

Site-Specific Relaxation and Recombination by the Tn3 Resolvase: Recognition of the DNA Path between Oriented *res* Sites

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Summary

We studied the dynamics of site-specific recombination by the resolvase encoded by the Escherichia coli transposon Tn3. The pure enzyme recombined supercoiled plasmids containing two directly repeated recombination sites, called *res* sites. Resolvase is the first strictly site-specific topoisomerase. It relaxed only plasmids containing directly repeated *res* sites; substrates with zero, one or two inverted sites were inert. Even when the proximity of *res* sites was ensured by catenation of plasmids with a single site, neither relaxation nor recombination occurred. The two circular products of recombination were catenanes interlinked only once. These properties of resolvase require that the path of the DNA between *res* sites be clearly defined and that strand exchange occur with a unique geometry. A model in which one subunit of a dimeric resolvase is bound at one *res* site, while the other searches along adjacent DNA until it encounters the second site, would account for the ability of resolvase to distinguish intramolecular from intermolecular sites, to sense the relative orientation of sites and to produce singly interlinked catenanes. Because resolvase is a type 1 topoisomerase, we infer that it makes the required duplex *bdNA* breaks of recombination one strand at a time.

Introduction

A variety of topological changes and primary structure rearrangements can occur during a transient interruption of the DNA backbone, with profound regulatory as well as structural consequences. Since the integrity of the DNA is jeopardized during breakage and reunion, these alterations must be carefully and efficiently executed. Topoisomerases are the best characterized of the enzymes that break and rejoin DNA in a concerted fashion (reviewed by Wang and Liu, 1979; Cozzarelli, 1980; Gellert, 1981). They catalyze the interconversion of various topological isomers of DNA by passing a DNA segment through an enzyme-bridged interruption of a second DNA segment. Type 1 topoisomerases transiently break one DNA strand, and type 2 enzymes coordinately break both strands of the helix (Liu et al., 1980). Topoisomerases remain bound to the broken ends during strand passage; one of the bonds is covalent and stores the energy of the

broken phosphodiester bond, allowing resealing of the break in the absence of an external energy source. This mechanism ensures that topological alterations are conservative, quantized and controlled.

We report a study of site-specific recombination, a breakage and reunion reaction that changes primary as well as tertiary structure. Recombination is a much more complicated process than topoisomerization, requiring pairing of the two recombination sites (synapsis), four strand scissions, coordinated rearrangement of the eight broken ends and four ligation events. The entire set of reactions, however, can be performed in vitro with small pure proteins, a divalent or polyvalent cation and no other cofactor. The first in vitro recombination system modeled the site-specific integration of phage λ DNA into host DNA and excision of the prophage (reviewed by Nash, 1981; Echols and Guarneros, 1983). Integration requires the Int protein of λ and integrative host factor of Escherichia coli, whereas the reverse reaction of excision requires in addition the phage protein Xis. Both intermolecular and intramolecular recombinations are efficient, and the intramolecular reactions are insensitive to the relative orientation of the recombination sites, called *att* sites. When the *att* sites are present in a head-to-tail configuration in a supercoiled plasmid, recombination generates two catenated daughter rings. Int alone cannot mediate recombination, but does bind specifically to the *att* sites and can relax supercoiled plasmids. Curiously, the topoisomerase activity does not require *att* sites.

Recently, site-specific recombination has been achieved with just the purified resolvase encoded by the $\gamma\delta$ transposon (Reed, 1981). Transposition of members of the Tn3 family such as $\gamma\delta$ involves two sequential breakage and reunion events (Shapiro, 1979; Heffron, 1983). The first is a replicative and nonhomologous recombination that duplicates the transposon and a 5 bp sequence at the site of insertion and links the donor and recipient DNA via Tn3 bridges. The second event, carried out by resolvase, is a reciprocal recombination at the resolution, or *res*, site of the directly repeated transposons that restores the original arrangement of donor and recipient, except Tn3 now interrupts the recipient sequence as well. Purified $\gamma\delta$ resolvase efficiently converts a supercoiled plasmid containing *res* sites in a directly repeated orientation into catenated recombinant rings (Reed, 1981). Unlike the λ reactions, resolvase does not efficiently recombine sites in a head-to-head orientation. The energy of the phosphodiester bonds broken during recombination is stored in a covalent complex between resolvase and the DNA (Reed and Grindley, 1981), as for topoisomerases.

We have purified the resolvase of Tn3 from an overproducing strain. We discovered that it is a strictly site-specific topoisomerase, the first of its kind. In both relaxation and recombination, resolvase distin-

guishes both the relative orientation of *res* sites separated by thousands of base pairs and intramolecular from intermolecular *res* sites, and in recombination it produces singly linked catenated product rings. These features demand that both the geometry of the DNA at synapsis and the dynamics of strand exchange be rigidly defined.

Results

Resolvase Purification and Recombination Activity

We prepared large quantities of pure resolvase from an overproducing strain. Since resolvase is an autogenous repressor (Chou et al., 1979; Gill et al., 1979), the resolvase promoter was replaced with the P_L promoter of phage λ using standard cloning techniques. P_L is an exceedingly strong promoter that is conveniently controlled by the thermolabile repressor, cI857. When cells containing the recombinant plasmid were induced for 90 min at 42°C, 5% of the cellular protein was resolvase (Figure 1).

Resolvase was purified by two related procedures. Native resolvase precipitates unless the ionic strength is high (Reed, 1981), and one procedure involved chromatography in the presence of denaturing levels of urea and renaturation of resolvase by dialysis. The second procedure maintained resolvase in its native form throughout; both preparations had the same specific activity. Details of the purification are described in the Experimental Procedures, and samples from various stages are displayed by gel electrophoresis in Figure 1. Sixty milligrams of homogeneous resolvase was prepared from 150 g of cells.

The purified Tn3 resolvase, like the related $\gamma\delta$ enzyme, efficiently recombines DNA. Incubation of resolvase with pRR51, a 5800 bp plasmid containing two directly repeated *res* sites, generated two catenated daughter rings, mono51A (3200 bp) and mono51B (2600 bp). Recombination was detected by treatment of the reaction products with endonuclease Bam HI, which cuts the starting material and smaller recombinant once, thus severing the linkage of the daughter circles (Figure 2A). Recombination was linear with time for the first half of the reaction and then gradually reached a plateau of about 65% recombinants (Figure 2B, bottom), a level that was not increased by fresh addition of resolvase. The dependence on enzyme concentration was sigmoidal, but about 15 resolvase promoters were required for each reciprocal recombination event. The large amounts of enzyme were not required to favor formation of a free resolvase multimer, because at constant enzyme concentration raising the concentration of DNA actually decreased the number of molecules that recombined (data not shown). The critical factor is the ratio of enzyme to *res* sites, because raising the resolvase concentration to reestablish the original ratio restored the fraction of molecules that recombined. Thus, like several other recombination proteins, RecA, Int, Xis

