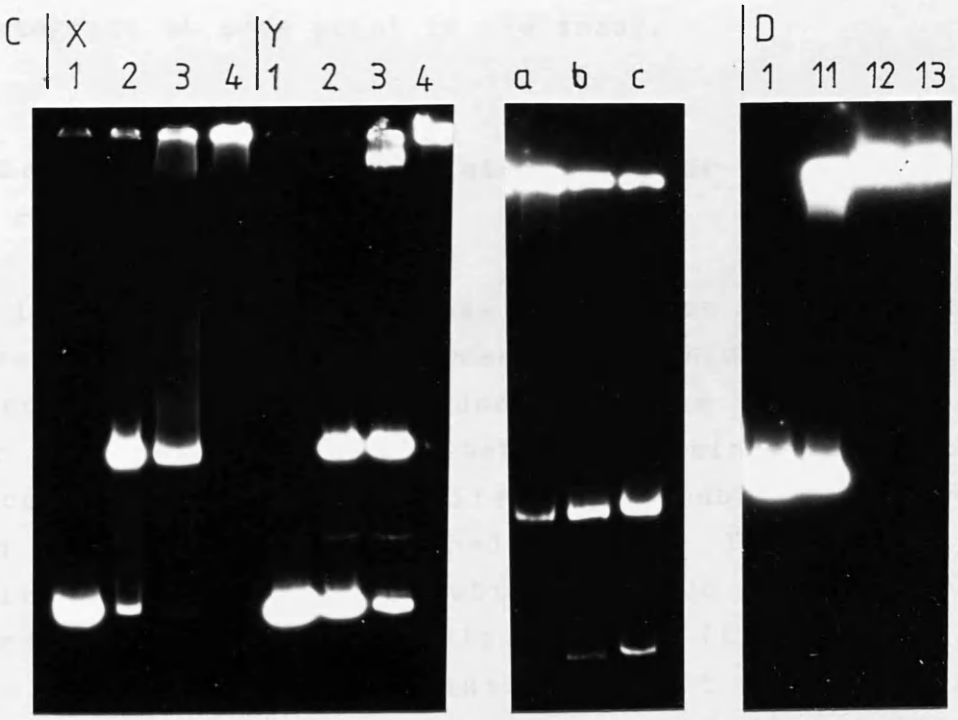
3.7 The effect of carrier DNA on the binding reaction.

In all the gel assays mentioned so far, the binding reactions included non-specific carrier DNA (pUC18 or pMTL23 supercoiled DNA at approximately 25 ug/ml). Aggregation of the fragments (at approximately 0.1ug/ml) in the wells of the gel occurred if the carrier DNA was omitted, but only at the higher resolvase concentrations used. At the lower resolvase concentrations, the usual six complex pattern was observed for the wt-res fragment, and two complexes for subsite I, but the first complex(es)

Figure 3.15. The effect of a titration of <u>res</u> fragments under different binding conditions.
All the following reactions were incubated with the indicated amounts of resolvase for 10 min, at 37°C, in binding buffer B unless otherwise stated. 6% gel; conditions B.
(A) Titration of a sym-<u>res</u> fragment (D1) with resolvase.
(B) Titration of a wt-<u>res</u> fragment with resolvase with (lanes 1-4) and without carrier DNA (lanes 5-12).
(C) Titration of a subsite I fragment without carrier in either recombination buffer D (lanes 1-4 X) or binding buffer B (lanes 1-4 Y). The sample with the highest resolvase concentration was subsequently diluted in binding buffer B (plus 40 mM NaCl) and incubated for a further 10 min (lanes a,b and c respectively:- 2, 4 and 8-fold dilution).
(D) Titration of a Tn21 <u>res</u> fragment with Tn3 resolvase, without carrier DNA.

Lanes	nM resolvase	Lanes	nM resolvase
1 + 5	0	6	3.5
2 + 9	28	7	7
3 + 10	56	8	14
4 + 11	112	12	224
		13	448


A
1 2 3 4
B
1 2 3 4
5 6 7 8 9 10 11 12


C
X
1 2 3 4
Y
1 2 3 4
a b c
D
1 11 12 13

were seen at four-fold lower resolvase concentration than in the presence of carrier DNA (figure 3.15B & C). Without carrier DNA, the binding pattern also changes less with increasing resolvase. Although the presence of the carrier DNA has an effect in the assay, the concentration of this DNA was much greater than that of the fragment, and implies that resolvase is very selective for its site, as non-res DNA did not compete efficiently for binding of the protein.

3.8 Can Tn3 resolvase form stable complexes with Tn21 res?

Binding reactions of a Tn21 res fragment and Tn3 resolvase without carrier DNA revealed rapid aggregation of the fragment (as for Tn3 res in these conditions) but no stable complexes at the lower resolvase concentrations (figure 3.15D). It is possible that Tn21 res does not form stable complexes with Tn3 resolvase, and that they disintegrate at some point in the assay.

3.9 Gel retardation of an intermolecular recombination reaction.

In our model, recombination between two res sites proceeds via a synaptic intermediate in which the subsites II and III are wrapped around resolvase tetramers. In other systems, interactions between protein dimers which are complexed with their sites can produce structures which are looped or sandwiched together. These forms of complexes have peculiar mobilities and are severely retarded in native polyacrylamide gels (Kramer et al, 1987). So far, the gel assay has not revealed any abnormal structures in the gel assay, all complexes resulting from resolvase occupying res, and no inter-site contacts between res sites on different molecules. To

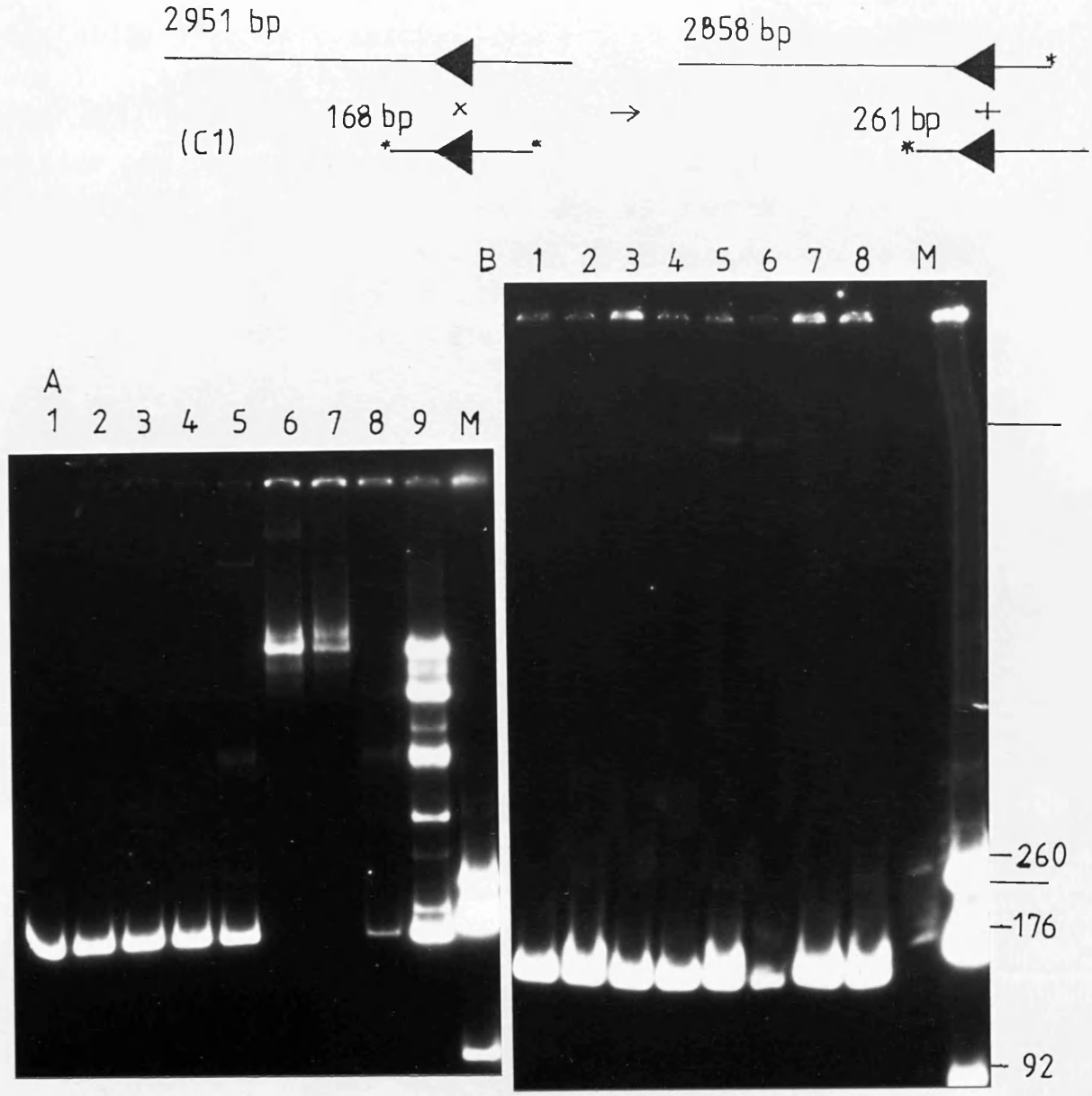


Figure 3.16. Titration of two wt-res linear fragments in recombination buffer D. A purified, labelled wt-res fragment (C1) was incubated with HindIII-linearised, unlabelled pMA2856 (wt-res) and resolvase for 19 hours at 37 °C. The labelled fragment was also incubated (with 224nM resolvase) alone for 19 hours under the same conditions (lane 8) or for 10 min in binding buffer B (lane 9) prior to loading onto the gels. After incubation the reaction samples were split and loaded onto one of two gels.
(A) Native polyacrylamide gel, 6%, conditions B.
(B) Denaturing polyacrylamide gel, 5%, 1x TBE. The samples were denatured by protease K/SDS.
Lanes 1-7 respectively:- 0, 14, 28, 56, 112, 224 and 448 nM resolvase.

investigate if synaptic intermediates could be trapped by the gel assay, an intermolecular recombination reaction using the end-labelled 168bp wt-*res* fragment (C1) and a second unlabelled substrate (pMA2856) was attempted. Both substrates were incubated in recombination reaction conditions (recombination buffer D) differing slightly from binding conditions by the inclusion of 5mM spermidine and 10 mM $MgCl_2$. This change in conditions does not affect binding as shown for a subsite I fragment (figure 3.15C). Intermolecular recombination reactions require incubation times of several hours and thus this reaction with resolvase proceeded for 22 hours. Each sample was split into two and loaded onto either a native or a denaturing polyacrylamide gel (figure 3.16A & B respectively). Complexes of the end-labelled substrate and product were detected. These products were still stable after several hours, although some of the complexes had dissociated, compared to a fresh 10 minute incubation on the same gel. Again, this is consistent with the idea that dissociation is slow under our binding and gel conditions. The non-denaturing gel did not show any unusually retarded complexes, which would have been indicative of a higher *res*/resolvase structure, the synapse predicted to form during recombination.

3.10 Can subsites II and III form a synaptic structure?

If recombination proceeds via a synaptic complex predicted in our model, then isolated accessory sites should be able to form such a structure without the presence of a crossover site. One of our approaches to detect a synaptic intermediate involved binding reactions with fragments containing subsites II and III. Low concentrations of the end-labelled subsites II and III fragment (B1) did not exhibit any unusually retarded complexes (figure 3.5). Increasing the concentrations of

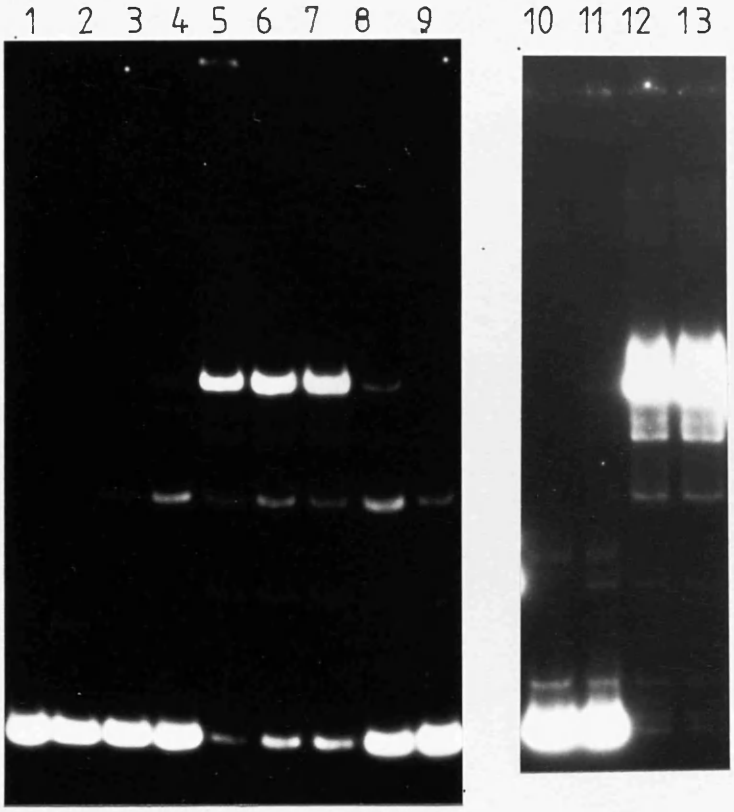
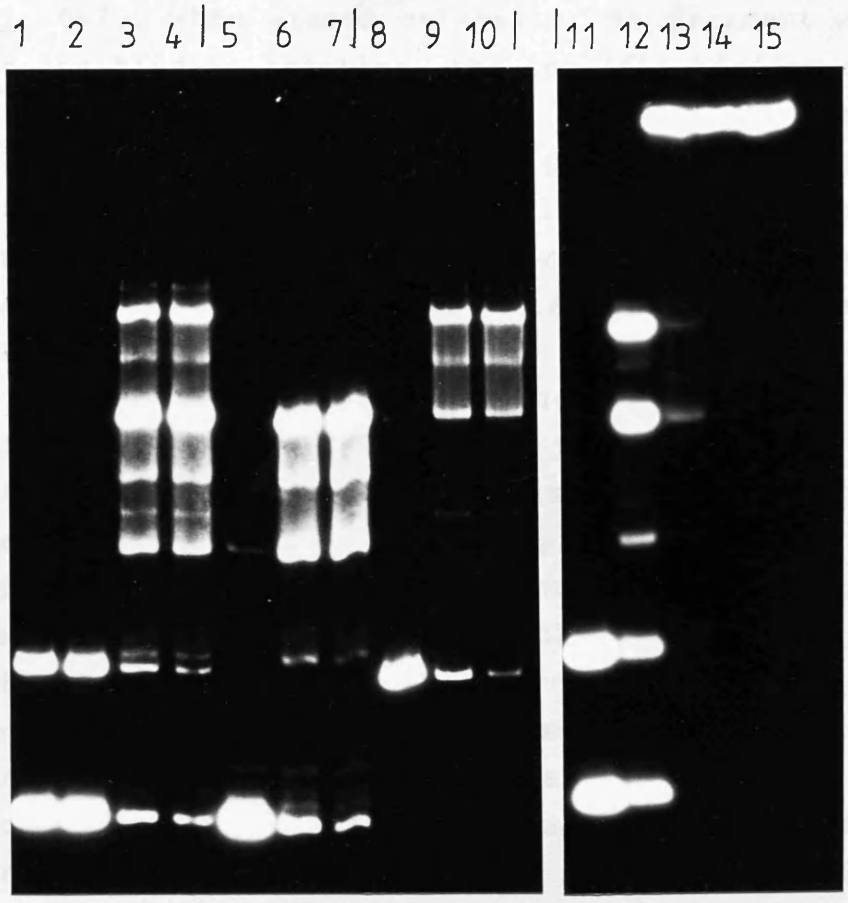


Figure 3.17. Binding of resolvase to subsites II and III. (A) A labelled subsites II and III fragment (B1) was incubated under binding conditions B, without additional supercoiled carrier DNA, with the indicated concentrations of resolvase, for 10 min at 37°C. 56nM resolvase was added to samples containing increasing amounts of a EcoRI digest of pAL2195 (see figure 5.15), i.e. an excess of the unlabelled subsites II and III fragment plus vector fragment. A titration of resolvase was continued for the highest concentration of unlabelled fragment.

Lane	nM TnpR	ug/ml cold B1	Lane	nM TnpR	ug/ml cold B1
1	0		10	0	3
2	7		11	112	3
3	14		12	224	3
4	28		13	448	3
5	56				
6	56	3			
7	56	0.75			
8	56	0.19			
9	56	0.047			



(B) Two different subsites II and III fragments, B1 and D2 (ges) were incubated separately or together, with the resolvase, under binding buffer B conditions, with carrier DNA (10 min, 37°C). The mixture of fragments was also incubated without carrier DNA. All gels were 6% polyacrylamide, conditions B.

Lane	nM TnpR	Lanes	
1 + 11	0	1-4 B1/D2	
2, 5, 8 + 12	28	5-7 B1	
3, 6, 9 + 13	56	8-10 D2	
4, 7, 10 + 14	112	11-15 B1/D2	without carrier
15	224		

both the protein and the specific binding site changes the likelihood of intermolecular complexes forming (Kramer *et al*,1987). When excess unlabelled B1 fragment was included in the binding reaction, the specific binding of resolvase was competed out (figure 3.17A). Increasing the concentration of resolvase produced the four complex pattern, but no extra complexes were observed. This suggests that at the concentrations of protein and DNA used, no inter-fragment complexes were either formed or stable in the gel assay.

A second line of investigation involved mixing two different subsite II and III fragments in the gel assay (B1 and D2). The *ges* site (see chapter 5) provided a second source of the accessory sites, as ges is composed of subsites II and III adjacent to the *gix* crossover site from the G-inversion system. The presence of *gix* did not alter the four complex pattern observed for subsites II and III, and therefore resolvase did not form any stable interactions with *gix* in the gel assay (figure 3.17B). The complexes of the mixture of fragments were assigned to one or other of the fragments by comparison with those from individual fragments (figure 3.17B), and no novel complexes were observed, in conditions with and without carrier DNA. In the latter case, a smearing of complexes was produced between the final complexes of each fragment and the aggregated complexes in the wells of the gel. Within this smear were hints of extra complexes which were not as stable as the less retarded complexes. Further investigation of these complexes is required, although it is unknown if they are the result of non-specific contacts with *gix*.

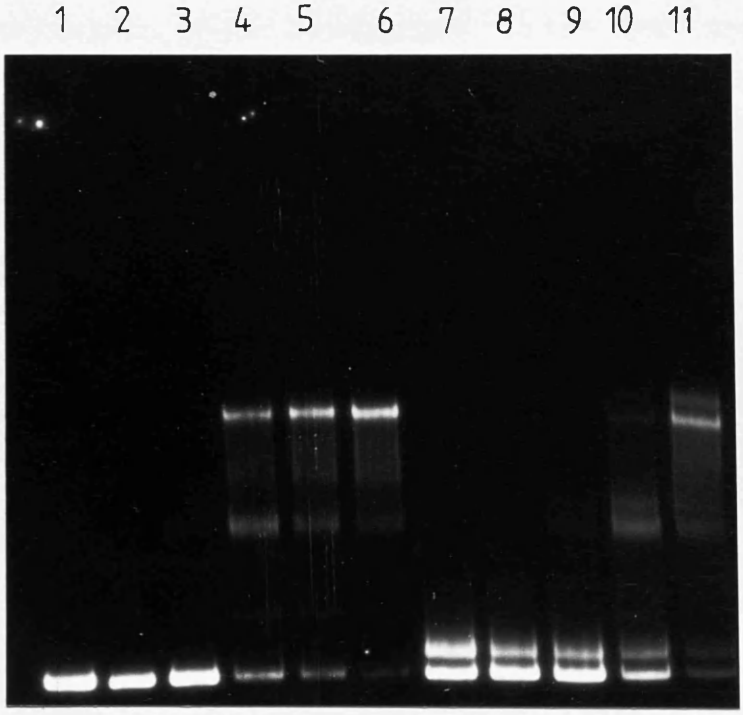


Figure 3.18 Binding of resolvase to fragments containing two copies of subsite I. Fragment AA3 alone, or mixed with fragment AA4 (both containing 2x subsite I) were incubated in binding buffer A for 10 min, 37°C, with the indicated amounts of resolvase. 5% gel, conditions A (pH 8.2).

Lanes	nM resolvase
1 + 7	0
2 + 8	5
3 + 9	10
4 + 10	20
5 + 11	39
6	79

Lanes 1-6 AA3, 7-11 AA4 + AA3

3.11 Binding of resolvase to two identical subsites on one fragment.

A partial EcoR1 digest of the duplicated subsite I sequence from pAL214 produced a fragment containing two copies of subsite I, which was subcloned into the pMTL23 polylinker to facilitate isolation of different sized fragments containing two copies of subsite I (pAL243; figure 3.3). The binding of resolvase to two copies of a subsite enabled several lines of investigation. Firstly, can two subsites of *res* separated by about 200 bp form a loop structure, as seen for other DNA site-specific proteins (lambda repressor, Hochschild and Ptashne, 1986: *lac* repressor, Kramer *et al*, 1987: *deo* repressor, Mortensen *et al*,1989)? Secondly, does resolvase bind two identical sites stepwise, or can it shuffle between the sites (i.e. are two or four complexes formed; figure 3.7 A & D)? Thirdly, will there be any cooperativity in the binding to two identical subsites that are separated by distances greater than the subsites within *res*?

Resolvase binding to a fragment containing two copies of subsite I is shown in figure 3.18. More than two complexes were formed. Under the higher pH conditions, the number of sharp complexes was four. This was interpreted as resolvase binding rapidly to the second subsite, to form a major fourth complex. A similar observation was made when the protein was binding to other fragments containing more than one intact subsite (figure 3.5). The protein, therefore, is capable of rapidly binding a second subsite at a distance, and will bind any number of subsites in a fragment to give two complexes per site. The absence of any complexes shifted further than complex 4 suggests that no inter-site complexes were formed or were stable in the gel system. This was confirmed by mixing two different fragments, both containing two copies of subsite I. The complexes observed were of the pattern expected for each fragment occupied by resolvase independently, since

no abnormally retarded complexes were detected (figure 3.18).

SUMMARY

Resolvase binding to wt-*res* and partial *res* regions exhibited a pattern of two separate retarded complexes per subsite. Although this is inconsistent with the three complexes observed for gamma-delta resolvase binding its wt-*res* site, the gel binding assay developed here for Tn3 resolvase is different from the TBE gel conditions and lower pH binding conditions used for gamma-delta resolvase, which may account for differences in the stability of *res*/resolvase complexes (Hatfull and Grindley, 1986).

The combinations of *res* subsites on the fragments used in the gel assay suggest that no shuffling of resolvase between subsites is occurring (at least for complexes that have entered the gel) and that the subsites are occupied in a random order. We do not know to what extent the complexes on a gel represent the complexes present in solution, and whether some dissociation occurs once the reaction enters the gel matrix. Identification of which subsite(s) is bound in each complex awaits the footprinting of specific complexes.

No evidence for higher ordered structures was found in the gel binding assay for Tn3 *res*/resolvase interactions. In the gamma-delta system, loop structures have been indicated by footprinting and in the gel assay for a single wt-*res* site (Salvo and Grindley, 1988). gamma-delta resolvase was shown to bend each of the subsites, which, in part, provides a suitable explanation for the protein accommodating the different spacings of the three subsites. However, the direction of the bending of the subsites with respect to each other within *res* has not yet been deduced. Our model predicts that resolvase

bends each subsite within *res* in the formation of a wrapped synaptic intermediate of the recombination reaction. Photofootprints of *res* sites with resolvase on linear and supercoiled DNA were similar, and with live reactions (supercoiled substrates containing two *res* sites) few alterations of photoreactivity within *res* were observed (J.L. Brown, 1986). This suggested that resolvase bends each subsite within *res* into a structure that is most accessible to forming a productive synaptic structure. The `loop' structure proposed for a gamma-delta site may have resulted from resolvase bending *res* in a way that enabled protein:protein contacts between resolvase bound at subsites I and III (Salvo and Grindley, 1988). Such a structure may not contribute directly towards the formation of a synaptic complex, but may also be indicative of resolvase bending an individual *res* in a similar way that it would be bent when wrapped around a second *res* site in the synapse. Any similar loops that may have formed for the Tn3 *res* site may not have been stable in the gel system, but even for gamma-delta *res*, the presence of loop structures was enhanced when the distance between the subsites I and II was increased by integral turns of the helix (approximately 10 bp increments).

Recombination reactions did not reveal any abnormally retarded complexes expected for the proposed synaptic structure. Since products of recombination were observed for an intermolecular reaction, the absence of observable synaptic intermediates may have been a consequence of their instability in the gel assay, and/or their instability in intermolecular reactions. Interactions between proteins bound to operator sites in `loop' or `sandwich' structures were captured in the gel assay for the *lac* and *deo* repressors (Kramer *et al*,1987; Mortensen *et al*,1988). At least for *lac* repressor, the affinity for its operator site is much greater than the affinity of resolvase for *res* (the dissociation constants for *lac* repressor is in the order of 10^{-14}M, whereas for resolvase

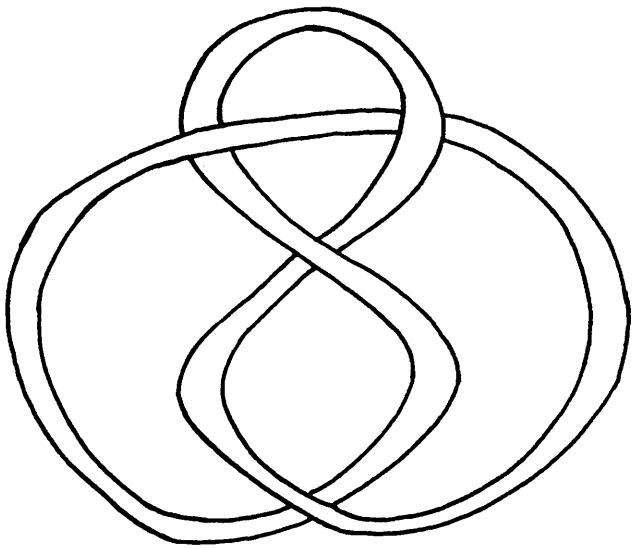
the value is 10^{-8}M). Also, *lac* repressor is associated into tetramers in solution, whereas resolvase is in dimers. Both these features of *lac* repressor may contribute to the stability of anomalously retarded higher order structures when this protein binds operator sites and provide an explanation for the failure to observe similar structures for *res*/resolvase complexes.

Although intermolecular events are possible in the Tn3 system, a more successful isolation of a synaptic intermediate might be seen for two *res* sites (or two copies of subsites II and III) in *cis*. Our model predicts the same synaptic intermediate for inverted *res* sites, which provides a topological barrier to recombination of inverted sites. Inverted *res* sites in *cis* can recombine on linear substrates, where synapsis of sites may or may not have proceeded as expected. The gel assay could be used to capture synaptic complexes between inverted sites. None of the experiments in the gel assay were performed on closed circular molecules, which are the preferred substrates for recombination. Retardation of circular molecules induced by protein binding has been shown for the *lac* repressor (Kramer *et al*,1988).

Synaptic intermediates might only be trapped when a crosslinking agent is added to the reaction (Benjamin and Cozzarelli, 1988). If a *res*/resolvase synapse could be trapped by the gel binding assay as an anomalously shifted complex, footprinting the complex should help reveal the structure of the DNA around the resolvase.

CHAPTER FOUR

RECOMBINATION PROPERTIES OF AN ISOLATED SUBSITE I OF Tn3 *res*



INTRODUCTION

Site-specific recombinases promote reciprocal exchanges of DNA between limited specific regions of homology. Different systems employ a wide range of different DNA sites and accessory proteins to promote recombination events. Two recombination sites are recognised as having a relative orientation and are aligned in a parallel sense for strand cleavage and exchange, such that the left hand of one site is joined to the right hand of the other and *vice versa* (figure 4.1A). Polarity within a site is achieved by some asymmetrical element, which defines a left and right end to the site. The result of recombination is deletion (or excision) between directly repeated sites and inversion between inverted sites (figure 4.2). Polarity can be lost when essential orientation-determining sequences are symmetrized. Antiparallel events are then also permitted, as the recombinase no longer distinguishes the left and right ends of a site (figure 4.1B).

