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# FLP recombinase is an enzyme

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**ABSTRACT** **The FLP protein of the yeast 2-μm plasmid catalyzes intermolecular site-specific recombination with a turnover number of ≈0.12 $min^{-1}$ (per FLP monomer) for relaxed DNA substrates. Under conditions that enhance its stability, the protein can be used in catalytic rather than stoichiometric amounts. The reaction rate exhibits a strong dependence on FLP protein concentration even when the protein is present in excess relative to available recombination sites.**

Site-specific recombination events play crucial roles in DNA transposition, partitioning of extrachromosomal elements, gene regulation, and generation of genetic diversity (1). A number of site-specific recombination systems have been characterized *in vitro*. These include the bacteriophage λ integration system (2), the resolvases derived from the Tn*3* class of bacterial transposons (3, 4), the bacterial invertases (5, 6), the *Cre-lox* system of bacteriophage P1 (7), and the FLP system of the 2-μm plasmid of *Saccharomyces cerevisiae* (8, 9).

Studies of these systems have provided detailed information about the architecture of the recombination sites, protein–DNA interactions, juxtaposition of two recombination sites, and key reaction intermediates (1, 10–14). A potentially useful approach to many questions that remain is the application of classical enzyme kinetic methods. No detailed kinetic description of a site-specific recombination reaction has appeared. A number of factors contribute to the lack of information about kinetic parameters in these systems. The assays are often tedious, instability of some recombinases may affect reproducibility, and the reactions themselves are complex. Perhaps most important is the fact that it is not clear that these proteins are enzymes. The "recombinase" in each system appears to be required in stoichiometric rather than catalytic amounts (1, 3, 15–17). Enzymatic turnover has not been demonstrated in any case. The focus of this report is turnover in the yeast FLP recombination system.

The yeast 2-μm plasmid encodes a site-specific recombination system (FLP) that inverts the two unique regions of the plasmid (18). This inversion event is central to the strategy by which the plasmid amplifies its copy number (19–21). The only protein required is the plasmid-encoded FLP protein (22), which has recently been purified to near homogeneity (23, 24). The relatively small recombination site (FLP recombination target or FRT) has been characterized extensively. The FLP protein will promote inversions, deletions, or intermolecular recombination efficiently if appropriate substrates are provided. Supercoiled and relaxed substrates react efficiently *in vitro*.

The minimal FRT site consists of two 13-base-pair (bp) repeats separated by an 8-bp spacer (Fig. 1). There are two 12-bp FLP protein binding sites within FRT (25–30). These include the external base pair of the spacer on each side plus the proximal 11 bp of each repeat. A number of mutations are well tolerated when present in one FLP protein binding site but have a much greater than additive effect when present in both (30). The 6 central bp of the spacer have no essential contacts with FLP protein. The sequence here exhibits considerable flexibility. However, there is a requirement for homology between spacers of two reacting FRT sites. A number of FRT sites with spacer alterations have been constructed that are as functional as the wild-type sites in homologous reactions (31–33). The alignment of two sites during recombination is also mediated by asymmetry in the spacer. FRT sites with symmetrical spacers remain functional but permit either of the two possible alignments of two sites during recombination (32). Several of the properties of the FRT site described above were employed in our studies of FLP protein turnover.

## MATERIALS AND METHODS

**Bacterial Strains and Reagents.** Plasmids were maintained in *Escherichia coli* HB101 (34). The restriction enzymes *Pst* I and *Eco*RI were purchased from New England Biolabs. Agarose and optical-grade cesium chloride were from Bethesda Research Laboratories. Bovine serum albumin (Pentax fraction V) was from Miles. PEG 6000 was purchased from Sigma. 3-[tris(hydroxymethyl)methylamino]-1-propanesulfonic acid (Taps) buffer was from Research Organics (Cleveland, OH). All other reagents were the highest grade possible and were purchased from Malinckrodt or Sigma.

