Mechanism of Tn3 Resolvase Recombination in Vivo*

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To determine the physiologically important features of site-specific recombination by Escherichia coli Tn3 resolvase we analyzed the salient properties of the reaction in vivo. A two-plasmid system in which one plasmid served as substrate while the other encoded both resolvase and a thermolabile repressor of resolvase transcription provided controlled, synchronous recombination. Recombination proceeded rapidly and was promoted by (−) DNA supercoiling. The structures of the in vivo recombination products were predominantly the same as those previously identified in vitro. By examination of the products of successive rounds of recombination of a four-site substrate, we ruled out a transduction but ultimately reached a higher extent than found in vitro. We propose that inversion and fusion exploit topologically interlinked substrates that occur at low levels in vivo. This proposal is supported by the unexpected specificity of fusion. Our data imply that supercoiled DNA, the resolvase synaptic complex, and the mechanism of strand exchange are fundamentally similar in vivo and in vitro, but that the repertoire of resolvase substrates and products is expanded in vivo by the action of other enzymes that alter DNA topology.

Transposition of the Tn3 family of mobile genetic elements occurs in two steps. In the first step, a cointegrate intermediate is formed which consists of the donor replicon fused to a target replicon by directly repeated transposon bridges (reviewed in Ref. 1). This process requires the Tn3-encoded transposase. In the second step, site-specific recombination by resolvase, the product of the transposon tnpR gene, separates the cointegrate at sites designated res to yield the donor and target plasmids, each containing a Tn3 element.

The resolvase reaction has been extensively studied in vitro. Purified resolvase efficiently recombines supercoiled plasmids containing two res sites with the amount of recombination proportional to the superhelical density of the substrate (2–4). With a standard substrate, the reaction occurs only if the res sites are directly repeated in the same molecule, just as they are positioned in a natural cointegrate. The major product is a singly linked negative catenane (5). Inversion and intermolecular recombination (fusion), once considered forbidden reactions, are now known to occur efficiently with knotted and catenated substrates, respectively (6, 7).

This site specificity of resolvase may be explained by topological effects. Prior to DNA exchange, the two res sites come together in an elaborate nucleoprotein structure called the synaptic complex, which contains three tightly interwound (−) supercoils (7, 8). The synaptic complex contains a particular interwound geometry of the res sites which is favored by negative supercoiling of directly repeated sites, knotting of inverted sites, and multiple catenation of sites on separate DNA rings.

It is important to establish the properties of resolvase action in vivo, as in vitro studies show that the requirement for negative supercoiling and the unidirectionality of the reaction may be defeated somewhat by changes in reaction conditions (6, 8–10). As well, inside of cells the ionic composition differs from in vitro conditions and the supercoiled structure of the substrate DNA is altered by the binding of a number of proteins (11, 12). In turn, the comparison of the properties of resolvase in vivo and in vitro can provide information about the cellular milieu.

Toward these ends, we developed a system that provides controlled, efficient resolvase expression and recombination in Escherichia coli cells. From a comparison of the products formed in vitro and in vivo, we conclude that recombination in the cell displays three of the topological properties previously determined by rigorous in vitro biochemical analyses: 1) The highly preferred substrate has directly repeated sites contained on a single molecule; 2) the product of the reaction is almost exclusively a singly linked catenane; and 3) recombination is promoted by negative supercoiling. From these observations and from the structure of products from multiple rounds of recombination, we conclude that the mechanism of site synapsis and strand exchange are the same in vivo as established in vitro. However, the intracellular environment did influence aspects of the reaction. The product catenanes were instantly converted to free circles by the action of DNA gyrase present in the cell. Inversion and intermolecular reactions did occur in vivo, albeit slowly, and we propose that these reactions result from the transient formation of knotted and catenated substrates, respectively, in the cell.

EXPERIMENTAL PROCEDURES

Bacteria—The Escherichia coli strains used were MG1655.10 (lac recA) (5) and W3101 (rpsL red114 xis1 c857) (12). Both strains were propagated on L-broth plates.

