Binding of Tn3 Resolvase to Its Resolution Site

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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.

CONTENTS

Abbreviations.

Units

k	-	10^3	m	-	10^{-3}	μ	-	10^{-6}
n	-	10^{-9}	p	-	10^{-12}	f	-	10^{-15}
bp	-	base pairs			kb	-	kilo base pairs	
A	-	Ampères			V	-	Volts	
W	-	Watts			Ci	-	Curies	
℃	-	degrees Centigrade			g	-	grammes	
l	-	litres			m	-	metres	
M	-	molar			mol	-	moles	
h	-	hours			min	-	minutes	
sec	-	seconds			rpm	-	revolutions per minute	
cps	-	counts per second			cpm	-	counts per minute	
dpm	-	disintegrations per minute						

Chemicals/Reagents.

APS	-	ammonium persulphate
ATP	-	adenosine triphosphate
BSA	-	bovine serum albumin
DMF	-	dimethyl formamide
DMS	-	dimethyl sulphate
DNase I	-	deoxyribonuclease I
dNTP	-	deoxynucleoside triphosphate
DTT	-	dithiothreitol
EDTA	-	ethylenediaminetetraacetic acid (disodium salt)
EtBr	-	ethidium bromide
EtOH	-	ethanol
IPTG	-	isopropyl β-D-thiogalactopyranoside
MNNG	-	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
MOPS	-	3-[*N*-morpholino]propanesulphonic acid
OPA	-	o-phthaldialdehyde
PIPES	-	piperazine-*N,N'*-bis[2-ethanesulphonic acid]
RHB	-	resolvase dilution buffer
SDS	-	sodium dodecyl sulphate
TMED	-	*N,N,N',N'*-tetramethylethylenediamine
Tris	-	tris-(hydroxymethyl)aminoethane

Other Terms

X^r - resistance to X. X^s - sensitivity to X.
ori - origin of replication. FIS - factor for inversion stimulation.
PAGE - polyacrylamide gel electrophoresis.
s.c. (plasmid) - supercoiled. o.c. (plasmid) - open circular.

Mutants of Gin or Cin are identified by the original amino acid, the substitution made and its position in the protein sequence, e.g. GinMV114 has a methionine to valine substitution at position number 114.

Mutants of γδ resolvase are denoted by the original amino acid, position and substitution made, e.g. γδ M106C resolvase has a methionine to cysteine substitution at amino acid position 106.

Unless stated otherwise, base positions within a (specified) subsite of *res* are indicated by the general term 'XnB', where X can be L or R, indicating the left or right half of the subsite, respectively; n is the base position, numbered from each end of the subsite (as shown in Fig. 3.20); and B is the base being described (i.e. A, C, G or C).

Acknowledgements.

I would like to thank Marshall Stark for providing advice on a day to day basis, for helping me to "leave this place", and for all the fine malt that I could wrestle free from Martin McBride. I would also like to thank Martin Boocock for p.m. resolvase wisdom, protein preps. and pie-shop provisions. I am grateful to Dave Sherratt for the opportunity to work in his group, and for encouragement along the way. Thanks also to Mary Burke for making things run 1000-fold more smoothly than they otherwise would have. 'Attenborough-esque' levels of thanks to all the (thousands of) members of the Sherratt group, for introducing me to various molecular biology incantations, and for early pots as required: especially to the 'oldies' (Amy, George, Karen, Liz, Sean), Gerhard, Richard and Stephen.

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A great deal more than thanks is due to my family for continued faith and support, and, of course, to Jen for always being a friend.

SUMMARY

Tn3 resolvase binds to three subsites within its DNA target, *res*. Complexes of resolvase bound to *res* can be isolated using non-denaturing polyacrylamide electrophoresis (PAGE). Six complexes are generated with Tn3 *res* and resolvase, but only three complexes have been reported for the related γδ resolution system. By varying the conditions used for non-denaturing PAGE it was demonstrated that the different methods used were not solely responsible for the observed differences in the patterns of complexes produced.

In vivo overexpression of Tn3 resolvase in the presence of several tritiated amino acids was used to produce tritiated resolvase. Substantial purification of the radiolabelled resolvase was achieved using a method adapted for use with small amounts of material, but based on a previously published resolvase purification method. The ^{3}H-resolvase sample produced was used, with ^{32}P end-labelled DNA fragments, to determine the relative and absolute stoichiometry of the protein/DNA complexes observed for Tn3. Although with *res* one complex (complex 5) was not isolated, the observed results indicate that the stoichiometry of the least retarded complex (complex 1) is one resolvase monomer per *res* site, and that this value increases by one monomer per *res* for each successively more retarded complex, to a value of six resolvase monomers per *res* site for the most retarded complex (complex 6). This is consistent with the prediction that each subsite of *res* can be bound by a dimer of resolvase.

A synthetic DNA was used to generate subsite II in isolation from subsites I and III. Binding of Tn3 resolvase to subsite II produced two (or sometimes three) complexes. The stoichiometry of complexes of subsite II with resolvase was consistent with results produced for *res*, i.e. the first complex had a ratio of one monomer of resolvase per DNA site and this value increased by one monomer per site for successively more retarded complexes. Synthetic derivatives of a subsite II sequence were also produced, with an additional 5 or 10 base pairs (bp) of DNA sequence inserted at the subsite centre (named subsite (II + 5) and subsite (II + 10), respectively). Tn3 resolvase was shown to bind to these altered subsites, but the distribution of complexes produced was different from those for subsite II. Complexes of subsite (II + 10) had the same stoichiometry as equivalent complexes of subsite II, as did the first two complexes of subsite (II + 5). DNase I footprinting and methylation interference analysis of Tn3 resolvase binding to subsites II, (II + 5) and (II + 10) suggested that the protein/DNA contacts made were equivalent to those made by resolvase binding

to a normal subsite within *res*. Hence it was concluded that the differences in complex formation observed with subsites (II + 5) and (II + 10) are likely to result from an alteration of contacts made between resolvase molecules bound at these sites.

Binding analysis of several different DNA fragments carrying subsite II or subsite (II + 10) was used to show that binding of a monomer or a dimer of Tn3 resolvase induces a bend at the centre of each subsite. From previous studies it seems likely that resolvase monomer-induced bending of DNA is a common feature for binding to each subsite of *res*.

Purified samples of γδ resolvase and of a γδ resolvase mutant, γδ M106C, (both supplied by Nigel Grindley) were also used in binding experiments. The amino acid substitution present in γδ M106C allows the formation of covalently linked dimers of resolvase. Comparison of binding of Tn3, γδ and γδ M106C resolvases to subsites II, (II + 5) and (II + 10) showed that γδ resolvase binds as a predominantly dimeric species, and that Tn3 resolvase binds as a predominantly monomeric species. This comparison also suggested that binding of Tn3 resolvase to subsite II is co-operative.

A model of Tn3 resolvase binding to *res*, based on these results, is proposed, in which the majority of resolvase is monomeric and occupies the *res* site in six successive steps, each representing binding of a resolvase monomer to one half of a subsite, until each subsite is bound by a dimer of resolvase. The subsites are not occupied in only one specific order, but in a variable order, probably reflecting the affinity of resolvase for each target site. It seems likely that resolvase bound to one half of a subsite can 'shuffle' between binding positions of *res* during non-denaturing PAGE, without complete dissociation of the retarded complex.

A mutagenesis strategy was developed for the production and isolation of mutants of Tn3 resolvase capable of directing resolution between isolated subsite I elements. Initial attempts to produce such mutants were unsuccessful. A new vector for mutagenesis and expression of resolvase was produced and the resolution substrates, used to screen for mutants, were improved.

Chapter One

Introduction

1.1 Transposon Tn3.

The transposition mechanism of transposon Tn3 has two distinct steps (Fig. 1.1), each directed by a different transposon-encoded protein (reviewed in Ref. 77). The *tnpA* gene product, transposase, mediates intermolecular replicative transposition of Tn3, resulting in fusion of the donor and target molecules and duplication of the transposon. Although this increases the number of copies of the transposon, the number of Tn3-carrying DNA molecules has not been increased. Resolvase, the *tnpR* gene product, can direct recombination between specific DNA sites, called *res*, on each Tn3 element, producing discrete recipient and donor DNA molecules, each now carrying a copy of Tn3. The product of resolution is a DNA catenane and DNA gyrase is believed to separate the interlinked circles *in vivo*[10]. The structure of Tn3, its mechanism of transposition and the structure of Tn3 *res* are summarised in Fig. 1.1.

1.2 Prokaryotic Site-specific Recombinases.

Prokaryotic site-specific recombination systems are classified into two families[4,71,82], based on similarities in the protein sequences (Fig. 1.2), recombination site organisation (Fig. 1.3) and reaction mechanisms of the various systems (Fig. 1.4). The resolvase and DNA invertase family (to which Tn3 resolvase belongs) has several members for which *in vitro* recombination has been studied. The most extensively investigated member of the other family is the integrase system of bacteriophage λ[87]. This family also includes the *Saccharomyces cerevisiae* 2μm plasmid FLP/FRT inversion system, the bacteriophage P1 *lox*/Cre system, the *Escherichia coli* type I fimbrial phase variation system and the XerC/XerD recombination system (also of *E. coli*) which is required for efficient segregation of the *E. coli* chromosome and the naturally occurring ColE1 plasmid (and other related plasmids)[4,18,78].

A further subdivision can be made within the resolvase family, based on the type of reaction mediated by each system[29]. Resolvases act on directly repeated sites giving resolution (deletion or excision) of the intervening DNA, while the DNA invertases act on inverted repeat sites to give, as the name suggests, inversion of the DNA sequence in between (Fig. 1.4).

1.3 Tn3 Resolvase and Tn3 *res*.

Resolution by Tn3 resolvase of a negatively supercoiled plasmid carrying two directly repeated *res* fragments has been demonstrated both *in vivo* and *in vitro*[40,46]. *In vitro* resolution of an appropriate supercoiled substrate only requires resolvase and one of a number of simple buffering systems, indicating that there

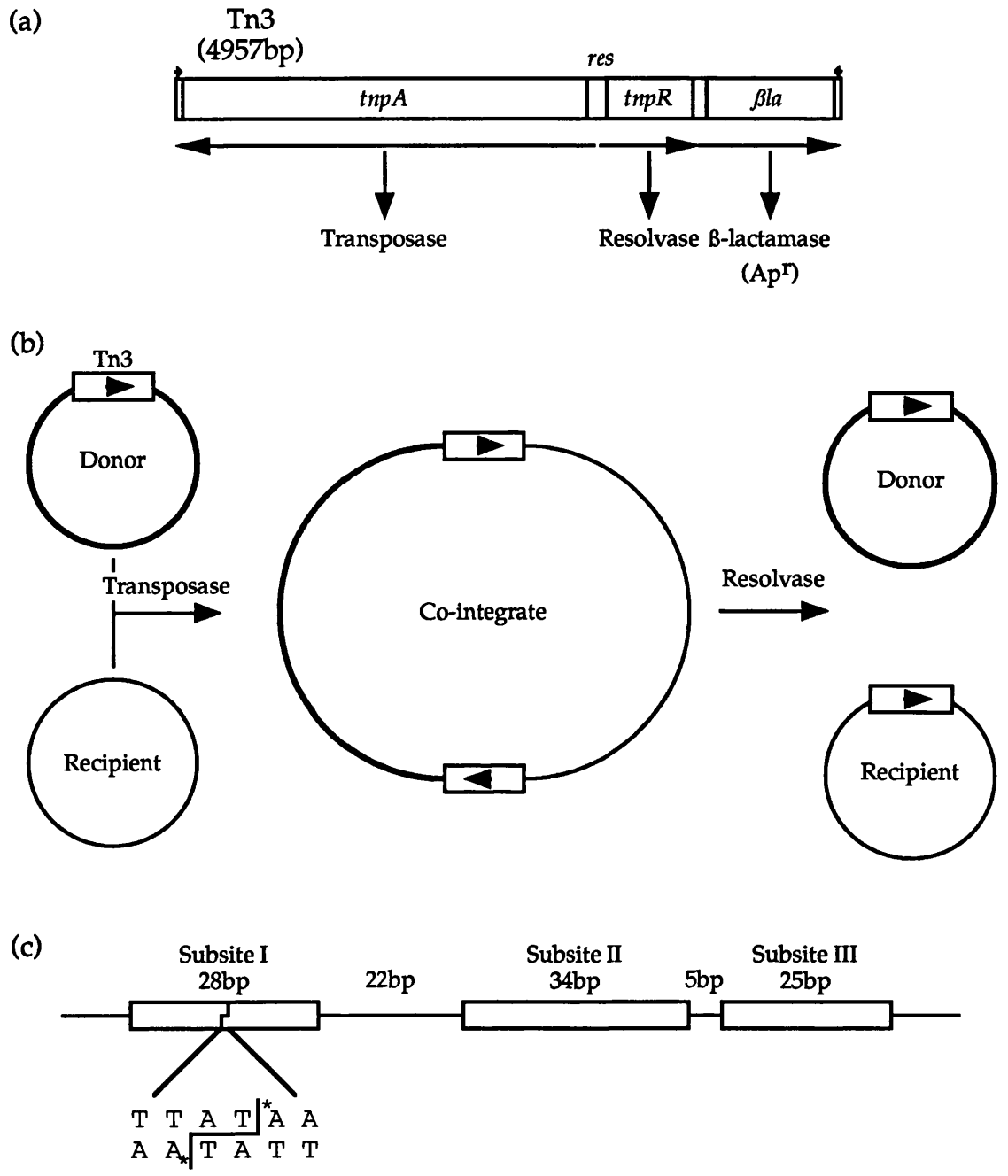


Figure 1.1. Transposon Tn3.

(a) Structure of Tn3. Terminal inverted repeats (38 bp each) are denoted by small arrows. Directions of transcription are as shown.

(b) Replicative Transposition of Tn3. The relative orientation of the Tn3 elements is indicated by an arrowhead. The transposition step also requires DNA polymerase for replication of Tn3, and the resolution step requires DNA gyrase to decatenate the products of co-integrate resolution.

(c) Structure of Tn3 *res*. Subsites I, II and III are indicated by boxes and the position of DNA cleavage by resolvase is also shown. An asterisk is used to denote positions at which resolvase becomes covalently linked to the DNA. All sizes/distances are in base pairs.

Figure 1.2. Alignment of Predicted Amino Acid Sequence of Representative Members of the Resolvase and DNA Invertase Family of Site-Specific Recombinases.

Position numbering is of Tn3 resolvase sequence. Every tenth position is underlined. Where possible numbers are given. Identical residues are denoted by an asterisk. The active site serine (serine 10 in Tn3 resolvase) is denoted by an arrow. Also shown is the position of the proposed helix-turn-helix DNA binding motif. (Based on Ref. 70 and 77).

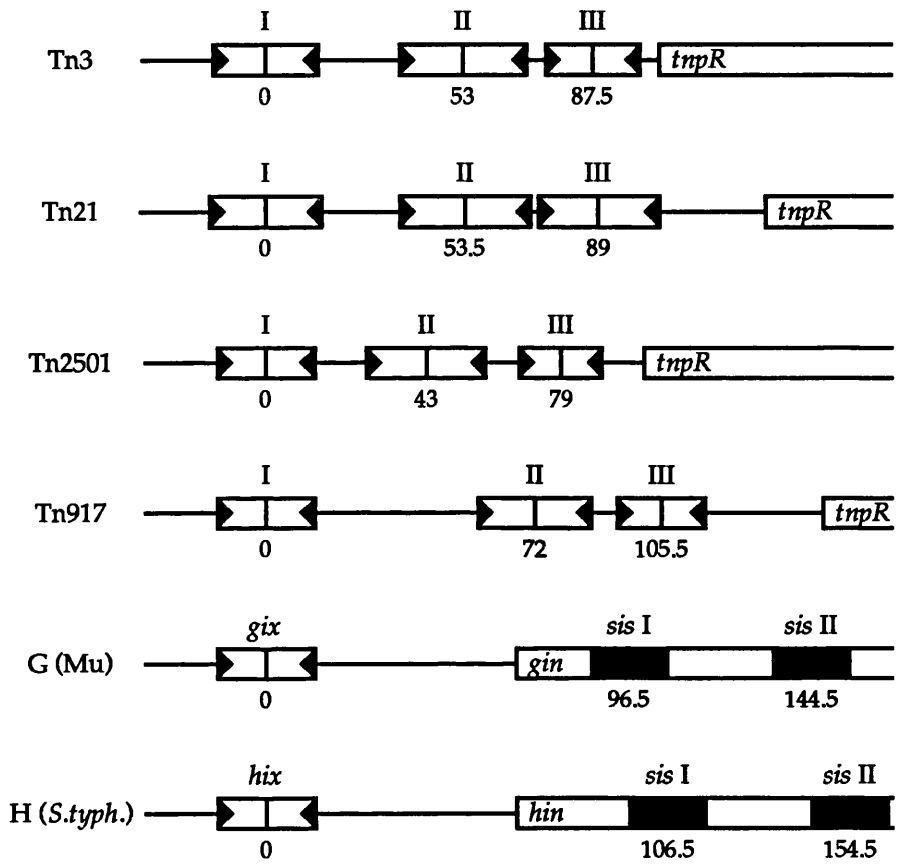


Figure 1.3. Organisation of recombination sites of members of the resolvase/invertase family.

Subsites of *res* are arranged as shown, where subsite I is the crossover site. For the inversion sites both the crossover sites (*gix* or *hix*: unfilled boxes) and accessory (FIS-binding) sites (*sis*: filled boxes) are shown. Phosphodiester co-ordinates are defined from the centre of the crossover sites. The number below each site indicates the co-ordinate of the site centre, and the (sub)site name is shown above. Arrowheads denote partial inverted repeat sequences within a site. The ends of the recombinase genes (i.e. *tnpR, gin, hin*) are also shown where possible.

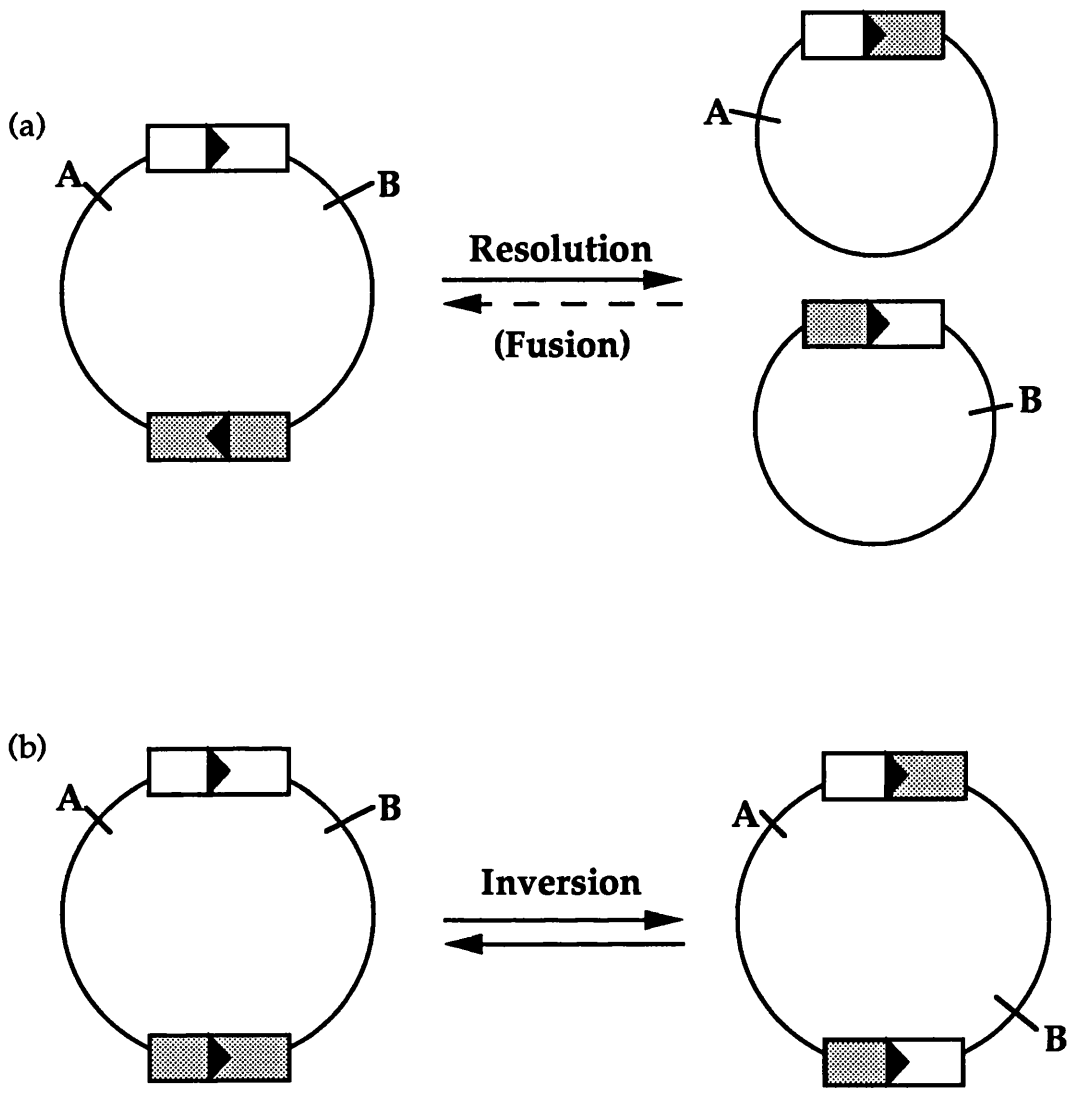


Figure 1.4. Simple representation of the outcomes of resolution and inversion.

(a) resolvase: resolution of a circular substrate carrying directly repeated sites. (the reverse reaction is fusion, which resolvase does not normally catalyse).

(b) invertase: inversion of a circular substrate carrying inverted repeat sites. (See Fig 1.7 and 1.8 for topological details of the specific reactions catalysed). Recombination sites are indicated by blank or shaded boxes, with arrow heads to show their orientation, defined by sequence asymmetry. A and B are sequence markers, e.g. restriction endonuclease recognition sites.

is no absolute requirement for other specific factors[81]. Footprinting and methylation protection have been used to demonstrate sequence-specific interactions of resolvase with three regions within the *res* site (these are named subsites I, II and III)[41]. Fig. 1.3 shows the arrangement of the Tn3 *res* site, in comparison with the recombination sites of some members of the same family. The subsites are of unequal length and are unevenly spaced but each has imperfect dyad symmetry. UV photofootprinting experiments have shown that resolvase binds to *res* on a supercoiled substrate much as it does to a linear *res* fragment[15]. Band shift analysis of Tn3 resolvase with *res* produced six discrete complexes and such studies suggest that each subsite might be bound by a dimer of resolvase[7]. Tn3 resolvase shows significant homology with other resolvases and invertases in the family (Fig. 1.2) and appears to contain two distinct structural domains (by partial proteolysis; see section 1.4). A putative 'helix-turn-helix' DNA binding motif lies close to the C-terminus[59] (Fig. 1.2). Similar motifs in λ Cro and λ repressor have been shown to be responsible for binding of the protein to its target DNA sequence, primarily by insertion of one α helix into the major groove of the DNA, to allow sequence-specific protein/DNA interactions[13,35]. Experiments in which the C-terminal region of Tn21 resolvase was replaced by that of Tn3 resolvase further support the importance of these sequences for DNA binding[5].

1.4 γδ Resolvase and γδ *res*.

Tn3 resolvase and *res* are very similar to those of the γδ transposable element (Tn1000). Indeed Tn3 and γδ resolvase and *res* (and those of Tn1 and R46) are functionally interchangeable[19,41,63]. Hence many features of the Tn3 resolution system are thought to also apply to γδ, and vice versa. Footprinting analysis of γδ resolvase bound to *res* gives equivalent results to those produced for Tn3[25]. An alignment of the DNA sequence of the Tn3 and γδ *res* sites is shown in Fig. 1.5.

Limited proteolysis of γδ resolvase produces a 43 amino acid C-terminal fragment and a large N-terminal fragment (of about 140 amino acids)[1]. Purified C-terminal domain has been shown to bind to *res*, and footprints to the ends of each subsite[67]. Hence it is expected to be primarily responsible for the DNA binding activity of resolvase. The N-terminal domain is believed to contain the catalytic site[1]. The crystal structure of the N-terminal domain of γδ resolvase has been determined at 2.7 Å[74]. Only one of the four dyad-related dimers observed had the two catalytic serine residues at a distance appropriate for cleavage at *res* [74]. Mutational analysis of γδ resolvase suggests that this is not the dimer responsible for binding subsites of *res*. The active sites in the dimer believed to

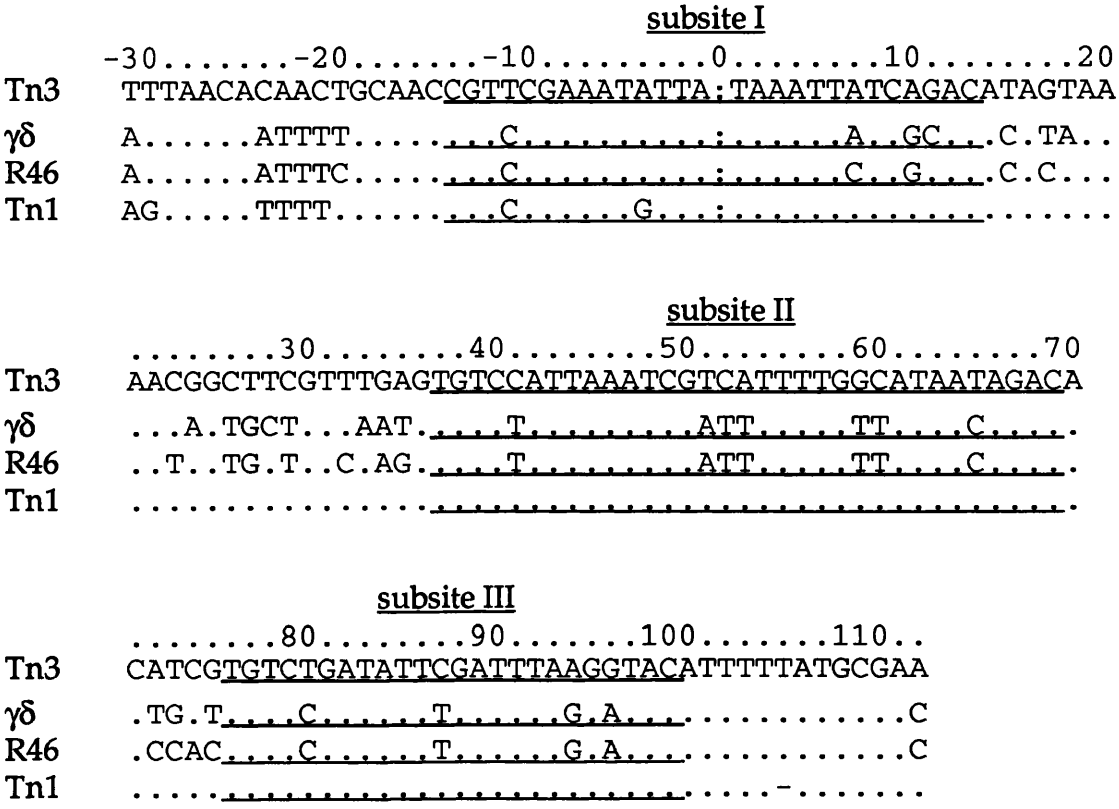


Figure 1.5. Sequence of the Tn3 *res* site.

The Tn3 sequence shown represents nucleotide positions 3074 to 3216 of Tn3. Base positions given above the *res* sites are numbered relative to the phosphodiester bond at the centre of the crossover site (subsite I) which is marked as position 0 and indicated by a colon (:). Note: this is not the position at which resolvase cleaves this DNA strand, but the central point around which the 2 bp staggered break is made (see Fig. 1.1).
Subsites of *res* are denoted by underlining of the relevant sequences. Shown beneath the Tn3 sequence are the base variations which are found in the γδ, R46 and Tn1 *res* sites. Sequence identical to Tn3 *res* is denoted by dots (.). Spaces inserted for the purposes of sequence alignment are denoted by a dash (-).

bind to each subsite were more than 3 nm apart[34]. Although the crystals studied were produced from intact γδ resolvase the C-terminal domain was disordered, and so its position could not be determined.

Non-denaturing polyacrylamide gel electrophoresis of binding reactions of *res* with resolvase has indicated a potential difference between the Tn3 and γδ resolution systems, as six protein/DNA complexes are generated for Tn3[7] but γδ produces only three[28]. *In vitro* recombination of substrates containing wild type and mutant *res* sites suggests that the γδ synapse may be more stable than that of Tn3[81].

1.5 The Resolution Reaction.

Resolvase-directed cleavage and strand exchange of *res* sites occurs at subsite I only. A double stranded DNA break is made at the central AT dinucleotide of subsite I, creating 2 bp 3' protruding ends (Fig. 1.1 and 1.6). A 5'-phosphoserine bond covalently joins the *res* site to resolvase (at the active site serine 10 residue)[28,65]; the resolvase/DNA bond is broken on re-ligation of the DNA ends (Fig. 1.6).

The major product of *in vitro* intramolecular recombination of two directly repeated *res* sites (on a supercoiled substrate) is a -2 catenane[89]. It is believed that this is also the product of *in vivo* resolution, and that the DNA rings are decatenated by DNA gyrase[10]. *In vitro* recombination conditions can also be adjusted to allow intramolecular or intermolecular reactions of linear or relaxed substrates, but these reactions are inefficient.

The specificity of the product created by resolution is believed to reflect topological selectivity in the resolvase recombination mechanism[80]. Analysis of minor *in vitro* resolution products, resulting from multiple rounds of strand exchange without synapse dissociation, led to the deduction that, during resolution, three negative interdomainal DNA nodes (or crossings) are trapped between the recombination sites, and that strand exchange proceeds in a right-handed sense[89,90] (Fig. 1.7). It has been demonstrated that a linkage change, ΔLk, of +4 accompanies conversion of a supercoiled resolution substrate to a -2 catenane product[11]. The reverse reaction, resolvase-directed fusion of a -2 catenane, can occur only at low superhelical densities. A ΔLk value of -4 has been determined for this reaction[80]. These results are consistent with models of synapsis based on plectonemic interwrapping of the *res* sites (Fig. 1.7), but not those involving a solenoidal synapse.(For reviews of Tn3 resolution see Ref. 79 and 82).

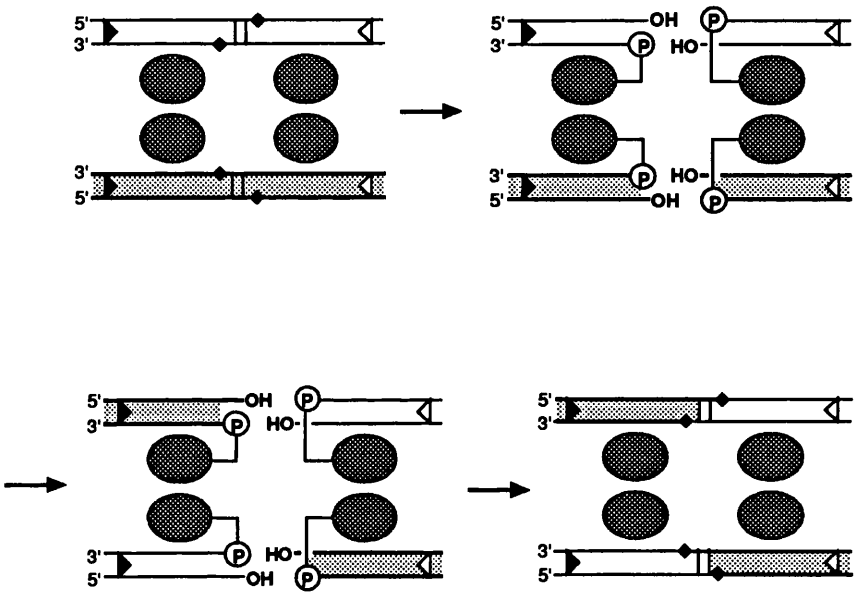


Figure 1.6. Events at the crossover sites during catalysis of recombination by resolvase.

Resolvase subunits are represented by shaded ovals; the ends of the crossover region by inverted black and white arrowheads; the two base pairs in the overlap region by vertical lines; and the phosphates that are attacked by the resolvase by black diamonds. The DNA strands are shown as thick and thin lines, with shading to differentiate the two substrate crossover sites. (Adapted from Ref. 82).

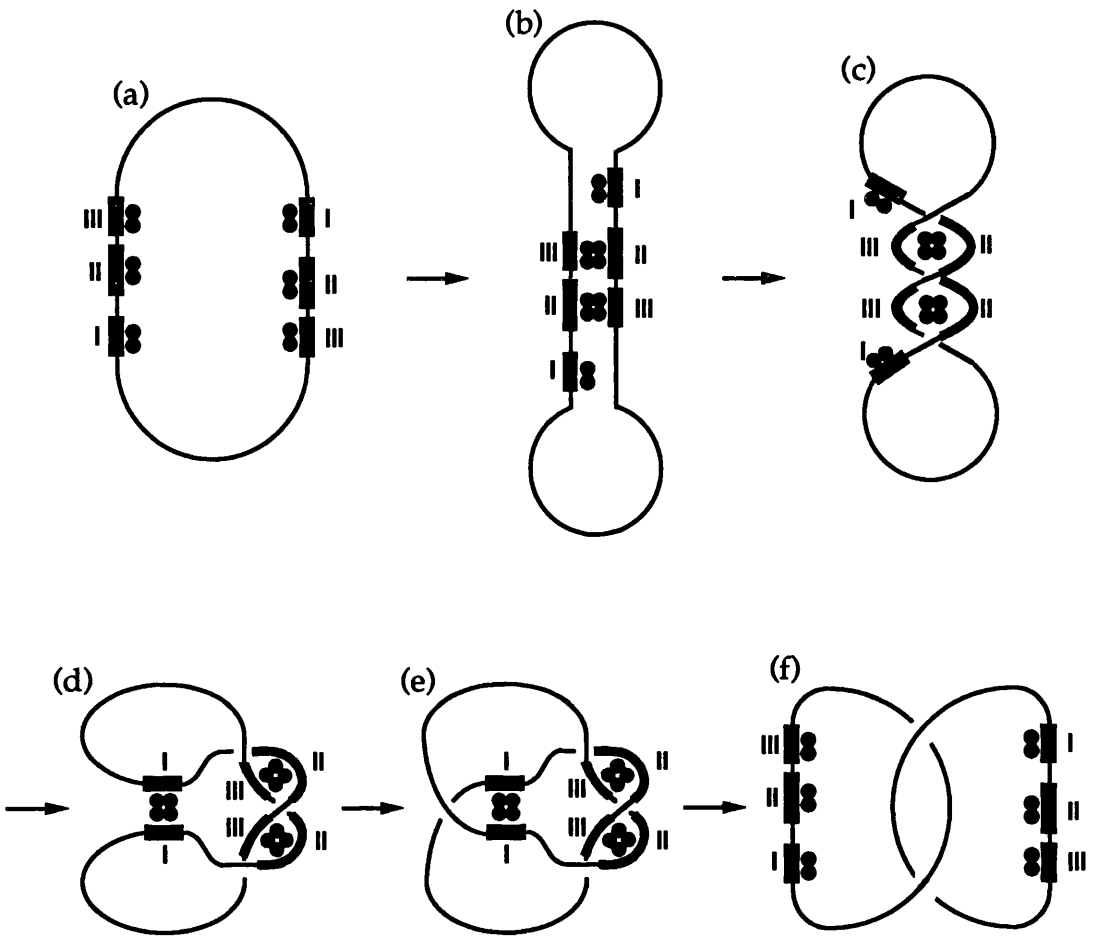


Figure 1.7. Two-step synapsis model for resolvase-catalysed recombination.
Resolvase monomers are represented by filled circles; DNA by thin lines; and subsites of *res* by thick lines. Subsites are numbered (I, II, III) as for Fig. 1.1.
(a) Binding of resolvase to each *res* site (a dimer of resolvase at each site).
(b)-(c) Step 1 of synapsis; antiparallel alignment of subsites II and III of *res* by wrapping of subsites around resolvase tetramer 'cores', formed by resolvase dimer-dimer interactions.
(c)-(d) Step 2 of synapsis; synapsis of crossover subsites (I) by a third resolvase dimer-dimer interaction, to give a recombination proficient synapse with three (-) interdomainal nodes between the crossover subsites.
(d)-(e) Cleavage, strand exchange and re-ligation at subsite I (see Fig. 1.6).
(e)-(f) Dissociation of the synaptic structure.
(See Ref. 79 for further details).

1.6 The DNA Invertase Sub-family.

The best characterised members of the invertase family (reviewed in Ref. 23) are phage Mu Gin, phage P1 Cin and *Salmonella typhimurium* Hin. Gin is responsible for inversion of the G-segment of Mu, altering the expression of tail fibre genes, and hence the phage host range. Tail fibre gene expression in phage P1 is altered by inversion by Cin. In *Salmonella typhimurium* the expression of flagellin genes is switched by Hin-directed inversion of promoter sequences. These invertases are functionally complementary[61]. The recombination sites at which they act (*gix*, *cix* and *hix*, respectively) are structurally similar to a single *res* subsite, i.e. they are similar in size and have imperfect dyad symmetry (Fig. 1.3).

1.7 The Inversion Reaction.

In addition to the invertase protein, efficient inversion of a supercoiled DNA substrate carrying two inverted repeat sites requires host-encoded FIS, supplied *in trans*, and FIS binding sites (*sis*) on the DNA molecule[36,45]. In the absence of FIS (or *sis*) the *in vivo* rate of inversion is reduced at least 100-fold. Each inversion site is believed to bind a dimer of invertase (R. Kahmann; unpublished data). Gin is believed to bind to *gix* in monomer steps (R. Kahmann; unpublished data). The C-terminal domain of an invertase is also expected to be largely responsible for binding of the invertase to DNA. The inversion sites do not have a central AT dinucleotide but the invertase does produce a double stranded 2 bp staggered break at the centre of the site and the protein is also linked to the DNA via a 5′-phosphoserine bond[42] (to the equivalent of serine 10 in resolvase; see Fig. 1.2).

The inversion enhancer complex formed by binding of FIS to *sis* is believed to stabilise the -2 synapse required for recombination between the inversion sites[43]. Strand exchange proceeds by a right handed 180° rotation, giving an unknotted product and a linkage change of +4[37].

Data produced for inversion reactions are consistent with a simple rotation model of recombination based on a plectonemically wrapped synapse, equivalent to that proposed for resolution by resolvase[11,38] (Fig. 1.8). In these models subsites II and III of *res* act as accessory sites, stabilising the required synapse, much as the inversion enhancer complex acts during inversion. Invertase mutants have been produced which can direct efficient inversion in the absence of FIS or *sis*[26,43]. Hence the possibility of making mutants of resolvase which can direct resolution at subsite I in the absence of subsites II and III is an inferred consequence of these comparative models of resolution and inversion.

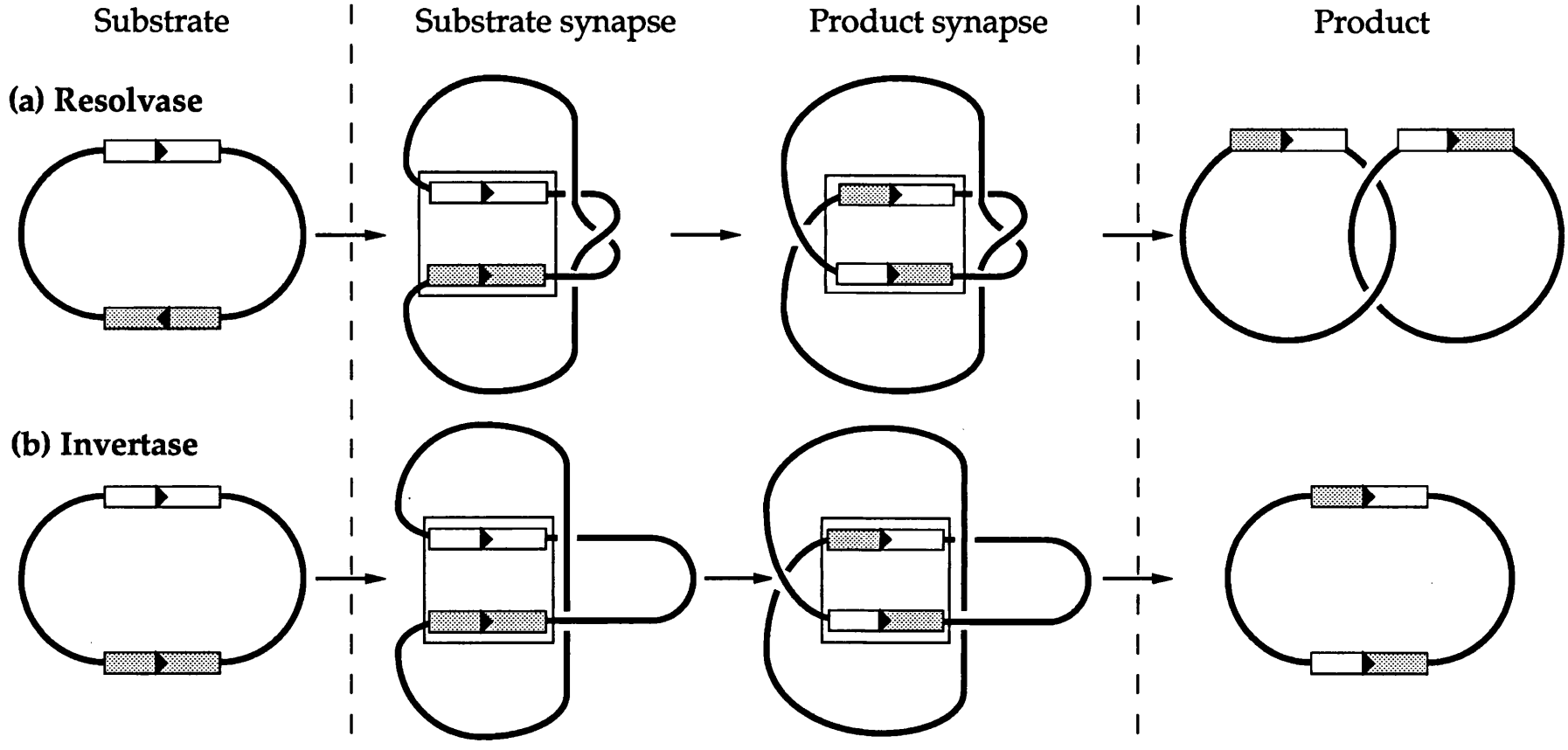


Figure 1.8. Comparative models of recombination by the resolvases and invertases.
Topology of strand exchange from recombination by (a) Tn3 resolvase; (b) Gin/Hin.
Boxes containing arrows represent crossover (sub)sites. Recombinases and accessory sites/proteins are not shown in this diagram. These features are indicated, for resolvase, in Fig. 1.7. The position (and function) of the FIS/*sis* enhancer in the invertase synapse is proposed to be analogous to resolvase bound at subsites II and III of *res*, i.e. to trap a specific number of (-) interdomainal nodes between the crossover sites (2 (-) nodes in the case of inversion). (Adapted from Ref. 82).

1.8 Project Aims.

A long term aim of this work is the production of mutants of resolvase which can recombine subsite I sequences without accessory sites. The creation of such mutants requires the production of an appropriate resolvase expression vector, to act as a mutagenesis target, and of one or more *in vivo* resolution substrates which can be used to screen for the desired recombinase. It is desirable that the mutagenesis strategy adopted should be compatible with the generation of further mutants of resolvase in the future.

All other objectives arise from the general aim of increasing our understanding of the interactions required for binding of resolvase to *res*, prior to synapsis.

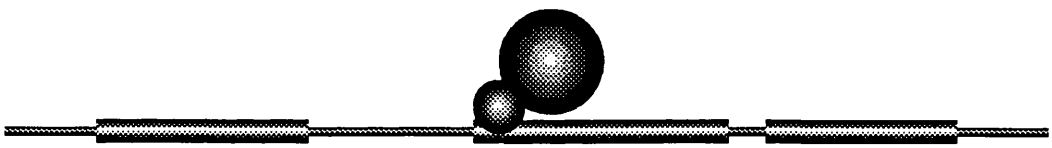
At the outset of this work, interpretation of band shift assay results was based upon predictions of the protein and DNA content of each complex. No direct evidence had been produced regarding this matter. It was considered appropriate to determine the stoichiometry of complexes produced with resolvase and *res* (and other DNA sites, if possible), particularly as several mutants of *res* and resolvase had been categorised according to the number and mobility of complexes they generated. We hoped that a combination of protein and DNA detection methods could be found which would prove suitable for this, and future stoichiometric determinations (e.g. with altered resolvases or DNA sites, or for synaptic complexes). Whether or not this was the case the data produced would serve as a reference point for other binding analysis.

In order to extend such comparisons to band shifts of γδ resolvase and *res*, another aim was to determine the basis of the differences in complex formation observed for the Tn3 and γδ resolution systems.

One further, more general objective was to investigate interactions of resolvase with a DNA site by the production of altered *res* sequences. This matter was somewhat simplified by considering one subsite of *res* (subsite II) in isolation.

Chapter Two

Materials and Methods



2.1 Bacterial Strains.

Most bacterial strains used were derivatives of *Escherichia coli* K-12. The only exception is BL21, an *Escherichia coli* B derivative. All strains used are listed in Table 2.1.

Strain.	Genotype.	Source.
AB1157	*thr*1, *leu*6, *his*G4, *thi*1, *ara*14, *pro*A2, *arg*E3, *gal*K2, *sup*37, *xyl*15, *mtl*1, *tsx*33, *str*31	
AB2463	AB1157, but *rec*A13	D.J.Sherratt
BL21	*hsd*, *gal*	W.Studier
BL21(DE3)	*hsd*, *gal*, (λ*cI ts* 857, *ind*1, *Sam*7, *ini*5, lac_{UV5}-T7 gene-1)	W.Studier
DS941	AB1157, but *rec*F143, *sup*E44, *lac*ZΔM15, $lacI^q$	D.J.Sherratt
EM1	DS941, but *mut*S	E.Morrell
JM101	*sup*E, *thi*, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*ZΔM15, $lacI^q$]	M.R.Boocock
JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, λ^-, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*ZΔM15, $lacI^q$]	Promega

Table 2.1 Bacterial Strains.

2.2 Plasmids.

Plasmids which were used (or constructed) in this work are listed in Table 2.2.

2.3 Synthetic Oligonucleotides.

The oligonucleotides listed below were all synthesised on an Applied Biosystems 391 PCR-Mate oligonucleotide synthesiser using standard reagents supplied by Applied Biosystems or Cruachem Ltd. After synthesis all oligonucleotides were deprotected by incubating the glass support in 1 ml of fresh 30% aqueous ammonia for 1-2 h at room temperature. The sample was vortexed and the supernatant was removed following centrifugation. 1 ml of fresh 30% aqueous ammonia was added to the supernatant which was then incubated overnight at 50 °C in a tightly sealed tube (not Eppendorf tubes). After cooling, the deprotected DNA solution was transferred to several Eppendorf tubes, ammonium acetate was added to 0.5 M, and the DNA was precipitated with 2 volumes of ethanol.

Table 2.2 (part 1 of 3).

Plasmid	Size (kb)	Marker	Origin	Description or Derivation.	Source or Reference.
pAA3	5.8	ApKn	ColE1	800 bp RI/*Sal* I Tn21 *res* & 1500 bp P Kn^r & 800 bp P Tn21 *res* in pUC19	Ref. 2.
pAL211	4.848	ApTc	ColE1	2381 bp P/A (I) pAL11 + 2467 bp P/A (*res*) pMA19	Ref. 8.
pAL225	4.908	ApTc	ColE1	2526 bp P/A (I) pAL13 + 2382 bp (I) pAL215	Ref. 8.
pAL265	4.848	ApTc	ColE1	2381 bp P/A (I) pAL15 + 2467 bp P/A (*res*) pMA2615	Ref. 8.
pAL3054	2.861	Ap	ColE1	Tn3 subsite I (from exonuclase III deletion of *res*) in pUC18	Ref. 8.
pBend2	2.689	Ap	ColE1	Cloning vector for bend analysis (derived from pBR322)	Ref. 39.
pBR322	4.361	ApTc	ColE1	Cloning vector derived from pMB1	Ref. 85.
pCIA70	5.346	Kn	p15A	Tn3 resolvase expression vector (derived from pACYC177)	P.Haffter.
pCS349	5.248	Cm	λ*dv*	930 bp *Sph* I/*Acc* I *argR* fragment in pCB19B	Ref. 83.
pDB4	6.7	Ap	ColE1	1900 bp H/*Acc* I *galK* + 4771 bp H/RV pMA21	Chapter 6.
pDB5	6.7	Ap	ColE1	1900 bp H/*Acc* I *galK* + 4752 bp H/RV pAL225	Chapter 6.
pDB6	6.6	Ap	ColE1	1900 bp H/*Acc* I *galK* + 4692 bp H/RV pAL211	Chapter 6.
pDB7	6.6	Ap	ColE1	1900 bp H/*Acc* I *galK* + 4692 bp H/RV pAL265	Chapter 6.
pDB22	2.834	Ap	ColE1	164 bp *res* RI/*Hae* III pMA1426 + 2670 bp RI/*Sma* I pUC18	Chapter 5.
pDB341	8.5	Kn	pSC101	3060 bp N/P (Partial) Kn^r pDB703 + 5400 bp N/P pDB4	Chapter 6.
pDB351	8.4	Kn	pSC101	3060 bp N/P (Partial) Kn^r pDB703 + 5300 bp N/P pDB5	Chapter 6.
pDB361	8.4	Kn	pSC101	3060 bp N/P (Partial) Kn^r pDB703 + 5300 bp N/P pDB6	Chapter 6.
pDB371	8.4	Kn	pSC101	3060 bp N/P (Partial) Kn^r pDB703 + 5300 bp N/P pDB7	Chapter 6.
pDB441	8.5	Kn	pSC101	3060 bp N/P (Partial) Kn^r pDB704 + 5400 bp N/P pDB4	Chapter 6.
pDB451	8.4	Kn	pSC101	3060 bp N/P (Partial) Kn^r pDB704 + 5300 bp N/P pDB5	Chapter 6.

Table 2.2 (part 2 of 3).

Plasmid	Size (kb)	Marker	Origin	Description or Derivation.	Source or Reference.
pDB461	8.4	Kn	pSC101	3060 bp N/P (Partial) Kn^r pDB704 + 5300 bp N/P pDB6	Chapter 6.
pDB471	8.4	Kn	pSC101	3060 bp N/P (Partial) Kn^r pDB704 + 5300 bp N/P pDB7	Chapter 6.
pDB703	3.485	Kn	pSC101	1260 bp Kn^r B pUC4K + 2225 bp *Hinc* II/*Rsa* I *ori* pSC101 (with *Bam* HI linkers)	Chapter 6.
pDB704	3.485	Kn	pSC101	1260 bp Kn^r B pUC4K + 2225 bp *Hinc* II/*Rsa* I *ori* pSC101 (with *Bam* HI linkers)	Chapter 6.
pDB2004	2.724	Ap	ColE1	50 bp X/P oligo (II) + 2674 bp X/P pUC18	Chapters 3 and 5.
pDB2054	2.734	Ap	ColE1	48 bp X oligo (IIβ) + 2686 bp X pUC18	Chapter 3.
pDB2060	2.737	Ap	ColE1	48 bp X oligo (IIβ) + 2689 bp X pBend2	Chapter 3.
pDB2107	2.744	Ap	ColE1	58 bp X oligo (II + 10) + 2686 bp X pUC18	Chapters 3 and 5.
pDB2118	2.747	Ap	ColE1	58 bp X oligo (II + 10) + 2689 bp X pBend2	Chapter 3.
pDB2201	2.734	Ap	ColE1	48 bp X oligo (IIBN) + 2686 bp X pUC18	Chapter 3.
pDB2507	2.735	Ap	ColE1	55 bp X/*Sal* I oligo (II + 5) + 2680 bp X/*Sal* I pUC18	Chapters 3 and 5.
pDB6001	6.9	Tc	ColE1	1324 bp RI/P Tn3 *tnpR* + 5600 bp RI/P pSelect-1	Chapter 6.
pDB6020	6.4	Tc	ColE1	758 bp RI/*Ssp* I Tn3 *tnpR* + 5600 bp RI/*Sma* I pSelect-1	Chapter 6.
pKO500	4.0	Ap	ColE1	*galK* promoter probe derived from pK01	Ref. 55.
pLS134	6.0	Cm	p15A	2 x Tn3 *res* in pACYC184	Ref. 86.
pLS135	4.0	Cm	p15A	ori^+ resolution product of pLS134	Ref. 86.
pMA21	4.927	ApTc	ColE1	1065 bp P/H pLS139 + 3826 bp P/H pMA44	Ref. 8.
pMA1441	2.986	Ap	ColE1	282 bp *res Pvu* II pLS138 + *Sma* I pUC18	Ref. 15.
pMA6111	5.9	Ap	ColE1	1324 bp RI/P Tn3 *tnpR* + 4600 bp RI/P pKK223-3	Chapter 4.

Table 2.2 (part 3 of 3).

Plasmid	Size (kb)	Marker	Origin	Description or Derivation.	Source or Reference.
pRW80	5.5	ApTc	ColE1	566 bp RI γδ *res* & 573 bp *Rsa* I γδ *res* (pRR51) in pBR322	Ref. 28.
pSelect-1	5.68	Tc	ColE1	Cloning vector for directed mutagenesis (derived from pBR322)	Ref. 62.
pUC4K	3.966	ApKn	ColE1	Cloning vector derived from pBR322	Pharmacia.
pUC18	2.686	Ap	ColE1	Cloning vector derived from pBR322	Ref. 95.

Table 2.2 **Plasmids.**
Only antibiotic resistance markers are listed under 'Marker'. The origin of replication of each plasmid is listed in the 'Origin" column (pSelect-1, pDB6001 and pDB6020 also have phage f1 single stranded replication origins). The following abbreviations are used in the description/derivation section;
A = *Ava* I, B = *Bam* HI, H = *Hin* dIII, N = *Nde* I, P = *Pst* I, RI = *Eco* RI, RV = *Eco* RV, X = *Xba* I,
oligo = oligodeoxynucleotide, (*res*) = Tn3 *res* site, (I) = Tn3 subsite I. Other subsite oligodeoxynucleotides are named as in section 2.3. (Partial) indicates a restriction endonuclease partial digestion product.

(1) Tn3 subsite II derivatives.

The design and use of these oligonucleotides is further described in Chapters 3 and 5. Key to sequences:

Tn3 sequences are bounded by (parentheses).

UNDERLINED text denotes subsite II sequence.

ITALICISED text denotes substitutions or insertions.

(a) Subsite II

Two complementary oligonucleotides, producing subsite II on a 50 bp *Xba* I/ *Pst* I fragment.

Top strand (IIAXP54):

5'CTAGA(TTGAGTGTCCATTAAATCGTCATTTTGGCATAATAGACACATCG)CTGCA3'

Bottom strand (IITPX46):

5'G(CGATGTGTCTATTATGCCAAAATGACGATTTAATGGACACTCAA)T3'

(b) Subsite (II + 5)

Two complementary oligonucleotides, producing subsite II with an additional 5 bp inserted at its centre. Gives a 55 bp *Xba* I/ *Sal* I fragment.

Top strand (+5GXS55):

5'CTAGA(TTGAGTGTCCATTAAATCGTCA*TTTCA*TTTTGGCATAATAGACACATCG)G3'

Bottom strand (+5TSX55):

5'TCGAC(CGATGTGTCTATTATGCCAAAA*TGAAA*TGACGATTTAATGGACACTCAA)T3'

(c) Subsite (II + 10)

Two complementary oligonucleotides, producing subsite II with an additional 10 bp inserted at its centre. Gives a 58 bp *Xba* I fragment.

Top strand (5'+10T58):

5'CTAGA(TGAGTGTCCATTAAATCGTCA*TTTATCGTCA*TTTTGGCATAATAGACACATC)T3'

Bottom strand (3'+10X58):

5'CTAGA(GATGTGTCTATTATGCCAAAA*TGACGATAAA*TGACGATTTAATGGACACTCA)T3'

(d) Subsite II (β)

Two complementary oligonucleotides, producing subsite II on a 48 bp Xba I fragment.

Top strand (5'WTIIX48):
5'CTAGA(TGAGTGTCCATTAAATCGTCATTTTGGCATAATAGACACATC)T3'
Bottom strand (3'WTIIX48):
5'CTAGA(GATGTGTCTATTATGCCAAAATGACGATTTAATGGACACTCA)T3'

(e) Subsite IIBN
Two complementary oligonucleotides, producing subsite II containing a *Bgl* II and an *Nsi* I restriction site. Gives a 48 bp *Xba* I fragment.
Top strand (5'IIBNX48):
5'CTAGA(TGAGTGTCCATTAGATC*T*TCATTT*AT*GCATAATAGACACATC)T3'
Bottom strand (3'IIBNX48):
5'CTAGA(GATGTGTCTATTATGC*AT*AAATGA*A*GATCTAATGGACACTCA)T3'

(2) pSelect-1 Mutagenesis Oligonucleotides.
Details of the use of these oligonucleotides is given in Chapter 6.
Key to sequences:

UNDERLINED text denotes changes from target sequence.
Text in *ITALICS* denotes sequence synthesised using mixed DNA precursors, in an attempt to introduce degeneracies.

(a) Ampicillin Repair Oligo (GAp^{r}27).
This oligonucleotide contains a four base insertion in the pSelect-1 target sequence. When this change is made in pSelect-1 the resultant plasmid gains ampicillin resistance. A *Pst* I site is also introduced by this insertion.
5'GTTGCCATTGCTGCAGGCATCGTGGTG3'

(b) Tn3 *tnpR* Degenerate Oligonucleotide (GXho66).

This 66 bp oligonucleotide was designed to anneal to the Tn3 *tnpR* sequence coding for amino acids M103 to E124 of resolvase. The 6 bases at each end of the oligonucleotide were synthesised as normal. A translationally silent base change (from *tnpR* sequence) was produced at the 3' end of the oligonucleotide which would create a unique *Xho* I site in mutagenised *tnpR* derivatives, allowing easier identification of plasmids produced from the mutagenesis protocol. The remaining 54 bases were synthesised using nucleotide precursor reagents which were contaminated with a small amount of the other three precursors (about 1.5% of normal concentrations of each). This should produce an average of one degeneracy in each full length DNA molecule synthesised[31,73]. The sequence of this oligonucleotide is shown beneath the protein sequence coded.

M G Q M V V T I L S A V A Q

5'ATG GGG *CAA ATG GTG GTC ACC ATC CTG TCG GCT GTG GCA GAG*

A E R R R I L E

GCT GAA CGC CGG AGG ATC CTC GAG3'

2.4 Chemicals.

Chemicals	Source
General chemicals, biochemicals and organic solvents.	Aldrich/Sigma, BDH, Fluka, May and Baker.
Media.	Difco, Oxoid.
Agarose.	BRL, Flowgen.
Radiochemicals.	NEN-DuPont.
10x restriction enzyme buffers.	BRL, Boehringer Mannheim, Promega.
5x ligation buffer.	BRL.
Deoxynucleotide triphosphates.	Promega.

2.5 Proteins.

Tn3 resolvase	gift from Martin Boocock
γδ resolvase	gift from Nigel Grindley (Yale).
γδ resolvase mutant, M106C	gift from Nigel Grindley (Yale).
Tn21 resolvase	gift from Steve Halford (Bristol).
Eco RI restriction endonuclease	gift from Steve Halford (Bristol).

Other DNA restriction and modification enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs, Promega and United States Biochemical.

2.6 General Stock Solutions.

(a) DNA Electrophoresis.

50x TAE: 1210 g Tris, 410 g sodium acetate, 93 g $Na_2EDTA.2H_2O$, 71 ml glacial acetic acid, made to 5 litres in deionised water. 1x TAE should be pH 8.2.

10x TBE: 109 g Tris, 55 g boric acid, 9.3 g $Na_2EDTA.2H_2O$, made to 1 litre in deionised water. Gives pH 8.3.

5x 'single colony' final sample buffer: 10% ficoll, 5% SDS, 0.05% bromophenol blue, 0.25% orange G in 1x TAE. Diluted 5-fold in 1x TAE and RNase A added to 10 µg/ml (pretreated by boiling for 10 min to inactivate DNase) for 1x stock.

Final Sample (loading) Buffer: 10% ficoll, 0.5% SDS, 0.05% bromophenol blue, 0.25% orange G in 1x TAE.

4x K mix: 25% sucrose, 0.2 mg/ml protease K, 0.01% bromophenol blue in deionised water. Only for use in agarose gel electrophoresis.

(b) Protein Electrophoresis.

10x Laemmli gel buffer: 30 g Tris, 144 g glycine, 10 g SDS, made to 1 litre in deionised water.

(c) *In vitro* DNA manipulation.

restriction digest buffers: 10x stocks as recommended (and supplied) for each restriction enzyme; stored at -20 °C. Appropriate restriction digest buffers were used for Klenow polymerase and CIP treatment.

5x KGB: 0.5 M potassium glutamate, 125 mM Tris-acetate (pH 7.6), 50 mM magnesium acetate, 250 µg/ml BSA, 2.5 mM 2-mercaptoethanol. Stock was filter sterilised and stored at -20 °C.

5x ligase buffer: BRL, stored at -20 °C.

10x TE: 0.1 M Tris.HCl (pH 8.0), 10 mM EDTA, in deionised water.

(d) Other Stocks.

Phenol: Buffered against 0.5 M Tris.HCl (pH 8.0). Contained 0.1% 8-hydroxyquinoline.

2.7 Conditions for *Escherichia coli* Growth and Selection.

(a) Media.

L-Broth: 10 g bacto-tryptone, 5 g bacto-yeast extract, 5 g NaCl, made to 1

litre in deionised water, adjusted to pH 7.5 with NaOH. Where required, supplemented with 0.2% glucose.

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

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

M^{ac}Conkey Agar: 40 g/l M^{ac}Conkey agar base (Difco), 1% appropriate sugar (in this case galactose) in deionised water.

4x Davis and Mingioli salts (D&M salts): 500 ml deionised water, 28 g $K_2HPO_4.3H_2O$, 8 g KH_2PO_4, 4 g $(NH_4)_2SO_4$, 1 g tri-sodium citrate.$2H_2O$, 0.4 g $MgSO_4.7H_2O$, made to 1 litre with deionised water.

