Knot with six crossings is depicted in this Roman bas relief from about the third century A.D. The identical knot form was observed in electron micrographs of DNA and the information from it can be used to postulate mechanisms of genetic recombination. See page 171. [From the private collection of Ilya Prigogine, Center for Statistical Mechanics and Thermodynamics, University of Texas, Austin 78712]

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.

STEVEN A. WASSERMAN
JAN M. DUNGAN
NICHOLAS R. COZZARELLI
Department of Molecular Biology,
University of California,
Berkeley, California 94720

The resolvase proteins encoded by transposons Tn3 and γ6 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.

12 JULY 1985
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 resolvas 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 catenane, of which there are only two isomers. Resolvas are 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 resolvas 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 resolvas 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).

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 resolvas (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 resolvas 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 resolvas action.

The DNA predicted to be a six-noded knot represented about 0.1 percent of the resolvas 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 mol-
cules is shown in Fig. 5. All 11 knots
had exactly the structure expected from
the processive pathway (Fig. 1). If resol-
vase, 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 1/811 or
1.2 x 10^-10. By analyzing only a limited
number of molecules we were thus able
to reliably distinguish between alterna-
tive pathways for knot formation.

The fact that resolvase-generated six-
noded knots are of the predicted stereo-
structure verifies that processive recom-
bination occurs. Resolvase and DNA
must therefore form an activated com-
plex that persists throughout strand ex-
change, since nascent catenated prod-
ucts can undergo further recombination
whereas catenanes free in solution can-
not.

We believe that the strand exchange
mechanism used in the processive path-
way 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 ne-
cessitate another synaptic configuration,
because for a given interdominal 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 synap-

sis.

We have shown that resolvase intro-
duces a single interdominal node during
recombination. For interdominal link-
age change to be odd, the reaction sites
must align in parallel during recombin-
ation (4) and thereby bring homologous
sequences into register. Thus, for synap-

sis, topology determines geometry.

Resolvase must introduce a (+) inter-
dominal node rather than remove a (-)
one. In a single round of recombination
these are indistinguishable, but the ap-
pearance of (+) nodes in the knotted and
catenated products of multiple rounds
can only be explained if resolvase intro-
duces 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 de-
scribed (11).

The results of our work on strand ex-
change 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 interdominal com-
ponent, which indicates the change in
strand arrangement across the site of
recombination. We have also deduced
the number of substrate supercoils that
are interdominal; it is these that are
critical to description of the synaptic
complex.

The three (-) supercoils between re-
combination sites must be stabilized
directly by resolvase because the predomi-
nant product remains the singly linked
catenane over a wide range of substrate
supercoil densities (13). We have dis-
cussed previously how a simple solenoi-
dal wrapping of each res site around
resolvase could entrap two, but not three,
interdominal supercoils; the third
would have to be fixed by another means
(8). If, however, each resolvase-DNA
complex involves plectonomically inter-
wind 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 asymmetrically
disposed protein-DNA complexes, each
entrapping 1.5 (-) interdominal super-
coils. Additional data are needed to de-
cide among the alternative mechanisms
for entrapment.

In the course of this work we have
turned the recA protein-coating tech-
nique for determining DNA topology
into a reliable method with high sensi-
tivity. With the improved recA tech-
nique, knotted or catenated double-stranded
DNA is first converted to a topologically
equivalent (4) single-stranded form.
Single-stranded DNA is a much better
substrate for reaction with recA protein
than is double-stranded DNA, yet com-
plexes 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 exten-
sively interlinked molecules. Lastly, the
unit length of complexes of single-
stranded DNA with recA protein can be
varied by the choice of nucleotide cofac-
tor (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 electro-
phoresis, the structure of minute
amounts of key intermediates and prod-
ucts can be readily determined.

References and Notes
1. R. R. Reed, Cell 25, 713 (1981); —— and N.
D. F. Grindley, ibid., p. 721.
2. M. A. Krassnow and N. R. Cozzarelli, ibid. 32,
1313 (1983).
3. H. A. Nash and T. J. Pollock, J. Mol. Biol. 170,
4. N. R. Cozzarelli, M. A. Krassnow, S. A.
28, 562 (1972); K. Mizuochi, M. Gellert, R. A.
10. The six-noded compound catenane is most likely the product of two different productive encounters between resolvase and substrate. In the first encounter, resolvase acts processively, mediating two rounds of strand exchange, and releases a four-noded knot. This knot still contains directly repeated res sites and can therefore undergo a normal resolution reaction in a second encounter. The product is a singly linked catenane in which one ring contains a four-noded knot. The second reaction has been demonstrated in vitro, with a purified four-noded knot (S.A.W. and N.R.C., unpublished).
14. The structure of a duplex DNA knot is an invariant property of the helix axis. Since each strand of the duplex is deformable to the axis, the knots defined by each single strand and by the axis are topologically identical.
18. We thank K. McEntee for a gift of E. coll recA protein. Supported by NIH grants GM 31655, 31657, and 07222. S.A.W. is a fellow of the Miller Institute for Basic Research in Science.

22 March 1985; accepted 22 May 1985