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Isolation and characterization of the Tn*3* resolvase synaptic intermediate

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We have isolated in quantitative yield the synaptic intermediate formed during site-specific recombination by Tn*3* resolvase and characterized it by restriction endonuclease mapping, electron microscopy and topological methods. The intermediate accumulates at low reaction temperatures and is stabilized by crosslinking of the resolvase protomers with glutaraldehyde. The DNA–resolvase complex that maintains the structure of the intermediate (the synaptosome) is ~100 Å in diameter, forms specifically at resolution (*res*) sites, and requires two *res* sites in a supercoiled DNA molecule. Resolvase bound to individual *res* sites protects ~ −0.5 supercoil per site from relaxation by a topoisomerase, whereas the formation of the synaptosome protects −3 supercoils and condenses the associated DNA to a supercoil density 2.5 times that of the non-complexed substrate. Although recombination requires two directly repeated *res* sites, both direct and inverted sites form synaptosomes. We conclude that the specificity of recombination is achieved by a three-stage recognition system: binding of resolvase to separate sites, formation of the synaptosome and determination of site orientation from within the complex.

Key words: Tn*3* resolvase/site-specific recombination/synaptic complex

Introduction

Site-specific recombination is the genetic exchange between particular DNA sequences. It plays an important role in a number of biological contexts including transposition, viral integration and excision, transcriptional regulation, the modulation of cell cycle control of plasmid replication and the generation of antibody diversity (Tonegawa, 1983; Weisberg and Landy, 1983; Plasterk and Van de Putte, 1984; Grindley and Reed, 1985; Volkert and Broach, 1986). In addition to this intrinsic physiological importance, site-specific recombination has been instrumental in the elucidation of basic concepts of DNA structure and DNA–protein interactions (Nash, 1981; Wasserman and Cozzarelli, 1986).

The resolvase recombination system encoded by the Tn*3* family of transposons has been well characterized (review in Grindley and Reed, 1985). Resolvase is a multimer made up of 21 000-dalton protomers. It performs the site-specific exchange that is the final step in Tn*3* transposition and functions as its own transcriptional repressor. Both activities are mediated by the binding of resolvase to a specific 113-bp sequence called *res* (Grindley *et al.*, 1982). The *res* site in turn consists of six copies of a 9-bp consensus sequence to which resolvase binds. No high-energy cofactor is required for recombination but substrates must be negatively supercoiled (Reed, 1981).

Two stages of recombination are site synapsis, the proper alignment of two sites on the surface of the recombination enzyme, and strand exchange, the actual breakage and crossed reunion of DNA. Both processes are tightly controlled by resolvase. A rapid and quantitative resolution occurs both *in vivo* and *in vitro* but only if the *res* sites are directly repeated (head-to-tail orientation) in the same molecule (Reed, 1981). Moreover, the number, sign and distribution of substrate supercoils at synapsis are uniquely determined (Wasserman *et al.*, 1985). Because this specificity holds independent of both the distance between *res* sites in the primary sequence and the supercoil density of the substrate, a synaptic intermediate of unique geometry is implicated (Benjamin *et al.*, 1985). Strand exchange is equally precise. A total of four DNA strands are broken and rejoined to yield a singly linked catenane with the loss of four supercoils (Krasnow and Cozzarelli, 1983; Boocock *et al.*, 1987; J.M.Dungan and N.R.Cozzarelli, unpublished data).

This paper focuses on the structure of the synaptic intermediate, which is schematized in Figure 1. The limits of the two DNA domains that will be separated by recombination are the crossover points in the *res* sites. These domains are shown as thick and thin lines. The substrate supercoils can be characterized as intradomainal or interdomainal depending on whether they are made by crossings of DNA, called nodes, within or between domains. We have argued that the mechanism of synapsis ensures that there are exactly three negative interdomainal supercoils (Wasserman *et al.*, 1985; Wasserman and Cozzarelli, 1985). During strand exchange, one positive node is introduced between the domains resulting in the observed product, a negative singly linked catenane. Any variation in the number of supercoils among substrate molecules will be entirely intradomainal and these, in contrast to interdomainal supercoils, are not converted to catenane interlinking. The degree of interdomainal supercoiling of DNA can not be observed directly, but was deduced from the structure of the singly linked catenane and several minor products of recombination. It was assumed that the minor products are derived from a single synaptic event followed by several successive strand exchanges before dissociation of the synaptic complex (Wasserman *et al.*, 1985).

We have developed a simple method for isolating the synaptic complex in quantitative yield. Topoisomerase relaxation of a resolvase-bound substrate does indeed result in a linking number difference of −3 as compared to unbound substrate. The simplest interpretation of the data is that our assumptions are correct and that resolvase stabilizes three interdomainal supercoils without appreciably altering

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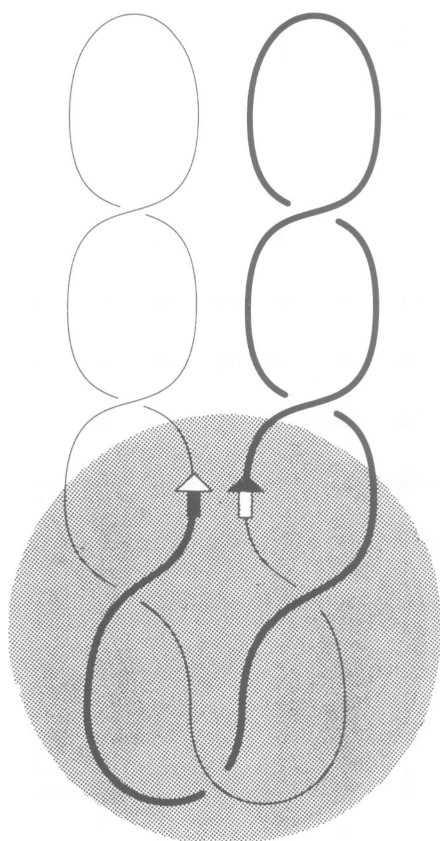

Fig. 1. Scheme for arrangement of supercoils in resolvase synaptic complex. A supercoiled resolvase substrate having two *res* sites, represented by arrows, is shown in plane projection. The two DNA domains that will be separated by recombination are defined by the thick and thin lines. Three interdomainal supercoils, those made by the crossing of domains, are surrounded by a grey circle representing resolvase. The remaining substrate supercoils are intradomainal, diagrammed as the crossing of thick over thick and thin over thin lines. The sign of supercoil crossings or nodes is dictated by the convention of Cozzarelli *et al.* (1986). The nodes shown are all (−); reversal of the position of overpassing and underpassing DNA would result in a (+) node.

