Determinants of correct *res* site alignment in site-specific recombination by Tn3 resolvase

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In site-specific recombination reactions catalyzed by Tn3 resolvase, the right and left arms of the *res* site are always religated to the correct partner. This poses the problem of how resolvase aligns the two sites correctly for the cleavage/religation reaction. We show that the "accessory" binding subsites II and III of *res* are important for correct alignment of the adjoining crossover subsite (subsite I). Deletion of subsites II and III from one of the two *res* sites removes a barrier to recombination between incorrectly aligned crossover subsites. Correct alignment does not require any DNA sequence asymmetry in the crossover subsite, DNA supercoiling, or covalent linkage of the two *res* sites. Our results suggest that correct subsite I alignment is determined by local, resolvase-mediated interactions of subsites II and III of both partners, consistent with a current model of the synapse. Surprisingly, the topological selectivity for intramolecular resolution in a supercoiled substrate does not require subsites II and III in both recombination partners.

[*Key Words:* Tn3 *res*/resolvase; site-specific recombination; site alignment; resolution selectivity]

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Site-specific recombination enzymes mediate precise rearrangements of DNA sequences (for review, see Sadowski 1986). They have many different functions, including integration and excision of phage into and out of the bacterial chromosome (Landy 1989), gene switching by inversion of DNA segments (Glasgow et al. 1989), plasmid monomerization and multimerization—processes important for the stable maintenance of plasmids at the correct copy number (Austin et al. 1981; Summers and Sherratt 1984; Fitcher 1986)—and resolution of the intermediates of replicative transposition to give the transposition end products (Reed 1981). In vertebrates, programmed DNA rearrangements reminiscent of site-specific recombination are important in the generation of antibody diversity (e.g., see Lewis and Gellert 1989).

The well-characterized yeast and prokaryote site-specific recombinases fall into two families: the phage λ integrase family (Argos et al. 1986) and the resolvase/DNA invertase family (Hatfull and Grindley 1988; Sherratt 1989). These two families of recombinases are not structurally related, but they share some features that distinguish them from general recombination systems, which act on extended segments of homologous DNA. Site-specific recombinases act at clearly defined DNA recognition sites. Their catalytic mechanism involves synapsis of the two sites, staggered cleavage of the DNA phosphodiester backbone, and religation of the DNA ends to give two recombinant DNA junctions. Resolvases and DNA invertases are thought to cut all four strands of the DNA before any ends are religated, whereas enzymes of

the λ integrase group initially cut, exchange, and religate one pair of strands to generate a Holliday junction intermediate. During strand exchange the phosphorylated DNA ends are transiently joined to a seryl residue (resolvases/DNA invertases) or tyrosyl residue (integrases) at the catalytic site of the enzyme (for review, see Hatfull and Grindley 1988; Landy 1989).

Many recombinases catalyze a complete recombination reaction only when the recognition sites are aligned correctly and in a specific topological linkage. Tn3 resolvase selectively catalyzes an intramolecular resolution reaction (see Fig. 2, below), which is required for the second step of Tn3 transposition (Reed 1981). The Tn3 *res* recombination site consists of three discrete binding sites for resolvase; the specialized subsite for strand exchange (subsite I, the crossover subsite) is adjoined by two accessory binding sites (subsites II and III) that are essential for efficient recombination in vivo and in vitro (see Fig. 5, below; Grindley et al. 1982; Kitts et al. 1983; Wells and Grindley 1984). Accessory binding sites for the recombinase or additional protein factors are a characteristic feature of other recombination systems with a stringent topological selectivity (Sadowski 1986). The DNA invertases are an exceptional case; they require two structurally simple crossover sites and a separate *cis*-acting recombination enhancer element that binds the *Escherichia coli* FIS protein (Johnson and Simon 1985; Kahmann et al. 1985; Koch and Kahmann 1986). A key objective of current research is to understand how the accessory binding sites and proteins modulate the

recombination reaction at the crossover sites (Hatfull and Grindley 1988; Klippel et al. 1988; Landy 1989).

In the *res*/resolvase system and in other microbial recombination systems (but not in the immunoglobulin system), religation of left ends to right ends is required (Sadowski 1986; Lewis et al. 1988). Distinguishing these ends may not be trivial, as the crossover sites have imperfect dyad symmetry and are believed to bind a symmetrical dimer of recombinase. Thus, one might expect recombinase-mediated synapsis of the crossover sites to be possible in either the "correct" (parallel) or "incorrect" (antiparallel) alignment, potentially giving both correct and incorrect recombinant junctions (see Fig. 1).