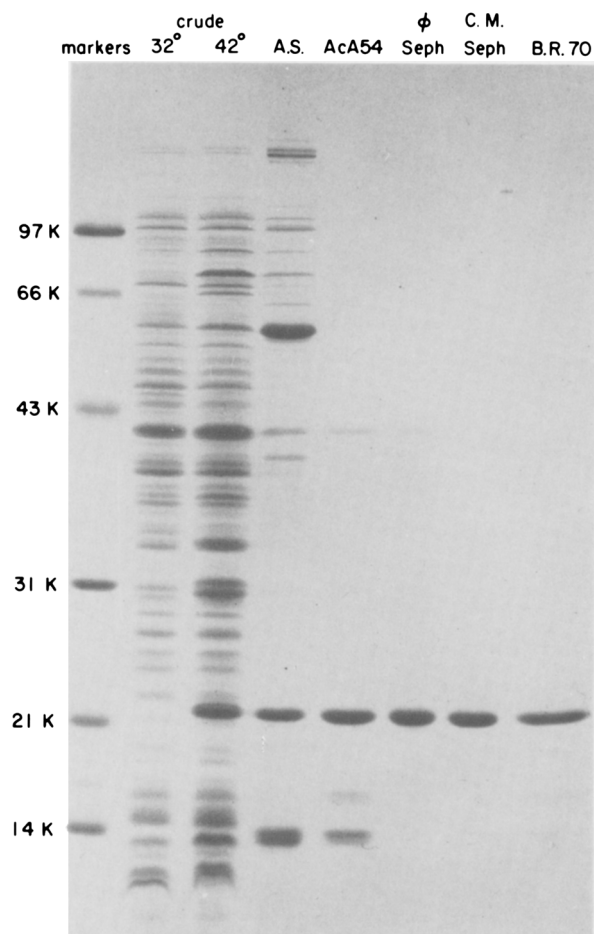


Figure 1. SDS Gel Electrophoresis of Resolvase Purification Fractions

Tn3 resolvase was purified by two procedures identical through the AcA54 sizing column as described in the Experimental Procedures. Samples containing 4 μ g of resolvase from various stages of the purification were analyzed by electrophoresis through a 15% polyacrylamide gel containing SDS (Laemmli, 1970) and stained with Coomassie blue. (Lane crude 32°) Cells grown at 32°C just before induction; (lane crude 42°) cells after induction at 42°C for 90 min; (lane A.S.) ammonium sulfate fraction; (lane AcA54) Ultrogel AcA54 pool; (lane ϕ Seph) phenyl-Sepharose CL-4B pool from purification procedure B; (lane C. M. Seph) CM-Sepharose CL-6B pool from procedure B; (lane B.R. 70) Bio-Rex 70 pool from procedure A; (lane markers) molecular weight standards of phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme, in order of decreasing size. The molecular weight of each standard is given at the left. Resolvase migrates slightly slower than soybean trypsin inhibitor.

and integrative host factor, stoichiometric levels of resolvase are required (Shibata et al., 1979; McEntee et al., 1979; Nash and Robertson, 1981; Abremski and Gottesman, 1982; Reed, 1981).

Resolvase Is a Topoisomerase

Recombination requires that resolvase break two DNA segments and rearrange the broken ends before rejoining. Topoisomerases also transiently break DNA, but they must pass a second DNA segment through a

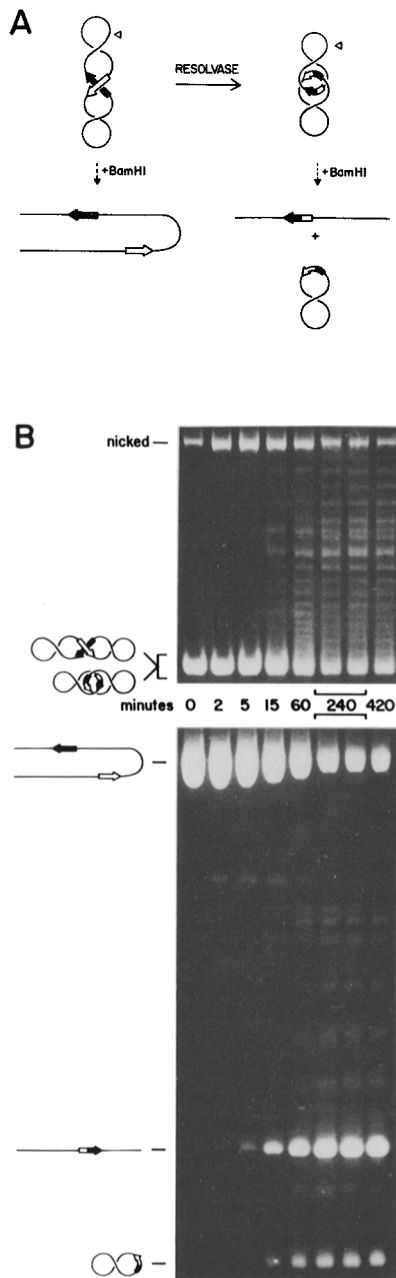


Figure 2. Time Course of Relaxation and Recombination by Resolvase

(A) Diagram of the recombination assay. Resolvase-mediated recombination of supercoiled plasmid pRR51, containing directly repeated *res* sites, generates the catenated recombinant rings mono51A and mono51B. Restriction at the unique Bam HI site (open triangle) linearizes the parental ring and mono51B, thus disrupting the interlinking of the products. The *res* sites are drawn as open and filled arrows for ease of visualization.

(B) Time course of relaxation (top) and recombination (bottom). pRR51 was incubated with resolvase under standard conditions. Samples were removed at the times indicated, and either quenched with 0.4% SDS to monitor relaxation (top) or first treated with excess Bam HI nuclease to monitor recombination (bottom). The material in the righthand lane of the 240 min reactions was treated with proteinase K after addition of SDS. The material was analyzed by electrophoresis through 1% agarose gels. The position of starting material and products is indicated; the twisted closed curves show the position

single break and rejoin the broken ends in their original configuration. To determine whether resolvase is also a topoisomerase, the reaction products were not treated with Bam HI nuclease but analyzed directly by electrophoresis. A ladder of slowly migrating bands was generated with the same time course as recombination (Figure 2B, top). Most of the bands comigrated with relaxed topoisomers of the parental plasmid generated by treatment with *E. coli* DNA topoisomerase I. It was unclear, however, whether the slowly migrating bands were catenated recombinants that had lost various numbers of supercoils during recombination, or whether resolvase is an authentic topoisomerase that had relaxed the parental plasmid. We used two-dimensional gel electrophoresis to decide between the alternatives. The products of a resolvase reaction were subjected to electrophoresis, and the DNA was digested with Bam HI in situ after excision of the lane from the gel. Electrophoresis in a second dimension distinguishes parent from recombinants because Bam HI unlinks the catenated products and greatly increases their mobility. Each discrete band in the first dimension resulted in a spot in the second dimension with the mobility of linear pRR51 substrate, showing that resolvase had relaxed the parental ring (Figure 3).

A diffuse set of spots with the mobility of the linear recombinant in the second dimension and retarded mobility in the first dimension indicated that the recombinant catenanes had also lost supertwists. These spots were not aligned with the discrete bands in the first dimension, but probably correspond to the blurry interband DNA. The smearing of the partially relaxed catenanes could result from differential segregation of parental supercoils among the two daughter rings, because catenanes with the same total number but a different distribution of supercoils would migrate slightly differently. Variable segregation of supercoils also explains why the closed circular recombinant is not seen in Figure 3. Overexposure shows that these forms were resolved in the second dimension into several different topoisomer spots (data not shown); the topoisomers of mono51A are visible in Figure 2B (bottom). Thus the distribution of supercoils at synapsis is not uniquely dictated.