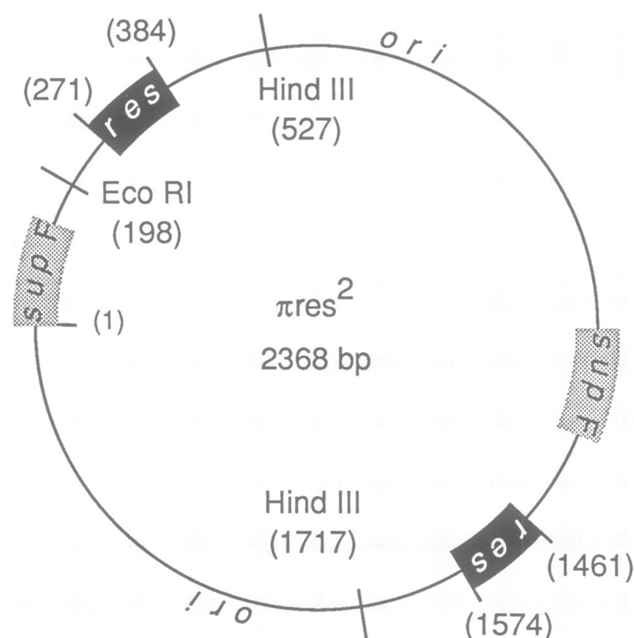

Fig. 2. Map of plasmid πres^2. The circle represents the DNA of plasmid πres^2, a dimer of πres. The two directly repeated *res* sites (black arcs), the *sup*F genes (grey arcs) and the origins of replication (*ori*) are marked. The nucleotide position for the boundaries of the *res* sites and the beginning of each restriction site is numbered clockwise from the beginning of the *sup*F gene at position 1.

intradomainal DNA structure. The synaptic intermediate forms in two steps. Resolvase first binds to each *res* site introducing ~ −0.5 supercoil per site; this may be the topological consequence of bending of the *res* site by resolvase (Hatfull *et al.*, 1987). Then the bound sites are brought together to form a nucleoprotein complex called a synaptosome, presumably by a continuous sliding motion of supercoiled DNA, termed slithering (Benjamin and Cozzarelli, 1986). Although recombination only occurs between directly repeated sites, synapsis occurs with both orientations and we propose that a third stage determines the ability of the synaptic complex to undergo recombination. Restriction enzyme mapping, electron microscopy and topological methods suggest that the *res* site DNA is packed into a hypersupercoiled structure in the synaptosome prior to the ordered process of strand exchange.

Results

Structure of the resolvase synaptic intermediate

Determination of the structure of the synaptic intermediate is critical to understanding the entire process of recombination by resolvase. It should provide evidence about the mechanism that generated the complex and about the distortion of DNA that precedes strand exchange. As an intermediate, it is expected to exist only transiently and thus two conditions had to be met to isolate amounts sufficient for structural analysis. First, recombination had to be synchronized so that the DNA−protein complex could be trapped. Second, the intermediate had to be stabilized so that it could be isolated and analyzed intact.

For stabilization, we crosslinked resolvase protomers in the complex with glutaraldehyde, a bifunctional reagent for primary amines. This reagent had been effective for stabilizing nucleoprotein complexes in the λ integration and excision reactions (Better *et al.*, 1982, 1983). Synchrony was achieved by simply lowering the reaction temperature from 37°C to 15°C. We omitted $MgCl_2$ to help ensure that recombination was minimal (Reed, 1981; J.M.Dungan and N.R.Cozzarelli, unpublished data); however, the intermediate could also be trapped in the presence of 10 mM $MgCl_2$. Under both reaction conditions, we detected almost no recombination while synapsis occurred efficiently and specifically.

The synaptic complex was first detected by the change in DNA electrophoretic mobility caused by binding of the protein. Resolvase was bound to πres^2, a very small substrate containing two *res* sites (Figure 2), for 5 min at 15°C, and the reaction was treated with 0.2% glutaraldehyde for an additional 5 min. The DNA and DNA−protein complexes were then purified by gel filtration. Resolvase bound to supercoiled πres^2 DNA had a lower electrophoretic mobility than DNA stripped of resolvase by treatment with the protein denaturant, SDS (Figure 3, lanes 1−3). The synaptic intermediate, labeled sc-Res, should have a supercoiled structure clamped together in the center. This form would be converted upon restriction at the single *Eco*RI site

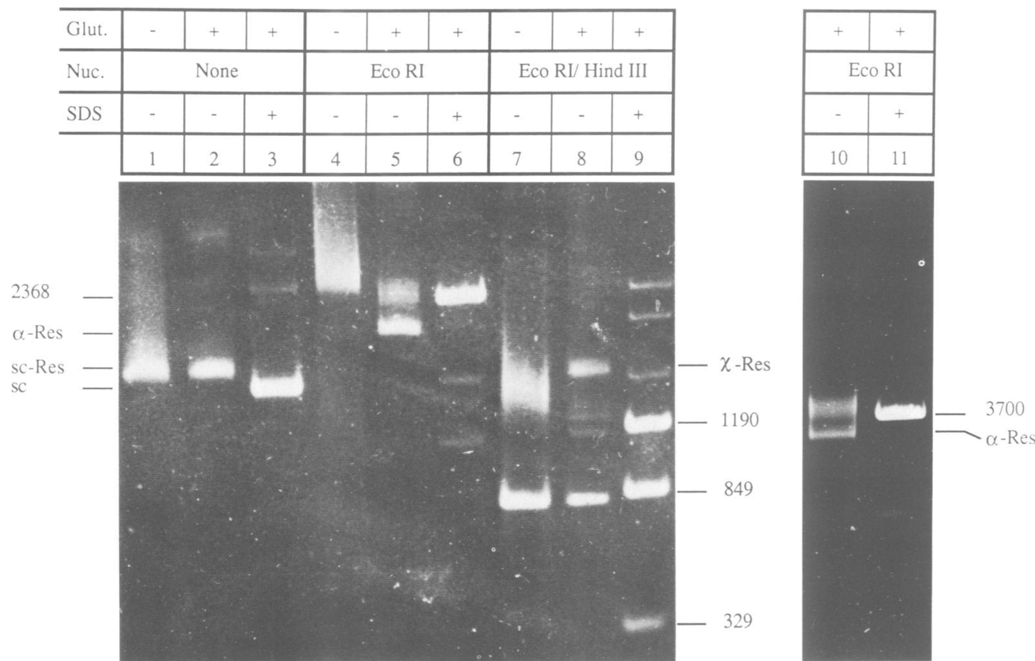

Fig. 3. Gel electrophoresis of resolvase complexes. The formation of synaptic complexes was assayed by the reduction of DNA electrophoretic mobility. Resolvase substrates with two *res* sites, πres^2 (**lanes 1–9**) and πinv (**lanes 10** and **11**) were preincubated with resolvase in binding buffer at 15°C for 5 min. Glutaraldehyde (Glut.) was added for an additional 5 min at 15°C, where noted, to stabilize the resolvase bridge that holds together synaptic complexes. After exchanging into restriction buffer by gel filtration, the DNA was cleaved with the indicated restriction endonuclease (Nuc.) prior to electrophoresis through a 0.8% agarose gel at 4°C and staining with ethidium bromide. The loading buffer contained 2% Ficoll and, where noted, SDS to disrupt complexes. Restriction sizes of unbound DNA fragments are shown along with the position of supercoiled πres^2 (sc) and supercoiled (sc-Res), α (α-Res) and χ (χ-Res) complexes having glutaraldehyde-fixed resolvase bridges. The minor species in lanes 6 and 9 are due to incomplete digestion of resolvase complexes and to a small amount of recombination (<5%).

