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Step-Arrest Mutants of FLP Recombinase: Implications for the Catalytic Mechanism of DNA Recombination

RONALD L. PARSONS, PARUCHURI V. PRASAD, RASIKA M. HARSHEY, AND MAKKUNI JAYARAM*

Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037

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The site-specific recombinase (FLP) encoded by the yeast plasmid 2μm circle belongs to the integrase (of phage λ) family of recombinases. The sparse homology within the members of this family contrasts with the invariance of three residues, His-396, Arg-399, and Tyr-433 (the numbers correspond to the family alignment positions), among them. We report here results on substrate recognition and catalysis by FLP proteins altered at these residues. Mutations of the conserved His and Tyr that aborted the reaction at specific steps of catalysis permitted genetic dissection of the possible biochemical steps of recombination. We provide indirect evidence that recombination by FLP proceeds through a Holliday junction intermediate.

Site-specific recombination is characterized by relatively short recombining sites and by mechanisms that require no DNA synthesis or exogenous high-energy cofactors. The reaction is often catalyzed by a single protein with the help, in some instances, of one or two accessory proteins. The reaction involves recognition of the DNA substrate by the recombinase, followed by cleavage, exchange, and ligation of the participant DNA strands. During cleavage, the phosphodiester bond's energy is conserved via a phosphoprotein linkage. Strand exchange is catalyzed similarly by the topoisomerases (for a review, see reference 14). The procaryotic site-specific recombinases can be broadly classified into two families: the integrase (Int) family and the Hin family (3). The Int family includes site-specific recombinases encoded by phage λ (Int), by the λ-related phages 80, P22, 186, P4, and by phage P1 (Cre). The FLP recombinase of the yeast plasmid can also be grouped with the Int family (3). In contrast to the more homogeneous Hin family (invertases encoded by *Salmonella* spp. [Hin], *Escherichia coli* [Pin], phage Mu [Gin], and P1 [Cin], and resolvases encoded by Tn*3* and γδ), members of the *int* family are highly divergent in their primary sequences. The amino-terminal regions of the Int family members show little homology, whereas the carboxyl-terminal regions (including that of FLP) are moderately related. However, all members of the Int family share three conserved amino acids: His-396, Arg-399, and Tyr-433, which correspond to His-305, Arg-308, and Tyr-343, respectively, of FLP. This strict invariance is probably related to the similarity in the chemistry of reactions catalyzed by these proteins. The proteins Int, Cre, and FLP nick their DNA substrates at specific positions to create 3′ protein-bound termini and 5′ overhangs with free hydroxyl groups. The 5′ protruding ends are 6 base pairs (bp) long for Cre, 7 bp for Int, and 8 bp for FLP (2, 8, 15). Furthermore, Int and FLP establish a transient covalent linkage to DNA through a phosphotyrosine (6; C. Pargellis, S. Nunes-Duby, L. Vargas, and A. Landy, personal communication).

We showed earlier that alterations of Tyr-343 of FLP had little or no effect on substrate recognition (19). However, they abolished DNA cleavage and consequently recombination. Thus, Tyr-343 might indeed form a covalent linkage with DNA. We wished now to examine the possible roles of His-305 and Arg-308 in the substrate recognition and catalytic steps of recombination. Our results show that His-305 is not required for DNA cleavage but is essential for subsequent strand transfer and ligation. They imply that Arg-308 contributes to the tightness of the FLP-DNA complex and may play a role in DNA cleavage. We also provide indirect evidence that, analogous to recombinations catalyzed by Int and Cre (8a, 11, 16, 18), FLP recombination also probably proceeds via a Holliday intermediate.

MATERIALS AND METHODS

Plasmids. The construction of plasmids that express FLP in *E. coli* from the λp_R promoter and of the substrate plasmid (pJK) used for in vivo recombination assays has been described previously (10).

Mutagenesis. Mutations at His-305 and Arg-308 of FLP were introduced by the double primer method (17). Details of mutagenesis have been described (19). The accuracy of the mutations introduced was confirmed by DNA sequencing (13, 20).

Purification of FLP. Wild-type and mutant FLP preparations were partially purified by the protocol of Prasad et al. (19). These preparations, which were 25 to 40% pure, were used in in vitro DNA binding, DNA cleavage, and recombination assays. To determine the purity of FLP, preparations were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels, stained by Coomassie brilliant blue, and scanned by an LKB Ultrascan XL laser densitometer. In this gel system, FLP migrates with an apparent mobility of 45 kilodaltons (kDa). Actual FLP concentrations were then derived after estimation of total protein by using the Bio-Rad Laboratories kit with bovine serum albumin as the standard.

Recombination assays. Recombination assays in vivo were carried out as described previously (10). In vitro recombination assays were done by the method of Prasad et al. (19). The reaction mixtures (50 μl) contained 50 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 1 to 2% glycerol, 50 mM NaCl, 0.5 pmol each of the two substrate molecules, and 4 to 8 monomers of FLP (for the data in Fig. 1) per recombination site. Reactions were stopped with sodium dodecyl sulfate (0.2%) and processed further as described previously (19).

DNA binding and DNase footprinting. Binding reactions were done essentially as described by Prasad et al. (19).

* Corresponding author.

Approximately 0.02 to 0.05 pmol of the end-labeled substrate was incubated with FLP or the FLP mutants in a 50-μl volume containing 50 mM Tris hydrochloride, pH 7.5, 50 mM NaCl, 200 μg of bovine serum albumin per ml, 100 μg of denatured calf thymus DNA per ml, and 1 mM dithiothreitol for 30 min at 30°C. After addition of heparin (5 μg/ml) and incubation for 5 min at room temperature, 5 μl of a solution containing 10 mM Tris hydrochloride, pH 7.5, 30% (vol/vol) glycerol, and 0.1% bromophenol blue was added to the reaction mixture. Portions of the sample were electrophoresed on 5% polyacrylamide gels (30:1, cross-linking) at 4°C in 1× TBE (25 mM Tris hydrochloride [pH 7.4], 25 mM boric acid, 1 mM EDTA).