The Topoisomerase Is Uniquely Site-Specific

Resolvase is exceptional in its ability to distinguish the relative orientation of recombination sites. In vitro and under some conditions in vivo, recombination is efficient only between directly repeated *res* sites (Reed, 1981; Kostriken et al., 1981; Chiang and Clowes, 1982). Recombination of pRR55, identical to pRR51 except for the relative orientation of the *res* sites,

of the most highly supercoiled rings. The doublet that interrupts the regular spacing of the topoisomer ladder in the top gel (easily seen in the 15 min reaction) is catenanes with a nick in one of the two rings.

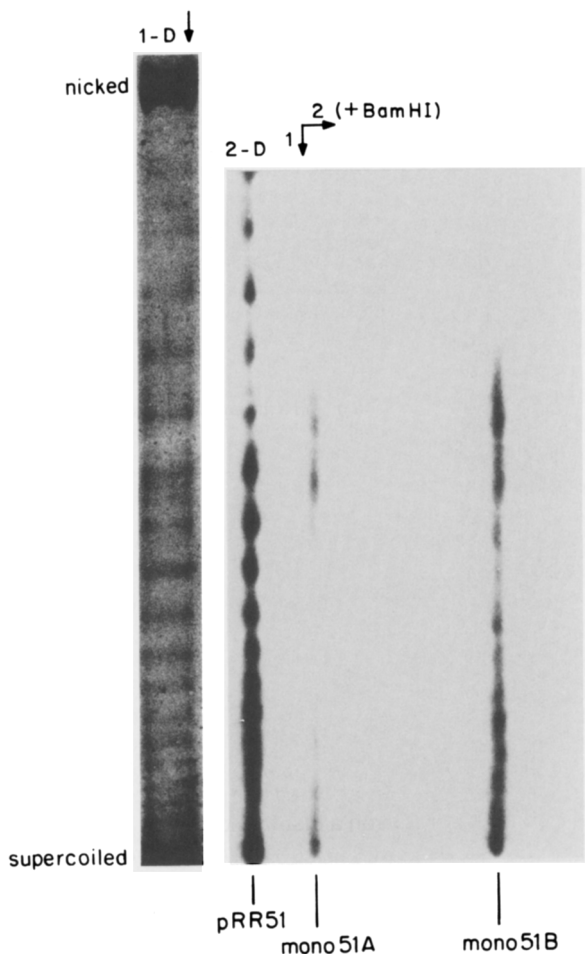


Figure 3. Analysis of Resolvase Products by Two-Dimensional Gel Electrophoresis

pRR51 was incubated with resolvase for 3 hr and analyzed in a 1% agarose gel for the first dimension (top to bottom). The lane was excised from the gel, the DNA was digested *in situ* with nuclease Bam HI and the products were subjected to electrophoresis in a second dimension (left to right). Southern blotting and hybridization to ³²P-labeled pRR51 and autoradiography were used to detect the products. A portion of the resolvase products run in an adjacent lane was stained with ethidium bromide and photographed after the first dimension (1-D) and is shown aligned with the two-dimensional analysis (2-D). The positions of the nicked DNA and the most highly supercoiled parental plasmid are shown in the first dimension. The positions of linear parent (pRR51), linear recombinant (mono51B) and open circular recombinant (mono51A) are shown in the second dimension.

inverts the DNA segment between the two sites; this occurred to only 1%–2% of the extent of the pRR51 reaction (data not shown). Relaxation is a simpler reaction, since topological alterations require breakage and reunion at only a single site and since the substrate is altered in only a single structural parameter. We tested the sequence specificity of the topoisomerase using substrates containing different numbers and relative orientation of the recombination sites. Relaxation of pBR322 containing zero, one or two *res* sites in inverted orientation could not be

detected (Figure 4, lanes a–h). This was so even when gels contained the DNA-intercalating agent chloroquine to separate the native topoisomers and allow detection of a shift in distribution by only a single supercoil (Figure 4, lanes a'–d') or when twice the standard concentration of resolvase was used and the reactions were continued for 1 day. The enzyme retained activity during this prolonged incubation, since substrates containing directly repeated *res* sites added after 1 day were recombined, although less well. The specificity was not due to subtle sequence differences between the plasmids. The 3200 bp recombinant ring (mono51A) containing a single hybrid of the two parental *res* sites was not relaxed by resolvase (Figure 4, lanes i and j). However, the 6400 bp concatamer of this plasmid, mono51A², which maintains the precise nucleotide sequence of the monomer, was a fully competent substrate for relaxation (Figure 4, lanes k and l), whereas the dimeric pBR322 control was not (data not shown). Thus the sole requirement for relaxation is the direct repetition of *res* sites.

Three conclusions can be drawn. First, the topoisomerase activity is intrinsic to the breakage and reunion activity that marks recombination, since both have the same unique substrate requirements. Second, the functional resolvase must have at least two active centers, both of which must be occupied by *res* sites for either to break and rejoin DNA. Furthermore, the activated resolvase does not act *in trans*, since resolvase relaxed only the competent substrate in mixtures of two different *res*-containing plasmids (Figure 4, lane m). Third, even though the *res* sites are kilobases apart, their relative orientation on the enzyme surface must be fixed and must reflect their orientation in the DNA sequence.

Resolvase Does Not Act on Intermolecular *res* Sites

The failure of resolvase to relax or recombine plasmids with a single *res* site is not simply a consequence of the distance separating two recombination sites. Even when two single-site plasmids (mono51A) were locally constrained by topological interlinks introduced by DNA gyrase, the catenated rings were neither relaxed (Figure 5, lanes c and d) nor recombined (lanes c' and d') by resolvase. The same result was obtained when purified dimeric catenanes from a native preparation of mono51A were tested (data not shown). Thus there is a specific proscription against intermolecular reactions. This experiment also implies that the reversal of cointegrate resolution is extremely inefficient even though the sequence of the *res* sites is unchanged by recombination.

Resolvase Is a Type 1 Topoisomerase

Topoisomerases are divided into two classes based on the number of strands that are broken during each

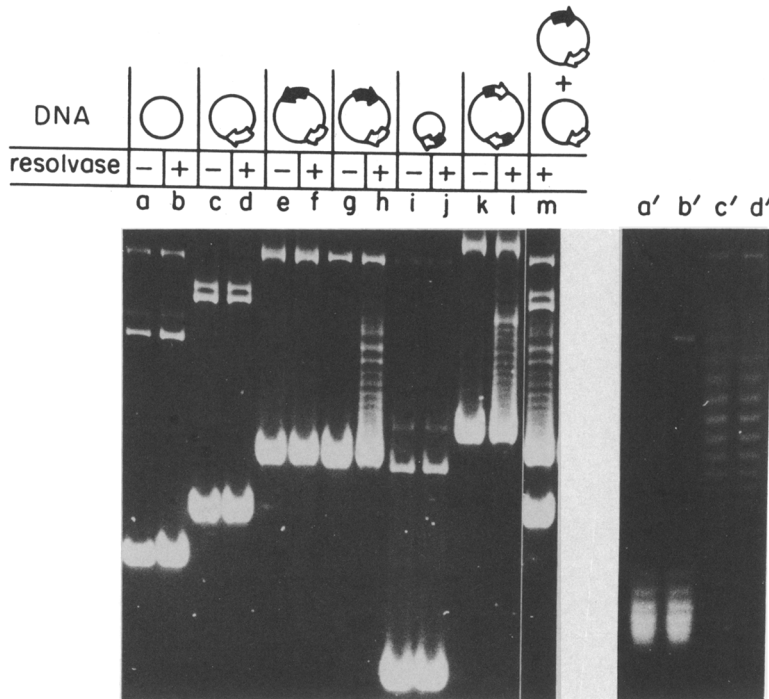


Figure 4. Site Specificity of Relaxation by Resolvase

Supercoil relaxation by resolvase was assayed using pBR322 or derivatives containing one or two *res* sites. The number and relative orientation of *res* sites are shown by the arrows. The products were displayed by electrophoresis through an agarose gel (lanes a-m) or an agarose gel containing the DNA-intercalating agent chloroquine phosphate to separate the native topoisomers (lanes a'-d'). The DNA substrates were: pBR322 (lanes a, b, a' and b'); mono51C (lanes c, d, c' and d'); pRR55 (lanes e and f); pRR51 (lanes g and h); mono51A (lanes i and j); mono51A², the concatameric direct repeat of mono51A (lanes k and l); and a mixture of mono51C and pRR51 (lane m).

