

# Recombination Site Selection by Tn3 Resolvase: Topological Tests of a Tracking Mechanism

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## Summary

**In vitro recombination by Tn3 resolvase of plasmids containing two directly repeated recombination (*res*) sites generates two singly interlinked catenated rings. This simple product catenane structure was maintained over a wide range of substrate supercoil densities and in a reaction mixture in which phage  $\lambda$  Int-mediated recombination generated its characteristic multiply interlinked forms. Using substrates containing four *res* sites, we found that resolvase recombined neighboring *res* sites with high preference. This position effect implies that resolvase searches systematically along the DNA for a partner site. Intervening *res* sites in the opposite orientation did not prevent translocation. We analyzed the geometric arrangement of the interlocked rings after multiple recombination events in a four-site substrate and the pattern of segregation of nonspecific reporter rings catenated to the standard substrate. The results of these novel topological tests imply that the translocating enzyme may not make continuous contact with the DNA.**

## Introduction

Knowledge of the molecular mechanism of recombination has come primarily from the study of site-specific recombination, the only recombination process that has been reconstructed in vitro with purified components (Nash, 1981; Reed, 1981; Abremski et al., 1983). Site-specific recombination has also been the vehicle for discoveries of general importance, such as the origin and role of negative supercoiling of DNA (Mizuuchi and Nash, 1976). The two best-studied recombination proteins are phage  $\lambda$  Int and the resolvase encoded by the highly related transposons, Tn3 and  $\gamma\delta$ .

We are investigating the *E. coli* Tn3 resolvase, the 21,000 dalton product of the *tnpR* gene. Transposition of Tn3 from a donor plasmid to a recipient proceeds through a cointegrate intermediate consisting of the donor replicon fused to the recipient via directly repeated Tn3 bridges (Heffron, 1983). Site-specific recombination by resolvase at a *res* site within each Tn3 restores the original structure of the donor and generates a recipient replicon with a copy of Tn3. Within the 120 bp *res* site, multiple resolvase promoters bind three adjoining homologous regions 30 to 40

bp long, readying the site for breakage and reunion (Grindley et al., 1982; Kitts et al., 1983).

Resolvase recombines, in vitro, model cointegrates containing directly repeated *res* sites (Reed, 1981). The reaction requires a supercoiled substrate and generates two catenated progeny rings. Resolvase is also a type 1 topoisomerase. Supercoil relaxation, like recombination, requires two directly repeated *res* sites and is an alternate expression of the enzyme's breakage and rejoining function (Krasnow and Cozzarelli, 1983).

A chief conclusion of our previous report was that the recombination and topoisomerase activities of resolvase cannot be initiated simply by specific binding at the *res* sites and random collision of two sites (Krasnow and Cozzarelli, 1983). Rather, resolvase must also recognize the DNA between the sites, even though they can be at least 6 kb apart (Krasnow et al., 1983). We presented three pieces of evidence. First, resolvase acts 50 times better on sites with the same orientation along the DNA, even though synapsed direct and inverted sites differ only in the path of the connecting DNA. Second, recombination and relaxation require that both *res* sites are on the same piece of DNA. Two rings each with a single *res* site will not recombine even if held together by a catenane interlink. Third, the intertwining by supercoiling of the domains of the substrate that will be separated by recombination is expected to cause extensive and variable interlocking of the product rings (Mizuuchi et al., 1980), yet the resolvase products are uniquely singly linked catenanes. Thus, the enzyme must ensure that the supercoil intertwining is segregated within and not between domains. This is difficult to achieve if only the sites are recognized. In all three respects the reactions are different from Int recombination. Int operates both intermolecularly and intramolecularly, on inverted as well as directly repeated *att* sites, and produces multiply linked products (Mizuuchi et al., 1980; Pollack and Nash, 1983; Spengler et al., 1984).

The model that best fits the data is called tracking or looping (Krasnow and Cozzarelli, 1983), and a version is shown in Figure 1. (We will reserve the term tracking for movement along DNA, and the model will be referred to as looping because its characteristic feature is a bivalent tracking that generates a DNA loop). Resolvase binds initially to DNA through two sets of nonspecific interactions and translocates randomly along the DNA, much as other repressors are envisioned to locate their operators (von Hippel et al., 1984). Upon contacting a *res* site, one binding site of resolvase interacts tightly and specifically. As DNA continues to slide through the second binding site, a loop of DNA forms between the binding domains. The loop expands and contracts as the one-dimensional search along the DNA for another *res* site proceeds. The second *res* site could be empty or bound by another resolvase as shown in Figure 1. Contact between two resolvase-bound *res* sites in correct orientation induces a conformational change leading to scission, strand exchange, and rejoining. By tracking between *res* sites,

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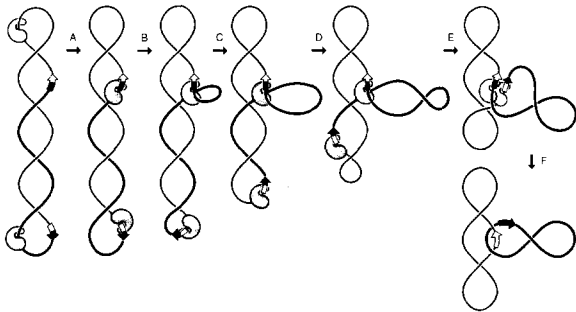


Figure 1. The Looping Model for Resolvase

A negatively supercoiled resolvase substrate is shown with half-filled arrows representing the *res* sites. The two DNA domains that will be separated by recombination are differentiated by thick and thin lines. Two asymmetric resolvases, depicted as cashew-shaped structures, initially bind to DNA through nonspecific ionic interactions. They translocate along the DNA by a random one-dimensional walk until they recognize a *res* site and bind tightly and specifically (A). Another part of resolvase remains nonspecifically bound and, by continuing the walk, forms a loop of DNA between the specific and nonspecific interaction (B). The loop is negatively supercoiled, and its formation is compensated by a diminution in supercoiling elsewhere. For clarity, only one resolvase is shown forming a loop. As further translocation expands the loop (C and D), interdomainal supercoils are segregated within domains. At synapsis (E), the two resolvase-bound sites are held in parallel orientation by protein-protein interactions and all supercoils are intradomainal except those constrained by the protein. Breakage and crossed reunion results in recombination products that are joined by a single interlock (F). This model is the same as that proposed previously (Krasnow and Cozzarelli, 1983) except for two details. First, the sites are synapsed parallel rather than antiparallel to each other as favored by a theoretical analysis of recombination (Cozzarelli et al., 1984). This orientation is achieved in this diagram by a single negative supercoil entrapped by the enzyme between the sites. Second, two resolvases rather than one are shown tracking simultaneously; this is reasonable because of the vast excess of enzyme over sites in our standard conditions.

resolvase ensures that reaction occurs only between intramolecular sites. Because both the proteins and the sites are asymmetric, a fixed orientation relative to the intervening DNA is maintained by the enzyme during translocation. Thus, direct and inverted site orientation is distinguished. The end product of looping consists of two enzyme-filled *res* sites directly across from each other, with the supercoil interwindings segregated within the DNA segments that will be separated by recombination. This precludes extensive interlocking of the catenated products.