Plasmid Constructions—Plasmid transformations and isolations were carried out as described (13). The resolvase expression plasmid, pBRESc1 (Fig. 1A), was constructed as follows. pACYC184 (14) was cleaved with XhoI, and the largest fragment (2300 bp) containing the p15A origin of replication (15) and the chloramphenicol resistance

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*These experiments were supported by grants from the National Institutes of Health. This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research (to H. W. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: bp, base pair(s); SDS, sodium dodecyl sulfate.
gene (Cam\(^{R}\)) was isolated. The overhangs were repaired with DNA polymerase, and synthetic BamHI linkers were ligated to the ends. The resultant fragment was cyclized with DNA ligase, transformed into MG1655.10, purified, and reopened at the BamHI site by cleavage with XhoII. The tnp\(_R\) gene under control of the pJ promoter was isolated from pMK17 (3) as follows. The unique BamHI site in tnp\(_R\) was destroyed by XhoII cleavage by methylase with BamHI methylase. The plasmid was then cleaved with XhoII and the largest fragment (1570 bp) was purified, ligated into the XhoII-cut pACYC184 derivative, and transformed into W3101A. The plasmid containing the tnp\(_R\) gene in the same transcriptional orientation as the Cam\(^{R}\) gene was then isolated. This plasmid, designated pJBR5, was linearized with XhoII after methylation protection of the BamHI sites. A 2400-bp BglII fragment containing the clts gene was isolated from pRK248 and its transcription orientation with respect to tnp\(_R\) was repressed during induction. Resolvase expression was repressed further inactivated the repressor and rapidly led to high levels of resolvase. Resolvase expression was repressed during

**RESULTS**

**Resolvase-mediated Resolution in Vivo**—To control resolvase expression in vivo, we constructed the plasmid pJBR531 (Fig. 1A) containing both the resolvase gene, tnp\(_R\), expressed from the \(\lambda\)p promoter, and clts, which encodes a thermolabile repressor of tnp\(_R\). A recA\(^{-}\) E. coli strain devoid of the Tn3 and \(\lambda\) transposons (MG1655.10) was transformed sequentially with pJBR531 and pA\(^{Δ}\)ARI (Fig. 1B), a substrate plasmid for resolvase. Resolvase expression was repressed during growth of the cells at 28°C. Shifting the culture to 42°C inactivated the repressor and rapidly led to high levels of resolvase synthesis. Returning the induced cells to 28°C repressed further tnp\(_R\) transcription (19) while allowing recombination to continue. The use of short induction times of usually just a few minutes' duration synchronized recombination. Resolvase was assayed by cutting at the single EcoRI site in pA\(^{Δ}\)ARI, which converts the substrate into full length linear DNA and the resolved products into half-length linear and circular molecules, and separating the DNA species by gel electrophoresis (Fig. 2A). Negligible recombination was detected prior to induction, demonstrating the effectiveness of repression of transcription from pJ, by the clts product at 28°C. However, after only a 2.5-min induction at 42°C, 35% of the substrate was recombined. This corresponds to about four plasmids recombined per min/cell, assuming 30 copies of the plasmid substrate/cell. If, after the 2.5-min induction, the cultures were returned to 28°C for 17.5 min, 93% of the substrate was resolved. The extent of catenation of the substrate was recombined. This corresponds to about four plasmids recombined per min/cell, assuming 30 copies of the plasmid substrate/cell. If, after the 2.5-min induction, the cultures were returned to 28°C for 17.5 min, 93% of the substrate was recombined. The extent of catenation of the substrate was recombined. This corresponds to about four plasmids recombined per min/cell, assuming 30 copies of the plasmid substrate/cell. If, after the 2.5-min induction, the cultures were returned to 28°C for 17.5 min, 93% of the substrate was recombined.
cells harboring pJBREScI and pA'ARI were grown at 28°C. Because 35% of the substrate was recombined at the time of a 2.0-min induction (Fig. 2B, lanes 5 and 7). The reduction in recombination was even greater (24%) when norfloxacin was added immediately after a 2.0-min induction (Fig. 2B, lanes 4 and 7). Because gyrase inhibition occurred after the induction was terminated by downshift of the growth temperature to 28°C, the diminution of recombination was not due to reduced transcription of tnpR. In addition, norfloxacin had no direct effect on resolvase recombination in vitro (data not shown). Instead, we attribute the drop in recombination to the relaxation of the plasmid substrate by topoisomerase I which results after inhibition of gyrase supercoiling activity (12) and the requirement for negative supercoiling for resolvase function (4).