10x M9 salts: 128 g $Na_2HPO_4.7H_2O$, 30 g KH_2PO_4, 10 g NH_4Cl, 5 g NaCl, 2.5 g $MgSO_4.7H_2O$, made to 1 litre with deionised water.

10x M63 salts: 136 g $K_2HPO_4.3H_2O$, 20 g $(NH_4)_2SO_4$, 5 g $FeSO_4.7H_2O$, 2 g $MgSO_4.7H_2O$, made to 1 litre with deionised water.

Minimal Broth: D&M, M9 or M63 salts diluted in sterilised deionised water, to give 1x concentration. Supplemented with 5 μg/ml thiamine (vitamin B_1), an appropriate carbon source (generally 0.2% glucose), and amino acids if required (at 40 μg/ml).

Minimal Agar: As for minimal broth but with the addition of 1.5% agar.

Phage Buffer: 7 g $K_2HPO_4.3H_2O$, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4.7H_2O$, 15 mg $CaCl_2.2H_2O$, 1 ml 1% gelatin, made to 1 litre in deionised water.

Tetrazolium Agar: 25.5 g Antibiotic Medium 2 (Difco), 50 mg 2,3,5-triphenyl-2H-tetrazolium chloride, 950 ml deionised water. Supplement with 50 ml of a 20% sugar solution. Rehydrated Difco Antibiotic Medium 2 contains 1.5 g/l bacto-beef extract, 3.0 g/l bacto-yeast extract, 6.0 g/l bacto-peptone and 15.0 g/l bacto-agar.

Prior to the addition of supplements growth media were sterilised at 120 °C for 15 min and minimal salts at 120 °C for 20 min. $CaCl_2$ was sterilised at 114 °C for 10 min and other supplements at 108 °C for 10 min.

(b) Indicators.

X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside): used (at 20 to 40 μg/ml in solid growth medium) to test for expression of the α subunit of lacZ from derivatives of pUC18 (or pSelect-1) in DS941. Blue colonies are formed by lacZ expressing cells which can cleave X-gal, to give a blue product. The colourless X-gal is not cleaved by $lacZ^-$ cells which give white colonies. Stock X-gal (40 mg/ml in DMF) was stored at -20 °C.

The indicators used in MacConkey medium and Tetrazolium medium both respond to the pH of colonies grown on the plates. These are discussed further in Chapter 6. Tetrazolium Chloride 1% solution (in deionised water) was stored at 4 °C.

(c) Antibiotics.

The following concentrations of antibiotics were used for both liquid and solid selective media.

Antibiotic	Stock Solution	Selective Conditions
Streptomycin (Str)	10 mg/ml in H_2O	100 μg/ml
Ampicillin (Ap) *	5 mg /ml in H_2O	50 μg/ml
Tetracycline (Tc)	1 mg/ml in 10 mM HCl	10 μg/ml
Chloramphenicol (Cm)	2.5 mg/ml in Ethanol	25 μg/ml
Kanamycin (Kn)	5 mg/ml in H_2O	50 μg/ml

Table 2.3 Antibiotics.

* : For production of Tn3 resolvase from JM101 pMA6111 ampicillin was used at a final concentration of 100 μg/ml.

Stock solutions were stored at 4°C. For agar plates, molten agar was stabilised at between 50 °C and 60 °C prior to the addition of antibiotic.

(d) Growth Conditions.

Liquid cultures were grown at 37 °C with vigorous shaking. Growth on agar plates was at 37 °C for at least 12 h, with plates inverted. Antibiotics, indicators and inducers were used as required. Bacterial strains to be stored were grown overnight in L-Broth and diluted 2-fold with 40% glycerol, 2% peptone prior to storage at -70 °C.

(e) Induction.

Induction of expression from IPTG-inducible promoters was generally produced by the addition of IPTG at 0.1 - 0.5 mM final concentration, to the appropriate media. IPTG stocks (100 mM in deionised water) were stored at -20 °C

2.8 Plasmid DNA Preparation.

(1) Large Scale DNA Purification.

The method used is derived from that of Birnboim and Doly[9].

Solution B.D.#1 50 mM glucose, 25 mM Tris.HCl pH 8.0, 10 mM EDTA.

Solution B.D.#2 0.2 M NaOH, 1.0% SDS (made freshly).

Solution B.D.#3 0.6 vol 5M CH_3COOK, 0.115 vol CH_3COOH, 0.285 vol H_2O.

L-broth (200 ml), with appropriate antibiotics, was inoculated with plasmid-containing cells and incubated at 37 °C, with vigorous shaking, for approximately 12 h (or until stationary phase is reached). The cells were harvested by centrifugation (12,400g, 5 min, 4 °C). The cell pellet can be stored at -20 °C for several weeks. The pellet was resuspended in 4 ml of B.D.#1 by mixing for 5 min at 0°C. 8 ml B.D.#2 was added, the sample was mixed by gentle inversion and then cooled for 4 min on ice. 6 ml cold B.D.#3 was added and the solution was mixed with cooling. The plasmid DNA solution was separated from the cell debris by centrifugation (39,200g for 30 min at 4 °C, stopped without braking). Each supernatant was poured into 12 ml isopropanol and the DNA was precipitated for about 15 min at room temperature. The precipitated material was pelleted by centrifugation (27,200g, 10 to 30 min at 20 °C). The pellet was washed with 70% ethanol, vacuum dried and resuspended in 2.09 ml of 1x TE buffer to allow further purification by CsCl/EtBr gradient ultracentrifugation. 5 g of CsCl was dissolved in 3 ml H_2O and then mixed with the DNA solution. 270 µl of 15 mg/ml ethidium bromide was added and the solution was placed in an ultracentrifugation tube. The tubes were placed in a Beckman Ti70 fixed angle rotor and spun at 200,000g for 16 h at 25 °C. DNA bands were visualised using a long wave UV source (365 nm). Generally two bands were visible, the upper containing chromosomal and nicked plasmid DNA, the lower containing the desired supercoiled plasmid DNA. The lower band was removed using a 1 ml syringe and the ethidium bromide removed by repeated extraction with 0.5 vol. butanol. In some cases the DNA was then dialysed twice in 1x TE. Alternatively the solution was diluted four fold with H_2O, then ethanol precipitated, dried and resuspended in 1x TE buffer.

(2) Small Scale DNA Preparation.

An Eppendorf microcentrifuge was used for all centrifugation steps described in the following protocols.

(i) Alkaline Lysis Mini-prep.

This method is adapted from the above large scale prep. and uses the same B.D.#1, B.D.#2 and B.D.#3 solutions.

Colonies picked from plates were used to inoculate 5 ml of 2x YT broth (with antibiotics). The cultures were grown (37 °C with shaking) to stationary phase (normally overnight). 1.5 ml of culture was harvested by centrifugation (14,000 rpm for 30 sec) and the supernatant removed. The cell pellet was resuspended in 100 µl of cold B.D.#1 and incubated for 5 min at room temperature. Next 200 µl of B.D.#2 was added and mixed, then placed on ice for 5 min. 150 µl of cold B.D.#3 was added and the solution was mixed gently on ice for 5 min. Cell debris was pelleted by centrifugation (14,000 rpm for 5 min) and the supernatant was transferred to an equal volume of 1:1 phenol/chloroform. This was mixed by vortexing and the phases separated by centrifugation (14,000 rpm for ≥ 2 min). The aqueous (upper) phase was transferred to 2.5 vols. ethanol and incubated at room temperature for ≥ 2 min to allow the nucleic acid to precipitate. The precipitate was pelleted by centrifugation (14,000 rpm for 5 min) and the pellet was washed with 1 ml of 70% ethanol. The pellet was dried (5 min at room temp) and then resuspended in 30 to 50 µl of 1x TE.

(ii) STET preparation (adapted from Holmes and Quigley[32]).
STET buffer : 8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris.HCl pH 8.0
Cells were grown to stationary phase in 10 ml L-broth (37 °C with shaking) with selection. Cultures were harvested by centrifugation (14,000 rpm for 10 min) and the pellets resuspended in 700 µl of STET buffer and 50 µl of a 10 mg/ml lysozyme solution was added. The samples were boiled for 45 sec and then centrifuged (14,000 rpm for 15 min at 4 °C). The pelleted cell debris was removed using a toothpick and the remaining supernatant was extracted once with an equal volume of phenol and twice with equal volumes of chloroform (taking the aqueous phase in each case). The solution was then mixed with 1 vol isopropanol and incubated for 15 min at room temperature. The nucleic acid precipitate was pelleted by centrifugation (14,000 rpm for 15 min) and the pellet was dried at room temperature before being resuspended in 50 µl of 1x TE.

2.9 Bacterial Transformation with Plasmid DNA.

(1) High Efficiency Transformation Using Rubidium Chloride.
(a) Preparation of Competent Cells.

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

TFB I : 30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% v/v glycerol, adjusted to pH 5.8 with 0.2 M AcOH

TFB II : 10 mM MOPS (or 10 mM PIPES), 75 mM $CaCl_2$, 10 mM RbCl, 15% v/v glycerol, adjusted to pH 6.8 with 0.1 M HCl or KOH as required.

TFB I and TFB II were filter sterilised.

4 ml of stationary culture, grown in 20 ml of Ψ broth was added to 200 ml prewarmed Ψ broth in a large flask. The culture was grown to give OD_{600} = 0.46 to 0.6. The cells were rapidly cooled and then harvested by centrifugation (3,840g for 10 min at 4 °C). The pellet was gently resuspended in 40 ml of cold TFB I and then placed on ice for 30 min. The cells were repelleted by centrifugation (3,840g for 10 min at 4 °C) and resuspended in 8 ml of cold TFB II, then placed on ice for 15 min. 200 µl aliquots of cells were then dispensed to prechilled 1.5 ml Eppendorf tubes which were then capped and placed in liquid nitrogen to freeze the cell suspension. The aliquots of frozen cells were stored at -70°C.

(b) Transformation Procedure.

Aliquots of competent cells were allowed to thaw on ice. Once thawed 50 µl of cells was added to ≤ 10 µl of plasmid DNA in 1x TE. The samples were heat shocked, i.e. 5 min at 37°C (or 2 min at 42 °C) then cooled to 0 °C. 5 vols. of 2x YT were added and the samples were incubated at 37 °C for 1 h, before plating the cells on selective media and incubating at 37 °C overnight.

(2) Transformation Using Calcium Chloride.

(a) Preparation of Competent Cells.

1 ml of stationary culture, grown in 2.5 ml L-broth, was diluted 20 fold in fresh L-broth. This culture was grown at 37 °C with shaking to give A_{600nm} of 0.4 to 0.5. The cells were harvested by centrifugation (12,100g, 1 min, 4 °C) and resuspended in 10 ml of cold 50 mM $CaCl_2$. The cells were repelleted (12,100g, 1 min, 4 °C) and resuspended in 1 ml of cold 50 mM $CaCl_2$. At this point 15% v/v glycerol was mixed with the cells which were then dispensed to 200 µl aliquots and stored at -70 °C. However, if the cells were required within 48 h then the cells were kept on ice (for at least 30 min) until needed.

(b) Transformation.

200 µl of competent cells (kept or thawed on ice) was mixed with ≤ 50 µl of DNA in 1x TE. The sample was incubated on ice for 15 min prior to heat shock (5 min at 37 °C, or 2 min at 42 °C) and then returned to ice for another 15 min. 200 µl L-broth was added and the sample was incubated at 37°C for 60 min. 50 to 100 µl of culture was spread on selective plates and grown overnight.

Note : The incubation step prior to plating of the transformation mixture is to allow expression of plasmid-encoded resistance genes, required for growth under the selective conditions. As ampicillin does not directly prevent this gene expression, transformants being selected just by ampicillin resistance can be plated directly after the second cooling of the heat shock step.

2.10 Restriction Endonuclease Digestion of DNA.

Restriction digests generally used a 2-fold to 10-fold excess of enzyme to ensure complete cleavage of the DNA. Digests were incubated at the suppliers' recommended temperature for >1 h and were terminated by the addition of a loading buffer, or by heating (≥70 °C), or phenol extraction. The buffer used for digests was either that suggested by the supplier or an appropriate concentration of KGB buffer[54]. KGB was mainly used where more than one restriction endonuclease was included in a digest.

Partial restriction digests were produced using dilutions of stock enzyme. An appropriate level of DNA cleavage was determined prior to digesting large amounts of purified plasmid DNA.

2.11 Calf Intestinal Phosphatase (CIP) Treatment of DNA.

Approximately 0.5 units of CIP was added to some restriction digests (to remove 5′ phosphate groups from linearised DNA fragments) to prevent ligation of certain fragments to each other, or themselves. The digests were then incubated for 15 min further at 37 °C. CIP was then separated from the sample by organic extraction, or by electrophoresis and recovery of the DNA fragment.

2.12 Filling of 3′ Recessed DNA Fragment Ends.

Digested DNA was incubated at room temperature in KGB buffer (1.0x, 1.5x or 2.0x) with 100 μM each of dATP, dCTP, dGTP and dTTP, and 2 units of Klenow fragment of DNA polymerase I, in a total volume of 25 μl. The reaction was stopped after 15 to 30 min by heating the sample to 70 °C for 5 min.

2.13 5' Phosphorylation of Oligonucleotides.

T4 polynucleotide kinase was used to phosphorylate oligonucleotides as described in the 'Altered Sites' technical manual[62].

2.14 Ligation of DNA Fragments.

DNA fragments (1-2 μg) were ligated (normally in a volume of 10 μl) in 1x BRL ligation buffer with 1 unit of T4 DNA ligase. Samples were incubated at 16 °C overnight, or for 2-4 h at room temperature. Aliquots (~3μl) of the ligation mix

were used to transform competent cells. If more DNA was used then reactions were scaled up accordingly. The ratio of insert to vector was (very roughly) 3:1 for 'sticky end' ligations and 10:1 for blunt end ligations.

2.15 DNA Precipitation.

Where necessary the salt concentration of the DNA solution was made to 0.3 M with sodium acetate. 2 vols EtOH were added and the sample was mixed and incubated at 0℃ or -20℃ for >15 min (sometimes overnight). The precipitated material was pelleted by centrifugation. The pellet was rinsed with 70% EtOH and dried prior to further manipulation.

2.16 Gel Electrophoresis

(1) Agarose Gels.

0.7-1.5% agarose gels were produced by dissolving agarose powder in 1x TAE by boiling (steam bath or microwave oven). The solution was allowed to cool to about 60 ℃ before pouring it into a gel former, and leaving to set at room temperature.

(a) Purified DNA samples.

Isolated plasmid DNA, restriction digest products and recombination products were analysed using submerged horizontal 1x TAE gels. The most commonly used gels were ≥15 cm long and were run overnight at about 1.5 V/cm.

(b) 'Single colony' samples.

A single colony was patched to a fresh plate (patches >1 cm^2). After overnight growth a sterilised toothpick was used to scrape the cells from the plate to 100-150 µl of 'single colony' final sample buffer. After mixing, the sample was incubated at room temperature for 15 min and then spun in a microcentrifuge (full speed) for 30 min. Avoiding the sticky pellet, 30-50 µl of supernatant was loaded on to a 1.2% agarose gel which was placed in, but not under, 1x TAE buffer. Once all the samples were loaded electrophoresis was started (50-80 V). Once the samples had been given time to enter the gel (separation of the two dye bands was used to indicate this) 1x TAE was added to submerge the gel. Electrophoresis was then completed as for other agarose gels.

(2) Polyacrylamide Gels

(a) Non-denaturing Polyacrylamide Gels for DNA Analysis.

These gels were mostly used for restriction analysis of plasmid constructs but were also used for purifying small double stranded DNA fragments. The concentration of acrylamide used varied with the size of fragments being run.

Acrylamide [% (w/v)]	Effective Range of Separation (bp)
3.5	1,000 - 2,000
5.0	80 - 500
8.0	60 - 400
12.0	40 - 200
15.0	25 - 150
20.0	6 - 100

Table 2.4 Effective Range of Separation of Double Stranded DNA on Polyacrylamide Gels.

A number of different sized vertical gel kits were used, commonly with 0.75 mm spacers. These gels were sealed around the edges with plastic tubing, which was clamped between the gel plates. The acrylamide gel mix was poured between the plates and left to set at room temperature after the insertion of a well former. Once the gel was set, the clamps and tubing were removed and the formed gel was clamped into the gel kit. Buffer was generally added before removing the well former. The gels were run at room temperature in 1x TBE at a constant voltage, normally between 100 and 200 V. Depending on the material run, the gels were ethidium bromide-stained, silver-stained, or bands were visualised by autoradiography of the wet gel.

The different sized kits used required 10, 30 or 45 ml of acrylamide mix. The following recipe for a 30 ml gel was directly adjusted for other gel volumes.

Reagent	Volume Added
30% acrylamide : 0.8% bisacrylamide (w/v)	X ml
10x TBE	3 ml
H_2O	(27 - X) ml
10% APS (w/v)	360 µl
TMED	18 µl

Table 2.5 Components of an X% (w/v) Polyacrylamide Gel (for DNA).

(b) Non-denaturing Polyacrylamide Gels for Analysis of DNA/Protein Complexes.

These gels were used to separate protein/DNA complexes. The most frequently used type of gel was a 6% polyacrylamide, 50 mM Tris.Glycine pH 9.4, 0.1 mM EDTA gel (called TG 9.4) , e.g. Table 2.6 shows the components for 30 ml of TG 9.4 gel mix.

Reagent	Volume Added
30% acrylamide : 0.8% bisacrylamide (w/v)	6 ml
0.5 M Tris.Glycine pH 9.4	3 ml
0.2 M EDTA	15 µl
H_2O	20.5 ml
10% APS (w/v)	360 µl
TMED	18 µl

Table 2.6 Components of a 6% (w/v) Polyacrylamide Gel (TG 9.4).

Other gel buffers used were 10 mM Tris.HCl pH 8.2, 0.1 mM EDTA (called TH 8.2) and 0.5x TBE. After setting the gels were placed in the appropriate buffer and prerun at 200 V constant voltage at 4 °C for 60 to 90 min. Samples were loaded with the gel running at 100 V constant voltage and after loading, the gels were run at 200 V constant voltage for 3 to 4 h. The method of detecting bands was, again, dependent on the nature of the material run. For binding experiments using small amounts of ^{32}P end-labelled DNA, the gels were transferred to three layers of 3 MM filter paper and dried under vacuum (80 °C for 45 - 60 min). The DNA bands were then visualised by autoradiography of a sheet of Kodak XS1 or Fuji RX100 film. Large scale band shift assays were either EtBr- or silver-stained.

(c) Denaturing Polyacrylamide Gels for DNA.

This type of gel was used for plasmid sequencing, purification of synthetic single stranded oligonucleotides, DNase I footprinting and methylation interference analysis. The products of plasmid sequencing, DNase I footprinting and methylation interference experiments were all analysed using an IBI Base Runner sequencing gel kit. All the gels used contained 0.5 g/ml urea and 1x TBE. The acrylamide used was a 40% (w/v) stock solution containing a 19:1 ratio of acrylamide to bisacrylamide. Gels were prerun in 1x TBE running buffer for 15 to 60 min to allow the desired temperature (55 °C) to be achieved (this prevents renaturation, and anomalous migration of the single stranded DNA). Other variant features of the gels used are summarised in Table 2.7.

	Plasmid Sequencing.	DNase I Footprinting.	Methylation Interference.
Gel Thickness.	0.2 mm	0.2-0.4 mm wedge	0.2 mm
Gel Length	45 cm	60 cm	45 cm
% Acrylamide	8%	8%	20%
Power	45 W	55 W	45 W
Run Time	1-2 h	2-3 h	~4 h

Table 2.7 Features of 'Sequencing Type' Polyacrylamide Gels Used.

After running, these gels were fixed in 10% acetic acid and dried under vacuum. Bands were visualised by autoradiography.

Polyacrylamide gels used for purification of synthetic oligonucleotides also contained urea as a denaturant. These gels were used as described in Sambrook *et al.*[73].

(d) Denaturing Polyacrylamide Gels for Proteins (Laemmli gels).
The protein SDS-PAGE system used was that of Laemmli[48].

(3) Staining of Gels.
(a) Ethidium Bromide Staining of DNA.
All agarose and some polyacrylamide DNA gels (and some large scale band shift gels) were stained by soaking for 20-30 min in a solution of 0.6 μg/ml EtBr in running buffer (or water). The gels were then destained by soaking in water for 2 x 10 min. Bands were visualised by exposure to a 254 nm UV source. Where DNA was to be isolated for further manipulation (e.g. for cloning) a longer wave length UV source (365 nm) was used to reduce photochemical damage of the DNA sample.

(b) Coomassie Staining of Proteins.
Some Laemmli gels were stained by soaking in a solution of 1% Coomassie Blue, 50% MeOH, 10% AcOH for 1-1.5 h with shaking, followed by destaining in 10% MeOH, 10% AcOH as required.

(c) Silver Staining of Protein and/or DNA.
Some Laemmli gels and non-denaturing DNA polyacrylamide gels were stained using this method. Most of the large scale band shift assays described (Chapters 3 and 5) were also stained this way.

Solution A: 0.2 g/l silver nitrate in deionised water.
Solution B: 4.2 ml 1.8% NaOH, 1.4 ml 30% aqueous ammonia.

Solution C: Drop-wise addition of 4 ml Soln. A to Soln. B, with stirring (use within 5 min of preparing).

Solution D: 2.5 ml 1% citric acid, 0.25 ml 38% formaldehyde, made to 0.5 l with deionised water.

The gel was soaked, with shaking, in 50% MeOH for 1 h and then placed in Soln. C for 15 min. Next the gel was washed in deionised water for 5 min and then placed in Soln. D until bands had developed sufficiently (about 10 min). The reaction was stopped by transferring the gel to a 10% acetic acid solution for 10-20 min. After washing in deionised water, the gel was photographed and then dried under vacuum.

(d) 'Stains-All' Staining of Synthetic Oligonucleotides.

'Stains -all' (1-ethyl-2-[3-(1-ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-*d*]thiazolium bromide; from Aldrich) is a cationic carbocyanine dye which can be used for staining of DNA, RNA or protein analysed by PAGE. Protein, RNA, double stranded DNA, and single stranded DNA all stain differently with 'Stains-all'.

70 ml water and 20 ml isopropanol were mixed with 10 ml of a 0.1% (w/v) solution of 'Stains-all' (in formamide). The gel was soaked in this solution until sufficient staining was observed (5-20 min). The gel was then destained in water as necessary. Single stranded DNA oligonucleotides gave cyan or purple bands, depending on their size (smaller molecules stained cyan, larger molecules stained purple). This is a suitable method of staining for DNA which is to be extracted after electrophoresis, as the gel is not exposed to a UV source.

(4) Photography.

All gels were photographed using Polaroid type 667 Land film or a Pentax 35 mm SLR camera loaded with Ilford HP5 or Pan F film. EtBr-stained gels were photographed under illumination from a 254 nm UV source, with a Kodak Wratten filter No.23A on the camera. Coomassie-stained and silver-stained gels were photographed on a fluorescent light box.

2.17 Extraction of DNA After Electrophoresis.

(a) Agarose Gels.

EtBr-stained DNA bands were visualised using a long wave UV source (365 nm) and cut from the gel with a scalpel. The DNA was extracted using the centrifugation method of Heery *et al.*[30]. The DNA produced was purified by phenol extraction and EtOH precipitation, before further manipulation.

(b) Polyacrylamide Gels.

(1) Synthetic Oligonucleotide Purification.

Oligonucleotide purification from denaturing polyacrylamide gels was as described in Sambrook *et al.*[73], except that bands were visualised using 'Stains-all' (see section 2.16 part 3d).

(2) Double Stranded DNA Purification.

After staining, bands were cut from the gel using a scalpel. DNA was then eluted by the 'crush and soak' method of Maxam and Gilbert[53].

2.18 End-labelling of DNA fragments.

The DNA fragment to be labelled was produced as a restriction digest product with at least 1 3' recessed end. Most labelling reactions were in a volume of 20 µl, containing 1-2 µg of linear DNA, three unlabelled dNTP's (each at 10 µM final), one $\alpha(^{32}P)$dNTP (at 5 µCi per pmol of DNA ends to label) 1 unit of Klenow fragment of DNA polymerase I and appropriate buffer. As DNA was often added directly from a restriction digest buffers used were 1.5x KGB, 1x BRL REact2, 1x BRL REact4 or 0.5x BRL REact3. The sample was incubated at room temperature for 15-20 min before adding 1 µl of 1 mM dNTP stock (unlabelled dATP, dCTP, dGTP and dTTP at 1 mM each), followed by 10 µl of 0.2 M EDTA to stop the reaction. Labelled DNA was separated from enzymes and unincorporated dNTP by phenol extraction and EtOH precipitation or by gel electrophoresis and elution of the desired DNA band.

2.19 Band Shift Analysis (Gel Binding Assays).

Dilutions of Tn3, Tn21, γδ and γδ M106C resolvases were made at 0 °C with 1x resolvase dilution buffer [1 M NaCl, 10 mM Tris.HCl pH 7.5, 0.2 mM EDTA, 50% glycerol (v/v)]. Dilutions were stored at -20 °C. Some dilutions of Tn3 resolvase were made in 1 M NaCl, 50 mM Tris.HCl pH 8.2, 0.1 mM EDTA, and were stored on ice for up to 1 week.

Binding Buffers:

2x TG 9.4: 20 mM Tris.glycine pH 9.4, 0.2 mM EDTA, 20% (v/v) glycerol.

2x TH 8.2: 20 mM Tris.HCl pH 8.2, 0.2 mM EDTA, 20% (v/v) glycerol.

(1) Small scale reactions.

Purified pUC18 DNA (freshly diluted to about 50 µg/ml) was mixed with an equal volume of 2x binding buffer. An appropriate amount of end labelled DNA fragment was added (approximately 0.1 ng/µl *res* fragment) and the binding mix was dispensed to 10 µl samples (stored at room temperature). 0.4 µl of a

resolvase dilution (or resolvase dilution buffer) was added to each sample, which was mixed and incubated at 37 °C for 10 min (or as stated). After incubation samples were transferred to ice and loaded onto the gel as soon as possible (about 10 min maximum).

(2) Large scale reactions.
(a) With unlabelled DNA.
As for small scale reactions except; no pUC18 carrier DNA was included and target DNA concentration was much greater, i.e. 10 µl aliquots were prepared containing 1x binding buffer, about 100 ng unlabelled *res* fragment (and about 900 ng vector fragment).
(b) For stoichiometry determinations.
As for (a) above except; some target DNA was labelled; amounts of material used were all as described or all increased 5-fold (i.e. 50 µl reactions, no change in concentrations of components.

2.20 *In Vitro* Recombination.

Tn3 resolvase dilutions were prepared as for binding reactions (section 2.18).
1x C9.4 recombination buffer: 50 mM Tris.HCl pH 9.4, 10 mM $MgCl_2$, 0.1 mM EDTA, 20% (v/v) glycerol.
40-80 µl samples containing approximately 1 µg plasmid DNA in 1x C9.4 were mixed with 1-2 µl of diluted resolvase (or resolvase dilution buffer). The reactions were incubated at 37 °C for 0.5-18 h and then stopped by incubation at 70 °C for 5 min. Half of each sample was digested with an appropriate restriction endonuclease (after adjusting the reaction buffer). Where appropriate 1 µl of each sample was used to transform competent DS941, and the remaining material was extracted against butanol (to reduce sample volume). Samples were then mixed with 5 µl of K mix. Digested and undigested material was analysed by agarose gel electrophoresis.
Note: an 18 h incubation time was used when recombination of some substrates examined was expected to be poor. For an efficiently resolved substrate 30 min incubations were more typical.

2.21 Direct Sequencing of Double Stranded Plasmids.

The method used was mainly as described in the Sequenase T7 DNA polymerase technical manual[88] based on the method of Sanger *et al.*[75]. The exception to this was the way in which plasmid template was prepared. 2-3 µg of double stranded plasmid DNA was incubated in 30 µl of 0.2 M NaOH for 15 min at room temperature. 5 pmol of sequencing primer was added and the sample was

vortexed briefly. Next 120 µl of ice cold EtOH and then 12.75 µl of 5 M ammonium acetate (pH 7.5) were added and the sample was incubated at -70 °C for 5 min. The DNA was pelleted by centrifugation (for 30 min at 4 °C), washed in 70% EtOH and then pelleted again. After drying briefly the template DNA was resuspended as described in the Sequenase protocol.

2.22 DNase I Footprinting.

Tn3 resolvase dilutions were as for band shift assays (section 2.18). Dilutions of 1 mg/ml DNase I were made in DNase I dilution buffer and stored at -20 °C.
DNase I dilution buffer: 50 mM Tris.HCl pH 8.0, 50% (v/v) glycerol.
C8.2 buffer: 50 mM Tris.HCl pH 8.2, 10 mM $MgCl_2$, 0.1 mM EDTA.
The method used was as derived from that of Galas and Schmitz[21].
End-labelled target DNA (labelled on one strand only) was purified after PAGE. The DNA was resuspended in C8.2 binding mix to give an activity of >25 cps, and then dispensed in 4 µl aliquots. 0.2 µl of diluted resolvase (or dilution buffer) was added to the DNA which was incubated at room temperature for 5 min. Next 1 µl of diluted DNase I (or dilution buffer) was added and the sample was mixed thoroughly. After 5 min at room temperature the reaction was stopped by the addition of 4 µl of stop mix (3 vol Sequenase loading dye: 1 vol 100 mM EDTA). The samples were heated to 80 °C before loading <4 µl onto a sequencing gel.
Optimal concentrations of DNase I (6.25 µg/ml) and resolvase (see chapter 3) were determined by preliminary experiments. No carrier DNA was included in these binding reactions.

2.23 Methylation Interference Experiments.

Plasmid containing the site of interest was digested and end-labelled to give a small target DNA fragment and larger vector fragment, each labelled on one strand only. About 20,000 counts per second of this material was resuspended in 20 µl of C8.2. After adding 2 µl of 10% (v/v) DMS (freshly diluted in water) the samples were incubated for 5 min at room temperature. Methylation was terminated by the addition of 1 µl of 2-mercaptoethanol. The sample was made to 70 µl with 1x TE and then extracted with an equal volume of phenol, then 1:1 phenol/chloroform and finally chloroform. The DNA was precipitated with 2.5 vol of EtOH and resuspended in 10 µl of 1x TE.
Methylated and unmethylated DNA samples were used in TG 9.4 band shift assays (each sample being used for two resolvase reactions and one 'blank' control). An autoradiograph was made of the wet gel and bands of interest were cut from the gel. DNA from the gel chips was eluted using an adapted 'crush and

soak' method (elution buffer consisted of 0.5 M ammonium acetate, 1 mM EDTA, 20 µg/ml yeast tRNA). After elution DNA samples were EtOH precipitated and resuspended in 5 µl deionised water.
The DNA was cleaved at modified positions by addition of 50 µl 10% (v/v) piperidine followed by incubation at 90 °C for 30 min. The samples were lyophilised and resuspended three times, finally resuspending in 5 µl of deionised water. The resuspended DNA was mixed with 4 µl of Sequenase loading dye, heated to 80 °C and loaded to a sequencing gel. An attempt was made to balance the activity loaded to adjacent tracks to simplify interpretation of the bands produced.

2.24 Expression and Purification of Tritiated Tn3 Resolvase.

The materials and methods used are described fully in Chapter 4.

2.25 *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine Mutagenesis.

The method used was as described by Miller[56]. The recommended MNNG treatment of cells is with 50 µg/ml MNNG (final) for 30 min. In addition to this other samples were treated using final MNNG concentrations of 100 µg/ml and 200 µg/ml. Different exposure times of 10, 30 and 60 min were used with each MNNG concentration.

2.26 pSelect-Based Directed Mutagenesis.

The method used was as described in the 'Altered Sites' technical manual[62] except that the mutagenesis template was produced by denaturing double stranded plasmid DNA which had been nicked by DNase I in the presence of EtBr to give an average of one single stranded nick per plasmid molecule[81].

2.27 Preparation of Protein Samples for Amino Acid Analysis.

A known volume of protein sample was mixed with a known volume (and concentration) of taurine solution in a large glass tube. The samples were made to 500 µl with deionised water. 500 µl of concentrated HCl was added to each, and the tubes were sealed. After degassing the samples, the sealed tubes were incubated at 105 °C overnight. After hydrolysis the samples were lyophilised (overnight), resuspended in about 500 µl deionised water, lyophilised again and finally resuspended in a small volume (50 or 100 µl).

Between 10 and 20% of the sample was taken for OPA derivatisation for each analysis. Derivatised amino acids were analysed by D. Mousedale and E. O'Neil using HPLC separation.

2.28 Concentration of Resolvase Samples.

Resolvase.	Resolvase Fraction	Monomer Concentration	Method of Estimation.
Tn3	R14 f45	~250 μM	comparison (& Bradford's)
"	R14 f46	~250 μM	comparison (& Bradford's)
"	R14 f47	~150 μM	comparison (& Bradford's)
"	R17 f47	20 μM	amino acid analysis
"	R17 f48	~160 μM	comparison with R17 f47
"	R17 f49	~500 μM	comparison with R17 f47
"	RD 8 f7	62 μM	amino acid analysis
"	RD 9 f6	19 μM	amino acid analysis
γδ		~40 μM	comparison with R17 f47
γδ M106C		~100 μM	comparison with R17 f47
Tn21		101 μM	amino acid analysis

Table 2.8 Estimated (monomer) Concentration of Resolvase Samples.

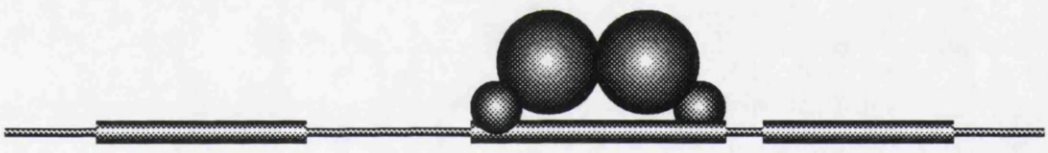
All values are estimations, but those derived from amino acid analysis are likely to be most accurate. All other values were estimated comparatively by silver- or Coomassie-stained Laemmli gels and, where appropriate, by comparison of resolution or binding activities. R14 samples were also measured by Bradford's assay[12], but resolvase is believed to give low results by this method, when compared with BSA standards.
The only experiments for which an absolute resolvase concentration was required were those to determine the absolute stoichiometry of complexes of resolvase with *res* (i.e. that of RD 9 f6).

2.29 Preparation of Polyacrylamide Gel Chips for Scintillation Counting.

Material was cut from the gel using a scalpel. Each gel chip was placed in a sealed scintillation vial with 1 ml of H_2O_2, and incubated at 55 °C for 24-72 h (until the gel chip was dissolved). After cooling, 10 ml of 'Ecoscint' scintillation cocktail was added. The re-sealed vials were mixed by vortexing and then scintillation counted as required.

Chapter Three

Binding of Resolvase to *res* and Its Subsites



3.1 Introduction.

Non-denaturing polyacrylamide gel electrophoresis (PAGE) has been used to allow the detection of complexes of a wide variety of nucleic acid binding proteins bound to their target sites, including Tn3 resolvase bound to the *res* site. Six discrete, reproducible complexes were observed on binding of Tn3 resolvase to Tn3 *res*[7], but only three complexes were produced by equivalent analysis of the γδ *res*/resolvase system[28] and of the Tn21 *res*/resolvase system[5]. It was not clear whether these differences reflected variation in the mechanism of binding of the resolvases or resulted from something trivial, such as the difference in binding and gel conditions used for each system.

Analysis of the γδ resolvase used in these experiments indicated that the major species present was approximately the size expected for a resolvase dimer[34]. Considering the binding pattern observed, this suggests that each complex could result from binding of successive resolvase dimers (or possibly tetramers) to the *res* site (Fig. 3.1b). However, it was not proved that the major species observed during size analysis was also the major species present under the different conditions used for the formation, or electrophoresis, of complexes.

The band shift assay allows investigation of early steps within the resolution reaction and, as such, is very useful for analysing the effects of altered resolvases or sites which prove incapable of completing recombination, but may still exhibit some binding activity. An isolated subsite I sequence was produced by exonuclease III digestion of the Tn3 *res* site. A plasmid containing subsites II and III, but not subsite I, was produced using the same method. Binding analysis of these substrates showed that two complexes were produced for each intact subsite present[7]. A DNA fragment containing just over half of subsite I, produced by further dissection of *res* , generated one complex with resolvase. However, this result alone is insufficient to show a general relationship of one complex produced for each half subsite present in the DNA fragment examined.

The general pattern of binding of Tn3 resolvase to *res*, or its subsites, has been described as co-operative, due to the small range of resolvase dilutions over which large increases in binding are produced. However experiments have not been conducted to specifically investigate the co-operative nature of this process.

To account for the complexes observed with the Tn3 resolution system, different resolvase binding models were considered[7]. If the resolvase bound as a dimer (as predicted for γδ) and the order of occupation of half sites was random, then

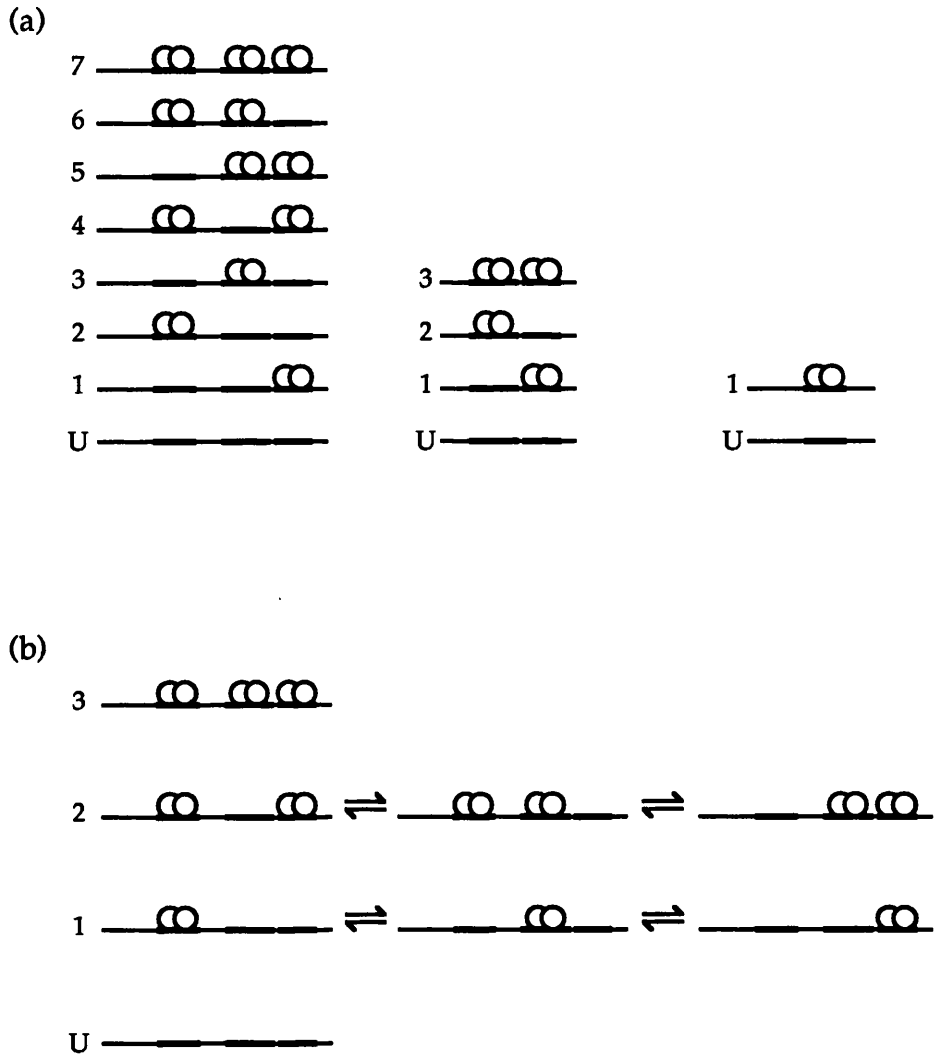


Figure 3.1. Schematic Representation of Models for Dimers of Resolvase Binding to a *res* Site.

'U' indicates an unbound DNA fragment. Complexes are numbered in order of decreasing mobility. Resolvase is represented by circles, and the DNA by lines, with thicker lines indicating the subsites.

(a) Random order occupation of subsites by a dimer binding unit, without shuffling. This model predicts that 7 complexes would be generated with a full *res* site; 3 complexes with 2 subsites and 1 complex for an isolated subsite.

(b) Random order occupation of subsites by a dimer binding unit, with shuffling. 3 retarded bands would be expected from binding to *res* (as shown). 2 retarded species would be seen with two subsites and 1 with a single subsite (not shown).

seven complexes would be expected from binding to a *res* site, three complexes from two subsites, and one complex from a single subsite (Fig. 3.1a). Complexes resulting from resolvase dimers binding to different subsites of *res* would be expected to have different mobilities, due to the differences in the size and position of the bend produced (section 3.6). However, if the bound resolvase can switch between free subsites on the same fragment, or 'shuffle'[49], then the mobility of each complex would result from the average position of the resolvase molecules bound, reflecting the affinity of resolvase for each subsite. In this case (still considering resolvase binding as a dimer) one complex would be expected for each subsite on the DNA fragment investigated, e.g. *res* would produce three complexes (Fig. 3.1b), as seen for γδ resolvase. Methylation interference analysis of the least retarded γδ complex gave partial interference patterns at subsites I and II[28]. This is believed to be the result of the resolvase 'shuffling' between all three subsites, but having a comparatively low affinity for subsite III. Observed features of γδ binding do fit this model (Fig. 3.1b), but Tn3 binding does not.

Rather than a dimer, Tn3 resolvase could bind as a monomer, to one half of a subsite in each step. If the order of occupation was random, and if no shuffling occurred, binding to *res* in monomer steps would produce sixty-three different complexes. In a model including shuffling, six complexes would be predicted for binding to a *res* site, and more generally two complexes would be expected for each subsite in a DNA fragment (Fig. 3.2a). Alternatively the order of subsite occupation could follow a non-random path in such a way that six complexes would be generated from a *res* site (Fig. 3.2b). This model is believed to be incorrect due to the similar behaviour of different isolated subsites, and because of the previously mentioned evidence for shuffling.

Gel filtration analysis of Tn3 resolvase indicates that it is dimer-sized under the conditions used (M. Boocock; pers. comm.). It could bind each subsite as a dimer, to give a weak complex, before undergoing a conformational change (e.g. Fig. 3.3a), producing a second, more retarded complex. Alternatively a resolvase dimer bound to a subsite could, by dissociation of a monomer, break down to give another complex containing a bound monomer (Fig. 3.3c). These mechanisms (incorporating shuffling) could account for the binding patterns observed with Tn3 *res* and resolvase. Two other models which would also account for the observed two complexes per subsite are also shown in Fig. 3.3. Models of dimer binding in which only one half of the dimer contacts the DNA (or one half of the dimer, and then the other) could be proposed. From such models the predicted patterns of complexes would be as described for monomer

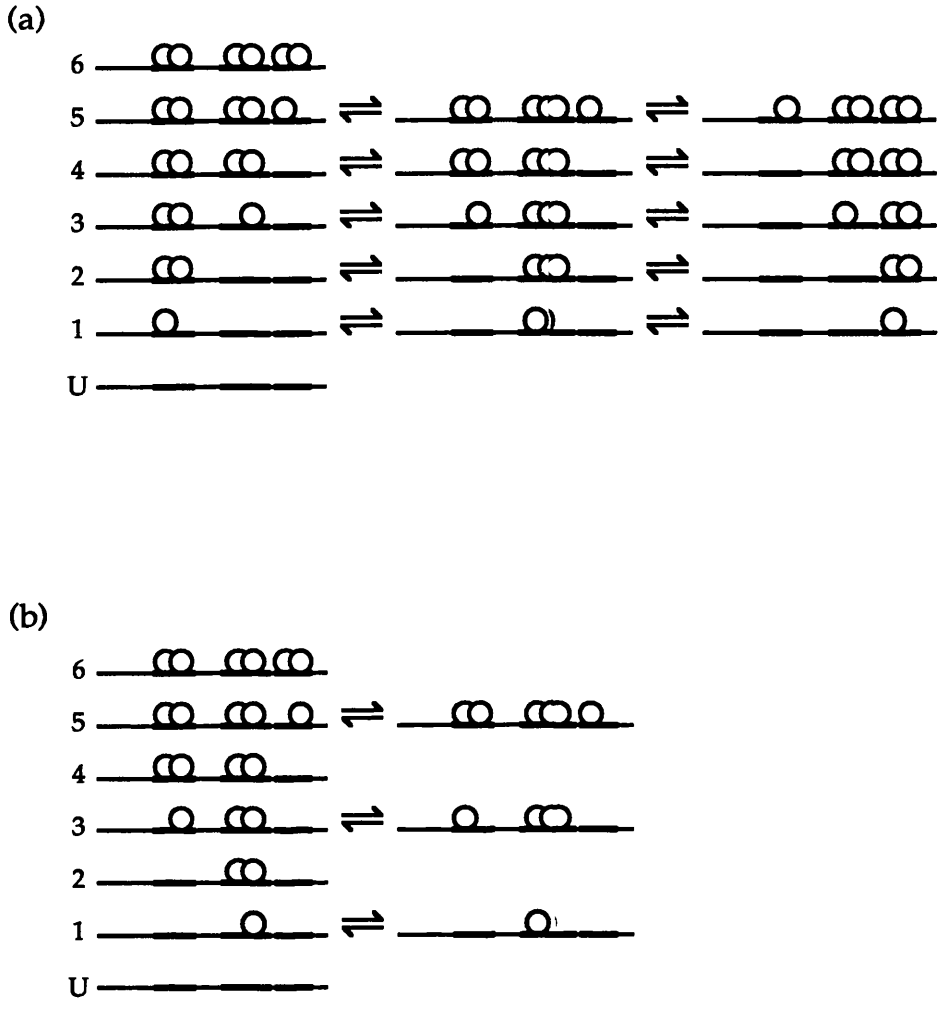


Figure 3.2. Schematic Representation of Models for Monomers of Resolvase Binding to *res*.

'U' indicates an unbound DNA fragment. Complexes are numbered in order of decreasing mobility. Resolvase is represented by circles, and the DNA by lines, with thicker lines indicating the subsites.

(a) Random order occupation of subsites by a monomer binding unit, with shuffling. Different species created by shuffling within a subsite are not shown. 6 different complexes would be produced with a full *res* site. 4 complexes would be expected for two subsites, and 1 complex with one subsite (not shown).

(b) Ordered occupation of subsites by a monomer binding unit, without shuffling between subsites, but allowing shuffling of monomers between the two halves of a subsite, as shown. As for the model presented in (a), 6 complexes would be observed with *res*. 4 complexes would be expected for two subsites; and 1 with a single subsite (not shown). The same patterns of complexes would be expected for ordered occupation without shuffling between halves of a subsite.

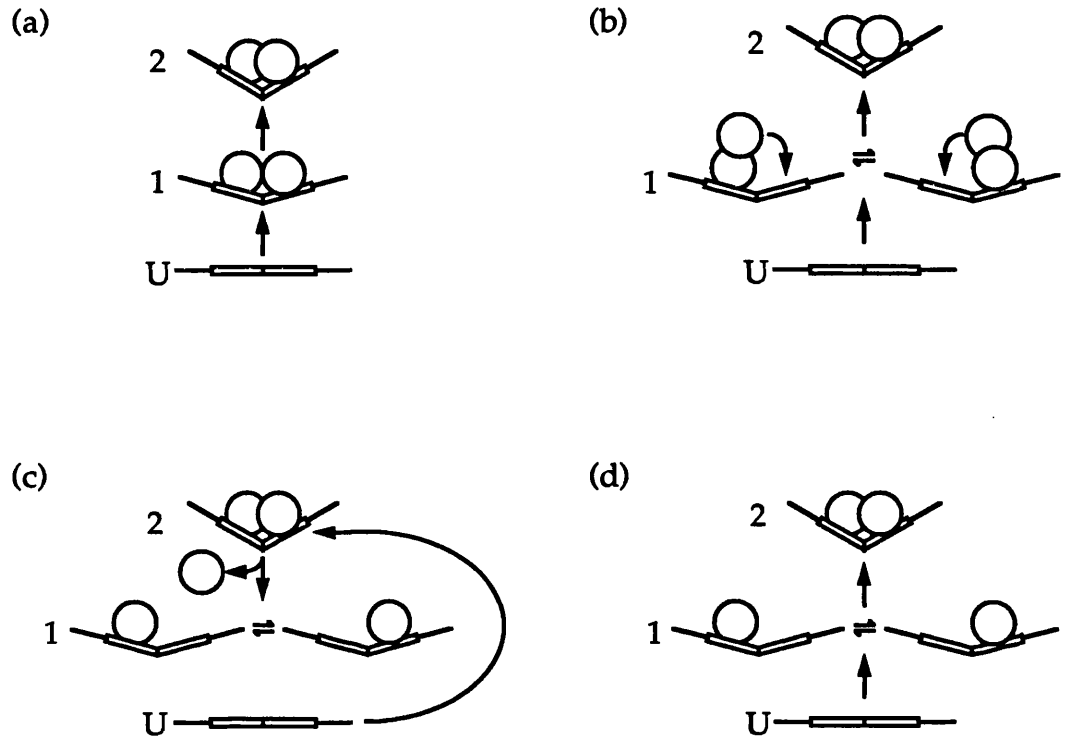


Figure 3.3. Schematic Representation of Models for Binding of Resolvase in Two Steps per Subsite of *res*.

'U' indicates an unbound DNA fragment. Complexes are numbered (and positioned) in order of decreasing mobility. Resolvase is represented by circles, and the DNA by lines, with boxes indicating the subsites.

(a) Binding of a resolvase dimer to the entire subsite, followed by a conformational change to produce a second, more retarded complex.

(b) Binding of a resolvase dimer to one half of the subsite (with shuffling), followed by a conformational change to allow binding of the same dimer to the other half of the subsite, to generate a more retarded complex.

(c) Binding of a resolvase dimer to the entire subsite, followed by dissociation of one half of the dimer, leaving a resolvase monomer bound (with shuffling between halves of the subsite).

(d) Binding of a resolvase monomer to the subsite (with shuffling), followed by binding of a second monomer, to form a bound dimer.

binding models. Tetramer binding models, in which only two resolvase monomers end up in contact with the DNA, would give patterns of complexes as predicted by dimer binding models.

At the time of starting this work, our favoured model for Tn3 resolvase binding to *res* had resolvase binding in monomer steps, with an apparently random order of occupation, and incorporating shuffling (Fig. 3.2a).

Intrinsically bent DNA sequences have been shown to migrate anomalously during gel electrophoresis[52]. Similarly, protein-induced bending of a DNA fragment would be expected to cause retardation of the resulting complex during electrophoresis[49]. However the presence of bound protein would also cause migration of the complexed material to be slower than that of the naked DNA fragment. The degree of retardation observed for a bent DNA fragment (with an intrinsic or an induced bend) is dependent on both the size of the bend, and its position within the fragment. The centre of a DNA bend can be estimated by comparison of the gel mobilities of a number of fragments, each containing the proposed DNA bend at a different point within the same sequence context. Plotting the relative mobility of the fragments indicates the point of maximum retardation, at which the bend is predicted to be at the centre of the DNA fragment[93]. Such experiments have been used to detect protein-induced bending of DNA sites[7,47,69,93], but not all systems examined exhibited a variation in the mobility of the complexes produced[24,93]. This suggests that, where observed, these differences in mobilities are not just a result of changing the position of the bound protein within the DNA fragment. Obviously the unbound DNA species must also be examined in these experiments, to eliminate the effect of any intrinsic bend. As bending of subsites, induced by resolvase binding, is predicted to play an important role in the structure and formation of the *res* synaptic complex[79], examining the position and extent of resolvase-induced bending may yield valuable information.

Work described in this chapter will also include footprint analysis and methylation interference analysis of Tn3 resolvase bound to Tn3 subsite II, and some subsite II derivatives.

3.2 Binding of Tn3 Resolvase to Tn3 *res*.

Band shift analysis of Tn3 resolvase bound to a Tn3 *res* site (using a 262 bp *Eco* RI fragment of pMA1441 and TG 9.4 conditions) gives rise to six complexes (Fig. 3.4), named complex 1 to 6, as indicated. The alternating weak and strong nature of the complexes (with decreasing mobility) is consistently observed. Prolonged

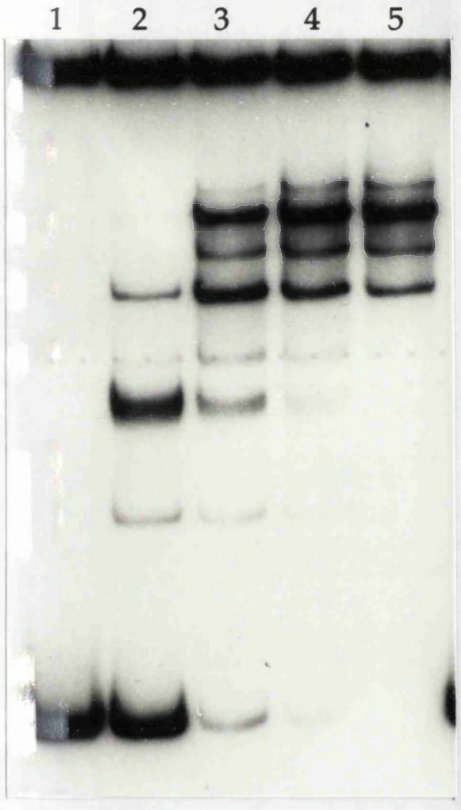


Figure 3.4. Binding of Tn3 Resolvase to Tn3 *res*.

End-labelled *res* fragment (pMA1441 *Eco* RI) was incubated for 10 min at 37 °C with dilutions of Tn3 resolvase, as indicated, using TG 9.4 conditions.

Lane	Resolvase	
1	R.H.B.	
2	2^{-8}	R14 f45
3	2^{-7}	"
4	2^{-6}	"
5	2^{-5}	"

incubation on ice gives increased levels of weak complexes (i.e. complexes 1, 3 and 5) without significantly affecting strong complex levels (i.e. complexes 2, 4 and 6). This effect is shown in Fig. 3.5a.

3.3 Comparison of Binding of the Tn3, γδ and Tn21 Resolvases Using Different Band Shift Conditions.

As mentioned, the pattern of binding described for Tn3 resolvase/*res* was different to that reported for the γδ and Tn21 systems. In an attempt to rule out any differences arising from variations in the experimental approaches used, band shift assays of Tn3 resolvase with Tn3 *res* were conducted under different conditions. Fig. 3.5a shows the retarded complexes observed with TG 9.4 conditions, and Fig. 3.5b shows equivalent samples prepared using the TH 8.2 system. The complexes on the TG 9.4 gel are much sharper than those on the TH 8.2 gel, possibly indicating an increased stability of the *res*/resolvase interaction. Six complexes are visible on each gel. Aliquots of the samples run on the TH 8.2 gel were also run on a 0.5x TBE gel, with 0.5x TBE electrophoresis buffer, (Fig. 3.5c). In this case four diffuse complexes are produced, indicating that the gel conditions used can affect the number and nature of the complexes observed. This is not surprising as measurements of the half-life of *res*/resolvase complexes in solution suggest that it is much less than the length of time for which these gels are run[7]. It is believed that discrete complexes would not be observed without the 'cage' effect of such gels[49].

Although γδ complexes have been detected with 0.5x TBE gels, the experiments described so far have not exactly replicated the band shift conditions used to examine one resolution system in the analysis of another. Gifts of γδ resolvase and pRW80 DNA (containing a γδ *res* site) from Nigel Grindley, and of Tn21 resolvase and pAA3 DNA (containing a Tn21 *res* site) from Steve Halford allowed this omission to be redressed. TG 9.4 conditions were used to examine the binding of the γδ and Tn21 resolvases to their respective *res* sites (Fig. 3.6). It is clear that each set of reactions produces more than (the previously observed) three complexes, but it is not clear that the pattern observed is comparable to that of Tn3 (Fig. 3.5a).

3.4 Binding of Tn3 Resolvase to an Isolated Subsite II.

The absence of restriction endonuclease sites between subsites II and III of Tn3 *res* prevented examination of the binding of these subsites in isolation. Such analysis was required to further test the general observation that two complexes were produced for each intact subsite present on the DNA fragment used.

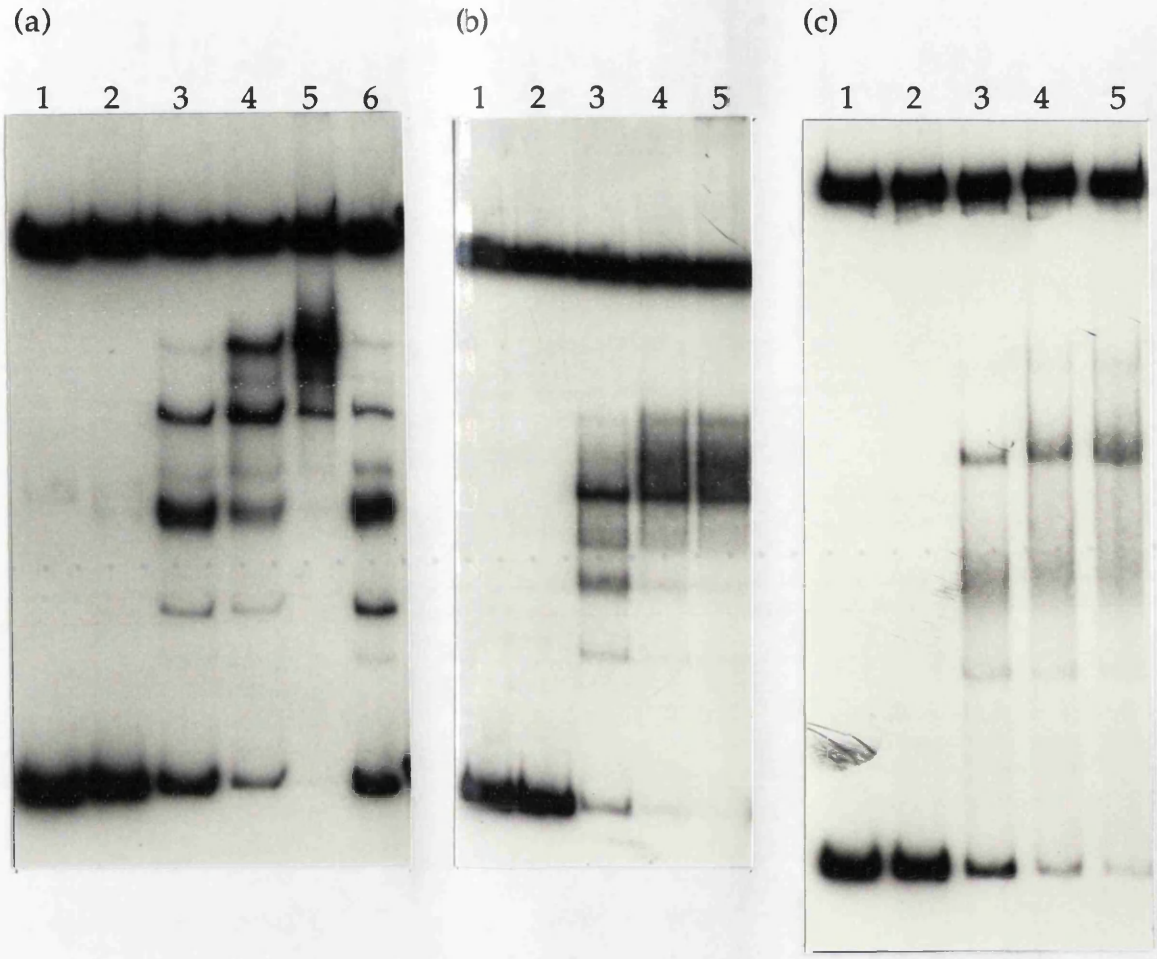


Figure 3.5. Effects of Different Gel Conditions on Complexes Observed using Tn3 Resolvase and Tn3 *res*.

End-labelled *res* fragment (pMA1441 *Eco* RI) was incubated for 10 min with dilutions of resolvase, as indicated. All incubations were at 37 ℃ unless stated otherwise. Binding and gel conditions were as described.

Lane	Resolvase	
1	R.H.B.	
2	2^{-8}	R14 f45
3	2^{-7}	"
4	2^{-6}	"
5	2^{-5}	"
6	2^{-7}	" (0 ℃ incubation)

(a) TG 9.4 binding reactions and gel.
(b) TH 8.2 binding reactions and gel.
(c) TH 8.2 binding reactions but 0.5x TBE gel.

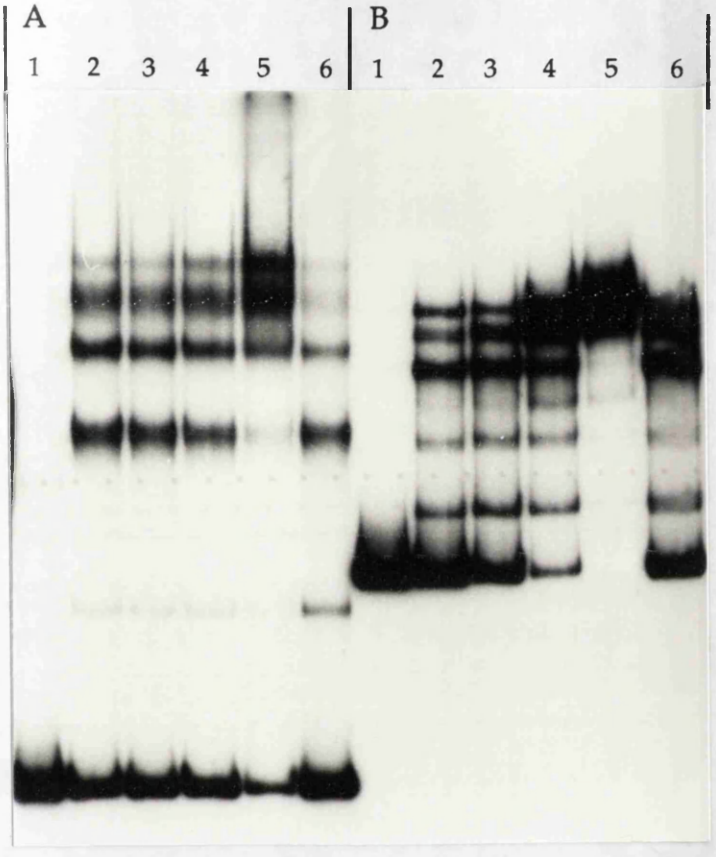


Figure 3.6. Binding of γδ Resolvase to γδ *res*, and of Tn21 Resolvase to Tn21 *res*.