For footprinting, the binding reactions were carried out essentially as described above, except that the incubation mixtures included 10 mM $MgCl_2$. The subsequent steps were the same as described by Andrews et al. (2). Footprinting of the top strand was done with the substrate labeled at the *Eco*RI end. The pUC19 vector containing this substrate was first cut with *Eco*RI and filled in by the Klenow reaction with [α-^{32}P]dATP and the other three unlabeled deoxynucleoside triphosphates. The plasmid was then cut with *Hin*dIII, and the FLP substrate was purified by gel isolation.

DNA strand cleavage. The reaction mixtures contained (in a total volume of 100 μl) approximately 0.05 to 0.1 pmol of labeled DNA, 50 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 75 μg of sonicated calf thymus DNA. After incubation with FLP or FLP mutants at 30°C for 30 min, the reactions were stopped by the addition of 1 μl of 20% sodium dodecyl sulfate. Samples were treated with proteinase K (1 mg/ml) for 2 h at 37°C, phenol-chloroform extracted, and ethanol precipitated. The DNA was dissolved in 15 μl of 80% formamide–10 mM NaOH–0.1% xylene cyanol–0.1% bromophenol blue, heated at 90°C for 3 min, and run on 10% denaturing polyacrylamide gels (19:1 cross-linking).

Generation of synthetic FLP site Holliday junctions. Holliday junctions were obtained from four DNA fragments derived from the A and B forms of 2μm circle (7), each of which includes the FLP recombination site. After denaturation and reannealing (3a, 9), the χ structures were separated from the parental fragments by electrophoresis in low-gelling-temperature agarose, excised from the gels, and recovered by adsorption to glass powder by the Geneclean procedure (Bio-101, La Jolla, Calif.).

General methods. Bacterial transformations, isolation of plasmid DNA, restriction enzyme digestions, and other miscellaneous methods were done by published procedures (12). DNA sequences were determined by the method of Maxam and Gilbert (13) or Sanger et al. (20).

RESULTS

Recombination activity of FLP mutants in vivo. FLP mutants in which Tyr-343 was replaced by Ser or Phe recognized the DNA substrate normally but failed to execute strand cleavage (19). For this study, we replaced His-305 with Leu, Pro, or Gln and Arg-308 with Gly by using site-directed mutagenesis. The mutated *FLP* genes were then placed in plasmid vectors designed to drive *FLP* expression from the λp_R promoter, which was controlled by the temperature-sensitive repressor *c*I857 (19). The recombination substrate was a plasmid (compatible with the FLP expression plasmid) in which two directly repeated FLP sites bordered the Tn*5*-derived kanamycin resistance gene (10). Recombination between the FLP sites should result in loss of the kanamycin marker on a circular piece of DNA that lacks a replication origin. The recombination event is thus manifested by the production of kanamycin-susceptible cells from a *recA E. coli* host harboring the substrate plasmid.

TABLE 1. FLP recombination in vivo[a]

FLP	No. of transformants	
	Amp^r Cam^r Kan^s	Amp^r Cam^r Kan^r
Wild type	428	6
His-305→Pro	0	342
His-305→Leu	0	412
Arg-308→Gly	0	286
His-305→Gln	15	320

[a] Recombination assays in vivo were performed as described by Jayaram (10). The source of FLP or the FLP variant was a pBR322-derived plasmid (Amp^r). The substrate plasmid was a pACYC derivative (Cam^r). The substrate also contained the kanamycin resistance (Kan^r) gene from Tn*5* bordered by two direct repeats of the FLP site. Recombination between these sites caused deletion of the Kan^r gene and was manifested as Kan^s colonies. Absence of Kan^s declared a lack of FLP recombination. For FLP His-305→Gln, four randomly picked Kan^r transformants were diluted, plated with ampicillin and choramphenicol, and, after 24 h of growth at 32°C, replica plated on plates with the same two drugs plus kanamycin. The plates were scored after 6 h of growth at 40°C. There were 65 Amp^r Cam^r Kan^s transformants and 452 Amp^r Cam^r Kan^r transformants from the original four transformants after this treatment.

Three of the four mutants, FLP(His-305→Leu), FLP(His-305→Pro), and FLP(Arg-308→Gly), were inactive in recombination (Table 1). However, FLP(His-305→Gln) showed a low level of activity. In the initial screen, fewer than 5% of the primary transformants obtained with FLP(His-305→Gln) were kanamycin susceptible (Table 1). When individual kanamycin-resistant transformants were retested after plating out appropriate dilutions, 10 to 15% of the resultant colonies were found to be kanamycin susceptible.

