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Catalytic residues of γδ resolvase act *in cis*

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The resolvase protein of the γδ transposon is a site-specific recombinase that acts by a concerted break-and-join mechanism. To analyse the role of individual resolvase subunits in DNA strand cleavage, we have directed the binding of catalytic mutants to specific recombination crossover sites or half-sites. Our results demonstrate that the resolvase subunit bound at the half-site proximal to each scissile phosphodiester bond provides the Ser10 nucleophile and Arg8, Arg68 and Arg71 residues essential for cleavage and covalent attachment to the DNA. Several other residues near the presumptive active site are also shown to act *in cis*. Double-strand cleavage at one crossover site can proceed independently of cleavage at the other site, although interactions between the resolvase dimers bound at the two crossover sites remain essential. An appropriately oriented heterodimer of active and inactive protomers can in most cases mediate either a 'top' or 'bottom' single-strand cleavage, suggesting that there is no obligatory order of strand cleavages. Top-strand cleavage is associated with the topoisomerase I activity of resolvase, suggesting that a functional asymmetry may be imposed on the crossover site by the structure of the active synapse.

Keywords: heterodimer/resolvase/recombinase/site-specific recombination/γδ transposon

Introduction

In site-specific recombination, transposition and retroviral integration, phosphoryl transfer reactions involving four DNA strands are coupled in time and space. This is achieved in a variety of systems through the combined catalytic action of four recombinase subunits (Stark *et al.*, 1992; Landy, 1993; Grindley, 1994). It is not yet well understood how the strand transfer reactions are coordinated and confined to properly aligned DNA substrates. In Mu transposition, catalysis is contingent on formation of a stable transposase tetramer (Lavoie *et al.*, 1991; Baker *et al.*, 1993); in FLP recombination, each catalytic site is a composite of two recombinase subunits (Chen *et al.*, 1992), while in Tn*7* transposition and Xer site-specific recombination, two catalytically active proteins work together and have distinctive roles in the reaction (Blakely *et al.*, 1993; N.Craig *et al.*, personal communication). To address these issues for site-specific recombination by γδ resolvase, we first needed to identify which binding sites in the DNA deliver the resolvase subunits involved in specific strand cleavages.

The resolvase encoded by transposon γδ catalyses a site-specific recombination reaction that resolves the cointegrate intermediate formed during γδ transposition (reviewed in Grindley, 1994). Although the chemistry of DNA cleavage and religation appears straightforward (Figure 1), the reaction takes place within an elaborate synaptic structure comprising 240 bp of DNA and probably six dimers of resolvase. A wealth of structural information has been revealed by crystallographic studies of the catalytic domain of resolvase (Sanderson *et al.*, 1990; Rice and Steitz, 1994b) and, most recently, the entire resolvase dimer bound at the 28 bp crossover site (Yang and Steitz, 1995), but we know few details of the molecular architecture of the catalytically active complex, and the mechanism of recombination remains enigmatic (Hughes *et al.*, 1993; Rice and Steitz, 1994a; Soultanas *et al.*, 1995).

Recombination by resolvase *in vitro* normally requires a negatively supercoiled DNA substrate containing two directly repeated 120 bp *res* sites (Reed, 1981). Each *res* contains three binding sites for the resolvase dimer; the crossover point is located at the centre of site I (Figure 3). The 21 kDa γδ resolvase polypeptide consists of two structural domains (Abdel-Meguid *et al.*, 1984). The 140-residue N-terminal domain contains the catalytic site and is responsible for dimerization (Hughes *et al.*, 1993; Rice and Steitz, 1994b), while the 43-residue C-terminal domain recognizes inverted 12 bp elements found at the left and right arm of each dimer-binding site (Figure 2; Abdel-Meguid *et al.*, 1984; Rimphanitchayakit and Grindley, 1990). The N-terminal domain participates in additional resolvase interactions that are essential for synapsis and recombination (Hughes *et al.*, 1990) .

Within the resolvase–*res* synaptic complex, strand exchange apparently occurs by concerted cleavage of all four DNA strands at the centre of site I, rearrangement of the eight DNA ends, and concerted religation of four recombinant strands (Figure 1; Reed and Grindley, 1981). Synapsis of the two *res* sites is very fast relative to the observed rate of strand exchange (Parker and Halford, 1991). Because the active synapse traps three interwound supercoils between the crossover sites (site I) by interwrapping of *res* sites II and III, the recombinant product is a catenane of two interlocked circles (Wasserman *et al.*, 1985; Stark *et al.*, 1989; Benjamin and Cozzarelli, 1990; A.Bednarz, C.Koch, R.Kahmann and M.R.Boocock, unpublished).

Resolvase recombination intermediates with broken or incompletely rejoined DNA strands do not accumulate

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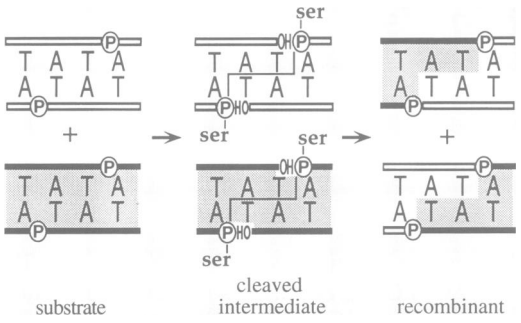

Fig. 1. Proposed role of covalent resolvase–DNA intermediate in recombination by resolvase. The four central base-pairs of *res* site I are shown. Each of the scissile phosphodiester bonds is attacked by the Ser10 residue of a resolvase protomer which becomes covalently linked to the recessed 5′ DNA end; a free 3′ hydroxyl DNA end is released. Cleavage of all four DNA strands is thought to be completed before the half-sites are rearranged and religated in the recombinant configuration.

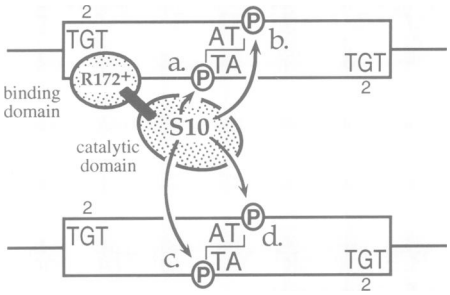

Fig. 2. Four possible modes of DNA strand cleavage by a resolvase protomer bound at one half of *res* site I. The Ser10 nucleophile in the catalytic domain of resolvase could potentially attack any of the four scissile bonds at either of the two crossover sites (a–d). We distinguish between cleavage at the bonds 'proximal' (a) or 'distal' (b) to the binding site of the recognition domain, and we refer to both (a) and (b) as cleavage '*in cis*' (i.e. at the same site as DNA binding). We refer to cleavage at the other site (c or d) as cleavage '*in trans*'. Note that other residues in the catalytic domain do not necessarily act at the same cleavage site as Ser10.

under optimal reaction conditions. However, in reaction buffers lacking Mg^{2+} or an alternative multivalent cation (and especially in buffers containing glycerol or ethylene glycol), recombination is severely inhibited, and a 'cleaved intermediate' gradually accumulates. In this species, there is a staggered double-strand cut at both crossover sites, and the recessed 5′-phosphorylated DNA ends are covalently joined to four resolvase subunits, through phosphodiester linkages to Ser10 in the active site (Figure 1; Reed and Grindley, 1981; Reed and Moser, 1984; Hatfull and Grindley, 1986).

The four-strand cleaved species is believed to be an authentic recombination intermediate, although it has not been isolated in a stable and active form, as has been done for a nicked synaptic intermediate in the phage Mu integration reaction, for example (Lavoie *et al.*, 1991). Cleavage and recombination by resolvase both require a supercoiled DNA substrate with two directly repeated *res* sites, consistent with cleavage being on the reaction pathway of recombination. Cleaved intermediates made by γδ resolvase, or the related DNA invertase Hin, can be chased into religated substrate or recombinant by altering the reaction conditions (Reed and Grindley, 1981; Johnson and Bruist, 1989). It should be noted that the topoisomerase I activity of resolvase is also specific for supercoiled substrates with two directly repeated *res* sites (Krasnow and Cozzarelli, 1983). Topoisomerase I activity persists in several situations where the complete recombination reaction is blocked; the mechanistic relationship between the two activities is not clear (Falvey *et al.*, 1988; Castell and Halford, 1989; Stark *et al.*, 1989; Leschziner *et al.*, 1995).