How might resolvase ensure correct crossover site alignment during recombination? Recombinases of the integrase family test the alignment of the homologous sites using direct DNA–DNA interactions. To do this, they rely on an asymmetric 6- to 8-bp "overlap" sequence at the center of the crossover site (Hoess et al. 1986). The alignment is not tested during synapsis of the sites but during the strand exchange reaction, in which a Holliday junction migrates through the asymmetric core sequence (de Massy et al. 1989; Nash and Robertson 1989; Nunes-Duby et al. 1989). The Tn3 *res* crossover site, however, is cut with only a 2-bp stagger at the center of a perfectly symmetrical hexanucleotide, TTATAA (Reed and Grindley 1981). The right and left arms of the crossover site differ at 6 of 11 positions (see Fig. 5, below). We wished to test whether resolvase recognizes these differences to align the sites correctly. Alternatively, resolvase could recognize the right and left arms of the crossover site indirectly, through the asymmetric location of subsites II and III; specific protein–protein contacts between different resolvase subunits could be used to align the sites. A third possibility is that the correct alignment is defined indirectly because

Figure 1. Alignment of crossover sites in site-specific recombination. The cartoons illustrate the synapsis of two crossover sites by protein–protein interactions between the recombinase subunits, with the sequences aligned in a parallel or antiparallel sense, thus giving rise to correct (*left–right*) or incorrect (*left–left*) recombinant junctions. Note that we use correct and incorrect rather than parallel/antiparallel or left–right/left–left; these latter terms are potentially misleading, as they have been used by others to describe different phenomena.

Figure 2. Resolution selectivity in site-specific recombination. (A) Resolution and inversion in a circular substrate containing two recombination sites in direct repeat. If the sites recombine in the parallel alignment, giving correct (*left–right*) junctions, the substrate is resolved into two circular products. If the sites recombine in the antiparallel alignment, giving incorrect (*left–left*) junctions, a segment of the substrate is inverted. For a substrate with sites in inverted repeat, these outcomes are reversed. (B) Topology of the resolution reaction between complete *res* sites in direct repeat (Wasserman and Cozzarelli 1985; Wasserman et al. 1985). Three negative interdomainal supercoils are trapped between the synapsed crossover sites; the recombination product is a simple catenane of two circles with a specific stereochemistry.

the mechanism of synapsis traps exactly three interdomainal supercoils (Fig. 2B).

Resolvase acts efficiently only on directly repeated *res* sites in *cis* and not on inverted sites or sites in separate molecules. Recombination between two directly repeated *res* sites always "resolves" the circular substrate into two circular products (rather than inverting a segment), because the crossover subsites are cut and rejoined in the "parallel" alignment (Fig. 2A; Reed 1981). We refer to this behavior as the resolution selectivity of the system. The closely related *Hin*/*Gin*/*Cin* DNA invertases exhibit an analogous inversion selectivity. Contrasting models to account for resolution selectivity emphasize processive "tracking" or "scanning" of resolvase along the DNA (Kitts et al. 1983; Krasnow and Cozzarelli 1983), "slithering" of plectonemically supercoiled DNA (Benjamin and Cozzarelli 1986), and topological restrictions on the formation of an interwrapped synapse (Boocock et al. 1986).

Precisely three interdomainal supercoils are trapped between the crossover sites in the Tn3 resolution reaction (Fig. 2B; Wasserman and Cozzarelli 1985; Wasserman et al. 1985). A current model suggests that the topological selectivity is associated with a specific plectonemically interwrapped synaptic complex (Boocock et

al. 1986; Stark et al. 1989). This model predicts an essential role for the accessory subsites II and III of both partners in synapsis, alignment and resolution selectivity (see Fig. 8A, below). To investigate the importance of different regions of *res* for these processes, we studied the effect of deleting subsites II and III and the effect of replacing the wild-type crossover subsite with a perfectly symmetrical sequence. Our data indicate that subsites II and III of both partners are needed for correct crossover site alignment but are not essential for resolution selectivity.

Results

Resolvase recognizes the isolated crossover subsite from Tn3 res

To compare the properties of the complete Tn3 *res* site and the isolated *res* crossover subsite (subsite I), we deleted subsites II and III using exonuclease III. The sequence of the isolated subsite I and its flanking DNA is shown in Figure 5 (below), aligned with the sequence of the complete *res* region. Binding of resolvase to the complete *res* site and to the isolated subsite I was compared using gel retardation assays (Fig. 3; Fried and Crothers 1981). Under our binding conditions, six discrete resolvase–DNA complexes are formed from the complete *res* site (Fig. 3, lanes 5–8; presumably two complexes for each subsite; A.L. Bednarz and M.R. Bocock, unpubl.). Two complexes are obtained from the isolated subsite I (Fig. 3, lanes 1–4). Full occupation of the isolated subsite I occurs at resolvase concentrations comparable to those required for full occupation of the complete *res* site (Fig.