TG 9.4 band shift assays of γδ resolvase/*res* and of Tn21 resolvase/*res*. All incubations were for 10 min at 37 ℃ unless stated otherwise.

Lane	Resolvase
1	R.H.B.
2	2^{-8}
3	2^{-7}
4	2^{-6}
5	2^{-5}
6	2^{-7} (0 ℃ incubation)

A γδ resolvase dilution with γδ *res* (pRW80 *Sal* I/*Hin* dIII)

B Tn21 resolvase dilution with Tn21 *res* (pAA3 *Eco* RI/*Bam*HI)

Subsite II was of particular interest as bending predictions for the *res* site suggested that subsite II would be bent most on binding of resolvase, and this feature was incorporated in the Glasgow model of Tn3 *res* synapsis[11].

As it could not be cloned from Tn3 *res*, an isolated subsite II element was produced synthetically.

3.4.1 Construction of Subsite II.

The sequences of the two complementary oligonucleotides used to make subsite II are given in Fig. 3.7 (and in Chapter 2). Their sequences were designed to give subsite II flanked at each end by 5 bp of adjacent Tn3 DNA, which was directly next to the restriction sites used for cloning. The oligonucleotides were deprotected, purified and annealed (as described; Chapter 2) to give a 50 bp DNA fragment, with overhanging *Xba* I and *Pst* I ends. This fragment was cloned between the *Xba* I and *Pst* I sites of pUC18, giving the plasmid pDB2004 (Fig. 3.7). The cloned subsite II DNA was checked by Sanger di-deoxy sequencing.

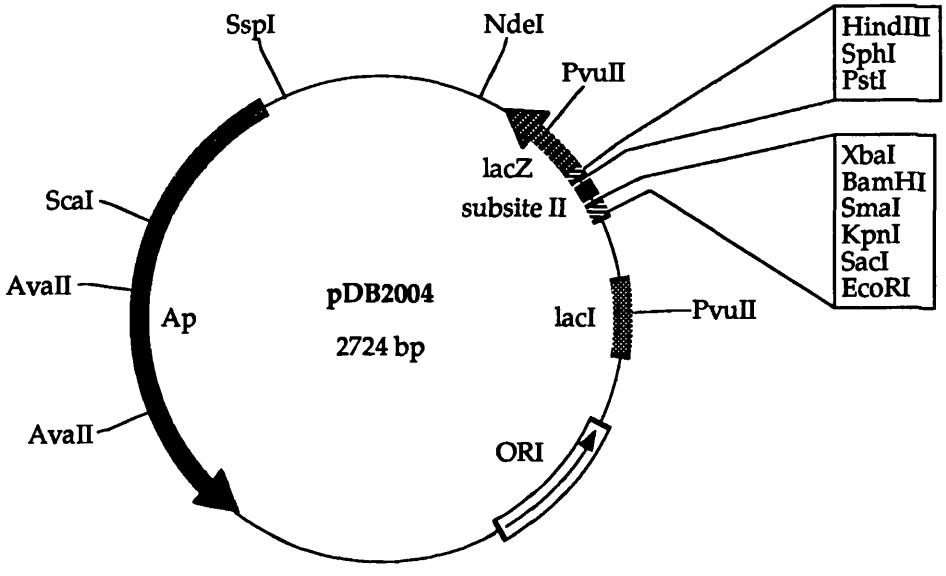
3.4.2 Band Shift Analysis of Tn3 Resolvase with Subsite II.

The plasmid pDB2004 was used as a source of isolated subsite II for band shift analysis. The pattern of complex formation (Fig. 3.8) appeared equivalent to that previously observed for Tn3 *res*, for subsites II and III, and for subsite I[7], i.e. two major complexes were produced and these were alternately weak and strong, with decreasing mobility. These complexes were observed using a 'normal' range of resolvase dilutions. However, a third, more retarded complex was produced at higher resolvase concentrations.

To gain more information about the complexes formed, a binding time course experiment was conducted using subsite II (Fig. 3.9). A single binding reaction was prepared, containing ten times the normal amount of material. This was incubated at 23 °C, and a control sample was removed (and loaded to the gel) prior to the addition of resolvase. A second sample was transferred to the gel directly after the addition of resolvase. Incubation was continued at 23 °C, with further time points taken at 20 sec and then at 1, 2, 4, 8 and 16 min.

Some binding of resolvase is visible at the first time point. Even in experiments where samples were incubated on ice, some complex formation was observed in the first samples taken. At the earliest time point approximately equal amounts of complexes 1 and 2 are present. However, the amount of complex 2 present increases with each time step (and the amount of unbound DNA decreases), whereas complex 1 levels remain fairly constant throughout. This gel supports

(a)



(b)

EcoRI SstI KpnI SmaI BamHI XbaI Subsite II

GAATTCGAGCTCGGTACCCGGGGATCCT**CTAGATTGAG**TGTCCATTAAATCGTCA

CTTAAGCTCGAGCCATGGGCCCCTAGGAGATC**TAACTC**ACAGGTAATTTAGCAGT

Subsite II PstI SphI HindIII

TTTTGGCATAATAGACACATCGCTGCAGGCATGCAAGCTT

AAAACCGTATTATCTGTGTAGCGACGTCCGTACGTTCGAA

Figure 3.7. Map of pDB2004.

(a) Map of pDB2004.

(b) Sequence of polylinker region of pDB2004, showing inserted subsite II element (in bold type).

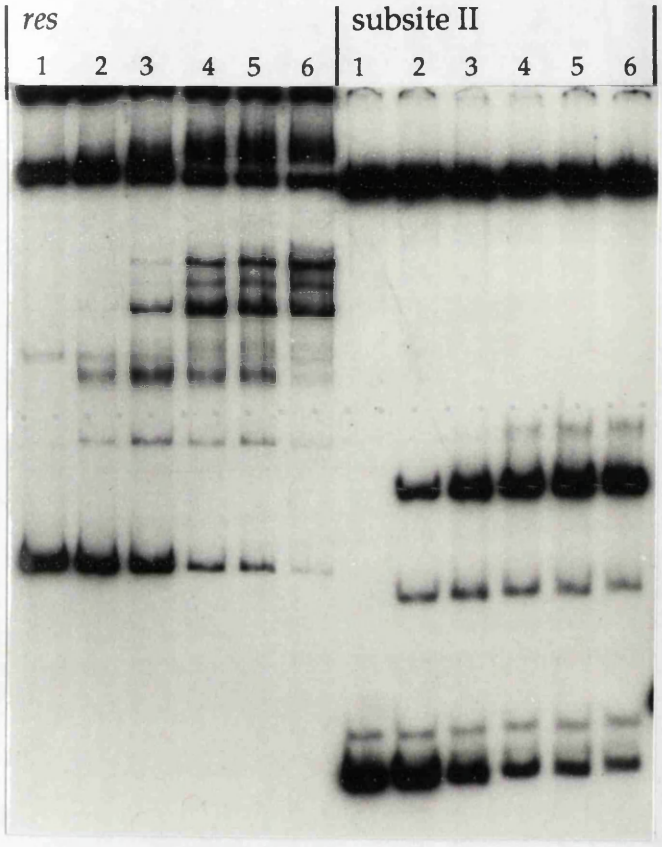


Figure 3.8. Binding of Tn3 Resolvase to an Isolated Subsite II.
TG 9.4 band shift assays of Tn3 resolvase (dilutions of R14 f46) with *res* (pMA1441 *Eco* RI) or an isolated subsite II (pDB2004 *Eco* RI/*Hin* dIII). All incubations were for 10 min at 37 °C.

Lane	Resolvase
1	R.H.B.
2	2^{-8}
3	2^{-7}
4	2^{-6}
5	2^{-5}
6	2^{-4}

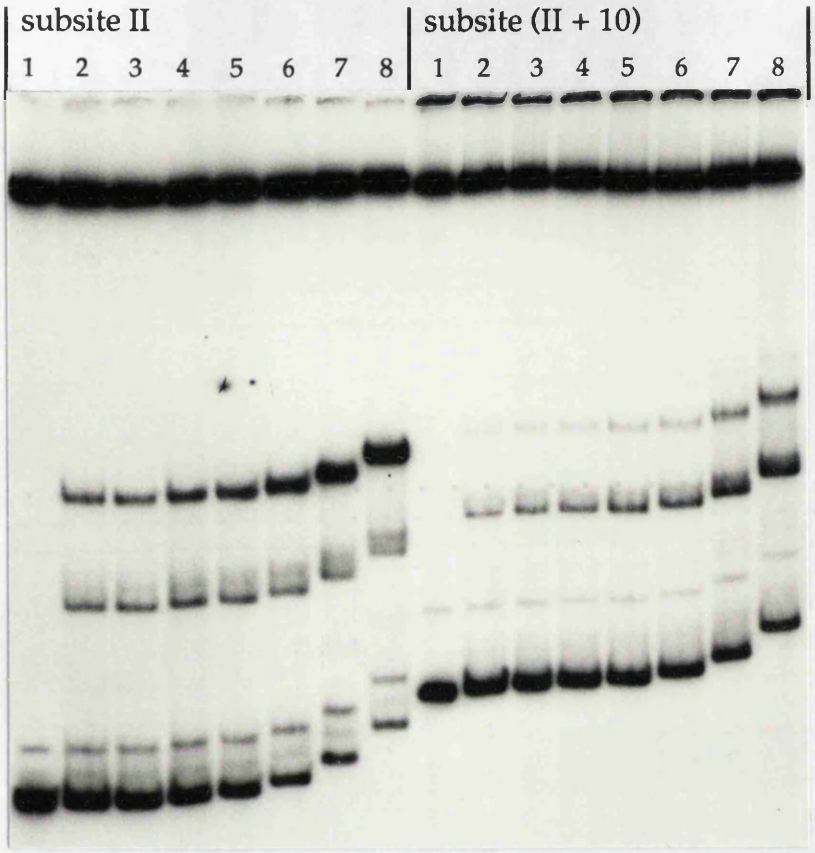


Figure 3.9. Time Course of Binding to Subsite II and to Subsite (II + 10).

TG 9.4 band shift assays of Tn3 resolvase (2^{-5} dilution of R14 f46) with subsite II (pDB2004 *Eco* RI/*Hin* dIII) or subsite (II + 10) (pDB2107 *Eco* RI/*Hin* dIII). All incubations were at 23 ℃ for the time indicated. Two 'zero' time points were produced for each subsite. One before the addition of resolvase, the other was taken immediately after adding resolvase to the binding reaction. Subsite (II + 10) is described in section 3.5.

Lane	Incubation time.	
1	0 sec	(no resolvase)
2	'0 sec'	
3	20 sec	
4	1 min	
5	2 min	
6	4 min	
7	8 min	
8	16 min	

the general assumption that other binding reactions, incubated at 37 °C for 10 min, have reached equilibrium (complex formation was observed to be slower at 23 °C than at 37 °C; data not shown).

3.5 Binding of Resolvase to Derivatives of Subsite II with Altered Spacing.

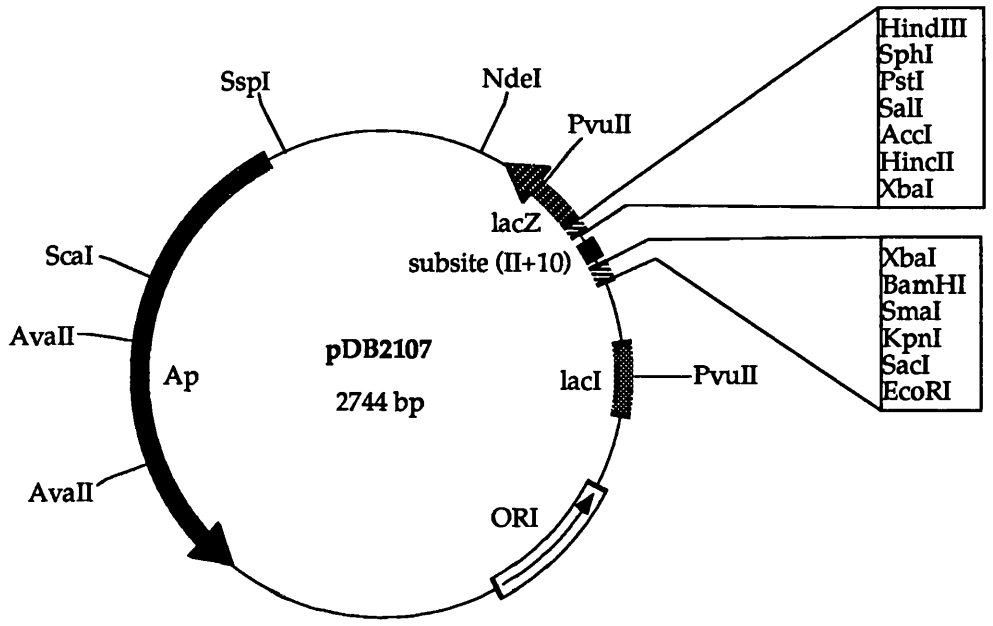
Our interpretation, at the time, of the complexes formed with subsite II was that complex 1 comprised subsite with resolvase bound at one half site only, but complex 2 had the subsite fully occupied (and exhibited greater stability). In addition, it was possible that complex 1 was an intermediate in the formation of complex 2, and that, in the time course experiment reported in section 3.4.2, larger amounts of complex 1 do not accumulate over time due only because of the equilibrium between complexes 1 and 2. Binding to form complex 2 might be co-operative (see section 3.11)

Two derivatives of subsite II were produced in an attempt to disrupt the apparent co-operative nature of binding to subsite II. One derivative, subsite (II + 10), contained an additional 10 bp of DNA inserted at the centre of the subsite II sequence. The other, subsite (II + 5), contained only 5 bp of additional DNA, at the same position. If subsite II forms a helical structure with 10 - 11 bp per turn, then the ends of the subsite (i.e. the proposed resolvase binding sites) will be separated by three full helical turns of DNA, and so will lie 'in phase' with each other. Insertion of another full helical turn (~10 bp) should not disrupt the phase of the subsite ends. Reduced binding caused by such an insertion would be expected to result from the change in the distance between the subsite ends. Insertion of a half helical turn of DNA (~5 bp) would be expected to dramatically alter the phase of the two binding sites, such that the relative position of the bound resolvase would be very different from when bound to subsite II. Aligning resolvase units bound to subsite (II + 5) as for binding to subsite II (perhaps by resolvase/resolvase interactions) would be predicted to result in major distortion of subsite (II + 5) and/or resolvase.

3.5.1 Construction of Subsite (II + 5) and Subsite (II + 10).

The sequence of subsite (II +10), shown in Fig. 3.10 (and in Chapter 2), was designed to produce an altered subsite II flanked by 4 bp of adjacent Tn3 DNA on each side. This additional Tn3 sequence was placed directly next to the restriction enzyme sites (*Xba* I sites) used to clone the subsite. The change from the natural sequence within the subsite was the insertion of an additional 10 bp of new sequence, positioned centrally within the subsite. The inserted sequence was chosen in an attempt not to disrupt the periodicity of the predicted

(a)



(b)

EcoRI SstI KpnI SmaI BamHI XbaI Subsite (II+10)

GAATTCGAGCTCGGTACCCGGGGATCCT**CTAGATGAGTGTCCATTAAATCGTCATTTAT**

CTTAAGCTCGAGCCATGGGCCCCTAGGAGATC**TACTCACAGGTAATTTAGCAGTAAATA**

Subsite (II+10) XbaI SalI PstI SphI HindIII

CGTCATTTTGGCATAATAGACACATCTCTAGAGTCGACCTGCAGGCATGCAAGCTT

GCAGTAAAACCGTATTATCTGTGTAGAGATCTCAGCTGGACGTCCGTACGTTCGAA

Figure 3.10. Map of pDB2107.

(a) Map of pDB2107.

(b) Sequence of polylinker region of pDB2107, showing inserted subsite (II + 10) fragment (in bold type). The shaded region within the subsite indicates the 10 bp not present in subsite II.

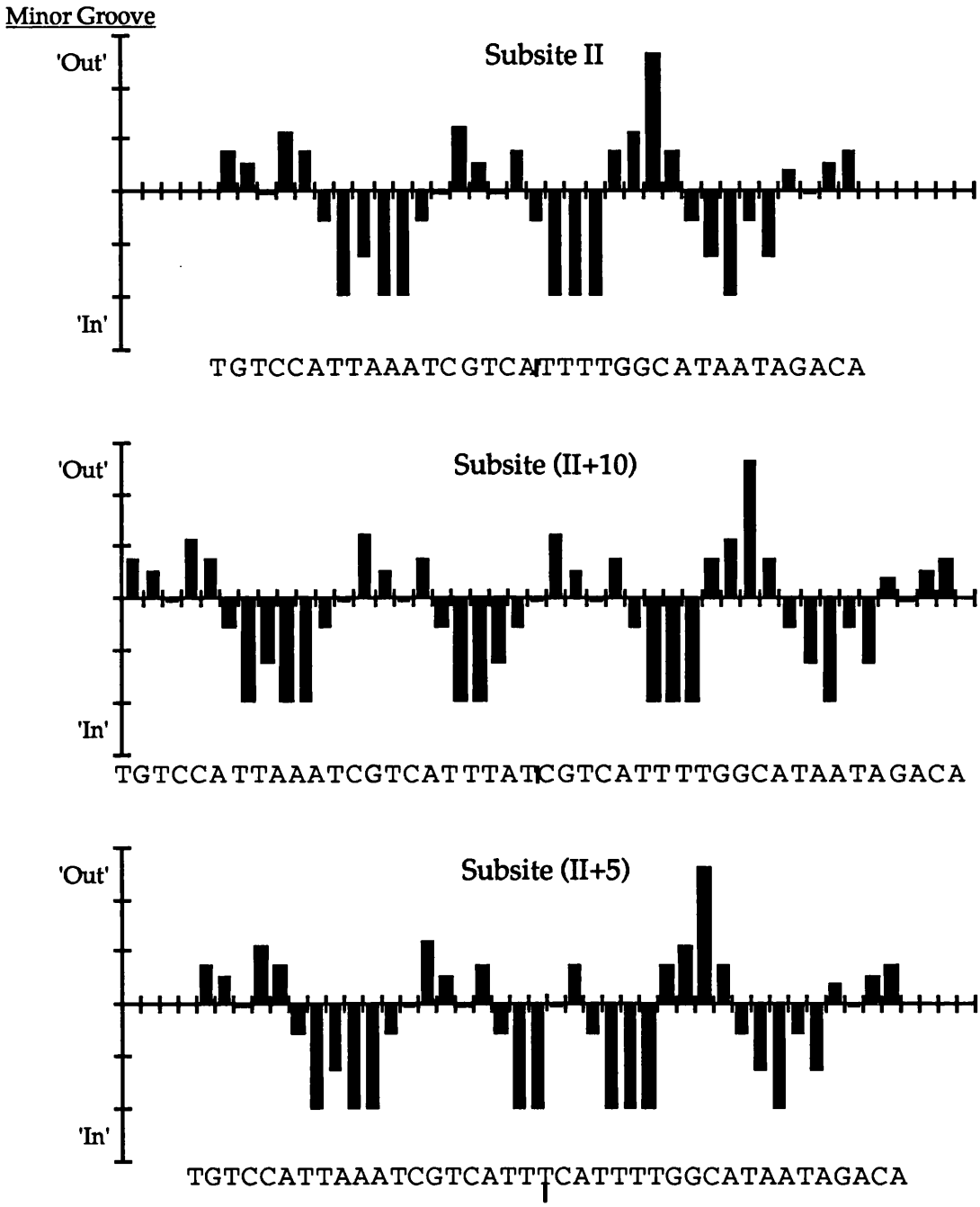


Figure 3.11. Predicted 'Bending Preference' of Subsite II and Its Derivatives.

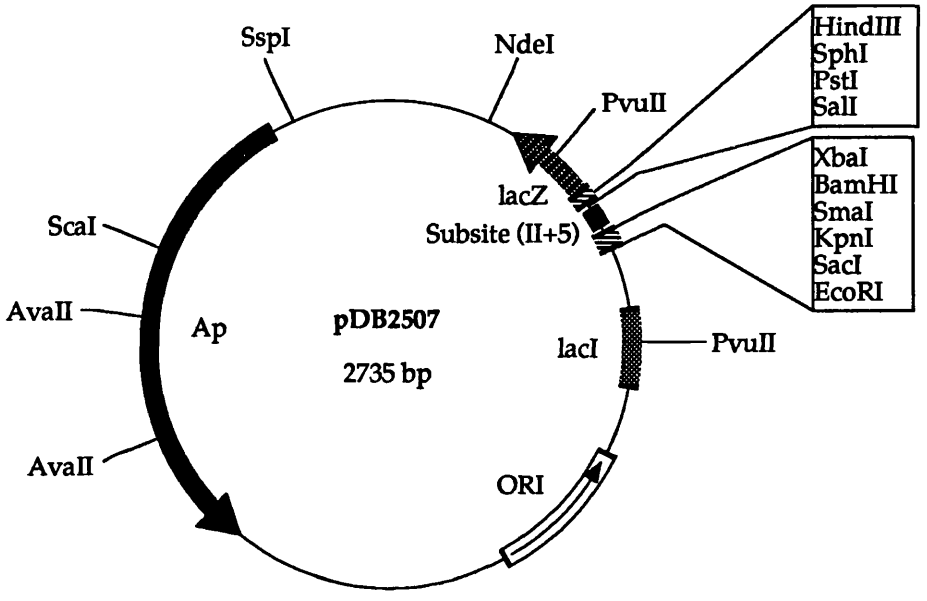
Bend preference values have been plotted for each dinucleotide in the sequence of subsites II, (II+10) and (II+5). These values were calculated using the formula

$$f.\cos(\theta)$$

where f, the fractional variation in occurrence, and θ, the phase angle(°), are as defined and determined by Satchwell *et al.*[76].

The sequence of each subsite is aligned below the relevant plot, and the centre of each subsite is marked with a small line. The predicted position of the minor groove within a DNA bend is indicated, e.g. minor groove 'in' means the minor groove would lie to the inner side of the DNA bend.

(a)



(b)

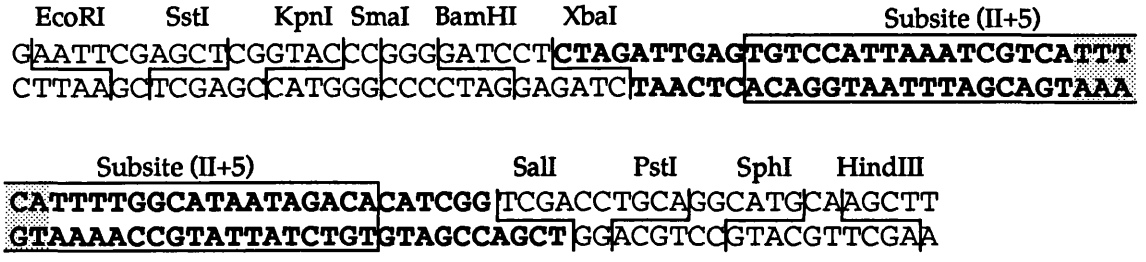


Figure 3.12. Map of pDB2507.

(a) Map of pDB2507.

(b) Sequence of polylinker region of pDB2507, showing inserted subsite (II + 5) fragment (in bold type). The shaded region within the subsite indicates sequence not present in subsite II.

'bendability' of subsite II (Fig. 3.11). The 'bendability' predictions presented are based on data from studies of chicken nucleosome core DNA[76]. The sequence of subsite (II + 5), shown in Fig. 3.12 (and Chapter 2), was designed to conform to that of subsite (II + 10), i.e. the sequence of the 5 bp inserted at the centre of subsite (II + 5) was derived by deleting the central 5 bp of the 10 bp at the centre of subsite (II + 10). These oligonucleotides have 5 bp of additional Tn3 sequence flanking the subsite, and this sequence is directly adjacent to the Xba I and Sal I restriction endonuclease sites used to clone the subsite.

Each pair of complementary oligonucleotides was deprotected, purified and annealed (as described; Chapter 2). Subsite (II + 10) was cloned to the *Xba* I site of pUC18, giving the plasmid pDB2107 (Fig. 3.10), and subsite (II + 5) was cloned between the *Xba* I and *Sal* I sites of pUC18, to give pDB2507 (Fig. 3.12) The cloned subsite sequences were checked by Sanger di-deoxy sequencing.

3.5.2 Band Shift Analysis of Tn3 Resolvase with Subsite (II + 10) and with Subsite (II + 5).

Plasmids pDB2107 and pDB2507 were used as a source of subsite (II + 10) and subsite (II + 5), respectively. Binding of Tn3 resolvase to each subsite gave a quite different pattern of complexes from that expected. The lowest levels of resolvase used with subsite (II + 10) produced two complexes (Fig. 3.13), but in this case, the less retarded complex (complex 1) was the major species. In the presence of higher resolvase concentrations the amount of both complexes increased. At the highest resolvase concentration almost equal amounts of each complex were formed, enough for each to be considered 'strong' complexes. At the higher resolvase concentrations, a third, fainter complex was formed, which had the lowest mobility of the complexes observed. Band shift assays of Tn3 resolvase with subsite II, using unlabelled DNA (and no additional carrier DNA) are also shown in Fig. 3.13.

At the lowest concentration of resolvase used, only one complex (complex 1) was produced by binding of resolvase to subsite (II + 5), (Fig. 3.13a). In the presence of greater amounts of resolvase three other, more retarded, complexes were detected. Complex 1 remained the major species present, although large amounts of complex 2 were also produced. The two remaining complexes (3 and 4) ran as a doublet, a little more slowly than complex 2. The pattern of complexes produced with subsite (II + 5), and their order of appearance over the resolvase titration, resembles that of subsite (II + 10), i.e. complex 1 and complex 2 of subsite (II + 5) seem similar to those of subsite (II + 10), and complexes 3 and 4 of

(a)

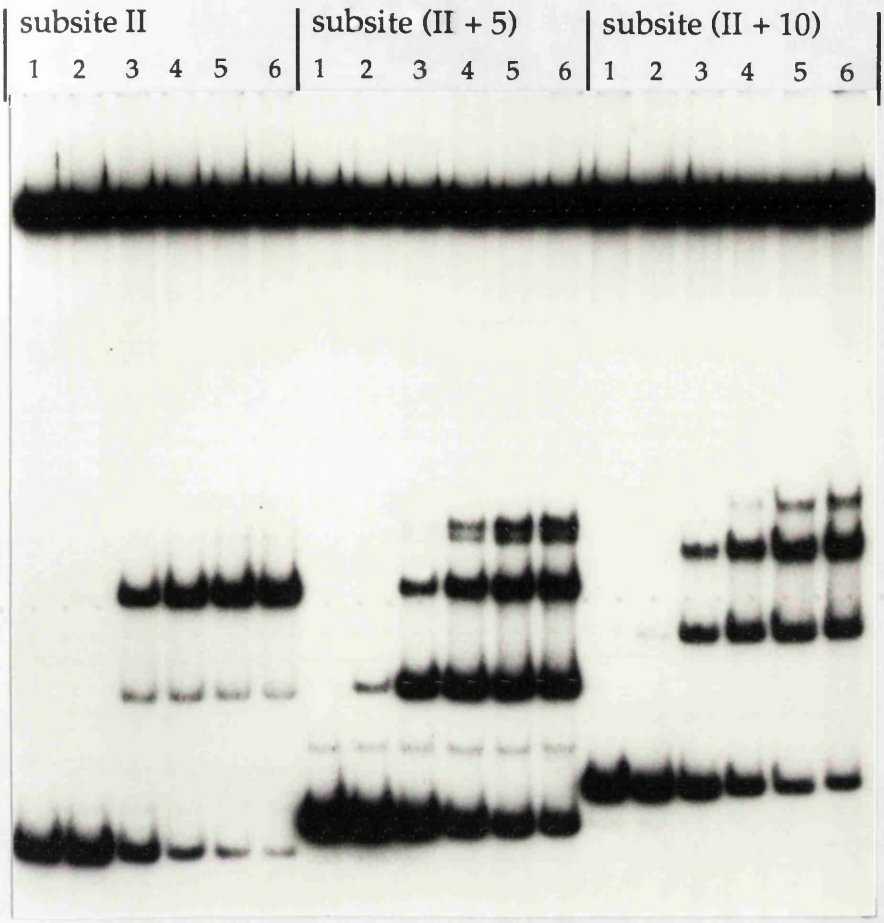
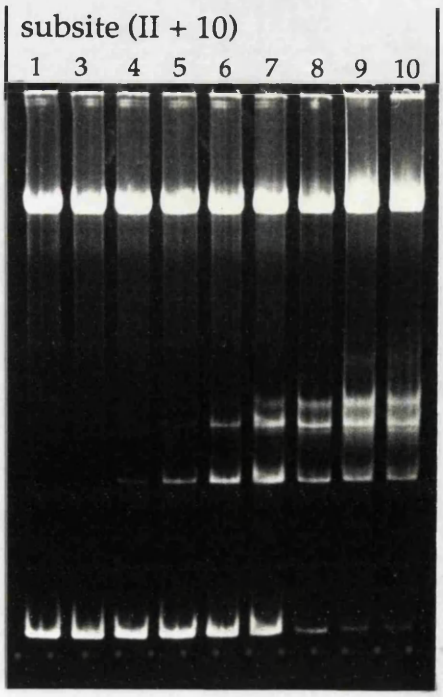


Figure 3.13. Binding of Tn3 Resolvase to Subsites (II + 5) and (II + 10).

TG 9.4 band shift assays of Tn3 resolvase with subsite II (pDB2004 *Eco* RI/*Hin* dIII), subsite (II + 5) (pDB2507 *Eco* RI/*Hin* dIII) or subsite (II + 10) (pDB2107 *Eco* RI/*Hin* dIII). All incubations were for 10 min at 37 ℃.
(a) End-labelled DNA fragments with dilutions of R14 f46.
(b) Unlabelled DNA with dilutions of R17 f47 (no carrier DNA, EtBr-stained gel).

Lane	Resolvase
1	R.H.B.
2	2^{-8}
3	2^{-7}
4	2^{-6}
5	2^{-5}
6	2^{-4}
7	2^{-3}
8	2^{-2}
9	2^{-1}
10	neat

(b)



subsite (II + 5) behave like complex 3 of subsite (II + 10). This was somewhat surprising as the inserted sequence of subsite (II + 10) was designed to disrupt binding as little as possible, whereas the inserted sequence of subsite (II + 5) was expected to interfere with binding by the resultant rotational displacement of the half-sites, relative to each other.

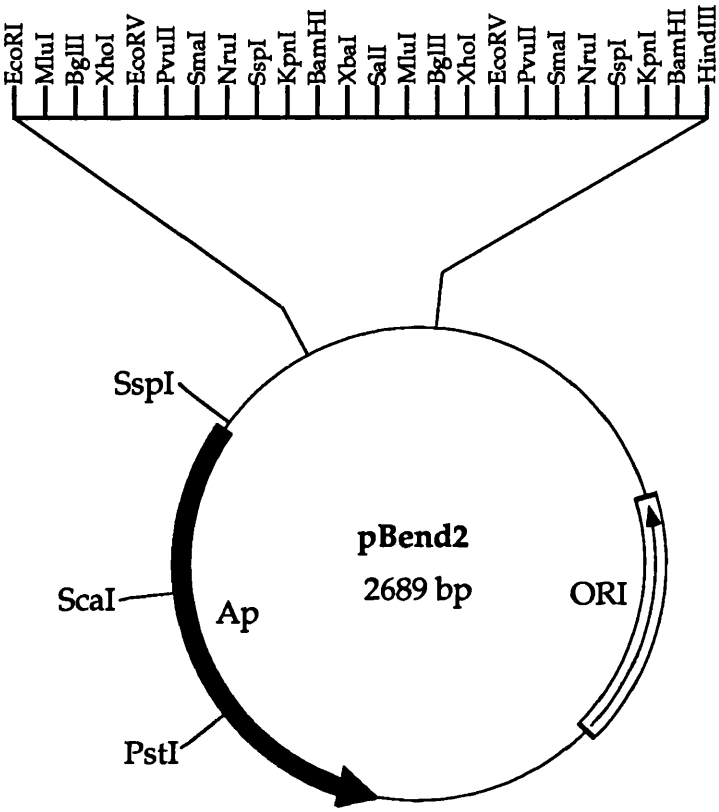
3.6 Resolvase-Induced Bending of Subsite II and Subsite (II + 10).

The plasmid pBend2 (Fig. 3.14) is designed to allow the production of circularly permuted DNA fragments[39], without creating directly repeated copies of the sequences which are being examined. Plasmids containing large directly repeated sequences are often prone to rearrangement. With pBend2 the sequence of interest is cloned to the centre of the polylinker and then re-isolated on a number of different restriction fragments in which the relative position of the insert (to the fragment ends) varies, but its sequence context remains the same.

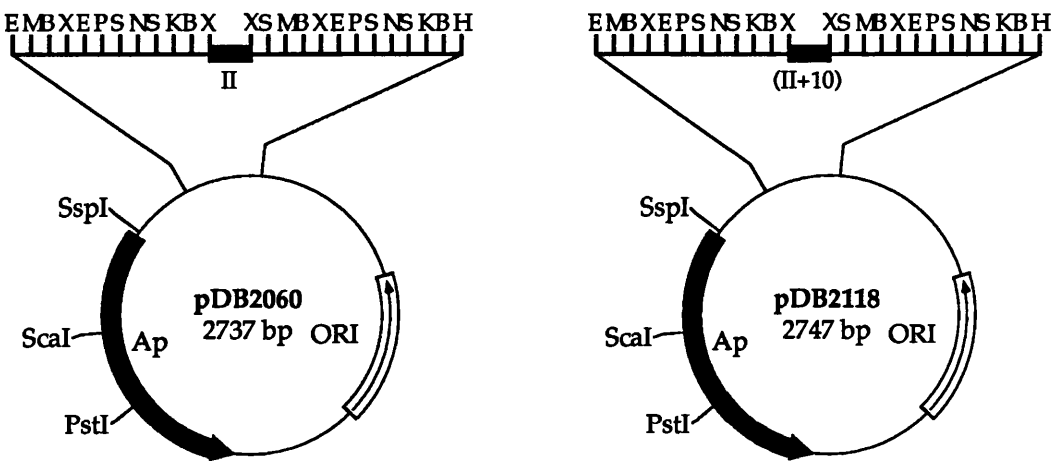
Subsite II and subsite (II + 10) were each cloned to pBend2. The plasmid pDB2118 (Fig. 3.14) was produced by cloning subsite (II + 10), as a synthetic *Xba* I fragment, to the *Xba* I site of pBend2. The fragment used was as described in section 3.5.1. The sequence and orientation of subsite (II + 10) within pDB2118 was checked by Sanger di-deoxy sequencing. Several attempts were made to subclone subsite II from pDB2004 to pBend2. Clones with the expected restriction fragment pattern were obtained each time, but in all cases these plasmids proved unstable, and underwent rearrangement before large scale plasmid preparations could be produced. Because of this, a second pair of complementary oligonucleotides was synthesised, to give a 48 bp *Xba* I fragment ('subsite II(β)', Chapter 2) containing subsite II. The arrangement of this subsite II fragment is similar to that of the subsite (II + 10) fragment described. A stable subsite II construct, pDB2060 (Fig. 3.14), was produced by cloning subsite II(β) to the *Xba* I site of pBend2. The sequence and orientation of the inserted DNA was checked by Sanger di-deoxy sequencing.

The plasmids pDB2060 and pDB2118 were each digested using several restriction endonucleases, to produce a series of DNA fragments, for band shift analysis, in which the relative position of the resolvase binding target was varied, but its sequence context remained constant. The DNA was prepared and used for non-radioactive band shift assays (described in Chapter 2) using TG 9.4 conditions. The complexes generated using pDB2060 (subsite II) were visualised by silver staining the gel produced (Fig. 3.15) and the mobilities of the complexes, and unbound DNA bands, were measured directly from the stained gel. The subsite

(a)


EcoRI
MluI
BglII
XhoI
EcoRV
PvuII
SmaI
NruI
SspI
KpnI
BamHI
XbaI
SalI
MluI
BglII
XhoI
EcoRV
PvuII
SmaI
NruI
SspI
KpnI
BamHI
HindIII
SspI
ScaI
Ap
pBend2
2689 bp
ORI
PstI

(b)


EMBXEPSNSKBX XSMBXEPSNSKBH
II
SspI
ScaI
Ap
pDB2060
2737 bp
ORI
PstI
EMBXEPSNSKBX XSMBXEPSNSKBH
(II+10)
SspI
ScaI
Ap
pDB2118
2747 bp
ORI
PstI

(c)

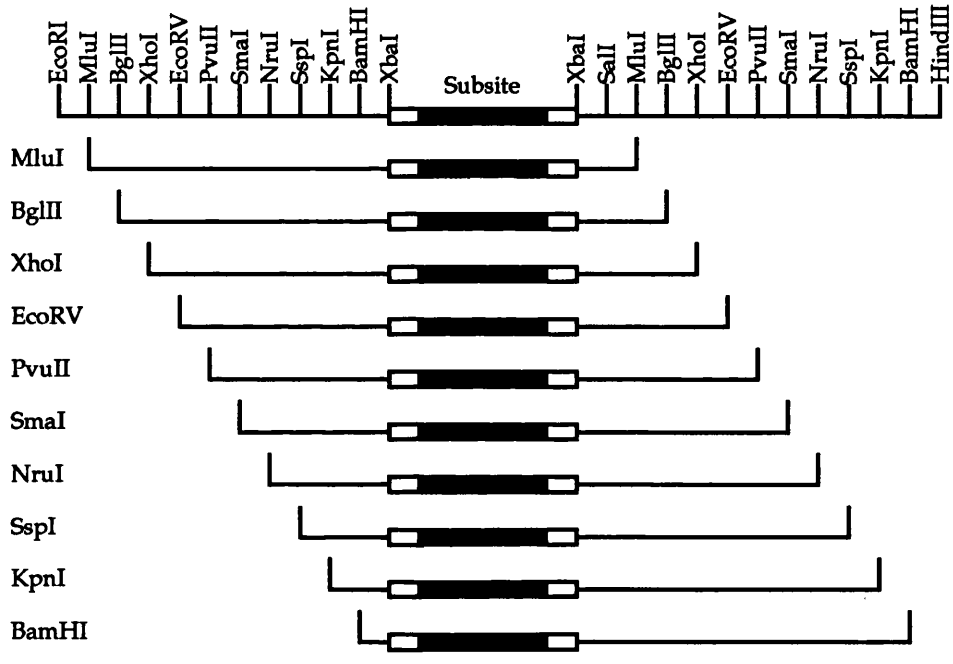


Figure 3.14. Map of pBend2, pDB2060 and pDB2118

(a) Map of pBend2. Not all pairs of restriction enzyme sites are shown in the polylinker, but all sites used are shown in the correct order.

(b) Map of pDB2060 and pDB2118. These two plasmids are identical to pBend2 with the exception of the subsite sequences inserted at the *Xba* I site. These are not shown to scale.

(c) Representation of DNA fragments produced, from pDB2060 and pDB2118, for bend analysis by the band shift assay.

(II + 10) gel was stained using EtBr, and the DNA was visualised by UV exposure. The mobilities of the different species generated from subsite (II + 10) (Fig. 3.16) were measured from a photograph of the gel. The mobilities, listed in Tables 3.1 and 3.2, were each measured from the bottom of the appropriate well to the band centre. It should be noted that the pattern of complexes produced for each subsite was as described previously in this chapter, and in Chapter 5.

Restriction Fragment	Inter-centre Distance (bp).	Mobility (mm)			Relative Mobility	
		U	c 1	c 2	c 1	c 2
Mlu I	- 48.5	105	77	60	0.73	0.57
Bgl II	- 42.5	104	74	57	0.71	0.55
Xho I	- 12.5	103	67	48	0.65	0.47
Eco RV	- 0.5	103	66	48	0.64	0.47
Sma I	11.5	103	68	49	0.66	0.48
Ssp I	29.5	104	72	53	0.69	0.51
Kpn I	35.5	104	74	56	0.71	0.54
Bam HI	54.5	104	81	65	0.78	0.63

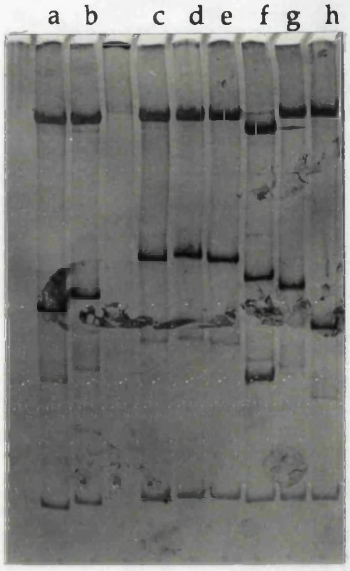
Table 3.1 **Relative Mobilities of Complexes Generated with Subsite II from pDB2060.**
The different subsite II DNA species are denoted by;
U = Unbound DNA fragment. c 1 = Complex 1.
c 2 = Complex 2.

Restriction Fragment.	Inter-centre Distance (bp).	Mobility (mm)				Relative Mobility		
		U	c 1	c 2	c 3	c 1	c 2	c 3
Mlu I	- 48.5	123	93	80	74	0.76	0.65	0.60
Bgl II	- 42.5	122	91	78	72	0.75	0.64	0.59
Xho I	- 12.5	120	85	73	68	0.71	0.61	0.57
Eco RV	- 0.5	119	84	72	67	0.71	0.61	0.56
Pvu II	5.5	119	85	73	68	0.71	0.61	0.57
Sma I	11.5	119	86	74	69	0.72	0.62	0.58
Nru I	23.5	120	88	75	70	0.73	0.63	0.58
Rsa I	35.5	121	92	78	73	0.76	0.64	0.60
Bam HI	54.5	123	100	85	79	0.81	0.69	0.64

Table 3.2 **Relative Mobilities of Complexes Generated with Subsite (II + 10) from pDB2118.**
The different subsite (II + 10) DNA species are denoted by;
U = Unbound DNA fragment. c 1 = Complex 1.
c 2 = Complex 2. c 3 = Complex 3.

It is clear from the mobility values listed for the two sets of unbound DNA that the subsite II and subsite (II + 10) fragments exhibit some degree of bending. The effects of this have been taken into account by calculating relative mobility values

(a)



(b)

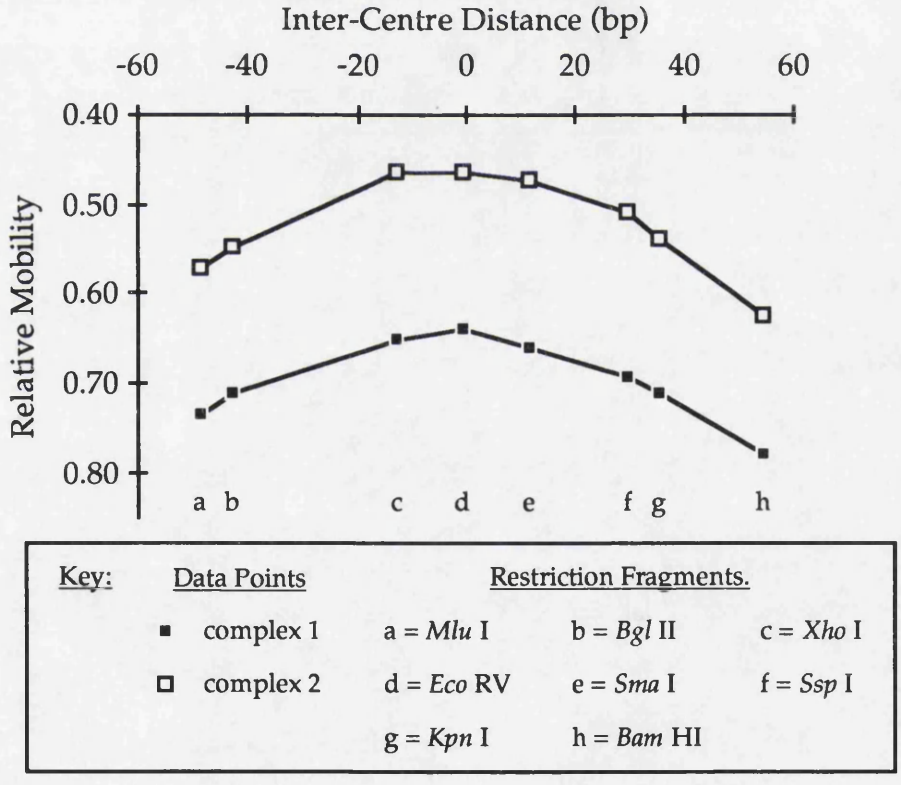
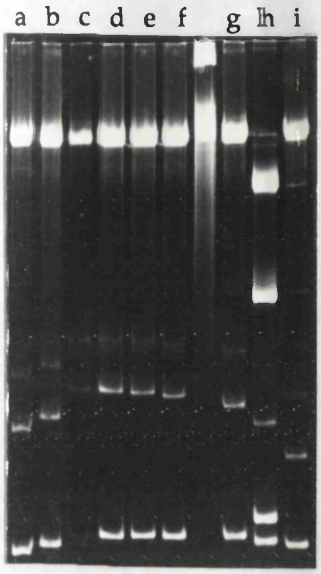


Figure 3.15. Analysis of Resolvase Induced Bending of Subsite II.

(a) TG 9.4 band shift assay of subsite II DNA fragments (generated from digestion of pDB2060) with Tn3 resolvase (2^{-4} dilution of R17 f49). Restriction endonucleases used for lanes 'a' to 'h' were as shown in the key.

(b) Plot of 'Relative Mobility', of each complex generated, against the 'Inter-Centre Distance' of the fragment used. Relative mobility values were calculated by dividing the measured mobility of a retarded complex by that of the unbound DNA species in the same track. The inter-centre distance is the number of bases by which the centre of subsite II is offset from the centre of the DNA fragment used. Points are marked 'a' to 'h' to indicate the different DNA fragments used.

(a)



(b)

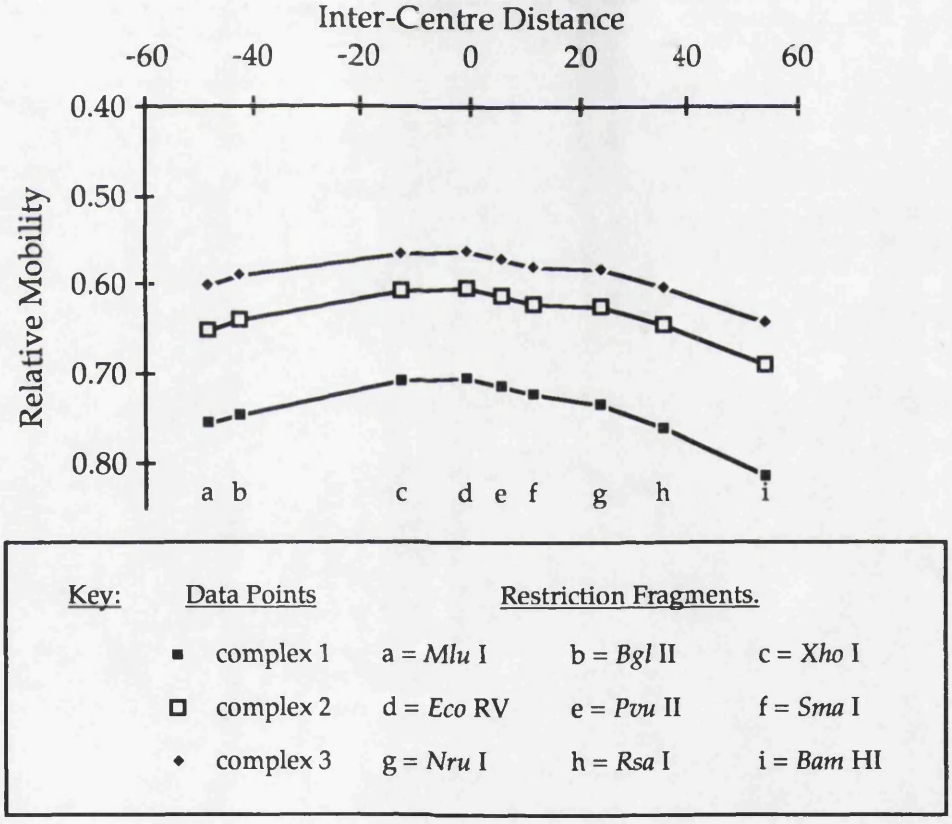


Figure 3.16. Analysis of Resolvase Induced Bending of Subsite (II + 10).

(a) TG 9.4 band shift assay of subsite (II + 10) DNA fragments (generated from digestion of pDB2118) with Tn3 resolvase (2^{-3} dilution, R17 f47). Restriction endonucleases used for lanes 'a' to 'i' were as shown in the key.

(b) Plot of 'Relative Mobility', of each complex generated, against the 'Inter-Centre Distance' of the fragment used. Relative mobility values were calculated by dividing the measured mobility of a retarded complex by that of the unbound DNA species in the same track. The inter-centre distance is the number of bases by which the centre of subsite II is offset from the centre of the DNA fragment used. Points are marked 'a' to 'i' to indicate the different DNA fragments used.

for each of the complexes. The relative mobility values listed in Tables 3.1 and 3.2 were calculated by dividing the measured mobility of a complex by that of the unbound DNA in the same track. Relative mobility values were plotted against the 'inter-centre distance', which is the distance by which the centre of the subsite is displaced from the centre of the restriction fragment used. From the graphs produced (Fig. 3.15 and 3.16) it can be seen that all the complexes analysed exhibit protein-induced bending, and it can be estimated that the bend centres for subsite II and subsite (II + 10) are at the centres of each subsite.

It is possible to examine whether the induced bending of complex 2 is greater than that of complex 1, by expressing complex 2 values relative to the mobility of complex 1; i.e. by dividing the mobility of complex 2 by that of complex 1 from the same track. Complex 2 of subsite II did appear to show a small degree of additional bending when analysed in this way. Complexes 2 and 3 of subsite (II + 10) did not show additional bending in the equivalent analysis. However, the small differences involved in this secondary examination require that it is interpreted with caution.

3.7 DNase I Footprinting of Subsite II and Derivatives of Subsite II.

DNase I footprinting (as described in Chapter 2) was used to test if the position of resolvase bound to an isolated subsite II was the same as for subsite II within the *res* site. Both strands of subsite II (from pDB2004) were footprinted (Fig. 3.17). The observed patterns of protection from, and enhancement of cleavage were as reported for subsite II within *res*[15,25]. This technique was also used to determine whether resolvase was binding to subsites (II + 10) and (II + 5) as it was to subsite II. Only one strand of each altered subsite was footprinted (as shown Fig. 3.19 and 3.20). This was considered an acceptable initial investigation because of the similarity of the footprints produced with each strand of subsite II. Footprinting gels of subsites (II + 10) and (II + 5) are shown in Fig. 3.18. Comparison of the footprints for all three subsites (Fig. 3.19) suggests that the resolvase is contacting the ends of each subsite in a similar fashion. Each half of the subsites analysed can be aligned as shown in Fig. 3.20. If the nucleotides are then numbered from the left hand side, as indicated, positions 1 to 10 are predicted to be most important in the interaction between the DNA and the C-terminal domain of the resolvase[68]. Enhanced DNase I cleavage was observed on the top strand (as written) at the bonds between nucleotides 4 and 6, and on the bottom strand at the bond between nucleotides 1 and 2 (an A-C dinucleotide in each case). This suggests that some sort of resolvase-dependent bending or distortion occurs at these positions. No other bonds within the first 10 bases of the half-sites showed

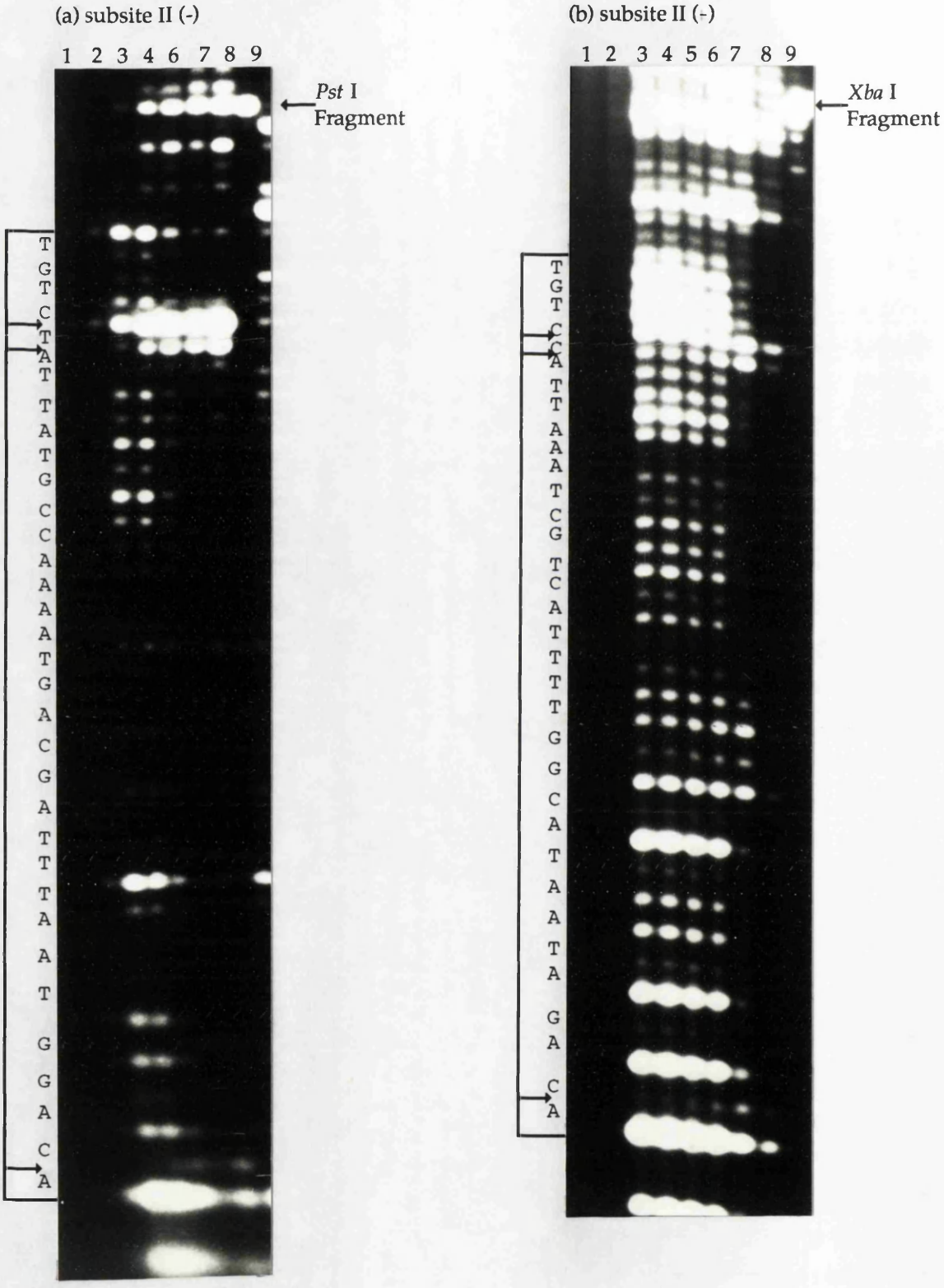

(a) subsite II (-)
1 2 3 4 6 7 8 9
Pst I
Fragment
TGTCTATTATGCCAAAAATGACGATTTTAAATGGACA
(b) subsite II (-)
1 2 3 4 5 6 7 8 9
Xba I
Fragment
TGTCCATTAAATCGTCATTTTGGCATAATAGACA

Figure 3.17. DNase I Footprint of Tn3 Resolvase Bound to Subsite II.

(a) Subsite II (-) strand. pDB2004 digested *Eco* RI/*Bam* HI, 3'end-labelled at *Bam* HI site. Sequence determined relative to *Pst* I digest of the same end-labelled material.

(b) Subsite II (+) strand. pDB2004 digested *Sma* I/*Hin* dIII, 3'end-labelled at *Hin* dIII site. Sequence determined relative to *Xba* I digest of the same end-labelled material.

For (a) and (b), binding reactions (containing an R17 f49 dilution, or resolvase dilution buffer) were incubated in C8.2 buffer for 5 min at room temperature, prior to the addition of DNase I (or DNase I dilution buffer). Samples were then incubated for a further 5 min at room temperature. The reactions were terminated by the addition of loading buffer, heated to 80 °C and then loaded to an 8% polyacrylamide sequencing gel.

Lane	R17 f49 dilution	DNase I
1	R.H.B.	D.D.B.
2	2^{-5}	"
3	R.H.B.	1 µl @ 6.25 µg/ml
4	2^{-9}	"
5	2^{-8}	"
6	2^{-7}	"
7	2^{-5}	"
8	2^{-3}	"
9	marker sample	

D.D.B. = DNase I dilution buffer.

The subsite sequence is placed approximately alongside the gel inside a box. Positions of enhanced cleavage are indicated by arrows. Regions of protection are indicated in Fig. 3.19 and 3.20.

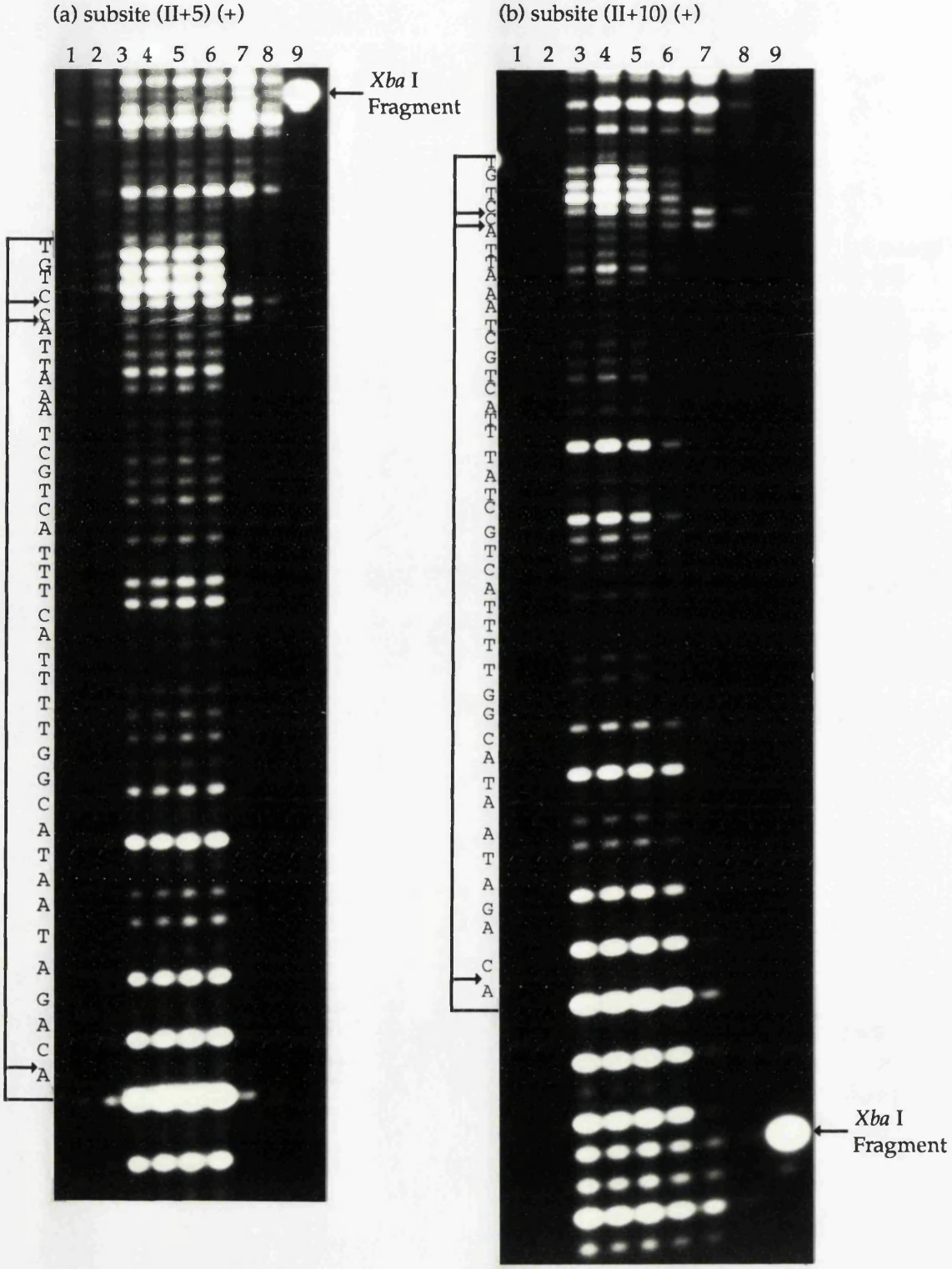

(a) subsite (II+5) (+)
1 2 3 4 5 6 7 8 9
Xba I
Fragment
TGTCCATTAATCGTCATTTCATTTTGGCATAATAGACCA
(b) subsite (II+10) (+)
1 2 3 4 5 6 7 8 9
TGTCCATTAATCGTCATTTATCGTCATTTTGGCATAATAGACCA
Xba I
Fragment

Figure 3.18. DNase I Footprint of Tn3 Resolvase Bound to Subsites (II + 5) and (II + 10).

(a) Subsite (II + 5) (+) strand. pDB2507 digested *Sma* I/*Hin* dIII, 3'end-labelled at *Hin* dIII site. Sequence determined relative to *Xba* I digest of the same end-labelled material.

(b) Subsite (II + 10) (+) strand. pDB2107 digested *Sma* I/*Hin* dIII, 3'end-labelled at *Hin* dIII site. Sequence determined relative to *Xba* I digest of the same end-labelled material.

For (a) and (b), binding reactions (containing an R17 f49 dilution, or resolvase dilution buffer) were incubated in C8.2 buffer for 5 min at room temperature, prior to the addition of DNase I (or DNase I dilution buffer). Samples were then incubated for a further 5 min at room temperature. The reactions were terminated by the addition of loading buffer, heated to 80 °C and then loaded to an 8% polyacrylamide sequencing gel.

