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Topological Selectivity in Xer Site-Specific Recombination

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Summary

The product topology of Xer-mediated site-specific recombination at plasmid sites has been determined. The product of deletion at pSC101 *psi* is a right-handed antiparallel 4-noded catenane. The ColE1 *cer* deletion product has an identical topology, except that only one pair of strands is exchanged. These specific product topologies imply that the productive synaptic complex and the strand exchange mechanism have fixed topologies. Further analysis suggests that synapsis traps exactly three negative supercoils between recombining sites, and that strand exchange introduces a further negative topological node in the deletion reaction. We present a model in which the requirement for a specific synaptic stucture, with two recombination sites interwrapped around the accessory proteins ArgR and PepA, ensures that recombination only occurs efficiently between directly repeated sites on the same DNA molecule.

Introduction

Interactions between proteins bound at distant sites on nucleic acids are important for a wide range of biological processes, including regulation of gene expression, DNA replication, RNA processing, and recombination. One of the best understood processes involving communication between distant segments on DNA is site-specific recombination, which brings about a wide range of programmed DNA rearrangements in both prokaryotes and eukaryotes (reviewed in Sadowski, 1986). Intriguingly, some site-specific recombination systems require that the interacting DNA segments have a specific relative orientation, this requirement being important for their proper biological function.

The enzymes that carry out site-specific recombination fall into two unrelated families, based on protein sequences and reaction mechanisms (Stark et al., 1992). Enzymes of both families catalyze conservative DNA break–join reactions in which protein phosphodiesters are reaction intermediates. Members of the λ integrase (Int) family of site-specific recombinases employ a tyrosine residue as a catalytic nucleophile and bring about recombination via a Holliday junction intermediate (reviewed in Landy, 1993). Members of the resolvase/DNA invertase family use a serine residue as a catalytic nucleophile and cut all four strands in the recombining sites before exchanging partners and religating recombinant sites (reviewed in Hatfull and Grindley, 1988; Stark et al., 1989a).

Site-specific recombinases act at short asymmetrical DNA sites. Members of the resolvase/DNA invertase family of site-specific recombinases are able to distinguish between sites connected in inverted and direct repeat. Resolvases only act at directly repeated sites, resulting in deletion, whereas DNA invertases only recombine sites in inverted repeat, causing inversion of the intervening segment (Hatfull and Grindley, 1988; Johnson, 1991). In contrast, bacteriophage λ Int, yeast 2 μm FLP, and P1 Cre, which are members of the λ Int family of recombinases, do not generally discriminate between pairs of sites in direct or inverted repeat, or between inter- and intramolecular reactions (Gronostajski and Sadowski, 1985; Abremski et al., 1986; Landy, 1989). A further difference between these systems is that while λ Int, FLP, and Cre give products of varying topological complexity (Mizuuchi et al., 1980; Pollock and Nash, 1983; Abremski and Hoess, 1985; Beatty et al., 1986), the resolvases and DNA invertases give products of specific topologies. Resolvases give singly interlinked (−2) catenanes, and DNA invertases yield unknotted circles (Wasserman and Cozzarelli, 1985; Kanaar et al., 1988).

It has been proposed that members of the resolvase/DNA invertase class of site-specific recombinases can only carry out recombination in highly defined protein–DNA structures (reviewed in Stark and Boocock, 1995). For resolvases, the required protein-DNA structure can only readily be formed from sites in direct repeat on a negatively supercoiled DNA molecule. For DNA invertases, the required protein-DNA structure aligns the recombination sites such that only inversion takes place. A consequence of the defined protein-DNA structure is that a fixed number of supercoils are trapped between recombining sites. This, together with a fixed strand-exchange mechanism, leads to products of defined topology. In contrast, λ Int, FLP, and Cre carry out recombination with variable synapse topology, giving products of mixed topology.

In this study, we investigated the topology of plasmid monomerising Xer site-specific recombination at ColE1 *cer* and pSC101 *psi*. This recombination is catalyzed by XerC and XerD, two members of the λ Int family of site-specific recombinases (Blakely et al., 1993). Recombination occurs at an ~30 bp core site to which XerC and XerD bind, but also requires ~200 bp of accessory sequences adjacent to the core (Summers and Sherratt, 1988). In addition to the recombinase proteins, one accessory protein (PepA) is required for recombination at *psi* (Colloms et al., 1996), and two accessory proteins (ArgR and PepA) are required for recombination at *cer* (Stirling et al., 1988, 1989). PepA belongs to a widespread family of leucine amino-peptidases (Taylor, 1993). In addition to its peptidase activity, PepA has DNA-binding activity and is involved in pyrimidine-specific transcriptional regulation of the *carAB* operon of E. coli (Charlier et al., 1995). ArgR is an L-arginine-

dependent DNA-binding protein that acts as a transcriptional repressor of arginine biosynthetic genes in E. coli (reviewed in Glansdorff, 1987).

Recombination at *cer* and *psi* is preferentially intramolecular, and resolution is highly favored over inversion (Colloms et al., 1996). We show here that the products of recombination at *cer* and *psi* have a specific topology. We propose that there is a requirement for a specific synaptic complex, in which accessory sequences are interwrapped a fixed number of times around accessory proteins, before recombination takes place. The requirement for this specific protein-DNA complex ensures that recombination only occurs efficiently between directly repeated sites on the same circular plasmid.

Results

The Product of Recombination at *psi* Has a Specific Topology

To understand fully the results presented in this study, a few topological terms must first be introduced. When viewed in plane projection, the path followed by a DNA molecule may cross itself. Such crossings are described as nodes and are designated as (−) or (+), according to the convention shown in Figure 2A. A knot is a single closed DNA ring, whereas a catenane consists of two interlinked rings. The node number of a knot or catenane is the minimum number of nodes in a plane projection of the knot or catenane and is a topological invariant, i.e., it cannot be changed without cutting the DNA.

Recombination between directly repeated *psi* sites on a supercoiled circular molecule yields two smaller catenated supercoiled circular products; no free product rings are formed (Figures 1A and 1B, lane 6) (Colloms et al., 1996). To determine the extent of interlinking of the component rings of the *psi* recombination product, the plasmid pSDC134, containing two directly repeated *psi* sites, was incubated with PepA, XerC, and XerD ("treated with Xer"), and the products were then nicked with DNaseI to remove supercoiling. The resulting catenane was analyzed by high resolution gel electrophoresis adjacent to a ladder of nicked pSDC134 knots. In this type of gel, the electrophoretic mobility of nicked knots and catenanes is determined largely by the number of nodes they contain (Dröge and Cozzarelli, 1992; Stasiak et al., 1996). The fully nicked recombination product comigrated with the 4-noded knot (Figure 1B, lanes 4 and 5). Therefore, the product of recombination at *psi* is a 4-noded catenane. The other bands seen in lane 4 of Figure 1B came from partial digestion and over-digestion with DNaseI. No free circles were seen in the sample, which had not been treated with DNaseI (Figure 1B, lane 6), and therefore the free circles seen in lane 4 came from over-digestion with DNaseI. We are confident that at least 95% of the product of recombination at *psi* is 4-noded catenane.

