PROPERTIES AND ACTION OF Tn3 RESOLVASE

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by

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Dedicated to Mum and Dad
for their love and support.

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Abbreviations

Chemicals

TEMED - N,N,N',N'- tetramethylethylenediamine

Repelcote - dimethyl-dichlorosilane in trichloroethane

DNA - deoxyribonucleic acid

RNA - ribonucleic acid

EDTA - ethylenediaminetetra-acetic acid (disodium salt)

EtBr - ethidium bromide

EtOH - ethanol

SDS - sodium dodecylsulphate

DTT - dithiothreitol

ATP - adenosine triphosphate

Tris - tris (hydroxymethyl) amino ethane

DMS - dimethylsulphate

APS - ammonium persulphate

X-gal - 5-bromo-4chloro-3indolyl-Bgalactoside

IPTG - isopropylthio-B-D-galactoside

Antibiotics

Ap - Ampicillin

Cm - Chloramphenicol

Rif - Rifampicin

Str - Streptomycin

Tc - Tetracycline

Phenotypes

X^r - resistance to X

X^s - sensitivity to X

am - amber

ori - origin of replication

res - resolution site

Measurements

bp - base pairs

kb - kilo base pair (10^3bp)

cpm - counts per minute

mA - milliamps (10^{-3}Amps)

^{o}C - degrees centigrade

g - centifugal force equal to gravitational acceleration

g - gramme

mg - milligramme ($10^{-3}g$)

ug - microgramme ($10^{-6}g$)

ng - nanogramme ($10^{-9}g$)

l - litre

ml - millilitre ($10^{-3}l$)

ul - microlitre ($10^{-6}l$)

M - Molar (moles per litre)

mM - millimolar

pH - acidity [negative $\log_{10}$ (Molar concentration H^{+} ions]

V - Volts

W - Watts

min - min

sec - second

cm - centimetre ($10^{-2}m$)

mm - millimetre ($10^{-3}m$)

uCi - microcuries ($10^{-3}Ci$)

Miscellaneous

SC - supercoiled plasmid

OC - open circular plasmid

ds - double stranded DNA

UV - ultraviolet light

RT - room temperature

Fig - Figure

Tn - transposon

WT - wild type

LMP - low melting point

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Summary

Under standard *in vitro* reaction conditions resolvase-mediated recombination is strictly intramolecular, acting only on supercoiled substrates with 2 directly repeated *res* sites to generate singly linked catenated products. These unique properties are not explicable by random collision of the *res* sites, making the mechanism of site synapsis an intriguing problem. This study has attempted to distinguish between a number of proposed models for site synapsis.

A wide range of multiple *res* site constructs were made and the resolution characteristics examined. The results cannot be accounted for by the 'tracking' model for synapsis, based on 1-dimensional diffusion of resolvase along the DNA intervening between the 2 *res* sites. The resolution characteristics of these multiple *res* site plasmids are explicable by a 'pairing model' which arises as a natural consequence of the '2-step' model for site synapsis; the bias against non-adjacent events can be explained if there is a tendency to maximise the number of synapsed sites and if there are conformational difficulties in forming more than one non-adjacent synapse. The bias can be relieved by increasing the number of intervening sites; non-adjacent events can occur at a frequency equal to adjacent events when the recombining sites are separated by 2 intervening sites on either side. Inverted sites have been shown to cause 'shadowing' in the same way as directly repeated sites.

Methylation protection experiments have shown resolvase recognises and binds to each of the 6 half sites of *res* in essentially the same way, making major groove interactions with bases of the concensus sequence. Photofootprinting results also imply a similar mode of resolvase binding at each subsite and a similar although not identical pattern of changes in the base stacking and helix geometry at the centres of each of the subsites. It is proposed that these changes represent resolvase-induced bending of the *res* site DNA. These resolvase-induced changes in base photoreactivity are also detected in *in vivo* photofootprinting experiments.

CHAPTER 1

INTRODUCTION

Genetic recombination in prokaryotes can be broadly categorised into 3 classes depending on the extent of homology between the parental DNAs and in addition, the proteins and cofactors involved in the recombination reaction.

1. General recombination.

General recombination requires extensive DNA homology between the recombining partners and is difficult to detect with sequences having anything less than a few hundred base pairs in common with each other. Numerous attempts have been made to reproduce homologous recombination *in vitro* with limited success. In *Escherichia coli* RecA is central to the process however *in vitro* it will only mediate homologous pairing and generate heteroduplex recombination intermediates requiring accessory proteins to complete the process and stimulate earlier steps in the pathway (Radding 1982). The high energy cofactor ATP and single-stranded DNA regions as one of the substrates are essential requirements. Although unsuccessful as a complete *in vitro* recombination system, analysis of intermediates during RecA-mediated reactions (Stasiak *et al* 1984) and the heteroduplex intermediates produced has contributed towards a greater understanding of several aspects of recombination and refinement of the recombination models.

2.Transpositional recombination

This type of recombination is associated with the mobility of many insertion sequences, transposons and bacteriophage Mu (Kleckner 1981, Shapiro 1983, Grindley and Reed 1985). Recombination is independant of the general recombination processes of the cell and instead requires a 'transposase' function encoded by the element. This type of recombination is site-specific in so far as it requires the two specific sequences which mark the end of the element (usually short inverted repeat sequences ranging in size from 8-40bp), but recombination is not specific for the target sequence and the target sequences have little or no homology with the ends of the element. Transpositional recombination is non-reciprocal and replicative, in all cases generating a few base pairs (the number being specific for the element) duplication of the target sequence and in some cases causes replication of the entire element. *In vitro* systems for transpositional recombination have been elusive, although Mizuuchi (1983) has now set up an *in vitro* system for

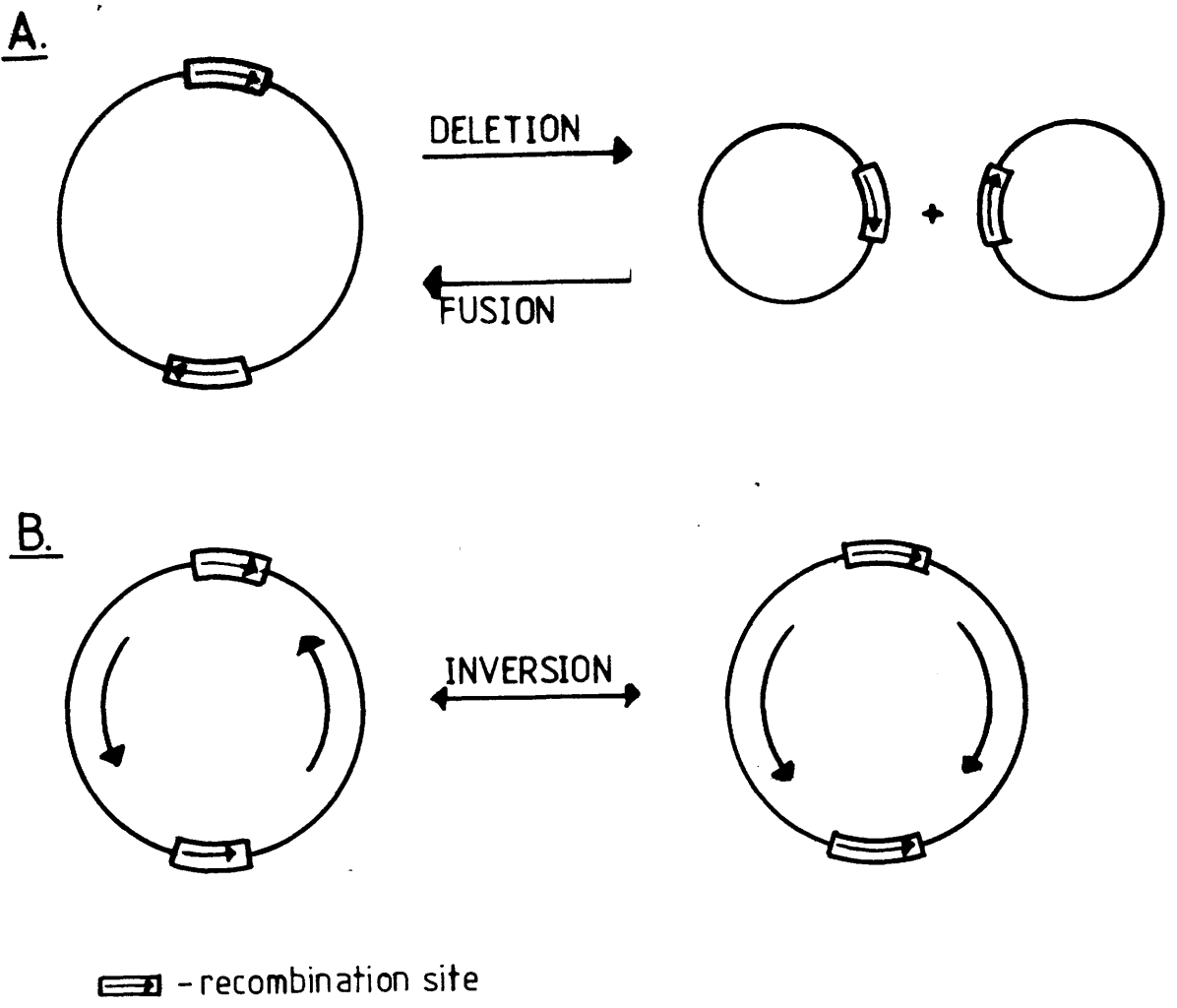


Figure 1.1 The consequences of site-specific recombination
A. Deletion - this results from intramolecular recombination between directly repeated sites and is commonly referred to as the resolution reaction.
Fusion - this results from intermolecular recombination.
B. Inversion - this results from intramolecular recombination between inverted sites.
(see text for further details).

phage Mu which generates cointegrate structures and branched DNA transpositional intermediates consistent with the Shapiro (1979) and Arthur and Sherratt (1979) models for transposition (Mizuuchi 1984, Craigie and Mizuuchi 1985).

3. Site-specific recombination

In contrast to general recombination, site-specific recombination requires only limited homology between two 'special' sites. Recombination is reciprocal between these two regions and requires no net loss or synthesis of DNA. Recombination is independant of RecA function and usually requires the activity of a protein encoded by genes adjacent to the recombining site. These proteins are not themselves in any way involved in general homologous recombination.

Site-specific recombination is of 2 types, intermolecular when the recombining sites are located on different DNA molecules and intramolecular when the recombining sites are located on the same DNA molecule. The recombining sites although superficially symmetrical have inherent asymmetry which imparts directionality to the sites. As a result, intramolecular recombination has one of 2 consequences depending on the orientation of the recombining sites with respect to each other; when the sites are in direct repeat recombination mediates excision of the DNA intervening between the 2 sites (commonly referred to as the resolution reaction); when sites are in inverted repeat recombination leads to inversion of the DNA intervening between the 2 sites, as shown in Figure 1.1.

Site-specific recombination is involved in a number of diverse processes. In prokaryotes it is involved in :- the integration and excision of phage Lambda (Nash 1981), the resolution of cointegrates formed during the 2 stage transposition of the Tn*3*-like family of elements (Arthur and Sherratt 1979), stable maintenance of phage P1 and the plasmid ColE1 (Austin *et al* 1981, Summers and Sherratt 1984), as a genetic switch, switching the host range of bacteriophage Mu, P1 and P7 (Kamp *et al* 1978, Iida *et al* 1982) and switching of flagellar antigens of *Salmonella typhimurium* (Simon *et al* 1980). The proteins involved in swithching host range and flagellar antigens are collectively called the 'invertases'. In eukaryotes site-specific recombination has been implicated in the stable maintenance of the 2 micron circle of *Saccharomyces cerevisiae* (Broach *et al* 1982), generation of diversity of

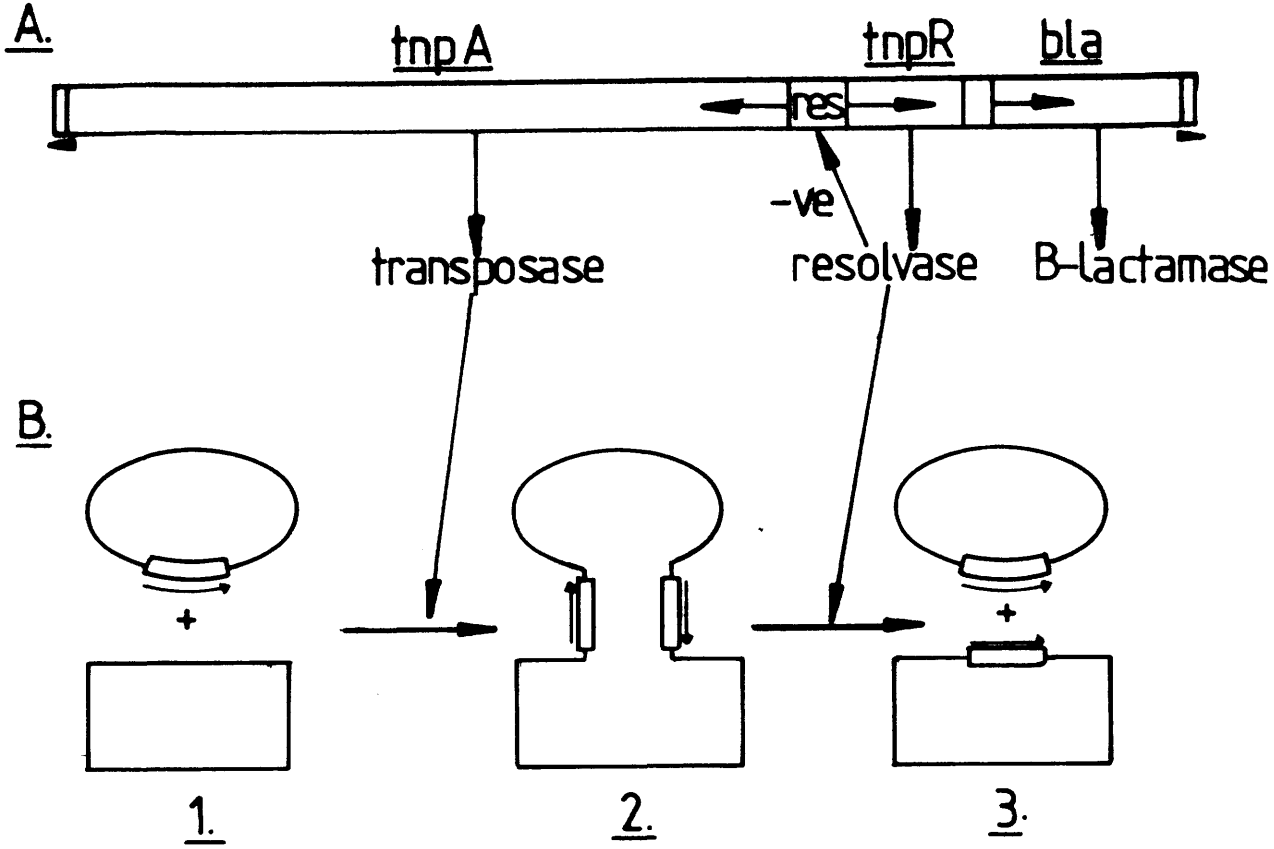


Figure 1.2 structural organisation and transposition pathway of Tn3.
A. Structural organisation As shown, Tn3 encodes three functional gene products:- (a) B-lactamase, conferring Ampicillin resistance. (b) Transposase, which mediates the formation of cointegrate intermediates by 'replicative' recombination requiring the two 38bp inverted repeat sequences which mark the end of the transposon. (c) Resolvase, which resolves cointegrate intermediates by mediating site-specific recombination between 2 directly repeated res sites. TnpA and tnpR are divergently transcribed from res and by binding here resolvase autoregulates its own synthesis and negatively represses the transcription of tnpA.
B. Transposition pathway of Tn3 (from Arthur and Sherratt 1979).
1. Donor replicon (containing the transposon) and the target replicon.
2. Cointegrate intermediate with donor and target replicons fused by directly repeated copies of the transposon.
3. Normal transposition end products; donor and target replicons each with a copy of the transposon.

antibodies (Bernard *et al* 1978, Early *et al* 1980) and T cell receptors of the immune system (Gasgoine *et al* 1984).

Of these systems the 5 best characterised are discussed here: phage Lambda integrative/excisive recombination (Lambda integrase (Int) acting at *attP*/*attB* and Xis acting at *attR*/*attL*), Tn*3* or Gamma delta cointegrate resolution (Tn*3* or Gamma delta resolvase acting at *res*), bacteriophage P1 stability and recircularisation (Cre acting at *lox*), 2-micron plasmid stability/inversion (FLP acting on *flp*) and the inversion systems of phage Mu (Gin acting at *gix*), *S.typhimurium* (Hin acting at *hix*), phage P1 (separate from Cre/*lox* with Cin acting at *cix*) and phage e14 (Pin acting at *pix*).

Following the lead of Nash (1975) using Lambda integrative recombination the systems listed above (with the exception of the Pin/*pix* reaction) have been successfully reproduced *in vitro*. The development of these *in vitro* systems in recent years has produced an astounding wealth of information which has highlighted a number of common properties and distinct differences between some of the systems and give some insight to the biological function these systems have evolved to serve.

The Tn*3* resolvase-mediated resolution reaction is the system of interest in this thesis and as a consequence will receive the major emphasis in the remainder of this introduction. The properties of the other systems are discussed with a view to highlighting features which are general properties of the site-specific recombinases and those which are unique to the resolvase-mediated recombination reaction.

Tn*3* resolvase-mediated site-specific recombination

Tn*3* and its relatives form a distinct class of transposons which share a common 2-step transposition mechanism (Grindley 1983) as diagrammed in Figure 1.2. The first step requires transposase together with the two terminal inverted repeat sequences, and by replicative recombination generates a cointegrate structure in which the donor and target replicons are fused by directly repeated copies of the transposon. In the second step resolvase mediates site-specific recombination between two directly repeated copies of the *res* site within the Tn*3* sequences of the cointegrate to separate the donor and target replicons each now containing a copy of the transposon (Kitts *et*

al 1982a,1982b).

Based on the genetic organisation and complementation ability, the Tn3 family of transposons can be divided into 2 sub-groups:-

1. In the Tn3 and Gamma delta subgroup the tnpA (transposase) and tnpR (resolvase) genes are divergently transcribed from the tnpA-tnpR intergenic region res. The -10 and -35 sequences of these 2 genes are an integral part of the recombination site res and by binding here resolvase can autoregulate its own synthesis and negatively repress the transcription of tnpA (Chou et al 1979a, 1979b, Reed et al 1982). The resolvase functions of Tn3 and Gamma delta cross complement, however the transposase functions do not .

2. In the Tn501 sub-group the region containing res and tnpR is inverted compared to the Tn3 structural organisation and by binding at res resolvase only autoregulates its own synthesis. In the best characterised members of this sub-group (Tn501, Tn21, Tn1721) the resolvase genes cross complement with each other but not with the Tn3 sub-group (Diver et al 1983).

The resolution reaction. Resolution in vitro was originally set up by Reed (1981a) using resolvase purified from Gamma delta, and subsequently using resolvase purified from Tn3 (Symington 1982, Kitts et al 1983, Krasnow and Cozzarelli 1983), Tn21 (Halford et al 1985) and Tn1721 (Rogowsky and Schmitt 1985).

In vitro under 'standard' buffer conditions (in the presence of a divalent cation) purified resolvase will efficiently mediate resolution of a synthetic cointegrate (a supercoiled plasmid with 2 directly repeated copies of the recombination site, res). The properties of this system exemplify 3 general features of the other site-specific recombination reactions:-

1. The remarkably simple protein/enzyme requirement of the in vitro systems, although Lambda integrative/excisive recombination, the inversion reactions and perhaps FLP-mediated recombination require the presence of one or more accessory proteins in addition to the recombinase.

2. There is no requirement for high energy cofactors and in all the systems examined to date the recombinase forms a covalent intermediate with one of the DNA termini of the break to conserve the energy of the phosphodiester bond for the subsequent ligation reaction. In this way

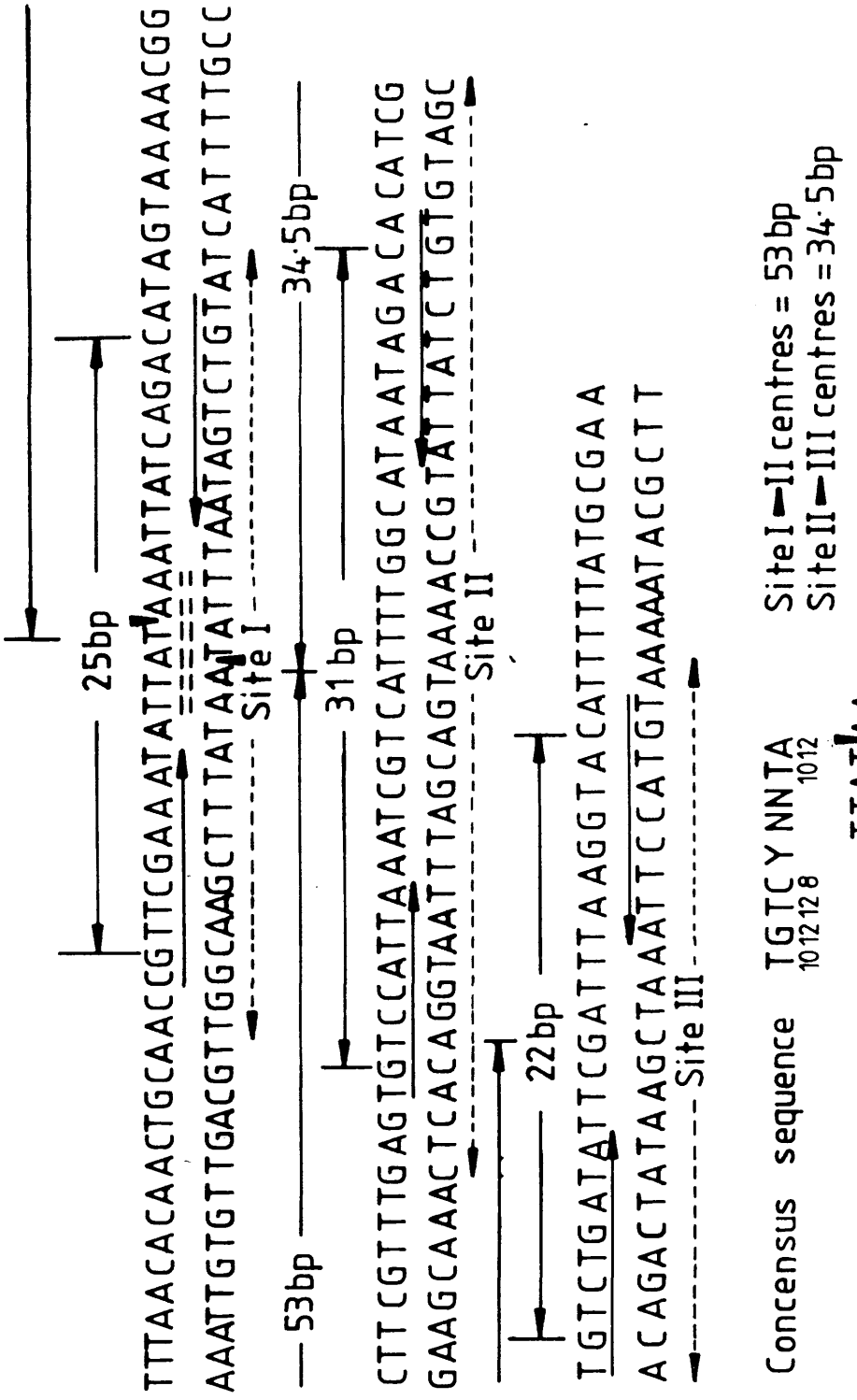


Figure 1.3 Sequence and organisation of the *res* site of Tn*3*.
The *res* site of Tn*3* is comprised of 3 subsites, I, II and III. As shown, each subsite contains inverted copies of the 9bp concensus sequence (the numbers under the concensus sequence show the number of half sites out of a possible 12 between Tn*3* and Gamma delta which have that particular base in this position) separated by a variable spacer region. The sites thus vary in size and as measured from the G positions of the concensus sequence are 25, 31 and 22bp for sites I, II and III respectively. Site II therefore spans 3 turns of the helix compared to only 2 turns for site III. The centres of sites I and II are separated by 53bp (5 helical turns) and sites II and III centres by 34.5bp. The sequence of strand exchange lies in the centre of site I and during recombination resolvase generates a 2 base 3' overhang with resolvase covalently linked to the 5' end via a phosphoserine linkage. All 3 subsites of *res* are required for efficient resolvase-mediated resolution. (further details are given in the text)

the recombinases resemble topoisomerases (Wang 1985) and indeed Int and resolvase have been shown to have a site-specific topoisomerase activity (Kikuchi and Nash 1979, Craig and Nash 1983b, Krasnow and Cozzarelli 1983) In the case of resolvase this activity is specific for the 2 *res* site synaptic complex but is distinct from the strand exchange reaction (Krasnow and Cozzarelli 1983).

3. The recombinases act stoichiometrically rather than catalytically.

Substrate requirements. Although the *in vitro* resolution reaction appears to have relatively simple protein/enzyme requirements it has very strict specificities regarding substrate topology and under standard reaction conditions resolvase-mediated recombination is strictly intramolecular acting only on supercoiled substrate with 2 directly repeated *res* sites. Recombination between inverted sites is very inefficient both *in vitro* and *in vivo* which together with the intramolecular specificity is in keeping with the biological role of the resolution reaction. These specificities are not general properties of the recombinases (Reed 1981a, Krasnow and Cozzarelli 1983, Kitts *et al* 1983).

The recombination site *res*. As shown by DNAaseI protection experiments of resolvase bound at *res* (Grindley *et al* 1982, Kitts *et al* 1983) the *res* site has considerable complexity and spans approximately 120bp within which resolvase specifically binds to 3 sites (sites I, II and III). Comparison of the nucleotide sequences of the Gamma delta and Tn3 *res* sites showed each subsite consists of inverted copies of a 9bp concensus sequence (TGTCYNNTA) separated by a variable spacer region. The 3 subsites (as measured by the distance between the G position of the 2 concensus sequences) are therefore 25, 31 and 22 base pairs in length for sites I, II and III respectively. The spacing between the sites also varies (see Figure 1.3). The spacing between the centres of site I and II is 5 turns of the helix, assuming 10.5bp per turn.

All 3 sites are required for efficient recombination and the 2 *res* sites are equal partners in the recombination reaction although minor deficiencies in one of the partners can be tolerated (Kitts *et al* 1983, Wells and Grindley 1984). SiteI alone contains the sequence of strand exchange within the palindromic sequence TTAT|AA in the centre of the site and on breakage 2bp 3' extensions are generated with resolvase

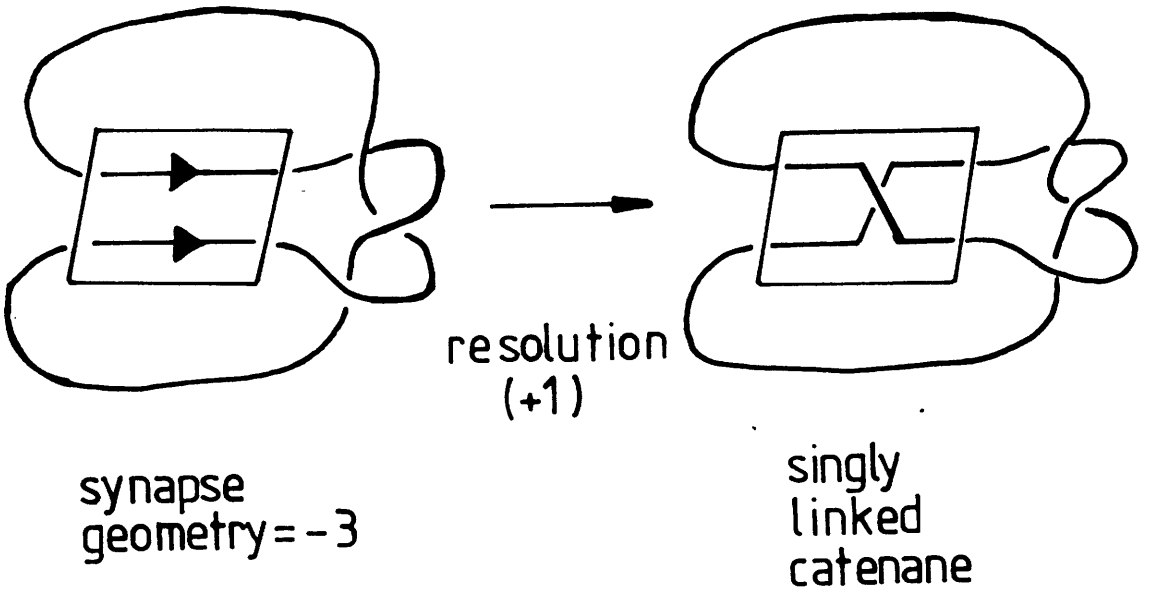


Figure 1.4 Topology of the resolvase/res synaptic complex and the topological change during resolution
This shows the 3 (-) supercoils trapped between the 2 res sites at synapsis. At strand exchange two of these interdomainal supercoils are converted into the unique catenane interlock (Wasserman and Cozzarelli 1985). See text for further details.

covalently linked to the 5' terminus via a phosphoserine linkage (Reed 1981b, Reed and Grindley 1981, Reed and Moser 1984). Linkage via a phosphoserine is unusual and most of the topoisomerases as well as FLP (the only other site-specific recombinase to be examined in this way) form a phosphotyrosine linkage.

The structural organisation of the *res* site has posed some interesting questions; the possible functional role of sites II and III given that site I alone contains the sequence of strand exchange, the recognition determinants at each binding site and the mode of resolvase binding which can accommodate the different spacing within and between sites to form the synaptic complex.

The importance of the 3 site organisation and the precise spacing within siteI and II and between the site I and II centres is exemplified by their evolutionary conservation throughout the Tn*501* subgroup of elements (Rogowsky *et al* 1985). This spacing may well play an important role in aligning the 2 *res* sites at synapsis. The Tn*501* subgroup all exhibit homology and are related by the concensus sequence YGTCAYRa/tTA in each half site.

***in vitro* resolution products.** A unique feature of the *in vitro* resolvase-mediated resolution reaction is that the major resolution products are two supercoiled circles catenated via a single interlink (Reed 1981, Krasnow and Cozzarelli 1983). These have recently been shown to have unique stereochemistry (Krasnow *et al* 1983a, Wasserman and Cozzarelli 1985), indicating resolvase strictly defines the DNA topology both on formation of the synaptic complex and during the strand exchange reaction and must somehow prevent the inclusion of random superhelical twists between the two sites at synapsis. In addition to these major products a number of minor species (1% of total) of more complex topology have been observed (Krasnow *et al* 1983b) which are considered to arise as a result of iteration of the basic strand exchange event prior to dissociation of the synaptic complex (Wasserman and Cozzarelli 1985, Wasserman *et al* 1985). This topological analysis of the major and minor resolution products together with the theoretical analysis of recombination topology (Cozzarelli *et al* 1984, Sherratt *et al* 1984) has led to very strong predictions regarding both the extent and direction of rotation about the synaptic axis during strand exchange. It is now widely accepted resolvase must align sites at synapsis with 3 negative

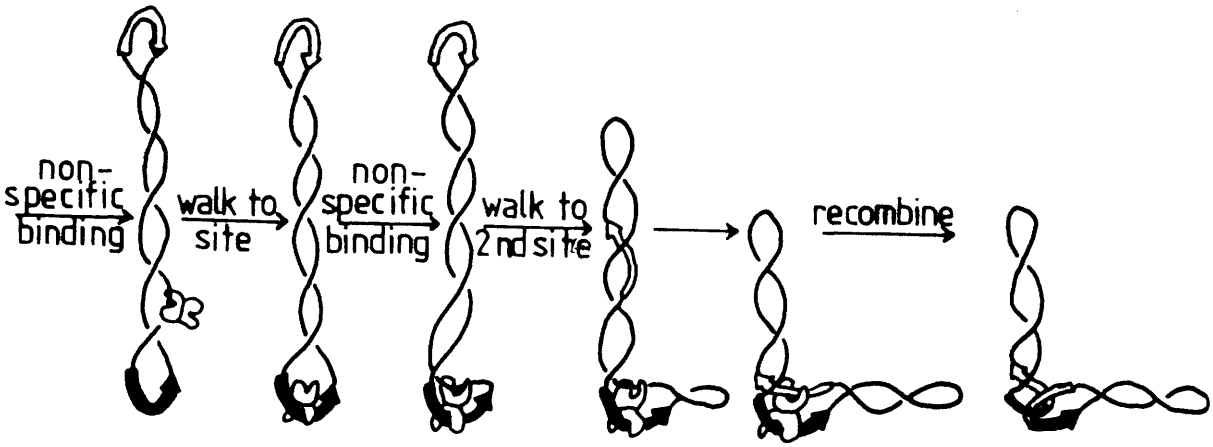


Figure 1.5 Tracking model for resolvase-mediated recombination as proposed Krasnow and Cozzarelli (1983).
A functionally dimeric resolvase binds non-specifically and translocates along the DNA until reaching a res site (solid arrow) where it binds tightly and specifically. The second subunit binds the adjacent DNA backbone in a single orientation, trapping a small loop of DNA between the 2 resolvase binding sites. 1-dimensional diffusion of the second subunit along the DNA causes the loop to expand and contract. When this subunit reaches a second res site in the correct orientation (open arrow), resolvase changes conformation and effects breakage and reunion. Strand exchange is directed by resolvase with a specific geometry resulting in singly interlinked recombinants.

supercoils between the synapsed sites with one supercoil being lost during strand exchange and the other two being converted into the unique catenane interlock (Wasserman and Cozzarelli 1985). Resolvase therefore introduces one positive interdomainal node at strand exchange (Figure1.4).

Models for resolvase-mediated site synapsis. Clearly, the resolvase-mediated recombination reaction exhibits a number of unique features which are not explicable merely by random collision of the 2 recombining sites. Briefly these features include:- intramolecular specificity, supercoiling requirement, specificity for directly repeated res sites, alignment of the 2 sites at synapsis with precisely 3 supercoils between the recombining sites, the simple yet unique stereochemistry of the products and the need for 3 binding sites rather than just one. How then can the 2 sites be brought together in such a specific way? To explain these unique directional and topological properties of the resolvase-mediated recombination reaction a number of models have been proposed. At the onset of the work described in this thesis the 'tracking' model was considered the most plausible candidate. During the work described here, in light of the elucidation of the topology of the recombination reaction the 2-step model for resolvase-mediated site synapsis was proposed (Boocock et al 1986). These two models are now briefly discussed in turn.

1. The tracking model. This model, as originally proposed by Krasnow and Cozzarelli (1983) and Kitts et al (1983), is based on processive searching of resolvase along the DNA intervening between the two res sites (see Figure 1.5). One subunit of a functionally dimeric resolvase is proposed to bind non-specifically to the DNA backbone and randomly translocate along the DNA in the same way as other repressors are envisaged to find their operators (Winter et al 1981, Berg et al 1982). Once a res site is found it will be tightly and specifically bound and translocation by that subunit will cease. The second subunit can then bind to the adjacent DNA in a unique orientation defined by the protein-protein interactions with the first subunit. In doing so it traps a small loop between the two binding sites which expands and contracts as the second subunit diffuses along the DNA in search of another res site. When the second subunit encounters a res site in the correct orientation

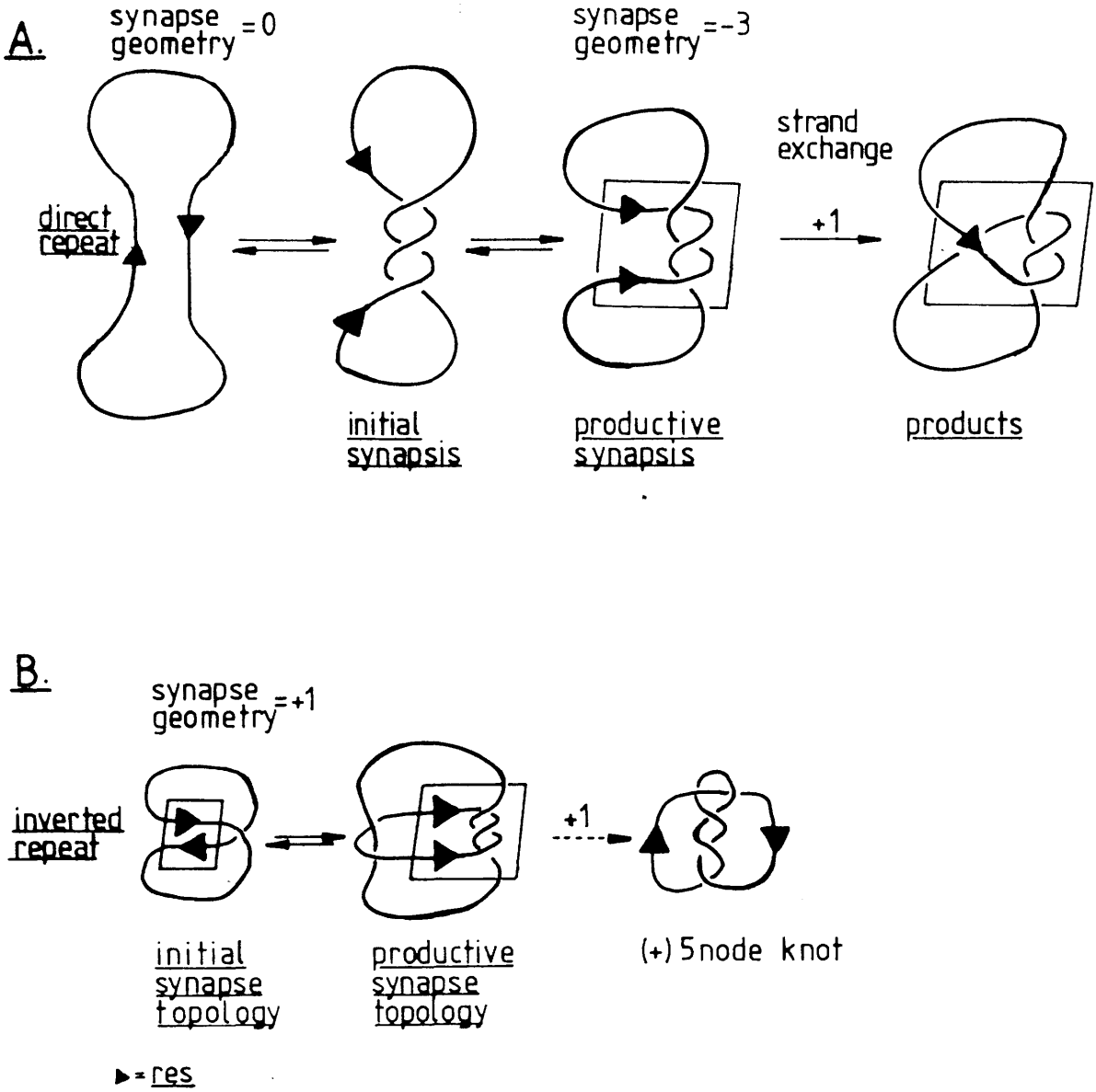


Figure 1.6 The 2-step model for resolvase-mediated recombination (as proposed by Boocock et al 1986).
See text for details.

A. Synapsis of directly repeated res sites.

1. Initial antiparallel alignment of the res sites with no trapped supercoils.
2. Twisting of the DNA helices around each other to introduce -3 interlinks.
3. A second topological step aligns the 2 sequences of strand exchange in parallel with the necessary -3 synapse geometry.
4. Strand exchange with a right-handed sense generates the singly linked product catenanes (-2).

Introducing twisting into complexes with anything other than an initial synapse geometry of zero is predicted to be energetically unfavourable leading to dissociation of the synapse.

B. Synapsis of inverted sites

With inverted sites antiparallel alignment of sites requires a minimum synapse complexity of +/-1. As detailed in the text twisting of such a complex is expected to be energetically unfavourable and the complexes are expected to dissociate. The structure of the predicted products would be as shown, if inversion were to proceed.

it will also bind tightly and specifically inducing a conformational change in resolvase, activating the strand scission, strand exchange and ligation activities.

Such a model predicts resolvase actively directs both synapsis and strand exchange thus providing an explanation for the barrier to intermolecular reactions, the directional specificity and the simplicity of the products.

2. The 2-step model This model is based on random collision rather than one-dimensional diffusion of resolvase along the DNA. Resolvase is considered to determine the precise organisation of *res* site DNA at synapsis. It is proposed that plectonemic wrapping of the 2 *res* sites around resolvase fixes 3 negative supercoils between the 2 sites. The exclusion of additional supercoils and the directional and intramolecular specificity are considered to be a consequence of the energetics of introducing wrapping into a negatively supercoiled substrate, rather than a direct function of resolvase.

This model (illustrated in Figure 1.6) requires initial anti-parallel alignment of 2 *res* sites. Random collision will thus generate a whole spectrum of possible complexes differing in the number of super-helical twists which are trapped between the two *res* sites at synapsis. Theoretical analysis has shown complexes which have no trapped super-helical twists (synapse geometry of zero, Sherratt *et al* 1984) have the unique ability to twist the DNA helices around each other in the axis of synapsis without 'tangling' the rest of the molecule. A second topological step could then align the two sequences of strand exchange in parallel with the necessary -3 synapse geometry (Boocock *et al* 1986).

For more complex initial synapse geometries, including the anti-parallel alignment of inverted *res* sites and intermolecular synapsis, twisting the DNA helices around each other would introduce 'tangling' into the rest of the molecule, a situation which is expected to be energetically unfavourable in a negatively supercoiled substrate. The 2-step model therefore envisages reversible association of the *res* sites with energetic constraints acting as a filter to prevent productive synapsis and strand exchange except in those complexes with an initial synapse geometry of zero. This model therefore provides an explanation for both the directional and intramolecular specificity as well as the simplicity yet unique nature of the resolution products. In addition,

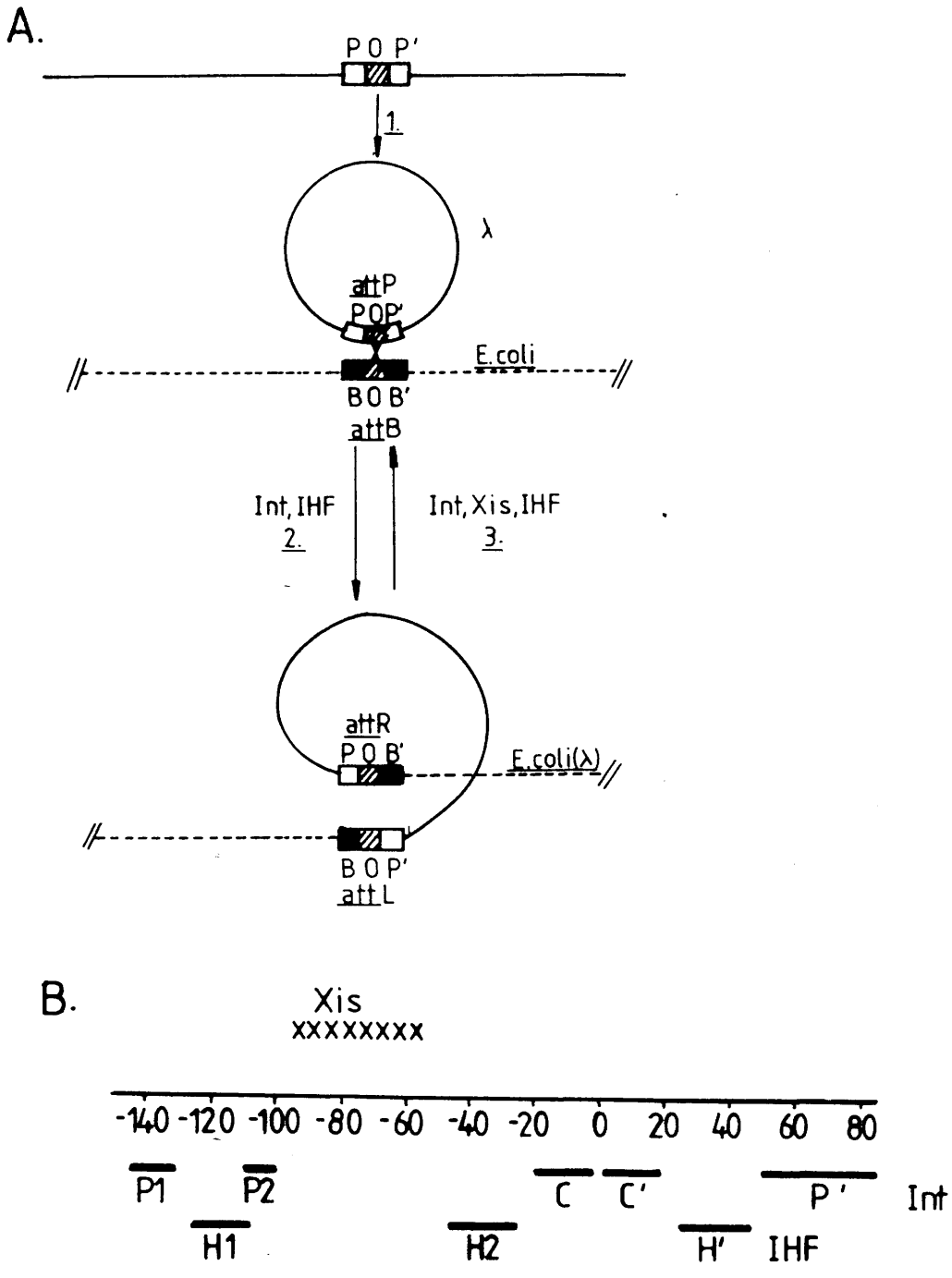


Figure 1.7 Lambda site-specific recombination.

A. The mechanism of integration and excision of phage Lambda (reviewed by Sadowski 1986).

1. Circularisation of the Lambda genome.

2. The phage attachment site (attP, POP') and the bacterial attachment site (attB, BOB') undergo reciprocal site-specific recombination promoted by Int and IHF to generate two unique hybrid attachment sites (attL and attR).

3. The integrated Lambda phage DNA can be cut out by reversal of reaction 2 in the presence of Int, IHF and Xis proteins.

The crossover takes place within the common core region (O) of each site. The sites are not drawn to scale and attP is 240bp in length containing multiple binding sites for Int and IHF (see below) in sharp contrast to attB which is only 25bp in length with only 2 Int 'core' type binding sites.

B. Protein binding regions in attP (Yin et al 1985)

The phage DNA is represented by the continuous line. The coordinates are indicated with zero being the centre of common core sequence, positions in the P arm are assigned negative numbers and positions in the P' arm are assigned positive numbers. Solid bars mark regions protected by Int (P1, P2, C, C' and P') and IHF (H1, H2 and H') proteins. xxx marks the Xis-protected region.

this model provides a possible role for 3 resolvase binding sites rather than just the site carrying the sequence of strand exchange.

Both these models make explicit experimentally testable predictions which are addressed in detail in chapter 3.

Lambda integration and excision

Lambda integrative recombination was the first recombination system to be successfully reproduced *in vitro* (Nash 1975). Elucidation of a number of features of this system together with those of resolvase-mediated recombination (as detailed above) has highlighted a number of differences between these two systems.

Recombination site In contrast to the resolvase-mediated resolution reaction, the recombining partners in Lambda site-specific recombination are non-equivalent (Mizuuchi *et al* 1981, Weisberg and Landy 1983). Integration occurs by reciprocal recombination between phage (*attP*) and bacterial (*attB*) attachment sites of 240 and 25 base pairs respectively (Campbell 1962, Landy and Ross 1977, Hsu *et al* 1980, Mizuuchi and Mizuuchi 1980). Each site is comprised of a 15bp core sequence flanked by unique arms of the *att* sites P, P', B and B' (see Figure 1.7). Strand exchange occurs within the 15bp core sequence (Mizuuchi *et al* 1981). Integrative recombination generates two hybrid sites *attL* and *attR* and requires both the phage-encoded protein Int (Nash 1975) and the *E.coli*-encoded integration host factor, IHF (Kikuchi and Nash 1978, Nash and Robertson 1981). Int has been shown to bind to 5 sites within *attP* (Ross *et al* 1979, Hsu *et al* 1980), 3 arm type binding sites and 2 junction type binding sites (Ross and Landy 1982, 1983), *attB* only has 2 junction type binding sites. IHF binds to 3 additional sites in *attP* as shown in Figure 1.7 (Craig and Nash 1984). Excisive recombination occurs between the hybrid prophage sites and in addition to Int and IHF this reaction requires the phage-encoded protein Xis (Guarneros and Echols 1970, Bushman *et al* 1984). Xis has been shown to bind to two adjacent directly repeated sites in the P arm of *attP* or *attR* (Abremski and Gottesman 1982, Yin *et al* 1985). Binding of Xis to *attP* inhibits integrative recombination.

Substrate specificities, recombination products and models for site synapsis. Efficient *in vitro* integrative recombination, also has a

specificity for substrate topology and normally requires attP be present on a supercoiled substrate (Mizuuchi et al 1978, Mizuuchi and Mizuuchi 1979).

In contrast to the resolvase-mediated recombination reaction the Lambda integrative and excisive recombination systems will mediate inter- or intra-molecular recombination and are insensitive to the relative orientation of the recombining sites. Intramolecular recombination between directly repeated sites generates catenated products (Mizuuchi et al 1980) whilst the products of the inversion reaction are knots (Pollock and Nash 1983). The products show a complexity which is related to the superhelical density of the substrate (in general, the products of excisive recombination are simpler than those of integrative recombination, Pollock and Nash 1983). Therefore unlike the resolvase-mediated recombination reaction, there is no mechanism which acts to exclude random superhelical twists from between the 2 recombination sites at synapsis and on strand exchange these superhelical twists are converted into interlinks in the products, in accord with synapsis of the 2 recombining sites by random collision (Mizuuchi et al 1980, Craig and Nash 1983a, Pollock and Nash 1983) However, results of a recent analysis of product topology (Spengler et al 1985, Griffith and Nash 1985) together with theoretical analysis of the recombination topology (Cozzarelli et al 1984, Spengler et al 1984) suggests the situation is slightly more complex with the recombination complex itself introducing non-random components into the reaction which become interlinks in the products. This is consistent with the proposal that Int and IHF organise attP into a nucleosome-like structure with wrapping of the DNA around a protein complex. Subsequent random collision with attB would then explain the observed topology of the products. Condensed Int, IHF and attP specific complexes have been observed by electron microscopy (Better et al 1982, Craig and Nash 1983a, Griffith and Nash 1985). Supercoiling of attP may be necessary to energetically favour the wrapping process (Griffith and Nash 1985).

Strand exchange. Int makes a 7bp staggered cut within the 15bp core region and during strand exchange becomes covalently linked to the 3' phosphoryl terminus (Craig and Nash 1983b). The mechanism of strand exchange is still uncertain, however Nash et al (1981) proposed a model for strand exchange based on the formation of a 4-stranded intermediate

of 2 synapsed duplex recombination sites with recombination taking place via a sequential set of concerted breakage, rotation and reunion reactions among the 4 strands at the site of exchange. An intermediate in this process is a Holliday junction as has been implicated in general recombination pathways implying these 2 very different processes may share a common intermediate, although in the Lambda system such a Holliday junction would be static due to the limited homology between the recombining att sites.

Hsu and Landy (1984) have shown Int can resolve synthetic Holliday junctions and this together with the requirement for homology in the core region (rather than a precise recognition sequence) and identification of a +/- 2 change in linking number (Nash and Pollock 1983) has been taken as indirect support of the Nash et al (1981) model. However, unlike resolvase-mediated recombination (and as predicted for the Nash et al (1981) strand exchange mechanism) Int-mediated recombination does not undergo iteration and the direction of rotation at strand exchange has not been determined and may equally well be the reverse of that predicted by the Nash et al (1981) model.

Resolvase-mediated recombination does not proceed via such an intermediate since this type of model cannot explain the change in linking number observed for the products of resolvase-mediated resolution reaction (M.Boocock unpublished results) which are in accord with a simple breakage-rejoin mechanism. This also appears to be the case for the invertases (R.Kahmann unpublished results) implying these 2 systems may be more closely related than was previously thought. The other systems described here have properties closer to the Lambda integration and excision reactions.

Therefore in contrast to the resolvase system, Lambda-specified site-specific recombination shows no intra or intermolecular specificity, no directional specificity and a complexity of interlinked products dependant on the superhelical density of the substrate plasmid. Despite these contrasting features, it is possible that 2-step models may also have some relevance to Lambda site-specific recombination (M. Boocock, pers. comm.). For example, Lambda integrative recombination may involve plectonemic wrapping of the attP arms (P and P') around the Int and IHF proteins thus trapping a precise number of interlinks, followed by random collision with attB. A variable number of interlinks would be passively trapped as a consequence of the substrate supercoiling. This

type of recombination would not be subject to the stringent energetic considerations of the resolvase-mediated recombination reaction since only one of the two recombining sites would be involved in the wrapping.

In addition to the integration/excision reactions detailed above, under certain specialised circumstances an Xis-independant excision reaction has been detected. The properties of this reaction are quite different from those of the Xis-mediated excision reaction and indeed this system exhibits specificities identical to those of the resolvase-mediated resolution reaction, a specificity for directly repeated recombining sites (attL and attR) on the same supercoiled substrate. As in the Tn3 resolution reaction the products are topologically simple, and are in this case free circles. Craig and Nash (1983a) have proposed the sites in this reaction may be synapsed by some kind of tracking process. However as with resolution this result could equally well be explained by an adaptation of the 2-step model as proposed for resolvase. The product topology would again be dictated by the energetic consequences of wrapping 2 recombining sites around each other, together with the strand exchange mechanism.

Cre-mediated recombination

This site-specific recombination system normally acts between two loxP sites to monomerise P1 dimers and in this way increase the 'plasmid' stability (Austin et al 1981) and additionally it is involved in circularising the phage genome on entry into the host cell (Sternberg et al 1981a). The two recombining sites are normally equivalent however occasionally, P1 can integrate into the chromosome by recombination between the phage (loxP) and the bacterial (loxB) 'attachment' sites which are different (Sternberg et al 1981b). The loxP site in contrast to attP of Lambda and res of Tn3 is remarkably simple spanning only 34bp, comprised of two 13bp inverted repeat sequences separated by an 8bp spacer region (Hoess and Abremski 1984, Hoess et al 1984). The directionality of the site is thought to reside in the asymmetry of this spacer region. Cre has been shown to generate 5' protruding ends with a 6bp overhang and during recombination Cre becomes covalently bound to the 3' phosphoryl terminus (Hoess and Abremski 1985). As with Lambda integration/excision homology at the site of strand exchange is an important factor and may imply a similar recombination mechanism via a

Holliday junction intermediate. Indeed, these 2 systems together with the FLP-mediated site-specific recombination system may be more closely related than was previously suspected and it has now been shown that there is considerable homology both between the recombining sites and the Int, Cre and FLP proteins themselves (Argos *et al* 1986 in press). These three systems do not however, cross complement.

Recombination *in vitro* requires only purified Cre and a suitable buffer with no requirement for high energy cofactors (Abremski and Hoess 1984). *In vitro*, this system recombines *lox*P sites organised either intermolecularly or intramolecularly and intramolecular recombination is insensitive to site orientation. In this respect the absence of such directional specificities mirrors the Int/Xis site-specific recombination systems but, unlike Int/Xis there is no requirement for either of the recombining sites to be present on a supercoiled substrate perhaps indicating extensive wrapping of the short recombining sites may not be necessary in this system. Cre-mediated intramolecular recombination between directly repeated *lox*P sites generates a spectrum of products including free circles, simple and multiply interlinked catenanes (Abremski *et al* 1983). Inversion generates both knotted and unknotted recombinants. The complexity of the products is influenced by the substrate topology with a reduction in the level of knots and catenanes on using an open circular plasmid. The complexity of catenanes and knots unlike Int/Xis is not influenced by the distance between the recombining sites (Abremski and Hoess 1985). Tracking models have been proposed to explain the high proportion of free circular products (Abremski *et al* 1983) however, neither tracking nor simple random collision models can fully account for the observed results and the system has properties intermediate between what is expected for the 2 possibilities.

FLP-mediated site-specific recombination

FLP may also act to maintain the copy number of the yeast 2 micron circle (Sadowski 1986) although it was originally identified as an inversion system (Vetter *et al* 1983). The binding site *flp*, like *lox* is comprised of two 13bp inverted repeat sequences separated by an 8bp spacer region; an additional 13bp sequence is also present in direct repeat with the left inverted repeat sequence and is also protected by

FLP in DNAaseI protection experiments (Andrews et al 1985). The function of the extra element is not known but it does not seem to be responsible for determining the directionality of the site, this function seems be determined by the asymmetry of the 8bp spacer region (Gronostajski and Sadowski 1985b, Jayaram 1985, Senecoff et al 1985). FLP cuts within the 8bp core region to generate 8bp 5' overhang with FLP covalently linked to the 3'phosphoryl terminus via a phosphotyrosine linkage (Gronostajski and Sadowski 1985c).