**Purification of FLP Recombinase.** FLP recombinase was purified to >95% homogeneity, based on $NaDodSO_4$/PAGE, by using a modification of published procedures (23, 24) (C.A.G., P. J. Rossmeissl, and M.M.C., unpublished). Protein concentrations were determined spectrophotometrically by using the Warburg–Christian equation (35), which assumes an extinction coefficient of 1.0 $ml \cdot mg^{-1} \cdot cm^{-1}$ at 280 nm. This agrees well with a preliminary measured extinction coefficient of 0.84 $ml \cdot mg^{-1} \cdot cm^{-1}$ and a calculated extinction coefficient (based on the tyrosine and tryptophan content of FLP protein) of 1.2 $ml \cdot mg^{-1} \cdot cm^{-1}$ (P. J. Rossmeissl, personal communication). A monomeric molecular weight of 48,600 g/mol was assumed. Protein concentrations are expressed in total monomers. Concentrations of protein used in various experiments are noted in the figure legends.

**Isolation and Manipulation of DNA Substrates.** The construction of the plasmids used as substrates is described elsewhere (30, 32). Plasmid DNA used in the recombination assays was in all cases purified by banding twice in cesium chloride gradients in the presence of ethidium bromide. Linear DNA substrates were obtained by incubation of supercoiled DNA with the appropriate restriction enzyme; this was followed by phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol), extraction, ethyl ether extraction, and ethanol precipitation. DNA concentration was determined by assuming 50 μg of double-stranded DNA per ml per absorbance unit at 260 nm. DNA concentrations are expressed in total

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Abbreviations: FRT, FLP recombination target; Taps, 3-[tris(hydroxymethyl)methylamino]-1-propanesulfonic acid.

nucleotides. All substrate plasmids used in this study were 2826 bp in length and contained one FRT site. FRT site concentration was obtained by dividing the DNA concentration by 5652. The plasmid pXf3 (3160 bp; ref. 34) does not contain an FRT site and was used in one experiment.

Substrate plasmids used in this study were pJFS36, pJFS39, and pJFS46. The plasmid pJFS36 (32) contains a recombination site that is wild type in sequence. The recombination site in pJFS39 is altered at five positions in the spacer to produce a symmetrical spacer sequence (32). The recombination site in pJFS46 contains a G → T transversion at position 10, within one of the two FLP binding sites (30), as well as the symmetrical spacer of pJFS39. All of these recombination sites are 34 bp in length.

**FLP Recombination Assays.** *In vitro* recombination assays contained 25 mM Taps (pH 8.0 at 25°C) (29% anion), 1 mM EDTA, 200 mM NaCl, 20% (vol/vol) glycerol, 2.5 mg of bovine serum albumin per ml, 10% (wt/vol) PEG, and variable concentrations of linear or supercoiled DNA substrates and FLP protein. Total reaction volume was 100–200 μl.

Reactions were initiated by addition of FLP protein after preincubation of the rest of the assay components for at least 5 min. FLP protein was diluted as appropriate in 25 mM Taps (pH 8.0 at 25°C), 1 mM EDTA, 1.0 M NaCl, and 2.5 mg of bovine serum albumin per ml. Reactions were performed at 30°C. Aliquots containing 0.2 μg of DNA (10–20 μl) were removed at indicated time points. For linear substrates, the reaction was terminated by addition of 8 μl of 3.4% (wt/vol) $NaDodSO_4$/50 mM EDTA/50% (vol/vol) glycerol/0.01% (wt/vol) bromophenol blue. For supercoiled substrates, the reaction was stopped by heating the individual sample containing 0.2 μg of DNA at 70°C for 10 min. Control experiments indicated the reaction was halted as rapidly by heat treatment as with $NaDodSO_4$ treatment (not shown). After allowing the sample to cool to room temperature for 10 min, 7 μl of a solution containing 43 mM $MgCl_2$, 7 mM dithiothreitol, 114 mM Tris·HCl (pH 7.8 at 25°C), and 0.7 unit of *Pst* I per μl was added to each sample, which was then incubated for 1 hr at 37°C. Digestion was stopped by addition of 8 μl of 6.8% $NaDodSO_4$/0.01% (wt/vol) bromophenol blue/50% (vol/vol) glycerol. Digestion of supercoiled DNA treated with FLP recombinase by *Pst* I resulted in substrates and products identical with those produced when linear DNA substrates, precut with *Pst* I, are used.

Products of the reaction were resolved by electrophoretic separation on a 1% (wt/vol) agarose gel in Tris/acetate/EDTA buffer (34) and visualized by ethidium bromide staining and subsequent illumination with a shortwave UV transilluminator. Gels were then photographed with Polaroid type 55 film, which produces a positive and a negative image.