Recombination activity of FLP mutants in vitro. To verify the in vivo results, in vitro recombination assays were done with partially purified wild-type and mutant FLP proteins. The assay is based on the fact that recombination between FLP sites on two suitably chosen DNA fragments (P1 and P2; Fig. 1) should produce two new fragments (R1 and R2) that differ in size from the parent molecules. The results of this assay agreed well with those of the in vivo assay. Preparations of FLP(His-305→Gln) fractions were quite variable in activity; some failed to yield detectable recombination in the in vitro assay. The results shown in Fig. 2 represent the most active of our FLP(His-305→Gln) preparations. In the assays with FLP(His-305→Leu) and FLP (His-305→Pro), unexpected DNA bands with lower electrophoretic mobility than the substrate DNA molecules were detected (Fig. 1). These reactions were stopped with sodium dodecyl sulfate-EDTA but not subjected to phenol extraction preceding electrophoresis. In phenol-treated samples, the extra bands, possibly related to specific catalytic defects of these mutants (discussed later in this section), were conspicuously absent (data not shown). They may represent substrate molecules with one or more FLP promoters covalently linked to them. Similar intermediates in FLP recombination have been observed earlier by Senecoff et al. (21). Alternatively, they may represent noncovalent protein-DNA complexes that were not completely dissociated. The results shown in Fig. 2 correspond to approximately 4 and 8 monomers of *FLP* per recombination site. For FLP(His-305→Pro), FLP(His-305→Leu), and FLP(Arg-308→Gly), no recombination could be detected up to a protein monomer-

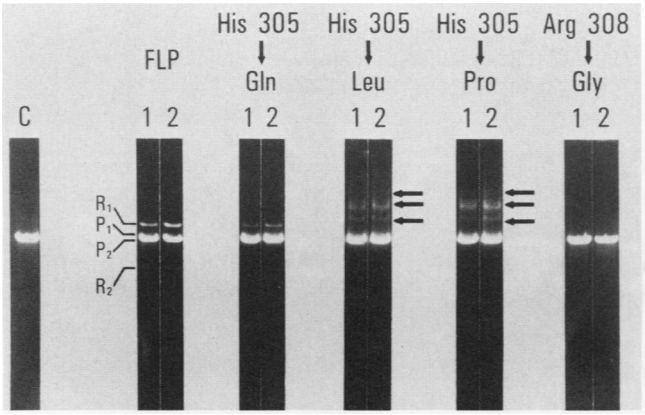

FIG. 1. In vitro FLP recombination. The substrate plasmid harboring the FLP site was linearized with two different restriction enzymes, and an equimolar mixture (0.5 pmol each) of the two types of linear molecules was incubated with partially pure (25 to 40%) FLP or FLP variant (19). The reaction mixtures contained approximately 4 to 8 promoters or monomers of FLP per substrate molecule. Since the relative locations of the FLP sites with respect to the ends of the molecules differed in the two linear types, recombination between them produced two DNA molecules of different sizes from their parents. The recombinants were fractionated by agarose gel electrophoresis and visualized by ethidium bromide staining. The parental DNA fragments are labeled P1 and P2, and the recombinant fragments are labeled R1 and R2. Arrows indicate the unexpected bands seen with FLP(His-305→Pro) and FLP(His-305→Leu). Total protein amounts were 0, 0.5, and 1.0 μg in lanes C, 1, and 2, respectively.

to-recombination site ratio of 40:1. Under our assay conditions, maximal recombination with the wild type was only about 15 to 20% conversion of substrates into products. The most active of the FLP(His-305→Gln) preparations gave no more than 3 to 4% recombination.

Substrate binding by the FLP mutants. The ability of the FLP mutants to bind normally to the substrate was assessed by gel retardation assays (5). Interaction between wild-type FLP and the substrate has been shown to result in three well-defined DNA-protein complexes, labeled CI, CII, and CIII in Fig. 2 (1, 19). These correspond to the binding of one, two, and three protomers of FLP, respectively, to the three symmetry elements of the FLP substrate (Fig. 2) (1). All of the mutants were capable of binding an FLP site-containing DNA fragment to generate the expected three complexes. The mutation of His-305→Gln had little or no effect on the binding affinity; all the other mutations resulted in weaker association between FLP and its substrate. The binding data in Fig. 2 can be used to obtain approximate estimates of the dissociation constants for each of the three FLP-DNA complexes. The values with wild-type FLP were 1×10^{-8} to 2×10^{-8} M for CI and 2×10^{-9} to 3×10^{-9} M for CII and CIII. While the change His-305→Gln did not alter these values significantly, substitution of His-305 by Pro or Leu increased them 4- to 10-fold. Among the mutants tested, FLP(Arg-308→Gly) showed the poorest binding to substrate. Strict quantitative comparison of the substrate affinities of the mutants was not possible, since the preparations used in these studies were rather crude.

DNase footprints of the mutants. We wished to determine whether the DNA-protein complexes formed by the FLP variants were qualitatively different from those of the wild-type complex. DNase footprinting of the mutants was done on the same substrate that was used for the gel retardation experiment. The results obtained for the His-305 mutants (with the substrate end labeled on the bottom strand) are shown in the top panel of Fig. 3. The footprints of the mutants were essentially identical to that of wild-type FLP. The patterns for FLP(His-305→Pro) and FLP(His-305→Leu) contained prominent bands corresponding to substrate cleavage by FLP. This band was significantly less intense in the footprints of wild-type FLP and FLP(His-305→Gln) (see also results on DNA cleavage by the mutants). Similar footprinting results were obtained with the substrate end labeled on the top strand as well (data not shown). No obvious footprint could be observed with FLP(Arg-308→Gly). While some weak DNase protection was apparent with the bottom strand, little or no protection was seen with the top strand even at protein concentrations sufficient to convert approximately 40 to 50% of the input substrate into DNA-protein complex as determined by the gel retardation assay (Fig. 3, bottom panel).