If intramolecular recombination occurs following random collision of sites on a supercoiled molecule, a variable number of supercoils can be trapped and converted into catenation or knotting of the product. Thus, recombination of directly repeated *att* sites by bacteriophage λ Int yields catenanes, whose complexity depends on the extent of supercoiling of the substrate and the relative positions of the recombining sites (Mizuuchi et al., 1980; Pollock and Nash, 1983; Bliska and Cozzarelli, 1987). The specific topology of the *psi* recombination product implies that recombination at *psi* occurs with a specific synapse topology. To show that this recombination does not vary with site spacing, we tested substrates in which directly repeated *psi* sites were separated by 0.4 and 3.0 kb (pSDC153), 1.6 and 3.0 kb (pSDC134), and 1.2 and 1.2 kb (pSDC170); all of these

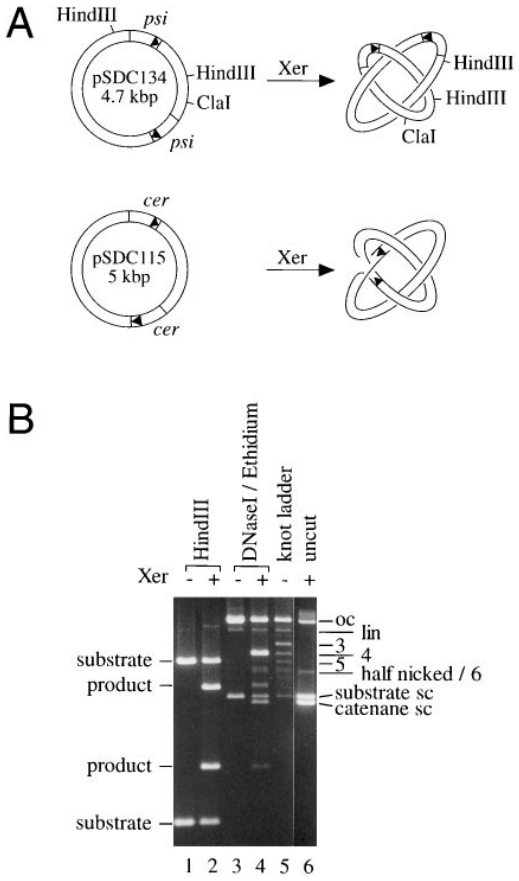

Figure 1. Recombination between Directly Repeated *psi* Sites Generates a 4-Noded Catenane

(A) Recombination substrates and products. Recombination between the two *psi* sites on pSDC134 (4.7 kb) produces 2 catenated circles of 3.05 and 1.65 kb. XerC-mediated strand exchange between the two *cer* sites on pSDC115 (5.0 kb) yields a Holliday junction-containing structure in which the recombined strands form catenated rings of 2.65 and 2.35 kb, and the unrecombined strand remains as a 5.0 kb unknotted circle.

(B) pSDC134 was treated with PepA, XerC, and XerD (lanes 2, 4, and 6; + Xer) or left untreated (lanes 1 and 3; − Xer), cleaved with HindIII (lanes 1 and 2), singly nicked with DNaseI (lanes 3 and 4) or left uncut (lane 6), and run on a 0.7% agarose gel. Lane 5 contains a nicked pSDC134 knot ladder produced by topoisomerase II. The positions of unknotted nicked circular pSDC134 (oc), linear pSDC134 (lin), supercoiled pSDC134 (substrate sc), and supercoiled catenane (catenane sc) are indicated to the right of the gel, as are the positions of nicked 3-, 4-, 5-, and 6-noded knots. HindIII-cleaved substrate and product bands are indicated to the left of the gel. The species seen in lane 4 are as follows: open circle, linear, and supercoiled forms of the unrecombined substrate; fully nicked 4-noded catenane; half-nicked 4-noded catenane in which the 1.6 kb circle remains supercoiled; fully supercoiled catenane; and a small amount of linearized and nicked free product rings released by over-digestion with DNaseI.

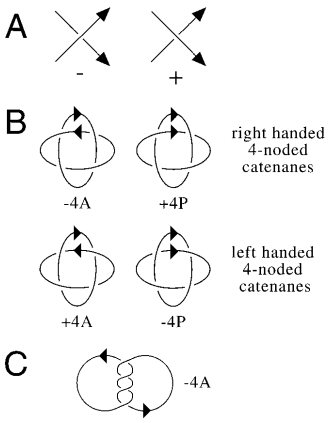

Figure 2. The Four Different 4-Noded Catenanes

(A) Nodes, where one segment of DNA crosses over another in a planar projection, are assigned as either positive or negative according to the convention shown.

(B) The two rings in a 4-noded catenane can either follow a right-handed helical path (−4A and +4P) or a left-handed helical path (+4A and −4P) around each other. The orientation of the two rings, as defined by the direction of the recombination sites, can be either parallel or antiparallel. There are therefore only four topologically distinct 4-noded catenanes, all of which belong to the torus class of catenanes. They are denoted here as −4A, +4P, +4A, and −4P, according to the sign and number of nodes they contain (−4 or +4) and the relative orientation of the two rings, i.e., parallel (P) or antiparallel (A).

(C) An alternative representation of the −4A catenane.

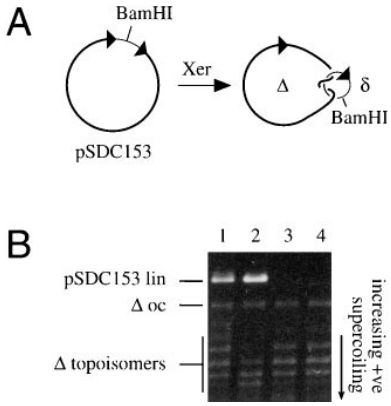

Figure 3. Determination of the Handedness of the *psi* Recombination Product

(A) The substrate pSDC153 contains two closely spaced *psi* sites separated by a BamHI site. In the presence of PepA, XerC, and XerD, pSDC153 recombines to form a 4-noded catenane consisting of two rings: pSDC153Δ (3036 bp) and pSDC153δ (395 bp). The diagram shows a −4A catenane in which the large ring follows a right-handed path around the small ring.

