Mutations in the 2- μ m circle site-specific recombinase that abolish recombination without affecting substrate recognition

(strand cleavage/protein–DNA complexes/DNase “footprinting”)

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ABSTRACT The site-specific recombinase encoded by the yeast plasmid 2- μ m circle (FLP) forms a transient covalent linkage with its substrate DNA via a tyrosine residue, which appears to be located near its COOH terminus. The homology of the COOH terminus of FLP with that of the Int family of recombinases suggests that tyrosine-343 of FLP could be involved in forming the DNA–protein bridge. We have mutated tyrosine-343 to a phenylalanine or serine. We demonstrate that the binding of each of the two mutant proteins to its substrate is indistinguishable from that of wild-type FLP. However, both mutant proteins are incapable of catalyzing strand cleavage and recombination.

Site-specific recombination, unlike generalized recombination, is characterized by relatively short DNA substrates of specific sequences, by the requirement of rather limited homology of the recombining partners, and by strand exchange mechanisms that require no DNA synthesis or exogenous high-energy cofactor (1). The recombination reaction involves cleavage, swapping, and religation of the participant DNA strands. The energy of the phosphodiester bond is conserved during cleavage in the form of a phosphoprotein linkage. This is reminiscent of the mode of strand exchange mediated by topoisomerases. The best-studied site-specific recombination systems among prokaryotes are those mediated by the Int protein of phage λ , the resolvase encoded by the related transposons Tn3 and $\gamma\delta$, and the Cre protein of phage P1 (2–4). Recently, considerable information concerning the site-specific recombination mediated by the FLP protein of the 2- μ m yeast plasmid has become available (5–10). Like most topoisomerases, FLP and Int form a phosphotyrosine bridge with their DNA substrates during catalysis (refs. 11 and 12; C. Pargellis, S. Nunes-Duby, L. Vargas, and A. Landy, personal communication); in contrast, the link between the resolvase and its substrate is a phosphoserine (13).

The remarkable similarity in the chemistry of the reactions catalyzed by the prokaryotic site-specific recombinases and the FLP protein is contrasted by the unexpectedly large diversity among these proteins. However, in spite of this global diversity, the site-specific recombinases encoded by bacteriophage λ , ϕ 80, P22, 186, P4, and P1 can be aligned in their COOH-terminal halves (14). A 40-residue region near the COOH terminus is well-conserved in all these proteins and shares a conspicuous degree of homology with a region near the COOH terminus of the FLP protein. This family of recombinases (the integrase family) does not appear to be related to other site-specific recombinases, such as the prokaryotic invertases or the $\gamma\delta$ resolvase (3, 15–18). Within the integrase family, three residues (histidine, arginine, and tyrosine) located at alignment positions 396, 399, and 433,

respectively, are perfectly conserved. It has been speculated that these residues form part of the active site of this family of recombinases and that tyrosine-433 forms the transient covalent linkage to DNA during strand breakage and reunion (14).

In this communication, we describe the conversion of tyrosine-343 (family alignment position 433) of the FLP protein to serine or phenylalanine. We demonstrate that both the mutant proteins are inactive in recombination *in vivo* in *Escherichia coli*. They are also incapable of catalyzing recombination or strand cleavage *in vitro*. However, like wild-type FLP, both mutant proteins bind to the DNA substrate to give three hierarchical DNA–protein complexes. Furthermore, the DNase “footprints” left by the mutant proteins on the substrate are indistinguishable from those left by the wild-type FLP.

MATERIALS AND METHODS

Plasmids. The construction of plasmids pMI and pJK (Fig. 1) has been described (5). The structure of pMI is almost identical to that of pMJ, which was extensively used as the source of FLP in recombination assays *in vivo* (5). The pMI homologs, pMS and pMP (which contain serine-343 and phenylalanine-343, respectively) were obtained by directed mutagenesis using synthetic oligodeoxynucleotides.