Figure 3. Binding of resolvase to subsite I and to the complete *res* site. Gel retardation assays used a 203-bp *Eco*RI fragment carrying subsite I of *res* (lanes 1–4) and a 168-bp *Bam*HI–*Eco*RI fragment carrying the complete *res* site (lanes 5–8). Fragments labeled with ³²P were incubated with resolvase as described in Materials and methods; the protein–DNA complexes were separated on a 6% nondenaturing polyacrylamide gel and are designated 1–6 (vertically). (u) The unbound DNA fragment. (Lanes 1–5) 112 nM resolvase; (lanes 2–6) 56 nM resolvase; (lanes 3 and 7) 28 nM resolvase; (lanes 4 and 8) no resolvase.

Figure 4. Arrangement of *res* sites in the substrates used for intramolecular recombination. Subsites I, II, and III of *res* are represented as rectangular boxes (for DNA sequences, see Fig. 5). The open and solid arrowheads represent the right and left arms of subsite I, respectively. The pBR322 vector sequences are not shown to scale. (H) *Hind*III site. The constructions are detailed in Materials and methods.

3). Subsites II and III are therefore not necessary for avid binding of resolvase at subsite I.

Recombination between subsite I and the complete res site

To test the recombination properties of an isolated subsite I, we constructed a family of pBR322-derived substrates containing the complete *res* site and the isolated subsite I in different combinations and configurations (Fig. 4). Recombination was assayed *in vitro* using negatively supercoiled plasmid substrates, purified Tn3 resolvase, and reaction conditions that favor the recombination of poor substrates (buffer A or B; Materials and methods). A substrate containing two intact *res* sites in direct repeat, pMA21, resolved to completion within 1 hr. Under the same conditions, we observed slow recombination between the isolated subsite I and a complete *res* site in direct repeat, using the substrate pAL211 (<20% product in 19 hr; Fig. 6A, below). We estimate that the rate of recombination is <1% of that for the reaction between two complete *res* sites. Recombination between two copies of the isolated subsite I, either in direct repeat (pAL225) or in inverted repeat (pAL221), was not detectable in supercoiled substrates under any reaction conditions tested. Thus, subsites II and III greatly stimulate *in vitro* recombination between the crossover sites but are not essential in both recombination partners.

We also attempted to recombine a substrate, pAL215, that contains an isolated subsite I and a complete *res* site in inverted repeat (Fig. 4). pAL215 recombined *in vitro* with a similar efficiency to pAL211 (subsite I and complete *res* in direct repeat). Restriction analysis after these reactions revealed only resolution products (Fig. 6B, below) and no detectable level of inversion products (<1% of total product). Thus, the selectivity for resolution over inversion is maintained in recombination reac-

tions between subsite I and the complete *res*, regardless of the relative orientation of the two sites in the substrate.

In the resolution reaction of pAL215, the right arm of the isolated subsite I was religated to the right arm of the complete *res* site. The circular plasmid containing this recombinant junction was isolated from the reaction products. DNA sequencing of the junction revealed a novel recombinant *res* site with a perfectly symmetrical subsite I, as expected if the two crossover sites are cleaved at the usual positions and the two right arms are religated to each other. This site was called sym-*res* (Fig. 5). (The junction between the two left arms was not sequenced because it could not be maintained stably in vivo; the predicted junction sequence is a 300-bp palindrome, longer than can be stably maintained in a *recA* strain; see Boissy and Astell 1985). The structure of the resolution products of pAL215 is thus indicative of incorrect alignment of the crossover sites during strand exchange (Fig. 1). Note that our assay for site alignment requires the formation and analysis of recombinant products; incorrectly aligned sites that fail to recombine will not be detected.

To check that the vector DNA sequences that replace subsites II and III do not affect the alignment or reactivity of the isolated subsite I, we compared four substrates with the recombination site cassettes inserted in different configurations: pAL211, pAL261, pAL265, and pAL215 (Fig. 4). In these substrates the left arm of subsite I adjoins the natural upstream sequence from Tn3, while the right arm adjoins one of two different vector sequences (Fig. 5). The four substrates all recombined with a similar low efficiency, and all four gave resolution products and no detectable inversion products. Correct junctions were formed when the two recombination sites were in direct repeat (pAL211 and pAL265); incorrect junctions were formed when the sites were in inverted repeat (pAL261 and pAL215). We conclude that the alignment of the crossover sites in the cleavage/religation reaction is determined solely by the relative orientation of the subsite I and the complete *res* within the

substrate. The differences between the left and right arms of subsite I and their different flanking sequences did not determine which arm of subsite I was religated to the left arm of the complete *res*. Three different subsite I flanking sequences substituted equally inefficiently for the deleted subsites II and III in the resolution reactions of pAL211, pAL265, and pAL261 (Fig. 5). Because these sequences do not resemble each other, or the deleted *res* sequences, we conclude that the sequence of this flanking DNA is not important for the resolution reaction. Using a 203-bp DNA fragment containing the isolated subsite I cassette in the gel-binding assay, we were unable to detect any additional complexes attributable to specific binding of resolvase to the flanking sequences (Fig. 3).

