Holliday junctions in FLP recombination: Resolution by step-arrest mutants of FLP protein

Makkuni Jayaram, Karen L. Crain, Ronald L. Parsons, and Rasika M. Harshy

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

Communicated by E. Peter Geiduschek, July 12, 1988 (received for review November 20, 1987)

ABSTRACT The FLP “recombinase” of the 2-μm circle yeast plasmid can resolve synthetic FLP site–Holliday junctions. Mutants of the FLP protein that are blocked in recombination but are normal in substrate cleavage can also mediate resolution. The products of resolution by these mutants are almost exclusively nicked molecules with a protein-bound 3’ end. There is no significant asymmetry in strand cleavage (top versus bottom) by the mutants in linear or in circular FLP substrates; nor is there a bias in resolution (toward parental or toward recombinants) of Holliday junctions (corresponding to top- or to bottom-strand exchange) by wild-type FLP. During normal FLP recombination, a small amount of the expected Holliday intermediate can be detected.

The Int ("integrase" of phage λ) family of site-specific "recombinases" is characterized by the highly divergent primary amino acid sequences of its members and the absolute conservation of only three residues, His-396, Arg-399, and Tyr-433, among them (1). Properties of three members of this family, Int of phage λ, Cre of phage P1, and FLP of the yeast 2-μm circle, suggest that the strict invariance of this triad relates to the similarity in strand cleavage and exchange catalyzed by these proteins (2–4). Both FLP and Int establish a transient covalent link between DNA and protein during recombination through a phosphotyrosine (5, 6). Alterations of Tyr-343 (family position 433) of FLP have little effect on substrate recognition but abolish DNA cleavage and, consequently, recombination (7). This result suggests that Tyr-343 is the residue involved in covalent attachment to DNA. The DNA–protein linkage in the Int reaction has now been mapped to the invariant tyrosine residue (6). Unlike the FLP variants mutated at the Tyr-343 position, the variants at the His-305 position cleave the substrate normally but are blocked in the subsequent steps of strand exchange and rejoining.

Does the FLP recombination reaction proceed by a concerted double-strand breaking and rejoicing mechanism or by a sequential two-step mechanism in which single-strand scission and reunion take place? The latter mechanism would predict that a Holliday junction is a normal intermediate in the reaction. For both Int and Cre, strong evidence supports formation of the Holliday intermediate in recombination (8–10). For FLP, which can cause both single-strand nicks and double-strand breaks in its DNA substrate in vitro (2, 11), the relative preponderance of single-strand cuts suggests that the more likely mode of strand exchange involves single-chain breaking and resealing.

We report here that FLP and His-305-mutant FLP (which are normal in substrate cleavage) can resolve synthetic FLP site–Holliday junctions. In addition, a small amount of Holliday structures can be detected during normal FLP recombination. Whereas the His-305-mutant FLP proteins show no significant strand asymmetry during cleavage of the substrate, wild-type FLP displays no bias in the mode of resolution of synthetic FLP site–Holliday junctions.

MATERIALS AND METHODS

The Step-Arrest Mutants of FLP. Variants of FLP protein mutated at His-305 or at Tyr-343 were obtained by site-directed mutagenesis as described (7). The mutant FLP genes were cloned in Escherichia coli plasmids and expressed from the phage λ Pλ promoter.

Purification of FLP and FLP Variants. Partially purified proteins were obtained from E. coli strains expressing them by the procedure outlined by Prasad et al. (7). The preparations used in these studies were 20–40% pure.

Generation of Synthetic FLP Site–Holliday Junctions. Holliday junctions were obtained from four DNA fragments derived from the A and B forms of the yeast 2-μm circle plasmid (12), each of which includes the FLP recombination site. After denaturation and hybridization (13, 14), the chi structures were separated from the parental fragments by repeated electrophoresis in low-gelling agarose, were excised from the gels, and were recovered by adsorption to glass powder using the GeneClean kit (ref. 15; Bio101, La Jolla, CA).

