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Discovery of a Predicted DNA Knot Substantiates a Model for Site-Specific Recombination

Abstract. *The mechanism of site-specific genetic recombination mediated by Tn3 resolvase has been investigated by a topological approach. Extrapolation of a detailed model of synapsis and strand exchange predicts the formation of an additional DNA product with a specific knotted structure. Two-dimensional gel electrophoresis of DNA reacted* in vitro *revealed a product, about 0.1 percent of the total, with the appropriate mobility. A technique for determining DNA topology by electron microscopy was improved such that less than a nanogram of DNA was required. The structure of the knot was as predicted, providing strong evidence for the model and showing the power of the topological method.*

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The resolvase proteins encoded by transposons Tn3 and γδ efficiently promote site-specific recombination of supercoiled DNA containing directly repeated resolution (*res*) sites (*1, 2*). Distant sites are first brought together in an ordered fashion; the strands of this synapsed DNA are then broken in four locations, generating eight free ends, each of which undergoes defined reorientation and ligation. Such movements of recombining DNA strands often result in knotted or catenated products from whose structure it is possible to deduce critical features of a reaction pathway (*3, 4*).

The fact that the topology of DNA knots or catenanes, unlike that of supercoils, is invariant in both intact and nicked duplex DNA has made electron microscopy particularly useful in analyzing these products of genetic rearrangement. No amount of stretching, twisting, or other physical distortion can alter the stereostructures of knotted or catenated products; the topological signature of the recombination mechanism remains distinct.

The topological concepts used to analyze recombination are those of nodes and domains (*5*). Most easily defined for molecules viewed in plane projection, nodes are simply the crossings of DNA segments. The number, arrangement, and sign of nodes together define topology, whether that of supercoils, catenanes, or knots. Each node is assigned a value of either +1 or −1; by convention, nodes of naturally occurring supercoiled DNA are negative.

For a resolvase substrate, the two regions of DNA between the *res* sites define two domains (Fig. 1). Alterations in DNA topology inherent to recombination have radically different consequences depending on whether the nodes involved are intradomainal (self-intersections of a domain) or interdomainal (intersections of different domains) (*4*). Only interdomainal nodes contribute to knotting or catenation, whereas both types of nodes contribute to changes in superhelicity.

The principal products of resolvase action are singly linked catenanes (*1, 2*). With sensitive detection methods, other products have been found at the level of 1 to 3 percent (*6, 7*). We have recently presented a model that provides a unitary mechanism for formation of all the observed products (*4, 8*). According to this scheme (Fig. 1), resolvase usually acts dispersively, mediating only a single round of strand exchange on binding and then releasing as sole product the singly linked catenane. We postulate, however, that occasionally—about once every 20 productive encounters—resolvase acts processively to promote additional strand exchanges prior to dissociation, generating the more complex products. The rotation of strands about each other during each exchange introduces a single (+) interdomainal node.

The detailed topology of two of the complex products, the four-noded knot and the figure-8 catenane (*6, 7*), but not of the singly linked catenane were available in constructing the model. Implicit in the scheme, however, was the prediction that the two nodes of the singly

