THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 35, Issue of December 15, pp. 21779-21788, 1990
Printed in U.S.A.
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Protein-based Asymmetry and Protein-Protein Interactions in FLP Recombinase-mediated Site-specific Recombination*

(Received for publication, July 13, 1990)

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When the FLP recombination target (FRT) is cut in half so that only one FLP protein-binding site is present, FLP protein forms a complex in which two such sites are linked head to head. Although held together exclusively by noncovalent interactions, this complex survives electrophoresis in an agarose gel and exhibits a half-life that can be measured in hours. Characterization of this complex indicates that a very stable, asymmetric dimeric complex of FLP protein monomers bound to the FRT is a likely early intermediate in FLP-mediated site-specific recombination. The apparent asymmetry is a property of the protein components of the complex. Even though the DNA components form a perfect palindrome, only one of the two possible DNA cleavage steps takes place in the course of complex formation. Formation of this complex does not occur with half-FRT site DNA substrates that preclude head to head monomer contact or when a FLP mutant protein is used that binds the FRT site but cannot cleave it. Trimeric and tetrameric complexes are also observed, the latter at very low frequency. These results are discussed in terms of an expanded model for early events in FLP-mediated site-specific recombination.

Conservative site-specific recombination is a process that generates precise and efficient DNA rearrangements at well-defined DNA sites (for reviews, see Craig, 1988 and Landy, 1989). The yeast 2-μm plasmid encodes a site-specific recombination system. This system permits the plasmid to circumvent host cell cycle control of plasmid replication for the purpose of increasing plasmid copy number (Futcher, 1986; Volkert and Broach, 1986; Murray *et al.*, 1987; Reynolds *et al.*, 1987). The recombinase involved is encoded by the plasmid and is designated FLP. The FLP recombinase is a member of the integrase family of site-specific recombinase, and its mechanism of action is similar in many respects to that of the bacteriophage λ integrase and the cre recombinase of bacteriophage P1 (for reviews, see Argos *et al.*, 1986; Sadowski, 1986; Craig, 1988; Landy, 1989; Cox, 1989).

On a chemical level, the reaction carried out by these recombinases is straightforward. Four specific phosphodiester bonds in the two DNA target sites are cleaved, and then four new phosphodiester bonds are formed in such a way that the DNA on either side of the target sites is rearranged. A transient covalent bond is formed between the recombinase and the DNA (a 3′-phosphotyrosine link in the case of integrase-class recombinase) (Sadowski, 1986; Craig, 1988; Landy, 1989) that preserves the phosphate ester and renders high energy cofactors such as ATP unnecessary. On this level, the recombinases can be likened to a biological fusion and streamlining of the reactions carried out by restriction enzymes and DNA ligase.

The requirement that the cleaved DNA ends be rejoined in a predictable manner with new partners adds an important element of structural organization and complexity to this reaction. The cleavage steps occur at specific points that are staggered by 6–8 bp[1] on the two DNA strands. The region between the cleavage sites is referred to as an overlap, core, or spacer region, and this is generally flanked by binding sites for the recombinase. In addition, the four cleavage events are not concerted. Cleavage and strand exchange occurs on one side of the core regions of two aligned sites, followed by strand exchange to form a Holliday intermediate (Nunes-Düby *et al.*, 1987; Kitts and Nash, 1988; Hoess *et al.*, 1987; Jayaram *et al.*, 1988; Meyer-Leon *et al.*, 1988, 1990). This isomerizes to bring the other two cleavage sites into position for the second set of cleavage and exchange reactions needed to give products.

The tight spatial organization of the DNA substrates required for this sequence of events is readily apparent in the λ integrase system, where multiple proteins are required in a complex that binds to and condenses a region extending over 200 base pairs in one of the two recombination targets (Landy, 1989). Some other integrase-class recombinases, however, utilize simpler recombination sites and require no proteins except for the recombinase itself. These include the cre-lox and FLP systems. These recombinases therefore not only promote cleavage and religation, but also orchestrate a complex and sequential series of motions that bring DNA strands and phosphodiester bonds into the right positions at the right times.

The minimal FRT site required for FLP-mediated recombination *in vivo* and *in vitro* consists of an 8-base pair core region flanked by 13-base pair inverted repeats. The two FLP recombinase-binding sites within the FRT include the external base pairs of the core and the 11 contiguous base pairs in the adjacent repeats (Andrews *et al.*, 1985; Bruckner and Cox, 1986; Senecoff *et al.*, 1988; and Fig. 1*A*). There are no identified protein-DNA interactions in the central six base pairs of the core, and the sequence here can be altered substantially without affecting FRT function as long as two reacting sites have homologous cores. The core sequence of the FRT site in the plasmid pJFS39 (Fig. 1*B*) is palindromic. Although this sequence differs from the wild-type sequence at five of the central six positions, this site is fully functional in FLP-

* This work was supported by National Institutes of Health Grants GM 37835 (to M. M. C.) and GM 14711 (to R. B. I.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: bp, base pairs; FRT, FLP recombination target; SDS, sodium dodecyl sulfate; TAPS, 3-[tris(hydroxymethyl)methylamino]-1-propanesulfonic acid; kbp, kilobase pairs.

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mediated recombination (Senecoff and Cox, 1986). Assuming one FLP monomer binds to each binding site, they would be bound head to head. There is no direct evidence for contact (cross-core) between recombinase units bound at the two sites, although some cooperativity is evident in the binding studies carried out by Sadowski and co-workers (Andrews *et al.*, 1986, 1987; Beatty and Sadowski, 1988). A cross-core interaction is also suggested by the result that increasing the size of the core by 2 base pairs abolishes FRT function (Senecoff *et al.*, 1985).

We have begun to study early events in this reaction up to and including the first cleavage events. A start has been made by Sadowski and colleagues (Andrews *et al.*, 1986, 1987; Beatty and Sadowski, 1988), who have provided evidence that FLP protein acts primarily as a monomer and loads onto the FLP-binding sites within the wild-type FRT in a prescribed sequential manner. This binding is accompanied by bending of the FRT site (Schwartz and Sadowski, 1989).[2] In addition, Jayaram and colleagues (Prasad *et al.*, 1987) have constructed a FLP protein mutant in which the nucleophilic active site tyrosine is changed to phenylalanine (Tyr^{343}→Phe). This mutant has been shown to bind to but not cleave the FRT site, and it is particularly useful in analyzing early steps in the reaction pathway (Prasad *et al.*, 1987). Some changes in the core sequence, in particular those that increase GC content, also permit binding of FLP protein but prevent or inhibit one or more steps preceding cleavage (Umlauf and Cox, 1988).[3]

We have begun to examine these early steps by using modified FRT sites lacking DNA on one side of the spacer region. This is an approach recently used successfully by Landy and colleagues (Nunes-Düby *et al.*, 1989) for the λ integrase system. In the λ integrase system these half-sites for recombination are competent for recombination steps up to the second set of cleavage and ligation steps, even though there is no λ integrase present on the opposite side of the core region. In contrast, we find that FLP protein bound to appropriately constructed half-FRT sites form cross-core dimeric complexes, mediated by strong noncovalent protein-protein interactions, and no reaction occurs with half-sites that do not permit complex formation. These complexes and their implications for early steps in FLP-mediated site-specific recombination are described in this report.

MATERIALS AND METHODS

Enzymes and Chemical Reagents—FLP protein was purified by a published procedure (Meyer-Leon *et al.*, 1987; Gates *et al.*, 1987) with the exception that preparations were further purified by means of standard chromatography on orange A-agarose (Amicon Corporation). FLP protein obtained in this manner is greater than 70% homogeneous as determined by SDS/polyacrylamide gel electrophoresis. FLP protein concentration was determined using the method of Bradford (1976), and the reported concentrations have been corrected in all cases for the degree of purity of the preparation. Mutant FLP protein (Tyr^{343}→Phe) was a gift from Dr. M. Jayaram (University of Texas, Austin). The mutant protein was approximately 6.2% pure as determined by polyacrylamide gel electrophoresis. The correct concentration of the mutant protein was estimated based on a comparison of its band intensity with a standard curve generated with several lanes on the same gel containing appropriate concentrations of bovine serine albumin. Restriction enzymes were purchased from New England BioLabs. Avian myeloblastosis virus reverse transcriptase was from Life Science Inc. Restriction enzymes and reverse transcriptase were used under conditions recommended by their manufacturer. Cytochrome *c* was purchased from CalBiochem. T_4 poly-nucleotide kinase was from Promega. Agarose was from Bethesda Research Laboratories. Radioactive nucleotides were from Amersham Corp. Bovine serum albumin (Pentax fraction V) was from Sigma. Oligonucleotides were synthesized by the Protein Sequence-DNA Synthesis Facility within the University of Wisconsin Biotechnology Center. The plasmids pSWU11 and pSWU14 have been described previously (Umlauf and Cox, 1988), and features important to this work are summarized in Fig. 1. DNA concentrations are reported in terms of DNA fragment concentration. Unless otherwise noted, this is equivalent to FRT site or half-FRT site concentration.