Strand cleavage by FLP mutants. We showed earlier that Tyr-343 variants of FLP were blocked in DNA cleavage (19). Substitution of Arg-308 with Gly also abolished cleavage (data not shown). However, FLP variants with substitutions of His-305 were active in DNA cleavage (Fig. 4). This result was not unexpected for FLP(His-305→Gln), since this protein was weakly active in recombination (Table 1, Fig. 1). The results with FLP(His-305→Leu) and FLP(His-305→Pro), although surprising, were quite significant. These proteins carried out the first step of recombination, DNA cleavage, but failed to accomplish subsequent strand exchange and religation. All mutants of His-305, FLP(His-305→Gln), FLP(His-305→Leu), and FLP(His-305→Pro), accumulated the cleavage product at higher levels than wild-type FLP (Fig. 4). Among the mutants, the extent of cleavage decreased in the order FLP(His-305→Pro) > FLP (His-305→Leu) > FLP(His-305→Gln). Since the strand union step between substrates is either blocked or severely hindered in these FLP variants, it is not surprising that they produce the cleavage intermediate at higher levels than normal. However, attempts to derive the entire input substrate into the cleaved product by FLP(His-305→Pro) have not been successful (Parsons, unpublished results). This would suggest that in the absence of strand exchange, the nicked strand can be resealed by a reversal of the cleavage reaction. The requirement of higher levels of FLP(His-305→Pro) and of FLP(His-305→Leu) for cleavage saturation (Fig. 4) probably reflects the lower substrate affinities of these proteins. As with wild-type FLP, cleavage by the His-305 mutants apparently resulted in a free 5′-OH (Fig. 4) and a 3′ protein-bound phosphate. When the substrate used for the cleavage assay was labeled at the 5′ phosphate (by the polynucleotide kinase reaction in the presence of [γ-^{32}P]-ATP), no cleavage band could be detected unless the samples were proteinase K treated (data not shown). We assume that DNA-protein attachment for the mutants takes place through the same Tyr residue as for FLP.

Strand exchange mechanism during FLP recombination. The Holliday junction intermediate predicted for a single-strand exchange mechanism has not been observed during normal FLP reactions. To test whether FLP can resolve a predicted Holliday intermediate, we constructed synthetic FLP site Holliday junctions by a procedure analogous to that used to create λ *att* site Holliday junctions (Fig. 5) (9). The four DNA fragments used for this construction were derived from the A and B forms of 2μm circle (7), which arise as a result of intramolecular FLP recombination. The Holliday junctions (χ structures) that denaturation and reannealing of