to an 'α form' held together by resolvase, designated as α-Res. This complex migrated between supercoiled and nicked πres^2 DNA (Figure 3, lane 5). After relaxation by wheat germ topoisomerase I (data not shown), its migration was slightly slower than in nicked DNA, as expected for a complex that maintains a topologically closed domain. The control reactions, either without fixation or with SDS treatment to denature the resolvase bridge (Figure 3, lanes 4 and 6), did not show the α-Res band. The very faint recombinant bands, migrating as 1178-bp linear and as 1190-bp circular molecules (Figure 3, lane 6), indicate that <5% recombination had occurred. (The 12-bp difference in monomer size is introduced by the cloning procedure described in Materials and methods.)

The synaptic intermediate was further characterized by cutting at the single *Eco*RI site and the *Hin*dIII site in each domain. The products are an asymmetric 'χ form' consisting of 329- and 1190-bp *res* site fragments joined by resolvase and a 849-bp vector fragment (Figure 3, lane 8). The χ-Res band migrated, as expected, near the position of 1500-bp linear DNA. SDS disruption of the χ complex revealed the 329- and 1190-bp fragments in addition to the 849-bp vector piece (Figure 3, lane 9). Without fixation, the resolvase-bound DNA migrated not as a χ structure but as a smear behind unbound DNA (Figure 3, lane 7). Because the addition of SDS disrupts the supercoiled α and χ complexes, glutaraldehyde crosslinking can not form a continuous covalent linkage from *res* site to *res* site by way of resolvase; rather we infer that the synaptic intermediate is stabilized by crosslinking of proteins to proteins (Hayat, 1975).

Quantification of the experiment shown in Figure 3 and similar experiments involving resolvase substrates used in previous studies, pRR51 and pA^2 (Reed, 1981; Krasnow and Cozzarelli, 1983), showed that the amount of DNA recovered as intermediate forms sc-Res, α-Res and χ-Res varied between 40 and 100% of the substrates. Because topological analyses presented below indicate that the true yield neared 100%, we conclude that variable losses occurred during isolation and electrophoresis. The incubation temperature is critical for trapping the intermediate; at 20, 30 and 37°C, the amount of complex recovered dropped to 40%, 10% and undetectable respectively. Formation of the complex, like recombination, required supercoiling, because with nicked or linear πres^2 no synaptic complexes were detected. Formation of resolvase complexes also had the same sigmoidal dependence on resolvase concentration as recombination.

Resolvase does not promote intermolecular recombination and we do not observe intermolecular synaptic complexes with two-site plasmids. This was indicated by the absence of 658 (2 × 329) and 1698 (2 × 849) bp bands in lane 8 of Figure 3. We detected some intermolecular pairing at a low (10%) level when plasmids that contain an odd number (one or three) of *res* sites were used (data not shown). Presumably, the site in these substrates which cannot pair intramolecularly, can interact with a *res* site on another molecule, though inefficienlty. Because intermolecular recombination does not occur under these conditions, these intermolecular structures may be maintained by alternative protein–protein interactions.

πinv DNA contains two *res* sites in inverted orientation and is recombined only minimally (Grindley and Reed, 1985). None the less, after binding of resolvase, fixation with glutaraldehyde and linearization with *Eco*RI, α-Res structures were readily detected (Figure 3, lanes 10 and 11).

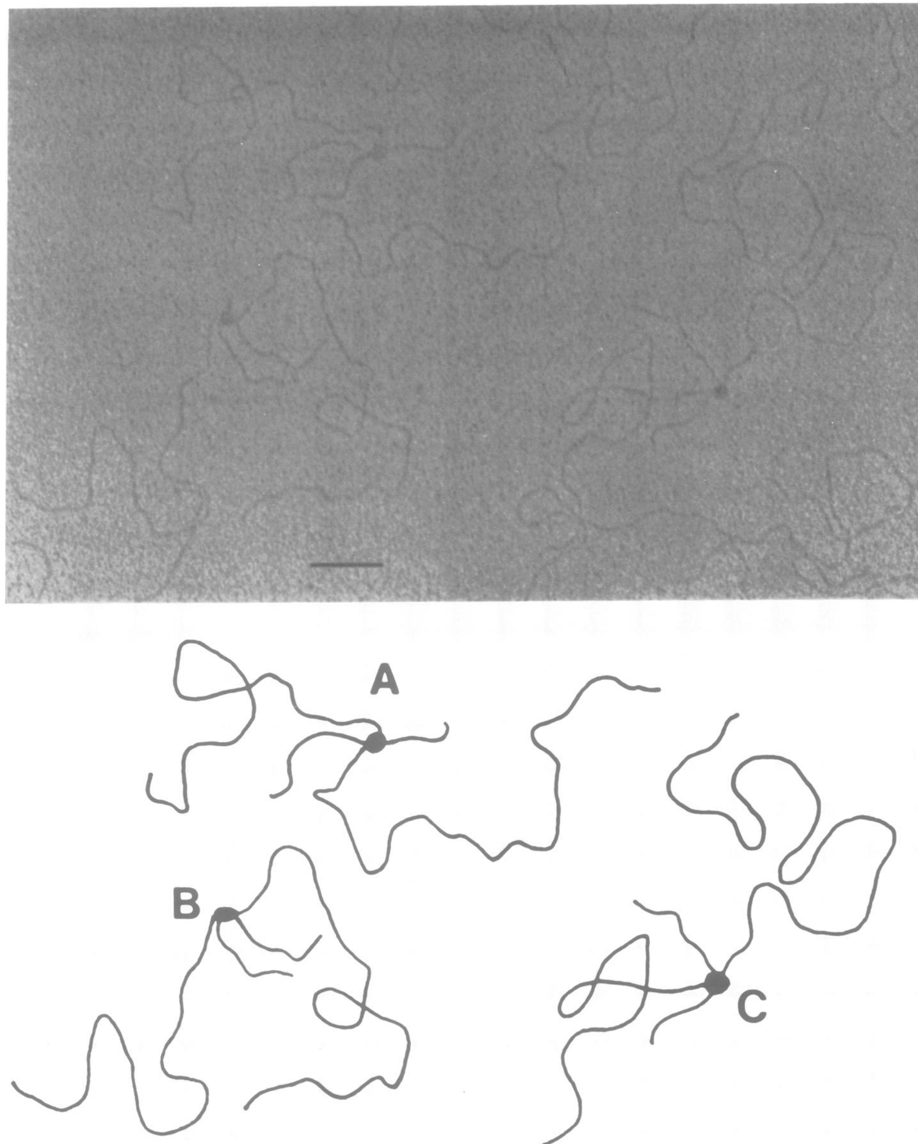

Fig. 4. Electron microscopy of resolvase synaptic complexes. χ-Res complexes of pRR51 were restricted with endonuclease *Eco*RI, purified by gel filtration, spread on a layer of polylysine on carbon-coated grids and rotary shadowed with tungsten. The electron micrograph is displayed along with a tracing of the three complete χ-Res complexes in the field. The resolvase synaptosome appears as an electron-dense mass at the intersection of four DNA arms. The two shorter arms (400 and 450 bp) in molecules A and C are separated by larger arms (2200 and 2700 bp), whereas the shorter arms are next to each other in molecule B. The bar represents 1000 Å.