Lane	R17 f49 dilution	DNase I
1	R.H.B.	D.D.B.
2	2^{-5}	"
3	R.H.B.	1 µl @ 6.25 µg/ml
4	2^{-9}	"
5	2^{-8}	"
6	2^{-7}	"
7	2^{-5}	"
8	2^{-3}	"
9	marker sample	

D.D.B. = DNase I dilution buffer.

The subsite sequence is placed inside a box (approximately) alongside the gel . Positions of enhanced cleavage are indicated by arrows. Regions of protection are indicated in Fig. 3.19 and 3.20.

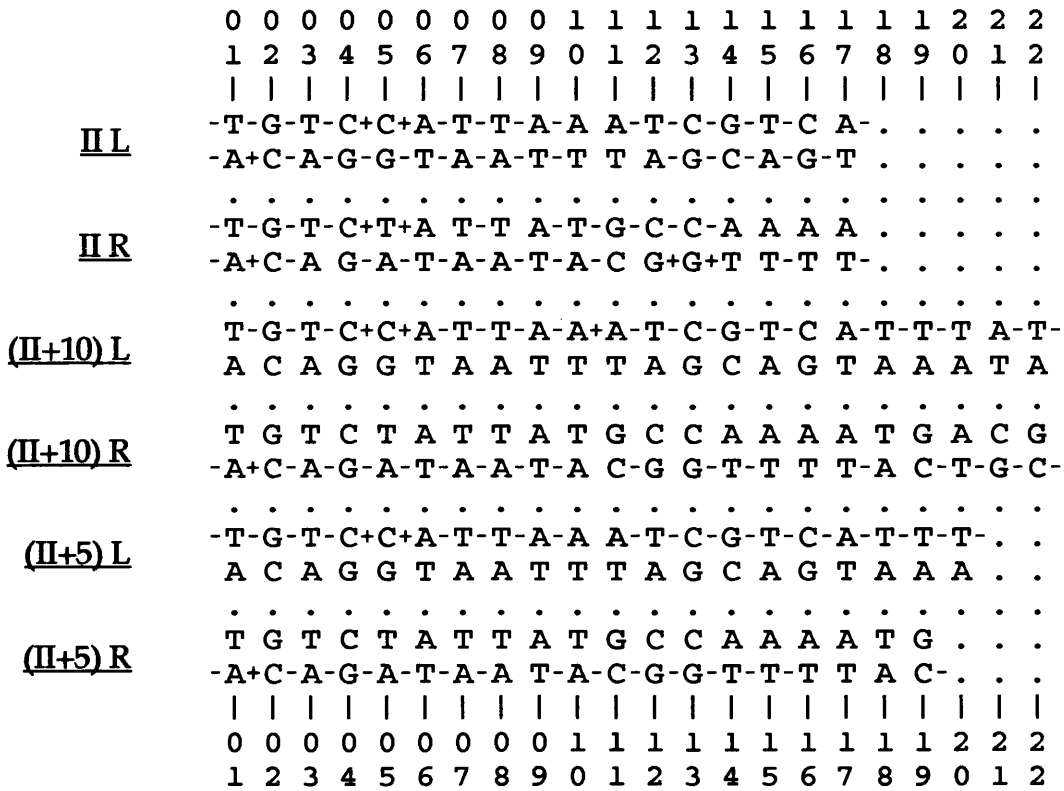


Figure 3.20. Alignment of Halves of Subsite II and Its Derivatives, Showing Footprint Data.

Subsite halves are aligned from the first T of the TGT motif corresponding to position 1 of the top strand. Each half site is named according to the subsite from which it has come, and L or R, to indicate the left or right end. Descriptions of cleavage patterns (in the main text) use the numbering system shown. An approximation of the cleavage patterns observed is indicated by the symbols placed at bond positions within each sequence;

- \+ indicates enhanced DNase I cleavage in the presence of resolvase.
- \- indicates reduced DNase I cleavage (i.e. protection) in the presence of resolvase.

Where no symbol is present either no change was seen in DNase I cutting at that position, or no data were obtained for that bond. Dots are to assist comparison of the aligned sequences.

A more detailed representation of the same data is shown in Fig. 3.19.

increased cleavage, and almost all exhibited some degree of protection.

3.8 Methylation Interference Experiments Using Subsite II and Derivatives of Subsite II.

Methylation interference experiments were used to give further comparison of binding of resolvase to subsites II, (II + 10) and (II + 5), and to investigate the parts of each subsite required for the formation of each complex produced. The method used is described in Chapter 2, but the important features are outlined in Fig. 3.21. The principle of a modification interference experiment is that modification of a nucleotide which is involved in binding will result in the disruption of that particular interaction. To produce a clear interference pattern it is necessary to achieve approximately one modification per DNA fragment used.

The modification experiments were conducted using dimethyl sulphate to methylate the G residues of each subsite. Only one strand of each subsite was analysed (shown in Fig. 3.22 and 3.23). The results of the methylation interference experiments for subsites II, (II + 10) and (II + 5) are shown in Fig. 3.22. These results are summarised in Fig. 3.23. Again the results were similar for each of the subsites investigated. Methylation of the G residue at position R2G of each subsite prevented formation of complex 2 (and other more retarded complexes), but appeared not to interfere with complex 1. The production of complexes was affected by the methylation of only one other G residue. Modification of the G residue at position L5G of each subsite prevented formation of all complexes. Both of these positions have been shown to be important in the binding of resolvase to subsite II of an intact *res* site, as have the equivalent nucleotides on both strands of subsites I, II and III[15,25]. However, differences in the importance of each residue were not previously observed as the experiments did not involve analysis of material from complex 1. Methylation of some residues in subsite (II + 5) gave enhanced binding of resolvase. The position of methylation of these bases is unknown.

3.9 Binding of γδ Resolvase, and γδ Mutant M106C, to Subsites II, (II + 5) and (II + 10).

To allow further comparison of binding of the Tn3 and γδ resolvases, dilutions of γδ resolvase were used in TG 9.4 band shift assays with subsites II, (II + 10) and (II + 5), (described in sections 3.4.1 and 3.5.1). A mutant of γδ resolvase, M106C (supplied by Nigel Grindley), was also used.

M106C has a methionine to cysteine substitution at amino acid position 106 of the γδ sequence. This residue is at the subunit interface in the crystal 1-2 dimer[74]. It is

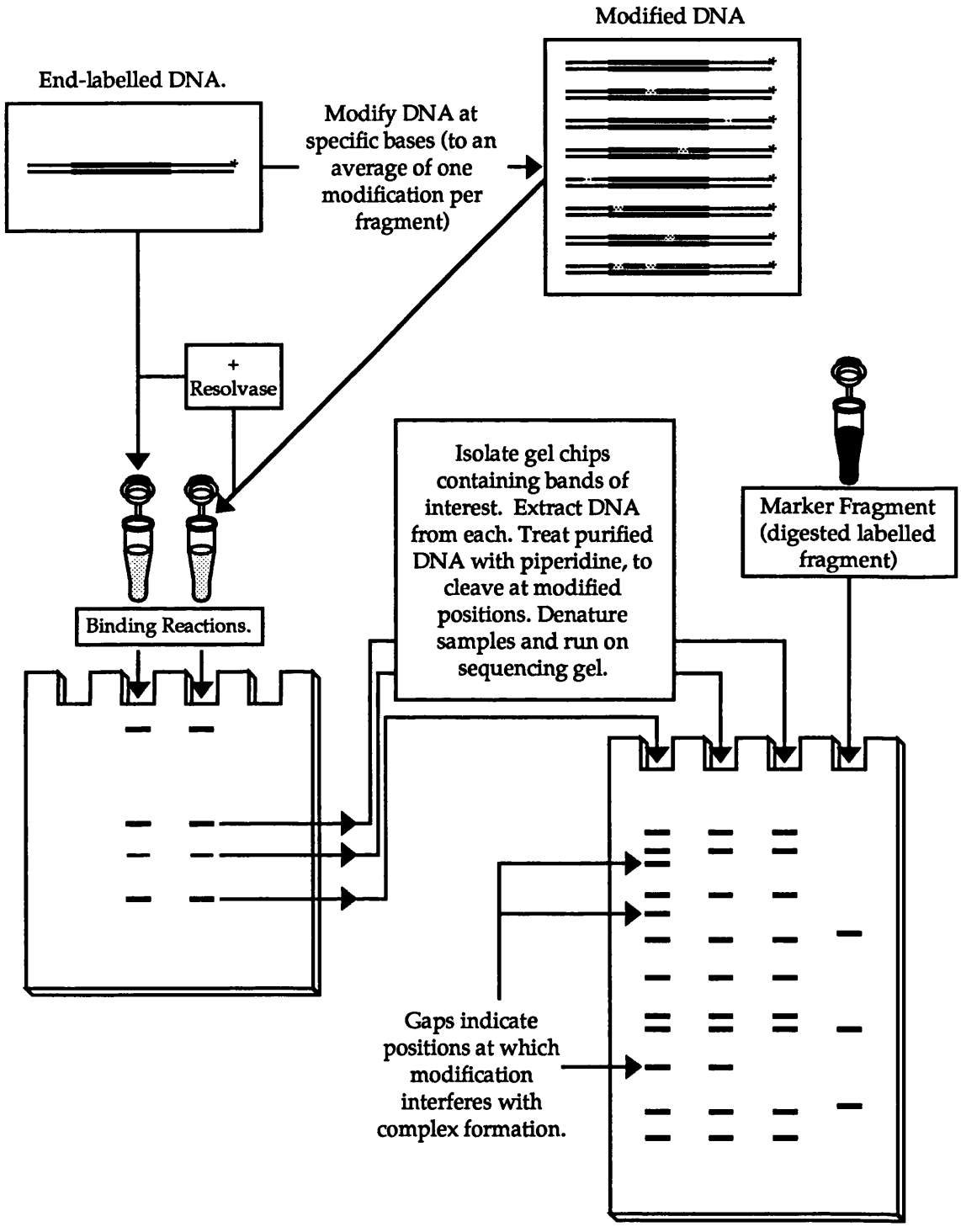


Figure 3.21. Outline of Modification Interference Experiments.
DNA molecules are represented by double lines, with thicker lines indicating the binding site. * indicates ^{32}P labelled DNA strands, and ~~ is used to denote points of modification, with some of the possible modifications being shown for one strand only. DNA species on gels were visualised by autoradiography.
In the experiments described in this chapter the form of modification used was methylation (mainly of G residues) by dimethyl sulphate.

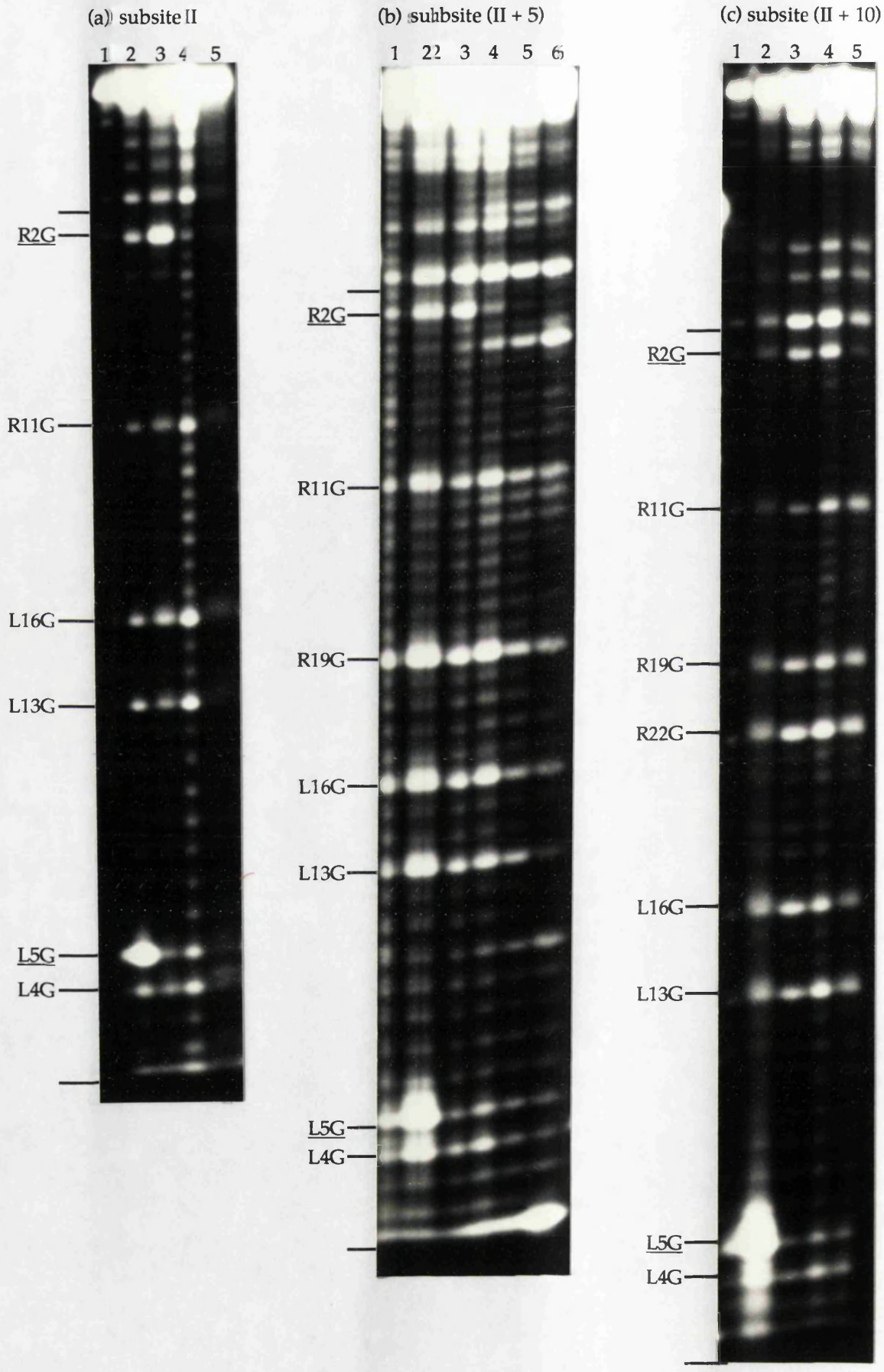

(a) subsite II
1 2 3 4 5
R2G
R11G
L16G
L13G
L5G
L4G
(b) subsite (II + 5)
1 2 3 4 5 6
R2G
R11G
R19G
L16G
L13G
L5G
L4G
(c) subsite (II + 10)
1 2 3 4 5
R2G
R11G
R19G
R22G
L16G
L13G
L5G
L4G

Figure 3.22. Methylation Interference of Tn3 Resolvase Binding to Subsites II, (II + 5) and (II + 10).

Bam HI/*Pst* I fragments carrying subsite II (pDB2004), subsite (II + 5) (pDB2507) or subsite (II + 10) (pDB2107) were 3'end-labelled at the *Bam* HI end (giving labelled (-) strand). Methylation, complex formation and sample preparation were as described in Chapter 2, and summarised in Fig. 3.21. These samples were run on an 8% polyacrylamide sequencing gel. *Xba* I digested DNA samples were also run (not shown) as markers of sequence position.

Lane	Sample
1	methylated DNA stock
2	unbound DNA band
3	complex 1 band
4	complex 2 band
5	complex 3 band
6	complex 4 band

G residues are marked and numbered as described in Fig. 3.20. Those which are shown to be required for complex formation are underlined. The start and end of each subsite sequence is marked by an unlabelled line.

(a) Subsite II.

(b) Subsite (II + 5).

(c) Subsite (II + 10).

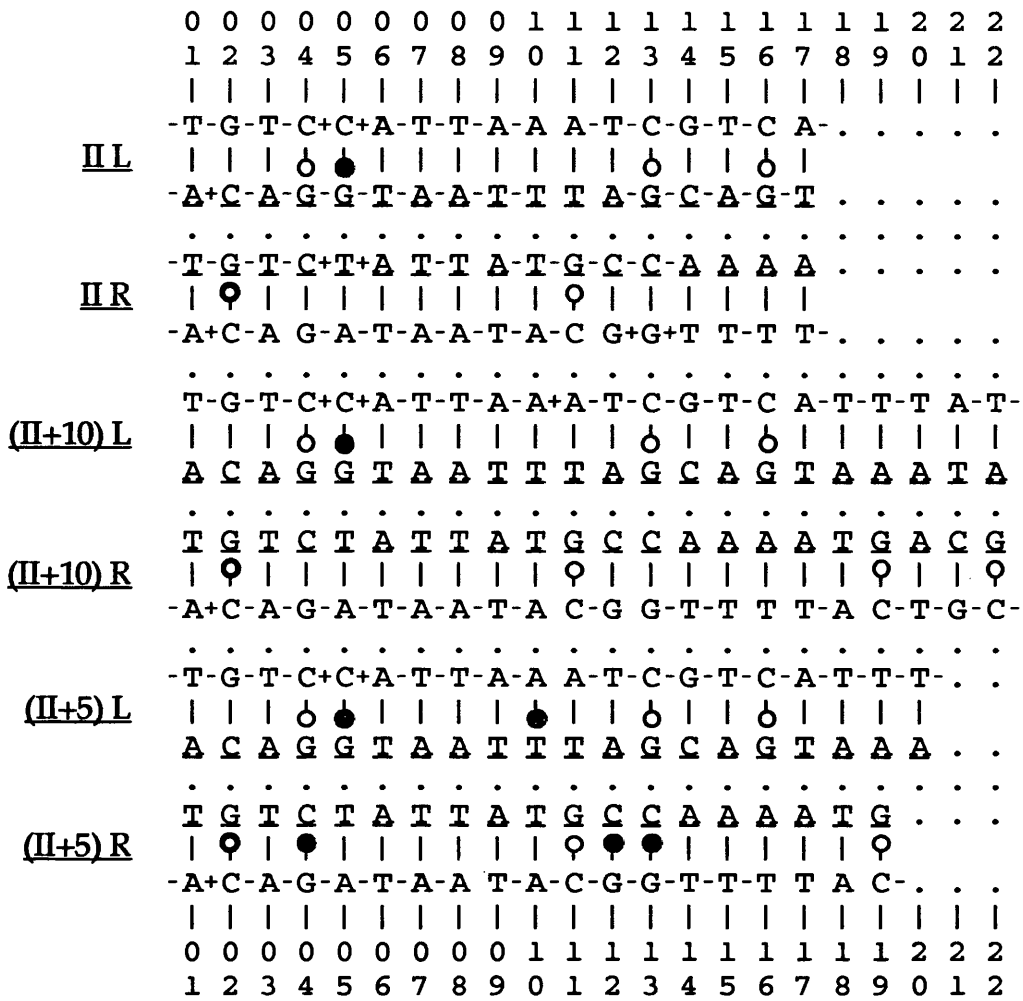


Figure 3.23. Summary of Data From Methylation Interference Experiments.

Subsite halves are aligned from the first T of the TGT motif corresponding to position 1 of the top strand (as in Fig. 3.20). Each half site is named according to the subsite from which it has come, and L or R, to indicate the left or right end. DNase I protection patterns (as described in Fig. 3.20) are retained for reference.

Methylation interference data are indicated as follows:

- ● methylation inhibits formation of all complexes.
- ◎ methylation inhibits formation of all complexes except complex 1.
- ○ no effect of methylation observed.
- ● methylation gives enhanced formation of some, or all, complexes.

Methylation symbols are placed directly above or below the modified base. Sequences for which methylation interference was investigated are indicated by underlining. For G residues the interfering modification is expected to occur at position N-7, facing into the major groove. For other modified bases the position of the modification is unknown.

proposed that a 1-2 dimer binds to *res*[34]. The sample of M106C used had been oxidised to create a disulphide bond between the subunits of the dimer. Fig. 3.24 shows Tn3 resolvase, γδ resolvase and M106C, run on denaturing polyacrylamide gels, under reducing and non-reducing conditions. Under reducing conditions all three proteins run as monomer sized species. The same is also true for Tn3 and γδ resolvase under non-reducing conditions, but the M106C sample predominantly contains a resolvase dimer sized species (although some monomer sized material is present). This would suggest that the cysteine residues at position 106 of M106C have been linked to create dimers.

Binding of γδ and M106C resolvases to Tn3 subsite II, and subsites (II + 10) and (II + 5) is shown in Fig. 3.25, 3.26, 3.27 and 3.28. Binding of Tn3 resolvase is also shown in Fig. 3.25, for comparison. It is very striking that for all three subsites examined the same pattern of complexes is generated with the γδ and M106C resolvases. This supports the proposal that the crosslinked dimer observed for M106C is the dimer involved in binding of γδ resolvase. Although such results do not prove that the γδ resolvase binding unit is a dimer, they certainly support this argument. To interpret the pattern of complexes produced in this way requires that the monomeric resolvase observed in M106C is also capable of binding (see Chapter 7). As with Tn3, the complexes produced are numbered in order of increasing retardation. This is not meant to suggest that all complexes with the same name are necessarily thought to be equivalent species.

For subsite II the complexes generated with γδ and M106C resolvases appear similar to those observed with Tn3 resolvase. Complex 1 appears faint at quite low resolvase concentrations, and remains faint in the presence of more resolvase. Complex 2 appears as the major complex for all tracks in which binding was observed. A third complex is produced with the highest resolvase levels, as was sometimes observed using Tn3 resolvase. However, complex 3 is also visible at lower concentrations of γδ and M106C resolvase, and appears as a stronger band than with Tn3 resolvase. The concentration range over which M106C binding is observed is much larger than with Tn3 resolvase, while the γδ range appears to be somewhere in between.

The range of concentrations over which M106C resolvase bound to subsites (II + 10) and (II + 5) also appeared greater than for Tn3 resolvase (with γδ in between again). However, the complexes produced seemed dissimilar to those generated with Tn3 resolvase. Binding of the γδ and M106C resolvases to subsite (II + 10) produced at least three complexes. Complex 1, unlike that for Tn3, was a

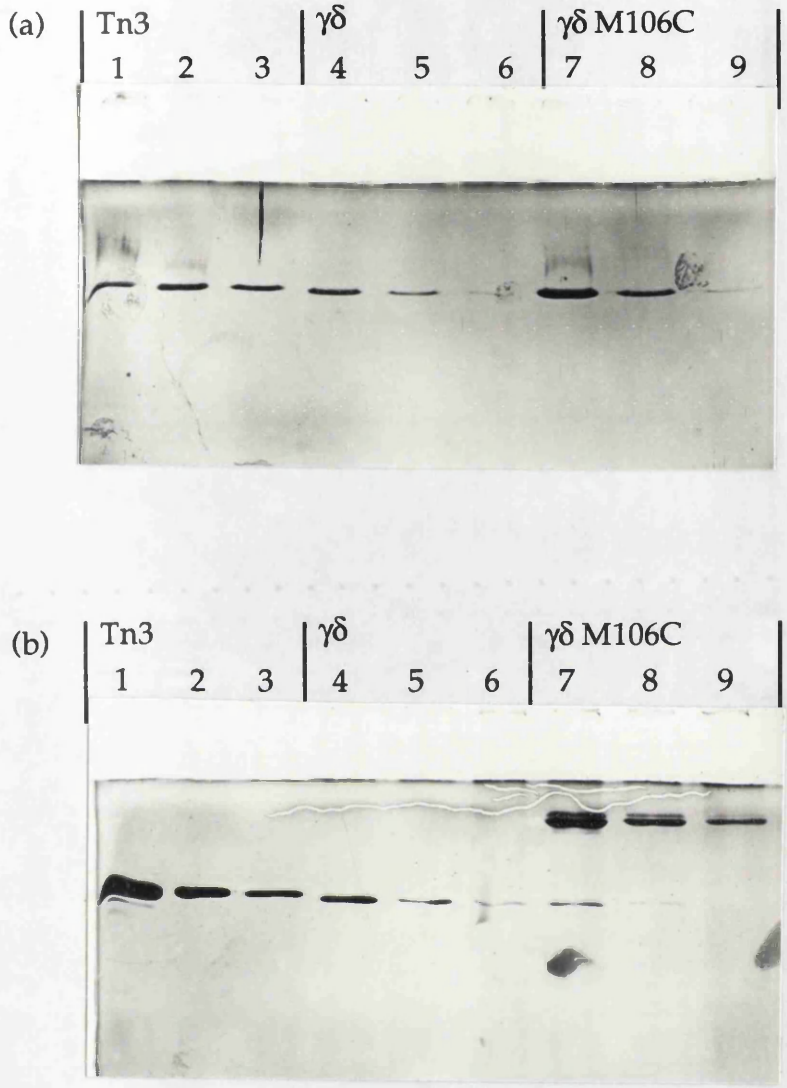


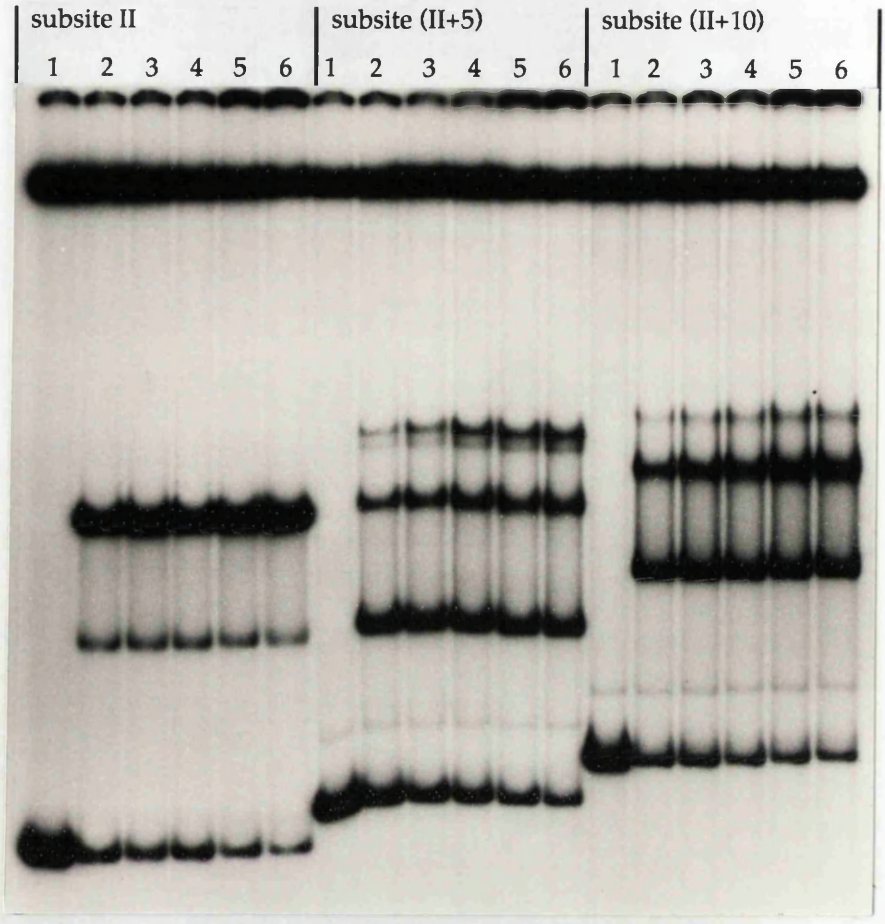
Figure 3.24. Denaturing PAGE of Tn3, γδ and γδ M106C Resolvase Samples.

(a) Dilutions of resolvases with 2-mercaptoethanol as a denaturant.
(b) Dilutions of resolvases with urea as a denaturant.

Lane	Resolvase	
1	neat	R17 f48
2	2^{-1}	"
3	2^{-2}	"
4	neat	γδ
5	2^{-1}	"
6	2^{-2}	"
7	neat	γδ M106C
8	2^{-1}	"
9	2^{-2}	"

Gel (a) does not give a comparison of the relative resolvase concentration of each sample as some material leaked from the wells after loading.

(a)


subsite II
1 2 3 4 5 6
subsite (II+5)
1 2 3 4 5 6
subsite (II+10)
1 2 3 4 5 6

(b)

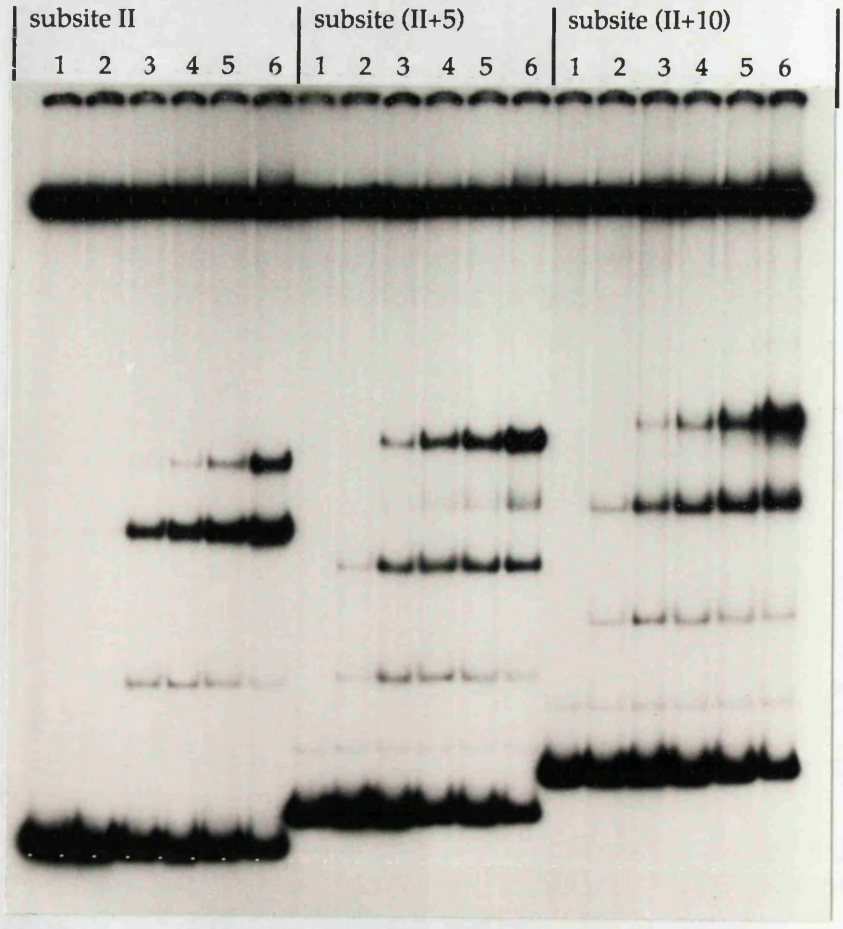

subsite II
1 2 3 4 5 6
subsite (II+5)
1 2 3 4 5 6
subsite (II+10)
1 2 3 4 5 6

Figure 3.25. Comparison of Tn3 and γδ Resolvase Binding to Subsites II, (II + 5) and (II + 10).

(a) Binding of Tn3 Resolvase (R17 f49).

(b) Binding of γδ Resolvase.

End-labelled subsite II (pDB2004 *Eco* RI/*Hin* dIII), subsite (II + 5) (pDB2507 *Eco* RI/*Hin* dIII) or subsite (II + 10) (pDB2107 *Eco* RI/*Hin* dIII) was incubated for 10 min at 37 °C with a resolvase dilution (as indicated) using TG 9.4 conditions.

Lane	Resolvase Dilution
1	R.H.B.
2	2^{-8}
3	2^{-7}
4	2^{-6}
5	2^{-5}
6	2^{-4}

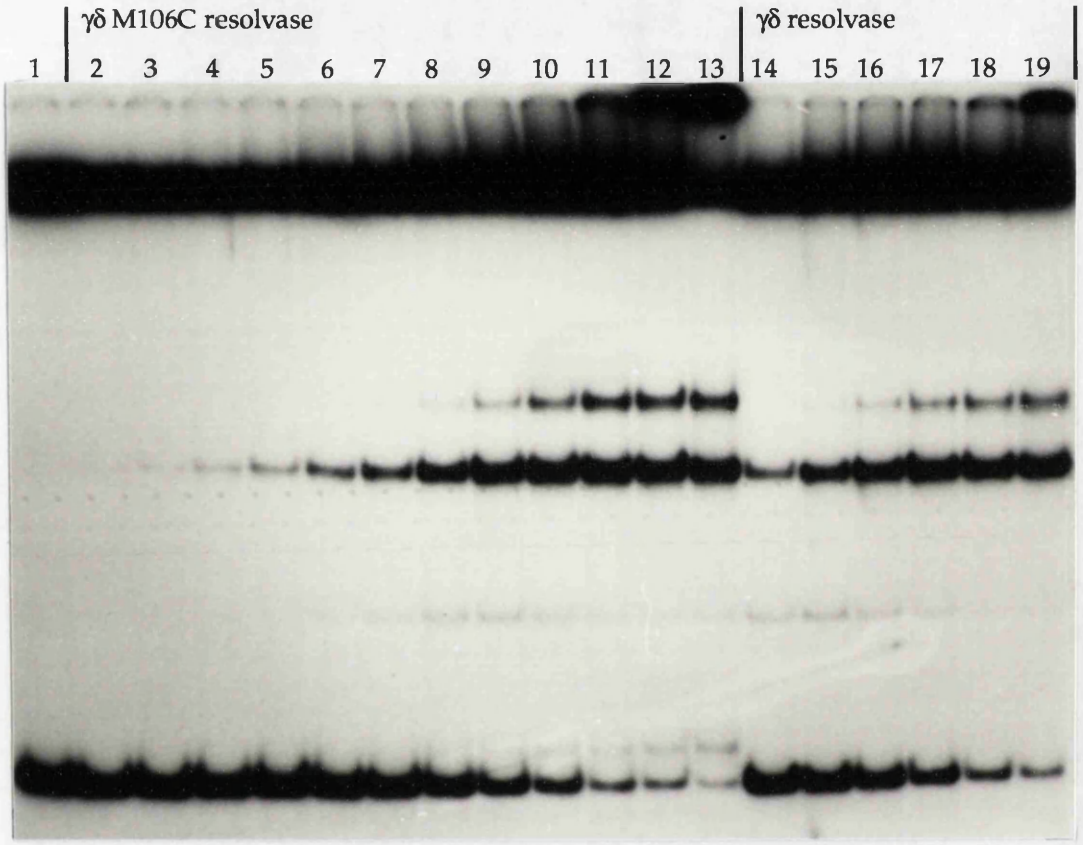


Figure 3.26. Binding of γδ and γδ M106C Resolvases to Subsite II.
End-labelled subsite II (pDB2004 *Eco* RI/*Hin* dIII) was incubated for 10 min at 37 °C with resolvase, as indicated, using TG 9.4 conditions.

Lane	Resolvase sample		Lane	Resolvase sample	
1	R.H.B.		11	2^{-5}	γδ M106C
2	2^{-14}	γδ M106C	12	2^{-4}	"
3	2^{-13}	"	13	2^{-3}	"
4	2^{-12}	"	14	2^{-8}	γδ
5	2^{-11}	"	15	2^{-7}	"
6	2^{-10}	"	16	2^{-6}	"
7	2^{-9}	"	17	2^{-5}	"
8	2^{-8}	"	18	2^{-4}	"
9	2^{-7}	"	19	2^{-3}	"
10	2^{-6}	"			

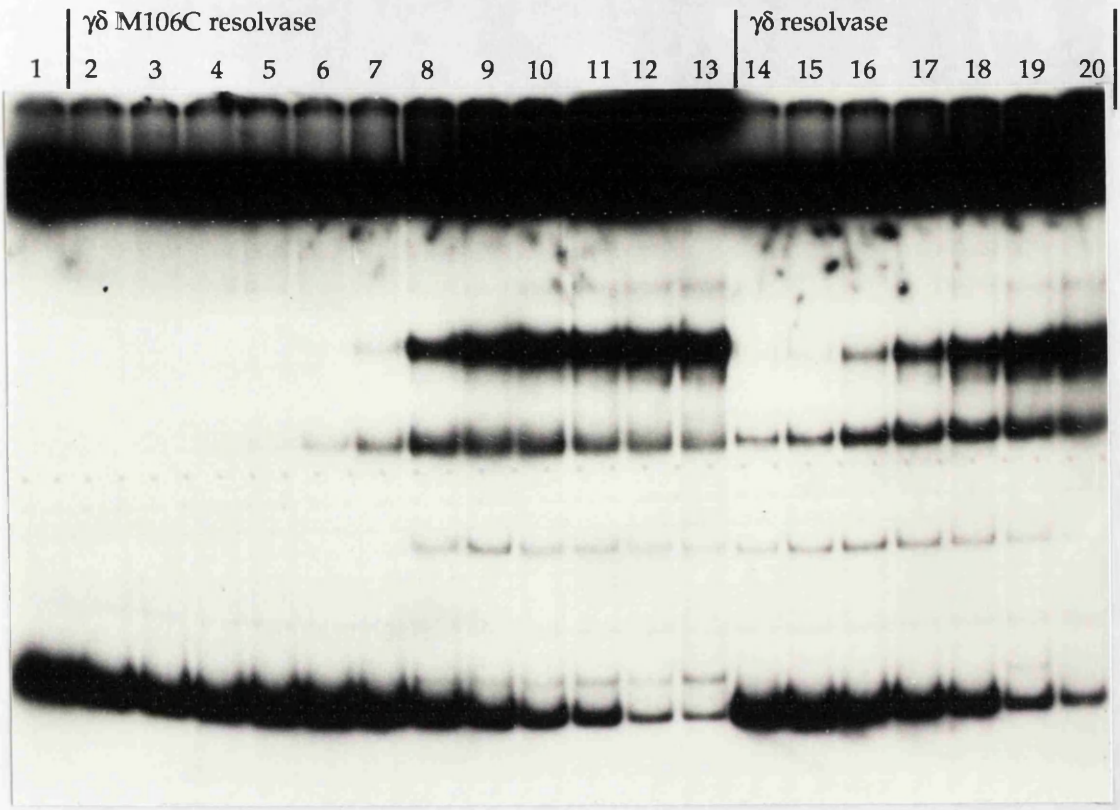


Figure 3.27. Binding of γδ and γδ M106C Resolvases to Subsite (II + 10).
End-labelled subsite (II + 10) (pDB2107 *Eco* RI/*Hin* dIII) was incubated for 10 min at 37 °C with resolvase, as indicated, using TG 9.4 conditions.

Lane	Resolvase sample		Lane	Resolvase sample	
1	R.H.B.		11	2^{-5}	γδ M106C
2	2^{-14}	γδ M106C	12	2^{-4}	"
3	2^{-13}	"	13	2^{-3}	"
4	2^{-12}	"	14	2^{-9}	γδ
5	2^{-11}	"	15	2^{-8}	"
6	2^{-10}	"	16	2^{-7}	"
7	2^{-9}	"	17	2^{-6}	"
8	2^{-8}	"	18	2^{-5}	"
9	2^{-7}	"	19	2^{-4}	"
10	2^{-6}	"	20	2^{-3}	"

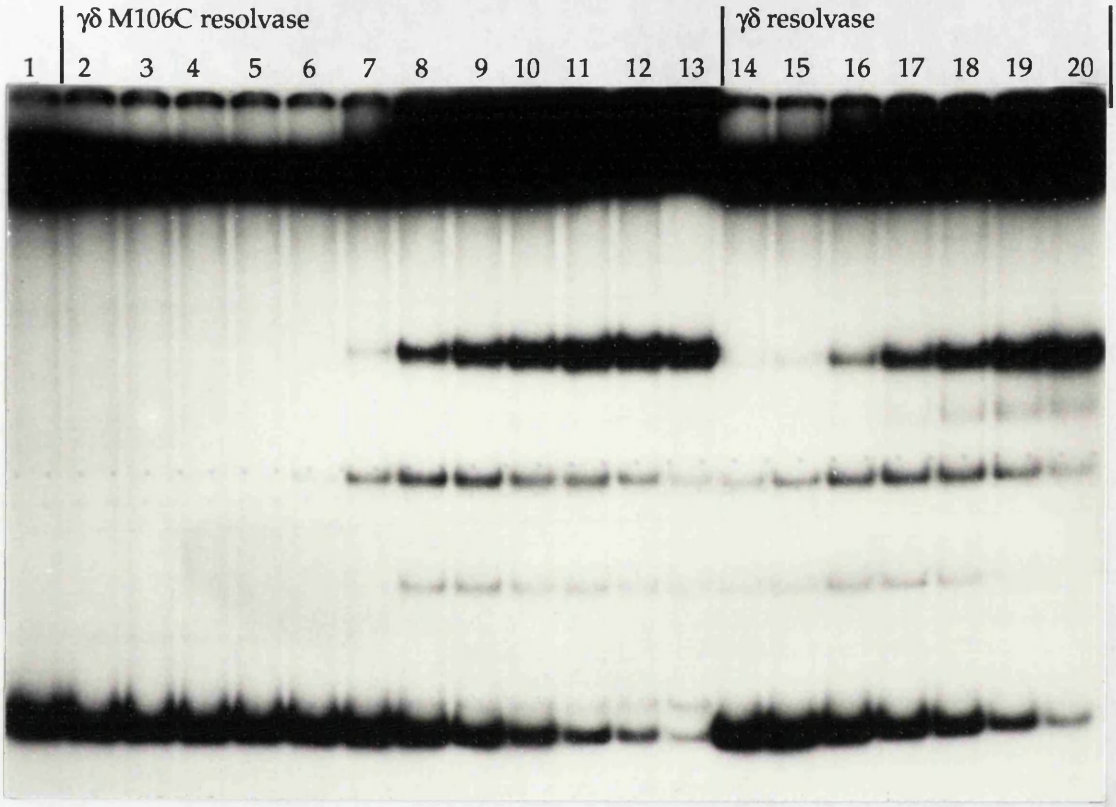


Figure 3.28. Binding of γδ and γδ M106C Resolvases to Subsite (II + 5).
End-labelled subsite (II + 5) (pDB2507 *Eco* RI/*Hin* dIII) was incubated for 10 min at 37 °C with resolvase, as indicated, using TG 9.4 conditions.

Lane	Resolvase sample		Lane	Resolvase sample	
1	R.H.B.		11	2^{-5}	γδ M106C
2	2^{-14}	γδ M106C	12	2^{-4}	"
3	2^{-13}	"	13	2^{-3}	"
4	2^{-12}	"	14	2^{-9}	γδ
5	2^{-11}	"	15	2^{-8}	"
6	2^{-10}	"	16	2^{-7}	"
7	2^{-9}	"	17	2^{-6}	"
8	2^{-8}	"	18	2^{-5}	"
9	2^{-7}	"	19	2^{-4}	"
10	2^{-6}	"	20	2^{-3}	"

minor complex, when present. Although the second complex was the strongest at low resolvase concentrations, complex 3 clearly became the major species as resolvase levels were increased. Some faint, highly retarded complexes were visible in many of the tracks.

Four complexes were observed on binding of γδ, M106C or Tn3 resolvase to subsite (II + 5). However, as with subsite (II + 10), complex 1 for the γδ and M106C resolvases was a minor species, where present. Again complex 2 was the major band only at low resolvase concentrations, with complex 4 the major species in most tracks. The increase in amounts of complex 3 trailed that of complex 4, but complex 3 remained faint at all times.

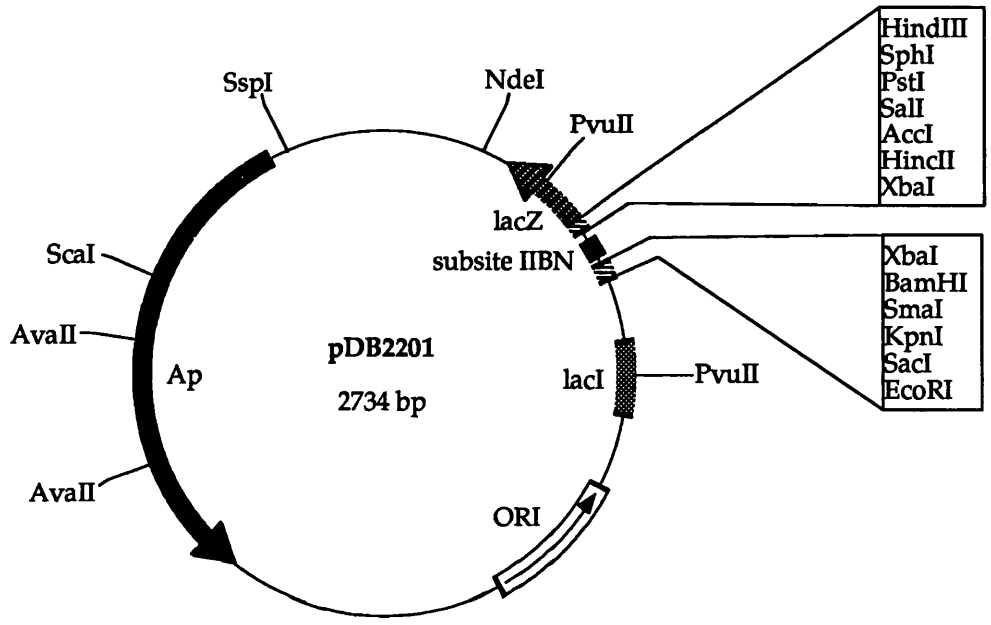
The results described in this section are very suggestive regarding models of binding for γδ resolvase, and also Tn3 resolvase. However, the results presented in Chapter 5 are also very significant, and so binding models will be discussed, in light of all the results presented, in Chapter 7.

3.10 Binding of Resolvase to Each Half of an Altered Subsite II.

Binding of intact resolvase to one isolated half of a *res* subsite has not been demonstrated. Tn3 resolvase has been shown to generate one complex on binding to a fragment containing subsite I sequence lacking the first eight bases (of a total of twenty-eight), from the left hand end[7]. The C-terminal domain of γδ resolvase has also been shown to produce one complex on binding to a DNA fragment containing all of subsite I, bar the first five bases of the left end[68].

A derivative of subsite II (subsite IIBN, Fig. 3.29) was designed with two particular aims in mind. By introducing two restriction endonuclease sites (by base substitutions) at specific points within the subsite II sequence, DNA fragments could be produced containing pieces of the subsite which contain slightly more, or slightly less, than half of the subsite. However, with two restriction sites, each end of the subsite could be produced as the smaller, or larger, fraction of the subsite. Secondly the restriction endonuclease sites could be used to reduce the spacing between the ends of subsite II (by site cleavage with filling or polishing of sticky ends, followed by re-ligation), or to increase the spacing [as with subsites (II + 10) and (II + 5)] by the insertion of additional DNA. The subsite was cloned to pUC18 such that restriction endonuclease digestion (i.e. *Bam* HI and *Bgl* II or *Nsi* I and *Pst* I), followed by ligation, could be used to produce derivatives in which one half of subsite IIBN is deleted. From these derivatives fragments containing a half-site, surrounded by non-Tn3 sequence, could be produced.

(a)



(b)

EcoRI SstI KpnI SmaI BamHI XbaI Subsite IIBN Bgl II

GAATTCGAGCTCGGTACCCGGGGATCCT**CTAGATGAGTGTCCATTAGATCTTCA**

CTTAAGCTCGAGCCATGGGCCCCTAGGAGATC**TACTCACAGGTAATCTAGAAGT**

Nsi I Subsite IIBN XbaI SalI PstI SphI HindIII

TTTATGCATAATAGACACATCTCTAGAGTCGACCTGCAGGCATGCAAGCTT

AAATACGTATTATCTGTGTAGAGATCTCAGCTGGACGTCCGTACGTTCGAA

Figure 3.29. Map of pDB2201.

(a) Map of pDB2201.

(b) Sequence of polylinker region of pDB2201, showing inserted subsite IIBN fragment (in bold type). The shaded region within the subsite indicates substitutions for subsite II sequence.

3.10.1 Construction of Subsite IIBN.

The design and synthesis of subsite IIBN was as described for subsite II(β) (section 3.6), except the sequence of subsite IIBN contained the following substitutions (numbered from left or right end of subsite II, as indicated in Fig. 3.20);

A to G at position L10A.	G to T at position L14G.
G to T at position R13G.	T to A at position R14T.

The complete sequences of the two oligonucleotides synthesised are given in Fig. 3.29 (and Chapter 2). The substitutions in the left hand side of the subsite produce a *Bgl* II site, while those on the right create a *Nsi* I site (Fig. 3.29). The *Xba* I fragment produced was cloned to the *Xba* I site of the pUC18 polylinker, giving pDB2201 (Fig. 3.29). The sequence and orientation of subsite IIBN in pDB2201 was checked by Sanger di-deoxy sequencing.

3.10.2 Band Shift Analysis of Tn3 Resolvase with Subsite IIBN.

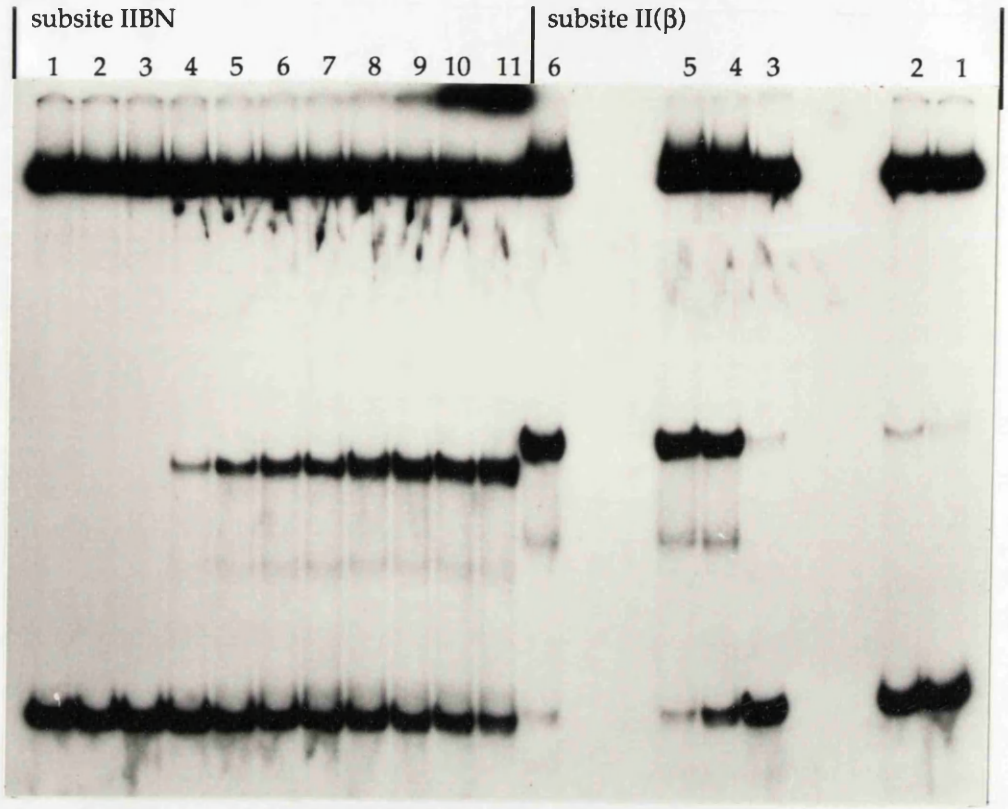
Tn3 resolvase bound less well to subsite IIBN than to subsite II, under TG 9.4 band shift assay conditions. This was not a result of differences in the preparation of the DNA fragments used, as the same effect was observed when the two fragments were mixed in a binding reaction (Fig. 3.30). Tn3 resolvase failed to bind to each of the partial subsites produced, by restriction endonuclease digestion, from subsite IIBN (Fig. 3.30).

Substitution of the A at position 10 left (to G, used to introduce the Bgl II site) has probably had the greatest effect on the binding of resolvase. Substitution of position 10 Right of γδ subsite I was shown to greatly reduce the affinity of the C-terminal domain of γδ resolvase for the subsite[68]. None of the other substitutions introduced have been shown to greatly affect the binding of resolvase, and so the lack of binding of resolvase to the right end fragments of subsite II is still considered to be significant.

3.11 Co-operativity of Resolvase Binding.

A similar degree of binding to subsite II was observed at the highest concentrations of Tn3 resolvase (Fig. 3.13a) and γδ M106C (Fig. 3.26) used (i.e. at a 2^{-4} dilution of each stock solution). However a 2^{-8} dilution of Tn3 resolvase produced very little complex with subsite II, yet with an equivalent dilution of M106C almost 50 % of the subsite II fragment present was in retarded complexes. Even with a 2^{-13} dilution of M106C resolvase some subsite II complex was generated. The absolute concentration of the M106C resolvase stock is unknown, but comparison with Tn3 resolvase (Fig. 3.24) suggests that the Tn3 resolvase

(a)


subsite IIBN
1 2 3 4 5 6 7 8 9 10 11
subsite II(β)
6 5 4 3 2 1

(b)

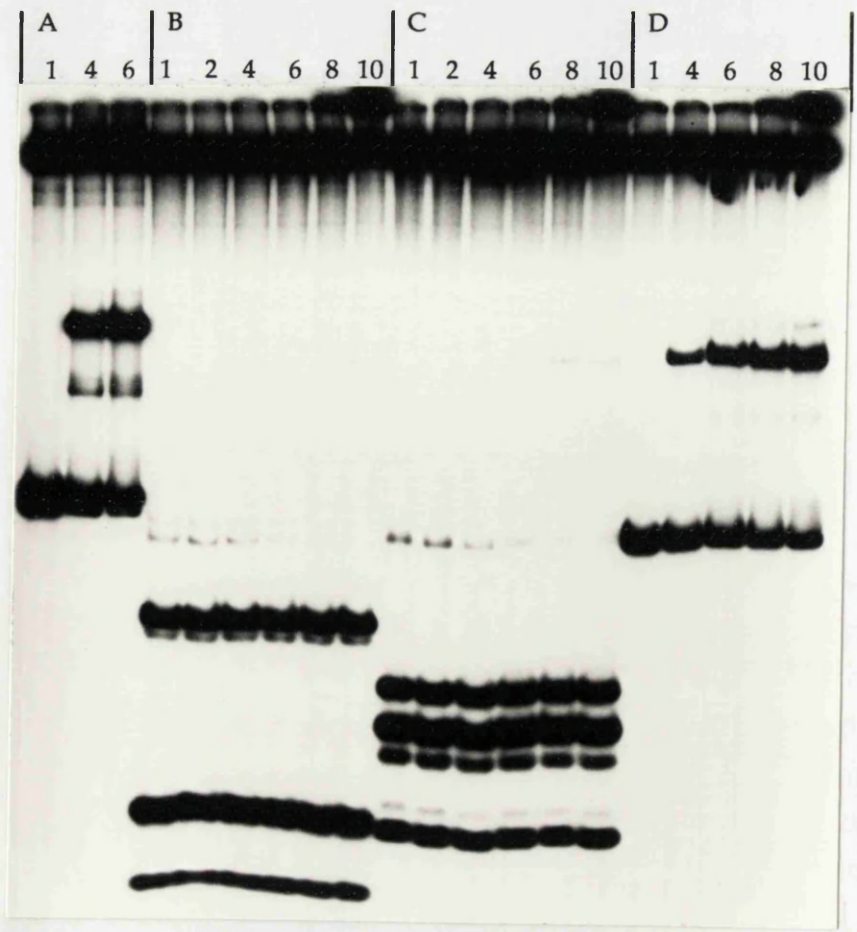

A
1 4 6
B
1 2 4 6 8 10
C
1 2 4 6 8 10
D
1 4 6 8 10

Figure 3.30. Binding of Tn3 Resolvase to Subsite IIBN.

(a) Binding to subsite IIBN.

End-labelled subsite II(β) (pDB2054 *Eco* RI/*Hin* dIII) or subsite IIBN (pDB2201 *Eco* RI/*Hin* dIII) was incubated for 10 min at 37 °C with Tn3 resolvase (dilutions of R17 f49, as indicated) using TG 9.4 conditions.

Lane	Resolvase	Lane	Resolvase
1	R.H.B.	7	2^{-5}
2	2^{-10}	8	2^{-4}
3	2^{-9}	9	2^{-3}
4	2^{-8}	10	2^{-2}
5	2^{-7}	11	2^{-1}
6	2^{-6}		

(b) Binding to Partial Subsites.

End-labelled DNA was incubated as in (a). Lane numbers correspond to resolvase dilutions as for (a). End-labelled DNA fragments were as follows:

A subsite II (pDB2004 *Eco* RI/*Hin* dIII) +
subsite IIBN (pDB2201 *Eco* RI/*Hin* dIII)

B subsite IIBN (pDB2201 *Bam* HI/*Hin* dIII) digested by *Bgl* II

C subsite IIBN (pDB2201 *Bam* HI/*Hin* dIII) digested by *Nsi* I

D subsite IIBN (pDB2201 *Bam* HI/*Hin* dIII)

All resolvase-dependent complexes in tracks containing partial subsites are the result of binding to undigested subsite IIBN fragment.

stock is about 2-fold more concentrated than the M106C stock. Hence the most likely explanation for the observed binding differences is that Tn3 resolvase is binding to subsite II in a co-operative manner. Binding of the M106C dimer (to give complex 2) would be expected to be non-co-operative, unless more than 1 dimer is bound to each subsite.

The amounts of DNA in complex 2 and in the unbound band were measured for each lane containing Tn3 resolvase with subsite II (Fig. 3.13a) and M106C with subsite II (Fig. 3.26). Measurements were made by analysing an autoradiograph of each gel, using a scanning densitometer. The values produced were used to calculate the ratio c2/c0, where c2 is the amount of DNA in complex 2, and c0 is the amount of DNA in the unbound band. A graph of $\log_2(R)$ against $\log_2(c2/c0)$ was plotted for each set of binding reactions (Fig. 3.31), where R is the relative concentration of resolvase used (i.e. the resolvase dilution factor). The densitometer did not detect any complexed material in the 2^{-8} Tn3 resolvase track, but some complex 2 was visible on the autoradiograph. An estimate was made of the upper limit of c2/c0 for this sample, and this point was also plotted. If this point is included, the Tn3 data set gives a line with a gradient of >1, whereas the M106C data give a gradient of about 1. Inclusion of estimated values for M106C samples which were below the sensitivity of the densitometer did not alter the gradient of the line produced.

For a simple binding system in which the protein, P, binds to DNA site, D , to give complex, PD, following the equation

$$P+D \rightleftharpoons PD$$

the dissociation constant, K_D, is defined as;

$$K_D = \frac{[P]\cdot[D]}{[PD]}.$$

When the protein is in large excess it can be assumed that $[P] \approx [P]_0$, where $[P]_0$ is the initial concentration of unbound protein, and so;

$$\frac{[PD]}{[D]} = \frac{[P]}{K_D} \approx \frac{[P]_0}{K_D}$$

If the amount of bound DNA, b, and unbound DNA, u, can be determined (the absolute concentrations are not required) then

$$\left(\frac{b}{u}\right) = \frac{[P]}{K_D} \approx \frac{[P]_0}{K_D},$$

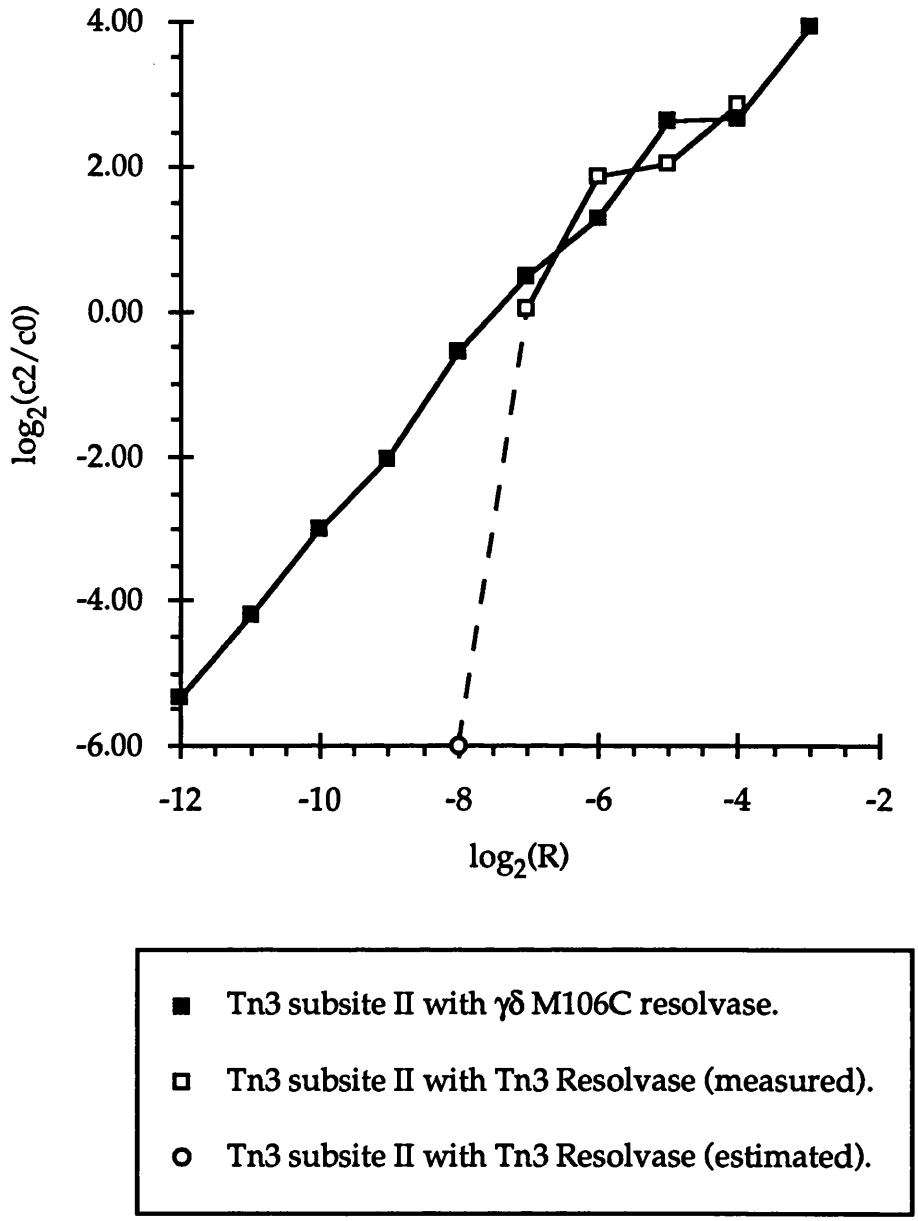


Figure 3.31. Comparison of Tn3 and γδ M106C Resolvases Binding to Subsite II.

The ratio of DNA in complex 2 (c2) to unbound DNA (c0) in each track was calculated from values produced by measuring the intensity of bands on the relevant autoradiographs, using a scanning densitometer. The tracks for which measurements were made are shown in Fig. 3.13 (Tn3 resolvase) and Fig. 3.26 (γδ M106C resolvase). R is the dilution factor of (stock) resolvase added to the binding reaction.