Similar models have been proposed for other recombination processes (Abremski et al., 1983; Craig and Nash, 1983; Kitts et al., 1983; Wood and Tonegawa, 1983). However, even for resolvase, where the case for looping is strongest, direct evidence is scant. We therefore tested several key aspects of the looping scheme. First, we showed that resolvase produced simple catenanes even as substrate supercoil density was varied widely and in a mixture where *Int* generated complex products. Second, we demonstrated the striking propensity of resolvase to recombine neighboring *res* sites on a substrate with multiple sites, thereby validating a key prediction of the model. Finally, we performed several novel topological tests, which contradicted the continuous sliding aspect of loop-

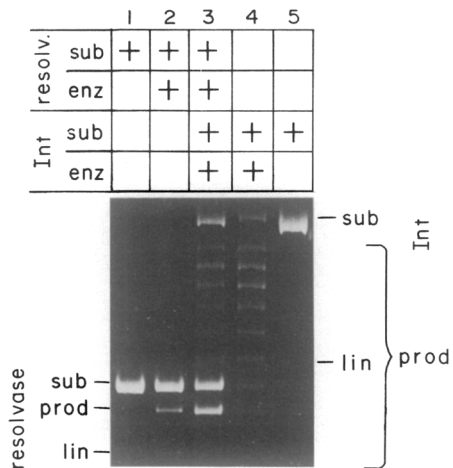


Figure 2. Comparison of the Interlocked Products of Resolvase and *Int*

The 50  $\mu$ l reactions contained, as indicated: resolvase (resolv) substrate (sub), pA<sup>2</sup>; *Int* substrate, pBP86; and the enzymes (enz) resolvase and *Int*. After 75 min at 30°C, the reactions were stopped by heating at 65°C for 10 min. The products were nicked with DNAase I to remove supercoils and separated by high-resolution gel electrophoresis; the ethidium-stained gel is shown. The positions of the nicked substrates and catenated products (prod) are indicated as is the small portion of linearized substrate (lin) produced by DNAase I. The resolvase substrate and products migrate within the envelope of *Int* products.

ing. The simplest explanation is that the enzyme can avoid DNA and protein barriers during translocation by some kind of gating or DNA segment transfer, although we present alternative explanations in the Discussion.

## Results

### Catenanes Made by Resolvase and *Int* Differ in Complexity

The singly interlocked catenanes produced by resolvase contrast with the complex products of the  $\lambda$  *Int* system. Because both use negatively supercoiled substrates, this difference implies that DNA structure alone cannot account for product structure. A caveat is that the distinct product structures could have resulted from differences in reaction conditions, notably the spermidine in the  $\lambda$  system and the stoichiometric amounts of three DNA binding proteins, resolvase, *Int*, and IHF.

We found, however, that both reactions could be carried out efficiently in the presence of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10 mM MgCl<sub>2</sub>. The product profiles were compared using high-resolution gel electrophoresis in which the mobility of nicked dimeric catenanes increases with number of interlocks (Figure 2). *Int* still generated a broad distribution of catenated products centered around 4 to 5 interlocks (lane 4), whereas resolvase produced only singly linked catenanes (lane 2). Even when both reactions occurred in the same mixture, the products retained their characteristic linkage (lane 3). Indeed, the interlocking patterns of the recombinants do not overlap because the least complicated *Int* recombinant is doubly interlocked (S. J. Spengler, A. Stasiak, and N. R. Cozzarelli, unpublished data). The topological differences of

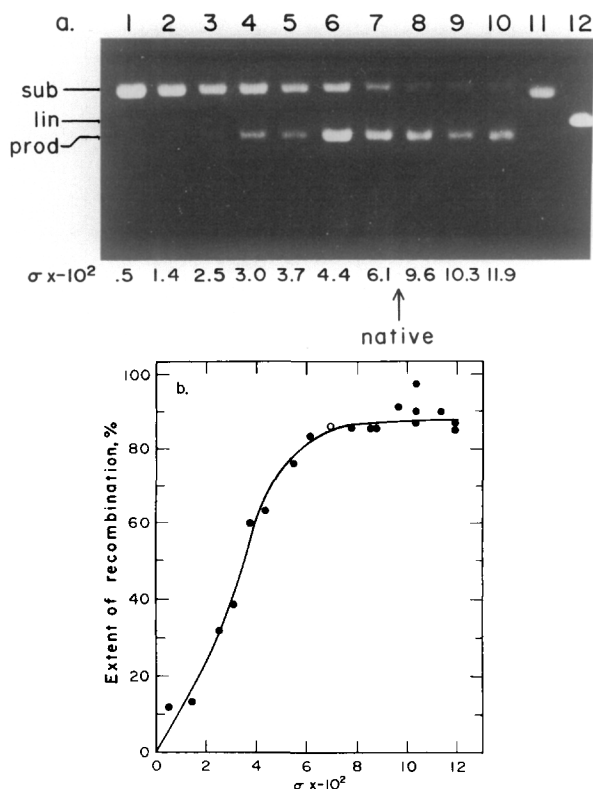


Figure 3. The Effect of Substrate Supercoil Density on the Complexity and Amount of Catenanes Produced by Resolvase

(a) Electrophoretic analysis of resolvase catenanes. Preparations of pRR51 differing in superhelical density were reacted with resolvase and then nicked with DNAase I. A portion of the products (prod) (lanes 1-10), nicked pRR51 (sub) (lane 11), and linear (lin) pRR51 (lane 12) were displayed by high-resolution gel electrophoresis. The superhelical density ( $\sigma$ ) of each substrate is given below each lane. The  $\sigma$  of native pRR51 is  $-0.064$  and is indicated by an arrow between lanes 7 and 8.

(b) The extent of recombination. A portion of the reactions in part (a) were treated with Bam HI to decatenate progeny rings and linearize the substrate. The extent of recombination was calculated from the relative amount of the linear forms as determined by gel electrophoresis. Results with a different batch of substrates, which included native DNA (open circle), are also shown. The 5%-10% of nicked DNA in the substrate accounts for the majority of unreacted material at higher supercoil densities.

the products demonstrate that the enzymes respond characteristically to their substrate.

#### Substrate Supercoil Density Affects the Amount but Not the Complexity of Resolvase Catenated Products

Although resolvase produces singly linked catenanes when acting on substrates of native supercoil density, we tested whether higher supercoil densities would force more complex products and lower densities would lead to unlinked rings. With substrate superhelical densities ranging from relaxed ( $\sigma = 0.0$ ) to almost twice that of native substrate ( $\sigma = -0.12$ ), the product was a singly interlocked catenane (Figure 3a). The results again contrast with Int, where product complexity increases with supercoiling (Pollack and Nash, 1983).

Although product structure remained constant, the extent of recombination by resolvase decreased steadily at

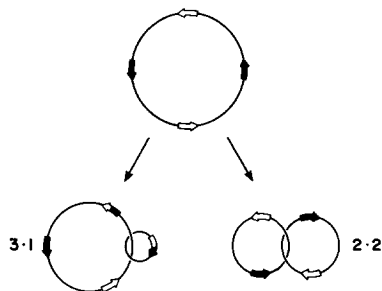


Figure 4. Neighboring Site Preference for Recombination by Resolvase

A substrate with four directly repeated *res* sites, p51<sup>2</sup>, is shown with sites as alternately open and filled arrows. Recombination can occur between neighboring sites (left) or opposing sites (right) to form 3-1 and 2-2 products, respectively. To test this, 2.3  $\mu$ g of p51<sup>2</sup> were treated with 2  $\mu$ g of resolvase and analyzed by electron microscopy after nicking with DNAase I. From a 15 min reaction, 54 molecules were identified as 3-1 and 3 as 2-2. From a 180 min reaction, 26 catenanes were scored as 3-1 and 2 as 2-2. The products of a 20 min resolvase reaction were also separated by gel electrophoresis. The first-round products were isolated from the gel and decatenated with DNA gyrase, and the released rings with one two and three *res* sites were separated by electrophoresis. The results showed that 95% of the products were 3-1 and 5% were 2-2.

supercoil densities below  $\sigma = -0.061$ , a value close to that of native, and was absent with a relaxed substrate (Figure 3b) or a nicked substrate (Reed, 1981). When, on average, only three supercoils (8% of native density) were present, 10% of the substrate still recombined. The almost linear decline in extent of reaction with diminishing supercoils agrees with the first-order decrease in free energy associated with loss of a fixed number of supercoils (Hsieh and Wang, 1975), and the relationship is similar to that of most other supercoiling responsive proteins (e.g., Liu and Wang, 1979). The plateau value of 85%-90% recombination beginning at native supercoil density ( $\sigma = -0.064$ ) was not an artifact due to substrate depletion, because it took the same time to half-complete recombination at supercoil densities of  $-0.03$ ,  $-0.056$ , and  $-0.10$ , values that range from the midpoint of the curve to well along the plateau.

