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Synthesis of an Enzymatically Active FLP Recombinase In Vitro: Search for a DNA-Binding Domain

ANTHONY A. AMIN AND PAUL D. SADOWSKI*

Department of Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada

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We have used an in vitro transcription and translation system to synthesize an enzymatically active FLP protein. The FLP mRNA synthesized in vitro by SP6 polymerase is translated efficiently in a rabbit reticulocyte lysate to produce enzymatically active FLP. Using this system, we assessed the effect of deletions and tetrapeptide insertions on the ability of the respective variant proteins synthesized in vitro to bind to the FLP recognition target site and to carry out excisive recombination. Deletions of as few as six amino acids from either the carboxy- or amino-terminal region of FLP resulted in loss of binding activity. Likewise, insertions at amino acid positions 79, 203, and 286 abolished DNA-binding activity. On the other hand, a protein with an insertion at amino acid 364 retained significant DNA-binding activity but had no detectable recombination activity. Also, an insertion at amino acid 115 had no measurable effect on DNA binding, but recombination was reduced by 95%. In addition, an insertion at amino acid 411 had no effect on DNA binding and recombination. On the basis of these results, we conclude that this approach fails to define a discrete DNA-binding domain. The possible reasons for this result are discussed.

The 2μm plasmid of *Saccharomyces cerevisiae* encodes a protein, the FLP recombinase, that promotes an inversion event across a specific site within two 599-base-pair (bp) inverted repeats of the plasmid (8, 45). This reaction results in two isomeric forms of the plasmid and appears to be essential for amplification of the plasmid copy number in the cell (14, 35, 41, 46).

The FLP recombination system serves as an attractive model to study the molecular events in the pathway of site-specific recombination (6, 15, 16). The first step in the reaction involves a specific interaction of FLP protomers with the target sequence called the FRT (FLP recognition target) sequence (4, 9). The FRT site consists of three 13-bp symmetry elements, two of which are in inverted orientation and separated by an 8-bp core region (4, 38). Results obtained by using a gel mobility shift assay and various footprinting techniques (3, 7) suggest that a single symmetry element of 13 bp is the basic unit to which a FLP protomer binds. Results from these same experiments indicate that FLP protomers are assembled in an ordered manner onto the FRT site.

Crystallographic studies of several sequence-specific DNA-binding proteins have already provided considerable insight into the molecular interactions between some proteins and their target sequences (reviewed in references 2, 32, 36, and 48). The DNA-binding domains of proteins for which there is no crystallographic information have been identified by limited proteolysis and purification of functional polypeptides (1, 12, 19, 20, 43) or by assays of truncated proteins whose synthesis has been directed from deletion variants of their genes in vitro (18, 42) and in vivo (24, 34).

To understand the interactions important for the FLP binding reaction, we were interested in identifying elements of the protein that are required for site-specific DNA binding. To this end, we used in vitro transcription and translation of mutant FLP genes to assess the enzymatic activity of the respective FLP proteins. We found that the DNA-binding activity of the FLP protein is remarkably sensitive to perturbation of its structure.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, DNA modification enzymes, and vectors. *Escherichia coli* HB101 [F⁻ *hsdS20* r_B^- m_B^- *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Smr) *xyl-5 mtl-1 supE44*] and K802 (*hsdR hsdM*⁺ *gal met supE*) were used to propagate plasmids. Cells were made competent for transformation with calcium chloride as described by Mandel and Higa (29). Plasmid vector pSP64 was obtained from Promega Biotec. A derivative of pSP64 that has the alfalfa mosaic virus coat protein leader sequence adjacent to the SP6 promoter was obtained from Lee Gehrke (23). This leader sequence effects efficient translation of heterologous mRNA in both the rabbit reticulocyte lysate and wheat germ extracts (23). This plasmid was designated pPS672. Plasmids pBA104 and pBA112 are described by Andrews et al. (4). pBA104 contains two FRT sites in direct orientation and was used as an excisive recombination substrate for the FLP protein (16). A 100-bp fragment containing the FRT site used in gel mobility shift assays was obtained from plasmid pBA112. DNA restriction and modification enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), Pharmacia, Inc. (Piscataway, N.J.), and Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and used according to the specifications of the manufacturers. Preparation of plasmids from 1-ml cultures was done as described previously (21). Cesium chloride density gradient purification of plasmids was performed from 1-liter cultures as described elsewhere (11, 40). The following duplex oligonucleotide linkers were used in this study: *Bam*HI–5′-CCGGATCCGG-3′ (Bethesda Research Laboratories), *Bam*HI–5′-CCGGATCC TGG-3′ (New England BioLabs, Inc., Beverly, Mass.), *Sal*I–5′-GGTCGACC-3′ (New England BioLabs), *Nco*I–5′-CATGCCATGGCATG-3′ (New England BioLabs), and *Xho*I–5′-CTCGAGCTCGAG-3′ (kindly provided by Keith Schappert).