To test whether truncation of the accessory subsites is needed to allow recombination with the incorrect alignment of the crossover subsites, we compared the recombination properties of pMA2631 (two complete *res* sites in inverted repeat) and pAL261 (one site truncated). Using supercoiled pMA2631, we failed to detect any products of a resolution reaction between the *res* sites under conditions that gave up to 20% resolution of pAL261 (Fig. 6A). Thus, the second set of accessory sites in pMA2631 appears to block the resolution reaction between the crossover subsites. This contrasts with the result obtained for *res* sites in direct repeat; the second set of accessory sites greatly stimulated the resolution reaction (e.g., in pMA21, compared with pAL211). Inversion reactions of the supercoiled pMA2631 substrate were also undetectable, consistent with previous observations that inverted sites recombine only in nonsupercoiled substrates (Boocock et al. 1987; J.L. Brown et al., unpubl.) or in knotted supercoiled substrates (Droge and Cozzarelli 1989).

Our results for in vitro recombination of supercoiled substrates are summarized in Table 1. Recombination between two isolated crossover sites was not detectable. When one of the crossover sites was adjoined by subsites II and III, resolution proceeded slowly, and inversion was not detectable, regardless of the relative orientation of

Figure 5. Sequence of the complete Tn3 *res* site, the isolated subsite I and flanking sequences, and the sym-*res* site. Bold lines indicate the partial dyad symmetry of each subsite. The flanking sequences shown to the right of subsite I are the sequences that would need to substitute for subsites II and III in a resolution reaction with the complete *res* site, in the substrate indicated. The sequences are presented in the same orientation as the complete *res* site in that substrate; the subsite I sequence therefore appears in the reverse orientation for pAL261 and pAL215. The cartons at right clarify the relationships between the flanking sequences, designated a, b, and c, on both sides of subsite I. Subsites I, II, and III of *res* are shown as in Fig. 4. The wild-type Tn3 *res* and pBR322 sequences are taken from Heffron and McCarthy (1979) and Sutcliffe (1979).

Figure 6. In vitro recombination between subsite I and a complete *res* in supercoiled substrates. Substrates were incubated with resolvase in buffer A (gel A) or buffer B (gels B, C, and D) containing 25 mM NaCl, at 37°C for 19 hr (except gel C; 16 hr). Reaction products were analyzed on 1.2% (A–C) or 0.7% (D) agarose gels. (A) Restriction analysis of reaction products from pMA21, pMA2631, pAL211, and pAL261 (see Fig. 4). Reactions contained 0, 200, 400, and 800 nM resolvase (lanes a–d). All samples were cut with *Pst*I and *Hind*III. (S) Restriction fragments corresponding to unrecombined substrate; (P) restriction fragments with sizes corresponding to resolution products; (m) size markers 3234, 2817, 2466, and 1129 bp. The small amounts of inversion product obtained with pMA2631 (lanes b–d with pMA2631; restriction fragments of a similar size would be diagnostic for inversion of pMA21, pAL211, pAL261, pAL215, and pAL265) are due to traces of nicked substrate in the reaction. (B) Characterization of resolution products from pAL215 and pMA21. Reactions contained 0, 50, 100, and 200 nM resolvase (0, +, ++, and +++). Products were cut with either *Pst*I (P) and/or *Hind*III (H) to reveal supercoiled circular resolution products (C) and the corresponding linear DNA fragments (L). The free supercoiled circles were not detectable in the uncut products of pAL215 recombination; this was confirmed by Southern hybridization of similar gels. (C) Characterization of resolution products from pAL265 and pMA21. Reactions contained 400 nM (pAL265) or 200 nM (pMA21) resolvase (+), or no resolvase (0). Products were cut with *Pst*I (P) and/or *Hind*III (H), as indicated. (S) Restriction fragments corresponding to unrecombined substrate; (C) supercoiled circular resolution products; (L) linear restriction fragments corresponding to circular resolution products. (D) Topological analysis of resolution products of pAL211. Reactions contained 0 or 400 nM resolvase, as shown; the products were nicked with 5, 10, or 100 ng/ml DNase I. Marker tracks contain nicked knots (K) and simple catenanes (C) made from pMA21 using T4 topoisomerase and resolvase, respectively. (C) Supercoiled pAL211; (O) unknotted nicked circles; (P) nicked resolution product; (H) half-nicked resolution products.

the two sites. In substrates containing two complete *res* sites in direct repeat, resolution proceeded efficiently; when the sites were in inverted repeat, neither resolution nor inversion was detectable.

The isolated Tn3 *res* crossover site did not recombine with a complete *res* site at a significant rate when complemented in vivo with a resolvase-overproducing plasmid (A.L. Bednarz and D.G. Blake, unpubl.) in agreement with previous results for $\gamma\delta$ *res* (Wells and Grindley 1984).