#### Resolvase Acts Preferentially on Neighboring *res* Sites

The findings that resolvase recombination directed catenane complexity and required intramolecular directly repeated sites provided evidence that resolvase does not simply bind to *res* sites but also interacts with the DNA between these sites. This is accomplished in the looping model by resolvase sliding from one *res* site to another while remaining bound to the first site. If the enzyme tracks along the DNA starting from one *res* site, it should sequentially encounter other sites according to their position in the primary sequence. The prediction is that adjacent sites in a multi-site substrate will recombine before more distant ones. We tested this with plasmid p51<sup>2</sup> (see Table 3) which has four directly repeated *res* sites spaced 2.7 to 3.2 kb apart. As diagrammed in Figure 4, recombination between opposing sites generates a catenane of two rings, each containing a pair of sites, whereas a neigh-

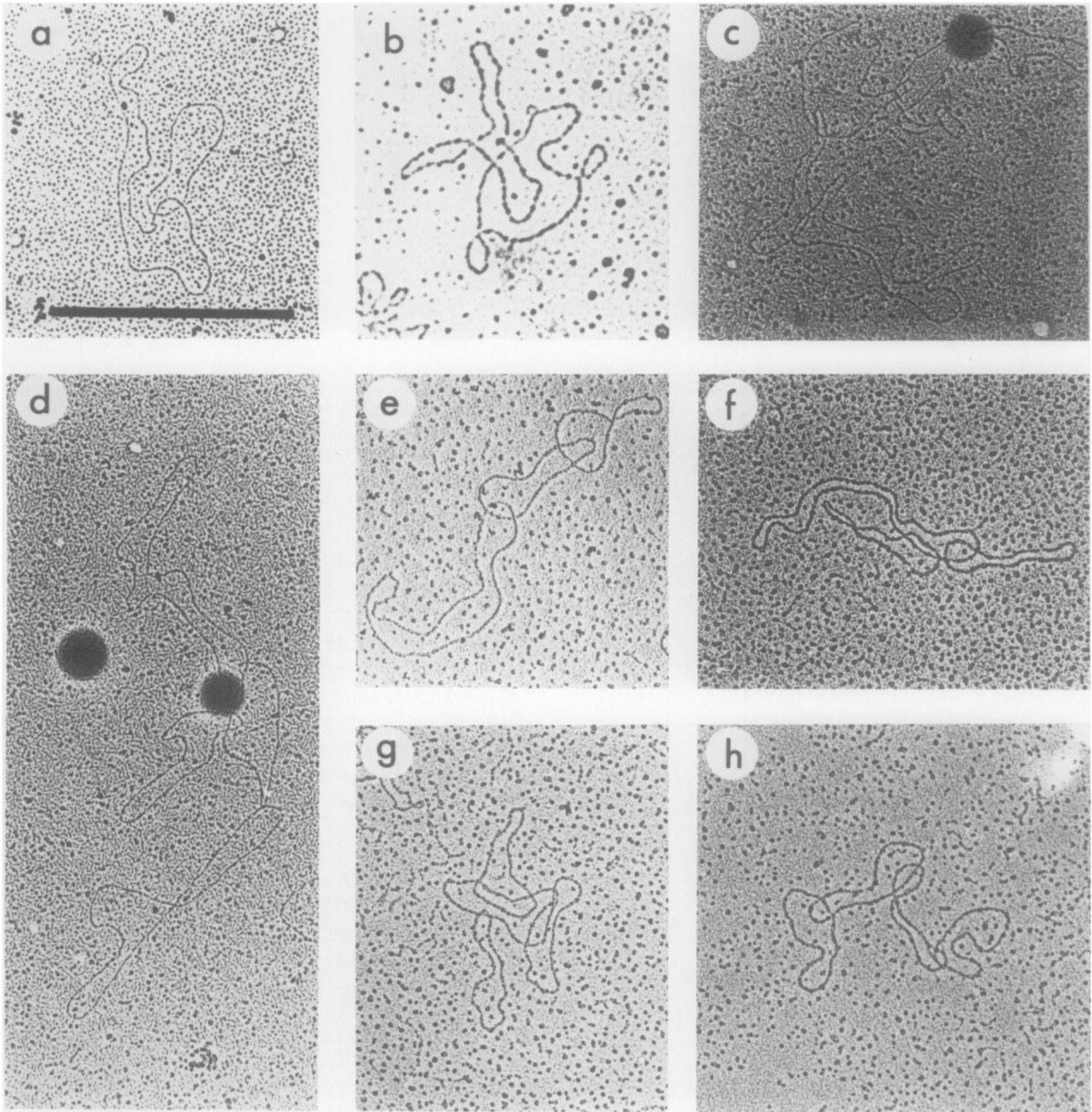


Figure 5. Electron Micrographs of Products of Resolvase Recombination

The nicked resolvase products were spread for electron microscopy in the presence of cytochrome C and formamide. The first recombination products from  $p51^2$  were a dimeric catenane between a three-site and a one-site ring, symbolized 3-1 (a) and, rarely, a 2-2 catenane (b). Recombination of  $\phi X \cdot 51 \cdot \phi X$  generated tetrameric catenanes in which pA and pB were each linked to a  $\phi X$  molecule (c) or both  $\phi X$  molecules were linked to either progeny pA or pB (d). Recombination within the symmetric four-site substrate,  $pA^4$ , yielded  $pA \cdot A \cdot A^2$  (e) and  $pA \cdot A^2 \cdot A$  (f) in the second round and  $p/iso A_4$  (g) and  $pn A_4$  (h) in the third. Bar = 1  $\mu m$ .

boring site reaction produces a ring with a single *res* site linked to a ring with three sites. These two products are symbolized 2-2 and 3-1. The products of recombination were nicked with DNAase I and their structure was determined by both electron microscopy and gel electrophoresis (Figure 4).

By electron microscopy, a total of 85 molecules were scored as products of a single recombination event because they appeared to be composed of two linked rings whose composite size equalled that of the substrate. Only five of these were classified as 2-2, and the relative ratio

of 3-1 and 2-2 catenanes was the same after 15 and 180 min reactions. Representative micrographs are shown in Figures 5a and 5b. A control with unreacted substrate showed that molecules occasionally fold over to look like catenanes so that a rare class such as 2-2 catenanes may be overestimated.

