Gin-mediated DNA inversion: Product structure and the mechanism of strand exchange

(site-specific recombination/bacteriophage Mu/DNA topology)

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ABSTRACT Inversion of the G loop of bacteriophage Mu requires the phage-encoded Gin protein and a host factor. The topological changes in a supercoiled DNA substrate generated by the two purified proteins were analyzed. More than 99% of the inversion products were unknotted rings. This result excludes synapsis by way of a random collision of recombination sites, because the resulting entrapped supercoils would be converted into knots by recombination. Instead, the recombination sites must come together in the synaptic complex in an ordered fashion with a fixed number of supercoils between the sites. The linking number of the substrate DNA increases by four during recombination. Thus, in three successive rounds of inversion, the change in linking number was +4, +8, and +12, respectively. These results lead to a quantitative model for the mechanism of Gin recombination that includes the distribution of supercoils in the synaptic complex, their alteration by strand exchange, and specific roles for the two proteins needed for recombination.

Site-specific recombination systems promote genetic exchange between specific DNA sequences. They are found in many organisms and play roles in differential gene expression, reversible integration of viral DNA into host chromosomes, and transposition (for reviews see refs. 1-3). Many of the systems have been established *in vitro* with pure proteins.

Recombination between two sites that are directly repeated within a single DNA molecule (head-to-tail orientation) causes deletion of the DNA between the sites, whereas recombination between inverted sites (head-to-head orientation) inverts the sequence between the sites. An important example of the latter is the Gin system of phage Mu. Within the DNA of this phage is the 3-kilobase (kb) G region that occurs in two orientations, G(+) and G(-). This region is responsible for the alternative expression of two sets of genes whose products are involved in tail-fiber synthesis, thereby determining the absorption properties of the phage (2). The G region is bordered by two 34-base-pair recombination sites, *gixL* and *gixR*, in inverted orientation (2). It has been shown that another sequence is needed in cis to obtain high levels of inversion (4). This recombinational enhancer, or *sis* sequence, functions in a manner that is independent of its orientation and distance from the *gix* sites. The very similar Hin invertase system found in *Salmonella typhimurium* also requires an enhancer (5). The two proteins of the Gin system are the phage-encoded Gin protein and a host factor named Fis or Gin host factor (GHF) (4, 6); Gin binds to the *gix* sites, and GHF binds to *sis* (7). We hereafter refer collectively to these proteins as Gin, unless otherwise stated.

Although the Gin family of invertases is $\approx 30\%$ similar to the amino acid sequence of the resolvases of the Tn3 transposon family (2), there are striking differences between the two. First, the orientation of recombination sites is opposite and noninterchangeable for the two families. Second, whereas the invertases need both a host factor and a recombinational enhancer, the resolvases require neither (8, 9). Finally, each *gix* site consists of a single inverted repeat, whereas a resolvase site is composed of three tandemly arranged pairs of inverted repeats (9). Despite these distinctions, the results presented in this paper imply that invertases and resolvases share critical common mechanistic features.

We have shown (6) that the Gin system not only recombines DNA but also removes negative supercoils. This relaxation is tightly coupled to recombination. It requires two inversely oriented *gix* sites, Gin, and GHF. We find that the linking number (Lk) of the DNA increases by four during each round of inversion by Gin and that the products are unknotted. From these results, we propose a model for the number and distribution of supercoils in the Gin synaptic complex, their alteration by strand exchange, and the role of enhancers in inversion.

MATERIALS AND METHODS

DNA. The substrate DNA used was pGP238(-) (6). The *gix* sites in this 5.3-kb plasmid are in inverted orientation and separated by 1.5 kb; the enhancer sequence is at wild-type spacing to the *gixR* site. Plasmid DNA was isolated from *Escherichia coli* strain DH1.

To isolate pure topoisomers, 25 μ g of plasmid DNA was electrophoresed through a $17 \times 15 \times 0.35$ cm agarose gel (SeaKem Laboratories, Rockland, ME) containing chloroquine at 27 μ g/ml. After ethidium bromide staining, topoisomer bands were excised from the gel, and the DNA was electrophoresed into dialysis bags in the presence of tRNA at 5 μ g/ml. After phenol and chloroform extraction, the DNA was concentrated with 1-butanol, precipitated with ethanol, and filtered through a Sephadex G-50 column in 10 mM Tris-HCl, pH 7.5/1 mM EDTA.