(B) Xer-reacted pSDC153, containing a mixture of unreacted pSDC153 and catenated product, was singly nicked with DNaseI. One half of the sample (lane 1, precut) was treated with BamHI to linearize the small ring and then treated with DNA ligase to seal the nick in the large ring. The other half (lane 2, postcut) was treated first with DNA ligase and then with BamHI. As a control, the plasmid pSDC153Δ, produced from pSDC153 by recombination in vivo, was treated in an identical fashion and loaded in lanes 3 (precut) and 4 (postcut). The DNA was subjected to electrophoresis on a 0.7% agarose gel containing 0.5 μg/ml chloroquine to ensure that the pSDC153Δ topoisomers were positively supercoiled during electrophoresis.

substrates yielded exclusively 4-noded catenane (data not shown). We also looked at the effect of varying superhelical density on recombination of pSDC134. Fully relaxed DNA failed to recombine (Colloms et al., 1996), whereas pSDC134 with superhelical density ranging from approximately 30% to 150% of native superhelical density recombined to give 4-noded catenane (data not shown).

The Product of Recombination at *psi* Is a Right-Handed Catenane

The product of recombination at *psi* could be any one of four possible 4-noded catenanes, all of which belong to the torus class of catenanes (Figure 2B). In two of these catenanes (−4A and +4P), each ring follows a right-handed path around the other; in the other two forms (+4A and −4P), each ring follows a left-handed path around the other. If the component rings of a catenane are nicked and then resealed, the supercoiling remaining in each ring will be due only to the helical path followed by the axis of each DNA ring around the other. If the rings are intertwined in a right-handed fashion, this supercoiling will be positive; if the intertwining is left-handed, the supercoiling will be negative. When the rings of a catenane are of uneven size, the smaller ring adopts a more planar (less supercoiled) configuration, and the larger ring adopts a more supercoiled configuration (Wasserman et al., 1988; Vologodskii and Cozzarelli, 1993). We therefore used a substrate (pSDC153) with unevenly spaced *psi* sites to determine the handedness of the *psi* catenane. Xer treatment of pSDC153 produced a 4-noded catenane consisting of 1 ring of 395 bp and another of 3036 bp (pSDC153δ and -Δ, respectively; Figure 3A). Both rings of this catenane were nicked once using DNaseI in the presence of ethidium bromide. The nicked catenane was either treated with ligase to reseal the nicks and then with BamHI to linearize the small ring (postcut), or treated with BamHI before resealing the large ring with ligase (precut). Topoisomers of the large ring, resealed while catenated (Figure 3B, lane 2), migrated faster on a gel containing chloroquine than the same ring resealed while free (Figure 3B, lane 1). The concentration of chloroquine present in the gel ensured that the topoisomers produced were positively supercoiled during electrophoresis. Therefore, the ring resealed while catenated was more positively supercoiled than that resealed while free, and hence the two circles of the catenane must be intertwined in a right-handed fashion.

To show that this difference in mobility was not caused by any variation in buffer and temperature during ligations before and after BamHI digestion, pSDC153Δ, produced by transforming pSDC153 into an Xer^+ E. coli strain, was treated in a similar fashion. No difference in mobility was observed (Figure 3B, lanes 3 and 4); therefore, the difference observed in lanes 1 and 2 of Figure 3B is due only to catenation of the two rings.

The Product of Recombination at *psi* Is a Right-Handed Antiparallel Catenane

Given that the *psi* recombination product is a 4-noded right-handed catenane, the relative orientation of the

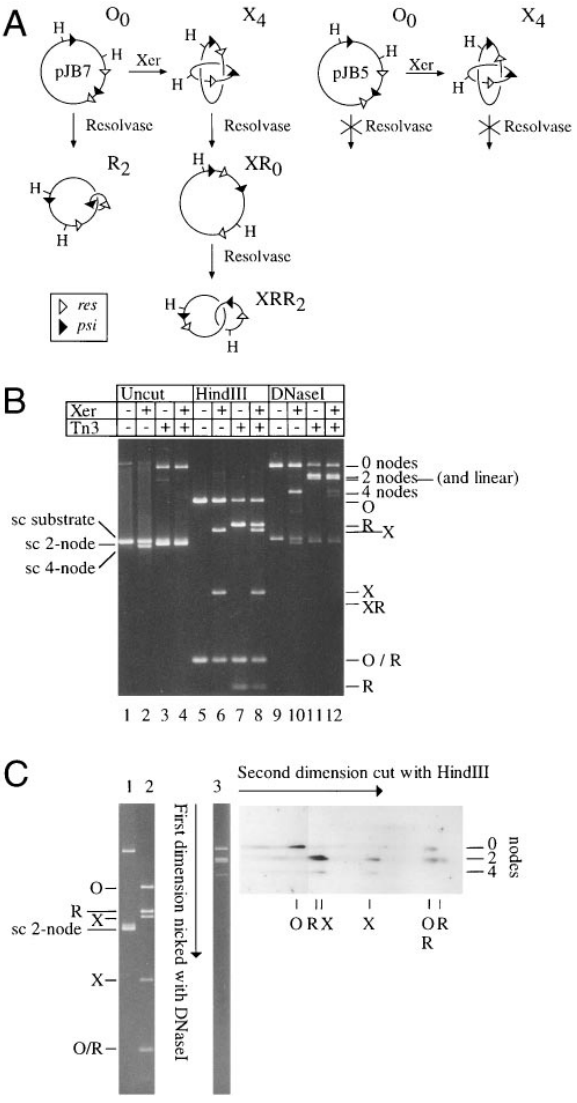

Figure 4. Determination of the Relative Orientation of the Rings in the *psi* Catenane Using Tn3 Resolvase

(A) The plasmid pJB7 contains two directly repeated *psi* sites (solid triangles) and two directly repeated *res* sites (open triangles); pJB5 contains two directly repeated *psi* sites and two inverted repeat *res* sites. If the product of recombination at *psi* is a −4A catenane (X_4, as depicted here), then the *res* sites on the pJB7 Xer catenane will be antiparallel, and those on the pJB5 catenane will be parallel. Alternatively, if the product of recombination at *psi* is a +4P catenane, then the *res* sites on the pJB7 Xer catenane will be parallel, and those on the pJB5 catenane will be antiparallel. Antiparallel *res* sites in a right-handed catenane can recombine efficiently, whereas parallel *res* sites cannot (Benjamin and Cozzarelli, 1990). One round of recombination at antiparallel *res* sites on a −4A catenane yields an unknotted circle with directly repeated *res* sites (XR_0); a subsequent round of recombination yields a −2 catenane (XRR_2). Resolvase can also act on unrecombined pJB7 to produce a −2 catenane (R_2). Note that X_4 and XRR_2 are catenanes of exactly the same two rings and therefore have identical restriction patterns.