To selectively label one of the two possible χ structures, denaturation and hybridization were carried out with three unlabeled fragments and one fragment that was radioactively end-labeled on the top or the bottom strand.

Resolution of Holliday Junctions. The conditions were the same as those for in vitro recombination assays described previously (7), with 0.02–0.05 pmol of 32P-labeled χ structure per reaction. Approximately 0.2 pmol each of two unlabeled FLP substrates were also included in several of these reactions so that the extent of resolution could be monitored on agarose gels by ethidium bromide staining. Reactions were stopped by adding NaDodSO4 (0.2%). The samples [some of which were treated with proteinase K (1 mg/ml for 1 hr at 37°C) were phenol/chloroform-extracted, and ethanol-precipitated prior to electrophoresis. For denaturing agarose gels, samples were made 0.25 M in NaOH and 10 mM in EDTA, and were run in E buffer (40 mM Tris·HCl, pH 7.8/20 mM sodium acetate/2 mM EDTA).

Recombination Reactions. In vitro recombination were carried out essentially as described by Prasad et al. (7) except that the reaction mixtures contained 25% (vol/vol) glycerol and were incubated for 10 min at 30°C.

Strand Cleavage on Linear Substrates. The reactions were done with 0.05–0.1 pmol of labeled DNA as described (7). After incubation with FLP, samples were treated with proteinase K (1 mg/ml) for 1 hr at 37°C and then 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 crosslinking).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
Strand Cleavage on Circular Substrates. The conditions were essentially identical to those for linear substrates except that reactions were carried out in 50 μl with ~1 pmol of unlabeled DNA. After incubation with FLP (10–20 molecules of FLP monomer per FLP site), the reaction mixtures were desalted on a spin-dialysis column (Sepharose CL-6B, Pharmacia) and treated with phage T4 polynucleotide kinase in the presence of [γ-32P]ATP (>3000 Ci/mmol; 1 Ci = 37 GBq) to phosphorylate the 5' OH resulting from cleavage by FLP. The reaction was stopped by heating the samples at 65°C for 5 min. After removal of unreacted ATP by spin-dialysis through Sepharose CL-6B, samples were digested with restriction enzymes that flank the FLP cleavage sites. Digests were heat-inactivated (65°C, 10 min), freed of salt by spin-dialysis on Bio-Gel P-30 (Bio-Rad), and vacuum-dried. Dissolution of the samples and electrophoresis in denaturing polyacrylamide gels were as described for the linear substrates.

Electron Microscopy. DNA samples were prepared by the basic protein film procedure (16) and platinum-shadowed before examination by electron microscopy.

General Methods. Bacterial transformations, isolation of plasmid DNA, restriction enzyme digestions, and other miscellaneous methods followed published procedures (17).

RESULTS

Formation of Holliday Structures in FLP Recombination. Can we detect the predicted single-strand exchange interme-
diate (Holliday junction) in an in vitro FLP-mediated recombination reaction? To locate this putative intermediate, we constructed marker Holliday structures (chi structures) from DNA fragments I through IV derived from the A and B forms of the 2-μm circle (Fig. 1A; refs. 12 and 18). Fragments I and IV represent the products of FLP recombination between fragments II and III. The results of a recombination reaction between 32P-labeled fragments II and III with increasing concentrations of wild-type FLP are shown in Fig. 1B. A small amount of radioactivity comigrated with the marker chi structure. The amount of this radiolabeled band increased proportionally as the extent of recombination rose. In this reaction system, the various possible recombinations can result in only four products, fragments I, II, III, and IV.