enzymatic cycle (Liu et al., 1980; Cozzarelli, 1980). The unit change in linking number is the best operational distinction of the two classes and thus provides key mechanistic information on the breakage and reunion event. Treatment of a unique topoisomer of pRR51 with resolvase generated a set of relaxed bands differing in linking number from the starting material in increments of one (Figure 6). We confirmed that these bands were indeed topoisomers of the parental plasmid by treatment with Bam HI in situ and electrophoresis in a second dimension. Thus both known recombinational topoisomerases, resolvase and Int (Nash et al., 1980), are type 1 enzymes. Intermediates in type 1 reactions can often be fixed by addition of protein denaturants, resulting in single-strand scissions and covalent attachment of the enzyme to one broken end (Wang and Liu, 1979). This may be true for resolvase because in some experiments, such as Figure 2B (top), nicked rings were seen after SDS addition early in the reaction.

The Catenated Products Are Interlinked Once

No free product rings are generated by resolvase-mediated recombination; each recombinant is catenated to its sibling (Reed, 1981; Figure 2). Intramolecular recombination could directly convert the interwound supercoils of the substrate into intertwinings of the product rings as a simple geometric consequence of partitioning interwound segments of parental DNA into two separate rings (Mizuuchi et al., 1980; Pollock and Nash, 1980). As illustrated in Figure 7, the number of links maintaining the catenane would depend

on the extent of interwinding of the inter-*res* site segments, i.e., the segments that are separated by recombination. Given a distribution in both the relative position of the *res* sites (the two extremes of which are shown in Figure 7) and the number of plectonemic supercoils, this passive model predicts that the catenanes would be extensively and heterogeneously interlinked. Neither was observed for resolvase.

To characterize the linkage of the resolvase products, we took advantage of the finding of Sundin and Varshavsky (1980) that the electrophoretic mobility of catenated open circular rings of a given size depends only on the number of interlinks between the two rings. SV40 replicative intermediates, consisting of multiply intertwined dimeric catenanes, are resolved into a ladder of discrete bands because each interlink further compacts the catenane and increases its mobility (Sundin and Varshavsky, 1980; Figure 8A, lane b). Using this ladder as a standard, we determined the complexity of catenanes of SV40 DNA produced by *E. coli* DNA gyrase, and then characterized the resolvase products using as reference catenanes of the monomeric recombinant rings generated by gyrase.

When form I SV40 DNA was catenated by gyrase and then nicked with DNAase I, all of the dimeric product comigrated in high-resolution gels with the singly interlinked SV40 replicative intermediate (Figure 8A, lanes a and b). Even after an extended fluorographic exposure, which would have revealed gyrase products present at 1%–2% of the simple catenane, no additional dimeric species could be detected (Figure 8A, lane c). Thus, although gyrase can act on

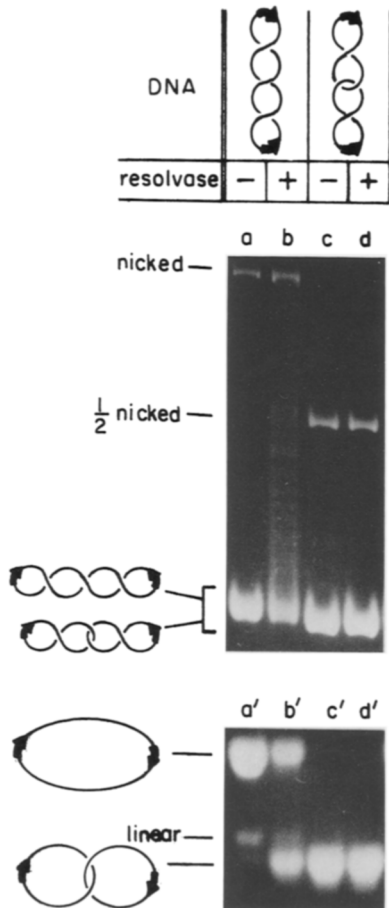


Figure 5. Catenated Single *res* Site Rings Are Not Relaxed or Recombined by Resolvase

mono51A² (lanes a, b, a' and b') or purified dimeric catenanes of mono51A formed by DNA gyrase (lanes c, d, c' and d') were treated with resolvase under standard conditions and analyzed directly on agarose gels to monitor relaxation (lanes a–d) or nicked with DNAase I and run on high-resolution agarose gels to monitor recombination (lanes a'–d'). The mono51A² substrate was prepared by an identical treatment of native mono51A² with DNA gyrase followed by purification of the residual monomer to control for supercoil alterations during the catenation reaction. Mixing controls showed that neither of the purified DNAs inhibited the standard resolvase reaction.

a supercoiled ring more than once to generate trimeric and tetrameric catenanes (Figure 8A, lane c), it does not introduce multiple interlinks between rings.

We then catenated the monomeric product of resolvase recombination (mono51A) using gyrase. The products were treated with DNAase I and run on a high-resolution gel next to the nicked products of a resolvase reaction with mono51A² as substrate (Figure 8B). Nearly all the resolvase products (Figure 8B, lane a) migrated as a single discrete band with the same mobility as the dimeric catenane produced by gyrase (Figure 8B, lane b). The same result was obtained in an analogous experiment with pRR51. Thus the resolvase products are interlinked once. Since any variation in the intertwining of the inter-*res*

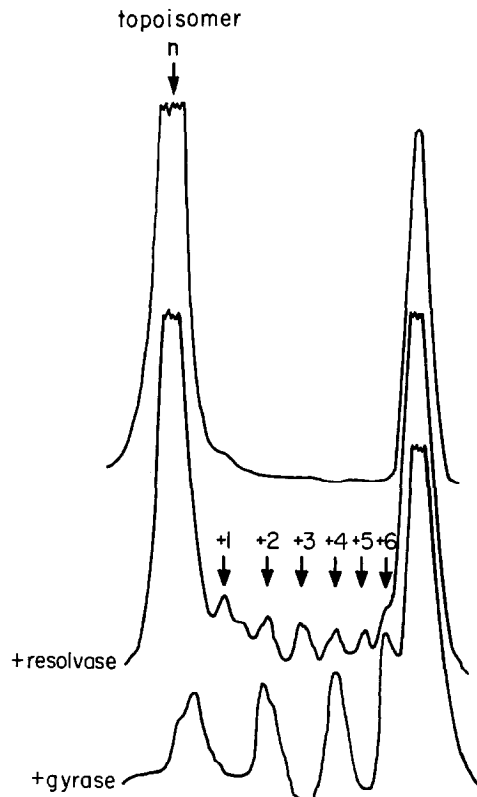


Figure 6. Resolvase Relaxes DNA Rings in Steps of One

A single topoisomer of native pRR51 (topoisomer n) was incubated with resolvase for 3 min (+ resolvase). The products were analyzed on a chloroquine-containing agarose gel, and a densitometric tracing of a photographic negative of the ethidium-bromide-stained gel is shown. A set of relaxed topoisomers differing in linking number from the starting material in multiples of two generated by DNA gyrase (+ gyrase) and native pRR51 were run in adjacent lanes to index the relaxed topoisomers, which are designated n+1–6.

site segments at synapsis or during strand exchange would be reflected in the linkage of the products, both processes must be rigidly defined to generate homogeneous catenanes. This could be easily accomplished if resolvase plays an active role in directing synapsis and strand exchange.