The FLP and Cre-mediated systems have minimal crossover sites (FRT and *lox* sites, respectively) composed of inverted recombinase recognition elements flanking a central asymmetric core region (figure 1.8). Core homology between two crossover sites, in either of these systems, is essential for a complete recombination reaction. When symmetrical core sequences were engineered into FRT and *lox* sites (the entire *lox* site was symmetrized), it was found that the respective recombinase no longer recognised asymmetry within the sites and both the antiparallel and the parallel recombination events proceeded equally efficiently (for *in vitro* intermolecular recombination of linear substrates) (Senecoff and Cox, 1986; Hoess *et al*, 1986). It was concluded that the left-to-right polarity of the FRT and *lox* sites was defined by asymmetry within the core sequence.

Core sequence asymmetry may not be the sole

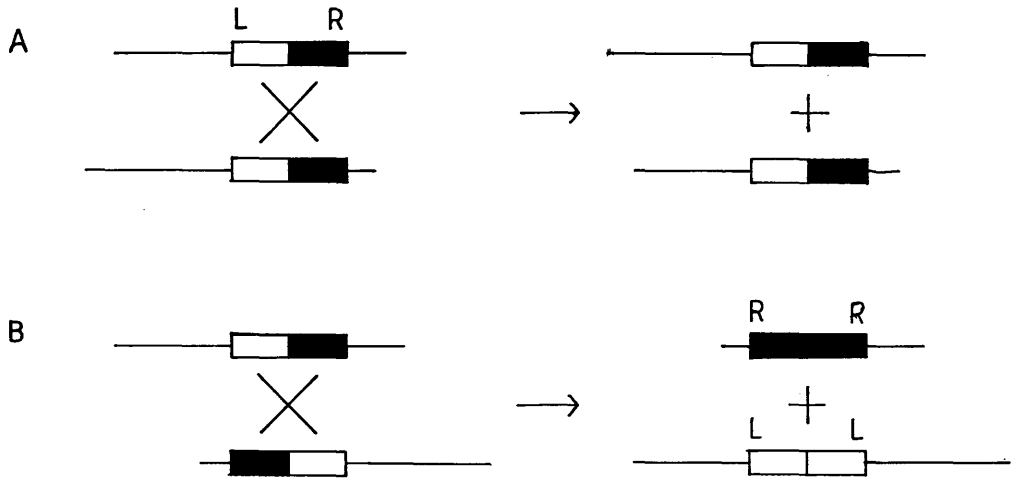


Figure 4.1. Diagrammatic representation of recombination products expected for different alignments of sites.
(A) The products of a `parallel' recombination event retain the left-to-right polarity of each site.
(B) The products of an `antiparallel' recombination event form two novel sites in which either two left halves of a site or two right halves are joined.

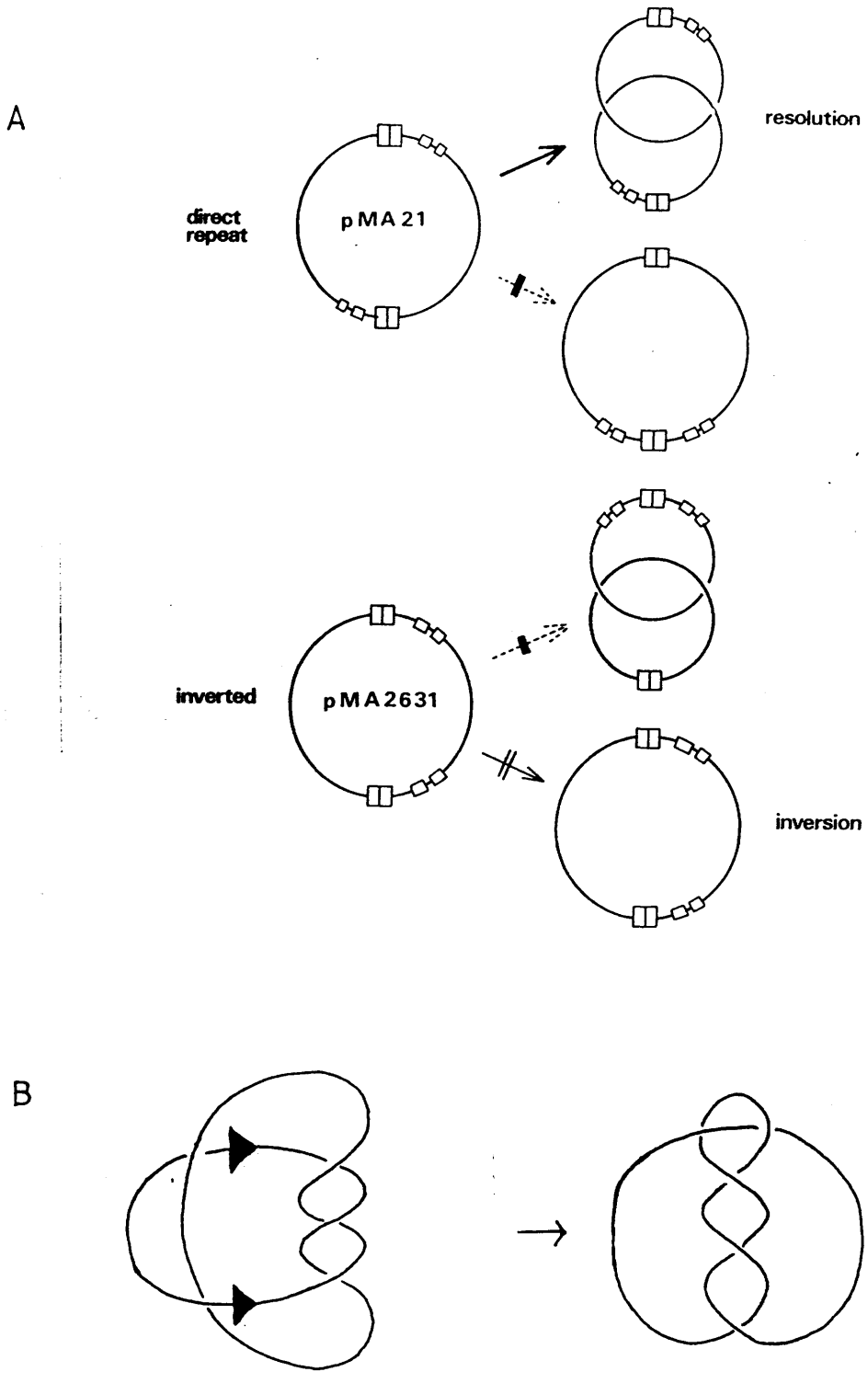


Figure 4.2. Expected recombination events and proposed synaptic intermediates for wt-res substrates.
(A) Directly repeated res sites result in deletion of the substrate to form a -2 catenane. Inverted res sites would be expected to invert the DNA between the sites.
(B) The proposed synaptic intermediate and product topology for an inversion event mediated by resolvase.

determinant of site polarity. In the Hin invertase system, crossover sites in direct repeat do not recombine. When the asymmetrical core sequence AA was converted to the symmetrical AT sequence in both sites, inversion was still selected (Johnson and Simon, 1985). It is not known if the imperfect symmetry within the arms of a hix site contributes towards polarity, but completely symmetrized gix sites also preferentially invert in the related Gin invertase system (Mertens et al, 1988; R. Kahmann, personal communication). This suggests that some other factor may be involved in the selection for inversion in these systems. A candidate for the factor effecting the selection of recombination event is the host protein FIS, and its site of action, the enhancer or sis site, which has been shown to be required by the Hin, Gin and Cin invertase systems (Johnson and Simon, 1985; Kahmann et al, 1985; Huber et al, 1985). These invertases, in common with other recombinases that act on complex recombination sites, not only require additional proteins and binding sites (to the two minimal recombination sites), but also require a supercoiled substrate. FLP and Cre recombinases do not require substrate supercoiling and do not precisely specify the substrate or product topology, only selecting an event on the basis of core sequence asymmetry in the site alignment. For the Gin/Hin/Cin inversion systems, FIS may be involved in the selection of sites in inverted repeat, particularly when core asymmetry is removed.

The lambda integrase system also requires accessory proteins and their binding sites flanking both sides of attP (figure 1.9). The resulting hybrid sites from the integrative event (attL and attR) have either one or other of the flanking arms. The Int and IHF sites around the attP crossover site are required for the formation of a functional intasome (Richet et al, 1986). In the integrative reaction, attP must be on a supercoiled substrate to recombine with attB, which need not be supercoiled (Mizuuchi et al, 1980). In the excisive

reaction, attL and attR can recombine without supercoiling in an Xis-dependent manner. Xis can be partially replaced by the FIS protein required in the invertase systems; this protein recognises a site within the P arm, overlapping the Xis binding site (Thompson et al, 1987).

Asymmetric arrangements of sites flanking the attP crossover site are required to determine the site of the initial strand exchange event, which generates the Holliday intermediate. When the Int crossover site was inverted with respect to the arm sites in attP, the initial strand exchange event was still adjacent to the P arm (Nunes-Duby et al, 1987; Kitts and Nash, 1988). The structure of attP somehow determines which side of the crossover core is cleaved first, implying that the intasome structure is asymmetrical in function. In excisive events, an alternative synaptic structure between attL and attR may be formed, with Xis bound to its site within the P arm of attR, but the initiation of strand exchange still occurs adjacent to the P arm. Xis-independent recombination of directly repeated attL and attR sites is still possible, but only if the substrate is supercoiled (Craig and Nash, 1983). This reaction may be the reverse of the integration event, initiation of strand exchange occurring at the opposite side of the crossover core sequence (adjacent to the P' arm). It is unknown how the position of the P arm influences the choice of the strand cleavage site.

The defined selectivities of the resolvase systems established in vitro (Reed 1981; Kitts et al, 1983) can be partly broken down by changing the standard buffering conditions. Under `permissive' conditions (in buffers containing spermidine and/or glycerol), some selectivity is lost, but other aspects of selectivity are maintained.

1. The supercoiling requirement is removed. Linear, nicked and relaxed circular molecules are recombined.

2. Substrates with inverted sites are recombined, but only if supercoiling is removed.

3. Intermolecular events are permitted if at least one substrate is not supercoiled.

4. Catenanes will fuse if they are relaxed.

5. The direction of strand exchange rotation can be reversed, at least for the fusion of the circles in a relaxed catenane.

6. The two *res* sites continue to be aligned in the 'correct' parallel sense (so as to regenerate both *res* sites) even for recombination in intermolecular reactions between non-supercoiled substrates.

For non-supercoiled substrates only, the selection for directly repeated *res* sites in *cis* is removed under permissive conditions. In all these previously 'illegitimate' events, the correct parallel alignment of *res* sites is maintained, suggesting that resolvase still recognises a left-to-right polarity within *res*. It is possible that resolvase preferentially forms an interwrapped synaptic complex, when the substrate is no longer supercoiled and even when the *res* sites are on separate molecules, thus aligning the two crossover sites in parallel for strand exchange. Product topology and strand exchange rotation in the fusion of a closed relaxed catenane agree with the idea that such a plectonemically wrapped synapse is produced in these substrates (Stark *et al*, 1989a).

Resolvases share approximately 30% amino acid homology with the Hin, Gin, Cin and Pin invertases (figure 1.2), but they have the opposite selectivity in recombination. The accessory components for the resolvase-mediated system are two additional resolvase binding sites, subsites II and III , within *res*. Subsite I of *res*

resembles an invertase crossover site; both have 2 bp central crossover core sites for strand exchange, and both have imperfect symmetry within the arms of the site (figure 1.5). Supercoiled substrates are the usual requirement for both resolvase and invertase. An unusual feature of the crossover site of Tn3 *res* is the presence of a symmetrical core sequence, which suggests that at least this does not define the polarity of *res*, nor the selection for resolution, and in this respect, is similar to the mutant *hix* site containing a symmetrical core sequence. Wild type invertases do not recombine without FIS and the enhancer sequence, but FIS-independent mutants of Gin and Cin no longer select inversion (Klippel *et al*, 1988b; Haffter and Bickle, 1988). Substrates for the mutant Gin no longer need to be supercoiled. If resolvase could recombine crossover sites without the requirement for accessory sites, the selection for resolution and supercoiling might be similarly broken. We propose that accessory functions in these systems assign asymmetry to the crossover sites, influencing the reaction selectivity by their ability to form specific synaptic complexes. The differences of selectivity within the two systems may be attributed to differences in the synaptic complexes that the different accessory functions can form (figure 1.6).

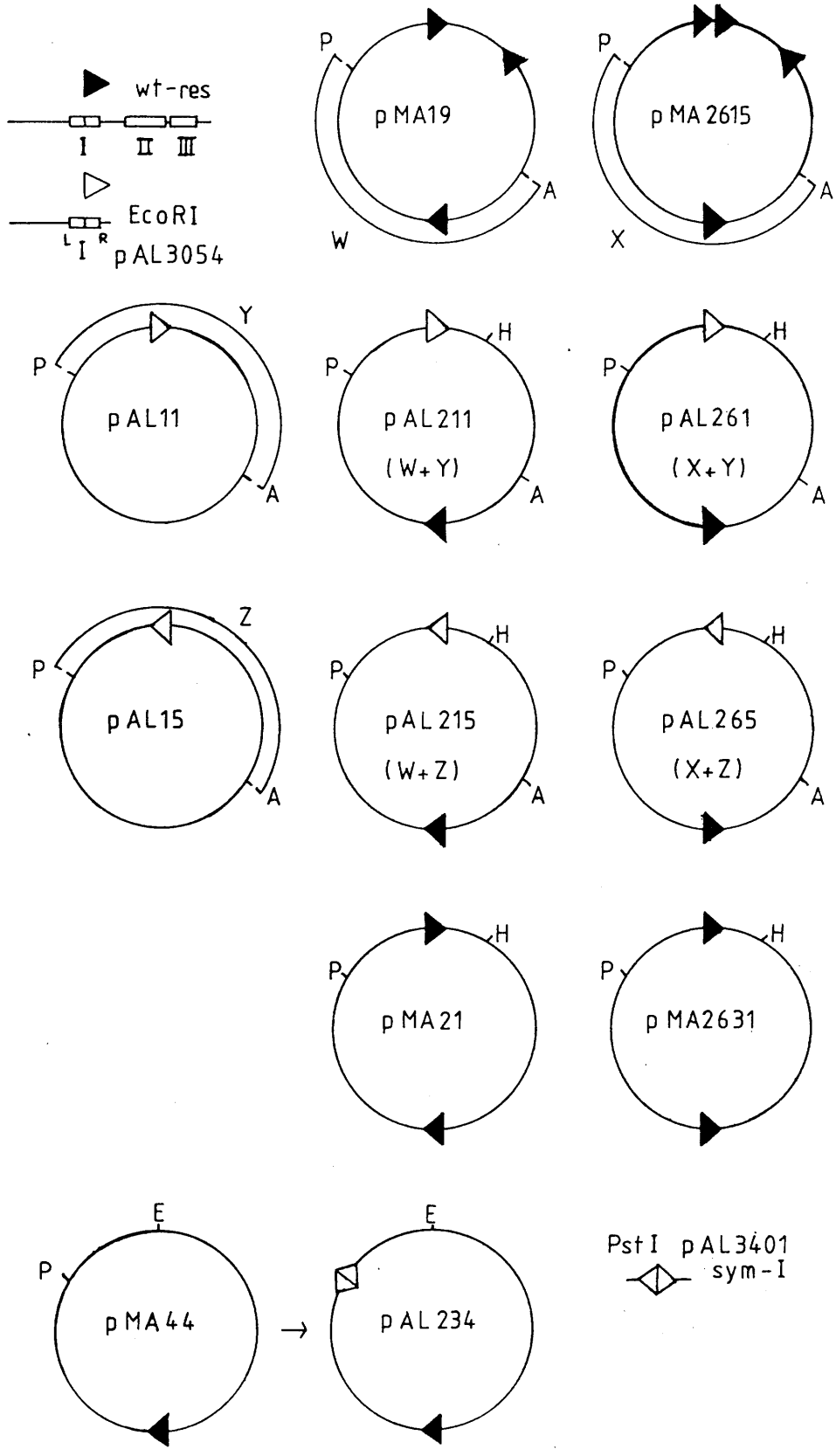
We predict that the structural organisation of *res* is important in defining the selectivity of the system. The effect of removing subsites II and III is expected to be the loss of selectivity for resolution, because these subsites would no longer be available to form the plectomemically wrapped structure predicted in our model (shown in figure 4.2B). Therefore, inversions of inverted sites as well as resolution of directly repeated sites are expected.

Previous attempts at reducing the functional length of *res* revealed that a slight deletion into subsite III is tolerated in one partner, but not in both (P. Kitts, 1982; P. Dyson, 1984). Further deletion as far as central

Figure 4.3. Schematic diagram to show the pBR322-derived fragments exchanged to construct subsite I x wt-*res* substrates. PstI-AvaI wt-*res* fragments from pMA19 or pMA2615 (both Tc^S) were ligated to PstI-AvaI fragments of pAL11 or pAL15 (containing a 203 bp EcoRI subsite I from pAL3054; figure 3.2). Subsite I x wt-*res* substrates were selected for Tc^r and on the basis of their size. A 34 bp PstI fragment of symmetrical subsite I was inserted into pMA44 to construct pAL234 (see section 4.4 and figure 5.3).

P = PstI, A = AvaI, H = HindIII
PstI-HindIII sizes (bp):-

	Substrate	Resolution		Inversion
		H	P	
pMA21	1065, 3862	2311,	2616	3090, 1837
pMA2631	" "	2331,	2596	3110, 1817
pAL211	986, 3826	2232,	2616	3090, 1758
pAL215	" "	2369,	2479	2953, 1895
pAL261	" "	2252,	2596	3110, 1738
pAL265	" "	2389,	2459	2973, 1875


wt-res
I
II
III
EcoRI
L
I
R
pAL3054
P
pMA19
A
W
P
pMA2615
A
X
P
Y
pAL11
A
P
H
pAL211
(W+Y)
A
P
H
pAL261
(X+Y)
A
P
Z
pAL15
A
P
H
pAL215
(W+Z)
A
P
H
pAL265
(X+Z)
A
P
H
pMA21
P
H
pMA2631
E
P
pMA44
E
pAL234
PstI pAL3401
sym-I

subsite II of gamma-delta *res* abolishes recombination (Wells and Grindley, 1984; Grindley *et al*, 1982). In light of `illegitimate' events now possible under permissive conditions, deleted *res* sequences may still be observably recombinogenic. Deleting subsites II and III from one *res* site enables us to ask what selectivities of the recombination reaction are removed.

RESULTS

4.1 Substrates lacking subsites II and III in one recombination site

To test the recombination properties of an isolated subsite I, a family of substrates containing this subsite and a wt-*res* was constructed in analogy to the pBR322-derived resolution substrate pMA21. Each construct was made by a PstI-AvaI fragment exchange of different pBR322-based plasmids containing the relevant site (figure 4.3). Resolution of pMA21 was used as a positive control in every assay. PstI-HindIII digestion of all substrates in this family gave a restriction pattern that is diagnostic for a recombination event.

In vitro recombination assays of these substrates were performed on negatively supercoiled molecules with purified Tn3 resolvase under variations of the permissive conditions used for `illegitimate' reactions. Recombination products were initially detected in recombination buffer B, but initial levels of recombination product observed were enhanced by altering the conditions, omitting the spermidine and raising the pH (5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 50 mM Tris/HCl pH 9.4, 25 mM NaCl: recombination buffer E). Reactions were incubated for up to 19 hours at 37°C and stopped at 70°C for 5 minutes. Digestions with PstI and HindIII were performed in the same buffer as the recombination assay

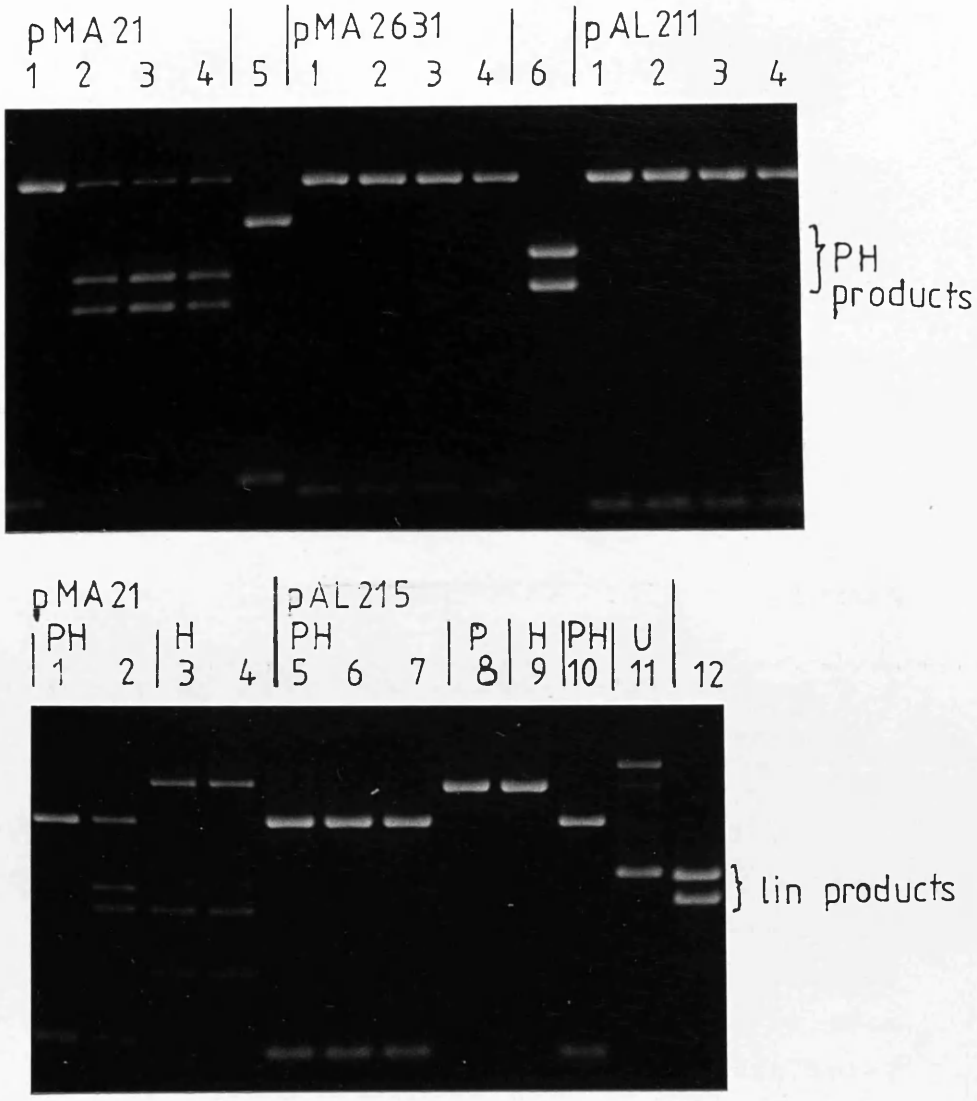
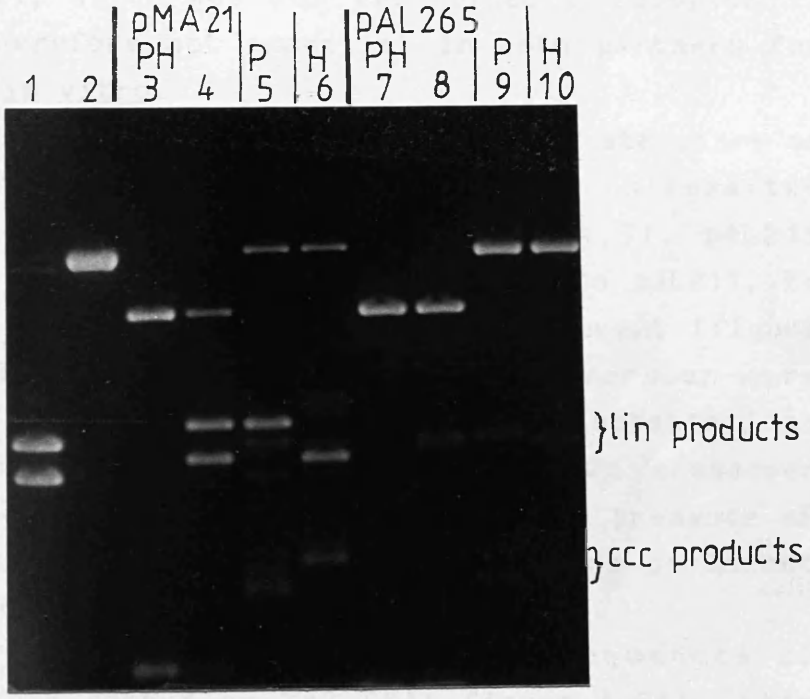


Figure 4.4. <u>In vitro</u> **recombination of subsite I x wt-<u>res</u> substrates.** Resolution products and positions of expected inversion products are indicated. Products were restricted by either PstI or HindIII or both enzymes; digestion with a single enzyme gives in a linear and circular resolution products of characteristic sizes. 1.2% agarose gels.
(A) Titration of pMA21, pMA2631 and pAL211 with Tn3 resolvase in recombination buffer E, 25mM NaCl for 19 hours at 37°C.
Lanes 1-4 have 0, 197, 394 and 789 nM resolvase respectively. Lane 5 is a BamHI-PstI digest of pBR322 (3234 and 1129 bp); lane 6 is a PstI-AvaI digest of pMA19 (2817 and 2466 bp).
(B) Titration of pAL215 with Tn3 resolvase in recombination buffer B, 25mM NaCl, for 19 hours at 37°C. Lane 12 has pMA19 PstI-AvaI fragments (2817 + 2466 bp).

Lanes	nM resolvase
1 + 5	0
6	49
7,8 + 9	98
2,3,4,10 + 11	197



(C) Titration of pAL261 and pAL265 with Tn3 resolvase in recombination buffer B, 25mM NaCl, for 19 hours at 37°C. Lanes 1-4 contained 0, 98, 197 and 394 nM resolvase respectively.

(D) Titration of pMA21 and pAL265 with Tn3 resolvase in recombination buffer B, 25mM NaCl, for 16 hours at 37°C. Lane 1 is a PstI-AvaI digest of pMA44 (1897 + 2466 bp); lane 2 is linear pAL265 (4848 bp; PstI). Lanes 3 and 7, 0nM resolvase; lanes 4-6, 197 nM; lanes 8-10, 394 nM.

after the NaCl concentration had been raised to 100 mM. Recombination products were virtually undetectable under our standard high pH buffering conditions for all the subsite I x wt-*res* constructs, compared to pMA21, which recombined in all buffers tested.

When subsite I was placed in direct repeat with wt-*res*, pAL211, incubation with purified Tn3 resolvase and subsequent restriction revealed products of resolution, but no products from an inversion event were detected (figure 4.4). This substrate is equivalent to pMA21 except that subsites II and III are missing from one of the *res* partners. The recombination efficiency of pAL211 was clearly reduced (not more than 20% recombined in 19 hours, figure 4.4) in comparison to the intact resolution substrate pMA21, which was 80% recombined . Subsites II and III are therefore not essential in both partners for recombination *in vitro*.

A second construct, pAL215, has the same structure as pAL211, except that the isolated subsite I is inverted with respect to the wt-*res* site (figure 4.3). pAL215 recombined *in vitro* at a similar efficiency to pAL211, to give restriction products of the resolution event (figure 4.4). Again, no restriction products of inversion were observed. Tn3 resolvase therefore recognises subsite I as symmetrical and is capable of recombining two crossover regions aligned in an antiparallel sense. The presence of subsites II and III in one *res* partner appears to direct the reaction for resolution only.