Strand exchange by resolvase induces a change in DNA topology equivalent to a right-handed (or left-handed) 180° rotation of the two left half-sites relative to the right half-sites in the cleaved intermediate (Stark *et al.*, 1989; Stark and Boocock, 1994). It is suggested that the rearrangement of the resolvase-linked half-sites (Figure 1) may be coupled to a rotational exchange of subunits within a resolvase tetramer formed at the crossover sites (Stark *et al.*, 1989). A contrasting proposal is that the 1,2 dimers bound at the crossover sites do not dissociate during catalysis, and that strand exchange is accomplished by localized changes in the conformation and topology of the DNA (Rice and Steitz, 1994a). The main objection to the subunit rotation model is that unprecedented flexibility in the resolvase–resolvase interactions would be needed to prevent catastrophic dissociation of the half-sites during recombination. An attractive way to sidestep this problem is to suppose that each resolvase subunit binds to one half-site by its recognition domain, and covalently joins to a different half-site by its catalytic domain (Figure 2B). Such molecular bridges could prevent dissociation of the left and right half-sites during strand exchange.

It has previously been shown that the serine nucleophile (Ser10) is required only in the resolvase subunits bound at site I of *res* (Grindley, 1993). There is evidence that resolvase preferentially becomes covalently linked to the *res* site to which it is initially bound (Dröge *et al.*, 1990), but bridging between the two halves of the same crossover site has not been tested. Here we use site-directed complementation between mutant resolvases (Grindley, 1993) to determine the source of the enzyme subunit(s) involved in particular strand cleavages, and we also investigate how these reactions are coupled.

Results and discussion

Coordinated cleavage and religation at the two crossover sites

To examine the relationship between the cleavage reaction and recombination, we followed the kinetics of cleavage, and the topology of subsequent religation reactions. Under typical 'cleavage' conditions (no Mg^{2+}, 5% glycerol), products of double-strand cleavage at both crossover sites accumulated slowly over 240 min, and were at all times more abundant than any partial cleavage products (Figure 4). The four strand cleavages are thus highly coordinated.

Recombination was not completely blocked under 'cleavage' conditions. Nicking with DNase I revealed that the main recombination product was a fully ligated two-noded catenane (Figure 4; the nicked catenane was not seen in significant amounts prior to DNase I cleavage). This indicates that religation of the strands is also well coordinated, and gives normal recombinant products. Although the resolvase covalent intermediate is stabilized

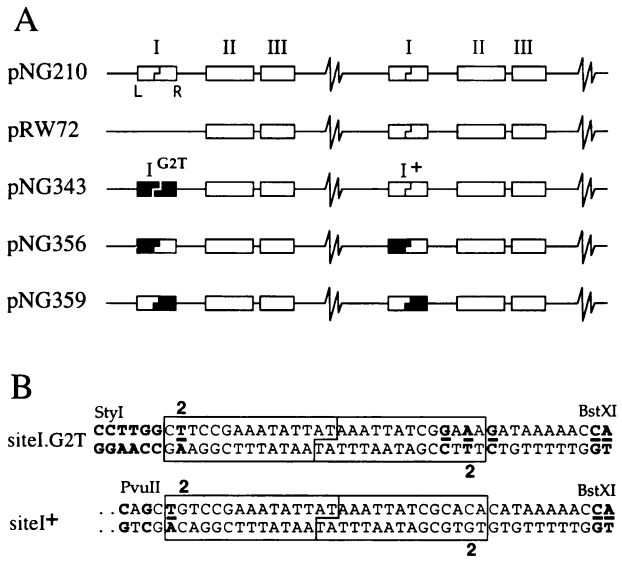

Fig. 3. (**A**) Structure of the γδ *res* site, and structure of plasmid substrates containing two *res* sites with various combinations of site I^+ and site I^{G2T}. The three resolvase binding sites in *res* are shown as boxes, and the left and right arms of site I are indicated. Open boxes represent binding sites in *res* with wild-type sequences; halves of site I containing the G2T specificity mutation are shown in black. The plasmid substrates were all pBR322 derivatives, and differ slightly in the *res*–vector junctions (see Materials and methods). (**B**) Sequence of site I^{G2T}, and comparison with the synthetic wild-type site I used in these experiments. Bases that differ from the natural γδ sequence are in bold type, including the G to T changes at position 2 in the left and right arms of site I^{G2T}, and (part of) the engineered *Bst*XI site that was used to replace site I^+ with site I^{G2T} (see Materials and methods).

under $-Mg^{2+}$ conditions, we did not see products from processive rounds of recombination, as were seen in $-Mg^{2+}$ reactions with the Gin invertase (Kanaar *et al.*, 1990). The relatively low yield of free recombinant circles suggests that uncoupled religation reactions are less frequent, and that the resolvase-linked DNA ends do not normally escape from the synaptic complex. When the 'cleavage' reaction was supplemented with 10 mM Mg^{2+} after 240 min, the cleavage products were mostly consumed, and the two-noded catenane appeared in high yield (Figure 4). This result supports the idea that the cleaved form is an authentic reaction intermediate in which the four DNA ends are held in place by resolvase subunit interactions, but does not show that the cleaved form is an immediate precursor of the recombinant product.

We wished to test whether site I is needed in both *res* partners for the cleavage reactions, since sites II and III are sufficient to direct synapsis and interwinding (M.Watson, M.R.Boocock and W.M.Stark, submitted; A.Bednarz, C.Koch, R.Kahmann and M.R.Boocock, unpublished). The plasmid pRW72 carries one complete *res* site and one with site I deleted (Figure 3; Wells and Grindley, 1984). Under standard cleavage conditions, pRW72 was not significantly cut or nicked by resolvase at the remaining crossover site (not shown). Formation of a stable cleavage intermediate thus requires the second crossover site and, presumably, the resolvase dimer bound to it.

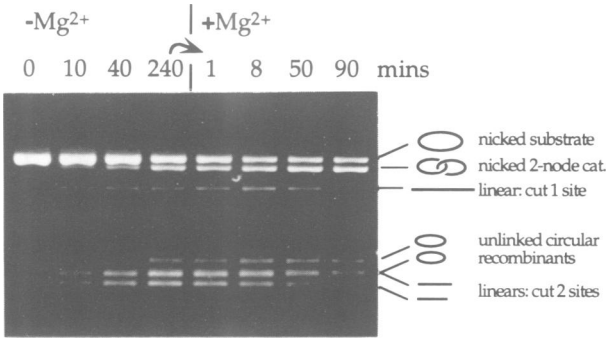

Fig. 4. Cleavage and religation of DNA by resolvase. Supercoiled pNG210 (see Figure 3) was incubated with resolvase in 1 mM EDTA, 100 mM NaCl, 5% glycerol, 50 mM Tris–HCl pH 8.2 at 37°C. After 4 h, the reaction was supplemented with 10 mM $MgCl_2$. Samples taken at the times indicated were quenched with 300 μg/ml EtBr, nicked with DNase I and treated with SDS–protease K. Cleavage products and nicked forms of the substrate and recombinant circles were separated on a 1.2% agarose gel, as shown (the nicked circular species were generated mainly by DNase I nicking of closed-circular products of the resolvase reaction).

Targeted cleavage at one crossover site

Which DNA strands are cut when only one of the two crossover sites is occupied by catalytically active resolvase? The substrate pNG343 carries the G2T specificity mutation in the left and right arms of one crossover site, and has wild-type ($G2^+$) recognition sequences at the other crossover site and at both copies of sites II and III (Figure 3). The active R172L specificity mutant of resolvase can be selectively 'targeted' to the G2T crossover site (its optimal binding site) by complementing with mutant proteins carrying the wild-type $R172^+$ DNA binding domain, which bind relatively weakly to this site (Grindley, 1993). The R172L and wild-type resolvases act synergistically on pNG343 under cleavage and recombination conditions, confirming that the G2T mutant crossover site is fully functional in the presence of R172L resolvase (Figure 5B, track 2).