**Data Analysis.** Assays employing each of the substrate plasmids were designed to produce two discrete products resulting from intermolecular recombination as illustrated in Fig. 1. In each case half of the recombination events produces products identical to substrates, which are not observed. Rates of reaction as a function of either protein or substrate concentration were determined by using the plasmid pJFS39, either supercoiled or linearized by digestion with *Pst* I. The discrete products produced by pJFS39, linearized by *Pst* I either before or after reaction with FLP protein, are 4096 bp and 1556 bp.

The relative amounts of the larger discrete product formed during reaction with FLP protein and the substrate band were determined by densitometric scanning of the negative images of the photographed gels. To correct for differences in band intensity due to the different lengths of the DNA fragments and other variables related to photographic reproduction, etc., internal standards were included in each gel. The internal standards were derived from a single large reaction of pJFS39 driven to a stable end point with excess FLP protein. This end point was assumed to represent a 50% conversion of the substrate DNA to new products. This assumption has been verified with $^{32}P$-labeled substrates. The FRT sites are distributed 1:2:1 in the three bands from top to bottom. Four or five dilutions of this DNA were included in separate lanes in each gel, ranging from 20 to 200 ng of total DNA. The relative intensities of the large new product band, P1, and the substrate band generally varied by <10% over this range. This indicates that the densitometric data are approximately linear for reactions in which 5% or more of the substrate has been converted to new products. The average ratio of P1:S band intensities (designated X) for a given series of internal standard varied from X = 0.66 to 0.91 in the experiments described in this report. If DNA length were the only significant variable (P1 is 4096 bp and should bind more ethidium bromide than the 2826-bp S), X should be 0.72. By using the observed ratios obtained in each gel, the densitometric data for each time point were used to calculate % conversion to the P1 and P2 bands by dividing the apparent P1:S ratios at each time point by X and then multiplying by 50.

## RESULTS

**Assays.** The assay illustrated in Fig. 1*A* involves a wild-type FRT site with the unaltered, asymmetric spacer also shown in Fig. 1. A plasmid (pJFS36) containing this FRT site is linearized separately with two different restriction enzymes, producing two substrates identical in length but differing in the placement of the FRT site relative to the ends. Two of these sites must be aligned in parallel during recombination. Recombination between two molecules of one of these substrates will result in products identical to the substrate, but a reaction between one of each of the two different substrates will produce two discrete products, as shown in Fig. 1*A*.

The assay in Fig. 1*B* involves an FRT site with a symmetrical spacer. Only one substrate, produced by linearization of the plasmid pJFS39 with a single restriction enzyme (*Pst* I), is used. Alignment of two sites during recombination can be either parallel or antiparallel in this case. The former generates products identical to substrates, whereas the latter generates discrete products shown in Fig. 1*B*. The plasmid pJFS39 reacts as efficiently as pJFS36 in recombination but does not crossreact with pJFS36 (32). The new products of the two reactions also differ in size and are readily distinguished on a gel.

The assay in Fig. 1*C* involves an FRT site with the symmetrical spacer of the plasmid pJFS39 as well as a point mutation in one of the two FLP protein binding sites (a G → T change at position 10). This mutation has a small effect on recombination efficiency. That effect is much greater, however, when the mutation is present in both FLP binding sites (28, 30). The plasmid (pJFS46) is linearized with *Pst* I, as in the reaction in Fig. 1*B*. The large product of the antiparallel reaction illustrated in Fig. 1*C* will contain an FRT site effectively inactivated because it contains this mutation in both FLP binding sites. The new products of the reaction in Fig. 1*B* can potentially participate in additional recombination events; the large product of the reaction in Fig. 1*C* cannot.

The reaction conditions include PEG, glycerol, and bovine serum albumin. PEG was used to enhance the efficiency of intermolecular recombination. Rates were increased up to several orders of magnitude when 10% PEG was used. Bovine serum albumin and glycerol were added to stabilize FLP protein. In their absence, nearly complete loss of FLP protein activity was observed at 30°C over 10–30