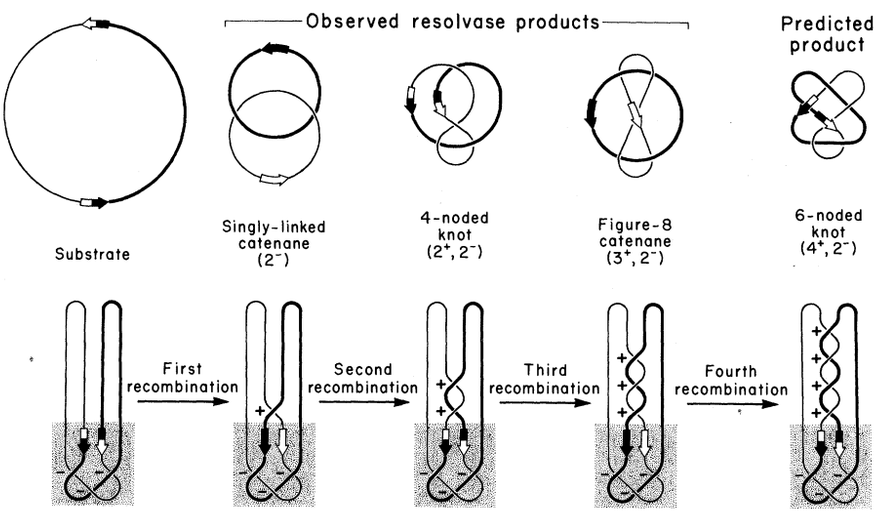


Fig. 1. Scheme for resolvase-mediated recombination. In the upper row, the duplex DNA substrate and products are represented in standard topological form as they might appear after nicking. In the lower row the DNA's are depicted as intact forms bound to resolvase (stippled rectangle) with the two directly repeated *res* sites (thick arrows) dividing the substrate into two domains (thick and thin regions). The substrate at synapsis has three (−) supercoils that entail crossing of the two domains. Successive rounds of recombination, each introducing a single (+) interdomainal node, are drawn in the lower row. Bound resolvase maintains the three synaptic supercoils. After dissociation from resolvase at any stage, product supercoil nodes either cancel with ones of opposite sign or are removed by subsequent nicking. Node composition is indicated in parentheses.

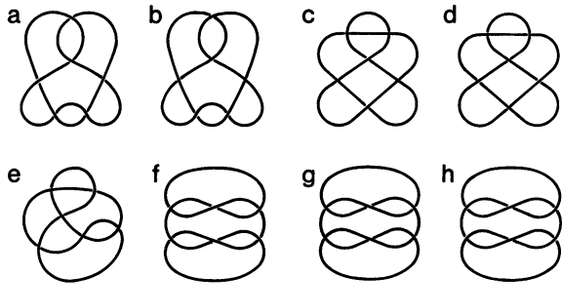


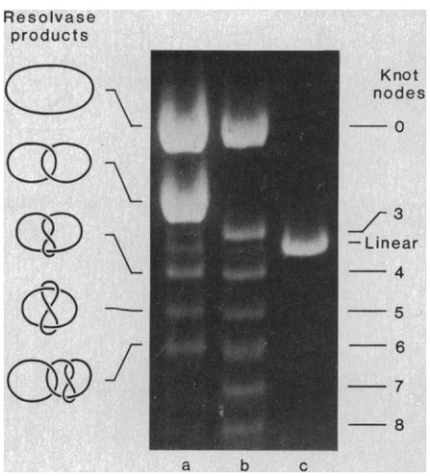
Fig. 2. Structures of the eight possible six-noded knots. The three pairs—**a** and **b**, **c** and **d**, and **g** and **h**—are enantiomers; knots **e** and **f** are amphichiral (equivalent to their own mirror image). Knot **2d** is the predicted product of recombination shown in Fig. 1.

interlinked catenane would be negative; this was subsequently confirmed (*8*).

Despite the consistency of this model with available data, difficulties remained. The model in effect postulates that a catenated product occasionally recombines in situ, even though resolvase and free catenanes do not react (*2*). The scheme also assumes, without biochemical evidence, that there are always exactly three interdomainal supercoils at synapsis, regardless of substrate superhelicity. We therefore set out to test the model more rigorously, using it to predict the product of an additional round of strand exchange and then looking for such a DNA product.

Extrapolation of the model indicates that four rounds of processive strand exchange, a very rare occurrence, should yield a specific six-noded knot (see Fig. 1). Since there are seven other possible structures for a six-noded knot (Fig. 2), this affords a much more stringent test of the model than was possible with the singly linked catenanes, of which there are only two isomers. Resolvase is predicted to make only that six-noded knot with four (+) and two (−) nodes in the configuration of Fig. 2d. In contrast, *Escherichia coli* topoisomerase I makes all eight six-noded knots (*9*).

