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The γδ resolvase bends the *res* site into a recombinogenic complex

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We have characterized complexes between the γδ resolvase and its recombination site, *res*, using both a gel retardation assay and DNase I cleavage. The mobility of resolvase–*res* complexes in polyacrylamide gels is sensitive to the location of *res* within the DNA fragment and is at a minimum when *res* is at its center. This behavior is characteristic of a protein-dependent bend. By the same assay we have found that bends are induced upon the binding of resolvase to each of the three individual binding sites that constitute *res*. In the wild-type *res*, the centers of binding sites I and II are 53 bp apart and the central section of the intersite DNA is sensitive to DNase I cleavage. We find that insertions of 10 or 21 bp (one or two turns of the DNA helix) have no discernible effect on the ability of *res* to recombine or to form complexes with resolvase. However, insertions of short segments (e.g. 6 or 17 bp) equivalent to non-integral numbers of helical turns, inhibit recombination and prevent the formation of the normally compact resolvase–*res* complex. Complexes of resolvase with *res* containing 10 or 21 bp insertions exhibit a pattern of enhanced and suppressed DNase I cleavages that suggest that the intersite segment is curved. This curvature requires both that site I and II are appropriately spaced, and that site III is also present and occupied.

Key words: γδ resolvase/recombinogenic complex/site-specific recombination

Introduction

The transposon γδ is one of a number of prokaryotic transposable elements related to Tn*3* that encode a site-specific recombination system (for reviews, see Heffron, 1983; and Grindley and Reed, 1985). The role of this recombination is to resolve a cointegrate molecule, the fusion product of intermolecular transposition, into the two final products: target molecule with inserted transposon and regenerated donor replicon. This recombination is catalyzed by resolvase, the product of the transposon *tnpR* gene, acting at a site called *res* that lies within the transposon, just upstream of *tnpR*. Three distinct resolvase binding sites are contained within the 115 bp that span *res* (Grindley *et al.*, 1982). Recombination occurs in the center of site I, the most *tnpR*-distal site (Reed and Grindley, 1981), but all three sites are required for efficient resolution *in vivo* or *in vitro* (Wells and Grindley, 1984). The *res* site itself is highly asymmetric; no two binding sites are of the same size and the spacing between sites is different. However, DNase protection experiments have shown that all three sites in *res* are protected at about the same resolvase concentration (Grindley *et al.*, 1982; Abdel-Meguid *et al.*, 1984). Co-operativity between sites and/or their specific spatial arrangement in *res* directs the organization of a highly ordered protein–DNA complex that supplies recombinational potential.

Resolvase is sensitive to the topological structure of the cointegrate substrate. Two copies of *res* must lie in the same orientation on the same negatively supercoiled molecule if efficient recombination is to occur *in vitro* (Reed, 1981b). In addition, the products isolated after resolution *in vitro* are nearly all singly linked catenanes (Reed, 1981b; Krasnow and Cozzarelli, 1983). Combined, these observations suggest that *res* sites are brought into synapsis and DNA strands exchanged in a topologically constrained manner. The detailed analysis of resolution products has led Cozzarelli and co-workers to propose that three negative interdomainal nodes must be trapped prior to strand exchange (Cozzarelli *et al.*, 1984; Wasserman and Cozzarelli, 1985; Wasserman *et al.*, 1985). These nodes could result from the *res* sites being bent into toroidal loops, or alternatively from plectonemic interwinds that would result from wrapping one *res* about the other (Boocock *et al.*, 1986; Craigie and Mizuuchi, 1986).

Examination by electron microscopy of resolvase–*res* complexes (both single *res* complexes and the synapsed *res* sites of a cointegrate molecule) indicated that the *res* DNA was significantly condensed (Salvo and Grindley, 1988; Benjamin and Cozzarelli, 1988). In this report we have used polyacrylamide gel electrophoresis and DNase I footprinting to gain a more detailed picture of the distortion of *res* DNA that results from resolvase binding. We have detected two types of DNA bending: (i) intrasite bends at each individual binding site, which result from the resolvase–DNA interaction; and (ii) an intersite bend of the DNA segment between sites I and II, which results from resolvase–resolvase interactions, requires the presence of site III and is dependent on the appropriate helical phasing of the binding sites.

Results

Resolvase bends the res site

Wu and Crothers (1984) have shown that a bent DNA fragment migrates abnormally slowly through a polyacrylamide gel. Moreover, the degree of retardation is position-dependent, the lowest mobility occurring when the bend is at the center of the fragment, thus allowing determination of the site of bending. Both sequence-specific bends (as found in kinetoplast DNA) and protein-induced bends (as demonstrated with the *Escherichia coli* cAMP receptor protein) exhibit this characteristic effect. We have made use

of polyacrylamide gel electrophoresis to probe the structure of the resolvase−*res* complex. For the DNA substrates we cloned a 320 bp *res*-containing fragment as a tandem repeat in pNG16 (see Materials and methods). Using a variety of restriction enzymes that cut just once within the 320 bp sequence, we isolated identically sized, circularly permuted fragments which differ only in the location of *res*. Labeled fragments were equilibrated with resolvase, and the complexes were analyzed electrophoretically (Figure 1A).

Several resolvase−DNA complexes of different mobility are formed with each DNA fragment. These appear to correspond to a variety of partially and fully occupied *res* sites with the final, slowest migrating complex consisting of *res* with all three specific binding sites filled (Falvey and Grindley, 1987; Hatfull and Grindley, 1986). It is clear that the slowest complexes exhibit a position-dependent mobility that is characteristic of a protein-induced bend. A plot of mobility versus position shows that the lowest mobility occurs when *res* lies at the center of the fragment, indicating that the center of the bend lies near to the center of *res* (Figure 1B).