Mutagenesis. The tyrosine-343 of FLP was mutated to serine or phenylalanine by the double-primer method (19). The oligodeoxynucleotides were synthesized in an A-380 Applied Biosystems (Foster City, CA) nucleic acid synthesizer by the phosphoramidite method (21). Mutagenesis was carried out on an *Sph* I/*Xba* I segment of 2- μ m circle that encompasses FLP, cloned into M13mp18. The mutations were then transferred to pMI by replacing the *EcoRV/Xba* I fragment of pMI encoding the COOH-terminal two-thirds of FLP with the corresponding mutant fragments.

Purification of FLP. The wild-type and mutant proteins were purified to 25–40% homogeneity by modifications of published procedures (22, 23). The crude extracts prepared as described (23) were subjected to streptomycin sulfate precipitation (4%), followed by ammonium sulfate precipitation of the resulting supernatant (40%). The pellet was dissolved in 50 mM Tris-HCl, pH 7.5/20 mM 2-mercaptoethanol/200 mM NaCl/1 mM EDTA, desalted with Sephadex G-25, and chromatographed on Accell CM cation exchanger (Waters Associates) and on double-stranded DNA-cellulose (Pharmacia). On NaDodSO₄ polyacrylamide gels, FLP migrated at $\approx$ 43 kDa. Transfer of gel-fractionated proteins to nitrocellulose and probing with FLP antibodies were done by published procedures (24).

Recombination Assays. *In vivo* recombination assays were done as described by Jayaram (5). *In vitro* recombinations were done according to Prasad *et al.* (6).

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FIG. 1. Plasmids that express wild-type or altered FLP in *E. coli*. (A) In pMI, the FLP gene is expressed from the λP_R promoter; the plasmid also harbors the temperature-sensitive repressor *cl857*. At 42°C, the repressor is inactivated and FLP expression is turned on. Plasmids pMS and pMP are derivatives of pMI in which tyrosine-343 of FLP has been mutated to serine and to phenylalanine, respectively. Mutations were produced by the double-primer method on a single-stranded FLP template cloned in M13mp18 (19). The sequences of the mutagenic primers and the second primer are shown. The coordinates are according to the 2- μ m circle numbering scheme of Hartley and Donelson (20). The 2- μ m circle portion of pMI is represented as a thick line. The wavy line stands for the FLP coding region. (B) After mutagenesis, the accuracy of the mutations was verified by DNA sequencing. The altered bases in pMS and in pMP are indicated by the arrows. (C) The pJK plasmid (5), which is the substrate for FLP recombination in *E. coli*, is a pACYC derivative. This plasmid is compatible with the ColE1 plasmid pMI or its mutant homologs pMS and pMP. The consequence of recombination between the directly repeated FLP sites is the loss of the kanamycin-resistance (Kan^R) marker. Cam^R , chloramphenicol resistance; Amp^R , ampicillin resistance; ORI, origin.

Strand Cleavage, DNA Binding, and DNase Footprinting. All three assays were carried out using an ≈ 100 -base-pair (bp) *Hind*III/*Eco*RI fragment that includes the complete FLP recombination site. The fragment was labeled at the *Hind*III end on the bottom strand by the Klenow polymerase reaction in presence of [α - ^{32}P]dCTP and the other three unlabeled deoxynucleoside triphosphates.

Approximately 0.1–0.2 pmol of the labeled fragment was incubated at 30°C for 30 min with 0.5–1.0 μ g of partially purified FLP in 50 mM Tris-HCl, pH 7.5/50 mM NaCl/5 mM $MgCl_2$ /1 mM dithiothreitol/75 μ g of sonicated and phenol-extracted calf thymus DNA per ml, in a total vol of 50 μ l. The reaction was stopped by the addition of 1 μ l of 20% NaDodSO₄. After phenol extraction and ethanol precipitation, the DNA was dissolved in a small vol 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). Since cleavage of the DNA by FLP results in a free 5'-OH and a protein-bound 3'-phosphate, no proteolysis was required to detect the labeled breakage product resulting from the 3'-end-labeled substrate.