However, there would be nine possible Holliday structures with differing arm lengths corresponding to self and nonself interactions between these four fragments. These additional chi structures or some other recombination intermediates could account for the extra bands seen in the vicinity of the reporter chi. However, all of the chi structures, except for chi1 and chi2, should be relatively unstable because of the freedom for uni- or bidirectional branch migration. We pooled the DNA from 10 reactions corresponding to lane 3 of Fig. 1B, and, after electrophoresis, eluted the presumed chi band from a gel in the presence of a 30-nucleotide-long synthetic oligodeoxynucleotide as carrier. Electron microscopy of this sample revealed the expected cross-stranded structures with the predicted arm lengths.

![Fig. 1. Holliday junctions in FLP recombination.](image)

(A) Fragments I through IV, each containing the FLP site (hatched area), were denatured and rehybridized to obtain the Holliday junctions chi1 and chi2 (χ1 and χ2) (13). The 1506-base-pair (bp) AvaI fragment I and the 912-bp HindIII fragment IV were derived from the A form of the yeast 2-μm circle; the 1287-bp (II) and the 1131-bp (III) AvaI–HindIII fragments were obtained from the B form (12). Notice that fragments I and IV are the products of FLP-mediated recombination between fragments II and III. (B) Recombination reactions were carried out with 0.2 pmol each of fragments II and III end-labeled by the Klengow reaction in the presence of all four deoxynucleoside [α-32P]triphosphates. The samples were fractionated on 1.2% agarose gels. Lanes 1–4 correspond to 1, 2, 4, and 6 μl (~10–20 monomers of FLP per recombination site) of protein, respectively. The synthetic chi structures obtained from 32P-labeled fragments I–IV were run for reference. (C) Pooled DNA from 10 reactions corresponding to lane 3 in B was gel-fractionated, and the band that comigrated with the synthetic chi structure was excised, eluted in the presence of a 30-base synthetic oligodeoxynucleotide as carrier, and subjected to electron microscopy. The mottled background is due to the carrier oligodeoxynucleotide.
(Fig. 1C). Approximately 50–60% of the observed molecules accounted for \( \chi \) structures; the remainder consisted of a mixture of linear structures, three-armed structures, and molecules that were difficult to interpret. Apparently, then, Holliday structures are indeed formed during normal FLP recombination, although their concentration does not exceed 1–2% of that of the recombinants.

**Resolution by FLP of \( \chi \) Structures Harboring Top or Bottom Strand Exchange.** Assuming that a Holliday structure is a normal intermediate in FLP recombination, is there a prescribed order for the bottom- and top-strand exchanges? Such a temporal sequence in strand transfer would imply that each of the two synthetic \( \chi \) structures shown in Fig. 1A represents the intermediate specific to the forward or to the backward steps of the reaction \( I + IV \rightleftharpoons II + III \). This, in turn, should reveal itself as an asymmetry in the mode of resolution of \( \chi 1 \) and \( \chi 2 \) by FLP. To test this expectation, we selectively labeled \( \chi 1 \) or \( \chi 2 \) and used each of them as substrates in resolution reactions by FLP (Fig. 2 A and B). Both \( \chi 1 \) and \( \chi 2 \) were resolved with roughly equal facility in the parental or in the recombinant mode. Thus, Holliday structures generated from linear FLP substrates were symmetric in resolution.

**Resolution of Holliday Junctions by Step-Arrest Mutants of FLP.** The strand-cleavage activity of FLP, even in the absence of the strand-joining function, should be sufficient to mediate resolution of a FLP site—Holliday structure. Thus, one would expect the Tyr-343-mutant FLP (7) to be inactive and the His-305-mutant FLP (18) to be active in \( \chi \)-structure resolution. When a mixture of \( ^{32} \)P-labeled \( \chi 1 \) and unlabeled \( \chi 2 \) (constructed using fragment II labeled on the bottom strand; see Fig. 1A) was used as substrate, \([\text{Phe}^{143}]\text{FLP and [Ser}^{143}]\underline{\text{FLP failed to resolve the \( \chi \) structure, but [Pro}^{165}]\underline{\text{FLP and [Leu}^{165}]\underline{\text{FLP gave rise to the predicted resolution products (Fig. 2B). These products were apparent on agarose gels only if the reaction mixtures were protease-treated preceding phenol extraction and gel fractionation. These results imply that strand cleavage within the \( \chi \) structure by the His-305-mutant FLP resulted in nicked DNA molecules in which protein was covalently attached to the 3' end of the nick. Alkaline agarose electrophoresis (Fig. 2C) revealed a labeled product that corresponded in size with the expected position of the nick (see also strand-nicking described later).