res Site Selection in Vivo—A plasmid such as pA4 with four directly repeated res sites can undergo three successive rounds of recombination. As shown in Fig. 3, there are two possible products in each round. If recombination occurs at adjacent sites in the first round, the product is a singly linked catenane, designated pA1-A1, in which a ring with three res sites is linked to a ring with a single site. Exchange via opposite sites would instead yield pA2-A2, a singly linked catenane with two sites in each ring. The possible second round products consist of a ring with two res sites linked to two rings each with a single site (pA-A2-A) and a ring with a single res site linked to another such ring and a ring with 2 sites (pA-A-A2). The two possible products of the third and final round, pisoA and pna, are, respectively, branched and straight chain catenanes of four rings, each of which contains a single res site.

The distribution of the products formed in each round of recombination provides key information about the mechanism of res site selection. For example, tracking models in which recombination bound to one res site slides along the DNA until a second site is captured were originally considered to explain the direct site specificity of resolvase (2, 4). With a 4-site substrate a tracking mechanism would generate only the products on the left-hand side of Fig. 3 (pA-A2-A, pA-A-A2, and pisoA). The discovery of substantial amounts of the other products in vitro in the second and third rounds provided the first evidence against tracking models (4). For reasons which are still not understood, resolvase demonstrated a marked preference for adjacent sites in the first round, such that the product pA1-A1 was 20 times more abundant than pA2-A2 (4).

We constructed a derivative of pA4 that lacked two of the four EcoRI sites to simplify product analysis. The resolvase recombination products generated in vitro from this substrate were nicked by DNase I and displayed by high resolution gel electrophoresis. The result is the characteristic irregularly spaced ladder of bands (Fig. 4, lane 1) whose topological structure (indicated at the left of the figure) was previously determined by a combination of high resolution gel electrophoresis and electron microscopy.
Fig. 3. Recombination products formed from a substrate containing four res sites. The resolvase substrate pA4, which contains four res sites (bold arrows; not to scale) in direct orientation and two EcoRI sites (RI), is shown. For simplicity, the positions of the EcoRI sites in the products are omitted. Thin arrows denote possible pathways for formation of the products. Superscripts indicate the number of pA copies in a substrate or catenane; a center dot indicates catenation, pA a straight chain catenane, and piso a branched chain catenane. A tracking mechanism predicts that only the pathway on the left would be used.

The number of pA copies in a substrate or catenane; a center dot indicates catenation, pA a straight chain catenane, and piso a branched chain catenane. A tracking mechanism predicts that only the pathway on the left would be used.

We then performed the analogous experiment in vivo to study the physiological mechanism of site selection. Cells harboring pA4 and pJBREScl were induced for 2 min by a shift to 42°C after which the cells were returned to 28°C for 28 min and the DNA analyzed. Addition of norfloxacin at 1.5 min proved optimal for blocking decatenation while still allowing multiple rounds of recombination. Under these conditions, the products of the second and third rounds (Fig. 4, lane 2) had the same electrophoretic mobilities as the products formed in vitro (Fig. 4, lane 1).