(B) pJB7 was left untreated, treated with Xer, treated with resolvase, or treated first with Xer and then with resolvase as indicated. Samples were run uncut (lanes 1–4), cut with HindIII (lanes 5–8), or nicked with DNaseI (lanes 9–12). Products in lanes 1–4 are indicated as follows: supercoiled pJB7, sc substrate; 2-noded catenanes, sc 2-node; 4-noded catenane, sc 4-node. Products in lanes 5–8 are indicated as in (A) above (O, R, X, and XRR = X). The product of two *psi* sites in the catenane could either be parallel (+4P) or antiparallel (−4A) (see Figure 2B). We used the well-characterized Tn3 *res*/resolvase site-specific recombination system to distinguish between these two possibilities. Supercoiled right-handed torus catenanes containing antiparallel *res* sites are substrates for resolvase, whereas right-handed torus catenanes with parallel *res* sites are not (Benjamin and Cozzarelli, 1990). We therefore constructed the plasmids pJB7 and pJB5 (Figure 4A), containing two directly repeated *psi* sites and two *res* sites in direct (pJB7) or inverted (pJB5) repeat. Xer treatment yielded 4-noded catenane in which each ring contained one *res* site and one *psi* site. These 4-noded catenanes were then treated with resolvase to ascertain the relative orientation of the *res* sites.

A single round of resolvase-mediated recombination acting on antiparallel *res* sites in a 4-noded right-handed catenane (X_4; Figure 4A) is expected to yield an unknotted circle with a novel restriction pattern (XR_0; Figure 4A). This unknotted circle contains two directly repeated *res* sites and can undergo a second round of recombination to yield a −2 catenane consisting of exactly the same two rings as those in the 4-noded catenane (XRR_2; Figure 4A) (Benjamin and Cozzarelli, 1990).

pJB7 was treated with Xer to produce a 4-noded catenane (X_4; Figure 4A); the reaction was then extracted with phenol and treated with resolvase. As controls, pJB7 was left untreated, or treated with Xer or with resolvase alone (Figure 4B, lanes 1–4). The supercoiled 4-noded catenane produced by Xer migrated distinctly ahead of the supercoiled substrate (Figure 4B, lane 2), whereas the 2-noded catenane produced by resolvase migrated only slightly ahead of the substrate (Figure 4B, lane 3). The 4-noded catenane produced by Xer was apparently converted to 2-noded catenane by the action of resolvase (Figure 4B, lane 4). DNaseI nicking confirmed this result: resolvase- and Xer-treated pJB7 contained mainly 2-noded catenane and very little 4-noded catenane (Figure 4B, lane 12). However, the HindIII digest revealed that the restriction pattern of the pJB7 *psi* catenane was left unchanged by the action of resolvase (Figure 4B, lanes 6 and 8).

Two-dimensional gel electrophoresis was used to confirm this result (Figure 4C). pJB7 was treated first with Xer, then with resolvase, and then nicked with one round of Xer recombination followed by one round of resolvase recombination (XR) was not detected. The 984 bp fragment (O/R) is present in both pJB7 (O_0) and in the pJB7 resolvase catenane (R_2). Note that the nicked 2-noded catenanes in lanes 11 and 12 have slightly different mobilities owing to the different sizes of their component rings (XRR_2 runs just ahead of R_2).

(C) Two-dimensional gel with DNaseI nicking in the first dimension and HindIII cutting in the second dimension. All lanes contain pJB7 treated first with Xer and then with resolvase. Samples were run uncut (lane 1), cut with HindIII (lane 2), and nicked with DNaseI (lane 3). A duplicate of lane 3 was cut from the gel, incubated with HindIII, cast into a new gel, and run in the second dimension. The positions of nicked 0-, 2-, and 4-noded molecules are indicated. Restriction fragments are indicated as in (A) and (B) above. Note that the supercoiled small ring (807 bp) of the resolvase catenane R(2) stains poorly and is not visible in lane 2, but can be clearly seen (now nicked) at the 2-node position in the 2-dimensional gel.

DNaseI and run on an agarose gel. The lane containing this sample was cut from the gel and incubated with HindIII prior to electrophoresis in the second dimension. Two distinct restriction patterns were observed at the 2-node position. One of these (X) was identical to that of the original 4-noded catenane, while the other restriction pattern came from the action of resolvase on unrecombined pJB7 substrate (R). Thus, the pJB7 *psi* catenane was a good substrate for two rounds of recombination by resolvase, producing a −2 catenane with an unchanged restriction pattern (XRR_2) as shown in Figure 4A.

We also carried out a similar experiment with pJB5, containing two *psi* sites in direct repeat and two *res* sites in inverted repeat. When this substrate was treated with Xer, it gave a 4-noded catenane, which was not a substrate for resolvase (Figure 4A; data not shown).

The catenane produced by the action of Xer on pJB7 was a good substrate for Tn3 resolvase and gave exactly the products predicted for antiparallel *res* sites on a right-handed 4-noded torus catenane. The catenane produced by the action of Xer on pJB5 was not a substrate for Tn3 resolvase, as expected for a right-handed torus catenane with parallel *res* sites. Therefore, the product of recombination at *psi* is a 4-noded right-handed torus catenane with antiparallel *psi* sites; the pJB7 Xer catenane has antiparallel *psi* sites and antiparallel *res* sites, whereas the pJB5 Xer catenane has antiparallel *psi* sites and parallel *res* sites (Figure 4A).

The product of a single round of resolvase-mediated recombination on the pJB7 *psi* catenane (XR_0) was not detected. It appears that any 4-noded catenane that undergoes one round of resolvase-mediated recombination to give unknotted product rapidly undergoes a second round of recombination yielding −2 catenane. To see the product of one round of resolvase recombination on the pJB7 *psi* catenane, we singly nicked one ring of the catenane using the restriction enzyme ClaI in the presence of ethidium bromide. As expected, this half-nicked catenane was a good substrate for resolvase (see Benjamin et al., 1996), yielding nicked unknotted circular product with the predicted restriction pattern (data not shown). This product was not supercoiled and was therefore not a good substrate for further rounds of resolvase-mediated recombination.