Using a 2^{-8} dilution of Tn3 resolvase (with subsite II) a small amount of complex 2 was observed. However no complex 2 was detected when this track was scanned using the densitometer. An estimated value (upper limit) has been plotted for this track, demonstrating a difference between the two sets of binding reactions.

$$\Rightarrow \log\left(\frac{b}{u}\right) = \log[P] - \log K_D \approx \log[P]_0 - \log K_D$$

Hence a plot of $\log\left(\frac{b}{u}\right)$ against $\log[P]_0$ should give a straight line with an x-axis intercept of $-\log K_D$. For a simple binding mechanism a line with a gradient of 1 is expected (i.e. a two-fold increase in resolvase concentration corresponds to a two-fold increase in the ratio of bound to unbound DNA). A gradient of >1 is expected for co-operative binding.

For a number of reasons these equations can not be applied directly to information produced by scanning autoradiographs of the band shift assays described in this chapter.

(i) Sensitivity of Scanning Densitometer.
The scanning densitometer used does not give accurately values for the faintest or most intense bands observed. This can give misleading results.

(ii) Sensitivity of Autoradiograph Film.
The response of the autoradiograph film is not linear for all activities detected. The faintest and most intense bands produced lie outwith the linear response range of the film. This will compound the problems associated with the sensitivity of the scanning densitometer.

(iii) Detection of Complexes by the Band Shift Analysis Method.
The complexes observed by the band shift assay may not accurately reflect those present in solution. Unstable complexes could be lost during electrophoresis. If such complexes remain intact for the time it takes to enter the gel matrix they may dissociate to give DNA smearing. In this case the amount of unbound material is likely to be accurately detected, but not the total DNA present.

(iv) Presence of Competitor DNA.
Including competitor DNA in binding reactions can alter the actual free resolvase level in each sample, which may not be in vast excess over the target DNA fragment. The actual free resolvase concentration may not change linearly with the concentration of resolvase added when competitor DNA is present.

(v) Differences from Simple Binding Model.
Binding of resolvase, even to subsite II, does not fit the simple equation

$$P+D \rightleftharpoons PD$$

For M106C resolvase, ignoring complexes of the monomeric resolvase (i.e. complex 1; see Chapter 7), more than one complex is produced. Complex 2

is proposed to contain dimer-bound subsite, but the nature of complex 3 is unknown. Hence an equation such as

$$nP_2+D \rightleftharpoons (n-1)P_2+DP_2 \rightleftharpoons DP_{n(2)}$$

where P_2 is a resolvase dimer; n is an unknown number; DP_2 is complex 2 and $DP_{n(2)}$ is complex 3, may better describe this binding reaction. Binding of Tn3 resolvase to subsite II can probably be described by the equation

$$2P+D \rightleftharpoons P+DP \rightleftharpoons DP_2$$

where P is a monomer of resolvase; DP is complex 1 and DP2 is complex 2. It has not been shown conclusively that complex 1 is an intermediate in the formation of complex 2.

If the stoichiometry and the relationship of the complexes formed in these binding reactions is known, then measurements of the amounts of DNA in bands produced can be used to examine the nature of the binding which produces them. Removing carrier DNA from resolvase binding reactions does not assist analysis as, for the conditions used, this can cause aggregation of the complexed material.

Looking at Fig. 3.13a, it is clear for subsite II that the amount of complex 2 produced at low Tn3 resolvase concentrations is more than doubled by a 2-fold increase in the concentration of resolvase added. If the amount of complex 1 present is considered insignificant, and the presence of carrier DNA does not give a non-linear relationship between the actual and assumed free resolvase concentration, then this shows that Tn3 resolvase binding to subsite II to produce complex 2 is co-operative. The contrast to the behaviour of M106C resolvase in the equivalent assay suggests that the non-linear concentration dependence of Tn3 resolvase is unlikely to be an artefact of the binding assay. The data from M106C (e.g. Fig. 3.31) are consistent with non-co-operative binding to subsite II.

Due to the presence of more than 1 major complex in most of the other binding samples produced (and the lack of information regarding the relationship of these complexes) this method of interpretation is unsuitable for the other band shift assays described in this chapter.

3.12 Conclusions.

Band shift assays of the Tn3 *res*/resolvase system gave different patterns of complexes when gel conditions were varied. The same was true for the γδ and Tn21 systems, but this alone did not account for observed differences of the Tn3 pattern from those of the γδ and Tn21 systems. Tn3 resolvase bound to subsite II

sequence when isolated from the rest of *res*, and the insertion of 10 bp or 5 bp of additional DNA at the centre of the isolated subsite II sequence (giving subsite (II + 10) and subsite (II + 5) respectively) did not appear to inhibit binding of Tn3 resolvase to the subsite. However band shift assays of the two altered subsites did produce patterns of complexes different from that of normal subsite II. DNase I footprinting of subsites II, (II + 10) and (II + 5) suggested that resolvase was binding to each end of each subsite in a similar way. Methylation interference experiments, using the same subsites, suggested that the same residues (towards the outer ends of the subsites) were involved in the resolvase/subsite interactions of each subsite.

Bend analysis of subsite II and subsite (II + 10) showed resolvase-induced bending in all complexes observed for both subsites, but it was not evident that the degree of bending was increased in any complex, beyond that observed for complex 1.

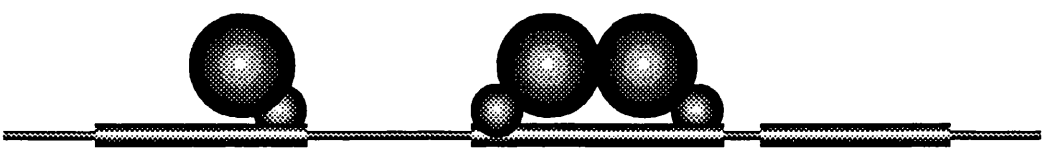
No retarded complexes were observed in band shift assays of Tn3 resolvase with (approximate) halves of a modified subsite II, but this may have been a result of the sequence modifications made, as binding of Tn3 resolvase to the complete element was poorer than to a normal subsite II sequence.

Purified γδ resolvase was shown to bind to Tn3 subsite II and to subsites (II + 10) and (II + 5). A mutant of γδ resolvase, M106C, which consisted mainly of covalently linked resolvase dimers, was also shown to bind to subsites II, (II + 10) and (II + 5). The patterns of complexes produced with γδ resolvase were remarkably similar to those observed with M106C, supporting the beliefs that dimers are the predominant species in γδ resolvase in solution, and that the dimer formed by the covalent linking of two M106C monomers is equivalent to the dimer involved in binding of γδ to the *res* site. The differences in the patterns of complexes produced by binding of γδ resolvase, or of Tn3 resolvase, to subsite II [or (II + 10), or (II + 5)] are consistent with the idea of γδ resolvase being mostly dimeric, and of Tn3 resolvase being predominantly monomeric in this assay.

By the qualitative criteria defined, co-operative binding was observed in the formation of complex 2 of Tn3 resolvase with subsite II.

Chapter Four

Production and Purification of ^{3}H-Resolvase



4.1 Introduction.

Determination of the stoichiometry of *res*/resolvase complexes requires that DNA and protein can each be quantified by distinct, yet compatible means. ^{32}P end-labelling of DNA fragments is a technique commonly used in molecular biology and, as such, was the method of choice for this work. However, the means by which resolvase levels should be evaluated was not so obvious.

Having access to purified resolvase, *in vitro* ^{125}I labelling was an option[27]; however it was possible that the degree of protein modification involved in this technique may have affected the binding activity of the labelled species. Another approach, using purified protein, would have been to raise antibodies against resolvase and use radiolabelled or enzyme-linked antibodies to quantify the amount of protein present[50]. This approach has potential problems due to the circuitous nature of protein quantification by immunoblotting. A third option was to radiolabel resolvase as it was translated, prior to purification. This could be done *in vivo* by adding labelled amino acids to medium which is then used to grow an expression strain[94]. In theory this method is also applicable to *in vitro* translation systems, but the availability of a proven *in vivo* expression strain made this unnecessary.

The choice of isotope for protein tagging was set by two main criteria; compatibility with ^{32}P for dual counting, and availability of affordable modified amino acids. ^{35}S-methionine was available within the department, but ^{35}S and ^{14}C both have decay energy spectra considerably overlapping that of ^{32}P. Quite a large number of tritiated amino acids could also be purchased, and the decay spectra of ^{3}H and ^{32}P can be easily distinguished, if the ^{3}H activity can be kept in excess of ^{32}P activity. For these reasons pilot resolvase expression experiments were conducted using both ^{35}S- and ^{3}H-labelled amino acids.

The proposed approach to resolvase purification was to adapt the large scale resolvase purification method used to prepare the Tn3 resolvase fractions used in Chapter 3 (M. Boocock; pers. comm.). It was necessary to considerably reduce the scale of the procedure in order to produce radiolabelled protein of a high specific activity, without using excessive amounts of isotope in the induction steps. The procedure was expected to be adaptable to lower resolvase levels, as it was previously scaled up during its adaptation from other published methods[46,64]. The large scale protocol had been used successfully on more than one occasion but, although the general features remained constant, no one purification had been reproduced exactly.

4.2 Tn3 Resolvase Expression Vector pMA6111.

The plasmid pMA6111 (Fig. 4.1) was used to express Tn3 resolvase in the *E. coli* strain JM101. This 4.5 kb plasmid was constructed (by M. R. Boocock) by subcloning a 1.3 kb $tnpR^+$ *Eco* RI/*Hin* dIII fragment from pMA498[15] to pKK223-3[14], such that resolvase expression is driven by the IPTG inducible *tac* promoter upstream of the *Eco* RI site.

Over-expression of resolvase from this plasmid (generally by growth in liquid media containing between 0.1 mM and 1 mM IPTG) was associated with very poor growth of the host cells (compared with an identical culture without induction). Where growth rates were not reduced upon IPTG induction it was often found that plasmid rearrangements had occurred, resulting in reduced resolvase expression. The decrease in post-induction growth rates was used as an indirect indicator for the selection of cultures to be used for resolvase expression. This 'induction test' is described in section 4.4.1.

4.3 Pilot Expression of ^{3}H-Resolvase.

Pilot experiments were conducted in order to determine appropriate conditions for the expression of resolvase with sufficient incorporation of the isotope used. The only resolvase expression strain used was JM101 pMA6111, and all inductions used 10 ml cultures. Both ^{35}S (^{35}S-methionine) and ^{3}H (a mix of tritiated arginine, isoleucine, leucine, threonine and valine) were tested as candidate isotopes, using minimal media based on D & M salts, M9 or M63 media (defined in Chapter 2). The effects of varying the point of induction, the point at which isotope was added and the total expression time were investigated.

Levels of resolvase expression were estimated by comparing induced and uninduced samples on Laemmli gels. Incorporation of isotope was checked by isolating bands of interest from such gels, dissolving the polyacrylamide chip (as described in Chapter 2), adding scintillant and then counting the sample. The conditions favoured, as a result of these comparative experiments, were those described in section 4.4.

4.4 Small Scale Resolvase Purification.

Once suitable conditions had been found to produce radiolabelled resolvase, it was necessary to purify the protein on a scale compatible with the levels of isotope which could be used. The growth media (liquid or solid) used throughout the expression protocol for the maintenance and induction of JM101 pMA6111 cells contained :

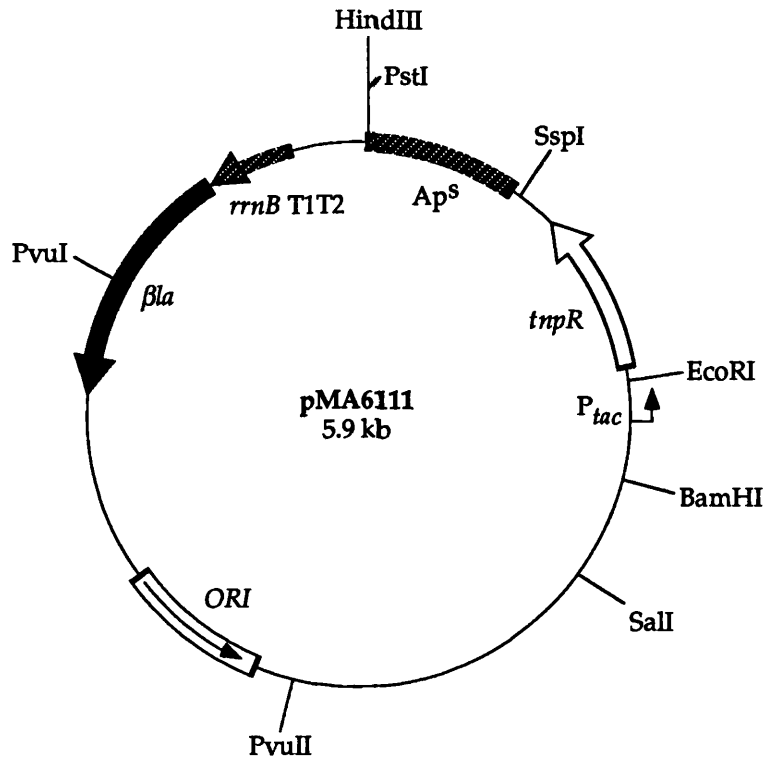


Figure 4.1. Tn3 Resolvase Expression Vector pMA6111.
Resolvase expression from the *tac* promoter is IPTG-inducible, but occurs (at lower levels) in the absence of IPTG. The Tn3 sequences inserted to pKK223-3 include some, but not all, of the ampicillin resistance gene, *βla*, also present in pKK223-3.

1 x Davis and Mingioli minimal salts
0.2 % glucose
5 μg/ml vitamin B_1
100 μg/ml Ap.

For simplicity this is referred to as MA medium during the description of these experiments.

The method of resolvase induction used for all attempts at small scale purifications was similar to that of RD 8 (section 4.4.1). The steps involved in the resolvase expression and purification are outlined in Fig. 4.2 and Fig. 4.3, respectively.

4.4.1 Expression of Resolvase in Induction RD 8.

JM101 pMA6111 was streaked from -70 °C stocks on to MA plates and incubated at 37 °C for 2 days. Colonies from these plates were streaked onto fresh MA plates and grown (37 °C) for another two days. Individual colonies were used to inoculate 4 x 10 ml aliquots of MA broth (one for each isolate of JM101 pMA6111). These cultures were grown overnight at 37 °C with aeration (by shaking). In the tables of optical density values (Tables 4.1 and 4.2) italics are used to indicate the sample used for the resolvase induction.

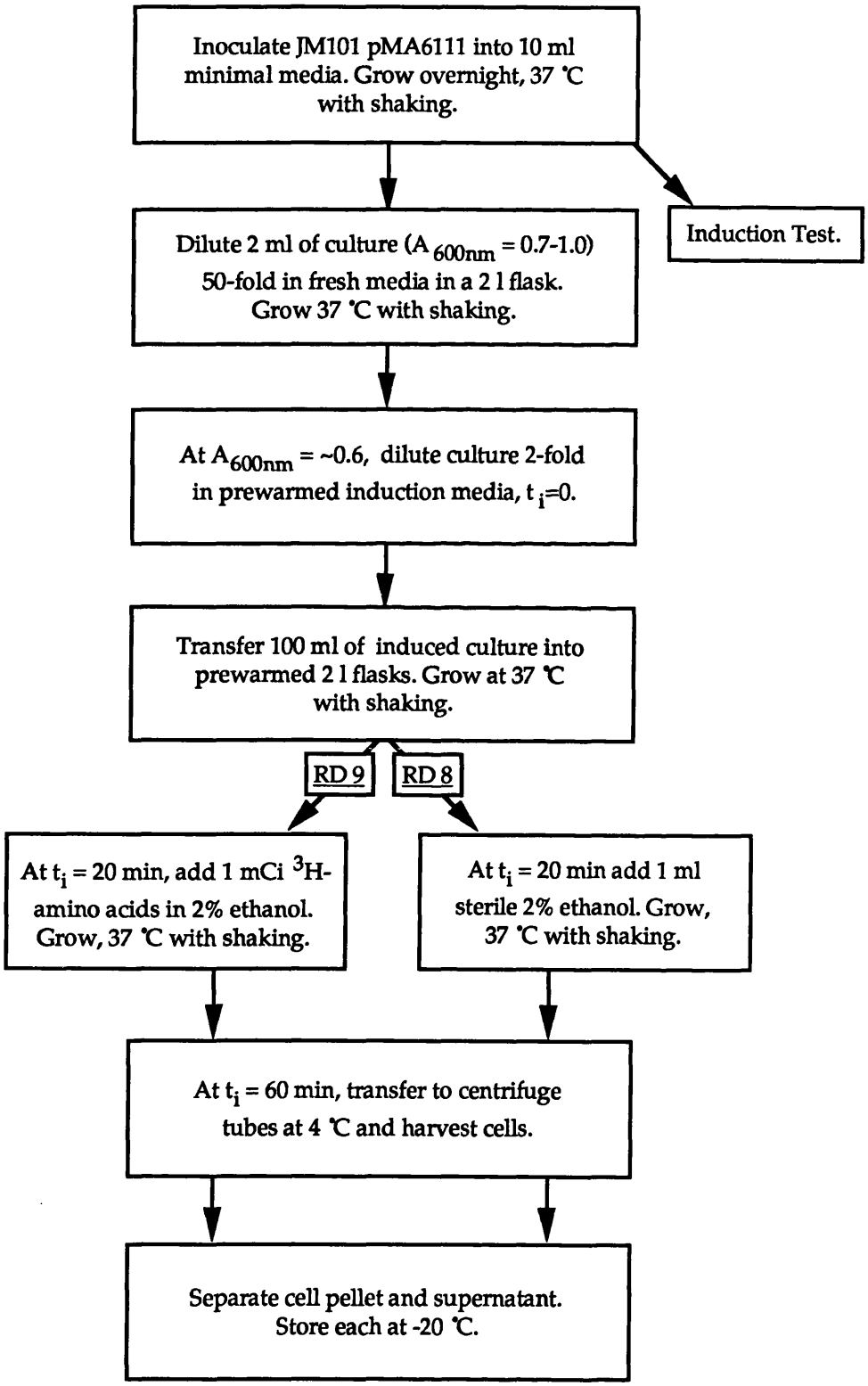
Isolate	A_{600nm}
JM101 pMA6111 #1a	0.672
JM101 pMA6111 #1b	0.750
JM101 pMA6111 #3a	*0.702*
JM101 pMA6111 #3b	0.758

Table 4.1 Optical Density of Overnight JM101 pMA6111 Cultures for RD 8.

The A_{600nm} of the cultures was measured (Table 4.1) and 2 ml of each sample was diluted 50 fold in 100 ml of MA broth and then grown with shaking at 37 °C in a 2 l flask. At the same time 200 μl of each sample was diluted 50 fold into 10 ml MA broth and into 10 ml MA broth containing 0.2 mM IPTG. Both dilutions were grown at 37 °C with shaking in 20 ml boiling tubes. This was to check that the isolates of JM101 pMA6111 grew well in the MA broth, indicating few plasmid-free cells, but grew very poorly in the presence of IPTG, as previously observed with high levels of resolvase expression from pMA6111. These 'induction tests' were grown for 6 h and then compared by eye.

The desired point of induction was at an A_{600nm} of around 0.6; therefore the

(a)


Inoculate JM101 pMA6111 into 10 ml minimal media. Grow overnight, 37 °C with shaking.
Induction Test.
Dilute 2 ml of culture (A_{600nm} = 0.7-1.0) 50-fold in fresh media in a 2 l flask. Grow 37 °C with shaking.
At A_{600nm} = ~0.6, dilute culture 2-fold in prewarmed induction media, t_i=0.
Transfer 100 ml of induced culture into prewarmed 2 l flasks. Grow at 37 °C with shaking.
RD 9
RD 8
At t_i = 20 min, add 1 mCi ^{3}H-amino acids in 2% ethanol. Grow, 37 °C with shaking.
At t_i = 20 min add 1 ml sterile 2% ethanol. Grow, 37 °C with shaking.
At t_i = 60 min, transfer to centrifuge tubes at 4 °C and harvest cells.
Separate cell pellet and supernatant. Store each at -20 °C.

(b)

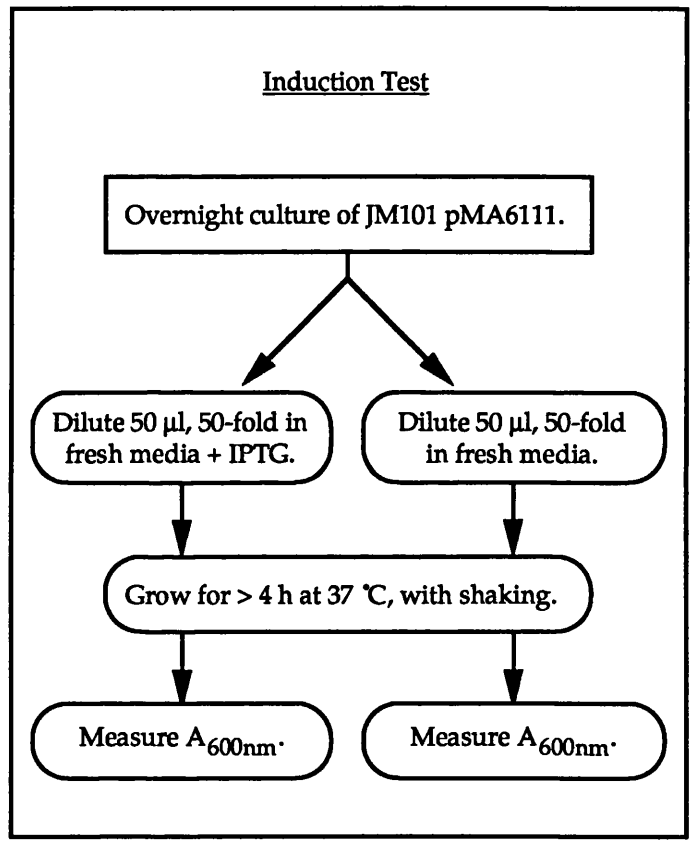


Figure 4.2 **Summary of Resolvase Expression in Induction RD 8 and RD 9.**

(a) RD 8 and RD 9 Induction.
The control for RD 9 (RD 10) was produced by the method shown for RD 8.

(b) The 'Induction Test'.

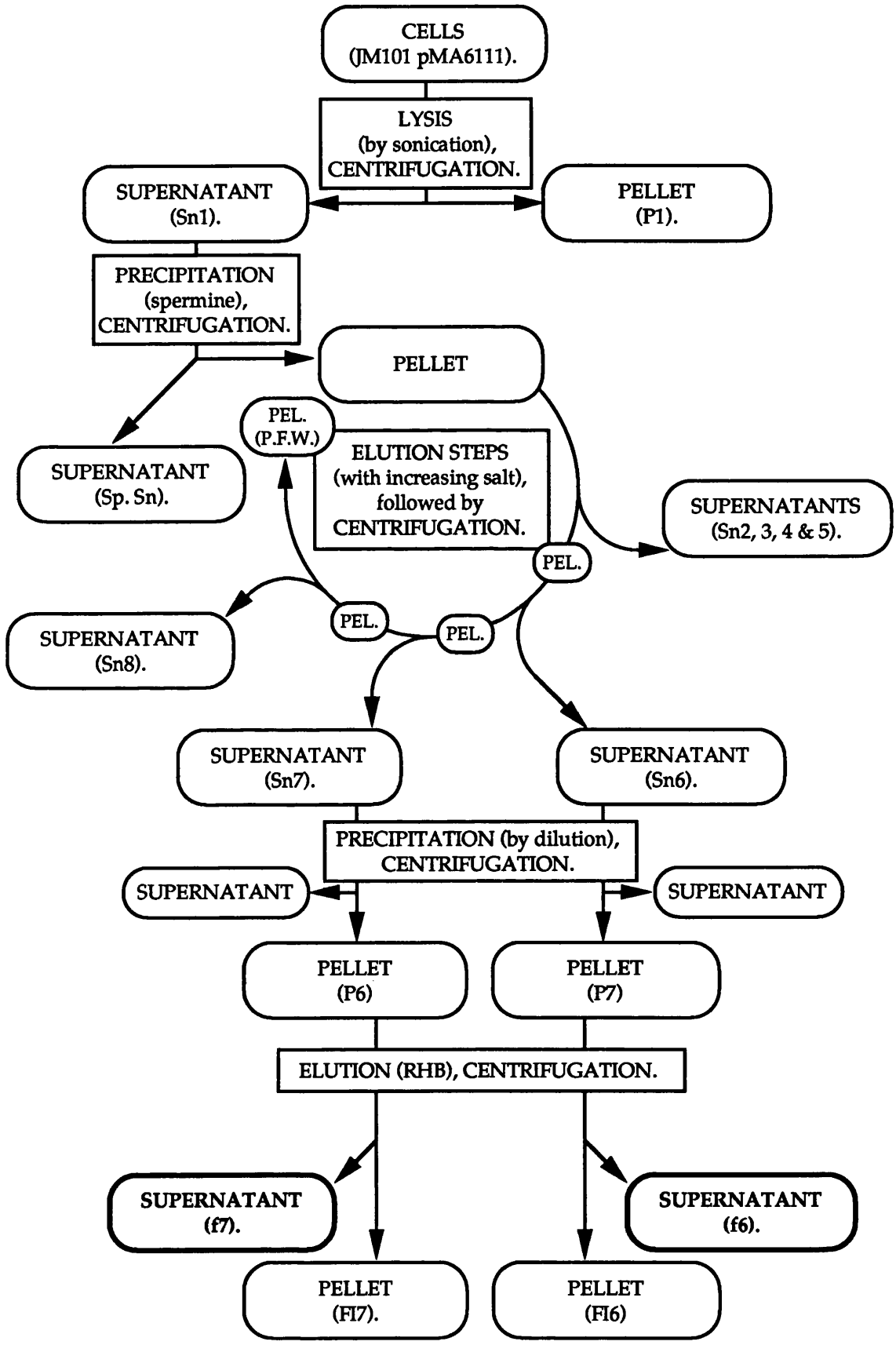


Figure 4.3 Outline of Small Scale Resolvase Purification.
Sample names are as defined in section 4.4. and 4.5. 'PELLET' was abbreviated to 'PEL.' where space was limited.

A_{600nm} of the main cultures was measured during their growth.

Time of Growth	A_{600nm} of :			
	JM101 pMA6111 #1a	JM101 pMA6111 #1b	*JM101 pMA6111 #3a*	JM101 pMA6111 #3b
4 h 20 min	0.188	0.179	*0.191*	0.177
6 h 25 min	0.484	0.460	*0.559*	0.477
6 h 55 min	0.549	0.531	*0.653*	0.550

Table 4.2 Optical Density of JM101 pMA6111 Cultures for Resolvase Induction RD 8.

Sample JM101 pMA6111 #3a was selected for the resolvase induction on the basis of the induction test and the densities of the main cultures. 6 h 55 min after the initial dilution, 50 ml of JM101 pMA6111 #3a was mixed with an equal volume of prewarmed MA broth containing 0.3 mM IPTG. The diluted culture was then incubated at 37 °C with shaking. This induction point was defined as $t_i = 0$ min.

The ^3H amino acids to be used for the tritiated induction were supplied in sterile 2% ethanol, and so some sterilised 2% ethanol was warmed to 37 °C, then at t_i = 20 min, 1 ml of 2% ethanol was added to the resolvase induction culture, RD 8. This was returned to the shaker and continued incubation at 37 °C. At $t_i = 60$ min the sample was transferred to a chilled 250 ml centrifuge tube and the cells were harvested by centrifugation (3,840 g for 3 min at 4 °C). The supernatant was decanted to a separate tube and the supernatant and cell pellet were stored overnight at -20 °C.

4.4.2 Purification of Resolvase from Induction RD 8.

The important features of the purification are that the resolvase remains soluble after the cells are lysed, that the resolvase co-precipitates with the DNA on addition of spermine, and that the resolvase can eventually be eluted (with little contamination) from this pellet. Two factors which appear to affect these features are the concentration of cell components in the cell lysate (due to the volume in which the cells are broken) and the approximate fragment size of the DNA present (in turn affected by the method of cell lysis).

In the large scale protocol the cells are broken by three passes on a French Press, resulting in significant shearing of chromosomal DNA. The small scale purification required the cells to be lysed in much too small a volume to allow use of the French Press. In experiments where cells were broken in an ultrasonic waterbath, or by lysozyme and repeated freeze/thaw treatment, the resolvase

was precipitated on centrifugation of the lysate. This was presumed to be the result of insufficient shearing of the DNA present (as the approximate DNA size, examined by agarose gel electrophoresis, was greater than that observed in directly sonicated samples). Treatment of such a lysate with further mechanical shearing steps did not produce soluble resolvase. Cell lysis by direct sonication of the sample did produce soluble resolvase. A number of tests were required to produce a balance of sample size, volume of lysis buffer and total sonication time which could reproducibly result in good cell lysis (without the vial breaking), and resolvase eluting at the desired salt concentration (without excessive contamination). The correct spermine concentration for precipitation, and then elution of resolvase, was another important consideration.

Solutions and Buffers :

1x KPM :	25 mM KH_2PO_4 pH 7.0, 5 mM $MgCl_2$, 1 mM EDTA, 0.4 mM DTT, 1 mM benzamidine.
RHB :	2 M NaCl, 20 mM Tris.HCl pH 7.5, 0.1 mM EDTA, 0.2 mM DTT.
PMSF	10 mg/ml in ethanol.
spermine	20 mM in H_2O.

The induced cells were thawed on ice, washed in 50 ml of 50 mM KH_2PO_4 (pH 7.0) and pelleted by centrifugation (12,100 g for 1 min at 4 °C). The cell pellet was resuspended in 2 ml of 1x KPM with 20 µl of PMSF, and then transferred to a glass sonication vial which had been stored on ice. A 20 µl sample of RD 8 cells was taken at this point. The remaining material was placed on ice and set up for sonication as shown in Fig. 4.4. The cells were broken by 6 x 10 sec bursts of sonication, separated by breaks of 30 sec to prevent overheating. After every second sonication step 20 µl of PMSF was added. Once sonication was completed a 20 µl sample was removed and the remaining lysate was transferred to a small centrifugation tube. At this point the lysate appeared almost iridescent, indicating good cell lysis.

The protein content of 1 µl of supernatant from the sonicated and unsonicated samples was compared using Bradford's reagent. The supernatant from the unbroken cells contained almost no protein (compared by eye with B.S.A. standards at 0, 1, 2, 3, 4 and 5 mg/ml) but the lysate supernatant gave a reading of about 4-5 mg/ml of protein. The sonicated material was spun at 50,400 g for 10 min at 4 °C to remove cell debris, and any intact cells. The supernatant was then split between two 1.5 ml Eppendorf tubes and spun, at 14,000 rpm, in a microfuge for a further 3 min (4 °C). The supernatant produced, Sn 1, was mixed

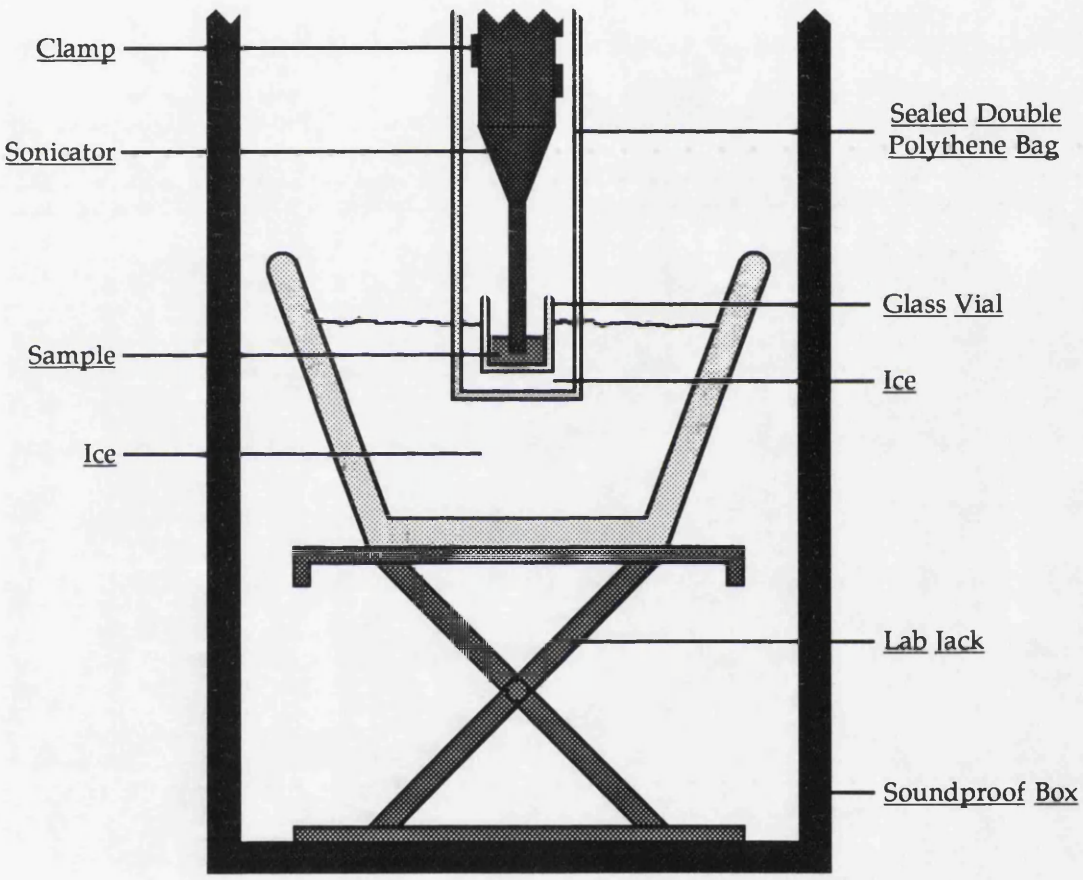


Figure 4.4 Arrangement of Samples for Sonication.

with 20 µl of PMSF and a 20 µl sample was removed and stored on ice. Sn 1 was placed in a small, chilled glass bottle and 4 x 5 µl of 20 mM spermine were added, with mixing, to precipitate the nucleic acid (and some other material, including resolvase). The sample was incubated on ice for 5 min and then transferred to Eppendorf tubes. The precipitated material was collected by spinning in a microfuge (14,000 rpm, 4 °C) for 2 min. The supernatant (Sp. Sn) was removed and stored on ice. The two pellets were washed for 5 min in a total of 400 µl of 1x KPM, 1 mM spermine and 0.2 mg/ml PMSF. In this and subsequent steps, washing consisted of adding the wash buffer, breaking the pellet by pipetting and vortexing for 1 min, 3 min on ice, and then 1 min further pipetting, to 5 min total. The sample was then centrifuged for 2 min (14,000 rpm, 4 °C) and the supernatant, Sn 2 was removed and stored. Washing and centrifugation steps were repeated with the following buffers, producing the supernatants indicated :

1x KPM/1 mM spermine/0.2 mg/ml PMSF	+ 50 mM NaCl	(Sn 3)
1x KPM/1 mM spermine/0.2 mg/ml PMSF	+ 100 mM NaCl	(Sn 4)
1x KPM/1 mM spermine/0.2 mg/ml PMSF	+ 200 mM NaCl	(Sn 5)
1x KPM/1 mM spermine/0.2 mg/ml PMSF	+ 400 mM NaCl	(Sn 6)
1x KPM/1 mM spermine/0.2 mg/ml PMSF	+ 1 M NaCl	(Sn 7)
1x KPM/1 mM spermine/0.2 mg/ml PMSF	+ 2 M NaCl	(Sn 8)

The pellet remaining after the 2 M NaCl wash step was stored as 'P.F.W.'.

The bulk of the resolvase should elute in Sn 7, but it was previously observed that the resolvase sometimes eluted at slightly lower salt concentrations. Therefore Sn 6 and Sn 7 were each diluted 6 fold with H_2O (after removing a 40 µl sample from each). The dilutions were placed on ice for 1 h to precipitate any resolvase present, and then spun in a microfuge at 14,000 rpm, 4 °C for 20 min. The supernatants, SD 6a and SD 7a, were removed and the pellets, P6 and P7, were washed in $\frac{1}{6}$ x [1x KPM/ 1 M NaCl/ 1 mM spermine/ 0.2 mg/ml PMSF] by 2 min of gentle pipetting followed by 5 min on ice. The samples were spun in a microfuge for 10 min (14,000 rpm, 4 °C) and the supernatants, SD 6b and SD 7b, removed. The pellet produced from Sn 6 was small, but Sn 7 produced a large, white pellet. Each pellet was resuspended in 25 µl of RHB by gently pipetting the samples, then returning them to ice, for a total of 20 min. Insoluble material was removed by centrifugation in a microfuge (14,000 rpm, 5 min, 4 °C).

In the next stage of the large scale purification the resolvase-containing fraction is

further purified using a size filtration column. However the potential loss of material expected with a smaller sample made such a filtration step impractical at this point.

The two final fractions, RD 8 f6 (from Sn 6) and RD 8 f7 (from Sn 7) were transferred to small Eppendorf tubes and mixed with an equal volume of glycerol. The insoluble pellets, FI 6 and FI 7, were stored on ice, along with samples from all other stages, with the exception of RD 8 f6 and RD 8 f7 which were stored at -20 °C. Samples from most steps were run on a Laemmli gel to check for the presence and purification of resolvase throughout (Fig. 4.5).

This gel shows that the pellet produced from the high speed spin (Fig. 4.5, lane 4) contained very little resolvase (but most of a second induced species). The resolvase had mostly purified in the expected fraction, i.e. Sn 7 and then RD 8 f7, and had only small amounts of contaminating material (c.f. R17 f48; Fig. 4.5, lane 1).

The binding activity of RD 8 f7 was tested by binding to a subsite II fragment, and was comparable with that of a purified fraction, R17 f47, from a large scale resolvase preparation (data not shown). The recombination activity of RD 8 f7 was also comparable to that of R17 f47, as tested by *in vitro* resolution of the substrate pMA21 (data not shown).

4.5 Expression of ^{3}H-Resolvase in Induction RD 9.

Overnight cultures of JM101 pMA6111 were produced from -70 °C stocks as described for RD 8. Italics are used in Tables 4.3, 4.4 and 4.5, to indicate the culture used for the resolvase induction.

Isolate	A_{600nm}
JM101 pMA6111 #1a	*0.854*
JM101 pMA6111 #1b	0.748
JM101 pMA6111 #3a	0.898
JM101 pMA6111 #3b	0.742

Table 4.3 Optical Density of Overnight JM101 pMA6111 Cultures for RD 9.

The A_{600nm} of the stationary cultures was measured (Table 4.3) and each culture was diluted 50 fold in 100 ml of MA broth which was grown with shaking at 37 °C in a 2 l flask. 'Induction tests' were prepared as for RD 8 and grown for 5 h 50 min, then A_{600nm} values were measured for each sample (Table 4.4).

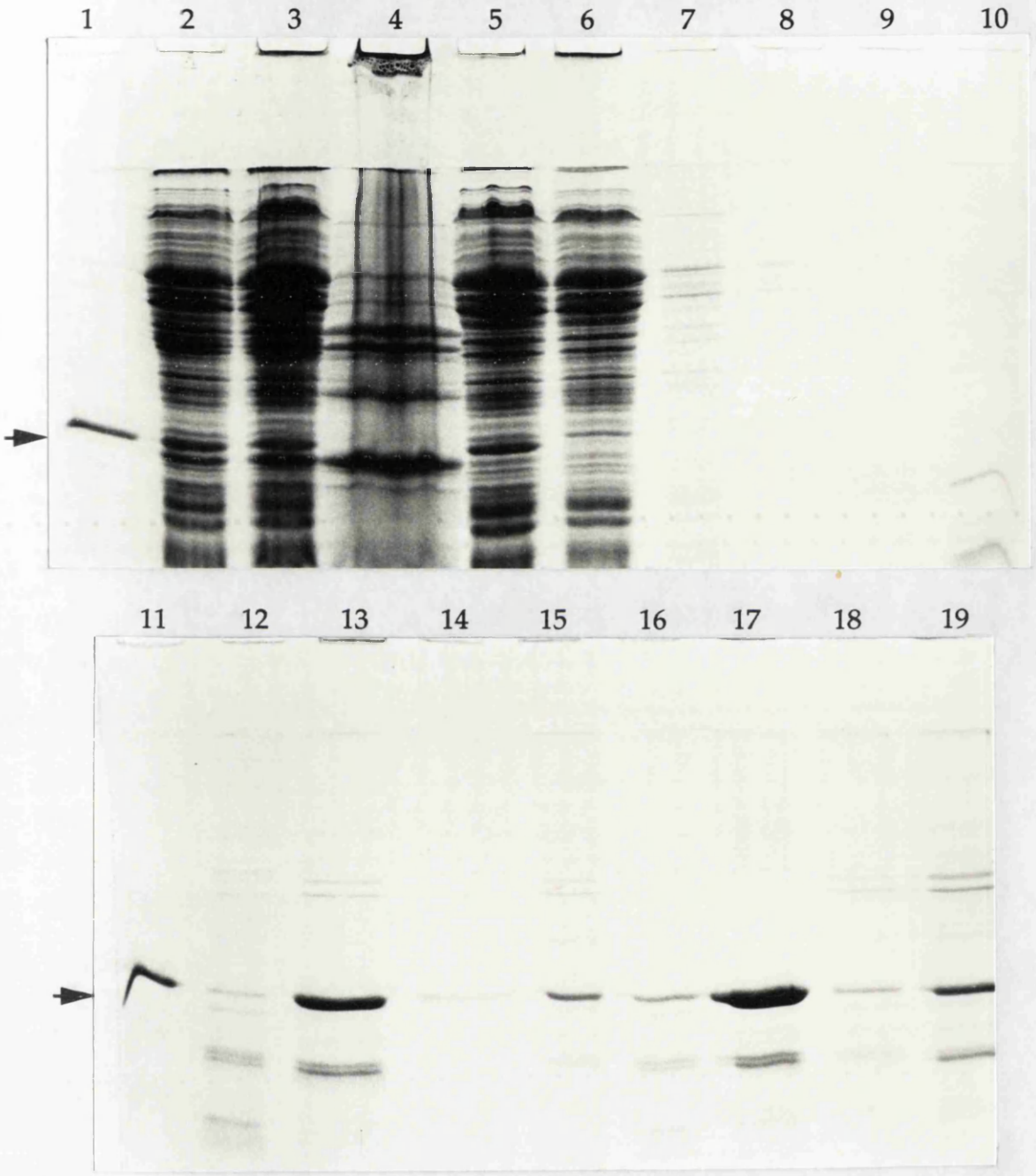


Figure 4.5. Small Scale Resolvase Purification RD 8.
Resolvase is marked by an arrow.

Lane	Sample	% run	Lane	Sample	% run
1	1 µl R17f48		11	1 µl R17f48	
2	induced cells	0.5	12	supernatant 6	2.5
3	sonicated cells	0.5	13	supernatant 7	2.5
4	pellet P1	1.0	14	supernatant 8	1.2
5	supernatant 1	0.5	15	pellet P.F.W.	2.5
6	spermine sn.	0.5	16	RD 8 f6	10.0
7	supernatant 2	2.5	17	RD 8 f7	10.0
8	supernatant 3	2.5	18	pellet FI6	20.0
9	supernatant 4	2.5	19	pellet FI7	20.0
10	supernatant 5	2.5			

Isolate	A_{600nm} of uninduced	A_{600nm} of induced
JM101 pMA6111 #1a	*0.583*	*0.042*
JM101 pMA6111 #1b	0.513	0.047
JM101 pMA6111 #3a	0.734	0.049
JM101 pMA6111 #3b	0.483	0.050

Table 4.4 Optical Density of RD 9 'Induction Test' Samples.

As for RD 8, the A_{600nm} of the main cultures was measured during their growth (Table 4.5).

Time of Growth	A_{600nm} of :			
	JM101 pMA6111 #1a	JM101 pMA6111 #1b	JM101 pMA6111 #3a	JM101 pMA6111 #3b
4 h 00 min	*0.186*	0.167	0.257	0.152
5 h 50 min	*0.519*	0.466	0.770	0.426
6 h 15 min	*0.588*	-	-	-

Table 4.5 Optical Density of JM101 pMA6111 Cultures for Resolvase Induction RD 9.

Sample JM101 pMA6111 #1a was selected for the resolvase induction on the basis of the induction test and the density of the main cultures. 6 h 15 min after the initial dilution JM101 pMA6111 #1a was mixed with an equal volume (100 ml) of prewarmed MA broth containing 0.3 mM IPTG. The diluted culture was split between two prewarmed 2 l flasks which were then incubated at 37 °C with shaking. This induction point was defined as t_i = 0 min.

^{3}H-Amino Acid	Specific Activity (Ci/mmol)	Amount Added (nmol)
L-[2,3-^{3}H]-arginine	58.4	3.42
L-[4,5-^{3}H(N)]-isoleucine	111.7	1.79
L-[3,4,5-^{3}H(N)]-leucine	158.0	1.27
L-[4,5-^{3}H(N)]-lysine	97.4	2.05
L-[3,4-^{3}H]-valine	65.0	3.08

Table 4.6 Specific Activities of Tritiated Amino Acids Used in Resolvase Induction RD 9.

200 µl each of 5 tritiated amino acids (arginine, isoleucine, leucine, lysine, and valine, all at 1 µCi/µl in 2% ethanol) were mixed together and warmed to 37 °C.

The specific activities of the tritiated amino acids are shown in Table 4.6. At t_i = 20 min the 1 ml (1 mCi) of tritiated amino acids was added to one of the induced cultures, labelled RD 9, which was then returned to the shaker and continued incubation at 37 ℃. Immediately after this, 1 ml of 2% ethanol was added to the control culture, RD 10 which was also returned to the shaking platform. At t_i = 60 min each sample was transferred to a cold 250 ml centrifuge tube and the cells were harvested by centrifugation (50,400 g for 3 min at 4 ℃). The supernatants were decanted to separate tubes and the supernatants and cell pellets were stored overnight at -20 ℃.

4.6 Purification of ^{3}H-Resolvase from Induction RD 9.

The purification of resolvase from RD 9 was conducted exactly as described for RD 8 with the following exceptions :

(i) Sonication;
Cells were set up for sonication as shown in Fig. 4.4, and sonicated as for RD 8. However once sonication was completed the lysate did not appear iridescent, suggesting incomplete cell breakage. The protein content of 1 µl of supernatant from the sonicated and unsonicated samples was also compared using Bradford's reagent. The supernatant from the unbroken cells did contain almost no protein (compared by eye with B.S.A. standards at 0, 1, 2, 3, 4 and 5 mg/ml) but the lysate supernatant gave a reading of about 2-3 mg/ml of protein (c.f. 4-5 mg/ml in RD 8). It was believed that sonicating the sample too much may have been as detrimental to the purification as insufficient sonication. Therefore additional steps were not taken.

(ii) Low salt precipitation;
The pellet produced from Sn 6 was pale yellow, but the pellet from Sn 7 was barely visible.

The two final fractions produced, RD 9 f6 (from Sn 6) and RD 9 f7 (from Sn 7) were equivalent to RD 8 f6 and RD 8 f7, respectively. Again material from most steps was run on a Laemmli gel to check for the presence and purification of resolvase (Fig. 4.6).

This Laemmli gel shows that the pellet produced from the high speed spin (Fig. 4.6, lane 4) contained substantial levels of resolvase, compared with, for example, RD 8. The amount of resolvase present, compared with that in the supernatant (lane 5), gives some indication of the proportion of cells that were not broken. As

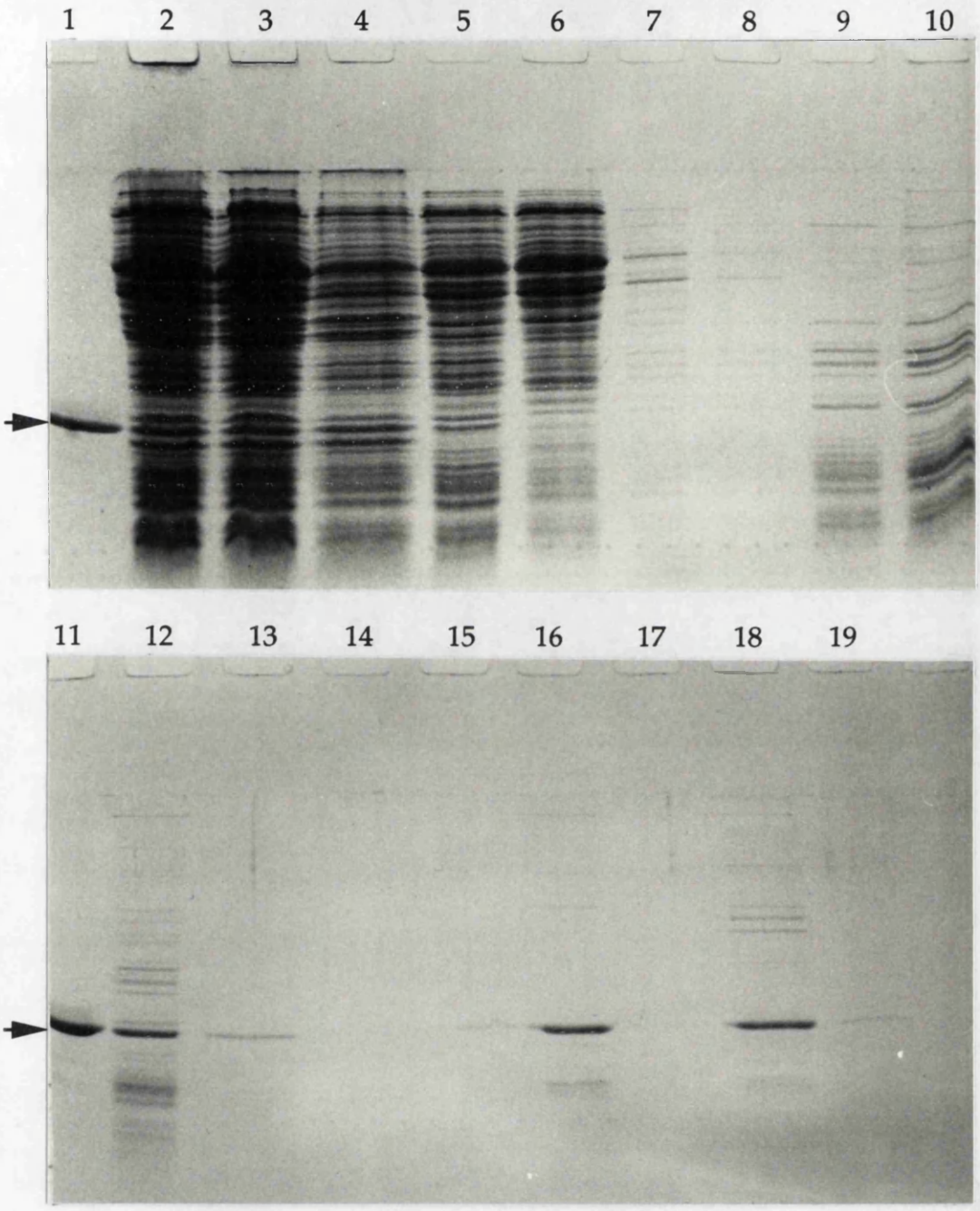


Figure 4.6. Small Scale ^{3}H-Resolvase Purification RD 9.
Resolvase is marked by an arrow.

Lane	Sample	% run	Lane	Sample	% run
1	1 µl R17f48		11	1 µl R17f48	
2	induced cells	0.5	12	supernatant 6	2.5
3	sonicated cells	0.5	13	supernatant 7	2.5
4	pellet P1	0.5	14	supernatant 8	1.2
5	supernatant 1	0.5	15	pellet P.F.W.	1.2
6	spermine sn.	0.5	16	RD 9 f6	10.0
7	supernatant 2	2.5	17	RD 9 f7	10.0
8	supernatant 3	2.5	18	pellet FI6	10.0
9	supernatant 4	2.5	19	pellet FI7	10.0
10	supernatant 5	2.5			

a result of this the resolvase did not purify in the expected fractions. This may be a result of a lower concentrations of total cellular material, from Sn 1 onwards. Although Sn 7 (lane 13) gave a very poor resolvase yield, the resolvase present in Sn 6 (lane 12) and then RD 9 f6 (lane 16) proved sufficient for the experiments planned.

The main problem in the sonication of such a small volume of cells is ensuring that the button probe makes good contact with the cell suspension but does not touch the glass vial, which is easily cracked. This becomes somewhat harder when everything must be contained to prevent the aerosol of tritiated material from escaping; i.e. the containment during cell lysis probably reduced the ability to fine tune the apparatus, resulting in poor contact between the probe and the sample during some steps of sonication.

The binding activity of RD 9 f6 was tested by the execution of the experiments for which it was produced. These are described in Chapter 5. The resolution activity of RD 9 f6 was comparable to that of R 17 f47 (data not shown).

4.7 Determination of Resolvase Concentration of RD 9 f6.

The concentration of resolvase in fraction 6 of RD 9 was determined by amino acid analysis of a hydrolysed sample, after OPA derivatisation (Chapter 2). This method of analysis was believed to be considerably more accurate than others which quantify protein levels using Bradford's reagent, Coomassie stain or direct spectrophotometric analysis, especially given the small amount of material available. Immunological methods alone could not provide a direct measure of resolvase concentration. Many thanks are due to Dr D. Mousdale and Ms E. O'Neil, of Bioflux Ltd, who conducted derivatisation and analysis of the samples described in this section.

Before RD 9 f6 was analysed, fractions from other resolvase stocks were analysed to estimate the amount of RD 9 f6, and the ratio of internal standard (taurine) that should be used in the determination of the composition of RD 9 f6. The samples used for this purpose were from RD 8 f7 (section 4.4.2), and from a fraction of a large scale preparation, R 17 f47.

In using this method of analysis (or the others mentioned in this section) the assumption is made that the resolvase samples are free of any contaminating material, e.g. other polypeptides. Whilst this is unlikely to be entirely true of any protein purified from an *in vivo* expression system, denaturing PAGE of R 17 f47 indicated that resolvase accounts for the vast majority of the total material

present. The comparative manner used to convert the results of amino acid analysis to a resolvase concentration also helped to reduce the influence of any contaminating material on the final value produced.

As described in Chapter 2, a known volume of the resolvase sample was mixed with a known concentration of the internal standard, taurine, prior to hydrolysis. This material was lyophilised and resuspended several times and finally resuspended in a small, known volume. The dilution factor of the stock resolvase sample is calculated by:

$$\text{Resolvase Dilution Factor} = \frac{\text{Vol. Resolvase Added } (\mu l)}{\text{Final Sample Volume } (\mu l)}$$

If no loss of material (or inaccuracy in volume measurement) occurs, the concentration of taurine expected in the analysis is:

$$\text{'Expected' [tau] } (\mu M) = \frac{\text{Amount of Taurine Added (pmol)}}{\text{Final Sample Volume } (\mu l)}$$

Example :

In the analysis of R 17 f47, a 5 µl sample of R 17 f47 was prepared for analysis. 4 µl of 1 mM taurine (4000 pmol) was included as an internal control. The hydrolysed material was finally resuspended in 100 µl of solvent.

$$\text{Resolvase Dilution Factor} = \frac{5\ \mu l}{100\ \mu l} = 20.$$

$$\text{Taurine concentration} = 40\ \mu M.$$

Derivatised material was analysed and the area of the peak produced for each residue was calculated. By comparison with peaks produced from derivatised standards, these areas were converted to concentration values using the following formula:

$$\text{Observed } [\alpha.\alpha.] = \left[\frac{(\text{peak area of } \alpha.\alpha.)\cdot([\text{standard}])}{(\text{peak area of standard})}\right]$$

The taurine internal standard was used to correct amino acid concentrations, taking account of loss of material during hydrolysis, as follows:

$$\text{Corrected } [\alpha.\alpha.] = \left(\frac{(\text{Expected [Tau]})\cdot(\text{Observed } [\alpha.\alpha.])}{(\text{Observed [Tau]})}\right)$$

If the assumption is made that resolvase is the only polypeptide present in the sample analysed, then a resolvase concentration value can be calculated from the

concentration of each amino acid by:

$$\text{Stock}\,[\text{Tn3 TnpR}] = \left(\frac{(\text{Corrected}\,[\alpha.\alpha.]) \cdot (\text{Resolvase Dilution Factor})}{\text{No. of Residues in Tn3 TnpR}} \right)$$

This assumption is clearly not true (e.g. Fig. 4.5 and Fig. 4.6). However, as resolvase is the major species present in all the samples examined, considering only the residues which occur frequently in Tn3 resolvase should give the best estimation of the actual resolvase concentration. Additionally some of the contaminating species are believed to be derived from intact resolvase and, if complementary fragments are represented equally, will have the same specific activity as the active resolvase.

The results of amino acid analysis of R 17 f47, RD 8 f7 and RD 9 f6 are given in Table 4.7, 4.8 and 4.9 respectively. Residues are listed in the order in which they were detected, with the exception of taurine. The 'Corrected [α.α.]' value for taurine is also the 'Expected [tau]'. Two sets of data, A and B, are listed in Table 4.9. These were produced by parallel analysis of two RD 9 f6 samples, allowing an average resolvase concentration to be determined.

Amino Acid	Observed [α.α.] (μM)	Corrected [α.α.] (μM)	Residues in Tn3 TnpR	[R17 f47] (μM)
Asx	8.77	9.13	19	9.61
Glx	0.72	0.75	21	0.72
Ser	15.49	16.13	11	29.32
His	3.57	3.71	2	37.13
Gly	58.87	61.30	15	81.73
Thr	14.34	14.93	12	24.88
Arg	*19.76*	*20.57*	*20*	*20.57*
Ala	*11.93*	*12.43*	*16*	*15.53*
Tyr	1.64	1.71	2	17.12
Met	0.00	0.00	5	0.00
Val	*15.64*	*16.29*	*14*	*23.27*
Phe	5.49	5.72	5	22.87
Ile	*17.25*	*17.96*	*15*	*23.95*
Leu	*16.20*	*16.87*	*16*	*21.09*
Lys	*9.17*	*9.55*	*12*	*15.92*
Tau	38.41	40.00	-	-

Table 4.7 Amino Acid Composition of R 17 f47.

Amino Acid	Observed [α.α.] (μM)	Corrected [α.α.] (μM)	Residues in Tn3 TnpR	[RD 8 f7] (μM)
Asx	26.57	34.77	19	36.60
Glx	9.02	11.80	21	11.24
Ser	55.00	71.98	11	130.87
His	12.74	16.67	2	166.71
Gly	178.21	233.20	15	310.93
Thr	49.40	64.65	12	107.74
Arg	*45.85*	*60.00*	*20*	*60.00*
Ala	*36.00*	*47.10*	*16*	*58.88*
Tyr	2.98	3.90	2	38.96
Met	5.12	6.70	5	26.81
Val	*34.08*	*44.60*	*14*	*63.71*
Phe	16.47	21.55	5	86.21
Ile	*37.52*	*49.10*	*15*	*65.47*
Leu	*38.69*	*50.63*	*16*	*63.29*
Lys	*26.84*	*35.12*	*12*	*58.54*
Tau	15.28	20.00	-	-

Table 4.8 Amino Acid Composition of RD 8 f7.

Amino Acid	Observed [α.α.] (μM)		Corrected [α.α.] (μM)		Residues in Tn3 TnpR	[RD 9 f6] (μM)	
	A	B	A	B		A	B
Asx	19.89	14.97	24.72	20.99	19	13.01	11.05
Glx	28.60	8.61	35.54	12.07	21	16.93	5.75
Ser	36.96	25.49	45.94	35.76	11	41.77	32.51
His	25.97	16.23	32.28	22.77	2	161.42	113.83
Gly	99.12	80.33	123.21	112.69	15	82.14	75.13
Thr	25.93	18.71	32.23	26.25	12	26.86	21.88
Arg	*14.73*	*14.38*	*18.30*	*20.18*	*20*	*9.15*	*10.09*
Ala	*12.58*	*14.29*	*15.64*	*20.04*	*16*	*9.77*	*12.53*
Tyr	4.68	4.99	5.81	7.00	2	29.07	34.98
Met	3.78	3.10	4.70	4.35	5	9.40	8.70
Val	25.27	31.06	31.41	43.58	14	22.44	31.13
Phe	7.37	9.48	9.16	13.29	5	18.33	26.58
Ile	*31.34*	*28.13*	*38.96*	*39.47*	*15*	*25.97*	*26.31*
Leu	*34.68*	*29.76*	*43.11*	*41.75*	*16*	*26.95*	*26.10*
Lys	*20.99*	*18.47*	*26.10*	*25.92*	*12*	*21.75*	*21.60*
Tau	32.18	28.51	40.00	40.00	-	-	-

Table 4.9 Amino Acid Composition of RD 9 f6.

In Table 4.7, 4.8 and 4.9 italics indicate the samples which were used to calculate

the concentration of resolvase for each fraction. The resolvase concentration results are listed in Table 4.10.

The residues used in the determination of the resolvase concentration were selected on using the following criteria;

(i) Occurrence in Tn3 Resolvase.

As mentioned, results derived from frequently occurring residues should be affected less by minor contaminating species.

(ii) Stability.

Certain residues have been empirically determined to be more reliable due to an appropriate stability during hydrolysis. An 'appropriate' stability requires that, for the hydrolysis conditions used, all peptide bonds present will be broken, but that the amino acid itself should remain intact.

(iii) Peak Separation.

The separation of material on the column is directly reflected by the distribution of the peaks produced on the trace. The most accurate values will be produced from large, discrete peaks.

Because of these criteria, the resolvase concentrations of R 17 f47 and RD 8 f7 in Table 4.10 are an average of the values produced for arginine, alanine, valine, isoleucine, leucine and lysine. A sample of Tn21 resolvase was also analysed (data not shown) and good agreement of concentration values was observed with the same six amino acids. The value for RD 9 f6 (Table 4.10) does not include valine results due to the discrepancy between samples A and B. The data used to calculate a [Tn3 TnpR] value for RD 9 f6 showed greater variation than that of the other fractions examined. This may be a result of greater contamination in RD 9 f6, but there is no indication of which values, if any, are incorrect.

Purified Fraction	[Tn3 TnpR] (μM)
R 17 f47	20.06
RD 8 f7	61.65
RD 9 f6	19.02

Table 4.10 Resolvase Concentration of R 17 f47, RD 8 f7 and RD 9 f6.