Half-site Construction—The plasmid pXHQ101 (2,827 bp) has a minimal FRT site with a symmetrical spacer flanked by two FLP protein-binding sites. It is identical to the plasmid pJFS39 (Senecoff and Cox, 1986) except that there are 7 additional bp between the FRT site and the *Bam*HI cleavage site (Fig. 1). To generate the half-site plasmid pXHQ01, 5 μg of pXHQ101 was digested with *Xba*I and *Eco*RI in 50-μl solution for 4 h. This cleaved pXHQ101 into fragments of 20 and 2,807 bp. To this mixture was added 2 μl of 2 mM dNTP and 16 units of reverse transcriptase, and incubation was continued for 1 h at 37 °C to fill in the sticky ends. The large fragment was purified by agarose gel electrophoresis and circularized by ligation of the blunt ends (Maniatis *et al.*, 1982). The resulting 2,811 bp plasmid was named pXHQ01 (Fig. 1*C*), and its sequence was confirmed by dideoxynucleotide sequencing (Schultz and Reznikoff, 1990).

DNA Manipulation—All plasmid DNA were purified by banding twice in cesium chloride gradients in the presence of ethidium bromide. DNA fragments with *Xba*I-cut ends were 3′-end-labeled with [α-^{32}P]dCTP and avian myeloblastosis virus-reverse transcriptase. In a 200-μl solution were included 25–35 pmol of *Xba*I-cut ends, 10 μg of acetylated bovine serum albumin, 33 pmol of [α-^{32}P]dCTP, 165 pmol of nonradioactive dCTP, and 100 units of avian myeloblastosis virus-reverse transcriptase (Life Science). The reaction buffer contained 50 mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, and 40 mM KCl. The reaction was incubated at 37 °C for 2 h, then subjected to phenol/chloroform/isoamyl alcohol (25:24:1) extraction once. The DNA was further purified by means of a Sephadex G-50 spun column twice and finally precipitated with ethanol/sodium acetate (Maniatis *et al.*, 1982). At least 80% of the DNA fragments treated in this way have one deoxycytidine incorporated at the 3′-end. The *Eco*RI-cut fragment was labeled in the same way except that [α-^{32}P]dATP was used. The 5′ blunt ends of *Nru*I-cut pXHQ01 was dephosphorylated (Berger and Kimmel, 1987), labeled with [γ-^{32}P]ATP (Maniatis *et al.*, 1982), cut with *Xba*I, and the resulting 0.6-kb *Xba*I- and *Nru*I-cut half-site was isolated on an agarose gel (Maniatis *et al.*, 1982; Dretzen *et al.*, 1981). Another half-site, the 1.7-kb *Dra*I-cut pSWU11, was also isolated after purification on an agarose gel.

Reaction Conditions—Reactions with half-FRT sites were carried out in a solution (20 μl) containing 25 mM TAPS buffer, pH 8.0, 1 mM EDTA, 5% (w/v) PEG (polyethylene glycerol, molecular weight 8,000), 10% glycerol (v/v), 200 mM NaCl, 5.4 nM half-site DNA, and a variable amount of FLP protein as indicated. The reaction sample prepared for electron microscopy did not include PEG and glycerol.

Unless otherwise stated, experiments were carried out according to the following protocol: FLP protein was diluted as needed in a buffer containing 25 mM TAPS, pH 8.0 at 25 °C, 1 mM EDTA, and 1.0 M NaCl. Another solution containing $n \times 18$ μl (where n was the number of aliquots to be taken in a given experiment) of all components except FLP protein were prepared and incubated at 30 °C for at least 5 min. Then 9 μl of solution was removed to serve as a zero time control. The reaction was initiated by adding $2n - 1$ μl of appropriately diluted FLP protein. Aliquots (10 μl) of reaction solution were removed at the indicated time, and these were treated as indicated below.

Detection of Complexes Formed with Half-FRT Sites by Nondenaturing Agarose Gel Electrophoresis—A 0.6% (w/v) 14.5 × 13.5 × 0.8 cm agarose gel was prepared in 1 × TBE (0.089 M Tris-HCl, pH 8.0, 0.089 M boric acid, and 0.002 M EDTA) in a standard horizontal gel box maintained at room temperature (20–23 °C). The gel was prerun for 10 min at 100 V in the 1 × TBE buffer before sample was loaded. The following four different substrates were used: pXHQ01 cut with either *Xba*I or *Eco*RI, pSWU14 cut with *Sal*I, and the 1.7-kbp half-site fragment purified from *Dra*I-cut pSWU11. For each substrate, 36 μl of reaction solution without FLP protein was prepared as described above under Reaction Conditions. From each solution, 9 μl was removed and loaded directly onto the gel prior to reaction, then 3 μl of 2-fold diluted FLP protein was added to initiate the half-site reaction to give a final FLP protein concentration of 42 nM. After 5

[2] C. J. E. Schwartz and P. D. Sadowski (1990), manuscript submitted.

[3] C. J. E. Schwartz and P. D. Sadowski (1990), manuscript submitted.

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min of incubation at 30 °C, 10-μl aliquots were removed from each reaction and loaded directly onto the gel without adding anything. Another 10-μl solution was removed and 1 μl of 10% SDS was added prior to loading onto the gel. To generate DNA size markers (2.82 and 5.59 kbp in length), a 40-μl reaction containing 5.4 nM *Bam*HI-cut pXHQ101 and 84 nM FLP protein was incubated under standard reaction conditions for 15 min at 30 °C. The resulting recombination reaction was stopped with 4 μl of 10% SDS, and 10 μl was loaded on the same gel. In addition to the marker bands seen, this reaction generates a 54-bp product that is not observed in these gels. Electrophoresis was carried out at 100 V for 105 min. Electrophoresis was always carried out immediately after the samples were loaded. For nondenaturing agarose gel experiments subsequent to that shown in Fig. 3, this protocol was modified in that electrophoresis was carried out in mini gel box (7.5 × 5 × 0.3-cm gel) at 70 V, 30–40 mA for 70–90 min.

To quantify complexes, the gel was stained with 0.5 μg/ml EtBr for 15 min, destained with H_2O for 15 min, and photographed with polaroid Type 55 film. Gel band intensity was quantified with a Zenith soft-laser scanning densitometer SL-504-XL from Biomed Instruments, Inc. A standard curve relating the amount of DNA with band intensity was obtained from a series of diluted DNA samples loaded on the same gel.

Electron Microscopy—Half-site reactions were carried out as described under Reaction Conditions, except that PEG and glycerol were omitted. The *Xba*I-cut pXHQ01 was used as substrate. FLP protein and half-site DNA concentrations in the 40-μl reaction solutions were 42 and 5.4 nM, respectively. After 5–10 min of incubation at 30 °C, the sample was prepared for electron microscopy. For experiments in which DNA was not denatured, 10 μl of the reaction mix was added to 7 μl of 2.5% formaldehyde in reaction buffer (minus PEG and glycerol), incubated at 37 °C for 30 min, then dialyzed for 5 h against a solution containing 20 mM NaCl and 5 mM Na_2EDTA (solution A). The dialyzed sample was diluted 20-fold into solution A, and 7 μl of this was mixed with 2 μl of cytochrome *c* (0.1%) and 5 μl of a spreading solution (0.041 M Na_2CO_3, 0.0064 M Na_2EDTA, 0.026 M HCl, 18.9% HCHO, and 37.2% formamide), and spread by the drop method (Inman and Schnos, 1970). For experiments in which DNA was partially denatured, 3 μl of the reaction was mixed with 4 μl of 0.02 M NaCl, 0.005 M Na_2EDTA, and 5 μl of partial denaturation buffer (0.067 M Na_2CO_3, 0.01 M Na_2EDTA, 0.052 M NaOH, and 31% HCHO). This mixture was incubated at 30 °C for 40–60 min, and then cooled on ice. To this was added 10 μl of formamide and 3 μl of cytochrome *c* (0.1%), and this mixed sample was spread by the drop method.