Recombination at *cer* Occurs with the Same Topology as Recombination at *psi*

In contrast to recombination at *psi*, recombination at *cer* requires ArgR, as well as PepA, XerC, and XerD, and leads to the exchange of only one specific strand of each site in our in vitro conditions (Colloms et al., 1996). The product of "recombination" at *cer*, therefore, contains a Holliday junction (Figure 1A). Since there are no previously published data on the gel mobility of nicked Holliday junction-containing species of different topological complexity, we compared Holliday junction-containing products from recombination at *cer* to those from recombination at *psi*. During recombination at *psi*, XerC exchanges one pair of strands to make a Holliday junction intermediate, and XerD exchanges the second pair of strands to make catenane (Colloms et al., 1996). We used a catalytically inactive XerD (XerDY279F) to make Holliday junction-containing products by strand exchange at *psi*, reasoning that these products would have a topology analogous to that of the 4-noded catenane produced by wild-type XerC and XerD acting at *psi*.

Holliday junction-containing products were made by the action of PepA, ArgR, XerC, and XerD at *cer* (pSDC115), and PepA, XerC, and XerDY279F at *psi* (pSDC134). The topology of these products was investigated by DNaseI nicking and high resolution gel electrophoresis adjacent to a knot ladder. In both cases, two major products were seen (Figure 5A, lanes 2 and 3); one comigrated with the 4-noded knot, the other migrated between the linear substrate and the 3-noded knot. It appears that XerC-mediated strand exchange at *cer* and *psi* produced a 4-noded catenated figure-8, in which the recombinant strands formed two circles catenated by four interlinks, and the nonrecombinant strand remained unknotted (Figure 5B). When this product was nicked in both domains to remove all supercoiling, the product migrated at the 4-node position. However, if one of the recombinant strands was nicked in the region of homology between the two recombination sites, the Holliday junction could branch migrate to the nick. The catenation could then be released by passing one recombinant strand through the nick in the other recombinant strand, giving a more open figure-8 structure that migrated between the linear substrate and the 3-noded knot (Figure 5B).

Further evidence that the product of recombination at *cer* is a 4-noded catenated figure-8 came from experiments with the decatenating enzyme E. coli topoisomerase IV (Peng and Marians, 1993). Treatment of the *cer* Holliday junction-containing product with topoisomerase IV, prior to DNaseI nicking, efficiently converted the catenated figure-8 into the slower migrating open figure-8 (Figure 5A, lane 5). It seems that topoisomerase IV can recognize and remove the catenation nodes of the *cer* recombination product.

We also analyzed the *cer* recombination product using the Holliday junction-cleaving enzyme T4 endonuclease VII (Kosak and Kemper, 1990). Treatment of an in vitro *cer* recombination reaction with T4 endonuclease VII yielded a product that comigrated with the 4-noded knot (Figure 5A, lane 6). Restriction analysis confirmed that this product was a 4-noded catenane (data not shown). Thus, T4 endonuclease VII cleaves the nonrecombinant strands in the *cer* Holliday junction, yielding 4-noded catenane.

These results suggest that strand exchange at *cer* occurs with the same topology as strand exchange at *psi*. XerC-mediated top strand exchange at both *cer* and *psi* produced a 4-noded catenated figure-8, which in the case of *psi* could be resolved by XerD-mediated strand exchange to form a 4-noded (−4A) catenane.

The Topology of Inversion at *psi*

Substrates containing *cer* or *psi* sites in inverted repeat are poor substrates for Xer recombination in vitro (Colloms et al., 1996). However, a low level of inversion was seen when the *psi* inverted repeat substrate pSDC154

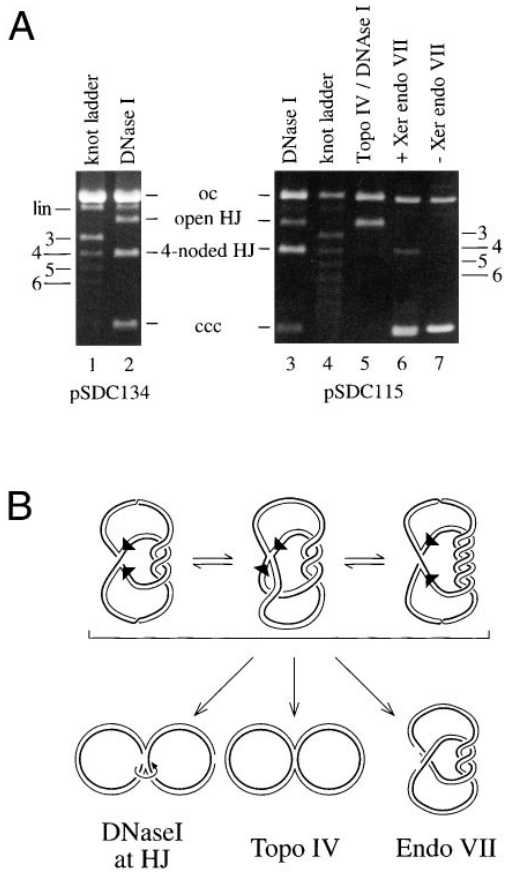

Figure 5. Topology of Holliday Junction-Containing Products

(A) pSDC134 was incubated with PepA, XerC, and XerDY279F, singly nicked with DNaseI (lane 2), and run adjacent to a nicked pSDC134 knot ladder (lane 1) on a high resolution agarose gel. pSDC115 was incubated with ArgR, PepA, XerC, and XerD, singly nicked (lane 3), and run adjacent to a nicked pSDC115 knot ladder (lane 4). In each case, the major product is a 4-noded catenated figure-8. Treatment of the *cer*-catenated figure-8 with topoisomerase IV prior to nicking with DNaseI converts all of the 4-noded structure to the uncatenated form (lane 5). T4 endonuclease VII cleaves the nonrecombinant strands of the *cer* product, yielding a 4-noded catenane (lane 6). Lane 7 contains unrecombined pSDC115 treated with T4 endonuclease VII as a control.

(B) The Holliday junction-containing products of XerC-mediated strand exchange at *cer* and *psi* can be represented as containing either a left-handed crossed, a square planar, or a right-handed crossed Holliday junction with three, four, or five interdomainal nodes. In all cases, the recombinant strands form a −4A catenane. Nicking both domains with DNaseI away from the Holliday junction releases the supercoiling without changing the topology of these structures. If one of the recombinant strands is nicked with DNaseI in the region over which the Holliday junction can branch migrate, a more open figure-8 structure is formed (DNaseI at HJ). Topoisomerase IV removes the catenation nodes to produce an open figure-8 (Topo IV). Endonuclease VII cleaves the recombinant strands at the Holliday junction, releasing a nicked 4-noded catenane (Endo VII).