When R172L was complemented by wild-type resolvase under cleavage conditions, over 50% of the input pNG343 suffered double-strand cleavage at both *res* sites; a relatively small amount was cut at only one site (Figure 5A). When R172L resolvase was complemented with the S10C catalytic site mutant, cleavage of pNG343 was again very efficient, but only one *res* site was cut (the S10C protein is inactive under these reaction conditions). The cleavage mapped exclusively to the G2T crossover site (36% of substrate), and not to the $G2^+$ site (<1%; Figure 5A, track 4). The simplest interpretation is that the catalytically active R172L protein binds selectively to the G2T site, and cleaves this site when complemented by S10C protein bound at the $G2^+$ site. Alternatively, the G2T mutant site could be intrinsically more susceptible to cleavage in complementation experiments. This possibility was dismissed by complementing the double mutant S10C/R172L with wild-type resolvase, so that the S10C catalytic domains were now directed to the G2T site of pNG343. Cleavage was seen exclusively at the $G2^+$ site (42% of substrate) and not at the G2T site (<1%; Figure 5). However, when the wild-type protein was combined with the wild-type specificity S10C mutant, so that a particular

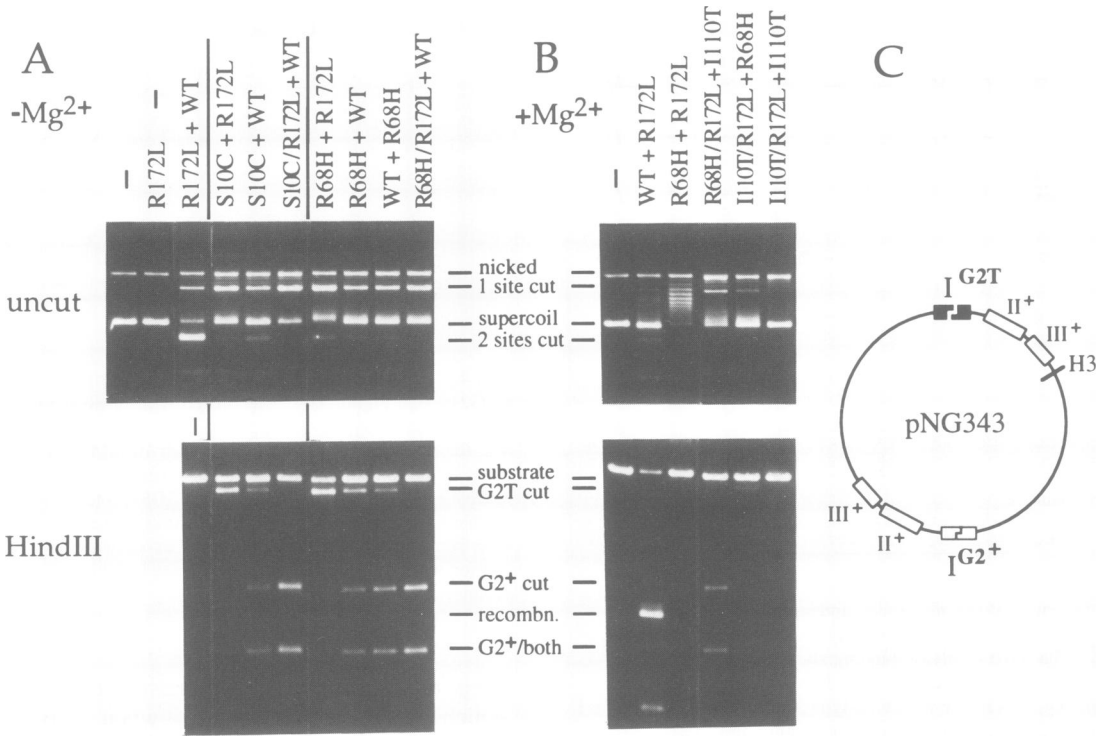

Fig. 5. Targeted double-strand cleavage of pNG343 by various combinations of resolvase mutants. (**A**) Cleavage reactions in a buffer containing no Mg^{2+} and 40% ethylene glycol (buffer 2). After 8 h at 37°C, reactions were quenched by addition of SDS–protease K: samples were run uncut on a 1.2% agarose gel (top panel), or were restricted at the unique *Hin*dIII site and end-labelled before electrophoresis (lower panel); reaction products were quantitated by phosphorimaging of the lower gel. The mutants are listed in the order of addition to the reactions (~30 s between enzyme additions). The resolvase concentrations were: 110 nM R172L, 155 nM (track 3) or 78 nM (tracks 5, 6, 8 and 9) wild-type, 250 nM S10C, 160 nM S10C/R172L, 155 nM R68H, 100 nM R68H/R172L. (**B**) Similar reactions in a buffer containing 10 mM Mg^{2+} (buffer 1): resolvase concentrations as for A, with 160 nM I110T and 160 nM I110T/R172L. (**C**) Structure of pNG343 (4798 bp), showing the location of the unique *Hin*dIII (H3) site with respect to the G2T and $G2^+$ crossover sites of *res* (*res* sites not shown to scale).

crossover site would have a similar affinity for both proteins, the plasmid was cut in similar yield at the two *res* sites (15% G2T only; 9% $G2^+$ only).

Our data for pNG343 show that single crossover site cleavages occur exclusively at the site with the higher affinity for the active, $S10^+$ resolvase, or at either site if the active and inactive proteins have the same recognition specificity. Therefore we conclude that the two Ser10 nucleophiles required for double-strand cleavage at a given crossover site are provided '*in cis*' by the resolvase dimer bound to that crossover site. Our conclusion confirms and strengthens that of Dröge *et al.* (1990), who observed some preference for cleavage *in cis* in experiments where complete res^+ sites were preloaded with wild-type and S10C resolvases.

In the context of *res*×*res* reactions, the R172L and $R172^+$ enzymes do not exhibit absolute selectivity for G2T and $G2^+$ sites respectively, although the cocrystal structure reveals that Arg172 makes the predicted direct contacts to G2 in the recognition sequence (Yang and Steitz, 1995). Significant cleavage at both sites ($G2^+$ and G2T) was seen when pNG343 was incubated with wild-type-specificity resolvase (Figure 5A, tracks 8 and 9), or with R172L resolvase alone, especially at elevated concentrations of enzyme (not shown). Binding and catalysis by R172L resolvase at the 'heterologous' $G2^+$ sites is likely to be aided by cooperative interactions between the six resolvase binding sites in the synapse. In an attempt to prevent unwanted binding of $R172^+$ resolvase at the G2T crossover sites, we have for certain experiments used the S173G mutant, which has a greatly reduced affinity for all sites, but retains the wild-type recognition specificity (N.D.F.Grindley and G.Hatfull, unpublished). As expected, complementation of the R172L protein with the double mutant S10C/S173G gave cleavage exclusively at the G2T site of pNG343, while complementation of the S10C/R172L double mutant with S173G gave cleavage exclusively at the $G2^+$ site (data not shown). However, for all of the 'targeting' experiments, the appropriate absolute and relative concentrations of the two enzymes had to be determined empirically (Figure 6).

Residues required in cis for cleavage

In addition to Ser10, a number of other residues are suspected to be important for the strand cleavage and religation reactions, including Tyr6, Arg8, Gln14, Gly40, Asp67, Arg68, Arg71 and Glu118 (Hatfull *et al.*, 1987; Hughes *et al.*, 1990; Leschziner *et al.*, 1995). These residues (except for Glu118) are on the same face of the catalytic domain as Ser10 (Sanderson *et al.*, 1990; Rice and Steitz, 1994b), and are highly conserved among 44 members of the resolvase–invertase family (Leschziner *et al.*, 1995); substitution mutants are inactive or severely defective in recombination, yet retain near-normal affinity for all three sites of *res* (except for the E118K mutant) (Hatfull and Grindley, 1986; Hatfull *et al.*, 1987; N.D.F.Grindley *et al.*, unpublished). To determine whether these residues are required at one or both crossover sites

for DNA cleavage, mutant proteins were targeted to the $G2^+$ crossover site of pNG343, as described above for the S10C mutant.

Complementation of R172L resolvase by the R68H mutant induced pNG343 cleavage at the G2T crossover site (53% G2T; <1% $G2^+$; Figure 5). Conversely, when the R68H/R172L double mutant was combined with wild-type resolvase, cleavage was exclusively at the $G2^+$ site (<2% G2T; 33% $G2^+$). Thus in both cases, cleavage was specifically blocked at the crossover site to which the R68H protein was targeted. We conclude that, as with Ser10, the Arg68 residues needed for cleavage are provided by resolvase subunits bound at the same crossover site. Similar conclusions can be drawn regarding residues Tyr6, Arg8, Gln14, Gly40, Asp67, Arg71 and Glu118. The Y6F, R8Q, Q14L, G40D, D67G, R71H and E118K mutant resolvases all efficiently complemented cleavage by R172L resolvase at the G2T site of pNG343, and cleavage was selectively blocked at the $G2^+$ site to which the mutant proteins were targeted (data not shown). The wild-type residues at these positions are evidently important for cleavage at the crossover site to which the resolvase dimer is bound. Since they are dispensible for double-strand cleavage at the other crossover site, we deduce that none of these residues is essential for synapsis of the two crossover sites, nor of sites II and III.