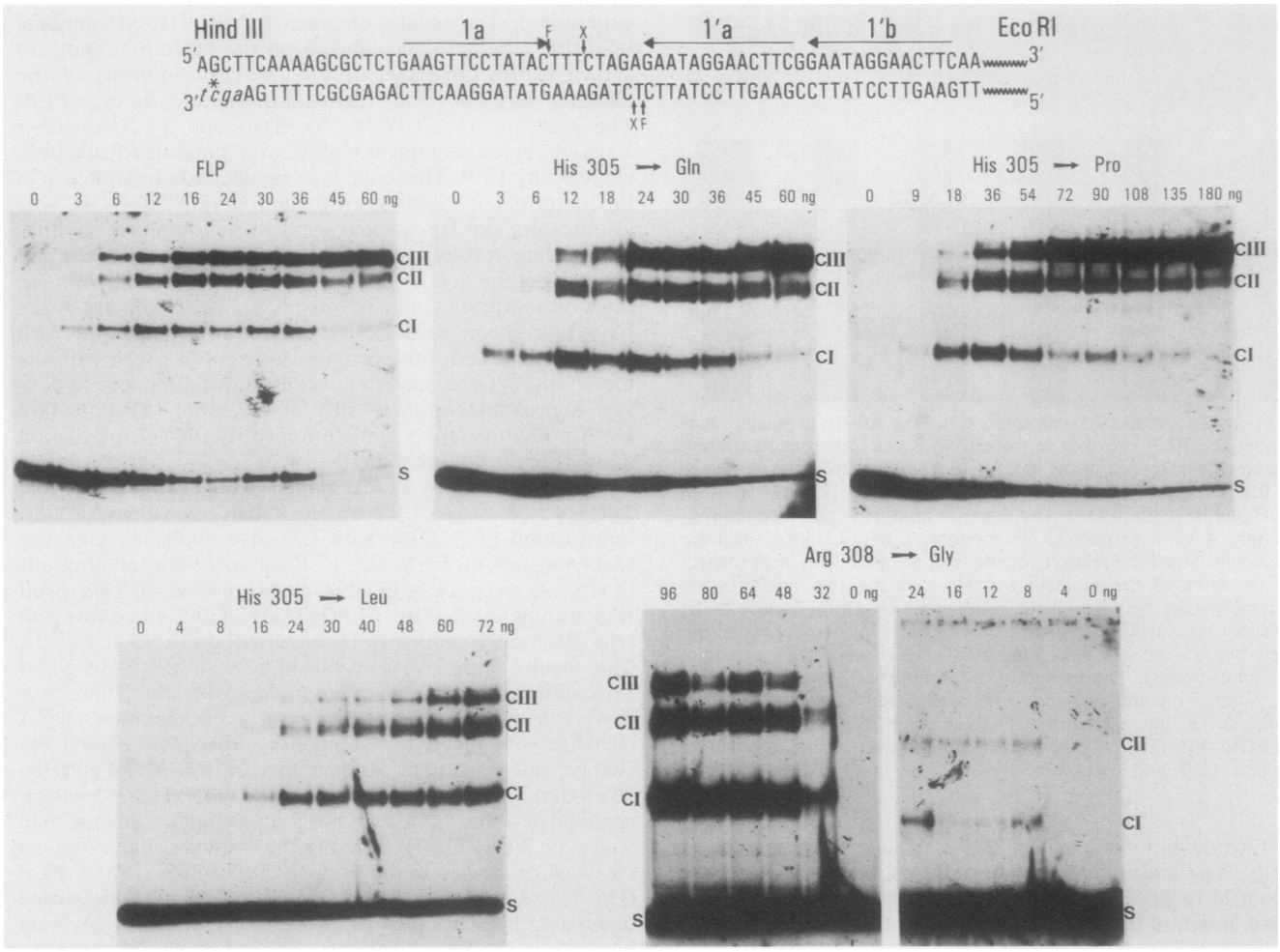

FIG. 2. Substrate binding. The substrate used for the binding assays has been described earlier (19) and is shown in the top panel. This 100-bp substrate includes the complete FLP site consisting of the symmetry elements 1a, 1'a, and 1'b plus the 8-bp spacer of the 1a-1'a dyad. The wavy line indicates sequences derived from the vector. The positions of cleavage by FLP (F) and *Xba*I (X) within the spacer are indicated. Binding reactions were carried out with 3'-end labeled substrate (0.02 to 0.05 pmol per reaction), labeled on the bottom strand with [α-^{32}P]dCTP by the Klenow reaction. The bases introduced by the Klenow enzyme are italicized, and the position of the label is indicated by an asterisk. The details of the binding reactions were described in Prasad et al. (19). Samples were run on 5% polyacrylamide gels at 4°C in Tris-borate buffer and autoradiographed. S, Substrate; the FLP-DNA complexes are labeled CI, CII, and CIII. Concentrations of FLP or of FLP variants are indicated above each lane.

the DNA fragments should produce are shown schematically in Fig. 5 (top) (3a). The gel-purified χ structures, when incubated with wild-type FLP, gave rise to the expected linear recombinant products (Fig. 5, bottom). In addition, in the gel assay for recombination reaction between ^{32}P-labeled fragments II and III (Fig. 5), a small amount of radioactivity migrated at a position expected for the χ intermediate (data not shown). The amount of DNA present in this band was not sufficient for recovery from the gel and electron microscopy. Attempts are being made to scale up the reaction so as to isolate enough of the presumed χ structure for electron microscopic examination. When a chemically synthesized Holliday structure (a gift from Fred Heffron and Merl Hoekstra) was used as the substrate, no resolution by FLP was observed (data not shown). Thus, FLP cannot resolve any Holliday junction; furthermore, our FLP preparations were not contaminated with nonspecific Holliday-resolving activities. Finally, cleavage-incompetent *FLP* mutants were also inactive in Holliday resolution (Parsons and Jayaram, unpublished results).

DISCUSSION

The mutational analyses of His-305 and Arg-308 of FLP described here, together with our previous results on alterations of Tyr-343, shed further light on the possible significance of the absolute conservation of these three residues within the *int* family of recombinases. Mutations of Tyr-343 did not affect DNA binding but blocked cleavage of DNA. This Tyr is most likely involved in strand breakage and covalent attachment to DNA. The one mutation of Arg-308 examined here (Arg→Gly) also abolished DNA cleavage. However, this mutant was affected in its substrate recognition. Although it gave the apparently normal three DNA-protein complexes (CI, CII, and CIII, Fig. 2) in gel retardation assays, no clear DNAse footprints could be obtained for this protein. Earlier studies had shown that with wild-type FLP, complexes CII and CIII were footprintable while CI was not (1). We conclude that the lack of Arg-308 interferes with some important DNA contact that is essential for DNase protection. It is likely that the positive charge on the