Plasmid construction. Two plasmids were used to direct the synthesis of FLP mRNA by SP6 polymerase. Plasmid

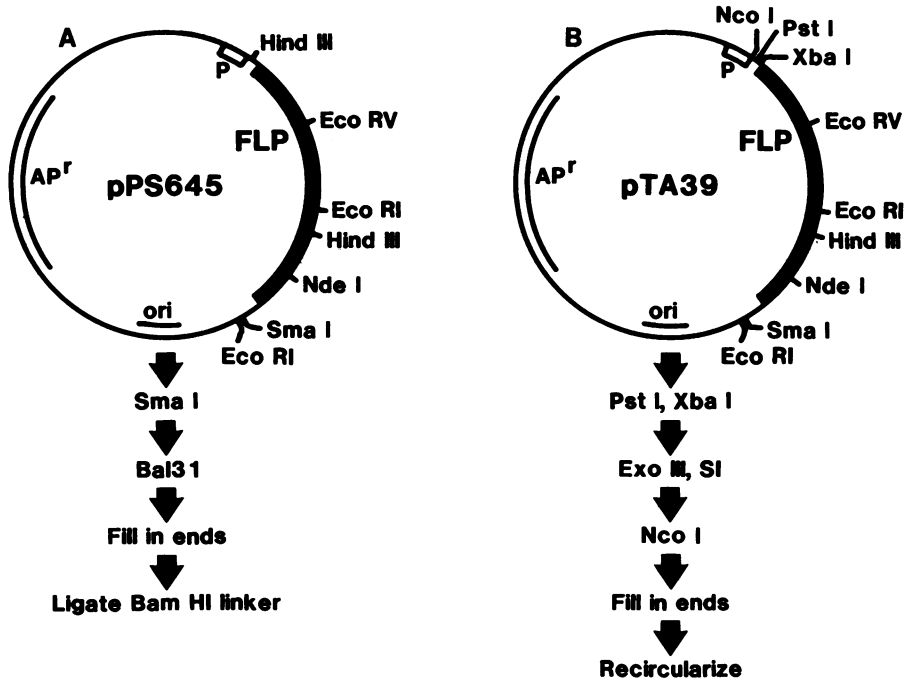

FIG. 1. Strategy used to generate carboxy-terminal (A) and amino-terminal (B) deletions in the FLP protein. See text for details. ▬, FLP gene sequences: P, SP6 polymerase promoter; AP^r, ampicillin resistance gene; ——, pSP64 sequences.

pPS645 was constructed by ligation of a 1.4-kbp *Bgl*II-*Bgl*II fragment from plasmid pDV56 (5), containing the FLP gene, into the unique *Bam*HI site of pSP64. The gene is in the sense orientation with respect to the SP6 promoter. Plasmid pTA43 was constructed by ligation of a 1.4-kbp *Sph*I-*Sma*I fragment from pPS645, carrying the FLP gene, into the filled-in unique *Nco*I site of pPS672. To facilitate this, the *Sph*I end was made blunt by the 3′ exonuclease of T4 DNA polymerase. Plasmid pTA39 was constructed by ligation of the *Nco*I linker, which has ATG codons in all three reading frames, into the filled-in *Hin*dIII site in the polylinker region of pPS645. Circular maps with relevant restriction sites are shown in Fig. 1.

Carboxy-terminal deletions of the FLP gene. A schematic illustration of the strategy used to construct BAL 31 deletions in pPS645 is shown in Fig. 1A. pPS645 DNA was linearized with *Sma*I and treated with BAL 31 nuclease according to the instructions of the manufacturer (Bethesda Research Laboratories). The termini were repaired with reverse transcriptase and four deoxynucleoside triphosphates, and the blunt ends were ligated to a *Bam*HI linker (5′-CCGGATCCTGG-3′). The circularized DNA was used to transform competent HB101 by selection for ampicillin-resistant colonies. Deletion endpoints were determined precisely by sequencing from the *Bam*HI site into the gene by the technique of Maxam and Gilbert (31).

Amino-terminal deletions of the FLP gene. A schematic illustration of the strategy used to construct amino-terminal deletions in plasmid pTA39 is shown in Fig. 1B. Plasmid DNA was digested with *Pst*I and *Xba*I and treated with exonuclease III (New England BioLabs), followed by S1 nuclease (Sigma Chemical Co., St. Louis, Mo.). Since the *Pst*I site is refractory to digestion with exonuclease III, unidirectional deletions were generated into the FLP gene sequences from the 5′-terminal end while leaving the SP6 promoter intact. The fragments were then digested with *Nco*I, the ends were repaired with reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) and the plasmids were recircularized by using T4 DNA ligase. Ampicillin-resistant colonies were obtained after transformation of HB101. The extent of deletion was determined by restriction digestion, followed by the double-stranded sequencing technique (47) with the SP6 promoter primer (5′-CATACGATT TAGGTGACACTATAG-3′) (New England BioLabs). Constructs that had the remaining FLP gene sequences in frame with the ATG supplied by the *Nco*I site were chosen for further analysis.

Linker insertion construction. All in-phase oligonucleotide linker insertions in the FLP gene contained in plasmid pTA43 were done according to the linker-tailing technique described by Lathe et al. (25). Plasmid pTA43 was linearized with *Eco*RV, *Hin*dIII, or *Nde*I, position 5917, 106, or 341, respectively, of the 2μm plasmid coordinates (17). The *Hin*dIII and *Nde*I ends were repaired with reverse transcriptase and ligated to *Sal*I (5′-GGTCGACC-3′) and *Bam*HI (5′-CCGGATCCGG-3′) linkers, respectively. The *Eco*RV end was ligated directly to the *Bam*HI linker. Plasmids containing the appropriate linkers in the *Eco*RV, *Hin*dIII, and *Nde*I sites are referred to as pTA209, pTA211, and pTA213, respectively. Insertion of an *Xho*I linker (5′-CTC GAGCTCGAG-3′) into one of two *Rsa*I sites (position 384) and into the two *Dra*I sites (positions 5807 and 6179) of the gene was achieved by partial digestion with the respective enzyme. Plasmids designated here as pTA251, pTA243, and pTA250 have the linkers inserted into the *Dra*I sites (positions 5807 and 6179) and *Rsa*I site (position 384), respectively.