Plasmid DNA (10 μ g/ml) was singly nicked by treatment with pancreatic DNase at 1.6 μ g/ml in the presence of ethidium bromide at 300 μ g/ml for 30 min at 30°C. After extraction with phenol and chloroform, the DNA was ethanol precipitated and resuspended in 10 mM Tris-HCl, pH 7.5/1 mM EDTA.

To obtain substrate DNA purified from naturally contaminating knotted molecules, 100 μ g of nicked pGP238(-) was electrophoresed through a 0.8% agarose slab gel ($13.5 \times 27 \times 0.7$ cm) at 2 V/cm for 40 hr. The electrophoresis buffer was 80 mM Tris-HOAc, pH 7.5/5 mM NaOAc/1 mM EDTA/0.03% NaDodSO₄. After ethidium bromide staining,

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Abbreviations: Lk, linking number; GHF, Gin host factor.

the band containing the open-circular form of the plasmid was excised, and the DNA was purified as described above. This DNA was incubated with T4 DNA ligase at 33 units/ μ l at 37°C in the presence of ethidium bromide at 7 ng/ml to obtain the supercoiled form. Finally, the ligated DNA was purified by CsCl/ethidium bromide density gradient centrifugation.

Enzymes. Gin protein was purified from *E. coli* strain PP1458 using a modification of our method (6). The Aca-54 pool was diluted to 0.2 M NaCl and loaded on a 1-ml Mono S column (Pharmacia). The column was washed with 4 ml of buffer A (20 mM Tris-HCl, pH 7.5/10 mM MgCl₂/1 mM dithiothreitol) plus 0.2 M NaCl and then with 3 ml of buffer A/0.4 M NaCl. Then a 10-ml linear gradient to 0.6 M NaCl/buffer A was applied. Gin protein eluted between 0.53 and 0.55 M NaCl. It was >95% pure as judged by Coomassie blue staining of NaDodSO₄/polyacrylamide gels and contained less GHF contamination than our previous preparations. GHF was purified as described (6). T4 topoisomerase (10) was a gift from B. Alberts (University of California, San Francisco). Restriction enzymes, T4 DNA ligase, and bovine pancreatic DNase were from commercial sources.

Reactions. The standard recombination reaction was performed at 37°C for 60 min and contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, DNA at 10 μ g/ml, Gin at 2.5 μ g/ml, and GHF at 0.5 μ g/ml. Recombination was assayed by gel electrophoresis after digestion with *Eco*RI and *Hind*III. This generates 3.9-kb and 1.4-kb fragments from the starting G(-) form and 3.6-kb and 1.7-kb fragments from the recombinant G(+) form.

To determine if the Gin-treated products were knotted, the gel system described above for the isolation of nicked open-circular DNA was used. DNA was transferred from the gel to nitrocellulose (Schleicher & Schuell BA85) and probed with ³²P-labeled DNA.

Marker knot ladders were made with T4 topoisomerase (11) and nicked with DNase I as described above.

RESULTS

The Predominant Gin Product Is Unknotted. To determine if the product of Gin recombination was knotted, 0.2 μ g of pGP238(-) was incubated with Gin, nicked with DNase I to remove supercoiling, and analyzed by high-resolution agarose gel electrophoresis. The only product observed was the simple open-circular form, even though restriction endonuclease digestion showed that 45% of the substrate was converted to the recombinant G(+) form. To detect minor products, the reaction was scaled up to 15 μ g of substrate and, after electrophoresis, the DNA was transferred to nitrocellulose and probed with ³²P-labeled DNA to improve sensitivity. For Gin-treated and untreated DNA, besides the predominant open-circle form, minor amounts of knotted species containing up to 10 nodes (crossings of DNA segments in plane projection) were seen. Thus, under these enhanced detection conditions, the level of *in vivo* knotted substrate molecules was too high to observe knots that might be formed by recombination.