Resolvase bends individual binding sites

The resolvase-induced bending of *res* DNA could result from wrapping *res* around a core of resolvase protomers in a structure analogous to a nucleosome. Alternatively, the total bending could result from summing individual bends induced by the interaction of resolvase with one or more of the three distinct binding sites that constitue *res*. To distinguish between these, we have performed a similar electrophoretic analysis of resolvase−DNA complexes formed with each of sites I, II and III (Figure 2). In all cases, mobility of the complex was found to be dependent on location of the binding site, indicative of a bend. From graphic representation of the data (Figure 2) we have determined the position of the centers of the bends. These lie near the centers of sites I, II and III. We note that the DNA fragments have a small inherent bend in the absence of protein. However most of the sequences responsible for this effect seem to be located in the flanking vector DNA, and are not derived from the *res* site.

Resolution is sensitive to the spacing between binding sites

All three resolvase binding sites in *res* are necessary for efficient resolution. However, it was not known whether their precise spatial arrangement was also important. Between sites I and II there is a 15 bp stretch of DNA that remains sensitive to DNase I when *res* is complexed with resolvase (Grindley *et al.*, 1982); the nucleotide sequence of this region is poorly conserved between γδ and Tn*3* (see Reed, 1981a). These data indicated that this region does not specifically interact with resolvase and suggested that it would provide an ideal position for making internal alterations to the geometry of *res*. We constructed a series of *res* derivatives, res^{sot} (sot = Spacing between sites One and Two) in which the spacing between sites I and II was systematically changed. Cointegrates with one modified and one wild-type *res* ($res^{sot} \times res^{+}$) or with two copies of a res^{sot} site were constructed and analyzed for recombination. Table I shows the results of resolution *in vivo* using the host strain MG1047 which contains three chromosomal copies of γδ. Two points are clear: (i) insertions that approximate

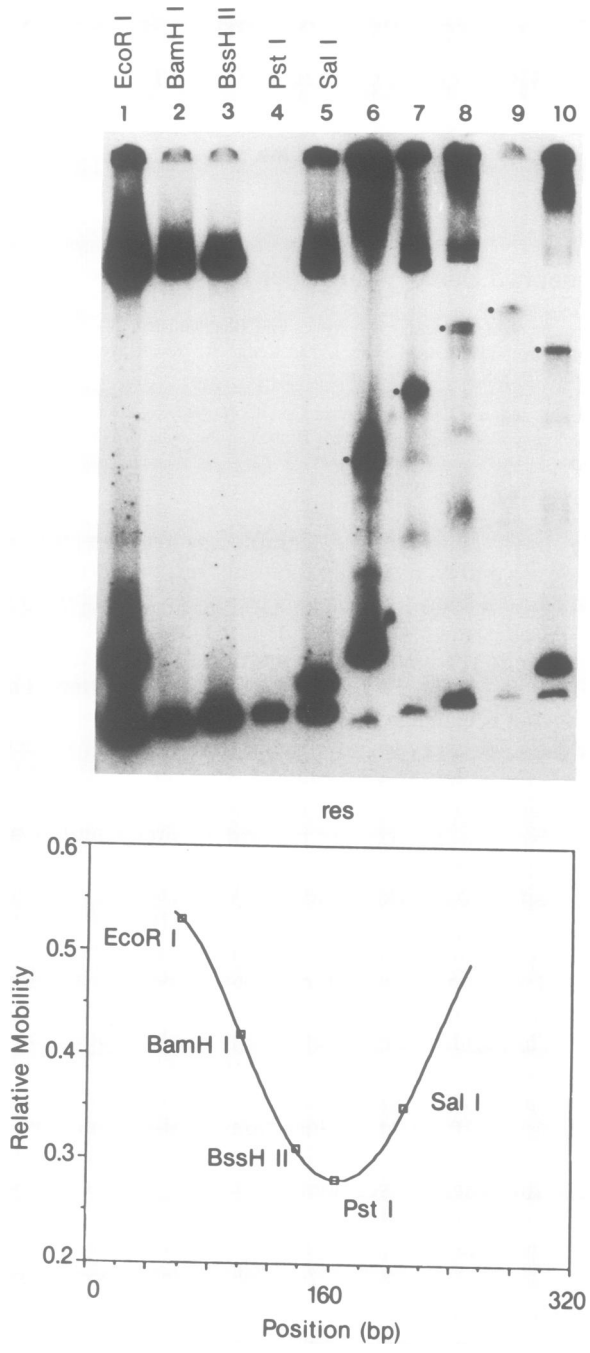

Fig. 1. Analysis of resolvase−*res* complexes by polyacrylamide gel electrophoresis. The plasmid pRW105 (which contains a tandem repeat of a 320 bp DNA fragment with *res*; see Materials and methods) was digested with a variety of different restriction enzymes (as listed below). Except in the case of the *Pst*I digest, fragments were 3′ end-labeled with ^{32}P and were used without further purification. The 320 bp *res*-containing fragment from the *Pst*I digest was gel-purified and 5′ end-labeled. (**A**) Uncomplexed DNA (lanes 1−5) and resolvase complexes (lanes 6−10) were analyzed on a 4% polyacrylamide gel. Restriction digests were: *Eco*RI, lanes 1 and 6; *Bam*HI, lanes 2 and 7; *Bss*HII, lanes 3 and 8; *Pst*I, lanes 4 and 9; *Sal*I, lanes 5 and 10. The restriction sites are respectively, 62, 101, 138, 164 and 210 bp to the right (site III—proximal) side of the center of the *res* sequence. The fully occupied *res* complex is marked with a black dot. Restriction digests with *Eco*RI, *Bss*HII and *Sal*I generated an additional large fragment containing *res* which accounts for the very slow moving resolvase−DNA complexes visible in lanes 6, 8 and 10. (**B**) Relative mobility of *res*−resolvase complexes (□) plotted as a function of the restriction site position relative to the center of *res*.