In vitro transcription and translation assays. In vitro transcription of templates by using SP6 polymerase and in vitro translation of capped mRNA in a rabbit reticulocyte lysate were carried out with kits and specifications of Promega Biotec. Plasmid DNA templates used for transcription were linearized with *Bam*HI or *Sma*I, extracted with phenol-chloroform, ethanol precipitated, and suspended in water at

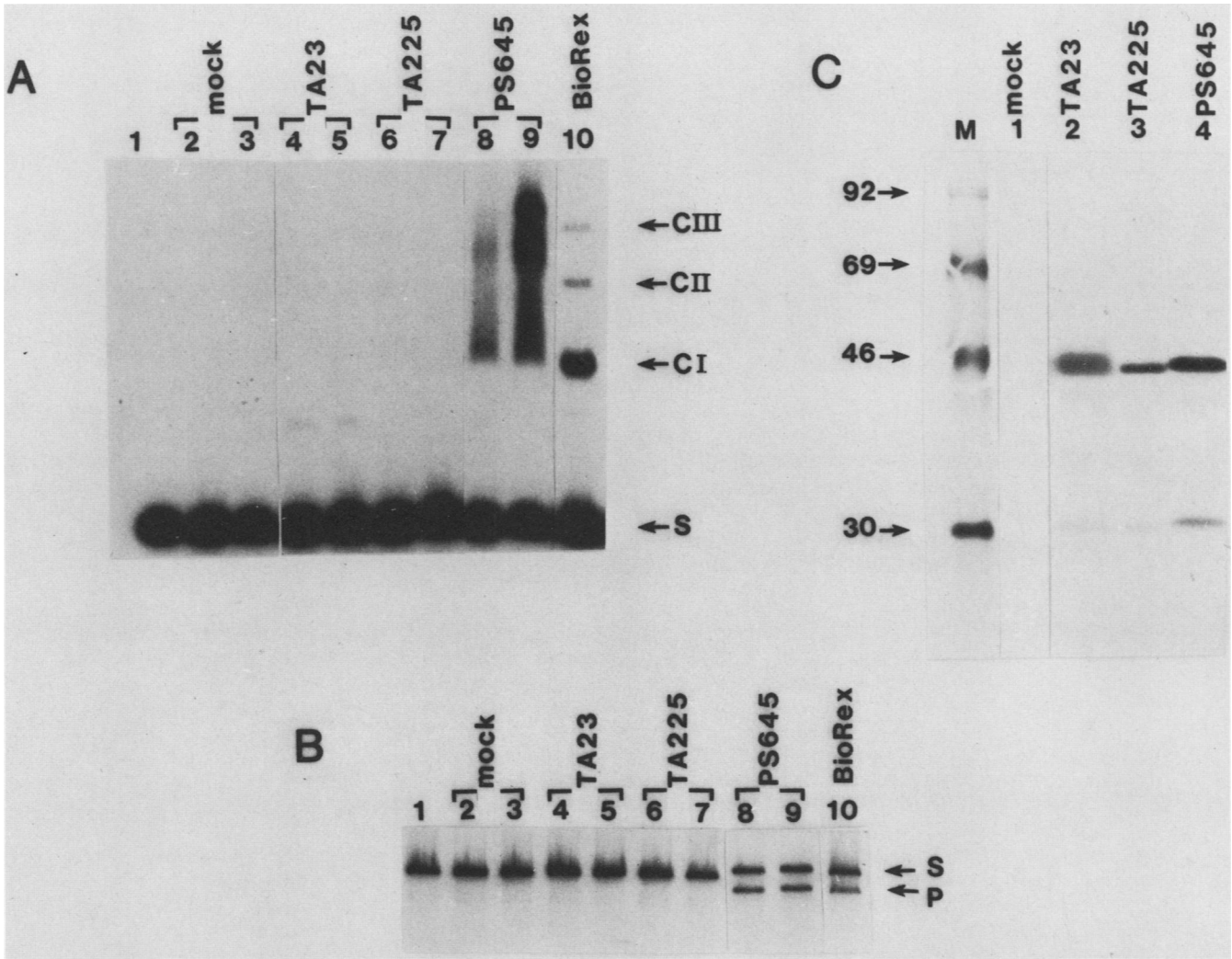

FIG. 2. Demonstration that FLP proteins without the carboxy-terminal six amino acids are not functional in the mobility shift and recombination assays. (A) Autoradiograph of mobility shift assay gel. Translation products were incubated with 0.02 pmol of a ^{32}P-end-labeled 100-bp *Eco*RI-*Hin*dIII fragment containing the FRT sequence for 20 min at 30°C. The reactions were subjected to electrophoresis on a 5% polyacrylamide gel. The gel was dried and exposed to film (see text for details). S, 100-bp *Eco*RI-*Hin*dIII fragment; CI, CII, and CIII, FLP-FRT complexes I, II, and III, respectively. Lanes: 1, no extract; 2 and 3, 8 and 16 μl of mock translation extract minus mRNA; 4 and 5, 8 and 16 μl of pTA23 translation product; 6 and 7, 8 and 16 μl of PTA225 translation product; 8 and 9, 8 and 16 μl of pPS645 translation product; 10, 0.2 U of a partially purified FLP protein (5). (B) Autoradiograph of recombination assay gel. Translation products were incubated with 0.01 pmol of ^{32}P-end-labeled 8.9-kbp *Eco*RI-*Eco*RI fragment of pBA104 (an excisive recombination substrate) for 20 min at 30°C. The reactions were subjected to electrophoresis on an 0.8% agarose gel. The gel was dried and exposed to film (see text for details). S, 8.9-kbp linear *Eco*RI-*Eco*RI substrate; P, 7.0-kbp *Eco*RI-*Eco*RI recombinant product. Lanes; 1, no extract; 2 and 3, 2.5 and 5.0 μl of mock translation extract minus mRNA; 4 and 5, 2.5 and 5.0 μl of pTA23 translation product; 6 and 7, 2.5 and 5.0 μl of pTA225 translation product; 8 and 9, 2.5 and 5.0 μl of pPS645 translation product; 10, 0.1 U of a partially purified FLP protein (5). (C) Autoradiograph of SDS-polyacrylamide gel. Translation products (40,000 cpm) were subjected to electrophoresis on a 15% polyacrylamide-SDS gel. The gel was impregnated with En^3 Hance, dried, and exposed to film. Lanes: M, molecular size markers (indicated in kilodaltons on the left); 1, 10 μl of mock translation extract minus mRNA; 2, pTA23 translation product; 3, pTA225 translation product; 4, pPS645 translation product.