Cleavage Point Determination—Plasmid pXHQ01 was cut with *Xba*I and *Pst*I, and labeled at the 3′-end with [α-^{32}P]dCTP (3,000 Ci/mmol). A 90-μl standard reaction solution was prepared without FLP protein. As a zero time control 9 μl was removed from this solution prior to FLP protein addition. Diluted FLP protein (9 μl) was added to initiate the half-site reaction (42 nM FLP protein final concentration). Aliquots (10 μl) of solution were withdrawn at the indicated times. These were immediately diluted with 10 μl of H_2O, heated at 90 °C for 2 min, put on ice for a few minutes, and finally spun briefly in an Eppendorf microcentrifuge. To phosphorylate the cleavage product, 4 μl was taken out from the heat-treated 120-min reaction aliquot, and incubated with 10 μM ATP, 70 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 1 μl (10 units) of T_4 polynucleotide kinase in a 20-μl solution at 37 °C for 1.5 h. The kinase reaction was stopped by heating at 65 °C for 10 min. Additional 4-μl samples were taken from the 0, 5, 30, and 120 min heat-treated reaction aliquots. These were diluted five times with 16 μl of H_2O to adjust the DNA concentration to be the same as that of the phosphorylated sample. To 4 μl of each of these samples was added 2 μl of loading dye (deionized formamide with 0.37% EDTA, 0.3% bromphenol blue, 0.3% xylene cyanol). They were then sealed, boiled for 3 min, then opened, and loaded onto an 8 M urea, 20% polyacrylamide gel (16 × 36 × 0.05 cm). The DNA markers, 3-, 8-, 12-, 13-, and 14-base oligonucleotides (obtained from the University of Wisconsin Biotechnology Center), were labeled at their 5′-ends with [γ-^{32}P]ATP and T_4 polynucleotide kinase, and appropriate amounts of these marker DNAs were loaded onto the gel. Electrophoresis was carried out at 30 watts (constant power) for about 3 h. The bands were visualized by autoradiography.

Attempt to Detect Oligonucleotide Exchange between Half-sites—pXHQ01 was digested with *Xba*I and *Pst*I, and labeled at the *Xba*I-cut 3′-end with [α-^{32}P]dCTP as described above. Additional *Xba*I-cut pXHQ01 was filled in with nonradioactive dCTP in the same way. The resulting unlabeled half-site was ~2.8 kbp in length, compared to 2.1 kbp for the labeled substrate. A reaction solution (108 μl) without FLP protein was prepared, containing 2.7 nM of the labeled half-site plus 2.7 nM of unlabeled half-site. A 9-μl aliquot of this solution was removed and treated with 1 μl of 10% SDS to serve as a zero time control. Diluted FLP protein (11 μl) was added to start the reaction (final FLP protein concentration was 42 nM). Aliquots (10 μl) of reaction solution were removed after 5, 30, and 120 min of incubation, and reactions were stopped by adding 1 μl of 10% SDS. The zero time control (10 μl) and SDS-stopped reaction aliquots (11 μl) were loaded onto a 0.8% (w/v) agarose mini gel as described above. To serve as a marker, 0.025 μg of *Xba*I-cut pXHQ01 (3′-end-labeled with [α-^{32}P]dCTP) was also loaded in a separate lane. Gel electrophoresis was carried out at 70 V for 100 min. The gel was stained with ethidium bromide and photographed with polaroid 57-film. Then the gel was dried over DE81 paper and visualized by autoradiography.

FLP-mediated DNA Cleavage—pXHQ01 was cut with *Xba*I and *Pst*I, and labeled at the *Xba*I-cut 3′-end with [α-^{32}P]dCTP as described in DNA Manipulation. For this experiment, specific activity of the labeled DNA was 446 Ci/mmol. Seven standard reaction solutions were prepared (90 μl each). An aliquot, 9 μl, was taken from each and diluted with 11 μl of H_2O as a zero time reaction control. Stock FLP protein was diluted as needed, and 9 μl of the appropriately diluted FLP protein was added to start each reaction. Aliquots (10 μl) were removed from each reaction at indicated times, immediately diluted with 10 μl of H_2O and heated at 90 °C for 2 min. Each aliquot was then put on ice until the experiment was completed. After the experiment, condensation in the tubes was collected by microcentrifugation. The samples were stored at −20 °C for no longer than 8 days and thawed as needed. To provide an internal standard, 0.43 pmol of the labeled half-site DNA were digested with *Bam*HI to completion in an 80-μl reaction volume. Digestion was stopped by heating at 90 °C for 2 min. The solution was diluted 2-fold by adding 80 μl of H_2O to adjust the half-FRT site concentration to be the same as that of half-FRT site cleavage reaction aliquots. Thawed reaction samples (5 μl) were mixed with 2–3 μl of loading dye (0.37% EDTA, 0.3% bromphenol blue, and 0.3% xylene cyanol in deionized formamide), boiled for 3 min, then loaded onto an 8 M urea, 12% polyacrylamide gel. The *Bam*HI-digested half-FRT site standard DNA solution was diluted further as needed with H_2O, and 5 μl of each of the diluted and undiluted standard solutions was combined with 2–3 μl of loading dye and loaded onto the gel in the same way as the reaction samples. Electrophoresis was carried out at 35 watts for 100 min in 1 × TBE. The gel was put onto DE81 paper and the bands visualized by autoradiography. Bands were localized on the gel with the aid of the developed x-ray film, and cut out. Quantitation of bands was provided by liquid scintillation counting (Bio-Safe II Fluor from Research Products International Corp.) on a Beckman LS-3801 counter. The extent of cleavage of half-FRT site DNA was estimated from the linear standard curve derived from the *Bam*HI-digested half-site DNA.

Extent of Cleavage in Complexes—pXHQ01 was cut with *Xba*I and *Pst*I and labeled at the *Xba*I-cut 3′-end with [α-^{32}P]dCTP as described in DNA Manipulation. Specific activity was 446 Ci/mmol. A 36-μl reaction mixture was prepared with standard component concentrations including 5.4 nM FRT half-sites, and reaction was initiated with 4 μl of diluted FLP protein (42 nM FLP protein final concentration). After 5 and 30 min of incubation at 30 °C, 10 μl of reaction solution was removed and loaded into agarose mini gel. Gel electrophoresis was performed as described above. After obtaining gel band intensities from densitometer scanning, the gel was dried over DE81 paper, and the amount of radioactivity in each band was also quantified by cutting the band out and scintillation counting. The amount of ^{32}P that would be present in each band without cleavage was determined by comparing the amount of radioactivity and the band intensity with standard curves for both parameters generated from samples of the unreacted labeled substrate loaded onto the same gel.

Complex Half-life Determination—Reaction mixtures were made up with standard reaction components including 42 nM FLP protein and 5.4 nM FRT half-site substrates. Two half-site substrates were used: *Xba*I-cut pXHQ01 and a 0.6-kbp substrate derived from *Xba*I- and *Nru*I-cut pXHQ01. The smaller substrate was labeled at the *Nru*I (distal to the half-site) end with [γ-^{32}P]ATP as described above. Reaction mixtures were made up with both substrates separately and incubated at 30 °C for 10 min to allow complex formation. One volume (6 μl) of the reaction mixture with the labeled small substrate was mixed with 19 volumes (114 μl) of the reaction mix with the large substrate. FLP and total substrate concentrations remained constant

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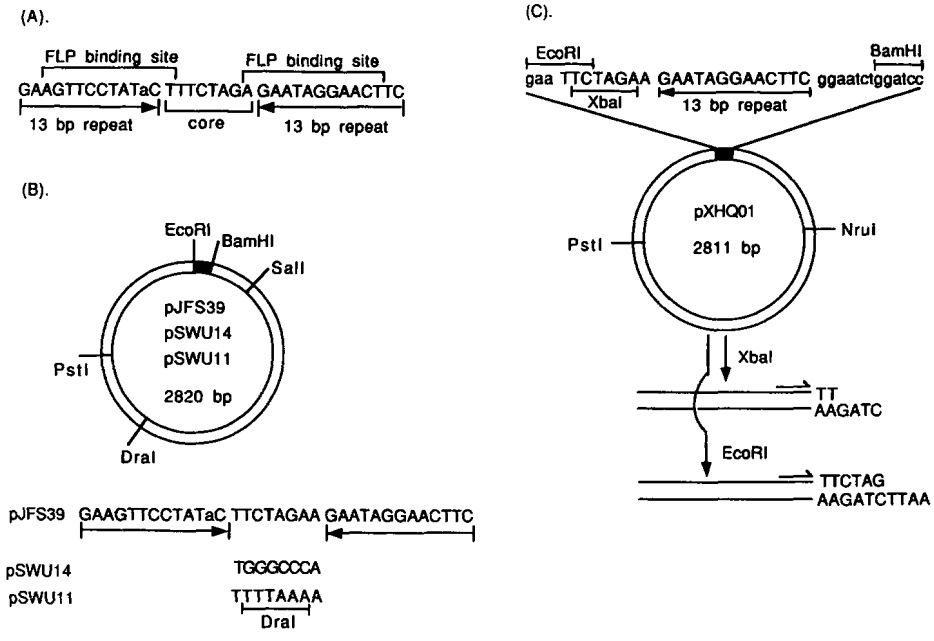


FIG. 1. **DNA substrates used in this study.** *A*, the minimal FRT site. *B*, plasmids pJFS39, pSWU14, and pSWU11; these plasmids are identical except for the core sequences in each FRT site as indicated, and the left and right 13-bp repeats are switched in the pSWU series relative to pJFS39. *C*, the plasmid pXHQ01, source of most of the half-site substrates. The FRT site in this plasmid represents a cloned half-site, with only one FLP protein-binding site. Cleavage with *Xba*I or *Eco*RI produces substrates terminating in core sequences as shown.

throughout. Aliquots (10 μl) of the reaction mix were removed and loaded onto a 0.8% agarose gel (nondenaturing) at the indicated times. Recycling of complexes was manifested by the transfer of label into larger species. Variations of this protocol for control experiments are noted in the figure legends. Electrophoresis, autoradiography, and band quantitation were carried out as described above.