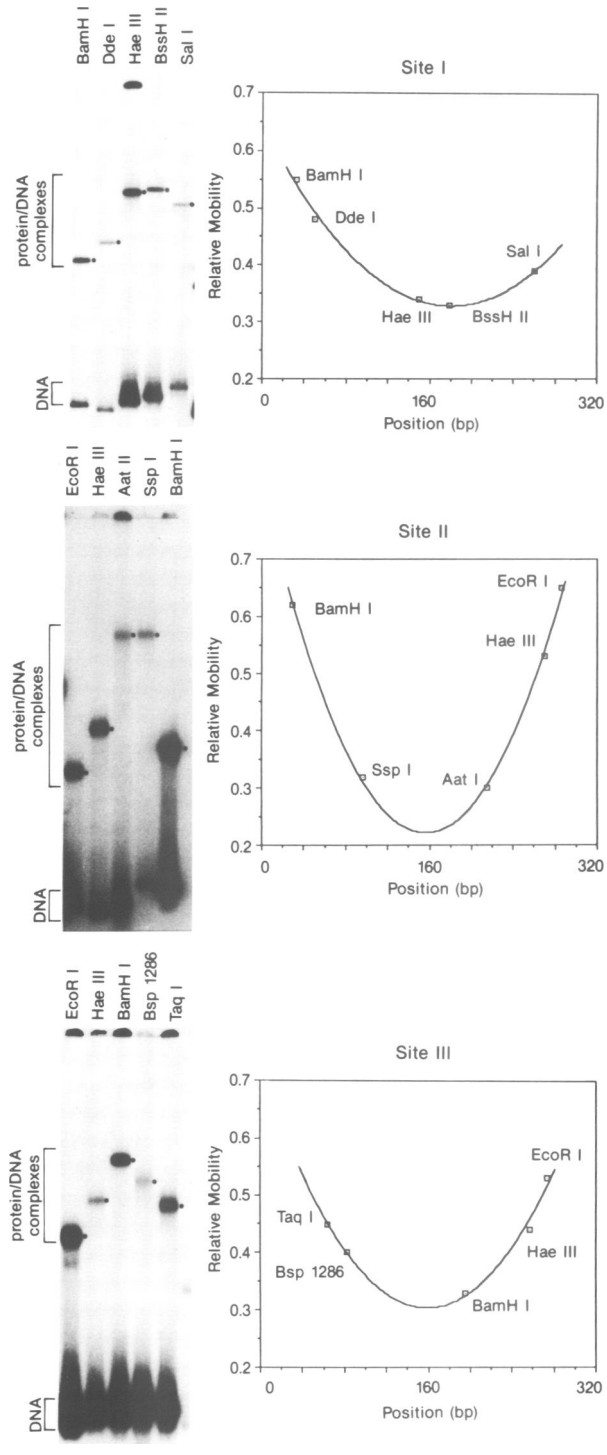

Fig. 2. Analysis of complexes between resolvase and its individual binding sites by polyacrylamide gel electrophoresis. **Top**. Site I complexes. The DNA fragments (all 320 bp) were obtained by digesting pJS110.Tan with the following restriction enzymes: *Bam*HI (which cuts 35 bp to the right of the center of site I), *Dde*I (52 bp), *Hae*III (150 bp), *Bss*HII (181 bp) and *Sal*I (253 bp). **Center**. Site II complexes. The DNA fragments (all 320 bp) were obtained by digesting pJS115.Tan with *Eco*RI (which cuts 287 bp to the right of the center of site II), *Hae*III (271 bp), *Aat*I (214 bp), *Ssp*I (98 bp) and *Bam*HI (32 bp). **Bottom**. Site III complexes. The DNA fragments (all 318 bp) were obtained by digesting pJS118.Tan with *Eco*RI (which cuts 275 bp to the right of site III), *Hae*III (257 bp), *Bam*HI (197 bp), *Bsp*1286 (81 bp) and *Taq*I (65 bp). The graphs show relative mobilities of the complexes plotted as a function of the restriction site position relative to the center of the binding site.

Table I. Effect of I–II spacing on cointegrate resolution

Change in I–II spacing (bp)	% Resolution *in vivo*[a]	
	$res^{sot} \times res^{+}$	$res^{sot} \times res^{sot}$
−2	6	4
0	100	(100)
+2	100	28
+4	26	0
+6	4	0
+7	0	ND
+8	20	ND
+10	100	100
+12	100	74
+17	3	ND
+19	96	ND
+21	100	ND
+23	100	ND
+29	9	ND
+33	80	5

[a]Ampicillin-resistant transformants of MG1047 (*recA*$^-$, $\gamma\delta^+$) were selected, patched onto ampicillin plates and replicated onto tetracycline plates. The numbers indicate the percentage of patches that had lost resistance to tetracycline, indicating resolution of the cointegrate structure.
ND Not determined.

to one turn of the DNA helix are well tolerated but insertions of only a part of a turn are strongly inhibitory to resolution; (ii) the inhibitory effects are magnified in the cointegrates with two copies of a modified *res* (e.g. res^{sot}*+2* and res^{sot} *+12*). Resolution reactions performed *in vitro* showed the same effects.

Spacing affects the conformation of resolvase – res complexes

Presumably the inhibition of resolution that results from alterations in the site I–II spacer is caused by the inability of res^{sot} mutants to form a recombinationally productive complex with resolvase. We have investigated whether differences between complex formation by some of the res^{sot} mutants could be detected by the gel assay (Figure 3). Short DNA fragments were used (~120 bp) to minimize the formation of DNA tails that might influence the migration rate of the final complexes. The striking result is that only res^{sot}*+10* and res^{sot}*+21* show a pattern of complex formation similar to res^{+}—a virtually quantitative conversion of *res* DNA into a final complex of characteristic electrophoretic mobility. Many of the other res^{sot} mutants (*+4*, *+6*, *+8* and *+17*) form final complexes (non-quantitatively at the highest resolvase concentration used) which have considerably reduced electrophoretic mobility suggestive of a more extended structure than the wild-type complex. With virtually all of the mutants (except *+10* and *+21*) complexes with partially occupied binding sites accumulate, suggesting that co-operative interactions between resolvase dimers at neighboring sites are lost or weakened. From the electrophoretic mobility of the final res^{+} complex we conclude that it is relatively compact and is stabilized by interactions between the three resolvase-occupied binding sites.