We, therefore, purified the substrate DNA from the knotted contaminants. The DNA was nicked with DNase, resolved by gel electrophoresis, electroeluted, and religated in the presence of ethidium bromide to give a superhelical density close to the native value. The purified DNA was recombined with Gin and analyzed by high-resolution agarose gel electrophoresis after renicking with DNase (Fig. 1). Under these conditions, the mobility of knotted DNA molecules increases with node number (12), as is shown by the marker knot ladder made with T4 topoisomerase (lane 3). Besides the major Gin product of open circles, there was a minute amount (0.1% of the total) of a three-noded knot

FIG. 1. Products of Gin-mediated DNA inversion. pGP238(-) DNA purified away from contaminating knots was recombined by Gin, and the structure of the products was analyzed by high-resolution gel electrophoresis after nicking the DNA. The DNA in the gel was transferred to nitrocellulose and hybridized with radioactive pGP238(-) DNA. Lanes: 1, linear DNA produced by *Pst* I digestion of the substrate; 2, unreacted DNA; 3, T4 topoisomerase knot ladder; 4, recombinant DNA; 4', overexposure of lane 4. oc, Open-circular substrate; lin, linear DNA; 3-7, number of irreducible nodes in the knotted molecules.

(trefoil) (Fig. 1, lane 4). In an overexposure (Fig. 1, lane 4'), even smaller amounts of four- and five-noded knots were detected. Thus, the recombinant product is almost entirely unknotted; the mechanistic significance of the trace knotting is discussed below.

Linking Number Change. We have shown (6) that a small number of negative supercoils were removed concomitantly with strand exchange by Gin. Moreover, because the product of recombination is unknotted, it is possible to measure the Lk change (ΔLk) without the complication of the change in structure by knotting (13). To measure ΔLk , the substrate has to be uniform in Lk.

Topoisomers of pGP238(-) were separated by preparative agarose gel electrophoresis in the presence of the DNA-intercalating agent chloroquine phosphate (Fig. 2A). The topoisomers were numbered in order of increasing negative supercoiling, -1 to -9; neighboring bands differ in Lk by 1. Topoisomers -3, -6, and -7 were excised from the gel, purified, and incubated with Gin, and the products were analyzed by electrophoresis.

The recombinant of topoisomer -6 (Fig. 2B, lane 3) is an unknotted circle comigrating with topoisomer -2. Thus ΔLk is +4. The DNA can undergo multiple rounds of recombination (Fig. 2C). Topoisomer -7 has, we infer, undergone three rounds (Fig. 2C, lane 3). The mobility of the first round product is identical to that of topoisomer -3, or again ΔLk is +4. The ΔLk of the other products was confirmed using gels with various chloroquine concentrations, because their positive supercoiling on the gel shown complicates indexing. The second round product migrates at the position of the +1 topoisomer, and the third round product migrates at the position of the +5 topoisomer. The total ΔLk is then +8 and +12 for the second and third rounds of recombination, respectively. Electrophoresis in the absence of chloroquine showed that these topoisomers are still negatively supercoiled. Two rounds of recombination for topoisomer -3 (Fig. 2C, lane 1) gave products with the expected ΔLk of +4 and +8; they lined up precisely with the second and third round products of the -7 topoisomer. When the GHF or Gin protein was omitted from the reaction mixture, neither recombination nor a change in Lk was detected.

To confirm the number of recombination rounds that yielded the product topoisomers in Fig. 2, the recombinant form [G(-) or G(+)] of the DNA in these bands was

FIG. 2. Change in Lk during Gin-mediated recombination. (A) pGP238(-) (25 μ g) was resolved by electrophoresis through an agarose gel containing chloroquine at 27 μ g/ml. The ethidium bromide staining pattern is shown. oc, Open-circular form; -1 to -9, topoisomers with increasing negative supercoiling. (B) Electrophoresis through an agarose gel containing chloroquine. Marker ladder of a mixture of supercoiled topoisomers of the substrate DNA (lane 1), topoisomer -3 (lane 2), topoisomer -6 incubated with Gin (lane 3), and topoisomer -6 alone (lane 4). (C) Electrophoresis through chloroquine-impregnated agarose gel containing topoisomer -3 (i) with (lane 1) or without (lane 2) Gin and topoisomer -7 (ii) with (lane 3) or without (lane 4) Gin.