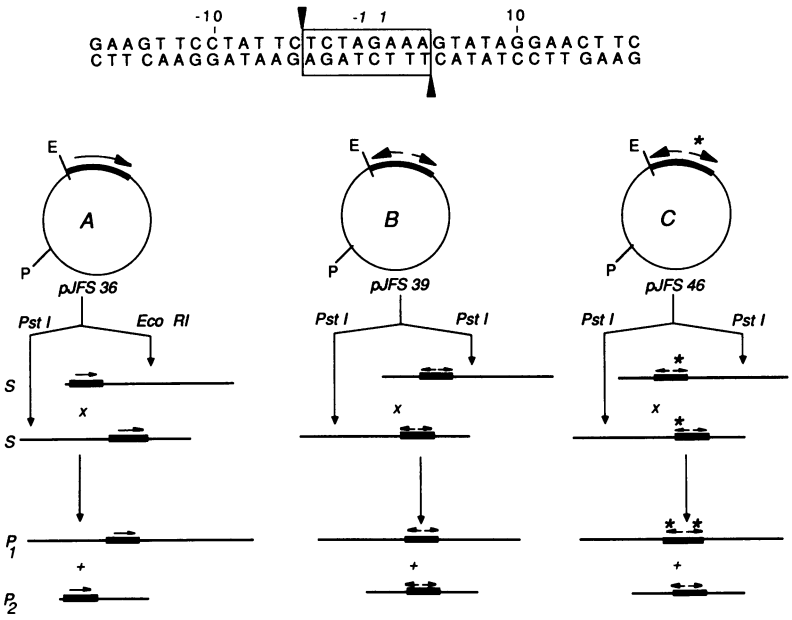



FIG. 1. FRT site and assays for recombination. The wild-type sequence of the FRT site in pJFS36 is illustrated at the top. Triangles indicate points of cleavage (25, 31, 36). The three assays used in this work are illustrated in *A–C*. (*A*) Reaction with linear pJFS36 substrates derived from separate digestion by two different restriction enzymes. Reactions occur only between FRT sites aligned in parallel. Reaction of one of the two substrates with an identical substrate generates products identical to substrates. Reaction between one of each of the two substrates generates the discrete products shown. (*B*) Reactions with linear substrates derived from *Pst* I digestion of pJFS39. The symmetrical spacer in this substrate permits either alignment in recombination. Parallel alignment generates products identical to substrates. Antiparallel alignment generates the discrete products shown. (*C*) Reaction of pJFS46 is identical to pJFS39 except for the presence of a mutation (asterisk) in one of the two FLP binding sites in the substrate. One of the discrete products contains two of these mutations.

min. Intermolecular recombination was measured in each case.

**Turnover in the FLP Recombination System.** Three strategies were employed to detect turnover of FLP protein and the FRT site. The first strategy is presented in Fig. 2. A stoichiometric amount of FLP protein (2:1 relative to available FRT sites) was added to a reaction mixture with the substrates derived from pJFS36. The reaction was allowed to proceed to an apparent end point (20 min). It was then challenged with an equal amount of pJFS39 substrate. Any reaction of pJFS39 will indicate movement or turnover of FLP protein from pJFS36. An efficient reaction of pJFS39 was observed. Fig. 2*B* presents a control in which FLP protein was incubated with pXF3 DNA (which lacks an FRT site) prior to addition of pJFS39. Fig. 2*C* presents the reaction of pJFS39 alone. The result was the same when pJFS39 was used in the first reaction and challenged with pJFS36 (not shown).

The experiment in Fig. 3 examines the recycling of the FRT site. The assays described in Fig. 1 *A* and *B* will reach an end point in which 50% of the substrates will be present as the products of discrete size. This will be true whether the reaction is limited to a single turnover of all substrates (one-half of the reaction will generate products indistinguishable from substrates) or if the reaction proceeds to a true equilibrium. With the plasmid pJFS46, however, the two possibilities can be distinguished. A single turnover will produce the normal 50:50 distribution of product and substrate-sized DNA molecules. In contrast, continued turnover will result in accumulation of the new product bands at the expense of substrate-sized DNA. This occurs because the large product is inactivated and each reaction that results in this product is effectively irreversible. An excess of FLP protein was used in this experiment to increase the rate of the slower pJFS46 reaction. The accumulation of product bands over time is evident in Fig. 3. After 12