The possible first round products, pA2-A2 and pA3-A, were not resolved. To try to determine their abundance and to confirm the structure of the subsequent products we used two-dimensional gel electrophoresis. A gel lane containing a ladder of nicked pA4 recombination products duplicate to that shown in Fig. 4, lane 2, was excised and the DNA was digested in situ with EcoRI. Electrophoresis in the second dimension yielded a characteristic pattern of spots consistent with the two-dimensional gel. The first dimension resolved pA3-A only partially from pA2-A2, but after restriction in situ with EcoRI the products in the second dimension help distinguish the two species. The IA restriction product consists of two overlapping spots, one derived from pA2-A2, and the other derived from the faster moving pA3-A2 (4). We calculated the amount of pA3-A2 by subtracting the contribution of pA2-A2 to the total amount of IA. The ratio of IA to nA provides a control for the accuracy of the method, and this ratio was within 15% of the expected 2:1 value. In addition, the in vitro first round product distributions calculated by this method agree to within 1% of the data previously determined by gel electrophoresis and electron microscopy (4).

**Table I**

| Recombination round | Product | Observed | Calculated |
|---------------------|---------|----------|------------|
|                     | In vitro | In vivo  | [site selection] |
| 1                   | pA4-A   | 94       | 71         | 67 |
|                     | pA2-A   | 6        | 29         | 33 |
| 2                   | pA-A2-A | 66       | 60         | 45 |
|                     | pA-A2-A2| 34       | 40         | 55 |
| 3                   | pisoA   | 27       | 29         | 23 |
|                     | pnA     | 73       | 71         | 77 |

The frequency of resolvase products is given for the three successive rounds of recombination of pA4 for the experiment shown in Fig. 4. Also shown are the product distributions expected if the res sites synapse randomly in each round without regard to distance between sites and site order. The ratio of pA4-A to pA2-A2 generated in the first round of recombination was estimated from the results of the two-dimensional gel. The first dimension resolved pA4-A only partially from pA2-A2, but after restriction in situ with EcoRI the products in the second dimension help distinguish the two species. The IA restriction product consists of two overlapping spots, one derived from pA2-A2, and the other derived from the faster moving pA3-A2 (4). We calculated the amount of pA3-A2 by subtracting the contribution of pA2-A2 to the total amount of IA. The ratio of IA to nA provides a control for the accuracy of the method, and this ratio was within 15% of the expected 2:1 value. In addition, the in vitro first round product distributions calculated by this method agree to within 1% of the data previously determined by gel electrophoresis and electron microscopy (4).
legend to Table I. The distributions of the second and third round products were determined by densitometric scans of one-dimensional gels. These results are summarized in Table I.

The primary conclusion is that the distribution of the products of recombination in the second and third rounds is essentially the same in vivo and in vitro. This shows that tracking by resolvase does not occur in vivo. The products of the first round of recombination in vivo are also consistent with this conclusion. These products appear to show little or no bias toward adjacent site selection, but the incomplete resolution of the two possible products, even after two-dimensional gel electrophoresis, makes the precise quantification uncertain.

**Resolvase-mediated Inversion and Intermolecular Recombination in Vivo**—With a standard supercoiled substrate, purified resolvase acts efficiently only on res sites oriented as direct repeats contained within the same DNA molecule (2, 3). It has been reported that high resolvase expression in vivo leads to recombination between inverted res sites (inversion), but that recombination between sites on different molecules (intermolecular recombination or fusion) remains rare under these conditions (1, 2). In order to assess inversion and fusion under our in vivo conditions, the inverted-site substrate pRR55 (Fig. 1C) was used in conjunction with the pBR322 expression plasmid.

Inversion was measured by cleavage of the DNA with EcoRI, followed by gel electrophoresis and filter hybridization. As evidenced by the appearance of the 3200-bp product fragment, inversion of pRR55 was clearly detectable only after 30 min of induction of resolvase (Fig. 5A, lane 2). Thereafter, we observed a slow but linear increase in recombination (lanes 3 and 4), at the rate of 3–5% of the substrate recombined per h. We can make only a rough comparison of this rate to the rate of resolution, because after the short induction times used in the resolution experiments, inversion was undetectable. After a 2.5-min induction, 35% of pA2ΔRI was resolved (Fig. 2A). We estimate by linear extrapolation that 0.2% of pRR55 was inverted after the same induction period. Therefore, in vivo, resolution was about 175 times faster than inversion. Inversion, like resolution, was inhibited by the addition of norfloxacin (data not shown); all the experiments shown here were done in the absence of the inhibitor.