Discussion

Breakage and reunion by the Tn3 resolvase can have one of two distinct consequences. Rearrangement of the broken ends at two *res* sites results in recombination. On the other hand, passage of a DNA segment through a transient nick followed by rejoining in the original configuration removes supercoils. Resolvase is thus an example of reunion-choice enzymes, enzymes that catalyze reactions in which DNA breakage is followed by alternative paths to reunion. Similarly, rejoining by eucaryotic topoisomerase I can either restore the original sequence or join separate DNA molecules (Been and Champoux, 1981). DNA replication proteins of small phages analogous to the ϕ X

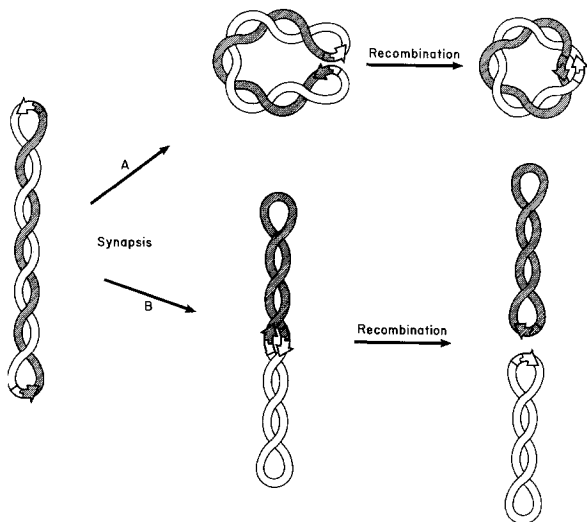


Figure 7. Passive Model for Catenation of Recombinant Rings
The plasmid substrate contains two directly repeated recombination sites (thick arrows). The positions of breakage and reunion within these sites define the boundaries of two domains (hatched and open) that are segregated by recombination. Recombination converts any net intertwining of the two domains at synapsis into interlinking of the product rings. Plectonemic supercoiling of the parental plasmid results in interwinding within and/or between the two domains. When the recombination sites are antipodal (left), all the interwindings are between the domains and are therefore converted into interlinks of relaxed product rings (path A). In the other extreme conformation of the substrate (path B), the *res* sites are directly across from each other; the interwindings are within each domain and are thus maintained as supercoils of the product rings. Neglected in this figure are linkage changes intrinsic to strand exchange, such as a loss of negative supercoils that could drive recombination, and a path that directs catenation of the products. These would be additive to the topological changes shown.

cisA protein can relax DNA, but breakage and reunion is typically interrupted by a round of DNA synthesis (Geider and Hoffmann-Berling, 1981).

Relaxation by resolvase, unlike any other known topoisomerase, is strictly site-specific. Only plasmids containing two *res* sites in a head-to-tail configuration were relaxed by resolvase, and the recombination reaction showed a similar preference. Since topological changes require breakage and reunion at only a single site, this unusual substrate requirement must reflect the need for proper pairing of *res* sites before breakage and reunion is initiated at either site. The *in vitro* results are concordant with the *in vivo* data showing recombination between intermolecular *res* sites to be much less efficient than between directly repeated sites, and that under some conditions inverted sites are recombined less well (Reed, 1981; Kostriken et al., 1981; Chiang and Clowes, 1982). These features of resolvase are nicely tailored to its biological function, because it will complete transposition by resolving fused chromosomes containing directly repeated transposons, but will not break and rejoin quiescent single copies of Tn3. Resolvase must still bind to an isolated *res* site because it also acts as

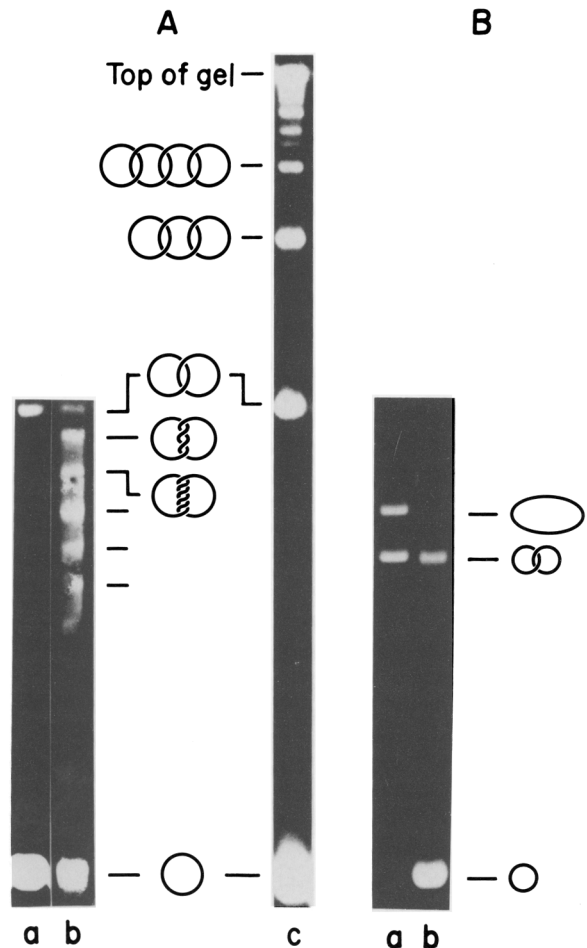


Figure 8. Complexity of Catenanes Made by DNA Gyrase and Resolvase

(A) ^3H -SV40 form I DNA was catenated by treatment with *E. coli* DNA gyrase and nicked with DNAase I, and the open circular catenanes were analyzed by electrophoresis through a high-resolution agarose gel and fluorography (lane a). The gel of a parallel reaction was exposed more extensively to detect minor products and the entire lane, including multimeric catenanes, is shown in lane c. ^3H -labeled SV40 DNA replication intermediates, composed of a series of open circular dimeric catenanes differing in the number of interlinks between the rings, were used as a standard (lane b). Horizontal lines: the positions (in order of increasing electrophoretic mobility) of the tetramer, trimer, dimers interlinked one to six times and the monomer SV40 ring.

(B) mono51A^2 was incubated with resolvase, nicked with DNAase I and analyzed in a high-resolution gel (lane a). Marker catenanes of mono51A were generated by treatment with DNA gyrase and nicked with DNAase I (lane b). The positions of the concatameric dimer (mono51A^2), singly interlinked catenane and monomer (mono51A) are shown.

a transcriptional repressor (Chou et al., 1979; Gill et al., 1979; Reed et al., 1982); in this role, covalent and topological alteration of the DNA are undesirable. Moreover, the specificity of resolvase for intramolecular sites ensures the irreversibility of the resolution reaction, since it disconnects the joined replicons.