Dilutions of RD 8 f7 (and R 17 f47) and an undiluted sample of RD 9 f6 were run on a Laemmli gel to allow comparison of their protein content (Fig. 4.7). The concentrations produced by amino acid analysis were compatible with the ratios of material on the gel, although the value for R 17 f47 appeared a little lower than

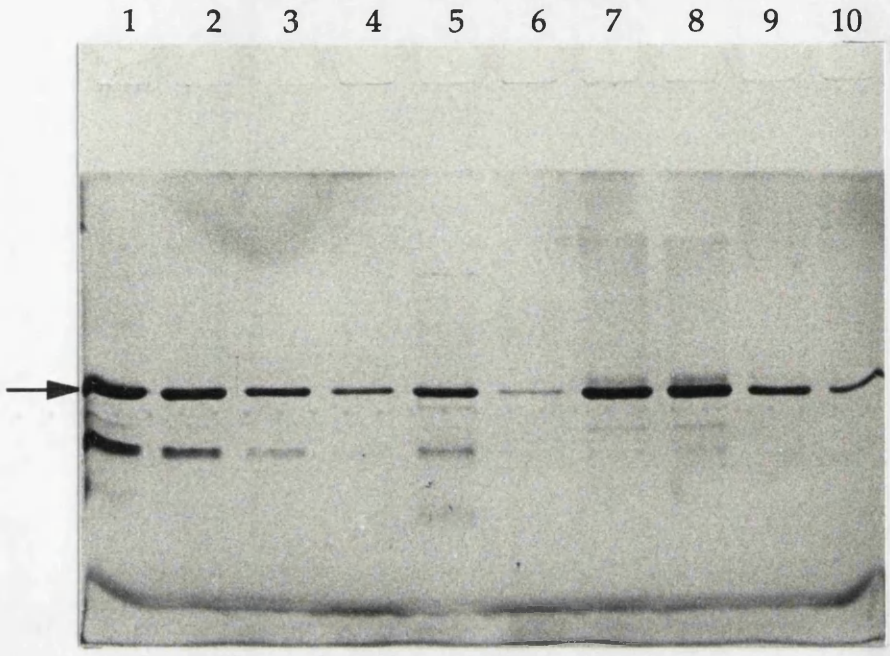


Figure 4.7. Comparison of Some Purified Tn3 Resolvase Fractions.
1 µl samples of a number of resolvase fractions were run on a Laemmli gel to allow comparison of the resolvase concentration of each. Dilutions were run in an attempt to produce tracks containing approximately equal amounts of resolvase. Resolvase is marked by an arrow.

Lane	Fraction	Dilution
1	RD 8 f7	neat
2	"	2^{-1}
3	"	2^{-2}
4	"	2^{-3}
5	RD 9 f6	neat
6	RD 8 f7	2^{-4}
7	R17 f48	2^{-3}
8	R17 f47	neat
9	"	2^{-1}
10	"	2^{-2}

the comparison suggested. This may have been due to the greater purity of R 17 f47.

4.8 Determination of Resolvase Specific Activity in RD 9 f6.

Given the concentration of resolvase in RD 9 f6, the specific activity, $A_{Sp.}$, can be calculated, once the volume-specific activity, A_V, of the resolvase is determined. The measurement and adjustment of RD 9 f6 activity was similar to that of stock DNA samples and isolated complexes, described in more detail in Chapter 5.

A known volume of RD 9 f6 (by weight) was placed in a scintillation vial with 10 ml of 'Ecoscint' fluid and 1 ml of 30% H_2O_2 (similar conditions to those in which activities of complexes were measured). The activity of the sample was counted (10 min, using a Wallac 1409/11 v1.4 liquid scintillation counter). The measured ^3H activity, ^3H A_X (counted at time T_X), was recorded in counts per minute (cpm) and converted (using a Wallac ^3H-spectra library and quenching data) to a measurement of disintegrations per minute (dpm).

^3H A_X (cpm) = 22668.5 cpm	^3H A_X (dpm) = 61878.4 dpm
T_X = 06:50, 22/11/92	Counting Efficiency = 36.6 %

The average ^3H A_X (dpm) value of a number of 'blank' samples was subtracted from the RD 9 f6 ^3H A_X (dpm).

'Blank' adjusted ^3H A_X = (61878.4 - 22.7) dpm = 61855.7 dpm

Finally this activity was adjusted (for decay) to a nominated counting date, T_0 (00:00, 1st October 1992), giving ^3H A_0 (dpm). If $\Delta T = T_X - T_0$, and the half life of the isotope is t, then ^3H A_0 is calculated by;

$$A_0 = A_X.2^{(\Delta T/t)}$$

i.e. ΔT = 52.28 days t = 4492.58 days

RD 9 f6 ^3H A_0 = (61855.7).[$2^{(52.28/4492.58)}$] = 62356.7 dpm

It was assumed that only the 50% glycerol in RD 9 f6 would significantly alter its density from that of H_2O. As glycerol has a density of 1.26, and H_2O has a density of 1.00, the density of RD 9 f6 was estimated to be 1.13. The RD 9 f6 sample counted weighed 0.76 mg and so the sample volume was (0.76/1.13)µl. Hence the volume-specific activity, A_V, of RD 9 f6 is:

$$A_V = \left[\frac{62356.7 \text{ dpm}}{(0.76/1.13)\ \mu\text{l}}\right] = 92714.5 \text{ dpm/µl}$$

The specific activity, $A_{Sp.}$, of the resolvase in RD 9 f6 is calculated using the formula;

$$A_{Sp.} = \left(\frac{A_V \text{ (dpm/µl)}}{\text{[Resolvase] (µM)}} \right) \text{ dpm/pmol}$$

i.e. $$\text{RD 9 f6 } A_{Sp.} = \left(\frac{92714.5 \text{ dpm/µl}}{19.02 \text{ µM}} \right) = 4874.4 \text{ dpm/pmol}$$

4.9 Sources of Error.

A number of small, inherent errors exist in the determination of the specific activity of resolvase in RD 9 f6. Accuracy of volume measurement, statistical errors in scintillation counting and accuracy of quenching and background adjustments are all factors to consider. These are discussed further in section 5.3. Comparison of data sets A and B in Table 4.9 shows that a significant source of error exists in the amino acid analysis itself, but this is difficult to quantify. However, the major errors in this value arise from the question of the purity of RD 9 f6.

Elsewhere in this work the terms 'RD 9 f6' and 'resolvase' have been used somewhat interchangeably, and this would be acceptable given the assumption that all the amino acid (and ^3H) content of RD 9 f6 is in the form of resolvase. Analysis of samples run on a Laemmli gel (Fig. 4.7) shows that this is untrue for R 17 f47 and RD 8 f7, as well as RD 9 f6. Whilst matters would have been improved if RD 9 f6 were as pure as R 17 f47 (or even RD 8 f7), the same problems would still require consideration.

In order to determine how much of the ^3H activity of RD 9 f6 was accounted for by resolvase, a sample of RD 9 f6 was run on a gel, and a gel chip containing the resolvase was isolated. The remainder of the lane containing RD 9 f6 was sectioned into equally sized gel chips. Each sample was dissolved, mixed with 'Ecoscint' fluid and then scintillation counted. The resolvase sample accounted for greater than 90% of the total ^3H activity detected.

The extent to which contaminating protein has affected the resolvase concentration value calculated for RD 9 f6 is less obvious. The small amounts of material available made quantification difficult. It is possible to estimate the relative intensities of bands on a Laemmli gel, but this would only introduce further inaccuracies due to methods of measurement and from unequal staining of different species. By deriving the resolvase concentration of RD 9 f6 from stable amino acids which are most abundant in resolvase (and give compatible values for a number of resolvase samples) the effect of contaminants will have been minimised, or even eliminated.

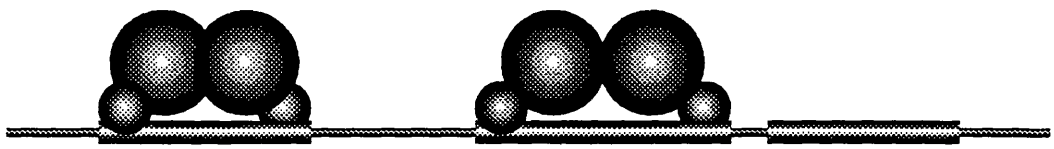
The resolvase specific activity of RD 9 f6 was not adjusted to compensate for the presence of contaminating protein as the effect of this material could not be quantified. However, the consequences of the errors mentioned here should be borne in mind when considering the absolute stoichiometry data (Chapter 5). Despite these reservations, this method of determination of resolvase concentration (this chapter), and the method used to quantify levels of resolvase (Chapters 4 and 5), are still believed preferable to the alternatives discussed.

4.10 Conclusions.

A method was developed for the production of small amounts of partially purified, tritiated resolvase from the expression strain JM101 pMA6111. The resolvase produced had a specific activity of 4874.4 dpm/µl, and exhibited the expected binding and resolution activities, although it was not as pure as other resolvase fractions previously produced using the same protocol. The concentrations, binding activities and resolution activities determined for different resolvase fractions (R 17 f47, RD 8 f7 and RD 9 f6) were consistent with the calculated resolvase concentration of RD 9 f6. The small scale resolvase preparation described will also be of use in the analysis of mutants of resolvase, as a simpler alternative to large scale purification.

Chapter Five

Stoichiometry of Complexes of Resolvase with *res*



5.1 Introduction.

The observation of discrete protein/DNA complexes by non-denaturing PAGE[49] is particularly useful when trying to dissect the resolvase resolution reaction into its component steps. However problems can arise when comparing altered DNA sites and subsites, or modified proteins, according to the pattern of complexes they produce. Evidence is required to show which different complexes, if any, can be considered equivalent, particularly in the case of *res*/resolvase, where so many complexes result from the action of one protein on a single site. Additionally, the investigation of the kinetics of protein/DNA interactions using such an assay[20] can be better understood if the nature of the complexes is known.

The band shift assay is a widely exploited technique (e.g.[6,7,17,49,58,60,83,84]) but there are comparatively few systems for which the stoichiometry of the retarded complexes has been determined (e.g.[16,20,22,27,50,94]). Despite the small number of examples a variety of techniques have been used in the determination of the protein component of complexes produced, and the choice of a method for protein quantification is discussed in Chapter 4. However in almost all cases, including this work, ^{32}P end-labelling was the favoured method for measuring the amount of DNA present. This technique was an obvious choice due to its familiarity, the availability of the reagents required and the properties of the ^{32}P isotope itself.

5.2 Preparation of DNA Samples for Stoichiometry Determinations.

The DNA fragments to be used in stoichiometric determinations were produced by restriction endonuclease digestion of plasmids pDB22, pDB2004, pDB2107 and pDB2507. The *res* site or *res* derivative present in each construct (described in more detail in Chapter 3) is as follows;

pDB22	Tn3 *res* site.
pDB2004	Tn3 subsite II.
pDB2107	Tn3 subsite II derivative, 'subsite (II + 10)', with an additional 10 bp inserted at the centre of subsite II.
pDB2507	Tn3 subsite II derivative, 'subsite (II + 5)', with an additional 5 bp inserted at the centre of subsite II.

The fragments used for binding are represented in Fig. 5.1 and the steps involved in DNA preparation and quantification are summarised in Fig. 5.2. An overview of the operations used to convert raw data to final stoichiometry values is shown in Fig. 5.3.

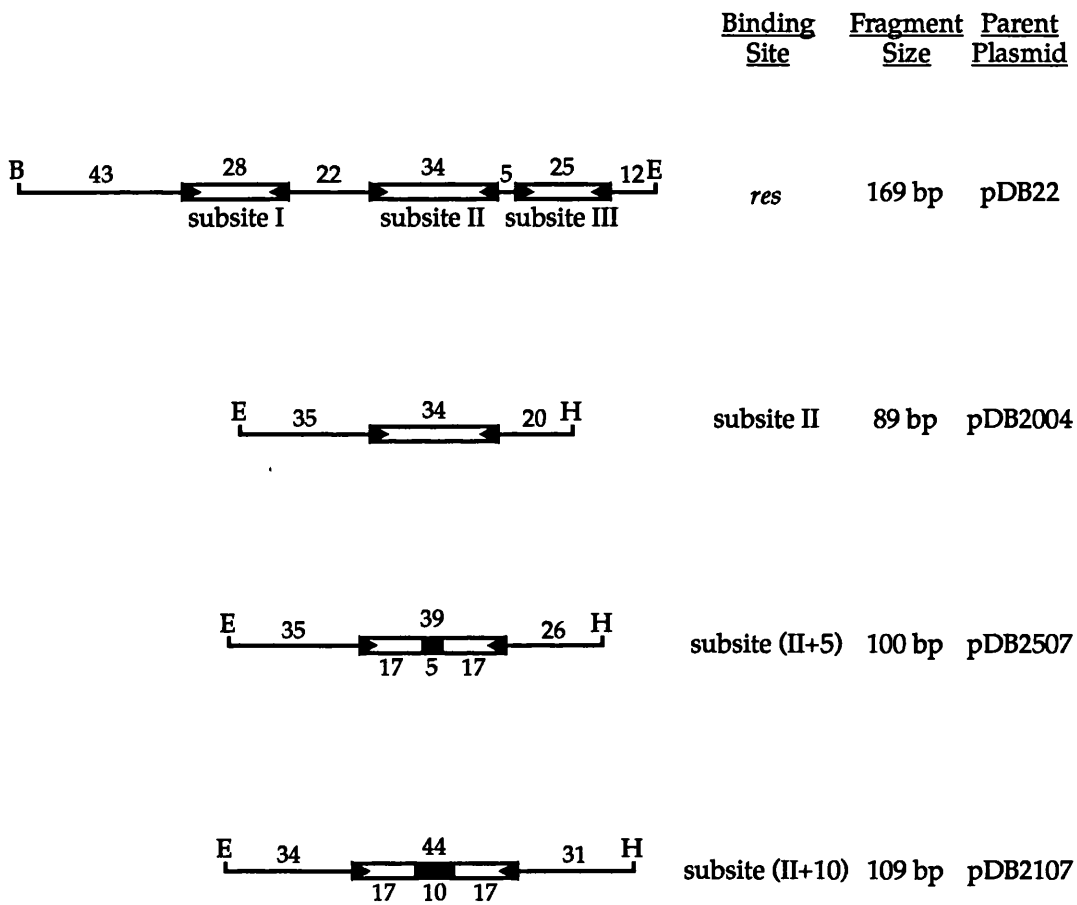


Figure 5.1 DNA Fragments Used in Stoichiometry Experiments.
Abbreviations of restriction enzyme sites; E=*Eco* RI, H=*Hin* dIII and B=*Bam* HI.

39 : sizes, in base pairs, of sites and inter-site distances.
▭ : binding site.
▶ : terminal inverted repeat.
■ : additional inserted sequence.
——— : other DNA.

Initially the approximate DNA concentration was determined for a large scale plasmid preparation of each construct to be used. This was done by measuring the A_{260nm} of a dilution of each sample, in order to determine the amount of DNA to be digested. About 100 µg of each plasmid (55 - 57 pmol) was digested with an excess of *Eco* RI (Enz. 1 in Fig. 5.2) which cuts each plasmid once to give full length linear product. After digestion each sample was extracted once with phenol and twice with chloroform prior to ethanol precipitation. The DNA pellets were dried, then resuspended in 50 µl of 1x TE. A fraction of this stock material was diluted in 1x TE in order to measure its absorbance at 260 nm (and 280 nm), allowing accurate determination of the DNA concentration. The assumption was made that the density of the DNA stocks and of 1x TE were effectively 1, and that the most accurate means of measuring the amounts of each added was by weighing them on a fine balance. 5 µl of each plasmid stock was transferred (using a Hamilton syringe) to a weighed Eppendorf tube and the weight was recorded. About 1 ml of 1x TE was added and the weight was noted again. The samples were then mixed and transferred to a 1 cm quartz cuvette for determination of the A_{260nm} and A_{280nm} values against a blank of 1x TE.

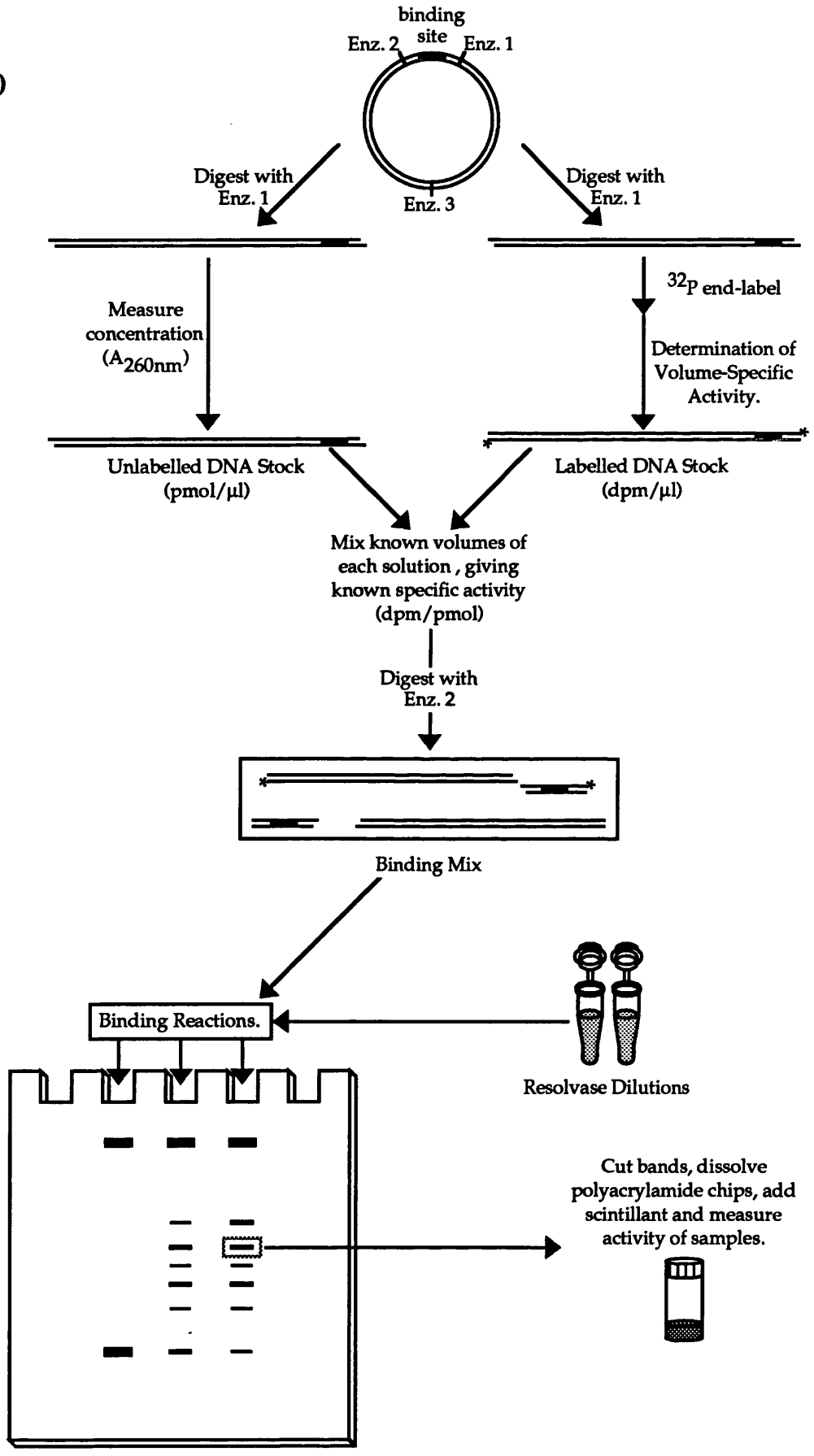
Plasmid	Size (bp)	Stock A_{260nm}	A_{260nm} / A_{280nm}	Cold [DNA] (µM)
pDB22	2,833	38.38	1.78	1.04
pDB2004	2,724	34.22	1.78	0.97
pDB2107	2,744	31.83	1.88	0.89
pDB2507	2,735	36.24	1.87	1.02

Table 5.1 **Concentration of Unlabelled Digested Plasmid Stock.** Concentration values for each linearised plasmid stock ("Cold [DNA]") were derived from the A_{260nm} value using an average molar extinction coefficient, ε, of 13,000 l/mol/cm per base pair. More accurate coefficients for A/T or G/C pairs within a 'typical' sequence were not found in the literature. However the base composition and sequence distribution of each plasmid was quite even and so the average value is acceptable.

The value for A_{260nm}/A_{280nm} should be 1.8 for double stranded DNA[73] and the observed values indicate that the samples are not significantly contaminated with RNA, dNTP's, protein or phenol, all of which would absorb at 260 nm and result in the observed DNA concentration being artificially high.

DNA from the same plasmid preparations was also ^{32}P end-labelled by digesting about 1 µg of each plasmid (about 0.5 pmol) with *Eco* RI and end-labelling using

(a)


binding site
Enz. 2
Enz. 1
Enz. 3
Digest with Enz. 1
Digest with Enz. 1
Measure concentration (A_{260nm})
^{32}P end-label
Determination of Volume-Specific Activity.
Unlabelled DNA Stock (pmol/µl)
Labelled DNA Stock (dpm/µl)
Mix known volumes of each solution, giving known specific activity (dpm/pmol)
Digest with Enz. 2
Binding Mix
Binding Reactions.
Resolvase Dilutions
Cut bands, dissolve polyacrylamide chips, add scintillant and measure activity of samples.

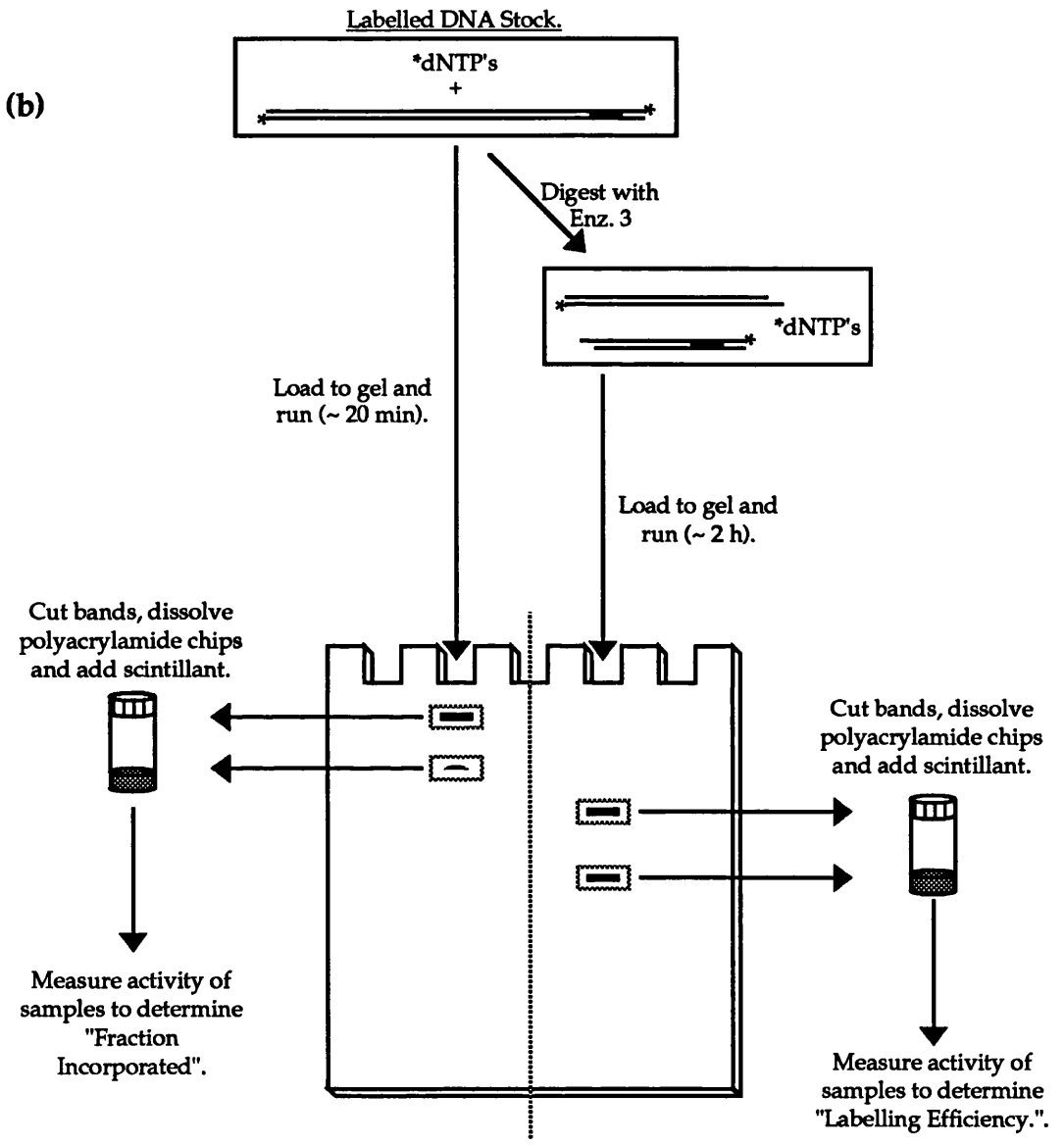


Figure 5.2 Preparation and Quantification of Samples in Stoichiometry Experiments.

(a) Producing the Complexes.

(b) Adjustment of DNA Volume-Specific Activities.

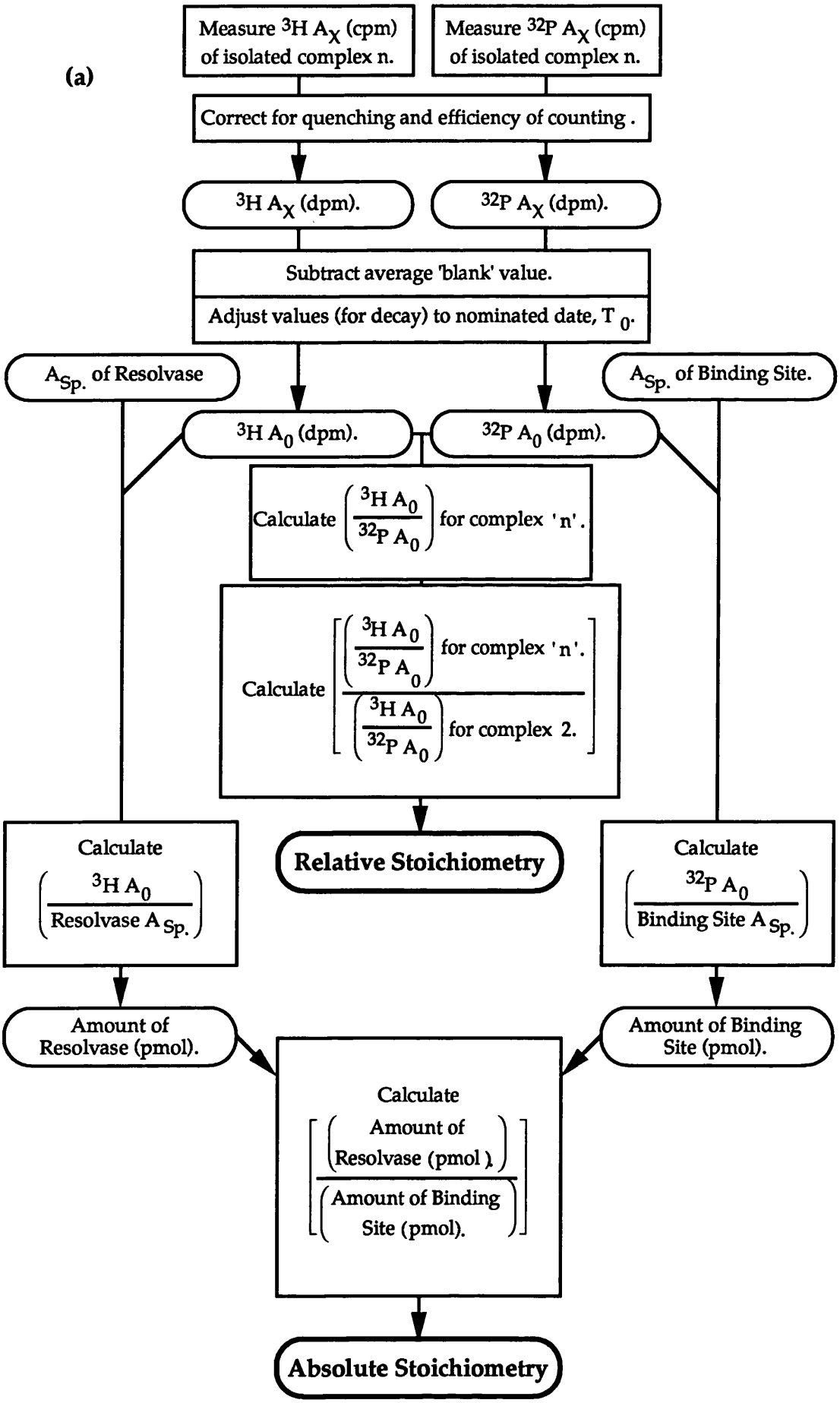
'*' is used to denote radioactive material and 'Enz.' (for enzyme) is used to indicate restriction endonucleases or their cut sites. For all plasmids used, Enz. 1= *Eco* RI and Enz. 3=*Sca* I. For pDB2004, pDB2107 and pDB2507, Enz 2=*Hin* dIII but for pDB22 Enz. 2=*Bam* HI.

the Klenow fragment of DNA polymerase I as described in Chapter 2. The labelled DNA was purified by subsequent extraction with phenol and then chloroform, ethanol precipitated and resuspended in 50 µl of 1x TE.

Dilutions were made from each of the labelled samples (10-fold serial dilutions, to 10^{-5}) and about 10 µl of each (volume measured accurately by weight) was added to 10 ml of scintillation fluid, mixed and counted to determine the volume-specific activity, A_V, of the labelled material, measured as counts per minute per microlitre (cpm/µl). In order to adjust this value with regard to the DNA fragment to be bound, some of each labelled sample was run on a polyacrylamide gel with and without further digestion by *Sca* I (Enz. 3 in Fig. 5.2). The bands were visualised by autoradiography and cut from the gel. The gel chips were dissolved in 1 ml of 30% H_2O_2, then mixed with 10 ml of scintillant and counted. The two bands cut from the *Eco* RI treated samples were full length linear plasmid and unincorporated dNTP's. These were measured to show the proportion of the activity present which was incorporated into the plasmid DNA. The *Eco* RI and *Sca* I digested material also gave two bands, of approximately 1.7 and 1.0 kb (the unincorporated material was run off the gel). The smaller of these two fragments contained the resolvase binding site in each case. These bands were scintillation counted to determine any difference in the efficiency of labelling at each end of the DNA.

Next, labelled and unlabelled linear material was mixed for each plasmid, the amount of each added being measured by weight. The mixed samples were digested with a second restriction endonuclease which would cut at only one position to release the binding site on a small DNA fragment (< 200 bp), leaving the majority of the plasmid intact. For pDB22 the enzyme used was *Bam* HI, for the other three plasmids it was *Hin* dIII (these are Enz. 2 in Fig. 5.2). After digestion the DNA was extracted and precipitated as described previously. The pellets were dried and resuspended in 2x TG 9.4 binding buffer. The amount of DNA added from the unlabelled stock was known, as was the activity (cpm and dpm) added from the labelled sample, and so the specific activity of the mixed material can be calculated in cpm/mol of binding site if the DNA contribution from the labelled sample is assumed to be insignificant in calculating the total DNA present. Approximate ratios of unlabelled to labelled plasmid ranged from a 'cold DNA' excess of at least 320 fold (pDB22) to a 'cold DNA' excess of at least 3000 fold (pDB2507).

(a)


Measure ^{3}H A_X (cpm) of isolated complex n.
Measure ^{32}P A_X (cpm) of isolated complex n.
Correct for quenching and efficiency of counting .
^{3}H A_X (dpm).
^{32}P A_X (dpm).
Subtract average 'blank' value.
Adjust values (for decay) to nominated date, T_0.
$A_{Sp.}$ of Resolvase
$A_{Sp.}$ of Binding Site.
^{3}H A_0 (dpm).
^{32}P A_0 (dpm).
Calculate $\left(\frac{^{3}H\ A_0}{^{32}P\ A_0}\right)$ for complex 'n'.
Calculate $\left[\frac{\left(\frac{^{3}H\ A_0}{^{32}P\ A_0}\right) \text{ for complex 'n'.}}{\left(\frac{^{3}H\ A_0}{^{32}P\ A_0}\right) \text{ for complex 2.}}\right]$
Relative Stoichiometry
Calculate $\left(\frac{^{3}H\ A_0}{\text{Resolvase } A_{Sp.}}\right)$
Calculate $\left(\frac{^{32}P\ A_0}{\text{Binding Site } A_{Sp.}}\right)$
Amount of Resolvase (pmol).
Amount of Binding Site (pmol).
Calculate $\left[\frac{\left(\text{Amount of Resolvase (pmol)}\right)}{\left(\text{Amount of Binding Site (pmol).}\right)}\right]$
Absolute Stoichiometry

(b)

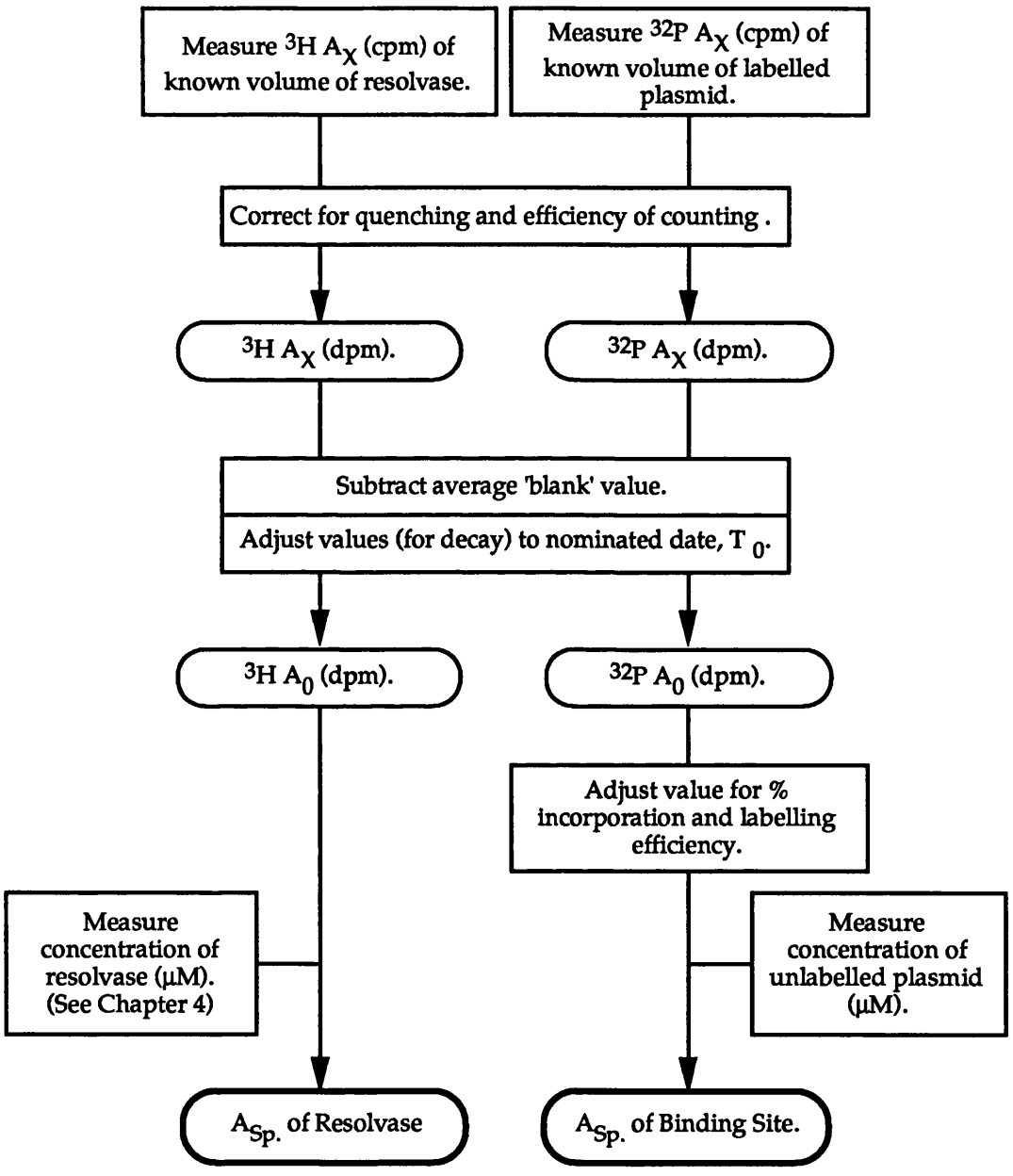


Figure 5.3. Summary of the Calculation of Relative and Absolute Stoichiometry Values for 'Complex n'.

(a) Stoichiometry Values.
(b) Specific Activities of Stock Material.
A_X=activity counted at time T_X.
A_0=activity adjusted to nominated time T_0.
$A_{Sp.}$=specific activity.
The terms used are described further in sections 5.2. and 5.6.

Plasmid	Digested By:	Stock ^{32}P A_0 (dpm)	Volume Counted(µl)	Stock ^{32}P A_V (dpm/µl)
pDB22	*Eco* RI/*Bam* HI	212,681.7	9.66	22016.7
pDB2004	*Eco* RI/*Hin* dIII	328,256.4	9.90	33157.2
pDB2107	*Eco* RI/*Hin* dIII	112,400.7	9.65	11647.7
pDB2507	*Eco* RI/*Hin* dIII	132,512.4	9.71	13647.0
Plasmid	Fraction Incorporated	Plasmid ^{32}P A_V (dpm/µl)	Labelling Efficiency	Site ^{32}P A_V. (dpm/µl)
pDB22	0.93	20,369.2	51.89%	10,570.4
pDB2004	0.91	30,263.5	45.89%	13,887.8
pDB2107	0.93	10,795.8	52.42%	5,659.4
pDB2507	0.93	12,756.1	47.96%	6,118.0

Table 5.2 **Activity of Binding Site Fragment in Labelled Stock Solution.** A_0 is the activity of given samples when adjusted from the measured activity, A_X at time, T_X, to the activity at a nominated time, T_0, in this case 00:00 on 1/10/92. A_V denotes the volume-specific activity of a sample. 'Fraction incorporated' shows what fraction of the total activity present is as end-labelled plasmid. 'Labelling efficiency' measures the proportion of incorporated counts represented by the DNA fragment containing the binding site. Both values are ratios of the activities of material cut from gels. The A_V values were calculated with regard to the binding site as follows:
Binding Site A_V = (Plasmid A_V).(Labelling Efficiency)
Plasmid A_V = (Stock A_V (dpm/µl)).(Fraction Incorporated)

Plasmid	Cold [DNA] (µM)	Volume Used (µl)	Site A_V (dpm/µl)	Volume Used (µl)	Site $A_{Sp.}$ (dpm/pmol)
pDB22	1.04	14.81	10,570.4	4.85	3,321.9
pDB2004	0.97	14.75	13,887.8	2.95	2,874.4
pDB2107	0.89	14.74	5,659.4	4.87	2,095.3
pDB2507	1.02	14.77	6,118.0	4.88	1,983.2

Table 5.3 **Specific Activity of Binding Site Fragment in Final Binding Mix.** 'Volume Used' is the amount of each linearised plasmid stock (unlabelled or labelled) mixed together for further digestion, prior to binding. The contribution of labelled DNA to the overall DNA concentration is assumed to be negligible.

5.3 Sources of Error.

The errors inherent in the data used to determine stoichiometry values are as follows;

(i) Measurement of sample volumes.

Sample volumes were determined by weight, using a balance accurate to

50 μg. This represents a relative error of less than ±2% for all samples considered.

(ii) Scintillation counting.

All cpm values listed were produced from counting samples for a minimum of 10 min each, therefore the total number of counts recorded was 10 fold greater than the cpm value. For a single measurement the standard deviation, σ, in counting a total number of counts, n, is approximately equal to $\sqrt{n}$. Therefore if n=10,000 counts (i.e. 1,000 cpm), then σ=100 counts, which is a relative error of ±1%. For all the values used in these calculations the relative error of counting was less than ±2%. Correction factors applied to cpm values to account for quenching and background activity ('blank' samples) may give rise to errors which are difficult to quantify, but are likely to be quite small.

(iii) Measurement of DNA concentration.

The spectrophotometer used in the determination of DNA concentrations read accurately to ±0.001 A. This results in a relative error of less than ±1% of the absorbance readings for the samples examined. The DNA stocks used were purified using CsCl/EtBr gradients, and so digested samples with an A_{260nm}/A_{280nm} ratio of about 1.8 are unlikely to contain significant contaminating material that would alter the A_{260nm} value, and hence the observed DNA concentration.

(iv) Measurement of resolvase concentration.

It is considerably more difficult to evaluate the error involved in determining the concentration of the resolvase used, other than to say that it is likely to be one of the largest errors in the production of an absolute stoichiometry value. This matter is discussed in Chapter 4.

Of the factors considered so far relative stoichiometry values are only affected by the statistical error in counting of radioactive samples, whereas all four of these factors are relevant to an absolute value. Other errors to consider are those which arise from the method of analysis itself. Such errors are more difficult to quantify but must still be taken into account when considering the results produced. For example;

(v) Gel artefacts.

In order to isolate the different complexes they must be cut from the different parts of the gel on which they have been run. However other material (smeared DNA or contaminating protein) may run in a similar position to the complex of interest, whilst other complexes may have a comparatively clean background. Levels of background material appeared

lowest for the less retarded complexes (e.g. Fig. 5.4).

(vi) Isolation of bands.

Isolating a sample by cutting a small gel chip involves the risk of excluding some of the material present, but taking a large gel chip includes a greater proportion of any background activity present. Also a variation in the size of gel slice taken may affect the counting characteristics of samples once dissolved. Whilst these factors will affect all material taken from gels they are particularly significant for ratios such as 'Fraction Incorporated' and 'Labelling Efficiency', shown in Table 5.2. Larger complexes are less affected by these problems.

Because of these sorts of errors more emphasis should be placed on relative stoichiometry results for major, less retarded complexes, i.e. complexes 1 and 2 of subsite (II + 10), complexes 1 and 2 of subsite (II + 5) and complexes 2 and 4 of *res*. However the reproducibility of the results produced using the different binding sites, and from different tracks of different gels (including the smaller scale experiments mentioned in section 5.5), suggests that these errors are quite small.

5.4 Preparation of Protein Samples for Stoichiometry Determinations.

Purification and quantification of tritiated resolvase for these experiments is discussed in Chapter 4.

5.5 Binding Reactions.

Initially binding reactions were carried out using approximately 0.5 pmol of plasmid (~ 1 μg) per 10 μl binding reaction, with no other carrier DNA present. 0.4 μl of the appropriate resolvase dilution was added to each sample which was mixed, incubated (10 min, 37 °C)and then placed on ice prior to loading to the gel, as for standard binding reactions. The omission of additional carrier DNA was acceptable as the resolvase is no longer in such great excess over the amount of DNA present; however the appearance of some of the tracks containing higher resolvase concentrations suggests that the large plasmid fragment was 'mopping up' some of the resolvase. The first gel produced (subsite II; not shown) was stained using ethidium bromide and bands were visualised on a 254 nm wavelength UV transilluminator. The expected band pattern could be seen in some photographs of the gel but the faintest complexes were not visible by eye. This problem increased on prolonged exposure to UV while bands were being cut from the gel (due to photobleaching of the stain) such that the last bands removed were located with reference to rulers in the photograph and their

absence from the gel was finally checked by a long exposure shot of the gel. All four sites investigated were used in binding reactions as described except that the complexes were identified by silver staining the gels. Photographs of silver stained band shift gels do not show the complexes very clearly but they were more obvious to the naked eye and could be cut with the gel on a light box and so were not subject to photobleaching. Although the silver staining method required soaking for a total of 90 min (c.f. 45 min for EtBr) the first hour of this was in a fixing solution, and therefore there was no concern of greater diffusion of complexes in this time. Each gel chip removed was placed in a 20 ml scintillation vial with 1 ml of 30% H_2O_2. The vial was sealed and incubated at 55 °C for up to 72 h to dissolve the polyacrylamide. 10 ml of 'Ecoscint' fluid was mixed with the sample which was then scintillation counted.

On the whole the cpm values produced for these complexes were below the levels required to give statistically acceptable results. However the data produced were analysed to, at least, give better estimates of the amounts and ratios of DNA and resolvase that should be used.

More DNA was prepared for binding by mixing labelled and unlabelled plasmid stocks (known volume by weight) and digestion as described previously. Once purified, the DNA samples were used for binding reactions as before except that the reaction volumes (but not concentrations) were increased 5 fold, i.e. 2.5 pmol of plasmid DNA was mixed in 50 µl of binding buffer to which 2 µl of a resolvase dilution was added. Mixing, incubation, loading, etc., remained the same but wells of a larger volume were used. Once loaded, the gels were run and silver stained in the same way, and bands were removed, dissolved and counted as before.

The definition of the complexes in these gels (Fig. 5.4 and 5.5) was somewhat reduced. The third and fourth complexes seen with subsite (II + 5) could not be distinguished from each other (Fig. 5.5) and complex 5 produced from the *res* site was not visible against the background staining in lanes containing RD 9 f6 (Fig. 5.4). This background is unlikely to be unbound resolvase running on the gel as the background was low in equivalent R17 f48 tracks, and no material was observed in attempts to run resolvase on such gels in the absence of DNA. The smearing in these tracks probably represents a small amount of contaminating protein from RD 9 f6, or could be retarded DNA as observed with high concentrations of resolvase in some other gels (e.g. Fig 3.13b). Some samples were taken from the smeared areas for counting, to check that levels of

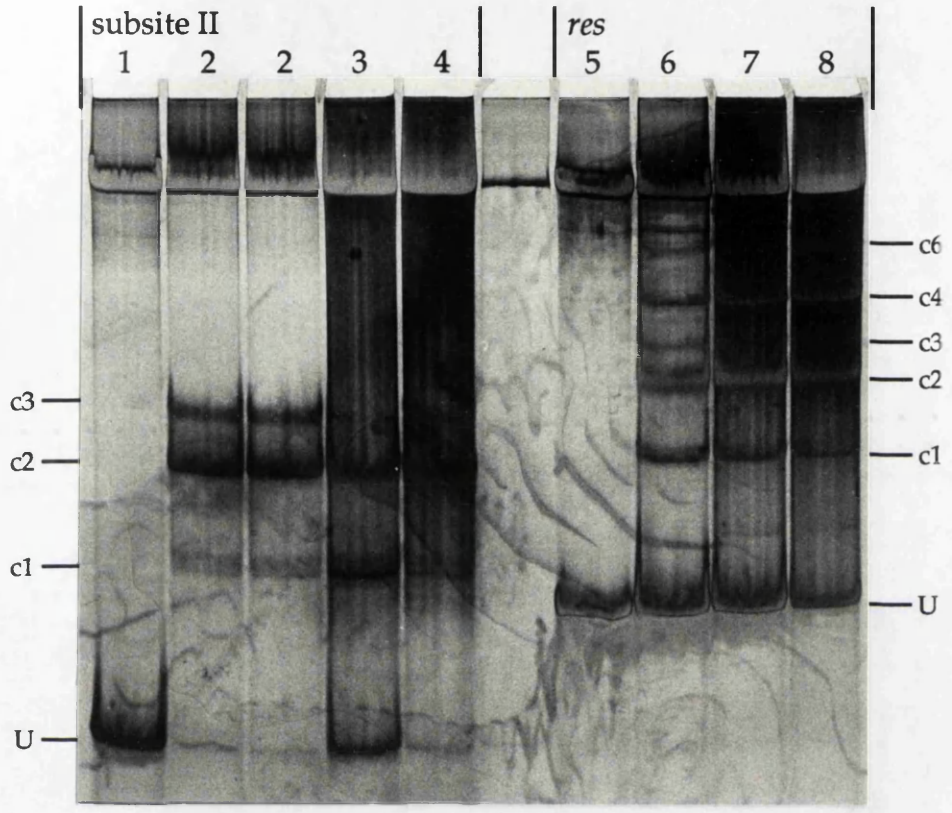


Figure 5.4. Large Scale Band Shift Assay with *res* and Subsite II.
'U' denotes unbound target DNA, complexes are indicated by cN where N is the complex number (see Chapter 3 for further details). Some silver-stained complexes were visible by eye but are not clear in monochrome photographs. The subsite II sample with R17 f48 resolvase was loaded between two lanes (both numbered 2 in this figure).

Lane	DNA	Resolvase
1	pDB2004 (*Hin* dIII/*Eco* RI)	R.H.B.
2	"	2^{-3} R17 f48
3	"	2^{-1} RD 9 f6
4	"	neat RD 9 f6
5	pDB22 (*Bam* HI/*Eco* RI)	R.H.B.
6	"	2^{-2} R17 f48
7	"	2^{-1} RD 9 f6
8	"	neat RD 9 f6

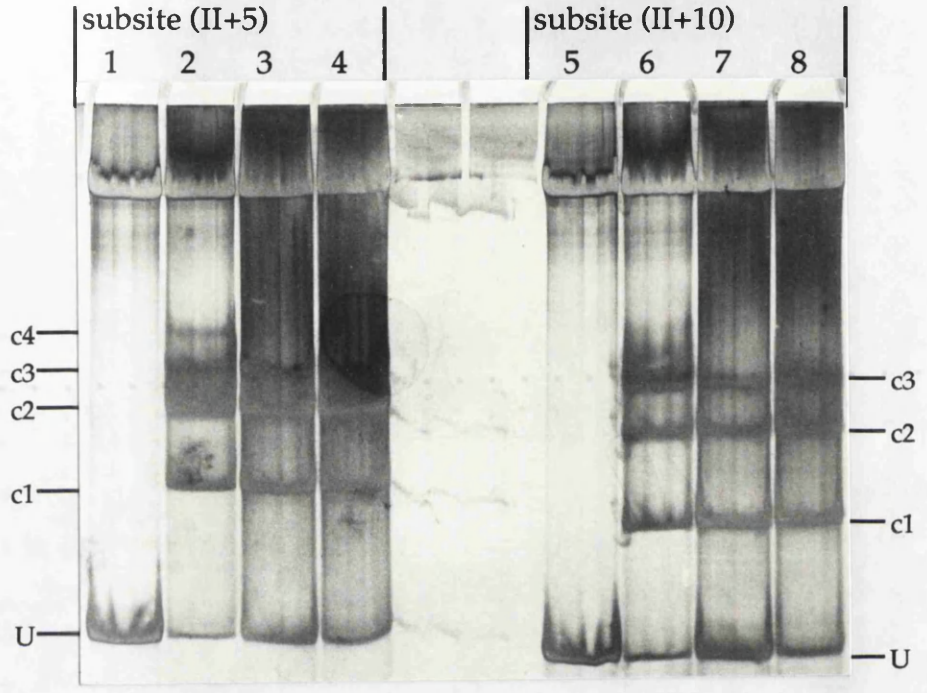


Figure 5.5. Large Scale Band Shift Assay with Subsite (II + 5) and Subsite (II + 10).

'U' denotes unbound target DNA, complexes are indicated by cN where N is the complex number (see Chapter 3 for further details). Some silver-stained complexes were visible by eye but are not clear in monochrome photographs.

Lane	DNA	Resolvase
1	pDB2507 (*Hin* dIII/*Eco* RI)	R.H.B.
2	"	2^{-3} R17 f48
3	"	2^{-1} RD 9 f6
4	"	neat RD 9 f6
5	pDB2107 (*Hin* dIII/*Eco* RI)	R.H.B.
6	"	2^{-3} R17 f48
7	"	2^{-1} RD 9 f6
8	"	neat RD 9 f6

contamination would not significantly alter results from complexes in those regions.

5.6 Subsite II Stoichiometric Determination.

The samples produced were counted for 10 min each using a Wallac 1409/11 liquid scintillation counter version 1.4. This is a windowless counter which was installed with the manufacturers own software to supply ^{3}H- and ^{32}P- spectrum libraries as well as quenching data for the appropriate scintillation fluid and vial type. Nonetheless dilutions of ^{3}H and ^{32}P stocks were mixed in different ratios and counted in 10 ml of scintillant to check the validity of the software to the amounts of material being used.

The isolated complexes were counted approximately every five days (for a period of 1 month) to check that the change in the assigned values of each isotope was consistent with the expected decay rate. All runs of counting included at least 3 blank samples containing H_2O_2 and scintillant, and the average value of the blanks was subtracted from each experimental value from the same run (giving 'blank adjusted' cpm and dpm values). Repeatedly counting the samples over a period of weeks meant that the ^{32}P values were highest and most accurate in the initial rounds of counting. However the ^{3}H values were likely to be more accurate in the final analysis as the decay in their activity was minimal on this time scale but the ^{32}P values were at their lowest, and so the ^{3}H peak was least affected by the tail of the ^{32}P energy peak. Because of this the calculations to determine the relative and absolute stoichiometry of the complexes were based on the ^{32}P values from the initial count of each sample, and the ^{3}H value from the last count. In order to check the observed decay rates of the isotopes in each sample all data were adjusted to the same start date (A_0 = 00:00 on 1st October 1992), which was essential for all data to be used in absolute stoichiometry determinations.

Manipulations of the data produced using subsite II are described below, with the steps involved summarised in tables and sample calculations where necessary. The data produced with the three other binding substrates were processed in the same manner and are shown in tabular form.

Although the samples were counted for 10 min each, values for ^{3}H and ^{32}P activities were recorded in counts per minute (cpm) and disintegrations per minute (dpm). The dpm values were calculated by the scintillation counter from the cpm values based on the programmed quench data and with respect to an external control source. The ratio of cpm to dpm for particular counting

conditions should be fairly consistent for any given isotope when the values of different samples are to be compared. ^{32}P is not subject to significant quenching and so the counting efficiency for all samples was close to 100%. ^{3}H is quenched and an efficiency of about one third is expected for ^{3}H counting. Almost all the ^{3}H data used in these calculations had counting efficiencies of 32% to 34%. Although some data shown in the activities tables have values outside this range, not all the data listed were carried forward to the final calculations.

Data were adjusted to a nominated date (00:00 on 1/10/92) as follows:
If A_X is the activity of a sample when counted at a time, T_X, and t is the half-life of the isotope then the activity of the sample can be calculated for a different time, T_0.
If $\Delta T = T_X - T_0$, and the activity of the sample at T_0 is A_0 then;

$$A_0 = A_X.2^{(\Delta T/t)}$$

The following blank adjusted values were produced for complex 2 of subsite II using undiluted RD 9 f6:

^{3}H: A_X = 20179.7 dpm	T_X = 29/10/92, 19:37	t = 4492.575 days
^{32}P: A_X = 4612.0 dpm	T_X = 4/10/92, 21:36	t = 14.3 days

For T_0 = 1/10/92, 00:00,

^{3}H: ΔT = 28.817 days	$A_0 = (20179.7).[2^{(28.817/4492.575)}] = 20269.6$ dpm
^{32}P: ΔT = 3.9 days	$A_0 = (4612.0).[2^{(3.9/14.3)}] = 5571.7$ dpm

The subsite II, c2, 365 nM f6 sample (where c 'n' is complex number 'n', and 'X' mM f6 represents a final RD 9 f6 concentration of 'X' mM) gave activities from different times of counting which were not consistent with each other once adjusted for decay. The ^{32}P value produced on the first counting was 5% lower than the other adjusted values (all within 1%). This may have been the result of incomplete mixing of the sample with the scintillation fluid as the counter used is not designed for Čerenkov counting. This would have been corrected when the samples were mixed again between measurements. The ^{3}H value for this sample showed the same discrepancy, but the difference in the values was about 30%, compared with less than 10%. A 10% variation in ^{3}H activities was a result of the progressive decay of ^{32}P in each sample allowing better detection of the ^{3}H present and so all ^{3}H values used were from the final rounds of counting. Because of this variation in the subsite II, c2, 365 nM f6 sample the ^{32}P activity quoted was derived from counting on 12/10/92 instead of 4/10/92.

Complex	[R]	A_X (cpm)	Efficiency of Counting	A_X (dpm)
c 1	730 nM	251.3	31.73%	792.0
c 1	365 nM	467.7	33.26%	1406.0
c 2	730 nM	6484.6	32.10%	20200.5
c 2	365 nM	6130.9	33.35%	18385.7
c 3	730 nM	1676.3	33.40%	5019.0
c 3	365 nM	587.9	33.98%	1730.3
Complex	[R]	A_X (blank adjusted dpm)	Time of Counting (=T_X)	A_0 (dpm)
c 1	730 nM	771.2	29/10/92 19:16	774.6
c 1	365 nM	1385.2	29/10/92 19:27	1391.3
c 2	730 nM	20179.7	29/10/92 19:37	20269.6
c 2	365 nM	18364.9	29/10/92 19:48	18446.7
c 3	730 nM	4998.2	29/10/92 19:59	5020.4
c 3	365 nM	1709.5	29/10/92 20:09	1717.1

Table 5.4 **^{3}H Activities for Subsite II Complexes (pDB2004).**
[R] is the final concentration of resolvase which was present in the binding reaction.

Complex	[R]	A_X (cpm)	Efficiency of Counting	A_X (dpm)
c 1	730 nM	336.9	99.26%	339.4
c 1	365 nM	647.8	99.37%	651.9
c 2	730 nM	4604.1	99.54%	4625.4
c 2	365 nM	2948.7	99.97%	2949.7
c 3	730 nM	813.2	99.32%	818.8
c 3	365 nM	380.4	99.37%	382.8
Complex	[R]	A_X (blank adjusted dpm)	Time of Counting (=T_X)	A_0 (dpm)
c 1	730 nM	326.0	4/10/92 21:15	393.5
c 1	365 nM	638.5	4/10/92 21:26	771.1
c 2	730 nM	4612.0	4/10/92 21:36	5571.7
c 2	365 nM	2936.3	12/10/92 19:22	5204.1
c 3	730 nM	805.4	4/10/92 21:58	973.7
c 3	365 nM	369.4	4/10/92 22:08	446.7

Table 5.5 **^{32}P Activities for Subsite II Complexes (pDB2004).**

Two binding reactions containing RD 9 f6 were prepared for each substrate to try to produce an optimal yield of each complex. Although the overall amount of complex in each track was not identical the ratio of protein to DNA should have been constant and so the average ^{3}H and ^{32}P activities were taken (where

appropriate) in order to determine the relative and absolute stoichiometry values.

Example: for complex 2 $^{3}H\ A_0 = (20269.6 + 18446.7)/2 = 19358.1$

$^{32}P\ A_0 = (5571.7 + 5204.1)/2 = 5387.9$

In some cases the amount of material present in complex from one reaction was considerably less than that of the other and so often the smaller sample may have been excluded from further calculations. An absolute limit was not placed on the dpm value required for a complex to be included so that results could be produced for all complexes detected. However it is obvious that greatest emphasis should be placed on the results derived from the largest values.

Example: for complex 2 $^{3}H\ A_0/^{32}P\ A_0 = 19358.1/5387.9 = 3.59$

Although the ratio $^{3}H\ A_0/^{32}P\ A_0$ is an indicator of the resolvase to DNA ratio, the $^{3}H\ A_0$ and $^{32}P\ A_0$ values do not represent real concentrations. The benefit of this ratio is to allow a comparison with the resolvase/DNA ratio in other complexes. The column "complex n/complex 2" in Table 5.6 shows how the amount of resolvase (relative to DNA) increases in successive complexes. These values were produced by dividing the $^{3}H\ A_0/^{32}P\ A_0$ ratio for a given complex, n, by that for complex 2.

Complex	$^{3}H\ A_0$ (dpm)	$^{32}P\ A_0$ (dpm)	$^{3}H\ A_0/\ ^{32}P\ A_0$	complex n / complex 2
c 1	1391.3	771.1	1.80	0.50
c 2	19358.1	5387.9	3.59	1.00
c 3	5020.4	973.7	5.16	1.44

Table 5.6 **Relative Stoichiometry of Subsite II Complexes (pDB2004).** Obviously the ratio 'complex n/complex 2' for complex 2 will be exactly 1.00 for all substrates investigated.

The absolute stoichiometry of a complex can be determined if $^{3}H\ A_0$ and $^{32}P\ A_0$ are converted into amounts of resolvase and subsite II fragment, respectively. This can be done by dividing each value by the appropriate specific activity.

The derivation of the specific activity of the resolvase in f6 of RD 9 is shown in Chapter 4 and the specific activity of the DNA samples is discussed earlier in section 5.2.

RD 9 f6; $A_{Sp.} = 4{,}874.39$ dpm/pmol.

subsite II; $A_{Sp.} = 2{,}874.38$ dpm/pmol.

Once the activities were expressed as pmol of resolvase or pmol of subsite II fragment the absolute stoichiometry is derived by dividing the amount of resolvase present by the amount of subsite II DNA.

Complex	Resolvase (pmol)	subsite II (pmol)	Resolvase : Subsite II
c 1	0.29	0.27	1.06
c 2	3.97	1.87	2.12
c 3	1.03	0.34	3.04

Table 5.7 Absolute Stoichiometry of Subsite II Complexes (pDB2004).

5.7 Relative Stoichiometry of Complexes of *res*.

The data for complexes produced with a full *res* site are presented in Tables 5.8, 5.9 and 5.10. As the calculations were equivalent to those described for subsite II fewer steps are shown. In some cases calculations are based on data from only one complex, e.g. where the cpm values of a complex in one track were significantly less than that in the other (c1; 730 nM [R]), or where the A_0 values produced from different times of counting showed unreasonable fluctuation (c3; 365 nM [R]) and, of course , in the case of c6; 365 nM [R] where complex was not visible against the background staining. No data are presented for complex 5 as it could not be detected on the gels. Again this was probably due to increased background in this area of the gel. However the mobility, distribution and relative intensity of the other complexes indicate their correct assignation and that the results observed under these conditions are relevant to the 'normal' smaller scale experiments described in Chapter 3.

Complex	[R]	^3H A_X (cpm)	^3H A_X (dpm)	Efficiency of Counting	^3H A_0 (dpm)
c 1	730 nM	168.2	468.8	35.88%	450.0
c 1	365 nM	279.3	838.1	33.33%	820.9
c 2	730 nM	2296.9	6875.4	33.41%	6885.2
c 2	365 nM	3680.9	10795.4	34.10%	10822.8
c 3	730 nM	732.5	2230.1	32.85%	2219.1
c 3	365 nM	820.5	2420.5	33.90%	2410.4
c 4	730 nM	5016.1	14965.2	33.52%	15011.3
c 4	365 nM	2610.1	7810.9	33.42%	7825.0
c 6	730 nM	5843.3	17631.0	33.14%	17689.1

Table 5.8 ^3H Activities of Wild Type *res* Site Complexes (pDB22).