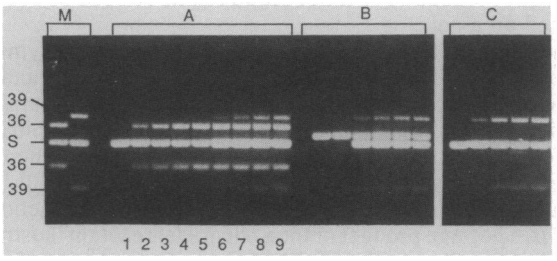



FIG. 2. FLP protein turnover: Challenge experiment. Concentration of FLP protein was 10.3 nM in each case. Lanes 1 and 2, markers for substrates (S) and product bands resulting from the pJFS36 (36) and pJFS39 (39) reactions, respectively, as illustrated in Fig. 1 *A* and *B*. (*A*) Challenge experiment. Lanes 1–5, pJFS36 (5.25 nM FRT sites total) reaction at 0, 5, 10, 15, and 20 min. A challenge, pJFS39 (5.25 nM final concentration in new FRT sites), was added at 20 min. Lanes 6–9, reaction at 5, 10, 15, and 20 min after the challenge. (*B*) Same as *A*, except that pXf3 DNA, linearized with *Pst* I, was substituted for pJFS36. The first two lanes are 0- and 20-min time points with pXf3 alone. (*C*) Reaction with pJFS39 alone (5.25 nM FRT sites and 5.1 mM FLP protein total) at 0, 5, 10, 15, and 20 min.

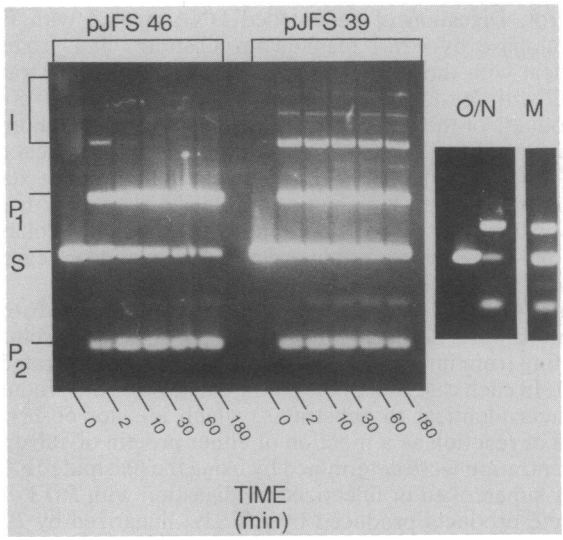



FIG. 3. FRT site turnover: Mutant product accumulation. Concentrations of FLP protein and FRT sites were 119 nM and 5.4 nM, respectively, in each experiment. pJFS46 and pJFS39 experiments are indicated; time points in each case are 0, 2, 10, 30, 60, and 180 min. P and S, new product and substrate bands, respectively. I and B, reaction intermediates and reaction by-products, respectively (to be described elsewhere). In the O/N panel, the first lane is an "S" marker ( t = 0 min), and the second lane is the pJFS46 reaction after incubating for 12 hr. M denotes the maximum pJFS39 level of product bands observed regardless of time of incubation.