Half-site Reaction with Mutant FLP Protein—Mutant FLP protein (Tyr^{343}→Phe) was diluted from the stock solution with 1 × dilution buffer (25 mM TAPS, pH 8.0 at 25 °C), 1 mM EDTA, and 1 M NaCl) to provide mutant protein concentrations indicated. The 2.1-kbp fragment from *Xba*I- and *Pst*I-cut pXHQ01 was used as substrate. Standard reaction conditions were used. The half-site reaction was started by adding 2 μl of appropriate diluted mutant FLP protein into 18 μl of half-site solution. In the control reaction, 2 μl of 2-fold diluted wild-type FLP protein (to final concentration = 42 nM) was added into 18 μl of half-site solution with the same DNA substrate. After 5 min of incubation, 10-μl aliquots of each reaction solution were removed and loaded onto agarose mini gel as described above. Electrophoresis was carried out at 50 V for 15 min, then at 70 V for 90 min.

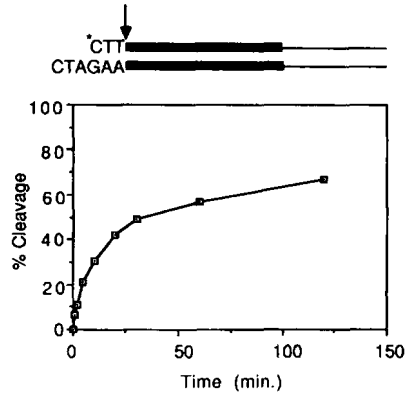


FIG. 2. **Cleavage of *Xba*I- and *Pst*I-cut pXHQ01 labeled with [α-^{32}P]dCTP.** Reactions were carried out as described under "Materials and Methods," with 5.4 nM half-site DNA and 42 nM FLP protein.

RESULTS

Experimental Design—Our strategy was to determine what reactions, if any, would occur with FRT sites that lacked one of the two FLP protein-binding sites. These are referred to as half-sites or half-FRT sites throughout this paper. The DNA substrates used in this study are outlined in Fig. 1. In most experiments, the linear half-site substrates were obtained by cleavage of the plasmid pXHQ01. The FRT site in this plasmid represents a cloned half-site and cleavage with either *Eco*RI or *Xba*I places the half-site at the end of a linear DNA molecule (2,811 bp) (Fig. 1*C*). The linear substrates terminate at a point that includes either half (*Xba*I) or nearly all (*Eco*RI) of the core sequence as shown. Note that a head to head joining of two *Xba*I-cleaved half-sites will produce a nominally complete FRT site with a normally sized (8 bp) core region. This is referred to as a pseudo FRT site. A similar joining of *Eco*RI-cut half-sites would produce a FRT with a 16-base pair core.

Half-site substrate was also produced from pSWU11, which also has a palindromic core sequence with a centrally positioned restriction site (Fig. 1*B*). Cleavage of this plasmid produces two linear DNA fragments of 0.4 and 1.7 kb, each with a half-site at one end. The 1.7-kb half-site DNA was gel-purified. The reaction of these linear half-site substrates was examined with a variety of methods described below.

Reactions of Half-FRT Sites—The first objective was to determine if these half-sites were cleaved by FLP protein. *Xba*I- and *Pst*I-cleaved pXHQ01 was used as a substrate, labeled at the 3′-end with [^{32}P]dCTP as shown in Fig. 2 and described under "Materials and Methods." Site-specific cleavage of this substrate should produce a labeled nucleotide trimer. The appearance of an appropriately sized band (characterized below) was observed and quantified using polyacrylamide gels run under denaturing conditions, and a typical time course is presented in Fig. 2. This result demonstrates that cleavage of these substrates occurs. In this case, about half of the DNAs are cleaved in 30 min, and a slower rate of cleavage is seen after 30 min. This result suggested that steps up to and including cleavage do not require interactions between FLP monomers bound to the FRT, but this perception was altered by the next two experiments.

To examine the reaction for any unusual recombination products, the reaction mixtures were loaded onto agarose gels and electrophoresis was carried out under nondenaturing conditions. As shown in Fig. 3, this experiment revealed the presence of three bands in addition to substrate that are produced when FLP protein is reacted with *Xba*I-cleaved

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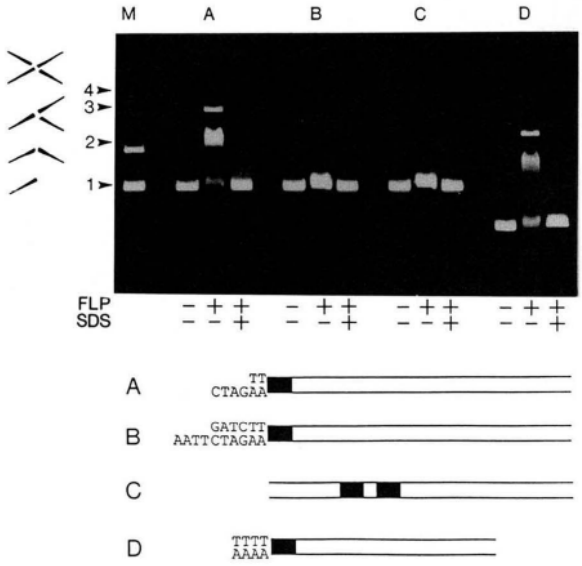


FIG. 3. **DNA species formed in FLP protein reactions with half-sites.** Nondenaturing agarose gel electrophoresis was carried out as described under "Materials and Methods," with 5.4 nM half-site DNA and 42 nM FLP protein. FLP protein and SDS are present as indicated in each lane. *Lane M* contains linear DNAs of 2.82 and 5.59 kbp, generated as described under "Materials and Methods" for use as markers. Reaction sets involved *Xba*I-cut pXHQ01 (*A*); *Eco*RI-cut pXHQ01 (*B*); *Sal*I-cut pSWU14 (*C*); and *Dra*I-cut pSWU11 (*D*). The core sequences found at the termini of the half-site substrates are shown. The *symbols* at the left of the figure are simplified renditions of the species present in each band, as seen in Fig. 4.

pXHQ01 (*reaction set A*). These are labeled *2*, *3*, and *4* in Fig. 3, and their intensity and rate of migration in the gel decrease in that order. Most of the DNA is found in band *2*, while band *4* is just detectable. These new species appeared within 5 min, and their relative amounts remained constant for 30 min.[4] All of the bands disappear when the sample is treated with SDS prior to loading on the gel, indicating the new bands involve noncovalent interactions between the macromolecules in the reaction.

The new bands did not appear when FLP protein was reacted with either *Eco*RI-cut pXHQ01 or with *Sal*I-cut pSWU14 (Fig. 3, *reaction sets B* and *C*, respectively). The latter substrate contains a full FRT site that is bound tightly by FLP protein but not recombined (Umlauf and Cox, 1988). Although the new species are not formed with these substrates, there is a slight retardation of band migration in both cases that may reflect FLP protein binding. These results indicate that the four extra base pairs in the *Eco*RI-cut substrate block formation of the new species, and that the retardation in migration is not simply a result of FLP protein binding. The band labeled *2* runs at a position corresponding to a DNA molecule twice the size of the linear pXHQ01, and these results suggest that FLP protein is linking linear DNA molecules by means of stable noncovalent interactions. Notably, no new species were detected in reactions with either uncleaved or *Bam*HI-cleaved pXHQ01 DNA,[4] reinforcing the idea that an appropriately sized core sequence at the terminus of the DNA is required to form these species.

Interestingly, when *Dra*I-cut pSWU11 (a linear half-site substrate that has four base pairs of core sequence that terminate in a blunt end) was used, the new species were formed almost as efficiently as with the *Xba*I-cut pXHQ01 (Fig. 3, *reaction D*). A head to head joining of two molecules of this substrate will produce a pseudo-full FRT site with an 8-bp core, as with *Xba*I-cleaved pXHQ01. In this case, however, there is no potential for stabilizing such a complex via base pairing between DNA sticky ends. This result indicates that formation of the observed species depends primarily upon protein-protein and/or protein-DNA interactions.