Complex	[R]	^{32}P A_X (cpm)	^{32}P A_X (dpm)	Efficiency of Counting	^{32}P A_0 (dpm)
c 1	730 nM	189.9	189.7	100.11%	214.1
c 1	365 nM	407.0	407.4	99.90%	478.7
c 2	730 nM	1610.1	1613.8	99.77%	1945.2
c 2	365 nM	2560.9	2561.7	99.97%	3098.5
c 3	730 nM	361.6	361.3	100.08%	423.2
c 3	365 nM	477.5	478.7	99.75%	566.2
c 4	730 nM	1734.8	1735.3	99.97%	2096.0
c 4	365 nM	989.5	987.2	100.23%	1185.8
c 6	730 nM	1450.4	1453.7	99.77%	1754.4

Table 5.9 ^{32}P Activities of Wild Type *res* Site Complexes (pDB22).

Complex	^{3}H A_0 (dpm)	^{32}P A_0 (dpm)	^{3}H A_0/ ^{32}P A_0	complex n / complex 2
c 1	820.9	478.7	1.71	0.49
c 2	8854.0	2521.9	3.51	1.00
c 3	2219.1	423.2	5.24	1.49
c 4	11418.1	1640.9	6.96	1.98
c 6	17689.1	1754.4	10.08	2.87

Table 5.10 Relative Stoichiometry of Wild Type *res* Site Complexes (pDB22).

5.8 Relative Stoichiometry of Complexes of Subsite (II + 10).

As with the *res* site data, extended calculations are not shown. Data from complex 3 were considered significantly more reliable from one of the two reaction tracks (730 mM [R]). All other ratios were derived from average values.

Complex	[R]	^{3}H A_X (cpm)	^{3}H A_X (dpm)	Efficiency of Counting	^{3}H A_0 (dpm)
c 1	730 nM	874.6	2607.9	33.54%	2598.7
c 1	365 nM	1393.5	4182.6	33.32%	4180.5
c 2	730 nM	4502.5	13694.9	32.88%	13735.6
c 2	365 nM	4333.0	12907.5	33.57%	12944.6
c 3	730 nM	4571.7	14150.7	32.31%	14193.5
c 3	365 nM	2632.4	7977.8	33.00%	7992.8

Table 5.11 ^{3}H Activities of Subsite (II + 10) Complexes (pDB2107).

Complex	[R]	^{32}P A_X (cpm)	^{32}P A_X (dpm)	Efficiency of Counting	^{32}P A_0 (dpm)
c 1	730 nM	784.3	782.0	100.29%	940.6
c 1	365 nM	1262.9	1260.7	100.17%	1527.0
c 2	730 nM	1995.7	1995.5	100.01%	2427.4
c 2	365 nM	1948.5	1949.8	99.93%	2372.3
c 3	730 nM	1510.5	1508.8	100.11%	1832.7
c 3	365 nM	931.9	934.4	99.73%	1129.1

Table 5.12 ^{32}P Activities of Subsite (II + 10) Complexes (pDB2107).

Complex	^{3}H A_0 (dpm)	^{32}P A_0 (dpm)	^{3}H A_0/ ^{32}P A_0	complex n / complex 2
c 1	3389.6	1233.8	2.75	0.49
c 2	13340.1	2399.9	5.56	1.00
c 3	14193.5	1832.7	7.74	1.39

Table 5.13 Relative Stoichiometry of Subsite (II + 10) Complexes (pDB2107).

5.9 Relative Stoichiometry of Complexes of Subsite (II + 5).

Only three complexes were visible with subsite (II + 5) presumably due to the inability to separate the doublet previously defined as complexes 3 and 4. Again this may be a result of the large amounts of material used, or the choice of stain. As with the subsite (II + 10) data not all calculation steps are shown, and complex 3 data are derived from the A_0 values of c3; 730 nM [R] only.

complex	[R]	^3H A_X (cpm)	^3H A_X (dpm)	Efficiency of Counting	^3H A_0 (dpm)
c 1	730 nM	1766.0	5208.3	33.91%	5210.9
c 1	365 nM	2697.0	8018.5	33.63%	8033.7
c 2	730 nM	3691.5	11085.4	33.30%	11114.5
c 2	365 nM	2973.8	8698.0	34.19%	8716.3
c 3	730 nM	6958.5	20687.0	33.64%	20759.5
c 3	365 nM	1825.1	5434.3	33.58%	5437.9

Table 5.14 ^3H Activities of Subsite (II + 5) Complexes (pDB2507).

Complex	[R]	^{32}P A_X (cpm)	^{32}P A_X (dpm)	Efficiency of Counting	^{32}P A_0 (dpm)
c 1	730 nM	1757.9	1762.2	99.76%	2149.4
c 1	365 nM	2760.0	2762.2	99.92%	3379.7
c 2	730 nM	1711.1	1708.6	100.15%	2085.0
c 2	365 nM	1474.9	1478.4	99.76%	1802.6
c 3	730 nM	2033.8	2037.5	99.82%	2491.3
c 3	365 nM	583.1	583.0	100.02%	701.3

Table 5.15 ^{32}P Activities of Subsite (II + 5) Complexes (pDB2507).

Complex	^3H A_0 (dpm)	^{32}P A_0 (dpm)	^3H A_0/ ^{32}P A_0	complex n / complex 2
c 1	6622.3	2764.5	2.40	0.47
c 2	9915.4	1943.8	5.10	1.00
c 3	20759.5	2491.3	8.33	1.63

Table 5.16 Relative Stoichiometry of Subsite (II + 5) Complexes (pDB2507).

5.10 Comparison of Absolute Stoichiometry Values of Sites Examined.

Site; Complex	Resolvase (pmol)	DNA Site (pmol)	Resolvase : DNA Site
II ; c 2	3.97	1.87	2.12
res ; c 2	1.82	0.76	2.39
(II + 10); c 2	2.74	1.15	2.39
(II + 5); c 2	2.03	0.98	2.08

Table 5.17 **Absolute Stoichiometry of Complex 2 of Sites Investigated.** Values shown are derived as described for subsite II (Table 5.7) from the activities in Tables 5.6, 5.10, 5.13 and 5.16, the DNA specific activities in Table 5.3 and the resolvase specific activity, $A_{Sp.}$ = 4,874.39 dpm/pmol, calculated in Chapter 4.

As discussed in section 5.3, the relative stoichiometry results for major complexes of each site are considered most reliable, however the results for other complexes all conform to the same pattern of binding. If the absolute resolvase/DNA stoichiometry value for complex 1 is likely to be an integer (other than 0) then it is probable that the absolute stoichiometry of complex 2 is an even whole number, as two binding units of resolvase associate with each DNA molecule in complex 2, and one resolvase binding unit associates with each DNA molecule in complex 1. This in turn simplifies the interpretation of the absolute stoichiometry results, i.e. if the value for complex 2 is expected to be 2, 4 or a larger even integer, then the results in Table 5.17 clearly show that this stoichiometry is 2 resolvase monomers per DNA site, for all four binding sites investigated.

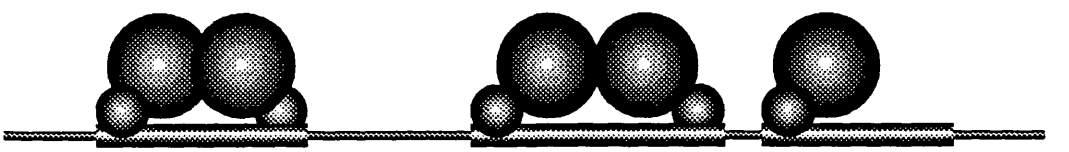
The relative stoichiometry values produced were in close agreement for equivalent complexes of different sites. The results shown in Table 5.17 were calculated from the same data as the relative stoichiometries, but also took into account resolvase and DNA specific activities. As the same resolvase specific activity value was used in each case, differences in the absolute stoichiometries shown must partly be due to differences in the observed DNA specific activities. This reflects some variation in the determination of DNA Stock volume-specific activities or, more probably, factors such as 'Efficiency of Labelling'.

5.11 Conclusions.

The relative stoichiometry data presented for *res*, subsite II, subsite (II + 10) and subsite (II + 5) show that each subsequent complex observed represents binding of a single additional resolvase unit. The calculation of absolute values shows that, for all four of these binding sites, the binding unit is a monomer of resolvase.

Chapter Six

Mutagenesis of the Tn3 *tnpR* gene



6.1 Introduction.

Mutants of the DNA invertase Gin have been produced which no longer require the accessory factor FIS to direct inversion of an appropriate substrate[43]. Some mutations of Cin, which can direct recombination in the absence of the enhancer element, have also been reported[26]. Although termed FIS-independent, inversion by the Gin mutants is still subject to stimulation by FIS. The FIS/enhancer-independent mutations identified are the result of single amino acid substitutions, which lie between positions 70 and 115 of the protein sequence.

In protein sequence alignments (Fig. 6.1), the positions of FIS-independence mutations of Gin, and of enhancer-independence mutations of Cin, coincides with the region in γδ resolvase which has been shown to be involved in protein/protein contacts between subunits of the 1-2 dimer, believed to bind subsites of γδ *res*, prior to synapsis[34]. The equivalent region is expected to be responsible for interactions between monomers of Gin bound to a *gix* site. Further mutagenesis of a FIS-independent Gin produced FIS-dependent revertants with a normal inversion phenotype. Amino acid substitutions which gave rise to FIS-dependent revertants were located in the N-terminal region of Gin. The same substitutions which create FIS-dependent revertants abolish recombination activity when introduced to (otherwise) wild-type Gin.

A model of Gin-mediated inversion has been proposed[38], which is related to models for resolution by Tn3 resolvase (see Chapter 1). In the Gin model the accessory factor (FIS bound to the enhancer) assists in the formation of a -2 synapse, to allow recombination of the two Gin-bound *gix* sites. By analogy, the subsite I sequences in a *res* synapse are considered equivalent to the *gix* sites, with subsites II and III serving, as accessory sites, to stabilise the -3 synapse.

Mutants of Tn3 resolvase were sought which could direct recombination of a substrate with two copies of subsite I only, to question the validity of this comparison.

6.2 Choosing a Resolvase Expression Vector as a Target for Mutagenesis.

The availability of a number of related resolution substrates, which could be adapted to allow *in vivo* screening for the desired mutants, dictated that the resolvase expression vector used should be compatible with these plasmids. It was also desirable to have an expression vector which could give reasonable rates of resolution *in vivo* (with wild type *res* sites) without greatly reducing cell growth rates because of very high levels of resolvase production. The resolvase

```
          .....10.....20.....30.....40.....50
γδ        MRLFGYARVSTSQQSLDIQVRALKDAGVKANRIFTDKASGSSSDRKGLDL
Tn3       MRIFGYARVSTSQQSLDIQIRALKDAGVKANRIFTDKASGSSTDREGLDL
Gin       MLI.GYVRVSTNDQNTDLQRNALVCAG..CEQIFEDKLSGTRTDRPGLKR
Cin       MLI.GYVRVSTNEQNTALQRNALESAG..CELIFEDKASGKKAERPGLKK

          .....60.....70.....80.....90.....100
γδ        LRMKVEEGDVILVKKLDRLGRDTADMIQLIKEFDAQGVSIRFIDDGISTD
Tn3       LRMKVEEGDVILVKKLDRLGRDTADMIQLIKEFDAQGVAVRFIDDGISTD
Gin       DLKRLQKGDTLVVWKLDRLGRSMKHLISLVGELRERGINFRSLTDSIDTS
Cin       VLRMLSRGDTLVVWKLDRLGRSMRHLVVLVEELRDRGINFRSLTDSIDTS

          .....110.....120.....130.....140.....150
γδ        GEMGKMVVTILSAVAQAERQRILERTNEGRQEAMAKGVVFGRKRKI..DR
Tn3       GDMGQMVVTILSAVAQAERRRILERTNEGRQEAKLKGIKFGRRRTV..DR
Gin       SPMGRFFFHVMGALAEMERELIIERTMAGLAAARNKGRIGGRPPKLTKAE
Cin       TPMGRFFFHVMGALAEMERELIVERTRAGLDAARAEGRIGGRRPKYQEET

          .....160.....170.....180.....190.....200
γδ        DAVL.NMWQQGLGASHISKTMNIARSTVYKVINESN
Tn3       NVVL.TLHQKGTGATEIAHQLSIARSTVYKILEDERAS
Gin       WEQAGRLLAQGIPRKQVALIYDVALSTLYKKHPAKRAHIENDDRIN
Cin       WQQMRRLLEKGIPRKQVAIIYDVAVSTLYKKFPASSFQS
```

Figure 6.1. Sequence Alignment of Tn3 Resolvase, γδ Resolvase, Mu Gin and P1 Cin.

Underlined residues in Gin and Cin sequences indicate positions at which substitutions giving FIS-independent Gin or enhancer-independent Cin were detected. The substitutions reported for each invertase were as follows;

Gin; SF75 IV94 FV104 HY106 MV114
Cin; RH71 HY106

The reported amino acid positions do not exactly match the positions shown here as a result of aligning the invertase and resolvase sequences.

The underlined γδ residue indicates the position at which a methionine is substituted by cysteine to create γδ M106C mutant resolvase, described in Chapter 3.

The region of γδ resolvase shown to make contacts between two resolvase subunits forming a 1-2 dimer is indicated (approximately) by a line above the γδ sequence. The equivalent regions are expected to provide a similar function in Tn3 resolvase, Gin and Cin.

expression vector pMA6111, described in Chapter 4, failed to meet either of these criteria, but a pACYC177 derivative, pCIA70 (Fig. 6.2) was acceptable on both counts. pCIA70 DNA was kindly supplied by P. Haffter. Resolvase expression from pCIA70 is driven by the lac_{UV5} promoter, which is induced in the presence of IPTG. Overnight growth of cells containing pCIA70 gave complete resolution of a pBR322-based resolution substrate, carrying two directly repeated *res* sites, when IPTG was included in the growth medium (see Fig. 6.5). Without IPTG in the medium resolution of the same substrate was detected, but some unresolved plasmid remained. No large scale resolvase purification protocol had been developed using pCIA70, but, if the desired mutants were isolated, it would have been possible to transfer the altered coding region to pMA6111, for overexpression and purification.

Directed mutagenesis techniques, or methods needing a single stranded DNA target, would have required the construction of a suitable new plasmid, which may not have satisfied the expression or compatibility criteria discussed. Hence it was considered reasonable initially to choose a method of mutagenesis which could be used with an acceptable resolvase source, i.e. pCIA70.

6.3 Construction of Substrates for Identification of Desired Mutants.

A substrate which would allow for screening of the desired mutants had already been produced by A. L. Bednarz[8]. pAL225 has two copies of subsite I cloned into pBR322, in direct repeat (Fig. 6.3). The design of this plasmid was based on pMA21, a pBR322 derivative containing two, directly repeated, copies of the Tn3 *res* site (Fig. 6.3). Resolution of pMA21 *in vivo*, followed by decatenation by DNA gyrase, would produce two smaller, unlinked DNA circles, each containing a *res* site (Fig. 6.4). One of these would carry the ampicillin resistance gene, *βla*, and the pBR322 origin of replication, and could be maintained *in vivo*. However the other product, coding for tetracycline resistance, would not be replicated and so would be lost after *in vivo* resolution. The same would be true of resolution of pAL225, if it occurred. Therefore resolvase mutants which directed this reaction could be screened by selecting for pCIA70 pAL225 transformants which were ampicillin resistant but tetracycline sensitive, and then checking for resolution by analysing the plasmid DNA present in the chosen isolates. This method requires replica plating of colonies, which could cause problems when looking for a small number of Ap^r Tc^s colonies amongst a very high Ap^r Tc^r background (i.e. many unresolved substrates). A more direct approach was made possible by subcloning a *galK* expression cassette into pAL225. This cassette was derived from the McKenney promoter probe vector, pKO500[55] and pCS349[83]. A 200 bp

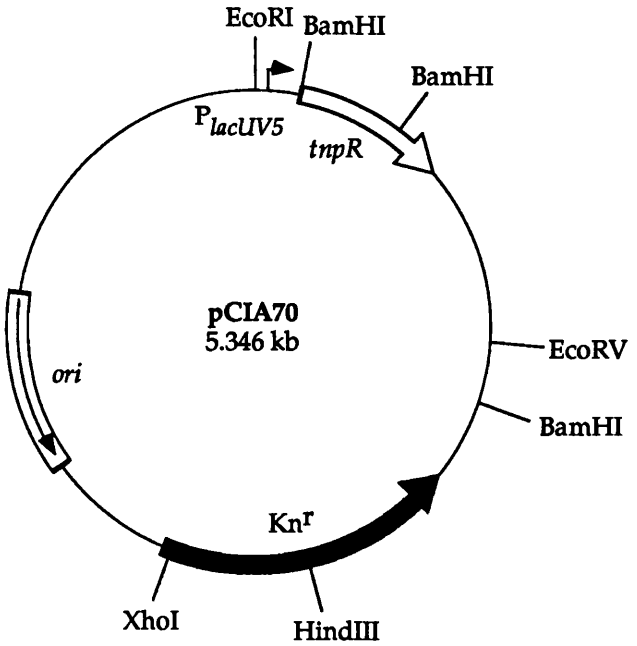
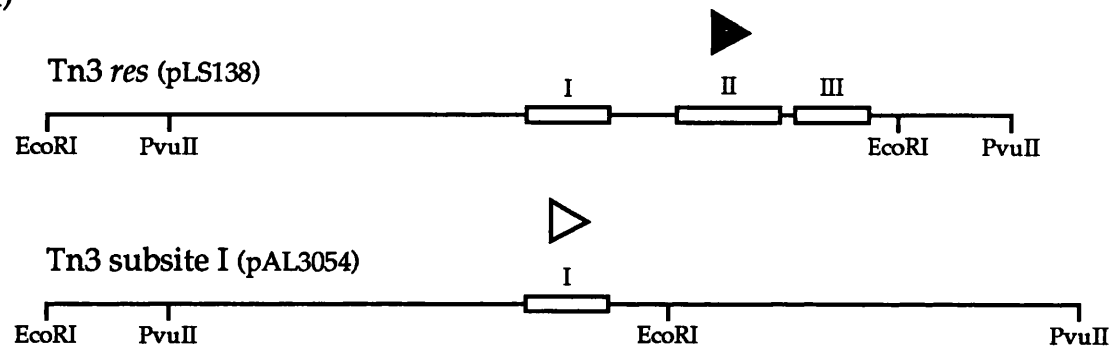
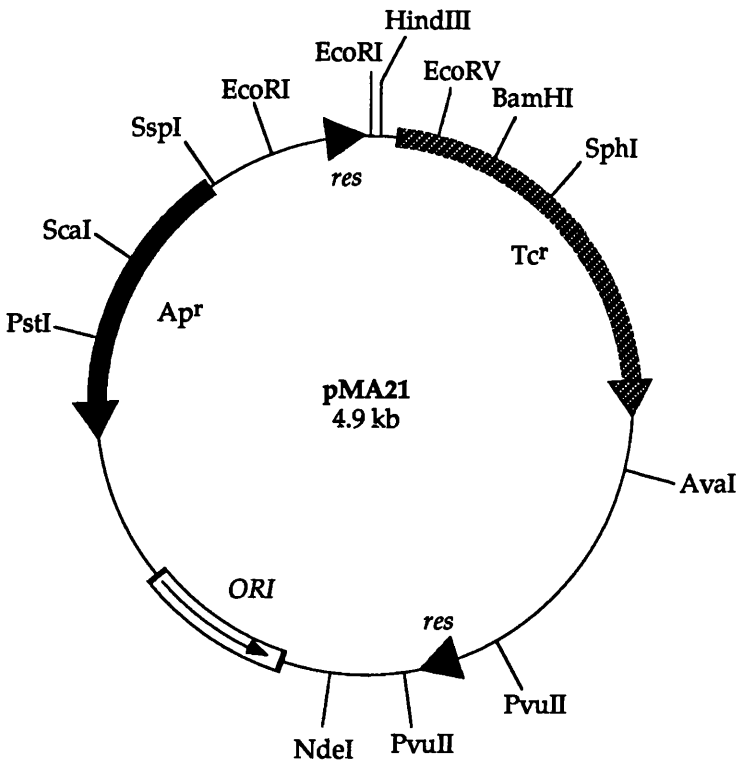


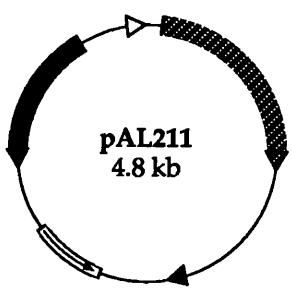
Figure 6.2. Map of Resolvase Expression Vector pCIA70.

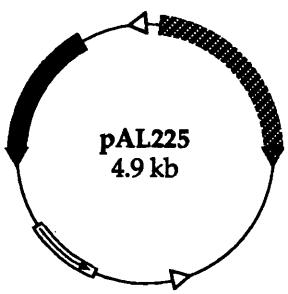
(a)

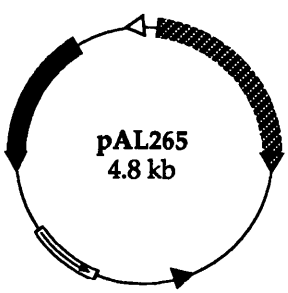

Tn3 res (pLS138)
I
II
III
EcoRI
PvuII
EcoRI
PvuII
Tn3 subsite I (pAL3054)
I
EcoRI
PvuII
EcoRI
PvuII

(b)


HindIII
EcoRI
EcoRV
BamHI
EcoRI
SspI
SphI
res
ScaI
Tcr
PstI
Apr
pMA21
4.9 kb
AvaI
ORI
res
PvuII
NdeI
PvuII


pAL211
4.8 kb


pAL225
4.9 kb


pAL265
4.8 kb

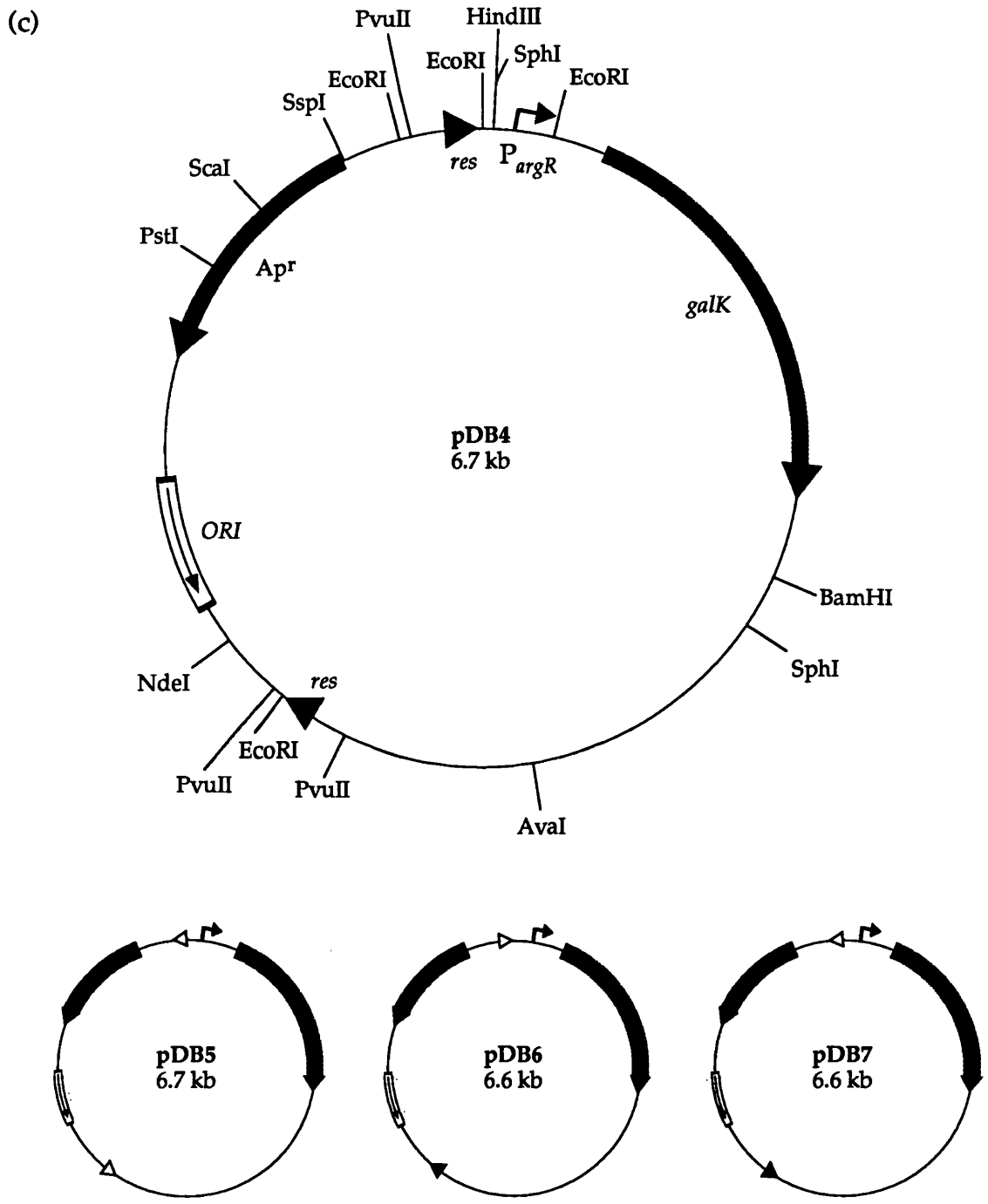


Figure 6.3. Resolution Substrates pMA21, pAL211, pAL225 and pAL265, and Their *galK* Derivatives.

(a) Source of Tn3 *res* site and Tn3 subsite I fragments used to produce pMA21, pAL211, pAL225 and pAL265.

(b) The map of pMA21 shows general features which it has in common with pAL211, pAL225 and pAL265. The relative orientation of the sites present in each of these plasmids is also indicated.

(c) A map of the pMA21 derivative pDB4 indicates restriction sites and genes common to each of the *galK* resolution substrates. Orientations of recombination sites in pDB5, pDB6 and pDB7 are also shown. pDB5 is derived from pAL225, pDB6 from pAL211 and pDB7 from pAL265.

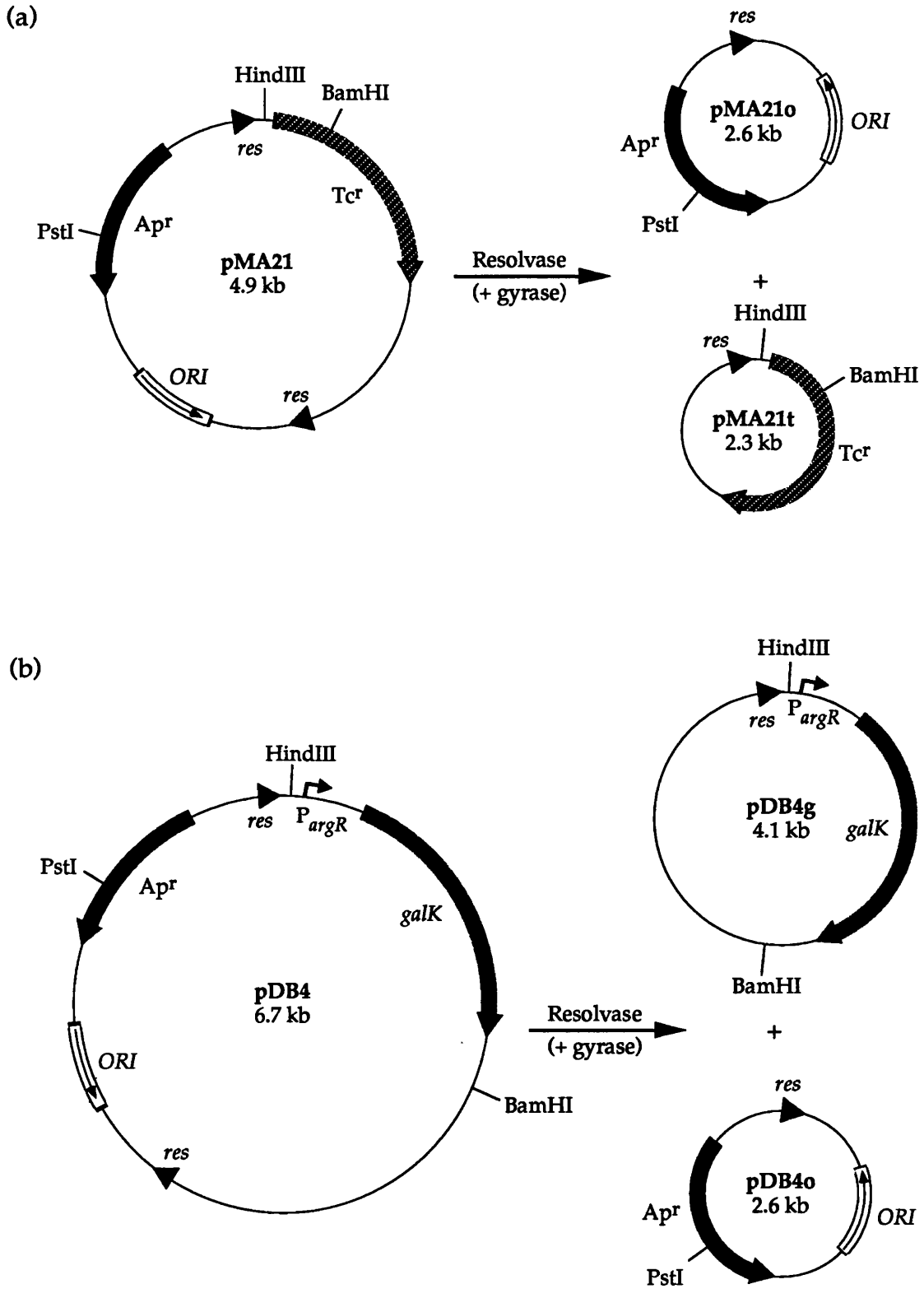


Figure 6.4. Resolution (and Decatenation) of pMA21 and pDB4.

(a) Resolution of pMA21. *In vivo* resolution results in deletion of pMA21t, as it lacks an origin of replication.

(b) Resolution of pDB4. As for pMA21t, pDB4g is lost following *in vivo* resolution.

Hin dIII/*Dra* I fragment, containing the *E. coli argR* promoter, was cloned from pCS349 to the *Hin* dIII and *Sma* I sites of pKO500, producing pXAP (M. Burke; pers. comm.). The *argR* promoter and *galK* gene were subcloned, as a *Hin* dIII/*Acc* I fragment, between the *Hin* dIII and *Eco* RV sites of pAL225 (producing pDB5; Fig. 6.3) and of pMA21 (to give pDB4; Fig. 6.3). The insertion of this fragment to each resolution substrate disrupted the tetracycline resistance gene. Similar constructs were produced by subcloning the same *galK* fragment to the same restriction endonuclease sites in the plasmids pAL211 and pAL265[7], giving pDB6 and pDB7 respectively (Fig. 6.3). Each of these plasmids has a copy of subsite I placed in direct repeat with a wild type *res* site. The difference between pDB6 and pDB7 is the orientation of the pairs of sites relative to the other plasmid sequence. The relative orientation of the sites in pDB4 is the same as pDB6, and in pDB5 is as for pDB7. The resolution product of pDB4 which retains the origin of replication (and *βla*) is named pDB4o, and the other product, with the *galK* gene is called pDB4g (Fig. 6.4). Similarly the (potential) products of pDB5, pDB6 and pDB7 are denoted by the suffix o (for origin) or g (for *galK*).

In vivo resolution of pDB4, pDB5, pDB6 or pDB7 can be assayed by selection of Ap^r *galK*$^-$ isolates, if using Ap^s *galK*$^-$ cells (that are wild type for other genes involved in the uptake and metabolism of galactose, e.g. *galP*, *galU*, *lacY*, etc.), such as DS941, because the *galK* cassette becomes covalently unlinked from the plasmid origin of replication following resolution of the substrate. pDB5 was constructed to allow screening for a resolvase mutant capable of directing subsite I by subsite I resolution. pDB4 was made as a control, to test that resolution could be detected. pDB6 and pDB7 were made to allow for selection of resolvase mutants giving increased levels of resolution between *res* and an isolated subsite I. If the desired phenotype (resolution between a pair of subsite I's) was not isolated from a single round of mutagenesis, mutants selected using pDB6 or pDB7 may have been good candidates for further modification, in an attempt to produce a resolvase which had no accessory site requirement.

6.4 Methods of Selection for Resolution of pDB4, pDB5, pDB6 and pDB7.

Two different approaches were considered for identifying deletion of *galK* from these resolution substrates. The presence of 2-deoxygalactose in growth medium results in an accumulation of 2-deoxygalactose-1-phosphate in *galK*$^+$ cells[3]. Accumulated 2-deoxygalactose-1-phosphate inhibits the utilisation of glycerol as a carbon source (bacteriostasis), and so a minimal medium containing 2-deoxygalactose, with glycerol as the carbon source, will select against *gal*$^+$ cells (D-fucose was also required to stimulate 2-deoxygalactose uptake in DS941, by

induction of *galP*). Although this approach could reduce the total number of colonies screened, an expression period would be required after the introduction of mutagenised pCIA70, to allow resolution of pDB5, and segregation of resolution products. The desired mutant resolvase may be very slow to resolve pDB5 (especially as it is a multicopy plasmid) and so growth over many generation times may be required. This in turn could result in a number of isolates being derived from the same original mutation. DS941 containing pCIA70 and pDB4 was grown in non-selective media to give a mix of *galK*$^+$ and *galK*$^-$ cells, which were then plated on selective media containing 2-deoxygalactose, D-fucose and glycerol. No obvious difference was noticed between colonies produced from cells containing resolved or unresolved pDB4, and so this is not a suitable selection method.

Cells which are *gal*$^+$ form red colonies when grown on M^{ac}Conkey agar plates containing galactose, but *gal*$^-$ cells give white colonies[56]. M^{ac}Conkey agar contains the pH indicator 2-methyl-3-amino-6-dimethyl-aminophenazine (neutral red) which is red at pH <6.8 and yellow at pH >8.0. Bacterial fermentation of reducing sugars (e.g. galactose) lowers the pH of the medium around the colony, giving red (or pink) cells. When galactose is the only sugar present *gal*$^-$ cells metabolise amino acids in the media, producing ammonia and resulting in an increase in pH. This causes the neutral red dye to become yellow, and so these colonies appear pale yellow or white.

M^{ac}Conkey galactose (Mac Gal) plates (containing ampicillin and kanamycin) were used to examine a mixed population of resolved and unresolved pDB4 in DS941 pCIA70. After overnight growth at 37 °C both red and white colonies were produced (Fig. 6.5). Single colony gel analysis of the different coloured colonies showed that all white colonies examined contained only pDB4o sized (and pCIA70 sized) plasmids, and were presumably *gal*$^-$. Red colonies were observed to contain either bands corresponding to pDB4 and pCIA70, or also contained a species of the size expected for resolution products of pDB4 (Fig. 6.5). In both cases the cells would be expected to be *gal*$^+$.

A second indicator medium containing 2,3,5 triphenyl-2H-tetrazolium chloride (TTC, red tetrazolium) was also tested. This indicator can be reduced to form a red precipitate (red formazan) only at high pH. Conversely to M^{ac}Conkey agar, cells metabolising galactose in this medium would give white colonies, as the TTC could not be reduced at the low pH produced, and so would remain colourless[56]. At the higher pH produced by growth of *gal*$^-$ cells the TTC would be

(a)

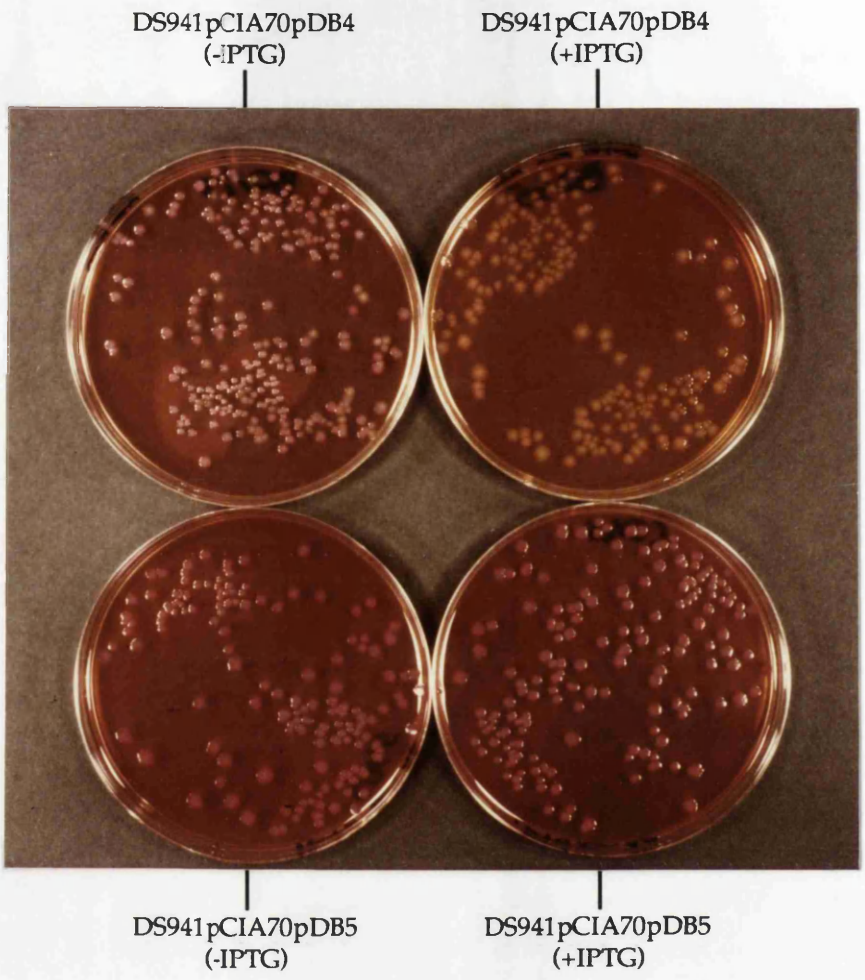


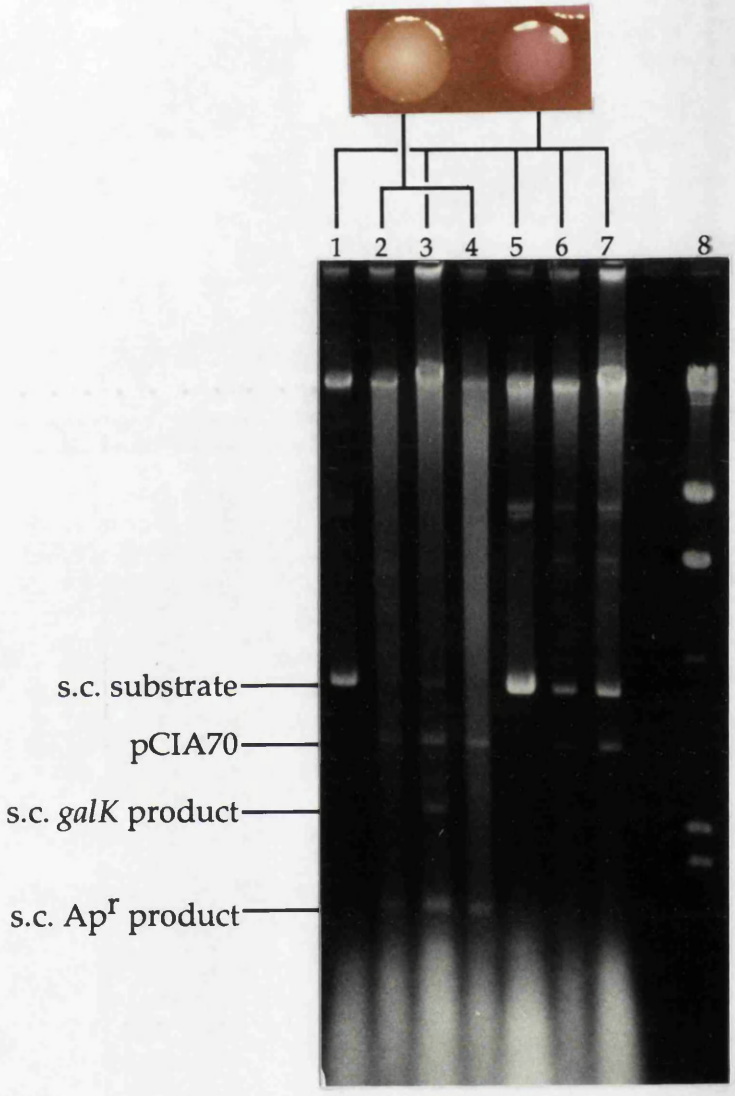
Figure 6.5. Detection of *In Vivo* Resolution Using M^{ac}Conkey Galactose Medium.

(a) *In vivo* resolution by resolvase expressed from pCIA70.

(b) Relationship of colony colour to resolution products.

Lane
1 - DS941pDB4 (+IPTG)
2 - DS941pCIA70pDB4 (-IPTG)
3 - DS941pCIA70pDB4 (-IPTG)
4 - DS941pCIA70pDB4 (+IPTG)
5 - DS941pDB5 (+IPTG)
6 - DS941pCIA70pDB5 (-IPTG)
7 - DS941pCIA70pDB5 (+IPTG)
8 -*Hin* dIII digested λ DNA marker.

(b)


1
2
3
4
5
6
7
8
s.c. substrate
pCIA70
s.c. galK product
s.c. Ap^r product

reduced to form a deep red precipitate. Unfortunately this effect was only observed when comparing chromosomal *gal*$^-$ and *gal*$^+$ *E. coli*. No difference was observed between DS941 pCIA70 pDB4 colonies and DS941 pCIA70 pDB4o colonies grown on the same plates.

For these reasons Mac Gal Ap Kn plates were considered the best selective media for distinguishing DS941 pCIA70 pDB5 from DS941 pCIA70 pDB5o.

6.5 *In Vivo* and *In Vitro* Resolution of pDB4, pDB5, pDB6 and pDB7 by Tn3 Resolvase.

As the resolution substrates produced were designed to test for resolution *in vivo* by mutants of resolvase, it seemed sensible to first check levels of resolution by wild type Tn3 resolvase, for each plasmid. Also the stability of each plasmid was checked over a number of cell generations.

pDB4, pDB5, pDB6 and pDB7 were each transformed to DS941. After transformation the cells were diluted in L-broth (+ Ap) and grown overnight, with shaking, at 37 °C. The following day samples were removed, plated to Mac Gal Ap plates and incubated at 37 °C overnight. Samples were also diluted 100-fold in fresh L-broth with Ap. These were again grown overnight at 37 °C with shaking. Cells from these cultures were diluted and grown another three times, giving a total of five periods of growth to stationary phase (estimated to be 50 to 100 generations). Samples from each culture were checked by plating to Mac Gal Ap, but no white colonies were detected (dilutions were plated as small colonies or overcrowding of the plate can affect the pH, and hence colony colour). DNA was isolated from plated samples and analysed by gel electrophoresis. No resolution products were observed.

To investigate resolution by Tn3 resolvase DS941 pCIA70 cells were transformed with pDB4, pDB5, pDB6 or pDB7. Transformants were subcultured, to give growth for approximately 60 generations, in L-broth with Ap and Kn, both with and without IPTG. Samples from each culture were plated to Mac Gal Ap Kn, and plasmid DNA was isolated from the remaining cells. Some DNA from each was examined by gel electrophoresis (Fig. 6.6).

Amongst the uninduced samples only resolution product of pDB4 was detected (i.e. a pDB4o sized species), but some pDB4 was still present. Similarly, for the induced samples pDB4o was the only resolution product observed, but in this case no pDB4 was seen, presumably having all been resolved.

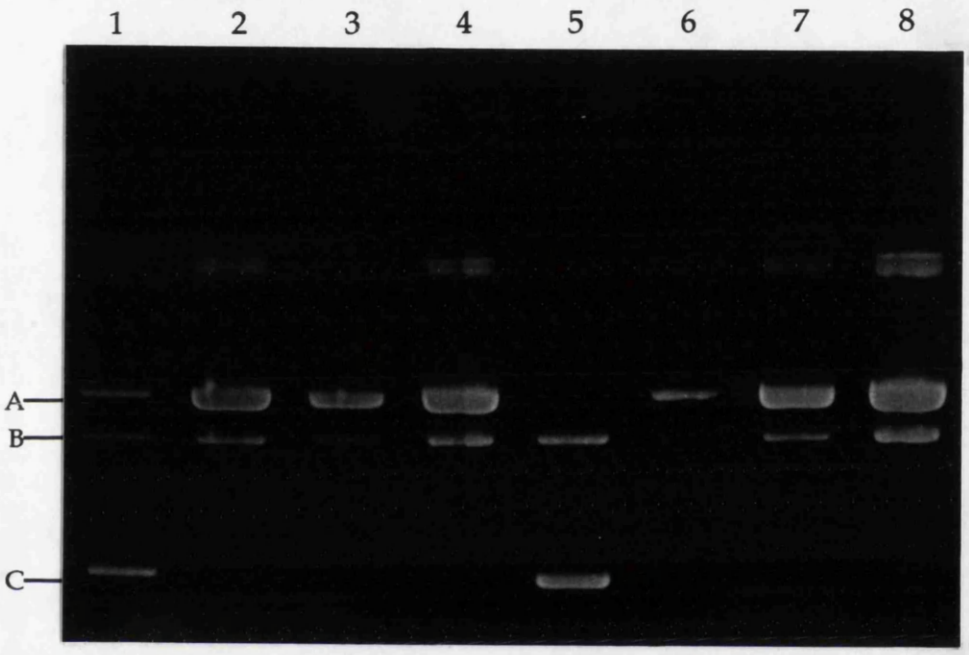


Figure 6.6. ***In Vivo*** **Resolution of pDB4, pDB5, pDB6 and pDB7.**
Strains were grown for approximately 60 generations, with or without induction, prior to isolation of DNA samples.
Lane 1 - DS941 pCIA70 pDB4 (-IPTG)
2 - DS941 pCIA70 pDB5 (-IPTG)
3 - DS941 pCIA70 pDB6 (-IPTG)
4 - DS941 pCIA70 pDB7 (-IPTG)
5 - DS941 pCIA70 pDB4 (+IPTG)
6 - DS941 pCIA70 pDB5 (+IPTG)
7 - DS941 pCIA70 pDB6 (+IPTG)
8 - DS941 pCIA70 pDB7 (+IPTG)
A = supercoiled substrate; B = supercoiled pCIA70; C = supercoiled product.

It was possible that small amounts of resolved product were present in the other samples, but could not be seen on the gel. Any resolution product present could also remain undetected using MacConkey plates if it had not been fully segregated from unresolved copies of the parental plasmid. The sensitivity of this assay can be improved by isolating the plasmid DNA from a sample, transforming to fresh DS941, and plating to Mac Gal Ap. This should increase the probability of isolating any resolution product present. If detection of such products is being inhibited by a very high background of parental plasmid, further steps can be introduced. The isolated plasmid DNA can be cut with a restriction endonuclease (e.g. *Hin* dIII) such that the parental plasmid and *galK*$^+$ product are both linearised, but the *ori*$^+$ product is not cleaved. As linearised DNA is a poor substrate for transformation (compared with supercoiled DNA[92]) any *ori*$^+$ product present will be more easily detected when the digested samples are transformed to DS941, and plated on Mac Gal Ap.

To test for low levels of resolution DNA isolated from the induced and uninduced samples described, was digested for 2 h with an excess of *Hin* dIII. Half of each digest was run on an agarose gel (Fig. 6.7), and the remaining DNA was used to transform DS941 cells. Transformation products were grown on Mac Gal Ap plates, and colonies were scored.

Plasmids in Sample.	IPTG Induction.	Red Colonies (*gal*$^+$).	White Colonies (*gal*$^-$).
pCIA70 + pDB4	-	0	>1500
	+	0	>2000
pCIA70 + pDB5	-	4	0
	+	0	1
pCIA70 + pDB6	-	1	1
	+	0	6
pCIA70 + pDB7	-	9	5
	+	6	20

Table 6.1. ***In Vivo* Resolution Products Detected by Transformation After *Hin* dIII Digestion.**

Plasmid DNA was prepared from some of the different colonies produced by transformation of the *Hin* dIII digested material, and examined by gel electrophoresis (Fig. 6.8). In all cases the DNA from red colonies contained only resolution substrate sized species, and the DNA from white colonies contained only an *ori*$^+$ resolution product sized species. Restriction endonuclease digestion analysis of these DNA samples supported the identification of these species. It is

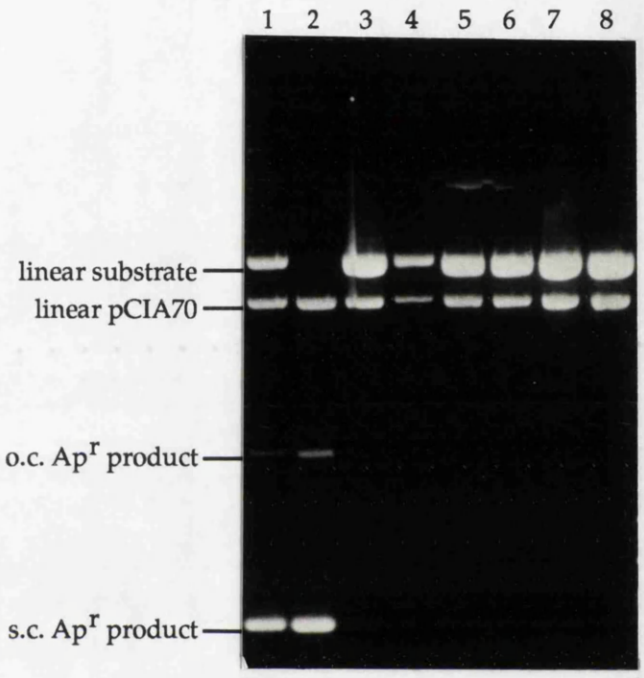


Figure 6.7. ***Hin* dIII Digestion of *In Vivo* Resolution Reactions.**
DNA samples shown in Fig. 6.6 were digested with an excess of *Hin* dIII for 2 h. This should linearise pDB4, pDB5, pDB6, pDB7 and pCIA70, but not pDB4o, pDB5o, pDB6o, pDB7o.

Lane 1 - DS941 pCIA70 pDB4 (-IPTG)
2 - DS941 pCIA70 pDB4 (+IPTG)
3 - DS941 pCIA70 pDB5 (-IPTG)
4 - DS941 pCIA70 pDB5 (+IPTG)
5 - DS941 pCIA70 pDB6 (-IPTG)
6 - DS941 pCIA70 pDB6 (+IPTG)
7 - DS941 pCIA70 pDB7 (-IPTG)
8 - DS941 pCIA70 pDB7 (+IPTG)

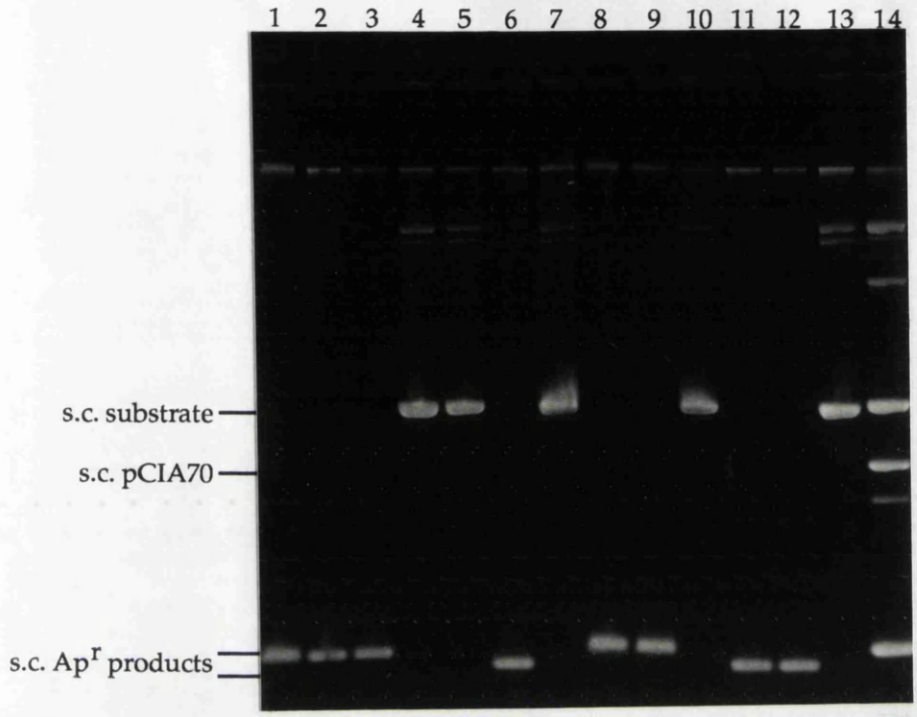


Figure 6.8. ***In Vivo*** **Resolution Products After** ***Hin*** **dIII digestion and Transformation.**

After *Hin* dIII digestion samples (see Fig. 6.7) were transformed to competent DS941. Transformants were plated to MacConkey Gal Ap. See main text (section 6.5) for further details.

Lane	"Reaction"	Induction	Colony Colour
1	pCIA70 pDB4	-	white
2	pCIA70 pDB4	+	white
3	pCIA70 pDB4	+	white
4	pCIA70 pDB5	-	red
5	pCIA70 pDB5	-	red
6	pCIA70 pDB5	+	white
7	pCIA70 pDB6	-	red
8	pCIA70 pDB6	-	white
9	pCIA70 pDB6	+	white
10	pCIA70 pDB7	-	red
11	pCIA70 pDB7	-	white
12	pCIA70 pDB7	+	white
13	pCIA70 pDB7	+	red

Lane 14 - DS941pCIA70pDB4 marker sample.

likely that the red colonies were produced from undigested parental plasmid, as most red colonies were generated from the samples containing the largest amounts of intact resolution substrate.

This method does not give a measure of the relative levels of resolution but does serve to show the presence of rare recombination events. Although these results suggest that some resolution of pDB6 and pDB7 occurs *in vivo*, and that this is probably resolvase dependent, the sample numbers produced are very low. The results produced with pDB5 can not be considered significant. Similar results were produced when a *recA* strain, AB2463, was used instead of DS941.

The same approach was used to study resolution of these plasmids *in vitro*, (to compare with the plasmids pMA21, pAL225, pAL211 and pAL265, from which they were derived). Purified pDB4, pDB5, pDB6 and pDB7 DNA was used for *in vitro* resolution reactions using C9.4 reaction buffer (described in Chapter 2). The reactions were incubated at 37 °C for 18 h and then stopped by heating to 70 °C for 5 min. Each sample was split in two, and one half was digested with *Hin* dIII, after adjusting the NaCl concentration to a suitable level for *Hin* dIII activity. Some digested and undigested material from each reaction was used to transform DS941 cells, which were then plated to Mac Gal Ap plates. The remaining material was run on an agarose gel (Fig. 6.9).

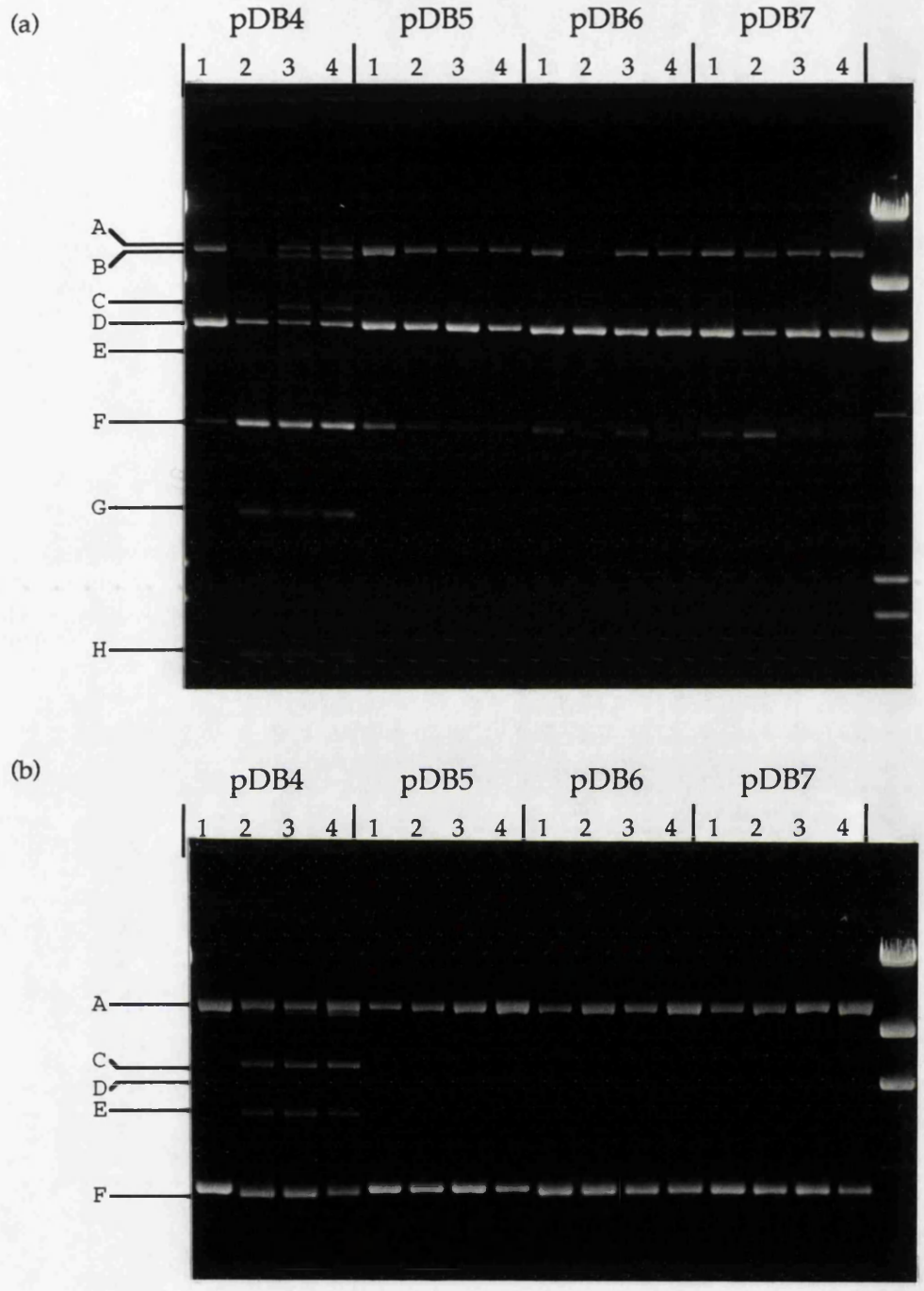


Figure 6.9. ***In Vitro*** **Resolution of pDB4, pDB5, pDB6 and pDB7.**

(a) *Hin* dIII digested material.

(b) Undigested material.

Resolution reactions were in C9.4 buffer at 37 ℃ for 18 h. Substrates were as indicated. Tn3 resolvase dilutions (R14 f46) for each lane were as follows: 1) R.H.B.; 2) 2^{-5}; 3) 2^{-4}; 4) 2^{-3}.

Bands are denoted as follows:

A= o.c. substrate; B= nicked catenane; C= semi-nicked catenane;

D= linear substrate; E= semi-nicked catenane; F= s.c. substrate;

G= o.c. Ap^r product; H= s.c. Ap^r product.

Where present, s.c. catenane or linear *galK* product co-migrate with s.c. substrate.

Resolution Substrate.	Resolvase Dilution.	Red Colonies (*gal*$^+$).	White Colonies (*gal*$^-$).	Plasmids Resolved (%)
pDB4	None	127	0	0
	2^{-5}	44	166	79
	2^{-4}	71	121	63
	2^{-3}	74	134	64
pDB5	None	164	0	0
	2^{-5}	185	0	0
	2^{-4}	159	0	0
	2^{-3}	177	0	0
pDB6	None	270	0	0
	2^{-5}	160	3	2
	2^{-4}	214	8	4
	2^{-3}	122	4	3
pDB7	None	75	0	0
	2^{-5}	256	8	3
	2^{-4}	103	2	2
	2^{-3}	-	-	-

Table 6.2. ***In Vitro*** **Resolution Products Detected by Transformation.**

Resolution Substrate.	Resolvase Dilution.	Red Colonies (*gal*$^+$).	White Colonies (*gal*$^-$).
pDB4	None	38	0
	2^{-5}	31	255
	2^{-4}	16	227
	2^{-3}	33	96
pDB5	None	109	0
	2^{-5}	8	0
	2^{-4}	16	0
	2^{-3}	10	0
pDB6	None	8	0
	2^{-5}	16	5
	2^{-4}	15	9
	2^{-3}	2	7
pDB7	None	33	0
	2^{-5}	16	1
	2^{-4}	13	12
	2^{-3}	2	19

Table 6.3. ***In Vitro*** **Resolution Products Detected by Transformation After** ***Hin*** **dIII Digestion.**
Again, red colonies are probably the result of transformation of DS941 by undigested resolution substrates.

Analysis of the resolution reactions by gel electrophoresis shows that about 80% of the pDB4 substrate has been converted to resolution product (Fig. 6.9). On close inspection of the original gel photographs some resolution products were also visible in the pDB6 and pDB7 reaction tracks, but no recombinant material was observed with pDB5. These results, in general, agree with the levels of resolution measured by detection of the *galK* reporter, after transformation (Table 6.2). Even the most sensitive method of detection used (transformation after *Hin* dIII digestion) failed to show resolution products of pDB5 (Table 6.3). The plasmid content of gal^+ and gal^- isolates was confirmed by gel electrophoresis of purified DNA, as described previously.

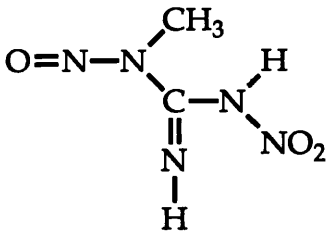
The levels of *in vitro* resolution observed here concur with those previously reported using related substrates[8], but products of *in vivo* recombination between an isolated subsite I and a *res* site were not detected previously.