The resolvase subunits bound at the crossover sites appear to be readily exchangeable. When a combination of R68H and wild-type resolvases was added to pNG343, double-strand cleavages were distributed about equally between the two crossover sites; moreover, the order of addition had little effect on the relative yields of the G2T and $G2^+$ cleavage products (Figure 5A, tracks 8 and 9). When the wild-type enzyme was added second, cleavage was not strongly targeted toward the G2T crossover site, although the inactive R68H enzyme might have been expected to dissociate more rapidly from the weaker G2T binding site than from the $G2^+$ site (Lee *et al.*, 1994). (The R68H and wild-type proteins have virtually indistinguishable binding properties, and both were present in excess over binding sites.) This suggests that the resolvase subunits bound at the crossover sites are freely exchangeable on the time-scale of the cleavage reaction. If synapsis of the two *res* sites is a very rapid, committed step, as reported for Tn*3* resolvase (Parker and Halford, 1991), this cannot preclude subsequent rearrangements of the resolvase subunits bound at site I. The facile exchange of the resolvase subunits bound at the crossover sites contrasts with observations from other systems, including FLP recombination (Lee *et al.*, 1994) and Mu transposition (Baker *et al.*, 1993).

Blocking cleavage at the $G2^+$ crossover site of pNG343 with R68H or S10C resolvase did not significantly reduce the rate of cleavage at the G2T site. However, when the same complementation experiment was repeated under recombination conditions (10 mM Mg^{2+}), double-strand cleavage was not observed. Nevertheless, multiple rounds of single- or double-strand cleavage and religation had taken place, as was revealed by the extensive relaxation of substrate supercoiling (Figure 5B, track 3). In the presence of magnesium, it seems that religation always outpaces the cleavage reaction, so the covalent intermediates do not accumulate.

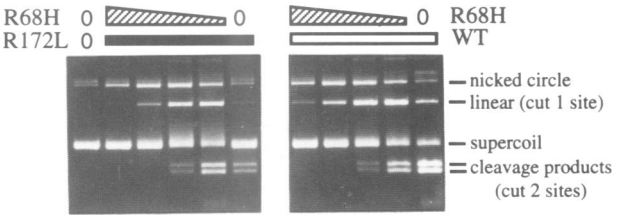

Fig. 6. Targeted single-strand cleavage of pNG359 (Figure 3) by premixed combinations of R68H and R172L resolvase (left panel), and untargeted 'control' cleavage reactions with R68H and wild-type resolvase (right panel). Reactions were in buffer 2 for 2.5 h at 37°C. Cleavage was quenched with SDS–protease K, and products were run on a 1.2% agarose gel. Estimated ratios of R68H to R172L protomers were (tracks 2–5): 5.6:1, 2.8:1, 1.4:1 and 0.7:1. Estimated ratios of R68H to wild-type protomers were (tracks 7–10): 4:1, 2:1, 1:1 and 0.5:1. The R172L and wild-type proteins were present at final (protomer) concentrations of 220 nM and 310 nM. Reactions similar to those in tracks 3 and 8 were used to measure the ratio of top- and bottom-strand cleavages (Figure 7 and Table I).

Trans-complementation of I110T resolvase

The I110T resolvase mutant (Hughes *et al.*, 1990) behaved very differently from the 'catalytic site' mutants in complementation experiments. When R172L was complemented by I110T resolvase, cleavage of pNG343 was not restricted to the G2T crossover site: there were similar yields of products with double-strand cuts at the $G2^+$ site only, or at the G2T site only (not shown). This result suggested that the I110T resolvase might be stimulated to cleave the $G2^+$ site by R172L resolvase bound at the G2T site. When I110T was complemented with the inactive R68H/R172L double mutant, cleavage at the $G2^+$ site was still observed under cleavage conditions (not shown), and also under recombination conditions (Figure 5B, track 4). Conversely, when the I110T/R172L double mutant was complemented with R68H, the cleavages mapped to the G2T crossover site (Figure 5B, track 5). Neither the I110T protein alone (Figure 5B, track 6) nor the R68H protein catalyses significant double-strand cleavage under these conditions. Cleavage at a site occupied by an I110T dimer thus appears to be complemented *in trans* by a catalytically inactive $I110^+$/R68H dimer (but not an I110T dimer) bound at the other crossover site.

Formally these data could be rationalized by suggesting that Ile110 is an essential residue required *in trans* for cleavage. However, Ile110 is not a surface residue, but is buried within the 1,2 dimer interface (Hughes *et al.*, 1993; Rice and Steitz, 1994b; see Figure 10). A more plausible hypothesis is that an I110T dimer can be activated through a conformational change induced by synapsis with an $I110^+$ dimer at the other crossover site.

It is notable that Ile110 and Thr109 of resolvase contact Ile97, at the edge of the β-sheet of the adjacent monomer in the 1,2 dimer. A comparison of the structures with and without DNA reveals some degree of plasticity in this interface (Rice and Steitz, 1994a; Yang and Steitz, 1995). The H106Y and I94V mutations in the Gin DNA invertase (at positions corresponding to T109 and I97 of resolvase) activate the protein, allowing it to function without FIS, and may stabilize the cleaved intermediate (Klippel *et al.*, 1988a,b, 1993; R.Kahmann, personal communication). In contrast, a disulfide crosslink that freezes the 1,2 dimer interface of M106C resolvase, or a similar crosslink in the Hin invertase, abolishes recombination and suppresses

Table I. Targeted single-strand cleavage by resolvase

Enzymes	A pNG359 ($I\text{-}R^{G2T}$)		B pNG356 ($I\text{-}L^{G2T}$)	
	top	: bottom	top	: bottom
1. WT + R68H	1.1	: 1	1.8	: 1
	1.0	: 1[a]	2.1	: 1[a]
2. R172L + R68H	9.7	: 1[a]	1	: 13[a]
3. R172L/R68H + WT	1	: 3.0	>10	: 1
4. R172L/R68H + S173G	1	: 8.7	>50	: 1
5. R172L + S10C	>10	: 1	1.2	: 1
6. R172L + S10C/S173G	>20	: 1	2.2	: 1
7. R172L/S10C + S173G	2.7	: 1	>50	: 1
8. R172L/S10C + WT	~2	: 1	>50	: 1
9. R172L + R8Q	>20	: 1	1	: 8.0
10. R172L + R72H	13	: 1[a]	1	: 5.6[a]
11. R172L + E118K	22	: 1[a]	1.2	: 1[a]
12. R172L + I110T	6.4	: 1	43	: 1
13. R172L/R68H + I110T	1	: 27	>20	: 1
14. R172L/I110T + R68H	>5	: 1	1	: >3

Relative efficiency of top and bottom single-strand cleavages of pNG356 and pNG359 by different combinations of (premixed) resolvase mutants. All cleavage reactions were in buffer 2 for 5 h at 37°C. Single-strand cleavage products were separated on sequencing gels and quantitated by phosphorimaging (see Figure 7). The cleavage ratios assume equal labelling efficiencies at the top- and bottom-strand nicks. Where a lower limit is given for the cleavage ratio, only one product was sufficiently abundant to quantitate: the other product was assigned a value equivalent to at least 2× the background reading in that part of the image. Data marked ([a]) are from an independent experiment using method 2 (see Materials and methods).

In the reactions analysed (similar to Figure 6, tracks 3 and 8) the yield of the single-strand cleavage products was generally <5% of input substrate (and in some cases much lower), and was not quantitated precisely. The final concentrations of resolvases were: 75 nM wild-type (160 nM for experiment 1[a]), 110 nM R172L, 600 nM (1. and 2.) or 160 nM (14.) R68H, 100 nM R172L/R68H, 160 nM S173G, 500 nM S10C, 800 nM S10C/S173G, 160 nM R172L/S10C, 900 nM R8Q, 1.3 μM R71H, 310 nM E118K, 160 nM (13.) or 310 nM (12.) I110T and 70 nM R172L/I110T.

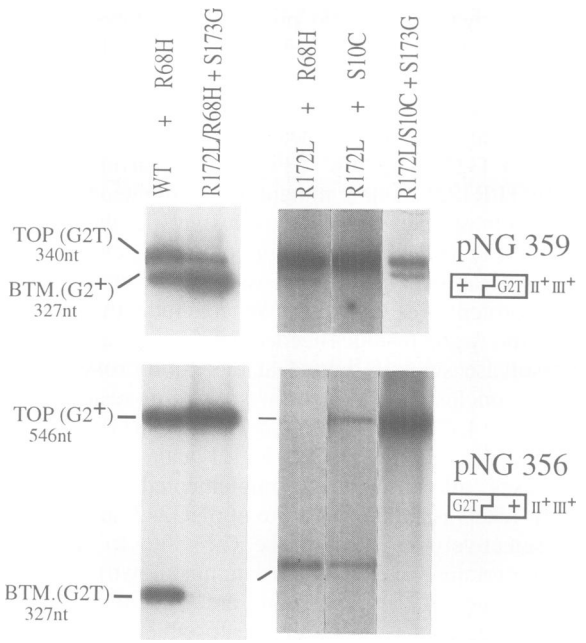

Fig. 7. Single-strand cleavage by active resolvase protomers targeted to the left or right crossover half-sites of pNG359 and pNG359. Cleavage reactions (5 h) were similar to those shown in Figure 6, tracks 3 and 8: resolvase concentrations were as for Table I (except S173G: 310 nM). Nicked circular reaction products were gel-isolated, labelled at the resolvase-induced nicks, then restricted with *Sca*I and *Hin*dIII (see Materials and methods). Labelled fragments diagnostic for the top- and bottom-strand cleavages were separated on a 6% sequencing gel, as shown.

double-strand cleavage (Hughes *et al.*, 1993; Lim, 1994). The '*trans*-complementation' of the resolvase I110T mutant, together with its defective ligation activity, provides further evidence that the conformation of the 1,2 dimer interface is critical for catalysis, and that it may be subject to external influences.