As a result of the experiment in Fig. 3, the species *2*, *3*, and *4* were tentatively identified as complexes containing 2, 3, and 4 substrate DNAs, respectively, linked at the half-sites. This assignment was confirmed by electron microscopy. The species in Fig. 4 (*set A*) were formed, fixed, and spread under partially denaturing conditions described under "Materials and Methods." Those formed under standard spreading conditions were identical except that they lacked the denaturation "bubbles" (data not shown). Of 120 molecules examined in one trial (standard spreading conditions), species *1*, *2*, *3*, and *4* represented 50, 38.3, 8.3, and 3.3% of the total, respectively). Adjusting the formaldehyde concentration used for fixation from 1.0% to either 0.5 or 2.0% had no significant effect on these results. Although the relative amounts of species *2*, *3*, and *4* are consistent with the observations in Fig. 3, the fraction of monomers converted to the other forms is significantly less when measured by EM. This could be due to less than quantitative preservation of these noncovalent macromolecular complexes during fixation or spreading, or to a reduced reaction efficiency resulting from the omission of PEG and glycerol in the reaction mixtures used for electron microscopy. In one trial in which glycerol (10%) was retained in the reaction mixture, the representation of the larger complexes increased somewhat; species *1*, *2*, *3*, and *4* represented 41, 43, 14, and 2.4% of the total, respectively. The assignment of the uppermost band in Fig. 3 as a tetrameric species is somewhat tentative since the band is barely visible and these species are rare in the EM measurements. However, tetramers are easy to find on the EM grids and much more prevalent than any other DNA species besides the monomers, dimers, and trimers, and their relative abundance in the EM is consistent with the weak band seen in the gel.

The DNAs in all species are clearly joined at a point where one end of each coincides. This is the same end in each case, as indicated by the denaturation bubbles in the DNA that serve as landmarks in samples spread under appropriate conditions (Fig. 4). A prominent denaturation tract is generally seen at the end of this DNA distal to the half-FRT site, manifested as a bubble or fork at that end. This feature was found in all cases at the end distal to the end where the DNAs came together. The positions of the denaturation bubbles in

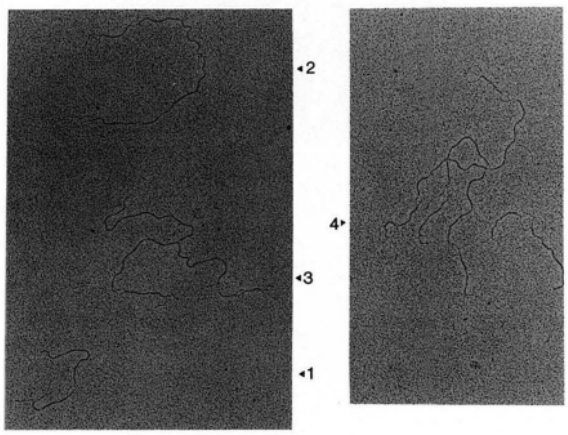


FIG. 4. **Electron microscopy of the species observed in reaction set A in Fig. 3.** Sample was prepared under partial denaturation conditions. Examples of monomeric (*1*), dimeric (*2*), trimeric (*3*), and tetrameric (*4*) molecules are shown.

[4] X.-H. Qian and M. M. Cox, unpublished data.

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45 of these molecules were mapped and the data used to generate a histogram to compare with a pattern of denaturation predicted on the basis of sequence analysis as described by Funnel and Inman (1979). The observed denaturation pattern corresponded exactly to that predicted on the basis of sequence analysis of the plasmid, confirming that all species with more than one monomer are joined at their FRT half-site ends (data not shown). Measurement of the length of the larger linear species confirmed that they were of dimeric length of pXHQ01. None of these species were observed by electron microscopy when *Eco*RI-cleaved pXHQ01 was used as substrate (data not shown), confirming the result with the agarose gel. Together, these results indicate that FLP protein forms tight noncovalent complexes that link two or more half-site substrates, and that formation of these complexes requires a partial core sequence capable of forming a pseudo-full FRT site when two are brought together.

It is worth noting that the band produced by the dimeric species in the electrophoresis experiment (Fig. 3) is broadened, skewed in the direction of larger species. This result was seen in all of the electrophoresis experiments and it may indicate a dynamic, reversible interaction between dimers that is occurring while the gel is run. This effect has not been characterized further.

The results presented in Fig. 5 indicate that cleavage of these DNA substrates occurs in a site-specific manner at the position expected for a FLP-mediated cleavage event. With *Xba*I- and *Pst*I-cut pXHQ01 labeled with [^{32}P]dCTP, a FLP-dependent cleavage product is seen on a denaturing polyacrylamide gel (*lanes 2–4*). When phosphorylated (*lane 5*), this product comigrates with a phosphorylated nucleotide trimer, as expected.

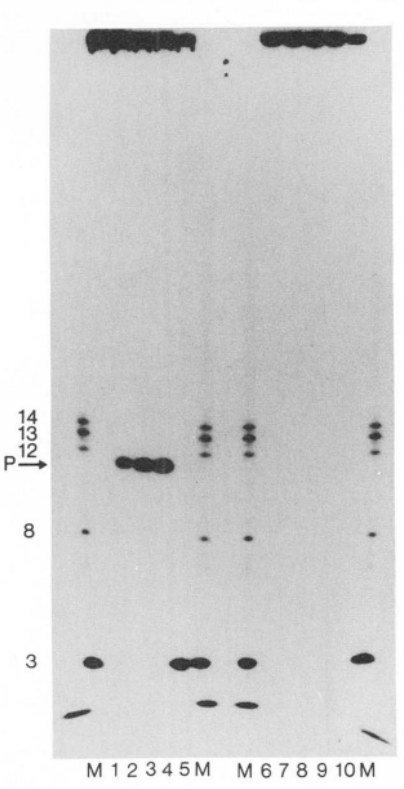


FIG. 5. **Cleavage of half-site DNA by FLP protein.** Reactions in *lanes 1–5* involve 5.4 nM [α-^{32}P]dCTP-labeled, *Xba*I- and *Pst*I-cut pXHQ01. *Lanes 6–10* involve reactions with 5.4 nM pXHQ01 cut with *Eco*RI, then labeled with [α-^{32}P]dATP. Time points (*lanes 1–4* or *6–9*) were 0, 5, 30, and 120 min, left to right. *Lanes 5* and *10* represent the samples in *lanes 4* and *9*, respectively, after phosphorylation. The *symbol P* indicates the migration position of the cleavage product prior to phosphorylation. The *markers* (*M*) are phosphorylated oligonucleotides with the lengths (in nucleotides) indicated to the left of the figure.

In this same experiment it is demonstrated that no FLP-mediated cleavage occurs with the *Eco*RI-cut pXHQ01 (Fig. 5, *lanes 6–10*). This experiment was carried out three times with the same result. It indicates that cleavage is coupled in some manner to formation of the complexes described above.

In Fig. 6, it is demonstrated that cleavage is not accompanied by detectable levels of strand exchange between half-sites, which would amount to an intermolecular transfer of an oligonucleotide trimer. Two linear *Xba*I-cut half-site substrates were prepared that differed in length. One was labeled with [^{32}P]dCTP and the other with unlabeled dCTP. Strand exchange (transfer of the labeled trimer from one substrate to the other) would be manifested by the appearance of label in the 2.8-kbp half-site. This does not occur (Fig. 6) although complex formation occurred efficiently in this experiment,[4] and the depletion of ^{32}P in the labeled substrate with time (relative to a labeled non-half-site control fragment) is consistent with an ongoing cleavage reaction. The oligonucleotide trimer produced in the cleavage reaction evidently leaves the complex as it is formed.

Characterization of the Cleavage and Complex Formation Reactions of Half-FRT Sites—A series of experiments was done to examine the extent of cleavage in the various complexes, the dependence of cleavage and complex formation on FLP protein concentration, and the stability of the complexes.