a concentration of 1 μg μl^{-1}. The transcription reaction was extracted with phenol-chloroform, and the RNA was precipitated with ethanol and suspended in 25 μl of water. A 250-ng (0.5-pmol) sample of this RNA was then translated in the rabbit reticulocyte lysate with 450 pmol of [2,3,4,5-^{3}H]leucine (110 Ci mmol^{-1}; ICN Radiochemicals, Irvine, Calif.) and incubated at 30°C for 1 h. The translation mixtures were used immediately for FLP-FRT complex formation and for recombination assays (see below). In all instances, the yields of trichloroacetic acid-precipitable counts were approximately the same.

Gel mobility shift and recombination assays. The 100-bp *Eco*RI-*Hin*dIII fragment of pBA112, containing the FRT site, and the 8.9-kbp *Eco*RI-*Eco*RI fragment of pBA104 were labeled at the ends by using [α^{-32}P]dATP and reverse transcriptase as described by Andrews et al. (4). In a typical mobility shift assay reaction, 8 or 16 μl of the translation reaction was combined with 0.02 pmol of the 100-bp ^{32}P-end-labeled *Eco*RI-*Hin*dIII fragment in 50 μl of 10 mM Tris chloride (pH 8.0)–1 mM EDTA containing 4 or 8 μg, respectively, of sonicated, phenol-extracted calf thymus DNA. The reaction mixture was incubated at 30°C for 20 min, and the FLP-FRT complexes were resolved on a 5% polyacrylamide gel as described by Andrews et al. (3). To