How can resolvase distinguish the relative orienta-

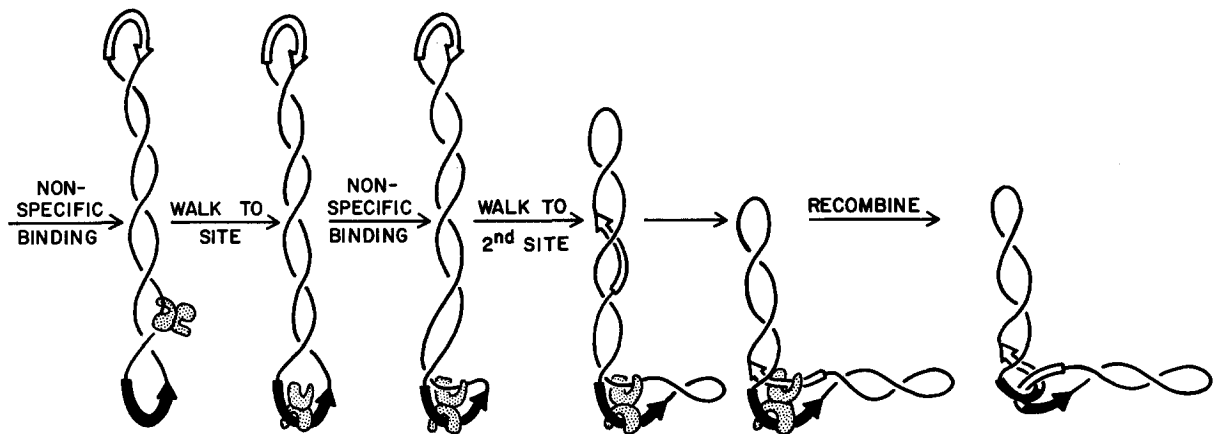


Figure 9. A Tracking Model for Resolvase-Mediated Recombination

A functionally dimeric resolvase (stippled) binds DNA nonspecifically and translocates randomly 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 two resolvase-binding sites. One-dimensional diffusion of the second subunit along the DNA causes the loop to expand and contract. When this subunit reaches a second *res* site (open arrow) in the proper orientation, the enzyme changes conformation, activating breakage and reunion. At synapsis, parental supercoils are maintained within each inter-*res* site domain, and there is no net intertwinning of the two domains. Strand exchange is directed by resolvase with a specific geometry, resulting in singly interlinked recombinants.

tion of *res* sites separated by thousands of nucleotides and complex tertiary writhing? Either the structure of the DNA maintains a fixed relationship between the orientation of the sites in space and in the primary structure, or the enzyme fixes it. The critical point is that recognition of the asymmetry of each individual *res* site is not sufficient; the coursing of the DNA between the sites must also be sensed.

We consider first enzyme-directed models. It is possible that resolvase molecules pack contiguously along the DNA between *res* sites, particularly if resolvase could condense long segments of DNA into nucleosome-like structures, as suggested for Int (Hsu et al., 1980; Better et al., 1982). Thus far, footprinting studies show only binding to three 30 bp regions encompassing the crossover point (Grindley et al., 1982). Resolvase could, however, move between the sites. In Figure 9, we represent an example of such a tracking model related to the schemes proposed for type I restriction endonucleases (Rosamond et al., 1979; Yuan et al., 1980). One subunit of a functionally dimeric resolvase binds nonspecifically to the DNA backbone. The enzyme translocates randomly along the DNA until reaching a *res* site, where it binds tightly and specifically. This is just how other DNA-binding enzymes such as Lac repressor are envisioned to quickly locate their specific site in an energy-independent manner (Winter et al., 1981); one kilobase of DNA can be scanned in a second. One resolvase subunit remains bound at the *res* site, repressing transcription, and the second subunit then binds to the adjacent DNA backbone in a unique orientation, trapping a small loop of DNA between the two binding sites. This subunit randomly searches along the DNA for a second *res* site, which causes the loop to expand and contract. When the second subunit encounters a

res site in the proper orientation, it is tightly bound and the enzyme changes conformation, activating breakage and reunion. The geometry of strand exchange is postulated to be such that a single interlink is introduced. If the second site is in the opposite orientation, it is not recognized by the asymmetric resolvase-binding site. It is reasonable that another resolvase molecule is already bound at the second site prior to arrival of the translocating enzyme; resolvase would have to displace the bound enzyme or recognize an asymmetric feature of the protein-DNA complex at the second site.

This model explains two additional results. First, the use of *res* sites on different DNA molecules is proscribed even when the proximity of the sites is ensured by catenation to be the same as in the standard substrate. By tracking between *res* sites, resolvase confines its search for the second site to a single dimension and determines whether sites are joined by a continuous piece of DNA. Since the second resolvase-binding site is occupied by the neighboring segment of DNA, it is unavailable for intermolecular interactions. Second, the products of resolution are singly linked catenanes, and therefore the inter-*res* segments must be uniquely and simply intertwined. The translocation mechanism ensures that there is no inter-*res* segment intertwinning at synapsis; all the intertwins are within each daughter domain, as shown in Figure 7B. Resolvase could then actively direct both synapsis and strand exchange, and the geometry of the latter would introduce the interlink. This is a radically different scheme for the catenation of the recombinant rings from the one proposed for λ site-specific recombination. Pollock and Nash (1980) suggested that the interlinking in the λ reaction is a passive result of the supercoiling of the parental plasmid, such as is

shown in Figure 7A. Depending on the length of DNA separating the recombination sites, the structure of the supercoils, such as their degree of interwinding and branching, and on dynamic fluctuations in the DNA structure, a variable and likely large number of supercoils would be converted to interlinks. Electron microscopic analysis of the products of the λ reaction suggested that the recombinants were linked many times (Mizuuchi et al., 1980), unlike resolvase products.

Supercoils of the substrate are not removed by translocation but merely segregated within each daughter domain. The segregation is not specific, however. Catenated products with the same total number of supercoils had reciprocal differences in the supertwisting of the sibling rings.

The tracking model is an attractive hypothesis because it explains three otherwise puzzling phenomena—the simplicity of catenated products, *res* orientation distinction and the proscription against intermolecular reactions. The model has not been tested directly, however, and all three phenomena need not have a single explanation. Any model that yields solely the synapsed structure in Figure 9 in which the inter-*res* site segments are not intertwined could explain the simplicity of the catenanes and the site orientation preference. If resolvase does not actively direct synapsis, then the structure of the DNA must be sufficiently rigid to define the path of DNA between *res* sites. For example, supercoils could keep the DNA stiff but aligned side by side and permit the DNA to move like a conveyor belt. The two *res* sites would then at some time face each other in a fixed manner and without intertwining of the inter-*res* segments, as in Figure 7B. Another example of this class of models denies the common view that supercoils are plectonemic. If instead they are toroidal or extensively branched, intertwining of the inter-*res* site segments would be precluded, and simple catenanes would result. Any model based solely on the structure of DNA must account for the lack of simplicity of the catenanes and site orientation preference in the λ system and the inability of resolvase to act on catenated single *res* site rings. These are more difficult to reconcile with a purely structural hypothesis, but in no way do they rule out a contributory role. For instance, resolvase-, but not Int-, mediated recombination might require a particular intimate juxtaposition of recombination sites afforded by plectonemic supercoils, as in Figure 7B, and thus would not act during the less intimate association shown in Figure 7A or on the catenated rings. It will be illuminating to compare the two recombination systems on the same substrate and under the same reaction conditions.