To examine cleavage in individual species, the complexes formed with [^{32}P]dCTP-labeled half-sites were cut out of a gel such as that in Fig. 3, and the label remaining in each complex determined as described under "Materials and Methods." The extent of cleavage in individual complexes found in a typical experiment varies characteristically. In the dimer species about half of the [^{32}P]dCTP label is absent, indicating that about half of the possible cleavage events had occurred. This suggests that one of the two possible cleavage events occurs in each dimeric species. This experiment was repeated three times, and each time with dimeric complexes isolated from both 5 and 30 min after FLP protein addition. The extent of cleavage found in the dimers was 50 ± 5% in all of the six separate measurements, with no pattern indicating a difference between 5- and 30-min time points. In the trimers, 69 ± 8% of the possible cleavage events are observed (*i.e.* 31%

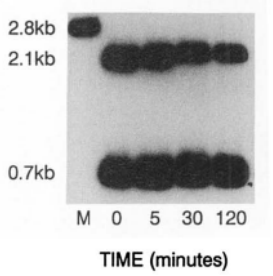


FIG. 6. **Oligonucleotide trimer products of FRT half-site cleavage are not transferred between half-sites.** Two half-site substrates of 2.8 and 2.1 kbp were produced from pXHQ01 as described under "Materials and Methods." The 2.1-kbp half-site was labeled with [^{32}P]dCTP, along with a 0.7-kbp *Xba*I-*Pst*I DNA fragment without a half-site that is produced as a byproduct of the cuts generating the 2.1-kbp substrate. A time course of the reaction involving both the 2.8 and 2.1 + 0.7-kbp substrates is shown. The 0.7-kbp fragment serves as an internal control against which to measure label depletion over time in the 2.1-kbp half-site. Reactions were carried out under standard conditions described under "Materials and Methods," with 5.4 nM total half-site substrates (2.7 + 2.7 nM) and 42 nM FLP protein.

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of the label is present in these species). In the tetramers, only a trace of label remains in the complexes, suggesting that most or all possible cleavage events have occurred within this complex. Note that the approximately 50% cleavage seen in the dimeric complex suggests a structural and functional asymmetry in these complexes.

As shown in Figs. 7 and 8, both the cleavage and complex formation reactions exhibit a somewhat cooperative dependence on FLP protein concentration. Rates of cleavage (Fig. 7) level off after 30 min. At lower FLP protein concentrations little cleavage is seen after 30 min. Above 33.6 nM FLP protein, a slow rate of cleavage is seen after 30 min. Notably, rates of cleavage continue to increase at FLP protein concentrations well above that required to saturate available FLP protein-binding sites. Complex formation is examined in Fig. 8. As FLP protein concentration increases, the reaction equilibrium shifts to favor the dimeric and trimeric complexes. Notably, higher FLP protein concentrations do not drive the reaction toward formation of tetramers. Instead, dimers become even more prominent at high FLP protein concentrations, and trimers are always much more prominent than tetramers. The pre-eminence of trimers relative to tetramers represents a second indication of structural and functional asymmetry in these complexes.

One caveat in these experiments is that the substrate used for the cleavage experiments, which includes a labeled cytidylate added at the *Xba*I-cut site, cannot form a pseudo-full FRT site with a normal 8 bp of core DNA. The addition of a deoxycytidylate has a maximal effect on the amount of FLP protein required to saturate the complex formation reaction, but the highest level of complex formation (monomers appearing as higher order forms in a nondenaturing agarose gel) is about 20% lower for this substrate than for *Xba*I-cleaved DNA. For this reason, the results in Figs. 7 and 8 are not directly comparable.

We also note that some activity loss occurs in our FLP protein preparations upon storage over a period of months, and the fact that an apparent 4–8-fold excess of FLP protein is required to obtain efficient cleavage and complex formation reactions may reflect the presence of some inactive FLP protein rather than an effect of binding affinity. Nevertheless, these experiments define the species prevalent at high FLP protein concentrations.

The stability of these complexes is evident from the fact that they survive agarose gel electrophoresis over a period of 1–2 h. Stability was examined further by forming complexes with half-site substrates, this time labeled at the end *distal* from the half-site, and determining how fast these complexes decayed after a challenge with a large excess of unlabeled half-site DNA reaction. The experiment is presented in Figs. 9 and 10. As for the experiment of Fig. 6, two half-site substrates with different lengths were prepared from pXHQ01. One was the *Xba*I-cut substrate used in many experiments above, and the other was a 0.6-kbp *Xba*I-*Nru*I fragment of pXHQ01. The latter was labeled with [γ-^{32}P] ATP at the *Nru*I end (distal to the half-site). Two reaction mixtures were prepared. The reaction volume used for the unlabeled substrate was 19-fold larger than that for the labeled substrate, although all reactants and solution components were present at the same concentrations. These reactions were incubated at 30 °C for 10 min to allow complex

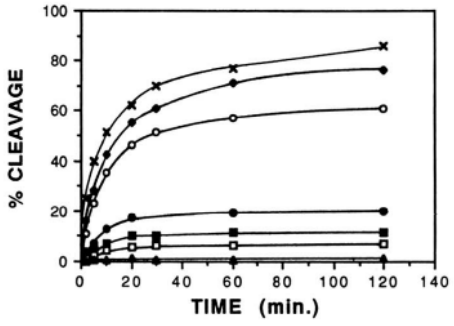


FIG. 7. **Rates of half-site cleavage as a function of FLP protein.** Reactions were carried out with 5.4 nM [α-^{32}P]dCTP-labeled *Xba*I- and *Pst*I-cut pXHQ01 half-site DNA and quantified as described under "Materials and Methods." FLP protein concentrations (in nM) were ▲, 8.4; □, 16.8; ■, 25.2; ●, 33.6; ○, 42; ◆, 63; ×, 84.

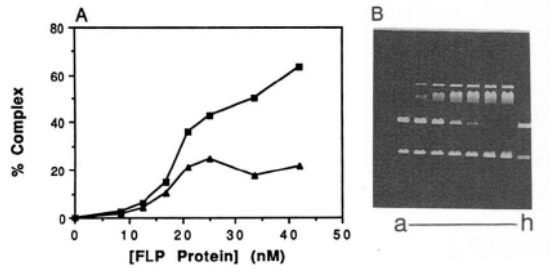


FIG. 8. **Complex formation as a function of FLP protein concentration.** The 5.4 nM half-site DNA substrate was the same as that used in Fig. 7, but without the [^{32}P]dCTP label. See "Materials and Methods" for details. *Panel A*, the symbols ■ and ▲ denote dimers and trimers, respectively. *Panel B*, FLP protein concentrations (in nM) for each lane are *a*, 8.4; *b*, 12.6; *c*, 16.8; *d*, 21; *e*, 25.2; *f*, 33.6; *g*, 42; *h*, 0. The upper and lower bands in *lane h* represent the 2.1- and 0.7-kbp DNA fragments produced by *Xba*I and *Pst*I cleavage of pXHQ01 (see Fig. 1). Only the larger fragment contains a terminal half-site. The smaller fragment serves as an internal control for any effects due to nonspecific protein binding.

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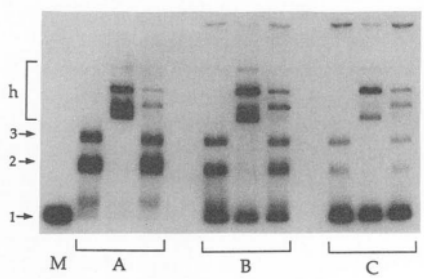


FIG. 9. **Stability of FLP-generated dimers and trimers of FRT half-sites.** ^{32}P-Labeled 0.6-kbp and unlabeled 2.8-kbp FRT half-site substrates were prepared as described under "Materials and Methods." Standard reaction mixtures were prepared with each including 5.4 nM half-site substrate and 42 nM FLP protein. An additional reaction mixture was prepared with *Hin*dIII-cut pXf3 DNA (unlabeled) and FLP protein at the same concentrations. Reaction sets *A*, *B*, and *C* represent 5-, 140-, and 380-min time points, respectively. *Lane M* is the labeled 0.6-kbp DNA prior to any reaction, and this marker serves as a zero time control. In each reaction set, the three lanes left to right are a reaction with one volume (2 μl) of the labeled 0.6-kbp half-site substrate combined with 19 volumes of the reaction mix containing *Hin*dIII-cut (unlabeled) pXf3 (DNA (*1*), a reaction in which one volume (1.8 μl) of the labeled half-site substrate was mixed with 19 volumes of the unlabeled 2.8 kbp half-site reaction mix prior to addition of FLP protein (*2*), and a reaction in which each reaction mixture was incubated at 30 °C for 10 min with FLP protein before mixing one volume (2 μl) of the labeled mix with 19 volumes of the unlabeled ones (*3*). The substrate (monomer), dimer, and trimer species produced in the control in the first lane of each set are indicated by the *numbers 1*, *2*, and *3* at the left of the figure. The *symbol h* denotes hybrid dimer and trimer species that incorporate at least one molecule of the labeled substrate in the second and third lanes of each set. Time points for the third reaction lane in each set are measured from the time of mixing the labeled and unlabeled samples. For the controls in the first two lanes of each set, $t = 0$ was defined as 10 min after the time of addition of FLP protein.