To check that particular flanking sequences of subsite I (either vector or Tn3 DNA: figure 4.5A) were not contributing to the selection for resolution, two further substrates were constructed in which the orientation of the wt-*res* site with respect to the vector sequence was reversed (figure 4.3). In these two new substrates, the left end of subsite I is in the same sequence context as for pAL211 and pAL215, and is used as either a left or right end depending on the relative

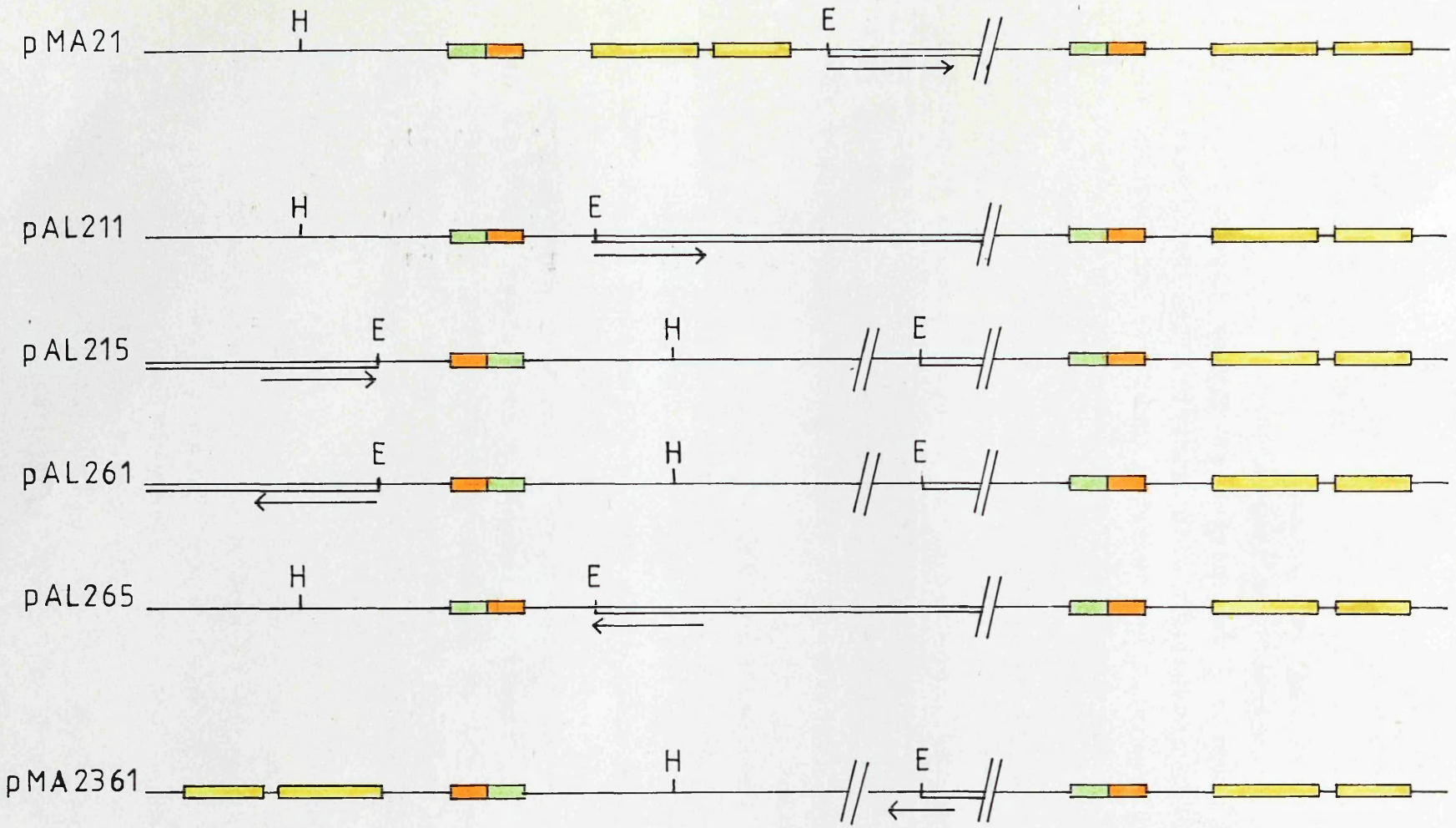


Figure 4.5. DNA sequences that replace subsites II and III in the subsite I x wt-<u>res</u> substrates . (A) Linear representation of these substrates, indicating the source of DNA adjacent to the isolated subsite I that replaces subsites II and III. H = HaeIII, E = EcoRI.

(B)

pMA21 CAACCGTTCGAAATATTATAAATTATCAGACATAGTAAAACGGCTTCGTTTGAGTGTCCATTAAATCGTCATTTTGGCATAATAGACACATCGTGTCTGATATTCGATTTAAGGTACATTTTTATG

pAL211 CAACCGTTCGAAATATTATAAATTATCAGACATAGTAAAACGGCTTCGGAATTCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCA

pAL215 ACTATGTCTGATAATTTATAATATTTCGAACGGTTGCAGTTGTGTTAAAAAAGCCGTCAGGCAGGGAGGCCGATATGCCCGTTGATTTTTTGACCACTGAGCAGGTTGAGAGTTATGGCAGGTTCA

pAL261 ACTATGTCTGATAATTTATAATATTTCGAACGGTTGCAGTTGTGTTAAAAAAGCCGTCAGGCAGGGAGGCCGATATGCCCGTTGATTTTTTGACCACTGAGCAGGTTGAGAGTTATGGCAGGTTCA

pAL265 CAACCGTTCGAAATATTATAAATTATCAGACATAGTAAAACGGCTTCGGAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACG

pAL234 CTGCAGTGTCTGATAATTTATAAATTATCAGACACTGCAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTG

CTGCAGTGTCTGATAATTTATAAATTATCAGACACTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGAT

(B) Sequence comparison of subsites II and III of Tn3 res and non-res DNA adjacent to the isolated subsite I in the indicated constructs. No obvious similarities between the sequences and subsites II and III are seen. The sequence shown for pAL215 is present in fragments from pAL214 used in the gel binding assays (figure 3.3).

orientation of the wt-*res* site. Both the inverted repeat substrate, pAL261, and the direct repeat substrate, pAL265, only gave resolution products in the *in vitro* assay (figure 4.4). All four subsite I substrates behaved in a similar way, as summarised in table 4.1. The sequences replacing the deleted subsites II and III were different in the different substrates and bore no resemblance to these accessory sites of Tn3 (figure 4.5B). Subsites II and III are normally located to the right of subsite I, but in the substrate pAL215, the crossover site is recognised in the opposite orientation for recombination and sequence originating from Tn3 replaces subsites II and III (i.e. about 100 bp to the left of subsite I, as shown in figure 3.3). A fragment containing this region did not have any detectable binding affinity for resolvase by the gel binding assay (figure 3.5 and 3.6).

In view of the data obtained for recombination between wt-*res* and an inverted subsite I, it was important to ask whether pMA2631, where the two wt-*res* sites are inverted with respect to one another, recombines in the same conditions. Note that pAL261 has the same structure as pMA2631, except that subsites II and III were removed from one *res* site in pAL261 (figure 4.3). pAL261 was capable of recombining *in vitro* when supercoiled, but only resolution products were detectable; pMA2631 failed to give detectable levels of any recombination product as a supercoiled substrate (figure 4.4). In summary, when subsites II and III are present in only one recombination site, resolution occurs, but not inversion, irrespective of the orientation of the isolated subsite I. When they are present in both *res* partners, recombination is prevented if the two partners are in inverted repeat. In our model of synapsis, for inverted *res* sites in a supercoiled substrate to form the same synapse as for directly repeated *res* sites (using subsites II and III from both *res* sites), extra tangling and interdomainal

Table 4.1 Relative recombination rates *in vitro*.

Substrate	Arrangement of crossover sites	Resolution	Inversion
pMA21	direct repeat (wt x wt)	100	< 0.06
pAL211/pAL265	direct repeat (wt x I)	1.0	< 0.06
pAL215/pAL261	inverted repeat (wt x I)	1.0	< 0.06
pMA2631	inverted repeat (wt x wt)	< 0.06	< 0.06

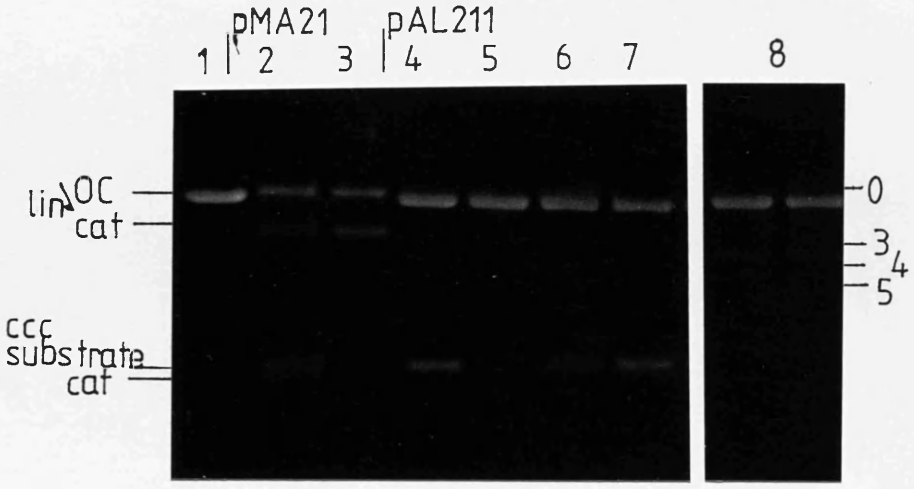


Figure 4.6. Topology of the products of *in vitro* recombination of subsite I x wt-*res* substrates. Supercoiled pMA21 and pAL211 were incubated in recombination buffer B, 25mM NaCl, with Tn3 resolvase, for 19 hours at 37°C and then nicked by DNase I. The position of a nicked catenane structure is compared to a knot ladder of pMA21 generated by T4 topoisomerase II (lane 8). Lane1is linear (PstI) pAL211.

Lanes	nM resolvase	ng/ml DNaseI
2 + 4	0	100
3	197	100
5	394	100
6	394	10
7	394	5

supercoiling would be introduced (figure 4.2B). Therefore, a productive synaptic complex might not form for inversion substrates, but attempts to form a synaptic structure might be preventing an alternative alignment of crossover sites for a resolution event. Removal of subsites II and III might prevent the formation of this unfavourable synapse and release subsite I for recombination.

4.2 Topology of products of subsite I substrates

Negatively supercoiled catenated products of pMA21 resolution run faster than the supercoiled substrate (Krasnow and Cozzarelli, 1983). Each circle can be released individually by restriction, to leave a faster-migrating supercoiled and a linearized product (figure 4.4). When the products of the subsite I substrates were restricted with either PstI or HindIII, both linear and supercoiled circular products of the predicted sizes were released. Unrestricted products did not migrate in the same position as released supercoiled circles (figure 4.4). Resolution products of subsite I substrates were therefore catenated and supercoiled. Sometimes the supercoiled catenated product for subsite I substrates was observed migrating faster then the supercoiled substrate, as for pMA21.

The degree of topological complexity of highly supercoiled molecules cannot be determined by gel electrophoresis, but specific catenanes or knots can be separated when they are nicked. By comparison with a reference ladder of knots made by topoisomerase II, specific catenanes or knots can be identified (Spengler et al, 1985). To determine the complexity of the catenanes, the recombination products were nicked with DNase I. This resulted in both the semi-nicked catenanes (where only one circle is nicked) and fully nicked catenanes for pMA21 and pAL211 (figure 4.6). After nicking, a novel species with

mobility slower than a 3-node knot was seen. On the basis of these results, it was concluded that resolution of the subsite I substrates proceeds as for the wild-type substrate to yield singly linked catenanes of the two product circles. When subsites II and III were absent from one recombination site, recombination still proceeds with the same selectivity as for the intact *res* substrates; inversion or fusion events were not observed for supercoiled molecules. The singly linked catenated products suggest that the resolvase is forming the wrapped synaptic structure required for resolution so that recombination selectivity is maintained (figure 4.12). It is possible that the presence of subsites II and III in both sites prevents the alternative synapsis with non-specific DNA if the two *res* sites are inverted.

4.3 Recombination properties of a novel *res* site, sym-*res*

The experiments described above suggest that the asymmetry of the DNA sequence of subsite I does not define the polarity of *res*. To test whether the accessory subsites II and III can impose polarity on the crossover site, the wild type subsite I was replaced by a perfectly symmetrical subsite I.

Resolution products of subsite I substrates, in which the sites are in inverted repeat, were expected to have a novel subsite I, made by joining either two left or two right halves of the wild type subsite I (figure 4.7). Both pAL215 and pAL261 were capable of such aberrant events. *In vitro* recombination of these substrates, followed by transformation into the *recA* strain, DS902, of HindIII restricted product and unrecombined substrate (only the product containing the origin remained circular, and was therefore likely to transform efficiently) enabled isolation of large quantities of the products. The recombination product of pAL215 retaining the pBR322

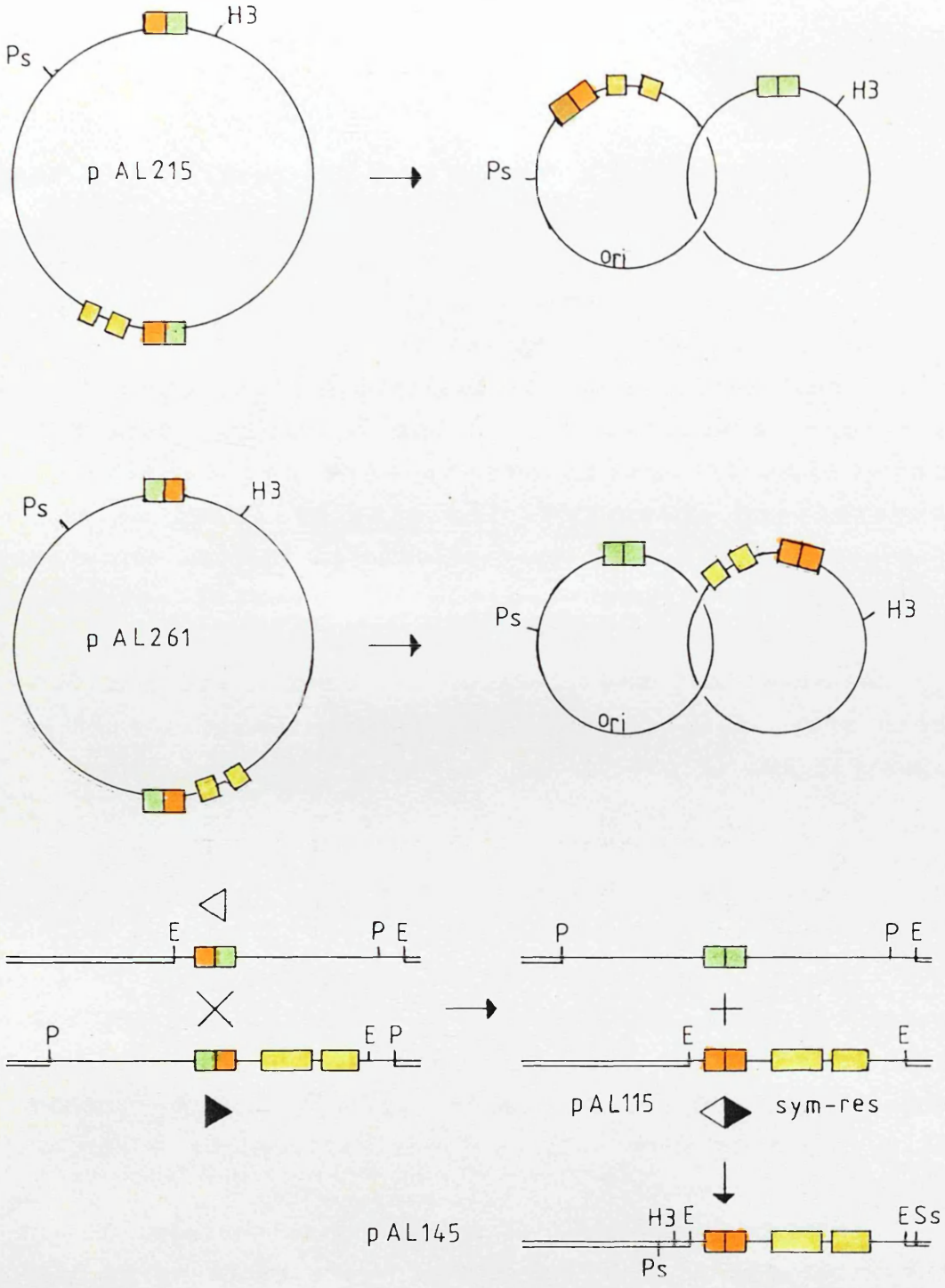


Figure 4.7. Diagrammatic representation of the resolution products expected from an `antiparallel' alignment of sites in pAL215 and pAL261.
(A) The resolution of pAL215 and pAL261 is expected to result in the joining of two left halves of res subsite I or two right halves to give symmetrical crossover sites. The product of pAL215 resolution containing the origin of replication (pAL115) has the novel sym-res site. A 145 bp EcoRI sym-res fragment from pAL115 was inserted into the EcoRI site of pMTL23 in the orientation indicated, to make pAL145.
E=EcoRI, P=PvuII, Ps=PstI, H3=HindIII.

(B)

sym-res

EcoRI +1

pAL115 GAATTCCGAAGCCGTTTTACTATGTCTGATAATTTATAAATTATCAGACATAGTAAAACGGCTTCGTTTG
CTTAAGGCTTCGGCAAAATGATACAGACTATTAAATATTTAATAGTCTGTATCATTTTGCCGAAGCAAAC

II III

AGTGTCCATTAAATCGTCATTTTGGCATAATAGACACATCGTGTCTGATATTCGATTTAAGGTACATTTT
TCACAGGTAATTTAGCAGTAAAACCGTATTATCTGTGTAGCACAGACTATAAGCTAAATTCCATGTAAAA

(B) The sequence of the sym-res site, as determined by direct plasmid sequencing of pAL145.

origin of replication was expected to contain a novel *res* site, with a symmetrical subsite I in place of the wild type sequence. Recombination of pAL215 gave a stable product pAL115, containing this novel *res* site, designated sym-*res*. The sequence of this site was determined by subcloning it into the pMTL23 polylinker (pAL145), and directly sequencing from the plasmid template (figure 4.7).

The product of recombination of the substrate pAL261 containing the pBR322 origin of replication was expected to have a novel subsite I composed of two left halves of the wild type crossover site (figure 4.7). However, transformation of this product into the *rec*A strain DS902 was unsuccessful, where the only transformant obtained was a size smaller than expected for the product of pAL261. We presume that the predicted aberrant product of pAL261 is unstable in a *rec*A background, because an inverted repeat is expected as a result of recombination (340 bp; figure 4.7). Palidromic sequences are stable in a *rec*BC, *sbc*B, *rec*F, strain, and the product of pAL261 recombination should be maintained in this strain background (Leach and Stahl, 1983; Boissy and Astell, 1985).

A simple resolution substrate containing two copies of sym-*res* (pAL2115) was made by dimerizing the product, pAL115. This was achieved by transforming the pAL115 into a strain with a *rec*BC, *sbc*A background (JC8679) to firstly multimerize the plasmid, then transform the isolated DNA into a *rec*A background (DS902) and screening for dimers in a single colony lysis gel. The sym-*res* dimer substrate (pAL2115) was resolved to monomer *in vivo* in a resolvase dependent manner, where either the *tnp*R gene of Tn3 was provided in *trans* (pPAK316: P. Kitts, 1982) or gamma-delta was provided on the F plasmid (in the strain JM101). pMA21 was shown to resolve similarly in the presence of *tnp*R in vivo. No breakdown of the pAL2115 substrate was observed in a *rec*A strain (DS902) without complementation by a *tnp*R$^+$ plasmid.

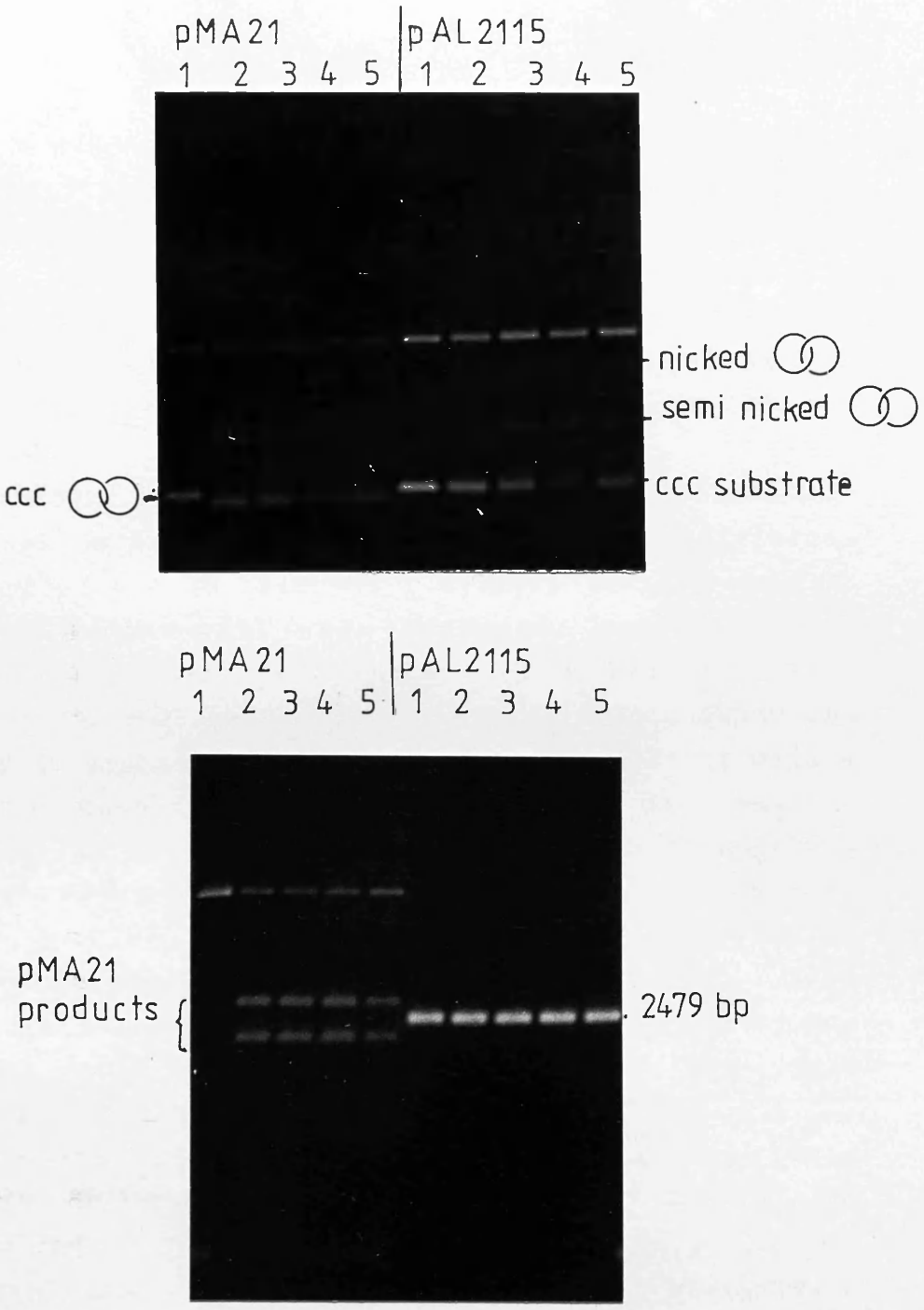


Figure 4.8. <u>In vitro</u> recombination of the supercoiled sym-<u>res</u> dimer substrate, pAL2115, by resolvase. A dimer of pAL115 was made as described in the text (pAL2115). Both pAL2115 and pMA21 were resolved by the addition of resolvase to the supercoiled substrates in recombination buffer D. The products of the reaction were subsequently nicked in the presence of DNase I (A). Recombination of these supercoiled substrates in recombination buffer A (standard conditions) gave similar results. Restriction of these products by PstI (B) revealed no fragments from pAL2115 that would indicate an `antiparallel' alignment of sites during recombination. Lanes 1-5:- 0, 69, 139, 278, 556 nM resolvase respectively.

In vitro recombination, in either standard or permissive conditions, of supercoiled pAL2115 did not give any detectable products as either supercoiled catenane or free circles on 0.7% agarose gels. However, once the products were nicked by DNase I, both semi-nicked and nicked catenated products were observed as for pMA21 (figure 4.8). The supercoiled sym-*res* substrate, pAL2115, recombined efficiently *in vitro*, under standard or permissive conditions, as seen for the wt-*res* substrate, pMA21. Since the restriction pattern for the monomer product of pAL2115 was identical to the dimer substrate, restriction of the products would only give a novel pattern of fragments if inversion between the two sites had occured. As shown in figure 4.8, restriction revealed no inversion products.

The symmetrical subsite I of sym-*res* contains an inverted repeat extending 60 bp. Although palindromic sequences of this length are stable *in vivo*, the isolated supercoiled DNA may have a potential to form a cruciform structure (Courey and Wang, 1983). Cruciforms of this size can extrude readily at temperatures varying from 0°C to 46°C, particularly if they are AT-rich. The AT-rich centre of the sym-*res* crossover site increases the potential of the sequence to form a cruciform at our recombination reaction temperatures (Zheng and Sinden, 1988: Courey and Wang, 1988). If sym-*res* extruded into a cruciform, this could interfere with recombination between the sites.

Formation of cruciforms can be detected by a two dimensional electrophoresis method (Courey and Wang, 1983). A mixture of topoisomers containing an equilibrium of molecules with and without a cruciform will run as a ladder in the first dimension on a native agarose gel. Cruciform extrusion removes negative supercoils, so molecules containing an extruded cruciform co-migrate with (non-extruded) topoisomers with fewer negative supercoils. Soaking the gel in an intercalating agent, chloroquine, relaxes each topoisomer. Electrophoresis in the presence

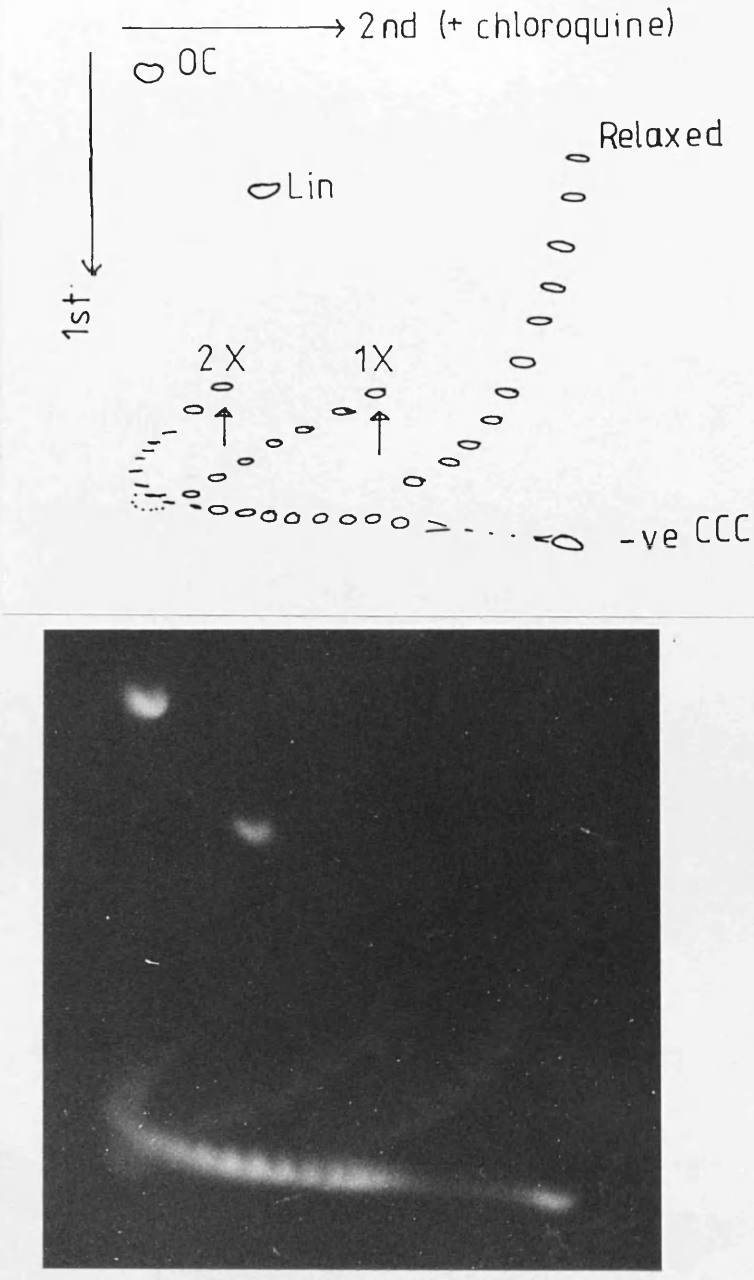


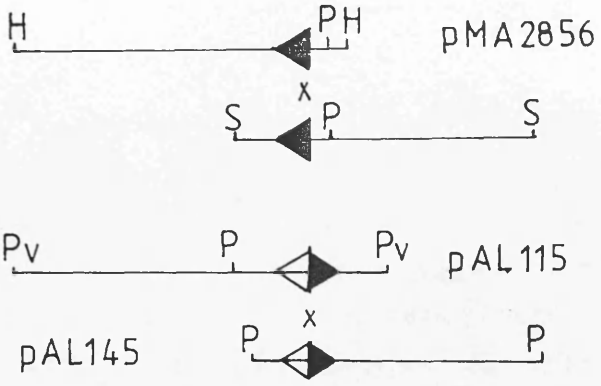
Figure 4.9. Two-dimensional electrophoresis of topoisomers of pAL2115. Topoisomers of pAL2115 were run in the first dimension on a native TAE agarose gel. After soaking in the presence of 25ug/ml chloroquine, the gel was turned 90° and run in a second dimension in the presence of the same concentration of chloroquine. Both sym-*res* sites had extruded a cruciform structure, as indicated by the migration of particular topoisomers, i.e. when supercoiling was decreased in the presence of chloroquine, those topoisomers that contained one (1X) and two (2X) cruciforms were separated from those that did not have any cruciform structure.

of chloroquine in a second dimension, separates non-cruciform topoisomers from those which had a cruciform prior to subjection to the intercalating agent.