Targeted cleavage at left and right half sites

To distinguish the functions of the resolvase subunits bound at the left and right arms of the crossover site, we constructed substrates carrying the G2T specificity mutation at one arm of this site. In pNG356, the G2T mutation is present at the left ends of both crossover sites, while in pNG359 it is at the right ends (Figure 3).

A heterodimer of R172L and R68H resolvase protomers should bind to a G2T–$G2^+$ hybrid crossover site in a unique orientation, and is expected to target single-strand cleavages to either the top or bottom strand. When pNG359 cleavage by R172L resolvase (at a high concentration) was complemented by the inactive R68H mutant, a variety of different products was obtained (Figure 6). At the lowest concentrations of R68H, double-strand cleavage occurred at both crossover sites. As the R68H concentration was raised, this reaction was suppressed, and products from double-strand cleavage at one site were seen. At higher concentrations, nicked circular products from single-strand cleavage at one or both sites became more abundant, and at the highest concentrations of R68H the cleavage reactions were suppressed altogether. Similar results were obtained with pNG356 (not shown) and also in an 'untargeted' control reaction with wild-type and R68H resolvase (Figure 6).

We believe that the single-strand cleavages seen in these reactions are due to the anticipated heterodimers of active and inactive subunits; the double-strand cleavages presumably occur when active subunits occupy three or four of the crossover half-sites. The ratio of these products was little altered by changing the order of addition or by premixing the proteins. Since γδ resolvase forms stable 1,2 dimers in buffers containing 1 M NaCl (Hughes *et al.*, 1993; Liu *et al.*, 1993), the premixed proteins were subjected to denaturation and renaturation to encourage the formation of heterodimers; this also had little effect on the product ratios for a given ratio of R172L and R68H subunits. We infer that resolvase mutants readily form heterodimers in solution, or at the crossover sites, under the conditions of the cleavage assay. It is possible that γδ resolvase can bind to its crossover site in two monomer association steps, like the related Tn*3* enzyme (Blake *et al.*, 1995)

To determine the ratio between the top and bottom single-strand cleavages, it was essential to exclude from the analysis all material that had been cut on both strands.

This was done either by isolating the nicked circular cleavage products from a preparative agarose gel, or by purifying a *Sca*I–*Hin*dIII fragment containing the *res* site. In both methods, cleavage products were labelled at the resolvase-induced nicks and were then digested with *Sca*I and *Hin*dIII: this ensured approximately equal labelling efficiencies at the top- and bottom-strand nicks, and prevented significant labelling of double-strand cleavage products.

In an 'untargeted' control experiment using a mixture of R68H and wild-type resolvases, the substrate pNG359 gave approximately equal yields of top- and bottom-strand cleavage products (Table I, line 1). The pNG356 substrate gave a modest (2:1) excess of top-strand cleavage. These single-strand cleavages are presumably due to heterodimers of R68H and wild-type subunits binding to the hybrid crossover site in both possible orientations. The results indicate that there is no obligatory order of strand cleavage, and that the intrinsic rates of top and bottom single-strand cleavage are little affected by the G2T mutation at the left or right half-site.

When the R172L resolvase was complemented with R68H resolvase, single-strand cleavage occurred predominantly on the top strand of pNG359, and on the bottom strand of pNG356 (Figure 7 and Table I, line 2). The strand bias in cleavage of these substrates was reversed when the R172L/R68H double mutant was complemented with wild-type or S173G resolvase (Figure 7 and Table I, lines 3 and 4). This clearly demonstrates that selective binding of R68H subunits at the right half-site suppresses top-strand cleavage, while selective binding at the left half-site suppresses bottom-strand cleavage. We conclude that the Arg68 side-chain is required only at the right half-site for top-strand cleavage, and at the left half-site for bottom-strand cleavage.

The R8Q and R71H mutants behaved similarly to R68H in complementation experiments with R172L (Table I, lines 9 and 10); we conclude that the arginines at positions 8, 68 and 71 are all required for cleavage at the bond proximal to the half-site occupied by the resolvase protomer.

Ser10 attacks the proximal cleavage site

Is the Ser10 nucleophile provided by the same subunit as the essential arginine residues? Targeting of the S10C catalytic domain to the left half-site of pNG359 (R172L and S10C resolvases), or to the left half-site of pNG356 (R172L/S10C and wild-type resolvases) almost completely suppressed bottom-strand cleavage, while allowing efficient top-strand cleavage (Table I, lines 5A and 8B). This result clearly associates the Ser10 at the left half-site with the bottom-strand cleavage (since it is specifically blocked by the S10C substitution) and implies that the subunit at the right half-site provides the Ser10 nucleophile for top-strand cleavage. Therefore we conclude that the catalytic Ser10 nucleophile for each of the four-strand cleavages is provided by the subunit bound to the proximal half-site. This deduction is based on the data summarized in Table I, on complementary results from targeting experiments with combinations of G2T(R), G2T(L) and $G2^+$ crossover sites in the same substrate (not shown), and on the data showing that the Ser10 nucleophiles for double-strand cleavage at a given crossover site are provided by the resolvase dimer bound at that site (Figure 5). In addition, we have not detected any complementation between the S10C and R68H mutants in single-strand cleavage and topoisomerase assays under $+Mg^{2+}$ conditions, as might have been expected if different subunits contribute the Ser10 and Arg68 residues to a composite active site (not shown).

Paradoxically, targeting S10C to the right crossover half-site did not greatly suppress top-strand cleavage relative to bottom-strand cleavage (Table I, lines 5B and 8A); in these experiments the total yield of single-strand cleavages was lower than when S10C was targeted to the left half-site, and there was no significant double-strand cleavage (not shown). (The same result was obtained when the wild-type DNA recognition domains were replaced by the S173G domain, in an attempt to improve the targeting selectivity in this experiment: Table I, 6B and 7A.) The simplest way to explain these data is to suggest that S10C at the right half-site suppresses cleavage of both strands, and that the observed reaction is due to a residual background rate of 'untargeted' cleavage by $S10^+$ catalytic domains (or possibly by the S10C domains: see below).

At present we do not understand why the S10C catalytic domain has dissimilar effects when targeted to the left and right arms of the crossover site. In experiments with several other mutants, it was noticeable that targeted suppression of top-strand cleavage was less complete than that of bottom-strand cleavage (Table I, lines 3A, 4A and 9B). It is possible that the 'targeting' of particular catalytic domains to the right half-site is less effective than it is to the left half-site, but it is not obvious why this should only affect reactions where an inactive catalytic domain is targeted to the right half-site. Although the S10C and R68H proteins are completely inactive in double-strand cleavage under the conditions of the cleavage assay (Figure 5A), they display a slow single-strand nicking activity (almost equally on the top and bottom strands) under $+Mg^{2+}$ recombination conditions (G.Hatfull and M.R.Boocock, unpublished). The S10C protein has a slightly reduced binding affinity at site I, while the S10L mutant has a severe and specific defect in binding at this site (Hatfull and Grindley, 1986), and was found not to be useful for targeting experiments. Similarly, the S9A mutant of Gin is severely defective in crossover site binding (Klippel *et al.*, 1988b). We speculate that the defective interaction of an S10C subunit with the top-strand cleavage site suppresses the activity of an $S10^+$ subunit at the bottom-strand cleavage site, or that cleavage by an S10C subunit at the top site is stimulated by an $S10^+$ subunit at the bottom cleavage site. Alternatively, a heterodimer with an inactive S10C subunit at the right half-site may be non-productively trapped in a configuration that is directed towards an initial attack on the top strand. Further experiments are needed to investigate the apparent functional asymmetry of the crossover site in these reactions.

Half-site complementation with I110T and E118K

The I110T and E118K mutations both affect residues on the 1,2 dimer interface (Hatfull and Grindley, 1986; Hughes *et al.*, 1990; Rice and Steitz, 1994b). A surprising bias in single-strand cleavages was seen when these mutants were targeted to one half of the crossover site.