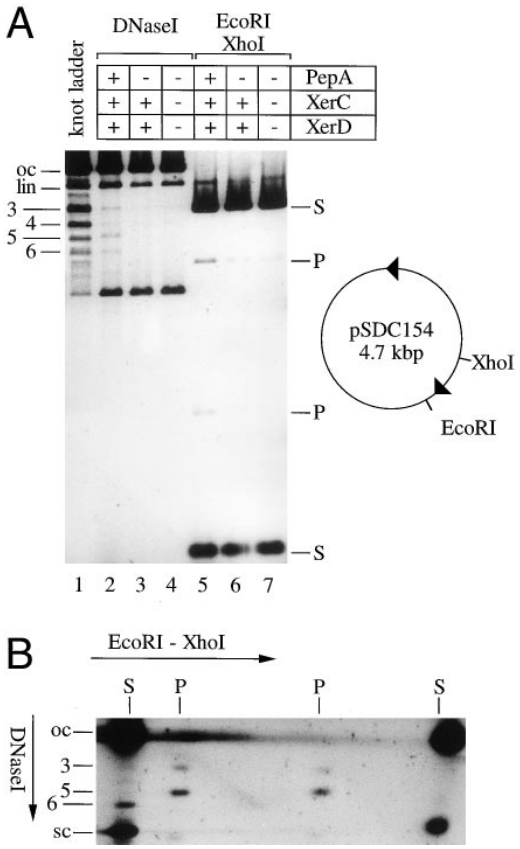

Figure 6. Topology of Inversion at *psi*

(A) pSDC154, containing two *psi* sites in inverted repeat, was incubated with PepA, XerC, and XerD, and cut with the restriction enzymes XhoI and EcoRI (lane 5) or singly nicked with DNaseI (lane 2) prior to electrophoresis on a 0.7% agarose gel. Untreated pSDC154 and pSDC154 treated with XerC and XerD in the absence of PepA were run on the same gel. Lane 1 contains a nicked pSDC154 knot ladder. DNA was stained with Sybr green I and visualized using a Fluorimager. Substrate (S) and inversion product (P) restriction fragments are indicated, as are open circle (oc) and linear (lin) substrate, and 3-, 4-, 5-, and 6-noded knots. Note that there is a small amount of unknotted inversion product in the substrate.

(B) The restriction pattern of pSDC154 knots was determined using two-dimensional gel electrophoresis. pSDC154 was treated with Xer, nicked with DNaseI, and subjected to agarose gel electrophoresis. The lane was excised from the gel and the DNA was cleaved in situ with EcoRI and XhoI prior to electrophoresis in a second dimension.

was incubated with PepA, XerC, and XerD (Figure 6A, lane 5). This inversion is PepA dependent (Figure 6A, lane 6). The major products of this inversion reaction were 3-, 5-, and 6-noded knots (Figure 6A, lane 2). Two-dimensional gel electrophoresis showed that the 3- and 5-noded knots had an inverted restriction pattern, whereas the 6-noded knot and the unknotted circle had the parental restriction pattern (Figure 6B). The 5-noded inversion product migrated just slower than the 5-noded twist knot in the knot ladder, consistent with it being a 5-node torus knot (Dröge and Cozzarelli, 1989).

Discussion

Using a variety of electrophoretic techniques, the product of recombination at *psi* was shown to be a right-handed 4-noded catenane. The well-characterized Tn3 *res*/resolvase system was used to confirm this result and to show that the *psi* sites are antiparallel on the product catenane. The product of strand exchange at

cer has an analogous topology but contains a Holliday junction.

Site-specific recombination reactions can be divided into two steps: synapsis, in which the recombining sites are brought together, and strand exchange, in which DNA strands are cleaved, exchanged, and religated. The topological outcome of a site-specific recombination reaction depends on two factors: the topology of the productive synapse (Syn) and the topology of the strand exchange mechanism (Xr). The defined product topologies imply that recombination at *cer* and *psi* occurs in a synapse with a fixed structure, and that strand exchange occurs via a fixed path (i.e., both Syn and Xr are fixed). Such a high degree of topological specificity has not been previously reported for a member of the λ integrase family of site-specific recombinases.

When two recombination sites on a circular substrate are brought together, they divide the substrate into two domains. Any crossings between these two domains are known as interdomainal crossings or nodes. Assuming parallel alignment of the recombination cores, there are only two ways to account for the observed topology of the *psi* deletion product. Either 3 negative interdomainal nodes are trapped in the productive synapse and strand exchange introduces a further negative node between the recombination cores (Syn = −3, 0; Xr = −1) (Figure 7A), or 5 negative interdomainal nodes are trapped in the synapse and strand exchange introduces a positive node (Syn = −5, 0; Xr = +1) (Figure 7A). Although both of these mechanisms yield identical (−4A) catenanes, it seems likely that only one pathway is specified by the recombination machinery.

How is the topology of recombination at *psi* and *cer* defined? We postulate that recombination can only occur in a defined nucleoprotein complex in which the accessory sequences of the two participating recombination sites are plectonemically interwrapped, in a right-handed sense, around accessory proteins, trapping three negative supercoils. ArgR binds to a site within the *cer* accessory sequences (Stirling et al., 1988), and PepA is now known to be a DNA-binding protein (Charlier et al., 1995). We now have direct evidence that the accessory sequences from two *cer* sites form a plectonemically interwrapped complex in the presence of ArgR and PepA (C. Alén, S. D. C., and D. J. S., unpublished data). A model for this interwrapped complex is presented in Figure 7C. Because the topology of recombination at *psi* is the same as that at *cer*, we believe that the accessory sequences of *psi* can form a similar interwrapped complex with PepA alone. The fact that PepA and ArgR can form such interwrapped complexes, in which distant segments of DNA are brought together, suggests that ArgR and PepA could form similar structures in their roles as transcription regulators and could even be involved in chromosome organization.

Recombination does not occur in the absence of accessory proteins and sequences, implying that strand exchange by the recombinases is activated by formation of the synaptic complex. The requirement for a specific (−3) synapse is sufficient to explain both the preference for intramolecular deletion over inversion and fusion reactions (topological selectivity) and the defined topology of the recombination products (topological specificity). To form a −3 synapse between sites in inverted repeat on a circular molecule, or between sites on separate supercoiled circles, unfavorable compensating positive nodes have to be introduced elsewhere. Therefore, a productive −3 synapse can only readily be formed between directly repeated sites on a supercoiled molecule, and recombination will only occur between such sites.