Topoisomers of the sym-*res* substrate, pAL2115, were made by the action of topoisomerase I on the negatively supercoiled DNA at different concentrations of ethidium bromide. The two dimensional electrophoresis of the topoisomers of pAL2115 can be interpreted as indicating extrusion of two cruciforms, one at each site (figure 4.9). Although this experiment demonstrated that pAL2115 can form cruciforms, it is unknown if they are present in a significant proportion of the negatively supercoiled DNA actually used in *in vitro* recombination reactions, or whether they were induced at some point when the topoisomers were made (there was a heating step to 70°C after topoisomerase I treatment). The properties of the sym-*res* cruciform are unknown, i.e. at which temperatures the cruciform extrusion occurs.

Symmetrical sites of the *lox*/Cre system have also shown a potential to form a cruciform (Hoess *et al*, 1986). Supercoiled substrates containing the symmetrical *lox* sites did not recombine *in vitro*. However, the recombination properties of these sites were investigated by using linear substrates, which remove the possibility of cruciform extrusion. Symmetrical *gix* sites have a lower efficiency of recombination compared to the wild type *gix* sites of the G-inversion system (Mertens *et al*, 1988), as in the supercoiled substrates required cruciform formation is possible. Linear substrates are recombined by a FIS-independent Gin mutant protein and could be used to investigate the recombination properties of the symmetrical *gix* sites (Klippel *et al*, 1988b).

Although recombination of supercoiled substrates demonstrated that resolvase will delete between two directly repeated sym-*res* sites, no sign of inversion or fusion events was seen. Intermolecular recombination between two sym-*res* sites would show how the sites could



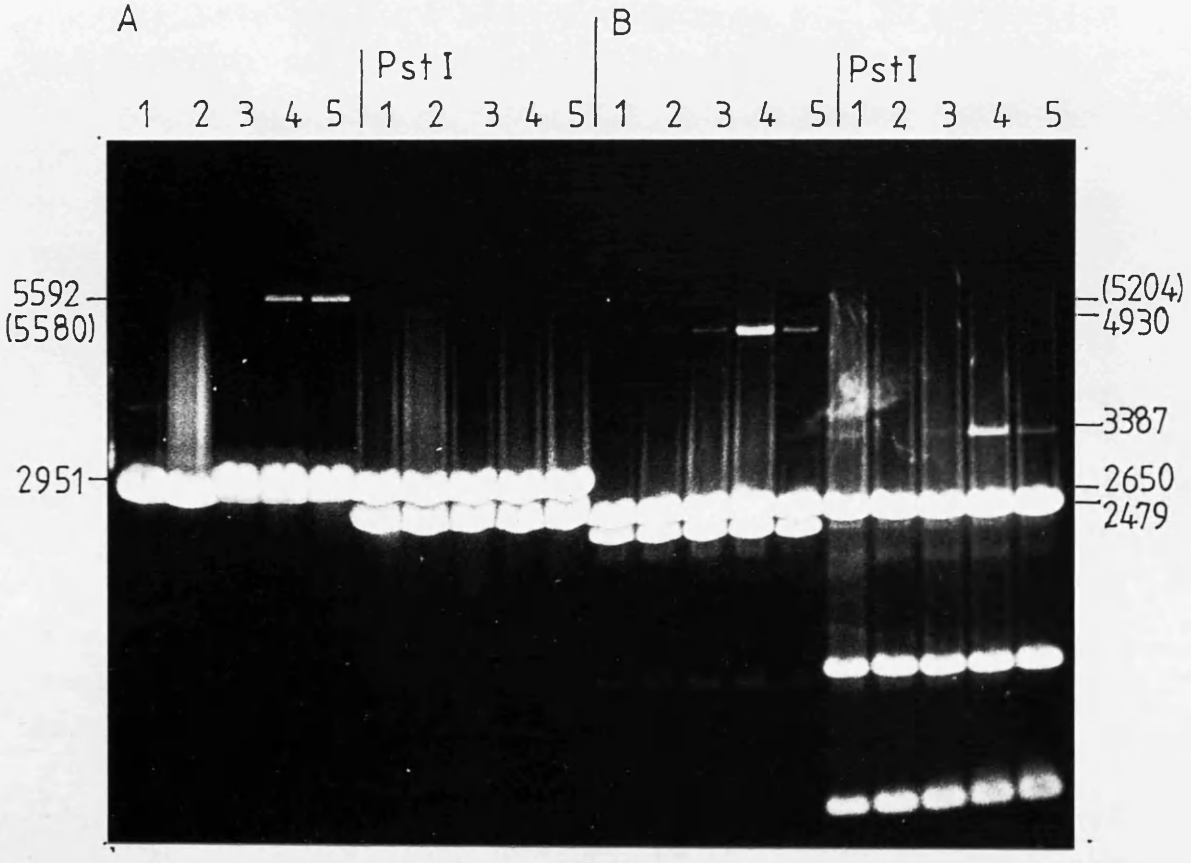


Figure 4.10. Intermolecular recombination of two different linear sym-res substrates. PstI-linearised pAL145 and PvuII-linearised pAL115 (both sym-res) in recombination buffer D were recombined by resolvase for 22 hours at 37°C. Two wt-res fragments (pMA2856 cut by either HindIII or SstI) were also recombined in the same conditions. The major products from both pairs of substrates were those of a `parallel' alignment of sites. Lanes 1-5:- 0, 139, 278, 556, 1112 nM resolvase respectively.

align when they are not in *cis* on a closed circular molecule. The parallel alignment of wt-*res* sites that resulted when two separate linear substrates recombined may have been influenced by the asymmetry in the crossover site. Since subsite I was shown to be functionally symmetrical, we would expect that subsites II and III in each site had aligned the crossover sites for recombination.

To check that asymmetry in subsite I cannot contribute to the alignment of two *res* sites, two different sym-*res* linear substrates (PstI cut pAL145 and PvuII cut pAL115) were recombined *in vitro*, under permissive conditions (figure 4.10). As a control, two different Tn3 wt-*res* linear substrates were also recombined in the same conditions (pMA2856 cut with either HindIII or SstI). For both pairs of substrates, the major product was that expected from the parallel alignment of *res* sites prior to strand exchange. We can therefore conclude that resolvase recognises the polarity of *res* when subsites II and III are present. For each pair of substrates, some products of the antiparallel aligment of sites were also observed, although these were less than 10% of the total recombination products. No more `antiparallel' recombination was observed for the sym-*res* substrates than for the wt-*res* substrates. Antiparallel alignment of sites had not been previously observed for intermolecular recombination between linear substrates with one *res* site each, but has also been observed for linear substrates carrying two *res* sites in *cis* (M. Stark, personal communication). Linearized pMA2631, where two wt-*res* sites are in inverted repeat, will permit inversion in permissive conditions, but a small amount of the incorrect `antiparallel' recombination product can be detected. We suggest that most of these aberrant products are a result of intermolecular events, as larger fusion products can be observed. The ability of resolvase to align two *res* sites correctly when they are in *trans* is therefore

usually maintained, but not always.

If synapsis of subsites II and III in both res sites is a requirement for correct site alignment, as suggested by our results, then removal of subsites II and III from one site should permit recombination in either the parallel or antiparallel sense in recombination of subsite I x wt-res linear substrates. When substrates pAL211 and pAL261 were linearized, no products were observed under permissive conditions, suggesting that both crossover sites required subsites II and III to synapse in linear molecules. However, it is unknown if the accessory sites are required or used in the antiparallel alignment of sites in non-supercoiled substrates.

4.4 An isolated symmetrical subsite I

A synthetic oligonucleotide of a symmetrical subsite I was subcloned from pAL3401 (figure 3.2) into the PstI site of a pBR322 derivative, pMA44 (figure 4.3) to give the construct pAL234. The symmetrical subsite I was composed from two right arms of the wild type subsite I, so that both arms contained the consensus sequence for resolvase recognition, and is equivalent to the perfectly symmetrical crossover site of sym-res. Binding affinities for the symmetrical subsite I and the isolated wild-type subsite I were shown to be similar by the gel binding assay (figure 3.14).

In light of the results using subsite I x wt-res substrates, pAL234 was expected to recombine in vitro, but in the resolution sense only. However, in vitro recombination of supercoiled pAL234 failed to give detectable products, even when incubated in the same reaction mix as a subsite I x wt-res substrate that recombined. The ability of an identical subsite in sym-res to act as a crossover site rules out any simple explanation for the failure of pAL234 to recombine. The

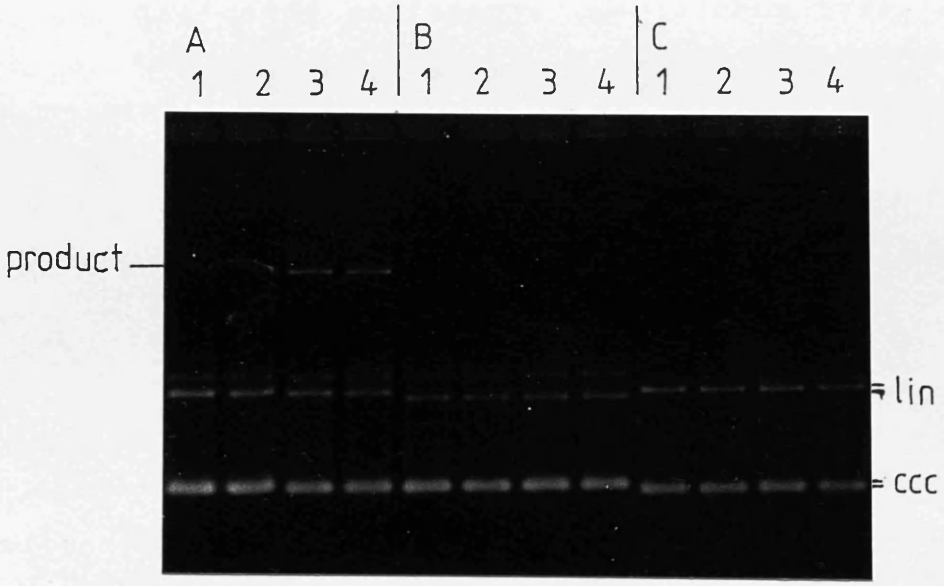


Figure 4.11. Intermolecular recombination between subsite I and wt-*res* sites, *in vitro*. Resolvase-mediated recombination between different combinations of circular and linear substrates was performed in recombination buffer C at 30°C for 19 hours. Products of recombination were only detected when both substrates contained a wt-*res* site.
Lanes 1-4:- 0, 139, 278, 556 nM resolvase.
(A) pMA2856 ccc x pMA2856 HindIII
(B) pMA2856 ccc x pAL3054 HindIII
(C) pMA2856 HindIII x pAL3054 ccc

position of the symmetrical subsite I (at the PstI site) in pAL234 is different from the position of the wild type subsite I in the other constructs (at the EcoRI site), but the sequences around the symmetrical subsite I also do not resemble subsites II and III when compared with *res* (figure 4.5B).

4.5 Intermolecular reactions using subsite I

In lambda integration, accessory sites flank both sides of the Int crossover site in *att*P. The recombinase Int and the accessory protein IHF bind these sites to form the synaptic intasome (Richet *et al*, 1986). The synaptic complex predicted for Tn3 resolution involves two complex recombination sites that interwrap with resolvase. A similar situation may arise in excisive lambda recombination, where the two crossover sites of *att*L and *att*R are flanked only to one side by accessory sites (figure 1.9). In excisive recombination, the proteins synapse the two sites for strand exchange in a supercoiling-independent manner.

A supercoiled and a linear molecule, each containing an intact *res* site, can be shown to recombine *in vitro* under permissive conditions. When either of these substrates was replaced by one containing just an isolated subsite I, no recombination products were observed under similar conditions (figure 4.11). It therefore appears that synapsis and recombination of *res* sites is only possible if both sites are intact, or if only one site is intact but both are on the same supercoiled substrate. It is possible that intermolecular events with an isolated subsite I are not feasible because the single wt-*res* alone cannot form an interwrapped structure and supercoiling of either (or both) substrates cannot favour interwrapping of non-specific DNA as it may for the intramolecular reaction.

An intermolecular reaction between a wt-*res* and a subsite I can be considered as analogous to the reaction between a lambda *att*L or *att*R with the simple *att*B site. Such combinations of sites have been shown not to recombine at high frequency, *att*B requiring an intact supercoiled *att*P partner which is capable of forming an intasome structure (Richet *et al*, 1988). The data for an isolated subsite I of *res* are consistent with the idea that wrapping is between two *res* sites rather than within one partner, as suggested for *att*P x *att*B.

DISCUSSION

1. Functional symmetry of *res*

Will resolvase fail to distinguish between resolution and inversion when subsites II and III are deleted? Unexpectedly, removing these subsites from one partner does not prevent resolvase from selecting resolution over inversion, and the recombination products are still singly linked catenanes. Instead, resolvase fails to ensure the correct alignment of the crossover sites in the supercoiled substrates. When the isolated subsite I was inverted with respect to the wt-*res* site, in pAL215 and pAL261, the `incorrect' antiparallel alignment of sites resulted, giving aberrant products in which two left ends were joined and two right ends were joined (figure 4.7). This is possible because there is a symmetrical central core dinucleotide, which permits complementarity within the short heteroduplex formed on strand exchange, regardless of the sense of site alignment. An isolated subsite I is therefore functionally symmetrical, even though it is not composed of perfectly symmetrical arm sequences. This implies that asymmetry within subsite I does not ensure the `correct' alignment of sites. The left and right arms of subsite I are treated as equivalent and

this is consistent with the idea that a dimer of resolvase binds to a site of dyad symmetry.

Tn3 *res* shares similarities to other *res* sites (figure 1.4). Although the central palindromic hexanucleotide of subsite I in Tn3 *res* enabled antiparallel recombination events to be observed, the core crossover sequence is not symmetrical in all *res* sites (e.g. IS101 *res*). In this respect, an asymmetrical core sequence is like the core crossover sequence of the related Hin, Gin, Cin and Pin invertase systems. The gamma-delta crossover site has been altered from the central AT dinucleotide to the CT dinucleotide with some effect on the strand cleavage and exchange by gamma-delta resolvase. However, this change was placed in a wt-*res* context, alongside subsites II and III, so what its effect on the alignment of crossover sites is unknown (Falvey and Grindley, 1987). We may predict that an asymmetrical crossover core sequence within *res* subsite I will prevent the aberrant antiparallel event in a substrate analogous to pAL215, but possibly only once the sites have been cleaved and when the strands fail to complement in the heteroduplex. Non-complementary core sequences in *hix* sites appear to be blocked for recombination. Once the sites have been cleaved by the Hin protein, the reaction appears to be stuck and cannot complete recombination (Johnson and Bruist, 1989). It has recently been shown that directly repeated *gix* sites are brought together by wild type Gin as for inverted sites, but in an antiparallel alignment for strand exchange (Klippel *et al*, unpublished). The strands are cleaved, but since the core sequences are not complementary, the recombinase rotates the strands 360^{o} before rejoining the sites, to generate knotted products (figure 1.6). When the symmetrical AT core sequence of the gamma-delta *res* site is replaced by the asymmetric AA, the altered site does not recombine with the wt-*res* site, but will recombine with an identical mutant site (Hatfull and Grindley, 1988).Therefore,

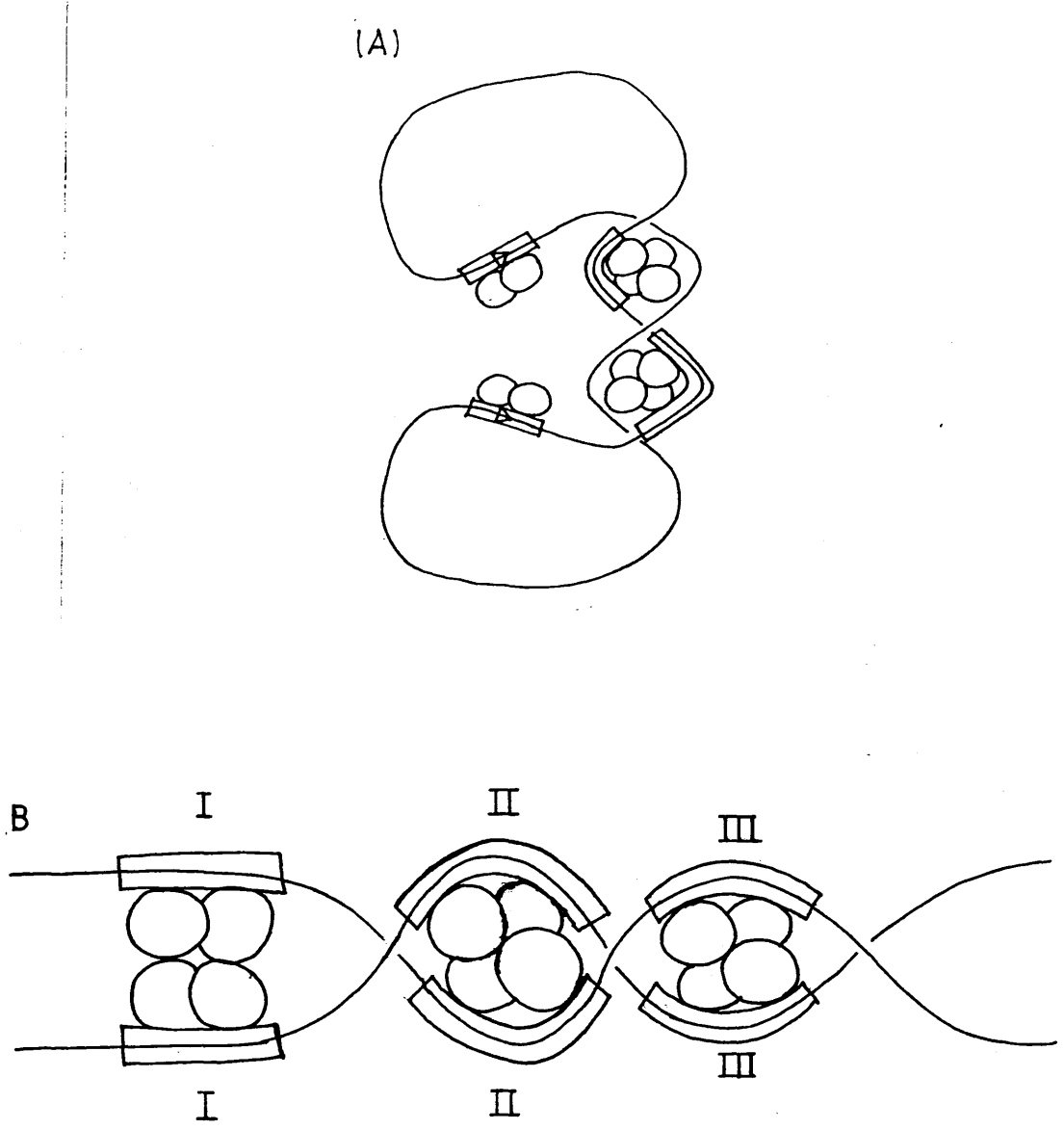


Figure 4.12.
(A) Diagrammatic representation of the predicted synapse structure for supercoiled subsite I x wt-*res* substrates. Resolution of these substrates gave simply catenated products, as seen for resolution between wt-*res* sites. Therefore, a similar synaptic structure can be proposed for substrates lacking subsites II and III in one *res* partner as for substrates with two intact wt-*res* sites.
(B) An alternative model of synapsis of *res* sites by wrapping of subsites II and III around resolvase.

recombination of *res* sites also appears to be blocked if the core sequences are not complementary.

Resolution is favoured when subsites II and III are removed from one partner, suggesting that they are not required in both partners to maintain topological selectivity of the reaction and products (a simple catenane). Although this is apparently inconsistent with the interwrapping model, the product topology suggests that three negative supercoils are trapped as in the wild type reaction. It is possible that vector DNA sequences wrap into the same synaptic complex as predicted for two wt-*res* sites, despite the absence of subsites. If this were possible, then clearly the wrapped complex is important in the reaction. In the invertase systems, FIS requires only one binding site, and in an analogous interwrapping event, may be employing non-specific sequences (figure 4.12). Alternatively, recombination between two crossover sites and just one set of subsites II and III may be by some completely different mechanism from that of the wild type reaction.

2. Do subsites II and III define the polarity of *res*?

We have already established that a wild type subsite I does not contribute to the polarity of *res*. The interwrap model hypothesis is that the presence of II and III in both partners ensures the `correct' alignment of sites, defining the left-to-right polarity of *res* (figure 4.2B). It is possible that subsites II and III define the polarity in some other way from our interwrap model (the arrangement of subsites within *res* is asymmetrical, and may impose polarity in some other way; figure 4.12). When a perfectly symmetrical subsite I is juxtaposed to subsites II and III in a wild type context, the resulting sym-*res* site is fully functional as a wt-*res* site. The presence of subsites II and III in both sites does, as expected, ensure the correct alignment of the now

perfectly symmetrical crossover sites, whose symmetry is disregarded. The asymmetry within subsite I found in the wt-*res* site is therefore not required for the function of *res* and is not essential for the `correct' parallel alignment of the two *res* partners. Subsite II and III can impose the correct alignment of *res* sites when present in both sites.

Although the asymmetrical arms of the *res* crossover site (subsite I) are not important for determining the polarity of *res*, asymmetrical arm sequences are found, and seem to have been conserved in other site-specific recombination systems. Each invertase system has two crossover sites composed of asymmetrical arm sequences and an asymmetrical core sequence. The whole crossover sites may (*gix* and *pix*) be perfect inverted copies of one another or imperfect inverted repeats (*hix* and *cix*). As already mentioned in the introduction to this chapter, the core asymmetry does not necessarily influence the selection of the inversion event, as this selection still holds when the core is symmetrized. However, asymmetry in the arms of the *hix* sites may still play a functional role in the polarity of the site.

It is possible that the invertase does not distinguish between the left and right arms of its crossover site and that the asymmetry of the site, as in the *res* crossover site, does not ensure the `correct' alignment of sites. Clearly, the asymmetrical core can influence the selection of the inversion event, but this is not the sole factor involved. Like the resolvase system, the invertase systems require factors in *cis* that lie outside the crossover site. The arrangement of the accessory functions with respect to the crossover sites in both the resolvase and invertase systems are themselves asymmetrical. Within *res*, the accessory sites ensure that the crossover sites are aligned in the parallel sense and this is consistent with the predictions of the interwrap model. For the invertase systems, the enhancer and FIS may

also impose the correct alignment of inverted sites, by the formation of a different interwrapped complex, but cannot correctly align directly repeated sites by the same complex (figure 1.6). Differences in the local structure of the synapse results in a preference for inversion instead of deletion. If FIS were to be used to stimulate synapsis of *res* crossover sites, we might expect a selection for inversion. Similarly, subsites II and III might be expected to impose a selection for resolution on the invertase sites. The results of mixing the accessory components of the two related systems are described in chapter 5.

Huber *et al* (1985) and Kahmann *et al* (1985) have shown that the enhancer site can be placed on either side of the crossover site in Cin or Gin inversion systems and efficiently promote inversions in a FIS-dependent manner. This suggests that invertases do not recognise their sites as asymmetrical with respect to the accessory enhancer site. For *res*, placing the accessory sites to the left of subsite I is expected to reverse the orientation of *res* but maintain the selection for resolution, as subsite I is functionally symmetrical. Indeed, placing subsites II and III adjacent to a perfectly symmetrical subsite I (sym-*res*) maintains the selectivity seen with wt-*res*.

Unlike the enhancer sequence of the invertase systems, the subsites II and III are located at a conserved position within *res* (figure 1.4). Accessory sites within *res* may be required for synapsis of two crossover sites and distancing them may prevent this synapsis, a possible reason for the conserved distance between subsites I and II. This distance has been altered by additional by 10 or 21 bp and still the resulting *res* sites were functional (Salvo and Grindley, 1988). However, the spacing between subsites I and II was not increased by more than 30 bp. Further increases in the space between subsites I and II, which allow recombination, may eventually become independent of the number of integral

turns of the helix, as the spacing becomes large enough to allow a degree of flexibility in aligning the sites. Synapsis of invertase crossover sites occurs independently of the distance of the enhancer site, but if the enhancer is located within 48 bp of the crossover site, then the substrate is unavailable for recombination (Johnson and Simon, 1985).

Even though the asymmetric flanking arms of lambda attP dictate the polarity of the crossover sites, with regard to the initial strand cleavage site, the core sequence is also asymmetrical. A symmetrical core sequence cannot be made for lambda att sites, as for FRT and lox sites, because the core is composed of an odd number of nucleotides (7 bp). The crossover site is composed of Int recognition arms flanking the asymmetric core. The left and right arms are not identical in the wild type att sites. Although each arm sequence has a different affinity for Int, where att sites have been constructed with identical Int-recognition arm sequences in the crossover site, recombination still proceeds as for wild type att sites (Nunes-Duby et al, 1987). Therefore, the asymmetry of the arm sequences of the Int crossover site do not contribute to the polarity of the sites.

If the core sequences of both attP and attB were symmetrical, they would be expected to recombine in either the parallel or antiparallel alignment in an integrative event. However, attL and attR might be expected to only recombine with the sites in the `correct' alignment, since this may be governed by the wrapping of arm sequences in the excisive synaptic complex. This would be similar to the alignment shown for sym-res sites. Similar intermolecular events between the symmetric lox and FRT sites were not influenced by any additional accessory proteins or binding sites, and thus recombination occured in all possible ways. However, in the wild type FRT site an additional arm of the crossover site is found adjacent to and in direct repeat to one arm of FRT. Although this

site is not required for recombination, its effect on the polarity of the site has not been investigated.

Transposition in the phage Mu has recently been shown to require an operator, or 'enhancer', site in addition to the inverted Mu ends (Leung *et al*, 1989). The transposase, MuA, only acts at inverted sites in *cis* on a supercoiled substrate (Craigie and Mizuuchi, 1986), but all these experiments included part of this enhancer site, which is also recognised by MuA. Transposition requires the operator in a particular orientation with respect to at least one of the ends, but can be positioned at a variety of distances. It remains to be seen how the accessory site influences the selection of inverted Mu ends.

3. Role of the subsites II and III in defining topology and in synapsis

In the synaptic complex proposed for *res*/resolvase, subsites II and III are needed in both recombination partners. This ensures that there is resolution of direct repeat sites only. In the *in vitro* recombination of substrates with subsites II and III in only one *res* partner, the isolated subsite I unexpectedly reacts to give resolution products only. The selection for resolution is maintained, surprisingly, when the subsite I is inverted with respect to the wt-*res* site, whereas inverted sites which both contain subsites II and III are blocked for recombination. The presence of subsites II and III in both sites, as direct repeats, gives very efficient resolution compared to subsites II and III in one site only. Subsites II and III are needed for resolvase to determine the relative orientation of the two crossover sites.

The bias towards resolution and the unique singly linked catenane products obtained when only one site contains subsites II and III, suggests that their presence in one site is sufficient to create a similar synaptic

complex as when they are present in both sites. We have previously suggested that synapsis is initiated by the random collision and wrapping of the accessory sites of the two partners. This would clearly not be possible if accessory sites are only present in one partner. Aligning the two crossover sites might be possible by random collision, but the strand exchange event could be prevented if the correct synaptic structure is not present. It is possible that resolvase bound at one site may be enough to bend the *res* site, so that it can plectonemically wrap with non-specific DNA adjacent to the isolated subsite I (figure 4.12). Thus, products are only observed when the predicted favourable resolution synaptic structure forms, resulting in the singly linked catenanes. Alternatively, the two sites could slither past one another in a supercoiled molecule and subsites II and III in one site plectonemically wrap around the second site as the crossover sites are aligned. In both cases, the alignment of crossover sites might be expected to be inefficient and could be a reason for the reduced efficiency of the reaction. Subsites II and III in the second *res* partner, which is inverted with respect to the first, will align the crossover sites as a result of the same local synaptic complex being formed, but which imposes unfavourable topology for the strand exchange reaction (figure 1.7). Inverted sites therefore prevent the crossover sites from aligning in any other way.