In order to identify any resolvase-generated six-noded knots, we subjected nicked reaction products to electrophoresis in tris-acetate buffer alongside a knot ladder produced by T4 topoisomerase. Beginning with the simplest knots, the three-noded trefoils, each consecutive rung of such a marker ladder is composed of knots with an additional node (*9*). Any six-noded knots produced by resolvase should therefore be aligned with the fourth rung of a T4 topoisomerase knot ladder. About 3 percent of the products did migrate in this region of the gel (Fig. 3). However, when a small number of these were analyzed they were found to be a different six-noded molecule: a singly linked catenane in which one of the rings is a four-noded knot (*7*).



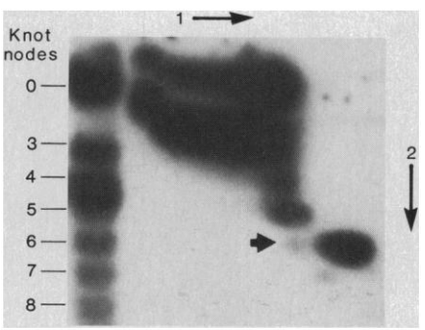


Fig. 3 (left). Complexity of resolvase products: ethidium bromide–stained gel. Supercoiled pA[2] substrate (*13*) was treated with resolvase and singly nicked with DNAse I (*9*). The DNA was subjected to electrophoresis with markers under conditions in which molecules migrate according to node number (*16*). (Lane a) Nicked resolvase products (1.8 μg); (lane b) substrate knots made with T4 topoisomerase (*17*) and then singly nicked; (lane c) linearized substrate. Fig. 4 (right). Complexity of resolvase products: Southern blot of a second-dimension gel. Singly nicked resolvase products (2.3 μg) were subjected to electrophoresis (left to right) in a first-dimension gel (1 percent agarose) at 3.5 V/cm for 40 hours in tris-borate buffer with 0.03 percent SDS (*10*). A slice from this gel was dialyzed against TE buffer (10 m*M* tris, *p*H 7.6; 1 m*M* EDTA) containing 0.03 percent SDS and again run (top to bottom) in a 0.9 percent agarose gel in tris-acetate buffer with 0.03 percent SDS at 3.2 V/cm for 18 hours. Reference knots were prepared as in Fig. 3, lane b. The DNA was transferred to nitrocellulose and probed with ^{32}P-labeled pBR322 generated by nick translation. The arrowhead indicates the faint spot species identified as a six-noded knot.

The appearance of this six-noded compound catenane can be accounted for by our scheme, but does not offer a critical test of the mechanism, since it involves two separate encounters with resolvase (*10*). In order to separate the predicted knot from the six-noded compound catenane we turned to two-dimensional gel electrophoresis. In a tris-borate first-dimension gel, the migration of six-noded knots produced by T4 topoisomerase was slower than that of the six-noded compound catenane. By combining a first-dimension tris-borate gel with a second-dimension tris-acetate gel, we could resolve a number of previously occluded resolvase products. One such DNA species, marked by an arrow, had a mobility identical to that of the T4 topoisomerase-generated six-noded knots (Fig. 4). This DNA was not seen on gel electrophoresis of unreacted substrate and was therefore not a preexisting in vivo species but an in vitro product of resolvase action.

The DNA predicted to be a six-noded knot represented about 0.1 percent of the resolvase product. Sections of ethidium bromide–stained one-dimensional and two-dimensional gels that contained this DNA were excised, and the DNA was electroeluted for structural analysis. The starting point for this analysis was a technique developed to make visible the path of a knotted DNA (*6*). Thickening of the DNA by coating with *E. coli recA* protein enhances the contrast of shadowed samples viewed in the electron microscope. Repeated trials are generally required, however, to obtain complete reaction between duplex knotted DNA and *recA* protein; this procedure is therefore unsuitable for use with scarce material.

The technique was improved by a simple change; the putative knots were converted to single-stranded form prior to treatment with *recA* protein. The DNA was denatured in the presence of glyoxal, which blocks renaturation without any apparent effect on reactivity with *recA* protein (*8*). The DNA was then coated with *recA* protein, shadowed, and photographed in the electron microscope.