In a more accurate procedure, the nicked catenated products were resolved by gel electrophoresis into bands with 1, 2, or 3 interlocks corresponding to the number of recombination events in the four-site plasmid. The singly interlocked DNA was isolated from the gel and decate-

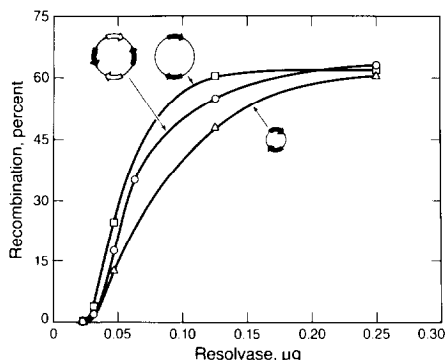


Figure 6. Resolvase Recombination Is Unaffected by Doubling the Standard Distance between Sites and by an Intervening Inverted *res* Site

The products from a standard reaction containing the indicated amount of resolvase were nicked and separated by high-resolution gel electrophoresis. The *res* sites are depicted as filled arrows for pRR51 ( $\Delta$ ) and  $p\lambda 51^2$  ( $\square$ ), which have two directly repeated sites separated by 2750 bp and 5950 bp, respectively, and as filled and open arrows for pR55<sup>2</sup> ( $\circ$ ), which has two pairs of direct sites separated by 5950 bp and oriented as shown. The extent of recombination was determined by comparison of product bands to unreacted material on a densitometric tracing of a photographic negative of an ethidium-stained gel.

nated by DNA gyrase. The unlinked products were analyzed by gel electrophoresis and the ratio of rings containing one or three sites to those with two sites was 20:1. Even this low estimate of 5% opposing site recombination may have been inflated by contamination of the gel-purified material with second-round products that, of necessity, contain two-site rings. Thus, as predicted by the looping model, resolvase recognizes the spatial arrangement of *res* sites and while conducting its search only rarely skips a site.

#### Recombination by Resolvase Is Not Strongly Distance-Dependent or Blocked by an Intervening Inverted Site

An alternative to the orderly search explanation for neighboring site preference is that recombination is strongly distance-dependent. To test this, we compared recombination with pRR51 that has two *res* sites separated by the same distance as the neighboring sites in  $p51^2$  to that with  $p\lambda 51^2$ , where the sites are spaced like the opposing sites in  $p51^2$ . The 2-fold greater distance between *res* sites in  $p\lambda 51^2$  did not affect the extent of reaction (Figure 6). Moreover, recombination with  $p\lambda 51^2$  and pRR51 had identical kinetics. Thus the striking preference for neighboring sites is not a trivial consequence of the greater distance between opposing sites.

We confirmed that the scarcity of opposing site recombination in  $p51^2$  is due to the intervening *res* sites that divert recombination.  $p55^2$  DNA has four *res* sites spaced as in  $p51^2$ , but all adjacent sites are in inverted orientation. At resolvase concentrations up to a 250-fold excess over DNA, recombination between opposing sites occurred to the same extent as with substrates with only directly repeated sites, pRR51 and  $p\lambda 51^2$  (Figure 6). If all four sites in  $p55^2$  were filled by resolvase before recombination initiated, then protein bound at an adjacent inverted site

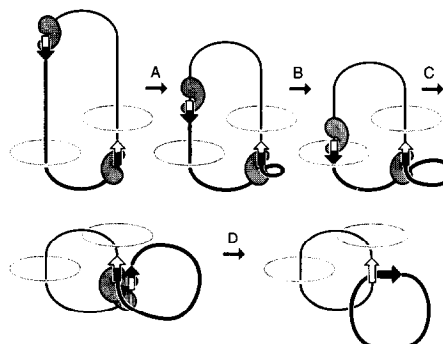


Figure 7. Reporter Ring Test of Looping Model

An abbreviated form of the looping model depicted in Figure 1 is shown except that two  $\phi X174$  RF molecules (grey circles) are catenated to the resolvase substrate and supercoils are omitted for clarity. The  $\phi X$  reporter rings are initially excluded from the DNA loop formed by resolvase at a *res* site (A) and they remain excluded during loop expansion (B and C) because resolvase seals off the base of the loop. The consequence is that after recombination (D) both reporter rings are segregated onto the same progeny circle. Segregation of one reporter to each recombinant is precluded by the model.

does not pose an important barrier to molecules searching for a properly oriented site.

#### The Reporter Ring Test of the Translocation Aspect of the Looping Model

The presence of catenated rings that we call reporter rings on a resolvase substrate provides a topological test of whether resolvase slides continuously from one *res* site to another (Figure 7). The loop of DNA entrapped by resolvase is assumed to be initially small because resolvase is sensitive to the relative orientation of *res* sites and only makes productive intramolecular contacts. DNA rings catenated to the substrate should be excluded from this small loop and remain excluded as the loop enlarges. Thus the looping model predicts that recombination would segregate both of these reporter rings to one of the recombinant circles and a lone reporter ring linked to a product circle would never result.

The substrate for this experiment was constructed by catenating  $\phi X174$  DNA to the resolvase substrate, pRR51, using DNA gyrase. The desired trimeric catenane with two  $\phi X174$  rings linked directly to pRR51, symbolized  $\phi X \cdot 51 \cdot \phi X$ , represented only a few percent of the gyrase products but was purified by preparative gel electrophoresis (Figure 8, lane 4). The substrate was reacted with resolvase and recombinant ring pB was cut with Bam HI to allow analysis by gel electrophoresis of reporter ring segregation to the intact recombinant ring pA (lane 3). The presence of the reporter rings diminished the reaction by at most 2-fold as determined from the relative amount of linear pB resulting from the reporter substrate (lane 3) and the pRR51 control (lane 1). We prepared markers for recombinant pA linked to one and two  $\phi X$  rings by catenating with DNA gyrase  $\phi X$  to pA (lane 2) and pA to itself (lane 5). A substantial proportion, about 40%, of the recombinant ring pA is linked to only one reporter ring (lane 3). Because the two reporter rings are not always segregated together, resolvase does not slide continuously between sites as originally postulated in the looping model.

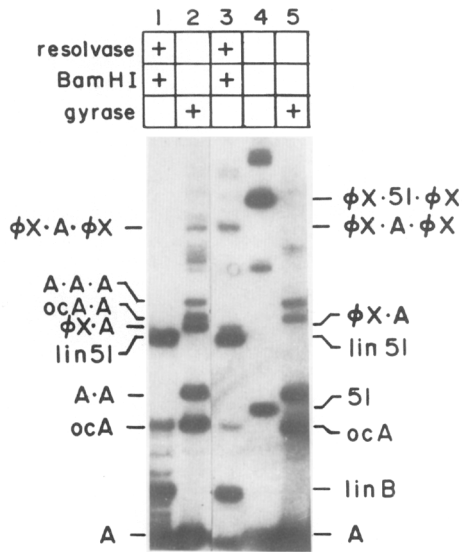


Figure 8. Reporter Rings Segregate Independently during Recombination

The substrate diagrammed in Figure 7 of two  $\phi$ X174 RF molecules catenated to pRR51,  $\phi$ X-51- $\phi$ X, was prepared using DNA gyrase and purified. It (lane 3) and a pRR51 control (lane 1) were reacted with resolvase, digested with Bam HI, and subjected to gel electrophoresis. Lane 4 contains untreated  $\phi$ X-51- $\phi$ X. A Southern blot of the gel was probed with labeled pRR51;  $\phi$ X174 released by Bam HI was therefore not detected. An autoradiogram is shown. The two important restricted products of  $\phi$ X-51- $\phi$ X are recombinant pA catenated to one or two  $\phi$ X molecules indicated by  $\phi$ X-A and  $\phi$ X-A- $\phi$ X, respectively, in the right margin. Markers for these were made by catenating with DNA gyrase pA and  $\phi$ X (lane 2) and pA alone (lane 5);  $\phi$ X-A and  $\phi$ X-A- $\phi$ X are the major additional bands in lane 2 compared to lane 5. Other bands in lanes 2, 3, 4, and 5 are catenanes with nicking of the various substituent rings. These are absent in the pRR51 control (lane 1), which provides markers for linear pRR51 (lin 51), open circular pA (ocA), linear pB (linB), and supercoiled pA (A), as noted in the right margin.  $\phi$ X-A in lane 3 is the upper band of the doublet with linear pRR51.