Topology of recombination between subsite I and complete *res* site

To investigate the effect of deleting subsites II and III from one partner on the topology of recombination, we analyzed the topology of the resolution products. Restriction with the appropriate enzymes revealed that both circular products are highly supercoiled (Fig. 6C, lanes 7 and 8). The circles were also exclusively catenated, as they were only released after restriction

Table 1. Summary of intramolecular recombination reactions

Substrate	Sites	Orientation	Resolution efficiency	Inversion efficiency	Alignment of sites (resolution)
pMA21	<i>res</i> + <i>res</i>	direct	100	<0.1	correct
MA2631	<i>res</i> + <i>res</i>	inverted	<0.1	<0.1	—
pAL211, pAL265	<i>res</i> + subsite I	direct	1	<0.1	correct
pAL215, pAL261	<i>res</i> + subsite I	inverted	1	<0.1	incorrect
pAL225	subsite I + subsite I	direct	<0.1	<0.1	—
pAL221	subsite I + subsite I	inverted	<0.1	<0.1	—

cleavage (Fig. 6B, lanes 8, 9, and 11); Electrophoresis of uncut reaction products revealed a species with a mobility slightly greater than the supercoiled substrate, as expected for a supercoiled catenane (not shown). To analyze the complexity of the catenanes, we nicked the reaction products with DNase I and compared the mobilities of the nicked species with a reference ladder of knots made with T4 topoisomerase II (Spengler et al. 1985). The major nicked product had the mobility of a simple two-noded catenane; significant amounts of unlinked circles or complex catenanes were not detectable (Fig. 6D). Thus, the resolution products were simple catenanes of supercoiled circles, as in the reaction between two complete *res* sites. This indicates that recombination between subsite I and a complete *res* is a concerted reaction in which all four DNA strands are cut and religated without releasing most of the supercoiling. Also, the topology of productive synapsis and strand exchange is strictly controlled, as it is in the standard reaction (Wasserman and Cozzarelli 1985).

Recombination properties of the *sym-res* site

The results described above indicate that resolvase fails to distinguish the left and right arms of subsite I when subsites II and III have been deleted and therefore fails to align the crossover sites correctly. To test whether the accessory subsites II and III are sufficient to direct correct alignment of the crossover sites, we investigated the recombination reactions of *sym-res*, a *res* site with a perfectly symmetrical crossover subsite. Plasmid pAL115 was obtained by in vitro resolution of pAL215; it carries the *sym-res* site, in which the wild-type subsite I is replaced by a perfectly symmetrical 60-bp sequence consisting of two right arms of subsite I and adjacent *res* sequences (Fig. 5). A recombination substrate with two *sym-res* sites in direct repeat was made by isolating a dimer of pAL115. Supercoiled pAL115 dimer recombined in vitro with a similar efficiency to pMA21 (two wild-type *res* sites). The recombination products from the pAL115 dimer were catenated circles, and restriction analysis revealed no detectable amount of any inversion products indicative of recombination between incorrectly aligned crossover sites (not shown). The dimer plasmid was also resolved efficiently in vivo, when complemented with a *tnpR*⁺ plasmid in a *recA* background. These results clearly indicate that the asymmetry of the wild-type subsite I sequence is not necessary for correct alignment and resolution selectivity or for efficient recombination.

We were concerned that the 60-bp palindrome in *sym-res* might extrude into a cruciform at in vitro levels of superhelicity and inhibit recombination, as has been reported for symmetrized recombination sites in the *lox/Cre* system (Hoess et al. 1986). We obtained no evidence of such inhibition with the pAL115 dimer.

To test whether subsites II and III are able to direct a particular alignment of two *sym-res* sites that are topologically unlinked, we investigated the intermolecular recombination reaction between two linear DNA sub-

strates carrying a single copy of *sym-res*. The substrates used were *Pst*I-linearized pAL145 and *Pvu*II-linearized pAL115; two control substrates carrying a single wild-type *res* site were made by linearizing pMA2856 with *Hind*III and *Sst*I. For both pairs of substrates, the major recombination products were linear molecules of the sizes predicted for correct alignment of the crossover sites during strand exchange (Fig. 7). For both pairs of substrates very small amounts of products (<10% of total recombinant) were detected with the sizes predicted for incorrect alignment of the crossover sites. Thus, the correct alignment of the crossover sites is strongly favored in intermolecular recombination reactions of *sym-res*, as was observed previously with the wild-type *res* site (J.L. Brown et al., unpubl.). This result demonstrates conclusively that subsites II and III can define the functional left and right arms of an adjoining crossover site that lacks any internal asymmetry. The very minor products attributable to incorrect crossover site alignment have not been observed previously in reactions with complete *res* sites and have not been characterized further.