These complexes are similar to those observed with $\gamma\delta$ resolvase and the inversion substrate, pRR55 (J.Salvo and N.Grindley, personal communication). Thus, the synaptic complex is also formed with inverted sites.

To summarize, synaptic complexes are trapped efficiently by lowering the reaction temperature and crosslinking the resolvase protomers wth glutaraldehyde. Two *res* sites in the same supercoiled DNA molecule are required for efficient pairing. The sites are joined by a protein bridge that separates the DNA into two topologically sealed domains that can be restricted independently, the complex is sufficiently stable that most of it survives even 10 h of gel electrophoresis at 4°C. The only notable difference between formation of the complex and recombination is that synapsis does not distinguish between direct and inverted sites.

Electron microscopy of synaptic complexes

The location of the protein bridge was identified directly by electron microscopy of fixed χ-Res and α-Res synaptic complexes. Figure 4 shows an electron micrograph of χ structures from *Eco*RI-cleaved pRR51 complexes. The resolvase synaptosomes, as we designate the nucleoprotein synaptic structure, appeared as an electron-dense mass at the junction of four DNA segments. We measured 34 shadowed synaptosomes and found it to be 155 ± 32 Å in diameter. The exact contribution of the shadow is not known, but shadowing increased the diameter of spherical 100 Å colloidal gold particles by ~70 Å. Taking into account the shadow and that the nucleoprotein complex is likely collapsed or shrunk by dehydration, our first-order approximation of the unshadowed synaptosome is a sphere 100 Å in diameter. πres^2 complexes cleaved with *Eco*RI, α forms, appeared as a supercoiled DNA domain joined to two DNA arms by a synaptosome. For 14 such α-Res complexes, the length of the arms, corrected for shadowing corresponded to 110 ± 18 and 940 ± 58 bp, whose sum is within 15 bp of the total

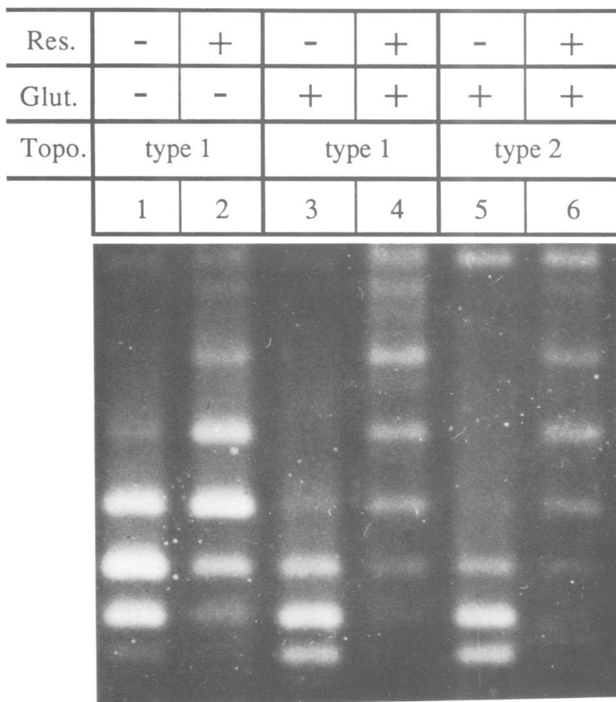

Fig. 5. Induced ΔLk of πres by resolvase binding. Complexes between supercoiled πres DNA which contains a single *res* site and resolvase (Res.) were formed and, where noted, reacted with glutaraldehyde (Glut.) and purified by filtration through a sepharose C1-4B column equilibrated with relaxation buffer. A 6-fold excess of wheat germ topoisomerase I (type-1) or T4 topoisomerase (type-2) was added and the mixture incubated at 15°C for 10 min. All samples were loaded with 0.2% SDS onto a 1.5% agarose gel containing 4.25 μg/ml chloroquine which makes the topoisomers positively supercoiled.

length of the DNA (1065) from the *Eco*RI site to the near edge of each *res* site (Figure 2). The boundaries of the *res* sites are taken as the far outsides of the inverted repeats identified by footprinting studies on linear fragments (Grindley *et al.*, 1982). The DNA arms of 20 χ-Res forms prepared by digestion of πres^2 with *Eco*RI and *Hin*dIII were also measured. The four arms are predicted to be 73, 143, 143 and 934 bp long. The observed lengths were 87 ± 18 bp for one arm, 130 ± 25 bp for two of the arms and 940 ± 110 bp for the fourth arm. The excellent agreement between predicted and observed DNA lengths demonstrates that the synaptosome is formed specifically at *res* sites.

The four arms of χ-Res complexes accounted for 1287 ± 118 out of the total of 1519 bp and thus ~232 bp are contained in the synaptosome. Because each *res* site is 113 bp long, the synaptosome must include all of the *res* site, but little if any additional DNA. Only 30 bp of B-type DNA per site would stretch straight across the diameter of the synaptosome. Thus, there is an ~4-fold compaction of DNA in the synaptosome, presumably because of extensive supercoiling. A similar condensation results from the binding of the λ recombination proteins Int and Xis to the recombination sites *att*P and *att*R (Better *et al.*, 1982, 1983).

The arms of DNA exciting the nucleoprotein complexes formed during λ recombination, as well as those formed between the Mu invertase, Gin, and its recombination sites (R.Kanaar and N.R.Cozzarelli, unpublished data), have a unique geometry. We tested whether resolvase complexes also had this characteristic. χ-Res complexes of pRR51 prepared by *Eco*RI digestion have two short arms of 400 and 450 bp arising from one *res* fragment and two long arms of 2200 and 2700 bp from the other (Figure 4). The two short arms could be either adjacent or separated by a long arm (Figure 4A and B respectively). We found that 17 out of 28 were arranged in the adjacent configuration. For χ-Res complexes generated by *Eco*RI and *Hin*dIII cleavage of πres^2, the position of the 73- and 934-bp arms was scored relative to the two 143 bp arms. The former two arms define the relationship between the sequences upstream to the *res* site to those downstream; 11 out of 18 were adjacent. Therefore, electron microscopy of the resolvase complexes, unlike those of Gin and Int, does not show a unique arrangement for the arms.