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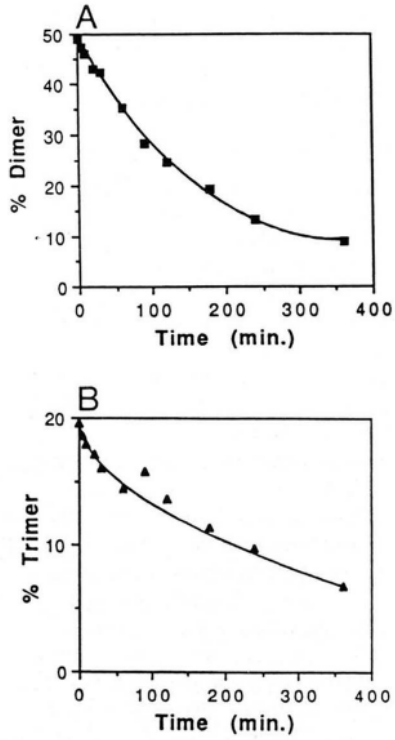


FIG. 10. **Dissociation of dimer and trimer complexes.** Decay of these complexes was measured as illustrated in Fig. 9. The values plotted represent the fraction of total labeled 0.6-kbp half-site DNA present as the dimeric or trimeric species derived from this substrate (as in the third lane of each reaction set in Fig. 9).

formation, mixed, and disappearance of the complexes formed with the labeled substrate alone was followed with time. In Fig. 9, reaction sets A, B, and C represent early (5-min), 140-, and 380-min time points, respectively. The first two lanes in each set are controls. The first lane is a complex formation reaction with the labeled half-site challenged with unlabeled linear pXf3 DNA (Sutcliffe, 1978) according to the same protocol. The plasmid pXf3 is a pBR322 derivative that lacks a binding site for FLP protein. The second lane represents a reaction in which the two DNA substrates were mixed prior to addition of FLP protein. Since the larger unlabeled DNA is present in 19 × excess, virtually all the label is seen in complexes involving this larger substrate. The second lane in each set therefore serves to define the hybrid complexes expected to form as complexes involving the small labeled substrate dissociate in the presence of the larger substrate. The last lane in each set is the challenge experiment in which labeled and unlabeled half-sites are allowed to form complexes prior to mixing, as described above. In set A, the third lane represents the status 5 min after mixing. Some label is present in the hybrid complexes, possibly derived from the small amount of uncomplexed labeled substrate evident in the first lane of the set. The dimeric and trimeric complexes of labeled substrate predominate and there is no evident difference in their intensities in lanes 1 and 3 of set A. Set B shows the same transfer has occurred after 140 min, reflected in the increased intensity of the hybrid bands relative to the original dimer and trimer complexes. After 380 min (set C), the hybrid bands are relatively more prominent than the original complexes.

Note that there is a general decrease in the intensity of the bands corresponding to all complexes with time that probably reflects a slow inactivation of FLP protein. In addition, the agarose gel assay used in this experiment introduces an unavoidable 5–10 min lag between the taking of a solution aliquot and the separation of the species on the gel. An estimate of the rate of dissociation of the dimeric and trimeric species, based on the observed decrease of the intensity of the bands representing the original dimeric and trimeric complexes at an expanded set of time points, is shown in Fig. 10. Since they include losses of complexes due to FLP inactivation, these curves represent an upper limit for the rate of dissociation. Although for reasons cited above only broad limits can be placed on the dissociation rates based on this experiment, it is clear that the rates can be measured in hours. The half-life of dimers (barring FLP inactivation) is at least 2 h. Trimers appear to be even more stable, with a half-life of 4 h or more.

The hybrid species evident in Fig. 9 are interesting in themselves and require further characterization. Preliminary characterization indicates the presence of at least two types of hybrid trimers. The hybrid species that predominates when the DNAs are mixed prior to FLP protein addition (*middle lane* in each set, Fig. 9) are somewhat different from those that are formed predominantly as dissociation of preformed complexes occurs (*third lane*, each set). The reasons for this are presently unclear.

Cleavage Is a Requirement for Complex Formation—One final experiment was carried out to see if complexes could be formed with a mutant FLP protein (Tyr^{343}→Phe) that can bind normally to FRT sites but lacks the active site tyrosine required for cleavage. As shown in Fig. 11, no multiple DNA complexes were formed with this mutant protein. The substrates in this experiment included two linear DNA fragments, and only the larger of the two had a terminal *Xba*I-cut half-site. The wild-type protein efficiently converted the half-site substrate to complexes as shown in the last lane. This experiment was repeated using additional concentrations of FLP protein spanning the range 18–54 nM (data not shown). In every experiment, the mutant protein produced no complexes. The slight change in band mobility evident at the higher mutant FLP protein concentrations represents nonspecific protein binding (possibly reflecting contaminants in the prep-

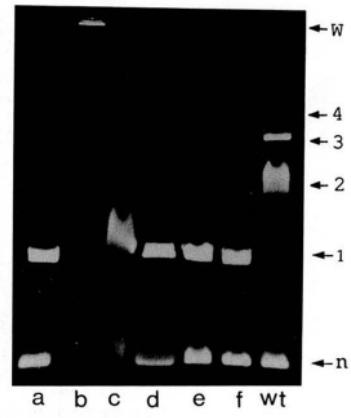


FIG. 11. **No complex formation observed with a mutant FLP protein.** The FLP (Tyr^{343}→Phe) protein was used as described under "Materials and Methods" with 5.4 nM *Xba*I- and *Pst*I-cut FRT half-site substrate (2.1 kbp). An equivalent amount (in DNA molecules) of the 0.7-kbp non-half-site DNA derived from this digestion of pXHQ01 was included as a nonspecific DNA control. The 2.1- and 0.7-kbp substrates are denoted *1* and *n*, respectively. The positions of dimeric, trimeric, and tetrameric species derived from the 2.1-kbp half-site substrate are shown with *arrows labeled 2*, *3*, and *4*. *W* denotes the position of the wells in the agarose gel. *Lanes a–f* denote 5 min reactions with 0, 72, 36, 36 (plus SDS treatment prior to electrophoresis), 18, and 9 nM mutant FLP protein, respectively. *Lane wt* shows the result of a standard 5-min reaction of this DNA with 42 nM wild-type FLP protein.

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aration) since it affects both the half-site and non-half-site substrates (*lane c*). The capacity of this mutant protein to bind specifically to FRT sites was confirmed independently.[5] This result therefore indicates that at least one cleavage event is a prerequisite for formation of the complexes observed in this study.

DISCUSSION

The primary conclusion of this study is that a very stable dimer of FLP protein is formed in reactions involving half-FRT sites. The stability of this species reflects strong noncovalent interactions between FLP monomers. This complex represents a possible early intermediate in FLP protein-mediated recombination and indicates that strong noncovalent cross-core interactions are an important feature of FLP protein-mediated site-specific recombination. The strong noncovalent interactions in this complex survive electrophoresis in an agarose gel and exhibit half-lives that are measured in hours.

The data suggests that the protein components of the dimeric complex are conformationally asymmetric. The palindromic pseudo-FRT site formed in these experiments presumably reflects a head to head interaction between two FLP monomers. There are two pieces of evidence in this study that indicate that the FLP monomers in the dimeric complexes are conformationally asymmetric. (a) About half of the possible cleavage events have occurred in the isolated dimers, and (b) among higher order complexes trimers are much more abundant than tetramers. One interpretation of these results is that the asymmetric cleavage of a FRT site required to generate a Holliday structure intermediate in a normal recombination reaction is inherent to the recombinase complex itself. Signals in the DNA sequence are not required (although they are likely to have some effect). This helps explain the efficient generation of Holliday structure intermediates with FRT sites that are perfectly palindromic. In this model, cleavage of these sites may occur on either side of the core, but only one of the two possible cleavage events will occur in any given complex in the population. The FLP protein monomers bound on either side of the core sequence are in different conformations, only one of which promotes cleavage. This conformational asymmetry would have to be coordinated between the FLP monomers bound to two reacting FRT sites in order to generate a Holliday intermediate.

Cleavage does not occur with half-sites that do not form complexes, suggesting that complex formation is required and precedes cleavage. The FLP (Tyr^{343}→Phe) mutant, on the other hand, does not form complexes, suggesting that cleavage is required for complex formation. This apparent paradox can be resolved, and the results of this paper summarized, with the aid of the hypothetical pathway for early events in the normal recombination reaction illustrated in Fig. 12. The FLP recombinase is shown as a monomer in solution based on the binding studies of Sadowski and colleagues (Andrews *et al.*, 1986, 1987; Beatty and Sadowski, 1988) and the behavior of the protein in a sucrose density gradient.[5] Binding of one monomer to a FRT site generates a species called complex I (Andrews *et al.*, 1987). The apparent absence of strong interactions prior to binding indicates that interaction with the FRT site induces at least one conformation change in the FLP protein. We suggest that two FLP monomers bind separately to the minimal FRT site creating a complex with a cross-core interaction. Formation of initial complex (*IIa*) probably correlates with the DNA bending observed by

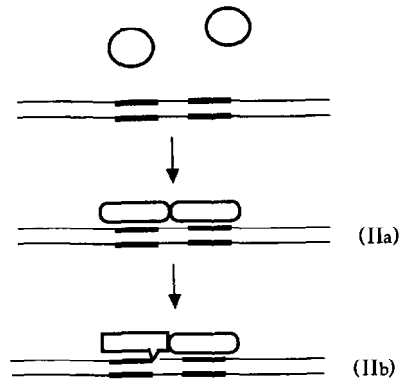


FIG. 12. **A model for early steps in FLP protein-mediated site-specific recombination.** See text for details.