Table 1. Distribution of Reporter Ring Experiment Products

Product	Analysis by	
	Electron Microscopy	Gel Electrophoresis
$\phi$ X-A-B- $\phi$ X	18%	80%
$(\phi$ X) <sub>2</sub> -A-B + $(\phi$ X) <sub>2</sub> -B-A	21%	
$\phi$ X- $\phi$ X-(A-B)	54%	
$\phi$ X <sup>2</sup> -(A-B)	7%	

$\phi$ X-pRR51- $\phi$ X was treated with resolvase for 15 min. A portion of the products was restricted with Bam HI and analyzed by gel electrophoresis by a procedure similar to that used in Figure 8. The remainder was spread for electron microscopy and 87 product molecules were scored. The nomenclature is  $\phi$ X-A-B- $\phi$ X, a  $\phi$ X174 ring catenated to each of the product pA and pB rings;  $(\phi$ X)<sub>2</sub>-A-B and  $(\phi$ X)<sub>2</sub>-B-A, both  $\phi$ X rings catenated to pA and pB, respectively;  $\phi$ X- $\phi$ X-(A-B),  $\phi$ X dimer catenane linked to pA or pB;  $\phi$ X<sup>2</sup>-(A-B),  $\phi$ X dimeric concatamer catenated to pA or pB. The three product catenanes containing two equivalents of  $\phi$ X were not resolved by the electrophoresis conditions used.

The precise ratio of actual recombinants with one and two reporter rings cannot be assessed by this experiment because of a technical limitation. The  $\phi$ X-51- $\phi$ X substrate is contaminated with about an equal amount of pRR51 catenated to a dimer  $\phi$ X catenane,  $\phi$ X- $\phi$ X-51, and, to a lesser degree, with pRR51 linked to a concatameric dimer

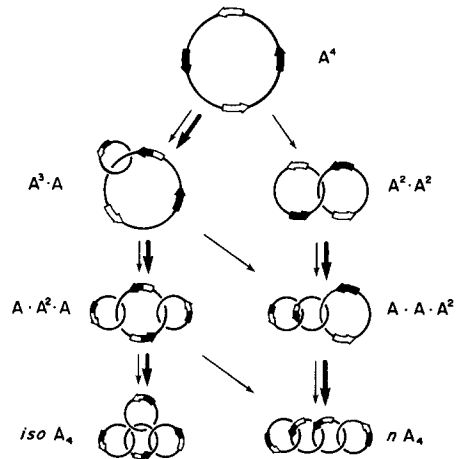


Figure 9. Recombination Pathways for a Substrate Containing Four *res* Sites

The resolvase substrate pA<sup>4</sup>, a plasmid with four *res* sites in head-to-tail orientation, is shown at the top. The identical sites are depicted as open and filled arrows. Recombination events predicted by the looping model are shown by bold pathway arrows, whereas light pathway arrows indicate events permitted by most other schemes. The looping model predicts that only the pathway on the left will be used. Superscripts and subscripts indicate the number of copies of pA in a concatamer and catenane, respectively; a center dot indicates catenation, *n* a straight chain catenane, and *iso* a branched chain catenane.

$\phi$ X because all three have nearly the same electrophoretic mobility. Recombination within the contaminating substrates will lead to pA linked to two or zero  $\phi$ X equivalents, but never one. This problem was avoided by using electron microscopy to distinguish the products. Using a different preparation of the reporter ring substrate, gel analysis showed that 20% of the restricted products had one  $\phi$ X ring catenated to progeny pA and the rest had two or zero equivalents of  $\phi$ X linked to pA. Visualization by electron microscopy of a portion of products before Bam HI digestion showed 16 (18%) molecules with one  $\phi$ X ring catenated to each progeny ring and 18 (21%) with both  $\phi$ X rings segregated to one of the product rings (Table 1; Figures 5c and 5d). Thus it is about equally likely that the reporter rings will segregate together as separately.

### Successive Rounds of Recombination on a Four-Site Plasmid

The major product of the first round of recombination on a four-site substrate is a 3-1 catenane. The structures of the products from subsequent rounds provide independent tests of the continuous sliding feature of the looping model because the linked monomeric ring products serve as reporters. The outcomes from successive rounds of recombination are diagrammed in Figure 9. The substrate depicted is pA<sup>4</sup>, which contains four copies of pA joined head-to-tail; the reactions predicted by the looping model are shown by bold pathway arrows. Recombination between opposing sites produces a catenane of two pA<sup>2</sup> rings, pA<sup>2</sup>-A<sup>2</sup>, and the reaction between adjacent sites generates pA<sup>3</sup>-A. In looping, the pA ring of a pA<sup>3</sup>-A would be excluded from the loop initiating the second round of recombination and thus segregate to the pA<sup>2</sup> product. Be-

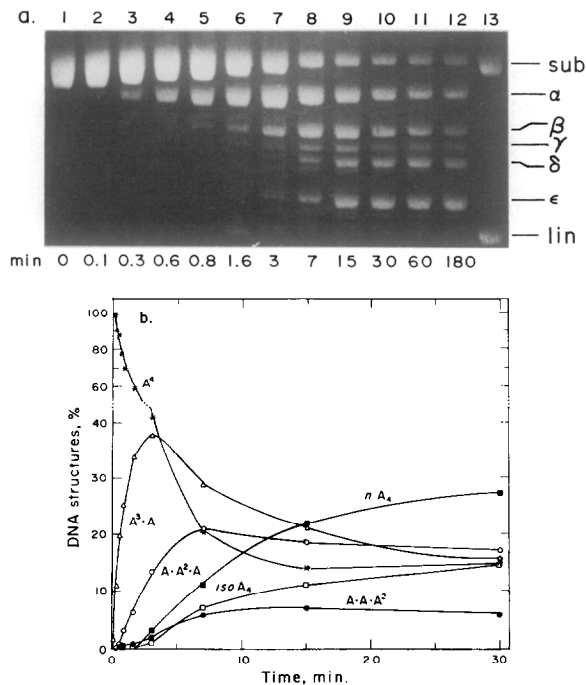


Figure 10. Kinetics of Recombination of  $pA^4$   
(a) At the times indicated below lanes 1–12, samples were removed from a 400  $\mu$ l resolvase reaction with  $pA^4$ , quenched, and nicked with DNAase I before separation by high-resolution gel electrophoresis. The ethidium bromide-stained gel is shown. Product bands are lettered  $\alpha$ – $\epsilon$  in order of increasing mobility. Markers of open circular (sub) and linear (lin)  $pA^4$  were generated by partial digestion with Eco RI (lane 13).  
(b) Quantitative analysis of the gel in (a) was by densitometric tracing of the photographic negative. The time scale is shown only to 30 min because the ratio of products in the second and third round of recombination remain essentially constant after 15 min. Note the scale change between 40% and 50% recombination.  $pA^4$  (\*),  $pA^3 \cdot A$  ( $\Delta$ ),  $pA \cdot A^2 \cdot A$  (O),  $pA \cdot A \cdot A^2$  ( $\bullet$ ),  $piso A_4$  ( $\square$ ), and  $n A_4$  ( $\blacksquare$ ).

cause the other second-round product, another  $pA$ , would also be linked to  $pA^2$ , the predicted structure is  $pA \cdot A^2 \cdot A$ . Precluded by looping is its geometric isomer,  $pA \cdot A \cdot A^2$ , in which two  $pA$  rings are linked to each other and only one is linked to  $pA^2$ . Analogously, in the third round of recombination, both  $pA$  reporter rings of a  $pA \cdot A^2 \cdot A$  substrate will partition to one of the product rings giving the branched tetrameric catenane symbolized  $piso A_4$ . Other mechanisms predict a substantial proportion of the unbranched isomer,  $pn A_4$ .