**His-305-mutant FLP Proteins Show No More Asymmetry in Strand-Nicking Than Does Wild-Type FLP.** The few single-strand nicked molecules seen during FLP recombination (2, 11) most likely represent substrates frozen in the act of exchanging strands. One simple way to assign a hierarchy of strand exchange in recombination (as in the Int reaction and apparently in the Cre reaction; refs. 8–10) is to impart asymmetry in the strand-cleavage step. If cleavage and exchange of one specific pair of strands (say top strands) are prerequisites for the cleavage of the other pair (bottom strands), prevention of the first exchange must abolish the second cleavage step. Therefore, we looked for possible asymmetry in strand cleavage by His-305-mutant FLP, which can cut the DNA but cannot accomplish strand exchange and ligation. We verified that DNA cleavage by the His-305-

![Fig. 2. Resolution of \( \chi \) (\( \chi _{1} \)) structures by normal and mutant FLP. (A and B) Selectively labeled \( \chi \) structures were prepared from a mixture of unlabeled fragments I, III, and IV, and fragment II was labeled selectively on the top or the bottom strand (indicated by the asterisk). The plasmid containing fragment II was first cut with HindIII (for labeling \( \chi 1 \)) or with Ava I (for labeling \( \chi 2 \)), treated with alkaline phosphatase, and labeled with \( ^{32} \)P-ATP by using T4 polynucleotide kinase. Fragment II was then excised by digestion with the second enzyme (Ava I or HindIII, respectively), gel-fractionated, purified, and used to build the \( \chi \) structure. Resolution assays were carried out under normal recombination conditions (\( \sim \)10–20 monomers of FLP or mutant FLP per recombination site). Products of resolution (proteinase K-treated for the mutants) were identified by electrophoresis in 1.4% agarose and autoradiography. The markers run for reference were a mixture of \( ^{32} \)P-labeled fragments I and IV (right-most lane in A) or \( ^{32} \)P-labeled fragments I and II (far right lanes in B). (C) After the resolution reactions, the proteinase K-treated samples were denatured (0.25 M NaOH and 10 mM EDTA) and electrophoresed in 2.0% agarose. Fragment II, \( ^{5} \)I labeled at the HindIII end, was digested with \( Xba I \) (which cuts close to the FLP cleavage sites; see Fig. 3) and run as a marker (right-most lane). The lanes marked "0" are controls with no protein added.**
mutant FLP was not, in any way, abnormal. In a linear substrate labeled selectively at the 5' or 3' ends, the site of cleavage was the same as that for wild-type FLP, and the consequence of cleavage (as with wild-type FLP) was the generation of a 3' phosphate covalently linked to protein (Fig. 3 A and B). With this linear substrate and the wild-type FLP protein, cleavage on the top strand was roughly double that on the bottom strand (Fig. 3 A and B). This slight asymmetry in cleavage was not significantly distorted for [α-32P]dATP or for [Leu323]FLP (Fig. 3 A and B). The lack of pronounced cleavage asymmetry was not a consequence of the topology of the substrate used. In a circular substrate, the extent of top- and bottom-strand cleavage was nearly equal (Fig. 3 C).