Binding reactions were done by slight modifications of the procedure of Prasad *et al.* (6). Approximately 0.1–0.2 pmol of the end-labeled fragment was incubated with 0.5–1 μ g of protein in 50 μ l containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 200 μ g of bovine serum albumin per ml, 100 μ g of denatured calf thymus DNA per ml, and 1 mM dithiothreitol for 30 min at 30°C. After addition of heparin (5 μ g/ml) and

incubation for 5 min at room temperature, 5 μ l of a solution containing 10 mM Tris-HCl (pH 7.5), 30% (vol/vol) glycerol, and 0.1% bromophenol blue was added to the reaction mixture. Aliquots of the samples were electrophoresed on 5% polyacrylamide gels (30:1 cross-linking; 16 $\times$ 14 $\times$ 0.3 cm) at 4°C in 1 $\times$ TBE (25 mM Tris-HCl, 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 as described by Andrews *et al.* (25).

General Methods. Bacterial transformations, isolation of plasmid DNA, restriction enzyme digestions, and other miscellaneous methods followed published procedures (26). DNA sequences were determined by the method of Maxam and Gilbert (27) or Sanger *et al.* (28).

RESULTS

In Vivo and in Vitro Recombination Activity of Serine-343 FLP and Phenylalanine-343 FLP. Replacement of tyrosine-343 of FLP by serine and phenylalanine was achieved by using mutagenic oligodeoxynucleotides as described (see Fig. 1A). Mutagenesis was carried out on an *Sph* I/*Xba* I segment from the 2- μ m circle genome (20) spanning the FLP coding region that had been cloned into M13mp18. After mutagenesis, the *Eco*RV/*Xba* I FLP fragment that includes the mutation was excised and used to replace the corresponding wild-type FLP fragment in plasmid pMI (5). In plasmid

Table 1. *In vivo* recombination assays for FLP, serine-343 FLP, and phenylalanine-343 FLP

Source of FLP	Number of transformants	
	<i>Amp^R Cam^R</i>	<i>Amp^R Cam^R Kan^R</i>
pMI (FLP)	216	48
pMS (serine-343 FLP)	362	362
pMP (phenylalanine-343 FLP)	412	412

Each of the FLP plasmids (pMI, pMS, or pMP) was introduced into a *recA E. coli* strain harboring the pJK plasmid. The resultant *Amp^R Cam^R* transformants were replica-plated on kanamycin plates at 42°C. The presence of functional FLP is manifest as kanamycin sensitivity. *Amp^R*, ampicillin resistance; *Cam^R*, chloramphenicol resistance; *Kan^R*, kanamycin resistance.

pMI, FLP expression is driven by the λP_R promoter, which is controlled by the temperature-sensitive repressor *cI857*. Unlike plasmid pMJ used in earlier *in vivo* recombination assays (5), pMI contains an intact FLP site within the 2- μ m circle repeat. The derivatives of pMI containing the FLP mutations, pMS (tyrosine-343 to serine-343) and pMP (tyrosine-343 to phenylalanine-343), were used for *in vivo* assays for FLP recombination as described (5). The rationale of the assay is that recombination between two directly repeated FLP sites bordering the Tn5-derived kanamycin-resistance

gene results in the loss of this marker on a circular piece of DNA that lacks a replication origin. The recombination event is thus manifest as kanamycin-sensitive colonies of a *recA E. coli* host harboring the substrate plasmid pJK (see Fig. 1C). Both serine-343 FLP and phenylalanine-343 FLP were inactive in this recombination assay (Table 1).

To confirm these *in vivo* results, partially purified wild-type and mutant FLP proteins were used in *in vitro* recombination assays. The assay is based on the fact that recombination between the FLP sites on two suitably chosen DNA fragments (P_1 and P_2 ; Fig. 2A) results in two new fragments (R_1 and R_2) whose sizes are different from those of the parents (6). The results of the assay are shown in Fig. 2A. While the wild-type protein catalyzed efficient recombination, both the mutants failed to do so.