Although relaxation and recombination follow identical early steps and are manifestations of the same breakage and reunion activity, they are distinct. Recombination may be driven by a concomitant loss of

a small number of negative supercoils (Nash et al., 1980). Relaxation of parental DNA seems not to be a consequence of such supercoil loss followed by recombination between the progeny to regenerate the parental ring but not the parental supercoils, because recombination between catenated progeny was not detected. Relaxation was not detected either, and thus the reduction in supercoil density of the catenated progeny must have taken place in the substrate before and/or during recombination. A necessary concomitant loss of supercoils seems likely, because in experiments using single topoisomers we detected no recombinants that had clearly conserved the parental supercoils (unpublished data).

There is a key distinction between breakage and reunion by Int and by resolvase. Whereas the properties of the topoisomerase and the recombination activity of resolvase are thus far indistinguishable, the characteristics of the two activities of Int differ in so many ways that it was not clear that they would share the same active site (Kikuchi and Nash, 1979; Nash and Robertson, 1981). Although integrative host factor and spermidine are required for Int-mediated recombination under standard conditions, both restrain the topoisomerase activity of Int. Second, although Int is required in at least 30-fold molar excess over substrate molecules in recombination, relaxation can occur at much lower levels of Int. Third, at the high concentrations of Int, relaxation is sluggish relative to recombination. Most striking, the topoisomerase activity of Int does not show the site specificity of recombination. Int relaxes substrates lacking the recombination site just as well as those that contain these sites. In contrast, relaxation and recombination by resolvase occur under identical conditions, are similarly affected by spermidine (unpublished data), have the same substrate and enzyme concentration requirements and show a similar time course.

There is, though, an important but unexpected similarity between the two recombinational topoisomerases. Although recombination requires duplex breaks, both Int (Nash et al., 1980) and resolvase (Figure 6) are type 1 topoisomerases and thus transiently interrupt only one strand during the relaxation. Reed and Grindley (1981) have shown that double-strand breaks are apparent after addition of a protein denaturant to extensive reactions with the $\gamma\delta$ resolvase in the absence of Mg^{2+} , where recombination is inhibited. However, a nicked species was also a major product in their experiment, and we find that Tn3 resolvase under similar conditions yields both nicked and cleaved products (unpublished data). Given the relaxation in steps of one, it is tempting to speculate that the nicked DNA is a fixed form of a recombination intermediate. If recombination involves a correlated pair of reciprocal single-strand exchanges, the omission of Mg^{2+} might result in occasional cleavage due to an artificial accumulation of two single-strand inter-

ruptions at a site. Under physiological conditions, the first exchange might generate a Holliday structure and the second mature the Holliday intermediate into the recombination products. Holliday intermediates might be a general feature of site-specific recombination enzymes, because genetic and biochemical evidence has implicated these structures in the λ integration reaction (Echols and Green, 1979; Enquist et al., 1979; A. Landy, personal communication).

Experimental Procedures

Enzymes

Cloning and purification of the Tn3 resolvase were similar to schemes used for its homolog from $\gamma\delta$ (Reed, 1981). The resolvase promoter was replaced with the strong leftward promoter (P_L) of phage λ by ligation of the Taq I-Pvu I fragment of Tn3 (Heffron et al., 1979) that contains the resolvase coding sequence to the Cla I-Pvu I P_L -containing fragment of the vector p λ 8 (Reed, 1981). The resultant plasmid, pMK17, was introduced into *E. coli* strain N4830 (Reed, 1981), which harbors a defective λ prophage containing cI857, a thermolabile repressor of P_L . Cells were grown in 300 liters of LB (Miller, 1972) and 50 μ g/ml ampicillin at 32°C to an OD₆₀₀ of 1, and transcription from P_L was induced by quickly (<5 min) raising the medium temperature to 42°C. After 90 min, 500 g of cells were harvested; extended incubation at 42°C did not increase yields of resolvase.

The first two steps in the purification took advantage of the binding of resolvase to DNA and the low solubility of resolvase below 0.5 M NaCl in the absence of DNA and followed those described for the $\gamma\delta$ resolvase (Reed, 1981). Briefly, 150 g of cells were treated with lysozyme and disrupted by sonication, and the lysate was clarified by centrifugation. Nucleic acids were precipitated with polyethyleneimine, and resolvase was eluted from the nucleic acids by a 1 M NaCl wash of the precipitate. Resolvase was precipitated by dialysis into 0.1 M NaCl and buffer A (20 mM Tris-HCl [pH 7.45], 10 mM MgCl₂, 1 mM dithiothreitol), resuspended to 225 ml with 1 M NaCl and buffer A, and fractionated with (NH₄)₂SO₄ to remove material that interfered with gel permeation chromatography. The material precipitated by addition of 31.5 g (NH₄)₂SO₄ was discarded, and resolvase was precipitated by addition of 0.15 g of (NH₄)₂SO₄ per milliliter of supernatant. The precipitate was resuspended in 1 M NaCl and buffer A to a final volume of 75 ml and filtered through a 7 cm² × 90 cm Ultrogel AcA54 (LKB) sizing column. Fractions containing resolvase activity had a K_{av} of 0.18 and were pooled. Two separate procedures were used subsequently. Procedure A maintained resolvase in its native conformation throughout, whereas procedure B utilized denaturing levels of urea in which resolvase is soluble even at low ionic strength. In procedure A, one fourth of the AcA54 pool was diluted with 0.2 M NaCl and buffer A to a final protein concentration of 20 μ g/ml; at higher concentrations resolvase readily aggregated. The sample was applied to a 4.9 cm² × 20 cm Bio-Rex 70 (Bio-Rad Laboratories) column. The cation exchange column was washed with 2 column volumes of 0.2 M NaCl and buffer A and developed with a 1 liter 0.2–1 M NaCl linear gradient containing buffer A. Resolvase activity eluted between 0.38 and 0.48 M NaCl and was stored in 1 M NaCl and buffer A. In procedure B, the remaining three fourths of the AcA54 pool was brought to 2 M NaCl by addition of 4 M NaCl and buffer A and loaded on a 1.8 cm² × 10 cm phenyl-Sepharose CL-4B (Pharmacia) column. The hydrophobic column was washed first with a 0.25 liter linear gradient from 2 M NaCl and buffer A to buffer B (5 mM MgCl₂, 20 mM Tris-HCl [pH 7.45], 1 mM dithiothreitol) and then with 2 column volumes of buffer B. Resolvase was eluted with 1 column volume of buffer C (6.3 M urea and buffer B). Fractions containing resolvase were loaded on a 4.9 cm² × 20 cm CM-Sepharose CL-6B (Pharmacia) column equilibrated with buffer C. The column was washed with one-half column volume of buffer C and developed with a 0.5 liter linear 0–0.3 M NaCl gradient in buffer C. Resolvase eluted between 45 mM and 75 mM NaCl, and this pool

was dialyzed against 1 M NaCl, 6.3 M urea and buffer A. Resolvase was renatured by extensive dialysis against 1 M NaCl and buffer A and stored at 0°C. Resolvase is stable under these conditions for at least 6 months. Procedure A was used because we anticipated that denaturation might affect activity, but we have not detected any loss or change in activity after the cycle of denaturation and renaturation. Procedure B is recommended because it removed several trace contaminants and can more easily be used on a large scale. Analysis of fractions from both procedures by SDS gel electrophoresis is shown in Figure 1. Resolvase concentrations are expressed in moles of the 21,000 dalton protomer, and were determined by comparison with bovine serum albumin standards after staining the gels with Coomassie blue.