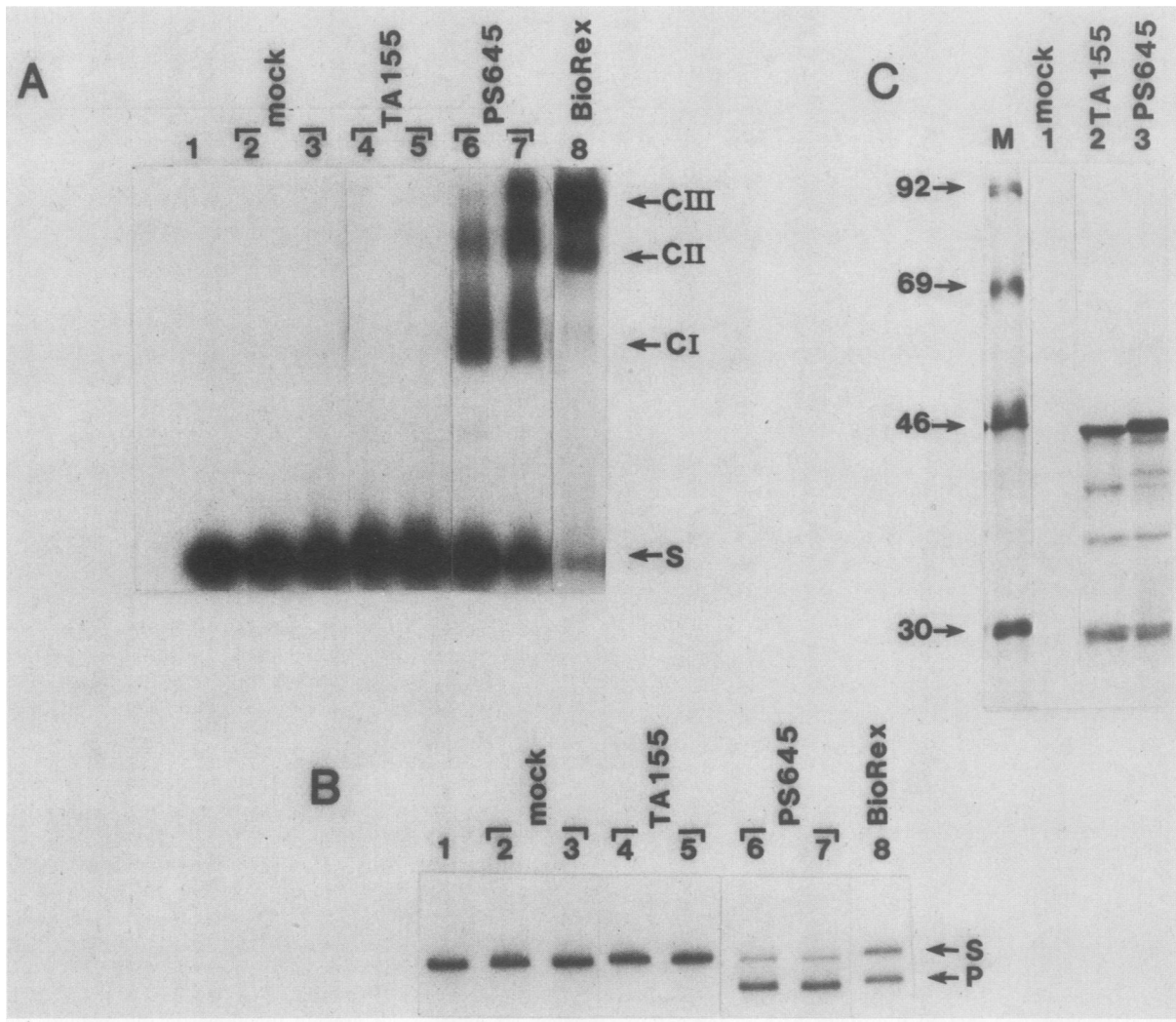

FIG. 3. Demonstration that FLP proteins without the amino-terminal six amino acids (amino acids 3 to 8) are not functional in the mobility shift and recombination assays. (A) Autoradiograph of mobility shift assay gel. Translation products were analyzed as described in the legend to Fig. 2A. S, 100-bp *Eco*RI-*Hin*dIII fragment; CI, CII, and CIII, FLP-FRT complexes I, II, and III, respectively. Lanes: 1, no extract; 2 and 3, 8 and 16 μl of mock translation extract minus mRNA; 4 and 5, 8 and 16 μl of pTA155 translation product; 6 and 7, 8 and 16 μl of pPS645 translation product; 9, 0.2 U of FLP protein (5). (B) Autoradiograph of recombination assay gel. Translation products were analyzed as described for Fig. 2B. S, 8.9-kbp linear *Eco*RI-*Eco*RI substrate; P, 7.0-kbp *Eco*RI-*Eco*RI recombinant product. Lanes: 1, no extract; 2 and 3, 2.5 and 5.0 μl of mock translation extract minus mRNA; 4 and 5, 2.5 and 5.0 μl of pTA155 translation product; 6 and 7, 2.5 and 5.0 μl of pPS645 translation product; 9, 0.1 U of FLP protein (5). (C) Autoradiograph of SDS-polyacrylamide gel. Translation products (40,000 cpm) were subjected to electrophoresis on a 15% polyacrylamide-SDS gel. Lanes: M, molecular size markers (indicated in kilodaltons on the left); 1, 10 μl of mock translation extract minus mRNA; 2, pTA155 translation product; 3, pPS645 translation product.

measure excisive recombination, 5 and 10 μl of the translation reaction were combined with 0.01 pmol of a ^{32}P-end-labeled *Eco*RI-*Eco*RI fragment of pBA104 in 20 μl of 50 mM Tris chloride (pH 7.4)–10 mM $MgCl_2$–100 μg of bovine serum albumin ml^{-1} and incubated at 30°C for 20 min. Reactions were analyzed by agarose gel electrophoresis (5).

Electrophoresis of labeled FLP. Electrophoresis of labeled proteins was carried out on 15% polyacrylamide-sodium dodecyl sulfate (SDS) gels as described by Laemmli (26). Gels were impregnated in En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.) and exposed to X-OMAT-AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C for 2 days.

RESULTS

Synthesis of enzymatically active FLP in vitro. To have a convenient means to assay the FLP protein encoded by various mutants, we developed an in vitro system for synthesis of enzymatically active FLP. First, FLP mRNA was synthesized in vitro, using SP6 polymerase to transcribe the *Sma*I-linearized template pPS645. The transcripts were then translated in vitro in a nuclease-treated rabbit reticulocyte lysate in the presence of [^{3}H]leucine. The results from SDS-polyacrylamide gel electrophoresis indicated that there was synthesis of full-length FLP protein (45 kilodaltons) in the extract (Fig. 2C, lane 4). A control experiment indicated

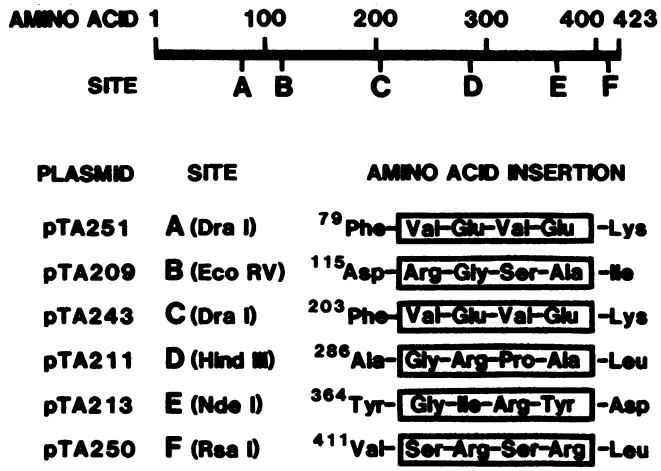

FIG. 4. Summary of locations of tetrapeptide insertions in the FLP protein, along with identification of the corresponding plasmids that encode these proteins.

that when FLP mRNA was omitted, there was very little background translation in the extract (Fig. 2C, lane 1).

The activity of the FLP protein synthesized in vitro was assessed first by testing for the formation of specific FLP-substrate complexes by using a gel retardation assay. We have previously shown that the FLP protein, expressed in and purified from *E. coli*, forms three distinct complexes when incubated with a DNA fragment containing an FRT site (3). FLP synthesized in vitro formed the same three complexes (Fig. 2A, lanes 8 and 9) as did FLP made in vivo (lane 10). The complexes formed from the FLP made in vitro generally migrated with a slightly slower mobility than did those formed with the Biorex II fraction of FLP. The reason for this may be that for each reaction, the wells in the acrylamide gel were markedly overloaded with protein (1.6 to 3.2 mg) derived from the translation lysate.

We then tested the ability of FLP synthesized in vitro to catalyze excisive recombination across two FRT sites that are in direct orientation. The substrate for this reaction was an 8.9-kbp end-labeled fragment (pBA104; 16). Recombination catalyzed by purified FLP protein was evidenced by the appearance of a 7-kbp labeled fragment (Fig. 2B, lane 10). The FLP protein synthesized in vitro generated the same fragment (Fig. 2B, lanes 8 and 9). The control extract from which the RNA was omitted did not support the formation of complexes (Fig. 2A, lanes 2 and 3), nor was it able to carry out recombination (Fig. 2B, lanes 2 and 3).