Schwartz and Sadowski (1989). The FLP (Tyr^{343}→Phe) mutant binds and bends the FRT site much the same as wild-type FLP protein (Schwartz and Sadowski, 1989), and we use the complex formed by this mutant protein to define complex IIa. Although complex IIa is shown as symmetric and the FLP protein is shown in a different conformation relative to free protein, this is only one of several possibilities. There is no evidence for or against conformation changes up to this point. Conformational asymmetry, perhaps dictated by the order in which the two FLP monomers bind, may also be a feature of complex IIa. Complex IIa also reflects the binding interaction observed in other studies with the FLP (Tyr^{343}→Phe) mutant. This is not, however, the stable dimeric complex we observe because the mutant protein does not support stable formation. One, and possibly both, of the FLP monomers in complex IIa apparently undergoes a conformation change to form an asymmetric complex IIb in which the two subunits are locked tightly together by strong noncovalent interactions. The first cleavage must occur subsequent to formation of complex IIa and may either precede or be concerted with formation of complex IIb. We speculate that the monomer that has cleaved its DNA substrate is covalently linked to its half-site in complex IIb. In this scenario, the stable dimeric species formed by FLP from half-sites is equivalent to complex IIb on the normal reaction path.

Formation of complex IIb may also involve interaction with other FLP protein-FRT complexes, leading in some cases to formation of the observed trimers and tetramers. If formation of complex IIb requires interaction with other complexes, then the stable dimeric species we observe here does not strictly reflect an intermediate in the reaction pathway but instead a stable decomposition product of another intermediate (*e.g.* a tetramer). In this alternative scenario, two complex IIas would form a tetramer within which a coordinated set of conformation changes would occur to form a tetramer consisting of two copies of complex IIb. If the interactions holding the two dimers together were weaker than the cross-core interactions stabilizing the individual dimers, the tetramers might decompose to dimers during electrophoresis to yield the result observed. Even if this is the case, however, the dimer species is nevertheless an indication that there is a very strong, noncovalent, cross-core interaction between FLP monomers after the first cleavage event.

FLP protein is an enzyme with a turnover number on the order of 0.1 min^{-1} (Gates and Cox, 1988). The very stable complexes observed here indicate that FLP complexes do not dissociate after a cleavage event and are effectively locked in at that point in the reaction cycle unless an appropriate acceptor (a DNA strand with a 5′-OH) is available to react with the 3′-phosphotyrosine protein-DNA linkage created in

[5] P. J. Rossmeissl and M. M. Cox, unpublished results.

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the first cleavage step. This is probably an important mechanistic feature of the system, since dissociation of the complex IIb stage in a normal reaction would result in an unproductive nicking of the DNA.

Evidence for the presence of an intact 3′-phosphotyrosine linkage in complex IIb is provided by the fact that these complexes react with normal (full) FRT sites to yield an array of recombinant products.[4] These products are still being characterized. It appears that half-sites can participate in nearly all steps of a complete recombination event as long as the core sequence permits formation of the head to head dimeric complex described here. These reactions provide additional evidence that the observations reported in the present study are relevant to the normal FLP-mediated recombination pathway.

Although FLP and the λ integrase are both in the integrase family of site-specific recombinases, the FLP and λ integrase systems appear to differ in one respect. Whereas half-sites must be joined in a head-to-head complex to form pseudo-full sites for reaction to occur in the FLP system, many of the same reactions occur readily with half-sites alone in the λ system (Nunes-Düby *et al.*, 1989). Strong cross-core interactions between FLP subunits appear to be a required part of the process that coordinates early steps in FLP-mediated recombination. In the λ integrase system this coordination is evidently supplied predominantly by other proteins bound on one side of the core in the much larger recombination site (Landy, 1989), although cross-core interactions may exist and play some as yet undetermined role in the λ reaction.

REFERENCES

Andrews, B. J., Proteau, G. A., Beatty, L. G.,and Sadowski, P. D. (1985) *Cell* **40,** 795–803

Andrews, B. J., McLeod, M., Broach, J. R., and Sadowski, P. D. (1986) *Mol. Cell. Biol.* **6,** 2482–2489

Andrews, B. J., Beatty, L. G., and Sadowski, P. D. (1987) *J. Mol. Biol.* **193,** 345–358

Argos, P., Landy, A., Abremski, K., Egan, J. B., Haggard-Ljungquist, E., Hoess, R. H., Kahn, M. L., Kalionis, B., Narayana, S. V. L., Pierson, L. S., III, Sternberg, N., and Leong, J. M. (1986) *EMBO J.* **5,** 433–440

Beatty, L. G., and Sadowski, P. D. (1988) *J. Mol. Biol.* **204,** 283–294

Berger, S. L., and Kimmel, A. R. (1987) *Guide to Molecular Cloning Techniques*, p. 99, Academic Press, Orlando, FL

Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254

Bruckner, R. C., and Cox, M. M. (1986) *J. Biol. Chem.* **261,** 11798–11807

Cox, M. M. (1989) in *Mobile DNA* (Berg, D. E., and Howe, M. M., eds) pp. 661–670, American Society for Microbiology, Washington, D. C.

Craig, N. (1986) *Annu. Rev. Genet.* **22,** 77–105

Dretzen, G., Bellard, M., Sassone-Corsi, P., and Chambon, P. (1981) *Anal. Biochem.* **112,** 295–298

Funnell, B., and Inman, R. B. (1979) *J. Mol. Biol.* **131,** 331–340

Futcher, A. B. (1986) *J. Theor. Biol.* **119,** 197–204

Gates, C. A., and Cox, M. M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 4628–4632

Gates, C. A., Meyer-Leon, L., Attwood, J. M., Wood, E. A., and Cox, M. M. (1987) *UCLA Symp. Mol. Cell. Biol.* **69,** 197–206

Hoess, R., Wierzbicki, A., and Abremski, K. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 6840–6844

Inman, R. B., and Schnos, M. (1970) *J. Mol. Biol.* **49,** 93–98

Jayaram, M., Crain, K. L., Parsons, R. L., and Harshey, R. M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7902–7906

Kitts, P. A., and Nash, H. A. (1988) *Nucleic Acids Res.* **16,** 6839–6856

Landy, A. (1989) *Annu. Rev. Biochem.* **58,** 913–949

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Handbook*, pp. 124, 466, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Meyer-Leon, L., Gates, C. A., Attwood, J. M., Wood, E. A., and Cox, M. M. (1987) *Nucleic Acids Res.* **15,** 6469–6488

Meyer-Leon, L., Huang, L.-C., Umlauf, S. W., Cox, M. M., and Inman, R. B. (1988) *Mol. Cell. Biol.* **8,** 3784–3796

Meyer-Leon, L., Inman, R. B., and Cox, M. M. (1990) *Mol. Cell. Biol.* **10,** 235–242

Murray, J. A. H., Scrapa, M., Rossi, N., and Cesarini, G. (1987) *EMBO J.* **6,** 4205–4212

Nunes-Düby, S. E., Matsumoto, L., and Landy, A. (1987) *Cell* **50,** 779–788

Nunes-Düby, S. E., Matsumoto, L., and Landy, A. (1989) *Cell* **59,** 197–206

Prasad, P. V., Young, L.-J., and Jayaram, M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2189–2193

Reynolds, A. E., Murray, A. W., and Szostak, J. W. (1987) *Mol. Cell. Biol.* **7,** 3566–3573

Sadowski, P. (1986) *J. Bacteriol.* **125,** 341–347

Schwartz, C. J. E., and Sadowski, P. D. (1989) *J. Mol. Biol.* **205,** 647–658

Schultz, V., and Reznikoff, W. S. (1990) *J. Mol. Biol.* **211,** 427–445

Senecoff, J. F., and Cox, M. M. (1986) *J. Biol. Chem.* **261,** 7380–7386

Senecoff, J. F., Bruckner, R. C., and Cox, M. M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7270–7274

Senecoff, J. F., Rossmeissl, P. J., and Cox, M. M. (1988) *J. Mol. Biol.* **201,** 405–421

Sutcliffe, J. G. (1978) *Nucleic Acids Res.* **5,** 2721–2728

Umlauf, S. W., and Cox, M. M. (1988) *EMBO J.* **7,** 1845–1852

Volkert, F. C., and Broach, J. R. (1986) *Cell* **46,** 541–550

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