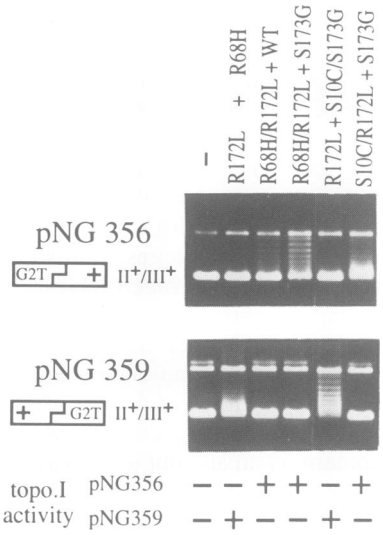

Fig. 8. Substrate relaxation by resolvase protomers targeted to the left or right crossover half-site in $+Mg^{2+}$ reactions. Reactions of supercoiled pNG356 or pNG359 with various combinations of premixed resolvase mutants were precisely as for the corresponding cleavage experiments documented in Table I, except that they contained 10 mM $MgCl_2$ and no ethylene glycol (buffer 1), and were for 4 h. Reaction products were separated on a 0.7% agarose gel; the relaxed circular products are seen as a ladder of bands between the negatively supercoiled substrate and the nicked circles.

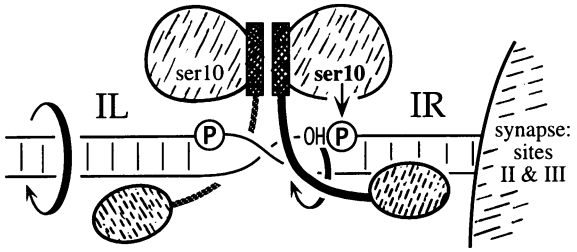

Fig. 9. Proposed 'swivelling' mechanism for the topoisomerase I activity of resolvase. The DNA at *res* site I is shown schematically as a ribbon with one positive half-twist at the centre (in fact there are two turns of the DNA helix between the recognition domains). The resolvase dimer is also represented in a simplified form: the catalytic domains, containing the Ser10 nucleophiles, are connected to the DNA recognition domains by the extended 'arm' region of the protein (Yang and Steitz, 1995). The resolvase protomer at the right half-site attacks and covalently joins to the 'top' strand of the DNA, releasing a free 3′ hydroxyl. In the topoisomerase I reaction, we suggest that the free 3′ hydroxyl swivels around the continuous DNA strand before religation of the single-strand nick. This could be facilitated by right-handed swivelling of the entire left half-site, after transient release of the left DNA-binding domain, as shown.

Cleavage was almost exclusively on the top strand when I110T was targeted to the right half-site, and primarily on the top strand when the same mutant was targeted to the left half-site. Our interpretation is that the defect in the 1,2 dimer interface leads to a preference for top-strand cleavage by the I110T/I110$^+$ heterodimers, irrespective of their orientation at the crossover site. This explanation is consistent with two further results. First, I110T subunits are competent for single-strand cleavage in heterodimers with the inactive R68H/R172 protein: cleavage was seen at the position proximal to the anticipated binding site of the I110T protein. (Table I, line 13). Secondly, single-strand cleavage by the I110T protein alone was readily detectable under $+Mg^{2+}$ conditions (Figure 5B, track 6) and mapped exclusively to the top-strand cleavage site (not shown). The top-strand bias may reflect a latent functional asymmetry in the crossover site, which is amplified by perturbation of the 1,2 dimer interface.

Targeting the E118K protein to the left half-site selectively blocked bottom-strand cleavage, while targeting the same mutant to the right half-site did not significantly suppress top-strand cleavage (Table I, lines 11A and B). Although this result suggests that Glu118 could be involved in cleavage at the bond proximal to the half-site, other data suggest that its role in specific strand cleavages may be indirect. The E118K mutant specifically fails to induce normal DNA binding at site I (Hatfull *et al.*, 1987), while in the cocrystal the side-chain of the adjacent residue, Arg119, closely approaches the proximal cleavage site in the DNA (Yang and Steitz, 1995).

Targeted top-strand cleavage induces topoisomerase I activity

Under recombination ($+Mg^{2+}$) conditions, targeting of catalytically defective mutants to the left or right half-site did not lead to the accumulation of single-strand nicks in pNG359 or pNG356. Instead, relaxation of substrate supercoiling was seen, but only with combinations of resolvases and substrate that directed the active catalytic domain to the right half-site (Figure 8). For example, with premixed R172L and S10C/S173G resolvases, pNG359 was relaxed but pNG356 was not; with premixed S10C/R172L and S173G the opposite result was obtained (Figure 8, tracks 5 and 6). Similar results were obtained with combinations of R68H and wild-type catalytic domains (Figure 8, tracks 2–4). Since the enzyme/substrate combinations that gave topoisomerase activity were precisely those that gave selective top-strand cleavage in the absence of Mg^{2+} (Table I), we conclude that top-strand cleavage is sufficient (and probably necessary) for the topoisomerase reaction. This conclusion is in line with data from previous experiments in which bottom-strand cleavage was selectively inhibited by mutations in the DNA sequence (Falvey and Grindley, 1987). Since relaxation persists when cleavage is completely blocked at one crossover site (Figure 5B, track 3), we deduce that the topoisomerase reaction takes place at a single top-strand cleavage site.

The relaxation reaction can be explained if the top-strand nick is religated after swivelling of either the enzyme-linked 5′ end, or the free 3′ hydroxyl end, around the continuous bottom strand. The latter mechanism appears more likely, since the 5′ end and the right half-site are expected to be topologically constrained by the synapsis of *res* sites II and III (Figure 9); this constraint might also account for the failure of bottom-strand cleavage to induce topoisomerase activity. In recombination reactions also, synapsis of sites II and III allows resolvase to distinguish the left and right halves of site I (Bednarz *et al.*, 1990).

Further discussion: cleavage in cis and recombination

We have shown that under cleavage conditions (no Mg^{2+}, with glycerol or ethylene glycol), the Ser10 nucleophile and presumptive catalytic residues Arg8, Arg68 and Arg71

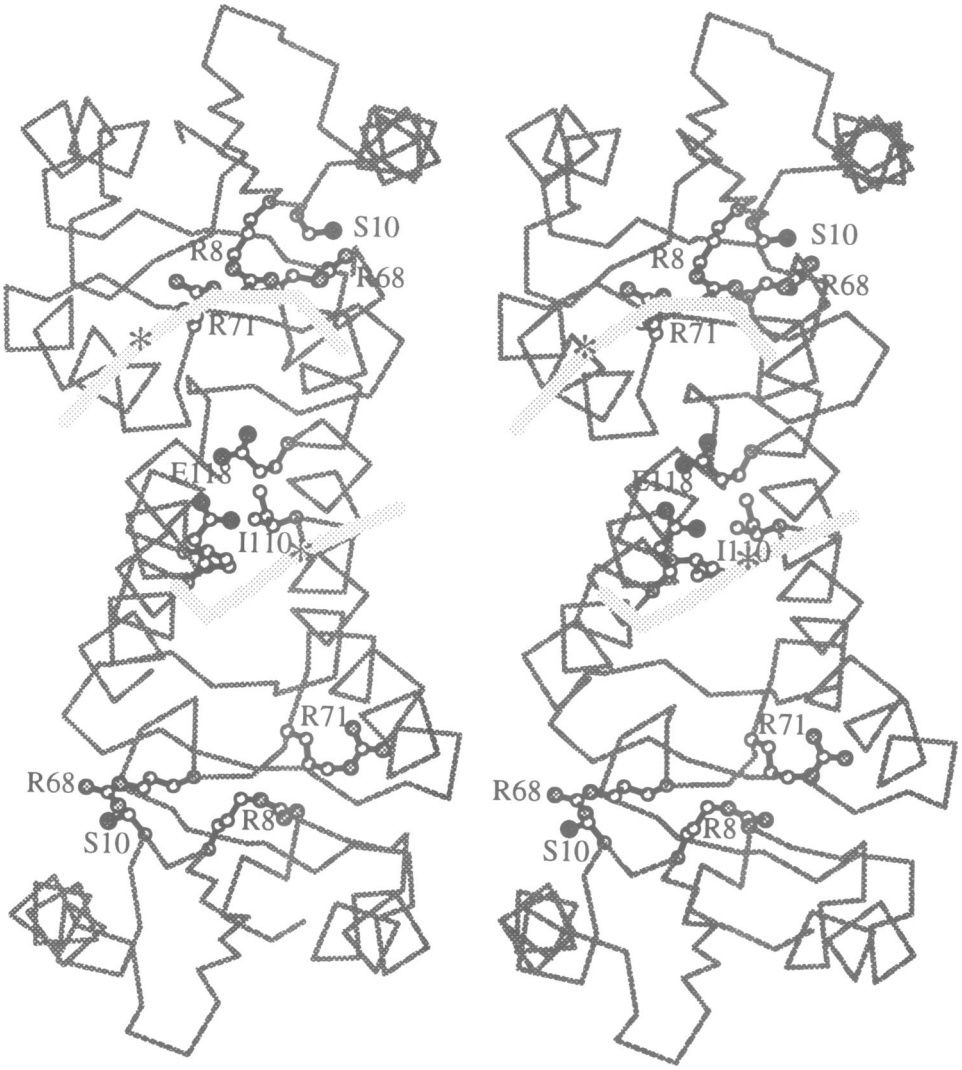

Fig. 10. Stereo view of the catalytic domain (residues 1–129) of γδ resolvase showing side chains discussed in this paper. This view is looking down the dyad axis of the resolvase dimer at the surface containing Ser10 and the three arginines at positions 8, 68 and 71. The two thick, lightly shaded lines indicate the backbones of the two DNA strands at the centre of the crossover region; the asterisks indicate the positions of the phosphates attacked by the Ser10 residues. This graphic was generated by Dr Wei Yang from the recently solved crystal structure of resolvase complexed with site I (Yang and Steitz, 1995). Note that the association between the catalytic region and DNA is much closer with the upper protomer than with the lower protomer.