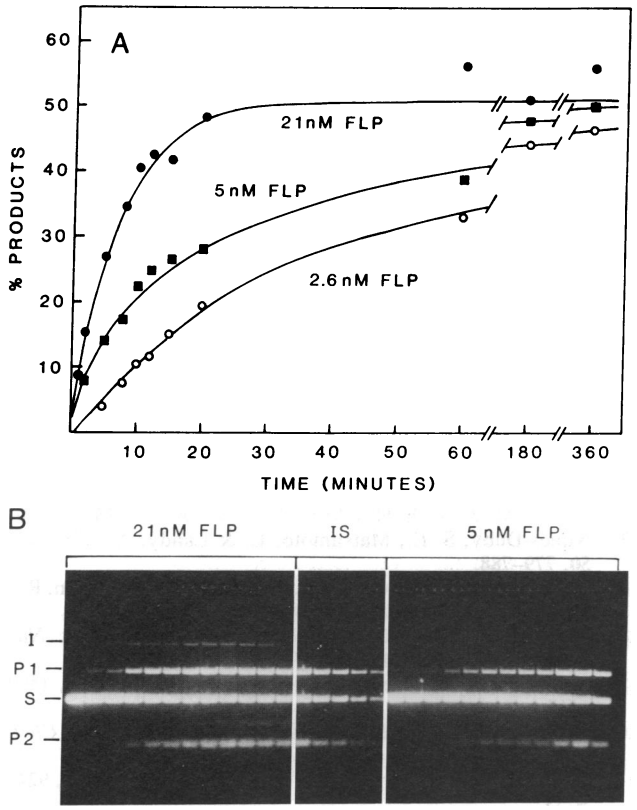



FIG. 4. Recombination time course: Subsaturating FLP protein. (*A*) The concentration of FRT sites (pJFS39) was 10.9 nM. FLP protein concentrations are indicated. "% PRODUCTS" refers to the fraction of FRT sites present in the discrete product bands. (*B*) Two of the assays used to generate the data in *A*. Time points for both reactions are 0, 1, 2, 5, 8, 10, 12, 15, 20, 60, and 180 min. IS, internal standards. P1, P2, and S, product and substrate bands (see text). I, reaction intermediate (to be described elsewhere).

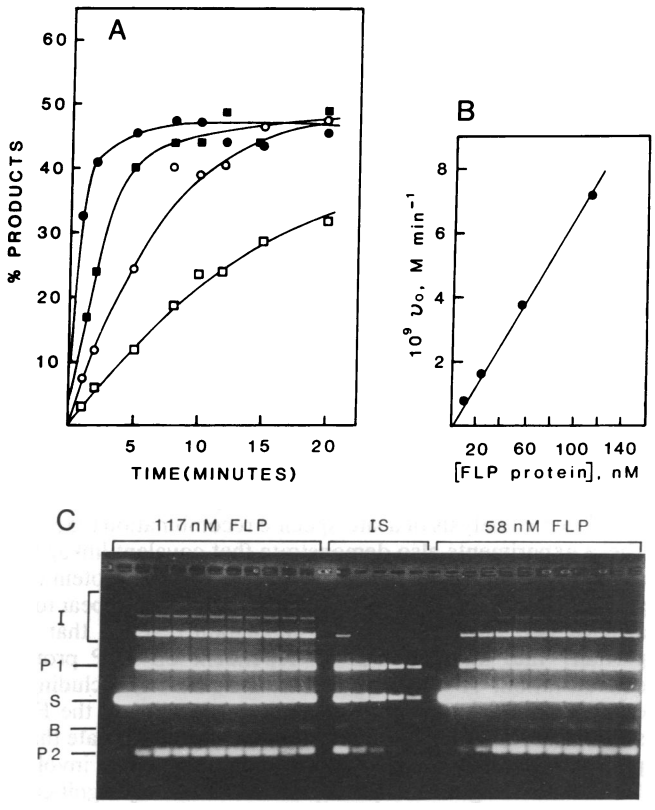



FIG. 5. Dependence of recombination rate on FLP protein concentrations. The concentration of FRT sites (pJFS39) was 10.9 nM in each experiment. (*A*) Reaction time courses. FLP protein concentrations were 117 nM (●), 58 nM (■), 25 nM (○), and 10.3 nM (□). (*B*) Initial rates of recombination estimated from data in *A*. (*C*) Two of the reactions plotted in *A* are illustrated. FLP protein concentrations are indicated. Time points were 0, 1, 2, 5, 8, 10, 12, 15, 20, 60, and 180 min. IS, internal standards. P1, P2, and S, product and substrate bands. I and B, reaction intermediates and by-products (to be described elsewhere).

hr, >95% of the total DNA was in the new product bands.* A control reaction run with pJFS39 reached an end point of 50% conversion to the discrete product-sized DNA, which was stable over the same time period. These results demonstrate that the normal FRT site is recycled after each reaction.

The final strategy is the most straightforward. In Fig. 4, the time courses of several pJFS39 reactions are presented. In two of them, the concentration of FLP protein is two to four times less than the concentration of FRT sites. Each reaction, however, proceeds to the same equilibrium end point. The FLP protein must carry out multiple reactions for this to occur. This result has been obtained in dozens of similar reactions carried out under these conditions with subsaturating FLP protein concentrations (not shown).

A turnover number was estimated from a number of time courses carried out with substrate in excess (not shown). With 5 nM FLP protein, the initial rate of the reaction exhibited saturation when the concentration of FRT sites exceeded 25 nM. Rates observed under the apparent $V_{max}$ conditions produced a turnover number for FLP protein of $\approx 0.12$ $min^{-1}$ for the FLP protein monomer, or $\approx 0.24$ $min^{-1}$ if the assumption is

hr, >95% of the total DNA was in the new product bands.* A

made that the active form of FLP protein is a dimer.† The apparent $K_m$ for FRT sites was in the range of 5–10 nM. These numbers are valid only for intermolecular recombination under a single set of conditions. A systematic examination of kinetic parameters will be presented elsewhere.