Micrographs were screened for molecules in which the path of the DNA and the overlay at each node could be discerned. Somewhat more than half of the molecules seen were linear, as expected from denaturation of molecules that are, on average, singly nicked. In the first-dimension sample all but five of the nonlinear molecules were six-noded compound catenanes of the type described previously. The remaining five were unit-length six-noded knots. In the

second-dimension sample there was very little DNA, but all six of the clearly defined nonlinear molecules were six-noded knots.

An example of the six-noded knot molecules is shown in Fig. 5. All 11 knots had exactly the structure expected from the processive pathway (Fig. 1). If resolvase, like topoisomerase I (*9*), tied with about equal probability each possible type of six-noded knot, the odds of all 11 knots being the same would be $1/8^{11}$ or 1.2×10^{-10}. By analyzing only a limited number of molecules we were thus able to reliably distinguish between alternative pathways for knot formation.

The fact that resolvase-generated six-noded knots are of the predicted stereostructure verifies that processive recombination occurs. Resolvase and DNA must therefore form an activated complex that persists throughout strand exchange, since nascent catenated products can undergo further recombination whereas catenanes free in solution cannot.

We believe that the strand exchange mechanism used in the processive pathway is the general resolvase mechanism. It is possible that most recombination products could result from a second mechanism with a different topological linkage change. Such a mechanism would have to yield negative singly linked catenanes but never be iterated to yield other products. It would also necessitate another synaptic configuration, because for a given interdomainal writhe only a single type of strand exchange will yield catenanes linked by two (−) nodes (*4*). Since the unitary scheme of Fig. 1 explains the structure of all known resolvase products, there is presently no reason to invoke such additional mechanisms and modes of strand synapsis.

We have shown that resolvase introduces a single interdomainal node during recombination. For interdomainal linkage change to be odd, the reaction sites must align in parallel during recombination (*4*) and thereby bring homologous sequences into register. Thus, for synapsis, topology determines geometry.

Resolvase must introduce a (+) interdomainal node rather than remove a (−) one. In a single round of recombination these are indistinguishable, but the appearance of (+) nodes in the knotted and catenated products of multiple rounds can only be explained if resolvase introduces a positive node in each round. The removal of a substrate supercoil during exchange by a unidirectional rotation of the strands is a likely driving force for recombination. Potential mechanisms for such strand rotations have been described (*11*).

The results of our work on strand exchange complement those of Nash and Pollock (*3*) and of Abremski and Hoess (*12*). They have measured the number of supercoils lost during recombination for two other enzymes. This number, with some assumptions (*4*), gives the overall change in writhe during recombination. We measure only the interdomainal component, which indicates the change in strand arrangement across the site of recombination. We have also deduced the number of substrate supercoils that are interdomainal; it is these that are critical to description of the synaptic complex.