for each strand cleavage are provided by the subunit bound at the proximal half-site. A number of other conserved residues, including Tyr6, Gln14, Gly40 and Asp67, are required *in cis* for double-strand cleavage, but we have not distinguished the specific role of subunits bound at the left and right half-sites (since the mutants available at these positions, although defective in double-strand cleavage, retain significant single-strand cleavage and topoisomerase I activity; Leschziner *et al.*, 1995 and our unpublished results). We infer that in the complete recombination reaction, the subunit at the proximal half-site also provides the key catalytic residues for each strand cleavage. At present we have no experimental data on the source of the catalytic residues (other than Ser10) needed to religate the recombinant strands.

Two results reported here reinforce the assumption that the disposition of the resolvase subunits is the same for recombination as it is under 'cleavage' conditions, when religation is severely inhibited. In recombination reactions initiated under 'cleavage' conditions, the strands were rejoined in an orderly fashion to give two-noded catenanes, suggesting that the integrity of the synapse is maintained despite the delayed religation of the strands. Occupation of both crossover sites by resolvase is essential for the cleavage reaction, although a catalytically inert dimer at one crossover site does not affect the rate of cleavage at the other site. We suggest that synapsis of resolvase dimers at the two crossover sites promotes a conformational change at the dimer interface that activates the cleavage reaction. An induced conformational change would account for the observed *trans*-activation of an I110T dimer for double-strand cleavage by a catalytically inactive dimer at the other crossover site. Since the cleavages normally appear to be concerted, it is likely that cutting at one site increases the rate of reaction at the second site.

Single-strand cleavages seem to be relatively poorly stabilized by resolvase. The fraction of substrate trapped in single-strand cleavage intermediates never approached the 50% level consistently achieved for targeted double-strand cleavage; in many top-strand 'cleavage' reactions,

despite the absence of Mg^{2+}, much of the input substrate was evidently cut and then religated with concomitant relaxation of negative supercoiling. The top- and bottom-strand cleavages at a given crossover site thus appear to be more tightly coupled than events at the two separate crossover sites.

Our data imply that Ser10 and Arg8, Arg68 and Arg71 from one subunit contribute to an autonomous catalytic site that can cleave a single DNA strand: we have no evidence that the Ser10 nucleophile can collaborate with residues 8, 68 and 71 from a different subunit. We note that Ser10 and the presumptive active site residues at positions 8, 14, 67, 68 and 71 are clustered on one face of the resolvase monomer, at least 18 Å from equivalent residues in the other subunit of the 1,2 dimer (Figure 10; Rice and Steitz, 1994b; Yang and Steitz, 1995). In the cocrystal stucture, the catalytic domains are positioned over the minor groove at the centre of site I, with the active sites closest to the proximal cleavage positions in the DNA. The Ser10 nucleophiles are 12 Å and 17 Å from the phosphates at the proximal cleavage sites and 18 Å and 28 Å from the phosphates at the distal sites (Figure 10; Yang and Steitz, 1995). The structure is fully consistent with cleavage at the proximal sites, although significant conformational shifts are still needed to bring the Ser10 nucleophiles within range of the relevant phosphodiester bonds.

The active site of resolvase seems to be organized differently from certain other site-specific recombinases. In strand cleavage reactions catalysed by the FLP recombinase, the tyrosine nucleophile comes from a subunit bound at the distal half of the crossover site, while the proximal subunit provides essential basic residues that activate the phophodiester bond (Lee *et al.*, 1994). Catalytic coupling between the subunits is suggested to be important for coordinating the strand exchanges (Chen *et al.*, 1992). In reciprocal exchange reactions catalysed by the related λ Int protein, however, the tyrosine nucleophile is provided by the subunit proximal to the cleavage site (Nünes-Duby *et al.*, 1994) and cleavage by the subunit at the distal half-site has also been ruled out for the XerC recombinase (Arciszewska *et al.*, 1995). There is, nevertheless, evidence that the tyrosine can be provided '*in trans*' for the cleavage of a suicide attL substrate (Han *et al.*, 1993). Our data for resolvase indicate that the Ser10 nucleophile and other catalytic residues are provided '*in cis*' by the subunit bound proximal to the scissile bond. While there is ample evidence of inter-subunit communication, as exemplified by the *trans*-complementation between the I110T and R68H mutants, we have no evidence for a composite catalytic site. However, the cocrystal structure raises the intriguing possibility that certain residues in the distal subunit may play a part in strand cleavage (Yang and Steitz, 1995).

The molecular mechanism of strand exchange by resolvase remains puzzling. Our data indicate that the catalytic domain of a resolvase protomer becomes covalently linked to the same half-site that is recognized by the DNA binding domain. The cocrystal structure shows in addition that the intervening 'arm' region of the protomer interacts with the same half-site (Yang and Steitz, 1995). This appears to rule out any scheme in which individual protomers form bridges between different

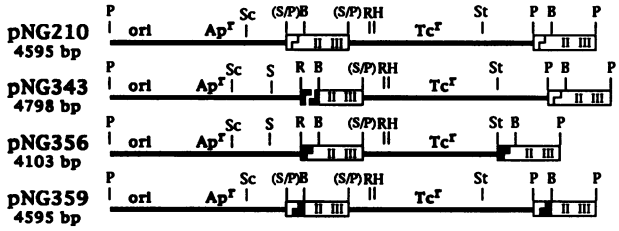

Fig. 11. Construction of plasmid substrates with various combinations of site I and site I^{G2T}. Black lines are pBR322 vector segments; boxes represent the *res* sites. Binding sites II and III (all with wild-type sequence) are indicated; site I has the crossover position marked. Halves of site I containing the G2T mutation are indicated in black; ori, Ap^r and Tc^r indicate plasmid segments encoding the replication origin, ampicillin resistance and tetracycline resistance respectively. Restriction sites are: P, *Pvu*II; B, *Bst*XI; H, *Hin*dIII; S, *Ssp*I; Sc, *Sca*I; St, *Sty*I; (S/P), the junction obtained by joining *Ssp*I and *Pvu*II ends.

half-sites, thereby facilitating the exchange of recombination partners, or preventing their dissociation. The efficient complementation between wild-type and R172L resolvases bound at $G2^+$ and G2T crossover sites discourages the idea that there is any dissociation and rearrangement of the DNA binding domains during recombination, since these domains would then be forced to bind to heterologous recognition sites. Crosslinking experiments have definitively shown that at least one of the DNA binding domains can be covalently linked to its recognition site throughout the recombination reaction (M.McIlwraith and W.M.Stark, personal communication). Our data suggest there is no obligatory order of single-strand cleavages, and we have seen no evidence that pairwise top- (or bottom-) strand exchanges can generate a Holliday junction when the other pair of strand exchanges is blocked (not shown). Instead, cleavage and religation at the top-strand sites is accompanied by relaxation of substrate supercoils (Figure 8). We suggest that the change in DNA linkage is accomplished by a rearrangement of the DNA strands (and possibly the resolvase subunit) at the left half-site, which takes place while the right half-site is covalently joined to resolvase (Figure 9). It is not clear how such a process might be related to the events in a complete recombination reaction.