In vitro recombination (Vetter et al 1983, Meyer-Leon et al 1984, Babineau et al 1985) may only require FLP (this has not yet been clarified) and an appropriate buffer and will mediate both inter- and intramolecular recombination and either excision or inversion, depending on the orientation of the recombining sites (Gronostajski and Sadowski 1985a). A recent report (Sadowski 1986) shows the products of recombination are multiply interlinked catenanes (directly repeated sites) or complex knots (inverted sites) which vary in complexity depending on the supercoil density of the plasmid in the same way as the Int/Xis recombination products suggesting a random collision mechanism. Additionally, the amount of intra and intermolecular recombination is influenced by the concentration of FLP present (Gronostajski and Sadowski 1985a).

The Invertases

A number of DNA inversion systems have been identified to date 4 of which will be discussed here;-

1. Gin-mediated inversion of the G loop of bacteriophage Mu switching the host range of the phage particles (Kamp et al 1978, van de Putte et al 1980).

2. Cin-mediated inversion of the C segment of phages P1 and P7, switching the host range of the phage particles (Iida et al 1982).

3. Hin-mediated inversion of a chromosomal segment of S.typhimurium which gives alternate expression of flagellar antigens (Simon et al 1980).

4. Pin-mediated inversion of the P segment of the e14 prophage in the chromosome of E.coli K12, the function of which is unknown to date (Plasterk and van de Putte 1985).

All of these inversion systems are closely related and the 4

recombinases Gin, Hin, Cin and Pin show about 60% homology with each other at the amino acid level and have the ability to cross complement (Plasterk *et al* 1983). These proteins are more distantly related to resolvase and no cross complementation has been detected (Plasterk *et al* 1983). *In vitro* systems have now been set up for Gin (Mertons *et al* 1984, Plasterk *et al* 1984), Hin (Bruist and Simon 1984, Johnson *et al* 1984) and Cin (Iida *et al* 1984). Like the resolvases under standard reaction conditions they show a strict requirement for a supercoiled substrate and will only efficiently mediate recombination between inverted sites present on the same molecule in a defined orientation with respect to each other, in this case in inverted repeat.

In contrast to the resolvase/*res* system the inverted repeat sequences across which recombination occurs are small being comprised of 26bp necessary for recombination from which an inversion concensus sequence has been derived (Plasterk *et al* 1983, Iida *et al* 1984). However, in the cases examined (Hin, Gin and Cin) inversion is dramatically stimulated by the presence in *cis* of a 60bp sequence of DNA derived from the coding region of the adjacently transcribed recombinase (Johnson and Simon 1985, Kahmann *et al* 1985, Huber *et al* 1985). In addition, recombination requires a host-encoded factor (Fis), which has not yet been identified. The stimulatory effect is independant of both distance and orientation from the recombining site and analogies have been drawn with the eukaryotic transcriptional enhancers (Craig 1985). The mechanism of action of Fis is not yet known. Earlier, it was speculated that the reaction specificities could be explained by a 'tracking' mechanism as was proposed for resolvase-mediated site-synapsis. With the discovery of the involvement of accessory factors it was then postulated that these additional sequences and proteins may act to provide a site of entry to the DNA for a 'tracking complex'.

Alternatively, these sequences and accessory proteins may be involved in the formation of the synaptic complex serving as an alternative to the 3 site organisation of *res*. Clearly, the 2-step model is equally relevant to this system, explaining the directional specificities as a consequence of the energetics of synapsis. The topology of the products of inversion are not yet known although on the basis of the directional specificity, these would be expected to have defined topology dependant on the number of interlinks between the two recombining sites at synapsis and the mechanism and direction of strand

Table 1.1 Comparison of properties of site-specific recombinases (as compiled by Sadowski 1986)

Property	Recombinase				
	Int	Resolvase	Invertases	P1Cre	2umFLP
Requirement for superhelicity	attP partner	Yes	Yes	No	No
Type of reaction	Excision or Inversion	Excision only	Inversion only	Excision or Inversion	Excision or Inversion
Complexity of site(s)	attP complex: attB simple	Complex	Recombination site simple, requires enhancer	Simple	Simple
Intermolecular recombination	Yes	No	No	Yes	Yes
Nature of breakage	7 base 5' overhang	2 base 3' overhang	Not known overhang	6 base 5' overhang	8 base 5' overhang
Covalent attachment of recombinase at site of break	$3'\text{-}PO_4$	$5'\text{-}PO_4$ phosphoserine	Not known	$3'\text{-}PO_4$	$3'\text{-}PO_4$ phosphotyrosine
Topology of products	Multiply linked catenanes or complex knots	singly linked catenanes	Not known	Singly linked and multiply linked catenanes, unlinked products knots	Multiply linked catenanes or complex knots

exchange.

Summary and objectives of present study

In summary, these site-specific recombination systems exhibit basic similarities yet also contrasting properties depending on the *in vivo* role of the recombination reaction (the properties of the various systems are summarised in Table 1.1, as compiled by Sadowski 1986). The site-specific recombination systems seem to fall into two distinct categories;-

1. Lambda Int/Xis/*att*, P1 Cre/*lox* and 2-micron FLP/*flp* recombination systems show considerable homology at the level of both proteins and 'core' recombining sites and *in vitro* are insensitive to site orientation generating an array of products, the complexity of which depends of the superhelical density of the substrate. In addition, there are implied similarities with regard to the mechanism of strand exchange and this may occur via a similar Holliday junction intermediate in each of the systems. Clearly the precise details will differ since each has a different specificity of cutting in the strand exchange sequence. The greater simplicity of the products of the Cre/*lox* reaction may imply a different site synapsis mechanism.

2. This group is comprised of the resolvases and the invertases which in contrast to the above mentioned systems under 'standard' reaction conditions have very strict directional and topological specificities and pose interesting questions as to the mechanism of site synapsis. Comparision of these two systems may prove informative because of their different directional specificities. Like the systems mentioned in 1. (above) these proteins show homology although they do not cross complement.

Clearly the precise details of the Cre/*lox*, FLP/*flp* and the inversion systems require further clarification before precise models can be proposed. Thoughts now appear to be turning towards models based on the energetic consequences of forming a synaptic complex on a supercoiled substrate and indeed a model very similar to the 2-step model for resolvase-mediated site synapsis has now been proposed for Mu transposition (Craigie and Mizuuchi 1986, submitted).

The system of interest in this laboratory was the Tn*3* resolvase/*res* recombination reaction. At the outset of the work described in this

thesis the topological details of the reaction were unknown. The properties which were known at this stage included the intramolecular specificity for a supercoiled substrate with 2 directly repeated res sites with recombination generating singly linked catenated products. In view of these unique properties the mechanism by which resolvase synapses res sites with directional specificity over a long range (yet excludes the random entrapment of superhelical twists between the two recombining sites) was of major interest and this problem was approached in two quite different ways:-

1. Designing a number of experiments initially to test the predictions of the tracking model for site synapsis and latterly to distinguish between tracking and the alternative models for resolvase-mediated site-synapsis which we developed during the course of the work described here.

2. By examining how resolvase recognises and binds to the 3 subsites of res with a view to highlighting why 3 binding sites are needed when siteI alone contains the sequence of strand exchange, how resolvase accommodates the different length within and between sites on forming an active synaptic complex and how it recognises the sequence of strand exchange. This was approached using a number of footprinting techniques on linear, supercoiled substrates and ultimately, on a 'live' resolution reaction.

CHAPTER 2

MATERIALS AND METHODS

Table 2.1 Bacterial Strains

Name	Relevant markers	Source or Reference
DS902 (AB1157)	*thr leu his pro arg recA*$^-$ Str^r	D.J. Sherratt
ΔM15	Δ[*lac pro*] Ø80d*lacZ*ΔM15	U. Ruther
DL283	*recB recC sbcB recA hsdR*$^-$ *hsdM*$^+$ Str^r	D. Leach
CB50	*ara* Δ(*lac pro*) *thi*	C. Boyd
LB1	AB1157 *lacI*^{q} ZΔM15 Rif^r	Chapter 6
BMH71-18	*supE thi* Δ(*lac proAB*) F' *proAB lacI*^{q} ZΔM15	C. Boyd

Table 2.2 Plasmids.

Name	Description	Phenotype	size(kb)	Source or reference
pBR322	vector derived from pMB1	Ap^r,Tc^r	4.36	Sutcliffe 1978
pUC8	vector derived from pBR322	Ap^r	2.67	Vieira and Messing 1982
pUC9	" " " "	Ap^r	2.67	" " " "
pUC18	" " " "	Ap^r	2.69	Yanish-Perron et al1985
ColEI	naturally occurring	Ce1,Ie1	6.6	D. Sherratt
pLS138	pPAK329 partial EcoRI + a 2nd 282bp EcoRI res^+ from pPAK329	Ap^r,Tc^r,2res^+	4.92	L. Symington
pLS139	pPAK329 BamHI + 375bp Sau3A res^+ fragment from RSF1365	Ap^r, 2res^+	4.91	L. Symington
pMA44	pBR322 PvuII + 282bp PvuII res^+ fragment of pLS138	Ap^r,Tc^r,1res^+	4.64	M. Boocock
pMA21	3.86kb H/P 1res^+ of pMA44 + 1.06kb H/P 1res^+ of pLS139	Ap^r,Tc^r,2res^+	4.92	M. Boocock
pMA34	2.47kb P/A 1res^+ of pMA44 + 2.81kb P/A 2res^+ of pLS139	Ap^r,3res^+	5.3	M. Boocock
pMA14	2.47kb P/A 1res^+ of pMA44 + 2.73kb P/A 2res^+ of pLS138	Ap^r,Tc^r,3res^+	5.2	M. Boocock
pMA431	3.95kb H/P 1res^+ of pLS139 + 1.34kb H/P 2res^+ of pLS138	Ap^r,3res^+	5.3	M. Boocock
pMA914	3.17kb P/A 2res^+ of pMA828 + 2.83kb P/A 2res^+ of pLS140	Ap^r,4res^+	5.91	M. Boocock
pMA422	4.22kb P/H 2res^+ of pMA34 + 1.34kb H/P 2res^+ of pLS138	Ap^r,4res^+	5.56	M. Boocock
pMA414	4.22kb P/H 2res^+ of pMA11 + 1.34kb H/P 2res^+ of pLS138	Ap^r,4res^+	5.56	M. Boocock
pMA453	2.81kb P/A 2res^+ of pLS139 + 3.17kb P/A 2res^+ of pMA249	Ap^r,4res^+	5.91	M. Boocock
pMA1534	282bp RI,2xPvuII res^+ of pLS138 into the EcoRI, SmaI and Hinc II sites of the pUC18 polylinker in direct repeat	Ap^r,3res^+	3.21	M. Boocock
pMA2350	362bp HaeIII and 282bp PvuII res^+ fragments cloned into the pUC18 polylinker in direct repeat (219bp spacing)	Ap^r,2res^+	3.33	M. Boocock
pLB42	pBR322 (PvuII) + 2.02kb HincII (3res^+) fragment of pMA414	Ap^r,Tc^r,3res^+	6.39	Chapter 3.
PLB43	" " + " " " " " "	Ap^r,Tc^r,3res^+	6.39	Chapter 3.
pLB44	" " + 1.67kb HincII (2res^+) fragment of pMA14	Ap^r,Tc^r,3res^+	6.03	Chapter 3.
pLB45	" " + " " " " " "	Ap^r,Tc^r,2res^+	6.03	Chapter 3.
pLB321	3.8kb P/A (2res^+) of pLB44 + 2.45kb P/A (1res^+) of pMA21	Ap^r,Tc^r,3res^+	6.31	Chapter 3.
pLB434	" " " " " + 2.81kb P/A (2res^+) of pMA34	Ap^r, 4res^+	6.51	Chapter 3.
pLB438	" " " " " + 2.73kb P/A (2res^+) of pMA14	Ap^r,Tc^r,4res^+	6.59	Chapter 3.
pLB440	" " " " " + 2.81kb P/A (2res^+) of pMA914	Ap^r, 4res^+	6.67	Chapter 3.

Table 2.2 continued

pLB466	4.21kb P/A (3*res*$^+$) of pLB42 + 2.45kb P/A (1*res*$^+$) of pMA21	Ap^r,Tc^r,4*res*$^+$	6.67	Chapter 3.
pLB553	" " " " " + 2.81kb P/A (2*res*$^+$) of pMA34	Ap^r, 5*res*$^+$	7.02	Chapter 3.
pLB566	" " " " " + 2.73kb P/A (2*res*$^+$) of pMA14	Ap^r,Tc^r,5*res*$^+$	6.95	Chapter 3.
pLB666	" " " " " + 2.81kb P/A (2*res*$^+$) of pMA914	Ap^r, 6*res*$^+$	7.3	Chapter 3.
pLB421	4.21kb P/A (3*res*$^+$) of pLB43 + 2.45kb P/A (1*res*$^+$) of pMA21	Ap^r,Tc^r,4*res*$^+$	6.67	Chapter 3.
pLB534	" " " " " + 2.81kb P/A (2*res*$^+$) of pMA34	Ap^r, 5*res*$^+$	7.02	Chapter 3.
pLB538	" " " " " + 2.73kb P/A (2*res*$^+$) of pMA14	Ap^r,Tc^r,5*res*$^+$	6.95	Chapter 3.
pLB638	" " " " " + 2.81kb P/A (2*res*$^+$) of pMA914	Ap^r, 6*res*$^+$	7.3	Chapter 3.
pLB514	3.1kb P/A (2*res*$^+$) of pMA914 + 3.09kb P/A (3*res*$^+$) of pMA414	Ap^r, 4*res*$^+$	6.19	Chapter 3.
pLB522	" " " " " + " " " " pMA422	Ap^r, 4*res*$^+$	6.19	Chapter 3.
pLB35	4.11kb SmaI/EcoRI pKK223.3 + 834bp PvuII/SphI(3*res*$^+$) pMA1534	Ap^r, 3*res*$^+$	4.9	Chapter 3.
pLB39	pLB35 PvuII + 282bp PvuII *res*$^+$ fragment of pMA44	Ap^r, 4*res*$^+$	4.22	Chapter 3.
pLB41	" " + " " " " " "	Ap^r, 4*res*$^+$	4.22	Chapter 3.
pLB03	pUC9 HaeII partial + 1.9kb HaeII *cer*$^+$ fragment of ColEI	Ap^r, *cer*$^+$	4.57	Chapter 4.
pLB16	pLB03 EcoRI + 282bp EcoRI *res*$^+$ fragment of pLS138	Ap^r,*cer*$^+$,*res*$^+$	4.85	Chapter 4.
pLB32	pUC9 SmaI-EcoRI + 163bp EcoRI-HaeIII *res*$^+$ fragment of pLB16	Ap^r,*res*$^+$	2.83	Chapter 4.
pLB36	pUC18 HincII + 170bp HaeIII *res*$^+$ fragment of pLB30	Ap^r,*res*$^+$	2.84	Chapter 5.
pLB37	" " + " " " " " "	Ap^r,*res*$^+$	2.84	Chapter 5.
pKK223.3	Ptac expression vector based on pBR322	Ap^r	4.58	K. Duncan
pMA6114	4.55kb EcoRI/H of pKK223-3 + 1.3kb *tnpR*$^+$ of pMA498	Ap^r,*tnpR*$^+$	5.85	M. Boocock
pLB34	pKK223.3 PvuII + 459bp PvuII *res*$^+$ fragment of pLB32	Ap^r, *res*$^+$	5.0	Chapter 6.
pLB33	pMA6114 PvuII + 459bp + " " " " "	Ap^r,*res*$^+$,*tnpR*$^+$	6.36	Chapter 6.
pLB51	" " + 523bp PvuII *res*$^+$ fragment of pLB37	Ap^r,*res*$^+$,*tnpR*$^+$	6.37	Chapter 6.
pLB52	pKK223.3 PvuII + 523bp + " " " " "	Ap^r,*res*$^+$	5.0	Chapter 6

P/A = PstI-AvaI H/P = HindIII-PstI

2.1 Bacterial strains

The bacterial strains used were all derivatives of *Escherichia coli* K-12 and are listed in Table 2.1. Genotype and phenotype symbols are those recommended by Bachman *et al* (1976) and Novick *et al* (1976).

2.2 Plasmids

The plasmids used in this study are listed in table 2.2. Nomenclature follows Novick *et al* (1976).

2.3 Chemicals and Biochemicals

CHEMICALS & BIOCHEMICALS	SOURCE
General chemicals	B.D.H., Hopkins and Williams,
and organic compounds	Kochlight Laboratories, May and Baker
Media	Difco, Oxoid
General biochemicals	Sigma
Hydrazine	Kodak
Dimethyl sulphate	Aldridge
Piperidine	Fluka-Garantie
Agarose	BRL
X-gal, IPTG	BRL
Atomlight Scintillant	NEN
Radiochemicals	NEN
10 x core buffer	BRL
Antibiotics	Sigma
Restriction enzymes	BRL, Boerhinger mannheim
Klenow polymerase	Boerhinger mannheim
Tn*3* resolvase	gift from Martin Boocock

2.4 Culture media

L-Broth: 10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose, 20mg thymine, made up to 1 litre in distilled water and adjusted to pH 7.0 with NaOH.

L-Agar: as L-broth without glucose and the inclusion of 15g/l agar.

Minimal Agar: 7g K_2HPO_4, 2g KH_2PO_4, 4g NH_4SO_4, 0.25g trisodium citrate, 0.1g $MgSO_4.7H_2O$, 17.5g agar, made up to 1 litre in distilled water.

Davis-Mingioli Salts (x4): 28g K_2HPO_4, 8g KH_2PO_4, 4g $(NH_4)_2SO_4$, 1g sodium citrate, 0.4g $MgSO_4.7H_2O$, made up to 1 litre with distilled water.

D/M Minimal Medium: 25ml D/M salts, 5mls 20% casamino acids, 250ul 20% glucose, 0.5ml vitamin B1 (1mg/ml), made up to 100mls with water.

M9 Salts (x10): 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl in 1 litre of distilled water.

M9 Minimal Medium: 10ml M9 salts, 2ml glucose (20% w/v), 0.1ml 1M $MgCl_2$, 0.1ml 100mM $CaCl_2$, 0.1 ml (1mg/ml) vitamin B1, 2.5ml (20%) casamino acids, made up to 100ml with distilled water.

Phage buffer: 7g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, 0.25g $MgSO_4$, 15mg $CaCl_2.2H_2O$, 1ml 1% gelatin made to 1 litre in distilled water.

Supplements: When required, supplements were added to minimal media at the following concentrations:

glucose 2mg/ml	amino acids 40ug/ml
thymine 50ug/ml	thiamine (vitamin B1) 20ug/ml
casamino acids 1%	

2.5 Sterilisation

All growth media were sterilised by autoclaving at 120°C for 15 minutes; supplements, gelatin solution and buffer solutions at 108°C for 10 minutes and $CaCl_2$ at 114°C for 10 minutes.

2.6 Buffer Solutions

(a) Electrophoresis

10x E Buffer: 48.4g Tris, 16.4g NaAc, 3.6g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water, pH adjusted to 8.2 with acetic acid.

10x TBE Buffer pH8.3: 109g Tris, 55g Boric Acid, 9.3g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water; pH is 8.3.

Single Colony Gel Loading Buffer: 2% Ficoll, 1% SDS, 0.01% Bromophenol Blue, 0.01% Orange G in buffer E.

Vertical Agarose Gel Loading Buffer: 1% Ficoll, 0.5%SDS, 0.06% Bromophenol blue, 0.06% Orange G in 1xTBE.

Horizontal Agarose and Polyacrylamide Gel Loading Buffer: 25% Sucrose, 0.06% Bromophenol Blue, 10mM Tris.HCl (pH8.0).

Sequencing polyacrylamide gel loading buffer: 80% (v/v) deionised formamide, 50mM Tris borate pH8.3, 1mM EDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue.

(b) Restriction and ligation buffers:

10 x Low salt: 100mM Tris/HCl pH7.5, 100mM $MgSO_4$, 10mM DTT. Stored at -20°C.

10 x Medium salt: 500mM NaCl, 100mM Tris/HCl pH7.5, 100mM $MgSO_4$, 10mMDTT. Stored at -20°C

10 x High salt: 1M NaCl, 500mM Tris/HCl pH8, 100mM $MgCl_2$, 10mM DTT.

10 x SmaI buffer: 200mM KCl, 100mM Tris/HCl pH8.0, 100mM $MgCl_2$, 10mM DTT. Stored at -20°C.

10 x SphI buffer: 6mM Tris/HCl pH7.5, 6mM $MgCl_2$, 6mM 2-mercaptoethanol, 50mM NaCl. Stored at -20°C.

10 x Ligation buffer: 660mM Tris/HCl pH7.6, 100mM $MgCl_2$, 10mM EDTA, 100mM DTT. Stored at -20°C.

4mM ATP: 4mM ATP in 4mM Tris/HCl pH7.5. Stored at -20°C.

TE buffer: 10mM Tris/HCl, 1mM EDTA; pH8.0.

2.7 Antibiotics

The antibiotic concentrations used throughout this work in both liquid

and plate selection were as follows:

Name	Source of Resistance	Selective conc.	Stock Soln
Ampicillin(Ap)	plasmid	50ug/ml	5mg/ml(dH_2O).
Streptomycin(Str)	chromosome	50ug/ml	5mg/ml(dH_2O).
Rifampicin(Rif)	chromosome	50ug/ml	5mg/ml(methanol).
Tetracycline(Tc)	plasmid	10ug/ml	1mg/ml(methanol).
Chloramphenicol(Cm)	plasmid	20ug/ml	2mg/ml(ethanol).

All stock solutions were stored at 4°C and when necessary added to molten agar pre-cooled to 55°C.

2.8 Indicators: X-gal (5-Bromo-4chloro-3indolyl-Bgalactoside) was stored at a concentration of 20mg/ml in dimethylformamide at -20°C and added to L-agar plates to a final concentration of 20ug/ml. This indicator was used in conjunction with the M15 host strain and the pUC vectors providing a screen for plasmids with inserts into the polylinker region (giving white colonies as compared to the blue colonies for the unaltered vector).

2.9 Growth conditions: Liquid cultures for transformation, or plasmid DNA preparation were routinely grown in L-broth at 37°C with vigorous shaking. Growth on plates was on L-agar or minimal medium plus supplements. Antibiotics were added as required. Plates contained 25mls of agar solution and were incubated for approximately 15 hours at 37°C unless otherwise stated. All dilutions for plating out cells were carried out in phage buffer.

Bacterial strains were stored in 50% L-broth, 40% glycerol at -20°C. Inocula from these were applied to selective plates and after growth at 37°C single colonies were selected.

2.10 Plasmid DNA isolation:

Birnboim and Doly (1976) DNA preparation (as modified by Chris Boyd, this laboratory): 200ml cultures of stationary phase plasmid containing cells were pelleted by centrifugation at 12000g, 5min at 4°C. The pellet was resuspended in 4mls of lysis buffer (50mM glucose, 25mM Tris HCl pH8.0, 10mM EDTA) and incubated 5min on ice. 8mls of alkaline-SDS

solution (0.2M NaOH, 1% SDS) were added, mixed and the lysate was left a further 5min on ice. 6mls of cold 5M KOAc pH4.8 (3M KOAc, 2M HOAc) was then added, gently mixed and the cell debris and chromosomal DNA was removed by centrifugation (32000g, 20min, 4°C). The supernatant was then deproteinised by phenol extraction (6mls) followed by chloroform extraction of trace phenol. Samples were spun at 12000g, 5min at each stage to separate the phases. The plasmid DNA was precipitated with 12mls of isopropanol for 15min at RT. The DNA was pelleted for 20min (39200g, 20°C) and the resulting pellet was rinsed with 70% EtOH, dried and then further purified by banding on a CsCl/EtBr gradient. The DNA was resuspended in 2.09mls TE (60min at 37°C) to which 270ul 15mg/ml EtBr was added together with 4.324 mls of TE containing 5g CsCl. The gradients were centrifuged in a Beckman Ti70 fixed angle rotor at 200,000g, 20°C for 16hours. Two bands were then visible, a lower super-coiled plasmid band and an upper chromosomal band. The plasmid band was removed with a hypodermic syringe by puncturing the tube at the appropriate point. The EtBr was removed by repeated extraction with butanol and the DNA was dialsyed against 2 x 1 litre of 1 x TE to remove the CsCl. The DNA was then ready for use.

Mini DNA preparation: Small quantities of restrictable DNA was isolated using a slight modification of the Ish-Horowitz and Burke method (1981). 1.5mls of stationary phase culture was pelleted at 12000g, 30secs. The cells were resuspended in 100ul of lysis buffer (50mM glucose, 25mM Tris/HCl pH8.0, 10mM EDTA). After 5 min incubation at room temperature, 200ul of alkaline-SDS solution (0.2M NaOH, 1%SDS) was added incubated 5min on ice and then mixed gently with 150ul of precooled 5MKOAc pH4.8 (3M KOAc, 2M HOAc). After a further 5min incubation on ice the cell debris and chromosomal DNA was pelleted by centrifugation at 12000g for 1 min. The supernatant containing the plasmid DNA was deproteinised by phenol extraction, followed by chloroform extraction with 1min centrifugation at 12000g at each stage to separate the phases. The supernatant was then mixed with 900ul EtOH and precipitated at RT 2min. After centrifugation at 12000g for 1min the pellet was rinsed with 70% EtOH, dried and resuspended in 20ul TE containing 2ug/ml RNAase. Generally, 10ul/restriction was used for low copy number plasmids and 2-5ul for high copy number plasmids.

2.11 Ethanol precipitation of DNA: The DNA solution was made 0.3M in NaOAc and 2 volumes of absolute ethanol were added. After mixing the nucleic acids were precipitated (-70°C 10min, or -20°C 45min) and were pelleted by centrifugation (27000g x 30min for large volumes or 12000g x 15min for volumes of 1.5mls and less). The pellet was washed with cold 70% EtOH and dried down under vacuum.

2.12 *In vivo* tritium labelling of DNA: 1ml of a fresh overnight culture of the plasmid strain grown in minimal media (with selection) was inoculated into 100mls of prewarmed minimal media (with selection) and incubated shaking for 2-3hours (mid-late log phase). 10uCi/ml ^{3}H thymidine was added together with deoxyadenosine to 1mM and 50ug/ml uridine. Deoxyadenosine was added because the cells were thy^{+} and required a source of deoxyribose-1-phosphate. Uridine was added to prolong the period of uptake of labelled thymidine. Plasmid DNA was then isolated using the modified Birnboim and Doly CsCl purification protocol described in section 2.9.

2.13 Restriction of DNA: Restrictions were usually performed in a total volume of 25ul containing 0.5 - 1ug of DNA, 2.5ul 10x restriction buffer and 1 unit/ug DNA of enzyme, the volume being made up to 25ul with distilled water. For restriction of larger quantities of DNA (as for restriction digests for end-labelling experiments) the volume was scaled up accordingly. The reactions were incubated at the appropriate temperature (as recommended by the suppliers) for 1-2 hours, after which time the enzyme was inactivated either by the addition of gel loading buffer or by phenol extraction and ethanol precipitation if subsequent manipulations were necessary.

2.14 End-labelling of DNA: Restriction fragments which produce a staggered cut with a recessed 3' hydroxyl group were labelled using the Klenow fragment of DNA polymerase I. Only one labelled (alpha-^{32}P) triphosphate needs to be incorporated adjacent to the recessed 3' hydroxyl group for end-labelling, (the labelled nucleotide used depends on the sequence of the restriction enzyme site). 2-5ug of appropriately restricted plasmid DNA was phenol extracted, precipitated, rinsed and

dried. The pellet was resuspended in 40ul distilled water, 5ul 500mM Tris/HCl pH7.9, 50mM $MgCl_2$, 100mM 2-mercaptoethanol and 3-5uCi (specific activity 3000Ci/mM) alpha-labelled dNTP in 5ul (alpha-^{32}PdATP for 3' end-labelling EcoRI sites and alpha-^{32}PdGTP for 3' end labelling BamHI sites), 1unit of Klenow polymerase was added per ug of DNA and incubated at 16°C, 60min. The samples were mixed with 20ul final sample buffer and electrophoresed through 5% polyacrylamide. The end labelled fragments were located by autoradiography of the gel.

2.15 Ligation of DNA fragments: The restriction fragments to be ligated were resuspended in 16ul distilled water, 2ul 10x ligation buffer and 2ul 4mM ATP (generally the concentration of fragment to vector was adjusted to approximately 3 to 1 for sticky ends and 10 to 1 molar excess for blunt end clonings). T_4-DNA ligase was added (0.01 units/ug DNA for 'sticky' ends, 1 unit/ug for blunt ends) and the mixture was incubated overnight at 16°C. Dilutions of the ligation mixture in TE (1 in 10, 1 in 20, 1 in 50) were used to transform competent cells.

2.16 Calf Intestinal Phosphatase (CIP) treatment: In an attempt to increase cloning efficiency phosphatase was used to remove the 5'-terminal phosphate groups from the linearised vector to prevent recircularisation of the vector. This technique is particularly useful when no direct selection or screening method is available for detecting insertion of the donor fragment. CIP operates in high, medium and low salt restriction buffers; 0.01 units were added to a vector DNA restriction digest and incubated 15min at 37°C. CIP was subsequently removed by phenol extraction followed by precipitaion of the DNA ready for ligation.

2.17 Transformation with plasmid DNA: Plasmids were introduced into different host strains by genetic transformation.

An overnight culture of the recipient strain was diluted 1 in 100 into 20ml L-broth and was grown to a density of approximately $2x10^8$ cells/ml (about 90min). The cells were harvested (12000g x 2min, 4°C), and were washed by resuspending in 10ml of cold (4°C) 50mM $CaCl_2$. The cells were pelleted again, resuspended in 0.2-1ml of cold 50mM $CaCl_2$ and kept on ice for at least 15min before being used.

200ul aliquots of the $CaCl_2$ treated cells were then added to the plasmid DNA in 50ul of TE buffer and the mixture was left on ice for 15min. The cells were heat shocked (42°C x 2min) and were returned to ice for a further 60min. 1ml of L-broth was added to the cell suspension and incubated at 37°C for 90 minutes to allow expression of the plasmid genes. For transformation to Ampicillin resistance no expression time was necessary. Aliquots of the transformation mixture were then spread onto selective plates.

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

2.18 Single colony gel analysis: This technique enables the plasmid content of an isolate to be observed without the need to purify DNA. A single colony was patched out (1cm square) on a selective plate and grown overnight. Using a toothpick a large scrape of cells was collected and resuspended in 150ul of single colony gel buffer. The cells were left to lyse at room temperature for 15min. Cell debris and chromosomal DNA was spun down (12000g x 15min, 4°C), and 50ul of the supernatant was loaded onto an agarose gel.

2.19 Curing strains of F: An overnight culture of the strain to be cured was diluted to 10^{-5} cells/ml. 50ul of this was inoculated into 2.5ml of L-broth (pH7.6) with 7.5, 15, 30 and 75ug/ml Acridine orange. These cultures were grown shaking at 37°C overnight in the dark. 10^{-2}, 10^{-4} and 10^{-6} dilutions were plated on L-agar. Colonies were tested for F^-/F^+ phenotype by testing for the ability to mate out. This was done by replica patching the colonies onto an L agar plate and a plate spread with a suitable F^- strain under conditions where only conjugants can grow.

2.20 Gel electrophoresis:

Agarose gels: Generally 0.8-1.2% agarose gels were used. Agarose powder (gelling temperature 36-42°C) was dissolved at 100°C in 100 or 200ml of buffer E and pre-cooled to 55°C prior to use.

Horizontal gels were of two types:-

(i)100ml gels - these were generally used to analyse restriction digests and were made by pouring 100ml of molten agarose into a 11 x 19cm

perspex gel former with a 13 space Teflon well former. After the gel had solidified the wells were wetted with E buffer, the comb was carefully removed and the gel was transferred to a gel tank with 800-900ml (sufficient to just cover the gel) buffer E. Samples were loaded in 25ul aliquots in horizontal gel loading buffer. Gels were usually run at 5V/cm for approximately 3 hours and then stained in 0.5ug/ml EtBr for 30min and photographed on a 254nM UV transilluminator.
(ii) 200ml gels - these were used to analyse restriction digests of samples of resolution time-course reactions. These gels were made by pouring 200ml of molten agarose into a 16.5 x 23cm gel former with a 19 space well former. The gels were run in 1 x E buffer in a gel tank with a buffering capacity of 3 litres at 5V/cm 12-15 hours, after which time the gels were stained in 0.5ug/ml EtBr and photographed on a 254nm UV transilluminator.

Low melting-point agarose gels: These gels were used to isolate restriction fragments for use in cloning reactions. The agarose (1%) was dissolved in 100ml 1 x E buffer at 100°C then pre-cooled to 37°C prior to pouring. Gels were poured in the same way as in (i) above and were run at 2V/cm 12 hours.

Vertical gels: Vertical agarose gels of 0.8-1.2% were used for single colony gel electrophoresis. The gel kits held two 16x15 cm glass plates separated by 3mm spacers. After sealing the gels with agarose 100mls of precooled (55°C) molten agarose was poured between the glass plates with insertion of a 10 or 15 toothed Teflon comb. When the gel had set, the top and bottom reservoirs were filled with sufficient E buffer to cover the gel and the comb was carefully removed. Single colony supernatants were loaded directly in 50ul aliquots. Marker plasmid DNAs were made up to 50ul using single colony final sample buffer. Gels were typically run at 6V/cm 3-5 hours or 2V/cm for 15 hours (at room temperature). Gels were stained in 0.5ug/ml EtBr for 30 minutes then photographed on a 254nm UV transilluminator.

The interpretation of unrestricted plasmid DNA was based on Dugaiczyk *et al* (1975). The fastest migrating and generally most abundant band was the supercoiled (SC) plasmid monomers. Behind this ran an open circular (OC) plasmid band often comigrating with

supercoiled plasmid dimers. Open circular dimers and higher forms if visible ran even more slowly. Plasmid linears could sometimes be detected running as a faint band between the SC and the OC forms of the plasmid. In single colony gels sheared fragments of chromosomal DNA ran as a thick band towards the top of the gel with any conjugative plasmids present running just behind the chromosomal band.

Polyacrylamide gels: These were of 2 types:-

5% polyacrylamide restriction gels: These gels were used to resolve fragments of 100-1000bp. Vertical gel kits were used which held two 16 x 15cm glass plates separated by 1.5mm spacers. The gel apparatus was sealed with 1% agarose in TBE and the 5% acrylamide gel mix was poured between the two plates followed by addition of a 13 space well former. 5% polyacrlyamide gel mix is made up by mixing 4mls 10 x TBE, 10mls 20% acrylamide/1% bisacrylamide (w/v), 23.5mls distilled water, 240ul 10% TEMED and 480ul 10% Ammonium persulphate. The gels were run in 1 x TBE at a constant current of 25-30mA at room temperature (2-3 hours). The DNA bands were visualised by staining in 0.5ug/ml EtBr for 30min and photographed on a 254nm UV transilluminator.

Polyacrylamide sequencing gels: These gels were used to separate fragments from 15-300bp. Two glass plates of 20 x 40 cm were used. The inner surface of both plates were swabbed with Repelcote and taped together with 2 x 0.3mm plasticard spacers. The sequencing gel mix was then poured between the two plates followed by insertion of a plasticard slot former, the gel was left to polymerise for at least 45min in a position raised slightly from the horizontal. The gels used in this work were 8% polyacrylamide made up as follows, 21g ultra pure urea, 10ml acrylamide stock (38% acrylamide, 2% bisacrylamide, deionised), 5ml 10 x TBE pH 8.3 made up to 50ml with distilled water. After the urea has dissolved 1.6ml of 1.6% APS and 50ul of TEMED were added, mixed and ready to pour into the gel kit. After polymerisation of the gel mix,the bottom seal was broken and the gel was clamped to the electrophoresis apparatus. The reservoirs were filled with 1 x TBE, the slot former was removed and the slots were washed out immediately with 1x TBE to remove unpolymerised acrylamide. The samples (after denaturation at 90^{o}C, 3min) were loaded in 2ul aliquots using a drawn out microcapilliary and

electrophoresis was commenced immediately at 40W. These gels were generally run for 2-3 hours or as appropriate.

After the gel run is complete, the gel plates were prised apart and the gel (on one of the glass plates) was fixed in 10% acetic acid for 10min, rinsed in water, transferred to 3mm filter paper and then dried down under vacuum. When dry X-ray film was placed on top of the gel and was sealed in a light tight container for 24 hours or (appropriate) before developing.

Photographing of gels: EtBr stained gels were viewed on a 254nm UV transilluminator and photographed using a Polaroid camera loaded with polaroid 4 x 5 Land film (no57) or a Pentax 35mm SRL loaded with Ilford HP5 film. Both cameras were fitted with a Kodak Wratten filter No.9.

Sizing of restriction fragments: The size of linear restriction fragments was estimated from graphs of the $\log_{10}$ molecular size plotted against the distance travelled according to the formula;

$\log M = A - B \times D$ (Helling *et al* 1974)

M = Molecular size in bp

D = Distance migrated

A = arbitrary constant

B = arbitrary constant

Molecular weight standards were obtained by restriction of Lambda cI857Sam7 (Philippsen *et al* 1978, Haggerty and Schleif 1975) or the pUC plasmids (Yanish-Perron *et al* 1985).

2.21 Extraction of DNA from gels:

Low melting-point agarose: After staining the gel was placed on a long-wave transilluminator (300-360nm) and the band of interest was excised. The agarose chip was added to 5 volumes of TE buffer and melted by heating to 65°C. After cooling to 37°C an equal volume of phenol was added, mixed vigorously and the phases separated by centrifugation (12,000g, 5min). The supernatant was collected phenol treated a further 3 times and then chloroform extracted twice to remove any traces of phenol. Following precipitation and drying of the pellet the fragments were ready for ligation.

Electroelution from polyacrylamide gels: The band to be isolated was

excised from the gel as above. The gel slice was then sealed in a short length of dialysis tubing with 250ul 1x TBE, secured across a horizontal gel kit, submerged in 1x TBE and electrophoresed for 2 hours at 60 Volts, after which time the current was reversed for 30 seconds. The TBE was removed, the dialysis sac and the gel slice were washed with 100ul 1x TBE and both fractions were pooled and precipitated. The dried pellet was then ready for use in ligation.

Crush-soak extraction from polyacrylamide gels: This was the method used for isolation of end labelled fragments. Bands on these gels were identified by alignment with the corresponding autoradiograph. The excised gel slice was homogenised in 500ul elution buffer (500mM NH_4OAc, 1mM EDTA, 0.1% SDS, 10ug/ml tRNA) and incubated at 42°C overnight. The eluate was collected by centrifugation through a glass wool plug and then precipitated with ethanol.

2.22 Isolation and counting of tritium labelled DNA from agarose: The gels (0.8%) were stained in EtBr and viewed on a 254nm UV trans-illuminator. The relevant bands were excised and as far as possible the size of the gel slices was kept constant (10 x 2.5mm). Each gel slice was added to 1ml of distilled water in a 20ml screw cap scintillation vial and the gel slice was melted by placing the vial in a boiling water bath for 15-20min. Thereafter, 10mls of Atomlight scintillation fluid was added and shaken vigorously. The samples were incubated at 4°C 1 hour in the dark and then counted in a scintillation counter. External standard ratios were measured for each sample. Slight quenching was seen due to the presence of the agarose however, this was essentially constant for gel slices of the sizes used throughout these experiments.

2.23 *in vitro* resolution reactions:

Resolvase units: these were defined in terms of the 2*res* site plasmid pMA106 (2.5kb). 1 unit is equivalent to the minimum amount of resolvase required for 50% resolution of 1ug plasmid in 1 hour under 'standard' reaction conditions (17.3ug/ml pMA106 in 50mM tris/HCl, 10mM $MgCl_2$, 1mM DTT, 0.2mM EDTA; pH9.4 at 37°C with resolvase dilutions added to 1/20 the volume).

The resolvase stock solution used in these experiments was at 44,000U/ml, and >2mg/ml. 1unit is estimated to be approximately equal

to 50ng of resolvase. The stoichiometry of a typical resolution reaction is estimated to be between 6-10 resolvase subunits/res site.

Titration of optimal resolvase:DNA ratio: Generally 4 reactions were set up with 20ug/ml DNA in 50mM tris/HCl, 10mM $MgCl_2$, 1mM DTT, 0.2mM EDTA; pH 9.4. To the 4 samples 1ul of 2^{-3}, 2^{-4}, 2^{-5} or 2^{-6} resolvase dilution (in 1M NaCl, 10mM tris/HCl pH8.0, 10mM $MgCl_2$) was added using a 1ul glass syringe. The reactions were incubated 2 hours, 37°C, heat inactivated 5min, 80°C and after cooling were restricted with an appropriate enzyme (generally PstI or HindIII were used since both enzymes cut efficiently in the resolution buffer; where other restriction enzymes are used the buffering conditions were altered as necessary). The restrictions were stopped by the addition of 5ul horizontal gel loading buffer containing 0.2ug/ml protease K and electrophoresed through 0.8% agarose. The resolvase dilution which gave the most extensive resolution was used for larger scale reactions.

Time course resolution reactions: a typical time-course reaction appropriate for 10 time points comprised of 20ug/ml plasmid DNA in 200ul 50mM tris/HCl, 10mM $MgCl_2$, 1mM DTT, 0.2mM EDTA; pH9.4 prewarmed to 37°C. A 20ul zero time point sample was removed and mixed with 1ul of resolvase dilution buffer. To the remainder of the reaction 9ul of the appropriate resolvase dilution was added, mixed quickly and incubated at 37°C. At the appropriate times, 20ul samples were removed and transferred immediately to fresh tubes preheated at 80°C, incubated 10min, cooled and restricted as described above.

2.24 Maxam and Gilbert sequencing: This is a chemical DNA sequencing method relying on limited base-specific chemical cleavage of end-labelled DNA fragments. The chemical cleavage procedure involves 3 steps; base modification, removal of the modified base from its sugar and finally strand scission of the DNA backbone at the sugar. 4 reactions (G, G+A, C, C+T) are generally carried out and the reagents or conditions which mediate each step in each reaction are summarised on Table 2.4 below.

Table 2.4

Cleavage	Base modification	Modified base displacement	Strand scission
G	Dimethylsulphate	Piperidine	Piperidine
G+A	Acid	Acid	Piperidine
C	Hydrazine	Piperidine	Piperidine
C+T	Hydrazine + salt	Piperidine	Piperidine

Sequencing protocol: The purified end labelled fragment was resuspended in 40ul of distilled water and divided up appropriately:

G-specific cleavage: To a 5ul aliquot of end-labelled fragment 200ul of 50mM Sodium cacodylate, 1M 2-mercaptoethanol, 100ug/ml tRNA was added, mixed and then supplemented with 1ul 10.7M DMS. The sample was incubated at 22°C 5-10min depending on fragment size (5min for 250bp fragment, 7min for 300bp fragment). The reaction was stopped by the addition of 50ul 1.5M NaOAc pH7.0, 1M 2-mercaptoethanol, 100ug/ml tRNA and precipitated with 750ul EtOH. The pellet was resuspended in 0.3M NaOAC, reprecipitated with EtOH and the rinsed, dried pellet was then subjected to piperidine treatment.

Purine-specific cleavage (G+A): A 10ul aliquot of end-labelled fragment was dried down under vacuum and the pellet was resuspended in 15ul of freshly made 20ug/ml diphenylamine, 66% formic acid, 1mM EDTA and incubated at 25°C for 7-15min depending on fragment size (9min for 250bp fragment and 11min for 350bp fragments). The reaction was stopped by the addition of 45ul of distilled water followed by 3 ether extractions. The sample was frozen at -70°C and dried down under vacuum ready for piperidine treatment.

C specific cleavage: to a 5ul aliquot of end-labelled fragment 15ul of 5M NaCl was added then mixed with 30ul of hydrazine (95% reagent grade). The sample was incubated at 22°C 5-15min depending on fragment size (7min for 250bp fragment, 9min for 350bp fragments). The reaction was

then stopped by the addition of 200ul of 0.3M NaAc, 0.1mM EDTA, 25ug/ml tRNA and precipitated with 750ul EtOH. The pellet was resuspended in 0.3M NaAc and reprecipitated. The resulting pellet was then rinsed and dried ready for piperidine treatment.

Pyrimidine-specific cleavage (C+T): 10ul of end-labelled fragment was mixed with 10ul of distilled water and treated with hydrazine as described for the C cleavage reaction (see above).

Piperidine treatment: the pellets were resuspended in 100ul of 1M piperidine and incubated at 90°C for 30min. The reactions were frozen at -70°C and then dried down under vacuum. The pellet was resuspended in 10ul distilled water, frozen at -70°C and dried under vacuum. This procedure was repeated two more times and then the pellets were resuspended in an appropriate volume of sequencing-gel loading buffer. The pellets were resuspended to give approximately 50 cpm/ul for the G and C reactions and 100cpm/ul for the G+A and C+T reactions, as crudely measured by holding the samples 1cm from a mini-monitor.

2.25 Footprinting techniques:

Methylation protection: 200ng of purified fragment end-labelled at one end was resuspended in 400ul of 50mM Sodium cacodylate pH8.0, 10mM $MgCl_2$, 0.1mM EDTA, 1mM DTT and 100mMKCl. This was then divided into 4 x 100ul aliquots. To 2 of the samples 7.5 units of resolvase was added at 0°C, mixed and incubated at 37°C for 5min to permit binding of resolvase. 1ul of 10.7M DMS was then added, mixed and incubated at RT for 5min (dependant on fragment size; this incubation time was adequate for the 250-300bp fragments used in these experiments). The mixture was then diluted with 1ml of pre-warmed (22°C) 10mM Tris/HCl pH7.9, 10mM $MgCl_2$, 100mM KCl, 1mM DTT, 0.1mM EDTA and 10mM 2-mercaptoethanol. The mixture was then immediately filtered through nitrocellulose and washed with a further 500ul of the buffer described above. The filter-bound DNA was then recovered by washing the filter with 500ul of 10mM Tris/HCl pH7.5, 10mM $MgCl_2$, 0.1mM EDTA, 0.5M Ammonium acetate. The eluate was precipitated with 2ug/ml tRNA and 2 volumes of EtOH, resuspended in 0.5M Ammonium acetate and reprecipitated. The pellet was resuspended in 200ul 0.3M NaAc, 5mM EDTA, reprecipitated with EtOH, rinsed and dried.

The 2 control samples were directly incubated with 1ul of 10.7M DMS and after 5min at RT the reaction was stopped by the addition of 1M 2-mercaptoethanol, 0.5M Tris/HCl pH7.5, 1.5M NaOAc, 10mM $MgCl_2$, 0.1mM EDTA and after the addition of 2ug/ml tRNA the DNA was precipitated from 2 volumes of ethanol. The pellet was then resuspended in 200ul 0.3M NaOAc, 5mM EDTA and reprecipitated with 2 volumes of EtOH, rinsed and dried.

One set of control and resolvase bound reactions were resuspended in 100ul of 20mM Ammonium acetate pH7.0, 0.1mM EDTA and incubated at 90°C for 15min (this protocol displaces both G and A bases) 10ul of 10M piperidine was then added incubated at 90°C for a further 30min. The remaining two samples were resuspended in 100ul of 1.0M piperidine and incubated at 90°C for 30min. Thereafter the remainder of the piperidine-induced strand scission protocol was followed (as described in section 2.22).

Equilibration of counts/ul: The final pellets were resuspended in 5 or 10ul of sequencing gel loading buffer, mixed vigorously and a fixed volume was transferred to a fresh 1.5ml Eppendorf tube. The counts in each sample were measured using Cerenkov counting and were adjusted to the same number of counts/ul in a volume appropriate to give 100 cpm/ul. 2ul aliquots of each sample were then run on an 8% polyacrylamide sequencing gel.

Methylation interference: 250ng of purified $\underline{res}^+$ fragment radioactively labelled at one end was resuspended in 500ul of 50mM sodium cacodylate pH8.0, 10mM $MgCl_2$, 0.1mM EDTA, 1mM DTT and 100mM KCl. This was divided into 5 x 100ul aliquots. Each aliquot was mixed with 1ul 10M DMS and incubated at 22°C, 5min (for 250bp fragments). The reaction was stopped by the addition of 25ul 1M 2-mercaptoethanol, 0.5M Tris/HCl pH7.5, 1.5M NaAc, 10mM $MgCl_2$, 0.1mM EDTA, 2ug/ml tRNA and precipitated with EtOH. The pellet was rinsed, dried and resuspended in 100ul 10mM Tris/HCl pH7.9, 10mM $MgCl_2$, 100mM KCl, 1mM DTT and 0.1mM EDTA. To 4 of the samples different concentrations of resolvase (in 1ul of resolvase dilution buffer) were added (1.25, 2.5, 5 and 10 units) and incubated 5min at 37°C, after which time a further 500ul of the above buffer was added, mixed and the samples were gently filtered through nitrocellulose and both the filtrate and filter bound fractions (by washing the

filter with 500ul of 10mM tris/HCl pH7.5, 10mM $MgCl_2$, 0.1mM EDTA, 0.5M NH_4Ac, 1% SDS) were collected. 2ug/ml tRNA was added and the samples were precipitated with 2 volumes of EtOH. The filter-bound fractions were resuspended in 0.5M NH_4Ac and reprecipitated with 2 volumes of EtOH. Both the filtrate and filter-bound fractions were resuspended in 200ul 0.3M NaAc, 5mM EDTA and reprecipitated with 2 volumes of EtOH. The rinsed, dried pellets together with the control sample were subjected to piperidine treatment as described in the Maxam and Gilbert sequencing protocol. Thereafter, 2ul of each sample (containing an equivalent number of cpm/ul) were electrophoresed through an 8% polyacrylamide sequencing gel and autoradiographed.

Photofootprinting: This protocol was adapted from the method of Becker and Wang (1984). Irradiation was carried out using an inverted 254nm shortwave transilluminator (without a filter), held 12cm above the sample to be irradiated. Samples were irradiated as small aliquots or as a 7.5 ml layer in the bottom of a petri dish. The irradiation procedure is different for the <u>in vitro</u> linear and supercoiled substrates and the <u>in vivo</u> substrates, consequently, the irradiation procedures are described individually:

Linear substrate: 200ng of purified <u>res</u> fragment selectively labelled at one end was resuspended in 108ul of 1x photofootprinting buffer (10mM tris/HCl pH7.4, 10mM $Mg(OAc)_2$, 10mM KCl, 0.1mM EDTA, 0.1mM 2-mercaptoethanol, 6% glycerol, 100ug/ml gelatin) divided into 3 x 36ul aliquots. To one aliquot 2ul of 2.5U/ul resolvase was added; to the other 2 aliquots 2ul of 1M NaCl, 10mM tris/HCl pH8.0, 10mM $MgCl_2$ was added to maintain the same final salt concentration as the sample containing Tn<u>3</u> resolvase. All samples were incubated 5min, 37°C. The +UV and +UV+resolvase samples were transferred to a petri dish and irradiated 2min at RT. 10ug tRNA was added to each sample. Resolvase was removed by phenol extraction and all 3 samples were precipitated with EtOH. The pellets were rinsed, dried and subjected to the photoproduct-specific cleavage protocol.

Supercoiled substrate: 3 x 2ug of plasmid DNA was precipitated with EtOH, rinsed and dried. The pellets were resuspended in 36ul 1 x photofootprinting buffer and treated as described for the linear substrate (see above). After UV irradiation and precipitation the DNA was

resuspended in 1 x restriction buffer, restricted appropriately and end-labelled. The end labelled *res*$^+$ fragment was purified and subjected to the photoproduct-specific cleavage protocol.

2*res* site plasmid: this was treated in the same way as for the single *res* site supercoiled substrate using 4ug plasmid DNA in 200ul of 1 x photofootprinting buffer plus 20ul of a 2^{-5} dilution of resolvase (5units).

***in vivo* photofootprinting:** 100ml of the appropriate plasmid-containing strains (*res*$^+$, +/- resolvase strains) were grown up to late log phase in L-broth (with selection) and then supplemented with IPTG to a final concentration of 0.5mM. The cultures were grown with shaking a further 60min, 50ml of the appropriate culture was spun down for the -UV, +UV, +UV+resolvase samples, resuspended in 25mls of 1 x M9 salts and the OD_{650} was adjusted to 1 for each culture. UV treatment was carried out in 7.5ml aliquots in petri dishes, on ice, for 4.5min. The cells were then immediately frozen at -70°C.

The cells were then fast-thawed and plasmid DNA was isolated using a scaled up Birnboim and Doly mini-prep protocol. The DNA was then restricted, end labelled and subjected to the photoproduct-specific protocol.

Procedure for selectively cleaving at positions of photochemical modification: Following the appropriate irradiation procedure purified end-labelled fragment was resuspended in 200ul 1mMTris/HCl pH7, 5mM NaCl and incubated at 50°C, 5hours to promote deamination of any photochemically modified cytosines. Following EtOH precipitation, the dried pellet was redissolved in 50ul of 5mM KPi (pH8.3), 5mM NaCl, 50mM thymidine, cooled to 0°C and then supplemented with 50ul of the same solution containing 1mg of freshly dissolved sodium borohydride. After vortexing the samples were allowed to stand unagitated and open to the air at 0°C in the dark. Thymidine is included during this reduction step to react with any borane generated by decomposition of $NaBH_4$ in water otherwise, borane would reduce non-photoreacted bases. The reactions were stopped after 15hours by the addition of 250ul 0.7mM NaOAc-HOAc pH5 and incubated 1hour at 22°C with occasional vortexing. The DNA was precipitated from 750ul 100% EtOH, rinsed and resuspended in 170ul 0.5mM NaOAc-HOAc pH5.0. Following 1hour at 22°C with occasional

vortexing the DNA solution was mixed with 100ul of water and precipitated from 750ul EtOH. Following precipitation the rinsed, dried pellets were dissolved in 2ul water plus 25ul 1M Aniline-HOAc pH4.5. After incubation 20min at 60°C in the dark the samples were frozen at -70°C and lyophilised under vacuum. Freezing and lyophilisation from 25ul of distilled H_2O was repeated 3 more times followed by 2 precipitations from ethanol. The final pellets were resuspended in 5ul 98% deionised formamide gel loading buffer, transferred to a new tube and adjusted to the same number of counts/ul in all samples prior to electrophoresis of the products through an 8% polyacrylamide sequencing gel.

CHAPTER 3

RESOLUTION CHARACTERISTICS OF MULTIPLE *RES* SITE PLASMIDS

Introduction

Chapter 1 describes the 'tracking' or 'looping model' for resolvase-mediated recombination as proposed by Krasnow and Cozzarelli (1983) and Kitts *et al* (1983), to explain the directional and intramolecular specificity of the reaction as well as the topological simplicity of the products. The model in this original form made a number of experimentally testable predictions:-

1. Resolution should preferentially take place between adjacent sites in a construct containing multiple directly repeated *res* sites.
2. Inverted *res* sites should not be recognised during the 1-dimensional searching process.
3. Extensive interlinking of the catenated products will be precluded by the 1-dimensional searching process, irrespective of the super-coil density of the substrate.
4. Non-specific 'reporter rings' catenated to a standard 2 *res* site substrate should always segregate together.
5. Only intramolecular resolution events will be seen.

The original aim of the experiments described in this chapter was to test the prediction of an adjacent site preference of resolution in substrates containing 4 directly repeated *res* sites and in addition, to examine the effect of inverting one *res* site on the resolution characteristics of the remaining 3 directly repeated sites.

In parallel to these experiments Benjamin *et al* (1985) independantly tested predictions 1-4 of the tracking model. By this stage, it was clear that the original prediction of non-recognition of inverted sites was rather naive and the tracking model was later adapted by Benjamin *et al* (1985) to accommodate the situation in which both *res* sites are resolvase-bound before synapsis. They then predicted an inverted site would block the translocation of resolvase along the DNA. The situation is in fact more complex and is addressed in more detail in section 3.7. The experimental results of Benjamin *et al* (1985) are summarised below:-

1. In a construct with 4 directly repeated *res* sites resolution preferentially occurs between adjacent sites.
2. In a 4 *res* site construct in which adjacent sites are inverted with

respect to each other the extent of resolution is unaffected.

3. Resolution products are always singly linked catenanes irrespective of the supercoil density of the substrate plasmid.
4. Reporter rings segregate independantly into the product catenanes in 3 independant experiments.