To measure quickly and quantitatively the six possible recombination products, we replaced the elaborate techniques of electron microscopy and decatenation of gel-purified products with high-resolution gel electrophoresis. Success in resolving geometric isomers involved two factors. First, the symmetric four-site plasmid,  $pA^4$ , was used because the difference in size of the progeny rings from  $p51^2$  resulted in overlapping mobilities. Second, electrophoresis was carried out extremely slowly, usually for 7–8 days.

Electrophoresis of nicked  $pA^4$  recombination products separated five open circular bands, lettered  $\alpha$  to  $\epsilon$  in order of increasing electrophoretic mobility (Figure 10a). No unlinked recombinant rings were produced. Bands  $\beta$  through  $\epsilon$  were excised from a preparative-scale gel and the DNA

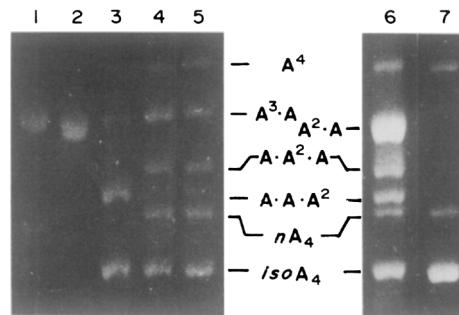


Figure 11. Catenanes of  $pA$ ,  $pA^2$ , and  $pA^3$  Made by DNA Gyrase—Substrates for Resolvase and Markers for Resolvase Products  
 $pA^4$  was incubated with resolvase and after 20 min the products were nicked and displayed by high-resolution gel electrophoresis (lane 5). Catenanes produced by DNA gyrase were nicked and run as standards; these reactions contained:  $pA$  (lane 7),  $pA$  and  $pA^2$  (lane 6),  $pA^2$  (lane 2), and  $pA$  and  $pA^3$  (lane 1). The gyrase catenanes made with  $pA^3$  and  $pA$  (lane 1) and  $pA^2$  (lane 2) were also treated with resolvase, nicked, and analyzed (lanes 4 and 3, respectively). Open circular and linear  $pA^4$  markers are in lane 1.

was examined by electron microscopy. A total of 236 molecules were scored and representative micrographs are shown in Figures 5e–5h. The predominant structures seen were  $pA \cdot A^2 \cdot A$  in band  $\beta$ ,  $pA \cdot A \cdot A^2$  in band  $\gamma$ ,  $piso A_4$  in band  $\delta$ , and  $pn A_4$  in band  $\epsilon$ .

Singly linked catenanes synthesized with DNA gyrase (Krasnow and Cozzarelli, 1983) were used to corroborate these band assignments, establish the electrophoretic mobility of first-round recombinants, and provide substrates for later rounds of recombination that are not generated by resolvase (Figure 11). With  $pA$  alone as the gyrase catenation substrate (lane 7), DNA bands comigrating with  $\delta$  and  $\epsilon$  (lane 5) were produced in a ratio of about 1 to 5. Because gyrase preferentially forms straight chain over branched catenanes (Kreuzer and Cozzarelli, 1980), this confirms that  $\delta$  is  $piso A_4$  and  $\epsilon$  is  $pn A_4$ . Catenation of  $pA^3$  with  $pA$  yielded  $pA^3 \cdot A$  (lane 1), which comigrated with band  $\alpha$ .  $pA^2 \cdot A^2$ , made using  $pA^2$  as the gyrase substrate (lane 2), migrated just ahead of  $pA^3 \cdot A$ , and all 45 first-round products analyzed by electron microscopy were  $pA^3 \cdot A$ . Therefore, neighboring-site recombination is clearly the rule in the first round and the finding of all four products in the second and third rounds of recombination of  $pA^4$  proves that continuous sliding between sites cannot be the exclusive recombination path.

The production of all four products in subsequent recombination rounds is not the result of an alteration in the enzyme or DNA (e.g., relaxation) during the first recombination round.  $pA^3 \cdot A$  synthesized with gyrase has a supertwist density higher than normal, yet all four possible second- and third-round structures were still formed by resolvase (lane 4). (The looping path may have been preferred in this experiment as evidenced by the increased ratio of  $pA \cdot A^2 \cdot A$  to  $pA \cdot A \cdot A^2$ ). The synthesis of  $pA^2 \cdot A^2$  by gyrase allowed us to examine a path that is typically not taken by resolvase. Because the only two products were  $pA \cdot A \cdot A^2$  and  $pn A_4$  (lane 3),  $pA \cdot A^2 \cdot A$  and  $piso A_4$  must derive from  $pA^3 \cdot A$  as shown by the pathway in Figure 9. The reactivity of  $pA^2 \cdot A^2$  and  $pA^3 \cdot A$  was not significantly

Table 2. Distribution of pA<sup>4</sup> Recombination Products

Recombination Round	Product Catenane	Observed	Expected			
			Looping Model (%)		Random Model (%)	
			Uncorrected	Corrected	Uncorrected	Corrected
1st	pA <sup>3</sup> ·A	≥95	100	100	67	67
	pA <sup>2</sup> ·A <sup>2</sup>	≤ 5	0	0	33	33
2nd	pA·A <sup>2</sup> ·A	70	100	95	45	64
	pA·A·A <sup>2</sup>	30	0	5	55	36
3rd	<i>piso</i> A <sub>4</sub>	35	100	70	23	35
	<i>pn</i> A <sub>4</sub>	65	0	30	77	65

The frequency of resolvase products for each round of recombination of pA<sup>4</sup> for the experiment shown in Figure 10 is tabulated for the 15 min time point; the product distribution holds constant after this time. Also tabulated are the results expected from the looping model and a model in which sites recombine randomly without regard to distance and order of sites. For both models a corrected prediction is included, which is based on the observed frequency in the previous round.

different, and thus the paucity of pA<sup>2</sup>·A<sup>2</sup> resolvase products is not a consequence of more rapid turnover.

We analyzed the kinetics of product formation (Figure 10b). The initial rate of the reaction is very fast, one-half of the substrate reacted once in 1.5 min. The extrapolated half-times are 3 min for the second recombination round and 5 min for the third. Thus the presence of one or two catenated pA rings does not have a major effect on the rate of the reaction, particularly given the possibility of decay of productive sites and of enzyme. pA<sup>3</sup>·A reaches a maximum at 3 min and diminishes 4-fold as later products are generated. A *res* site can be used more than once in a reaction because the end products require cumulative action at six sites in a plasmid that has only four sites. The kinetics confirm that pA<sup>2</sup>·A<sup>2</sup> is not turned over significantly more rapidly than pA<sup>3</sup>·A. The only products of pA<sup>2</sup>·A<sup>2</sup>, pA·A·A<sup>2</sup> and *pn* A<sub>4</sub>, should greatly predominate at earlier times if pA<sup>2</sup>·A<sup>2</sup> is processed preferentially, but they do not.

The appearance of pA·A<sup>2</sup>·A earlier than pA·A·A<sup>2</sup> may suggest that looping is somewhat preferred. However, in the third round there is more *pn* A<sub>4</sub> than the expected product of looping, *piso* A<sub>4</sub>. Indeed, after 15 min, the frequency of second- and third-round products fits much better a model in which sites collide randomly, rather than the looping model (Table 2). However, the evidence is overwhelming against a random collision of freely mobile sites. We therefore favor models in which the enzyme tracks along DNA, but avoids barriers so well that the segregation pattern of catenated rings approaches that expected by random collision.

## Discussion

By examining DNA topology during a reaction, one can make critical inferences about how distant DNA segments interact and how an enzyme manipulates its substrate. The power of this approach lies in both the preservation of transient aspects of the reactions through topological linkages and our expanding ability to analyze the stereostructure of DNA supercoils, knots, and catenanes. The approach was pioneered by Wang, who demonstrated the extent of DNA unwinding or wrapping attendant to the binding of key proteins (e.g., Saucier and Wang, 1972).