Substrates containing only one copy of subsites II and III were shown to recombine only when supercoiled. For an alignment of sites by the slithering mechanism, as proposed by Benjamin and Cozzarelli (1986), the substrates are required to be supercoiled and to have sites in *cis* Therefore, if slithering is required to align the sites via an interwrapped synaptic intermediate, subsite I x wt-res substrates may also only recombine as supercoiled molecules, with the selection for deletion only. However, the slither of supercoiled DNA does not account for many

other features of Tn3 recombination. Intermolecular reactions, *in vitro*, of unlinked molecules are still capable of aligning sites in a parallel sense in a subsites II and III dependent manner (as seen for sym-*res*). These events imply that subsites II and III can form some synaptic structure, but not necessarily the same one predicted for supercoiled intramolecular substrates and are thus imposing polarity on the crossover site of *res*. Intramolecular reactions where one *res* lacks subsites II and III have a reduced efficiency in supercoiled molecules. It may be that intermolecular events for subsite I x wt-*res* have a further reduced chance of collision and interwrapping and thus recombination drops to unobservable levels. Intermolecular recombination between two wt-*res* sites is normally reduced compared to supercoiled intramolecular resolution. An exception appears to be the efficient fusion of a relaxed simple catenane substrate (Stark *et al*, 1989a). Clearly, strand exchange can occur when supercoiling is absent, but the absence of supercoiling and its effect on strand exchange may be a contributing factor to the reduced recombination efficiency of intermolecular substrates.

In summary, the absence of subsites II and III from one partner does not prevent the same synaptic selection as for two intact *res* sites. The accessory sites in *res* may be implicated in the synapsis, the strand exchange or both.

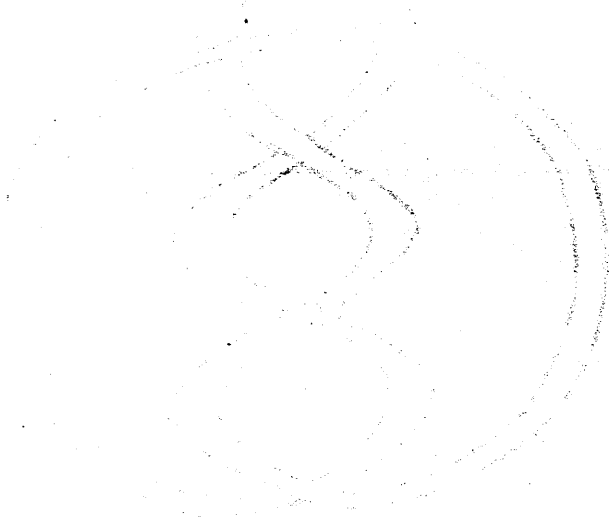
Whatever the mechanism for synapsis, resolvase recognises the relative orientation of *res* sites only when subsites II and III are present in both sites, but is still capable of directing the reaction for resolution when these subsites are absent from one *res* partner. There is evidence from other systems that isolated crossover sites may not be able to synapse without the presence of accessory sites. Cleavage can occur at *hix* sites by the Hin invertase in the absence of FIS and supercoiling, under conditions that include ethylene

glycol, but exclude Mg^{2+} ions (Johnson and Bruist, 1989). Adding back FIS will increase the amount of cleavage, but only if the substrate is supercoiled. When Mg^{2+} ions are added back, favourable recombination conditions resume, and cleaved molecules are religated. Recombinants result only if both FIS and supercoiling are provided. Hin is therefore capable of synapsing *hix* sites in the absence of accessory functions and also initiating strand exchange, but cannot generate a synaptic complex that is competent for recombination, as inversion is never observed. A productive inversion synapse will only result if FIS is supplied and the substrate is supercoiled. Cleavage of the sites also occurs under standard recombination conditions in substrates which do not have homologous core sequences in the crossover sites, suggesting that DNA homology at the crossover core is not essential for synapsing the sites and initiating the strand exchange reaction. These observations suggest that a FIS-dependent synapse controls the selection for inversion and correctly aligns the sites. A similar experiment has not been attempted with subsite I of *res*, where subsites II and III may be expected to enhance the cleavage at the crossover site in a way analogous to FIS enhancing cleavage at *hix* sites. Our wt-*res* substrates are capable of recombining in the absence of Mg^{2+}. Under these conditions, and adding ethylene glycol, some cleavage was observed for a resolution substrate, but which mainly recombined.

SUMMARY

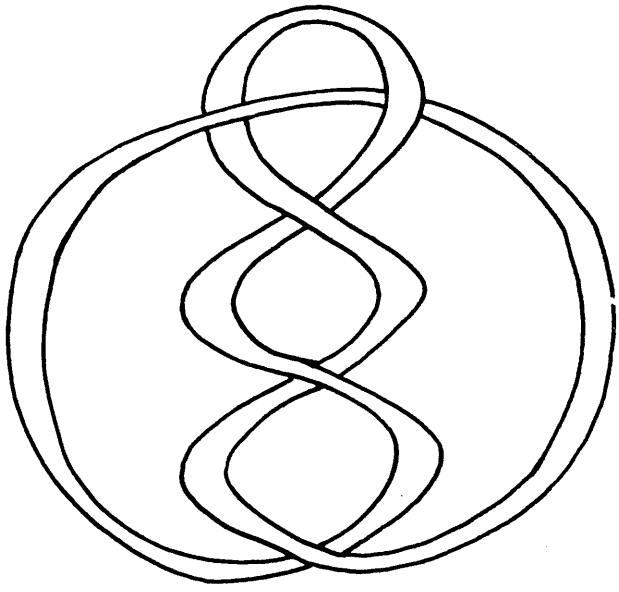
In the variety of site-specific recombination systems, many display similar properties in determining the selectivity of the reaction and in the alignment of sites. Where accessory binding sites and proteins are required, supercoiling is also usually essential, as if some intrinsic property of the accessory factors requires

supercoiling in its function. The accessory sites in res have now been shown to influence the alignment of otherwise symmetrically functional crossover sites, whereas for the FLP and Cre systems, only the asymmetry of the core sequence has been shown to influence the recombination event. For the Mu G- and P1 C-inversion systems, mutant Gin and Cin proteins are FIS-independent, and the inversion properties of the mutant system are more like the FLP and Cre systems. The next step for resolvase is to isolate similar mutants which can recombine independently of accessory sites and may help us to understand their role. Mutagenesis of resolvase is currently being pursued in our laboratory.



CHAPTER FIVE

THE FUNCTIONS OF ACCESSORY FACTORS



INTRODUCTION

The differences in the reaction selectivity of the resolvase and the Gin, Hin, Cin, Pin invertase systems may be a reflection of their requirements for different accessory proteins and sites. Resolvases and invertases have approximately 30% amino acid homology and act at similar crossover sites (figures 1.2 and 1.4). Resolution between two *res* sites requires two additional resolvase recognition sites (subsites II and III) at each *res* site for efficient recombination (chapter 4). For inversion between invertase crossover sites, a single enhancer site, *sis*, and a host protein FIS are necessary (Kahmann *et al*, 1985; Johnson and Simon, 1985; Huber *et al*, 1985). The enhancer site is required in *cis* with respect to the crossover sites, but does not need to be located at a fixed distance from or orientation to the crossover sites. In both the resolvase and invertase systems the accessory sites and proteins have been proposed to form a synaptic intermediate of a defined structure; the result of a precise strand exchange rotation in either case is a topologically unique product (figure 1.6).

If the accessory functions are essential for defining a specific synaptic intermediate (and, consequently, specific products), then an exchange of accessory functions between the systems would be expected to impose the reaction selectivity of the other system on the crossover sites (figure 5.1). By replacing a crossover site of *res* with an invertase crossover site, subsites II and III at each site would be expected to impose a resolution selectivity in intramolecular recombination of directly repeated sites, resulting in simple catenated products. An enhancer element placed in *cis* with two isolated *res* crossover sites should result in inversion between the sites and a free circular product, if both resolvase and FIS are provided. Substrates of both these types were designed and tested *in vivo* and *in vitro*. We

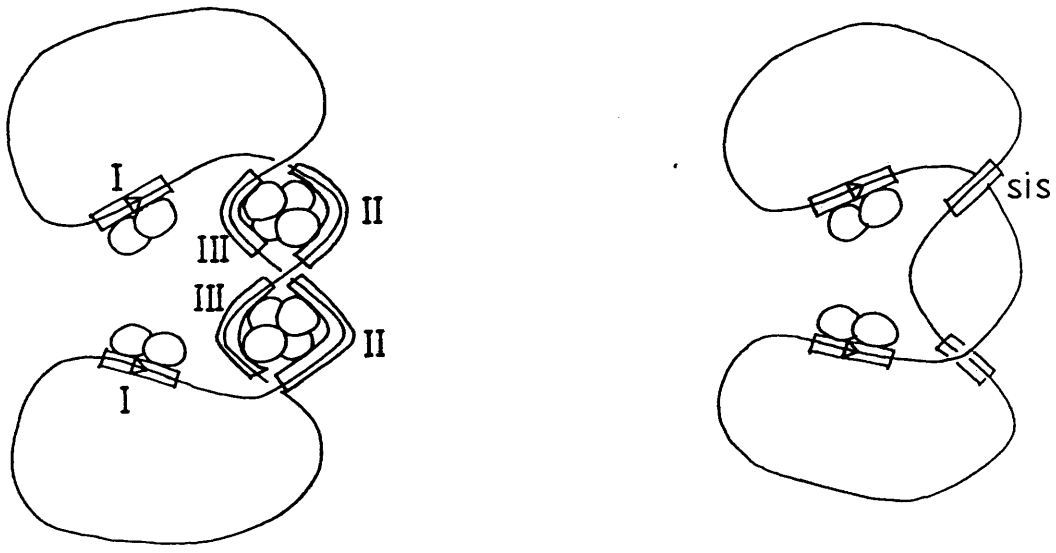


Figure 5.1 Comparison of proposed synaptic intermediates for the resolution and DNA inversion systems. Directly repeated res sites are proposed to align for recombination by plectonemically wrapping subsites II and III around resolvase. Inverted sites for DNA invertases (e.g. Gin) are proposed to require FIS and an enhancer site, sis, to form the synaptic structure depicted.

already know that *res* subsite I is functionally symmetrical (chapter 4) and therefore an inversion between these sites in preference to resolution would probably be a consequence of the enhancer and FIS protein.

Resolvase selection for resolution of directly repeated *res* sites is maintained for supercoiled molecules *in vitro*. Inversion between inverted *res* sites has also previously been reported to be inefficient *in vivo* (Reed, 1981; Chiang and Clowes, 1982; Kitts *et al*, 1983). Inverted products were detected, however, from recombination *in vivo* of substrates containing inverted R46 *res* sites and resolvase in *cis* (Dodd and Bennett, 1986). Since the presence of an enhancer site and FIS stimulates inversion between inverted crossover sites by an invertase, it was possible that FIS or another host factor can affect resolvase-mediated recombination between inverted *res* sites. Therefore, the effect of FIS on inversion between *res* sites *in vivo* and *in vitro* was investigated.

Plasmids containing four directly repeated sites exhibit, in general, an adjacent site preference of resolution. However, in some substrates with four or more sites, resolution between some non-adjacent sites occurred, but other non-adjacent events were prevented from recombining (J. L. Brown, 1986). This was interpreted as a consequence of one diagonal (non-adjacent) pairing of sites preventing or `shadowing' the intervening site from forming a productive synapse with a fourth *res* site. The interfering diagonal pair of sites can have the same shadowing effect in direct or inverted repeat, as both *res* sites are probably involved in a synaptic structure, interfering with the productive synapsis of the reciprocal non-adjacent sites (figure 5.1B). If subsites II and III are required to form a synaptic structure as the sites are aligned, then subsites II and III alone may be sufficient to cause a shadowing effect in multi-*res* site plasmids. Substrates were made to test the effect of *res* accessory

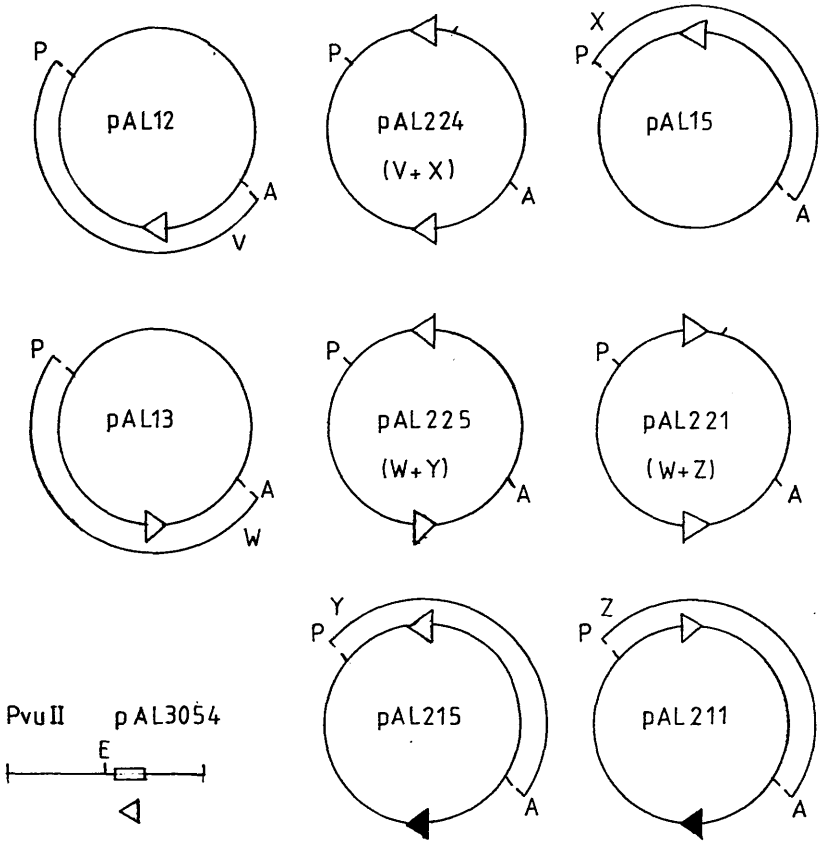


Figure 5.2 Construction of substrates containing two isolated crossover sites. A PvuII subsite I fragment from pAL3054 was inserted into the PvuII site of pBR322 to generate pAL12 and pAL13. PstI-AvaI fragments from pBR322-derived constructs containing subsite I in the EcoRI site (see figure 4.3) and from pAL12 or pAL13 were exchanged. Constructs were selected by size and checked by restriction.

Pst1-HindIII product sizes (bp) expected:-

	Substrate	Resolution H	Resolution P	Inversion
pAL221	986, 3922	2312,	2596	3170, 1738
pAL225	" "	2449,	2459	3033, 1738
pAL224	" "	2369,	2539	2953, 1955

P=PstI, H=HindIII, A=AvaI, E=EcoRI.

sites or an isolated *res* crossover site on resolution of multi-*res* site constructs, to gain insight into the minimum requirements for synapsis of sites.

RESULTS

5.1 *In vitro* recombination between two isolated *res* crossover sites

In an attempt to see if resolvase can recombine *res* crossover sites in the absence of subsites II and III, three substrates containing two isolated crossover sites (subsite I) of Tn3 *res* were constructed from pBR322-derived plasmids, by fragment exchange (figure 5.2). Two of these constructs contained two copies of subsite I in inverted repeat; they differ in the relative orientations of the pairs of sites with respect to the plasmid sequences (pAL221 and pAL224). Since subsite I is functionally symmetrical, all three substrates with two copies of the isolated subsite I were expected to participate in both deletion and inversion reactions, regardless of the relative orientation of the two sites.

In vitro recombination of supercoiled pAL221, pAL225 and pAL224 was attempted under both standard and permissive recombination conditions with purified resolvase. As in the recombination assays with subsite I x wt-*res*, any recombination products should have been detectable after a restriction enzyme digest. As a positive control for resolution, the substrate pMA21 with two wt-*res* sites in direct repeat was used alongside the subsite I x subsite I substrates, in all experiments. No products of recombination were detected for subsite I x subsite I constructs under any of the reaction conditions tested (figure 5.3).

When subsites II and III were removed from one *res*

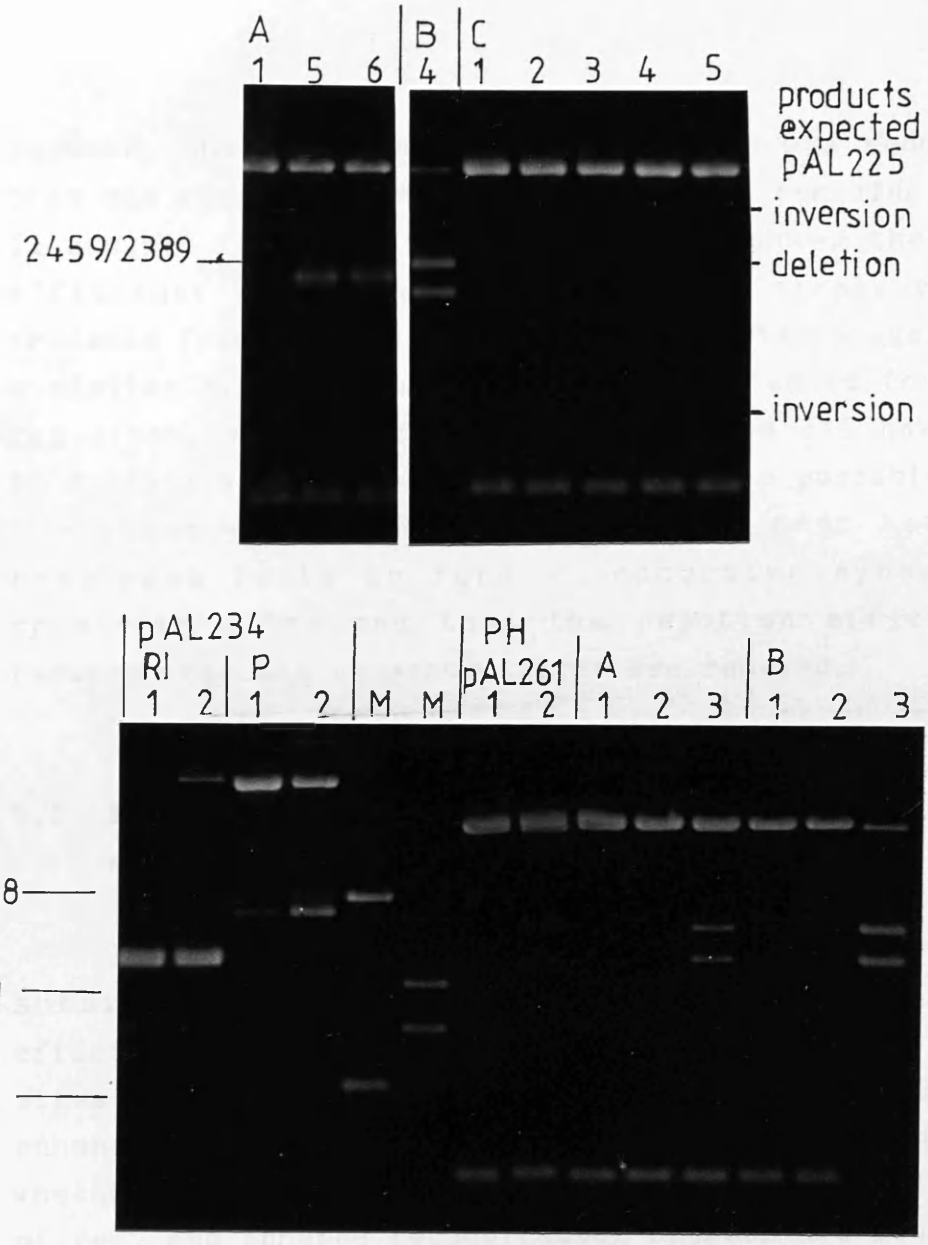


Figure 5.3 In vitro recombination of subsite I x subsite I substrates.
(A) Titration of pAL225 with Tn3 resolvase in recombination buffer B, 25mM NaCl, for 18 hours at 37°C. (lanes C 1-5). Recombination products for pAL265 (subsite I x wt-res) (A) and pMA21 (B) under the same conditions are also shown. Products were digested by PstI and HindIII and electrophoresed on a 1.2% agarose gel. Lanes 1-6: 0, 49, 98, 197, 394 and 789 nM resolvase respectively.
(B) pAL221 (A) and pAL224 (B) were incubated with 394 nM (except lane 1: 0 nM) Tn3 resolvase in recombination buffer C, 25mM NaCl, for 16 hours at 37°C. For pAL221, some pMA21 substrate was added for the final 35 min of incubation (lane 3) to show that the resolvase present in the reaction was still active. pAL234 was also incubated under the same reaction conditions. Deletion products of pAL234 are expected to be 1711 and 2928 bp (restricted by either EcoRI or PstI).

partner, the efficiency of the reaction was reduced, but products were still observable. However, removing subsites II and III from both sites apparently reduced the reaction efficiency to undetectable levels. The simple catenated products from subsite I x wt-*res* substrates suggested that a similar synaptic intermediate was formed as for two wt-*res* sites, and therefore subsites II and III have a role in forming a synaptic intermediate. It is possible that in the absence of subsites II and III from both sites resolvase fails to form a productive synapse with crossover sites and thus the reaction efficiency is reduced when the accessory sites are removed.

5.2 The effect of an enhancer site, *sis*, on the *in vitro* recombination of *res* sites

In a *res* recombination reaction, the presence of subsites II and III in both sites results in a high efficiency of reaction. Recombination between invertase sites is stimulated by the host protein FIS acting at an enhancer site, *sis*. It was therefore interesting to ask whether *sis* could functionally replace subsites II and III of *res*, and enhance recombination between two isolated *res* crossover sites. Substrates containing *sis* were constructed by simply inserting a *sis* fragment from the Mu *gin* sequence into the BamHI site of several existing substrates (figure 5.4). In addition to subsite I x subsite I substrates, it was also possible to test the effect of *sis* and FIS on the recombination properties of substrates with wt-*res* sites.

The addition of FIS to a supercoiled *sis*$^+$ subsite I x subsite I substrate might be expected to stimulate resolvase-mediated recombination as FIS stimulates invertase-mediated recombination. If FIS is involved in the formation of the synapse responsible for inversion selection, then inversion (but not resolution) of all

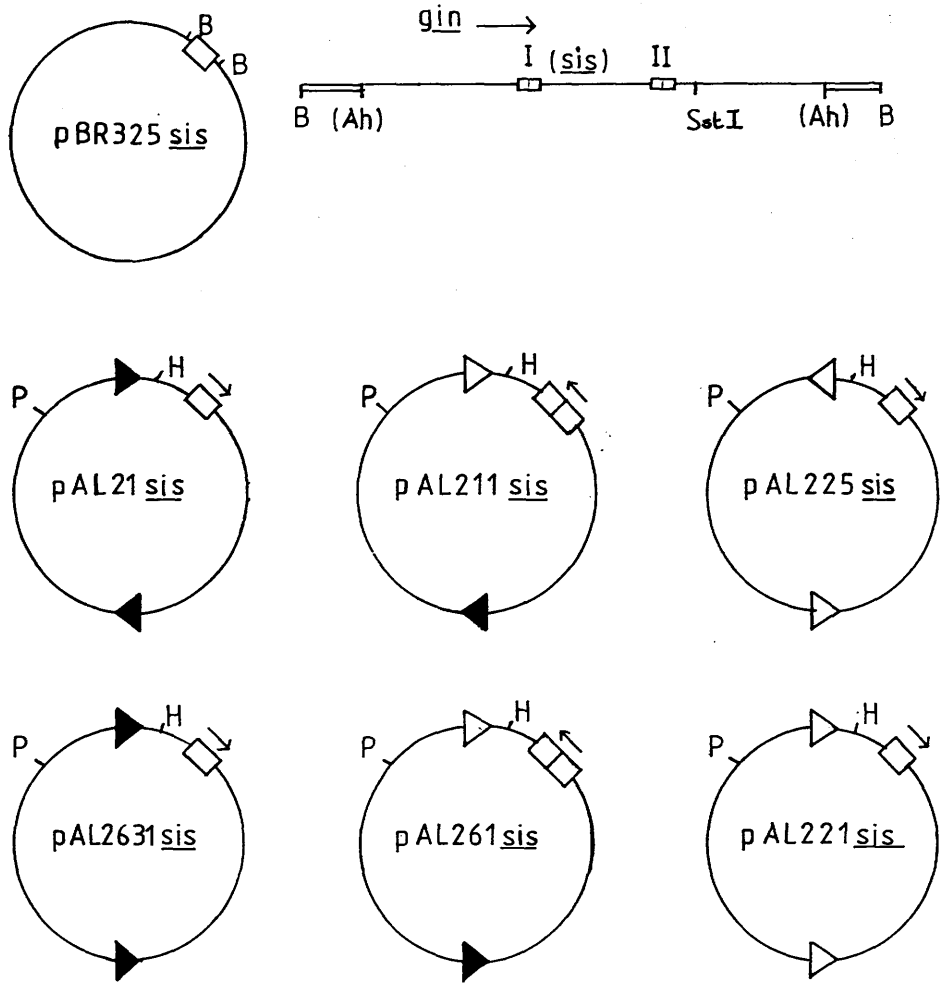


Figure 5.4 Construction of substrates containing an enhancer site, sis, from the gin gene sequence of Mu. BamHI linkers had been added to a 160bp AhaIII fragment which has the enhancer site. The BamHI fragment was removed from pBR325:sis and inserted into the BamHI sites of pMA21, pMA2631, pAL211, pAL261, pAL225 and pAL221. New sis^+ constructs were selected for the loss of Cm^r.

Approximate, expected PstI-HindIII product sizes (bp):-

	Substrate	Resolution H	Resolution P	Inversion
pAL21sis	1065, 4042	2311,	2796	3270, 1837
pAL2631sis	" "	2331,	2776	3290, 1817
pAL211sis	986, 4222	2232,	2949	3450, 1758
pAL261sis	" "	2252,	2929	3470, 1738
pAL221sis	" 4102	2312,	2776	3350, 1738
pAL225sis	" "	2449,	2693	3213, 1875

P=PstI, H=HindIII, B=BamHI, Ah=AhaIII.

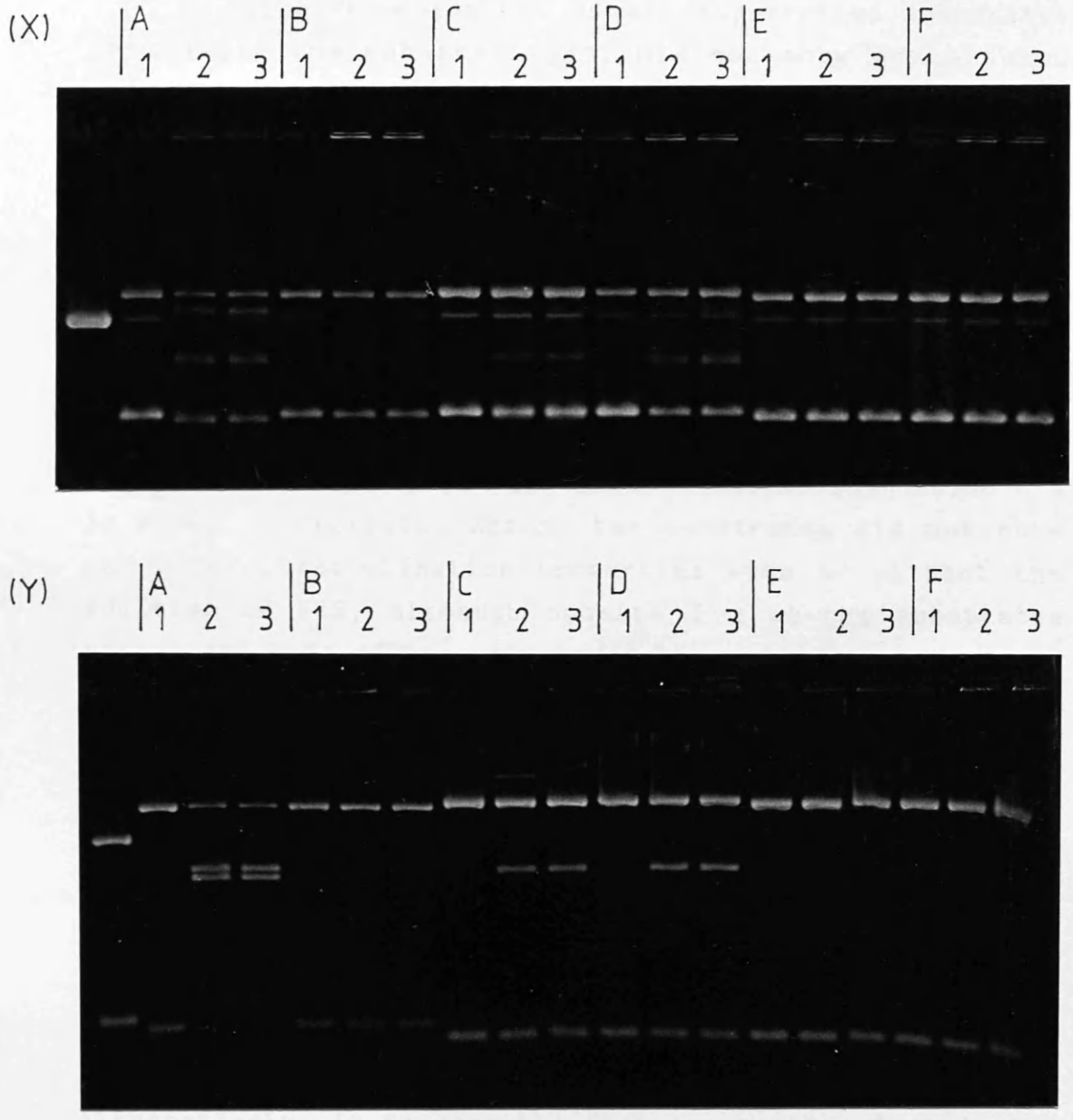


Figure 5.5 *In vitro* recombination of *sis*$^+$ resolvase substrates. pAL21*sis*, pAL2631*sis*, pAL211*sis*, pAL261*sis*, pAL225*sis* and pAL221*sis* were incubated in the presence of 0 or 315nM Tn3 resolvase (lanes 1 + 2 respectively), in recombination buffer C, 50mM NaCl for 22 hours at 37^{o}C. 0.5 ug/ml of FIS (Berlin) were added to reaction samples in lane 3. The uncut reaction products were electrophoresed on a 1% gel(X), PstI-HindIII restricted products on a 1.5% gel(Y).