The adjacent site preference of resolution and the simplicity of the products at different supercoil densities of the substrate is interpreted by Benjamin *et al* (1985) to be strong evidence in favour of the tracking model for resolvase-mediated site synapsis. However, the failure of the 'reporter ring' experiment which is diagnostic for continuous sliding of resolvase along the DNA to locate the second site, is strong evidence against tracking and the tracking model has now undergone several modifications to accommodate this result as well as non-recognition and translocation of resolvase through inverted sites. Of these, the simplest version is 'gated tracking' (Benjamin *et al* 1985). This model suggests that sliding of resolvase along the DNA is not continuous and can occasionally be interrupted to permit resolvase to bypass a region of DNA (reporter rings or resolvase-bound inverted sites). Conceptually, it is difficult to envisage how this process can occur in a controlled fashion to prevent bypass of a site in the correct orientation and how directional specificity can be maintained by an essentially non-specific process.

During the course of the experiments carried out in this laboratory, it became obvious that an adjacent site preference for resolution in a 4 *res* site plasmid is not diagnostic for tracking and can be adequately accounted for as a simple consequence of the 2-step model for site synapsis, as described in chapter 1 (Boocock *et al* 1986). This model relies on reversible pairing of *res* sites prior to recombination with selection of only those complexes with an initial synapse geometry of zero, all other complexes being non-productive as a result of conformational constraints on the system. This model also makes a number of predictions.

1. Pairing of sites is reversible.
2. Inverted sites can recognise and will interact with each other but recombination is blocked by conformational constraints.
3. The block on intermolecular recombination and intramolecular

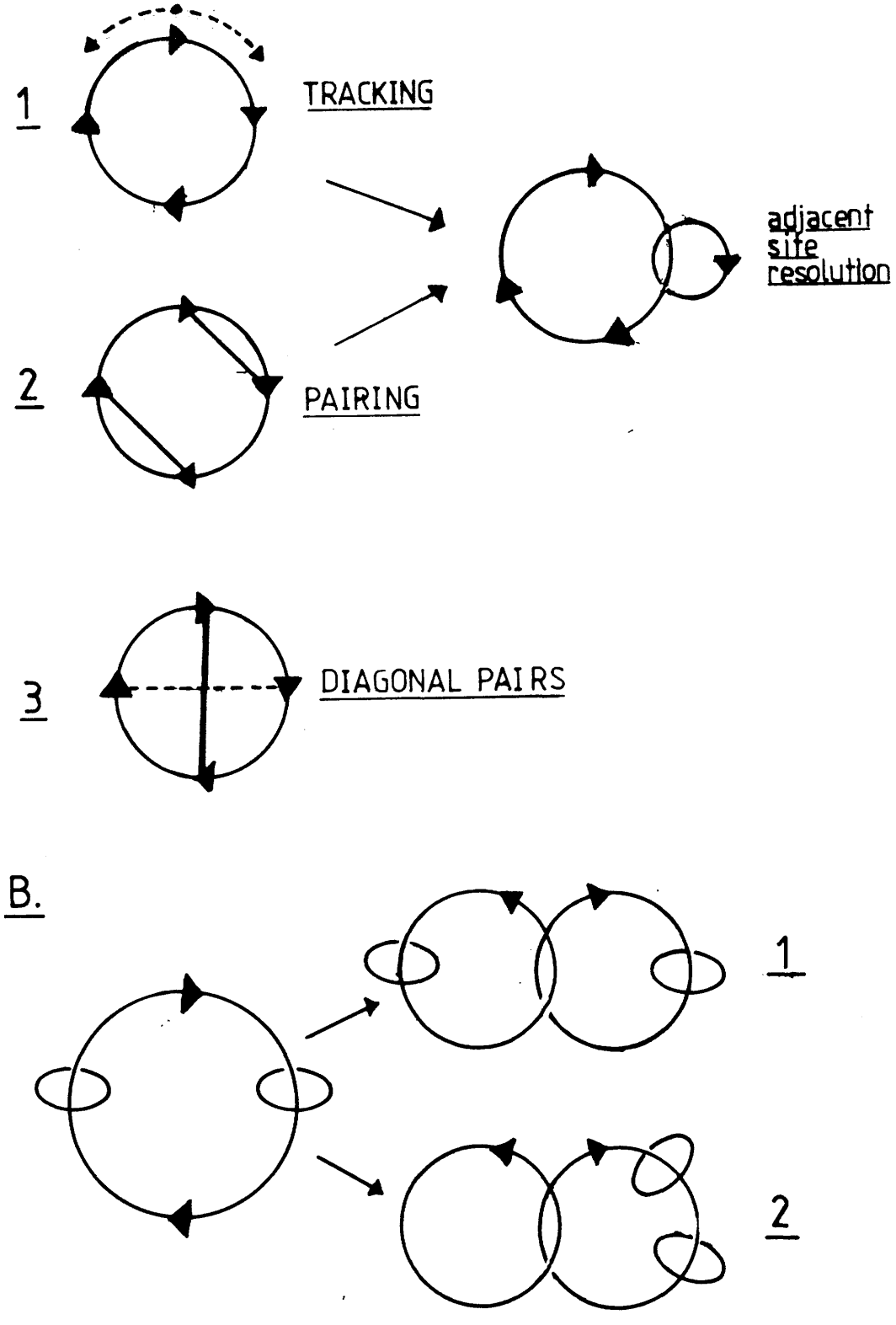


Figure 3.1 A. Tracking and pairing predictions for resolution of a construct containing 4 directly repeated res sites. 1. Shows the adjacent site preference prediction of the tracking model. Pairing models suggest that if the number of synapsed pairs is maximised then (2.) will be favoured over (3.) due to the potential conformation problems on forming more than one non-adjacent synapse (see text for details).

B. The reporter ring experiment. Models based on processive movement of resolvase along the DNA intervening between the 2 sites predict reporter rings will always segregate together as shown in (2.). Pairing models predict random segregation with equal proportions of forms (1.) and (2.) (see text for details).

inversion will be relieved on alteration of the substrate topology.

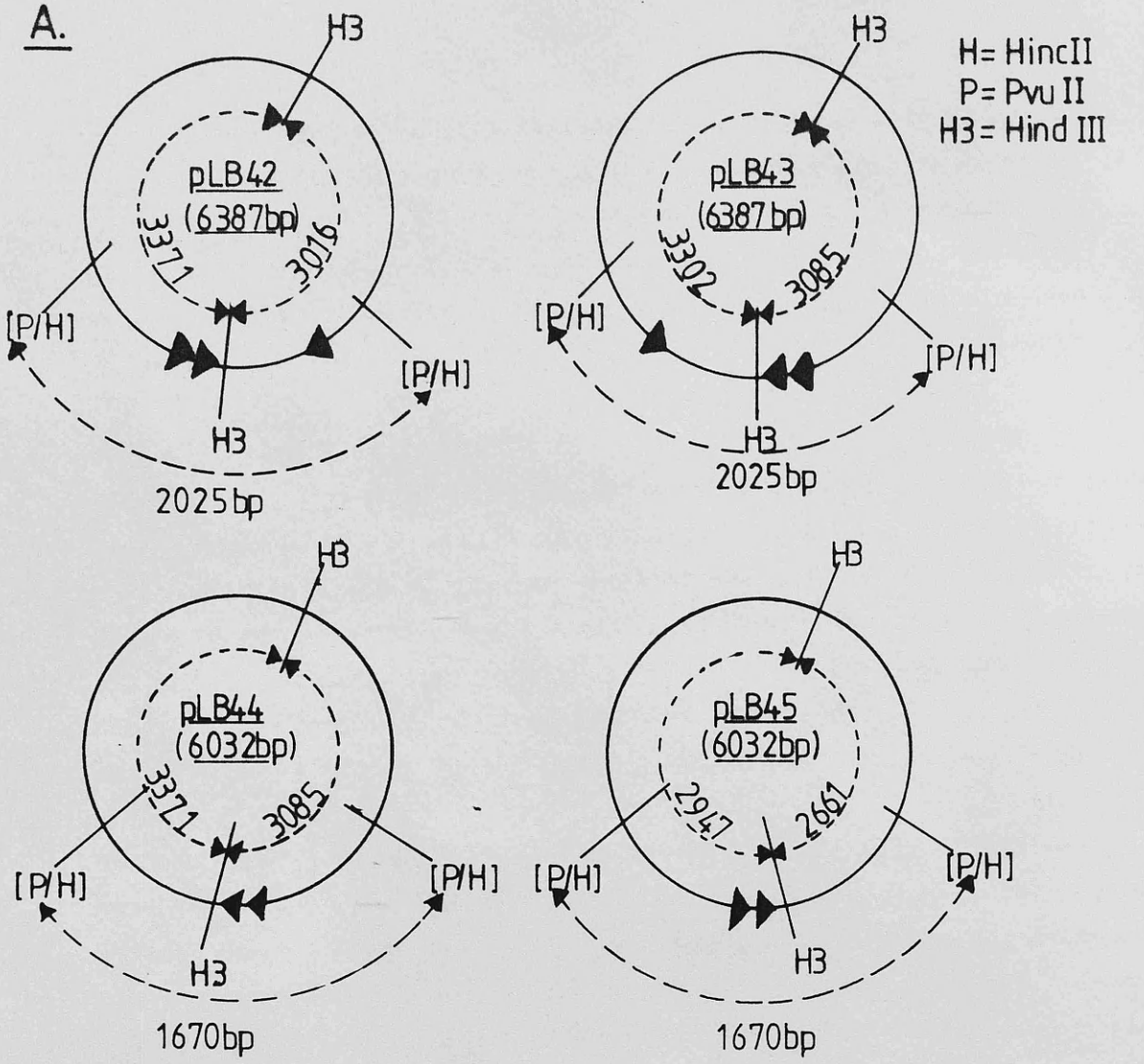
4. Sites II and III will play a major role in site alignment and in defining the polarity of the sites.
5. Resolvase will induce bending of *res* site DNA as it wraps the DNA around itself to form the synaptic complex with -3 synapse geometry.

In this model it is the proposal of reversible pairing which provides an alternative to tracking for any adjacent site bias in constructs with 4 directly repeated *res* sites. In such a 4 *res* site construct if there is a strong tendency to pair up all available sites, this would clearly favour adjacent site pairing because of the potential conformational problems in forming more than one non-adjacent synapse (Figure 3.1). It should be pointed out at this stage that such 'pairing' ideas are not in fact diagnostic for the 2-step model for site synapsis.

The advent of this alternative to tracking for resolvase-mediated site synapsis led to an extension of the original aim of these experiments to try to distinguish between the predictions of the two types of models. The end result is an extensive range of multiple-*res* site plasmids constructed to keep pace with evolving ideas on site synapsis and which ultimately provide evidence which rules out tracking as a model for resolvase-mediated site synapsis.

The approach taken in constructing the multi-*res* site plasmids described here was quite different from that of Benjamin *et al* (1985). Their constructs were highly symmetrical in structure and indeed were dimers or tetramers of plasmids containing 2 directly repeated, or single Gamma delta *res* sites. It was therefore not possible to analyse their constructs by restriction analysis as an alternative, Benjamin *et al* made impressive use of high resolution gel electrophoresis in conjunction with electron microscopy (Krasnow *et al* 1983b) and indeed, it is refinement of these techniques which permitted the detection and identification of the minor resolution products of 2 *res* site plasmids that result from iteration of the recombination reaction (Krasnow *et al* 1983b, Wasserman and Cozzarelli 1985, Wasserman *et al* 1985).

The design of the constructs used here depended on a highly asymmetric approach with an arrangement of restriction sites which



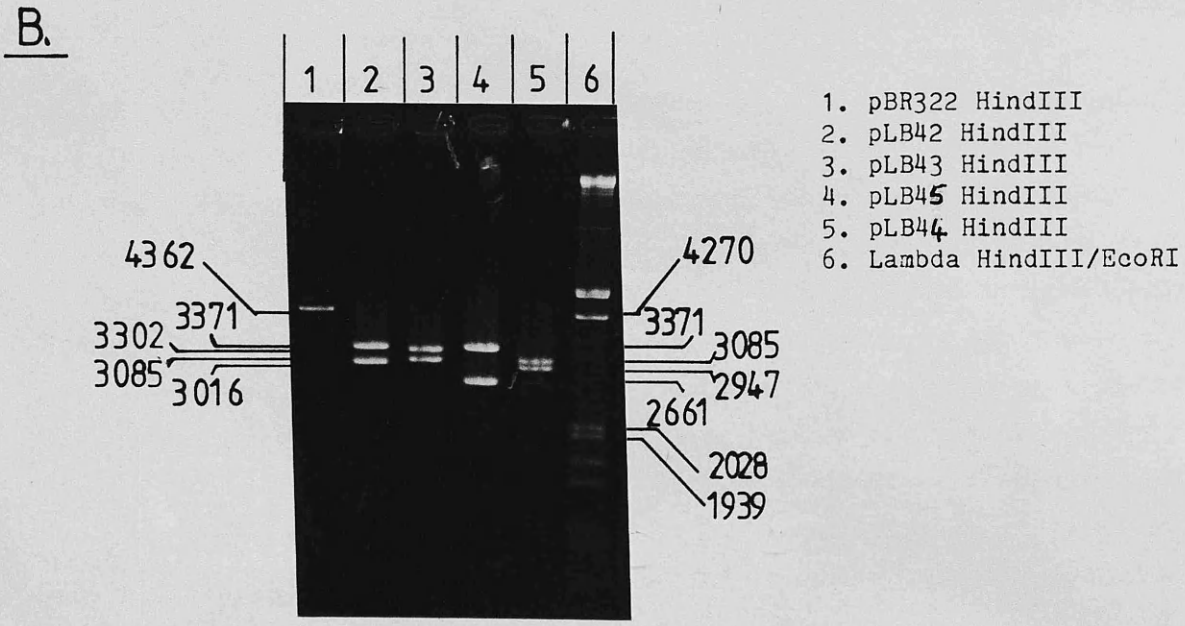


Figure3.2 A. Schematic diagram showing the organisation of pLB42, pLB43, pLB44 and pLB45. Details of their derivation are given in the text; the number and orientation of the res sites is shown. In all the constructs described in this chapter the res sites are represented by a filled arrowhead.

B. 1% agarose gel of HindIII restricted pLB42, pLB43, pLB44 and pLB45. These confirm the orientation of the inserts is as diagrammed in A. above. The expected fragment sizes are detailed in A.

permitted easy and selective analysis of the resolution products by restriction and agarose gel electrophoresis. This permits the resolution characteristics of a plasmid to be easily followed during a time-course reaction (as described in section 3.4).

The structure and resolution characteristics of the various constructs are now described and discussed in the context of the tracking and 2-step models for site synapsis and in relation to the results of Benjamin *et al* (1985).

Results and Discussion

3.1 Construction of pLB42, pLB43, pLB44 and pLB45

Martin Boocock and Lorraine Symington (this laboratory), generated a range of constructs into which various combinations of *res* sites had been cloned into the EcoRI-BamHI region of pBR322. Plasmids were available with 1, 2 or 3 *res* sites cloned into this region either all in direct repeat or a mixture of direct and inverted repeat and either with or without a single *res* site cloned into the unique PvuII site of pBR322. Some of these constructs are diagrammed in Figure 3.3(A). The aim here was to construct a range of plasmids with a similar array of *res* site combinations cloned into the unique PvuII site of pBR322. Once available, these could then be used in simple PstI-AvaI fragment exchanges with the other constructs to generate an extensive range of 3, 4, 5 and 6 *res* site plasmids with the *res* sites in various permutations.

As the first step towards making these plasmids the 1607bp HincII fragment of pMA14 (2 *res* sites in direct repeat) and the 2035bp HincII fragment of pMA414 (3 *res* sites, one inverted with respect to the others) were purified from 1% LMP agarose. These were then separately ligated to PvuII cleaved dephosphorylated pBR322. Ap^r transformants of DS902 were screened for the presence of the inserts by single colony gel analysis and the orientation of the insert was confirmed by Hind III restriction of plasmid DNA. Both orientations were isolated, pLB42 and pLB43 from the pMA414 fragment and pLB44 and pLB45 from the pMA14 derived fragment. These constructs are diagrammed in Figure 3.2.

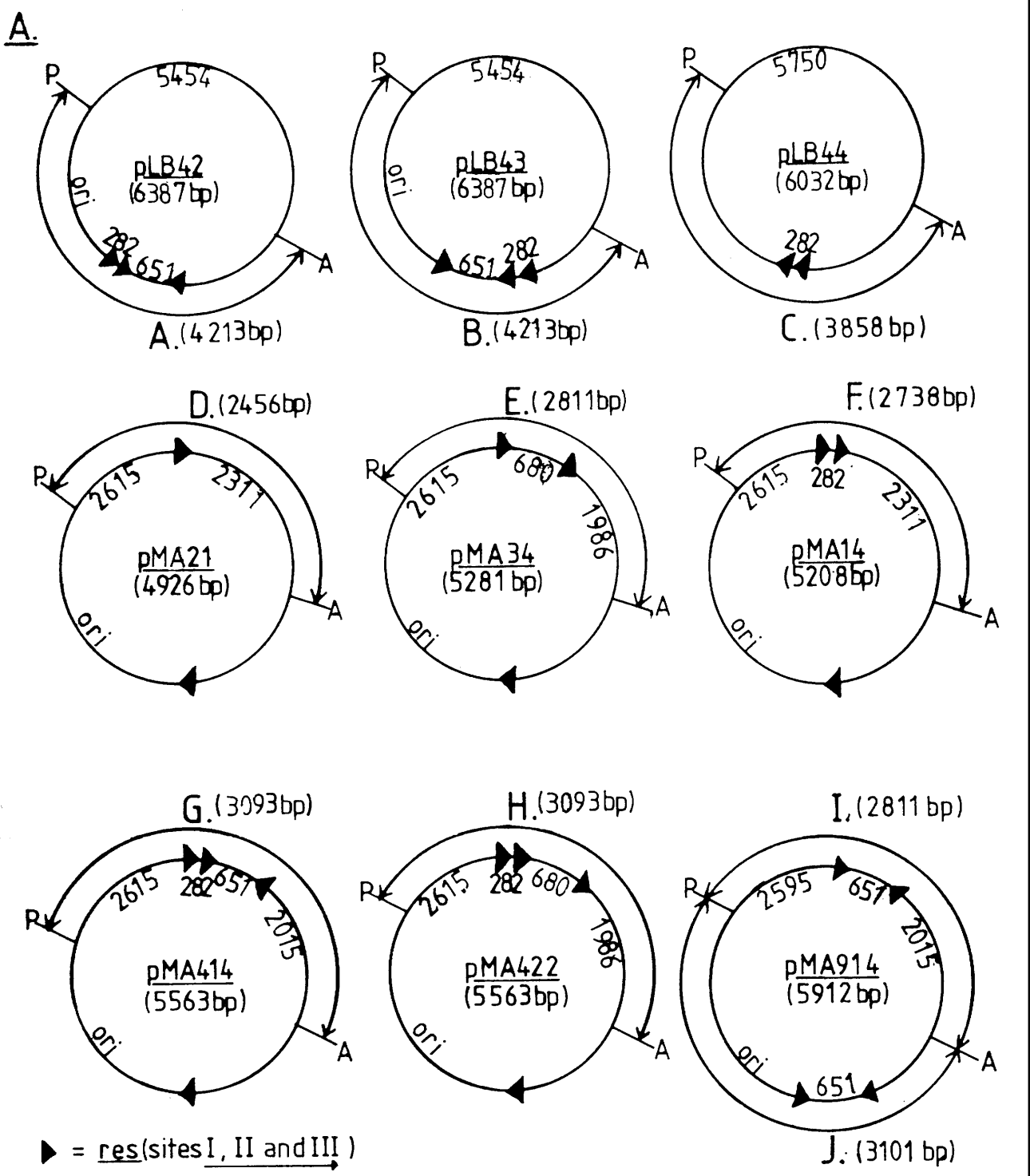


Figure 3.3

A. Diagrammatic representation of the plasmids used in the construction of the multiple res site constructs shown in B. (the strategy is detailed in the text). PstI-AvaI fragments used in the clonings are designated by the letters A to J. The sizes of the fragments, organisation, orientation and spacing of the res sites is indicated.

B. Diagrammatic representation of the range of multiple res site plasmids derived from selected PstI-AvaI fragment exchanges of the plasmids diagrammed in (A). The PstI-AvaI constituent fragments are denoted by a letter, their derivation can be traced by referring to Figure 3.3(A). The plasmid sizes, orientation and spacing of the res sites are indicated. A representative restriction digest confirming the composition of a number of these plasmids is shown overleaf in Figure 3.3C.

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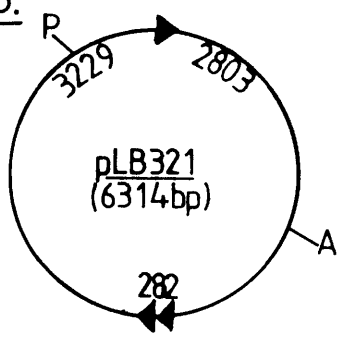
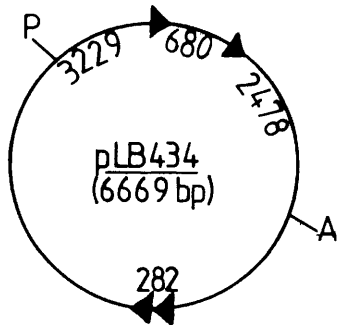
B.



C+D



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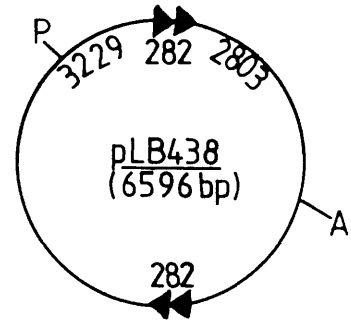

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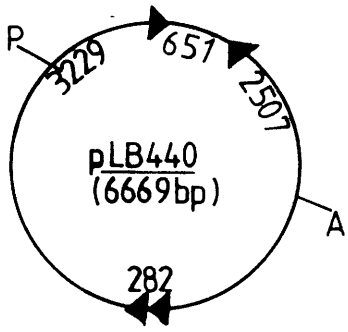

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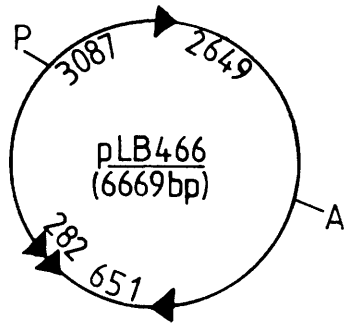
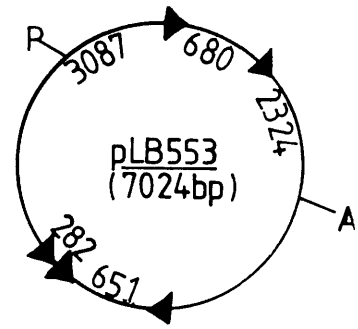


A+D



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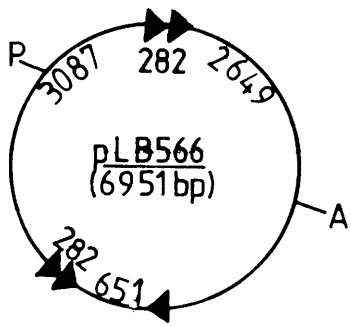

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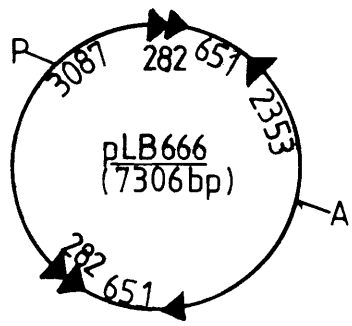
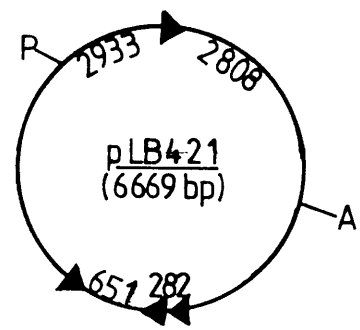


A+G



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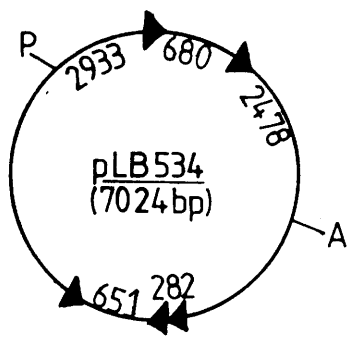

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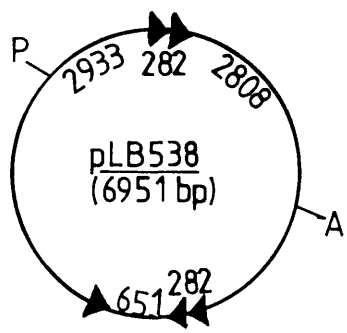

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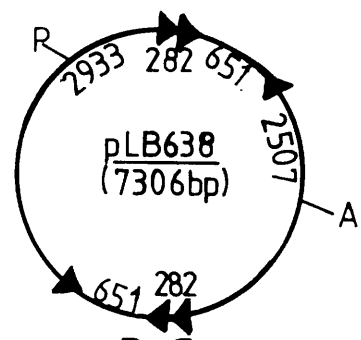

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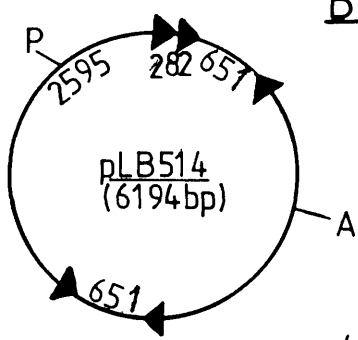

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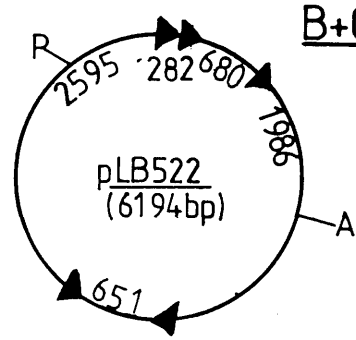

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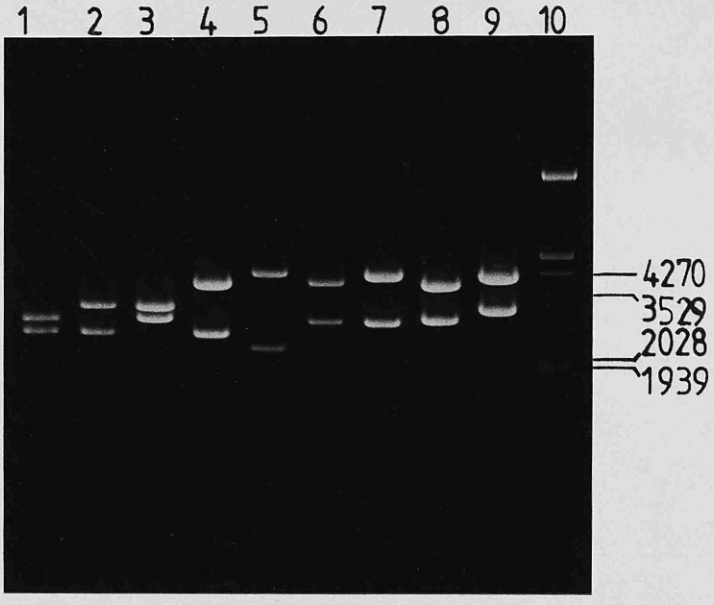


1. pMA14 PstI/AvaI
2. pMA414 " "
3. pMA914 " "
4. pLB321 " "
5. pLB42 " "
6. pLB438 " "
7. pLB566 " "
8. pLB440 " "
9. pLB666 " "
10. Lambda HindIII/EcoRI

Figure 3.3C PstI-AvaI restrictions of pLB434, pLB556, pLB440 and pLB666 PstI-AvaI restrictions of the parent plasmids used in the fragment exchanges are shown. These fragments are labelled as in Figure 3.3A and B. Comparison of the fragments comprising the plasmids listed above with the restriction digests confirms their composition to be as diagrammed. pLB321 was used here as a control instead of the 3858bp fragment of pLB44.

3.2 Construction of a range of 3, 4, 5 and 6 *res* site plasmids

Fifteen different constructs were made by fragment exchanges of the PstI-AvaI *res*$^+$ fragments of pLB42, pLB43 and pLB44 with the appropriate PstI-AvaI fragments of the Martin Boocock constructs. These exchanges are summarised diagrammatically in Figure 3.3. In each case, the required PstI-AvaI fragments were extracted from 1% LMP agarose, ligated and transformed into DS902. The constructions were confirmed by rerestricting with PstI and AvaI. Confirmatory restriction data is shown for some representative constructs (Figure 3.3C).

A number of these constructs have large blocks of sequence arranged in inverted repeat and as a consequence, even in a *recA* strain (DS902) a low level of resolvase-independant rearrangement is generally detectable. In addition, such instability has been the limiting factor in generating plasmids with more closely spaced inverted sites. The instability of perfect and imperfect palindromes has been examined by several groups with varying degrees of success (Boissy and Astell 1985). The most notable result was the enhanced stability of long palindromic inserts in plasmids and bacteriophage Lambda in *E.coli recBC sbcB* strains (Collins *et al* 1982, Leach and Stahl 1983), which are deficient in exonucleaseV and exonucleaseI, the *recBC sbcB* gene products respectively (Clark *et al* 1984). For this reason all the plasmids with extensive inverted repeat sequences were propagated in DL283, a *recBC sbcB recA* strain. This reduced the instability of these plasmids to some extent but not totally; zero time control lanes are therefore always included when analysing the resolution characteristics of these plasmids. Recent evidence (Boissy and Astell 1985) shows the stability of palindromes is increased in *recBC*, *sbcB*, *recF* strains and as a result of the mixed views regarding the effectiveness of a *recA* deficiency to inactivate the plasmidic *recF* recombination pathway (stimulated by the *recBC*, *sbcB* genotype, Clark *et al* 1984) the stability of the multiple *res* site plasmids is now being examined in a *recBC*, *sbcB*, *recF* strain (this laboratory).

3.3 Construction of pLB41

This construct is of a different derivation from those described in

section 3.2. This plasmid was specifically designed to incorporate a unique restriction site between each *res* site to permit easy and selective analysis of the resolution products. The construction is detailed in Figure 3.4.

The expression vector, pKK223.3 was used as the starting plasmid, the advantage over pBR322 being the presence of a polylinker region increasing the number of available cloning sites. As the first step in the cloning the 4108bp SmaI-SphI fragment of pKK223.3 was purified from 1% LMP agarose and ligated to the gel purified (1% LMP agarose) 834bp PvuII-SphI fragment of pMA1534. The resulting plasmid was named pLB35 in which the P_{tac} promoter fragment had been replaced by 3 closely spaced, directly repeated *res* sites.

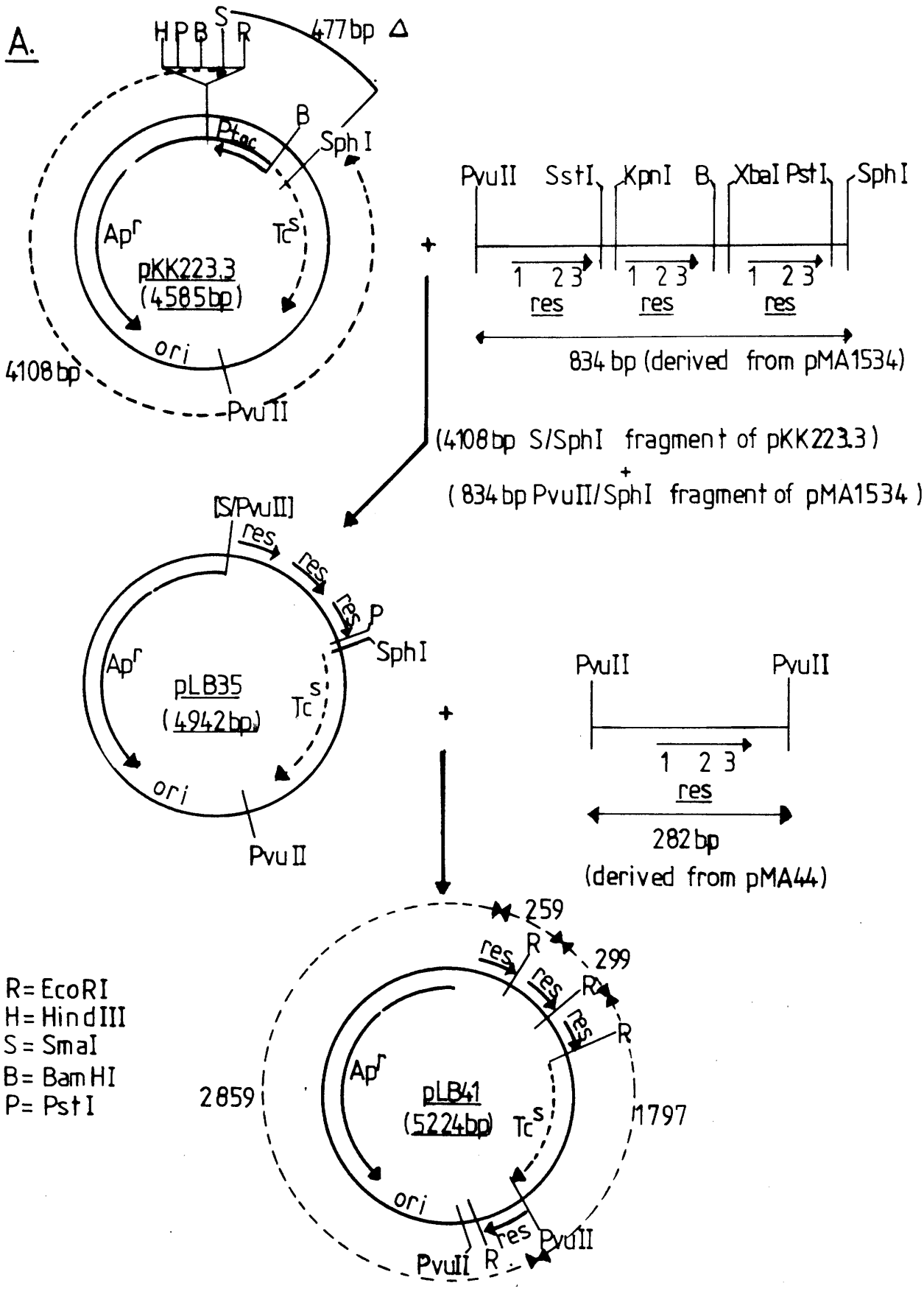
The plasmid pLB35 was then restricted with PvuII, dephosphorylated and ligated to a 282bp PvuII *res* fragment (electroeluted from 5% polyacrylamide) from pLS138. Ap^r transformants of DS902 were screened for the presence of the insert by single colony gel analysis and the orientation was confirmed by EcoRI restriction (see Figure 3.4).

The positions and nature of the unique restriction sites are detailed in Figure 3.4 along with the spacings between the *res* sites (defined as the distance between the 2 crossover sequences).

3.4 Analysis of the resolution characteristics of multiple-*res* site plasmids

The assay system used here depends on gel electrophoresis of restriction digests of samples from resolution time-course reactions. All the constructs described in section 3.2 are based on pBR322 and as a consequence they all have a unique PstI restriction site separating the 2 sets of cloned *res* sites. Therefore, restriction of unresolved plasmids generates a full length linear. Restriction of resolved plasmid will generate a variety of linear fragments and the supercoiled circles (plus catenanes) corresponding to the various resolution events. The relative ammounts of each product will depend on the extent of resolution (the number of primary, secondary and tertiary resolution events etc) and the resolution pathway to the various end products.

Figure 3.5 attempts to illustrate this assay system for the simple 3 *res* site construct pLB321. Section (A) shows the possible primary and


A.
H P B S R
477 bp Δ
B
Sph I
Ptac
Ap^r
Tc^s
pKK223.3
(4585bp)
ori
4108 bp
Pvu II
Pvu II Sst I Kpn I B Xba I Pst I Sph I
1 2 3
res
834 bp (derived from pMA1534)
(4108 bp S/Sph I fragment of pKK223.3)
+
(834 bp Pvu II/Sph I fragment of pMA1534)
[S/Pvu II]
res
P
Sph I
pLB35
(4942bp)
Pvu II
Pvu II Pvu II
282 bp
(derived from pMA44)
259
299
2859
1797
pLB41
(5224bp)
R = EcoRI
H = HindIII
S = SmaI
B = BamHI
P = PstI

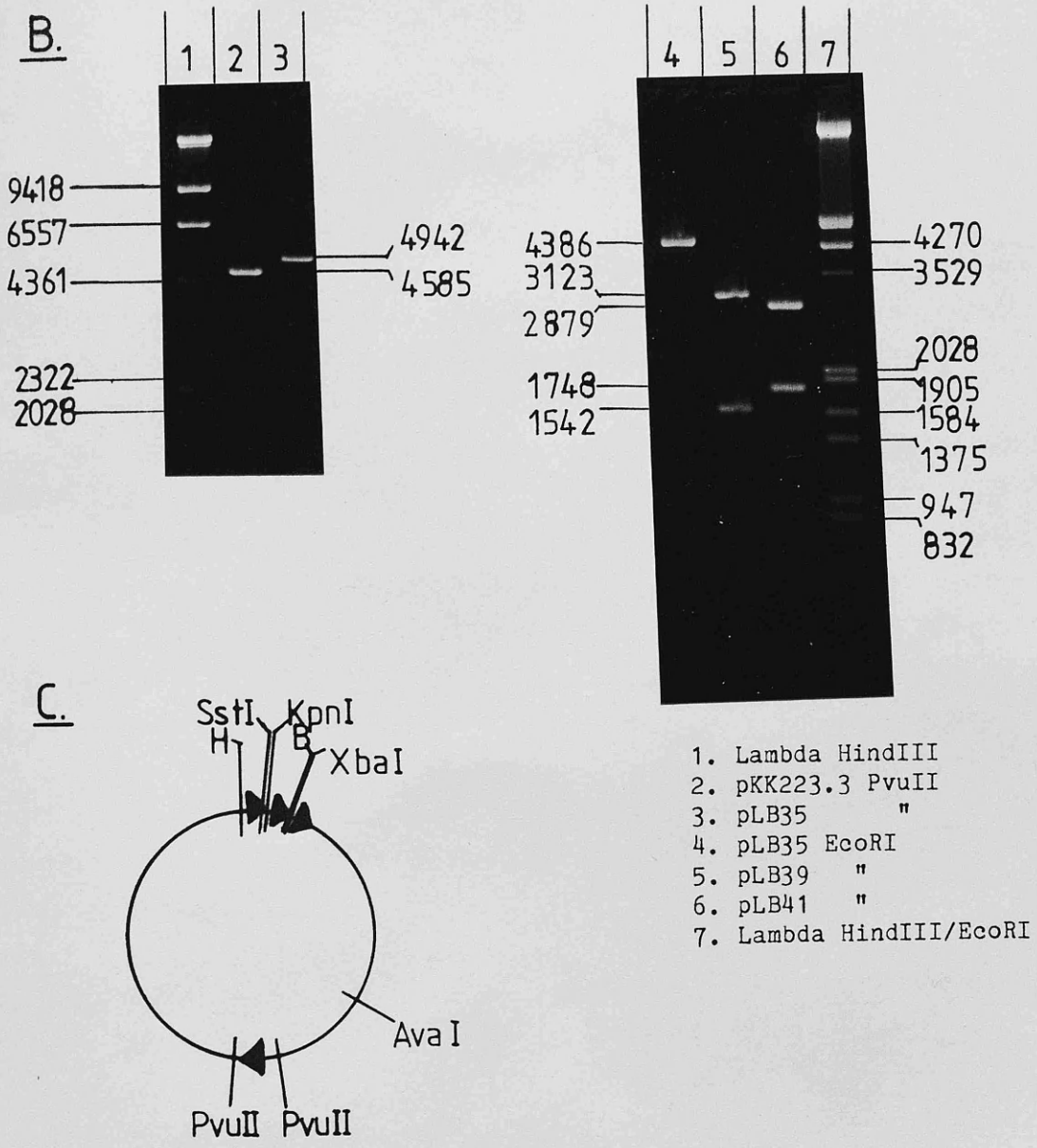
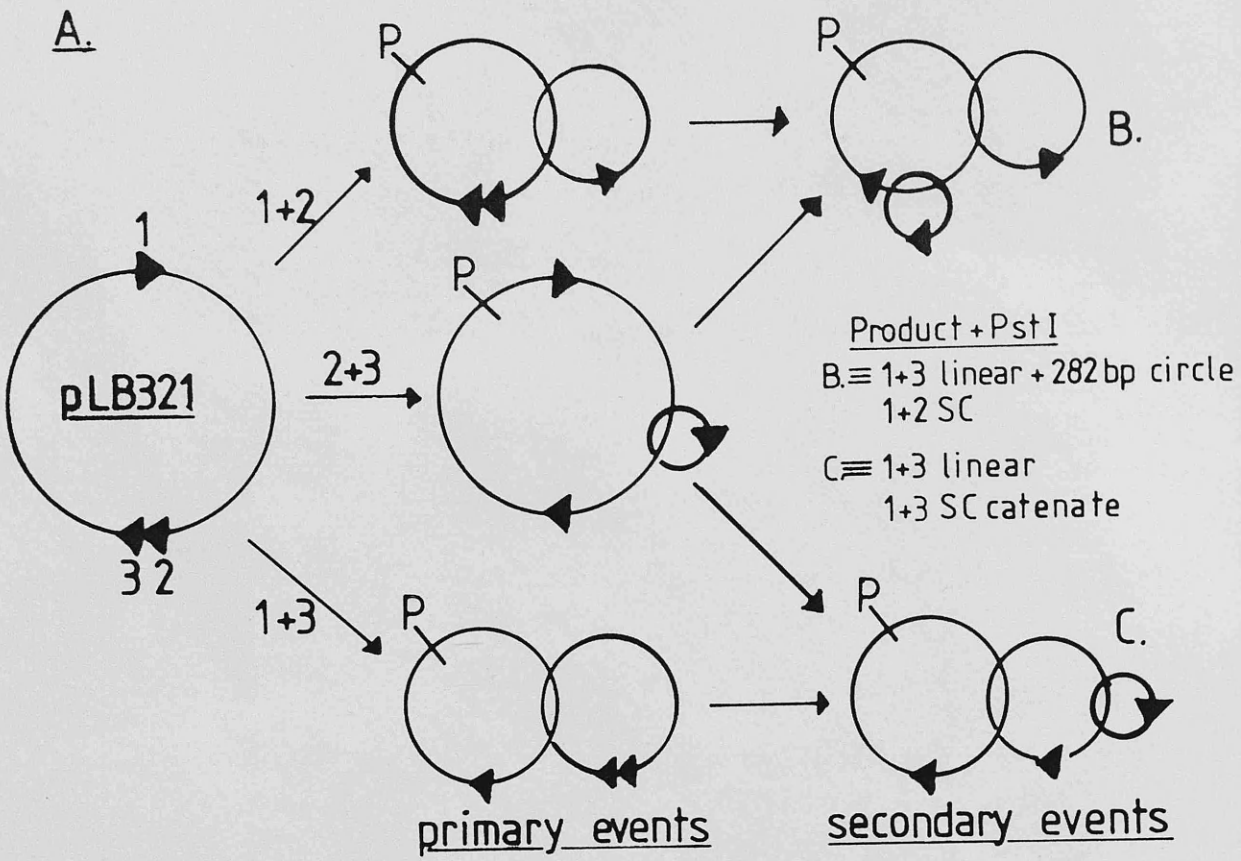


Figure 3.4 Construction of pLB41

A. Schemmatic diagram showing the strategy used to construct pLB41 (refer to text for details).

B. 1% agarose gels showing PvuII restrictions of pLB35 and pKK223.3 and EcoRI restrictions of pLB35, pLB39 and pLB41 confirming the insertion and orientation of the *res* site fragments. pLB39 has the PvuII *res*$^+$ fragment cloned into the unique PvuII site of pLB35 in the opposite orientation to pLB41.

C. Schematic diagram of pLB41 showing the distribution of unique restriction sites between the *res* sites.



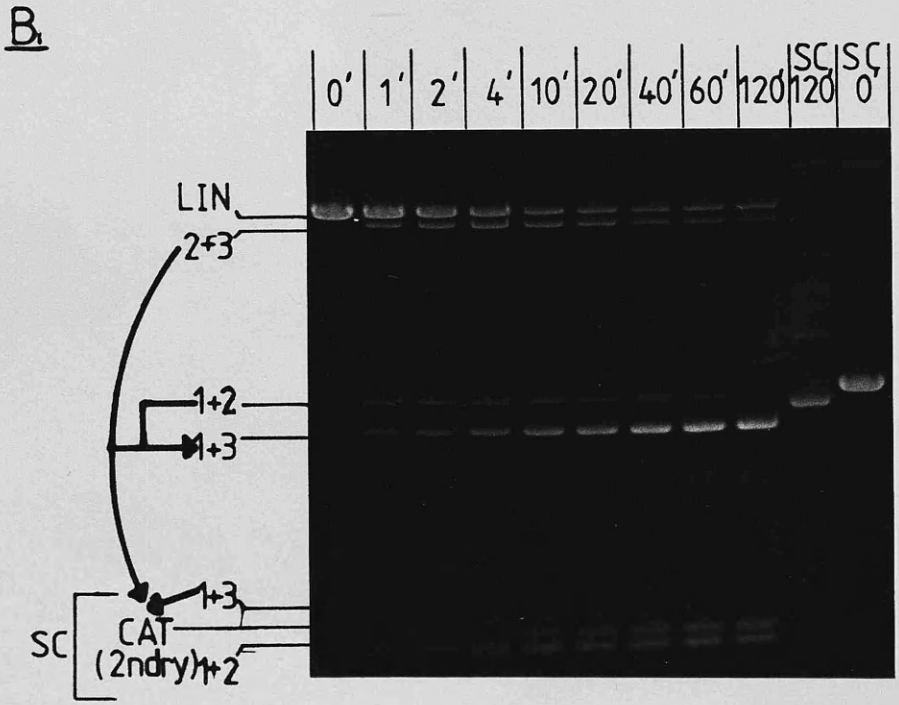


Figure 3.5 Resolution characteristics of pLB321

A. Schematic drawing of pLB321 showing the potential primary and secondary resolution events and the PstI restricted products they would generate (see text for details).

B. 0.8% agarose of PstI restricted samples of a resolution time-course reaction of pLB321. The primary resolution events are indicated. The arrows represent the pathways of breakdown of primary products. All primary products occur at an equal frequency (refer to text for details).

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secondary resolution events and details the PstI generated products arising from them. Figure 3.5(B) shows an actual PstI restricted time course resolution reaction of pLB321.

The major point to note about this assay system is the importance of the initial time points in analysing the primary resolution events; later time points show a build-up of secondary resolution products which band at the same positions as the primary resolution products and can cloud the interpretation, although analysis of these later time points can in some cases be informative.

The validity of using initial time points to examine primary resolution events is proven in the later sections in relation to some of the 4 *res* site plasmids. Most of the 4, 5 and 6 *res* site constructs have their own internal control for the extent of secondary resolution in the initial time points.

In the Figures diagramming the resolution characteristics and the annotations of gel photographs assigning the resolution products to specific bands refer only to the primary resolution events unless otherwise stated.

3.5 Resolution characteristics of constructs containing 3 directly repeated *res* sites

Figure 3.6(A) diagrams some of the 3 *res* site constructs available. The resolution characteristics of pMA14, pMA34 and pMA431 have been examined by Martin Boocock (this laboratory) and Figure 3.5(B) showing the restriction analysis of a resolution time-course reaction of pLB321 is representative of the result obtained in each case.

At initial time points, there is an even-handed distribution of all 3 types of products, implying there is very little distance bias in these asymmetric 3 site constructs, with closely spaced events occurring at a frequency equal to the widely spaced events. This result is not incompatible with the results and interpretation of Benjamin *et al* (1985) but it was rather unexpected on the basis of the 2 models for site synapsis.

One dimensional diffusion or 'tracking' models predict that certainly in 3-site constructs, 2 closely-spaced sites would 'locate' each other more efficiently than sites separated by a greater distance.

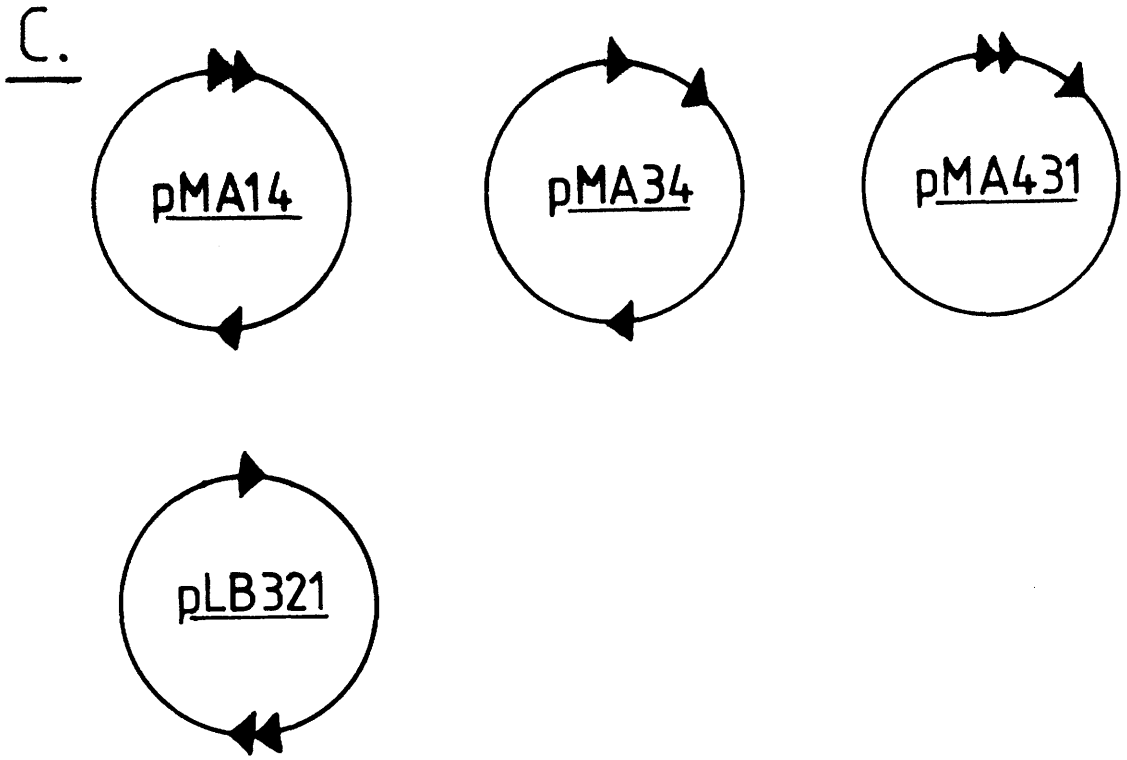


Figure 3.6 3 res site plasmids
Diagrammatic representation of some of the other 3 res site constructs available. As with pLB321, plasmids pMA14, pMA34 and pMA431 show no discrimination against close or widely spaced pairs of sites.

Similarly, the 2-step model for site synapsis predicts 2 sites would be more likely to come together with zero interlinks the shorter the distance between the sites.

Consequently, this absence of distance bias is difficult to reconcile with either model. Clearly, the situation is more complex than was originally realised and to date this result is not fully understood. However, the lack of distance bias has proved very useful and the convenient analysis of the asymmetric 4, 5 and 6 *res* site constructs depends on this property. A distance bias would certainly have complicated the analysis of the resolution characteristics of such constructs beyond reasonable comprehension.

3.6 Resolution characteristics of plasmids containing 4 *res* sites in direct repeat

3.6.1 Resolution characteristics of pMA422

This was the first plasmid used to test the adjacent site preference prediction for resolution and is diagrammed in Figure 3.7. During the discussion of these experiments the term 'shadowing' is adopted to define a pair of *res* sites which recombine less efficiently than other pairs of sites in the same construct. Shadowing correlates with the presence of an intervening *res* site on either side of the synapse and is not seen in 3 site constructs. For pMA422, both tracking and straightforward pairing models for site synapsis predict shadowing of resolution between sites 1+3 and 2+4 (non-adjacent pairs) with a predominance of products resulting from adjacent resolution events. PstI digests of samples from a pMA422 resolution time-course reaction are shown in Figure 3.7.

The results as predicted by both models show heavy shadowing of the non-adjacent event between sites 2+4. Unexpectedly, this shadowing is non-reciprocal and non-adjacent events between sites 1+3 occur at a frequency similar to resolution between adjacent sites. This 1+3 PstI restricted resolution product could arise as a result of secondary resolution events however, it occurs at a frequency much greater than that of the internal control for secondary resolution events. To rule out the possibility that the close spacing of sites 1, 2 and 3 may

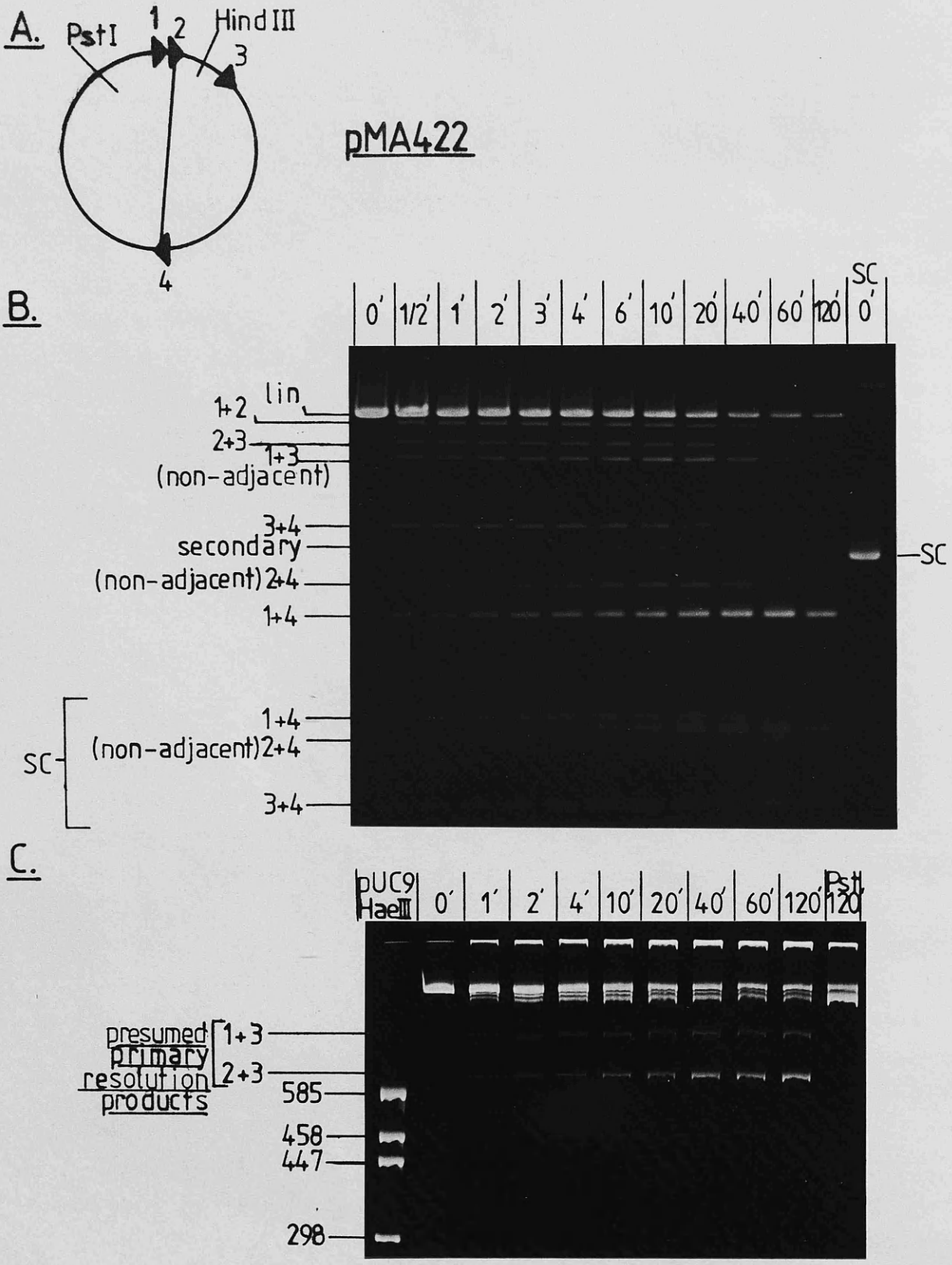


Figure 3.7 Resolution characteristics of pMA422

A. Diagrammatic representation of pMA422 showing the organisation of the res sites and relevant restriction sites. Resolution between sites 2+4 is shadowed as seen in B.