Elucidation of the characteristic change in linking number by topoisomerases led to the sign-inversion mechanism for these enzymes (Brown and Cozzarelli, 1979). Here we focused not on linking number but on the geometric arrangement of catenated rings, another topological invariant. Resolvase is an attractive model for studying protein movement along DNA because, unlike repressors and restriction enzymes, it yields products whose topology provides important information on movement.

Our four major findings will be enumerated and then discussed in turn. First, the products of resolvase action are singly linked catenanes over the range of substrate supercoiling tested (relaxed to almost twice native density). Second, this product topology contrasts sharply with Int-mediated recombinants that have a variable and greater number of interlocks even when the two reactions are carried out together. Third, recombination by resolvase shows a strong position effect since proximal sites are preferred at least 20-fold over distal sites in a four-site substrate. Finally, rings catenated to a resolvase substrate are not partitioned to the daughter rings as predicted by schemes in which the enzyme slides continuously between sites.

The supercoil density of the substrate was varied to challenge the ability of resolvase to, on one hand, prevent the wholesale incorporation of supercoil interwindings into catenane interlinks and, on the other, to ensure a single interlock when the supercoil density is low. We found no free rings or rings with two simple interlocks. This supports the looping mechanism. The end product of looping is two sites directly across from each other with the supercoil interwindings segregated into the domains that will be separated by recombination (see Figure 1). The interlocking of progeny rings is dictated by both the alignment of sites at synapsis and the mechanism of strand exchange and is oblivious to substrate supercoiling.

An alternative proposal is that supercoils are not interwound (plectonemic) as shown in Figure 1, but are highly branched or solenoidal (wound around an external axis) as in nucleosomes (Brady et al., 1983). The segregation of interwindings into recombinant domains could then be inherent in the DNA structure rather than imposed by the mechanism of synapsis. However, supercoil form is highly

dependent on supercoil density and ionic strength, whereas the interlocking of resolvase products is not. Resolvase recombinants remain singly linked even when the substrate supercoil density is twice that of native plasmids (Figure 3a) or when the NaCl concentration is 0.4M (H. Benjamin, unpublished data), conditions that favor plectonemic supercoils over solenoidal forms (Upholt et al., 1971). Moreover, Int substrate supercoils are plectonemic (S. J. Spengler, A. Stasiak, and N. R. Cozzarelli, unpublished data), and both Int and resolvase catenanes maintain their characteristic structure even when produced in the same reaction mixture.

Our tests of the systematic search and continuous sliding features of the looping model are novel. The former was tested by the position effect of sites on recombination. By three independent methods and for two different four-site substrates, we found at least a 20-fold preference for recombination of neighboring sites. The less interesting explanation that resolvase simply could not act at widely spaced sites was eliminated by showing that recombination took place normally when the intervening *res* sites were deleted or inverted. Neighboring site preference is difficult to reconcile with synapsis via random collision of sites as proposed for Int (Mizuuchi et al., 1980), but is a natural consequence of models in which the enzyme moves in an orderly way from one site to the other. In addition, recent preliminary analysis of the fate of a knotted resolvase substrate is also consistent with looping (S. A. Wasserman and N. R. Cozzarelli, unpublished data).

In the face of the impressive evidence for the looping model, the failure of the continuous sliding aspect of the model was surprising. There are four experiments not in accord with simple sliding.

The first test used p55<sup>2</sup>, a four-site substrate with two interleaved pairs of directly repeated sites but with neighboring sites in inverted orientation (Figure 6). Each pair of direct sites recombined as well as in the homologous substrate devoid of intervening inverted sites. Because there were up to 250 protomers of resolvase per substrate molecule, each site may have been coated with at least six protomers (Grindley et al., 1982; Kitts et al., 1983) and formed the base of an expanding loop of DNA. Nonetheless, these resolvase-tethered loops must readily pass one another in their search for a properly oriented site. Although the result was unexpected, the interpretation is qualified by the unproven site occupancy, and by the possibility that another step is limiting. Also, resolvase could contact predominantly one face of the helix and leave a passage on the opposite side.

The three reporter ring experiments gave the same answer; the substrate does not have a domain impenetrable to a catenated ring as implied if resolvase forms a loop at one *res* site and slides continuously to another. First, two  $\phi$ X rings catenated by DNA gyrase to a resolvase substrate segregated independently into progeny rings and not always together as predicted by looping. Second, with pA<sup>4</sup> as substrate, both the second and third rounds of recombination gave both possible geometric isomers as products, whereas looping predicts only a single product for each round. Third, recombination within pA<sup>3</sup>-A synthe-

sized with DNA gyrase gave similar results. Thus the intermolecular interwindings of catenated DNA are not segregated into each of the progeny rings as predicted by the model; this is in contrast to the segregation of the intramolecular interwindings of supercoils and knots.

The presence of reporter rings catenated to a substrate did not diminish appreciably either the rate or extent of recombination, and the geometric isomer products of pA<sup>4</sup> precluded by looping were generated at about the same rate as those predicted by looping. The catenated ring also did not diminish the site-orientation specificity that provides an important impetus for the looping model. With a substrate of two catenated rings, each with a pair of inverted sites, the frequency of inversion remained only about one percent.

The possibility remains that resolvase slides continuously over most of the distance between sites but that either at the beginning or the end of search the reporter ring enters the loop. The initial loop could contain a reporter ring frequently if for some reason, e.g., binding to resolvase, it tends to be sequestered there. At the end of the search, when neighboring loops may coalesce, the reporter ring could slip into the previously excluded domain. A third way to reconcile the data with continuous sliding is suggested by the water-locks-type mechanism that has been proposed for topoisomerases (Wang et al., 1980). Topoisomerases can be envisioned as having two portals for DNA that are open alternately, allowing a DNA segment to pass through the enzyme. If resolvase, a topoisomerase itself, can operate in this way, then a reporter ring could slip through the enzyme and into the loop. Qualifications such as these are difficult to rule out, but in the absence of direct evidence in their favor, we prefer the simpler interpretation that resolvase does not slide continuously between sites.

In consequence, the looping model must be modified or a different model adopted. An alternative model proposes that in a plectonemically supercoiled substrate DNA continually circles like a conveyor belt with the interwound segments slithering past each other (Krasnow et al., 1983). Distant *res* sites would eventually meet, triggering recombination. Singly linked catenanes could result because at the time of recombination supercoil interwindings are intradomain just as in the last step of the looping model; moreover, the orientation of sites is controlled by DNA structure. This model has difficulty explaining the differences from Int and particularly troubling is the neighboring site preference of resolvase, but elaborations can accommodate these results.