Resolvase-induced curvature of the DNA between sites I and II

Assembly of a resolvase – *res* complex might require not only

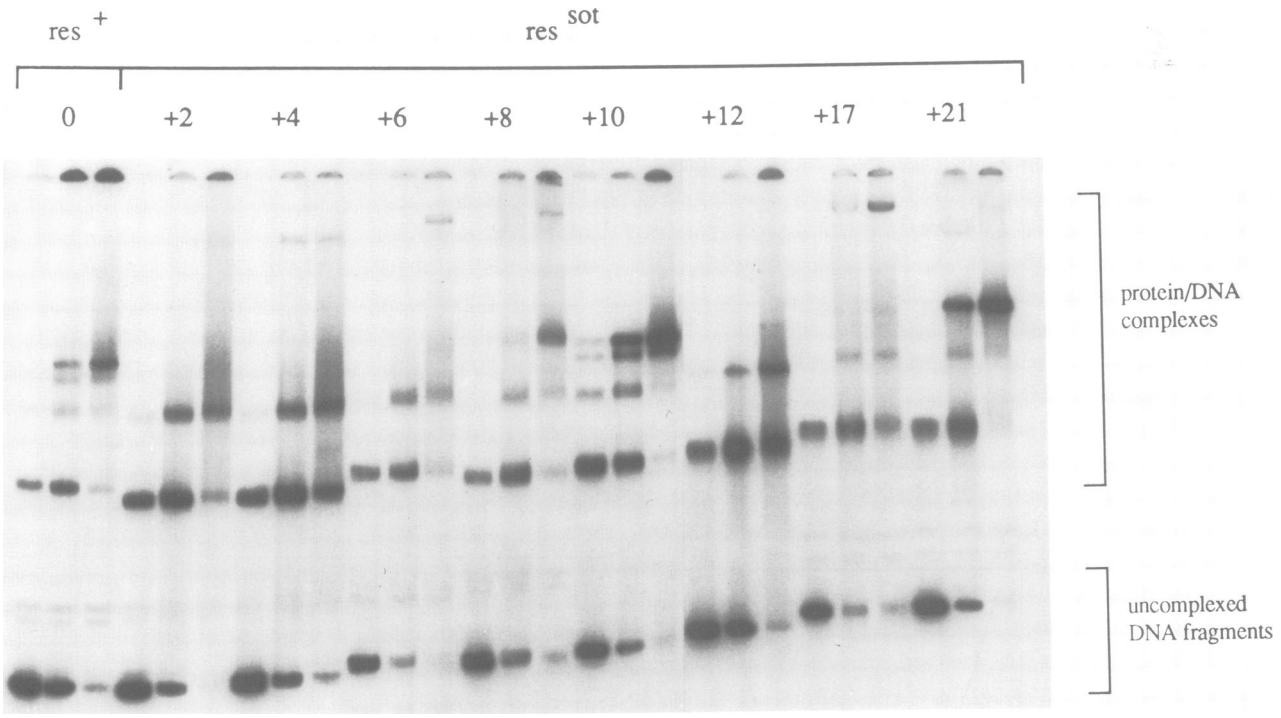

Fig. 3. Formation of complexes between resolvase and *res*^{sot} insertion mutants analyzed by polyacrylamide gel electrophoresis. Binding reactions contained ^{32}P-labeled DNA and resolvase at three different concentrations (~5, 25 and 125 nM from left to right for each *res* derivative). The DNA fragments were essentially minimal *res* sites; they extended 4 bp to the left of site I and 5 bp to the right of site III.

appropriate phasing of the individually bent sites, but also interactions between resolvase dimers at neighboring sites as suggested above. Such interactions, if essential, must be able to accommodate the acceptable insertions of 10 or 21 bp between sites I and II. One way to do this, as has been demonstrated in other systems (Hochschild and Ptashne, 1986; Kramer *et al.*, 1987) is to bend or loop the intersite DNA. A characteristic of a DNA loop is that the portion of the phosphodiester backbone exposed on the outside of the loop is particularly susceptible to DNase I whereas the inside portion is resistant to cleavage (Drew and Travers, 1985).

As can be seen in Figure 4(A) the DNase I cleavage patterns of *res*−resolvase complexes indicate that the DNA between sites I and II is bent provided that the sites are appropriately spaced. Complexes in which *res* has 21 or 10 bp insertions clearly showed an alternating pattern of enhanced and suppressed cleavages typical of a DNA loop. As expected, however, no enhancements were seen with the +17 bp insertion. More significantly, the presence of the non-adjacent site III is also required (Figure 4B), showing that the bending is not simply the result of an interaction between resolvase dimers on the adjacent sites I and II. A summary of the data obained with *res*$^{+}$ and with *res*^{sot}*+21* is shown in Figure 5. The data clearly define a face of the helix between sites I and II that, in a fully occupied complex between resolvase and *res*^{sot}*+21*, is readily accessible to DNase I cleavage. In the case of the wild-type *res*, no strongly enhanced DNase I cleavages are seen, but careful examination of the data by densitometry showed increased sensitivity of one phosphodiester bond on one strand and two bonds on the other. In addition, cleavage of one phosphodiester bond close to the right-hand boundary of site I (shown by an arrow in Figure 4A and B) is enhanced in