B. 0.8% agarose gel of PstI restricted samples of a resolution time-course reaction of pMA422. The non-adjacent 2+4 event is shadowed, the 1+3 event is not shadowed as confirmed in C.

C. 5% polyacrylamide gel of HindIII restricted samples of a resolution time course reaction of pMA422. This gel shows the 962bp non-adjacent 1+3 event occurs at approximately the same frequency as the 680bp 2+3 adjacent event (see text for details).

promote elevated levels of secondary resolution to generate the PstI 1+3 product, it is possible to examine the reciprocal 1+3 products by electrophoresing HindIII restricted samples of a resolution time-course reaction of pMA422 through a 5% polyacrylamide gel (Figure 3.7). HindIII cleaves between sites 2 and 3 to generate a 962bp fragment (1+3 product), a 680bp fragment (2+3 product) and a 282bp circular product (1+2 resolution, this product was not seen under the electrophoresis conditions used here). Unresolved material generates a full length linear fragment. The 962bp (1+3) fragment occurs at a frequency approximately equal to the 680bp (2+3) products as do the reciprocal 1+3 and 2+3 PstI restricted resolution products, implying the majority of the 1+3 product is derived from a true primary non-adjacent resolution event.

This result, shows non-adjacent events are not always shadowed. Can this result be accommodated by the 2 types of model for resolvase-mediated site synapsis? In terms of tracking, this result is very difficult to explain in the absence of any distance bias in the 3 res site constructs. If any type of 'hopping' were to be invoked due to the close spacing of sites 1 and 2 (282bp) or sites 2 and 3 (680bp), this would be expected to occur in any other construct containing the same spacing. This prediction fails for the 3 res site constructs and is tested in relation to other 4 res site plasmids in section 3.6.2.

The pairing model with some refinement can accommodate this result. The earliest version of the 'pairing' model suggested that 'diagonal pairs' (two similtaneously paired non-adjacent interactions) either can't form or are unstable and the sites rearrange to maximise the number of synapsed pairs, this can be referred to as the 'stable pairs model'. This model could explain non-reciprocal shadowing if the 1+3 synapse is exceptionally stable and causes relaxation of the normal requirement to maximise the number of synapsed pairs. However, this again would be expected to produce some sort of bias in 3 site constructs.

In a modified form of the 'pairing model' it is suggested that initial diagonal pairing does occur but interferes with productive synapsis of the sites (most simply by wrapping constraints on forming 2 non-adjacent synapses). This type of model would provide an explanation for non-reciprocal shadowing if one of the diagonal pairs does not

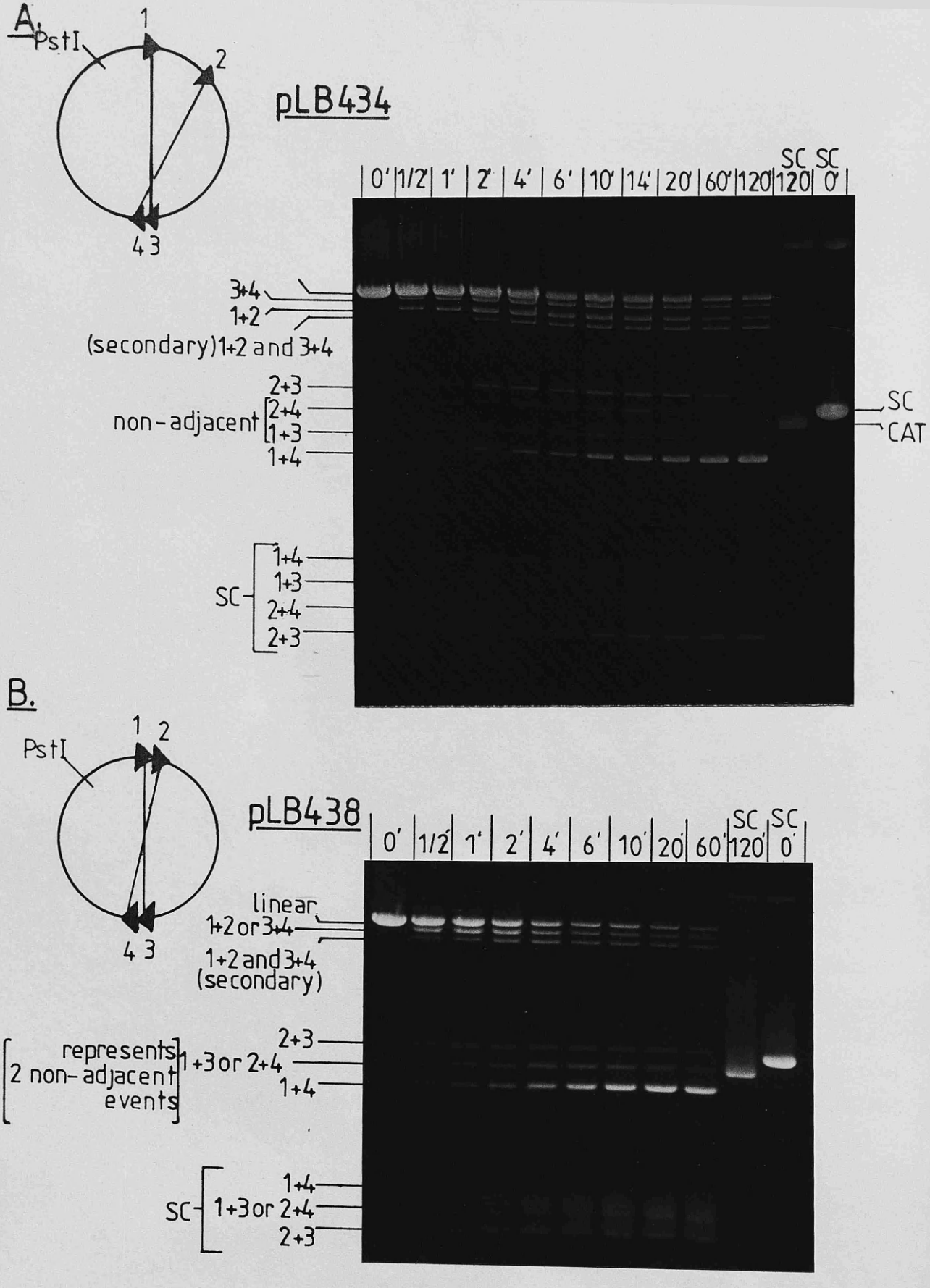
properly interfere with productive synapsis of the other non-adjacent pair of sites. Given the close spacing of sites 1, 2 and 3 in pMA422 this could be explained in 2 ways:-

1. If the intervening site 2 is masked by its close proximity to the synapsed site 1 and cannot interact with site 4 then the sites 1+3 event will be expected to proceed unhindered. This type of non-reciprocal shadowing should be maintained in any 4 res site construct containing the 2 closely spaced sites.
2. Alternatively, the intervening site 2 may be too close to both sites 1 and 3 and may be trapped on an inflexible loop of the 1+3 synaptic complex and be unable to pair with site 4. In this case non-reciprocal shadowing would be expected to breakdown if either of the neighbouring sites were moved further away.

Of the 3 models for adjacent site preference described - tracking, stable pairs and interfering pairs - only the interfering pairs model can plausibly explain the resolution characteristics of pMA422 in the absence of any distance bias in the 3 res site constructs. The two possible forms of the interfering pairs model make testable predictions which are addressed in the following section.

3.6.2 **Resolution characteristics of pLB434 and pLB438**

Rather than containing 3 closely-spaced res sites these plasmids contain 2 sets of 2 closely-spaced sites (maintaining either the 282bp spaced pair or both the 282bp and 680bp spaced pairs) widely separated from each other. This permits analysis of the effects of altering the organisation of the 3 closely spaced sites. Both the 'hopping' explanation of the tracking model and the close spacing proposal (1) of the interfering pairs model predict that non-reciprocal shadowing will be maintained in any 4 res site plasmid containing the 282bp spacing (assuming it is the closest-spaced pair which produces the effect however, the 680bp spacing can also be tested in one of these constructs). The alternative version of the interfering pairs model (2), predicts reorganisation of the sites away from the 3 closely spaced site organisation will relieve the non-reciprocal shadowing seen in pMA422. The stable pairs model also makes this prediction.



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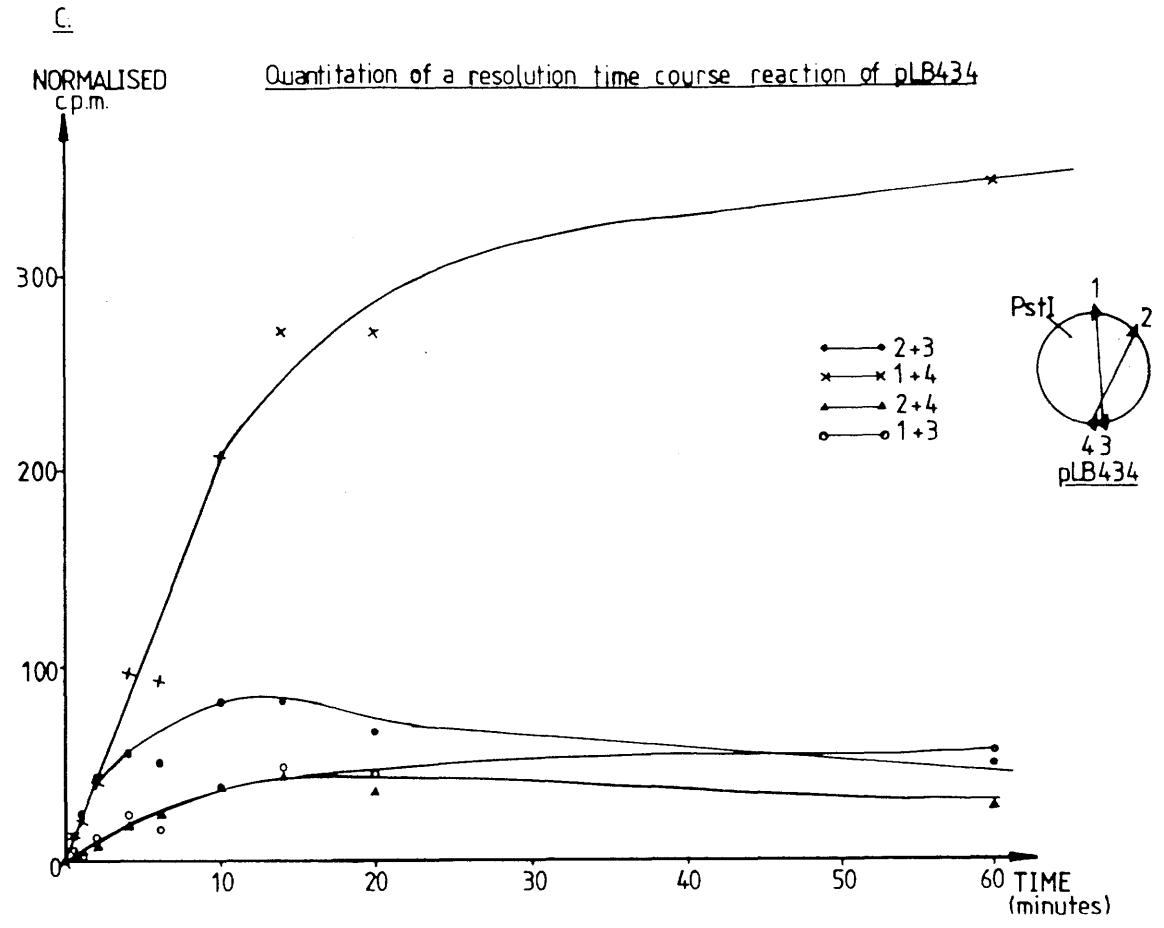

Figure3.8 Resolution characteristics of pLB434 and pLB438

A. 0.8% agarose of PstI restricted samples of a resolution time-course reaction of pLB434 using tritium labelled DNA. The positions of the primary resolution products are indicated as is the secondary product control. Resolution between sites 1+3 and 2+4 is shadowed.

B. 0.8% agarose of PstI restricted samples of a resolution time course reaction of pLB438. The primary resolution products are indicated as is the secondary resolution product control. Products from both non-adjacent events (1+3 and 2+4) band at the same position and are shadowed with respect to the adjacent events.

C. Quantitation of the PstI restricted resolution time-course reaction shown in Figure 3.8A. The data are plotted as normalised counts per minute (after correction for background and divided by fragment length) against time. Data is shown only for the 'long' adjacent and non-adjacent events. This plot confirms quantitatively shadowing of the 1+3 and 2+4 events (see section 3.11 for details). The assignment of the resolution products to gel positions again only refers to the initial time points, clearly thereafter fragments of this nature may also arise from secondary product formation.

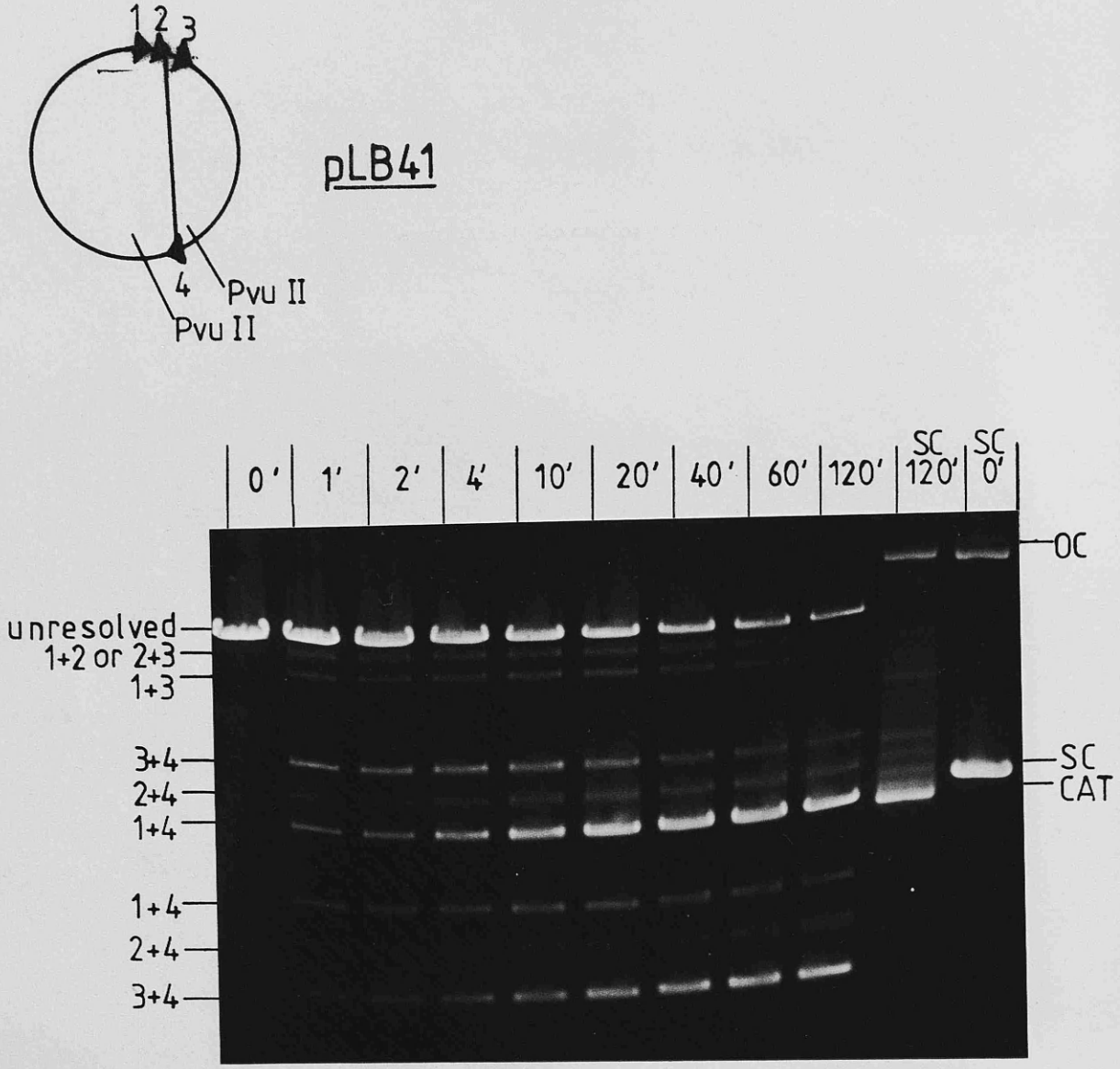

Figure 3.9 Resolution characteristics of pLB41 1%agarose gel of PvuII restricted samples of a resolution time-course reaction of pLB41. PvuII cleaves on either side of site4 and therefore all the fragments seen on this gel are linear. This gel shows shadowing of the 2+4 event and the preference of the 1+3 non-adjacent event over the two short spaced adjacent events and is also confirmed by XbaI and SstI digests on 5% polyacrylamide (data not shown). Details are given in the text.

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The resolution characteristics of these 2 plasmids can be seen in Figure 3.8. Both these plasmids show reciprocal shadowing more in keeping with the results of Benjamin *et al* (1985). These results indicate that non-reciprocal shadowing requires three closely spaced sites as predicted with the second version of the interfering pairs model or the stable pairs model. The resolution characteristics of these 2 plasmids again re-emphasises the lack of distance bias in resolution and rules out any model in which resolution preferentially occurs between pairs of sites that are nearest neighbours.

3.6.3 **Resolution characteristics of pLB41**

The plasmid pLB41 allows an independant test of an adjacent site preference of resolution in a construct containing 4 directly repeated *res* sites. This plasmid is completely asymmetric in design and was specifically constructed to incorporate a unique restriction site between each *res* site, thus permitting selective analysis of the resolution products. PvuII restricted samples of a pLB41 resolution time-course reaction are shown in Figure 3.9. As with pMA422 the long non-adjacent 2+4 event is shadowed. However this plasmid has an added complication in that the non-adjacent 1+3 event is not shadowed and indeed occurs at a frequency considerably greater than the adjacent 1+2 and 2+3 events. This is thought to be a consequence of the 259 and 299bp spacing between the sites since these sites have been shown to resolve very poorly either alone or together yet are fully active in long events in an asymmetric 3-site construct. Evidence that this is not merely a consequence of close spacing comes from plasmids with sites separated by shorter and intermediate spacings which show no such bias in similarly organised 3-site constructs (M.Boocock, unpublished results).

Forming a synaptic complex containing -3 interlinks with these close spaced sites leaves only a very short loop of DNA between the two synapsed sites. It is conceivable, that the ease of bringing together the two sequences of strand exchange in the manner conducive to recombination may then become dependant on the precise number of super-helical turns between the 2 sequences. An experimentally testable prediction of this type of proposal is a 10.5bp periodicity of the

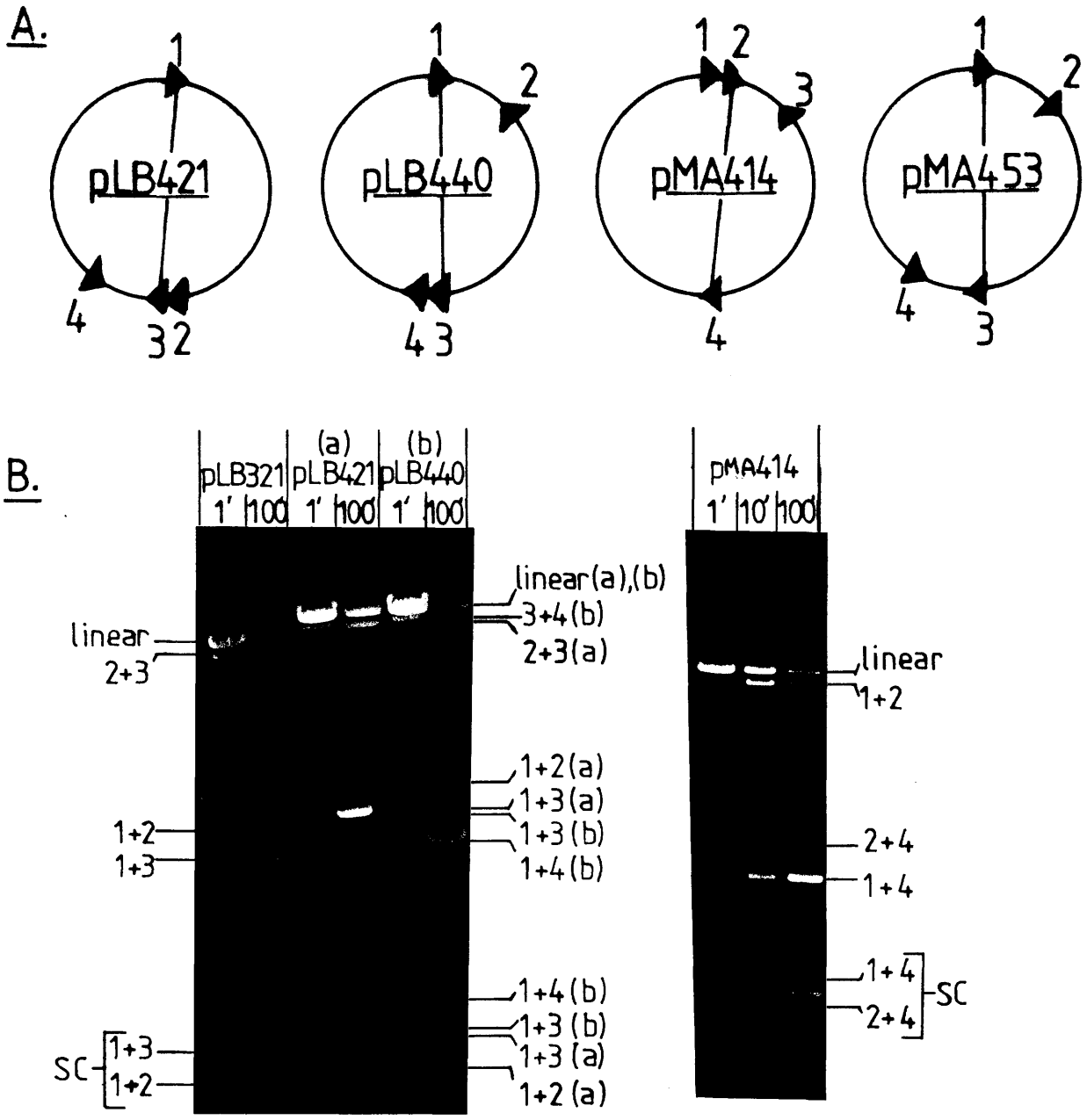


Figure 3.10 Resolution characteristics of 4 <u>res</u> site plasmids with one <u>res</u> site inverted with respect to the others

A. Schemmatic diagram of the 4 <u>res</u> site constructs of this type available showing the organisation and orientation of the <u>res</u> sites. The events which are shadowed are indicated by the solid line.

B. 0.8% agarose gel showing PstI restricted early and late resolution time points for pLB421, pLB440 and pMA414. The primary products are indicated using the numbers assigned in A. Two time points are also shown for pLB321 for comparison with pLB421. This shows the shadowing effects of introducing the inverted site. A resolution reaction of pLB453 is shown in Figure 3.11.

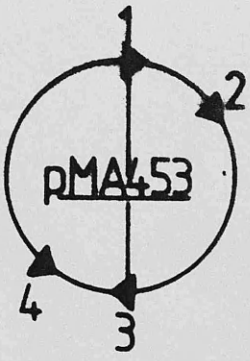
effect on precise alteration of the spacing between the sites. Primarily because of the inherent bias in site preference, pLB41 was used only to reaffirm the pMA422 result.

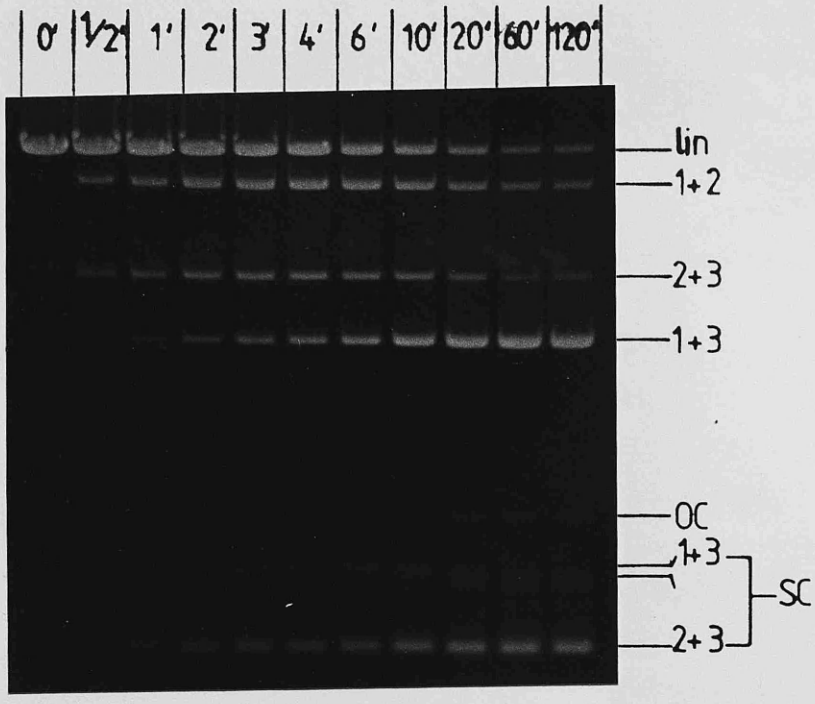
3.7 Resolution characteristics of 4 *res* site plasmids with one *res* site in inverted repeat with respect to the others

Four plasmids fall into this category and these are diagrammed in Figure 3.10. The 2-step model for site synapsis (Boocock *et al* 1986), makes the clear prediction that sites in inverted orientation can and will interact with each other. Recombination will be excluded due to conformational constraints on productive synapsis; synaptic complexes are expected to form but will never yield products. The 2-step model therefore predicts pairing will operate in these plasmids in the same way as in constructs containing 4 directly repeated sites to maximise synapsis of adjacent sites. The predictions of the tracking model are much less clear cut and differ depending on the assumptions made regarding the nature of the resolvase/*res* site complexes and whether only one or both *res* sites are initially resolvase bound. Benjamin *et al* (1985) now favour a tracking model in which both sites are resolvase bound. However, in this situation either shadowing or non-shadowing by an inverted site presents a dilemma for tracking; if resolution between 2 directly repeated sites is shadowed due to the formation of a complex between an inverted pair of sites why can these sites not then recombine? On the other hand, if resolution is shadowed for non-specific reasons simply because resolvase 'bumps' into a protein bound to the DNA, can this model really relate to the *in vivo* situation in which numerous proteins will be expected to be bound to the DNA (repressors, RNA polymerase etc)? If resolution between directly repeated sites is not shadowed by the presence of an inverted site and resolvase translocates through a resolvase/*res* site complex how does resolvase 'hop' over a protein bound site yet still maintain directional specificity by an essentially 'non-specific' process?

PstI restricted early and late samples of resolution time-course reactions of pLB440, pLB421 and pMA414 are shown in Figure 3.10 along with pLB321, the 3 *res* site control plasmid for pLB440 and pLB421. PstI restricted samples of a pMA453 resolution time course reaction are shown

A.

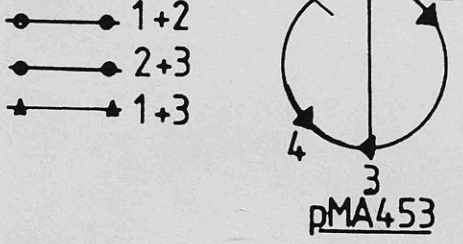




C.

Rate of product formation (arbitary units)

Rate plot of a resolution time course reaction of pMA453



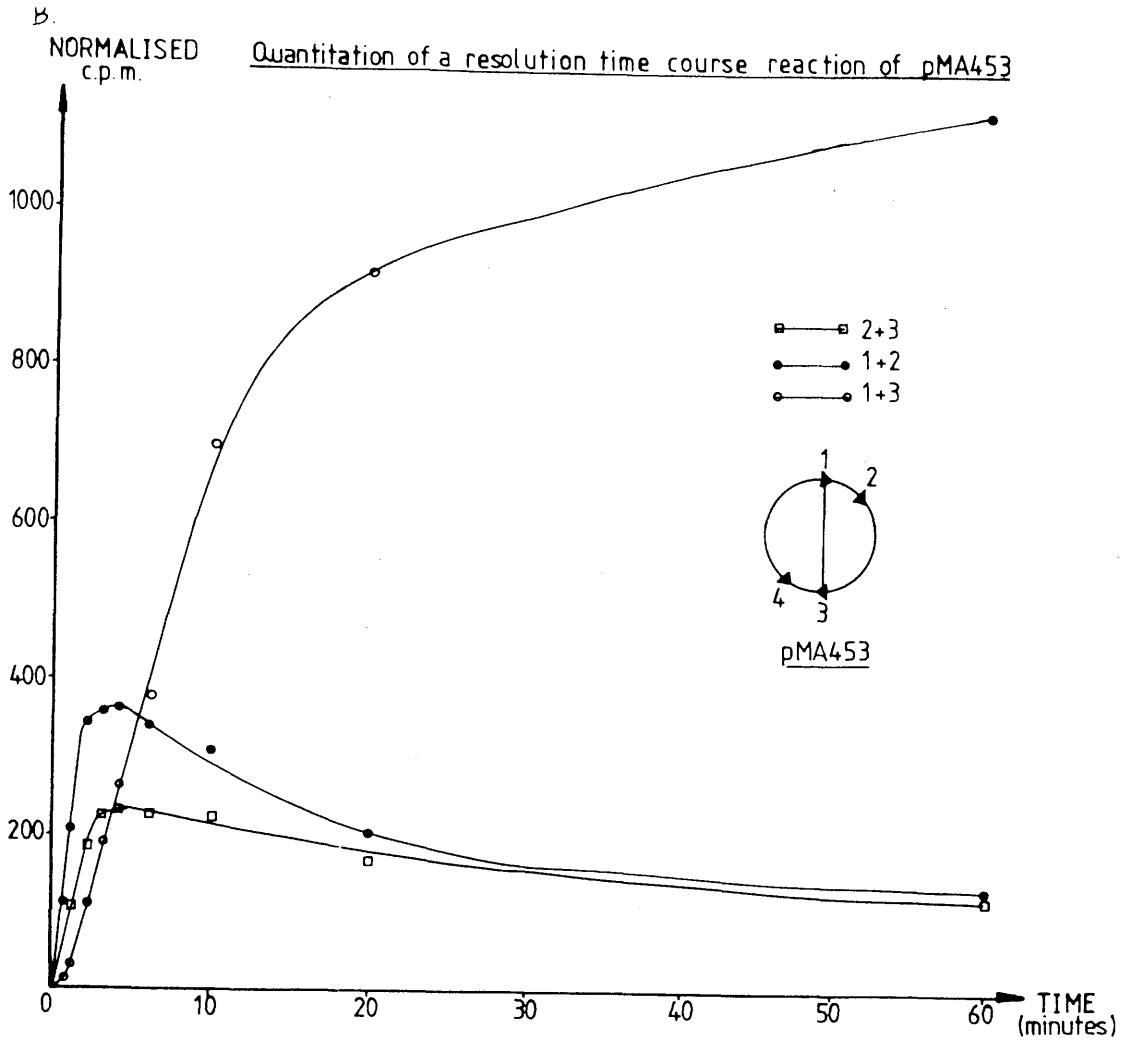


Figure 3.11 Resolution time course reaction of pMA453
A. 0.8% agarose gel of PstI restricted samples of a resolution time course reaction of pMA453 using tritium labelled DNA. The primary products are indicated. This demonstrates shadowing of the 1+3 event.
B. Tritium quantitation of a resolution time course reaction of pMA453. Normalised (by dividing the corrected counts by the fragment length) counts per minute are plotted against time. The data for the PstI linear 1+2, 2+3 and 1+3 products (referring to primary products, clearly at the later time points secondary products will also accumulate at these positions) are plotted. This result confirms shadowing of the 1+3 event as demonstrated by the initial lag before accumulation of this product (presumably mainly as a result of secondary events), details are given in the text.
C. Initial rate plot of resolution of pMA453. The rate of appearance of products is estimated by calculating the number of counts accumulated (or lost) in a particular time interval divided by the time interval. Points are plotted on the graph at the mid-point of the time interval. This result confirms the 1+3 event undergoes a lag in the initial time points before this product starts to accumulate (see section 3.11 for details).

in Figure 3.11 (this experiment was carried out using tritium-labelled DNA which has permitted quantitation of the reaction, this is discussed fully in section 3.11).

In all four cases there is very heavy shadowing of the non-adjacent resolution events, as seen with the similarly-organised plasmids with 4 directly repeated res sites. This is entirely in accord with the expectations of the 2-step model for site synapsis as well as the 'pairing ' predictions arising from it. However, these experiments alone do not provide definitive evidence for the 2-step model. Corroborative evidence that the 'shadowing' of non-adjacent events is due to specific interactions of inverted sites comes from two sources:-

1. Grindley and Reed (1985) have visualised 2 res site/resolvase complexes with both directly and indirectly repeated res sites using electron microscopy.
2. Boocock et al (1986) see inversion at an easily detectable rate on alteration of the substrate topology, this implies inverted sites can synapse but in a supercoiled substrate do not recombine due to conformational constraints. This is precisely the prediction of the 2-step model for resolvase-mediated site synapsis.

In terms of the tracking model, the only conclusion which can be drawn is resolvase cannot, under the experimental conditions used here, translocate through an inverted site. In view of the accumulating evidence that inverted sites can interact tracking does not provide an adequate explanation as to why these sites do not then recombine. Tracking suffers a further setback given the directional specificity can be relieved on relaxation of the substrate topology. Tracking cannot under any circumstance accommodate this result and indeed, the whole concept of tracking is based on the supercoiling dependence of resolution.

The detection of 'shadowing' by an inverted res site is superficially at odds with the results of Benjamin et al (1985). They use a 4 res site construct in which all adjacent sites are inverted with respect to each other. A possible reason for the failure to detect any effects of inverted sites on resolution of the sites in direct repeat lies in their experimental approach. The extent of resolution was measured after incubation at 37^{o}C for 60 minutes at a range of resolvase

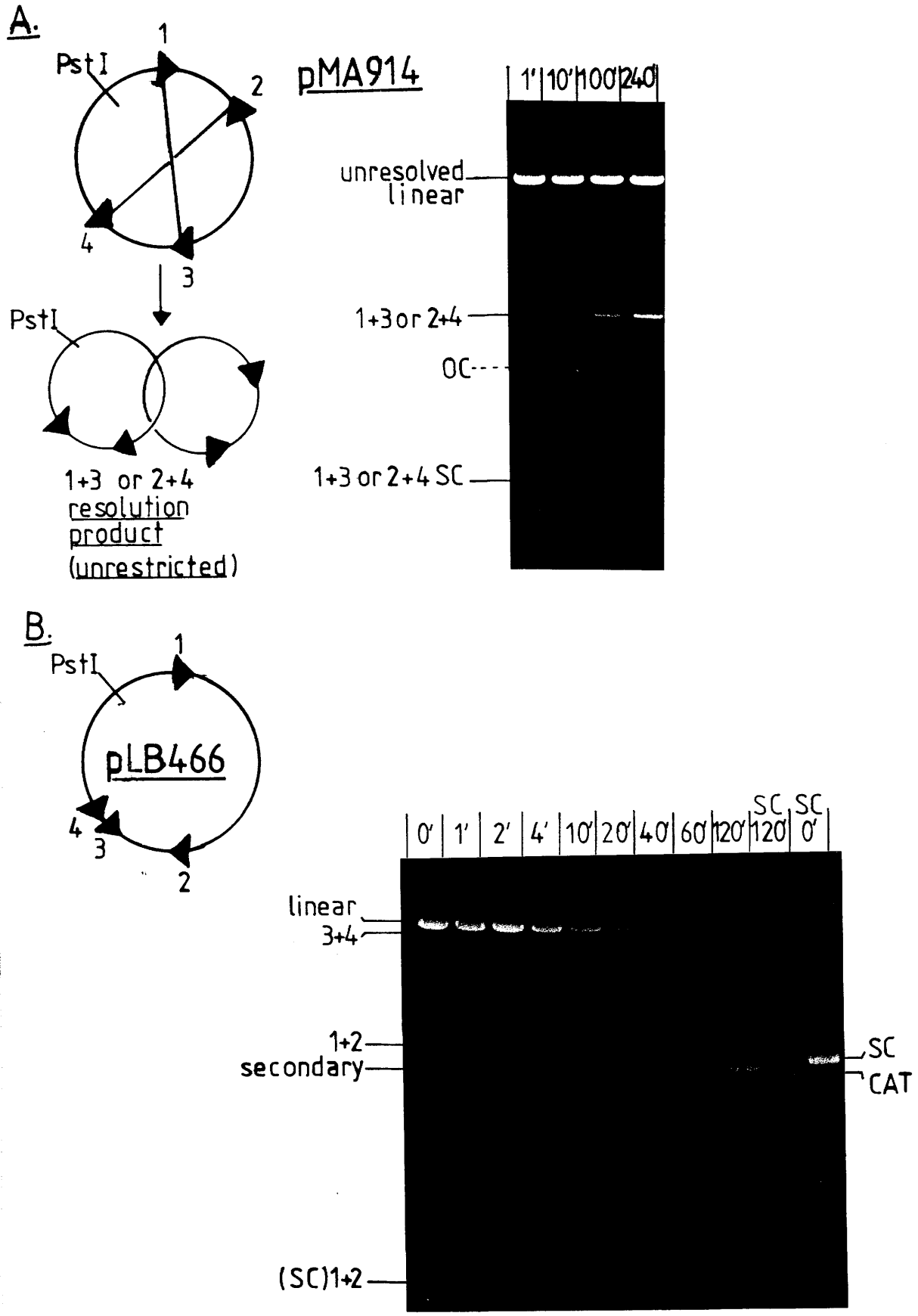


Figure 3.12 Resolution characteristics of pMA914 and pLB466
A. 0.8% agarose gel showing PstI restricted samples of a resolution time course of pMA914. This demonstrates the very poor resolution after 4 hours incubation at 37°C indicating both non-adjacent events are shadowed. The schemmatic diagram illustrates the product produced by resolution of this substrate.
B. 0.8% agarose gel showing PstI restricted samples of a resolution time course reaction of pLB466. This behaviour of this construct demonstrates the absence of distance bias of resolution. The primary and secondary resolution products at the initial time points are indicated.

concentrations. At no point were early resolution time points examined, (which from experience with the constructs described here is when the shadowing effects are most dramatic) and the rate of resolution was not measured. The effect of a single inverted site was not tested.

This highlights a crucial feature of the shadowing phenomenon. Shadowing of a particular nonadjacent event does not necessarily block recombination totally; infact all directly repeated pairs of sites can probably recombine to some extent, the efficiency depending on the site spacing, number and orientation of the intervening sites.

3.8 **Resolution characteristics of 4 *res* site constructs with 2 inverted and 2 directly repeated sites**

The resolution characteristics of pMA914 have been examined by Martin Boocock (this laboratory). This plasmid serves as a control for some of the 5 and 6 *res* site plasmids described in the following sections and is included here for completeness. PstI restricted samples of a resolution time-course reaction of pMA914 are shown in Figure 3.12. In pMA914 all resolution reactions are non-adjacent; resolution is slow and only reaches about 25% after several hours under standard reaction conditions. We do not understand why this result differs from that of Benjamin *et al* (1985) described above.

With regard to pLB466, this plasmid re-emphasises the absence of a distance bias of resolution as seen in Figure3.12. Both pairs of directly repeated sites resolve at approximately the same rate. The resolution profile of this plasmid nicely illustrates the validity of the initial time point analysis by the presence of an independant restriction fragment corresponding only to the secondary products.

3.9 **Resolution characteristics of substrates containing 5 *res* sites**

What are the predictions of the 3 models for site synapsis for the resolution characteristics of 5 *res* site plasmids?

1. Tracking predicts shadowing will be 'additive' in all 5 *res* site substrates (all non-adjacent events will be shadowed).
2. The stable pairs model predicts relief of shadowing in all 5 *res* site constructs.

3. As for all pairing models, the interfering pairs model in general predicts relief of shadowing in 5 res site constructs however, it is more fluid than the stable pairs model and based on the resolution characteristics of pMA422 there will be some dependence on the general organisation, spacing and interfering capabilities of the various pairs of res sites in relation to diagonal pairs. Therefore both additive shadowing and relief of shadowing are possible

The 5 res site constructs are summarised in Figure 3.13. Our ability to interpret the resolution kinetics of these constructs depends on at least one res site being inverted with respect to the others. The resolution characteristics fall into 2 categories.

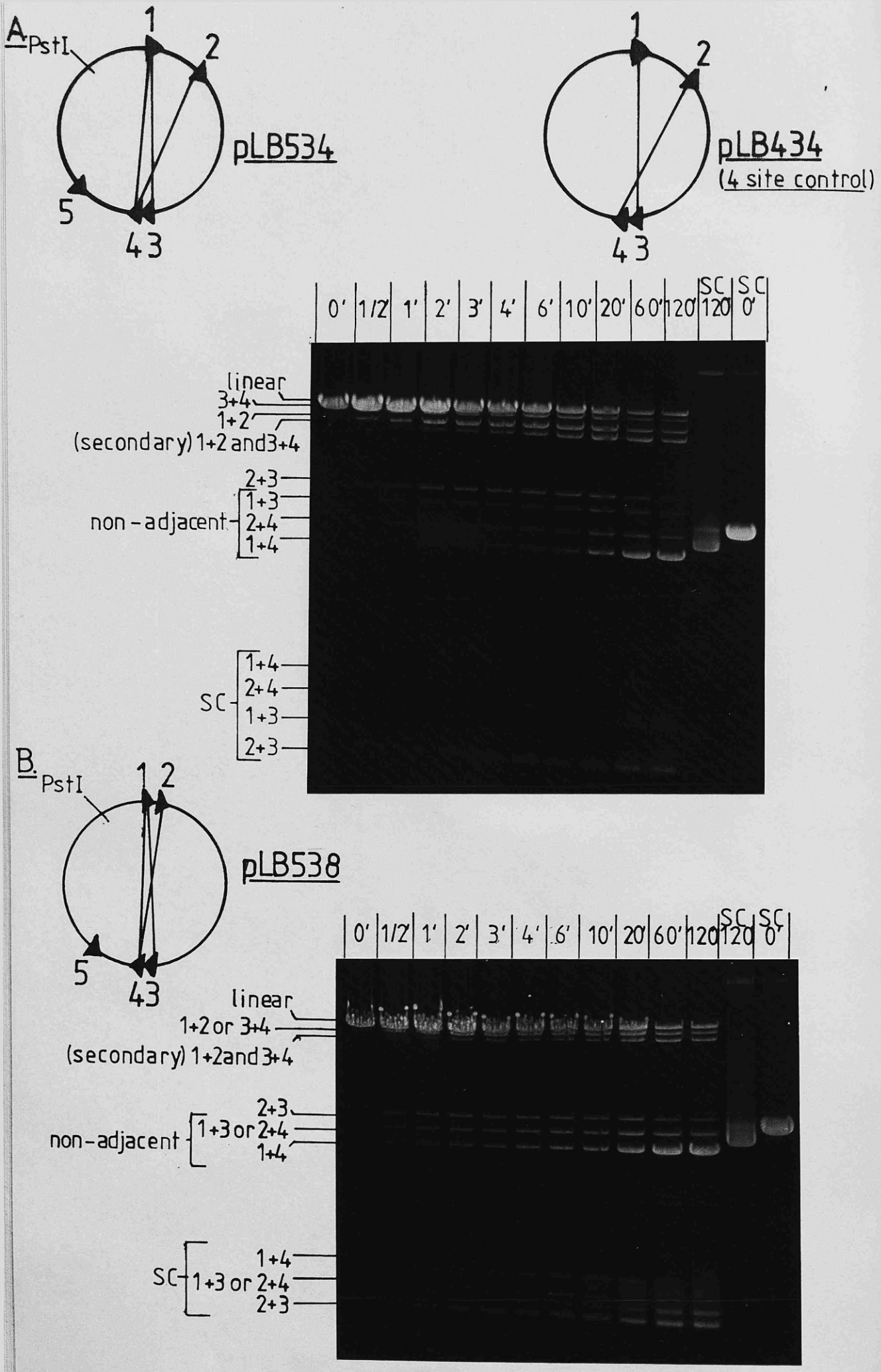
3.9.1 **Resolution characteristics of pLB522, pLB514, pLB534 and pLB538**

PstI restricted samples of resolution time-course reactions for these plasmids are shown in Figure 3.13. These four plasmids show additive shadowing when compared with their corresponding 4 res site control plasmids (pMA422 or pMA453 for pLB522; pMA414 or pMA914 for pLB514; pLB434, pMA453 or pLB421 for pLB534 and pLB438 or pLB421 for pLB538), with all non-adjacent resolution events shadowed except for the 1+3 type of pMA422.

3.9.2 **Resolution characteristics of pLB553 and pLB566**

PstI restricted samples of resolution time-course reactions are shown in Figure 3.14. Comparison of the resolution characteristics of pLB553 with those of pMA453 shows the non-adjacent 1+3 event does occur in pLB553 and at a frequency much greater than that attributable to formation by secondary resolution events (the resolution characteristics of both these plasmids have been quantitated, this is discussed in section 3.11). Similarly, pLB566 shows a distinct lack of discrimination against non-adjacent events as compared to a similarly organised 4-site control plasmid.

In relation to the models for site synapsis, the interfering pairs model is the only one which can accommodate both additive and non-


A
PstI
1
2
5
43
pLB534
pLB434
(4 site control)
0'
1/2'
1'
2'
3'
4'
6'
10'
20'
60'
120'
SC 120'
SC 0'
linear
3+4
1+2
(secondary) 1+2 and 3+4
2+3
1+3
2+4
1+4
non-adjacent
SC
1+4
2+4
1+3
2+3
B.
PstI
pLB538
linear
1+2 or 3+4
(secondary) 1+2 and 3+4
non-adjacent
1+3 or 2+4
SC
1+3 or 2+4

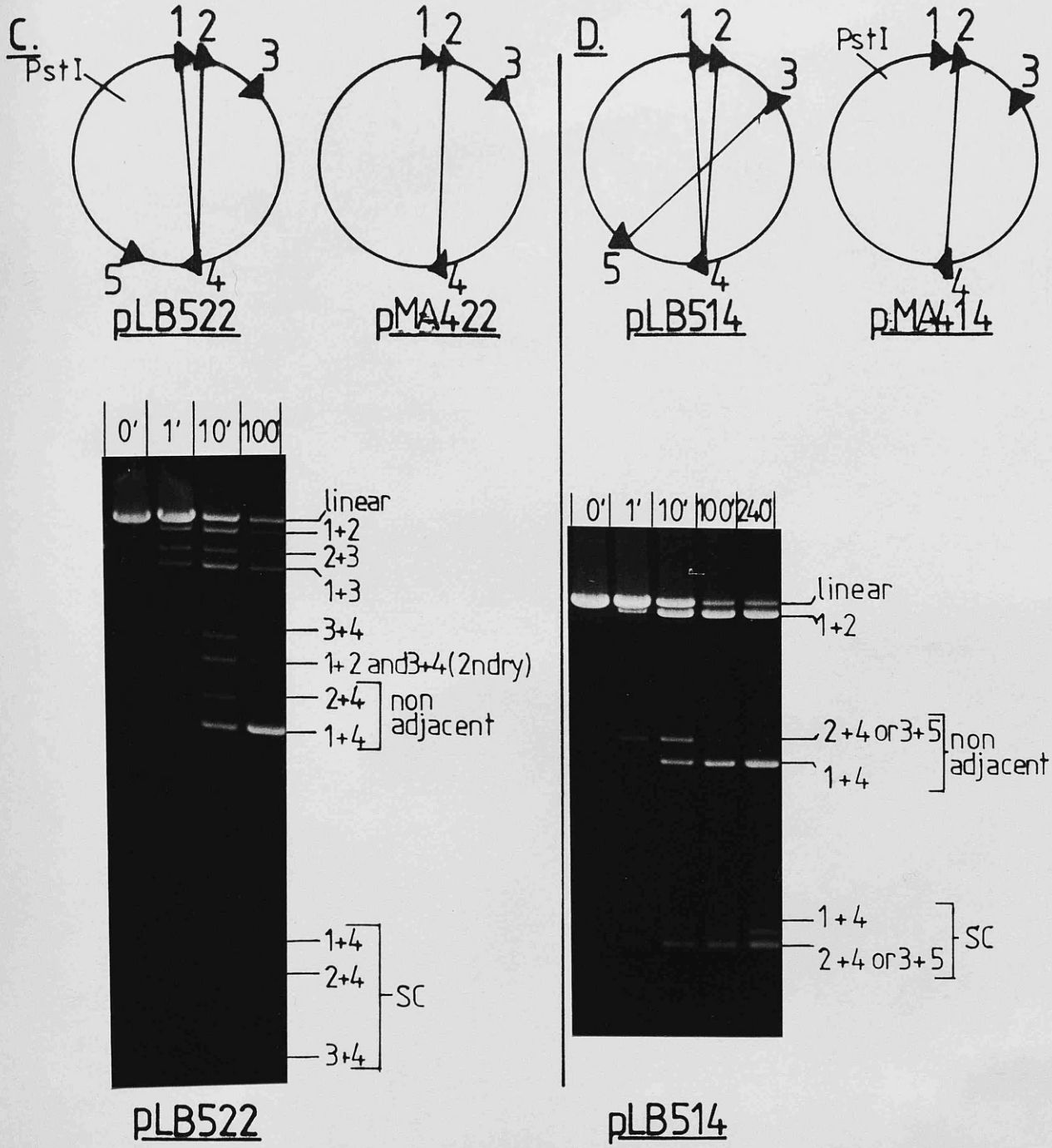


Figure 3.13 Resolution characteristics of pLB534, pLB538, pLB522 and pLB514 showing additive shadowing as compared to the 4 res site control plasmids

A. 0.8% agarose gel showing PstI restricted samples of a resolution time-course reaction of pLB534. All non-adjacent events are shadowed. Introduction of site 5 therefore introduces shadowing of the 1+4 event.

B. 0.8% agarose gel showing PstI restricted samples of a resolution time-course reaction of pLB538. Again all non-adjacent events are shadowed.

C. 0.8% agarose gel of PstI restricted samples of a resolution time-course reaction of pLB522. The introduction of site 5 introduces shadowing of the 1+4 event as compared to resolution of pMA422.

D. 0.8% agarose gel showing PstI restricted samples of a resolution time-course reaction of pLB514. Again the shadowing is additive as compared to the 4 res site control plasmids. A large proportion of the final products have resolved only the 1+2 event. This generates a plasmid identical to pMA914 which has been shown to resolve very poorly.

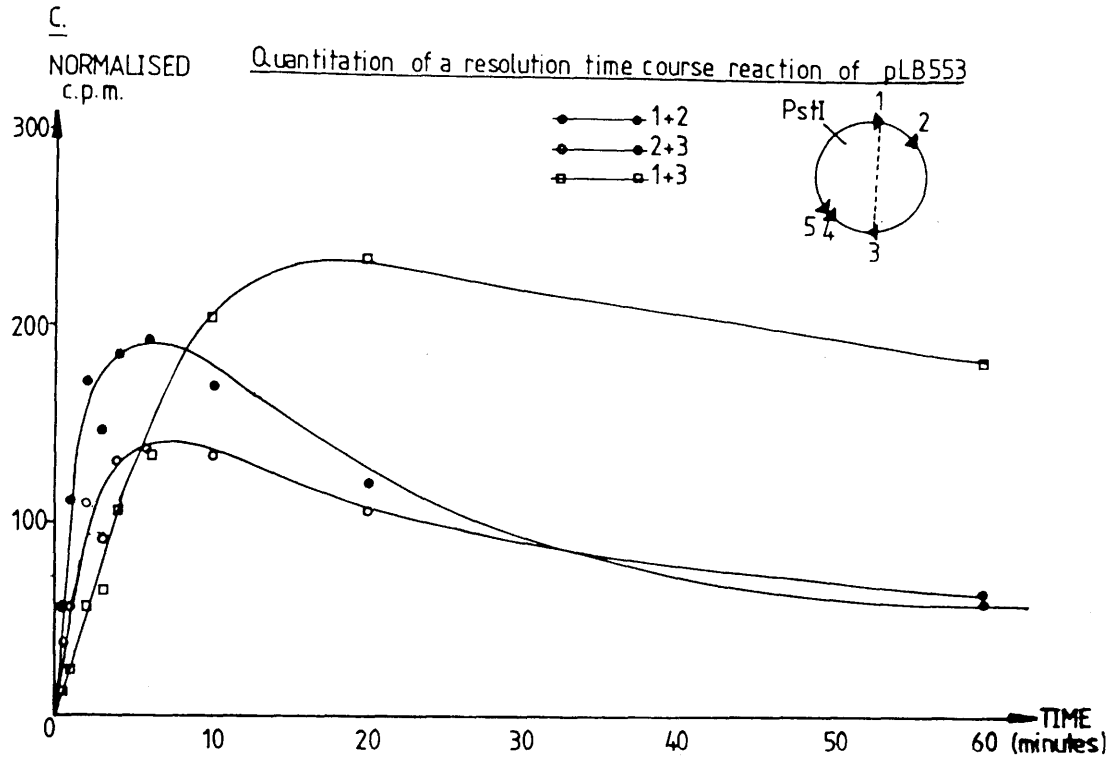
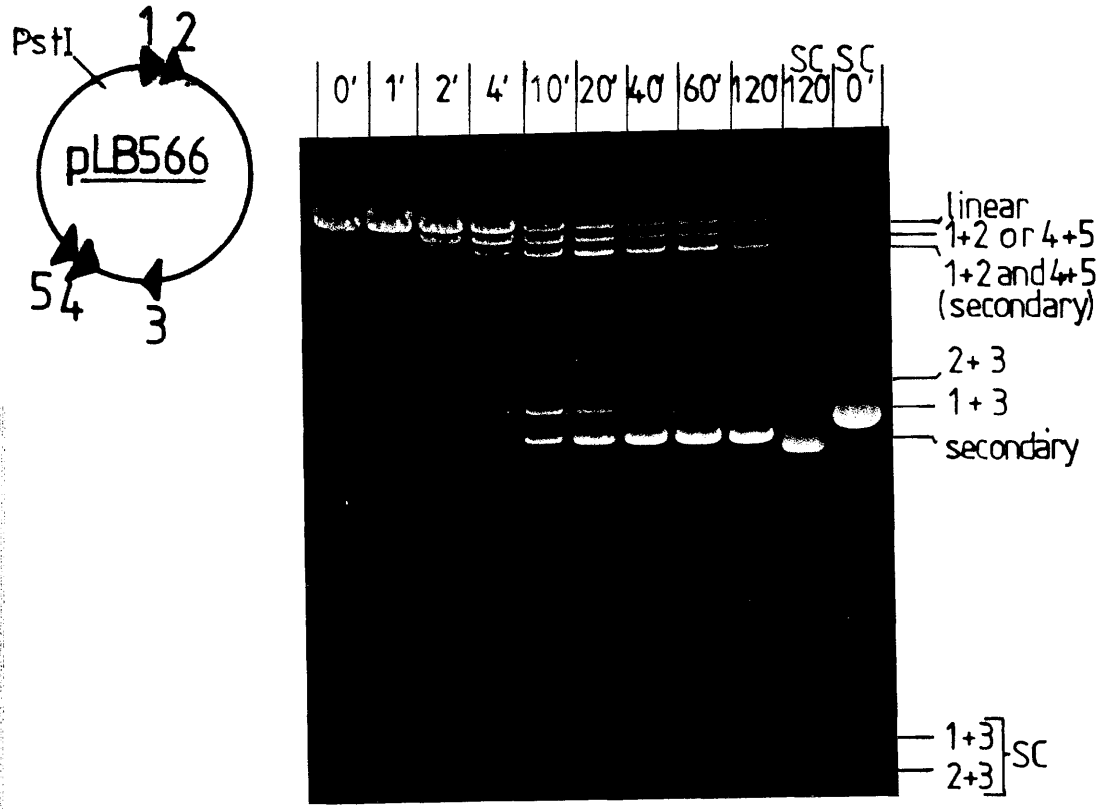
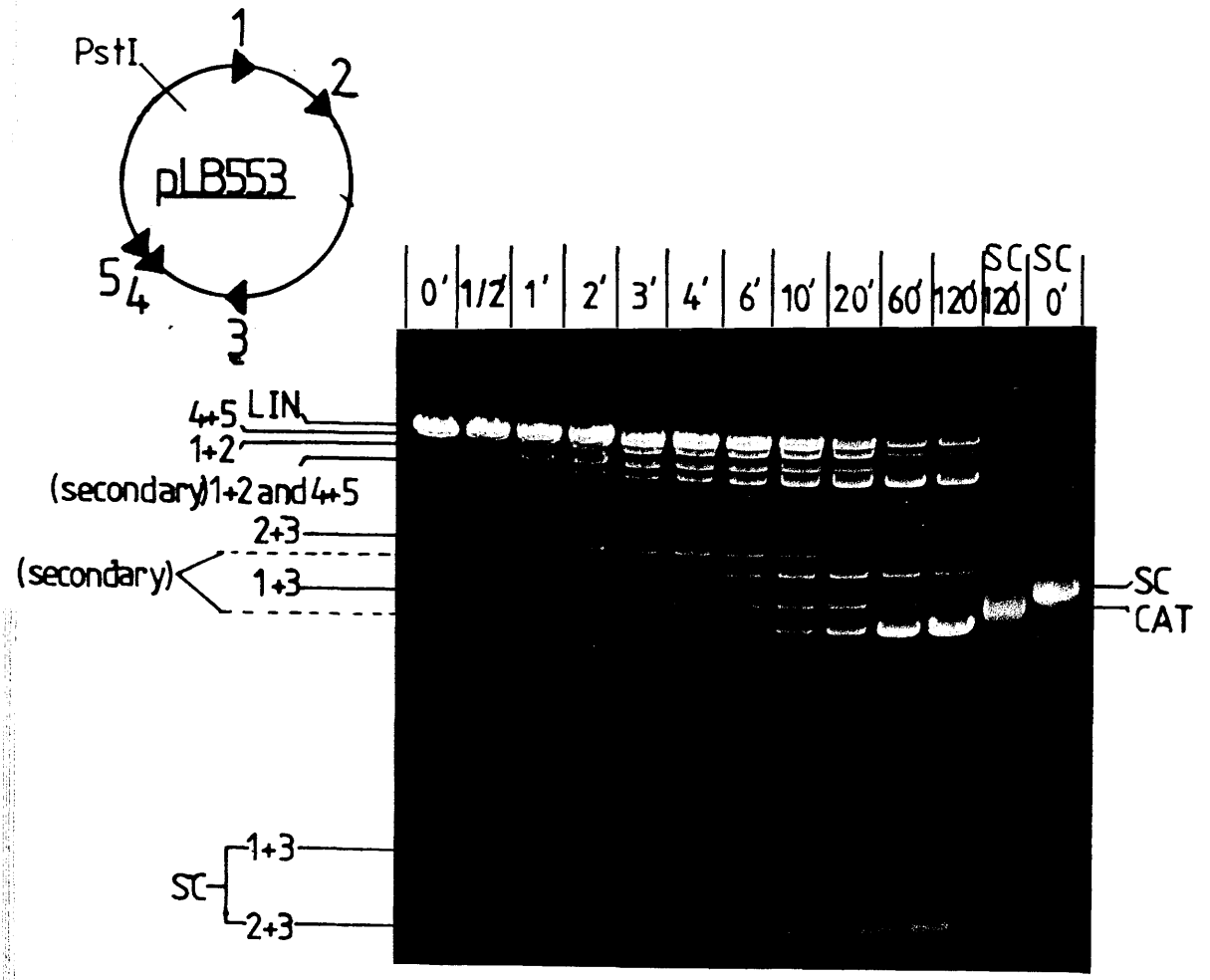


Figure 3.14 Resolution characteristics of pLB566 and pLB553
A. 0.8% agarose gel showing PstI restricted samples of a resolution time-course reaction of pLB566. This plasmids shows little or no shadowing of the 1+3 non-adjacent event unlike the similarly organised 4-site control plasmid pLB440.
B. 0.8% agarose gel showing PstI restricted samples of a resolution time-course reaction of pLB553 using tritium labelled DNA. The 1+3 non-adjacent event occurs at a frequency greater than that attributable to secondary product formation implying shadowing of this event may be only be partial.
C. Quantitation of the PstI restricted resolution time course reaction of pLB553 shown in Figure 3.14B. The data for the 1+2, 2+3 and 1+3 (initial events, clearly other products will accumulate at the same positions at the later time points) events are plotted as normalised (for fragment length) counts per minute against time. The 1+3 event is still shadowed but the shadowing is slightly less pronounced in the initial time points as compared to pLB453 although this will require further confirmation.