Materials and methods

Plasmid recombination substrates

Construction of the plasmids used to 'target' resolvase protomers to particular locations at *res* site I is described in Figure 11. The substrate with two 'wild-type' *res* sites, pNG210, contains two copies of a 116 bp synthetic res^+ *Pvu*II fragment, inserted at the *Ssp*I and *Pvu*II sites of pBR322. This differs from the natural γδ *res* site at six positions: four create the *Bst*XI site between sites I and II (see Figure 3B) and two create the *Pvu*II sites at each end of the fragment (the C/T change at the extreme left end of site I modestly increases the affinity for resolvase); none of the substitutions affects *res* site function. Substrates with site I^{G2T} (or portions thereof) contain segments derived from a synthetic *Eco*RI–*Bst*X fragment; the sequence is shown in Figure 3B except for the *Eco*RI–*Bam*HI–*Sty*I linker at the left-hand end (GAATTCGGATCC-TTGG), and the complete *Bst*XI site CCATGCTGTTGG (which is identical to the site engineered into the *Pvu*II res^+ fragment). In addition to the G2T substitutions in both halves of site I, this fragment has the G4C substitution in the right half of site I (this substantially increases the affinity for resolvase; Rimphanitchayakit and Grindley, 1990) and a C to G substitution immediately adjacent to the right end of site I (to eliminate a potential competing binding sequence for wild-type resolvase shifted two base pairs to the right of site I–R). The desired segments

were introduced into pBR322 or precursors of pNG210 by a combination of standard cloning methods and resolvase-mediated site-specific recombination (to generate the hybrid crossover sites present in pNG356 and pNG359). Supercoiled plasmid substrates were made by a large-scale alkaline lysis method, followed either by CsCl/ethidium bromide density gradient centrifugation, or by chromatography on a Qiagen column.

Enzymes

The single-site mutants of resolvase used in these experiments have been described previously (Newman and Grindley, 1984; Hatfull and Grindley, 1986; Hatfull *et al.*, 1987; Hughes *et al.*, 1993; Mazzarelli *et al.*, 1993; Leschziner *et al.*, 1995). The double mutants were constructed by appropriate fragment exchanges of the single-mutant expression plasmids or their precursors. The double-mutant proteins were over-expressed and purified as previously described (Hatfull *et al.*, 1989). All the protein preparations were essentially homogeneous with respect to polypeptide size, as judged by SDS–PAGE. The relative concentrations of mutant and wild-type proteins were estimated by careful comparison of Coomassie-stained samples and standards after SDS–PAGE. The absolute concentration of the wild-type protein was estimated from the absorbance at 280 nm.

Cleavage and recombination reactions

All reactions contained ~15 μg/ml supercoiled plasmid in one of the following buffers. Recombination and relaxation reactions were in buffer 1: 10 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 100 mM NaCl, 50 mM Tris–HCl pH 8.2 (final concentrations), normally for 30–40 min at 37°C, but for 2–4 h for relaxation assays. Cleavage reactions (except for Figure 4) were in buffer 2: 1 mM EDTA, 40% ethylene glycol, 5% glycerol, 100 mM NaCl, 50 mM Tris–HCl pH 8.2 (final concentrations), and were normally for 2–4 h at 37°C. Cleavage reactions in buffer 2 appeared to be more efficient than in buffer 3, although recombination was not completely suppressed even at these very high concentrations of ethylene glycol. For the kinetics experiment, the cleavage reaction was initially in buffer 3: 1 mM EDTA, 5% glycerol, 100 mM NaCl, 50 mM Tris–HCl pH 8.2. For DNase I nicking, the samples (15 μl) were supplemented with 300 μg/ml ethidium bromide and 10 mM $MgCl_2$ (where required), nicked for 10 min at room temperature at 2 μg/ml DNase I, then supplemented with 10% glycerol, 0.02% SDS and 200 μg/ml protease K, incubated briefly at room temperature, then extracted with 10 μl of 1:1 phenol:chloroform and loaded directly onto the agarose gel.

For recombination and cleavage reactions, resolvase was diluted into a buffer containing 50% glycerol, 1 M NaCl, 0.1 mM EDTA, 0.5 mM DTT, 10 mM Tris–HCl pH 7.5, at 0°C. The 'premixed' combinations of resolvase mutants were made by mixing these dilutions and were incubated at 0°C for at least 10 min before use. Cleavage and recombination reactions (typically 20 μl) requiring two resolvase mutants were normally initiated at room temperature by adding premixed mutants (2 μl per 20 μl reaction), or by first adding the inactive resolvase mutant (1 μl), mixing, then adding the active enzyme (1 μl), usually after an interval of between 30 s and 2 min; the reactions were then transferred to 37°C. Although the order of addition apparently had little effect on the reaction products, premixed mutants were used for the single-strand cleavage experiments. Resolvase dilutions and premixed mutants were routinely stored at –20°C for several weeks.

Analysis of double-strand cleavage products

Cleavage reactions of pNG343 were quenched by the addition of SDS to 0.02%, glycerol to 10% and protease K to 0.1 mg/ml [or by the addition of SDS only (0.1%) to leave intact the resolvase chains linked to the DNA ends]. Samples were either analysed directly on agarose gels, or were digested with *Hin*dIII after purification of the DNA on Promega 'Magic' resin (as described below), then 3′-end-labelled using *exo*⁻ DNA polymerase I Klenow fragment. The labelled fragments were separated on large-format 1.2% horizontal agarose gels run under a glass plate; gels were stained and photographed, then vacuum-dried. Labelled reaction products were quantitated by phosphorimaging.

Analysis of single-strand cleavage products

Method 1: the cleavage reactions (40 μl) were quenched by addition of SDS to 0.1% and protease K to 1 mg/ml. After 10–15 min at room temperature, samples (20 μl) were run on 1.2% GTG low melting point agarose gels (Seaplaque). After ethidium staining and photography by brief exposure to 300 nm light, gel slices containing the nicked circular products were excised. The agarose was melted with 100 μl of H_2O at 70°C for 15 min, mixed vigorously, and 0.5 ml of 'Magic' resin, as supplied in 6 M guanidinium thiocyanate (Promega), was then added. The resin was washed with 1 ml 80% isopropanol, and the DNA was recovered in 40 μl of prewarmed 0.1 mM EDTA, 10 mM Tris–HCl pH 8.0, as recommended by the supplier. Samples of the DNA (8 μl) were then labelled at the resolvase-induced nicks in a 12 μl reaction containing 0.6 U of the 3′–5′ exonuclease-deficient mutant (D424A) exo⁻ mutant of DNA polymerase I Klenow fragment (Derbyshire *et al.*, 1988; a gift from Cathy Joyce), 4 μCi of carrier-free [α-^{32}P]dATP, 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10 mM Tris–HCl pH 7.9 for 10–15 min at room temperature. The polymerase was then heat-inactivated (10 min at 70°C), unlabelled dATP was added to 250 μM, and the DNA was digested to completion with *Hin*dIII and *Sca*I, which cut at unique sites flanking the 'left' crossover site of pNG356 and pNG359 as shown in Figure 11. Strands labelled at the top- and bottom-strand nicks were then separated on 6% sequencing gels by standard methods. Dried gels were autoradiographed at –70°C with an intensifying screen. Products were quantitated using a Fuji BAS 2000 Phosphorimager and MacBas image analysis software.

Method 2: the cleavage reactions were quenched as above and mixed with 50 μl of water. The DNA was absorbed to 200 μl of 'Magic' resin (Promega) and recovered in 20 μl of TE, as above. Labelling at the resolvase-induced nicks and cleavage with *Sca*I and *Hin*dIII was also as described. The DNA was then run on a non-denaturing 5% TBE gel, and the 662 bp *Sca*I–*Hin*dIII fragment of pNG356 (or the 865 bp *Sca*I–*Hin*dIII fragment of pNG356) containing the nicked and internally labelled *res* site was located and excised from the gel. The DNA was recovered by the crush-and-soak method and run on a 6% sequencing gel, as described. Note that method 2 does not exclude from the analysis any reaction products that may have been cut on both DNA strands at the other *res* site. Analysis methods 1 and 2 gave similar top-: bottom-strand cleavage ratios for the R172L + S10C, R172L + R68H and WT + R68H experiments with pNG356 and pNG359 (data not shown).

The concentrations of active and inactive resolvase protomers required for single-strand nicking were in all cases determined empirically by pilot experiments such as those shown in Figure 6. In general, a 2:1 ratio of mutant $R172^+$ protomers to active R172L protomers was adequate for targeted double-strand cleavage, while a higher excess of mutant $R172^+$ protomers was often necessary for targeted single-strand cleavage.

Electrophoresis

Denaturing and non-denaturing polyacrylamide gels were run by standard methods in TBE buffer. Agarose gel electrophoresis was in E buffer (20 mM NaOAc, 1 mM EDTA, 40 mM Tris/acetate pH 8.2). Gels were run at ~5 V/cm, and were stained with ethidium bromide (0.5 μg/ml) and photographed with Polaroid film on a 300 nm transilluminator.

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