DISCUSSION
Do members of the Int family recombinases follow a common mechanism of two-step single-strand exchanges to accomplish recombination? The results obtained with FLP protein are consistent with Holliday junctions being normal intermediates during 2-μm circle recombination. Synthetic FLP sites—Holliday junctions (or chi structures) could be resolved by FLP and by mutant FLP arrested in recombination beyond the substrate cleavage step. Mutant FLP proteins that are inactive in DNA cleavage failed to mediate this resolution. By using synthetic junctions as markers, it could be shown that the amounts of these putative intermediates, though low, increased with increase in recombination. These findings compare favorably with the compelling evidence that Int and Cre proteins function via the Holliday intermediate in recombination (8–10, 19–21).

The efficiency of Holliday junction resolution by FLP was not as high as that reported for Int (14). In mixed reactions containing 32P-labeled chi structures and unlabeled linear FLP substrates, the relative levels of resolution and recombination were approximately 20–30% and 10–15%, respectively. Two possible reasons could account for this low efficiency of resolution. The repeated segments (599 bp; from the 2-μm circle plasmid) in the fragments used for the preparation of chi structures would permit a longer range for the branch migration of the cross than would be feasible in the sterically constrained state of normal recombination. Furthermore, the protein preparations used in recombination and resolution assays were only partially pure. The His-325-mutant FLP proteins were less effective in chi resolution than was wild-type FLP. This may have to do with purity of the mutant proteins, their relative binding affinities to chi structures, or the efficacy of proteinase K treatment required to reveal their resolution products.

A striking feature of Int-catalyzed recombination is the strict order in which bottom and top strands are exchanged. A plausible explanation for the polarity of strand exchange is the elaborate binding of Int and accessory proteins (IHF and Xis) to the phage recombination site (8). For the Cre reaction, although such asymmetric assembly of protein appears un-
likely, the hierarchy of strand exchange remains relevant (10). Asymmetry in this case could result from the local topology of the recombination complex or from the sequence asymmetry of the spacer within the recombination site. For the FLP system, a potential source of asymmetry is the extra copy of the FLP binding arm (Symmetry element 1b; Fig. 3) of the substrate or the sequence of the spacer. FLP showed no asymmetry in the mode of resolution of synthetic Holliday structures that corresponds to bottom-strand or top-strand exchange. However, apparent lack of asymmetry could have been caused by the freedom of branch migration beyond the minimal FLP site in the Holliday substrates. We also did not observe strong preferential strand cleavage by FLP or by the FLP mutants at His-305, which are defective in strand exchange. Nevertheless, these results do not necessarily preclude a prescribed order of strand swapping by FLP. Even when the initial cleavage by FLP is equally likely on both strands, the migration of each of the two Holliday junctions resulting from strand exchange away from their site of formation could be significantly different. The junction that migrates faster toward the opposite end of the overlap region is believed to escape resolution back to substrates better than the slower moving junction. One could envisage a conformational change of the recombination complex [Fig. 4; analogous to the Kitts-Nash model for Int recombination (9)] after strand exchange and branch migration that could alter the relative susceptibility of the cross-over points to cleavage and exchange by FLP. Some specialized structural feature of the spacer may account for the nonuniform rate of branch migration in opposite directions. The finding that, in addition to absolute spacer homology between recombining partners, there are certain sequence requirements of the spacer for efficient recombination is consistent with such a thought (22). The scheme shown in Fig. 4 can also account for the increased levels of the Holliday junctions seen with substrates containing symmetric spacers (23). The favored conformation of this junction with its fourfold symmetry (compare the twofold symmetry of the junctions in Fig. 4) would be such that all four sites are in the less susceptible state for cleavage and exchange. This must cause the persistence of the chi form. However, cleavage at each of the four sites is equally likely. Therefore, resolution can give rise to recombinants, parental, or Y-shaped structures (23).

Note Added in Proof. The amount of the Holliday intermediate in FLP recombination can be significantly increased if one strand of one of the substrates is eliminated from exchange by nicking it with His-305-mutant FLP and blocking the 5'-OH of the nick by phosphorylation.

We thank M. Cox for sharing results prior to publication. This work was supported by funds from National Institutes of Health.