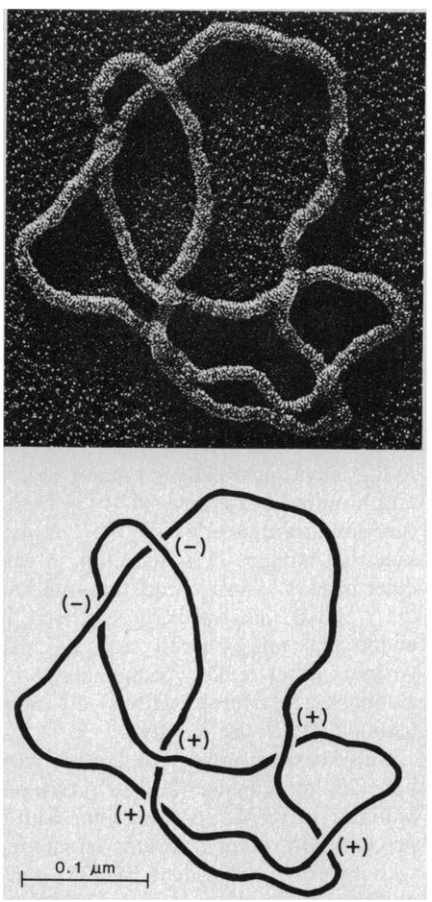


Fig. 5. Electron micrograph of six-noded knot made by Tn3 resolvase. Putative six-noded knot DNA was isolated by electroelution from an agarose gel similar to that in Fig. 3. The recovered DNA was concentrated with *sec*-butanol, and then passed over a 300-μl Sepharose CL-4B (Pharmacia) column in TE buffer. The nicked knots were denatured by glyoxal treatment (*8*) at 62° for 90 minutes. The resulting single-stranded DNA was desalted and then mixed with *E. coli recA* protein (80 μg/ml) in a 24-μl reaction containing 25 m*M* triethanolamine chloride (*p*H 7.6), 5 m*M* ATP, and 5 m*M* magnesium acetate. After 15 minutes, the complexes were fixed with glutaraldehyde and prepared for electron microscopy (*8*). Molecules were photographed at ×40,000 primary magnification and were scored by the procedures outlined in (*9*). Beneath the micrograph is a tracing of the knot showing the sign of each node.

The three (−) supercoils between recombination sites must be stabilized directly by resolvase because the predominant product remains the singly linked catenane over a wide range of substrate supercoil densities (*13*). We have discussed previously how a simple solenoidal wrapping of each *res* site around resolvase could entrap two, but not three, interdomainal supercoils; the third would have to be fixed by another means (*8*). If, however, each resolvase-DNA complex involves plectonemically interwound DNA wrapped on the resolvase surface, any number of supercoils could be fixed between the sites. For example, the three (−) supercoils shown in Fig. 1 could be held in place by symmetrically disposed protein-DNA complexes, each entrapping 1.5 (−) interdomainal supercoils. Additional data are needed to decide among the alternative mechanisms for entrapment.

In the course of this work we have turned the *recA* protein–coating technique for determining DNA topology into a reliable method with high sensitivity. With the improved *recA* technique, knotted or catenated double-stranded DNA is first converted to a topologically equivalent (*14*) single-stranded form. Single-stranded DNA is a much better substrate for reaction with *recA* protein than is double-stranded DNA, yet complexes of *recA* protein with single-stranded DNA provide shadowed images with as fine a level of detail as with duplex DNA. Also, the free rotation of the internucleotide bonds allows the ready polymerization of the *recA* along single-stranded DNA, even for extensively interlinked molecules. Lastly, the unit length of complexes of single-stranded DNA with *recA* protein can be varied by the choice of nucleotide cofactor (*15*). One can therefore adjust the length of the coated complex to obtain the most favorable display of nodes in the electron micrograph. By combining this method with the high resolution afforded by two-dimensional gel electrophoresis, the structure of minute amounts of key intermediates and products can be readily determined.

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Prostacyclin Synthesis Induced in Vascular Cells by Interleukin-1

Abstract: *Supernatants from cultures of human monocytes that had been stimulated with endotoxin or silica induced the synthesis of prostacyclin in endothelial and smooth muscle cells. The lymphokine mediating these effects on the cells of the blood vessel wall was identified as interleukin-1; interferons and interleukin-2 were inactive. Interleukin-1–induced prostacyclin synthesis represents a new aspect of the interaction between the immune system (as well as other tissues) and the vessel wall and may serve as a basis for the development of new strategies in antithrombotic therapy.*

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Although there is evidence of a bidirectional interaction between immunocompetent and vascular cells, the factors mediating this interaction are largely unknown (*1*). Products of lymphocytes or macrophages regulate various aspects of vascular cell function, including proliferation, procoagulant activity, expression of class II histocompatibility (Ia) antigens, and production of colony-stimulating factor (*2–6*). In vivo, stimulated macrophages induce new blood vessel formation, and capillary endothelial cells proliferate at sites of cell-mediated immune reactions (*7*). We showed earlier that lymphokine-containing supernatants of stimulated blood mononuclear cells induce prostacyclin (PGI_2) synthesis in vascular cells (*8*). Prostacyclin, the major metabolite of arachidonic acid in vascular cells, may have a crucial role in the physiology and pathology of blood vessels (*9*).