Discussion

Alignment of the crossover subsites

Our results implicate subsites II and III of *res* in the alignment of the crossover subsites. The experiments on intramolecular recombination in supercoiled substrates showed that an isolated subsite I can recombine, at a reduced rate, with a complete *res* site (subsites I, II, and III). The remaining subsites II and III were essential for detectable recombination but did not ensure correct alignment of the subsites I. In the reaction between a complete *res* site and a subsite I in inverted repeat, the incorrect alignment of crossover subsites was used exclusively. The deletion of subsites II and III from one partner thus reduces the overall efficiency of recombination and permits incorrect as well as correct alignment of the subsite I sequences in the cleavage/religation reaction.

The intermolecular recombination reactions between *sym-res* sites on linear substrates showed that two *res* sites can be aligned correctly when a region of 60 bp around subsite I has a perfectly symmetrical sequence. This result implies that the left and right arms of subsite I are recognized indirectly and identifies subsites II and III of both partners as the sequences required for this indirect recognition. Our data are consistent with the idea that the crossover site alignment is defined by resolvase-mediated interwrapping of subsites II and III of both partners during synapsis (Fig. 8A). (We cannot formally exclude the possibility that the alignment is sensed during the cleavage/religation reaction, but we cannot imagine how this would operate with symmetric crossover subsites.) Further work is needed to determine how the accessory subsites interact in the synapse and to elucidate the role of the individual subsites.

Substrate supercoiling and topological linkage were not required for correct alignment of two complete *res*

Figure 7. Intermolecular recombination between two linear substrates carrying sym-*res*. *Pst*I-linearized pAL145 (2650 bp) and *Pvu*II-linearized pAL115 (2479 bp) were incubated with resolvase in buffer C + 50 mM NaCl for 22 hr at 37°C. As a control, two linear substrates carrying the wild-type *res* site (pMA2856 linearized with either *Hind*III or *Sst*I, 2951 bp) were treated similarly with resolvase. Recombination products were separated on a 1.2% agarose gel uncut (lanes a–e and k–o) or after cleavage with *Pst*I (lanes f–j and p–t). Resolvase concentrations used were 0, 139, 278, 556, and 1112 nM in lanes a–e, f–j, k–o, and p–t. In each of these pairs of reactions, the plasmid substrate could recombine either with its partner or itself, in each of two alignments. There are therefore six possible products from each reaction (three for correct and three for incorrect alignment). Calculations of the expected sizes of substrate, as well as recombinant product, showed that DNA fragments of 5604 bp (pMA2856) and 5208 bp (pAL145 × pAL115), resistant to cleavage by *Pst*I, would be unambiguously diagnostic of incorrect alignment. DNA fragments corresponding to unrecombined substrate are marked S. Reaction products with the sizes predicted for correct alignment of the *res* sites are marked P; the 4932-bp product of the sym-*res* reaction yielded a fragment of 3389 bp, consistent with the expected structure. Similarly, the 5592-bp fragment resulting from recombination between wild-type *res* sites was cleaved by *Pst*I to give products of 2939 and 2653 bp. (Below) Outline plasmid maps: (P) *Pst*I; (Pv) *Pvu*II; (H) *Hind*III. Open arrowhead indicates wild-type *res*; solid arrowhead indicates sym-*res*. The bases of the arrowheads represent parts of *res* containing subsites II and III. The numbers indicate base pairs between restriction sites or restriction sites and the crossover site.

sites (sym-*res* or wild-type *res*). This indicates that assembly of the synapse and alignment of the sites does not need to be directed by the supercoil topology of the substrate. Supercoiling evidently stabilizes the synaptic structures (Benjamin and Cozzarelli 1988).

In its reactions with a complete *res* site, subsite I functioned as if it were perfectly symmetrical; resolvase disregarded the six sequence differences between the left and right arms of subsite I. This is consistent with the

idea that subsite I binds a symmetrical dimer of resolvase and synapses and recombines with a second subsite I using D2-symmetric protein–protein contacts (Stark et al. 1989).

Although subsite I is functionally symmetric, it did not recombine with a complete *res* site with a random alignment of the crossover sites. Only resolution products were obtained; inversion products, diagnostic for the opposite alignment of the crossover sites, were not detected—even when the sites were in inverted repeat. In these reactions, the topological alignment of the crossover sites in the synapse therefore takes precedence over the parallel or antiparallel alignment of their sequences. There is evidence that an analogous mechanism operates in the alignment of crossover sites by the DNA invertases (Kanaar et al. 1990). In the DNA invertase systems, however, the incorrect alignment of crossover site sequences does not lead to recombinant cleavage/religation products, because the sites have an asymmetric core.