Number of supercoils protected by resolvase binding

The ability to isolate the synaptic intermediates in high yield allowed the path of the DNA to be considered by topological methods. In the model for the synaptic complex shown in Figure 1 there are three negative interdomainal supercoils. The model is based on assumptions for the origin of the minor products and does not address whether or not resolvase binding changes helical twist or other intradomainal crossings outside of the synaptic complex. If the supercoiling of the synaptic structure in Figure 1 is correct and there is no additional net change in DNA structure, then after relaxation by a topoisomerase, the DNA will have a linking number difference (ΔLk) of −3 compared to unbound DNA. For convenience, we will describe binding reactions in terms of ΔLk, even though Lk only changes after the topoisomerase treatment.

To determine the effect of resolvase binding to individual *res* sites, we first measured ΔLk with the single *res* site plasmid, πres. The very small size of πres results in a narrow distribution of topoisomers after relaxation, which facilitates an accurate measurement of ΔLk. Resolvase was bound to the DNA and relaxed by wheat germ topoisomerase I. The resulting topoisomers were separated by electrophoresis through chloroquine-containing agarose gels into a ladder of bands differing in Lk by one. The DNA is positively supercoiled by the intercalating agent, and therefore faster migrating bands are more relaxed. The mean Lk for the population of topoisomers is the center of the Gaussian distribution determined by the quantification of the DNA in each topoisomer band. The ΔLk for πres was −0.53 (Figure 5, lanes 1 and 2; Table I). The same ΔLk was obtained if the complexes were purified by gel filtration prior to relaxation with topoisomerase I. The observed change in Lk represents the limit of relaxation because neither more topoisomerase nor longer relaxation periods changed Lk further. Resolvase had no effect on the Lk of a control plasmid, pBR322, which lacks *res* sites.

Experiments with multi *res* site plasmids were then performed. Relaxation of πres^2 and πinv bound by resolvase resulted in a ΔLk of −1.09 and −1.03 respectively, whereas πres^3 with three directly repeated *res* sites, showed a change of −1.85 (Figures 6 and 7, lanes 1 and 2; Table I). Therefore, resolvase binding at each *res* site leads to a ΔLk, on average of −0.55 ± 0.09 in a simple additive manner.

The results were dramatically different when the synaptosome was stabilized by glutaraldehyde treatment. Although ΔLk was −0.45 for πres, it increased to −3.07 for πres^2

Table I. Induced ΔLk by resolvase binding

Relaxation method	Type-1 topoisomerase				Type-2 topoisomerase				Nicking and ligation		
Substrate	πres	πres^2	πinv	πres^3	πres	πres^2	πinv	πres^3	πres	πres^2	πres^3
No fixation	−0.53 ±0.15 (3)	−1.09 ±0.11 (6)	−1.03 ±0.25 (2)	−1.85 ±0.36 (2)	ND	ND	ND	ND	−0.47 ±0.09 (2)	−1.13 ±0.37 (4)	−1.70 ±0.1 (3)
Fixation	−0.45 ±0.02 (2)	−3.07 ±0.26 (7)	−2.87 ±0.25 (6)	−3.45 ±0.36 (2)	−0.53 ±0.10 (2)	−2.96 ±0.19 (5)	−2.91 ±0.12 (5)	−3.34 ±0.20 (3)	ND	ND	ND

The DNA substrates were bound by resolvase, fixed with glutaraldehyde, where indicated, and relaxed by the method shown. The difference in linking number of resolvase bound and unbound DNA is shown along with standard deviations. The sample size is in parentheses. ND, not determined.

(Figure 5 and 6, lanes 3 and 4; Table I). These supercoils must be stabilized by direct binding of resolvase because all three also resisted relaxation (ΔLk = −2.96) by the type-2 topoisomerase of phage T4 (Figure 6, lanes 5 and 6; Table I). If these supercoils were merely constrained by a bridge between the crossover sites but were otherwise exposed to the topoisomerase, then they should be topologically equivalent to knot crossings and the double-strand passage activity of T4 topoisomerase should remove them. Because the distributions of topoisomers after type-1 topoisomerase treatment were slightly broader than those obtained with the type-2 enzyme, a small number of unbound but constrained supercoils may be infrequently trapped.

To test whether the number of trapped supercoils reflect a true intermediate or were an artifact of the small plasmid size used, we examined the ΔLk of fixed complexes with pRR51 and pA^2, both ~6 kb. Although the accuracy of ΔLk meausrements for these plasmids is limited, the observed values of ~ −3 are in good agreement with the πres^2 results and clearly preclude entrapment of interdomainal supercoils by random synapsis of *res* sites (Benjamin and Cozzarelli, 1986). πres^3 showed a shift of −3.45 and −3.34 after treatment with type-1 and type-2 topoisomerases respectively (Table I), again a change of ~2 negative supercoils from the unfixed control.

The synaptic complex of πinv which has two inverted *res* sites protected −2.87 supercoils from relaxation, a result similar to that obtained with πres^2 complexes (Figure 7, lanes 5 and 6; Table I). As discussed below, this implies that the crossover sites are mispaired in the πinv synaptic complex, in contrast to the proper parallel orientation in direct site plasmids.

Supercoiling is required for synaptosome formation. To determine if it is necessary for the assembly of complexes at individual *res* sites, we measured ΔLk by an alternative method in which the substrate DNA was relaxed. Resolvase was bound to nicked DNA, and the interruptions were then closed with DNA ligase. We found that ΔLk was −0.56 ± 0.14 times the number of *res* sites, the same after treatment with type-1 topoisomerase of unfixed complexes (Table I). We conclude that supercoiling is not needed for resolvase assembly at each individual site but that it is required for their juxtaposition into the synaptosome.

Discussion

We have described the isolation and characterization of the resolvase synaptosome, a structural and functional entity that is a key intermediate in recombination. It was obtained in essentially quantitative yield and characterized by gel electrophoresis, electron microscopy and linking number analysis. The results from these three techniques provide a clear description of the synaptic structure and suggest a mechanism for its assembly and the recognition of *res* site orientation.