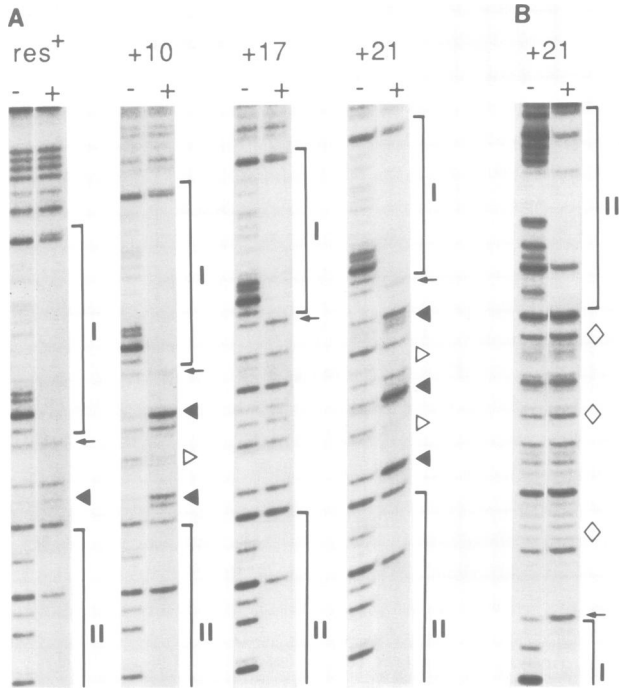

Fig. 4. DNase I susceptibility of the region between resolvase binding sites I and II in *res*$^{+}$ and *res*^{sot} mutants. (**A**) Footprinting of complexes of resolvase with whole *res* sites with *res*^{sot} insertions as indicated. −, no resolvase added; +, resolvase added. Protected regions corresponding to sites I and II are indicated. Sites of enhanced DNase cleavage are indicated by solid black triangles, sites of suppressed cleavage are indicated by open triangles. The small arrow indicates a cleavage site discussed in the text. (**B**) Footprinting of *res*^{sot}*+21* from which site III has been deleted. Open diamonds indicate sites where cleavage is enhanced when site III is present and *res* is fully complexed. The data are for the same DNA strand as in (A) ('bottom' strand of *res* as conventionally shown), but the sites are inverted [as a result of labeling the 3′ end in (B), the 5′ end in (A)].

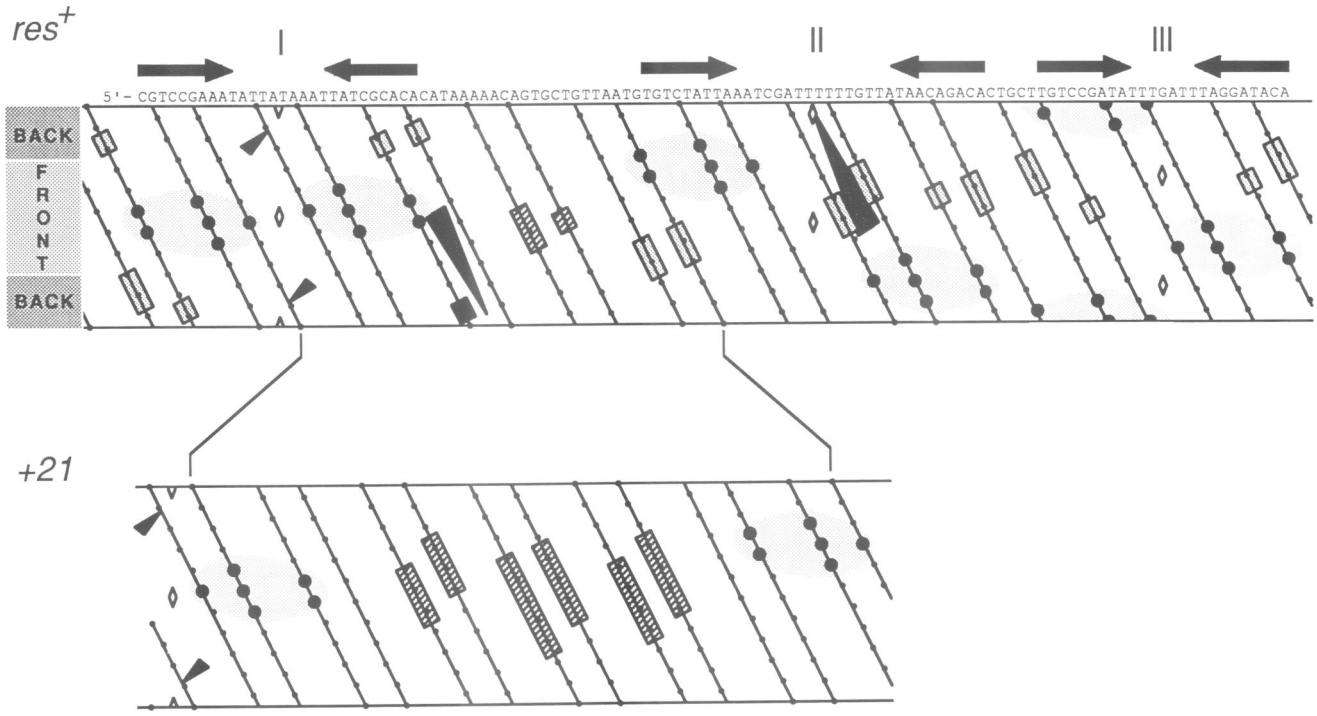

Fig. 5. Planar representation of the *res* DNA helix showing DNase I susceptibility of resolvase–*res* complexes. (**A**) *res*$^{+}$ with the normal spacing between binding sites I and II. DNA sequence is for the 'top' strand. The DNA helix is represented as a cylinder split down one side and unfolded. A helical repeat of 10.5 bp per turn is assumed. Black dots represent phosphates of the sugar–phosphate backbone. Cross-hatched rectangular areas between sites I and II indicate regions of the sugar–phosphate backbone which show enhanced sensitivity to DNase I in resolvase–*res* complexes. Stippled rectangular areas within the binding sites indicate sugar–phosphate bonds which remain susceptible to DNase I in resolvase–*res* complexes. The single black rectangle indicates a phosphodiester bond at which cleavage is inhibited in resolvase–*res*$^{+}$ complexes although enhanced in resolvase–site I complexes (see text for details). The lozenge-shaped stippled areas with the heavy black dots represent the areas contacted by the C-terminal domain of resolvase (black dots are phosphates that, when ethylated, inhibit complex formation) (Falvey and Grindley, 1987; V.Rimphanitchayakit and N.Grindley, unpublished observations). Large shaded triangles in the minor grooves to the right of site I and within site II indicate the progressive narrowing of the minor groove associated with A tracts (for details, see text). Small black arrowheads within site I indicate the cleavage site at the crossover point. The diamond symbols in major and minor grooves indicate the dyad axis of each binding site site. (**B**) *res*^{sot}*+21*. Symbols as in (A). Only the enhanced DNase I cleavage sites *between* sites I and II are shown.

complexes between resolvase and *res*^{sot}*+17* (or *res*^{sot}*+21* when site III is absent) but is inhibited in complexes with *res*$^{+}$, *res*^{sot}*+10* and *res*^{sot}*+21*. This cleavage site (black square, Figure 5) is predicted to be on the outside of the resolvase–site I complex but on the inside of the bend between sites I and II.