6.6 Production and Screening of Mutants of pCIA70.

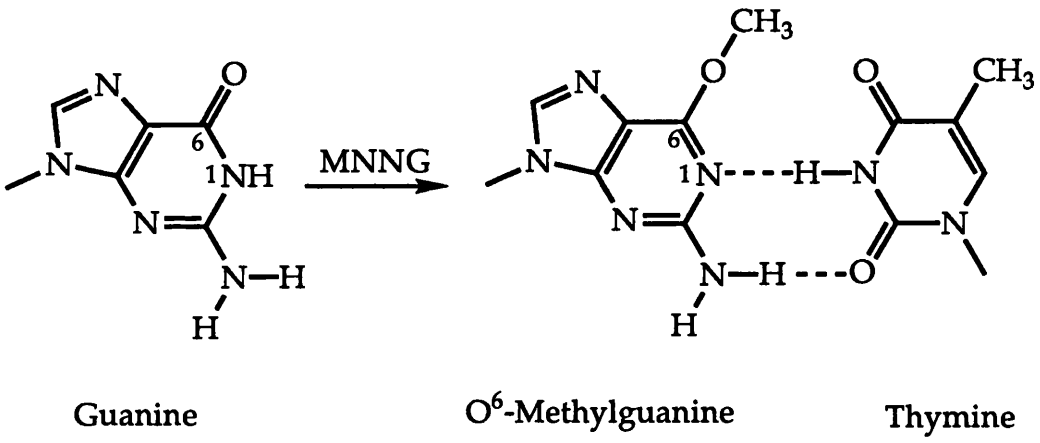
At the time of choosing a method of mutagenesis, directed (or site-specific) approaches were ruled out for three main reasons. Mutations thought to be responsible for FIS-independent phenotypes in Gin were not located in specific regions of the protein, in particular GinKR162 was believed to produce the strongest FIS-independent phenotype yet lay in the proposed DNA binding motif (however it was later shown that this phenotype was the result of a second substitution, GinMV114 , and that KR162 alone did not affect recombination). Secondly, some substitutions giving rise to FIS-independent Gin were already present at equivalent positions of the resolvase sequence, e.g. GinFV104. Finally, for the reasons listed in section 6.2, it was considered preferable to choose a mutagenesis protocol compatible with pCIA70, rather than trying to create new resolvase expression vectors for a particular mutagenesis approach.

In vivo chemical mutagenesis of a double stranded DNA template, using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Fig. 6.10), was used to produce FIS-independent mutants of Cin[26]. MNNG mutagenesis produces transitions[57,66] (i.e. G•C base pairs to A•T, and T•A to C•G; see Fig. 6.10) and so the possible range of coding changes produced is limited, but several FIS-independent derivatives of Cin were created. Hence MNNG seemed a reasonable candidate for initial attempts at mutagenesis of pCIA70.

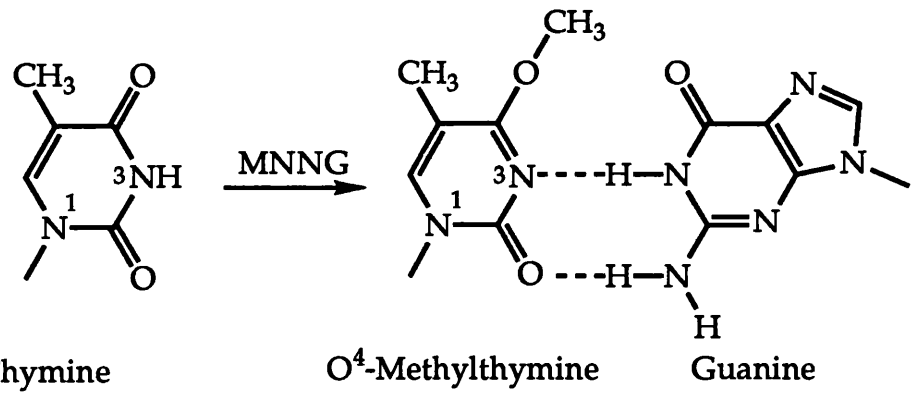
DS941 pCIA70 was treated with MNNG (Chapter 2) at a range of concentrations (0, 50, 100 or 200 μg/ml final) for a range of times (0, 10, 30 or 60 min). The mutagenised DS941 pCIA70 samples were grown overnight and then pCIA70



(a) *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)



(b) G•C ⟶ A•T



(c) T•A ⟶ C•G

Figure 6.10. Principal Consequences of Mutagenesis by MNNG. (from Miller, 1992)

(a) Chemical structure of MNNG.
(b) G•C to A•T Transition.
(c) T•A to C•G Transition.

DNA was isolated. Some pCIA70 DNA from each sample was transformed into DS941 pDB4 and plated to Mac Gal Ap Kn + IPTG, to assay for levels of mutagenesis. The pCIA70 from cells which were given a 10 min exposure to 200 μg/ml MNNG produced 20 to 30% red colonies. Exposure to less MNNG corresponded to a lower proportion of red colonies, and longer exposure corresponded to significantly fewer transformants. The DNA content of cells from these plates was checked. Red colonies contained little or no pDB4o (Fig. 6.11), suggesting reduced or abolished resolution activity due to the MNNG treatment of pCIA70. Mutations produced may have affected the function or expression of resolvase, but not the stability of pCIA70, as isolates were maintained on medium containing Kn. The number of independent *tnpR*$^-$ mutations produced was not determined.

The remaining pCIA70, treated with 200 μg/ml MNNG for 10 min, was used to transform DS941 pDB5. Following transformation, the cells were diluted in phage buffer (Chapter 2) and plated to Mac Gal Ap Kn IPTG medium. The samples were diluted to prevent overcrowding of plates, which could give small colonies which would not respond correctly to the screening method used. Approximately 40,000 colonies were screened.

Thirty-nine white (or very pale pink) colonies were isolated, and streaked to fresh Mac Gal Ap Kn plates. The sizes of plasmids present in each isolate was checked by gel electrophoresis of purified DNA. Four isolates contained plasmids corresponding to the size of pDB5o (but also contained some pDB5). The pCIA70 derivative from each was transformed to DS941, grown on Mac Gal Kn plates and then replica plated to Mac Gal Ap Kn. Kn^r Ap^s cells were pooled for each isolate, grown in L-broth with Kn, and used to prepare pCIA70 DNA (this should eliminate any pDB5o from later steps). This material was used to transform DS941 pDB4 or DS941 pDB5. Transformants were grown on Mac Gal Ap Kn IPTG plates. In all cases the transformants which had contained pDB4 gave exclusively white colonies, and those which had contained pDB5 gave red colonies. The plasmid content was analysed (by gel electrophoresis) and showed complete resolution of pDB4, but no recombination of pDB5.

Mutagenesis of pCIA70 was repeated using a 10 min treatment with 200 μg/ml MNNG, but desired resolvase mutants were not detected.

6.7 Production of a New Resolvase Expression Vector, to Allow Directed Mutagenesis.

When it became apparent that all the FIS-independent Gin (and enhancer-

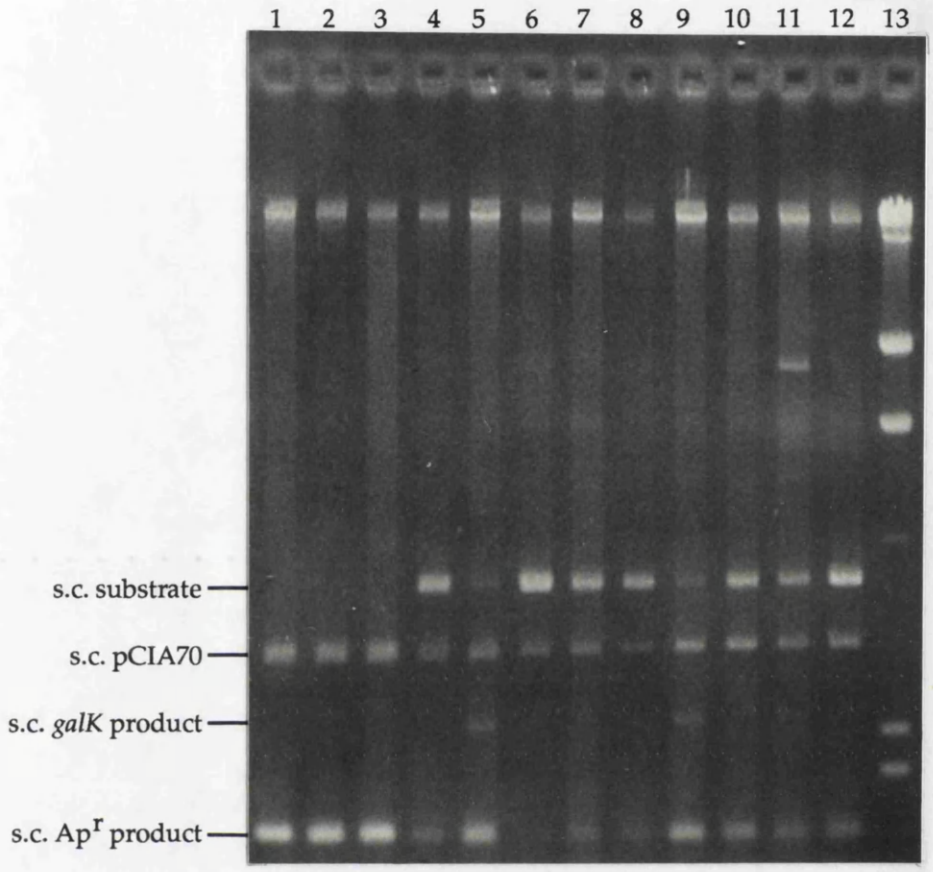


Figure 6.11. Screening of Mutagenised pCIA70 Using pDB4.
MNNG treated (200 µg/ml MNNG for 10 min) pCIA70 was used to transform competent DS941pDB4. Transformants were plated to MacConkey Gal Ap Kn +IPTG, and grown overnight. Red and white colonies were patched to fresh plates and then analysed by 'single colony' gel electrophoresis.

Lane	Colony Colour	pDB4 resolution
1	white	complete
2	"	"
3	"	"
4	red	very poor
5	"	poor
6	"	absent
7	"	very poor
8	"	"
9	"	poor
10	"	very poor
11	"	"
12	"	"

Lane 13 - *Hin* dIII digested λ DNA marker.

independent Cin) mutations were in the same area of protein sequence, it seemed preferable follow a mutagenesis strategy which was directed to the equivalent region of Tn3 resolvase.

To allow this, a 1.3 kb *tnpR*$^+$ *Eco* RI/*Hin* dIII fragment was subcloned (from pMA6111) between the *Eco* RI and *Hin* dIII sites of pSelect-1[62], to produce pDB6001 (Fig. 6.12). In addition to its pBR322 replication origin, the pSelect-1 plasmid contains a single stranded phage origin of replication, allowing preparation of single stranded circular DNA, which is an appropriate template for directed mutagenesis. In pDB6001 the resolvase reading frame was placed downstream of a T7 RNA polymerase promoter, which should not be recognised by *E. coli* RNA polymerases. T7 RNA polymerase was supplied using a strain (BL21) carrying a λ lysogen (DE3) which expresses T7 RNA polymerase from an IPTG-inducible promoter. Hence resolvase expression from pDB6001 would be expected only when present in BL21 DE3, grown in the presence of IPTG. In practice some T7 RNA polymerase is produced in BL21 DE3 without IPTG induction, and so some resolvase expression would be expected from uninduced cells. Resolvase expression from pDB6001 was tested in two ways. Competent DS941, BL21 and BL21 DE3 cells were transformed by pDB6001 and grown with and without IPTG induction. BL21 DE3 containing pDB6001 grew poorly under induction, a feature associated with resolvase overexpression from pMA6111. Whole cell extracts were prepared for all the induced and uninduced samples (including DS941, BL21 and BL21 DE3 without pDB6001) and analysed by denaturing PAGE (Fig. 6.13). The extract from induced BL21 DE3 pDB6001 showed a very slight increase in a band which ran in the same position as a purified resolvase marker. The level of expression was much less than that observed with pMA6111. Varying the growth time or point of induction may have improved this.

pLS134 is a Cm^r plasmid which contains two directly repeated copies of Tn3 *res*[86]. It is compatible with pDB6001 and so was used as a substrate for *in vivo* resolution, to test for expression of resolvase from pDB6001. Competent DS941, BL21 and BL21 DE3 cells were first transformed with pLS134. Next pDB6001 was used to transform DS941, BL21 and BL21 DE3, both with and without pLS134. Samples were grown overnight, with or without IPTG induction, and the plasmid content of each sample was checked by gel electrophoresis of isolated DNA (Fig. 6.14). pLS135, the *ori*$^+$ resolution product of pLS134, is approximately 4 kb in size, and pLS134 is about 6 kb[86].

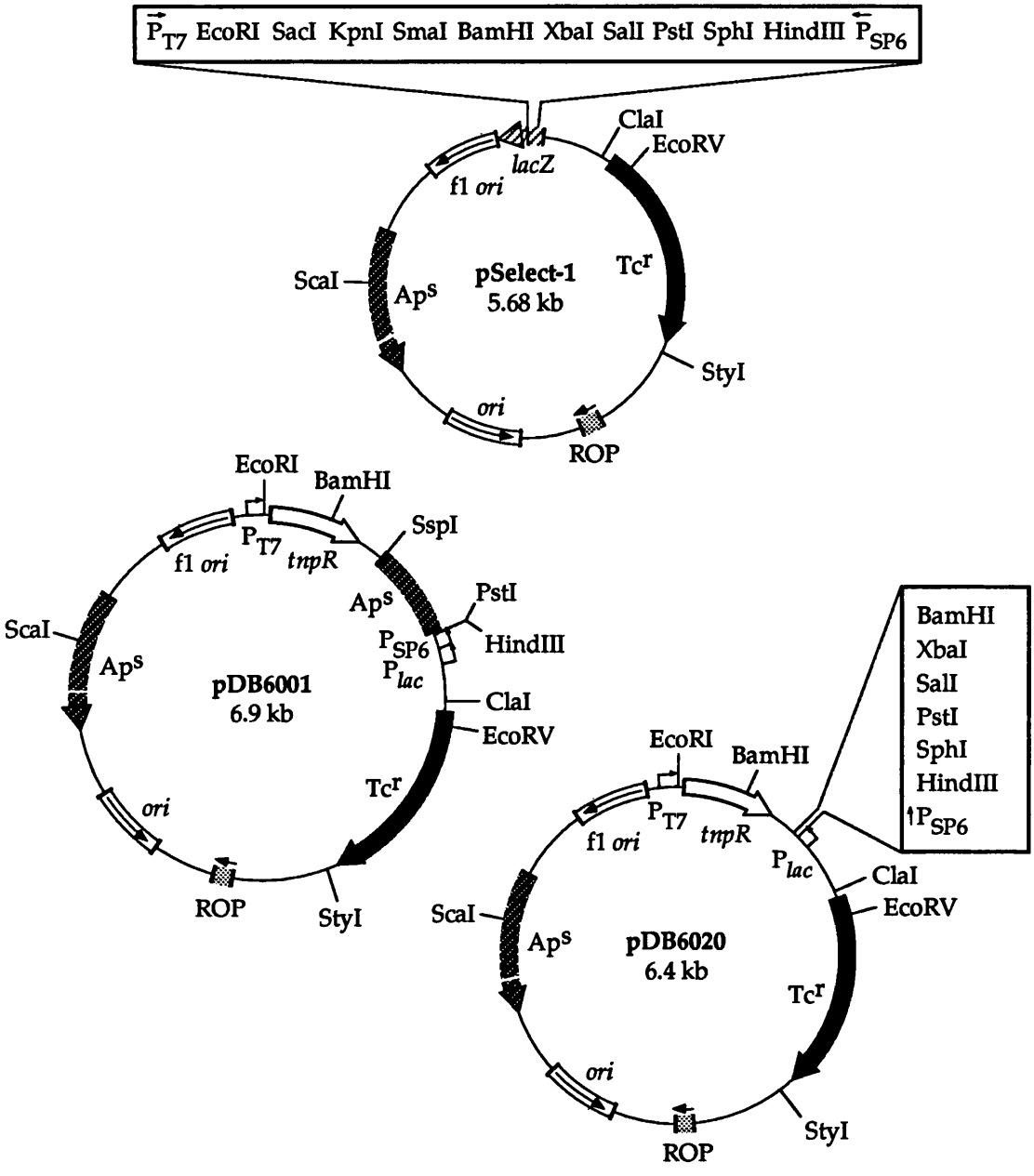


Figure 6.12. Maps of pSelect-1 and of Resolvase Expression Vectors pDB6001 and pDB6020.

To prevent overcrowding some promoter elements are shown, along with restriction enzyme sites, in polylinker boxes. The direction of transcription from these elements (when active) is indicated by a small arrow.

The *βla* gene (Ap^r) in each plasmid contains a single base substitution which removes a *Pst* I site and abolishes gene function. The approximate position of this substitution is denoted by a white line in the gene symbol. Other *βla* sequences are present in pDB6001, but this truncated gene does not confer ampicillin resistance.

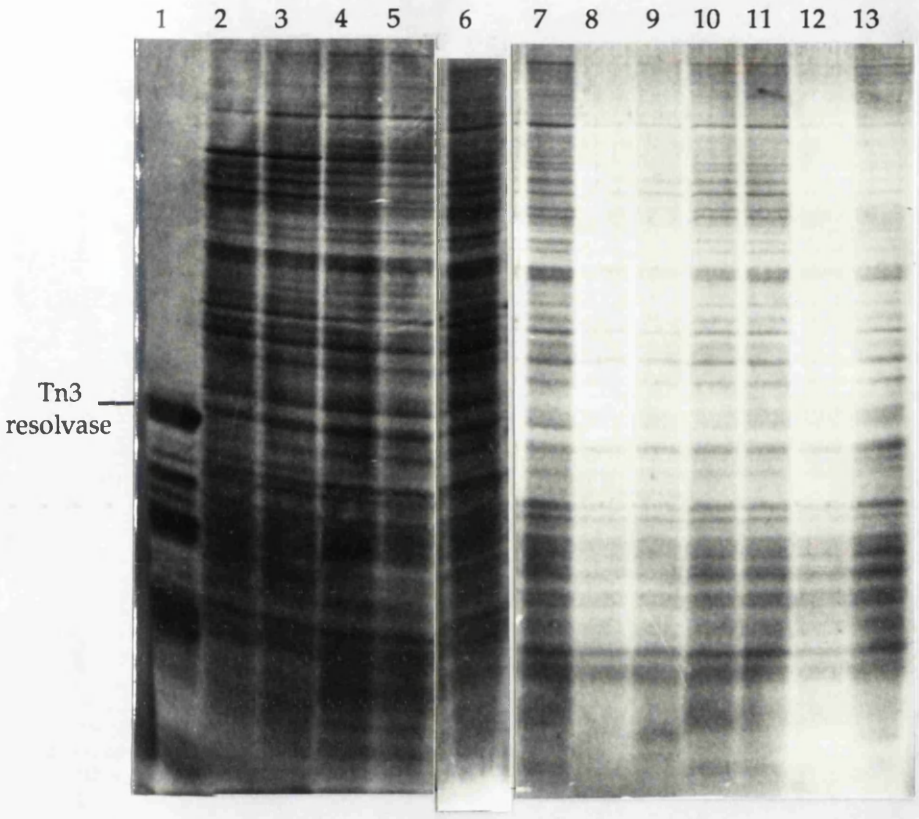


Figure 6.13. Expression of Tn3 Resolvase from pDB6001.

Samples were grown to stationary phase, diluted 10-fold in media with or without IPTG, and then grown for 2 h further. Cells from each sample were harvested, lysed, and then analysed by denaturing PAGE.

Lane	Sample
1	1 µl of R14 f46 Tn3 resolvase marker.
2	DS941
3	DS941 +IPTG
4	BL21
5	BL21 +IPTG
6	BL21 DE3
7	BL21 DE3 +IPTG
8	DS941 pDB6001
9	DS941 pDB6001 +IPTG
10	BL21 pDB6001
11	BL21 pDB6001 +IPTG
12	BL21 DE3 pDB6001
13	BL21 DE3 pDB6001 +IPTG

As mentioned previously, growth of BL21 DE3 pDB6001 was very poor in the presence of IPTG. In this case the same strain also grew poorly without IPTG induction. No resolution of pLS134 was observed in any of the strains used, without the presence of pDB6001. Surprisingly, inclusion of pDB6001 gave complete resolution of pLS134 in all three strains examined, regardless of IPTG induction. One possible explanation would be if DS941 and BL21 both contained a source of T7 RNA polymerase. However, other expression systems driven by T7 RNA polymerase have been shown to be inactive in DS941 (Fig. 6.14). An alternative explanation is that resolvase expression, in DS941 and BL21, is being driven by another promoter, upstream of the T7 promoter. No obvious candidate promoters were shown on maps of pSelect-1 but similar Tn7 polymerase-independent expression has been observed using other pSelect-1 derivatives (S. Bell; pers. comm.).

In some respects this basal expression of resolvase was fortuitous. As T7 RNA polymerase-directed expression of resolvase from pDB6001 inhibits cell growth, it is unsuitable for *in vivo* resolution assays. However pDB6001 in DS941 appears to give an acceptable resolution activity for screening of resolvase mutants.

6.8 Production of pDB6001 Compatible *In Vivo* Resolution Substrates.

Unfortunately pDB4, pDB5, pDB6 and pDB7 are incompatible with pDB6001, as they all have pBR322 replication origins. It was decided to produce derivatives of pDB4, pDB5, pDB6 and pDB7 which were compatible with pDB6001 but would allow the same general screening method to be used. The following features were considered desirable in the new set of resolution substrates;

(i) pBR322-compatible origin of replication.

(ii) Antibiotic resistance marker other than Tc^r or Ap^r. pDB6001 is Tc^r and would be selected as Ap^r, following the pSelect-1 mutagenesis strategy.

(iii) Low plasmid copy number. Selection of *gal*$^-$ cells containing *galK*$^-$ resolution products would be easier if segregation from a large number of unresolved resolution substrates was not required prior to detection.

The pSC101 plasmid occurs naturally in *Salmonella panama*, but can stably maintain six to seven copies per chromosome equivalent, in *E. coli*[51]. Although pSC101 encodes Tc^r (this is the origin of the Tc^r gene of pBR322) it is otherwise compatible with pBR322, and hence pDB6001 also. A 2,213 bp *Hinc* II/*Rsa* I fragment of pSC101, containing the replication region (for replication and partitioning of pSC101), was purified, and *Bam* HI linkers were attached at each end (by S. Rowland). The *Bam* HI fragment was ligated to a *Bam* HI fragment

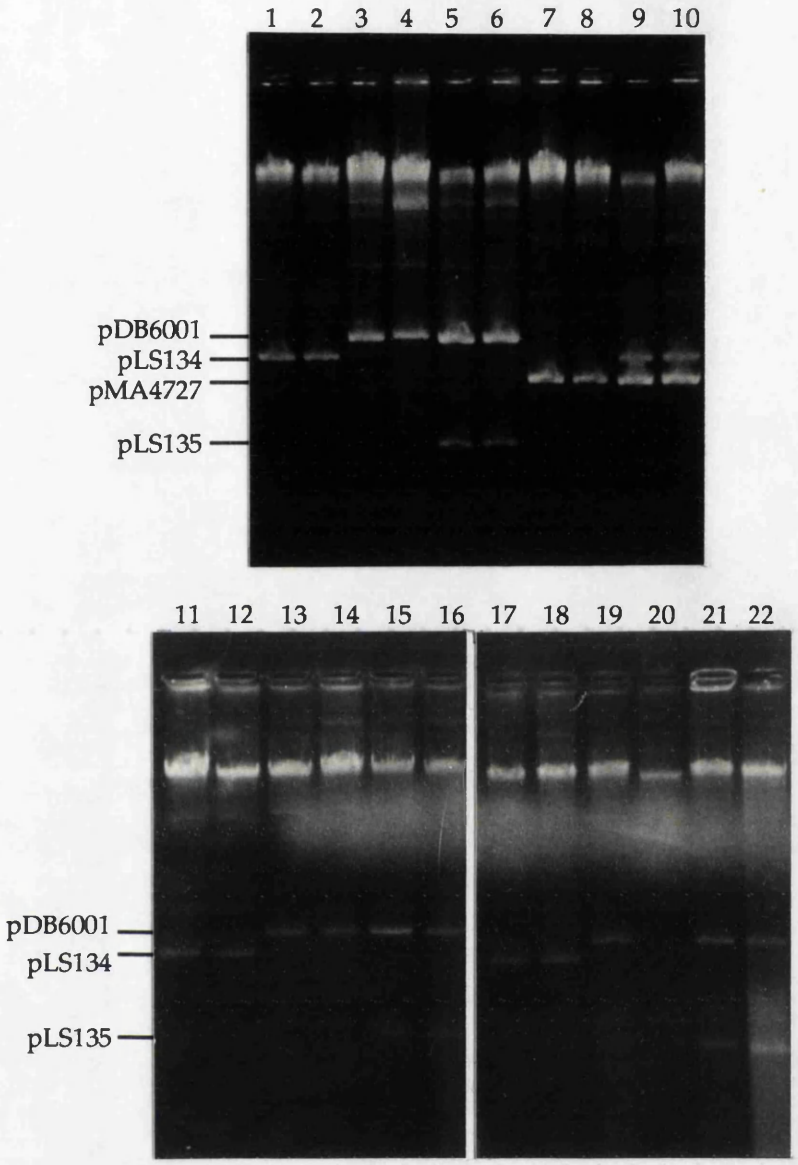


Figure 6.14. ***In Vivo*** **Resolution of pLS134 by pDB6001.**
Strains with or without the resolution substrate pLS134, were transformed by pDB6001 (or pMA4727) and plated to selective media. Transformants were patched to fresh plates, grown, and then analysed by 'single colony' gel electrophoresis. pLS135 is the *ori*$^{+}$ resolution product of pLS134. pMA4727 is an expression vector with resolvase expressed from a T7 polymerase promoter.

Lane	Sample	Lane	Sample
1	DS941 pLS134	11	BL21 pLS134
2	DS941 pLS134 +IPTG	12	BL21 pLS134 +IPTG
3	DS941 pDB6001	13	BL21 pDB6001
4	DS941 pDB6001 +IPTG	14	BL21 pDB6001 +IPTG
5	DS941 pDB6001 pLS134	15	BL21 pDB6001 pLS134
6	DS941 pDB6001 pLS134 +IPTG	16	BL21 pDB6001 pLS134 +IPTG
7	DS941 pMA4727	17	BL21DE3 pLS134
8	DS941 pMA4727 +IPTG	18	BL21DE3 pLS134 +IPTG
9	DS941 pMA4727 pLS134	19	BL21DE3 pDB6001
10	DS941 pMA4727 pLS134 +IPTG	20	BL21DE3 pDB6001 +IPTG
		21	BL21DE3 pDB6001 pLS134
		22	BL21DE3 pDB6001 pLS134 +IPTG

(~1.5 kb) of pUC4K, which carries a Kn^r gene. This generated two, similar Kn^r plasmids, pDB703 and pDB704 (Fig. 6.15). The difference between pDB703 and pDB704 is the relative orientation of the two cloned DNA fragments. Partial *Pst* I digestion, followed by complete *Nde* I digestion, of pDB703 and pDB704 allowed the production of two 3.06 kb DNA fragments, containing the pSC101 replication region and the Kn^r gene from pUC4K. Each of these fragments was ligated to a 5.5 kb *Nde* I/*Pst* I fragment (carrying both *res* (sub)sites and the *galK* cassette) of pDB4, pDB5, pDB6 or pDB7. The resulting plasmids were named as shown in Table 6.4.

Resolution Substrate Derived From:		Substrate Produced:
pDB703 +	pDB4	pDB34
	pDB5	pDB35
	pDB6	pDB36
	pDB7	pDB37
pDB704 +	pDB4	pDB44
	pDB5	pDB45
	pDB6	pDB46
	pDB7	pDB47

Table 6.4. Naming of New Resolution Substrates.

Constructs were made from both pDB703 and pDB704 in case transcription of the Kn^r gene in one direction caused problems. All substrates produced appeared viable and stable in DS941.

In vivo resolution of each new substrate was tested in DS941 pDB6001. Competent DS941 was transformed with one of each of the new substrates. These transformants were then transformed with pDB6001, and grown on Mac Gal Tc Kn plates. Cells containing pDB6001 with pDB34 or pDB44 gave white colonies (resolution products), but no red colonies. No resolution was detected using the other substrates. DS941 containing pSC101 derived resolution substrates, but no pDB6001, gave only red colonies. Sample colonies were picked and analysed by the 'single colony gel' method. In all cases red colonies showed no resolution products, and white colonies contained a resolution product sized species, but no resolution substrate.

6.9 Directed Mutagenesis of pDB6001.

pDB6001 was mutagenised following the pSelect-1 protocol (Chapter 2). The two mutagenic oligonucleotides used were a 27 bp 'Ampicillin Repair

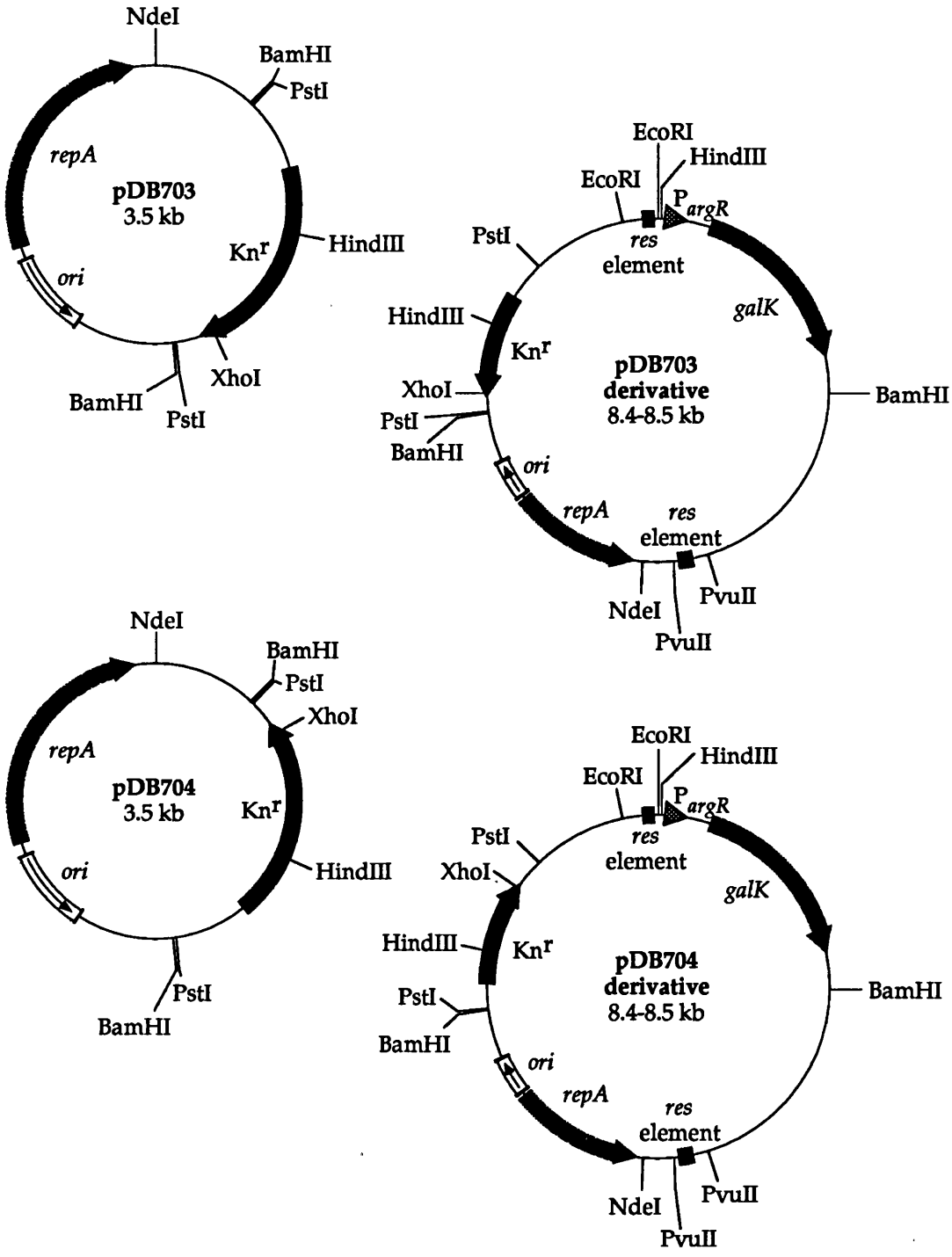


Figure 6.15. ***In Vivo*** **Resolution Substrates, produced from pSC101 Derivatives pDB703 and pDB704.**

Maps are shown for pDB703 and pDB704. Next to each map is a diagram showing the common features of the resolution substrates produced. The relative orientation of *res* sites in each plasmid is the same as for the substrate from which it was derived. For further details see Table 6.4 and Fig. 6.3.

Oligonucleotide' (identical to that supplied with the 'Altered Sites' kit[62]) and a 66 bp degenerate oligonucleotide, designed to mutate codons 105 to 123 of Tn3 resolvase (Chapter 2). Both oligonucleotides are complementary to the single stranded DNA produced from the f1 replication origin of pDB6001.

A single base change has been engineered in pSelect-1, such that a *Pst* I site is removed and the *βla* gene no longer produces a functional enzyme. If the ampicillin repair oligonucleotide is annealed, and used to prime transcription, plasmids produced from the newly replicated strand are Ap^r. After *in vitro* replication, the mismatched plasmid is transformed to a repair deficient strain of *E. coli*, and eventually Ap^r transformants are selected. If the second mutagenic oligonucleotide anneals correctly, then most Ap^r plasmids produced contain its new sequence also. The first (5') 6 bases of the 66 bp degenerate oligonucleotide were synthesised as for a normal oligonucleotide, and so should not produce any mutations. The next 54 bases were synthesised using phosphoramidite reagents, each contaminated with small amounts of the three other nucleotide precursor reagents, such that an incorrect base should be inserted at an average of once per complete DNA molecule produced[31]. The final 6 bases were synthesised using uncontaminated reagents.

These two oligonucleotides were used to mutagenise pDB6001, but the Ap^r products isolated were all smaller than pDB6001, and did not contain an *Xho* I site introduced by the incorporation of the degenerate oligonucleotide. The $tnpR^+$ fragment inserted to pSelect-1 to give pDB6001 (Fig. 6.12), also contained most of the Tn3 *βla* gene (from the 5' end through to the *Pst* I site). This sequence is in the opposite orientation to the mutagenised *βla* of pSelect-1, and so large regions of complementary sequence will exist on each strand of pDB6001. Results of restriction analysis of the mutagenised pDB6001 species were consistent with their having arisen from rearrangement between the two, non-functional *βla* sequences present, to generate an Ap^r product.

To overcome this problem the $tnpR^+$ *Eco* RI/*Ssp* I fragment (~0.9 kb) of pDB6001 was subcloned between the *Eco* RI and *Sma* I sites of pSelect-1, to give pDB6020 (Fig. 6.12). This fragment contains no Tn3 *βla* coding sequences. *In vivo* resolution of pDB34, pDB35, pDB36, pDB37, pDB44, pDB45, pDB46 and pDB47 by pDB6020 was as observed for pDB6001. However time did not permit further attempts at site-directed mutagenesis using pDB6020.

6.10 Conclusions.

Plasmids were produced to allow positive selection for *in vivo* resolution of a pair

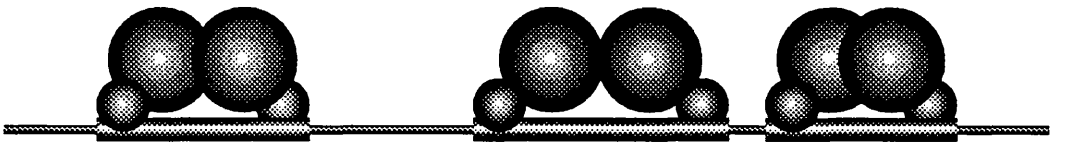
of *res* sites; a *res* site and an isolated subsite I; and a pair of isolated subsite I elements. Using these plasmids no resolution of two isolated subsite I elements was detected *in vivo* or *in vitro*. Resolution with subsite I and a *res* site was detected at less than 1% of the level observed with a two *res* substrate, both *in vivo* and *in vitro*.

Chemical mutagenesis of resolvase expression vector pCIA70 did not produce resolvase mutants capable of directing *in vivo* resolution of a substrate containing two isolated subsite I elements.

A resolvase expression vector suitable for directed mutagenesis, pDB6020, was produced. Compatible resolution substrates carrying either two *res* sites, one *res* site and an isolated subsite I, or two subsite I elements, were also produced to allow screening of mutagenised pDB6020.

Chapter Seven

Discussion



7.1 Stoichiometry of complexes of Tn3 resolvase with *res*, subsite II, subsite (II + 10) and subsite (II + 5).

Possible sources of error in the data used to determine stoichiometry values of complexes are discussed in Chapters 4 and 5. Relative stoichiometry values for complexes containing large amounts of material (e.g. complexes 1 and 2 for subsite (II + 10) or (II + 5)) should be least affected by such errors. The relative stoichiometry results suggest that the number of resolvase units per DNA site in each complex 2 is double that of complex 1. This resolvase/DNA value increases linearly for sequentially numbered complexes of *res*, with the possible exception of complex 5. The increase in the resolvase to DNA ratio between complexes 4 and 6 is the same as that between complexes 2 and 4, and 1 and 3. Results produced for complex 3 of subsite II and (II + 10) and for complexes 3 and 4 (combined) of subsite (II + 5) did not seem to fit so well to this general pattern. This may reflect genuine differences in the stoichiometry of (and nature of binding in) these complexes.

As calculated for complexes 1 and 2, the ratio of resolvase units per DNA site is 0.5:1. This is consistent with a resolvase unit which binds one DNA fragment to give complex 2 but makes contact with two DNA fragments in complex 1. This is unlikely as synapsed complexes would be expected to be more retarded than the complexes described. No complexes of intermediate mobility were detected in band shift assays containing different sized DNA fragments, carrying the same binding site. If the complexes are not generated by resolvase binding to more than one DNA fragment at a time (or by binding of truncated resolvase) then the binding unit must be a whole number of monomers, and the absolute stoichiometry of complex 2 must be 2, 4 or a larger even integer.

Even taking into account the difficulties of accurately determining resolvase and DNA specific activities, the absolute stoichiometry value for each complex 2 (Table 5.17) is clearly 2, rather than 4. Hence the binding unit is a resolvase monomer. For *res*, subsite II, subsite (II + 10) and subsite (II + 5) complex 1 has 1 resolvase monomer per DNA fragment, and complex 2 has two monomers per DNA site. Complexes 3, 4 and 6 of *res* have, respectively, 3, 4 and 6 resolvase monomers per DNA (complex 5 is expected to fit this pattern by containing 5 resolvase monomers per *res* site). Based on the relative stoichiometry values produced, complex 3 of subsite II and of subsite (II + 10) are expected to contain more than 2 resolvase monomers (possibly 3) per DNA molecule. The third complex isolated from subsite (II + 5) is also expected to have more than two

monomers per DNA molecule, and may have exactly three resolvase units per site. Alternatively the value determined may represent an average ratio produced from two complexes (complex 3 and 4, described in Chapter 3), which could not be differentiated in the large scale experiment used, but have different stoichiometries.

Subsites (II + 10) and (II +5) both produce large amounts of monomer bound complex, relative to subsite II. A time course of binding to subsite II showed similar levels of complex 1 in all samples, but complex 2 levels increased with time. The observed pattern of complex formation is consistent with complex 1 being an intermediate in the formation of complex 2 from unbound DNA (Fig. 3.3d). If complex 1 were only produced by dissociation of complex 2 (Fig. 3.3c) then the amounts of each complex would be expected to be directly related. In an equivalent analysis of binding to subsite (II + 10), levels of complex 1 and 2 both increased with time. These complexes could result independently from binding of resolvase monomer and dimer, respectively. Alternatively complex formation may be similar to that described for subsite II, but without the same degree of co-operativity of binding. As subsite (II + 10) complex 1 and 2 levels both increase with time, some or all of complex 1 could be a breakdown product of complex 2 (Fig. 3.3c).

7.2 Resolvase-Induced Bending of Subsites II and (II + 10).

Binding of a resolvase monomer to subsite II or to subsite (II + 10) produces a bend at the centre of the subsite. This may result from shuffling of the resolvase monomer (between positions on the subsite) producing bending at symmetrical points within the site, to give an averaged central bend (Fig. 7.1). With or without shuffling a resolvase monomer bound to part of the subsite could produce a bend directly at the subsite centre (or several bends, with an average at the centre). However, if resolvase binds primarily to sequences at the end of the subsite then the bend position of subsite (II + 10) would be expected to be displaced from that of subsite II, unless shuffling occurs, or the resolvase makes DNA contacts on both sides of the inserted sequences. As the actual position of resolvase-induced bends is not known it is difficult to interpret the slight increase in bending observed with subsite II complex 2. Similar analysis of subsite (II + 5) (or multiple copies of other subsites) may reveal information about the phase of the bend, or bends produced[49]. Resolvase-induced bending of *res*[15], and of subsite I[7] was previously demonstrated. It appears that induced bending by binding of a monomer of resolvase is a common feature of binding to each subsite of *res*.

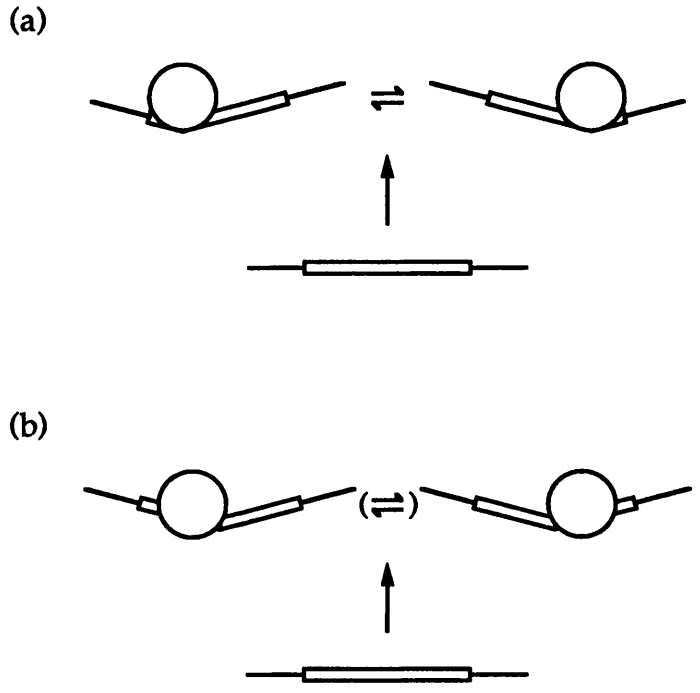


Figure 7.1. Resolvase-Induced Bending of a Subsite in Complex 1
Resolvase is represented by circles, and the DNA by lines, with boxes indicating the subsites.

(a) Binding of a resolvase monomer, inducing a bend at the point of binding. Shuffling produces bending at each side of the subsite, giving an average bend positioned at the subsite centre.

(b) Binding of a resolvase monomer at one point of the subsite, producing a bend at the centre of the subsite. This could be the result of several bends, with an average at the centre, or of resolvase binding to several parts of the subsite, either side of the centre. It is not possible to portray the range of potential contacts made, or bends induced. This model neither requires nor excludes the possibility of shuffling.

7.3 Binding of Resolvase to a Partial Subsite.

When complex 1 of subsite (II + 10) and complex 1 of subsite (II + 5) were each shown to contain monomer-bound DNA it seemed possible that high yields of a monomer-bound species might be generated by binding to a DNA fragment containing half of subsite II (if subsite II complex 1 material does not accumulate because of rapid conversion to a dimer bound species). However no complexes were observed with approximate half-sites of a modified subsite (subsite IIBN). This may partly be the result of DNA substitutions made to allow the isolation of half-sites, as binding to the whole modified subsite was poor (compared to subsite II). Comparison with mutations made in γδ subsite I[68] suggests that the mutations made to the right side of subsite II should not significantly reduce binding to that half. It has been shown that a DNA fragment containing more than half of Tn3 subsite I (right end 20 bp, of 28 bp total) generates only one faint complex with Tn3 resolvase[7]. The production of high yields of monomer-bound DNA may require more than one resolvase monomer target sequence per DNA fragment, to allow increased complex stability via shuffling. Alternatively a resolvase monomer bound at one 'half' of a subsite may normally contact sequences beyond the centre of the subsite. If shuffling does occur then the reduced binding of Tn3 resolvase to subsite IIBN could be accounted for by a reduced affinity of resolvase for the left end, and hence for the whole site.

7.4 Positioning of Resolvase on Subsites II, (II + 10) and (II + 5).

DNase I footprinting of subsites II, (II + 10) and (II + 5) does not yield information about specific complexes, but did show cleavage protection and enhancement at equivalent positions in each subsite. Where examined, much of the additional sequence at the centre of subsites (II + 10) and (II + 5) was also protected by resolvase, without resulting in exposed regions not present with subsite II. For subsites II, (II + 10) and (II + 5) the strongest DNase I cleavage enhancement was at bonds between R1A and R2C, and between L4C and L6A (see Fig. 3.20). Positions R2G and L5G (on the complementary strand; see Fig. 3.23) were shown, by methylation interference experiments, to be required for binding of resolvase. Methylation of L5G prevented any complex formation, but R2G is not required in the production of complex 1. This suggests that complex 1 of subsites II, (II + 10) and (II + 5) may be generated by a resolvase monomer binding to the left hand side of the site (i.e. no shuffling). If shuffling does occur then the resolvase bound to give complex 1 may not make the same contacts as that in complex 2 (i.e. R2G is not required for complex 1). Previous methylation protection experiments have shown G residues at these (or adjacent) positions to be important in binding to each end of subsites I, II and III[15,25]. Methylation

inhibition analysis of the complementary strand of each subsite should show if resolvase in complex 1 makes different contacts from that in complex 2 (i.e. whether L2G is required for complex 1).

7.5 Comparison of Binding of Tn3 Resolvase and γδ Resolvase.

As has been mentioned γδ resolvase is believed to be predominantly dimeric in solution[34], and to bind to subsites of *res* as a dimer. Comparison of oxidised and reduced samples of the γδ mutant resolvase M106C by denaturing PAGE clearly demonstrated that a dimer sized species can be created as a result of the M106C substitution. However some monomer sized resolvase was present in the oxidised sample. The substitution in γδ M106C may alter its ability to form unlinked 1,2 dimers, compared with wild type γδ resolvase. The similarity of the pattern of complexes generated by binding of γδ resolvase and γδ M106C was striking. All complexes generated with γδ resolvase were also produced with γδ M106C, although the titration range of γδ M106C appeared to be a little wider.

Table 7.1 shows the mobility of complexes generated by Tn3 or γδ resolvase with subsites II, (II + 10) and (II + 5), relative to the mobility of the unbound DNA. The same subsite DNA fragments were used with each resolvase. The complexes produced using γδ M106C ran in the same position as those for wild type γδ resolvase.

Binding Site.	Complex Number.	Mobility (mm).		Relative Mobility.		$Tn3_{Rm}/\gamma\delta_{Rm}$
		Tn3	γδ	Tn3	γδ	(%)
subsite II	3	-	62	(48.35)	48.06	(100.60)
	2	67	75	55.83	58.14	96.03
	1	86	100	71.67	77.52	92.45
	0	120	129	100.00	100.00	100.00
subsite II+10	3	50	55	48.08	47.83	100.52
	2	59	69	56.73	60.00	94.55
	1	74	88	71.15	76.52	92.99
	0	104	115	100.00	100.00	100.00
subsite II+5	4	52	59	46.85	47.97	97.66
	3	55	70	49.55	56.91	87.07
	2	64	79	57.66	64.23	89.77
	1	83	99	74.77	80.49	92.90
	0	111	123	100.00	100.00	100.00

Table 7.1 Relative Mobility of Complexes Generated with Tn3 or γδ Resolvase.
Mobilities were measured (in mm) from an autoradiographs of the dried gels. Relative mobilities (Rm) are expressed as percentages. A ratio of the relative mobilities of complexes produced with each resolvase ($Tn3_{Rm}/\gamma\delta_{Rm}$) is given, also as a percentage. Only two complexes (1 and 2) were detected for these Tn3 resolvase/subsite II samples. The complex 3 relative mobility shown in brackets was calculated from a different set of Tn3 resolvase/subsite II samples.

Complex 1 of subsite II with Tn3 resolvase appears similar to that produced with γδ or γδ M106C resolvase, suggesting that each may contain monomer bound DNA fragments. The slight difference in relative mobility may reflect a difference in the average bend induced on binding. The patterns of formation of complex 2 are consistent with monomers of Tn3 resolvase binding co-operatively to give a dimer bound site, for a dimer of γδ M106C to bind directly to the subsite, and for wild type γδ resolvase to form complex 2 by both of these routes. As the least retarded γδ resolvase complex is assumed to contain monomer-bound DNA it is likely that monomers of γδ resolvase are present in solution. By comparison with the pattern of complexes produced with γδ M106C (allowing for the presence of monomer in this sample) it is probable that the equilibrium between dimeric and monomeric γδ resolvase, in solution, favours the dimeric species. It is unlikely that Tn3 resolvase is entirely monomeric in solution, but it appears that monomers are the predominant species. The third complex generated by Tn3 resolvase with subsite II is believed to contain 3 resolvase monomers per DNA

fragment. Methylation interference experiments did not indicate that any additional interactions (with G residues) were required to form this complex and so, if present, the third resolvase molecule may form protein to protein and/or protein to DNA contacts. The third complex produced with γδ resolvase and γδ M106C has a similar mobility as the Tn3 equivalent but, as M106C gives quite a strong complex 3 band, it would be surprising if they all contained 3 resolvase monomers per DNA fragment.

The comparatively low levels of complex 1 observed for binding of γδ resolvase or M106C with subsites (II + 10) and (II + 5) strongly support the idea of γδ resolvase being mostly, but not exclusively, dimeric and of Tn3 resolvase containing high levels of monomer. The small amounts of complex 1 produced do have a similar relative mobility to those with Tn3 resolvase. The second complexes produced with the different resolvases also migrate at a similar rate. Despite the γδ proteins' dimeric state, complex 2 is not the major band produced with subsite (II + 10) or subsite (II + 5). This may be due to the inability of pre-formed dimers to fit to the altered spacing of the mutant sites (the subsite (II + 5) complex 2 band is weakest for both proteins). The equivalent complexes formed using Tn3 resolvase may not have interactions between the bound resolvase subunits. Such interactions, if present, are often associated with bending of the DNA site, but as binding of a resolvase monomer induces a DNA bend it is difficult to determine the presence or absence of further resolvase-induced bending.

Complex 3 of subsite (II + 10) with Tn3 resolvase is likely to be similar to that of subsite II. Complexes 3 and 4 of subsite (II + 5) might arise from different arrangements of the same number of molecules, or may, respectively, contain 3 and 4 monomers of resolvase per DNA subsite. Because of the intensity of the third complex generated by wild type or M106C γδ resolvase with subsite (II + 10), it seems probable that it will contain a whole number of dimers (presumably 2) per subsite. For subsite (II + 5) with γδ resolvase complex 3 is faint, but with M106C it is barely visible. Complex 4 appears strong for both, very much like the third complex generated with subsite (II + 10). These observations would be consistent with subsite (II + 5) complex 3 and 4 containing, respectively, 3 and 4 monomers of resolvase per DNA fragment.

7.6 Summary of Features of Binding of Tn3 resolvase.

Fig. 7.2 summarises the proposed composition of complexes generated by Tn3 resolvase with Tn3 *res*, subsite II, subsite (II + 10) and subsite (II + 5). It is

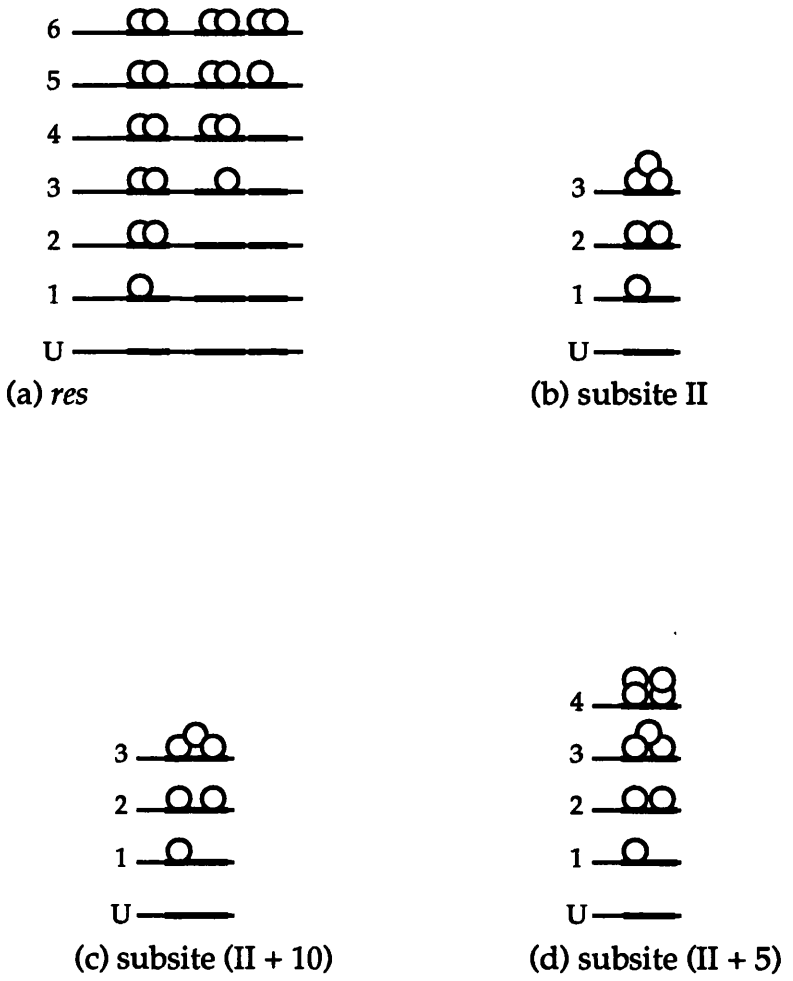


Figure 7.2. Summary of Composition of Complexes formed with Tn3 Resolvase.

'U' indicates an unbound DNA fragment. Complexes are numbered in order of decreasing mobility. Resolvase is represented by circles, and the DNA by lines, with thicker lines indicating the subsites.
Stoichiometries indicated for *res* complex 5; subsite II complex 3; subsite (II + 10) complex 3; and subsite (II + 5) complexes 3 and 4, are inferred as a result of successive monomer-binding steps. The positioning of resolvase on the DNA is not intended to exclude the possibility of other arrangements of resolvase (e.g. one monomer binding to several points across the subsite). As no additional methylation interference was observed with higher complexes of subsite II, (II + 10) or (II + 5), some resolvase in these complexes is shown not contacting the DNA. There is, as yet, no evidence to suggest whether or not this is the case.

believed that binding of a Tn3 resolvase dimer to each subsite of *res* is co-opertative but this may not be true of the subsite II derivatives produced. The arrangement of resolvase subunits in complex 3 of subsites II and (II + 10) and complexes 3 and 4 of subsite (II + 5) is unknown. It is expected that, as for γδ resolvase with γδ *res*, higher order interactions occur in fully occupied Tn3 *res* sites, and that shuffling of resolvase occurs between subsites of *res*[28,72].
It is proposed that the data presented indicate that each complex produced by binding of Tn3 resolvase *res* represents successive binding of a resolvase monomer to one half of a *res* subsite, until all subsites are bound by a dimer of resolvase. Complexes of subsite II (and its derivatives) are also believed to be formed by successive monomer binding, but higher complexes (i.e. complex 3 and 4) represent 'overloading' of the subsites, compared with those in *res*. The occupation of subsites (or half-subsites) by resolvase appears not to follow just one order, and probably varies in a manner reflecting the affinity of resolvase for each binding site. Shuffling of resolvase between subsite halves (on the same DNA fragment) is the most likely explanation of the stability of complexes during the band shift assay. However, simple interpretation of the results of methylation interference experiments disputes this feature of resolvase binding (section 3.8). Although this model of binding of Tn3 resolvase to *res* is based on binding of resolvase monomers, it is unlikely that Tn3 resolvase is exclusively monomeric in solution, but binding of resolvase dimers would not be expected to produce additional complexes to those described for monomer binding.

7.7 Band Shift Assay Conditions.

Experiments to compare the importance of conditions used for band shift assays have shown that the pattern of complexes observed does vary with the conditions used. However subsequent experiments have demonstrated a significant difference in the nature of binding of Tn3 and γδ resolvases. The differences between Tn3 and Tn21 binding patterns are also likely to result from differences in the ratio of monomeric and dimeric species present in solution.

7.8 Mutagenesis of Tn3 Resolvase.

The search for mutants of Tn3 resolvase which can direct resolution of two isolated subsite I's was unsuccessful (as were tests for equivalent mutants of γδ resolvase: M. Boocock; pers. comm.). The alignment of FIS-independent mutations[26,43] with the 1,2 dimer interface of γδ resolvase[74] suggests an obvious target region for future attempts to produce such mutants. Secondary mutations which give FIS-dependent revertants of FIS-independent Gin mutations were all positioned around the N-terminal end of Gin (R. Kahmann; unpublished data.),

i.e. GinMV114 is a FIS-independent mutant, GinMV114/KE34 is FIS-dependent but recombination proficient and GinKE34 exhibits no detectable recombination activity. Some of the mutations which give rise to FIS-dependent revertants are amino acid substitutions, which coincide with the position of substitutions in γδ resolvase which abolish recombination activity but do not prevent binding to *res*[33]. However these mutations do prevent formation of higher order structures such as the 'resolvosome'. These residues are positioned at the crystallographic 2,3/2',3' tetramer interface[74], and such an interaction could be involved in γδ synapsis. There are certain problems in interpreting the crystallographic data, particularly as the active site S10 residues in a 1,2 dimer are separated by more than 3 nm, too far apart to allow interaction with the correct phosphodiester bonds at the centre of subsite I[74].

Considering a simplistic model of inversion, it might be expected that FIS-independent mutants were made 'sticky ' enough to hold together a synapse, and direct strand exchange without the assistance of FIS[44]. It would be surprising if these mutations were strengthening the interface between Gin monomers bound to the same *gix* site (it has been indirectly demonstrated that Gin binds *gix* as a monomer: R. Kahmann; unpublished data.). However this interface may be important in stabilising the synapse during strand exchange, or at some other point, due to a conformational change during, or following, synapsis.

The range of iteration products created by γδ resolvase and by Tn3 resolvase with 'mismatch' substrates (described in Chapter 1) suggests that the γδ resolvase synapse remains intact under conditions in which the Tn3 synapse dissociates[81] (but can reform). Further analysis would be required to determine whether the γδ synapse is strengthened in a way which would make γδ resolvase a preferable subject for production of an accessory-site-independent resolvase.

7.9 Future Experiments.

Methylation interference experiments conducted on the second strand of subsites II, (II + 10) and (II + 5) would give more information about where complex 1 resolvase is bound. Use of other modification interference methods (e.g. ethylation) would give information about other bases in the subsites. By only considering G residues at present, our picture of the contacts that resolvase makes with the DNA may be somewhat misleading. Substitution of certain residues required for complex formation (e.g. L2G or R2G) could provide further information about the position of resolvase in different complexes. If shuffling is required for stable complex formation then it would be expected that both of the

substituted sites would fail to generate complex 1. However if a subsite containing both substitutions produced complex 1, then neither position would be involved in the contacts required for monomer binding. If only one of the mutated sites failed to generate complex 1 then it would appear that resolvase occupies the subsite in a non-random order. Substitution of other bases in parts of the subsite (by introduction of degeneracy during oligonucleotide synthesis, or by a forced misincorporation technique) may provide information for a greater number of the DNA positions than modification interference would allow. Mutant subsites may also reveal the smallest partial subsite required for binding of a single resolvase monomer. At present this would appear to require more than half of subsite II.

The alteration in the phase of the binding sites in subsite (II + 5) makes it an interesting substrate for bending analysis. Some indication of the phase of the resolvase-induced bends in subsite II could be produced by bend analysis of DNA fragments containing two copies of the subsite, separated by different lengths of sequence (e.g. by 5, 7, and 10 bp).

If bending of DNA in complex 1 is produced by resolvase binding at several points across the subsite, then it may be possible to produce truncated forms of resolvase which would not make the full range of contacts, and hence not induce the same bend.

Alteration of the *tnpR* gene to allow production of a mutant resolvase with additional C-terminal residues could assist in the identification of the stoichiometry of subsite (II + 10) complex 3 and of subsite (II + 5) complexes 3 and 4. A short run (about 20) of amino acids added to the C-terminal sequence of resolvase could alter the mobility of complexes generated by binding of the mutant (especially if charged residues are added)[49]. Band shift analysis using a mix of wild type and mutant resolvase would be expected to produce complexes of intermediate mobility, e.g. with subsite (II + 10) the following would be expected; two 'complex 1' bands (one for each resolvase), three 'complex 2' bands (two homodimer complexes, 1 heterodimer band); (possibly) four 'complex 3' bands (two homotrimers, two heterotrimers). Separation of complexes and identification of weak bands could be problematic, but conditions could be varied so that not all complexes were produced in all samples[7]. Such a mutation could affect the binding properties of the resolvase produced, but may not. A Gin mutant of this type of has been created, carrying an antibody epitope at its C-terminus (R. Kahmann; unpublished data.). This mutant invertase binds

normally and has been used in experiments which suggest that Gin binds as a monomer, and dimerises on the *gix* site. Similar results would be expected of equivalent experiments with Tn3 resolvase, but not γδ resolvase.

After further characterisation of binding to subsites II, (II + 10) and (II + 5), it may prove desirable to produce other sequence insertions (both larger and smaller), and perhaps deletions, at the centre of subsite II. Unfortunately the binding characteristics of subsite IIBN make it unsuitable for production of other subsite derivatives. At present there is no obvious rationale for substituting subsite II in *res* with subsite II derivatives.

The resolvase expression vector pDB6020 will hopefully prove suitable for many site-specific and site-directed mutagenesis strategies, particularly a further search for an accessory-site-independent resolvase. It may prove worthwhile to introduce unique restriction sites within the resolvase coding region of pDB6020, via silent mutations, as this would allow cassette mutagenesis of the 1,2 dimer interface region, and create a cassette mutagenesis template for future mutagenesis projects. Silent mutations which introduce restriction sites can be produced simply in pDB6020 using the pSelect mutagenesis strategy.

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