A = pAL21sis D = pAL261sis
B = pAL2631sis E = pAL225sis
C = pAL211sis F = pAL221sis

subsite I x subsite I substrates would be expected.

In vitro recombination of all supercoiled substrates containing the enhancer, *sis*, did not show any altered recombination properties in the presence or absence of 0.25 ug/ml FIS (gift from R.Kahmann) under standard or permissive conditions (figure 5.5). All substrates behaved in an identical manner to their non-*sis* equivalent parents, which were also not affected by the addition of FIS to resolvase reactions. Since our conditions differ from the invertase *in vitro* reaction conditions, resolvase reactions were also carried out under conditions similar to invertase conditions (10% glycerol, 50mM NaCl, 10mM $MgCl_2$, 50mM Tris/HCl pH 8.2, 5mM spermidine), in which FIS is known to function. Again, the substrates did not have any altered recombination properties with or without the addition of FIS, although subsite I x wt-*res* substrates recombined less efficiently under these conditions.

FIS did not interfere with the resolution of pAL21*sis* (directly repeated wt-*res* sites) and did not enhance any recombination between inverted wt-*res* sites of pAL2631*sis*. The presence of the enhancer site and FIS appears not to stimulate recombination between two *res* crossover sites, and does not have any effect on resolvase-mediated recombination when one or both sites have subsites II and III. It is possible that the proposed *sis*/FIS synapse cannot compete with the proposed wt-*res*/resolvase synapse and therefore the selection for resolution is held. Alternatively, it is possible that any synapse formed with FIS in substrates with isolated *res* crossover sites might not be functional for resolvase-mediated recombination.

5.3 The effect of the enhancer site on the *in vivo* recombination of *res* sites

In vivo recombination of pAL221 and pAL221*sis* was attempted in the presence of a compatible *tnpR*$^+$ plasmid,

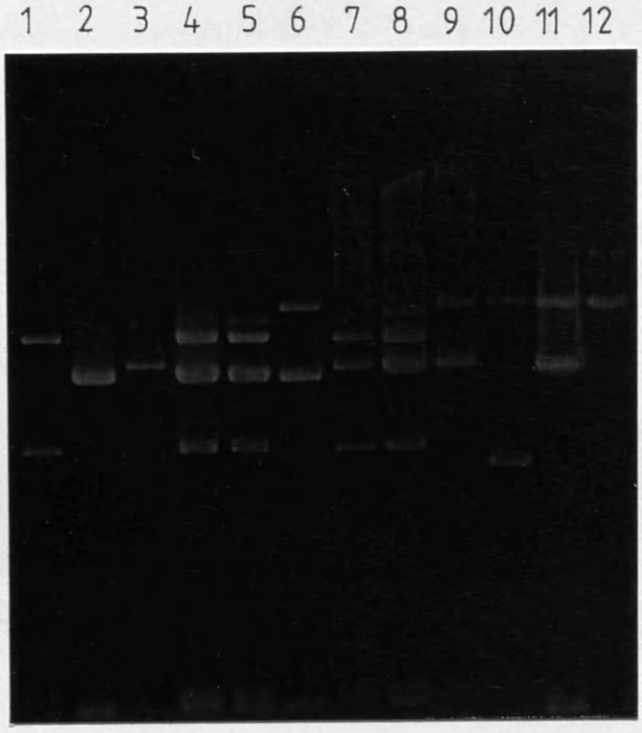


Figure 5.6 *In vivo* recombination of resolvase inversion substrates. Substrates pMA2631 and pAL2631*sis* were complemented with pPAK316 (see figure 5.16) for approximately 30 or 70 generations in strain DS902. The plasmid DNA was extracted and digested with PstI and HindIII. Inversion products are indicated. Both substrates also gave inversion products in the presence of pCIA70 for 30 generations. The DNA in each lane was PstI-HindIII digested except for lane 12, which was HindIII digested only (pCIA70 has no PstI sites). 1.2% agarose gel.

Lane			
	1	pPAK316	
	2	pMA2631	
	3	pAL2631*sis*	
	4	pMA2631 + pPAK316	30 generations
	5	pMA2631 + pPAK316	70 "
	6	pMA2631 + pCIA70	30 "
	7	pAL2631*sis* + pPAK316	30 "
	8	pAL2631*sis* + pPAK316	70 "
	9	pAL2631*sis* + pCIA70	30 "
	10	pMA21 + pCIA70	30 "
	11	pAL225 + pCIA70	30 "
	12	pCIA70	

pPAK316 or pCIA70, in DS902, but no products of recombination were detected after 100 generations (figure 5.12). No products had been observed in similar *in vivo* recombination experiments with substrates that lack subsites II and III at one *res* site; the failure to detect products *in vivo* for pAL221 was not unexpected. The presence of *sis* in the isolated site I substrate (pAL221*sis*) did not have an effect on the *in vivo* recombination properties of this substrate.

Two wt-*res* sites placed in inverted repeat on a supercoiled substrate (pMA2631: figure 5.11) did not recombine *in vitro* (chapter 4). However, in the presence of a compatible *tnp*R$^+$ plasmid, pPAK316, some inversion products were detected by restriction analysis of plasmids isolated from a *rec*A background (DS902). Approximately 5% of the pMA2631 substrate had inverted after 30 generations (figure 5.6). To test if the presence of an enhancer site, *sis*, and the FIS protein could stimulate this inversion reaction *in vivo*, the experiment was repeated as above but with the pAL2631*sis* substrate (we presume that DS902 is *fis*$^+$). Restriction of pAL2631*sis* isolated after 30 generations in the presence of pPAK316 revealed the same levels of inversion products as for pMA2631.

Inversion between two Tn3 *res* sites *in vivo* has also been investigated by P.Haffter and T.Bickle (personal communication). Two substrates, pCIA80 and pCIA83, were constructed containing inverted *res* regions from Tn3, and were identical except that pCIA83 also contained the enhancer sequence from the *cin* gene of bacteriophage P1 (figure 5.7). Their preliminary results indicated that the *sis*$^+$ plasmid inverted very efficiently *in vivo* when complemented with a compatible *tnp*R$^+$ plasmid, pCIA70. This phenomenon was subsequently investigated in our laboratory; the plasmids were kindly provided by P.Haffter. Both substrates inverted >70% in 30 generations, in the presence of pCIA70, with no detectable difference in frequency between the two substrates (figure

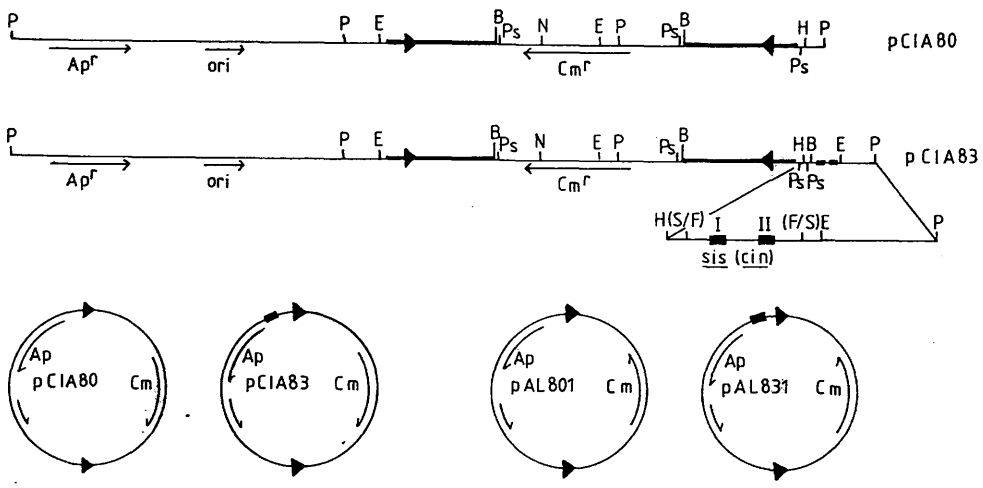


Figure 5.7 Diagrammatic representation of *res* inversion substrates. pCIA80 and pCIA83 are pUC18-derived and were constructed by P. Haffter in Basel. Both substrates contain 600 bp of Tn3 sequence, from the RsaI site in *res* subsite III and through some of the *tnpR* gene to a BamHI site. pCIA83 was made from pCIA80 by replacing a HindIII-PvuII section with a *sis*$^+$ fragment from the *cin* gene. The inverted forms of pCIA80 and pCIA83 (pAL801 and pAL831 respectively) were made by recombination *in vivo* (see figure 5.8). The directions of transcription of antibiotic resistance genes are indicated for the substrates (+) and their inverted (-) forms. pAL802 and pAL832 were made Cm^r by filling in the unique NcoI site within the *cat* gene.

P=PvuII, E=EcoRI, Ps=PstI, B=BamHI, N=NcoI, H=HindIII, F=Fnu4HI, S=SmaI

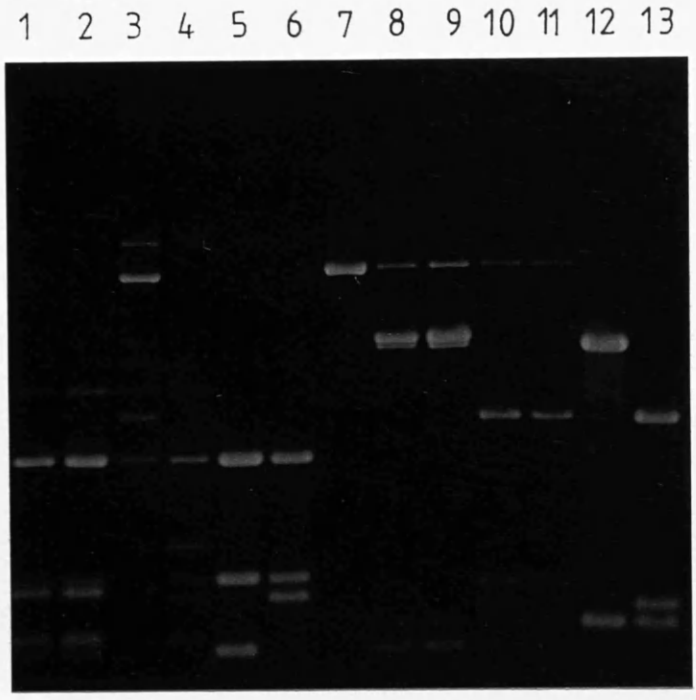


Figure 5.8 *In vivo* recombination of pCIA80 and pCIA83. Both substrates were recombined *in vivo* (in DS902 for 30 generations) in by complementation with pCIA70 (*tnpR*$^+$). For both pCIA80 and pCIA83, no difference in the amount of inversion products was detected when IPTG was included in the media to induce resolvase expression from the *plac*UV5 promoter of pCIA70. The 1.2% gel shows isolated plasmid DNA restricted by either EcoRI or PvuII.

Lane		
1	pCIA80 + pCIA70	PvuII digested
2	" " + IPTG	"
3	pCIA83 + pCIA70	"
4	" " + IPTG	"
5	pCIA80	"
6	pCIA83	"
7	pCIA70	EcoRI digested
8-13	as lanes 1-6, except digested with EcoRI	

PvuII	fragment sizes:-	pCIA80	1452, 1060, 2364
		pCIA83	1452, 1339, 2364
		pAL801	1127, 1385, 2364
		pAL831	1127, 1664, 2364
EcoRI	fragment sizes:-	pCIA80	1172, 3704
		pCIA83	1172, 1263, 2720
		pAL801	1051, 3825
		pAL831	1051, 1486, 2720

5.8). Therefore, the addition of an enhancer site to two different Tn3 *res* substrates (pMA2631 and pCIA80) did not alter the apparent rate of recombination between the inverted sites. However, the inversion frequencies of pMA2631 (complemented by pPAK316) and of pCIA80 (complemented by pCIA70) were different. One possible reason for this difference could be the different source of resolvase. In pPAK316, resolvase is expressed from its auto-regulated promoter in *res*, whereas in pCIA70, resolvase is expressed from a stronger promoter, *plac*UV5 (figure 5.16). Induction of the *lac*UV5 promoter by IPTG did not have an effect on the apparent inversion frequencies of substrates tested with pCIA70 in DS902 (figure 5.8). When pMA2631 was complemented with pCIA70 in DS902, the amount of inversion products detected after 30 generations increased slightly compared to complementation by pPAK316, but the inversion level was still not substantial (10%: figure 5.6). Thus resolvase appeared to be expressed at a higher level from pCIA70 than from pPAK316, but this did not account for the very efficient inversion seen with pCIA80 and pCIA83.

5.4 *In vitro* recombination of *res* inversion substrates

The differences between the *in vivo* inversion efficiencies of pMA2631 and its *sis*$^+$ derivative compared to pCIA80 and pCIA83 warranted further investigation *in vitro*. However, supercoiled pCIA80 and pCIA83 incubated with Tn3 resolvase *in vitro* under standard and permissive conditions (with or without FIS; gift from P.Haffter) failed to show any recombination products even when pMA21 resolved in the same reaction mix (figure 5.9). Therefore, these substrates did not behave any differently from pMA2631 *in vitro*, although they differ in their *in vivo* behaviour. *In vitro* recombination of supercoiled *res* inversion substrates is substantially different from that

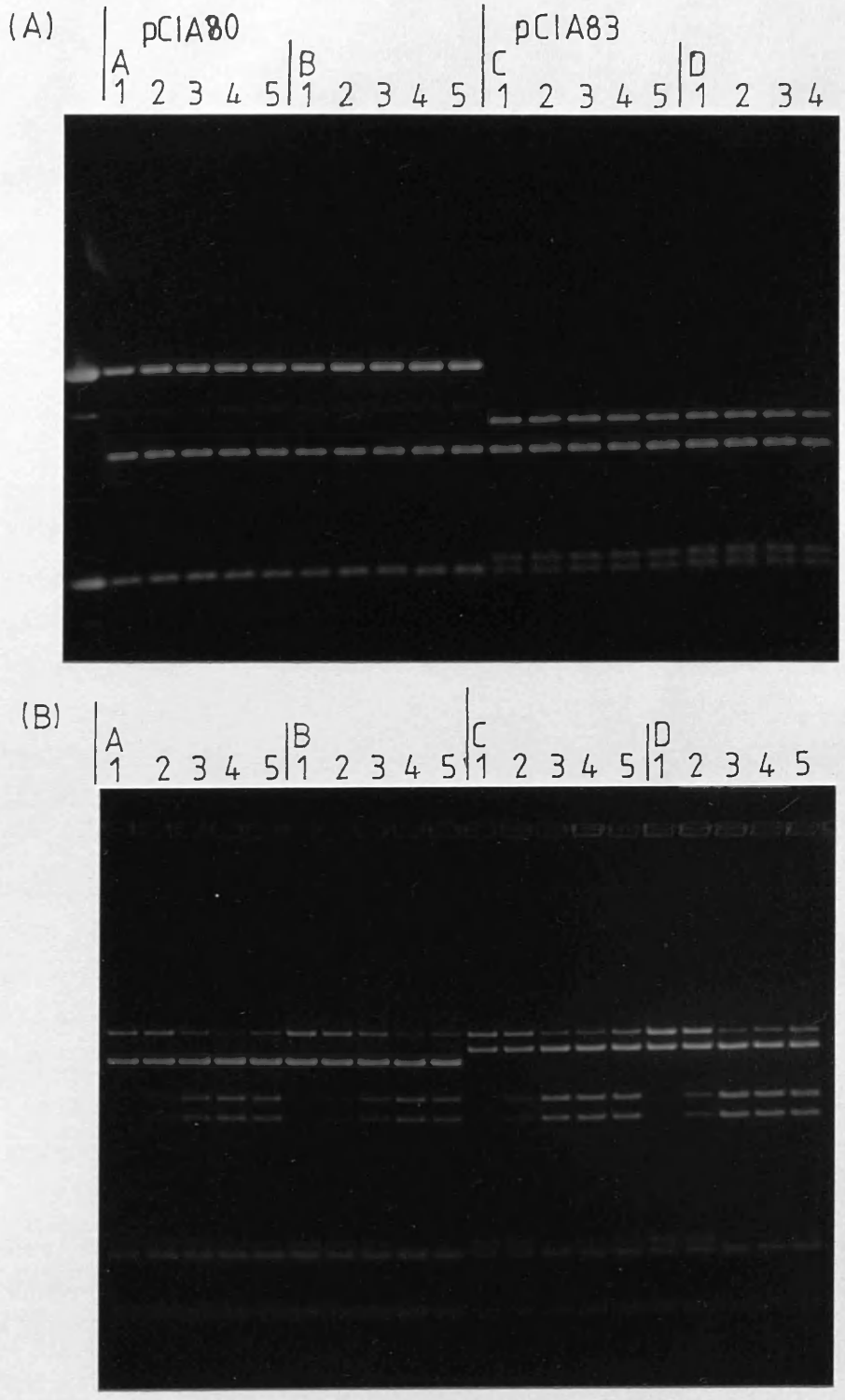


Figure 5.9 *In vitro* recombination of pCIA80 and pCIA83. Supercoiled pCIA80 and pCIA83 were incubated with Tn3 resolvase in recombination buffer B for 20 hours at 37°C, in which supercoiled pMA21 DNA was included as an internal positive control. 4ug/ml FIS (Basel) was added to samples in lanes B and D. FIS was diluted in resolvase dilution buffer. 25mM NaCl was added with the resolvase: extra NaCl was added to non-FIS samples (ca. 40mM final for all samples). Lanes 1-5 contained 0, 69, 139, 278 and 556 nM resolvase respectively. Both gels are 1.2% agarose. DNA was resticted with EcoRI (gel A) to reveal any recombination products of pCIA80 and pCIA83, or by PstI and HindIII (gel B) to show products of pMA21 resolution.

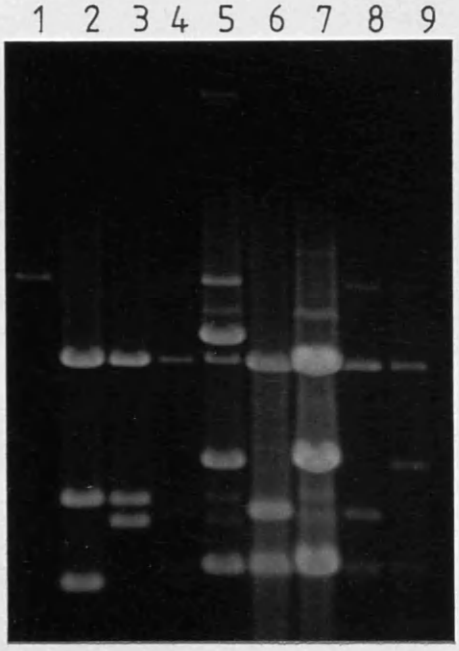


Figure 5.10. *In vivo* **recombination of pAL801 and pAL831.** pAL801 and pAL831 were recombined *in vivo* in the presence of pCIA70 (in strain DS902 for 30 generations). Plasmid DNA was isolated and restricted by PvuII. 1.2% agarose gel.

Lane	
1	pCIA70
2	pCIA80
3	pCIA83
4	pCIA80 + pCIA70
5	pCIA83 + pCIA70
6	pAL801
7	pAL831
8	pAL801 + pCIA70
9	pAL831 + pCIA70

exhibited *in vivo*.

5.5 Recombination properties of the inverted forms of plasmids

One possible complication for *in vivo* experiments is that the (+) and (-) inverted forms of the substrates may have different recombination properties, resulting in differences in the substrates for replication, transcription or recombination. An inverted (-) form of a plasmid may be favoured *in vivo*, possibly competing with its parental (+) form, resulting in an altered equilibrium between the two forms. Therefore, the inversion frequencies of the inverted forms of the substrates may differ from the parental forms. The observed differences of *in vivo* inversion frequencies between the tested *res* substrates could be a consequence of a favoured inverted form in one case (pMA2631) and not in the other (pCIA80). Since divergent transcripts in a plasmid can divide the molecule into more negative and more positive supercoiled domains, molecules with transcription of all genes in the same direction might be expected to be the favoured form *in vivo* (Wu *et al*, 1988). However, the arrangement and direction of transcripts of the parental forms of pMA2631 and pCIA80 are similar, and therefore, this explanation does not seem likely to be the reason for their differences in *in vivo* recombination (figures 5.7 & 5.11). The inverted forms of both pCIA80 and pCIA83 (pAL801 and pAL831, respectively) were isolated from *in vivo* recombination reactions after 30 generations, by selecting for substrate antibiotic marker only, and not pCIA70. The inverted form of pMA2631 was made artificially by inverting a partial EcoRI fragment of the substrate; a PstI-HindIII restriction digest revealed the inverted form, pRM2613 (figure 5.11).

Both pAL801 and pAL831 had inverted only 10% *in vivo*, after 30 generations, in the presence of pCIA70 (figure

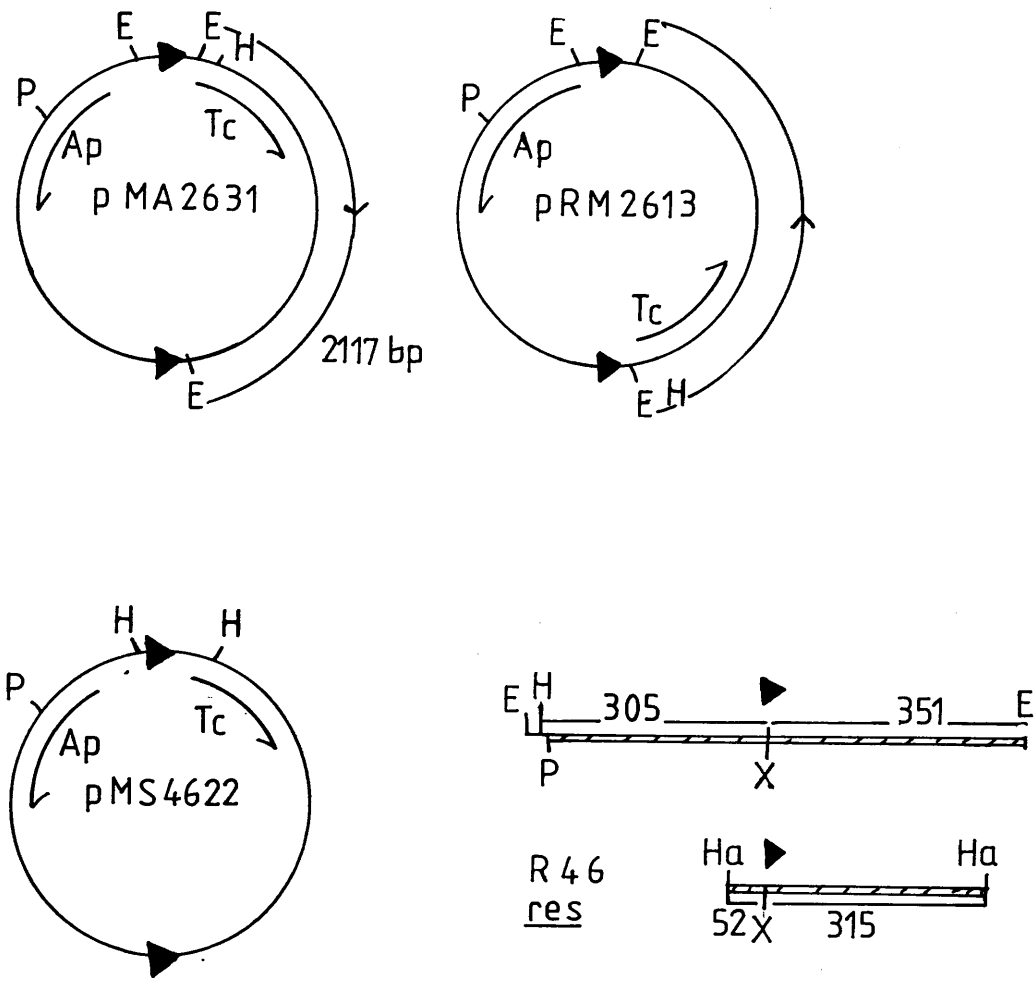


Figure 5.11 Structures of pRM2613 and pMS4611. The inverted form of pMA2631 was made by inverting the 2117bp EcoRI fragment to give pRM2613 (made by Richard McCulloch). A *res* site from R46 was inserted into the EcoRI and into the PvuII sites of pBR322, such that the two *res* sites were in inverted repeat (made by Marshall Stark).
P=PstI, H=HindIII, E=EcoRI, Ha=HaeIII,
X= centre of *res* crossover site.

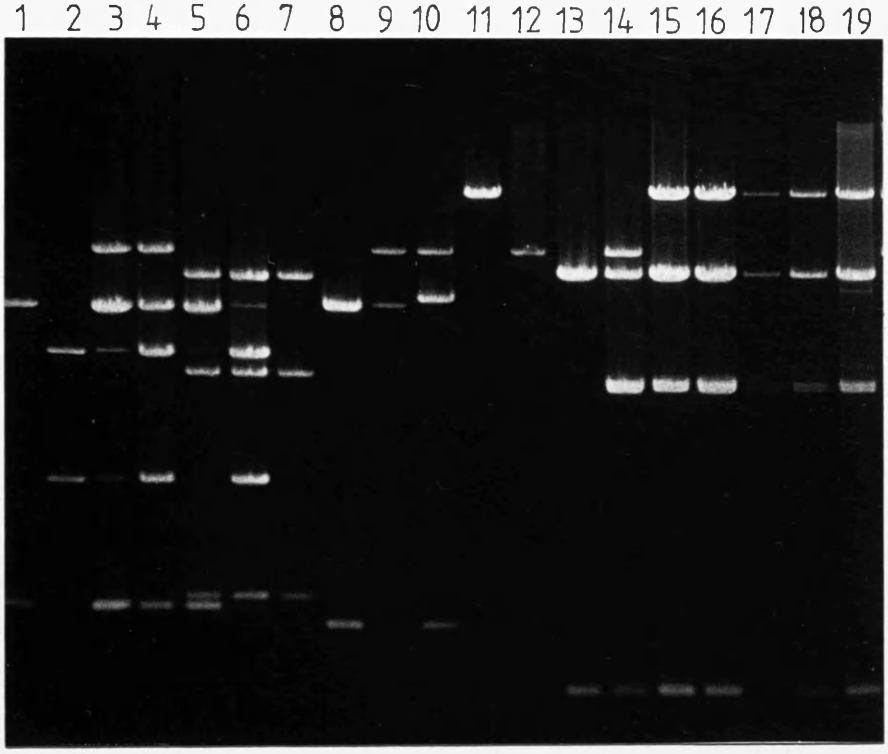


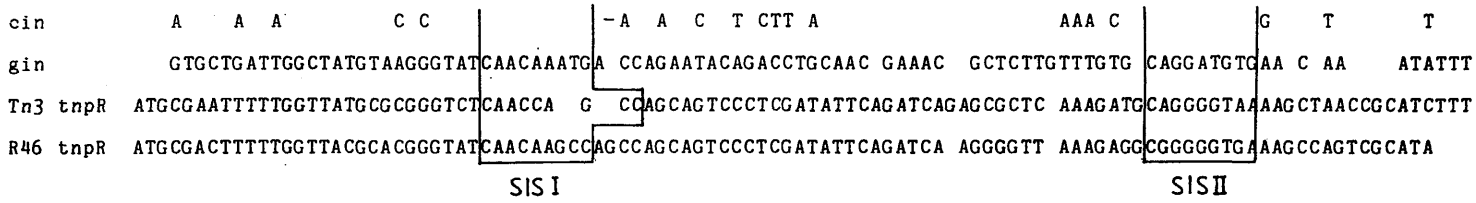
Figure 5.12 *In vivo* recombination of pRM2613 and pMS4622. Substrates pMA2631, pRM2613, pMS4611, pAL221 and pAL221*sis* were grown in DS902 for 30 generations in the presence of pCIA70 or pPAK316. pMS4622 was also grown with pPAK316 for 30 generations in CSH50 and CSH50 *fis*::Km. Isolated plasmid DNA was restricted, and run on a 1.2% gel.