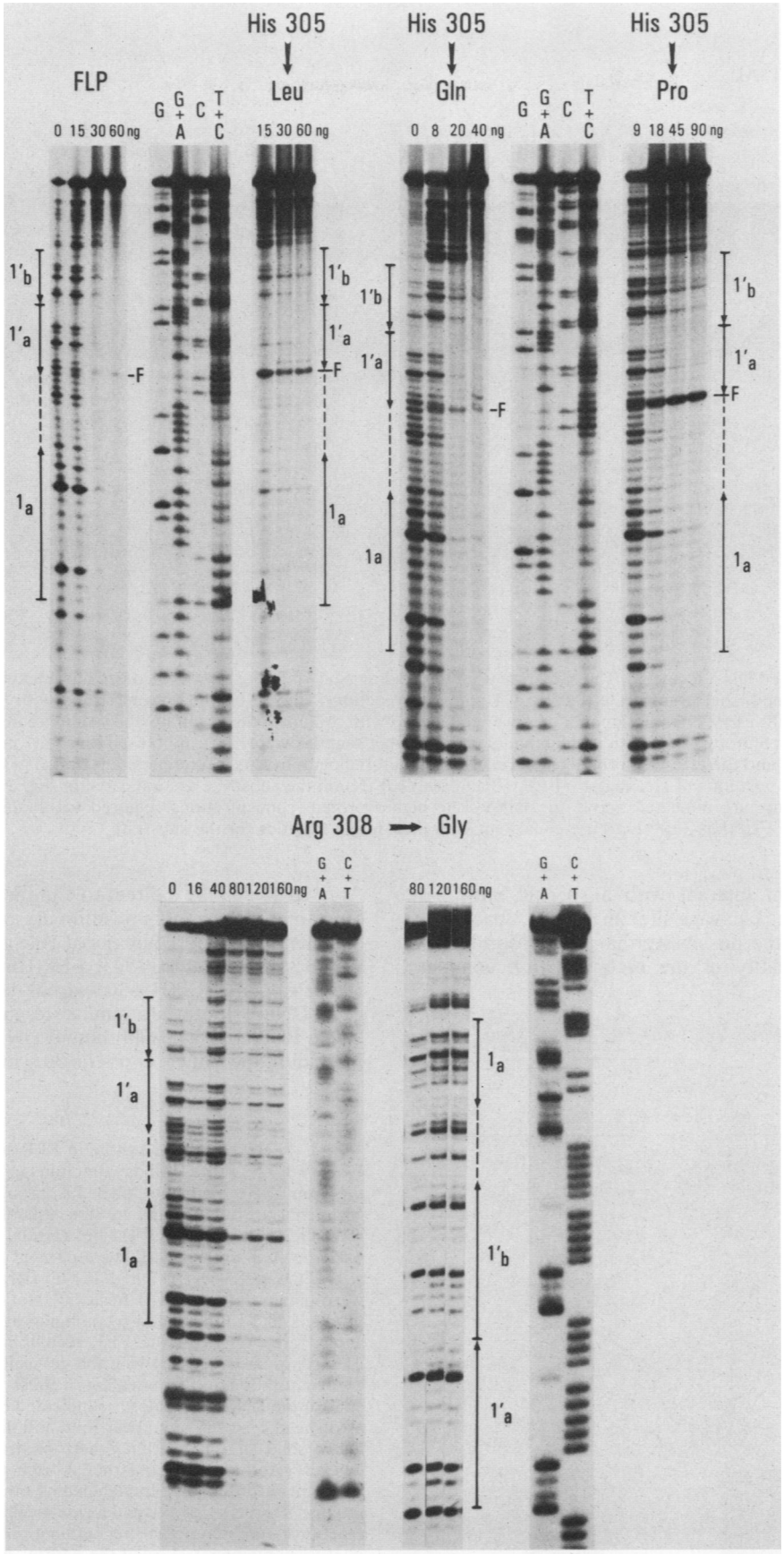

FIG. 3. DNase footprinting. The substrate used for the footprinting assays (see top panel of Fig. 2) was labeled with ^{32}P at the *Hin*dIII end on the bottom strand or at the *Eco*RI end on the top strand. In the top panel, the results of footprinting with the bottom strand are shown for wild-type FLP and the His-305 mutants. Samples were run on 12% sequencing gels. The band labeled F corresponds to FLP-induced cleavage of the substrate. This band is more prominent in the footprints of FLP(His-305→Pro) and FLP(His-305→Leu) than in the footprints of FLP or of FLP(His-305→Gln). In the bottom panel, the patterns obtained with bottom-strand (left) or top-strand (right) labeled substrate for FLP(Arg-308→Gly) are shown. The samples on the left (bottom-strand labeled) were run on a 12% sequencing gel, while those on the right (top strand) were run on a 10% sequencing gel. The amounts of FLP or FLP variants are shown above the corresponding lanes. A sequence ladder of the substrate (obtained by Maxam-Gilbert chemical sequencing) was run alongside the footprint lanes for alignment. The region corresponding to the FLP substrate (1a-1'a, 1'b; see Fig. 2) is indicated on the footprints.