In phage λ integrative recombination, accessory binding sites flanking the asymmetric *attP* crossover site determine which arm of this site initiates the strand exchange reaction with *attB* (Kitts and Nash 1988). In contrast, we have shown that the crossover subsite of Tn3 *res* is functionally symmetric and that the adjoining accessory subsites control the alignment of the crossover subsites in the cleavage/religation reaction. Recombination of incorrectly aligned *res* sites in vivo would generate a lethal palindrome and destroy the transposon. Our data imply that the correct alignment can be selected accurately during synapsis of the crossover sites. Thus, a mechanism for sensing the alignment during strand exchange is probably not needed, as it is in the integrase and DNA invertase systems.

Resolution selectivity

Surprisingly, resolution selectivity was maintained in intramolecular recombination reactions between an isolated subsite I and a complete *res* site in either direct or inverted repeat. Resolution selectivity therefore does

Figure 8. Models of the synaptic structures formed in the following reactions: (A) Resolution between two complete *res* sites in direct repeat; (B) resolution between subsite I and a complete *res* site in inverted repeat; (C) inversion mediated by DNA invertases. The crossover sites are represented as boxes with arrowheads, the accessory sites as open boxes, resolvase and invertase as shaded squares, and FIS protein as striped circles.

not require accessory binding sites in both recombination partners or correct alignment of the crossover sites. Deletion of subsites II and III from one partner did not alter the topology of the recombination products; they were supercoiled simple catenanes. Our data strongly suggest that three interdomainal supercoils are trapped, as in the reaction between two complete *res* sites. This appears to contradict the idea that the three interdomainal supercoils are trapped by interwrapping of subsites II and III of both partners (Fig. 8A). Nevertheless, the formation of an interwrapped structure between two complete *res* sites is supported by a number of independent experiments (Boocock et al. 1987; Stark et al. 1989; Benjamin and Cozzarelli 1990). We therefore propose that in the reaction between subsite I and a complete *res*, nonspecific DNA sequences from the supercoiled substrate can substitute inefficiently for subsites II and III of one *res* site. Critically, this model accounts for the selectively incorrect alignment of the crossover subsites in the reaction between subsite I and a complete *res* in inverted repeat (Fig. 8B). Direct characterization of the sequences within the synapse will be needed to test this explanation.

We are intrigued by a possible parallel between resolution selectivity in the reaction between subsite I and complete *res*, and inversion selectivity in the DNA invertase systems. Both systems control the number of interdomainal supercoils, -2 in the inversion reaction (Kanaar et al. 1989) and (by inference) -3 in the resolution reaction, but do not control the alignment of the crossover sites during synapsis. (Kanaar et al. 1990). Both reactions involve two crossover sites and two accessory sites: subsites II and III of *res*, or the two FIS-binding sites of the recombination enhancer *sis*. We speculate that in both bases, the synapse contains protein bridges between accessory binding sites and nonspecific DNA sequences (Fig. 8B,C). We suggest that these protein–DNA interactions at the accessory sites organize a local condensed structure that can stimulate recombination selectively between crossover sites in a specific topological alignment. It is possible that the proteins bound at the accessory sites do not need to make direct or specific contact with the recombinase at the crossover sites to stimulate the cleavage/religation reaction. In support of this idea, we note that the spacer between subsites I and II of *res* can be lengthened by at least two complete helical turns of DNA (Salvo and Grindley 1988). It will therefore be interesting to see whether the accessory sites and proteins can stimulate recombination in the heterologous system.

Materials and methods

Bacterial strains and plasmids

In vivo recombination was assayed in an *Escherichia coli* *recA* strain DS902 (Stark et al. 1989). Dimers of plasmids were made using the *recBC sbcA* strain JC8679 (Summers and Sherratt 1984) to multimerize the plasmid, followed by transformation of DS902 with the reisolated plasmid DNA. *res*⁺ substrate plasmids were generally derived from pBR322 (Sutcliffe et al.

1979); resolvase was supplied by a compatible pACYC184 derivative, pPAK316.

Subsite I of *res* was obtained from the complete *res* site by unidirectional exonuclease III digestion of a *res*⁺ pUC18 derivative (Henikoff 1984; Yanisch-Perron et al. 1985). A 203-bp *Eco*RI fragment from the resulting deletant, pAL3054, was inserted into the *Eco*RI site of pUC18 in tandem repeat, from whence the deletion endpoint was determined by DNA sequencing. Plasmid pMA21 (4927 bp) was obtained from pBR322 by insertion of 282-bp *Eco*RI and *Pvu*II fragments carrying Tn3 *res* into the *Eco*RI and *Pvu*II sites, respectively, with the *res* sites in direct repeat. pMA2631 is identical to pMA21 except that the *res* site at the *Pvu*II site is in the reverse orientation. Plasmids pAL211, pAL215, pAL261, and pAL265 (4848 bp) were constructed by inserting the 203-bp *Eco*RI subsite I fragment from pAL3054 into the *Eco*RI site of pBR322, in the required orientation, followed by a *Pst*I–*Ava*I fragment exchange with pBR322 derivatives carrying the complete *res* as a 282-bp *Pvu*II insert at the *Pvu*II site. Resolution of pAL215 gave a circular product, pAL115 (2479 bp), containing the sym–*res* site. pAL145 (2650 bp) was made by inserting the 145-bp *Eco*RI sym–*res* fragment from pAL115 into the *Eco*RI site of pMTL23, a pBR322-derived polylinker vector obtained from the CAMR laboratory (Porton Down, UK). Plasmid pMA2856 (2951 bp) is the resolution product of pMA2356 (Stark et al. 1989) and carries a 282-bp *res* insert between the *Sma*I and *Hinc*II sites of pUC18.