The Serine-343 FLP and Phenylalanine-343 FLP Cannot Mediate Strand Cleavage. The results of the *in vivo* and *in vitro* recombination assays demonstrate that replacement of tyrosine-343 of FLP by either serine or phenylalanine results in the loss of the recombination potential of FLP. It was possible that these proteins catalyzed cleavage of the DNA substrate but failed to complete the reaction by strand exchange and ligation. We tested this possibility by using the synthetic FLP substrate J3 (Fig. 3A; ref. 5). This substrate not only includes the minimal FLP recombination site con-

FIG. 2. *In vitro* recombination and strand cleavage. (A) The substrate for the *in vitro* recombination reactions was an equimolar mixture of *Pvu* II- or *Eco*RI-digested pJR1 (6). Plasmid pJR1 contains a 900-bp *Hind*III fragment from the A form of the 2- μ m circle (20), which includes a copy of the repeated segment cloned into the *Hind*III site of pBR322. An FLP-mediated cross-over between the two types of molecules results in two recombinant linear molecules. The parental molecules are labeled P_1 and P_2 ; the recombinant products are labeled R_1 and R_2 . The reaction conditions were those used by Prasad *et al.* (6). Protein concentrations were 0, 0.5, and 1.0 μ g in lanes 1, 2, and 3, respectively. (B) The substrate used in the strand cleavage assay (shown in Fig. 3A) was labeled close to its 3' end on the bottom strand. The position of the label is indicated by the C with the asterisk in Fig. 3A. Details of the assay are described in the text. The labeled fragment resulting from cleavage by FLP (F) was visualized by electrophoresis on a denaturing polyacrylamide gel followed by autoradiography. An *Xba*I digest of the labeled substrate (X) was run alongside for reference. The positions of strand cleavage caused by FLP and by *Xba*I are marked F and X, respectively, in Fig. 3A. Protein concentrations were 0 μ g (lanes 1), 0.5 μ g (lanes 2), and 1.0 μ g (lanes 3). (C) Approximately 40–50 μ g of the wild-type and mutant FLP preparations used in the assays described above were electrophoresed on a 10% NaDodSO₄/polyacrylamide gel (30:1 cross-linking), transferred to nitrocellulose (24), and probed first with anti-FLP antibodies and subsequently with peroxidase-conjugated anti-rabbit IgG. The blot was developed with 4-chloro-1-naphthol (Sigma) and H₂O₂ (Baker). A protein preparation from an *E. coli* host containing the vector without the FLP gene (purified by the same protocol used for FLP and the FLP mutants) shows the absence of the 43-kDa protein that reacts with anti-FLP antibodies. (Left) Coomassie blue-stained gel. (Right) Immunoblot probed with FLP antibodies.

FIG. 3. Substrate binding and DNase footprinting of FLP, serine-343 FLP, and phenylalanine-343 FLP. (A) The substrate for footprinting was an $\approx$ 100-bp *HindIII*/*EcoRI* fragment that includes the complete FLP site consisting of the three symmetry elements 1a, 1'a, and 1'b plus the 8-bp core of the 1a-1'a dyad symmetry. Wavy line indicates sequences derived from pUC19. The bottom strand was labeled at the *HindIII* end with [α - 32 P]dCTP using the Klenow reaction. The deoxynucleotides incorporated by the Klenow enzyme are italicized and the position of the label is marked by an asterisk. (B) Binding reactions were carried out as described in the text. Samples were run on 5% polyacrylamide gels at 4°C in Tris borate buffer and autoradiographed. The FLP-DNA complexes are called I, II, and III. The input substrate is indicated by S. Protein concentrations were 0 μ g (lanes 1), 0.5 μ g (lanes 2), 0.75 μ g (lanes 3), and 1.0 μ g (lanes 4). (C) The footprinted samples were run on 12% sequencing gels. The sequence ladder obtained by chemical cleavage of the substrate at G, G+A, C, and C+T is aligned with the DNase protection pattern. The band resulting from the FLP-induced cleavage of the substrate (F) is absent in the footprints of serine-343 FLP and phenylalanine-343 FLP. The region of the substrate strongly protected by FLP is shown as the solid bar below (see A); the weakly protected region is symbolized by the hatched bar. Protein concentrations were 0 μ g (lanes 1), 0.5 μ g (lanes 2), 1.0 μ g (lanes 3), 1.5 μ g (lanes 4), and 2.0 μ g (lanes 5).