This in vitro system has several advantages. There is no need to purify the FLP protein synthesized in the extract to carry out the DNA-binding and recombination assays. Also, the labeled protein makes possible an assessment of the amount of FLP protein added to the reactions. Finally, the protein made in vitro is not subject to proteolytic degradation that may affect aberrant proteins synthesized in *E. coli*.

Deletion of six amino acids from the carboxy-terminal region of FLP diminishes FRT recognition. We first determined whether the carboxy-terminal region of the 423-amino-acid FLP protein was required for recognition of the FRT site. Truncated polypeptides that lacked 60, 137, 173, or 308 amino acids from the carboxy terminus were made from runoff transcripts of linearized pPS645 DNA cut with *Nde*I, *Hin*dIII, *Eco*RI, or *Eco*RV, respectively. None of these polypeptides was able to form complexes with a 100-bp FRT-containing fragment, and no recombination was observed (data not shown). This suggested that the carboxy-terminal 60 amino acids specified information that is required for the FLP protein to bind to the FRT site. This preliminary observation prompted us to investigate in greater detail the importance of the C-terminal residues in FRT recognition. We constructed deletions of the 3′ end of the FLP gene that encoded proteins lacking less than 60 amino acids from the carboxyl terminus (Fig. 1A).

The smallest C-terminal deletion plasmid (pTA23) lacked DNA encoding six C-terminal amino acids. RNA synthesized from this template linearized with *Bam*HI did not support the synthesis of active FLP protein by the reticuloycte lysate. Plasmid pTA23 encoded an FLP protein that was unable to form specific FLP-DNA complexes (Fig. 2A, lanes 4 and 5) and to promote excisive recombination (Fig. 2B, lanes 4 and 5). The presence of the *Bam*HI linker at the 3′ end of the FLP gene in plasmid pTA23 led to the introduction of three amino acids at the end of the polypeptide. In the case of the protein encoded by pTA23, the addition was Ser-Arg-Ile (total length, 420 amino acids). Two plasmids encoding FLP proteins of 415 and 399 amino acids, respectively, likewise directed the synthesis of enzymatically inactive FLP protein.

The experiment with plasmid pTA23 used a template that was linearized with *Bam*HI. The 3′ end of the message synthesized in vitro would thus be formed by runoff transcription from the linearized template. To corroborate this result, a plasmid was constructed in which a termination codon was placed near the C terminus of the gene. The plasmid was then linearized at a site about 100 bp past the termination codon.

This plasmid (pTA225) was constructed by ligation of an *Xba*I linker (5′-CTAGTCTAGACTAG-3′; New England BioLabs) into the filled-in *Bam*HI site of pTA23. The *Xba*I linker contains stop codons in all three reading frames, and the second one would be used to terminate synthesis of the pTA225 protein. This plasmid was linearized with *Pvu*II, which cuts about 100 bases from the termination codon in the *Xba*I linker. For this template, the mRNA is predicted to allow synthesis of a 422-amino-acid protein. This protein does not contain the terminal six amino acids present in the wild-type protein but has Ser-Arg-Ile-Leu-Val at its carboxy-terminal end.

This gene likewise was found to encode an enzymatically inactive FLP protein (Fig. 2A and B, lanes 6 and 7). The autoradiograph of the SDS-polyacrylamide gel (Fig. 2C) indicates that the radiolabeled translation products encoded by pTA23 (lane 2), pTA225 (lane 3), and pPS645 (lane 4) were of the predicted sizes.

Deletion of six amino acids from the amino-terminal region of FLP diminishes FRT recognition. We next assessed the importance of the amino-terminal region of FLP in FRT site recognition. Deletions of the 5′ end of the FLP gene were constructed as described in Materials and Methods and the legend to Fig. 1B. One plasmid (designated pTA155) was chosen for further analysis. The mRNA synthesized from *Sma*I-linearized pTA155 would direct the synthesis of a polypeptide of 417 amino acids. It should be noted that the first two amino-terminal residues (Met-Pro) encoded by the wild-type FLP gene are preserved in this deletion mutant since these amino acids are encoded by the polylinker sequences.

The in vitro translation products of mRNA from pTA155 and pPS645 were assessed as described above for the ability to bind to the FRT site and to carry out excisive recombination. Results of the mobility shift assay showed that again,