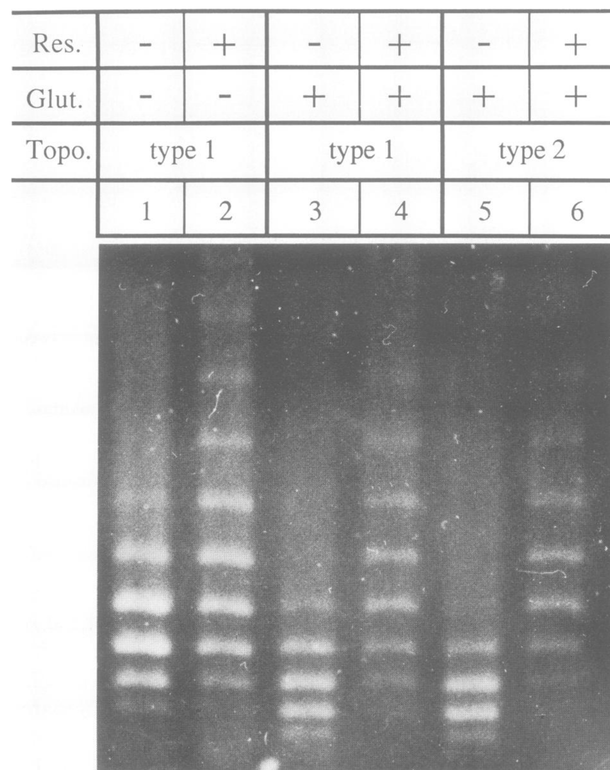

Fig. 6. Induced ΔLk of πres^2 by resolvase binding. The experiment was the same as in Figure 5 except the DNA substrate was πres^2 and the gel was 1.3% agarose. Because the substrate contains two *res* sites, a synaptic complex was formed that, when stabilized by glutaraldehyde crosslinking, caused a large change in linking number (**lanes 3–6**).

Resolvase binds to each *res* site to form a specialized nucleoprotein structure [a 'snup' (Echols, 1986)], and two snups come together to form a synaptosome. The resolvase snups are stable and form readily even with nicked (Table I) and linear DNA (J.Salvo and N.Grindley, personal communication). In contrast, the synaptosome is transient as befits a reaction intermediate. Synaptosomes were trapped by lowering the reaction temperature and treating with a protein crosslinking reagent, namely glutaraldehyde. Their efficient formation required that the two interacting *res* sites

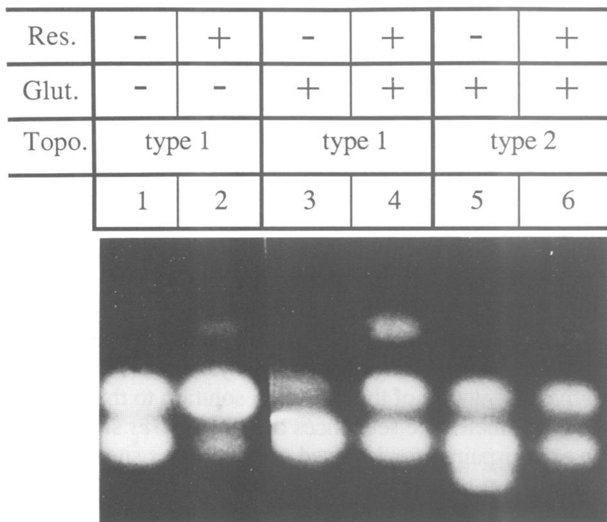

Fig. 7. Induced ΔLk of πinv by resolvase binding. The experiment was the same as in Figure 5 except the DNA substrate was πinv, which has two inverted *res* sites, and the gel was 1.1% agarose.

were on the same supercoiled molecule. Because SDS destroys the synaptosome, we conclude that glutaraldehyde crosslinking occurs betwen resolvase protomers and not between resolvase and the DNA. This protein bridge separates the substrate into two topologically sealed, supercoiled domains. Length measurements by electron microscopy limit the total DNA in the resolvase synaptosome to ~232 bp, the size of two 113-bp *res* sites. Thus the additional supercoiling upon juxtaposition of the individual snups does not require the recruitment of additional DNA. The many properties shared by recombination and synaptosome formation imply that glutaraldehyde fixation at low temperature preserves a true intermediate. These include the specificity for two intramolecular *res* sites and for substrate supercoiling, and the same sigmoidal dependence on enzyme concentration. Moreover, the ΔLk upon binding to individual *res* sites is unaffected by fixation.

The synaptosome was shown by electron microscopy to be an ~100-Å spherical mass at the intersection of four DNA arms. The arms of χ structures formed by restriction in each domain did not radiate in a unique order, unlike the complexes formed by Int and Gin. This is probably not a result of a lack of a specific geometry for the synaptosome because its topology and that of the exchange process are strictly determined. More likely, the four arms are arranged in some specific fashion, e.g. tetrahedrally, but depending on the orientation of the synaptosome with respect to the surface of the electron microscope grid, alternative configurations of the arms result. Perhaps the Int and Gin complexes have a more planar structure that limits the observed projections.

We measured the change in linking number, ΔLk, resulting from resolvase binding by relaxing free supercoils with a topoisomerase. The ΔLk for binding to individual *res* sites averaged −0.5, but for the synaptosome it was −3. Previous data had indicated that formation of the synaptic complex introduced exactly three negative interdomainal supercoils. Because ΔLk is also −3, we conclude that synaptosome formation stabilizes −3 interdomainal supercoils and has no other net effect on the structure of DNA.

Synaptosome formation does not simply preserve the typical supercoiled DNA structure of the substrate. On the contrary, the DNA in the synaptosome is under increased tension. This has two manifestations. Topologically, the supercoil density in the synaptosome ($\sigma = -0.14$) is 2.5 times that of typical substrates ($\sigma = -0.06$) due to the stabilization of three negative supercoils over only 232 bp of DNA. Concomitantly, supercoil density outside of the synaptosome is reduced (from a σ of −0.06 to −0.05 in the case of the small πres^2 plasmid). Therefore, synaptosome formation is predicted to be favored by negative supercoiling. In fact it is the formation of the synaptosome which requires negative supercoiling rather than snup formation at individual *res* sites that does not decrease supercoiling density outside of the snup. The second manifestation of severe distortion in the synaptosome is geometric: 232 bp of B-type DNA extends over 795 Å and the synaptosome diameter is only 100 Å. We will show elsewhere that the supercoils in the synaptic complex are wound plectonemically (H.W.Benjamin and N.R.Cozzarelli, in preparation) and Boocock *et al.* (1987) have proposed a model for resolvase synapsis that involves a very specific interwinding of the DNA. Plectonemic winding of the *res* site DNA does compact it, but not enough to give the observed packing. 232 bp of plectonemically supercoiled DNA with $\sigma = -0.06$ has a form that can be described as a cylinder ~320 Å long and 110 Å wide (T.Boles, J.White, and N.R.Cozzarelli, unpublished data). The dimensions of supercoiled DNA with a σ value of −0.14 have not been measured, but probably have a reduced width but not length. Thus, we suggest that additional compaction in the synaptosome is due to a supercoiling of the supercoiled *res* site DNA. The nature of the higher order coiling is unknown. We note, however, that if the bending of the U-shaped plectonemic axis, shown in Figure 1, is continued so that the *res* sites cross, then the necessary compaction and roughly tetrahedral orientation of the exciting DNA can be achieved. We presume that one function of the severe distortion of DNA in the synaptosome is to favor breakage and reunion by stabilizing a DNA form closer to the transition state.