The simplest modification of the looping model consistent with the bulk of the data is what we call gated looping. The basic idea is that sliding is occasionally interrupted and the enzyme bypasses a short region of DNA. For instance, this could occur by direct transfer to a nearby segment so that the enzyme does not detach from one segment until it has bound the second segment. If a reporter ring lies across the bypassed region, then the results are explained. The sequence skipped must be short and the gating controlled so that resolvase rarely skips *res* sites, transfers to a DNA segment either in the opposite orienta-

Table 3. DNA Substrates

Plasmid	Size (kb)	Description of Substrate (alias)	Reference or Source
pRR51	5.9	2 direct <i>res</i> sites, 2.7 and 3.2 kb apart	Reed, 1981a
pRR55	5.9	2 inverted <i>res</i> sites; inversion of one site in pRR51	"
pA	3.2	single <i>res</i> site; recombinant of pRR51 (mono51A)	Krasnow and Cozzarelli, 1983
pB	2.75	single <i>res</i> site; recombinant of pRR51 (mono51B) contains unique Bam HI site	"
pA <sup>2</sup>	6.4	2 direct <i>res</i> sites; concatamer of pA (mono51A <sup>2</sup> )	"
pA <sup>3</sup>	9.6	3 direct <i>res</i> sites; concatamer of pA	this work
pA <sup>4</sup>	12.8	4 direct <i>res</i> sites; concatamer of pA	"
p51 <sup>2</sup>	11.9	4 direct <i>res</i> sites; concatamer of pRR51	"
p55 <sup>2</sup>	11.9	4 <i>res</i> sites of alternating orientation; concatamer of pRR55	"
pλ51 <sup>2</sup>	11.9	2 direct <i>res</i> sites 5.9 kb apart, replacement of one set of opposing sites in p51 <sup>2</sup>	"
pBP86	9.8	2 direct <i>att</i> sites 1.5 and 8 kb apart	Mizuuchi et al., 1980

Concatemeric substrates were isolated by preparative agarose gel electrophoresis of plasmid preparations and propagated in the *recA*<sup>-</sup> strain, MG1655.10.

tion or in another molecule, or allows supercoils and knots to span the two progeny domains. Segment transfer is in fact expected for a protein with multiple DNA binding sites and there is physical evidence that it occurs for several other proteins such as the *lac* repressor and CAP that are also postulated as sliding along DNA (von Hippel et al., 1984). A clear difficulty, though, is that there is no obvious way that the segment transfer can be limited sufficiently to explain the resolvase data unless there are some unknown constraints on the structure of DNA. The gated looping model remains to be established but it would have the important advantage in vivo of allowing a tracking enzyme to avoid barriers and thus avoid the embarrassment of collisions with other tracking enzymes, DNA that lies across its path in its condensed intracellular state, and patches of cell membrane or RNA affixed to the DNA.

We have developed three topological methods to study the interaction between enzymes and DNA. First, by comparing catenated substrates made with gyrase or resolvase with concatamers one can distinguish the difference between covalent and topological linkage of DNA segments. One can determine, for example, whether an apparently *cis*-acting regulatory sequence must be on the same DNA molecule or can be on a second molecule held close by. Second, reporter rings linked to a substrate test the movement of any enzyme that entraps a DNA loop. Looping was first proposed for type 1 restriction enzymes (Rosamond et al., 1979), and electron microscopy has shown that other proteins bind together distant DNA segments (Moore et al., 1983). Third, high-resolution gel electrophoresis (Sundin and Varshavsky, 1981) separates catenanes that differ in not only the number but also the arrangement of interwindings. Thus, geometric isomers such as pA·A<sup>2</sup>·A and pA·A·A<sup>2</sup> were resolved. This improvement, along with the recent development of methods to determine the absolute structure of catenanes and knots (Cozzarelli et al., 1984), facilitates the topological approach to enzymology.

#### Experimental Procedures

##### DNAs

All DNA substrates used are described in Table 3. In our nomenclature, a superscript indicates the number of composite monomers in the con-

catamer and a subscript the number of molecules linked as catenanes. Catenated rings are also indicated by a centered dot. Thus, pA<sub>2</sub> and pA·A are equivalent names for a catenane of two pA rings and pA<sup>2</sup> is the concatameric dimer of pA. The trimeric pA<sup>3</sup> was isolated as an aberrant product after transformation of *E. coli* with pA<sup>4</sup>. pλ51<sup>2</sup> was constructed as follows. pRR51 was treated with Eco RI and Bam HI to remove a 1.3 kb piece containing one *res* site. This was replaced by the 1.3 kb Eco RI to Bam HI fragment of pPH201 that contains the λ *attL* prophage attachment site (Hsu et al., 1980). The resultant plasmid was dimerized to give pλ51<sup>2</sup>.

##### Enzymes

Tn3 resolvase (Krasnow and Cozzarelli, 1983) and *E. coli* DNA gyrase subunits A and B (Otter and Cozzarelli, 1983) were purified as described previously. Rat liver topoisomerase I was a gift of A. Sugino (University of Georgia, Athens). DNAase I and restriction enzymes were from commercial suppliers. Int was purified by a modification of the method of Kikuchi and Nash (1978) from *E. coli* strain P72, an over-producing strain constructed by H. Echols and J. Quan (University of California, Berkeley), which carries *F*'*lacI*<sup>Q</sup> and the plasmid λ *int* pGL101. *E. coli* integrative host factor, IHF, was prepared essentially by the method of Nash and Robertson (1981). Because all reactions containing the λ *int* product also contained IHF, the proteins are collectively referred to as Int.

##### pRR51 Preparations Differing in Supercoil Density

These were generated by treatment of pRR51 with rat liver DNA topoisomerase I in the presence of various concentrations of ethidium bromide up to 20 μg/ml. The supercoil density of each preparation was determined by electrophoretic band counting, using a series of agarose gels containing up to 30 μg/ml of chloroquine (Shure et al., 1977). The linking number deficit from relaxed DNA divided by double helical twist, the specific linking difference ( $\sigma$ ), is referred to as supercoil density.

##### Enzyme Reactions

Standard resolvase reactions were at 37°C for 1 hr and contained, in 20 μl: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.2 μg DNA, and 0.14 to 0.25 μg resolvase. The reactions were stopped by heating at 65°C for 10 min. For comparison of resolvase and Int, 75 min reactions at 30°C contained, in 15 μl: 20 mM Tris-HCl, pH 7.45, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, BSA at 50 μg/ml, 0.2 μg pBP86 or pA<sup>2</sup>, and either 42 ng IHF and 40 ng Int or 10 ng resolvase.

For analysis of catenane structure, products were nicked by DNAase I in the presence of ethidium bromide before high-resolution gel electrophoresis. To monitor recombination of pRR51, products were digested with Bam HI to disrupt the catenated recombinants before gel electrophoresis (Krasnow and Cozzarelli, 1983).

Catenation of DNA by *E. coli* DNA gyrase was at 30°C for 3 hr and contained, in 15 μl: 15 mM Tris-HCl, pH 7.6, 20 mM KCl, 2 mM MgCl<sub>2</sub>, 5 mM spermidine-HCl, 1 mM dithiothreitol, 1 mM ATP, BSA at 50 μg/ml,

0.2  $\mu$ g DNA, and 40 fmol of gyrase. Decatenation by gyrase was at 30°C for 1 hr and contained, in 20  $\mu$ l: 25 mM Tris-HCl, pH 7.6, 15 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, albumin at 50  $\mu$ g/ml, 1 mM spermidine-HCl, 1 mM ATP, 0.1  $\mu$ g DNA, and 40 fmol of gyrase.

#### Gel Electrophoresis

Standard and high-resolution analysis of DNA by electrophoresis through agarose gels and staining with ethidium were carried out as described (Krasnow and Cozzarelli, 1983). The high-resolution separation of nicked structural isomers on 27 cm long horizontal gels was at 1 V/cm for 7–8 days. Transfer to nitrocellulose and hybridization to [<sup>32</sup>P]pRR51 were used to enhance sensitivity. Densitometric tracing of photographic negatives of the ethidium-stained gels was used for quantitation of DNA forms.

#### Isolation of DNA from Agarose Gels and Electron Microscopy

DNA was purified from agarose gels by two methods. The glass bead method (Vogelstein and Gillespie, 1979) was used for preparation of DNA for decatenation by DNA gyrase. DNA to be visualized by electron microscopy and the substrate for the reporter ring experiment were isolated by electroelution (Krasnow and Cozzarelli, 1983). The reporter ring substrate was further purified by sedimentation through a 10%–30% sucrose gradient to remove an inhibitor of resolvase. DNA was prepared for electron microscopy by the method of Davis et al. (1971). The DNA on a parlodian-coated grid was stained with uranyl acetate, tungsten-shadowed, and examined with a Jeol-100B electron microscope.

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