sisting of the nearly perfect dyad symmetry (1a-1'a) plus the 8-bp core, but also a second copy of the symmetry element (1'b), which is not essential for recombination. Since J3 is bordered by *HindIII* and *BamHI* sites, a stock of this substrate was made in pUC19 digested with the above enzymes. However, during propagation of this plasmid stock, the *BamHI* site was lost due to the deletion of a single C-G. Therefore, the FLP site was excised from this plasmid by cutting with *EcoRI* and *HindIII* and was gel-purified. The bottom strand was labeled at the *HindIII* end by the Klenow polymerase reaction using [α - 32 P]dCTP and the other three unlabeled deoxynucleotides. The cleavage assay was done as described. While 2-4% of the input substrate was cleaved by wild-type FLP, no detectable cleavage could be observed with the mutant proteins (Fig. 2B). We have verified that the protein preparations used in the assay contained approximately the same concentrations of the wild-type and the mutant proteins by using immunoblots probed with anti-FLP antibodies (ref. 24; Fig. 2C).

Binding of FLP, Serine-343 FLP, and Phenylalanine-343 FLP to Its Substrate. The experiments described above establish that the two mutant FLP proteins are unable to catalyze not only complete recombination but also strand breakage, which is assumed to be the intermediate step in the reaction. Can these mutant proteins recognize and bind to the FLP substrate in a manner similar to the wild-type protein? To test this question, we used the same labeled FLP substrate as the one used for the strand cleavage assay. After incuba-

tion of this substrate with protein, the reaction mixtures were analyzed on polyacrylamide gels (29) to detect DNA-protein complexes. The wild-type FLP gave rise to three distinct complexes (I, II, and III) (see Fig. 3B). Kinetic experiments have shown that complexes I, II, and III are formed in a sequential order (data not shown). Furthermore, in the absence of the third symmetry element, 1'b, complex III was not formed. Thus, these complexes appear to be truly representative of hierarchical binding of protomers of FLP to its substrate. Similar results have been obtained by B. J. Andrews, L. G. Beatty, and P. D. Sadowski (personal communication). In addition, they have shown that complex I represents loose FLP binding, whereas complexes II and III represent tighter FLP binding and are recombination proficient. In our experiments, both serine-343 FLP and phenylalanine-343 FLP gave rise to the same three complexes as did FLP, as judged by the gel migration of these complexes (Fig. 3B).

Finally, we performed DNase footprinting experiments (30) to determine whether substrate binding by the mutant proteins is qualitatively different from that of the wild-type FLP. The footprints left by the three proteins on the bottom strand of the substrate (end-labeled as described in previous assays) are shown in Fig. 3C. The region normally protected by FLP consists of an $\approx$ 50-bp segment that spans the three symmetry elements (1a, 1'a, 1'b) as well as the core that constitutes the FLP site (Fig. 3A; ref. 25). The patterns of protection obtained with serine-343 FLP and phenylalanine-

343 FLP contained no obvious differences from that obtained with FLP. However, as expected, the band corresponding to strand cleavage by FLP was absent in the footprints of the mutant proteins.