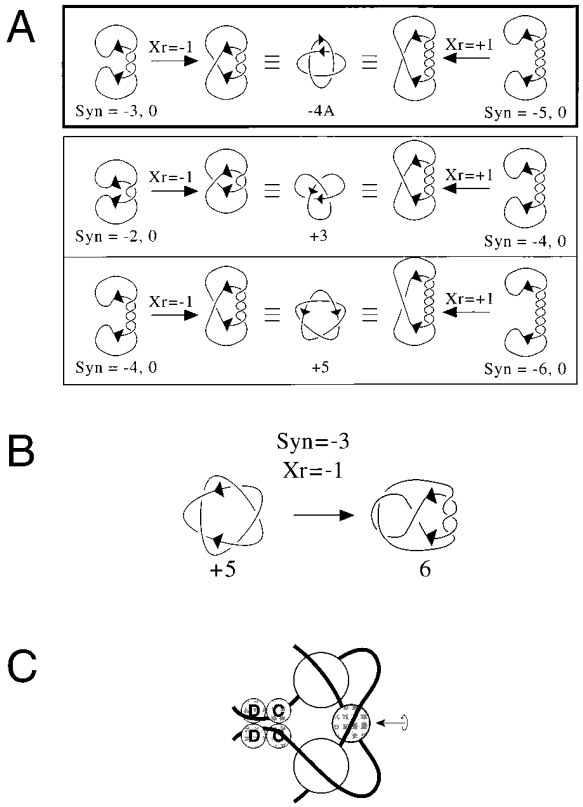

Figure 7. Topological Outcomes of Different Reaction Mechanisms

(A) A −4A catenane can be produced using a −3 synapse and a strand exchange mechanism that introduces a negative node between the crossover sites (Syn = −3, 0; Xr = −1), or a −5 synapse and strand exchange that introduces a positive node (Syn = −5, 0; Xr = +1). Inversion with Syn = −2, 0; Xr = −1 and Syn = −4, 0; Xr = −1 yields the observed +3 and +5 knots, as would inversion with Syn = −4, 0; Xr = +1 and Syn = −6, 0; Xr = +1. In order to simplify topological analysis, we arbitrarily chose to draw reactions in a projection where the recombination cores are aligned in parallel. Although strictly parallel alignment in space is unlikely, it is almost always possible to choose a projection in which sites appear parallel.

(B) Possible second round inversion pathway. If normally Syn = −3, 0; Xr = −1, then a +5 torus knot can invert to form a 6-noded knot.

(C) A model showing how interwrapping of *cer* accessory sequences around ArgR and PepA could trap three negative supercoils. The structure has a 2-fold axis of symmetry (arrow). One hexamer of ArgR (∼100 kDa; shaded circle) binds to ARG boxes located centrally in the accessory sequences of each *cer* site. Two hexamers of PepA (∼330 kDa; open circles) are shown binding to the accessory sequences on either side of each ARG box. The recombinases XerC (C) and XerD (D) bind to the recombination cores. It is possible to construct a similar model containing only one hexamer of PepA, with one of its 2-fold axes of symmetry lying on the symmetry axis of the synaptic complex. We do not rule out the possibility that there are protein–protein interactions between ArgR and PepA and the recombinases.

Similar topological filter models have previously been proposed to account for topological selectivity and specificity in the reactions catalyzed by resolvases, DNA invertases, and Mu transposase (reviewed in Stark and Boocock, 1995). For resolvase, there is a requirement for a specific synaptic complex that traps precisely 3 negative interdomainal nodes (Syn = −3, 0) (Wasserman et al., 1985; Dröge and Cozzarelli, 1989; Stark et al., 1989b). For the DNA invertases, there is a requirement for a complex that traps two negative interdomainal nodes (Syn = −2, 0) (Kanaar et al., 1988, 1989, 1990). In both cases, strand exchange occurs by a 180° right-handed rotation (Xr = +1). The requirement for a −3 synapse ensures that resolvase only acts at directly repeated sites, whereas the requirement for a −2 synapse ensures that invertases only bring about inversion.

How do we account for the topology of the inversion reaction at *psi*? We believe that there is some degree of flexibility in the *psi* productive synapse, such that one more or one less interdomainal node than normal can be tolerated. Thus, −2 and −4 synapses and strand exchange, so as to introduce a negative node between the recombination cores (Syn = −2, 0, and Syn = −4, 0; Xr = −1), yield the observed +3 and +5 torus knots as inversion products (Figure 7A). Alternatively, −4 and −6 synapses and strand exchange, so as to introduce a positive node between the recombination cores (Syn = −4, 0, and Syn = −6, 0; Xr = +1), yield +3 and +5 torus knots as inversion products (Figure 7A). Inversion at *psi* is a much less efficient reaction than deletion, suggesting that these alternate synapses are poorly tolerated for recombination. We have not observed strand exchange between *cer* sites in inverted repeat; perhaps ArgR increases the rigidity of the *cer* synaptic complex, ensuring that exactly three negative supercoils are trapped.

If recombination at *psi* normally utilizes a −3 synapse and strand exchange introduces a negative node (Syn = −3, 0; Xr = −1), we would expect the 5-noded inversion product to be a substrate for a further round of recombination, yielding a 6-noded knot with the parental restriction pattern (Figure 7B). A 6-noded knot with the parental restriction pattern was indeed observed (Figure 6). If recombination at *psi* normally utilizes a −5 synapse, and strand exchange introduces a positive node (Syn = −5, 0; Xr = +1), it is difficult to see how such a product could be obtained. We therefore believe that deletion at *psi* and *cer* occurs in a synapse with three trapped negative interdomainal nodes, and that strand exchange introduces a negative node (Syn = −3, 0; Xr = −1). However, we hope to confirm this using defined knots and catenanes as substrates for Xer recombination (e.g., see Dröge and Cozzarelli., 1989; Kanaar et al., 1989).

The introduction of one negative node by the strand exchange mechanism (Xr = −1) is consistent with a number of current models for strand exchange by members of the λ Int family of site-specific recombinases. These models all involve exchange of one pair of strands to form a Holliday junction, followed by Holliday junction isomerization and resolution to form recombinant products (see Stark et al., 1989b; Nunes-Düby et al., 1995). This type of model predicts that 1 unit of twist is added by the strand exchange mechanism (Xtw = +1), and overall 4 negative supercoils will be lost during recombination at *psi* (ΔLk = +4), making recombination at *psi* a highly favorable reaction in a negatively supercoiled substrate.