Discussion

Electron microscopic examination of the resolvase–*res* complex had indicated that it was a compact structure with highly condensed DNA (Salvo and Grindley, 1988; Benjamin and Cozzarelli, 1988). Here we have addressed the underlying mechanisms of this condensation. We have analyzed the complexes formed when resolvase binds to linear DNA fragments containing a *res* site. These complexes contain resolvase dimers loaded onto a single fragment and are not synaptic complexes (Benjamin and Cozzarelli, 1988; G.Hatfull and N.Grindley, unpublished observations) which, under the conditions used, require supercoiled DNA. We have found that there are two kinds of protein-dependent bends in *res* DNA: (i) intra-site bends that result from the interaction between a resolvase dimer and each of the three binding sites that constitute *res*, and (ii) an inter-site bend that depends upon protein–protein interactions between different protein-bound sites.

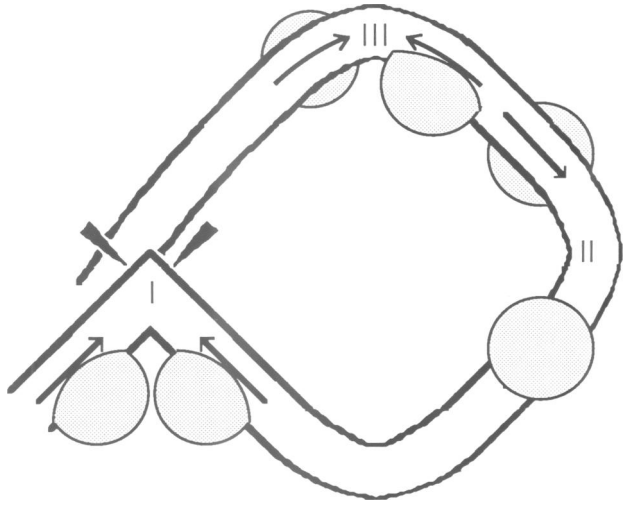

Fig. 6. A cartoon of a single *res*–resolvase complex. Only the C-terminal DNA-binding domains of resolvase are shown (stippled). The N-terminal domains which constitute more than three-quarters of the protein would be expected to fill the central area. The arrows indicate the conserved sequences to which the C-terminal domain binds. The black triangles indicate the crossover points on the outside of the site I kink.

The intra-site bends may well differ from each other as a result of the different configurations of each binding site: the inverted repeats of 9 bp which constitute each site are separated by 10 bp in site I, 16 bp in site II and 7 bp in site III (Grindley *et al.*, 1982; Falvey and Grindley, 1987). It seems likely that the different geometries of each binding site will affect both the extent and the direction of the induced bend. We have proposed that the site I bend consists largely of a localized kink which opens up the minor groove at the crossover point and renders it accessible to the intercalating agent methidiumpropyl EDTA [MPE.Fe(II)] (Hatfull *et al.*, 1987). Neither the site II nor site III bends, however, cause enhanced sensitivity to MPE.Fe(II) cleavage (Hatfull *et al.*, 1987). Although we have yet to determine the direction of the site II and III bends (see Salvo and Grindley, 1987; Zinkel and Crothers, 1987) we can infer the direction of the site II bend from the location of existing DNase I cleavage sites within the site II complex (Grindley *et al.*, 1982; data for this paper) (see Figure 5). Assuming that these cleavage sites lie on the outside of the bend, then the major groove at the center of site II lies on the outside of the bend. This contrasts with site I in which the minor groove is on the outside of the bend (Hatfull *et al.*, 1987; Salvo and Grindley, 1987).

The fully occupied *res*−resolvase complex appears to exhibit a bend between sites I and II in addition to the bends localized within the individual binding sites. Although it is most readily detected when the separation between sites I and II is increased (as in the +10 and +21 bp insertions), the DNase cleavage data suggest that the intersite bend exists with the wild-type I−II spacing. Formation of the intersite bend requires not only that the centers of sites I and II are separated by an integral number of helical turns, but also that site III is present and occupied. The requirement for a separation of I and II by integral helical repeats mirrors the results obtained with the araC protein (Dunn *et al.*, 1984), phage λ repressor (Hochschild and Ptashne, 1986), *lac* repressor (Kramer *et al.*, 1987) and some other proteins in which co-operative binding to two separated sites requires that the sites lie on the same face of the DNA helix; presumably the energy cost of bending the helix to allow protein−protein interactions is very much less than the energy cost of altering the helical pitch of the intervening DNA segment. Much more surprising was the requirement that site III be occupied. This result suggests that the looping of the I−II intersite segment is not simply a result of interactions between resolvase dimers bound at sites I and II. Several possibilities exist. The interaction could be between dimers at sites I and III, between dimers at all three sites, or between dimers at sites I and II, but in a site III dependent manner (e.g. occupancy of site III might stabilize the I−II interaction or might modify the site II complex to enable it to interact with the site I complex).