DISCUSSION

Homology alignments of prokaryotic site-specific recombinases assign them to two families: the Int-related and the Hin-related families. The Hin family is composed of the highly homologous invertases of *Salmonella* (Hin), phage Mu (Gin), phage P1 (Cin), and *E. coli* (Pin) (15–18). The resolvase proteins of the transposons Tn3 and $\gamma\delta$ (31, 32) also belong to this class of recombinases. The Int family consists of the site-specific recombinases encoded by bacteriophage λ and the lambdoid phage ϕ 80 and P22, the related phage P2 and 186, and the phage P4 and P1. The Int family differs from the Hin family in encompassing an unexpectedly high degree of sequence diversity (14). Nevertheless, there is a relatively high level of conservation within a 40-amino acid sequence located near the COOH-terminal portion of these proteins. This homology appears to extend to the COOH-terminal region of the 2- μ m circle FLP protein as well (14). We may therefore look upon FLP as a distant member of the Int family of recombinases. The most remarkable feature shared by the members of this family is the perfectly conserved trinity of histidine-396, arginine-399, and tyrosine-433. These family alignment positions correspond to residues 305, 308, and 343, respectively, of the FLP protein.

In spite of the individual differences among the Int-related recombinases, the reactions they catalyze share the same basic features, especially in regard to the chemistry of strand cleavage and exchange. The three proteins Int, Cre, and FLP nick their substrates at specific positions to create 3' protein-bound termini and 5' overhangs with a free hydroxyl group. The 5' protruding ends are 6 bp long for Cre, 7 bp for Int, and 8 bp for FLP (25, 33, 34). Presumably, the phosphoprotein bridge provides the energy required for the strand resealing step of recombination.

The fact that only three amino acid residues are invariant in a family of proteins that catalyzes reactions whose chemistry is almost identical implies that these residues are involved in recognition of specific DNA sequences and/or form part of the active sites of these proteins. We concentrated on one of these conserved residues, tyrosine-343 of the FLP protein, for the following reasons. First, the aromatic portion of the tyrosine residue could be involved in interactions with DNA. Second, for FLP as well as Int, the phosphoprotein linkage has been shown to occur via a tyrosine residue (ref. 12; C. Pargellis, S. Nunes-Duby, L. Vargas, and A. Landy, personal communication). Third, it is also known that, in FLP, this residue is in the COOH-terminal portion of the protein, although its precise location is undetermined (12). Based on these premises, one may expect the following properties for phenylalanine-343 FLP and serine-343 FLP. The change from tyrosine to phenylalanine would likely eliminate DNA cleavage and protein-DNA linking. The effects of the mutation on DNA binding, however, could be minimal. On the other hand, the tyrosine to serine mutations could have large effects on DNA binding; nevertheless, if substrate binding is not altered by this amino acid change, the mutant protein has the potential to form a phosphoprotein linkage. Indeed, in the case of the resolvase of Tn3 or of $\gamma\delta$ the DNA-protein linkage is established through a serine (13, 35). We have shown here that neither the tyrosine to phenylalanine nor the tyrosine to serine mutation significantly affects the binding of FLP to its substrate. Both mutations, however, abolish cleavage of the substrate and formation of the covalent protein-DNA complex and recom-

bination. The fact that the mutant proteins are not affected in stability or substrate binding rules out a general distortion in their secondary and tertiary structures. Thus, the results strongly suggest that tyrosine-343 forms part of the FLP active site but has minimal, if any, role in DNA binding. Our data are completely consistent with but do not prove the idea that tyrosine-343 is the residue that becomes linked to DNA during recombination.

Accepting that tyrosine-343 is part of the FLP active site, what is the role of the invariant histidine and arginine of the Int family in recombination? How do mutations of histidine-305 and arginine-308 of FLP (family alignment positions 396 and 399) affect DNA binding and/or catalysis by FLP? Experiments to answer this question using mutant FLP proteins (histidine to glutamine, arginine or leucine, arginine to isoleucine or glycine) are currently under way.

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