End labeling of DNA fragments

DNA restriction fragments were isolated by elution from polyacrylamide gels and end-labeled using Klenow fragment or T4 polynucleotide kinase. Labeled fragments were purified by phenol and chloroform extractions and precipitated with ethanol.

Gel-binding assays

A 168-bp *Bam*HI–*Eco*RI fragment from pMA2350 (complete *res*) and a 203-bp *Eco*RI fragment from pAL3054 (subsite I) were labeled for the binding assays. Resolvase was purified as in Stark et al. (1989), diluted in a buffer containing 1 M NaCl, 50 mM Tris-HCl (pH 8.2), and 1 mM EDTA. Binding reactions contained 10 mM Tris-glycine (pH 9.4); 0.1 mM EDTA, 10% glycerol (vol/vol), ~ 1 nM end-labeled DNA fragment, and 20 μ g/ml supercoiled pUC18 carrier DNA, in a total volume of 10 μ l, and were initiated by adding 0.4 μ l of diluted resolvase. The same volume of resolvase dilution buffer was added to reaction blanks. After mixing thoroughly, the reactions were incubated at 37°C for 10 min, transferred to ice, and loaded onto the gel almost immediately. Protein–DNA complexes were separated on 6% polyacrylamide gels containing 50 mM Tris-glycine (pH 9.4), 0.1 mM EDTA. Gels were prerun in the same buffer at 15 V/cm for 30–90 min at 4°C and run for 2–3 hr at 15 V/cm at 4°C. Gels were dried onto filter paper and autoradiographed against Kodak X-Omat S film for 1–3 days. The resolvase concentration required for a particular extent of binding to the complete *res* or to subsite I was essentially independent of the concentration of the labeled DNA fragment under these conditions.

In vitro recombination assays

Recombination reactions contained ~ 20 μ g/ml DNA substrate in one of the following recombination buffers: (A) 50 mM Tris-HCl (pH 8.2), 10 mM MgCl₂, 0.1 mM EDTA, 5 mM spermidine–

3HCl, 20% glycerol (vol/vol); [B] 50 mM Tris-HCl (pH 9.4), 10 mM MgCl₂, 0.1 mM EDTA, 20% glycerol (vol/vol); [C] 50 mM Tris-glycine (pH 9.4), 5 mM MgCl₂, 0.1 mM EDTA, 5 mM spermidine-trihydrochloride, 20% glycerol (vol/vol). Buffers A and C have been shown to support recombination of nonsupercoiled substrates (Boocock et al. 1987). Reactions were initiated by adding one-twentieth or one-fortieth of the volume of resolvase (prediluted in the same buffer as for binding reactions) to give a final NaCl concentration of 50 or 25 mM. Resolvase was purified as in Stark et al. (1989). Reactions were incubated at 37°C for up to 22 hr and stopped by heating to 70°C for 5 min. Samples were then treated with DNase I or restriction enzymes, as required. Sucrose loading buffer containing protease K was added prior to electrophoresis of samples on 1.2% and 0.7% agarose gels made up in 20 mM NaOAc, 1 mM EDTA, 40 mM Tris-AcOH (pH 8.2). Gels were run horizontally at ~3 V/cm, stained with ethidium bromide, and photographed onto Ilford HP5 film using short-wave UV illumination and a red filter.

DNase I nicking of DNA

DNase I was diluted in 10 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, and 50% glycerol (vol/vol) and stored at -20°C. Recombination reaction samples in recombination buffer A or C were treated with 5–100 ng/ml DNase I for 10 min at 0°C. Samples were then mixed with loading buffer containing 0.2% SDS and protease K.

DNA knotting by T4 topoisomerase II

Knotting reactions contained supercoiled DNA at ~20 µg/ml in topoisomerase II buffer (50 mM Tris-HCl at pH 7.5, 60 mM KCl, 40 µg/ml BSA, 10 mM MgCl₂, 0.1 mM EDTA). Knotting with T4 topoisomerase II was for 3 min at 30–32°C, and samples were DNase I-nicked on ice as described. T4 topoisomerase II was kindly supplied by H. Benjamin.

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