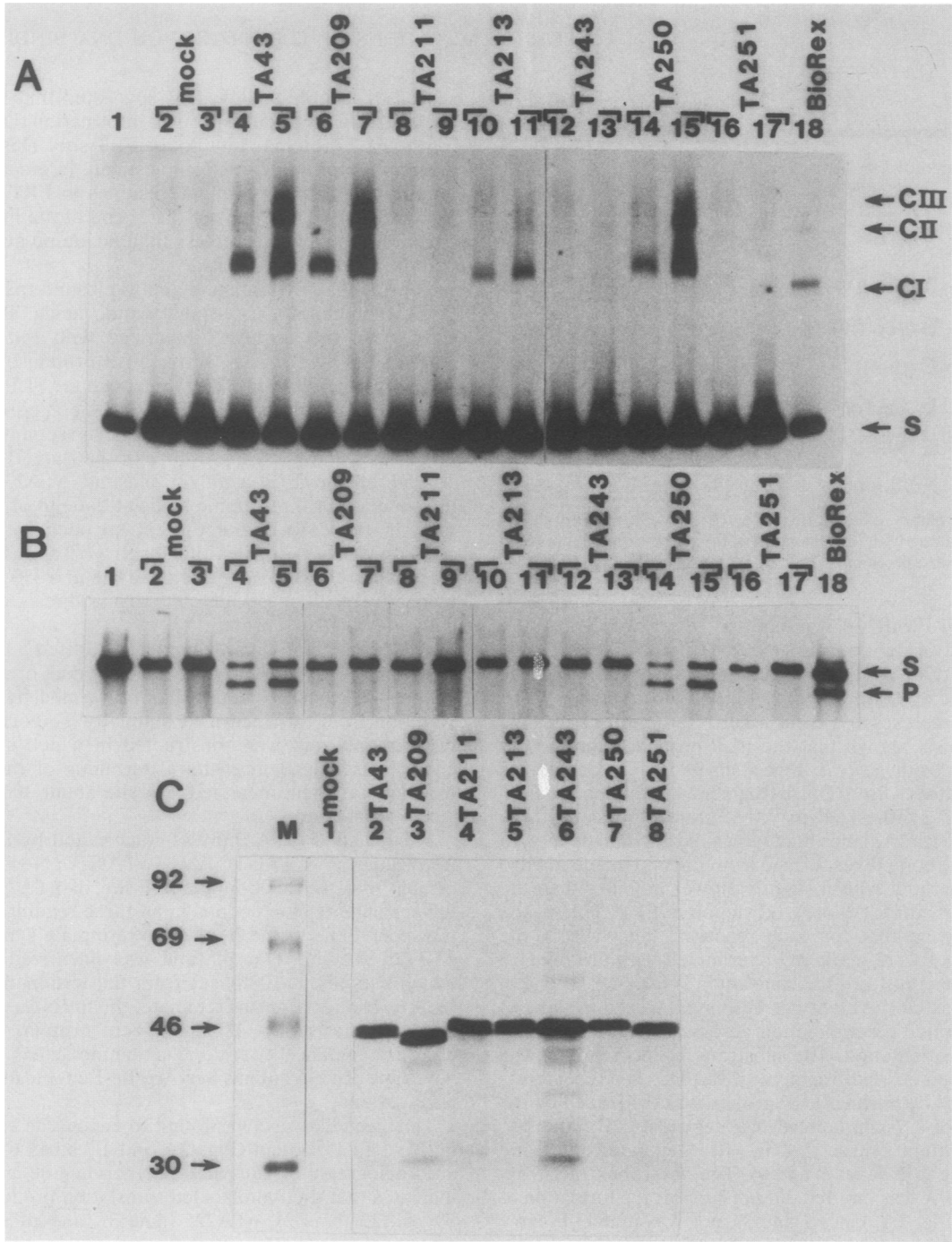

FIG. 5. Demonstration that FLP proteins with various tetrapeptide insertions show functional differences in the mobility shift and recombination assays. (A) Autoradiograph of mobility shift assay gel. Translation products were analyzed as described for Fig. 2A. S, 100-bp *Eco*RI-*Hin*dIII fragment; CI, CII, CIII, FLP-FRT complexes I, II, and III, respectively (complex III is barely visible in this experiment). Lanes: 1, no extract; 2 and 3, 8 and 16 μl of mock translation extract minus mRNA; 4 and 5, 8 and 16 μl of pTA43 translation product; 6 and 7, 8 and 16 μl of pTA209 translation product; 8 and 9, 8 and 16 μl of pTA211 translation product; 10 and 11, 8 and 16 μl of pTA213 translation product; 12 and 13, 8 and 16 μl of pTA243 translation product; 14 and 15, 8 and 16 μl of pTA250 translation product; 16 and 17, 8 and 16 μl of pTA251 translation product; 18, 0.2 U of FLP. (B) Autoradiograph of recombination assay gel. Translation products were analyzed as for Fig. 2B. S, 8.9-kbp linear *Eco*RI-*Eco*RI substrate; P, 7.0-kbp *Eco*RI-*Eco*RI recombinant product. Lanes: 1, no extract; 2 and 3, 2.5 and 5.0 μl of mock translation extract minus mRNA; 4 and 5, 2.5 and 5.0 μl of pTA43 translation product; 6 and 7, 2.5 and 5.0 μl of pTA209 translation product (the recombinant product is barely visible here, but with extended exposure of the autoradiograph a distinct band is clearly seen); 8 and 9, 2.5 and 5.0 μl of pTA211 translation product; 10 and 11, 2.5 and 5.0 μl of pTA213 translation product; 12 and 13, 2.5 and 5.0 μl of pTA243 translation product; 14 and 15, 2.5 and 5.0 μl of pTA250 translation product; 16 and 17, 2.5 and 5.0 μl of pTA251 translation product; 18, 0.1 U of FLP. (C) Autoradiograph of SDS-polyacrylamide gel. Translation products (40,000 cpm) were subjected to electrophoresis on a 15% polyacrylamide-SDS gel. Lanes: M, molecular size markers (indicated in kilodaltons on the left); 1, 10 μl of mock translation extract minus mRNA; 2, pTA43 translation product; 3, pTA209 translation product (see below); 4, pTA211 translation product; 5, pTA213 translation product; 6, pTA243 translation product; 7, pTA250 translation product; 8, pTA251 translation product. (Note: the pTA209 translation product migrated to a lower position than did the other translation products. An explanation of this aberrant migration may be that the SDS-bound polypeptide assumes a conformation different from those of the other polypeptides. Anomalous migration of proteins has previously been reported [18].)