E. coli DNA topoisomerase I (omega protein) and DNA gyrase subunits A and B were purified as described (Higgins et al., 1978; Sugino and Cozzarelli, 1980; Brown and Cozzarelli, 1981). Restriction enzymes, bovine pancreatic DNAase I and proteinase K were from commercial suppliers.

DNAs

The 5800 bp plasmid pRR51 is a model cointegrate with 900 bp and 600 bp fragments containing the $\gamma\delta$ *res* site inserted, respectively, into the Eco RI and Pvu II sites of pBR322 in a directly repeated orientation (Reed, 1981). The 900 bp fragment is in the opposite orientation in pRR55. Three plasmids containing a single *res* site are designated by the prefix mono. Mono51C (4900 bp) was prepared by removal of the 900 bp fragment from pRR51 by digestion with endonuclease Eco RI and religation. Mono51A (3200 bp) and mono51B (2600 bp) are the products of recombination of pRR51 by resolvase. Mono51A was prepared by treatment of the reaction products with endonuclease Bam HI, which cuts mono51B and pRR51 once but leaves mono51A intact, and transformation of *E. coli* to ampicillin resistance. Mono51B does not contain an origin of DNA replication, and was prepared by large-scale resolvase reactions followed by digestion with excess endonuclease TthIII I, which has a site in mono51A but not in mono51B. Concatameric head-to-tail dimers were isolated from native plasmid preparations by agarose gel electrophoresis and propagated in a *recA*⁻ strain (*E. coli* strain MG1655.10; *lac*⁻ *recA56* $\gamma\delta$ ⁻ *Tn3*⁻), to minimize conversion to monomeric forms. All other plasmids were also transformed into this strain to minimize recombination between *res* sites, and were isolated as described by Staudenbauer (1976). SV40 form I ³H-labeled DNA was purchased from Bethesda Research Laboratories. ³H-labeled SV40 DNA replication intermediates containing multiply interlinked catenanes (Sundin and Varshavsky, 1980) were a gift from A. Varshavsky.

To prepare a substrate uniform in linking number, the topoisomers of native pRR51 (40 μ g) were separated in a horizontal 1% agarose (Sigma, type II) slab gel (13.5 × 27 × 0.7 cm) containing the DNA-intercalating agent chloroquine phosphate at 5 μ g/ml to partially remove supercoils (Shure et al., 1977). Gels were run submerged in a recirculating Tris-acetate buffer (Shure et al., 1977) at 1–2 V/cm for 2–3 days. Bands were visualized after staining with ethidium bromide, excised, electroeluted into dialysis sacks, concentrated with sec-butanol and purified by banding in a CsCl density gradient in the presence of ethidium bromide. This last step removed an inhibitor of resolvase. Supertwisted dimers present in a native preparation of mono51A were isolated from a 1% agarose gel and purified in the same way. This preparation consisted of 80% catenanes; nearly all were interlinked once (H. Benjamin and N. R. Cozzarelli, unpublished data).

Reactions

Standard resolvase relaxation and recombination reactions were performed at 37°C for 1 hr and contained, in 20 μ l, 20 mM Tris-HCl (pH 7.45), 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.2 μ g of DNA and 0.04 μ g of resolvase. To monitor recombination of pRR51, incubation was continued for 5 min in the presence of 5 U of endonuclease Bam HI (Bethesda Research Laboratories) to disrupt the interlinked recombinants. Recombination of pRR55 was assayed

by treatment with endonuclease Eco RI, which generates 4900 bp and 900 bp fragments from the starting material and 3200 bp and 2600 bp fragments after inversion. Reactions were stopped by the addition of 1/6 volume of 20% (w/v) Ficoll 400, 2% SDS and 0.25 mg/ml bromophenol blue. Where indicated, proteinase K was added to 65 µg/ml and incubated for 15 min at 37°C. Products were analyzed by electrophoresis through 1% agarose gels, stained with 0.5 µg/ml ethidium bromide and photographed under ultraviolet illumination. When separation of native topoisomers was required, gels containing chloroquine were used.

To distinguish parental DNA from the catenated products, two-dimensional electrophoresis (Locker and Rabinowitz, 1981) was used. DNA was separated in the first dimension through agarose with or without chloroquine, stained with ethidium bromide and photographed. A gel slice containing the center portion of a single lane was shaken gently for at least 6 hr in three changes of buffer containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 6 mM MgCl₂, 1 mM dithiothreitol and bovine serum albumin at 100 µg/ml to saturate nonspecific protein-binding sites. The gel slice was surrounded with parafilm except at one end, filled with buffer (~1 ml) and endonuclease Bam HI at 250 U/ml and incubated for 2 hr at 37°C. The gel slice was laid across the top of a vertical 1.5% agarose slab gel (14 × 16 × 0.4 cm), embedded in 0.4% agarose and subjected to electrophoresis at 1 V/cm for 1 day. The higher percentage of agarose in the second dimension allowed stacking at the interface, and the bands from the first dimension thus appeared as more compact dots. Transfer to nitrocellulose and hybridization (Wahl et al., 1979) to pRR51 ³²P-labeled by nick translation (Kelly et al., 1970) were used to enhance sensitivity.

Catenation of DNA rings by *E. coli* DNA gyrase was carried out as described (Krasnow and Cozzarelli, 1982) except ³H-SV40 form I DNA, mono51A or a mixture of mono51A and mono51B was substituted for Col E1 DNA. In the preparation of the substrate to test the resolvase back reaction, both the monomeric starting material and the dimeric catenane product were separated on 1% agarose gels and purified as described above for individual topoisomers. For the analysis of catenane interlinking, catenanes were nicked with DNAase I (5–10 µg/ml) in the presence of 300 µg/ml ethidium bromide for 30 min at 30°C, phenol-extracted three times, ethanol-precipitated and resuspended in the electrophoresis buffer. High-resolution electrophoresis through 0.8% agarose gels (1–2 V/cm for 3–6 days in the presence of 0.03% SDS) and fluorography were carried out as described by Sundin and Varshavsky (1981).

A ladder of topoisomers from relaxed to native was produced by treatment of a native plasmid with *E. coli* DNA topoisomerase I. The reaction was performed at 52°C for 30 min and contained, in 20 µl, 30% (v/v) glycerol, 20 mM Tris-HCl (pH 7.6), 20 mM KCl, 6 mM MgCl₂, 0.5 mM dithiothreitol, bovine serum albumin at 50 µg/ml, 6 µg of plasmid DNA and 8 fmole of *E. coli* DNA topoisomerase I. A ladder of relaxed topoisomers differing in linking number from a homogeneous purified topoisomer in multiples of two was generated by relaxation with DNA gyrase (Brown and Cozzarelli, 1979). The reaction was carried out at 30°C for 30 min and contained, in 30 µl, 20 mM Tris-HCl (pH 7.45), 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.15 µg of purified topoisomer and 50 fmole of *E. coli* DNA gyrase reconstituted from its purified subunits.

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