Within the *res* site are two oligo A stretches both of which may assist the resolvase-induced condensation of *res*. Oligo A tracts have been shown to result in intrinsic (protein-independent) bending of DNA (Wu and Crothers, 1984; Hagerman, 1985; Koo *et al.*, 1986). From an analysis of the sensitivity of A tracts in duplex DNA to cleavage by hydroxyl radical, Burkhoff and Tullius (1987) have proposed that the minor groove narrows progressively from the 5′ to the 3′ end of the tract. Both of the A tracts in the γδ *res* site are positioned such that their 3′ ends lie at the inside of a curve where the minor groove is expected to be compressed (see Figure 5). One tract of five As lies just to the right of site I in the segment between site I and II and has its 3′ end on the inside of the intersite loop. The other tract, six As, is in the spacer within resolvase binding site II, with its 3′ end on the inside at the center of the site II bend. We are currently determining whether these A tracts assist in formation of the resolvase−site II complex (and its associated bend) and the intersite loop.

The path of the DNA within the resolvase−*res* complex is becoming apparent. The pattern of DNase I cleavage between sites I and II clearly defines the face of the DNA helix that lies on the outside of the intersite curve. As can be seen from Figure 5, this is the face that connects the *major* grooves at the centers of these two binding sites (assuming a helical repeat of 10.5 bp per turn). As discussed above, the DNA across site II appears to follow the same curvature, whereas the site I bend (kink) is in the opposite direction. Recently, Benjamin and Cozzarelli (1988) have demonstrated that a resolvase−*res* complex traps about one half a negative supercoil, suggesting either that the DNA enters and exits the complex along parallel paths, in opposite directions, or that the DNA helix within *res* is unwound by half a turn. Combining our bending data with these results, we suggest that the DNA in the resolvase−*res* complex may follow a path approximately as shown in Figure 6.

The existence of the intersite loop and its dependence upon interactions between resolvase protomers occupying different sites, indicates that the resolvase−*res* complex forms a closed structure—a loop of DNA closed into a ring by protein−protein interactions. It has been shown that when two *res* sites are paired to form a synaptic complex, three negative supercoils are trapped (Cozzarelli *et al.*, 1984; Wasserman and Cozzarelli, 1985; Wasserman *et al.*, 1985; Benjamin and Cozzarelli, 1988). It has been proposed that the three supercoils result from the wrapping of one *res* about the other, with sites II and III of *res* paired and interwrapped in anti-parallel fashion with sites III and II of the other (Boocock *et al.*, 1986, 1987). Evidence to support such a plectonemically wrapped synaptic structure has recently been obtained [unpublished results quoted in Benjamin and Cozzarelli (1988)]. Clearly, if a single resolvase−*res* complex takes the form of a closed loop, then interwrapping of two *res* sites to form a synaptic complex can only occur if the intra-*res* protein−protein interactions that hold the loop dissociate, presumably to be replaced by compensating inter-*res* interactions.

Materials and methods

Enzymes

T4 DNA ligase, polynucleotide kinase, S1 nuclease and calf alkaline phosphatase were purchased from Boehringer Mannheim; restriction endonucleases were purchased from New England Biolabs or Boehringer Mannheim. Resolvase was prepared according to the procedure described in Reed (1981b), and DNA polymerase I Klenow fragment was kindly provided by Cathy Joyce.

res site DNA fragments for electrophoretic analysis

We used the *resΔL* and *resΔR* deletions obtained by Wells and Grindley (1984) as the initial source of DNA fragments with individual resolvase binding sites. In the cases described, *Eco*RI sites that mark the deletion end points are joined to the *Eco*RI site of pBR322 with ΔL deletions adjoining the Ap^R-proximal region and ΔR deletions adjoining Tc^R.

res. A 308 bp *Hae*III−*Hin*dIII fragment from *resΔ101R* was isolated, the *Hin*dIII site was filled and *Bam*HI linkers (12mers) were added to both ends.

Table II. Construction of the *res*^{sot} mutations

Deletion combination		Change in I–II spacing		
Site I	Sites II and III	*Eco*RI joint	Filled joint (*Xmn*I)	S1 joint (*Hae*III)
Δ24R + Δ34L		−2	+2	
Δ24R + Δ26L		+6	+10	
Δ24R + Δ25L		+7		
Δ34R + Δ25L		+17	+21	
Δ34R + Δ34L		+8	+12	+4
Δ24R + Δ12L		+19	+23	
Δ34R + Δ12L		+29	+33	

pRW105 contains tandem copies of this 320 bp fragment in the *Bam*HI site of pNG16 (Grindley and Joyce, 1980).

Site I. A 310 bp *Bst*NI–*Eco*RI fragment from *resΔ24R* was isolated, the ends were filled and *Bam*HI linkers (10mers) were attached. pJS110.Tan contains tandem copies of this 320 bp fragment in the *Bam*HI site of pNG16.

Site II. The *res* site deletions *resΔ26L* and *resΔ82R* were first recombined *in vitro* using the common *Cla*I site in site II. This gave the plasmid, pRW113, in which the *res* sequences (*resΔ26LΔ82R*) are contained within a 62 bp *Eco*RI fragment cloned at the *Eco*RI site of pK01 (McKenney *et al.*, 1981). A 312 bp *Fnu*4HI–*Hpa*II fragment from pRW113 was isolated, the ends were filled and *Bam*HI linkers (8mers) were attached. pJS115.Tan contains tandem copies of this 320 bp fragment in the *Bam*HI site of pNG16.

Site III. A 314 bp *Aha*II fragment from *resΔ48L* was isolated, the ends filled, and then cloned into the filled *Bam*HI site of pNG16; this reconstitutes *Bam*HI sites at both ends of a 318 bp fragment. pJS118.Tan contains this 318 bp fragment recloned as tandem copies in the *Bam*HI site of pNG16.