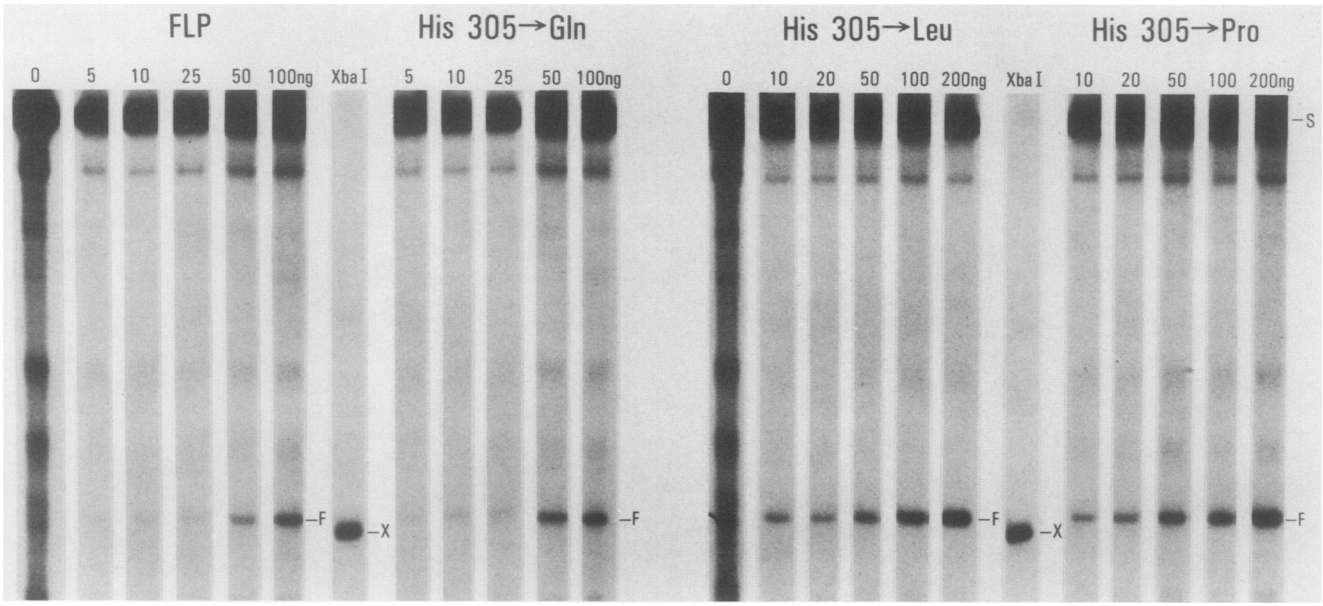

FIG. 4. FLP-promoted DNA cleavage. The substrate used for binding (shown in Fig. 2) was also used for cleavage assays. The reactions were done with the 3′-end-labeled substrate (0.05 to 0.1 pmol per reaction), labeled on the bottom strand with [α-^{32}P]dCTP (see Fig. 2). Following incubation with FLP (19), reaction mixtures were quenched with sodium dodecyl sulfate (0.2%) and treated with 1 mg of proteinase K per ml. After phenol-chloroform extraction and ethanol precipitation, samples were electrophoresed on a 10% denaturing polyacrylamide gel (19:1 cross-linking), and the cleavage product was revealed by autoradiography. As a reference, an *Xba*I digest of the labeled fragment was run alongside. The positions of cleavage by FLP (F) and by *Xba*I (X) on the substrate are indicated in Fig. 2. The amounts of FLP as well as the FLP mutants are indicated above the lanes. The higher protein concentrations required to obtain maximal cleavage with FLP(His-305→Pro) and FLP(His-305→Leu) are consistent with their lower affinities for the substrate.

Arg side chain might interact with a specific phosphate (perhaps the one at the cleavage site) on the DNA backbone. We cannot rule out the possibility that in addition to contributing to the stability of the recombination complex, Arg-308 could play a direct role in the cleavage step as well. More mutations at this position are required before we can confidently assign a functional role for Arg-308. The FLP variants altered at His-305, FLP(His-305→Gln), FLP(His-305→Pro), and FLP(His-305→Leu), bound the substrate and gave DNase footprints similar to that of wild-type FLP. While FLP(His-305→Gln) bound DNA as well as wild-type FLP did, the other two His-305 mutants showed lower

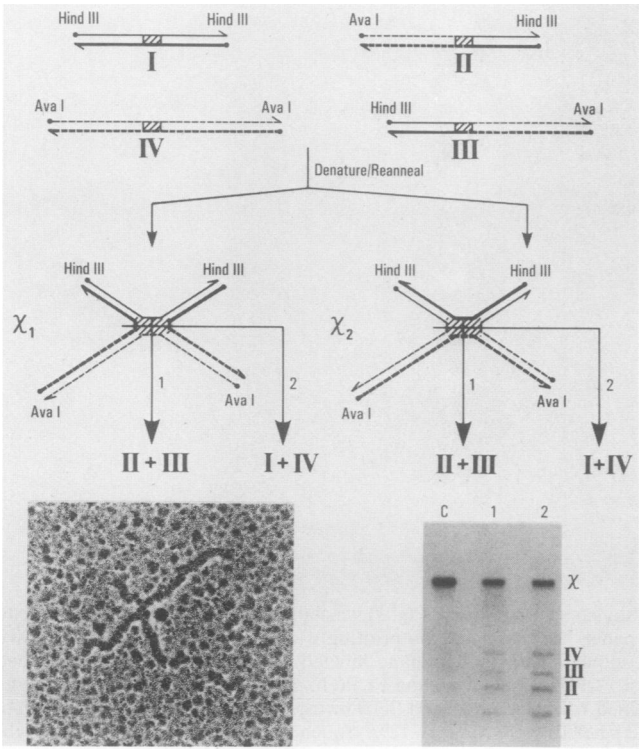

FIG. 5. Resolution of synthetic FLP site Holliday junctions by FLP. (Top) FLP site Holliday junctions χ_1 and χ_2 were created from the 2μm circle fragments labeled I through IV. Notice that these fragments are all unequal in size. All of them contain the FLP recombination site. The 912-bp *Hin*dIII fragment (IV) and the 1,506-bp *Ava*I fragment (I) were derived from the A form of 2μm circle; the 1,287-bp (II) and 1,131-bp (III) *Hin*dIII-*Ava*I fragments were obtained from the B form (7). Notice that I and IV are the products of FLP-mediated recombination between II and III. The stipled area refers to the FLP recombination site. (Bottom left) Electron micrograph showing the gel-isolated χ structure obtained by denaturation and reannealing of these fragments. The 5′ and 3′ ends of the DNA molecules are indicated by the solid circle and the arrowhead, respectively. Thin solid and dashed lines, Top strands; heavy solid and dashed lines, bottom strands. (Bottom right) ^{32}P-labeled χ forms were constructed from an equimolar mixture of fragments I through IV, end labeled by the Klenow reaction with all four α-^{32}P-labeled deoxynucleoside triphosphates. The resolution reactions were identical to recombination reactions. Each reaction mixture contained 0.25 μg of the χ form. After incubation with FLP for 30 min at 30°C, the reactions were phenol extracted, ethanol precipitated, and electrophoresed on 1.2% agarose gels. DNA was transferred from gels to nitrocellulose and visualized by autoradiography. Total protein amounts (20% FLP) were 0, 0.5, and 1.0 μg in lanes C, 1, and 2, respectively. The gel-isolated χ structures are slightly contaminated with the parental DNA fragments (lane C).