determined. The DNA in the bands in lanes 1 and 3 of Fig. 2C was analyzed. Since the substrate is in the G(-) form, the predictions are that the products whose Lk increased by 4 or 12 will be G(+), and those that have increased by 8 will be G(-). This is exactly the result found (Fig. 3). The trace amounts of the opposite forms are probably due to a slight topoisomerase activity of the Gin system that is uncoupled from recombination.

DISCUSSION

There are two major results presented in this paper. First, >99% of the products of Gin-mediated inversion are open circles. This finding excludes a random collision model for site synapsis, as found for some aspects of the Int system, because then the products would be knots with various complexities depending on the substrate supercoil density (14, 15). Instead, the data clearly implicate a much more ordered mode of site synapsis such as occurs with the Tn3 family of resolvases and the Mu transposase (11, 16, 17).

Our second major result is that ΔLk is +4 during a single round of recombination. The same conclusion has been reached by Kahmann and coworkers (cited in ref. 18). For

two other well-studied recombinases, Int and Cre, the ΔLk for an inversion reaction is +2 (13, 19). A discussion of the topological differences between these recombination systems is presented elsewhere (20). For the recombinases Int and resolvase, the Lk of the DNA substrate changes during recombination for two distinct reasons. One is relaxation in the absence of recombination. This is a true topoisomerase activity, because the only change observed in the DNA is in Lk (13, 21). The other change in Lk results from the sum of the DNA movements, such as rotations and passages, that accompany strand exchange. It is clear that the ΔLk of +4 for Gin recombination is due only to exchange. A distinctive reaction product was found in which $\Delta Lk = +4$, and the sequence orientation between the recombination sites had flipped from G(-) to G(+). When a second product was identified, its ΔLk was +8, and the orientation between the recombination sites had returned to G(-). For a third product found after even more extensive reaction, ΔLk was +12, and its orientation was G(+). Relaxation would not invert the sequence between the *gix* sites. Moreover, relaxation is only known to change Lk by 1 or 2 per reaction, and it is hard to see how it would give the discrete steps of four changes that were found. The finding of a distinctive ΔLk emphasizes that during exchange, when eight polynucleotide ends are exposed (double-strand interruptions at two sites), each of these are not free to rotate. If they were, then relaxation would ensue. Thus, the DNA must be firmly led through the motions that accompany recombination.

To explain precisely the mechanistic implications of these experimental results, we will introduce some topological concepts. More rigorous treatments of the application of topology to recombination can be found elsewhere (3, 22). DNA molecules can contain two type of nodes, which are designated negative and positive according to convention. The regions of DNA whose interrelation is altered by recombination define two domains that extend from one *gix* site to the other. They are shown as thick and thin lines in Fig. 4, which presents our scheme for the topology of Gin recombination. Nodes are either interdomainal or intradomainal depending upon whether they are made by a crossing between or within domains.

Three integral topological parameters describe the most important features of the mechanism of recombination (22). They are the interdomainal nodes of the synaptic complex (^{ter}Wr) and the interdomainal (^{ter}Me) and intradomainal (^{tra}Me) nodes introduced by recombination. These three parameters cannot be measured directly but can be com-

FIG. 3. Restriction analysis of the recombined topoisomers. To analyze the topoisomers in lanes 1 and 3 of Fig. 2C for their sequence orientation, G(-) or G(+), the bands in these lanes were isolated by electroelution and, after digestion with *Eco*RI and *Hind*III, electrophoresed on a 1.0% agarose gel. The DNA was transferred to nitrocellulose and probed with 32 P-labeled pGP238(-) DNA. The resulting autoradiograph is shown. The lane labels 0-3 refer to the putative rounds of recombination according to the decrease in Lk, and lane oc is the open-circular form. On the right, G(-) and G(+) indicate the expected mobilities of the respective restricted products.