Several models have been proposed to explain the specificity of resolvase recombination. An early protein-directed synapsis model, looping, proposed that resolvase tracks from *res* site to *res* site with continuous contact to the DNA (Krasnow and Cozzarelli, 1983; Kitts *et al.*, 1983). Looping restricted recombination to two intramolecular directly repeated *res* sites and limited interdomainal supercoils. Subsequent experiments involving the segregation pattern of DNA rings to the substrate demonstrated that resolvase could not maintain a loop by continuous contact (Benjamin *et al.*, 1985). As a result, models that invoke the structure of supercoiled DNA to direct recombination have been proposed. The 'plectosome' model (Craigie and Mizuuchi, 1986) proposes that the plectonemic structure of supercoiled DNA affords a unique geometry that certain recombinases can recognize, but only when proper site alignment is present. In our model, the continuous motion of plectonemic DNA about itself, called slithering, produces as its endpoint a plectosome. This model explains both resolvase specificity and certain features of phage λ recombination (Benjamin and Cozzarelli, 1986). Boocock *et al.* (1987) proposed as a mechanism a topological filter based

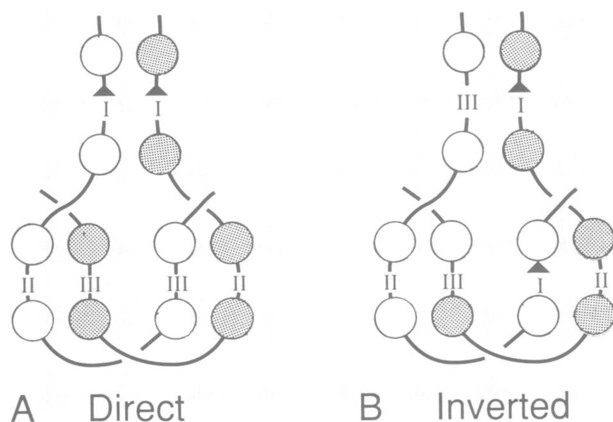

Fig. 8. Scheme for distinguishing direct from inverted *res* site orientation from within synaptic complexes with similar geometries. In these schematic representations of the synaptic complexes formed between direct (**A**) and inverted (**B**) *res* sites, only the region of DNA wrapped around resolvase is drawn. See Figure 1 for the entire molecule. The three tandem sets of inverted consensus sequences within each *res* site (labelled I, II and III) are bound by resolvase and are shown as pairs of circles; the circles of one *res* site are shaded. The asymmetric sequence between the consensus sequences for subsite I, where crossover occurs, is represented by an arrow which defines the direction of the *res* site. As long as only the resolvase binding to the consensus sequence is considered, the overall geometry of the complexes is basically identical for direct (A) and inverted (B) *res* sites. Both have all six consensus sequences paired and have entrapped three negative supercoils. However, when binding to the DNA in the crossover sequence between the repeats in subsite I is considered, then only for direct site substrates are the subsites I paired; their alignment is parallel as required by the properties of resolution. Subsites II and III are also paired with each other in this case, but the differences in their asymmetric sequences between the consensus sequences is either not recognized or not structurally important because neither has a competent crossover sequence. For inverted *res* sites, the position in which crossover normally takes place in subsite I is now aligned with II or III and therefore no recombination occurs.

on the unfavorable tangling of the DNA that is required to synapse non-recombining sites into a productive complex. All three models predict the same endpoint structure, but in the plectosome and topological filter models it is the essential feature. Our present results are in agreement with all of these DNA-directed synapsis models. We find that supercoiling is specifically required to juxtapose the snups into a synaptosome, a prerequisite of the plectosome and slithering model. Moreover, the hypersupercoiling in the synaptosome provides a concrete explanation for why the correct configuration is energetically favored by negative supercoiling of the substrate DNA. One may speculate that synaptosome formation for more permissive recombinases such as Int might not involve hypersupercoiling or be compensated by contacts within the synaptosome.

The formation of a snup by the binding of, presumably, six protomers of resolvase to a *res* site (Grindley *et al.*, 1982) produced a ΔLk of -0.5 per *res* site. This same value was obtained with four different plasmids (πres, πres^2, πres^3 and πinv) containing one, two or three sites in either direct or inverse orientation. For single site plasmids, a ΔLk of -0.5 was observed with or without fixation and using three methods of relaxation. Given this constancy, we suggest that -0.5 is the true ΔLk for snup formation at individual sites but we cannot eliminate the possibility that it reflects only the most stable binding interactions or the average ΔLk for a mixed but similar population. However, the fact that ΔLk is not an integer does not imply heterogeneity in snup structure. Although Lk must be integral for individual molecules, Lk for a population will usually not be. Two molecules with exactly the same writhe induced by binding of resolvase could have a different ΔLk because of variations in twist after relaxation.

Since inverted *res* sites do not recombine, the mechanism of synapsis could have proscribed their interaction. We have shown, however, that inverted sites form a synaptosome with the same ΔLk as with direct sites. This result was initially puzzling because all recombination systems distinguish the relative orientation of their sites. A solution to this paradox is that the nucleotide sequences that specify *res* site orientation are mispaired for inverted sites. The *res* site consist of three tandem subsites: I, II and III (Grindley *et al.*, 1982; Kitts *et al.*, 1983). These subsites, in turn, are made up of short inverted consensus sequences to which resolvase binds; each inverted repeat surrounds a unique asymmetric sequence that distinguishes the subsites from each other. Crossover occurs only in the asymmetric sequence of subsite I, whose orientation thus defines the orientation of the entire *res* site. Optimum protein–protein interactions in the synaptosome require that all three bound subsites are paired. This is shown for two direct *res* sites in Figure 8A; three negative supercoils are entrapped by the pairing of subsites I, II and III of one *res* site with subsites I, III and II in the other *res* site respectively. However, in order to get the three negative supercoils entrapped between inverted *res* sites, subsites I, II and III are now paired wtih subsites III, I and II respectively (Figure 8B). We suggest that the failure to pair subsites I prevents initiation of strand exchange but that synapsis is facilitated solely by orientation independent protein–protein interactions between resolvase-bound consensus sequences. Thus each paired tetramer of resolvase protomers bound to the DNA has a 2-fold rotational symmetry which reflects the 2-fold symmetry of the inverted repeat DNA sequence (Grindley *et al.*, 1982).

The argument is easily generalized to other site-specific recombination systems whose sites consist of a single inverted consensus sequence surrounding an asymmetric sequence (Sadowski, 1986). Here orientation specificity is based solely on the spatial relationship of the asymmetric sequence (parallel or antiparallel) because the dyad symmetry of the consensus sequences prevents specification of orientation. Thus, if synaptosomes for direct and inverse sites have the same number of entrapped supercoils, then only one them will have the spatial relationship which triggers exchange.