A.


PstI
1
2
pLB566
5
4
3
0'
1'
2'
4'
10'
20'
40'
60'
120'
SC 120'
SC 0'
linear
1+2 or 4+5
1+2 and 4+5
(secondary)
2+3
1+3
secondary
1+3
2+3
SC


PstI
1
2
pLB553
5
4
3
0'
1/2'
1'
2'
3'
4'
6'
10'
20'
60'
120'
SC 120'
SC 0'
4+5 LIN
1+2
(secondary) 1+2 and 4+5
2+3
(secondary)
1+3
SC
CAT
SC
1+3
2+3

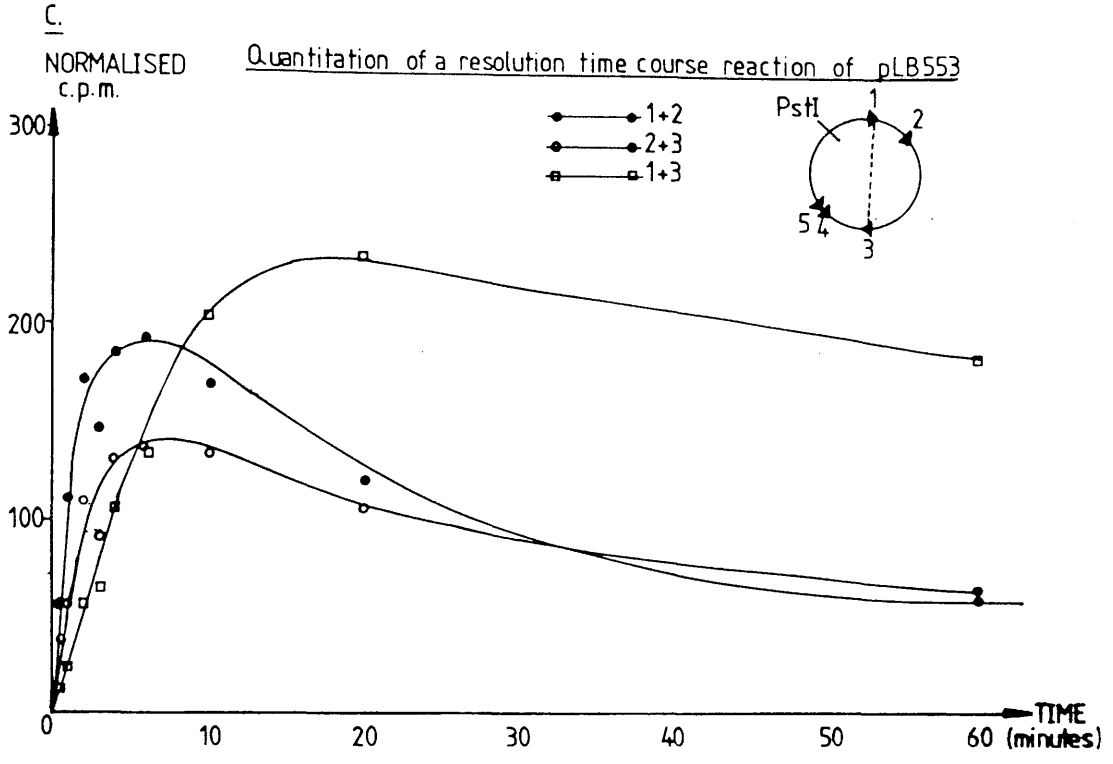


Figure 3.14 Resolution characteristics of pLB566 and pLB553
A. 0.8% agarose gel showing PstI restricted samples of a resolution time-course reaction of pLB566. This plasmids shows little or no shadowing of the 1+3 non-adjacent event unlike the similarly organised 4-site control plasmid pLB440.
B. 0.8% agarose gel showing PstI restricted samples of a resolution time-course reaction of pLB553 using tritium labelled DNA. The 1+3 non-adjacent event occurs at a frequency greater than that attributable to secondary product formation implying shadowing of this event may be only be partial.
C. Quantitation of the PstI restricted resolution time course reaction of pLB553 shown in Figure 3.14B. The data for the 1+2, 2+3 and 1+3 (initial events, clearly other products will accumulate at the same positions at the later time points) events are plotted as normalised (for fragment length) counts per minute against time. The 1+3 event is still shadowed but the shadowing is slightly less pronounced in the initial time points as compared to pLB453 although this will require further confirmation.

additive shadowing results depending on the spacing and general organisation of the res sites. Comparison of the structures of pLB534 (additive shadowing) and pLB553 (partial relief of shadowing) shows the main difference is the ability of sites 4 and 5 to recombine in pLB553 and not in pLB534. This seems to lessen the ability of these sites to interact with site 2 and consequently lowers the interference of the 1+3 event giving relief of shadowing.

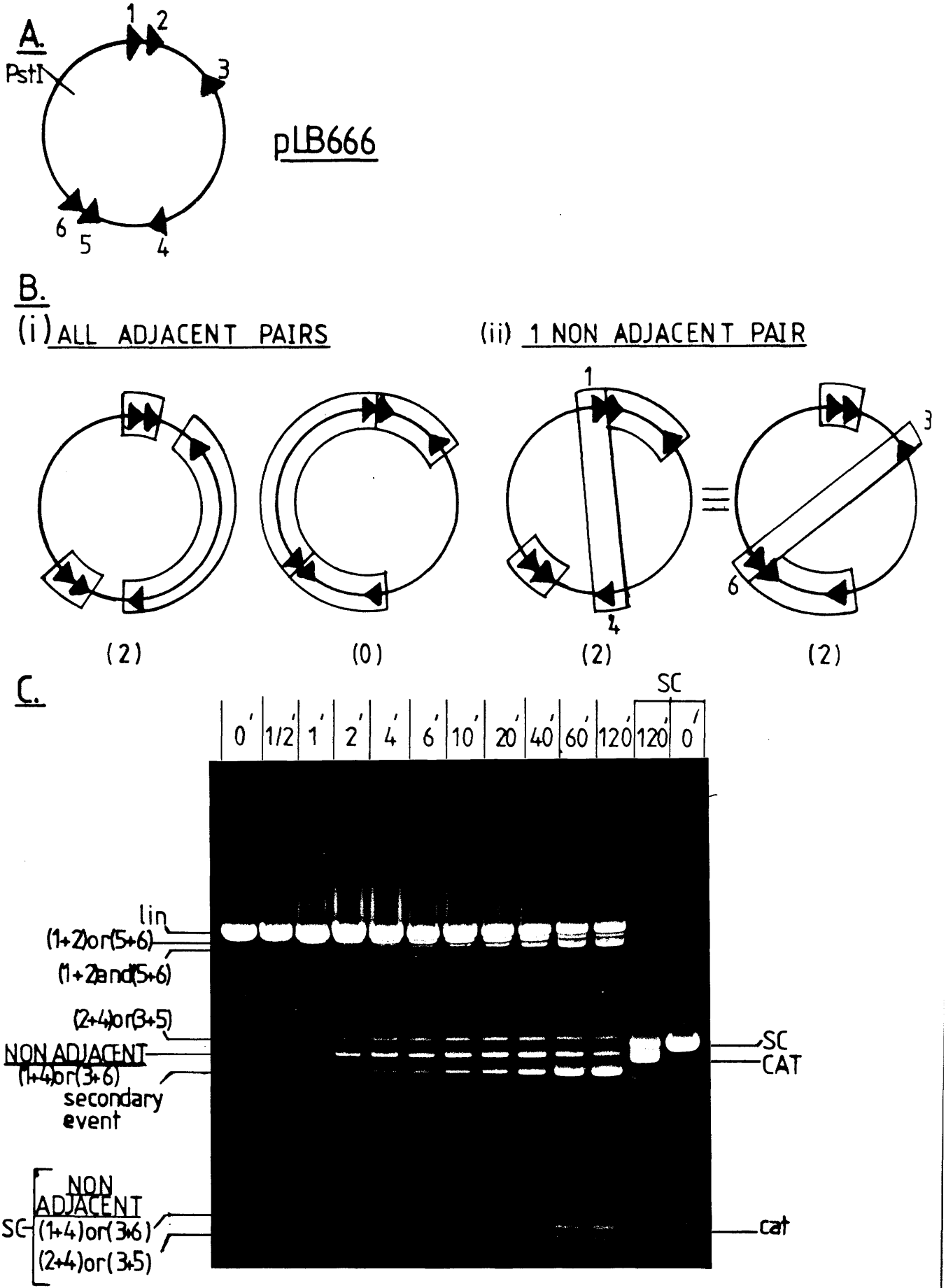
Again both tracking and stable pairs models fail to provide an explanation for the experimentally observed results. Tracking cannot under any circumstance account for relief of shadowing in 5 res site plasmids. The stable pairs model fails for the converse reason, it cannot account for additive shadowing in these constructs unless it is possible to see a corresponding distance bias in the 3 and 4 res site precursor plasmids.

3.10 **Resolution characteristics of 6 res site constructs**

The tracking and pairing models for site synapsis make quite different and indeed opposing predictions for the resolution characteristics of six site plasmids. For this purpose, the 6 res site constructs pLB666 and pLB638 were made and the predictions of the two models are best explained in the context of these two constructs. The first six site construct, pLB666, is diagrammed in Figure 3.15. This shows the various ways in which the number of synapsed sites could be maximised in the 'pairing' models. The predictions made by the tracking and pairing models are as follows (refer to Figure 3.15):-

1. The tracking model requires that 2 intervening sites will 'shadow' even better than one. Therefore all non-adjacent events will be heavily shadowed, particularly the 1+4 or 3+6 type. The only products expected on the basis of the tracking model are the adjacent 1+2 and 5+6 resolution products.

2. Pairing models predict that the pairing arrangements shown in Figure 3.15 should all be equally possible. Pairing as in (ii) would allow the non-adjacent 1+4 (or 3+6) event to occur, due to the presence of 2 intervening sites on each segment rather than just one (as in the 5 res site constructs). The ability of these 2 intervening sites to synapse with each other provides an alternative to the formation of


A.
PstI
1
2
3
4
5
6
pLB666
B.
(i) ALL ADJACENT PAIRS
(ii) 1 NON ADJACENT PAIR
(2)
(0)
(2)
(2)
C.
SC
0'
1/2'
1'
2'
4'
6'
10'
20'
40'
60'
120'
120'
0'
lin
(1+2)or(5+6)
(1+2)and(5+6)
(2+4)or(3+5)
NON ADJACENT
(1+4)or(3+6)
secondary event
SC
CAT
NON ADJACENT
SC
(1+4)or(3+6)
(2+4)or(3+5)
cat

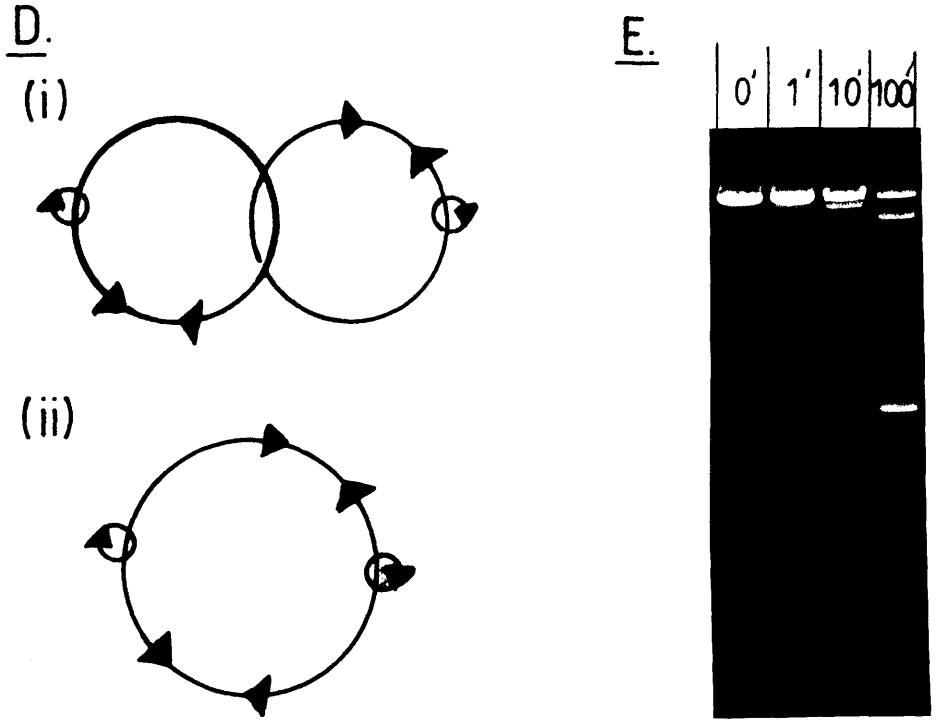


Figure 3.15 Resolution characteristics of pLB666

A. Schematic diagram of pLB666 showing the res site organisation and orientation and the position of the PstI restriction sites.

B. Shows the possible ways in which pairing type models could potentially maximise the number of synapsed sites (see text for details).

C. 0.8% agarose gel showing PstI restricted samples of a resolution time-course reaction of pLB666. The non-adjacent event separated on either side by 2 intervening res sites is one of the major resolution products. This gel does not give adequate separation of the short 282bp event for this purpose another short resolution time course reaction is shown in Figure 3.15E.

D. (i) The major product predicted from resolution involving an initial 1+4 or 3+6 non-adjacent event followed by the two 282bp spaced events.

(ii) This diagrams the plasmid generated by 2x282bp spaced resolution events. This plasmid would be unlikely to contribute greatly to the ammount of product diagrammed above (i) because it has a structure similar to pMA914 which has been demonstrated to resolve very poorly.

E. 0.8% agarose gel showing a short PstI restricted resolution time-course reaction of pLB666 to permit comparison of the ammount of the 282bp spaced event as compared to the 1+4 or 3+6 event (see text for further details).

'diagonal pairs' and the associated instability or interference effects are thus avoided. The pairing models therefore predict that the 1+4 synapse can potentially proceed to resolution when the synapse is productive. Pairing models also strongly predict the 2+4 (or 3+5) event will remain heavily shadowed compared to the 1+4 type event because pairing of site 2+4 leaves site 3 unpaired and with no option but to form a non-adjacent synapse with sites 1, 5 or 6 giving rise to the instability or interference effects of diagonal pairing. Given the possible outcomes diagrammed in Figure 3.15, pairing models predict the non-adjacent 1+4 event will occur at 1/2 the frequency of the 282bp 1+2 or 5+6 events.

Figure 3.15(C) shows PstI restricted samples from a pLB666 resolution time-course reaction. As predicted by the pairing models for site synapsis, one of the major initial products of resolution is the 1+4 or 3+6 product of the non-adjacent resolution event where the 2 recombining sites are separated by 2 intervening sites in either direction. As also predicted by pairing, the 2+4 and 3+5 resolution events are heavily shadowed.

The 1+4 event may occur at a frequency greater than expected implying the conformation with one non-adjacent synapse may well be favoured. One possible reason could be differences in the relative ease of pairing of inverted sites in different orientations, the site I to site I orientation as seen in (i) may be less favourable. Clearly, these aspects are worth further study using quantitative techniques.

Direct confirmation of the high frequency of occurence of the non-adjacent 1+4 (or 3+6) event should be possible by examining the unrestricted resolution products under the electron microscope using the recA coating technique of Krasnow et al (1983a). A large proportion of the products are expected to have the structure diagrammed in Figure 3.15(D) (corresponding to the strong catenane band in the PstI restricted resolution reactions). This structure could arise as a result of a tertiary event following the 1+2 and 5+6 events with independant segregation of the resulting small circles. This however is unlikely to occur at a high frequency because as shown in Figure 3.15, the 1+2 and 5+6 events generate a structure similar to pMA914 which is known to resolve very poorly (Figure 3.12), this is confirmed by the

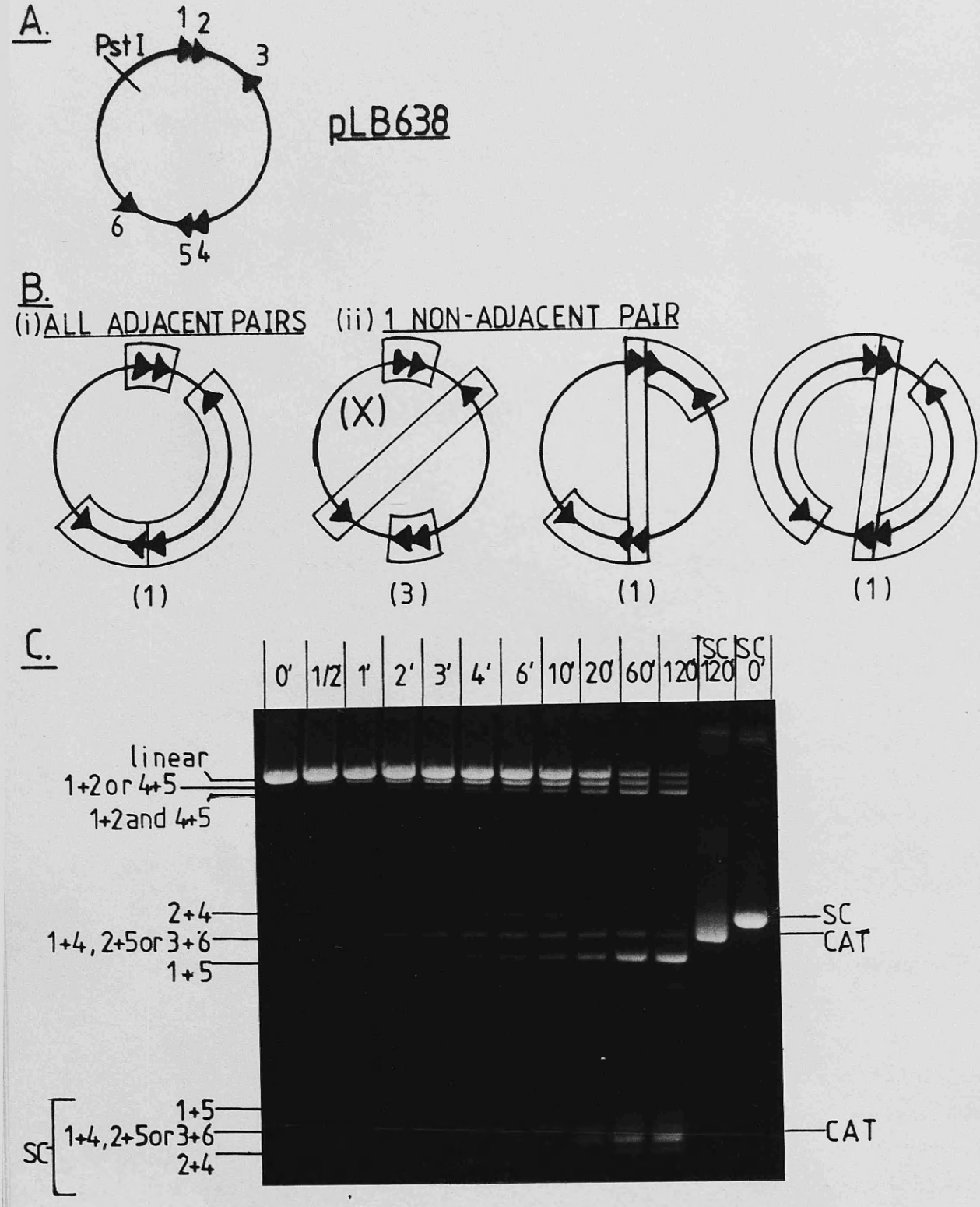


Figure 3.16 Resolution characteristics of pLB638

A. Schematic diagram of pLB638 showing the organisation of the res sites and the position of the PstI restriction site.

B. Possible ways in which pairing models could maximise the number of synapsed sites (see text for details).

C. 0.8% agarose gel showing PstI restricted samples of a resolution time-course reaction of pLB638. This demonstrates the 1+4 and equivalent events are present at a much higher frequency than attributable to secondary product formation (see text for details).

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accumulation of the 2 x 282bp event PstI linear products.

Unfortunately, time and availability of expertise did not permit electron microscopy of the resolution products of pLB666 to be carried out and this predicted structure remains to be confirmed. However, the restriction analysis shown here is diagnostic for the non-adjacent primary events. This data refutes the adjacent-site preference predictions of the tracking model whilst substantiating some very strong predictions of the pairing type models for site synapsis.

The six site result alone does not distinguish between the stable and interfering pairs models for site synapsis. However, when the resolution characteristics of pLB666 are compared with the relevant 5 res site control plasmids (pLB566 and pLB514) the results strongly favour the interfering pairs model for site synapsis.

The second construct, pLB638 is diagrammed in Figure 3.16 along with the ways in which 'pairing' could maximise the number of synapsed sites. The predictions of the tracking and pairing models are essentially the same as for pLB666:-

1. The tracking model again predicts that resolution will only occur between adjacent directly repeated res sites 1+2 and 4+5. All other events should be very heavily shadowed.

2. Pairing models predict that one of the major primary resolution products will arise as a result of a non-adjacent event with 2 intervening res sites(1+4, 2+5 or 3+6). Again as with pLB666 resolution events of the 2+4 and the 1+5 type should be very heavily shadowed. In view of the possibility of forming three potentially productive synapses it might be expected that the pairing format shown in X would be favoured. Unfortunately, it is not possible to detect such a preference in this plasmid since the products from all 3 non-adjacent events (1+4, 2+5 and 3+6) are indistinguishable from one another by restriction analysis. The individual non-adjacent events could only be distinguished by altering the plasmid organisation to incorporate a degree of asymmetry in the two 3-site cassettes. This type of experiment could be quite informative with regard to some aspects of the pairing models but is at present limited by the availability of inverted res site combinations that can be stably maintained in vivo.

PstI restricted samples from a resolution time-course reaction of pLB638 are shown in Figure 3.16. As predicted, the products from the

non-adjacent 1+4, 2+5 or 3+6 event are among the major primary resolution products and the 2+4 and 1+5 type products are indeed heavily shadowed. As discussed for pLB666 this result could be confirmed by examining the products of resolution under the electron microscope. The predictions are the same as for pLB666.

The resolution characteristics of pLB638 confirm the result obtained with pLB666 and again refute the adjacent-site preference prediction of the tracking model for resolvase-mediated site synapsis.

3.11 **Quantitation of resolution**

In order to analyse the finer details of the resolution characteristics of the extensive range of multiple res site plasmids available it was necessary to put the method of analysis on a more quantitative footing. This also allowed us to check the validity of visual interpretation of gel photographs, which can be complicated by variation in size and staining efficiency of the linear and supercoiled species. The method chosen was the use of tritium labelled DNA and scintillation counting of gel slices of restricted products of resolution time-course reactions. The resolution reactions were carried out in the usual way and samples were restricted and electrophoresed through 0.8% agarose. After staining and photography, gel slices of the various resolution products were isolated for each time point, dissolved in 1ml H_2O and mixed with 10mls of Atomlight scintillation fluid (high volume capacity). The bands corresponding to supercoils were not analysed in these experiments due to the difficulties in separating the catenanes and the potential inaccuracies arising as a result of varying degrees of relaxation of supercoils.

The samples were then counted in a scintillation counter against an external standard ratio to ensure the efficiency of counting remains constant. The counts were then corrected by subtraction of background and normalised by dividing the corrected counts by the length of the DNA fragment. The data were then plotted as normalised counts/minute against time. Time permitted use of this technique on only 3 constructs pLB434, pMA453 and pLB553 and the gels of restricted resolution time course reactions shown for these plasmids are the gels which were quantitated.

3.11.1 pLB434

The gel photograph and quantitation of the PstI restricted samples of a resolution time course reaction are shown in Figures 3.8. On this gel the 1+2, 3+4 and the secondary products of these 2 events did not separate sufficiently to permit clean isolation of the bands at the early time points; as a result these products were not included in the analysis.

The data for the two adjacent (1+4, 2+3) and the two non-adjacent (2+4, 1+3) events are plotted and the quantitated results are fully consistent with the visual interpretation of the gel photograph. The adjacent 1+4 and 2+3 events occur at an equal frequency as do the two non-adjacent events. However, the non-adjacent events are heavily shadowed compared to the adjacent events. Later time points show a build-up of the band at the 1+4 position, due to the accumulation of secondary products.

3.11.2 pMA453

Figure 3.11 shows a PstI restricted resolution time course reaction of pMA453. The data for the 1+2, 2+3 and 1+3 linear PstI products are plotted in Figure 3.11B. Again, various features clearly seen by visual inspection of the gel photograph are borne out by quantitation. Firstly, quantitation confirms shadowing of the non-adjacent 1+3 event the plot for this showing an initial lag at the first two time points, thereafter the plot shows a dramatic increase due to the accumulation of secondary products. The initial lag is confirmed in Figure 3.11C which shows a rate difference plot for the initial time points. This shows the 1+3 event exhibits an increasing rate of appearance of products in contrast to the 1+2 and 2+3 events which show a decreasing rate.

With respect to the adjacent events (1+2 and 2+3) there is a disproportionate amount of the 1+2 product apparent. The quantitation gives substance to this rather cautious conclusion drawn from the gel photograph. Visual interpretation of the resolution characteristics of the corresponding 3 <u>res</u> site plasmids does not show this bias and it must therefore be a consequence of the 4 <u>res</u> site organisation and may infact represent a pairing preference. As mentioned briefly in relation

to the 6 res site constructs perhaps the 1+4 type of inverted pair of sites (site I to site I) is less stable; rearrangement of the pairs in favour of the 1+2 and 3+4 pairs may thus favour the 1+2 event. Clearly, the examination of other constructs with these pairings is necessary.

3.11.3 pLB553

Figure 3.14 shows a PstI restricted resolution time course reaction of pLB553. The quantitative data for the 2+3, 1+3 and secondary event products is shown in Figure 3.14. As can be seen from the gel photograph the 1+3 product occurs at a frequency greater than that attributable to secondary product formation. Additionally, this product does not show a lag period in the initial time points; this is consistent with partial relief of shadowing as compared to the same event in pMA453. This relief of shadowing is not total since the 2+3 adjacent event product is still present in greater proportions.

In this construct, as in pLB434, the resolution products corresponding to the 282 and 680bp events have not separated sufficiently in this gel system to permit clean isolation and these products were not analysed here.

3.11.4 Summary

Quantitation of the resolution kinetics of these plasmids has confirmed the conclusions drawn based on visual interpretation of the gel photographs. Clearly this technique has the potential to provide detailed information on the resolution characteristics of these plasmids and may provide valuable information on the 'pairing' ideas on site synapsis. The interpretation here is rather cautious in the absence of quantitative information on the relevant 2, 3 and 4 res site plasmids; these are currently under investigation in this laboratory. Using this technique it is important to optimise the electrophoresis conditions for the plasmid concerned to maximise the separation of all the resolution products.

3.12 Possible unequal segregation of a resolution product

This result is very preliminary at present and requires quantitative confirmation. It is based on the resolution characteristics of some of the 4 *res* site plasmids with one *res* site inverted with respect to the others, as illustrated for pLB421 in Figure 3.10. Given the ammount of the resolution product corresponding to the 282bp primary resolution event observed throughout the time course reaction there does not seem to be as much of the 'shadowed' 1+3 catenated supercoiled product as is expected for random segregation of the small circle. This suggests that most of the 282bp product circle has segregated into the segment corresponding to the PstI linear fragment and hence is not detected here.

This is of interest because it may reflect intermolecular interaction of the small circle *res* site with the inverted *res*, site leading to non-random segregation of the small circle into the supercoil with the inverted site. This type of intermolecular interaction is predicted by the 2-step model for site synapsis. Indeed intermolecular recombination has been detected on alteration of substrate topology implying such interaction is entirely plausible. This type of interaction would not be detected in the reporter ring experiments of Benjamin *et al* (1985) since all the *res* sites were in direct repeat. Clearly this aspect of resolution is worthy of further investigation.

Concluding remarks and summary

The experiments described in this chapter were set up with the initial intention of testing the adjacent site preference prediction of the tracking model in a plasmid containing 4 directly repeated *res* sites. With the advent of the pairing models for adjacent site preference associated with the 2-step model for resolvase-mediated site synapsis (although not diagnostic for it), this analysis was extended in an attempt to distinguish between the 2 types of model. The end result was the construction of an extensive range of multiple *res* site plasmids the resolution characteristics of which refute tracking as a model for resolvase-mediated site synapsis and in doing so substantiate some very strong predictions of the pairing models.

The relative merits and shortcomings of the tracking, stable and interfering pairs models for resolvase-mediated site synapsis are

summarised below.

1. The tracking model

This model fails to provide an adequate explanation for several aspects of the resolution characteristics of the 4, 5 and 6 *res* site constructs examined here:-

1. The non-reciprocal shadowing seen in pMA422 in the absence of a similar bias in the other relevant 3 and 4 *res* site plasmids.
2. The partial relief of shadowing in some 5 *res* site constructs as compared to the corresponding 4 *res* site constructs.
3. The occurence of a non-adjacent resolution event at a frequency equal to and possibly even greater than equivalent adjacent resolution events in the same construct (6 *res* site plasmids).
4. Shadowing by inverted sites.
5. Inversion at an easily detectable frequency on alteration of the substrate topology (Boocock *et al* 1986).

The resolution characteristics of pLB666 and pLB638 very convincingly disprove the adjacent site preference prediction of tracking. This in addition to the failure of the reporter ring experiment (a truly diagnostic test for translocation of resolvase along the DNA) and the detection of inversion on alteration of substrate topology leaves tracking in a very weak position indeed. The only experimentally observed feature which is still consistent with tracking is the fixed product topology at all supercoil densities of the substrate. This result is not however diagnostic for tracking and is in fact a direct prediction of the 2-step model for resolvase-mediated site synapsis, arising as a consequence of the requirement for 2 sites to come together initially with zero interlinks, all other topologies being non-productive for conformational reasons.

Some of the results obtained here are superficially at odds with those of Benjamin *et al* (1985), this can be explained to some extent by the different experimental approaches (as described for examining the effect of inverted sites on the resolution characteristics of directly repeated sites in the same construct). One criticism of the approach of Benjamin *et al* is the use of Gamma delta *res* sites in experiments aimed at analysing the fine details of the Tn*3* resolvase-mediated

recombination reaction.

2.**The stable pairs model**

This model also fails to provide an adequate explanation for the resolution characteristics of some of the plasmids examined here. It cannot explain:-

1. The non-reciprocal shadowing seen in pMA422 in the absence of a similar bias in the other constructs.
2. Additive shadowing in some 5 res site constructs.

The resolution behaviour of the 6 res site constructs is consistent with the expectations of this model but is difficult to reconcile with the absence of a similar relief of shadowing in all 5 res site constructs.

3.**The interfering pairs model**

In contrast to the tracking and stable pairs models, this model can adequately explain the resolution characteristics of all the plasmids examined here. The interfering pairs model:-

1. Can explain the non-reciprocal shadowing in pMA422 due to the presence of 3 closely spaced sites.
2. Predicts the breakdown of non-reciprocal shadowing on reorganisation of the 3 closely spaced sites whilst still maintaining the 680bp spacing and/or the 282bp spacing. This was the result obtained with pLB434 and pLB438.
3. Can accommodate either additive or relief of shadowing in 5 res site constructs on the basis of the differing interfering capabilities of the various pairs of sites on diagaonal pairing.
4. Expressly predicts relief of shadowing of non-adjacent events separated by 2 sets of 2 intervening sites, as seen with pLB666 and pLB638.
5. Predicts shadowing by inverted sites in the same way as is seen for multiple directly repeated res sites (assuming inverted pairs of sites can interact as predicted for the 2-step model for site synapsis).

The interfering pairs model and the predictions arising from it are fully consistent with the experimentally observed results and provide a very compelling alternative to tracking for the results of Benjamin et

al (1985) showing an adjacent site preference of resolution in a symmetrical construct containing 4 directly repeated res sites. This type of model is not compatible with any version of the tracking model.

These results are fully consistent with, although not diagnostic for the 2-step model for resolvase-mediated site synapasis. However, when taken together with accumulating evidence from a number of other sources the argument for the 2-step model for site synapsis gains in strength and has superceded the tracking model as our favoured explanation for the unique properties of the resolvase-mediated recombination reaction.

Of the predictions of the 2-step model enumerated in the introduction to this chapter, experimental evidence has been found in support of several:-

1. Pairing of sites.
2. Interaction of inverted sites.
3. Inversion and intermolecular recombination on alteration of the substrate topology (Boocock et al 1986, M. Boocock unpublished results).
4. Resolvase-induced bending of res site DNA (discussed in detail in chapter 4).

Experiments to test the remaining predictions are currently underway in this laboratory:-

1. The 2-step model predicts a major role of sites II and III in site alignment and in defining site polarity. A prediction arising as a natural consequence of this is that sites II and III alone should be sufficient to cause the shadowing effects seen in the multi-res site plasmids.
2. Precise predictions can be made about the topology of the products of intramolecular inversion reactions in circular substrates.

The experiments described in this chapter, in addition to testing the predictions of the models for site synapsis have highlighted several aspects of the resolution reaction worthy of further investigation to understand the more detailed aspects of the reaction:

1. The use of the tritium labelling quantitation technique to investigate the kinetics of the resolution reaction to provide information regarding:

a. The strength or ease of formation of the various pairs.
b. The rate of appearance of secondary products.
c. The non-random segregation of the small catenated ring.
d. The pLB638 result (may confirm the visual interpretation of the resolution characteristics of this plasmid).

2. Electron microscopy of the 6 res site plasmid resolution products.
3. Investigate the possible 10.5bp periodicity of the ease of site synapsis of very closely spaced sites.

CHAPTER 4

METHYLATION PROTECTION AND INTERFERENCE EXPERIMENTS ON RESOLVASE BINDING AT *res*

Introduction

In view of the unique properties of the resolvase-mediated recombination reaction (as described in chapters 1 and 3), the structural organisation of the *res* site and the mechanism of recognition and binding by resolvase are of special interest. Indeed, investigation into these areas may contribute towards understanding both the directional and topological specificities of the reaction.

Questions of particular interest include:-

1. What functional role do sites II and III play given that site I alone contains the sequence of strand exchange?
2. Which aspect of resolution dictates the evolutionary conservation of an analagous 3 site organisation in the other Tn*3*-like elements (Grindley *et al* 1982, Rogowsky *et al* 1985)?
3. What are the recognition determinants at each site?
4. What mode of resolvase binding can accommodate the different spacing within and between sites to form an active recombination complex which can distinguish the sequence of strand exchange?
5. Does resolvase induce any DNA conformational changes on binding to single *res* sites or on the formation of the paired 2-*res* site recombination complex with the necessary -3 synapse geometry (Cozzarelli *et al* 1984, Wasserman and Cozzarelli 1985, Sherratt *et al* 1984)?

The 2 step model for synapsis (Boocock *et al* 1986), predicts a major role of sites II and III in forming the recombination complex along with some resolvase-induced conformational changes of the DNA to achieve the -3 synapse geometry.

The question of how specific DNA binding proteins recognise and bind to their target sequences has been approached using a variety of crystallographic, biochemical and genetic techniques (Pabo and Sauer 1984).

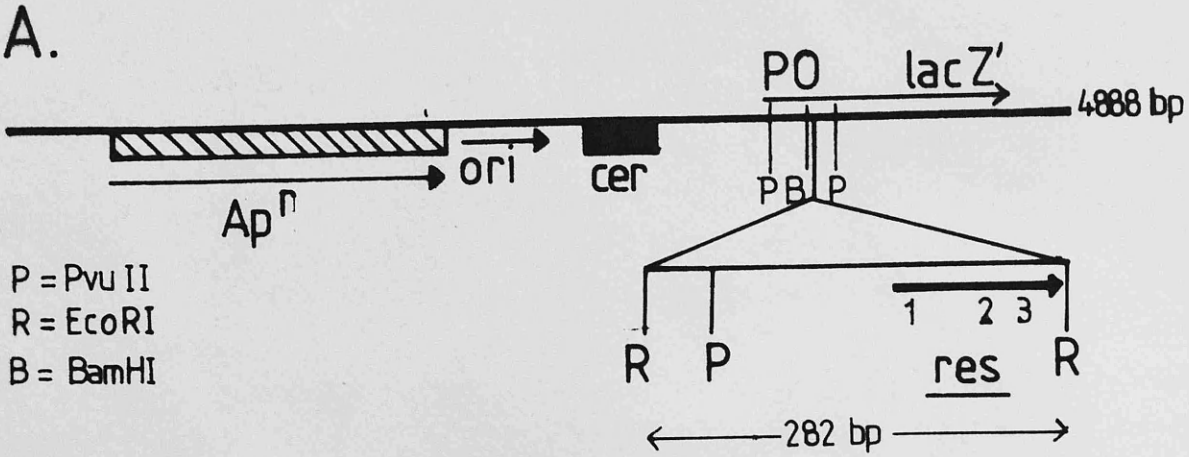
The ultimate approach is to examine co-crystals of the protein-DNA complex using X-ray diffraction. This has now been accomplished at low resolution (7A) for the phage 434 repressor-operator complex (Anderson *et al* 1985), and at high resolution (3A) for only one protein to date, the EcoR1 restriction endonuclease complexed with a synthetic oligonucleotide containing the recognition sequence (Frederick *et al* 1984).

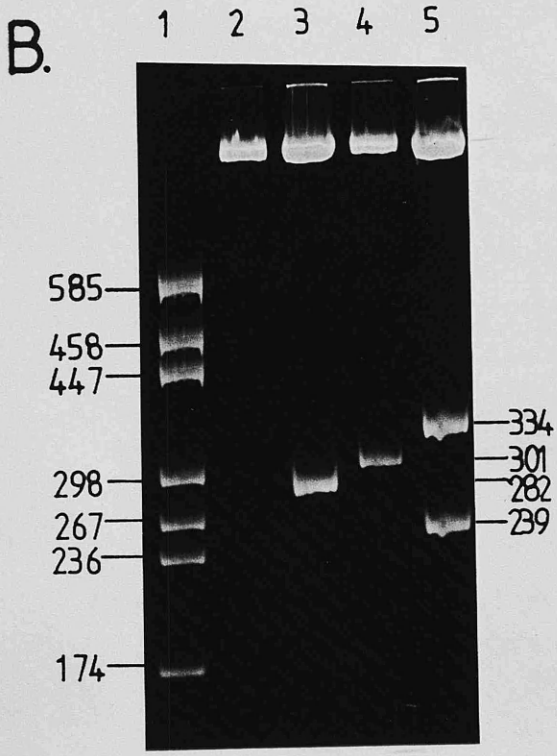
In lieu of such sophistication for most other DNA binding proteins, biochemical and genetic analysis of both the protein and the DNA binding site can yield useful information. Such data can be used in conjunction with crystallographic studies of the protein alone to generate computer derived protein-DNA binding models as has been done for cro, CAP and the Lambda repressor (Anderson *et al* 1981, McKay and Steitz 1981, Pabo and Lewis 1982). With the advance of *in vitro* systems, one of the most informative and easily applicable techniques is DNA footprinting of proteins complexed to their binding sites.

In this type of approach the pattern of chemically or enzymatically induced cleavage of the DNA is compared in the presence and absence of bound protein. Differences in the cleavage patterns may identify positions of protein interaction with the DNA.

In the first instance, footprinting techniques can be used to identify and define the limits of a DNA binding site. The external boundaries can generally be defined using Exonuclease III (Shalloway *et al* 1980) and the extent of binding within these limits using DNAaseI or the antibiotic Neocarzinostatin (Galos and Schmitz 1978). Indeed, DNAaseI is proving to be a more sensitive probe than was originally realised and is now considered to be sensitive to the width and accessibility of the minor groove of the DNA in the presence and absence of bound protein. DNAaseI may therefore have the potential to yield information on sequence or protein-induced variations in DNA structure (Drew 1984, Drew and Travers 1984, 1985b).

Further footprinting methods attempt to identify either bases or phosphates which are in direct contact with the protein. The alkylating agent, dimethylsulphate (DMS), can probe interaction of the binding protein with purine bases in the DNA (Gilbert *et al* 1976, Siebenlist and Gilbert 1980). DMS preferentially methylates the N7 position of Guanine in the major groove and the N3 position of Adenine in the minor groove of the DNA (Sun and Singer 1975). A protein in close approach, or interacting directly with these positions can alter the accessibility of DMS to the base. In general, these alterations are reductions in methylation, however enhancements are also known to occur. These are thought to arise either as a consequence of the formation of a hydrophobic pocket at the edge of a protein-DNA contact leading to a local increase in the DMS concentration (Johnsrud 1978, Siebenlist and Gilbert





1. pUC9 HaeIII
2. pLB03 EcoRI
3. pLB16 EcoRI
4. pLB03 PvuII
5. pLB16 PvuII

Figure 4.1 Construction of pLB16

A. Schematic diagram of pLB16 showing the general organisation, site of insertion and orientation of the 282bp EcoRI res$^+$ fragment from pLS138. The BamHI and PvuII restriction sites relevant to the end labelling reactions are indicated.

B. 5% polyacrylamide gel showing EcoRI and PvuII restrictions of pLB03 and pLB16. These confirm the insertion and orientation of the res$^+$ fragment is as diagrammed in Figure 4.1A.

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1980), or alternatively, to represent positions of local perturbation of the DNA configuration (Frederick *et al* 1984, Siebenlist 1979, Siebenlist *et al* 1980). DNA conformational changes may also be responsible for some of the weaker protected positions observed (M^{c}Ghee and Felsenfield 1979, Richmond *et al* 1984).

An adaptation of this approach is to methylate the DNA first and then attempt to bind the protein. A methylated A or G residue may interfere with protein binding, thereby identifying positions which are critical either for the formation or maintenance of the protein-DNA complex (Siebenlist and Gilbert 1980). A similar approach can be employed to examine the disposition of phosphate contacts along the DNA backbone using the ethylating agent, ethylnitrosourea (Siebenlist and Gilbert 1980, Bushman *et al* 1985). Ethylation neutralises the -ve charge and prevents ionic interaction between protein and DNA.

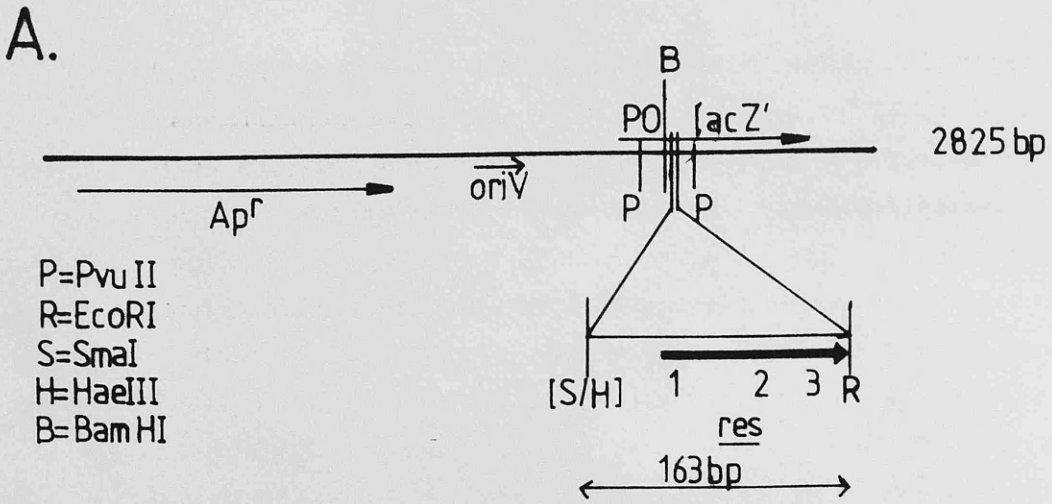
A method exsists which can probe protein interactions with the thymine bases in the major groove of the DNA. However, this method is unsatisfactory requiring the use of 5-bromouracil substituted DNA rather than the native form and has the potential to identify only a subset of the contact positions (Ogata and Gilbert 1977).

DNAaseI protection experiments have already given useful information on the resolvase-*res* system. This chapter describes experiments which aim at extending this footprinting analysis using the methylation protection and interference techniques described above.

Results

4.1 Resolution of pLS138 under the conditions of the methylation protection experiments

As a preliminary to the methylation experiments, it was necessary to ensure resolvase could bind to *res* site DNA and mediate normal resolution under the conditions used in the footprinting experiments (Siebenlist and Gilbert 1980). The buffer used for both protein binding and the subsequent methylation reaction is based on Sodium cacodylate, comprising of 50mM Na Cacodylate pH8, 10mM $MgCl_2$, 0.1mM EDTA, 1mM DTT and 100mM KCl.



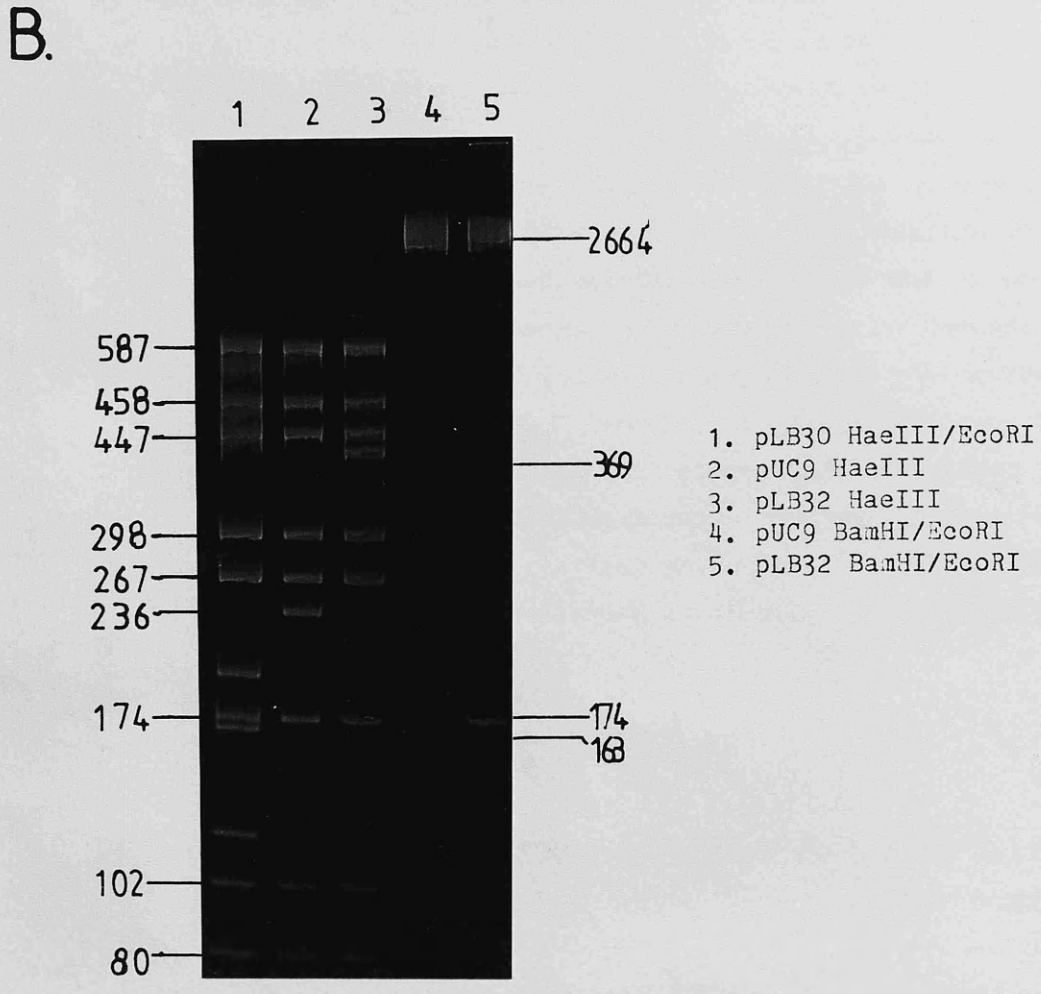


Figure 4.2 Construction of pLB32

A. Schematic diagram of pLB32 showing the general organisation, site of insertion and orientation of the 163bp HaeIII/EcoRI res$^+$ fragment of pLB30. The BamHI and PvuII restriction sites relevant to the end-labelling reactions are indicated.

B. 5% polyacrylamide gel showing HaeIII and BamHI/EcoRI restrictions of pUC9 and pLB32. These confirm the insertion of the HaeIII/EcoRI res$^+$ fragment into the polylinker region of pUC9 as diagrammed in A.

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The resolution characteristics of the 2 *res* site plasmid, pLS138, were examined in 1x methylation buffer using a pre-determined optimum DNA:resolvase ratio. The resolution products were analysed by agarose gel electrophoresis of BamHI restricted and unrestricted reactions. In this buffer, after 2 hours incubation at 37°C, resolution had proceeded to greater than 60% completion generating the normal, catenated end products (data not shown). A time-course reaction under these conditions exhibited the expected reaction profile.

4.2 **Construction of pLB16 and pLB32**

In order to isolate *res*$^+$ fragments suitable for uniquely end labelling either strand of the DNA, the *res* site was cloned into a restriction site rich background, leading to the construction of pLB16 (Figure 4.1). The 282bp *res*$^+$ EcoRI fragment from pLS138 was isolated from 5% polyacrylamide, extracted by electroelution and ligated to EcoRI cleaved pLB03 (a *cer*$^+$ derivative of pUC9, Summers and Sherratt 1984). The site of insertion and orientation of the insert is diagrammed in Figure 4.1.

pLB16 was an appropriate construct for carrying out methylation protection experiments on the (+) strand, 3'end labelled at the EcoRI site of the EcoRI-PvuII *res*$^+$ fragment however, using the 3' end labelled BamHI-HaeII fragment to footprint the (-) strand, the DNA of interest was too far removed from the labelled end to give adequate resolution on a sequencing polyacrylamide gel. As an alternative, pLB32 was constructed (Figure 4.2) by cloning a shortened *res* site into the pUC9 polylinker. A 163bp HaeIII-EcoRI *res*$^+$ fragment was isolated from 5% polyacrylamide, electroeluted and ligated to SmaI-EcoRI cleaved pUC9. DNA from Ap^r transformants was analysed for the presence of the insert by restriction. The 264bp BamHI-PvuII *res*$^+$ fragment was used for subsequent 3' end labellings at the BamHI site.