To assay for intermolecular recombination, a portion of each DNA sample analyzed for inversion in Fig. 5A was displayed by gel electrophoresis without restriction with EcoRI. The amount of the fusion product, dimeric pRR55, increased from 1 to 20% by 30 min, and over the next 90 min the amount remained constant (Fig. 5B, lanes 1–4). Because the dimer can be resolved by recombination back to monomer pRR55, we suggest that a steady-state of dimer formation and breakdown was achieved. Because of differences in the sequences flanking the two res sites in pRR55, two distinct intermolecular products can be formed (Fig. 6). In pathway a, recombination between sites in different positions in pRR55 generates a nonsymmetrical dimer. In pathways b and c, a perfect head-to-tail dimer is formed by recombination between res sites in equivalent contexts. The proportion of the two alternatives can be determined by cleavage at the single BamHI site in pRR55. Restriction of the symmetric dimer with BamHI releases two fragments the size of pRR55, whereas cleavage of the asymmetric dimer produces two fragments, one larger and one smaller than pRR55 (Fig. 6). After cleavage with BamHI, nearly all products (>99%) were pRR55 sized fragments (Fig. 5C). Remarkably, all the dimer detected in vivo was formed via equivalent-site exchange.

To measure the amount of resolvase that was produced during this experiment, the cellular proteins of the cultures were displayed by SDS-polyacrylamide gel electrophoresis (Fig. 7). By 30 min of induction (lane 2), resolvase was easily detected; by 120 min (lane 4), it constituted 4% of the total cellular protein. This corresponds to a resolvase to substrate ratio of about 6000:1, assuming that the weight of protein in a single E. coli cell is 1.56 × 10−13 g (22) and that the copy number of the substrate is 30/cell.

**DISCUSSION**

We have undertaken a topological analysis of the resolvase reaction in vivo to determine its physiologically important properties. Topological properties are ideal for the study of recombination in vivo, as they are unchanged during the isolation and processing of the DNA. We devised a system to provide controlled, synchronous resolvase recombination in E. coli. The products of recombination formed in this system in vivo were basically the same as those generated under...
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**FIG. 6. Products of intermolecular recombination.** The substrate, pRR55, has two res sites (arrows), one of which is close to a BamHI site (B). An intermolecular reaction mediated by resolvase can take one of three pathways. In pathway a, recombination occurs between res sites in nonequivalent positions, generating a nonsymmetrical dimer product. In pathway b, recombination between res sites in equivalent positions produces a perfectly symmetrical head-to-tail dimer. In pathway c, the substrate replicates but the produced rings are intertwined as a catenane; equivalent res sites are preferentially recombines by resolvase and generate symmetric dimers. Pathway a can be distinguished from pathways b and c by cleaving the DNA with BamHI, which releases two different sized fragments (6950 and 4950 bp) from the asymmetric dimer and two equal sized fragments (6950 bp) from the symmetric dimer.

**FIG. 7. Profile of resolvase induction.** Strain MG1655.10 harboring pJBREScI and pRR55 was induced and held at 42 °C to maximize resolvase expression. At the times indicated, total protein was isolated from cells and displayed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Lane 5 contains 1 μg of purified resolvase.

standard conditions in vitro, thereby ratifying the current view of synaptic structure and strand exchange derived from in vitro experiments using purified components (6–10, 23, 24). Moreover, because the activity of resolvase is highly sensitive to the structure of the substrate DNA, it appears that DNA supercoils in vivo and in vitro are functionally equivalent, despite the large differences in ligands.