Selective modulation of arachidonic acid metabolism in platelets as compared to that in vascular cells is a major goal of antithrombotic therapy (*9*). It is therefore important to identify the lymphokine that induces PGI_2 secretion in vascular cells. We now report that interleukin-1 (IL-1) induces PGI_2 synthesis in endothelial and smooth muscle cells, whereas other leukocyte mediators, including the interferons (IFN's) and interleukin-2 (IL-2), are inactive.

Culture supernatants of Percoll-purified (*10*) monocytes (2×10^6 cells per milliliter of RPMI 1640 medium with 5 percent fetal bovine serum) stimulated with lipopolysaccharide (LPS) (25 μg/ml) or silica (50 μg/ml) served as a source of IL-1 (*11*). The supernatants were precipitated with 75 percent ammonium sulfate and dialyzed. We refer to the resulting material as crude IL-1. Crude IL-1 was fractionated on a Sephacryl S-200 column or by chromatofocusing. Highly purified IL-1 (Ultrapure IL-1; Genzyme) was obtained from supernatants of *Staphylococcus albus*–stimulated monocytes by adsorption with rabbit antibodies coupled to a Sepharose 4B column. After elution from the column, the material, further purified by chromatography on Sephadex G-50, showed a homogenous band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*12*).

Interleukin-1 activity was evaluated in a costimulator assay, with C3H/HeJ thymocytes as responding cells and phytohemagglutinin (PHA) as stimulus. A partially purified (*13*) IL-1 preparation was assigned a value of 1000 units per milliliter and used as standard in each assay.

Results from triplicate cultures were plotted as a linear regression against the standard preparation and expressed in units per milliliter. Rat aortic smooth muscle cells and human umbilical vein endothelial cells, which are both components of vessel walls, served as indicators (*8, 14*). Briefly, intact confluent monolayers (2.5×10^5 to 3.5×10^5 cells in a 2-cm^2 culture well) were washed with 2 ml of phosphate-buffered saline (PBS) and then cultured for 24 hours at 37°C in the presence or absence of IL-1. In some experiments, acetylsalicylic acid (500 μ*M*) was added to cells throughout the incubation period. The amount of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) was measured by radioimmunoassay (*15*).

Vascular smooth muscle cells from rat aorta or endothelial cells from human umbilical vein were exposed to IL-1 for 24 hours, and PGI_2 production in the supernatant was measured as immunoreactive 6-keto-$PGF_{1\alpha}$ (Table 1). These vascular cells produce PGI_2 as their major arachidonic acid metabolite, as also assessed by thin-layer chromatography and mass spectrometry (*8, 16, 17*). Interleukin-1 preparations induced considerable release of 6-keto-$PGF_{1\alpha}$ in both rat aortic smooth muscle cells and human umbilical vein endothelial cells. Endothelial cells were consistently more sensitive to the stimulatory effect of IL-1, whether measured as minimal active concentration (usually 0.1 unit/ml) or amount of 6-keto-$PGF_{1\alpha}$ induced.

In six experiments with endothelial cells, IL-1 (1 unit/ml for 24 hours) induced the synthesis of 127.3 ± 25 pmol per 10^5 cells [mean ± standard error (S.E.M.); range 63 to 170] compared to baseline levels of 24.4 ± 9 (range 7.5 to 53). In six experiments with aortic smooth muscle cells, IL-1 (10 unit/ml for 24 hours) induced the synthesis of 6.6 ± 1.5 pmol of 6-keto-$PGF_{1\alpha}$ per 10^5 cells (mean ± S.E.M.; range 3 to 11.7) compared to baseline levels of 0.5 ± 0.1 (range 0.3 to 0.9). Induction of PGI_2 production by IL-1 was dependent on the integrity of the cyclooxygenase pathway of arachidonic acid metabolism, as shown by the complete blockade caused by acetylsalicylic acid (Table 1). Crude IL-1 preparations from LPS-stimulated monocytes contained LPS. The following observations suggest that LPS did not play an appreciable role in induction