4.3 **Calibration of Resolvase to DNA Ratio**

As the first step in these experiments, it was necessary to determine the concentration of resolvase required to saturate a fixed concentration of *res*$^+$ DNA. The DNA concentration was set at 50ng of purified

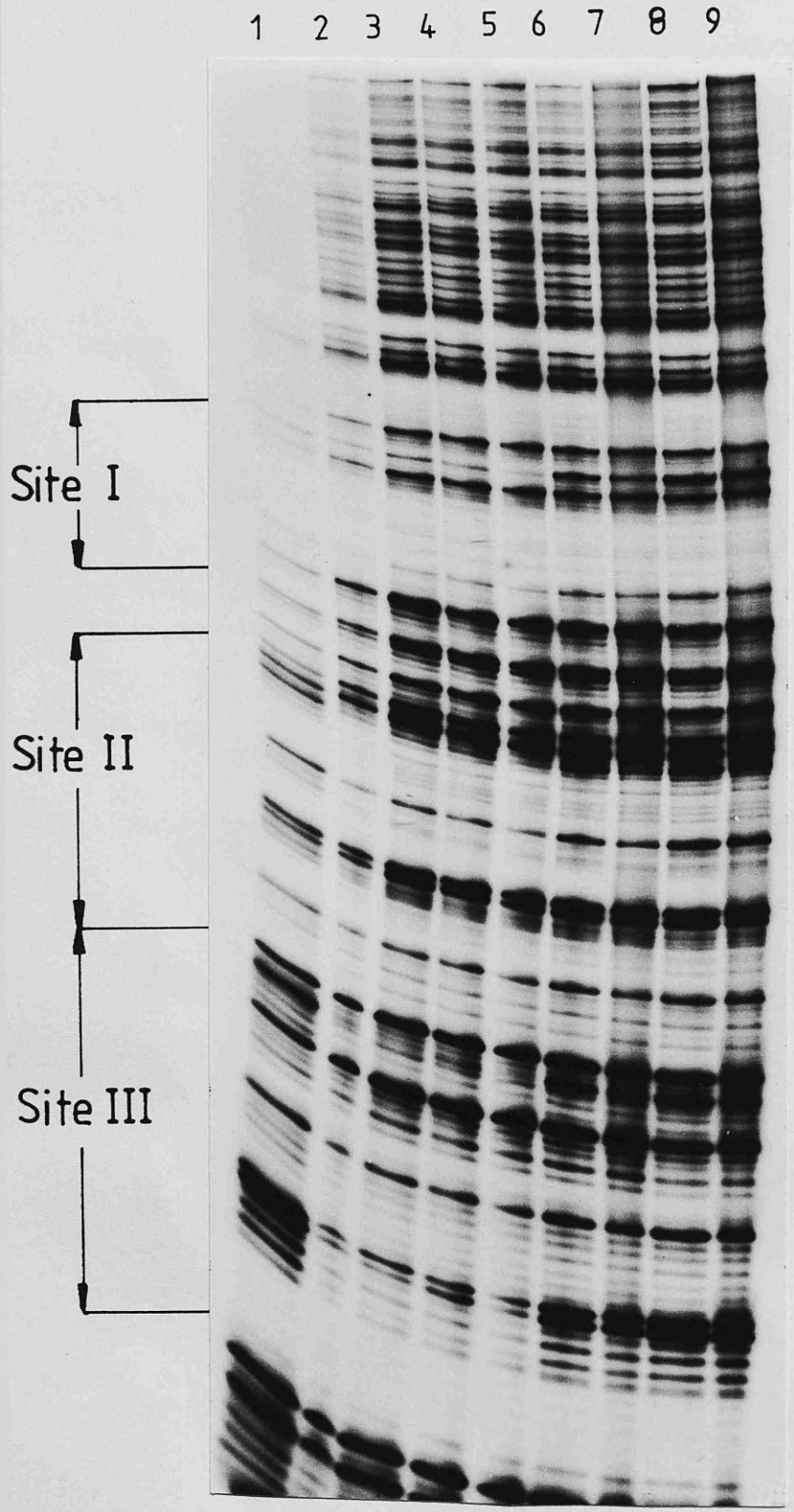

Figure 4.3 Determination of the optimum DNA:resolvase ratio for use in the methylation protection experiments of resolvase bound at <u>res</u>. The pLB16-derived EcoRI-PvuII <u>res</u>$^{+}$ fragment 3' end-labelled at the BamHI site was used as the substrate for this experiment. 50ng of purified fragment was used in each reaction and the concentration of resolvase was altered as indicated. Protection is seen at between 5-10 units of resolvase/50ng purified fragment and this was considered an appropriate range for further footprinting experiments (further details are given in the text). F = Filtrate, FB = Filter-bound.

plasmid per reaction using the pLB16 derived, EcoRI-PvuII *res*$^+$ fragment 3'end labelled at the EcoRI site. 250ng of end labelled fragment was purified and divided into 5 aliquots. One aliquot was methylated directly, serving as a control for the G specific cleavage profile in the absence of resolvase. Increasing ammounts of resolvase were added to the remaining 4 aliquots 1.25, 2.5, 5 and 10 units. Each sample was incubated at 37^{o}C for 5 minutes, to permit resolvase binding, then methylated in an identical way, approximating to the extent of one methylated base per end labelled fragment. Each reaction was then filtered through nitrocellulose, the protein-bound DNA being retained on the nitrocellulose filter and the unbound DNA passing straight through. The nitrocellulose bound fractions were recoverable by treatment with Ammonium acetate to disrupt the protein-DNA complex. Both fractions were isolated and subjected to the G specific cleavage conditions described in chapter 2. 2ul of each sample containing the same number of counts/ul were eletrophoresed through an 8% polyacrylamide sequencing gel. Figure 4.3 shows the resulting autoradiograph.

At 5 and 10 units of resolvase, the cleavage pattern in the filtrate and filter bound fractions were identical, although different from that of the G control cleavage pattern. This implies, that at the point of methylation, all the *res* sites were resolvase bound. The small fraction of the total counts recovered in the filtrate presumably represent fragments which have lost resolvase during the filtering procedure. At the 2 lower resolvase concentrations, very few counts were recovered from the filter-bound fraction and the pattern obtained is representative of unbound fragments. This is most likely to occur as a consequence of the dead space of the filtering unit and does not represent protein-bound DNA. From this experiment 5 to 10 units of resolvase/50ng DNA was deduced to be an appropriate resolvase:DNA ratio.

4.4 **Methylation protection experiments of resolvase bound at *res***

For footprinting the (+) strand of the *res* site, the 244bp EcoRI-PvuII *res*$^+$ fragment of pLB16 3'end labelled at the BamHI site was used as before. For the (-) strand, the 264bp BamHI-PvuII *res*$^+$ fragment of pLB32 was used following 3'end labelling at the BamHI site.

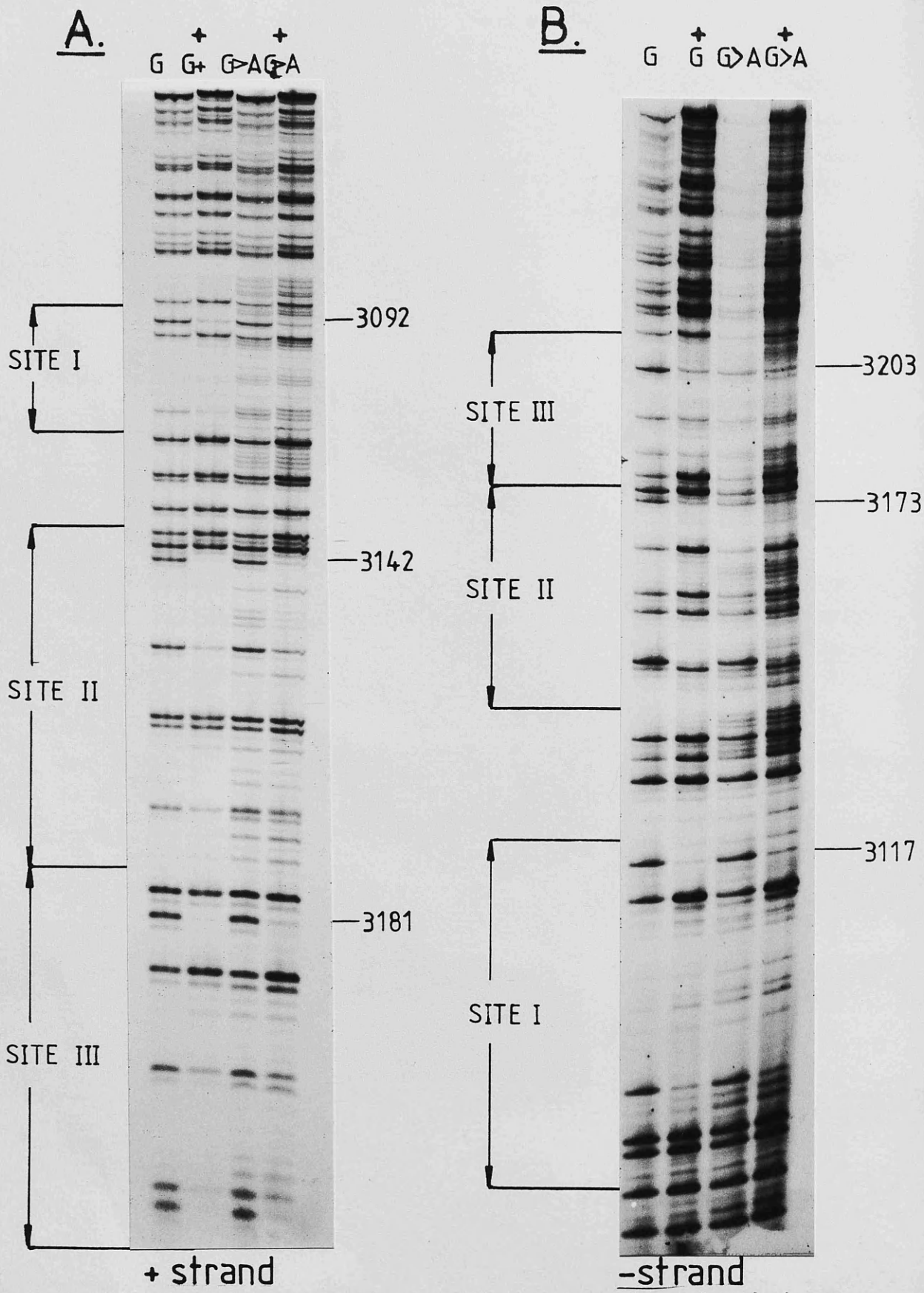

Figure 4.4 Methylation protection experiments on resolvase bound at res

A. Methylation protection of resolvase bound to the (+) strand of res using the EcoRI/PvuII res^+ fragment of pLB16 3'end-labelled at the EcoRI site.

B. Methylation protection of resolvase bound to the (-) strand of res using the BamHI-PvuII res^+ fragment of pLB32 3' end-labelled at the BamHI site.

In both A. and B. the site boundaries (as defined by DNAaseI protection experiments) are indicated and coordinates of the strongly protected G positions are given. (+) represents those reactions which were incubated with resolvase, the DNA:resolvase ratio used is detailed in the text. The data is summarised in Figure 4.5.

2 x 50ng aliquots were used as controls and methylated directly to an extent approximating to one methylated base per fragment, these were subsequently subjected to either G or G>A specific cleavage conditions. Two other 50ng aliquots were methylated to the same extent following incubation with 10 units of resolvase. The latter 2 aliquots were then filtered through nitrocellulose, the filter bound fraction was isolated and subjected to either G or G>A specific cleavage conditions. 2ul aliquots of each equilibrated sample were electrophoresed through an 8% polyacrylamide sequencing gel. The resulting autoradiographs can be seen in Figure 4.4.

Resolvase binding alters the extent of methylation of only a subset of purine residues within the res region, all of which lie exclusively within the 3 binding sites defined by DNAaseI protection experiments (Symington 1982, Kitts et al 1983). The positions of altered methylation are summarised in Figure 4.5 and in Figure 4.6. are plotted on a cylindrical representation of the double helix, assuming 10.5bp per turn of the helix (Wang 1979). The starting point for the plot is symmetry around the crossover sequence of site I.

The alterations in efficiency of methylation are largely reductions, either partial or total, presumably reflecting the close proximity and specificity of interaction of resolvase with the base in question. Examination of Figure 4.6. reveals that these putative contact points lie on only one face of the DNA helix at each of the 3 binding sites, a feature seen for many other DNA binding proteins (Siebenlist et al 1980, Jones and Tjian 1984, Johnson et al 1978). However, on examining their distribution over the entire res region, assuming 10.5bp per turn of the helix, site III is contacted on the opposite face of the helix from sites I and II . This is presumably a consequence of the spacing within and between the individual subsites of the res region, a feature which is precisely conserved between Tn3 and Gamma delta and is likely to be of functional significance.

The most strongly protected positions are a subset of Guanine residues in the concensus sequence, lying at the outer boundaries of the 3 sub-sites. Figure 4.7. aligns the 6 half sites of the res region at the TGT of the concensus sequence and comparison of the methylation profiles shows resolvase recognises and binds to each half site in essentially the same way. The most noticeable conserved feature is the

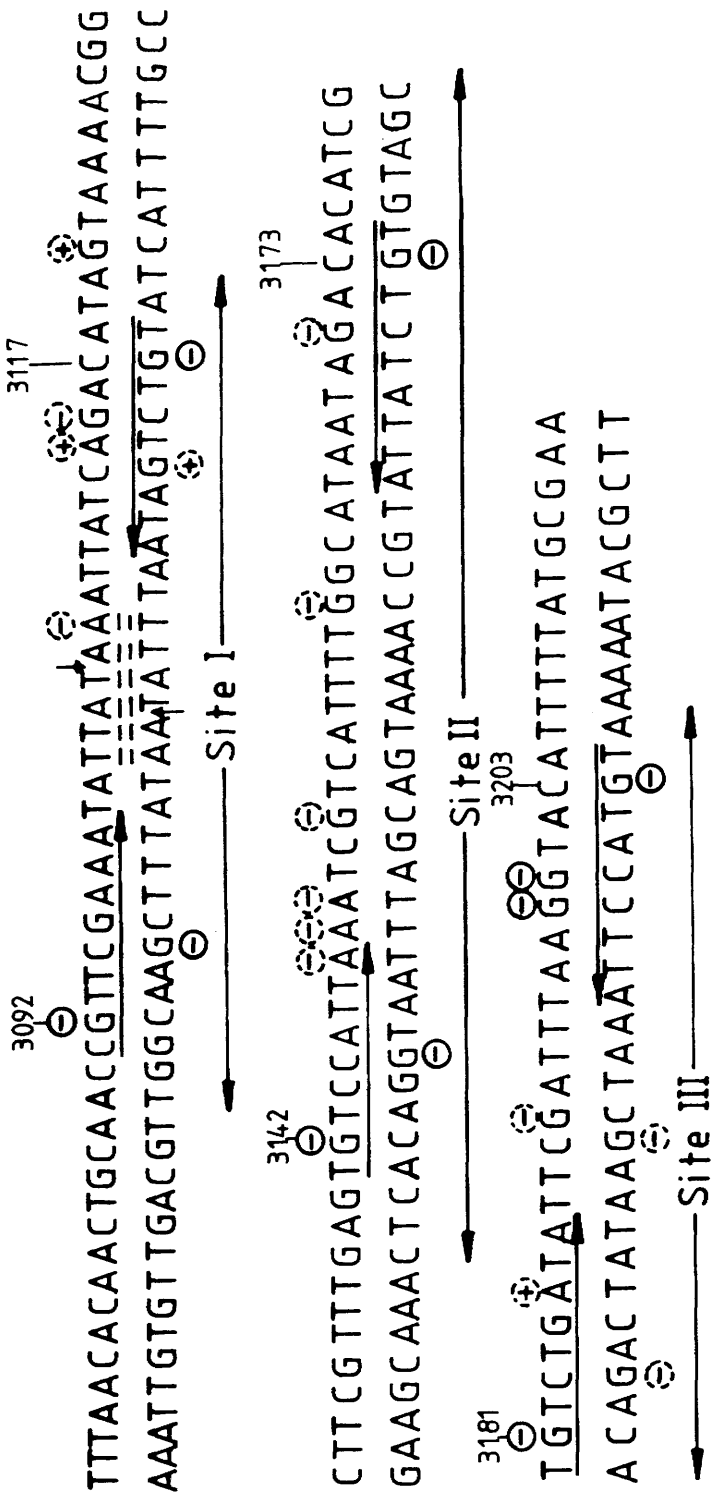

Figure 4.5 Methylation protection data
This Figure shows the DNA sequence of the res site with positions which show an altered extent of methylation on binding of resolvase to res indicated as appropriate. The boundaries of the sites are those defined by DNAaseI protection experiments (Symington 1982). The coordinates corresponding to the Tn3 sequence are indicated for each G position of the consensus sequence (see text for further details).

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absolute protection of the G in the TGT triplet of the concensus sequence, implicating this major groove interaction as one of the major recognition determinants for resolvase binding. Corroborative evidence for this comes from the cis_{10} mutation of Tn3 (Chou et al 1979) which has a G to A transition in the TGT triplet of the left half of site II. This TGT triplet constitutes part of the -35 region for the resolvase promoter and by binding here, resolvase can autoregulate it's own synthesis (Kostricken et al 1981). Autoregulation is drastically reduced in the cis_{10} mutation, indicating a reduced level of resolvase binding. Unfortunately, the effect of this mutation on resolution is unknown.

With regard to other similarities, the half sites fall into 2 categories. Sites IL, IIL and IIIR all exhibit very strong protection at the 5th position of the concensus sequence on the (-) strand. Sites IR, IIR and IIIL have no G in this position, instead, they show weak protection in the 4th position of the concensus sequence which resides in an AGA sequence in all three cases.

The significance of these 2 types of binding pattern is unknown; it may serve as a mechanism to distinguish the 2 half sites (site III being recognised the opposite way round from the other 2 sites) or, alternatively, and more likely, it is unimportant since this sequence grouping is not maintained in the Gamma delta sites.

In summary, resolvase recognises and makes major groove interactions with the concensus sequence at the outer limits of 3 binding sites. The distribution of contact positions seen at the concensus sequence is in keeping with the observed DNAaseI concensus cutting pattern (Grindley et al 1982), which indicates unhindered accessibility to the minor groove on the opposite face of the helix from the presumed contacts identified here. The methylation protection experiments described here, show minor groove interactions are weak and are confined to the centres of sites I and II. Weak major groove interactions are also seen at the centres of all 3 sites. Enhancements are few and show no ordered distribution other than a tendency to occur on the opposite face of the helix from the major groove interactions with the concensus sequence. In view of this distribution, either of the proposed mechanisms for methylation enhancements would be plausible although, perhaps DNA perturbation is more likely.

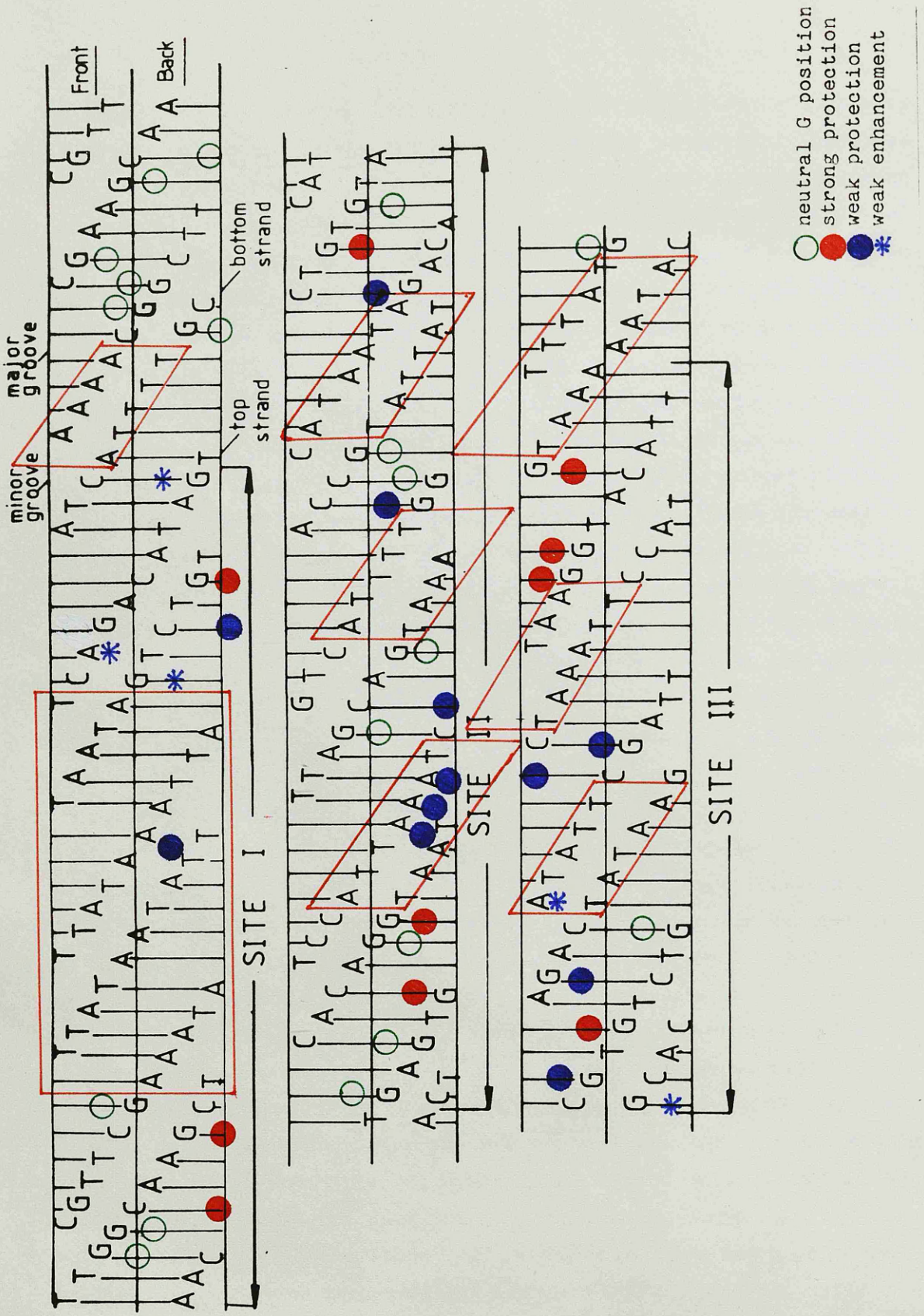

Figure 4.6 Methylation protection data for resolvase bound at res plotted on a cylindrical representation of res site DNA

This is a cylindrical representation of res site DNA assuming 10.5bp per turn of the helix. The starting point for the plot is symmetry around the centre of siteI. The sequences shown are contiguous and the boundaries of the sites are those defined by DNAaseI protection experiments (Symington 1982). The major and minor grooves and the (+) and (-) strands (top and bottom strands respectively) are indicated. Bases which show an altered methylation pattern have these positions marked in the major or minor groove as appropriate for alteration of G (N7 position) or A (N3 position) methylation. The regions enclosed in red boxes indicate the minor grooves of A plus T rich sequences which are potentially susceptible to resolvase-induced bending (see text for further details).

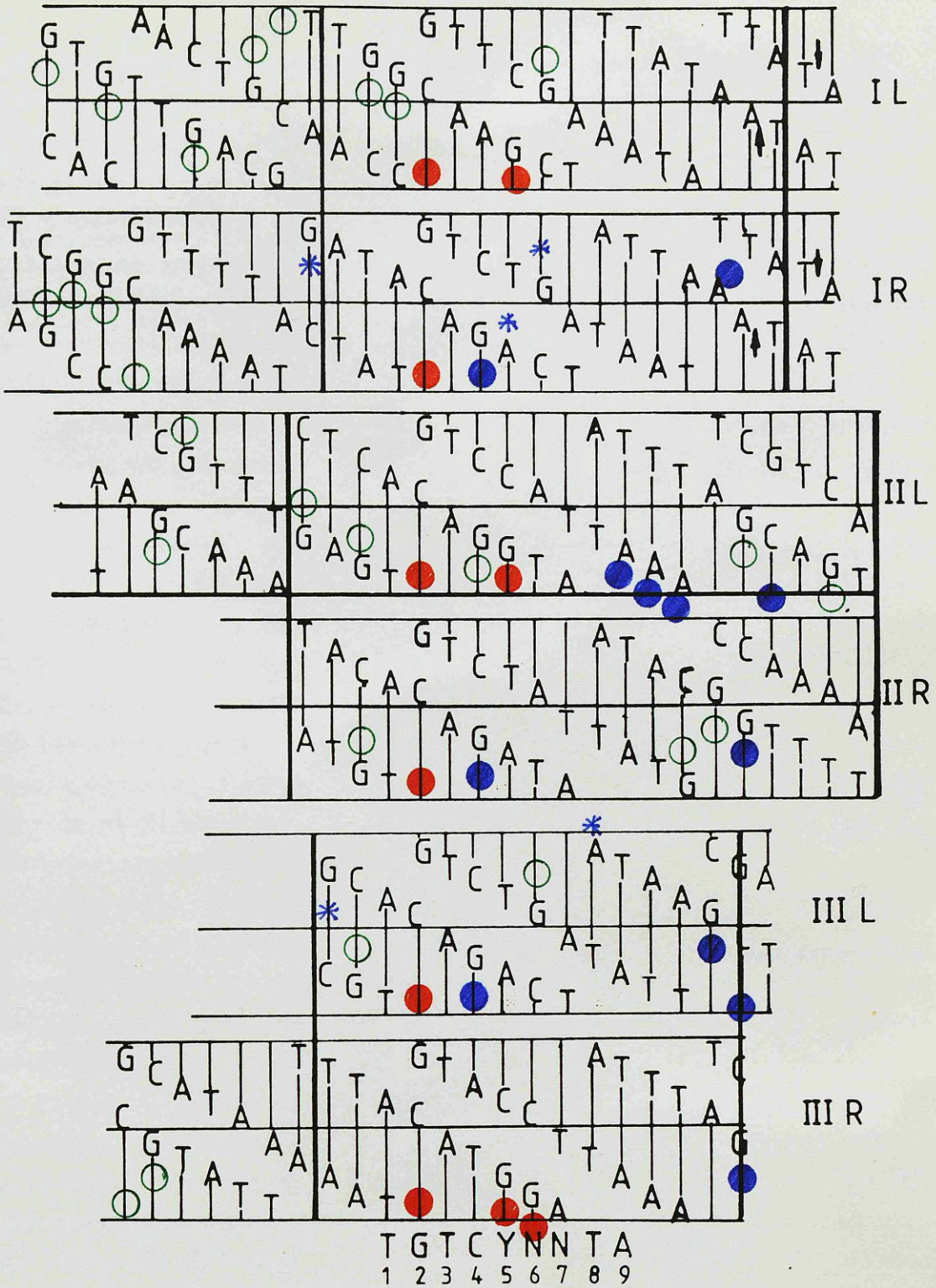

Figure 4.7 Methylation protection data plotted on a cylindrical representation of the res site DNA with half sites aligned
This is based on Figure 4.6, but each half site is now aligned at the TGT of the concensus sequence. This requires the 'right' half sites to be rotated through 180° with respect to Figure 4.6. This permits comparison of the methylation protection pattern at analagous positions in each half site. Boundaries are again based on the DNAaseI protection data (Symington 1982). (see text for further details).

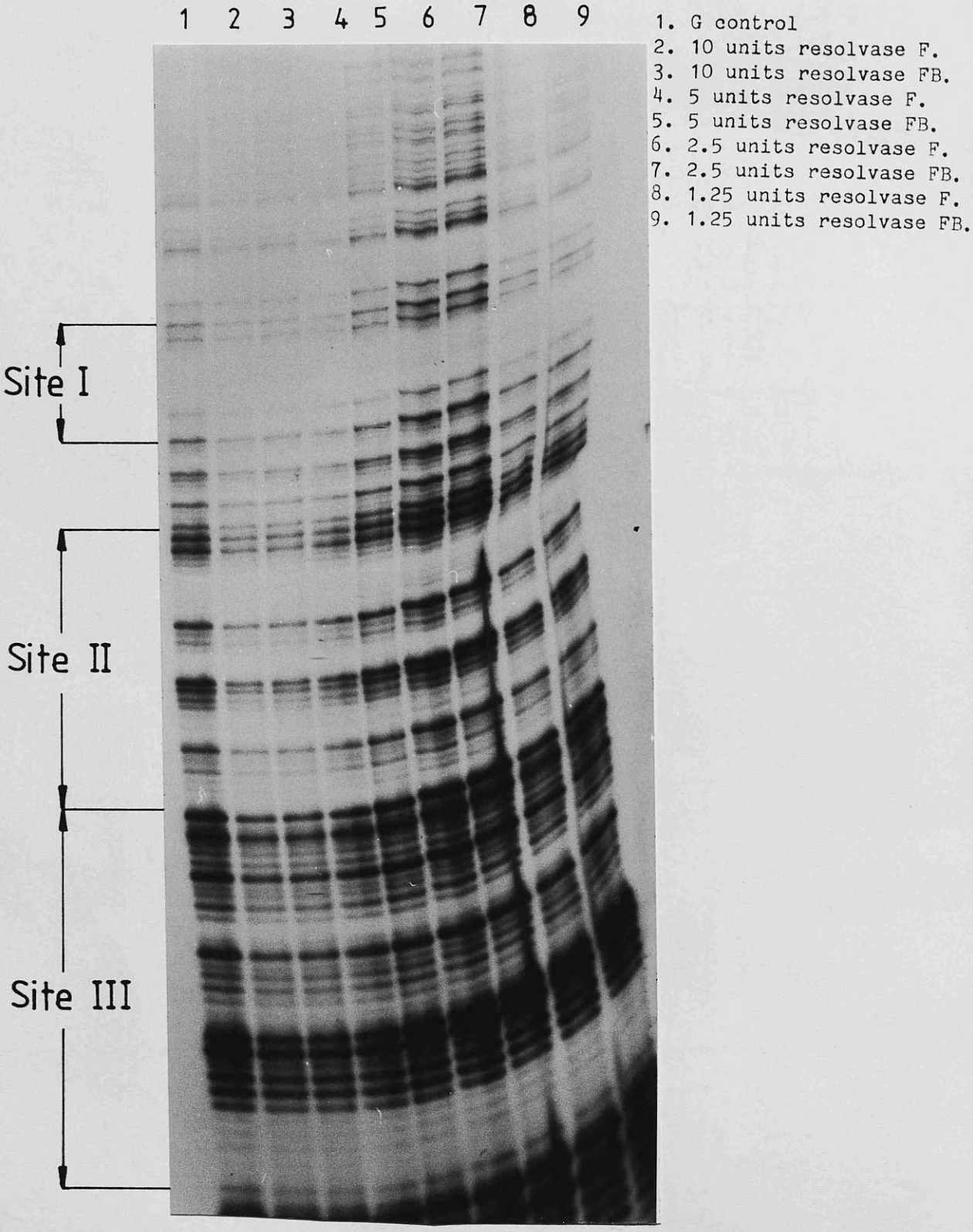

Figure 4.8 Methylation interference experiments on resolvase binding at res. The EcoRI-PvuII res$^+$ fragment of pLB16 3' end-labelled at the BamHI site was used as the substrate in this experiment. 50ng of purified fragment was used in each reaction, the DNA was methylated first and then bound with increasing concentrations of resolvase (as indicated above). F = filtrate, FB = filter-bound. In all cases the filtrate and filter-bound lanes gave identical patterns of methylation showing this experiment fails for resolvase binding at res; further details are given in the text.

4.5 Methylation interference experiments on resolvase binding to *res*

As described in the introduction to this chapter, the aim of this type of experiment is to identify purine residues which when methylated, can interfere with resolvase binding, thereby implicating these positions as being important either for the formation or maintenance of the protein-DNA complex (Siebenlist and Gilbert 1980). This experiment was attempted with the resolvase/*res* system using the end labelled 244bp EcoRI-PvuII *res*$^+$ fragment derived from pLB16.

5 x 50ng aliquots were used. Each sample was methylated in an identical way, to an extent approximating to one methylated base per fragment. One aliquot served as the G cleavage control sample, the remaining aliquots were supplied with increasing concentrations of resolvase 1.25, 2.5, 5 and 10 units and incubated at 37^{o}C for 5 minutes. Each sample was then gently filtered through nitrocellulose and both the filtrate and filter bound fractions were isolated. These, along with the control sample, were subjected to piperidine-induced cleavage of the DNA backbone at positions of methylated Guanines. 2ul aliquots of each sample containing the same number of counts/ul were electrophoresed through an 8% polyacrylamide sequencing gel. Figure 4.8. shows the resulting autoradiograph.

In theory, at a critical resolvase concentration necessary to saturate all the 'good' binding sites, the filtrate will only contain those fragments carrying a methylated base which interfered with resolvase binding. These positions should stand out substantially above background.

Figure 4.8 shows an autoradiograph representative of the result consistently obtained over a wide range of resolvase concentrations (additional data not shown). The filtrate and filter bound fractions give identical patterns of methylation, the relative ammounts of each fraction obtained merely reflecting the concentration of resolvase present.

In retrospect,it is perhaps not surprising that this experiment fails for resolvase binding at *res*. The *res* region contains 3 potential resolvase binding sites and on the basis of these results, presumably the inactivation of one site does not influence, to any great degree, the binding capabilities of the remaining 2 sites. Binding at only 1 or

2 of the 3 possible binding sites will still lead to retention of the res fragment on the nitrocellulose filter, rendering it indistinguishable from a fully functional res site.

That the straightforward approach to this type of experiment fails is disappointing, and precludes the use of similar techniques, such as ethylation interference experiments to probe the disposition of phosphate contacts along the DNA backbone (Siebenlist and Gilbert 1980).

It may be possible to make a success of this type of experiment by coupling it to the gel binding assay technique of Wu and Crothers (1984). Application of this technique to resolvase/res complexes identifies 6 discrete mobility shift bands, consistent with 2 step binding at each site (Boocock et al 1986). By running the methylation interference reaction mixes through such a gel system it may be possible to enrich for fragments which have been unable to bind resolvase at one of the 3 binding sites. Alternatively, these experiments could be attempted on isolated sub-sites of the res region. However, the restriction profile of res does not permit clean isolation of single sites and requires a more sophisticated approach, a task which is now underway in this laboratory using exonuclease III.

Methylation interference experiments have now been accomplished for site I of the Gamma delta res region (Grindley and Reed 1985). In this case, non-functional sites were enriched for using a resolution assay. Presumably, this required the initial methylation step to be carried out on a supercoiled 2 res site plasmid. The positions of methylation which interfered with resolution included the Gamma delta counterparts of the 4 protected G positions detected in the methylation protection experiments described here and re-emphasises the importance of the concensus sequence in resolvase binding to res.

Discussion

A set of general features for protein-DNA recognition have been derived based on the combined genetic, biochemical and physical studies of cro, CAP and the Lambda repressor (Sauer et al 1982, Matthews et al 1983, Ohlendorf et al 1983, Pabo and Sauer 1984). Evidence from experiments presented here and from experiments with Gamma delta (Abdel-

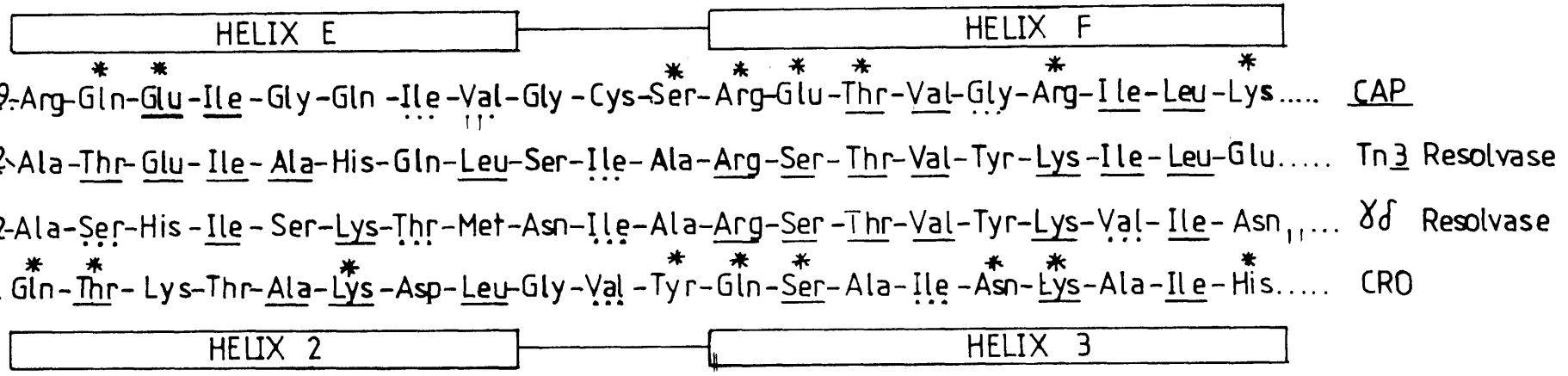

Figure 4.9 Amino acid sequences of Tn3 and Gamma delta resolvases which show homology with the alpha$_1$, alpha$_2$ helices of cro and the E and F helices of CAP which have been implicated in protein-DNA recognition (Abdel-Meguid et al 1984).
Residues which are identical to either cro or CAP are underlined with a solid line, broken lines represent residues which are similar. Residues of cro and CAP which are marked by an asterisk are those which are proposed to make contact with the bases in the DNA binding site.

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Meguid et al 1984), show resolvase exhibits many of these features.

Firstly, resolvase has 2 functional domains, separable by chymotrypsin cleavage (Abdel-Meguid et al 1984, M. Boocock pers. comm.). The large N-terminal domain mediates oligomer interactions as well as the strand exchange reaction (Newman and Grindley 1984), the small C-terminal domain mediates protein-DNA interaction. Indeed, it is within this C-terminal domain Pabo and Sauer (1984), identified a possible functional homology (Figure 4.9.) with the bi-helical domain of cro and CAP which would enable resolvase to adopt an analogous helix - turn - helix structure. This bi-helical domain is generally considered to carry the recognition determinants for protein binding to DNA, one helix making sequence-independant interactions with the phosphates of the DNA backbone, the other helix penetrating directly into the major groove of the DNA making sequence-specific, multidentate hydrogen bonds with the exposed edges of the base pairs.

Current evidence suggests the dyad symmetry of each sub-site of the res region is tailored for binding a dimer of resolvase. DNAaseI protection experiments of Abdel-Meguid et al (1984), using the small C-terminal domain of resolvase, demonstrated binding towards the outer region of each site in the vicinity of the concensus sequence. The methylation protection experiments described here, show this binding is a result of direct major groove interaction of resolvase with specific bases of the concensus sequence, in keeping with the predictions of a bi-helical recognition motif. This result re-emphasises the signifigance of the sequence conservation in all 3 sites.

Experiments described here also show weak major and minor groove interactions at the centres of each of the binding sites. The expected dimensions of the small domain along with the DNAaseI results of Abdel-Meguid et al (1984) imply these must arise as a result of close proximity of the large N-terminal domain, presumably in search of the sequence of strand exchange. On this basis, Abdel-Meguid et al (1984) proposed a model for a resolvase dimer binding at site I, where the C-terminal domains interact with the concensus sequence on one face of the DNA helix and the large N-terminal domains contact the DNA in the vicinity of the strand exchange sequence from the opposite face of the DNA helix. Results here are not in accord with this model, given that all the contact positions identified by the footprinting technique lie

on the same face of the DNA helix at each site. Of course, this may be explicable by any direct interaction of resolvase with the crossover sequence from the opposite face of the helix being purely transient, for the duration of the recombination event, a phenomenon which would not be detected by the techniques applied here. These 2 modes of binding would have significant consequences with regard the predicted structure of the synaptic complex.

Evidence from the experiments presented here suggests resolvase recognises and binds to each site in an analagous fashion. However, this is conceptually rather difficult to reconcile with the variation in length of each binding site, namely, 25bp,31bp and 22bp respectively. Flexibility of the C-terminal domain, with respect to the rest of the molecule, may play some role but is unlikely to be the sole contributory factor given the added discrepancy between the predicted size of a resolvase dimer and the much greater length of the DNA binding site (50A and 129A repectively, Rogowsky *et al* 1985). These factors together with the required -3 synapse geometry, predict a neccessity for resolvase-induced bending or wrapping of *res* site DNA, perhaps, in a manner similar to the wrapping of DNA around histones in formation of the nucleosome core particle (Richmond *et al* 1984).

Drew and Travers (1985a) have recently analysed DNA bending in relation to nucleosome positioning. Their findings suggest the ability of DNA to bend and the preferred direction of DNA bending is inherent in the nucleotide sequence of the DNA in question. From previous studies (Dickerson and Drew 1981, Drew and Travers 1984, M^{c}Call *et al* 1985), it appears A plus T rich and G plus C rich regions have helical conformations different from that of mixed sequence DNA. A plus T rich regions have a tendency to adopt rather narrow minor groove dimensions and G plus C rich regions to form a wide minor groove and vice versa with respect to the major groove of the DNA. Drew and Travers (1985a) have shown that on bending, there is a tendency for the naturally narrower minor groove edges of A plus T rich regions to face inwards towards the centre of curvature and for the wider minor groove edges of the G plus C rich regions to face outwards, away from the centre of curvature. Ideally, for bending, the 2 types of sequence blocks would be interspersed with a 5bp periodicity.

Examination of the *res* site DNA sequence of Tn*3*, reveals an uneven

distribution of A plus T rich sequences (refer to Figure 4.6). The central portion of site II reveals 3 blocks of A plus T rich sequences of 7,5 and 6 bp separated by 4 and 3bp of G plus C rich or mixed sequence. The centre of site III shows 2 A plus T rich blocks of 5 and 6 bp, separated by 2 bp of G plus C sequence.

From the cylindrical representation of the double helix shown in Figure 4.6, it can be seen the minor grooves of these A plus T rich regions show the same distribution as the putative contact positions, to lie, more or less, on only one face of the DNA helix at each site. Based on the Drew and Travers (1985a) result, one would predict that the minor grooves of these A plus T rich blocks would face inwards towards the bend centre thereby predicting the centre of curvature to be on the same face of the helix as the resolvase complex, a feature which would be expected for a model predicting wrapping of DNA around resolvase.

Presumably the degree and precise nature of bending will be dictated by the number and relative distribution of the A plus T rich blocks, thus predicting a crucial role for the relative length and general sequence composition of the 3 sites. It might perhaps be expected, that site II will bend more extensively than it's site III counterpart. Given the almost complete conservation of the spacing between sites I and II centres in all the Tn3-like elements (53bp or 53.5bp), the degree of bending at site II may be crucial in aligning the 2 regions of strand exchange in a manner conducive to recombination.

The conformation adopted by Site I cannot on this basis, be predicted. Rather than containing short runs of A plus T rich sequence interspersed with mixed or G plus C rich sequence as is proposed to be ideally required for bending, it contains a central 15bp A plus T rich region containing the recombination site. This sequence presumably adopts a conformation which favours strand exchange and the A plus T richness may favour unwinding, although not melting, since this would have been detected by the methylation protection experiments. Indeed, this 15bp region contains 4 of the TpA dinucleotides which Drew *et al* (1985b) have shown to be susceptible to unwinding when subjected to -ve superhelicity. This could perhaps explain, at least in part, the preference of the *in vitro* resolution reaction for supercoiling.

A similar examination of the sequence distribution in the Gamma delta *res* site, reveals an analagous arrangement of A plus T rich blocks

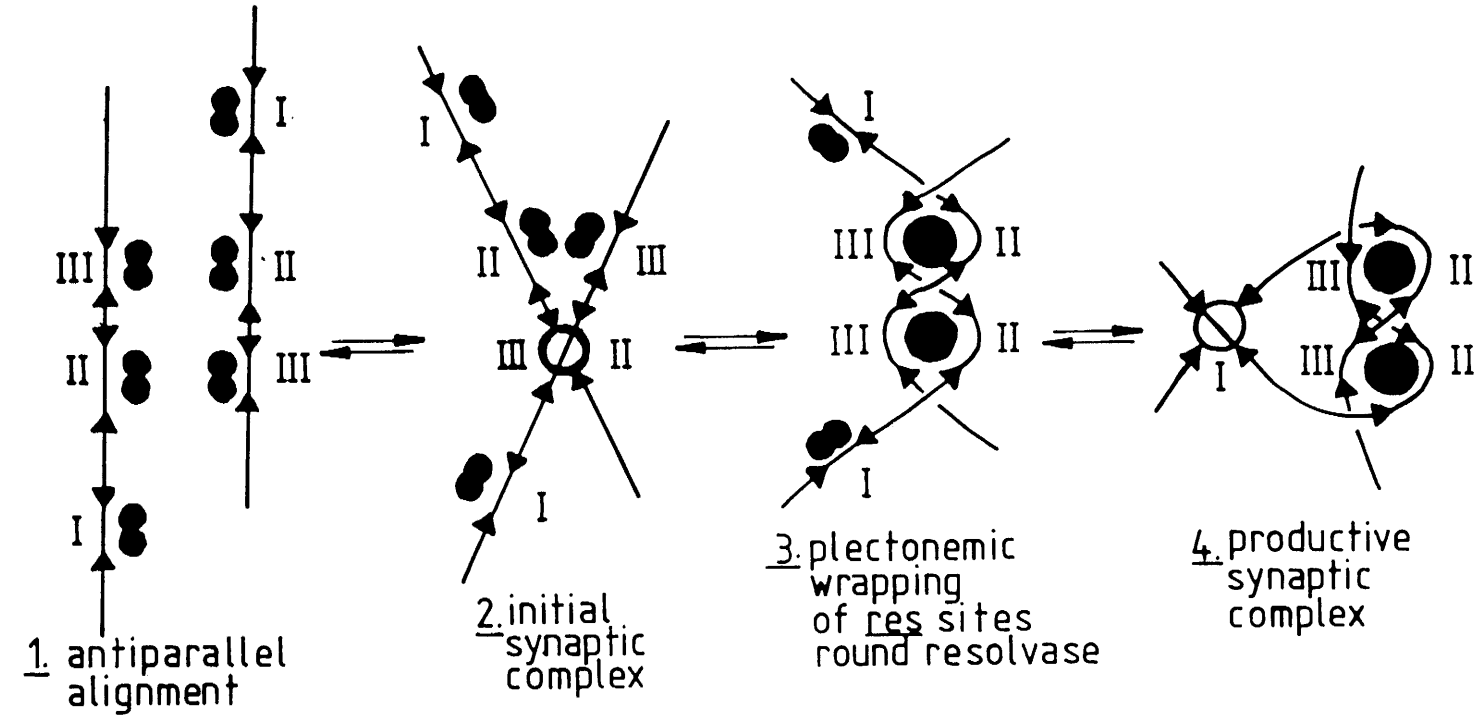


Figure 4.10 2-step synapsis model for Tn3 resolvase (Boocock et al in preparation)

1. Initial antiparallel alignment of resolvse bound res sites.
2. Initial synaptic complex with interaction of resolvase dimers to give tetramers with dihedral symmetry.
3. Plectonemic wrapping of sites II and III around resolvase introduces the necessary -3 interlinks between the two res sites. This requires resolvase-induced bending of res site DNA (for simplicity the DNA is drawn initially as a straight line; clearly this may be bent prior to synapsis).
4. The two strand exchange sequences are aligned in parallel to give the productive synaptic complex.

in each of the 3 sites. Both Tn3 and Gamma delta show an additional A plus T rich block lying just outside the end of the res site, which may represent some perturbation of the DNA configuration outwith the res region. The Tn501 family of Tn3-like elements (Rogowsky et al 1985) show a similar although, less marked tendancy towards A-T richness in the site centres.

Evidence for some resolvase-induced bending comes from preliminary analysis of circularly permuted res sites complexed to resolvase using the gel binding assay system of Wu and Crothers (1984), (M. Boocock, unpublished results). This result has led to the propopsal of a model for the synaptic complex (M. Boocock, unpublished) based on the anti-parallel alignment of sites predicted by the 2 step model. This model is shown in Figure 4.10. and relies on dihedral symmetry of the protein complex. It is important to note, that the type of bending expected here is rotational, in a way analagous to DNA in a nucleosome core particle rather than one dimensional however, the model for the resolvase/res site synaptic complex involves plectonemic wrapping (2 double helical strands wrapped around resolvase) rather than the solenoidal wrapping (involving only one double helical strand) in the nucleosome core particle.

This type of model for synapsis predicts the topological and directional specificities are largely fixed by sites II and III in addition to the energetic constraints on the system. The distribution of contact positions and the A plus T rich blocks is in keeping with this type of model, but does not provide any direct evidence for it. Clearly, the most compelling evidence for any binding model will come only from crystallographic studies of resolvase/res site complexes.

A greater understanding of resolvase-induced conformational changes may come from a more extensive analysis of resolvase/res complexes using the gel binding assay. In particular, examination of resolvase complexed to isolated sub-sites may permit the estimation of the extent of bending at each site. The behavioural characteristics of sites II and III (in the absence of Site I), in multi-res site constructs is a point of interest, since the binding model above predicts they alone would be responsible for the shadowing effects observed.

The question of sequence perturbation at the centre of each site is readdressed in the following chapter.

CHAPTER 5

in vitro PHOTOFOOTPRINTING OF RESOLVASE BOUND AT *res*

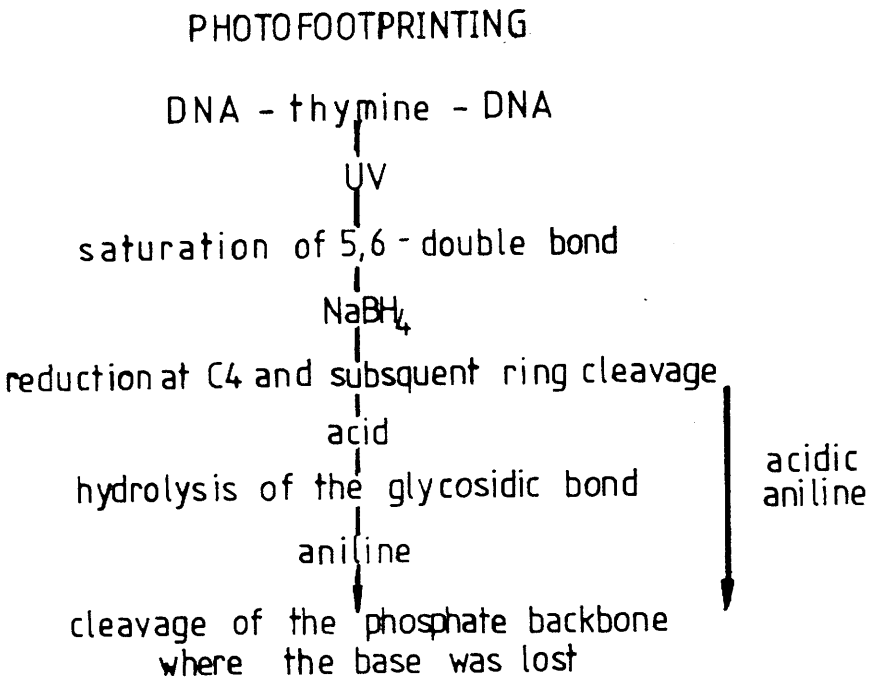


Figure 5.1 Photoproduct specific cleavage protocol (as summarised by Staryk 1985). This protocol (Becker and Wang 1984) exploits a feature of photoreacted bases which is absent in unreacted bases leading to breakage of the DNA backbone only at sites of photodamage. In each of the known photoproducts in DNA one or more of the 5,6-double bonds of a pyrimidine becomes saturated on photoreaction. As carbonyl groups conjugated to a double bond are poorly reduced by $NaBH_4$ the carbonyl groups at position 4 of photochemically modified thymines can be selectively reduced in the presence of other bases leading to ring cleavage. Subsequent breakage of the glycosidic bond and the phosphate backbone can be acheived in one step by treating with acidic aniline. Cytosines are also susceptible to this cleavage procedure because saturation of the 5,6-double bond leads to elevated lavels of spontaneous deamination to generate a carbonyl group at position 4.

Introduction

During the course of the methylation protection and interference experiments detailed in chapter 4, Becker and Wang (1984) described a new footprinting method which uses UV light to detect specific protein-DNA contact positions as well as protein-induced structural changes in the DNA at the base pair level.

This technique is based on the observation that a small but significant distortion of the DNA helix is required to form a UV photoproduct (Cameron and Cameron 1968). A protein interacting with the DNA may affect the geometrical changes necessary for photoproduct formation and consequently, exposure of protein-bound and unbound DNA to UV followed by photoproduct specific cleavage of the DNA backbone may generate two different patterns of fragments (the photoproduct-specific cleavage protocol is detailed in Figure 5.1).

Becker and Wang (1984) detected two types of alteration in photoreactivity:-

1. Reductions in photoreactivity on binding of *lac* repressor to it's operator site. Such reductions were considered to represent specific protein-DNA contact positions.
2. Enhancements of photoreactivity on formation of an 'open complex' by RNA polymerase. These enhancements were considered to represent gross alteration of the DNA conformation on conversion of DNA from the double-stranded to single-stranded form. Enhancements were not detected on *in vitro* photofootprinting the *lac* repressor bound to the operator sequence.

In relation to the resolvase/*res* system the attractions of this technique were numerous, providing the opportunity to:-

1. Probe the interaction of resolvase with a subset of pyrimidine residues within the *res* site.
2. Compare photofootprints of resolvase complexed to *res* on linear and supercoiled substrates.
3. Footprint an ongoing resolution reaction in the hope of identifying 2 *res* site intermediates at synapsis.
4. Compare photofootprints generated *in vitro* and *in vivo*.

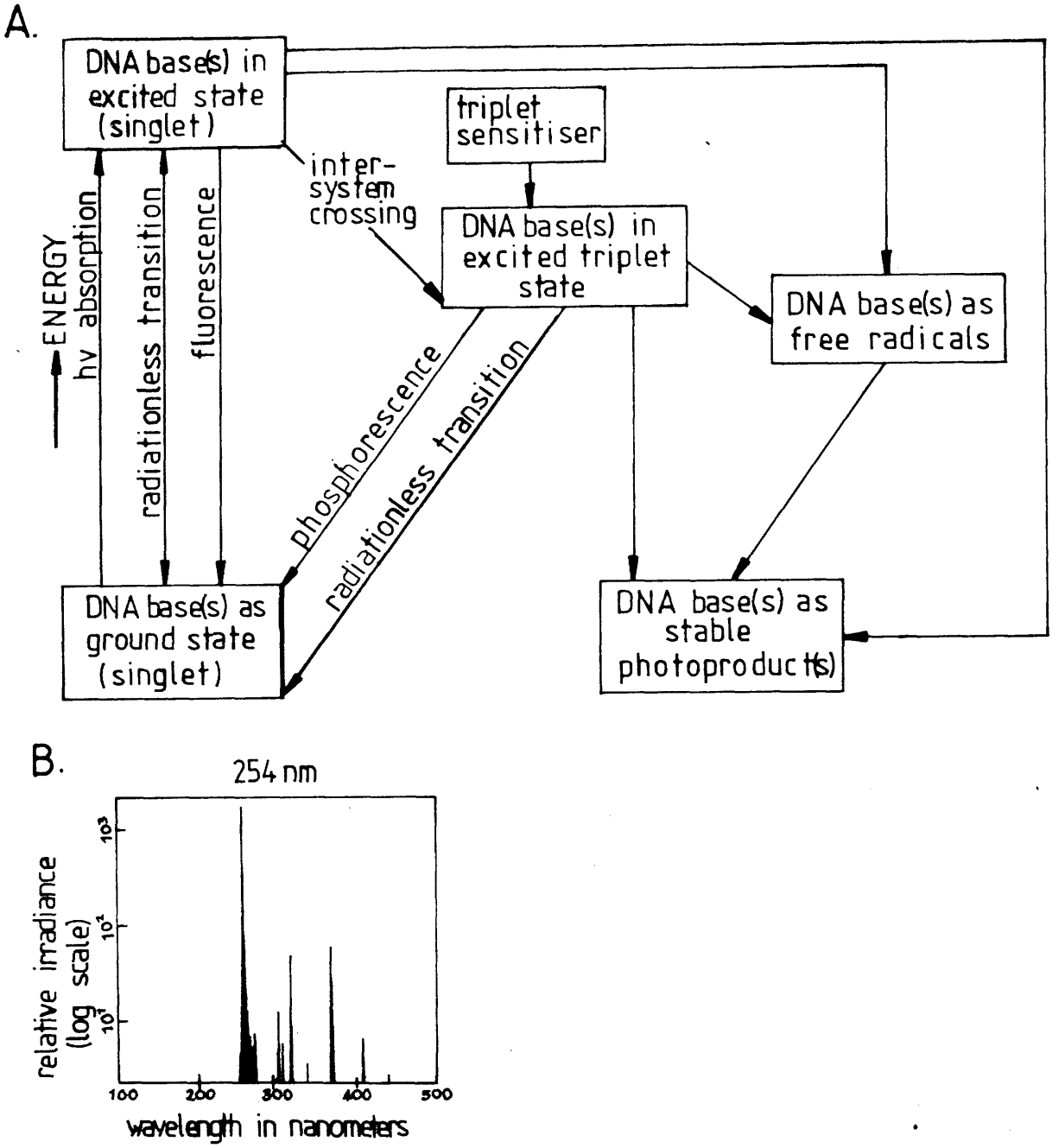


Figure 5.2
A. Pathways for dissipation of UV energy absorbed by DNA as detailed by Wang 1976. See text for further discussion.
B. Fluence profile for a 254nm transilluminator in the absence of a filter as used in the photofootprinting experiments (as detailed by UV products Ltd.)

This chapter describes photofootprinting of resolvase complexed to linear and supercoiled one or two *res* site substrates. Photofootprinting resolvase/*res* complexes *in vivo* is considered in chapter 6.

The interpretations by Becker and Wang (1984), of their results with the *lac* repressor and RNA polymerase (although adequate to explain their results) are too simplistic to explain the results obtained with resolvase bound at *res*. As a result, it is pertinent at this point to consider in more detail the process of photoproduct formation and the possible ways in which protein binding could alter photoreactivity.

Photoreaction involves 2 basic steps (Wang 1976), as illustrated in Figure 5.2.

1. Absorption of UV

Absorption of UV by bases in double-stranded DNA is relatively constant except at extreme conditions of pH, ionic strength or temperature, all factors which have profound effects on the DNA conformation. How could protein binding affect absorption?

1. Bound protein could absorb incident UV light, preventing excitation of the DNA at the binding site. This is very unlikely, since protein and DNA should absorb incident quanta independantly.

2. Specific interaction of protein with bases in the DNA and subtle changes in the structure of the double helix. This could render a normally reactive position unavailable for excitation or interaction with a neighbouring 'excited base'.

3. Extensive alteration of DNA structure. For example, single-stranded DNA behaves quite differently from double-stranded DNA.