Lane		
1	pMA2631	PstI-HindIII restricted
2	pRM2613	"
3	pMA2631 + pCIA70	"
4	pRM2613 + pCIA70	"
5	pMA2631 + pPAK316	"
6	pRM2613 + pPAK316	"
7	pPAK316	"
8	pAL221	"
9	pAL221 + pCIA70	"
9	pAL221*sis* + pCIA70	"
11	pPAK316	HindIII restricted
12	pCIA70	"
13	pMS4622	"
14	pMS4622 + pCIA70 (DS902)	"
15	pMS4622 + pPAK316 "	"
16	pMS4622 + pPAK316 (CSH50)	"
17-19	pMS4622 + pPAK316 (CSH50 *fis*::Km)	"

5.10), suggesting that the reverse reaction back to the parental form is less frequent than the apparently efficient forward reaction. In contrast, the inversion of pRM2613 was more efficient (complemented by pCIA70, 30 generations) than the inversion of the parental form, pMA2631 (figure 5.12). However, <20% of pRM2631 had inverted and therefore the preference for one inverted form of pMA2631 does not account for the difference between this pair of substrates and pCIA80 and its derivatives. To check that a difference in copy number did not contribute to the inversion frequency difference, a further pBR322 derivative was tested. An inversion substrate was made (pMS4622), with a structure similar to pMA2631, in order to also test the properties of the R46 recombination system. By transforming pMS4622 into DS902 with either pCIA70 or pPAK316, after 30 generations >50% of the re-isolated *res* plasmid was the inverted form (figure 5.12). Therefore, pMS4622 behaved in a similar way to pCIA80 and not like the analogous pMA2631 substrate.

5.6 Does the *tnpR* sequence contain a functional enhancer?

The apparent *in vivo* inversion frequency for pMA2631 and its derivatives is much slower than for pCIA80 and pCIA83 and the R46 *res* inversion substrate, pMS4622. These plasmids differ in the extent of Tn3 sequences flanking *res* used in the construction of the substrates. pMA2631 does not contain any *tnpR* sequence, but approximately 300 bp of *tnpR* is included in pCIA80, pCIA83 and pMS4622 (figures 5.7 & 5.11). The enhancer sequences within the invertase systems is located at the 5' end of the invertase genes. Since there is some considerable homology between resolvase and the invertases, it was possible that the 5' *tnpR* sequences found in the inversion substrates also contains a functional enhancer site (figure 5.13). To


cin A A A C C -A A C T CTT A AAA C G T T
gin GTGCTGATTGGCTATGTAAGGGTATCAACAAATGA CCAGAATACAGACCTGCAAC GAAAC GCTCTTGTTTGTG CAGGATGTCAA C AA ATATTT
Tn3 tnpR ATGCGAATTTTTGGTTATGCGCGGGTCTCAACCA G CCAGCAGTCCCTCGATATTCAGATCAGAGCGCTC AAAGATGCAGGGGTAAAAGCTAACCGCATCTTT
R46 tnpR ATGCGACTTTTTGGTTACGCACGGGTATCAACAAGCCAGCCAGCAGTCCCTCGATATTCAGATCA AGGGGTT AAAGAGGCGGGGGTGAAAGCCAGTCGCATA
SIS I
SIS II

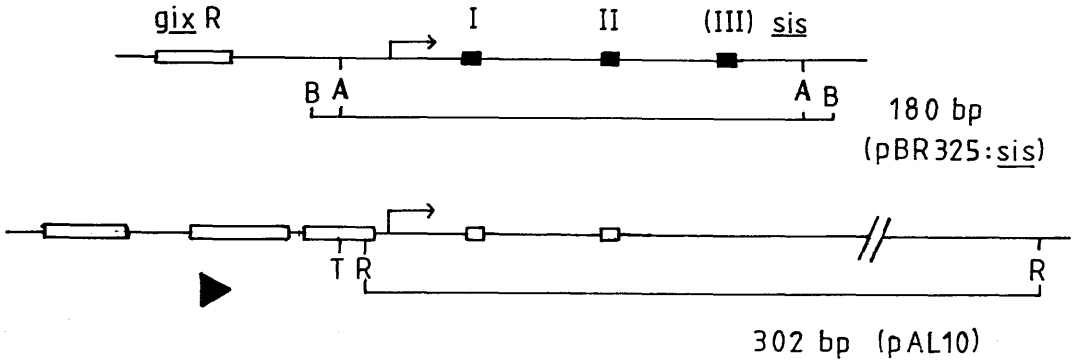


Figure 5.13 Comparison of the DNA sequence of the enhancer sites of gin and cin with an analogous region in tnpR genes. Two FIS binding sites in the gin and cin sequence are indicated. The positions of putative sis sites in the tnpR sequences of Tn3 and R46 are also indicated. The distance between sisI and sisII are conserved in invertase genes (Huber et al, 1985), but are not in tnpR genes. Fragments used for the FIS binding assay originated from pBR325sis and pAL10 (Tn3 sequences extend rightwards into the resolvase gene, from the TaqI site in res susbsite III).
A=AhaIII, B=BamHI, R=RsaI, T=TaqI.

check if inversion of these substrates depends on interactions between FIS and a putative enhancer, *in vivo* recombination by resolvase provided in *trans* was attempted in a *fis* background. Two strains, one wild type (CSH50) and one a *fis* mutant (CSH50 *fis*::Km), but otherwise isogenic, were obtained from R.Kahmann. These were used for *in vivo* recombination assays. Since pCIA70 carried the same resistance marker (Km^r) as the *fis* strain, pPAK316 was used to supply resolvase. It was not possible to complement pCIA80 and pCIA83 with pPAK316 as all these plasmids were chloramphenicol resistant. Both pCIA80 and pCIA83 were made chloramphenicol sensitive (pAL802 and pAL832 respectively) by filling in a unique NcoI site within the *cat* gene, but retaining their ampicillin resistance. Unfortunately, pAL802 and pAL832 were not stable in the presence of pPAK316 in either a *rec*A (DS902) or rec^+ (CSH50 and CSH50 *fis*::Km) background; both substrates, and their Ap^r marker were lost when attempting to grow the strains. It is unknown if the 600 bp of homologous DNA of the *res* and *tnp*R regions or the shared *cat* sequence of both the substrates and pPAK316 contributed to the apparent incompatibility of these plasmids.

The R46 *res* construct, pMS4622, provided an alternative substrate for testing the FIS dependence of *in vivo* inversion between *res* sites. Transformation of pMS4622 and pPAK316 into both CSH50 and its *fis* mutant, and subsequent growth for approximately 30 generations, gave similar levels of inversion of this substrate in both strains (by restriction analysis of isolated DNA; figure 5.12). These levels were similar to inversion of pMS4622 by resolvase from pPAK316 in DS902.

Substrates with a known functional enhancer sequence were also tested in the *fis* mutant strain. Both pMA2631 and pAL2631*sis* were recombined *in vivo* in CSH50 and CSH50 *fis*::Km in the presence of pPAK316. Restriction of products after 30 generations indicated no difference from

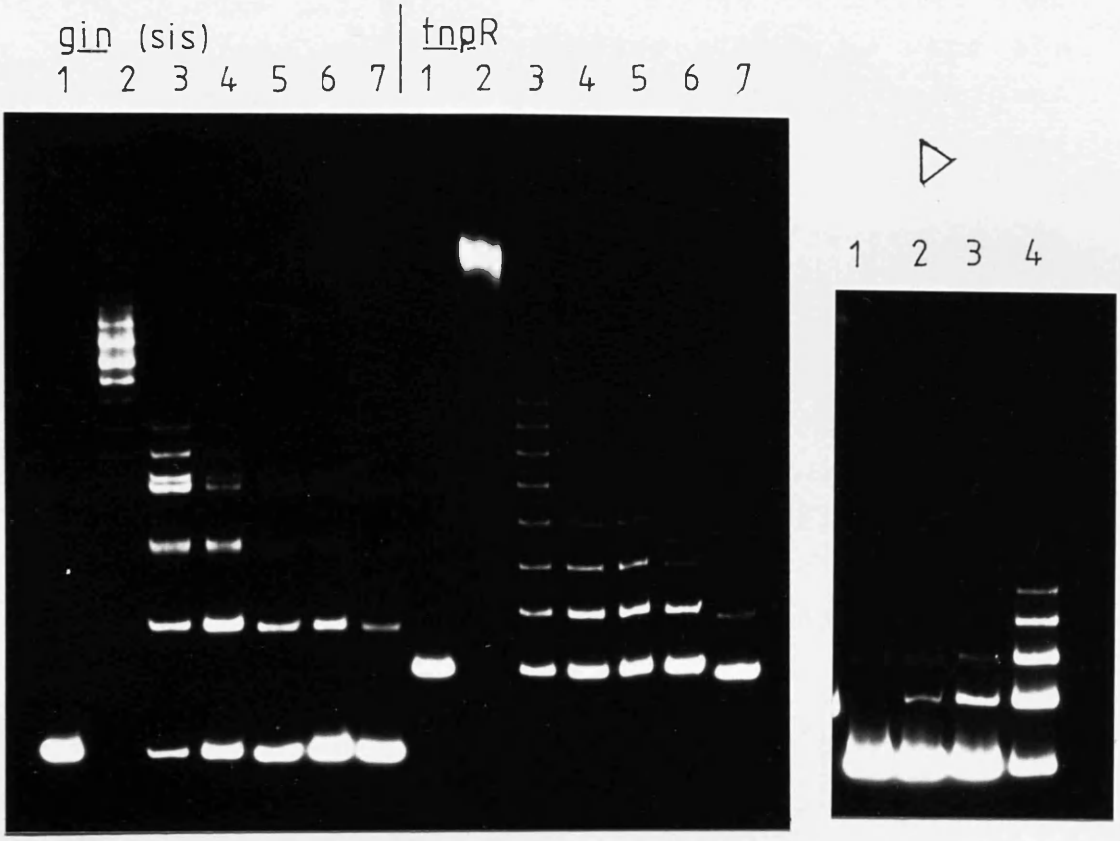


Figure 5.14 FIS binding to an enhancer site from gin, a putative enhancer site from Tn3 and a res subsite I fragment.

(A) DNA fragments from the gin enhancer sequence and Tn3 tnpR sequence (see figure 5.13) were incubated in binding buffer B (no carrier DNA) with 2.5ug/ml FIS protein (Berlin) at 37°C for 10 min. Competitor DNA (pAL211sis) was included in the binding reactions at 3.4 (lanes 3 + 10), 6.8 (lanes 4 + 11), 12.5 (lanes 5 + 12), 25 (lanes 6 + 13) and 50ug/ml (lanes 7 + 14). 6% polyacrylamide gel, conditions B.

(B) A res subsite I fragment (A1; see figure 3.3) was incubated in binding buffer B with FIS at 37°C for 10 min. Lanes 1-4 contained 0, 0.68, 1.25, 2.5ug/ml FIS (Berlin) respectively. 6% gel, conditions B.

the levels of inversion previously seen in DS902.

FIS therefore does not appear to have an effect on the *in vivo* inversion of *res* substrates, with or without a functional enhancer site. It is uncertain if the sequences within *tnpR* do contain a functional recombination enhancer; if so, it appears not to require FIS for its activity.

5.7 FIS binding to an enhancer site

With the development of the gel binding assay (chapter 3), it was possible to check if FIS can bind to the enhancer site from *gin* in the conditions used for the *in vitro* recombination assays. This was intended to give some indication that the purified FIS protein kindly provided by R.Kahmann was functional, since we had no purified invertase for a positive *in vitro* control recombination reaction. In addition, it was possible to ask if FIS recognises and binds to a putative enhancer region in the *tnpR* gene.

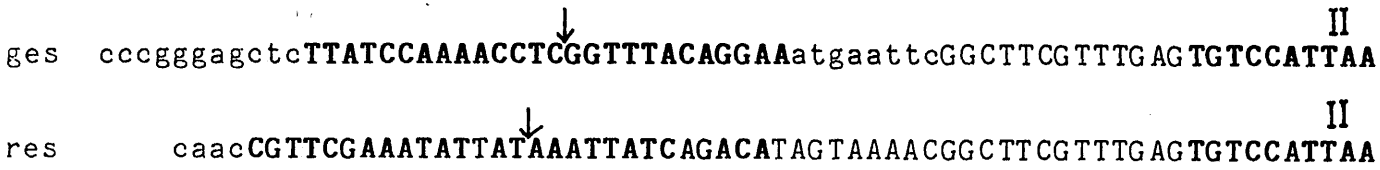
A DNA fragment containing the enhancer region of the Mu *gin* gene and a DNA fragment from the analogous position of the homologous *tnpR* gene of Tn3 were purified and end-labelled (figure 5.13). Conditions used for binding purified FIS (from Berlin) to the fragments were similar to those used in our *in vitro* recombination reactions with resolvase. Complexes of FIS binding to sequences from both *gin* and *tnpR* genes were observed (figure 5.14). In each case, the complexes were competed out by the addition of excess supercoiled pAL211*sis*, containing the functional *gin* enhancer sequence. A ladder of complexes was observed for both fragments; the *tnpR* fragment produced a uniform progression of complexes, but for the *gin* enhancer, some of the complexes were abnormally retarded. FIS binding to the fragments may be mainly successive additions of the protein binding to non-specific sites in the fragment,

giving a stoichiometric effect, displayed as a ladder of complexes. A similar ladder of complexes was also seen for FIS binding to a *res* subsite I fragment (figure 5.14). Footprints by FIS on the *cin* gene sequence have shown contacts at sites other than the enhancer site (Haffter and Bickle, 1988). However, the unusual ladder of complexes with the *gin* enhancer sequence suggests that FIS binding to certain sites within this sequence may be forming some specific bent structure that is anomalously retarded, important for a functional enhancer and that is not found for FIS binding to a putative enhancer site in the *tnpR* sequence. Not only are specific contacts by the FIS protein necessary for a functional enhancer, but the DNA sequences between the binding sites are also important and may possibly form a particular bend when FIS contacts its sites (Hubner and Arber, 1989). FIS binding to *sis* from the *gin* sequence may be capable of making a bend suitable for a synapse, but many sequence differences in *tnpR* may prevent this synapse structure from forming.

The gel binding results show that FIS can bind at *sis* in the permissive resolvase binding conditions. Although FIS can bind to both the *sis*-containing DNA fragment and the *tnpR* DNA fragment, we have no evidence that the resolvase gene has a functional enhancer sequence. This could be tested, however, by replacing a functional enhancer from an invertase gene with the putative enhancer region of *tnpR* and asking if invertase-mediated recombination is still stimulated in the presence of the FIS protein.

5.8 The effect of *res* subsites II and III on invertase recombination

If subsites II and III from *res* are responsible for the selection of a resolution event between two directly repeated *res* sites, then we might expect subsites II and


ges cccgggagctcTTATCCAAAACCTCGGTTTACAGGAAatgaattcGGCTTCGTTTGAGTGTCCATTAA
II
res caacCGTTCGAAATATTATAAATTATCAGACATAGTAAAACGGCTTCGTTTGAGTGTCCATTAA
II

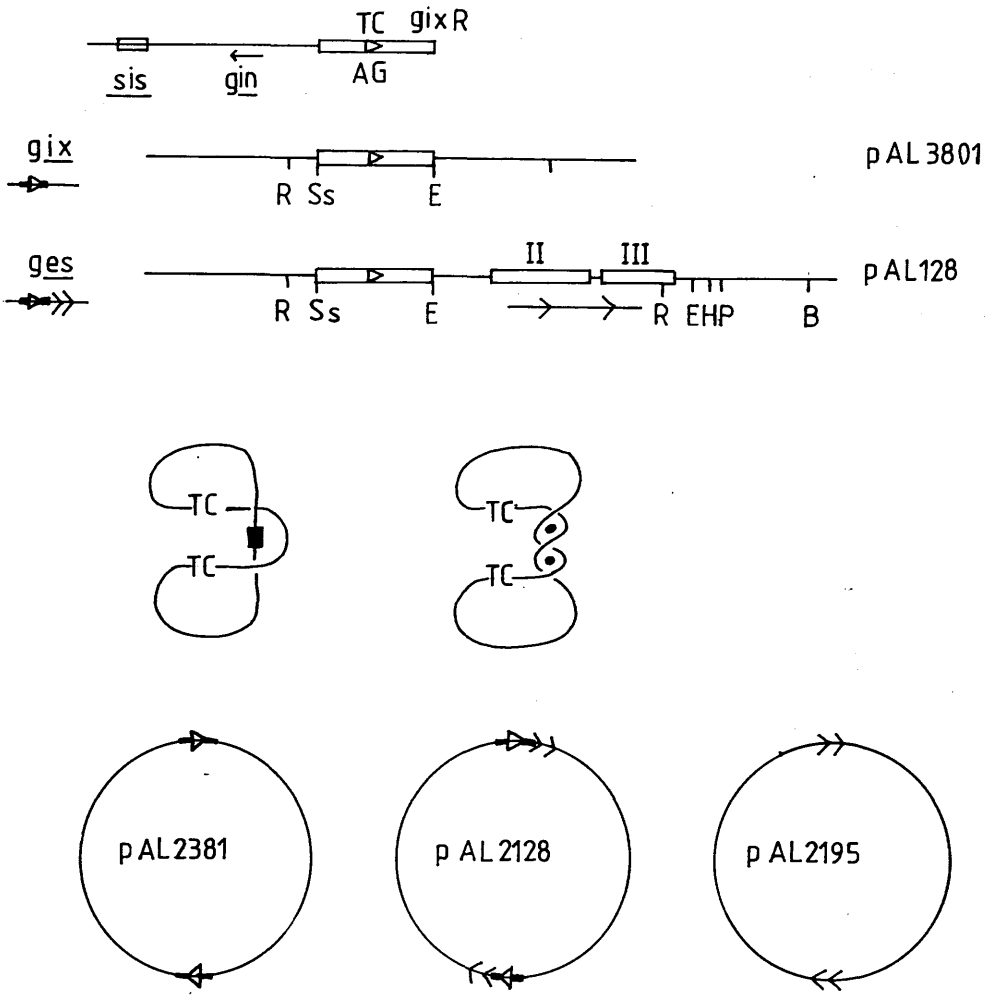


Figure 5.15 Construction of a hybrid recombination site (ges). The two synthetic oligonucleotide strands of the gix sequence were annealed to each other and cloned between the SstI and EcoRI sites of pMTL23 to give pAL3801. A 92 bp EcoRI subsites II and III fragment from pAL3151 (see figure 3.1) was inserted into the EcoRI site of pAL3801 to form a ges site (pAL128). pAL3801, pAL3151 and pAL128 were dimerised as described in the text. The sequence of ges was determined by directly using the plasmid pAL128 as a template. The proposed synaptic structures for inverted gix sites and for direct ges sites are also shown.

Ss=SstI, E=EcoRI, H=HindIII, B=BamHI, P=PstI, R=RsaI.

III to impose the same selectivity on different crossover sites. A gix site was chosen because of similarities of the invertase and resolvase systems and because wild type Gin cannot direct a deletion event between two directly repeated gix sites, regardless of the presence of FIS and sis. Therefore, any invertase-mediated deletion between gix sites that are both adjacent to res subsites II and III must be dependent upon and a consequence of the accessory functions.

To replace the crossover site of res with a gix crossover site, synthetic oligonucleotides for each strand of a gix site were designed to be cloned adjacent to subsite II of the res site in which subsite I had been deleted. The minimal gix site sequence used is found in both gixL and gixR, and the oligonucleotide was cloned into the polylinker of pMTL23 vector (pAL3801). Subsites II and III were then cloned into one side of gix, such that the orientation of the crossover site was towards subsites II and III, as gix is found with respect to the enhancer sequence in Mu (pAL128). The centre-to-centre spacing of the gix site and subsite II was designed to maintain the spacing found in wt-res. However, by sequencing directly from the plasmid template, it was found that the sequence of this hybrid site (designated ges) had a spacing 2bp shorter than intended, because of a sequencing error of the original subsite II and III deletion product (figure 5.15).

As shown in chapter 3, the ges site did bind resolvase, but only to give a retardation pattern of four complexes as found for subsites II and III alone. Therefore, Tn3 resolvase did not recognise the gix part of the ges site to form a stable complex.

To test the recombination properties of ges, dimers of pAL128, pAL3801 and pAL3151 (pAL2128, pAL2381 and pAL2195 respectively; figure 5.15) were made in vivo using a multimerising strain JC8679 (as for sym-res, chapter 4); dimers of the gix construct and for subsites II and III

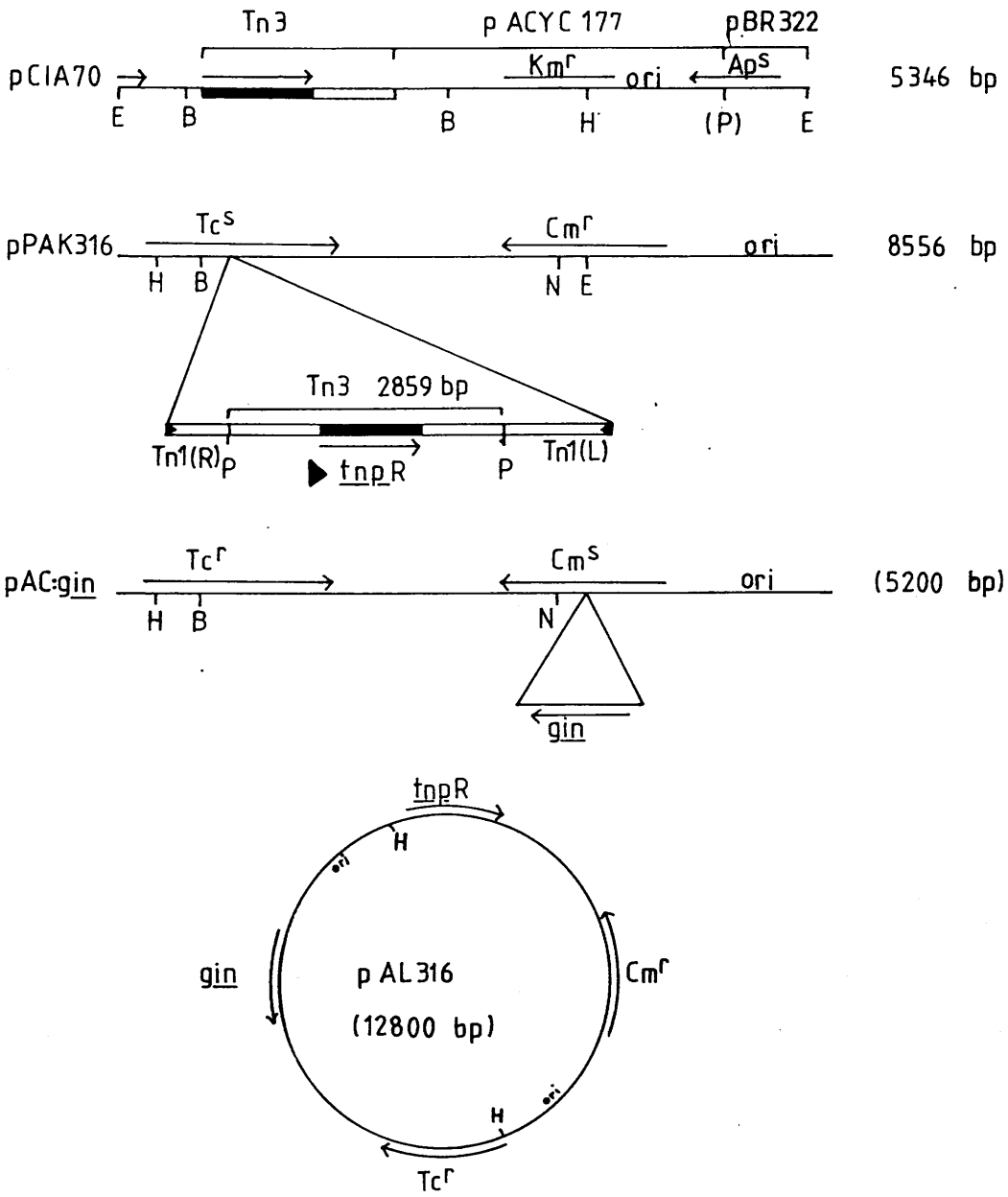
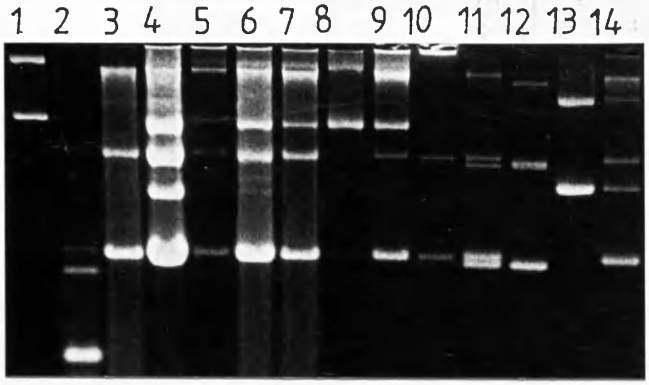


Figure 5.16 Diagrammatic representation of tnpR$^+$ and gin$^+$ constructs. pCIA70, pPAK316 and pAC:gin all have the p15A origin of replication. pPAK316 was derived from Tn1 transposition into pACYC184 (Kitts, 1892). pAL316 was made by fusing pPAK316 and pAC:gin at the HindIII site and selecting for both Tc^r and Cm^r.
Abbreviations as for figure 5.15; N=NcoI.



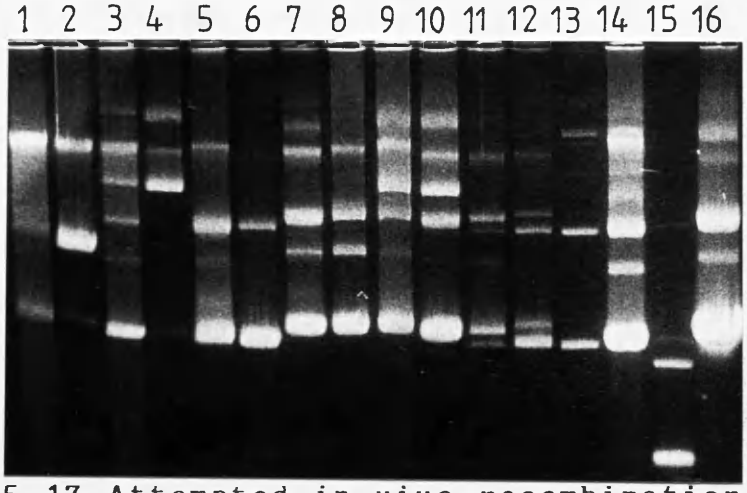


Figure 5.17 Attempted *in vivo* recombination of a *ges* substrate (pAL2128). Substrates pAL2128, pAL2381 and pAL2195 were grown in WA3782 with pAL316, pPAK316 or pAC:*gin* for 30 or 70 generations. 1.2% gels show isolated DNA.

(A)	Lane 1	pAL316		
	2	pAL128		
	3	pAL2128		
	4-7	pAL2128 + pAL316	30	generations
	8 + 9	as lanes 6 + 7, but	70	"
	10 + 11	pAL2128 + pAC:*gin*	30	"
	12	pAC:*gin*		
	13	pPAK316		
	14	pAL2128 + pPAK316	30	generations
(B)	Lane 1	pAL2381 + pPAK316	30	generations
	2	" "	70	"
	3	" + pAL316	30	"
	4	" "	70	"
	5	" + pAC:*gin*	30	"
	6	" "	70	"
	7	pAL2195 + pPAK316	30	"
	8	" "	70	"
	9	" + pAL316	30	"
	10	" "	70	"
	11	" + pAC:*gin*	30	"
	12	" "	70	"
	13	pAC:*gin*		
	14	pAL2381		
	15	pAL3801		
	16	pAL2195		

alone were made as control substrates. For an *in vivo* assay, both resolvase and Gin were required. Since both these proteins were provided on plasmids from the same compatibility group, the plasmids pPAK316 (*tnp*R$^+$) and pAC:*gin* were fused at their unique HindIII sites, to give pAL316 (figure 5.16).