The models for resolvase site specificity that best fit the in vitro data focus on the interwound (plectonomic) shape of supercoiled DNA (6, 7, 9, 23). Thus, correct site juxtaposition in the synaptic complex is favored by plectonemic supercoiling only when the sites are directly repeated (24). A nonproductive complex of inversely repeated sites is favored by the same superhelical driving force (7). Supercoiling has no direct effect on synopsis of sites in separate DNA molecules. A similar model has been proposed to explain site specificity in the phage Mu strand-transfer reaction and in the Gin DNA inversion reaction, although in these cases, the sites have an inverted orientation (25, 26). A synaptic complex in which the DNA sites are aligned properly by a specific supercoil geometry has been termed a plectosome (25).

Early genetic assays with transposons indicated that recombination is efficient only between directly repeated res sites (27). Later experiments utilizing cloned genes and multicopy plasmids suggested that in conditions of unusually high resolvase expression, inversion was also possible (1, 2, 28). The limitations of both approaches were that long time periods, even days, were needed for the assays and that the exact levels of resolvase and the immediate products of the reaction were unknown. Using our controlled recombination system, we were able to measure in this report the amount of resolution, inversion, and fusion using a variety of substrates, as well as to determine the structure of the immediate resolution products.

In vivo resolution is rapid and efficient. The rate of about four plasmids/min/cell resolved at 28 °C is 4 times greater than the rate we measured previously for Int recombination in vivo (12). The products of resolvase action, singly linked catenanes and smaller amounts of more complex forms resulting from processive recombination, are the same in vivo and in vitro to the limits of resolution of gel electrophoresis. This implies that both in vivo and in vitro, the synaptic complex has three (−) plectonemic supercoils and that the strand exchange mechanism introduces one positive supercoil and converts two substrate supercoils into a catenane interlink; any deviation from these specifications would lead to different products (21).

If, in vivo, the DNA linking deficit were expressed entirely in the form of solenoidal supercoils, the exclusion of multiply linked catenanes as products of recombination could be a trivial result of DNA structure, rather than the outcome of a controlled site-alignment mechanism. However, our previous experiments with λInt recombination in vivo demonstrated that about 40% of the linking deficit in plasmid DNA is in the form of plectonemic supercoils, and that complex catenanes with variable numbers of links were generated by Int (12). Thus, in vivo, resolvase relies on this fraction of plectonemic supercoils to supply specificity in the reaction but does not allow free supercoils to be converted to catenane interlinks.

Under standard conditions in vitro, the resolvase reaction is proportional to the superhelical density of the substrate (4). However, under modified reaction conditions, linear or relaxed substrates will recombine, albeit with loss of certain aspects of site specificity (6, 9, 10). In vivo, inhibition of DNA gyrase by norfloxacin reduced the extent of resolvase recombination. The earlier the inhibitor was added, the greater was the diminution of recombination. This reduction of the amount of recombination coincides with the decline in effective DNA supercoiling associated with inhibition of gyrase supercoiling activity by norfloxacin. The majority of the substrate was recombined in the first 5 min (Fig. 2), and about one third of plasmid supercoils were relaxed within 5 min of norfloxacin addition (12). We conclude that, in vivo, plecto-
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For the nonequivalent res sites to synapse properly, one must be looped back and this should be energetically unfavorable.

We conclude that resolvase can be "tricked" into performing the forbidden reactions of inversion and fusion by interlinking of the DNA substrate. The rate of these ancillary reactions, however, remains small compared to resolution. Thus, to preserve its biological specificity, resolvase relies heavily on topoisomerases to minimize the amount of the intracellular DNA present in a tangled form.

Our system provides a method for the controlled intracellular generation of singly linked catenanes via resolvase action. Because topologically linked DNA molecules are unavoidable intermediates of DNA replication and recombination, decatenation is an essential function in both prokaryotic and eukaryotic organisms (reviewed in Ref. 5). Our data show that DNA gyrase in vivo efficiently decatenates both multiply (12) and singly linked catenanes (Fig. 2B). The latter event allows the products of resolvase to replicate semiconservatively and thereby to complete the process of transposition.

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