2. Dissipation of energy

There are several ways in which an 'excited' base can return to 'ground state':-

1. Fluorescence (photon emission).
2. Emission of heat.
3. Reaction to form a new chemical entity, the end product of interest being the formation of a stable photoproduct. This step is very much dependant on the precise DNA conformation and is sensitive to the spatial arrangement of the base pairs including the precise separation distance, rotational orientation of the relevant groups and overlap of the donor and acceptor energy levels. In addition, the

degree of flexibility of the DNA may affect the ability of adjacent pyrimidines to undergo the geometrical changes necessary for photoproduct formation. For example, on joining 2 adjacent intrastrand thymines to form a thymine dimer the separation between the pyrimidines is decreased and they are rotated by some 8^{o} to allow formation of the cyclobutane ring (Camerman and Camerman 1968). The sugar-phosphate backbone must reorient to accommodate the rotational change, resulting in distortion of the DNA helix (Denhardt and Kato 1973).

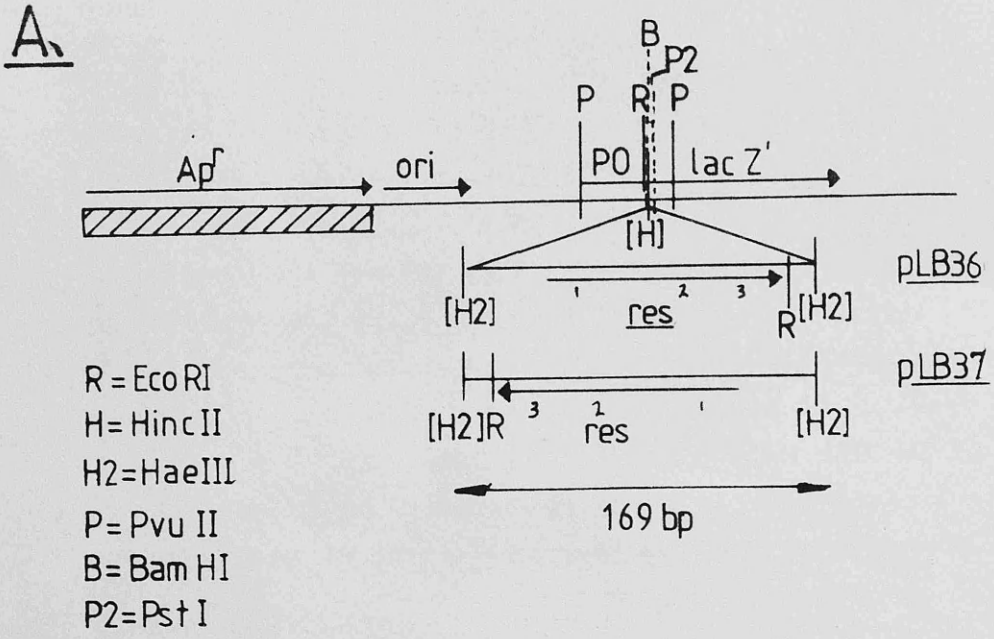
How could protein binding affect photoproduct formation?

1. As with absorption a specific protein-DNA contact could render a base unavailable for photoproduct formation.

2. Alterations of DNA structure. Protein binding could potentially alter the DNA conformation or the flexibility of the DNA. Either factor could reduce or enhance the ability of neighbouring bases to interact and undergo the geometrical changes necessary for photoproduct formation. Rather subtle changes in the DNA conformation may produce an observable effect. Factors which could potentially cause such alterations include:-

   a. Conversion of DNA from double to single-stranded this is known to increase the rotational freedom of the bases and would be expected to generate only enhancements of photoreactivity.
   b. Change the DNA conformation: for example a B to A form transition.
   c. DNA bending, this could subtly alter the spatial arrangement of the bases to cause either reductions or enhancements of photoreactivity. Protein-induced bending could potentially produce 'long-range' effects which are distanced from the actual protein contact positions.

As a consequence, the interpretation of photofootprinting may be more subtle than originally proposed by Becker and Wang (1984) and reductions in photoreactivity need not in all cases represent protein-DNA contacts and enhancements need not necessarily represent such gross structural changes in the DNA as unpairing of the base pairs. These points are addressed in more detail in relation to resolvase bound at res in the discussion section of this chapter.



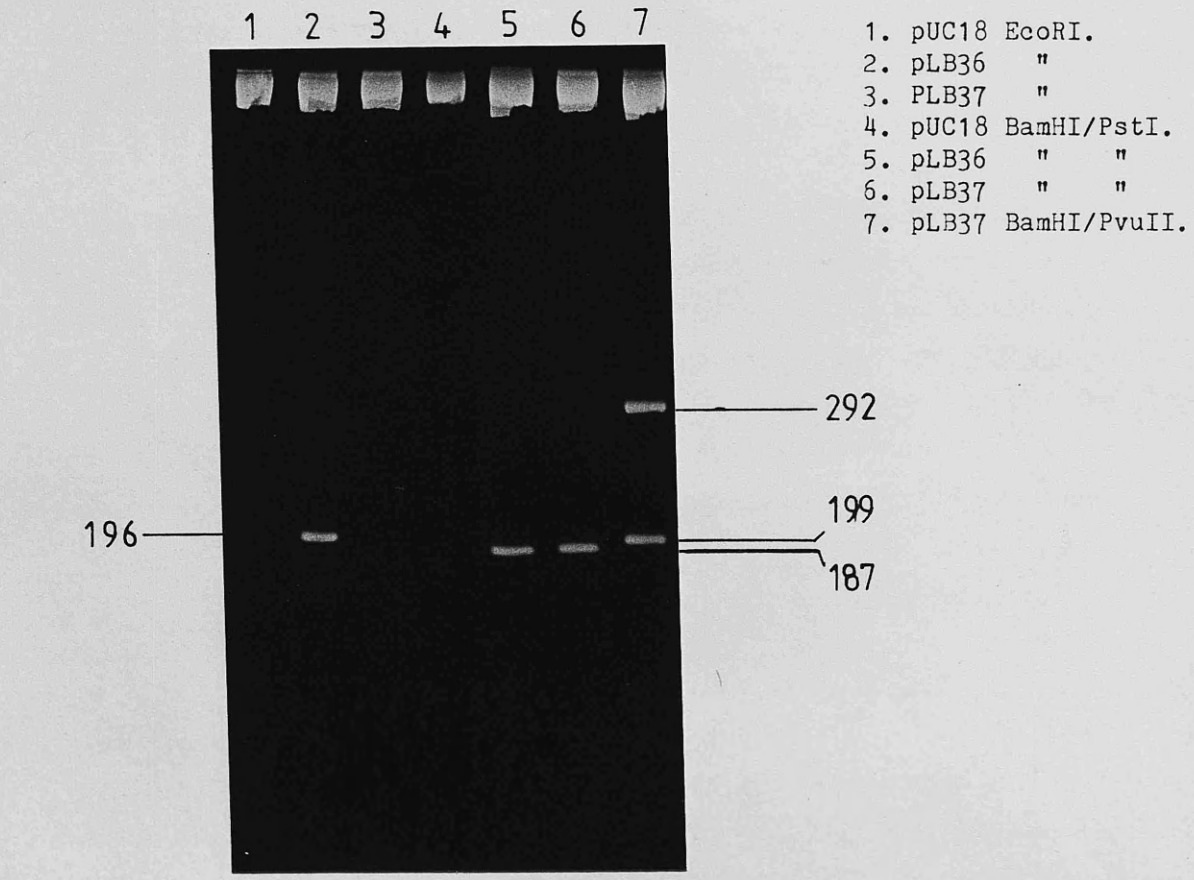


Figure 5.3 Construction of pLB37

A. Schematic diagram showing the general organisation, site of insertion and orientation of the 169bp HaeIII *res*$^+$ fragment in pLB36 and pLB37. BamHI and PvuII restriction sites relevant to the end labelling strategy are indicated.

B. 5% polyacrylamide gel of EcoRI and BamHI/PstI restrictions of pUC18, pLB36 and pLB37 confirming the insertion and orientation of the *res* site as diagrammed in A. The BamHI/PvuII restriction of pLB37 indicates the 292bp *res*$^+$ fragment used in the end labelling experiments. Size marker were run on this gel although they are not shown in this photograph.

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Results

5.1 Resolution characteristics of pLS138 under the conditions of the photofootprinting experiments

As with the methylation experiments, it was necessary to ensure resolvase could bind to *res* site DNA and mediate the normal resolution reaction under the conditions used in the photofootprinting experiments. For this purpose, the resolution characteristics of the 2 *res* site plasmid, pLS138 were examined in the photofootprinting buffer (10mM tris/HCl pH 8.0, 10mM $Mg(OAc)_2$, 10mM KCl, 0.1mM EDTA, 0.1mM B mercapto-ethanol, 6% glycerol, 100ug/ml gelatin). As before, the products were analysed by agarose gel electrophoresis of unrestricted and BamHI restricted reaction mixes. The reactions were precipitated prior to restriction due to the known glycerol sensitivity of many restriction enzymes. After 5 minutes incubation at 37°C resolution had proceeded to 10% completion and after 2 hours to greater than 60% completion, generating the normal catenated end products. A similar experiment is shown in Figure 5.11.

5.2 Construction of pLB37

The pLB32-derived BamHI-PvuII *res*$^+$ fragment 3' end labelled at the BamHI site was again used to obtain a footprint for the (-) strand of the DNA. To footprint the (+) strand, pLB37 was constructed to provide an alternative to the pLB16 derived EcoRI-PvuII *res*$^+$ fragment, which was proving problematic with regards to complete 3' end labelling of the EcoRI site.

The 170bp HaeIII *res*$^+$ fragment from pLB16 was isolated from 5% polyacrylamide, electroeluted and ligated to HincII cleaved, dephosphorylated pUC18. The site of insertion and orientation of the required recombinants is detailed in Figure 5.3.

5.3 Calibration of exposure to UV

Calibration of the UV source used here was necessary because both the source and conditions of exposure to UV differ from those used by

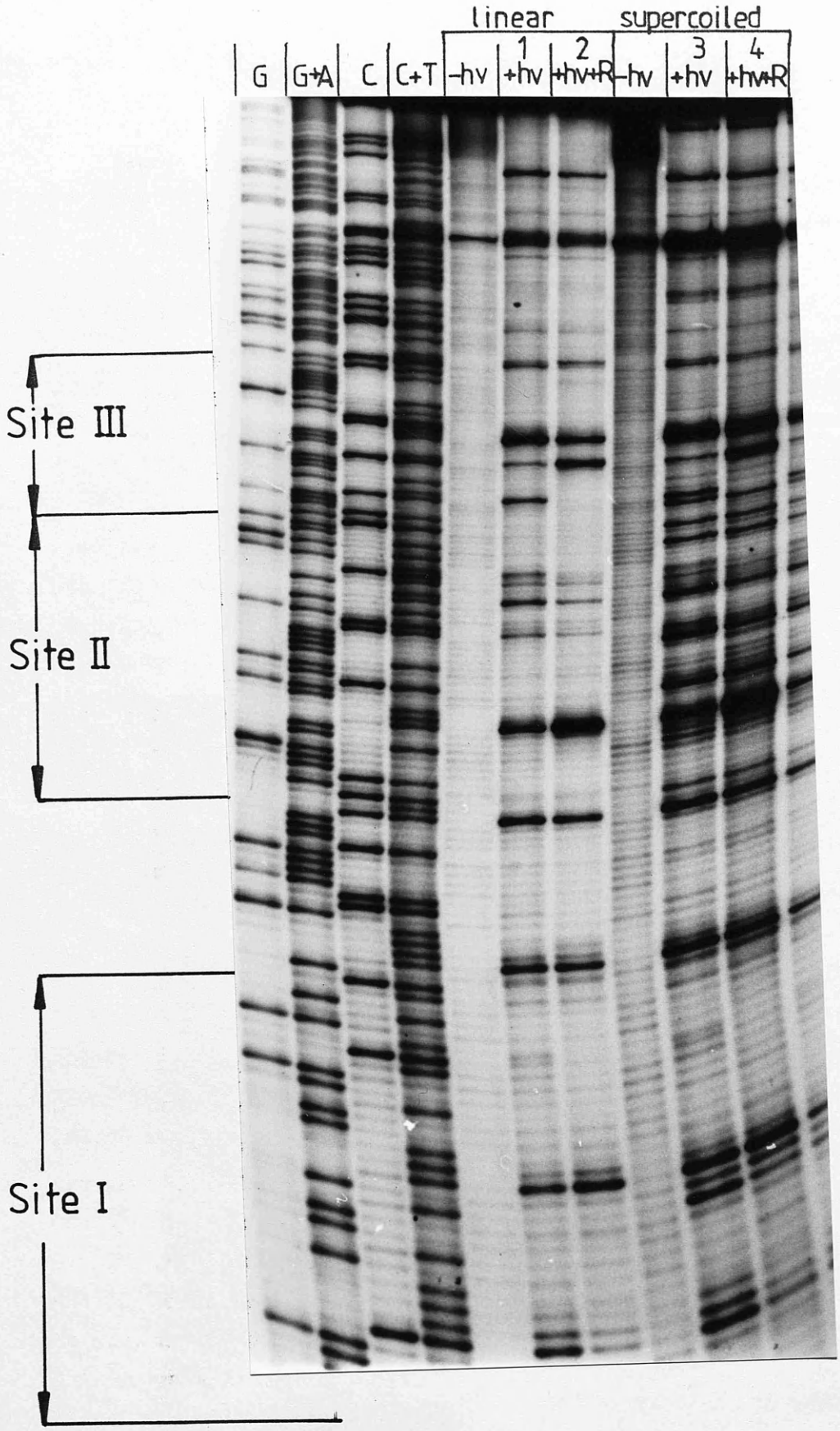

Figure 5.4 Autotradiograph of an 8% polyacrylamide sequencing gel showing a photofootprint of resolvase bound to the (-) strand of res. As indicated, this autoradiograph shows a photofootprint obtained with a linear and supercoiled pLB32 derived res^+ fragment. At the appropriate stage depending on the experiment, the BamHI-PvuII res^+ fragment was 3' end labelled at the BamHI site and purified from 5% polyacrylamide. For the linear substrate 5 units resolvase/0.5ng purified fragment was used. For the supercoiled substrate 5 units resolvase/2.5ng pLB32 DNA was used. A control Maxam and Gilbert sequence of the same fragment is run alongside to permit assignment of photoproducts to sequence positions within res. Further experimental details and discussion of the altered extent of photoreactivity on binding of resolvase to res are given in the text.

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Becker and Wang (1984). In the absence of a sophisticated lighting system, a 254nm shortwave transilluminator, without the filter, was used at a fixed 12cm from the sample. This was considered to be an adequate substitute based on the recommendations of M. Becker (pers. comm.) and observations of Setlow and Carrier (1966) and Varghese (1971), that 254nm UV mediates the type of photochemical reactions of both thymine and cytosine required by this technique. The fluence profile for the type of transilluminator used (without a filter) is shown in Figure 5.2.

For these experiments 50ng of the purified pLB32 derived 264bp BamHI-PvuII *res*$^+$ fragment, 3' end-labelled at the BamHI site was divided into 5 aliquots. One aliquot was subjected to the photoproduct-specific cleavage protocol without exposure to UV. The remaining aliquots were subjected to 2,4,6,or 8 minutes exposure to UV in 1x photofootprinting buffer. After photoproduct specific cleavage, aliquots of all 5 samples were electrophoresed through an 8% polyacrylamide sequencing gel (data not shown). The 2 minutes UV dosage was considered to be the most appropriate, generating UV-induced cleavage products along the entire fragment. Greater than 4 minutes UV treatment was excessive presumably due to the formation of greater than one photoproduct per fragment. The specificity of photoproduct formation is discussed in the following section.

5.4 **Photofootprinting linear resolvase/*res* site complexes**

Purified BamHI-PvuII *res*$^+$ fragments of pLB32 and pLB37, 3' end labelled at the BamHI sites were used as substrates to footprint the (-) and (+) strands of the DNA respectively. For each fragment 3 x 50ng aliquots were resuspended in 1 x photofootprinting buffer, 2 aliquots were subjected to UV treatment for 2 minutes, one in the presence and one in the absence of bound resolvase. All 3 aliquots were subjected to the photoproduct-specific cleavage protocol as described in chapter 2 and 2ul aliquots of each sample (containing an identical number of counts/ul) were electrophoresed through an 8% polyacrylamide sequencing gel alongside the appropriate set of Maxam and Gilbert (1977,1980) sequencing reaction cleavage products. Both techniques generate fragments terminating in a 5' phosphate permitting the photoproducts to be assigned to the relevant sequence positions within the *res* site. The

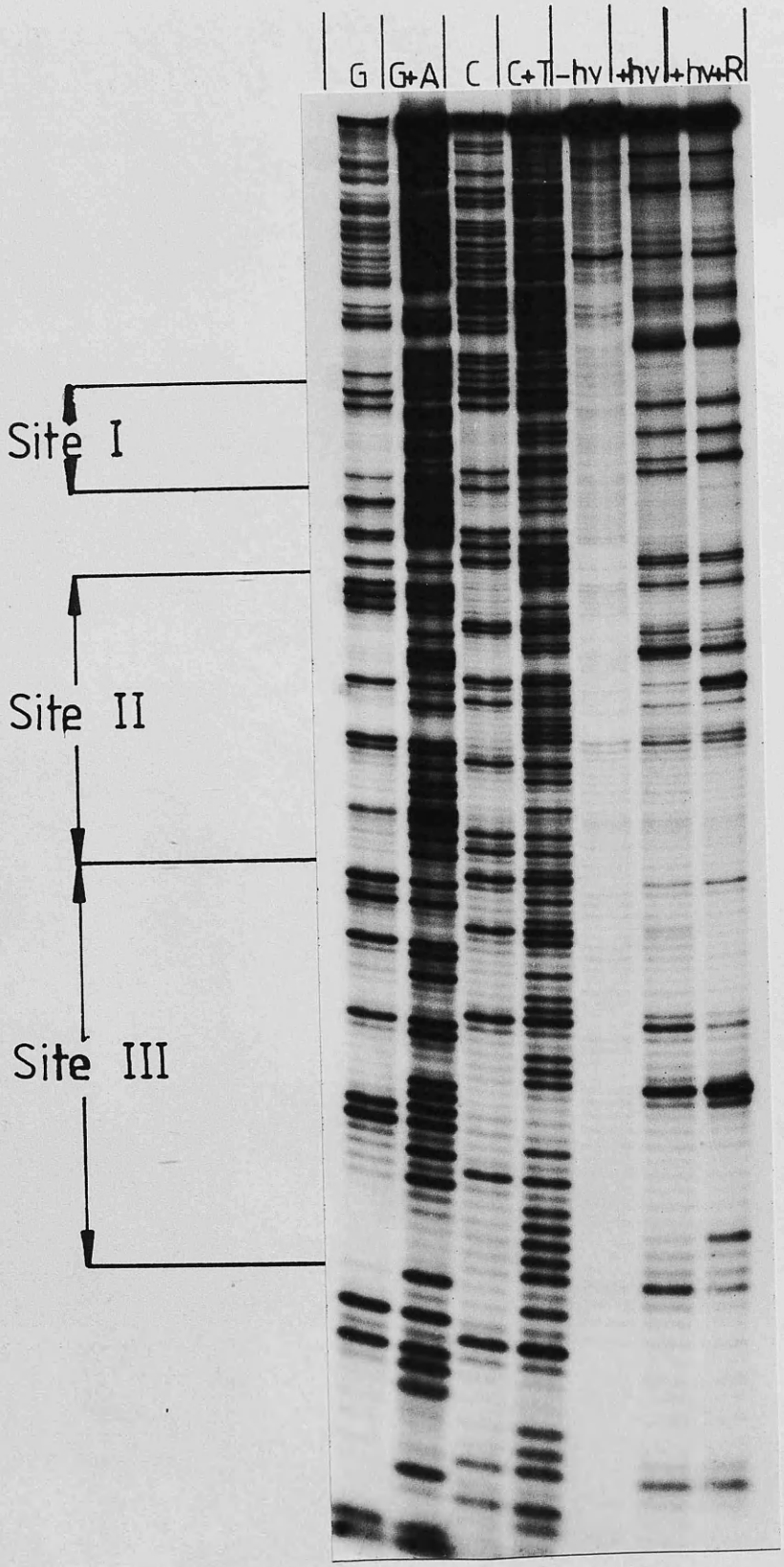

Figure 5.5 Autoradiograph of an 8% polyacrylamide sequencing gel showing a photofootprint of resolvase bound to the (+) strand of res.
The BamHI-PvuII res$^+$ fragment of pLB37 3' end labelled at the BamHI site was used as the substrate for these experiments. The lanes marked with R represent the samples which were incubated with resolvase. The site boundaries (as defined by DNAaseI experiments, Symington 1982) are indicated. A Maxam and Gilbert sequence of the res fragment is run alongside to permit assignment of alterations in photoreactivity to sequence positions within res. The reductions and enhancements of photoreactivity are plotted in Figure 5.8 and discussed in the text.

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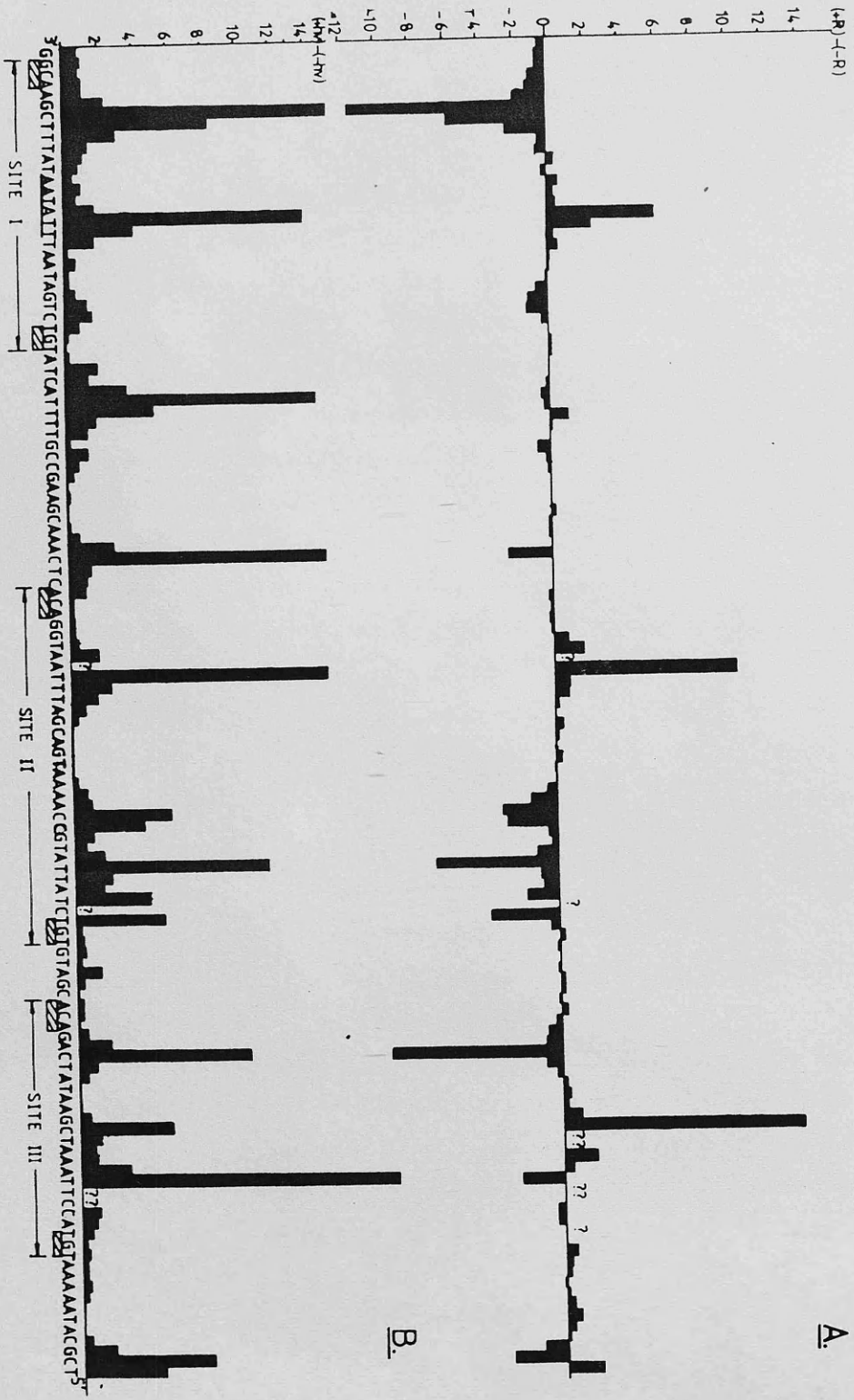


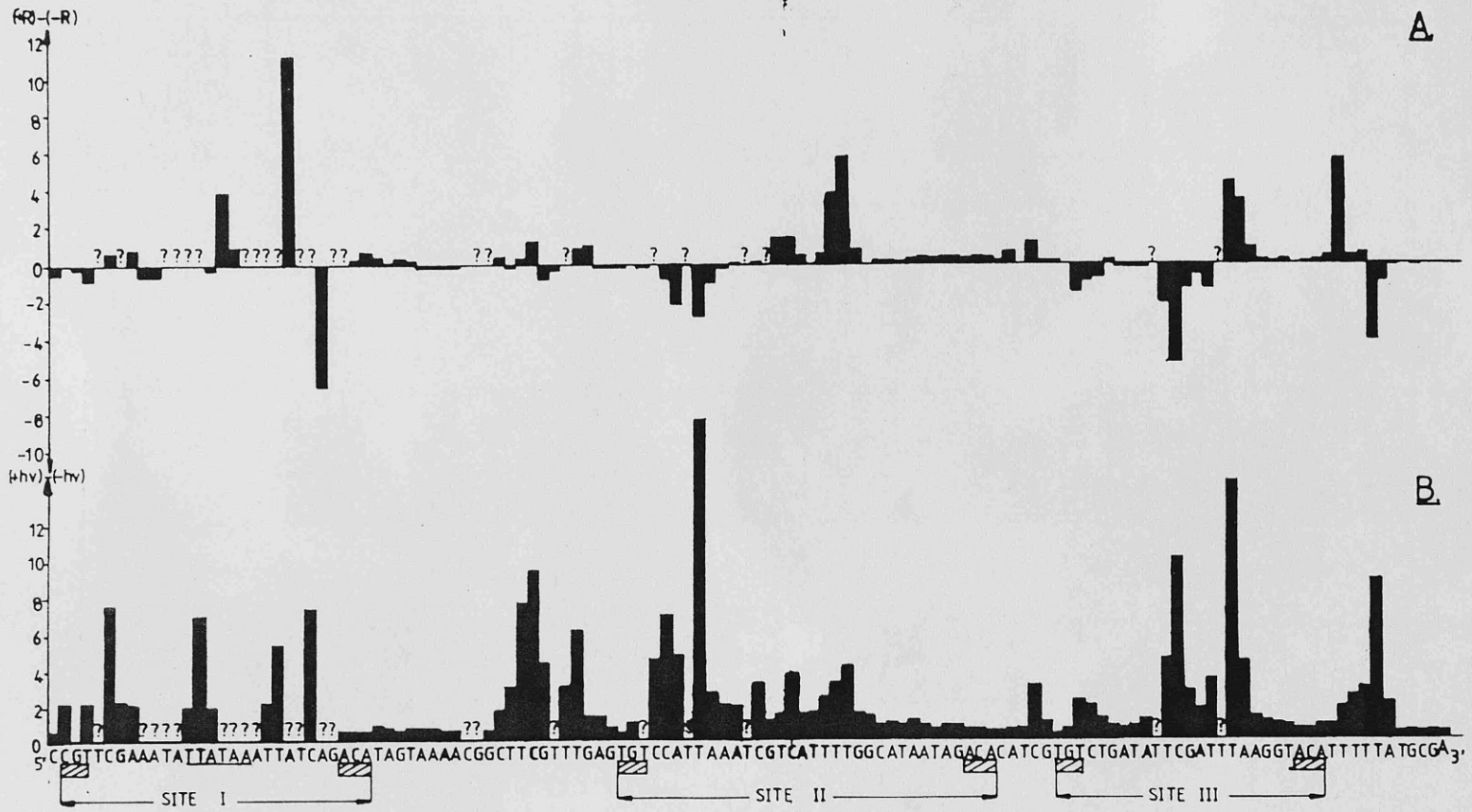
Figure 5.6

Figure 5.6 and 5.7 Analysis of the photofootprinting pattern of resolvase binding to the (+) and (-) strands of <u>res</u> using densitometry. The data plotted in Figures 5.6 and 5.7 represent the (+) and (-) strands of the DNA respectively. The data are plotted as:-

A. The extent of resolvase-induced alteration in photoreactivity as measured by the difference in peak heights between the resolvase (+) and (-) samples (after subtraction of the peak height in the absence of UV).

B. The extent of UV-induced cleavage at each position as measured by peak height after subtraction of background cutting in the absence of UV (cms). Question marks represent positions which cannot be assigned peak heights, normally because of very strong signals at an adjacent position.

Figure 5.7


(+R)-(-R)
A
(+hv)-(-hv)
B
5'CCGTTCGAAATATTATAAATTATCAGACATAGTAAAACGGCTTCGTTTGAGTGTCCATTAAATCGTCATTTTGGCATAATAGACACATCGTGTCTGATATTCGATTTAAGGTACATTTTTATGCGA3'
SITE I
SITE II
SITE III

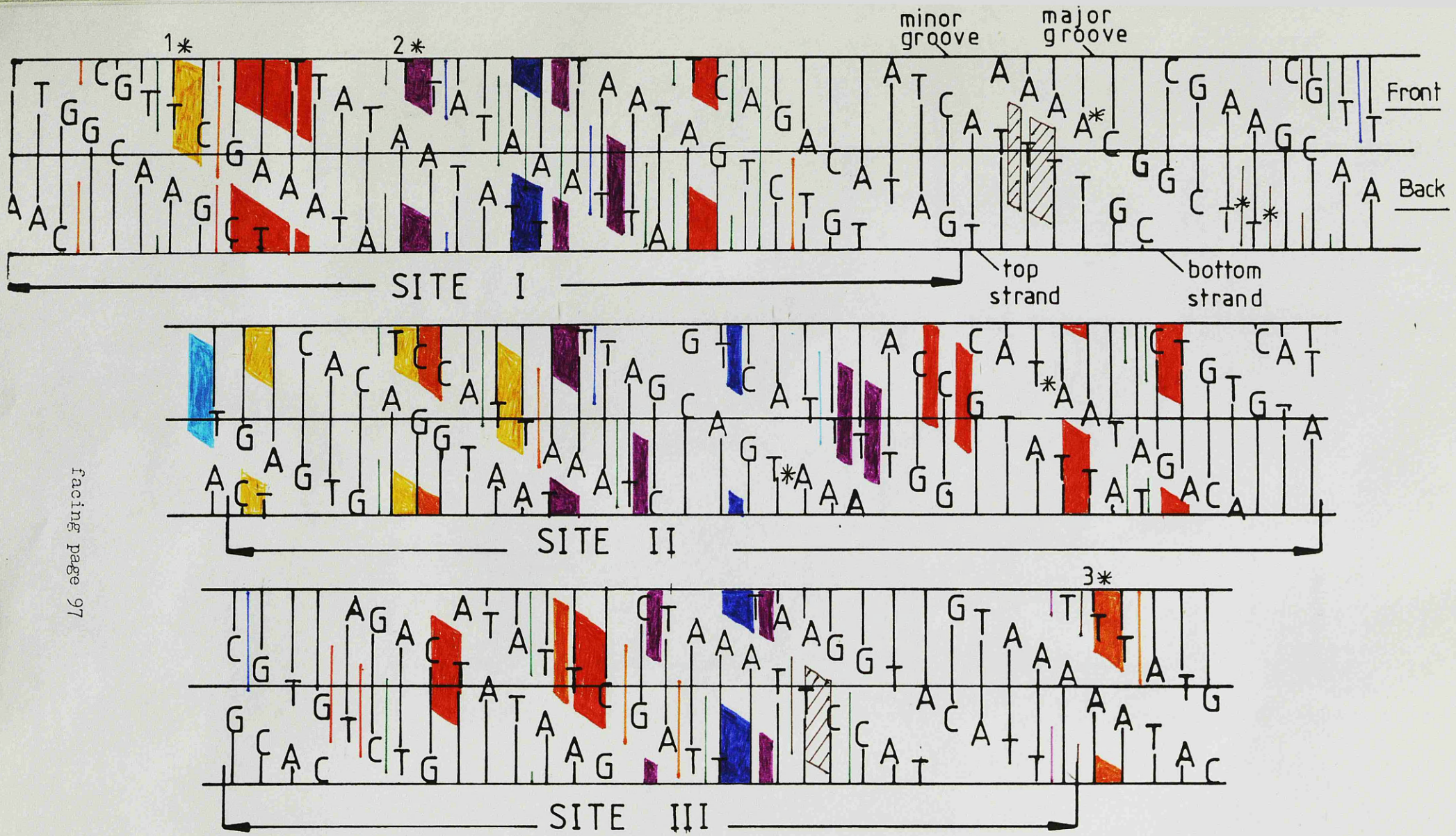


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Figure 5.8 Photofootprinting data of resolvase bound at res plotted on a cylindrical representation of the double helix.

This plot shows a cylindrical representation of res site DNA assuming 10.5bp per turn of the helix using the same criteria as detailed for Figure 4.7.

The data are plotted in colour coded categories assigned on the basis of the densitometric analysis shown in Figures 5.6 and 5.7. The resolvase-induced alteration of photoreactivity (diagrammed in Figures 5.6 and 5.7 B.) was calculated as a percentage change of the original UV-induced cleavage in the absence of resolvase (as diagrammed in Figures 5.6 and 5.7). Positions which did not show a peak height of greater than 2cms were not included in this analysis since this was the basal level of UV-induced cutting at positions which did not have adjacent pyrimidines. The categories were assigned a colour code:-

Reductions in photoreactivity	Enhancements of photoreactivity
weak 15-25% change	weak 15-25% change
intermediate 25-50% change	intermediate 25-50% change
strong >50% change	strong >50% change

— - peak height could not be assessed at this position
— - neutral position, pattern of photoreactivity is not affected by resolvase binding.
(changes of less than 15% were not plotted).

Additionally, the original extent of UV-induced cleavage at each position was also incorporated into this diagram to permit assessment of the possible signifigance of the resolvase-induced alteration in photoreactivity. The peak height is represented by the width of the coloured band between the two bases:-

C|T - weak cutting 2-3cm initial peak height
C▮T - intermediate cutting 3-6cms initial peak height
C▬T - strong cutting >6cms

The positions are plotted across the major and minor groove regions between the two bases involved in photoproduct formation since the cause of the altered extent of photoreactivity can not be localised any further at this point.

* - these positions represent the minor alterations in the photofootprinting pattern obtained with a linear substrate on application of the photofootprinting technique to a 'live' resolution reaction. The asterisks which have no number represent positions which show enhanced cleavage of a previously weakly cut position (mostly purine- pyrimidine interactions). Numbered positions represent a different resolvase-induced change in photoreactivity as compared to that of the single linear res site substrate:-

1. Now weakly enhanced photoreactivity rather than a weak reduction in photoreactivity.
2. No longer shows enhanced photoreactivity.
3. Now shows only weak reduction in photoreactivity.

(further details are given in the text).

Sequence of adjacent pyrimidines	Extent of cleavage at each sequence position						
3'----------5'	3'boundary	1st	2nd	3rd	4th	5th	5'boundary
ATTTTTA	-	+++	+	+	-	-	-
ATTTTG	++	+++	++	-	-		-
GTTTTA	-	++	+	+	-		-
ATTCC	+	+++	++	+	-		-
GCTTTA	+	+++	+++	+	-		-
GCTTCA	++	+++	+++	++	-		-
ATTTA	++	+++	++	++			++
ATTTA	-	+++	++	-			-
ATTTA	-	+++	+	-			+
GCTTA	+	+++	++	-			-
ACCTG	++	+++	++	-			-
ACTCA	+	+++	-	-			-
ATCTG	-	++	-	++			++
GTCTG	-	+	+	-			-
GTCTG	-	-	+	-			-
GTTTG	-	++	+	-			-
GCTTG	+	+++	++	-			-
ATTA	-	++	-				-
ATTA	-	+++	-				-
ATTA	+	+++	-				-
ATTA	-	+++	-				-
GCCG	-	+	-				-
GCCG	-	+	-				-
ACCG	-	+++	++				++
GCTA	-	++	-				-
ACTG	-	++	-				-
GCTA	-	+	-				-
ACTA	-	+++	-				-
ACTA	-	+	-				-
ACTA	-	+++	-				-
GCTA	-	+++	-				-
GCTA	-	+++	++				++

Table 5.1 Extent of UV-induced cleavage at runs of pyrimidines in res in the absence of resolvase.
This table shows the sequences within res which contain runs of adjacent pyrimidines. The 3' and 5' boundary sequences are also shown. The + symbols indicate positions showing UV-induced cleavage strong, intermediate and weak cutting for +++, ++ and + respectively. Positions where boundary purine residues are involved in the formation of photoproducts are indicated. - represents positions which do not show UV-induced cleavage, as detailed in the text these tend to occur towards the 5' end of a block of pyrimidines.

seen at pyrimidines in the absence of UV treatment.

5.4.3 **UV-induced cleavage profile**

Comparison of the cleavage profile obtained following exposure to UV with the background cutting pattern permits the photoproducts to be identified. As expected the majority of these are pyrimidine dimers TT, CT, CC and to a lesser extent TC. At runs of pyrimidines there is a consistent pattern in the extent of photoproduct formation (Table 5.1). Pyrimidine dimers preferentially form at the 3' pair in a run of pyrimidines and photoreactivity decreases towards the 5' end of the block. The most probable explanation for this observed preference is the consequence of the DNA conformation adopted by regions of purine-pyrimidine asymmetry on the ability of adjacent pyrimidines to form photoproducts. Under these circumstances, the DNA is thought to adopt a rather rigid conformation (Arnott et al 1983, Zimmerman and Pheiffer 1981, Drew and Travers 1984, 1985a), which may inhibit the rotational and other geometrical changes necessary for photoproduct formation (Wang 1976).

The most efficient photoproduct formation occurs at the 3' edge of a run of pyrimidines where the neighbouring purine residues may influence the structure. Calladine (1982) has proposed that interstrand steric hindrance between purine residues results in rotation of the bases to relieve these effects (propellor twist), this may result in alteration of the spatial arrangement of the corresponding pyrimidines which makes them more amenable to photoproduct formation.

As also found by Becker and Wang (1984), a small minority of the photoproducts arise from purine-pyrimidine interactions. Photoproduct formation is well documented between adjacent pyrimidines (Wang 1976), however photoreaction between purines and pyrimidines is not and the precise nature of these photoproducts is unknown. Of the examples of purine-pyrimidine interaction which occur in the res site there is no obvious concensus sequence and their only obvious common factor is the occurence at the junctions of pyrimidine blocks (as seen from Table 5.1). Clearly, additional factors other than the immediate boundary sequences must operate in determining their photoreactivity.

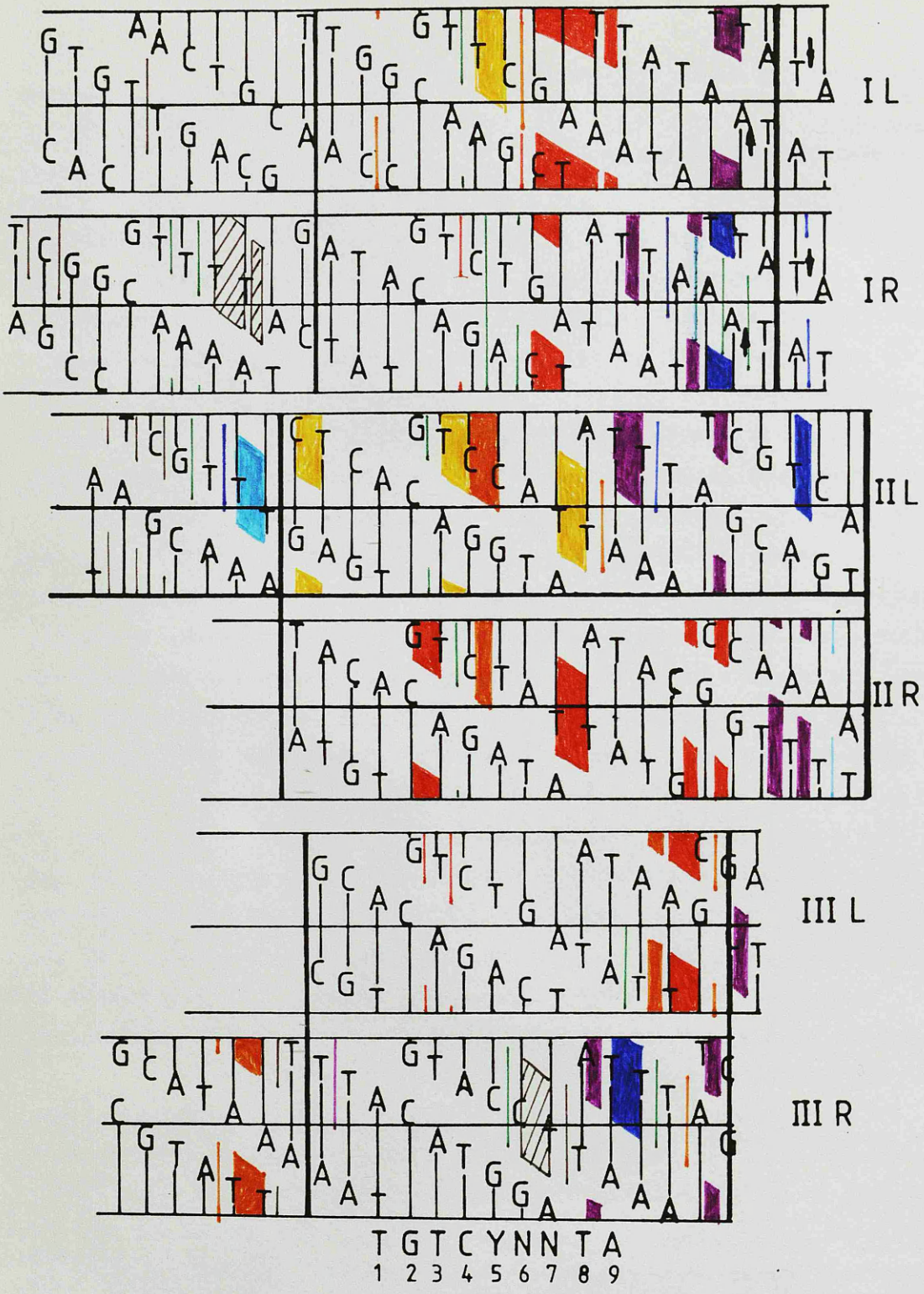

Figure 5.9 Photofootprinting data plotted on a cylindrical representation of the res site DNA with the half sites aligned.
This plot has the half sites aligned at the TGT of the concensus sequence (as with Figure 4.7) permitting comparison of the pattern of alterations of photoreactivity at equivalent positions in each half site. Note this comparison is limited to positions of identically distributed pyrimidine dimers. The categories are as defined for Figure 5.8.

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5.4.4 **Resolvase-induced alterations in photoreactivity**

As discussed in section 5.4.1, the resolvase-induced alterations in photoreactivity are plotted on a cylindrical representation of the res site DNA (Figure 5.8). Examination of Figure 5.8 shows the distribution of resolvase-induced alterations is similar in all 3 binding sites. There is a distinct tendency for reductions in photoreactivity to cluster in the outer regions of each site in the vicinity of the concensus sequence. Where the sequence and distribution of UV-induced cutting positions permit comparison, the pattern of reduced photoreactivity is generally very well conserved between the half sites. This pattern of reduced photoreactivity is in keeping with resolvase interacting with specific bases in the concensus sequence, as shown by the methylation protection experiments of chapter 4.

The most obvious candidates for bases which interact directly with resolvase are the most conserved bases of the concensus sequence. However, due to the scarcity of adjacent pyrimidines within the concensus sequence the role of these bases as specific contact positions cannot be probed by this technique. As a result of an unexpected purine-pyrimidine photoproduct formation at 2 out of 6 occurences of the invariant 2nd and 3rd (GT) positions of the concensus sequence the effect of resolvase binding can be tested in these 2 half sites. Resolvase binding induces a strong reduction in photoreactivity in both cases consistent with the interaction of resolvase with the G residue, as indicated by the methylation protection experiments of chapter 4.

The most striking reductions in photoreactivity occur at the 6th and 7th positions on the (-) strand of the concensus sequence in the three cases when the 6th and 7th position are adjacent pyrimidines. Clearly, outwith the concensus sequence, there is more variation in the precise details of the photofootprint which presumably reflects the different length and general sequence composition of each of the sites.

Reduction in photoreactivity extends beyond the concensus sequence in only 2 of the 6 half sites, site IIR and site IIIL. These 2 sites show reduction in photoreactivity at positions which in the other sites, although of different sequence composition, show extensive enhancement of photoreactivity. Comparison of Figure 5.9 with Figure 4.7 shows resolvase weakly protects G residues in these regions from methylation

by DMS. Both results suggest either weak interactions of resolvase, or resolvase-induced alterations in DNA structure in this region of both half sites. Additional reductions in photoreactivity are also seen lying outside the boundaries of site IIIR, as defined by DNAaseI protection experiments (Symington 1982, Kitts et al 1983), perhaps indicative of resolvase-induced distortions of the adjacent protein-free DNA. With regard to enhancements, these are almost exclusively confined to the central regions of the three sites. Enhancements detected by the photofootprinting technique are considered to represent protein-induced perturbation of the DNA conformation. The possible interpretations of resolvase-induced enhancements and reductions of photoreactivity are considered in more detail in the discussion section of this chapter.

5.5 **Photofootprinting of resolvase/*res* complexes on a supercoiled substrate**

Supercoiling has been defined as one of the requirements for efficient resolvase-mediated recombination under standard *in vitro* conditions (Reed 1981). Possible ways in which supercoiling may be involved in resolution include:-

1. Facilitation of 1-dimensional diffusion or 'tracking'. This is unlikely given the accumulating evidence against such models for resolvase-mediated site synapsis (discussed in detail in chapter 3).
2. To provide the necessary driving force for strand exchange (dissipation of supercoiling energy is not essential and supercoiling may only define the direction of strand exchange rotation).
3. Wrapping of *res* site DNA around resolvase to form the synaptic complex.
4. Unwinding of the sequence of strand exchange. Drew *et al* (1985) have shown that TA dinucleotides are susceptible to transitory supercoiling-induced unwinding (although not necessarily strand separation). Two TA dinucleotides occur within the sequence of strand exchange and a further two occur just outwith the strand exchange sequence.

The photofootprinting technique provides an opportunity to look for a role of supercoiling in *res* site recognition and binding by resolvase.

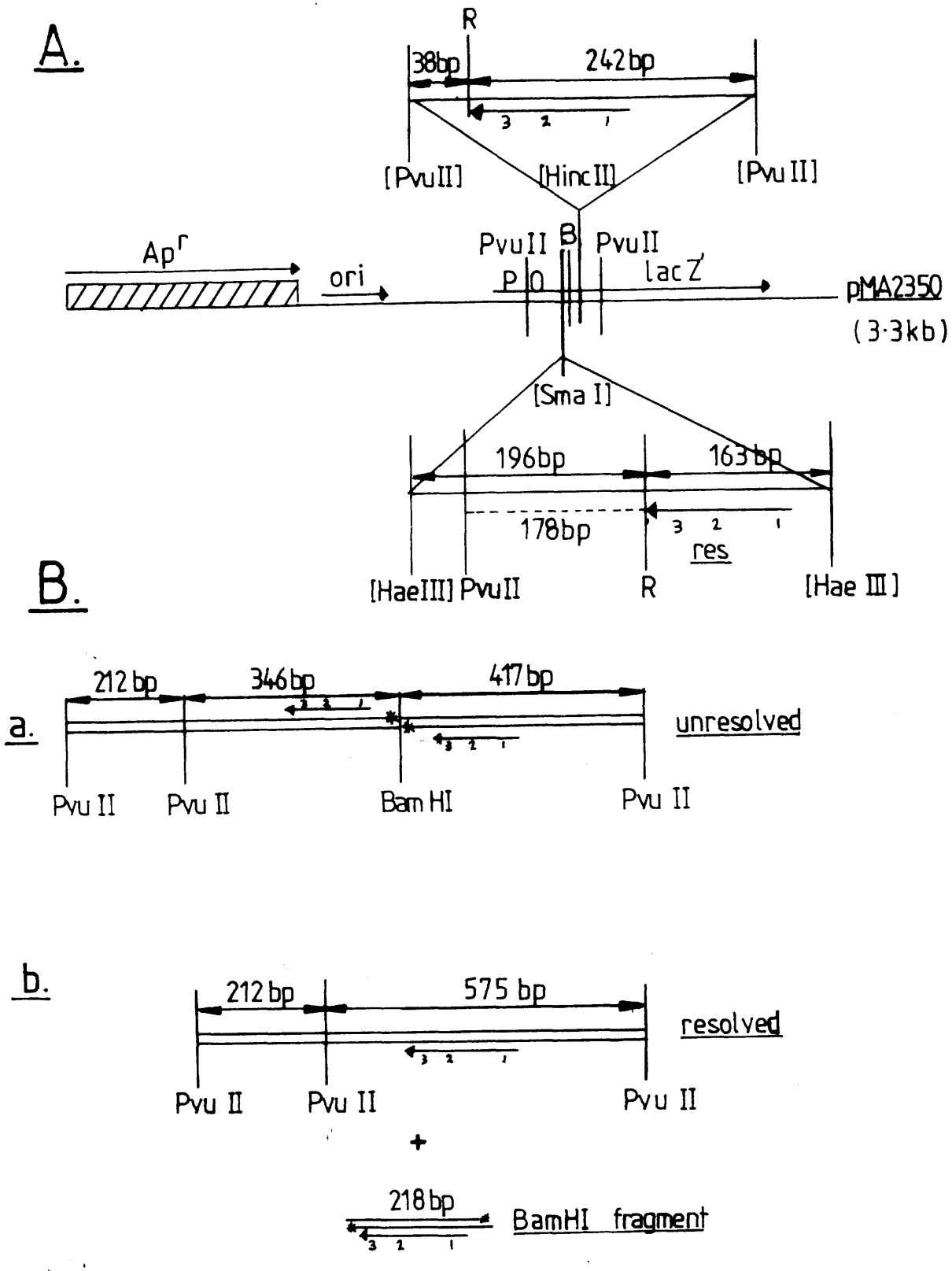
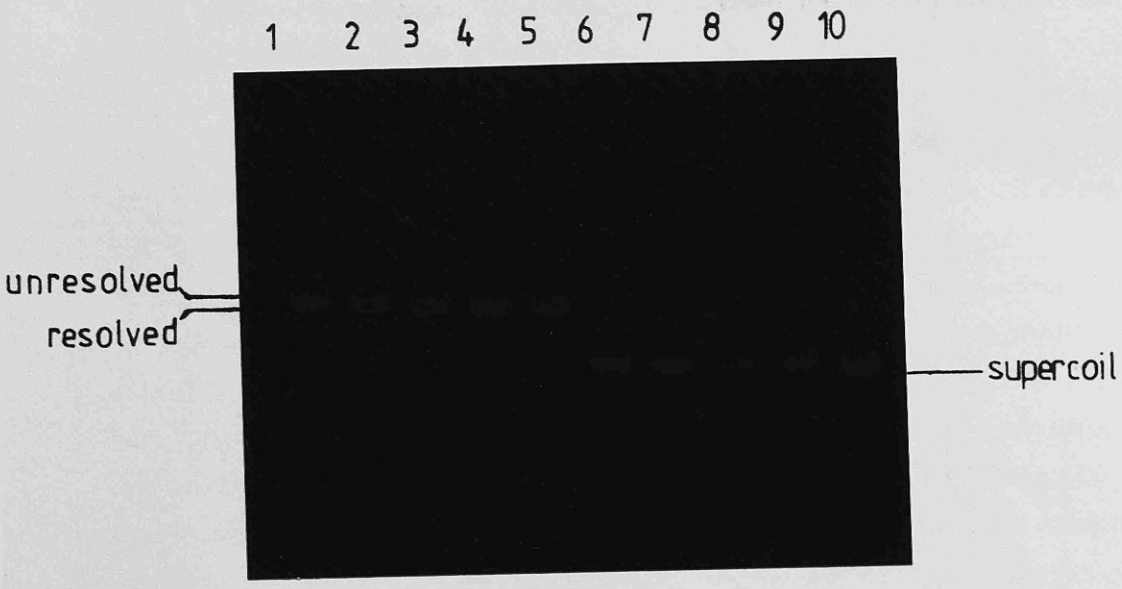


Figure 5.10 Organisation of pMA2350.
A. Schematic diagram of pMA2350 showing the general organisation, site of insertion and orientation of the two res sites. BamHI and PvuII sites relevant to the end-labelling strategy are marked.
B. This diagrams the end-labelling strategy used to isolate BamHI-PvuII res$^+$ fragments from a UV treated resolution reaction. (a) shows the BamHI-PvuII fragments which are isolated from unresolved (or paired) res sites, end labelled representatives of both the (+) and (-) strands will be isolated although they will be derived from different res sites.
(b) illustrates the small BamHI-PvuII fragments generated from resolved plasmids.

To this end photofootprints from linear and supercoiled substrates were compared. The protocol was essentially the same as for the linear substrates except binding of resolvase and UV treatment were carried out prior to restriction and end labelling of the plasmid DNA. A technical point of interest here is the electrophoretic mobility of UV treated restriction fragments through polyacrylamide. Rather than migrating as discrete bands these fragments exhibited retarded gel mobility and extensive smearing behind the band front. Presumably this is a direct consequence of the presence of photoproducts.

In 2 experiments with supercoiled substrates the pattern of UV-induced cleavage positions and the resolvase-induced alterations of photoreactivity were identical to the photofootprints obtained with linear substrates. However, in one experiment out of three, comparison of the linear and supercoil derived photofootprints showed a number of extra UV-induced cleavage positions in addition to the pattern obtained with linear substrates. These can be seen on comparing lanes 1 and 3 (the +UV controls of linear and supercoiled substrates respectively) of Figure 5.4. In all cases these arise as a result of purine-pyrimidine photoproduct formation. Clearly, their formation is dependant on some environmental variable of the system which was not identified in these experiments. It was, however, shown to be a salt, ethanol and glycerol independant effect.

With regard to these new UV-induced cleavage positions detected in one out of three experiments with a supercoiled substrate, resolvase binding alters the photoreactivity of only one position over the entire res site. This position is an intermediate strength reduction in photoreactivity at a strongly photoreactive position and maps at the TA position on the (-) strand in the sequence of strand exchange (AATATT). This is the first evidence of any resolvase-induced reduction of photoreactivity at the centres of the sites. Given the location, this alteration may indicate specific interaction of resolvase (presumably the N-terminal fragment) with bases of the strand exchange sequence. This 'possible contact' cannot on this basis be said to be supercoiling-dependant since supercoiling merely facilitates it's detection due to 'illegitimate' purine-pyrimidine photoproduct formation.



1. No resolvase + HindIII.
2. + resolvase at 37°C 5mins + HindIII.
3. + resolvase at 37°C 5mins + UV + HindIII.
4. + resolvase at 37°C 5mins + UV + 2 hours at 37°C + HindIII.
5. + resolvase at 37°C 2 hours + HindIII.
6. No resolvase - unrestricted.
7. + resolvase at 37°C 5mins - unrestricted.
8. + resolvase at 37°C 5mins + UV - unrestricted.
9. + resolvase at 37°C 5mins + UV + 2 hours at 37°C - unrestricted.
10. + resolvase at 37°C 2 hours - unrestricted.

Figure 5.11 1% agarose gel of HindIII restricted and unrestricted samples of pMA2350 at various points throughout photofootprinting of resolvase bound at res during a live resolution reaction. This set of reactions show resolution had proceeded to approximately 25% completion at the point of UV treatment. No further resolution occurs after UV treatment. In the absence of UV treatment after 2 hours incubation at 37°C resolution proceeds to greater than 75% completion. Superhelicity is maintained after UV treatment.