Plasmid DNA was prepared by the method of Birnboim and Doly (1979) from 200 ml overnight cultures. When necessary, restriction fragments were purified by electrophoresis on polyacrylamide gels in TBE (50 mM Tris–borate, pH 8.3, 1 mM EDTA). Fragments either were treated with calf alkaline phosphatase to remove the terminal 5′ phosphates and were 5′ end-labeled using [γ-^{32}P]ATP and polynucleotide kinase (Maxam and Gilbert, 1980), or they were 3′ end-labeled by filling with [α-^{32}P]dNTPs and the Klenow fragment of DNA polymerase I.

res mutants with altered spacing between resolvase binding sites I and II

Several of the deletions of *res* DNA constructed by Wells and Grindley (1984) have end points either to the right of site I (*Δ24R*, *Δ34R*) or to the left of site II (*Δ12L*, *Δ25L*, *Δ26L*, *Δ34L*). Since each deletion was terminated with an *Eco*RI site, we could join a Δ*R* and a Δ*L* deletion at their *Eco*RI sites to reconstruct a mutant *res* with all three binding sites intact but with varied spacers between sites I and II (Table II). In each case an *Eco*RI–*Bam*HI fragment from the Δ*L* deletion (~800 bp; containing sites II and III and the start of the *tet* gene of pBR322) was ligated to a *Bam*HI–*Eco*RI fragment from the Δ*R* deletion (~5460 bp; containing the rest of *tet*, a wild-type *res* site, the origin of replication and the *amp* gene, and site I). The resulting plasmids were *res*^{sot} × *res*$^{+}$ cointegrates with an *Eco*RI site between sites I and II of the mutant *res*. The remaining spacing mutants were constructed from this initial set by cutting at the *Eco*RI site and either filling with DNA polymerase I (Klenow fragment) or digesting back the 4 bp single-stranded ends with S1 nuclease. The resulting blunt ends were then ligated. Filling the *Eco*RI ends adds 4 bp to the I–II spacer and constructs an *Xmn*I site. The S1 nuclease treatment removes 4 bp and constructs a *Hae*III site (see Table II).

Cointegrates with two copies of a particular *res* mutation were constructed in two steps. First, a 990 bp *Xho*II fragment that contains the mutant *res*^{sot} was cloned into the *Bgl*II site of pNG105 (a plasmid with a *Bgl*II linker inserted into the *Pvu*II site of pBR322). Plasmids with *res* in the desired orientation (*tet*–site I–II–III–*ori*–*amp*) were detected by digestion with *Sal*I. In the second step a 1800 bp *Sca*I–*Bam*HI fragment from a *res*^{sot} × *res*$^{+}$ cointegrate (containing the start of *amp* and *tet* and the intervening mutant *res* site) was cloned into the 4470 bp *Bam*HI–*Sca*I fragment from the derivative of pNG105 with the corresponding *res*^{sot} mutation.

For electrophoretic analysis of resolvase–*res*^{sot} complexes we used fragments that were essentially the minimal *res* site. pNG139 contains *res* as a 123 bp *Eco*RI fragment; it was made by resolving a cointegrate with one copy of *resΔ–16L* and one copy of *resΔ101R* to give a single *resΔ–16LΔ101R*. A variety of *res*^{sot} insertions was introduced into this minimal *res* site by replacing the 54 bp *Ssp*I–*Cla*I fragment (which spans the entire I–II spacer segment) with the equivalent fragment from the mutant *res* sites. The plasmids were digested with *Eco*RI (using partial digestions for the *res*^{sot} mutants with an *Eco*RI site in the I–II spacer), 5′ end-labeled, and the desired fragments (123–144 bp) were purified by polyacrylamide gel electrophoresis. The same plasmids were used to generate fragments for DNaseI footprinting. For the footprinting experiments we also constructed as follows a plasmid, pNG183, with a *res* site that contained the *res*^{sot}*+21* insertion and lacked site III. The plasmid pNG142 contains *res* as a 130 bp *Eco*RI fragment with a *Sal*I site introduced by oligonucleotide mutagenesis between sites II and III. A *Pst*I–*Sal*I fragment from pNG142 (~850 bp, containing the start of the pBR322 *amp* gene and sites I and II of *res*) was cloned into the *Pst*I–*Sal*I replicon fragment of pNG16, to give pNG181. Finally, the *res*^{sot}*+21* insertion mutation was introduced into pNG181 by replacing the *Pst*I–*Cla*I fragment (~820 bp, containing the start of the *amp* gene plus site I and the proximal half of site II) with the equivalent fragment from the *res*^{sot}*+21* derivative of pNG139 (see above).

Gel electrophoresis of DNA – resolvase complexes

Binding reactions contained labeled DNA fragments in binding buffer (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl) and 100 μg/ml calf thymus DNA. Resolvase was added to a final concentration of ~100 nM (or as indicated) and reactions were incubated at 37°C for 5 min, then at room temperature for an additional 5 min. Loading buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 20% Ficoll and 0.1% xylene cyanol and bromophenol blue) was added, and samples were analyzed on 1.5 mm thick polyacrylamide gels (acrylamide:bis-acrylamide ratio of 30:1) in TBE. Electrophoresis was carried out at 6 V/cm.

Resolution assays

Resolution of mutant cointegrates *in vivo* was determined as described by Wells and Grindley (1984). Plasmids were introduced into the *E.coli* strain MG1047 (which contains three chromosomal copies of γδ) by transformation. Ampicillin-resistant transformants were selected and screened for loss of tetracycline resistance by replica plating. Resolution *in vitro* was as described by Reed (1981b).

DNase I footprinting experiments

End-labeled DNA fragments were equilibrated with resolvase (~0.4 μM) and then briefly digested with DNase I (0.3 μg/ml, 1 min, room temperature) essentially as described earlier (Grindley *et al.*, 1982). DNA fragments were obtained from pNG139 and its derivatives with the *res*^{sot} insertions (see above) and were labeled at the *Hin*dIII site 29 bp beyond the *Eco*RI site that abuts site III. Both strands were analyzed by using both 5′ and 3′ end-labeled fragments. For footprinting resolvase complexed to site I and II (in the absence of site III), pNG183 (see above) was 3′ end-labeled at the site I-proximal *Eco*RI site and at the site II-proximal *Sal*I site.

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