We conclude that resolvase recombination is divided into three stages. In stage one, the *res* sites are identified because of the strong selective binding of resolvase to the consensus sequences. A snup is formed and the DNA is bent, producing a ΔLk of -0.5. Stage 2 is the dimerization of these snups by protein–protein interactions to form the synaptosome, in which the DNA is under increased tension. In stage 3, the resolvase interaction with the crossover region in subsites I comes into play, and if the regions are properly paired in parallel, strand exchange results. It is not until this stage that inversion is proscribed, but intermolecular recombination is disfavored in stage 2.

Materials and methods

Enzymes

Tn3 resolvase was purified as described (Krasnow and Cozzarelli, 1983). T4 type-2 topoisomerase was a gift of B.Alberts at the University of California, San Francisco. Restriction enzymes were purchased from New England BioLabs and wheat germ type-1 topoisomerase was purified as described (Dynan *et al.*, 1981).

Strains

C1792, an *E.coli* C strain obtained from R.Calender at the University of California, Berkeley, is Sup^+, His^- and contains *arg* and *trp* amber mutations. It was made Sup^- to allow selection of πVX plasmids that contain a tRNA suppressor of amber mutations, *sup* F (Seed, 1983). A spontaneous mutant of C1792 to Sup^- was identified among survivors to plating with two different amber λ phage (Templin *et al.*, 1978). This strain, HWB126, was made $RecA^-$ by P1 transduction from strain GW5181 (a gift of G.Walker at MIT) that contains a gene conferring chloramphenicol resistance cloned into *rec*A. The resulting strain, HWB189, was transformed with πVX plasmids and maintained in minimal media with 0.2% glucose, 15 μg/ml histidine and 30 μg/ml chloramphenicol.

DNA

Very small resolvase substrates were constructed starting with plasmid πBeb obtained from K.Kreuzer at Duke University, which is πAN7 (a πVX derivative) containing an insert from phage T4 DNA cloned into the *Eco*RI and *Bam*HI sites of the polylinker (Seed, 1983). An insert in πAN7 greatly increases plasmid yields. The 303-bp *Sal*I−*Sau*3AI fragment from pRR51 (Reed, 1981), containing a *res* site, was cloned into the *Sal*I and *Bgl*II restriction sites of the polylinker of πBeb. The T4 DNA insert was then removed by cleavage with *Bam*HI, filling in of the ends with the Klenow fragment of DNA polymerase I, addition of *Eco*RI linkers, cutting with *Eco*RI and religation. This 1178-bp plasmid, πres, which has recovered both the *Bam*HI and *Eco*RI sites, is essentially πAN7 with a *res* site insertion and can still be obtained in high yields. A head-to-tail dimer of πres generated *in vivo* was isolated, and one *Eco*RI site was deleted by partial cutting with *Eco*RI, filling in of the ends, ligation of a *Pst*I linker that does not restore the *Eco*RI site and reclosure. The resulting 2368-bp plasmid, πres^2, is an exact dimer of πres except for this 12-bp insertion. Its structure is shown in Figure 2. πres^2 was then itself dimerized *in vivo* creating a tetramer. One of its two *Eco*RI sites was deleted by partial *Eco*RI digestion and addition of two single-stranded 15-mers that are complementary to the cohesive *Eco*RI ends but do not re-create the *Eco*RI site. The remaining 11 nucleotides contain a *Bgl*I site found at position 3482 of pBR322 and allow cyclization of the tetramer to create πres^4 (4747 bp) having one *Eco*RI site, two *Pst*I sites and a *Bgl*I site. πres^3 (3357 bp) was derived by transformation of resolvase recombinants of πres^4 into HWB189 and screening for trimers that contained a single *Eco*RI, *Pst*I and *Bgl*I site.

πinv, which has two *res* sites in inverted orientation, was constructed from πres and pRR51. πres was cleaved at the *Xmn*I site and, after *Pvu*I linkers were added, the DNA was digested with *Pvu*I and *Eco*RI. An *Eco*RI−*Pvu*I fragment from pRR51 that contains a single *res* site and the tetracycline resistance gene was then inserted resulting in a 3789-bp plasmid. Transformants were selected for tetracycline resistance.

Reactions

To bind resolvase to DNA, 5−50 μg/ml of enzyme was incubated with DNA at a mass ratio of 0.5 for 5 min at 15°C in a mixture containing 20 mM triethanolamine−HCl (pH 7.5), 150 mM NaCl and 5 mM EDTA. At 37°C and in the presence of 10 mM $MgCl_2$, this ratio produces maximal recombination. Crosslinking reactions were further incubated for 5 min at 15°C with 0.2% glutaraldehyde (Polyscience, Warrington, PA, EM grade) from freshly opened vials. Complexes, unfixed or fixed with glutaraldehyde, that were to be analyzed by restriction digestion were first purified at 4°C by gel filtration through 300-μl CL-4B (Pharmacia) columns, equilibrated with 20 mM triethanolamine−HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$ and 50 μg/ml bovine albumin. For ΔLk analyses, unfixed complexes were relaxed with topoisomerase I directly in binding buffer, whereas glutaraldehyde-fixed complexes were purified by gel filtration prior to relaxation. Albumin was omitted if complexes were to be examined by electron microscopy. For subsequent T4 topoisomerase reactions, the columns were run with 20 mM triethanolamine−HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 50 μg/ml albumin and 1 mM ATP. Endonuclease digestions were at 37°C for 10 min and contained excess enzyme; topoisomerase relaxations were at 15°C for 5−10 min.

Gel electrophoresis and DNA quantification

DNA−protein complexes were separated from unbound DNA by electrophoresis at 75 V on vertical agarose gels (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA). Samples were loaded at 4°C in restriction buffer plus 2% Ficoll-400, 0.02% bromophenol blue and 0.02% xylene cyenol. To disrupt complexes, 0.2% SDS was included in the loading buffer. Topoisomers were resolved by electrophoresis on horizontal agarose gels (50 mM Tris base, 30 mM glacial acetic acid, 10 mM EDTA) at 40 V after addition of loading buffer containing SDS. DNA quantization was achieved by densitometric tracings of autoradiograms or photographic negatives of gels stained with ethidium bromide. The center of the topoisomer distribution was determined either by summing the total DNA in each band multiplied by its relative topoisomer number and dividing by the total DNA in the distribution or by computing the center of the best-fit Gaussian distribution; the results of the two methods were indistinguishable.

Electron microscopy

The DNA−resolvase complexes purified by gel filtration were examined with a JEOL 100B electron microscope. The samples were spread on carbon-coated grids that had been glow discharged and layered with polylysine (Williams, 1977). All samples were stained with uranyl acetate and rotary shadowed with tungsten to allow both DNA and bound proteins to be visible. The length of the tungsten shadow was estimated to be 30−40 Å from the difference in radius of shadowed and unshadowed colloidal gold particles (M.Dodson, personal communication). We have added 10 bp to the measured lengths of DNA arms emanating from a synaptic complex to correct for the ~35-Å length of the shadow.

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