FIG. 4. Topological changes during Gin recombination. The arrows represent the *gix* sites, and the solid and open ribbons represent the domains between them. The substrate is shown with only the two essential (-) interdomainal supercoils (${}^{\text{ter}}Wr^S = -2$). Crossover at the sites is accompanied by the introduction of a (-) interdomainal node (${}^{\text{tra}}Me = -1$) and a (+) intradomainal node (${}^{\text{tra}}Me = +1$). Recombination inverts the relative orientation of the domains and thereby inverts the sign of the two substrate interdomainal nodes, making ΔLk ($Lk^P - Lk^S$) equal to +4, where Lk^P and Lk^S are the Lk of the product and substrate, respectively.

puted using equations that relate them to measurable quantities.

The change in Lk during recombination arises not only from the local changes in DNA made by the recombinase ($Me = {}^{\text{tra}}Me + {}^{\text{ter}}Me$), but also from changes in ${}^{\text{ter}}Wr^S$ that result from exchange. Whereas resolution preserves the sign of ${}^{\text{ter}}Wr^S$, inversion reverses it. Thus, for inversion,

$$\Delta Lk = Me - 2{}^{\text{ter}}Wr^S. \quad [1]$$

If for invertase reactions, we designate the algebraic sum of product interdomainal nodes as Kn^P , then

$$Kn^P = {}^{\text{ter}}Me - {}^{\text{ter}}Wr^S. \quad [2]$$

In the synaptic complex of both resolvase and Int, the sites are aligned in parallel (3). This arrangement will very likely be generally true, and we assume it is so for Gin. This geometry implies that Kn^P is an odd number. If the Gin product had three or more interdomainal nodes it would have been knotted. The product is almost entirely unknotted; therefore Kn^P must equal ± 1 .

We assume that the absolute value for both ${}^{\text{ter}}Me$ and ${}^{\text{tra}}Me$ is 1 for Gin, as it seems to be for all other site-specific recombination enzymes (20). Moreover, we explain below why the structure of the minor products of recombination suggests that ${}^{\text{ter}}Me$ is ± 1 . Therefore, from Eq. 2 and $Kn^P = \pm 1$, ${}^{\text{ter}}Wr^S$ can only have one of three values, -2, 0, and +2. However, from Eq. 1, the probable values of ${}^{\text{ter}}Me$ and ${}^{\text{tra}}Me$, and the finding that $\Delta Lk = +4$, ${}^{\text{ter}}Wr^S$ must equal -2 (Table 1). All the topological parameters for Gin are now set; ${}^{\text{ter}}Me = -1$, ${}^{\text{tra}}Me = +1$, and $Kn^P = +1$ (Table 1).

The mechanism implied by these parameters is shown schematically in Fig. 4. The two (-) interdomainal nodes of the substrate change their sign during inversion. Exchange introduces one (-) interdomainal node and one (+) intradomainal node; they can be thought of as arising from rotations of and around the helix axis, respectively. The value of ΔLk is +4, because the interdomainal and intradomainal terms of the mechanism cancel, and the two ${}^{\text{ter}}Wr^S$ nodes change sign from (-) to (+). It could not have been predicted that the change in Lk during recombination is unrelated to the local movements of the DNA strands during exchange and instead arises solely as a consequence of inversion of the sequence between the *gix* sites.

This scheme readily explains the trace amounts of trefoils produced by Gin. Resolvase is known to occasionally iterate, i.e., to repeat strand exchange without breaking down the synaptic intermediate (23). Two iterative rounds of

Table 1. Possible values for topological parameters for Gin recombination

${}^{\text{ter}}Wr^S$	${}^{\text{ter}}Me$	${}^{\text{tra}}Me$	ΔLk	Kn^P *
0	-1	-1	-2	-1
0	+1	-1	0	+1
0	-1	+1	0	-1
0	+1	+1	+2	+1
-2	-1	-1	+2	+1
-2	+1	-1	-†	+3
-2	-1	+1	+4	+1
-2	+1	+1	-†	+3
+2	-1	-1	-†	-3
+2	+1	-1	-4	-1
+2	-1	+1	-†	-3
+2	+1	+1	-2	-1

All the numbers were calculated using the equations in the text and checked by using a ribbon model for DNA (22). Only the set of values in bold type agrees with the experimental results.