In vivo recombination assays of the different dimer substrates were conducted in WA3782 with pAC:*gin* alone, pPAK316 alone or with pAL316, providing Gin, resolvase or both functions. We would expect only the *ges* dimer to break down, in a Gin and resolvase dependent manner; the *gix* dimer is not a substrate for Gin and the subsites II and III dimer lacks crossover sites. After 100 generations of the substrates with the different complementing plasmids, the products were analysed by isolating the DNA (figure 5.17). No monomer products were observed for the *ges* dimer (pAL2128), but both control substrates gave trace amounts of monomer, although not for all combinations of the complementing plasmids. The failure of the *ges* dimer to break down *in vivo* in a Gin and resolvase manner may have been due to the 'incorrect' spacing of the sites within *ges*. Altering the spacing between subsites I and II of Gamma-delta *res*, by an addition of 2 bp, showed a reduced recombination efficiency (Salvo and Grindley, 1988). Alternatively, the expression of *gin* from the *cat* promoter may have been too poor to be functional in a few generations, in which case the *ges* dimer would be expected to break down after more generations.

In collaboration with R.Kahmann and co-workers, the dimer substrates pAL2128, pAL2381 and pAL2195 were tested *in vitro* using their purified FIS-independent mutant Gin protein. This mutant Gin can catalyse recombination between directly repeated *gix* sites in the absence of an enhancer and FIS protein (Mertens *et al*, 1988). *In vitro* recombination of supercoiled pAL2128 and pAL2381 was observed with the mutant Gin alone. In both cases, the products of recombination were complex catenanes, as

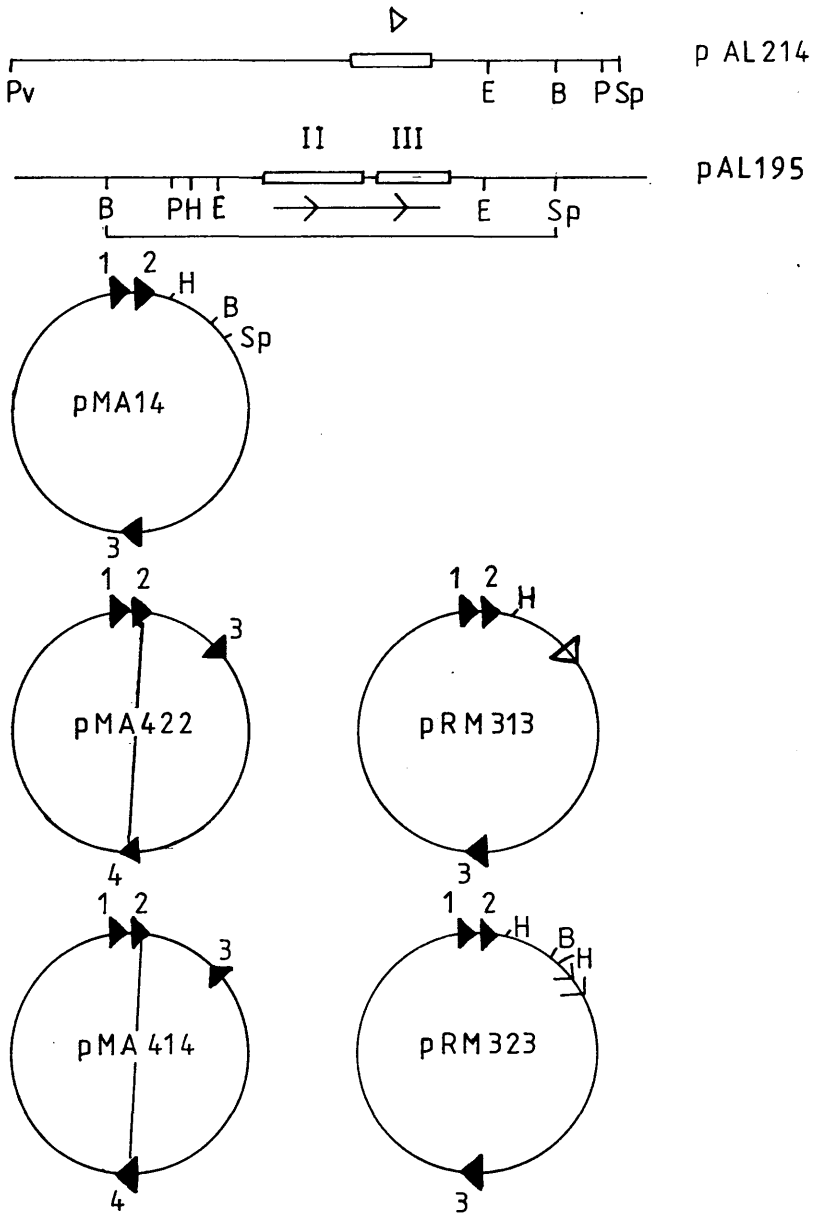


Figure 5.18 Multi-<u>res</u> site substrates containing isolated subsite I or subsites II and III. Both pRM313 and pRM323 were derived from pMA14 by inserting subsite I or subsites II and III between the BamHI and SphI sites respectively, and then selected for loss of Tc^r, and checked by restriction. Recombination between sites 1+3 of pMA414 and pMA422 were shadowed events (Brown, 1986).

Abbreviations as for figure 5.15; Sp=SphI, Pv=PvuII.

Expected resolution product sizes (bp):-

	1+2	2+3	3+1
pMA14	282, 4927	2898, 2311	2616, 2593
pRM313	282, 4940	2898, 2324	2616, 2606
pRM323	282, 4885	2898, 2273	2616, 2555

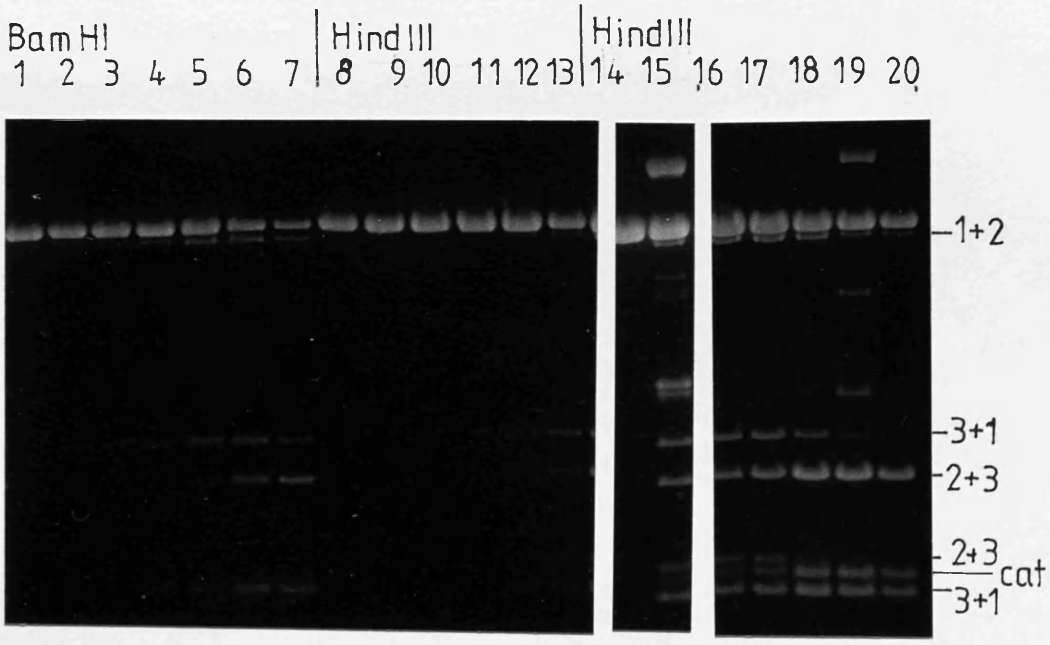


Figure 5.19 In vitro recombination of pRM313 and pRM323 with resolvase. Supercoiled pRM313 and pRM323 (see figure 5.18) were incubated (at 37°C; different time points) with 278nM resolvase (except lanes 1, 8 and 14) in recombination buffer A (50mM NaCl). Lanes 1-7, pRM323; lanes 8-13, pRM313; lanes 14-20, pMA14.

Lane		
	2,9 + 15	30s
	3,10 + 16	1 min
	4,11 + 17	2 min
	5,12 + 18	6 min
	6,13 + 19	20 min
	7 + 20	60 min

expected if there was no topological control of the reaction. The addition of Tn3 resolvase to the *in vitro* reaction was expected to result in simple catenated products from the *ges* dimer only; resolvase aligns two crossover sites for recombination, possibly by utilising subsites II and III in the formation of a synapse as proposed by our model, which should result in a defined product topology. When resolvase was also added to the reaction, the recombination rate of pAL2128 was reduced. Therefore, the presence of subsites II and III had an effect on the recombination of *gix* sites by the mutant Gin. Resolvase interactions with subsites II and III may be unable to support or may prevent Gin-mediated recombination between the *gix* sites. Again one possible reason for the failure to observe recombination between *ges* sites could be the spacing between *gix* and subsite II in the hybrid site. The experiments with *ges in vivo* and *in vitro* should be repeated for *ges* sites with different subsite spacing.

5.9 The shadowing effect of *res* subsites

To test for synapsis of different components of *res*, subsites II and III, or subsite I alone, was cloned into a 3-*res* construct pMA14 by Richard McCulloch, to make pRM323 and pRM313 respectively (figure 5.18). Both of these substrates had a design analogous to the shadowing substrates pMA422 and pMA414. In resolution reactions of these multi-*res* site substrates, a particular pair of *res* sites was prevented from recombining, regardless of the relative orientation of the interfering *res* site (J.L.Brown, 1986). The new substrates were recombined *in vitro* in a time-course with purified resolvase, under standard reaction conditions (initially tested by Richard McCulloch). Substrates pMA14, pMA422 and pMA414 were used as controls in the *in vitro* recombination assay. After

stopping the resolvase reactions, the substrates were restricted to indicate which products had formed at which time point and therefore, the order of res site pairing could be elucidated. As shown in figure 5.19, subsites II and III in pRM323, initially blocked the pairing of sites 1 and 3, as did a wt-res site in either direct (pMA422) or inverted repeat (pMA414). Subsite I did not block the pairing of sites 1 and 3, but may be blocking sites 1 and 2.

Subsites II and III, therefore, apparently have the same shadowing effect as wt-res on the pairing of res sites in a multi-res substrate. This implies at least that subsites II and III can interact with a wt-res site, which further supports the idea that res sites form a synapse by wrapping subsites II and III.

DISCUSSION

1. The function of res subsites II and III

Our interwrap model proposes that subsites II and III align the crossover sites for strand exchange (figure 5.1). As demonstrated in chapter 4, subsites II and III in only one res site can align the crossover sites. In these substrates, resolution and not inversion, between the sites is selected. Since the products of resolution of these substrates are also simply catenated, this suggested that the accessory sites are aligning crossover sites by forming the same synaptic intermediate as when both sites contain subsites II and III. When one res site lacks subsites II and III, the efficiency of reaction was reduced; the efficiency was further reduced to undetectable levels when subsites II and III were removed from both sites.

Subsites II and III are not only responsible for the alignment of the crossover sites and selection of the

resolution event, but also crossover sites do not appear to be able to form a productive synapse without these accessory sites. Resolvase, however, can bind a single subsite I (chapter 3) and will recombine at an isolated site, but presumably, only when subsites II and III have formed a wrapped synapse. Clearly, in substrates with subsites II and III absent from both sites, a similar synaptic structure cannot be formed by any resolvase interactions, and these therefore are much poorer substrates for recombination. The formation of a synaptic intermediate, provided in res by the wrapping of subsites around resolvase, appears to be critical for the recombination of res crossover sites.

Subsites II and III dc not need the crossover site to form a synapse. The evidence for this synapsis is twofold; a res site that lacked subsite I shadowed a recombination event in a multi-res site substrate, and the presence of subsites II and III in directly repeated ges sites interfered with gix site recombination. Subsite I x wt-res recombination suggested that resolvase can synapse subsites II and III with non-res DNA, but this was probably a consequence of the alignment of two res crossover sites. However, it is expected that two copies of subsites II and III can synapse when subsite I is removed from both sites. Such a synapse was not detected between subsites II and III on separate molecules in the gel binding assay, but intramolecular synapsis has not yet been attempted in this assay (chapter 3).

The failure of the mutant Gin protein to recombine at ges sites in vitro in the presence of resolvase suggested that subsites II and III were forming some synaptic structure, and possibly obstructing Gin from functioning. Resolvase might also be able to interfere directly with Gin-mediated recombination without synapsis. Although the spacing error between the sites within ges might be the reason for no observed recombination, the invertase itself might be affected by the resolvase at subsites II and III.

Although the mutant Gin is FIS independent, recombination between *gix* sites is still sensitive to the FIS protein; FIS can still stimulate inversion, but inhibits resolution by the mutant Gin protein (Klippel *et al*, 1988b). We do not know if resolvase at subsites II and III is influencing the protein at the crossover sites in a similar way.

2. The function of FIS and the enhancer site

An interwrap model for synapsis in the invertase system has also been proposed (Kahmann *et al*, 1986; Kanaar *et al*, 1988; figure 5.1). Recombination by invertases is dependent on the FIS and enhancer site. However, resolvase failed to recombine at isolated crossover sites in the presence of FIS and the enhancer sequence from the Mu *gin* gene. This was initially unexpected, as resolvases and invertases are 30% homologous. Perhaps recombinases may only function once the synaptic structure appropriate to the particular enzyme has formed.

3. *In vivo* inversion of *res* substrates

Not all *res* inversion substrates have the same *in vivo* recombination properties, although all those tested failed to recombine *in vitro* as supercoiled molecules. Although the Bin recombinase was initially characterised by its *in vivo* inversion properties, the location of a *bin* gene within Tn552 has suggested that this enzyme is a resolvase. Resolution between directly *bix* sites by has been shown by Bin *in vivo* (S.-J. Rowlands, personal communication). Therefore, the inversion properties of Bin *in vivo* might be similar to those found for Tn3 resolvase, and Bin might also fail to recombine inverted *bix* site substrates *in vitro*. In the Gin system, directly repeated *gix* site recombination is not possible *in vitro*, but a

similar level of deletion was detected *in vivo* as for *in vivo* recombination of pMA2631 (Plasterk *et al*, 1984). It is unknown if FIS and *sis* had an effect on this deletion. All the *res* substrates that recombined well *in vivo* contained 5' *tnp*R sequences in addition to the *res* sites. The presence of this additional sequence at both sites may have altered the *res* recombination properties by either extending the total inverted region of DNA in the plasmid, or by the action of some host factor at sites within the *tnp*R sequence. Replacing the *tnp*R sequences with different sequences should confirm or eliminate either possibility. We know that the observed *res* inversion *in vivo* is not an effect of FIS, as a *fis* mutant background had no effect on the recombination properties of *res* inversion substrates. Also, FIS did not stimulate inversion of these supercoiled substrates in *in vitro* recombination reactions. Although FIS bound to *tnp*R sequence that may contain an enhancer-like element, this did not suggest that this sequence can act as a functional enhancer site. The *tnp*R sequence may contain other sites for host factors; IHF is probably not involved as no sequences similar to the IHF consensus can be found in the *tnp*R sequence present in the inversion substrates.

Why should *res* inversion substrates invert at all *in vivo* but not *in vitro*? It is possible that supercoiling plays a role, as it has been suggested that the level of supercoiling *in vivo* is less than that for extracted DNA, as DNA-binding proteins are constraining cellular DNA (Lilley, 1986; Bliska and Cozzarelli, 1987). Resolvase inversion substrates that are not supercoiled can invert in reactions *in vitro*, but this is not as efficient as might be expected from *in vivo* observations. A further possibility for the *in vivo* observations is that inversions proceed via intermolecular reactions that could subsequently resolve to give `inversion' products.

The *in vivo res* inversion assays have shown that substrates can have a preferential inverted form, even for

a poorer inversion substrate. This suggested that there is some extra *in vivo* effect on inversion that may not be specific to *res* recombination, e.g. the formation of knots by gyrase.

SUMMARY

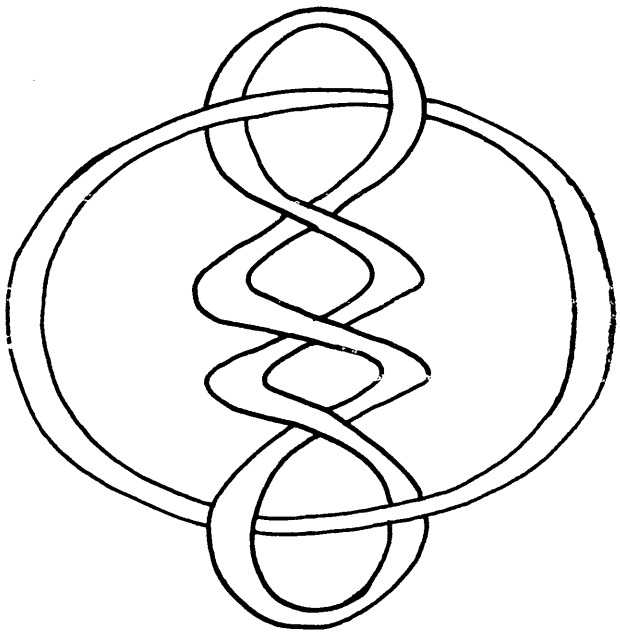
Many differences between the resolvase and invertase systems might account for the failure of this initial attempt to exchange accessory functions of the two systems. Communication between FIS and the invertase might be required for the formation of a synapse. FIS binds only one enhancer site and possibly contacts the invertase to anchor the synapse, although there is no evidence for this. Synapsis of *res* sites may be determined solely by the presence of subsites II and III in both partners; this interwrap structure may be sufficient for two crossover sites to recombine without contact with resolvase at subsites II and III. We do not know if *res* crossover sites can recombine if they are separated by more than an additional 30 bp from subsite II. The exchange of accessory functions between resolution and inversion systems might only be successful if hybrid recombinases are used, which can both contact the alternative accessory functions and recombine at their own crossover sites.

The capacity of subsites II and III from *res* to synapse each other with resolvase is in agreement with our model, as these subsites are expected to be able to align first. Although subsite I can bind resolvase, the protein appears to be unable to synapse crossover sites unless subsites II and III are present. Mutagenesis of resolvase is currently being attempted in our laboratory, to obtain a mutant resolvase that can recombine *res* crossover sites in the absence of subsites II and III. The evidence presented for synapsis of subsites II and III does not conclusively verify the predicted interwrap structure, as

an alternative synapsis of sites is possible (figure 4.12). If ges can be recombined by Gin in the presence of resolvase, simple catenated products should indicate that the predicted synapse can be formed. Gin should fail to recombine inverted ges sites on supercoiled molecules if the synapsis of subsites II and III imposes topological control on the reaction, as proposed for the resolvase system.

CHAPTER SIX

CONCLUDING REMARKS



Deciphering the Tn3 resolution system is not an easy task. The basic reaction requirements and specificities of the system were already established at the beginning of the research presented here, but the role of the three resolvase binding sites in res was still a question to be elucidated. Our model for synapsis (detailed in chapter 1) was proposed to explain how resolvase can select the resolution event, and to explain the requirement for res subsites II and III. Other models have been proposed to account for the selectivity of resolvase; these did not specifically propose a role for subsites II and III in the synapsis of sites, but an alternative model (`slithering') has been adapted to include accessory sites wrapping resolvase (Benjamin and Cozzarelli, 1986). Various predictions from the slithering model are different from those of our model.

An important objective of the work presented in this thesis stemmed from predictions made by our synapsis model; was it possible to demonstrate the division of functions between subsite I (strand exchange) and subsites II and III (synapsis, reaction selectivity)?

The polarity of res is determined by subsites II and III

When subsites II and III were removed from one res site, selectivity was kept, but the relative orientation of the crossover sites was now disregarded. Subsite I, in isolation, does not have any functional polarity and is treated as symmetrical by resolvase in recombination reactions. Replacing a wild type subsite I with a perfectly symmetrical subsite I, in the presence of subsites II and III (sym-res) resulted in subsites II and III imposing polarity on the crossover sites and aligning them `correctly' for strand exchange. The res sites do not have to be on supercoiled substrates, or in cis, for two

sites to be aligned in a parallel sense. Subsites II and III in both sites are presumed to direct the correct alignment of sites using a synaptic intermediate. When subsites II and III are absent from one site, products of recombination were only detected for supercoiled substrates and sites in *cis*. In this case, subsites II and III in one site were sufficient to promote recombination, presumably via the same synaptic intermediate as for two wt-*res* sites. It should be noted, however, that under certain reaction conditions minor products of an antiparallel alignment of wt-*res* or sym-*res* sites were also detected.

The function of accessory sites in aligning crossover sites may not be limited to the resolvase system. Invertases are dependent on an enhancer site, *sis*, and a host protein, FIS, to direct inversion between their inverted crossover sites. An interwrapping of sites and proteins has also been proposed for the invertase system, except that the structure (and product outcome) is different from that proposed for resolvase. In the experiments described in section 5.2, FIS and *sis* did not appear to have an effect on recombination of *res* sites, whether wt-*res* sites or isolated crossover sites were used. These accessory functions were expected to help resolvase direct inversion between two isolated *res* crossover sites. Similarly, when subsite I of *res* was replaced by the *gix* crossover site (a `*ges*' site; section 5.8), resolvase was expected to align *gix* crossover sites within *ges*, by using subsites II and III, such that only directly repeated *ges* sites could recombine. So far, no recombination between *ges* sites by the wild type Gin protein, in the presence of resolvase, has been observed. We do not know how the recombinase assesses the synapse formed by accessory functions, whether communication between proteins is required, or if a particular synaptic structure is required for a particular recombinase. Accessory factors may not, therefore, be easily

exchangeable between different systems.

Synapsis of *res* sites

The gel binding assays demonstrated that resolvase induces bending at *res* sites, and forms two complexes for each subsite present on a DNA fragment. Resolvase can bind an isolated subsite I with a similar affinity to a wt-*res*. However, without subsites II and III in both partners, resolvase fails to recombine two crossover sites. It is possible, therefore, that resolvase cannot synapse two isolated crossover sites.

An isolated crossover site will still recombine when subsites II and III are present in the other *res* partner. In these substrates, the resolution selectivity and product topology indicated that a similar intermediate synapse was formed as for two wt-*res* sites. Since subsites II and III are absent from one *res* partner, the synaptic structure presumably contains some non-*res* DNA, and it is unclear how it is formed. Resolvase bending subsites II and III at one *res* site may be sufficient to promote the formation of this synapse with non-*res* sequences adjacent to the isolated crossover site, but it is clear that a synapse is formed more effectively when subsites II and III are present in both sites. The only products detected for recombination of subsite I x wt-*res* substrates were simply catenated, suggesting that only when a specific synapse had formed, utilising subsites II and III from one site, is resolvase able to recombine crossover sites.

Our model proposes that during *res* site synapsis, the interwrap structure between subsites II and III is formed first. This ensures that the crossover sites are always aligned in parallel for strand exchange and that resolution is selected. Experiments with subsites II and III in multi-*res* site substrates (shadowing experiments; section 5.9) and recombination of *ges* sites have indicated

that synapsis between subsites II and III, independent of subsite I, is possible.

We do not know how resolvase manages to achieve the interwrap of subsites II and III. Each subsite of *res* is a different length and they are separated by different lengths of spacer DNA. These features and the arrangement of subsites are conserved in *res* sites of different transposons and plasmids, perhaps indicating their functional importance. Different sized subsites can accommodate resolvase by bending of the DNA and probably also by bending of the protein dimer. The bent structure induced by resolvase may result in a selective alignment of sites; our model proposes that subsites II and III interwrap with resolvase in an antiparallel alignment, such that subsite II pairs with subsite III from the other *res* site, although there is as yet no direct evidence for this alignment. An alternative parallel alignment of *res* sites may be possible, but this would not be able to explain the reaction selectivity and product topology of circular substrates.

It may be possible to generate a mutant of resolvase that is capable of recombining *res* crossover sites in the absence of subsites II and III. FIS-independent mutants of Gin and Cin have been isolated. Mutagenesis of resolvase is currently being attempted in our laboratory by David Blake, selecting for mutants that can recombine subsite I x subsite I substrates like those constructed for experiments in section 5.1.

Two copies of subsites II and III should be able to form a synaptic structure, if they are the functional site requirements for synapsis. We might have expected to capture a synaptic complex when there were no crossover sites, but attempts to trap and observe a synapse by intermolecular interactions in the gel assay failed when just subsites II and III were used, even though recombination products were detected when wt-*res* fragments were used. Synaptic complexes may be formed more

efficiently and stabilised by protein crosslinkers by using closed circular molecules (Benjamin and Cozzarelli, 1988). Therefore it might be possible to capture a synaptic intermediate using supercoiled substrates containing either two wt-*res* sites or two copies of subsites II and III. For synapsis of sites in inverted repeat, the substrate may need to be open circular. If a synaptic structure can be isolated using the gel retardation technique, footprinting of these complexes may detect subtle changes in DNA conformation that could indicate a particular interwrap structure.

By using X-ray crystallography, the structure of the amino-terminal domain of gamma-delta resolvase has been determined (Steitz *et al*, personal communication). Eventually, analysis of co-crystals of resolvase and subsite I should help indicate how resolvase can cleave the DNA and exchange strands. Co-crystal structures have been analysed for the phage 434 repressor (Anderson *et al*, 1987), *lac* repressor (Boelens *et al*, 1987) and nucleosomes (Richmond *et al*, 1984). However, the relatively poor affinity of resolvase for its site may not help these investigations.

Explicit predictions for inversion products of *res* substrates provide a test for the formation of our proposed synaptic intermediate. Analysis of inversion products of nicked substrates by electron microscopy is currently being undertaken, although evidence for the predicted 5n knotted product was provided by their migration on an agarose gel. Further evidence for the predicted interwrap structure was provided by the `reverse' recombination of catenated substrates (Stark *et al*, 1989a) and recombination of complex catenated substrates (Benjamin, personal communication).

In vivo inversions of *res* substrates.

The selection against inversion of supercoiled substrates is maintained for *in vitro* recombination reactions. Unexpectedly, inversion products were readily detected *in vivo*, when resolvase was provided in *trans* on a high copy-number plasmid. Not all *res* inversion substrates recombined *in vivo* with equal efficiencies, and the relative inversion frequencies of (+) and (-) forms of a particular substrate also differed. Although the presence of a functional enhancer sequence and host protein FIS did not apparently affect the inversion frequencies, there must be some other *in vivo* effect on the substrate. Supercoiling differences *in vivo* and *in vitro*, transcriptional effects, and other host factors may contribute to the detected rate of inversion of the *res* substrates. Any *in vivo* effect must overcome the constraints imposed by the synapsis of sites, if our synapsis model is correct. A possible competition effect that results in the selection of one inverted form over the other, could shift the equilibrium between the two forms. This competition (induced by some *in vivo* factor) may be more dramatic in certain substrates than others.

Models similar to the one proposed for synapsis of *res* sites have extended into other site-specific recombination systems (e.g. the Gin, Hin and Cin invertase systems, lambda integration and Mu transposition); topological constraints can also explain their reaction selectivities. The requirement for accessory functions, usually coupled to a requirement for supercoiling, has led to suggestions that synaptic complexes, utilising these accessory functions, are an intrinsic part of the reaction.

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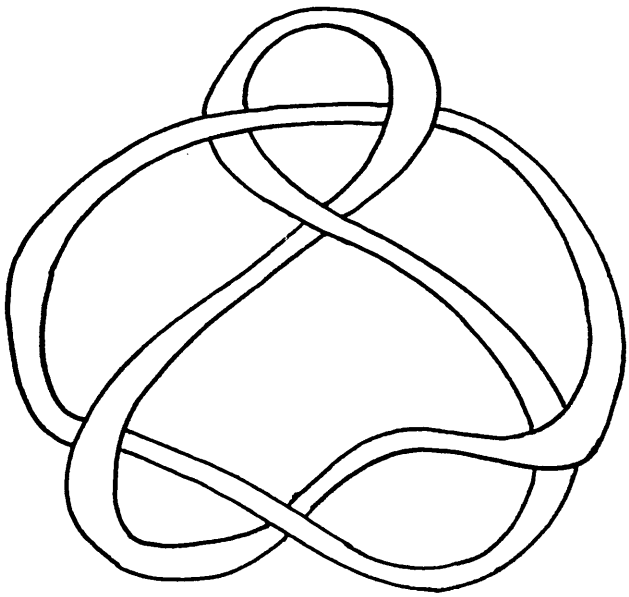

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