5.6 **Photofootprinting of an ongoing resolution reaction**

The aim of these experiments, was to attempt to photofootprint a 'live' resolution reaction, in the hope of detecting any differences in the profile of resolvase binding at *res* on formation of the synaptic complex. There are a number of reasons for expecting 2 site synaptic complexes will be a major constituent of the resolvase/DNA reaction mixture and will be moderately stable, in terms of photoreactivation times. Perhaps the most compelling, (although circumstantial), evidence comes from the 'shadowing' effects seen on examining the resolution characteristics of multi-*res* site plasmids as described in chapter 3. In addition, there is preliminary evidence for resolvase/*res* complexes from electron microscopy experiments of Grindley and Reed (1985).

The extreme rapidity of the photochemical reactions, 10^{-9} to 10^{-3} seconds from initial absorption of UV to the formation of a stable photoproduct (Wang *et al* 1976), makes photofootprinting a good method for attempting to characterise the 2 *res* site synaptic complex of resolution.

The substrate plasmid selected for use in these experiments was pMA2350. This plasmid contains 2 closely spaced, directly repeated *res* sites cloned into the polylinker region of pUC18. The sites of insertion, orientation and precise nature of the *res* sites is diagrammed in Figure 5.10. Selective use of restriction sites permits discrimination between resolved and unresolved plasmids. The end labelling strategy permits the isolation of representatives of both the (+) and (-) strands from the same reaction mixture although each is derived from a different *res* site. A resolvase:DNA ratio of 0.77units/ug pMA2350 plasmid was chosen after an initial titration experiment. For the two control samples, 20ug/ml pMA2350 DNA was used in 200ul of photofootprinting buffer. Double this was used in the sample with resolvase to facilitate the removal of 40ul aliquots at various stages throughout the reaction to assess the extent of resolution at each stage, as well as the effect of UV treatment on the superhelicity of the substrate plasmid. Each 40ul aliquot was divided into 2 samples. One set of samples was restricted directly with HindIII and then electrophoresed through 1% agarose alongside the corresponding set of uncut supercoiled samples. These can be seen in Figure 5.11. Incubation with

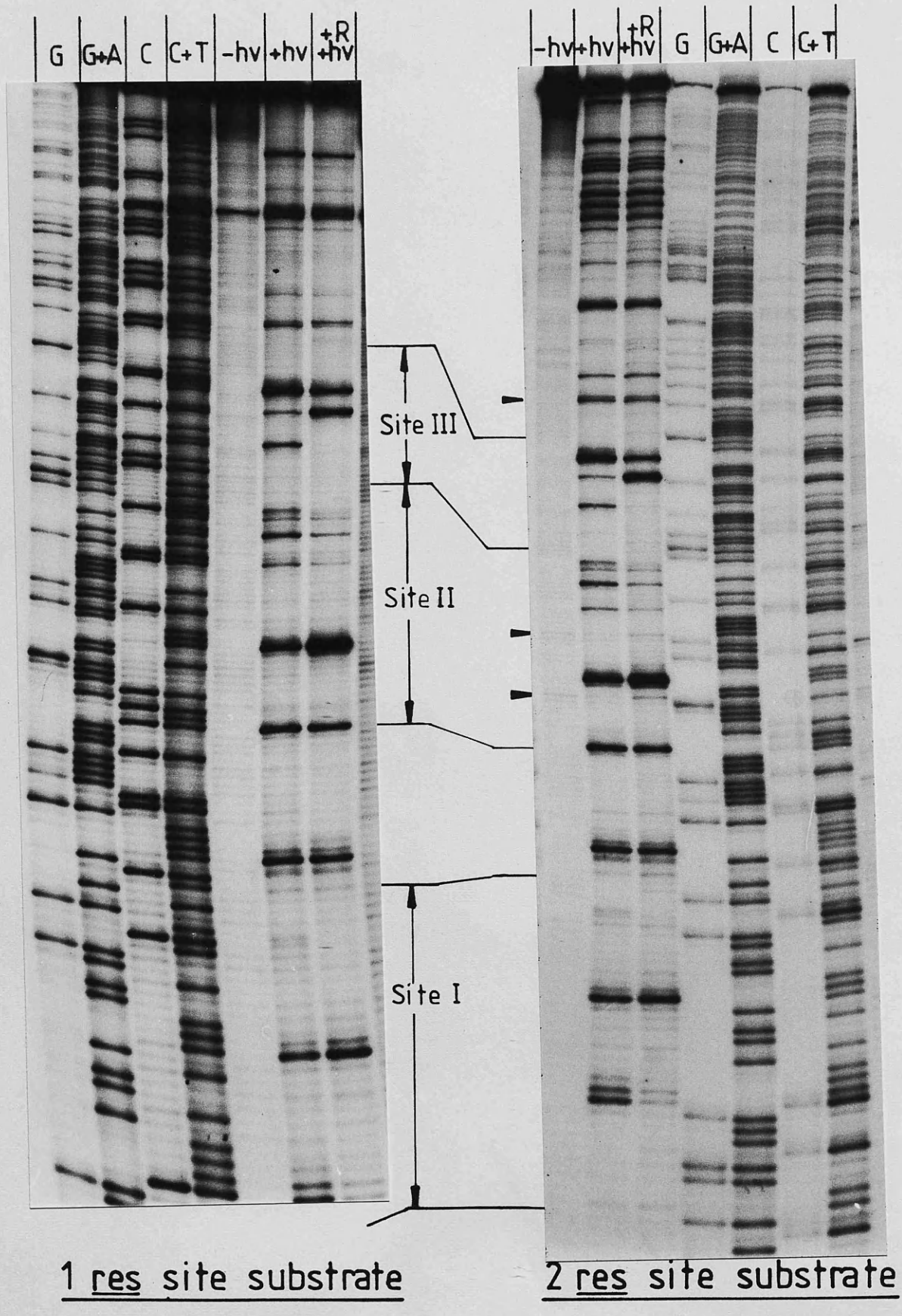


Figure 5.12 Autoradiograph of an 8% polyacrylamide sequencing gel of a photofootprint of resolvase bound to the (-) strand of res under conditions in which resolution was proceeding at the point of UV treatment. The 2 res site autoradiograph represents the (-) strand of a pMA2350 res site, a photofootprint from a single res site substrate is shown alongside for comparative purposes. Positions of noticeable differences are marked by arrows. These positions are discussed in the text and plotted on Figure 5.8.

resolvase was for 5 minutes, UV treatment was for 2 minutes.

Figure 5.11 shows that after 5 minutes incubation with resolvase resolution had proceeded to approximately 20% completion (the point of UV treatment). In the absence of UV treatment, after 2 hours at 37°C resolution had proceeded to greater than 70% completion. After UV treatment no further resolution occurs. Superhelicity is maintained throughout. However, photoproduct formation causes retarded mobility of supercoils through agarose due to a decrease in the -ve superhelicity of the plasmid, as a direct consequence of photoproduct formation (Denhardt and Kato 1973, Carriocchi and Pedrini 1981, Carriocchi and Sutherland 1983).

The 2 res site photofootprints are shown in Figures 5.12 and 5.13, the (+) and (-) strands of the DNA respectively. For comparative purposes the corresponding footprints from single res site linear substrates are shown alongside. Several differences in the pattern of resolvase-induced alterations of photoreactivity can be seen as compared to the photofootprint obtained with a linear or supercoiled substrate. Three alterations in the photofootprint are marked by a numbered asterisk in Figure 5.8 and the nature of the changes are detailed in the Figure legend. The remainder of the differences are enhancements where cleavage is seen at a previously uncut position. The majority of these occur either within site II or in the region intervening between sites I and II.

Clearly, the interpretation of these results must remain somewhat speculative until the proportions of single and 2 res site complexes in the reaction mixture at the point of UV treatment can be ascertained. A conclusion which can, however, be drawn is that either the resolvase/res site complexes responsible for the photofootprint obtained with linear and supercoiled substrates are also the major constituents of the 'live reaction mix, or that alternatively very few changes in the binding profile of resolvase are required to form the synaptic complex. Again this speculation requires investigation of the resolvase/res site complexes present in the linear and supercoil single res site experiments in addition to the complexes in the reaction mixture used in this experiment.

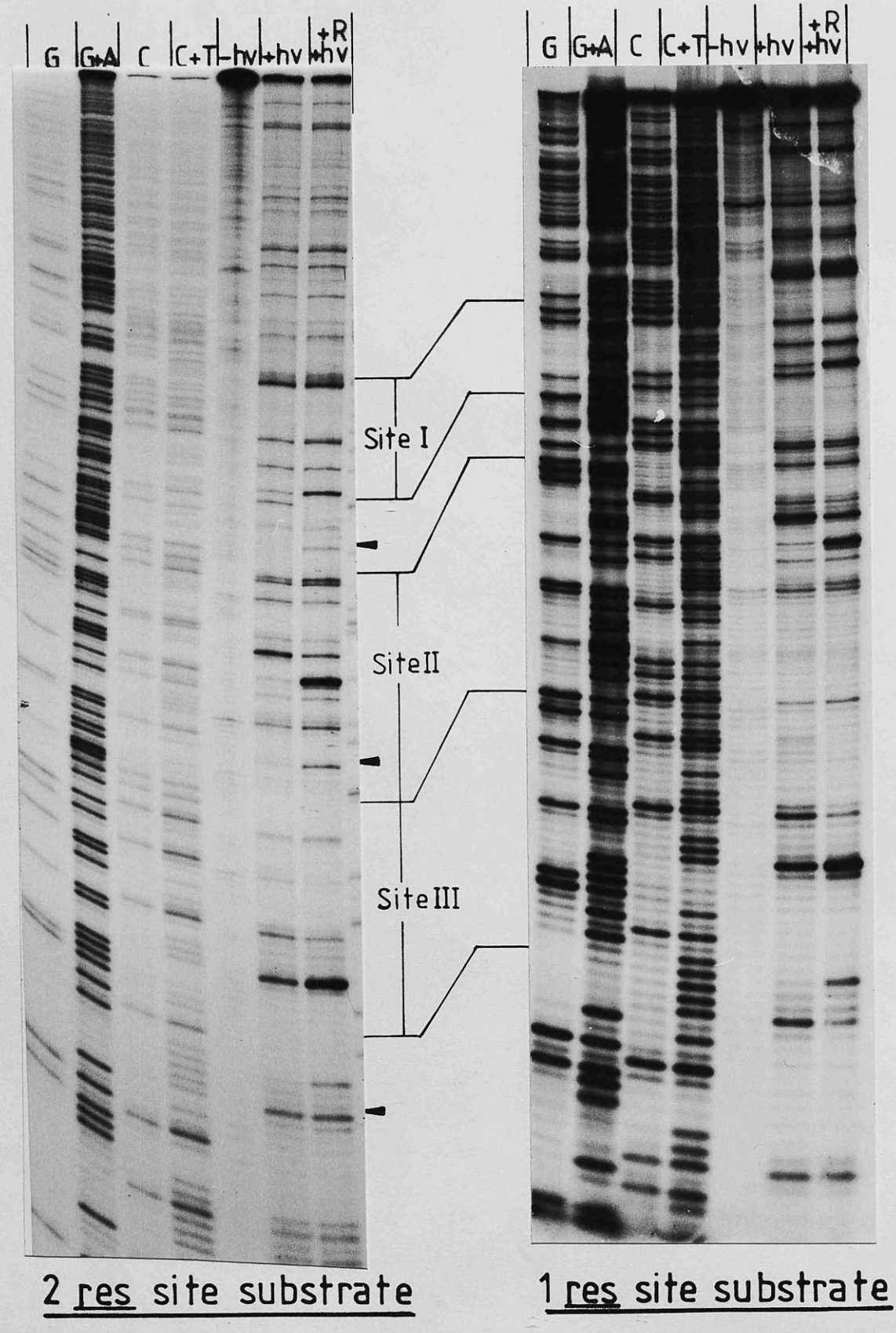


Figure 5.13 Autoradiograph of an 8% polyacrylamide sequencing gel of a photofootprint of resolvase bound to the (+) strand of res under conditions in which resolution was proceeding at the point of UV treatment. The 2 res site autoradiograph represents the (+) strand of a res site from pMA2350, a photofootprint form a single res site substrate is shown alongside for comparative purposes. Positions of noticeable differences in the photoreactivity are marked by an arrow. These positions are discussed in the text and plotted on Figure 5.8.

Discussion

Application of the photofootprinting technique to resolvase/*res* complexes shows resolvase induces a striking pattern of changes in the efficiency of photoproduct formation at numerous positions within the *res* site. Notably, reductions in photoreactivity cluster around the outer regions of each subsite in the vicinity of the concensus sequence and enhancements are almost exclusively confined to the centres of each of the 3 subsites. This is in keeping with the direct interaction of the DNA binding domain of resolvase with specific bases in the concensus sequence, as shown by the methylation protection experiments described in chapter 4. However, the assignment of definite contact positions on the basis of the photofootprinting results should be avoided, or at least approached with caution due to the potential sensitivity of the photofootprinting technique to rather subtle changes in DNA conformation; this could equally well explain some of the resolvase-induced reductions in photoreactivity observed. A case in point is the detection of some resolvase-induced reductions of photoreactivity at postions lying substantially outside the external boundary of site III, as defined by DNAaseI protection experiments (Symington 1982, Kitts et al 1983). Accumulating evidence from footprinting, (DNAaseI and methylation), as well as the organisation of the protein domains (Abdel-Meguid et al 1984), would argue very strongly against these positions being previously unidentified binding contacts, and would perhaps imply they are more likely to be a consequence of resolvase-induced deformations of the adjacent protein-free DNA.

Although the photofootprinting technique is very much more sensitive than the other available footprinting methods, and can be made even more sensitive by the use of triplet sensitisers (Becker and Wang 1984), further investigation will be needed before it is possible to interpret reductions and enhancements of photoreactivity in precise structural and photochemical terms.

Becker and Wang (1984), show that enhancements, in the case of RNA polymerase bound to the *lac*UV5 promoter at 32^{o}C, are indicative of the conversion of double-stranded DNA to single-stranded on the formation of a transcriptionally active 'open' complex. This interpretation is not, however, applicable to the enhancements detected in the centres of the 3

subsites within the res region, as proven by the methylation protection experiments of chapter 4. The methylation technique also has the ability to detect melting of the DNA strands by virtue of the extreme reactivity of the N1 position of adenine with DMS on conversion of DNA from double to single-stranded (Siebenlist et al 1980). Given the A plus T rich nature of the centres of each of the subsites of res, any melting of the DNA would have been detected. Over the entire res region there were only 2 very weak enhancements of adenine methylation, neither of which lie within the regions of enhanced photoreactivity as shown by these experiments. These positions of enhanced photoreactivity must therefore represent more subtle perturbations of DNA configuration, the precise nature of which must await a more extensive photochemical and structural analysis.

It is perhaps not unreasonable to speculate, (on the basis of the gel binding assay results, M.Boocock unpublished), that these enhancements and indeed a proportion of reductions in photoreactivity may reflect resolvase-induced bending of the DNA. In this regard, it would be of interest to apply the photofootprinting technique to systems in which DNA bending has been more extensively studied to see if the same type of patterns of altered photoreactivity are observed. A system which is of particular interest is the wrapping of DNA around histones in the formation of the nucleosome core particle (Richmond et al 1984), particularly in conjunction with the recent DNAaseI experiments of Drew and Travers (1985a) on the orientation of the minor grooves of A plus T and G plus C rich regions in bent DNA. Another potential candidate is CAP complexed to it's binding site, as originally used in the gel binding assay (Wu and Crothers 1984). Also, it would be of interest to apply photofootprinting to the restriction endonuclease, EcoRI, complexed to a fragment containing it's recognition sequence (although only 6bp in length), since X-ray diffraction of co-crystals of the protein-DNA complex has yielded detailed (3A resolution) information on the precise nature of the EcoRI-induced bending (Frederick et al 1984). In addition, it would be of interest to apply this technique to complexes of Lambda Int and IHF proteins complexed to attP since there is evidence to suggest these proteins may organise attP into a nucleosome-like structure (Better et al 1982, Pollock and Nash 1983).

If enhancements of photoreactivity do indeed represent DNA bending,

a prediction would be the breakdown of the consistent pattern of photoproduct formation at pyrimidine blocks, on application of this technique to naturally bent DNA, such as the kinetoplast DNA (Marini et al 1982).

Due to the scarcity of useful restriction sites within the res region, the gel binding assay has to date only been able to positively identify resolvase-induced bending at site II, (M. Boocock unpublished results). If these enhancements of photoreactivity do indeed represent resolvase-induced bending, a clear prediction of the results obtained here is the occurence of bending in all 3 subsites within the res region. It may be possible to test for resolvase-induced bending in each subsite of res by examining the mobility characteristics of resolvase complexed to isolated subsites of res in the gel binding assay. Experiments attempting to isolate single subsites of res are currently underway in this laboratory.

Comparison of the photofootprinting profiles obtained for resolvase binding at res on linear and supercoiled substrates shows an identical pattern of resolvase-induced changes of photoreactivity in both cases. This result eliminates a role of supercoiling in recognition and binding of resolvase to single res sites, implying a role either in the recombination reaction itself or, in alignment of the 2 sites for recombination. This result is reassuring in that it validates the use of linear res sites as substrates for the methylation experiments of chapter 4.

The application of photofootprinting to a 'live' resolution reaction revealed a small number of changes in the footprinting pattern, none of which correspond to the new photoproducts detected in some experiments using a supercoiled substrate. As mentioned earlier any interpretation of this data is purely speculative until a method is devised to ascertain the proportions of synapsed and non-synapsed resolvase/res complexes in the resolution reaction mixture at the point of UV treatment. One possible explanation is that the formation of the synaptic complex depends largely on protein-protein interactions between the large, N terminal domains of resolvase as the two res sites wrap around each other to form the synaptic complex with -3 synapse geometry. This would require un-synapsed resolvase-bound res sites to already be in the conformation required at synapsis needing only slight refinements of the binding profile to align the 2 strand exchange

sequences for recombination.

The problem of trapping true recombination intermediates cannot be approached by this technique due simply to the absence of adjacent pyrimidines at the centre of the strand exchange sequence. If bases with the potential to form pyrimidine dimers had been available, this technique would have been the most potentially useful candidate for detecting the melted strands due to the rapidity of photoreaction. However, in view of the known covalent linkage of resolvase to the DNA during recombination (Reed and Grindley 1981, Reed and Moser 1984), any such intermediates would almost certainly be lost during the photoproduct specific cleavage protocol (Langeveld et al 1978).

In summary, application of in vitro photofootprinting to resolvase bound at res shows a striking pattern of changes in the efficiency of photoproduct formation which goes some way towards substantiating the results of the methylation protection experiments of chapter 4. The results imply a similar mode of resolvase recognition and binding at each subsite and a similar, although not identical, pattern of changes in the base stacking and helix geometry in the centres of each of the 3 sub-sites. The results are consistent with the binding models and proposed structure of the synaptic complex discussed in chapter 4. However, they can provide no direct support for these models until the photofootprinting technique is better characterised. Once the structural basis for the resolvase-induced alterations in photoproduct formation is understood this data should be able to provide information on which side of the helix is contacted by resolvase and on the direction of bending in relation to the bound protein, two important considerations in deriving models for the synaptic complex and the mechanism of strand exchange.

In using the lac repressor as a model system in their initial studies on photofootprinting, a protein which does not induce bending to a degree detectable by the gel binding assay (Wu and Crothers 1984), Becker and Wang may well have under estimated the full potential and sensitivity of the technique. Indeed, enhancements and reductions of photoreactivity may be able to provide detailed information regarding protein-induced changes in the spatial arrangement of neighbouring bases. Clearly, a great deal more structural analysis is required. Photofootprinting is potentially a very sophisticated probe for protein-

DNA interactions limited only by it's dependance on adjacent pyrimidines, which results in a rather sketchy binding profile. Perhaps further investigation will identify conditions for efficient purine-pyrimidine photoproduct formation providing even more information. Clearly, caution must be applied in viewing this technique as simply another method to detect protein-DNA contacts.

CHAPTER 6

in vivo PHOTOFOOTPRINTING OF RESOLVASE BINDING AT *res*

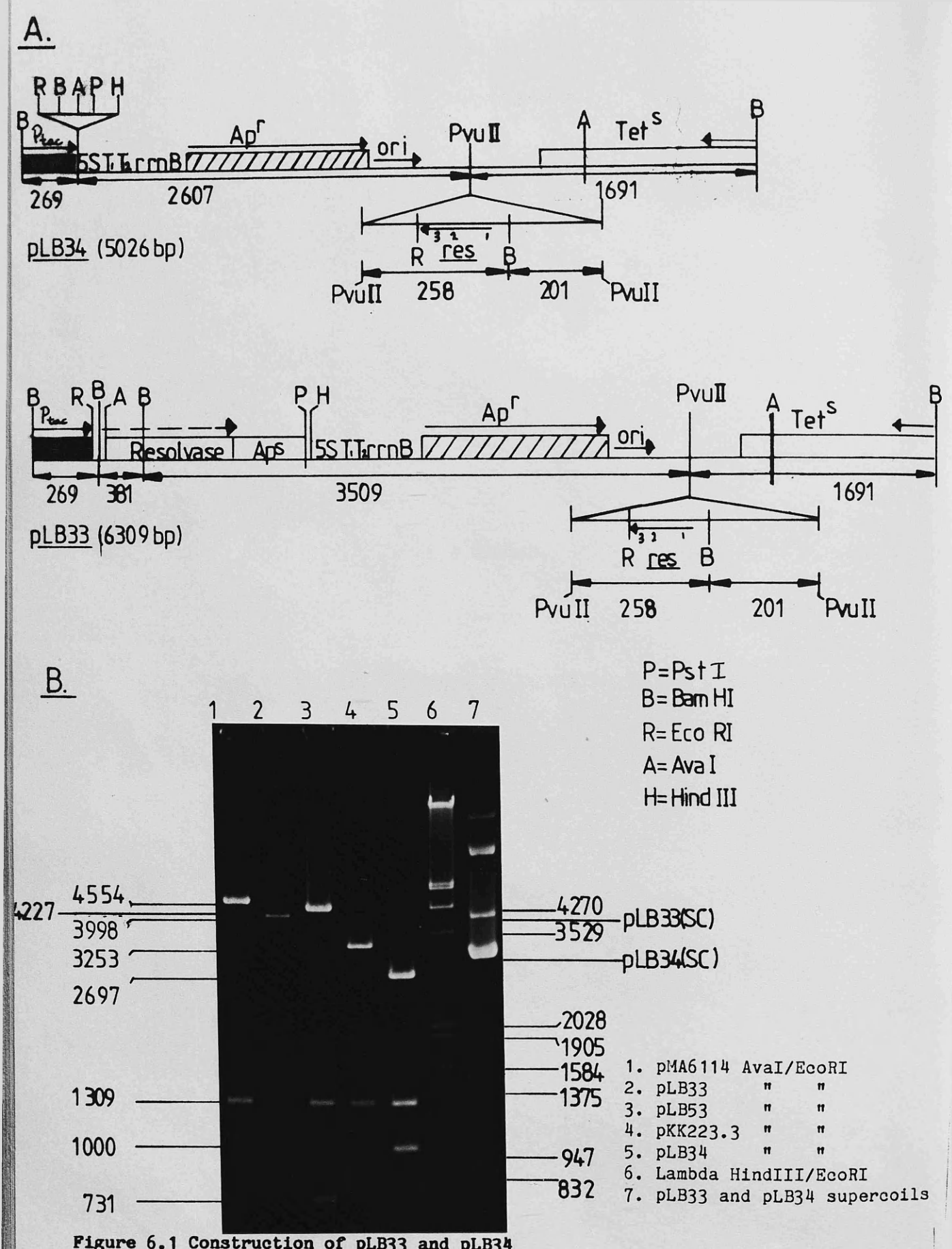


Figure 6.1 Construction of pLB33 and pLB34

A. Schematic diagram showing the general organisation of pLB33 and pLB34. The site of insertion and orientation of the res site is diagrammed. The BamHI and PvuII restriction sites relevant to end-labelling are shown as are the AvaI and EcoRI sites used to confirm the orientation of the insert.

B. 1% agarose gel showing AvaI/EcoRI restrictions of pLB33 and pLB34 confirming the orientation of the inserts is as diagrammed above in (A). pLB53 has the insert fragment cloned into pMA6114 in the opposite orientation to pLB33.

Introduction

One of the major concerns and indeed critisisms, of *in vitro* systems is the uncertainty with which they can accurately reflect the processes occurring in the more complex environment of the cell. This is a particular concern in the case of techniques such as footprinting which attempt to examine complex molecular interactions between proteins and DNA and which may be sensitive to a number of factors. As a consequence, there has lately been a trend towards the development of techniques which can be used to footprint protein bound to DNA *in vivo*.

The majority of footprinting techniques are too harsh or, too slow to be used on living cells without disrupting protein-DNA complexes. However, 2 of the exsisting *in vitro* footprinting techniques have now been successfully adapted for application to living cells. The first of these is an extension of the methylation protection technique described in chapter 4 (Nick and Gilbert 1985), the second an extension of the photofootprinting technique described in the preceding chapter (Becker and Wang 1984). Of these two, the photofootprinting technique has much greater potential and the advantages of this type of approach include:

1. The ease of permeability of light into cells.
2. The rapidity of photochemical reactions (Wang 1976).
3. The adaptibility for use under a wide variety of conditions.

This chapter describes the application of *in vivo* photofootprinting to the resolvase/*res* system in the hope of obtaining an acceptable degree of correlation with the binding profile obtained in the *in vitro* photofootprinting experiments described in chapter 5. The experimental approach was to compare the photo-induced cleavage pattern of *res* site DNA from cells producing and from cells deficient in resolvase. For end-labelling purposes, a construct was required which had the *res* region cloned into a background rich in restriction sites. Since resolvase is normally autoregulatory, the *tnpR*$^+$ plasmid used in these experiments had resolvase cloned under the control of the P_{tac} promoter. For this purpose pLB33 and pLB34 were constructed.

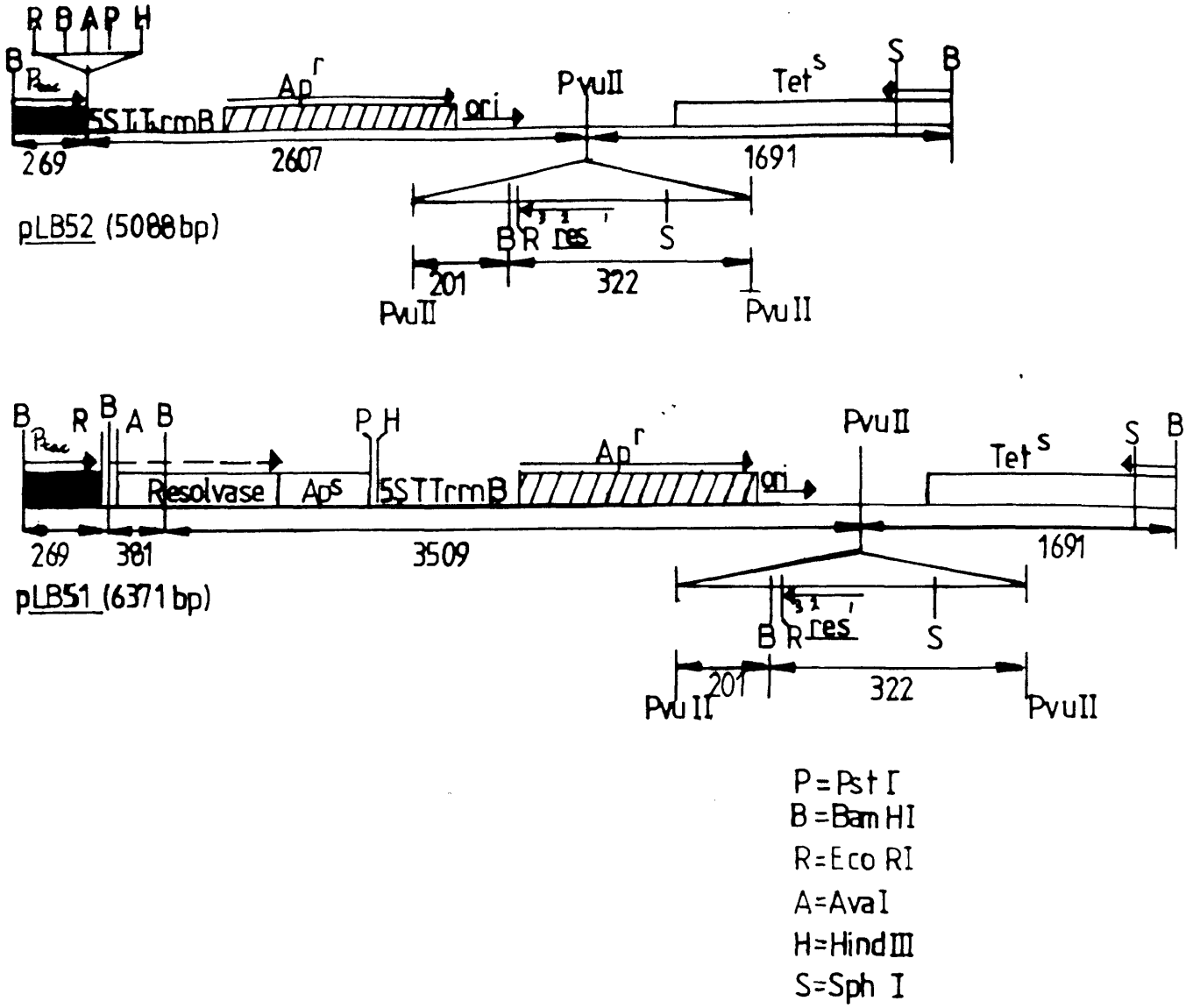


Figure 6.2 Structure of pLB51 and pLB52. Diagrammatic representation of pLB51 and pLB52 showing the general organisation including the site of insertion and orientation of the res site. The SphI and EcoRI restriction sites used to determine the orientation of the insert are shown (confirmatory restriction data not shown). Sites relevant to the end-labelling reactions are indicated.

Results

6.1 Construction of pLB33 and pLB34

As starting plasmids for these experiments the resolvase over-expression plasmid, pMA6114 and the tnpR$^-$ vector pKK223-3 were used. pKK223-3 is a Ptac expression vector, containing the optimised trp-lac derived promoter (DeBoer et al 1983). The plasmid, pMA6114 has the resolvase gene cloned into the polylinker region downstream from the tac promoter and resolvase expression can be suppressed in an appropriate lacI^q strain and induced with IPTG (Beckwith and Zipser 1970).

A res site was cloned into both of these plasmids in the same position and orientation. For this purpose, the 454bp res$^+$ PvuII fragment of pLB32 was isolated from 5% polyacrylamide, electroeluted and ligated to PvuII cleaved, dephosphorylated pKK223-3 or pMA6114. The ligation mix was transformed into LB1 (see below), and Ap^r transformants were screened for the presence of the insert by single colony gel analysis; the insert orientation was determined by restriction analysis (Figure 6.1).

The BamHI-PvuII res$^+$ fragments of these plasmids 3' end labelled at the BamHI site were suitable for footprinting the (-) strand of the DNA. However, problems with incomplete end-labelling at EcoRI sites necessitated the construction of pLB51 and pLB52 to footprint the (+) strand of the DNA.

6.2 Construction of pLB51 and pLB52

For these constructs the res site was isolated on a 492bp PvuII fragment from pLB37. As above the res$^+$ fragment was isolated from 5% polyacrylamide, electroeluted and ligated to PvuII cleaved, dephosphorylated pKK223-3 or pMA6114. Ap^r transformants of LB1 were screened for the presence of the insert by single colony gel electrophoresis, the insert orientation was determined by restriction analysis using SphI (data not shown). The res sites of pLB51 and pLB52 are in the same orientation as in pLB33 and pLB34 as diagrammed in Figure 6.2. For photofootprinting experiments the res site was isolated on a BamHI-PvuII fragment and 3' end labelled at the BamHI site.

1. Transfer of F' (*lacI*^{q}, *ZΔM15*, *pro*$^+$) into Rif^r, AB1157 (*pro*$^-$, *lacI*$^+$, *lacZ*$^+$, *rec*$^+$, *γδ*$^-$)
   Select Rif^r, *pro*$^+$ conjugants

2. Heterogenote intermediate

F'	*lacI*^{q}	*ZΔM15*	white on Xgal
AB1157	*lacI*$^+$	*lac Z*$^+$	blue on Xgal + IPTG

3. Homogenote for *lacI*^{q}, *ZΔM15* (gene conversion)

F'	*lacI*^{q}	*ZΔM15*	
AB1157	*lacI*^{q}	*ZΔM15*	white on Xgal + IPTG

4. Curing of F' with acridine orange

LB 1 *lacI*^{q}, *ZΔM15*, *rec*$^+$, *γδ*$^-$

Figure 6.3 Protocol for homogenetisation of the lacI^q, ZΔM15 genotype into Rif^r AB1157 as recommended by Miller (1972). See text for further details.

6.3 Construction of LB1

To prevent continual overexpression of resolvase from the P_{tac} promoter, pMA6114 and it's derivatives were maintained in a *lacI*^{q} strain where expression is suppressed but inducible on provision of IPTG. However, the readily available *lacI*^{q} strains such as JM101 contain a function capable of complementing the Tn*3* resolvase, attributable to the presence of Gamma delta on the F' factor carrying the *lacI*^{q} gene (Guyer *et al* 1978). On introduction of the 2 *res* site plasmid pLS134 into such a strain resolution products appear at a frequency much greater than that attributable to homologous recombination as judged by the rate of breakdown of plasmid multimers in the same strain (data not shown). As a consequence, these strains could not be used for *in vivo* photofootprinting since all the available *res* regions would be bound by Gamma delta resolvase rather than Tn*3* resolvase at the point of UV treatment.

To circumvent this problem the *lacI*^{q} marker was transferred into a Gamma delta-free strain using the technique of homogenotisation (Miller 1972). As the intitial step, an F'*lacI*^{q}, *lacZ*ΔM15, *pro*$^+$ episome was mated into a Rif^r resistant derivative of AB1157 (*lacI*$^+$, *pro*$^-$, *lacZ*$^+$, *rec*$^+$). As diagrammed in Figure 6.3 *pro*$^+$, Rif^r conjugants were selected. Gene conversion of the chromosome to *lacI*^{q}, ZΔM15 was detected by screening for the inability of colonies to turn blue on IPTG supplemented Xgal plates, indicative of the loss of a functional *lac Z* gene. The *lacI*^{q}, *lac* ZΔM15 genotype was tested by transformation of pUC8 into this strain; this reconstitutes the *lacZ*$^+$ phenotype in the presence of IPTG, as seen on X-gal indicator plates. Once isolated the homogenotised strain was cured of the F' factor using acridine orange and the absence of F was ensured by single colony gel analysis in addition to plate mating tests with the *pro*$^-$, *lac*$^-$, *ara*$^-$, Str^r CB50. The resulting strain was again tested for the absence of any Tn*3* resolvase-complementing functions in case of Gamma delta transposition or Hfr formation during the strain construction. The resulting Gamma delta-free, *lacI*^{q}, *lacZ*ΔM15 strain was named LB1.

6.4 *in vivo* Photofootprinting of resolvase bound at *res*

The *in vivo* photofootprinting experiments described by Becker and

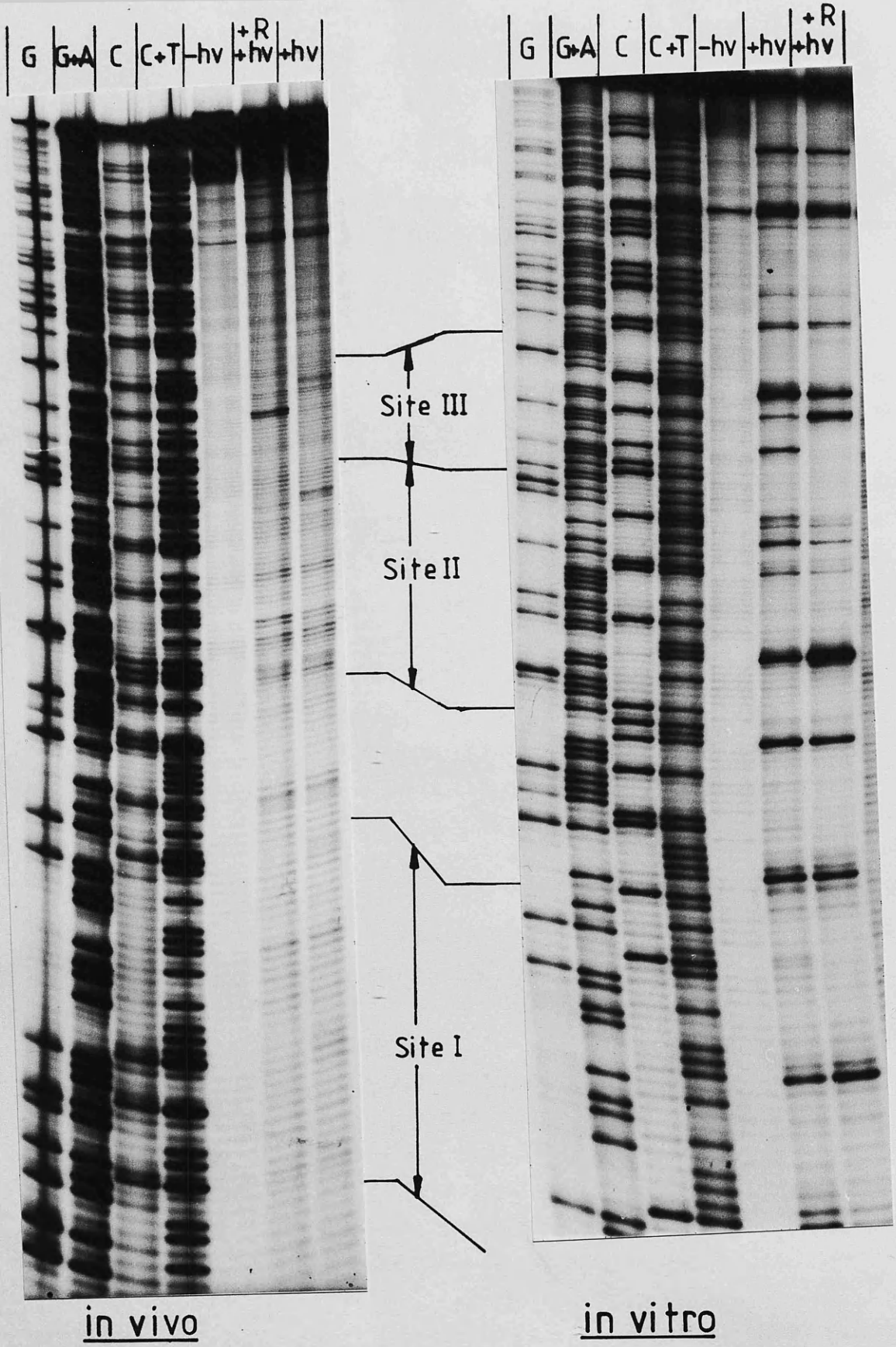


Figure 6.4 Autoradiograph of an 8% polyacrylamide gel of an <u>in vivo</u> photofootprint of resolvase bound to the (-) strand of <u>res</u> The -hv and +hv+resolvase controls were derived from pLB33-containing LB1 cells. The +hv control was derived from pLB34-containing LB1 cells. The <u>res</u> site was isolated on a 258bp BamHI-PvuII fragment 3' end-labelled at the BamHI site (see text for details). Exposure to UV was for 4.5 minutes. An <u>in vitro</u> generated photofootprint is shown alongside for comparative purposes. Note the photofootprinting tracks are arranged in a different order in each experiment (see text for further details).

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Wang (1984), use only 1.5mls of stationary phase culture. However, under the conditions used here, for efficient DNA isolation, end-labelling and subsequent manipulations 1.5mls was grossly inadequate. Consequently the entire procedure was scaled up for use on 50mls of late log-phase culture.

LB1 cells containing pLB33 (*tnpR*$^+$) or pLB34 (*tnpR*$^-$) were used to footprint the (-) strand of the DNA, for the (+) strand pLB51 (*tnpR*$^+$) or pLB52 (*tnpR*$^-$) containing LB1 cells were used. All 3 samples (-UV, +UV, +UV +resolvase), were treated with IPTG to exclude any alterations in the photoinduced cleavage pattern attributable to the effects of IPTG. The precise experimental protocol is detailed in chapter 2. For these experiments UV treatment was increased to 4.5 minutes on ice. Subsequent freezing and thawing steps were executed as quickly as possible to minimise any effects of the DNA repair enzymes of the cells.

Due to the peculiar mobility properties of UV treated DNA through polyacrylamide clean isolation of the end-labelled *res*$^+$ fragments was a problem. To counter this the end-labelling reactions were run on a sequencing length 5% polyacrylamide gel (of the standard 1.5mm thickness). Even this was insufficient for clean isolation of the 322bp pLB51 and pLB52 derived *res*$^+$ fragments from the 296bp, BamHI P_{tac} promoter fragment and as a result, after partial purification from polyacrylamide, the samples were restricted with HincII to shorten the contaminating fragment and then rerun through 5% polyacrylamide. Selective cleavage of the contaminating fragment was not possible prior to the first purification step due to the unsuitable disposition of HincII sites elsewhere in the plasmid. The resulting autoradiographs of the *in vivo* photofootprinting reactions are shown in Figures 6.4 and 6.5, the (-) and (+) strands of the DNA respectively. The corresponding *in vitro* generated photofootprints are also shown for comparative purposes, as well as a sequencing lane to permit precise alignment of each position.

From examination of Figures 6.4 and 6.5 the first point worthy of note is that the dosage of UV received by the plasmid DNA of interest *in vivo* is very much less than the dose received in the corresponding *in vitro* reaction, as judged by the rather poor level of UV-induced photoproduct formation. However, when selecting the dosage of UV treatment, a balance had to be struck between the efficiency of photoproduct

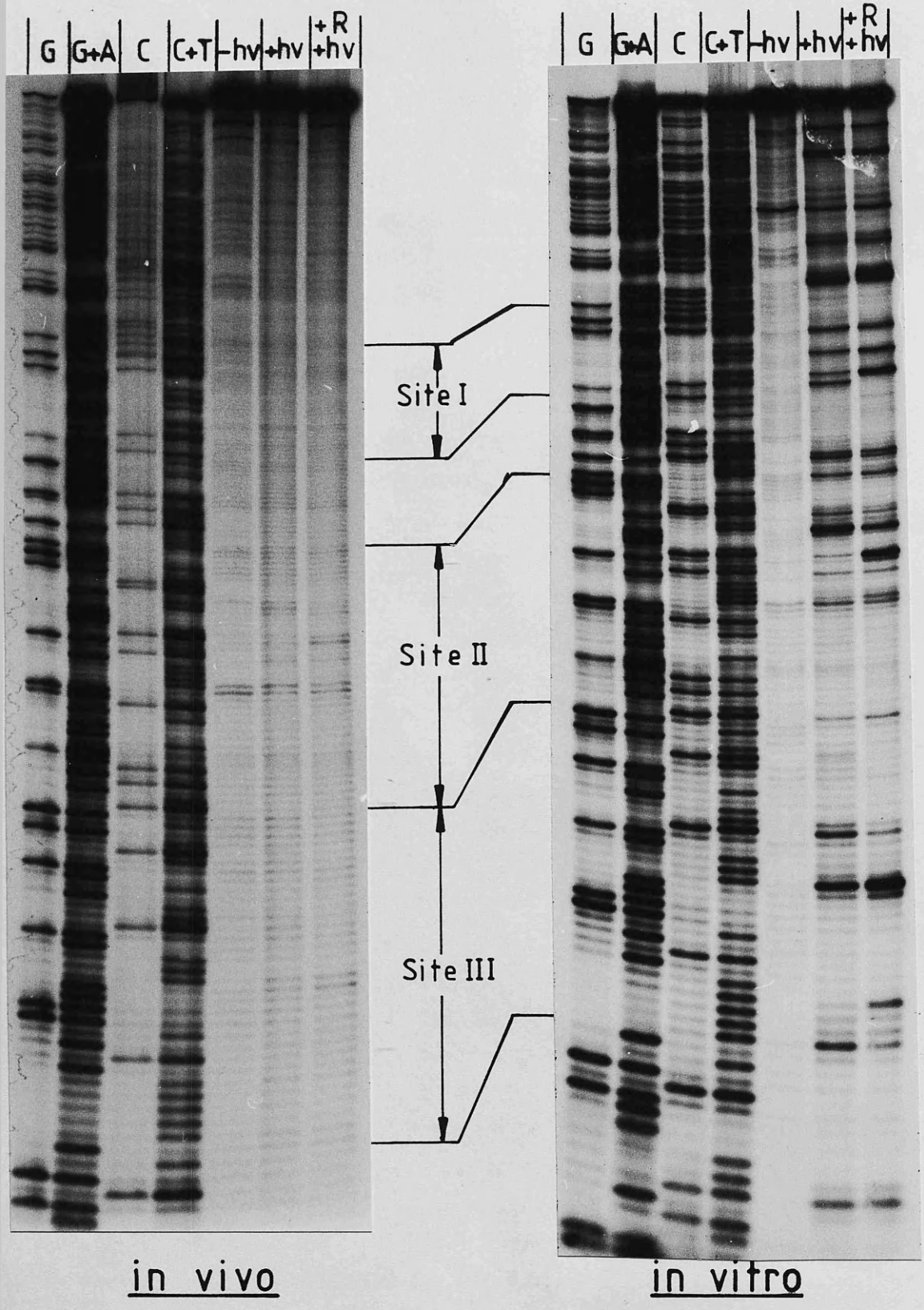

Figure 6.5 Autoradiograph of an 8% polyacrylamide sequencing gel of an *in vivo* photofootprint of resolvase bound to the (+) strand of *res*. The -hv and +hv+resolvase controls were derived from pLB51-containing LB1 cells, the +hv control from pLB52-containing LB1 cells. The *res* site was isolated on a 322bp BamHI-PvuII fragment 3' end-labelled at the BamHI site. An *in vitro*-generated photofootprint is shown for comparative purposes (see text for further details).

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formation and the efficiency of recovery of plasmid DNA amenable to restriction and end-labelling. Secondly, the level of non-specific background cutting on exposure to UV is very much greater *in vivo* than in the corresponding *in vitro* generated photofootprints. These 2 factors together have the effect of making the resolvase-induced alterations on binding much less obvious. However, using the sequence to align the footprints, careful comparison of the *in vivo* generated photofootprint with that of the corresponding *in vitro* experiment, reveals very similar patterns of resolvase-induced alterations in photoproduct formation. Due to the weak UV-induced cleavage in the *in vivo* photofootprinting experiments it has not been possible to detect positions which *in vitro* show only very weak reduction in photoreactivity. Additionally, the data for the (+) strand at the 5' end of *res* is not sufficiently clear for accurate comparison.

To test whether or not the alterations in photoreactivity detected are actually a consequence of specific interactions of resolvase with the *res* site, the 199bp BamHI-PvuII *res*$^-$ fragment from the *lac*OP region of pUC18 (see Figure 6.2), was also isolated from the pLB51 and PLB52 end labelling reactions and subjected to the photoproduct specific cleavage protocol. The resulting autoradiograph (data not shown), contrary to expectation, showed a cluster of photoinduced cleavage positions which are cut more efficiently in the *tnp*R$^+$ cells. By comparison with sequence run elsewhere on the gel, these alterations were deduced to lie at 111-114bp from the labelled end. This region, (Yanish-Perron *etal* 1985), lies exactly in the -35 region of the *lac* promoter of pUC18. The differences in photoproduct formation probably arise as a result of RNA polymerase binding to the -35 region of the *lac* promoter in the cells which are not producing resolvase. In the cells which are over-producing resolvase it is quite conceivable that the limited resources of RNA polymerase are channelled towards transcribing the resolvase gene also; overproduction of resolvase to the extent used here may disrupt the normal balance of such processes in the cell. Protection is not detected in the -10 sequence which on this (+) strand is devoid of adjacent pyrimidine dimers.

In view of this result an excellent control for this set of experiments would have been the inclusion of an uninduced, UV treated pLB52 (*tnp*R$^-$)-containing culture to look for alterations in the photofoot-

printing pattern at the binding site for the *lac* repressor. This would have permitted a direct comparison of the photoproduct-induced cleavage patterns obtained here with those of Becker and Wang (1984).

Discussion

Where the initial UV-induced cleavage profile is extensive enough to permit comparison, application of *in vivo* photofootprinting to resolvase bound at *res* shows the maintenance of the overall DNA binding profile obtained with *in vitro* experiments. Particularly encouraging is the maintenance of positions of enhanced photoreactivity on binding of resolvase, showing that if enhancements do indeed represent specific resolvase-induced perturbations of DNA conformation (as proposed in chapter 5), these conformational changes also occur in the more complex environment of the cell. These results reinforce conclusions drawn from the *in vitro* experiments on how resolvase recognises and binds to the *res* region.

With regards to the *in vivo* photofootprinting technique itself, its sensitivity is illustrated by the detection of possible RNA polymerase binding to the -35 region of the *lac* promoter in the experiments described above. However, the potential of this technique to identify uncharacterised protein binding sites is somewhat limited by its dependence on the presence of adjacent pyrimidines in the binding site. The extreme sensitivity of the technique does, however, highlight the dangers of overproducing the DNA binding protein of interest, since disruption of the balance of the normal housekeeping enzymes could potentially be responsible for some of the changes in the photofootprinting pattern observed. For example, in the case of resolvase binding at *res*, in the -UV control RNA polymerase may be binding at the -35 and -10 regions of the resolvase and transposase promoters. That changes in these regions are primarily due to differential binding of resolvase can be deduced from the close correlation between the *in vitro* and *in vivo* generated footprints in these regions of the sequence.

CHAPTER 7

CONCLUDING REMARKS

The aim of this thesis was to examine two aspects of the resolvase-mediated recombination reaction:-

1. To examine how resolvase recognises and binds to *res* site DNA.
2. To test some of the predictions of two of the models proposed for resolvase-mediated site synapsis.

(1.) Two footprinting techniques were used here in an attempt to determine how resolvase recognises and binds to the 3 subsites of *res* and how resolvase accommodates the different spacing within and between the individual subsites on formation of an active synaptic complex.

(i) Methylation Protection Experiments

The methylation protection experiments on resolvase bound at *res* have shown resolvase recognises and binds to all 3 subsites in essentially the same way, making major groove contacts with the concensus sequence at the outer ends of each half site. In addition, the invariant G of the second position of the concensus sequence has been identified as one of the major recognition determinants for resolvase binding.

Projection of the footprinting data on a cylindrical representation of the double helix (as discussed in chapter 4), implies resolvase contacts only one face of the DNA helix at each subsite and may contact site III on the opposite face of the helix from sites I and II. Examination of the *res* site sequence highlighted the marked AT richness of the centres of the three sites and in sites II and III a distribution of A plus T rich blocks interspersed with G plus C rich sequences which in view of the Drew and Travers (1985a) result are expected to have a preferred direction of bending. In view of the evidence for resolvase-induced bending in at least site II, DNA bending seems the most likely mechanism by which resolvase can accommodate the different sizes of the 3 subsites of *res*. The distribution of these A plus T rich blocks together with the disposition of the putative DNA contact positions identified by the methylation experiments here suggest resolvase binds on the 'inside' of the DNA bend, a point which is not trivial when deriving models for the synaptic complex and ultimately the mechanism of strand exchange. The footprinting data and the proposed direction of DNA bending are in accord with the structure of the synaptic complex proposed by M. Boocock (unpublished) however, they cannot provide any

direct evidence for it.

(ii) Photofootprinting

This technique may potentially be much more sensitive than was originally realised, being able to detect subtle changes in the DNA structure rather than merely protein-DNA specific contact positions or gross changes in the DNA conformation.

Resolvase binding at res generated a striking pattern of resolvase-induced alterations of photoreactivity in each subsite of res. Reductions of photoreactivity were clustered in the outer regions of each site in the vicinity of the concensus sequence consistent with the presumed bihelical binding domain of resolvase interacting specifically with the concensus sequence in each half site. Enhancements were confined to the centres of each site. In view of the evidence for resolvase-induced bending in at least site II, it was proposed these enhancements and a proportion of reductions in photoreactivity may represent the alterations in base stacking and helix geometry promoted on introducing a bend into the DNA. Clearly, more detailed characterisation of the photofootprinting technique itself is required before any firm conclusions can be drawn. However, some confirmation may come from the gel binding assay if it is possible to demonstrate bending in all three subsites of res as predicted from the results obtained here. If bending does occur, the extent of bending would be expected to be dictated by the size and nucleotide sequence of the subsites.

Experiments here have shown resolvase binds to linear and supercoiled substrates in the same way showing supercoiling is not required for binding and must be involved in some other part of the reaction; perhaps for the formation of the 2 res site/resolvase synaptic complex or in the strand exchange reaction. This result validated the use of linear substrates in the methylation experiments of chapter 4.

The application of photofootprinting to a 'live' resolution reaction demonstrated only a few fairly minor changes in the pattern of resolvase-induced alterations of photoreactivity as compared to footprints on linear or supercoiled single res sites. In the absence of information regarding the proportions of single resolvase-bound res sites and 2 site synaptic complexes in the reaction mixture at the point of UV treatment the interpretation of this result must remain purely

speculative however, perhaps one of the most attractive and indeed plausible explanations is the DNA of single *res* sites is already essentially in the conformation required for the synaptic complex requiring only slight refinements with protein-protein interactions playing the major role.

The maintenance of the pattern of resolvase-induced alterations in photoreactivity on footprinting resolvase presumed to be binding at *res in vivo* has been extremely encouraging, particularly the maintenance of enhancements of photoreactivity implying identical perturbations of *res* DNA conformation in the more complex environment of the cell. This lends credibility to the results derived from the *in vitro* system and any models for binding based on these results.

Footprinting techniques have been used here to their full potential. The way ahead for examining resolvase binding to *res* now lies in more detailed genetic and physical study of resolvase itself, particularly X-ray diffraction and ultimately X-ray diffraction of resolvase complexed to a subsite of *res*. Attempts are currently being made to examine the Gamma delta resolvase at resolutions higher than the 7A achieved by Abdel-Meguid *et al* (1984) (T. Steitz pers. comm.) and in addition, a number of mutant resolvases are being examined.

(2.) One of the major puzzles of the resolvase-mediated recombination reaction was how resolvase aligns the two *res* sites in a synaptic complex of uniquely defined topology prior to strand exchange and how resolvase maintains a directional specificity for directly repeated sites over such a long range. One of the models for resolvase-mediated site synapsis, 'the tracking model', was based on processive searching of resolvase along the DNA intervening between the two sites. This model made a number of experimentally testable predictions two of which were tested here, most notably the adjacent site preference for resolution in a substrate containing multiple directly repeated *res* sites.

The initial analysis with plasmids containing 4 directly repeated *res* sites did in general exhibit an adjacent site preference of resolution in accord with tracking however, with the realisation that 'pairing' models could equally well explain such a preference, this analysis was extended leading to the construction of 5 and 6 *res* site

plasmids. Analysis of the resolution characteristics of these plasmids demonstrated the breakdown of an adjacent site preference with in 6 *res* site plasmids, the occurence of a completely non-adjacent event at a frequency approximating to and possibly even greater than adjacent events in the same construct. These results are in complete agreement with the interfering pairs model for the behaviour of multiple *res* site plasmids.

In addition, analysis of these plasmids showed 'shadowing' by an inverted site in the same way as was seen with a directly repeated *res* site. Evidence that this is probably the result of the formation of a specific 2 *res* site/resolvase synaptic complex comes from the detection of inversion on alterating substrate topology (Boocock *et al* 1985).

These results, together with the failure of the reporter ring experiments of Benjamin *et al* (1985), make tracking no longer tenable as a model for resolvase-mediated site synapsis. The 2-step model (Boocock *et al* 1985) provides an attractive alternative to tracking models for resolvase-mediated site synapsis, providing an adequate explanation for the unique properties of the reaction. A number of experimentally testable predictions of this model have now been proven. The results described here in support of the 'interfering pairs' model for the behaviour of the multiple *res* site plasmids are fully consistent with although not diagnostic for the 2-step model.

A method for quantitatively examining the resolution characteristics of these constructs has been set up. This will permit a more detailed analysis of the resolution kinetics of these constructs which have been examined here.

With regard to the 2-step model for resolvase-mediated site synapsis probably the most rigorous test of the model will come from examination of the intramolecular inversion products on supercoiled substrates since the 2-step model for site synapsis makes explicit predictions about their topology. Additionally, the 2-step model predicts sites II and III (in the absence of site I) should be sufficient to cause shadowing in multiple *res* site constructs, due to the major role they are proposed to play in the formation of the synaptic complex. The 2-step model can also explain the differing specifities of the other site-specific recombination systems, including Lambda integration/excision and the inversion systems. Indeed, a

similar model has now been proposed for the specificities of Mu transposition based on the energetic constraints imposed by supercoiling implying such models may have a widespread application.

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