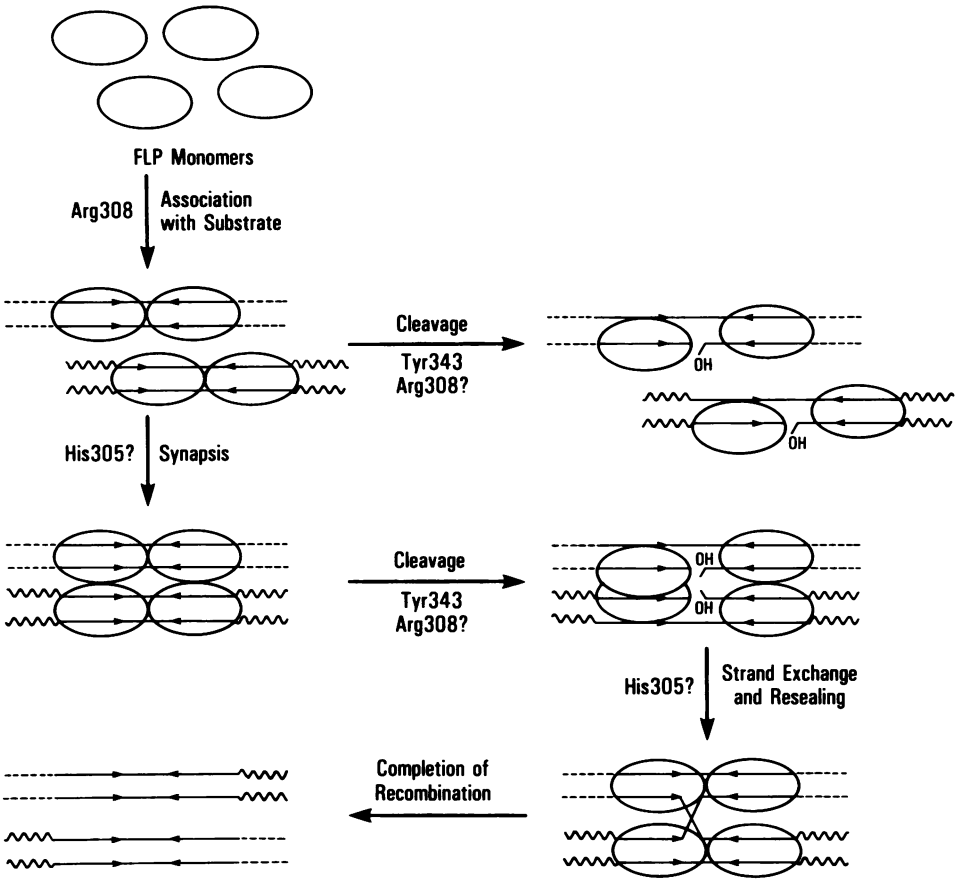

FIG. 6. Role of the conserved family residues of FLP in specific steps of recombination. The various steps in the FLP recombination pathway are diagrammed. The possible steps at which His-305, Arg-308, and Tyr-343 might function are indicated. Arg-308 is required for stabilizing the FLP-DNA complex but may also be required for the DNA cleavage reaction. Tyr-343 is critical to DNA cleavage and concomitant DNA-protein attachment. His-305 functions either at the synapsis step or at the strand reunion step or both, but is not required for DNA cleavage.

binding affinity. All three mutants were active in DNA cleavage but were severely affected in a subsequent step(s) of recombination, strand exchange or religation or both. With the Gln substituent, an extremely low level of recombination could be observed; with the Pro and Leu substituents, there was no detectable recombination. Two possible functional roles (not mutually exclusive) could be envisaged for His-305. His-305 may be necessary for the proper synapsis of the substrates in the recombination complex so as to allow normal strand transfer. Alternatively, His-305 may serve a catalytic function in the strand reunion step. In Fig. 6, we have schematically represented the various steps in the recombination pathway and indicated at which of these steps His-305, Arg-308, and Tyr-343 might function.

The recombination reaction can be considered to be the sum of two partial reactions: cleavage of DNA strands (which is analogous to the action of nucleases), and rejoining of two cut strands between the recombination partners (which, in its chemistry, is a direct reversal of the first step). The active-site residues and the mechanism of catalysis of bovine pancreatic RNase have been well established (reviewed in reference 4). Some important clues to the mechanism of staphylococcal nuclease are also available (4). It is interesting that two His residues and two Arg residues are key participants in catalysis by ribonuclease and staphylococcal nuclease, respectively. It would not be surprising if His-305 and Arg-308 played similar roles in *FLP* recombination. Here, we suggest that Tyr-343 of FLP leads a nucleophilic attack on the phosphodiester bond at the crossover site, causing DNA cleavage. Conceivably, Arg-308 could be hydrogen bonded to this phosphate (analogous to Arg-35 and Arg-87 of staphylococcal nuclease), activating it towards nucleophilic attack. Thus, a catalytic role for Arg-308 in recombination is not exclusive to but could result directly from its role in establishing a tight recombination complex (this study). Clearly, His-305 is not required for the strand cleavage step but is essential to complete recombination. It may act as a general acid-base catalyst in the *trans*-esterification reaction between the tyrosylphosphate and the 5′-hydroxyl of DNA following strand swap. Thus, the catalytic function of His-305 in recombination may be analogous to that of His-119 or His-12 in the cyclization and hydrolysis steps, respectively, of the RNase A reaction (4). The absolute conservation of the trio of His, Arg, and Tyr within the Int family suggests that the plausible mechanism of catalysis proposed here for FLP may be generally applicable to all members of this family.

In principle, one round of normal FLP recombination could proceed in a single step of concerted double-strand breakage and exchange or in two steps of single-strand breakage and union. In in vitro reactions, FLP can cause both single-strand nicks and double-strand breaks in its

substrate DNA, although the extent of nicks significantly exceeds that of breaks (2). Our results bear upon whether χ structures (the products of single-strand exchange) are possible intermediates in FLP recombination. The ability of FLP to resolve these structures into recombinant products provides one important test for their authenticity as recombination intermediates. Thus, the mechanism of strand exchange by FLP may mimic that mediated by Int and Cre, both of which generate Holliday intermediates (8a, 11, 18). These preliminary results encourage experiments to test whether FLP would resolve a synthetic FLP site Holliday junction equally well in the two possible modes. Assuming that Holliday structures are normal intermediates in the FLP recombination pathway, we may now design substrates to test whether FLP, like Int and Cre, executes the two steps of strand exchange in a strictly prescribed order (8a, 11, 18).

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