**FLP Protein Concentration Dependence.** Several titrations of FLP protein were carried out to determine the dependence of the initial rate of recombination on FLP protein concentration. If it is assumed that two FLP monomers bind to each recombination site, the titrations spanned the point at which FLP protein was stoichiometric (2:1) with available FRT sites. One goal was to determine if the reaction behaves stoichiometrically in any sense. Typical results are presented in Fig. 5. The rate of recombination increases (approximately linearly) over the range of FLP protein concentrations tested. Further increases in FLP protein concentration produced faster rates, which were difficult to estimate with the experimental design used here (not shown). No change in the pattern was observed at the point of stoichiometry. More FLP protein increases the rate of reaction even when protein is present in significant excess.

*The design of the experiment requires that both new product bands accumulate at the same rate, even though one is reactive and the other is not. A reaction between a "substrate" DNA molecule and a small "product" molecule will result in two recombinant molecules identical to the reactants. Continued reaction of the small product with itself or with the substrate-sized molecules will not alter the intensity of this band.

†These numbers are corrected for the fact that only half of the recombination events generated discrete products. The initial rate of "product band" formation has been multiplied by 2 before calculation of turnover number. The assumption that the FLP protein acts as a dimer is based on the organization of the FRT site. No evidence is available at present that provides information about the constitution of the active form of this recombinase.

## DISCUSSION

The FLP protein is an enzyme, catalyzing intermolecular site-specific recombination with a turnover number of $\approx 0.12$ $min^{-1}$ under one set of conditions. Three separate experiments demonstrate specifically that (*i*) after a reaction containing subsaturating amounts of FLP protein reaches an apparent end point, the protein moves freely to a second set of substrates added as a challenge and promotes additional recombination; (*ii*) the wild-type FRT site is recycled after each reaction; and (*iii*) recombination proceeds to a predicted equilibrium even when FRT sites are present in excess relative to the concentration of FLP protein. The reaction rate exhibits a strong dependence on FLP protein concentration even when the protein is present in excess relative to available recombination sites. The kinetics of the reaction exhibit saturation behavior for the DNA substrate but not for FLP recombinase.

These results open the way for the application of steady-state kinetic analysis of a site-specific recombination reaction. These experiments also demonstrate that covalent linkage of the FLP protein to the DNA has no effect on the protein that prevents subsequent reactions. At this point there appear to be at least two phases of the recombination reaction that are partially rate limiting. One is dependent on FLP protein concentration. A number of possibilities exist, including a conformation change or diffusion during binding to the FRT site. The effects of some spacer alterations (33) indicate that a phase of the reaction subsequent to binding, which involves DNA unwinding in the spacer, is also kinetically significant. Systematic kinetic studies should help to clarify these questions and contribute many details to our understanding of this process.

The failure to observe turnover in other site-specific recombination systems to date could be due to many factors. For complex systems involving several proteins, turnover may be tightly regulated. Turnover could also be very slow or may not occur at all. Alternatively, turnover could require another cellular factor yet to be introduced to the *in vitro* systems. Protein instability could also play a role. Initial attempts to carry out the experiment in Fig. 2 indicated that no turnover was occurring. This was traced, however, to protein instability that was inactivating the enzyme prior to addition of challenge DNA. This, and the lack of an accurate measure of FLP recombinase concentration, may explain the previous reports of a stoichiometric requirement for FLP protein in this system (1, 16). The resulting search for stabilizing conditions led to the inclusion of glycerol and bovine serum albumin in the reaction mixtures. Another problem could involve the kinetic mechanism of these reactions. If all systems share a kinetically slow phase dependent on the concentration of recombinase, the addition of excess recombinase may be necessary to provide reaction rates convenient for a given experiment. A final, obvious problem that could lead to high apparent stoichiometries would be inactive recombinase in a purified preparation. The procedure used to purify FLP protein includes site-specific DNA chromatography, which may tend to select for active enzyme. If inactive enzyme is present in our preps, we have *underestimated* the turnover number reported above.

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