*A product with $Kn^P = \pm 1$ is unknotted.

†When the product is knotted, Eq. 1 no longer holds (22). Since Gin products are unknotted, these cases are irrelevant.

recombination by Gin in this fashion will produce a (-) trefoil (Fig. 5). The frequency of trefoils is low because iteration is rare. The even scarcer higher-order knots could result from further iteration of the basic mechanism. If the knots were instead due to occasional entrapment of supercoils between recombination sites, then the knots would always have an odd number of nodes (15). The ladder of Gin products instead has steps spaced by one node (Fig. 1). This is the expected pattern from iteration, if, and only if, ${}^{\text{ter}}Me = \pm 1$.

The scheme is the exact topological analogue of the resolvase mechanism for excision. Resolvase and the Gin protein are $\approx 30\%$ similar in amino acid sequence despite the specificity of the Gin system for inversion and resolvase for excision. However, the same recombination mechanism operating on directly and inversely repeated sites will maintain the value of ${}^{\text{tra}}Me$ and the absolute value but not the sign of ${}^{\text{ter}}Me$ (22). This is exactly the relationship of the topological parameters for Gin and resolvase, because both ${}^{\text{ter}}Me$ and ${}^{\text{tra}}Me$ equal +1 for resolvase (refs. 16 and 23; J. M.

FIG. 5. Two rounds of iterative recombination by Gin. Only the two essential interdomainal nodes and the interdomainal change by the mechanism are shown. (Upper) Protein bound synapsed structures. (Lower) Nicked unfolded forms. Two processive rounds of recombination are shown in which the protein-DNA complex is not destroyed after the first round.

FIG. 6. A model for the formation of the Gin synaptic complex. (A) A negatively supercoiled substrate is shown with two arrows representing Gin-bound *gix* sites and an open box indicating the enhancer sequence bound by GHF. The two topological domains of the substrate are represented by thin and thick lines. In step one, both *gix* sites are brought together in a presynaptic complex at the base of the branch (B). In step two, the complete synaptic complex is formed by incorporation of the enhancer loaded with GHF (C). For both steps the DNA movement is shown as slithering, the sliding of segments across each other in a supercoiled molecule (24). Other motions that lead to the same structures would also suffice.

Dungan and N.R.C., unpublished data). Thus, the exchange mechanism of these homologous enzymes appears topologically equivalent, and their different specificity resides in $^{ter}Wr^s$. Parallel site alignment requires that $^{ter}Wr^s$ be odd for resolvases but even for invertases.

We can imagine two plausible ways to ensure that $^{ter}Wr^s$ equals -2 in the Gin synaptic complex. One has been described elsewhere (20) and postulates that one negative node results from the plectonemic crossing of *gix* sites in a supercoiled molecule, and the other results from an interaction between the enhancer and one, and only one, of the *gix* sites. Alternatively, branching of the DNA could allow the enhancer to interact equivalently with both *gix* sites, and one example of such a model is illustrated in Fig. 6. In Fig. 6A, Gin and GHF are bound at their respective sites. In step one, the bound *gix* sites come together to form a presynaptic complex at the base of a branch in the supercoiled substrate (Fig. 6B). Branches occur naturally in supercoiled DNA, and Gin or GHF may promote their formation or stabilize them by bending the DNA. In the presynaptic complex, the value of $^{ter}Wr^s$ is set at -2 . In step two, the complete synaptic complex is formed by the incorporation of the GHF-bound enhancer (Fig. 6C). Because of the symmetrical arrangement of the *gix* sites in Fig. 6B, reversal of the enhancer sequence orientation will not affect its activity, as found. Moreover, as long as step two is not rate-limiting, the distance between the enhancer and the recombination sites will not be important, a second feature of enhancers.

Clearly, additional evidence is needed to prove the mechanism of recombination in the Gin inversion system. The schemes presented, however, explain all available data and can easily be tested by topological and biochemical methods.

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