We remain puzzled as to why the −4A product catenane is not a substrate for further rounds of recombination, either immediately after its formation or following phenol extraction and readdition of recombinase proteins. The geometry of the proposed *psi* synapse (Figure 7C) is very similar to that proposed for the *res*/resolvase synapse, and we know that resolvase can synapse and recombine *res* sites on the *psi* product catenane. It seems likely that a −3 synapse can also form between *psi* sites on the −4A product catenane, but that there is some barrier to further rounds of recombination. Perhaps the decrease in supercoiling associated with this reaction (ΔLk is predicted to be +1) is not enough to compensate for the increased writhe in a 5-noded knot.

Selectivity is important for the proper in vivo role of *cer* and *psi*. Multimers formed by homologous recombination must be resolved by intramolecular deletion, but intermolecular fusion, including recombination between newly replicated sister chromosomes, must be avoided. λ Int is under a different sort of control; the differences between phage and bacterial attachment sites allow the system to differentiate between the integration reaction between *attP* and *attB* and the excision reaction between *attL* and *attR*, but not generally between sites connected in different configurations (Landy, 1989). Integration and excision require different subsets of accessory proteins, allowing the phage to respond to subtle differences in cellular physiology in its decision whether to integrate or excise (Thompson and Landy, 1989). In contrast, Xer recombination between plasmid resolution sites occurs between identical sites, and DNA topology must be used to distinguish between the resolution and fusion reactions.

A number of topological filter mechanisms have evolved independently. Mu transposition involves the formation of a specific synapse in which the transposon left and right ends and an internal enhancer sequence are brought together by MuA transposase and two accessory proteins, IHF and HU (Watson and Chaconas, 1996). The geometry of this synapse ensures that only Mu ends in inverted repeat can form an active transpososome (Craigie and Mizuuchi, 1986). The DNA invertases utilize a similar three-site synaptic complex involving two recombination sites, an enhancer sequence, the recombinase, and the accessory protein Fis. The *res*/resolvase and *cer*/*psi*/Xer systems both utilize accessory sequences adjacent to the recombination sites, but whereas resolvase binds to accessory sites as well as acting as the recombinase, the Xer system recruits PepA and ArgR to act as accessory proteins. These systems therefore use a wide variety of sites and proteins but use the same topological properties of supercoiled DNA to sense the relative orientation of two DNA segments. It seems likely that similar mechanisms also operate in other cases where proteins interact with multiple sites on supercoiled DNA molecules; for example, in the control of eukaryotic transcription by multiple transcription factors.

Experimental Procedures

Plasmids

pSDC134 and pSDC115 have been described previously (Colloms et al., 1996). pSDC153 contains two 360 bp *psi* sites separated by 395 bp, one in the SmaI site and one in the HincII site of pUC18. pJB5 and pJB7 are derivatives of pSDC134, containing a 282 bp EcoRI *res* fragment at the EcoRI site and a 282 bp SalI *res* fragment at the XhoI site. pSDC154 is pUC18 with two *psi* sites in inverted repeat separated by the Kan^r cassette from pUC4K (Pharmacia). Supercoiled plasmid DNA was prepared from DS984 by large scale alkaline lysis followed by CsCl-ethidium bromide density gradient centrifugation.

Reactions

Xer recombination reactions were carried out as previously described (Colloms et al., 1996). Resolvase reactions were in 50 mM Tris–HCl (pH 9.4), 50 mM NaCl, 10 mM $MgCl_2$, and 0.1 mM EDTA, as described by Stark et al. (1989b). DNaseI nicking reactions were in 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 0.3 mg/ml ethidium bromide, and 1 μg/ml DNaseI.

Knot ladders were produced by treating negatively supercoiled plasmid DNA with S. cerevisiae topoisomerase II in 50 mM Tris–HCl (pH 7.5), 120 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 200 μg/ml BSA, and 10 μM ATP. Reactions containing 20 μg/ml DNA and 30 μg/ml topoisomerase II were incubated for 4 min at 37°C, stopped with EDTA, phenol extracted, ethanol precipitated, and singly nicked with DNaseI as above.

Topoisomerase IV was reconstituted by mixing equimolar amounts of ParE and ParC in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM DTT, and 50% glycerol on ice for 30 min. Reactions were in 40 mM Tris–HCl (pH 7.5), 100 mM potassium glutamate, 6 mM $MgCl_2$, 10 mM DTT, 40 μM ATP, and 50 μg/ml BSA, and contained 300 ng DNA and 14 pmol of topoisomerase IV in a 20 μl reaction. Forty-minute reactions at 37°C were stopped by heating to 65°C for 15 min and nicked with DNaseI as above. Endonuclease VII reactions were in 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, and 100 μg/ml BSA, and contained 300 ng of DNA and 20 U of endonuclease VII. Thirty-minute reactions at 30°C were stopped by phenol extraction and loaded directly onto agarose gels.

Determination of Catenation Writhe

pSDC153 was reacted with Xer, nicked with DNaseI, ethanol precipitated, and resuspended in 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM ATP. One microliter (10 U) of BamHI was added to one aliquot (precut), and 1 μl of BamHI storage buffer was added to another (postcut). After 45 min at 37°C, 2 U of DNA ligase was added, followed by another 45 min at 37°C. DNA ligase was heat killed at 65°C for 10 min, and both reactions were digested with 10 U of BamHI. The DNA was subjected to electrophoresis on an agarose-TAE gel containing 0.5 μg/ml chloroquine phosphate.

Gel Electrophoresis

All gels were 0.7% agarose in Tris–acetate running buffer (40 mM Tris–AcOH [pH 8.2], 20 mM NaOAc, 1 mM EDTA) and were run at ~3 V/cm for 16–24 hr. For the two-dimensional gels, recombination reactions were nicked with DNaseI and run in duplicate on 0.7% Seakem GTG agarose gels. One lane was stained with ethidium bromide and visualized by UV illumination. The other lane was cut from the gel and equilibrated for 2 hr with 1 change in 1× restriction buffer containing 0.1 mM DTT and 100 μg/ml BSA. The gel slice was incubated at 37°C for 7 hr with gentle shaking in 5 ml of the same buffer with 0.5 U/μl of HindIII or 0.5 U/μl each of EcoRI and XhoI, cast into a new 0.7% agarose gel, and run in the second dimension. The gels were stained with Sybr green I (Molecular Probes) and visualized with a Molecular Dynamics Fluorimager 575.

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