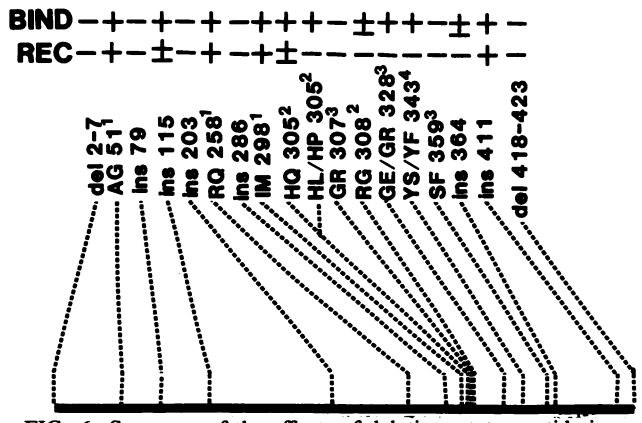

FIG. 6. Summary of the effects of deletions, tetrapeptide insertions, and amino acid substitutions on the ability of the FLP protein to bind to the FRT sequence and to carry out recombination. Numbers indicate positions in the amino acid sequence of protein with deletions (del), insertions (ins), and substitutions (the first letter signifies the amino acid present in wild-type FLP; the second letter indicates the substitution). Superscripts: 1, selected substitutions that result in proteins exhibiting elevated affinity for the FRT sequence in an in vivo assay system (27); 2, results described in reference 37; 3, results described in C. J. Schwartz and P. D. Sadowski, J. Mol. Biol., in press; 4, results described in reference 39.

only FLP protein encoded by plasmid pPS645 (wild-type FLP gene) formed complexes with the FRT site (Fig. 3A, lanes 6 and 7). The protein encoded by pTA155, which carries a deletion of amino acids 3 to 8, did not form complexes with the FRT site (lanes 4 and 5). As expected, only the protein encoded by pPS645 was proficient in recombination (Fig. 3B, lanes 6 and 7). Recombinant product was not detected in the reaction with the protein encoded by pTA155. The autoradiograph of the SDS-polyacrylamide gel (Fig. 3C) shows that the proteins encoded by pTA155 (lane 2) and pPS645 (lane 3) in the translation lysates were mostly of the predicted lengths. The lower-molecular-weight protein products present in Fig. 3C may be due to premature termination of translation. Their abundance varied with the particular reticulocyte lysate and did not appear to influence the activity of the FLP protein. Plasmids encoding FLP proteins that lacked 193 and 223 N-terminal amino acids likewise directed the synthesis of enzymatically inactive FLP polypeptides (data not shown).

Effects of tetrapeptide insertions on FRT recognition. To determine whether other regions of the FLP protein were important for FRT site recognition, we used the linker insertion mutagenesis technique (25). This technique has been used in other studies to define the protein kinase domain of the epidermal growth factor receptor (28) and certain functional domains of the oncogenic protein of Fujinami sarcoma virus (44). We therefore examined the effect of the tetrapeptide insertions on the ability of the modified FLP proteins to bind to the FRT site and to carry out recombination. The plasmids used that carry in-frame linker insertions in the FLP gene are listed in Fig. 4. Also shown are the sequences of the tetrapeptide insertions as well as their relative positions in the FLP protein. The mRNA was synthesized from the *Sma*I-linearized templates of these plasmids and used in the in vitro translation reaction.

The mobility shift and recombination assays were done with the in vitro-synthesized proteins encoded by the plasmids mentioned above. Results of the mobility shift assay (Fig. 5A) show that none of the three protein-nucleic acid complexes was formed with the proteins encoded by pTA251 (insertion at amino acid 79; lanes 16 and 17), pTA243 (insertion at amino acid 203; lanes 12 and 13), and pTA211 (insertion at amino acid 286; lanes 8 and 9). However, the protein encoded by pTA213 (insertion at amino acid 364) yielded the three complexes but at a reduced level (lanes 10 and 11) that we estimate to be 20 to 40% that of the wild-type FLP level (lanes 4 and 5). The proteins encoded by pTA209 (insertion at amino acid 115; lanes 6 and 7) and pTA250 (insertion at amino acid 411; lanes 14 and 15) produced about the same amount of all three FLP-FRT complexes as did the wild-type FLP protein encoded by plasmid pTA43 (lanes 4 and 5). The results of the recombination assay (Fig. 5B) show that the proteins encoded by pTA251 (lanes 16 and 17), pTA243 (lanes 12 and 13), and pTA211 did not carry out excisive recombination with the pBA104 substrate. However, the protein encoded by pTA250 gave yields of excision product (lanes 6 and 7) comparable to those of wild-type FLP made from pTA43 (lanes 4 and 5). The protein encoded by pTA209 produced an extremely low amount of excision product (lanes 14 and 15) that we estimate to be less than 5% of the level produced by native FLP. (The excision product was clearly noticeable only when the autoradiograph was exposed four times as long as usual.) As seen in the autoradiograph of the SDS-polyacrylamide gel (Fig. 5C), these translation products were of the predicted lengths. (The protein encoded by plasmid pTA209 had an anomalously fast migration, perhaps because of an aberrant conformation during electrophoresis.)

DISCUSSION

We have constructed a number of deletions and in-phase linker insertions in an attempt to determine whether the DNA-binding domain of the FLP recombinase is localized to a specific region of the polypeptide. An in vitro transcription-translation system that directs the synthesis of enzymatically active FLP protein was used to assess the effects of these mutations. Hope and Struhl (18) have used this basic approach successfully to localize the DNA-binding domain of the GCN4 protein to the carboxy-terminal 60 amino acids of the polypeptide. In a similar way, the DNA-binding domain of the glucocorticoid receptor has been delineated (42).

Unlike the situation for GCN4 and the glucocoritcoid receptor, we find that the DNA-binding activity of FLP, as assayed in a mobility shift assay, is diminished upon removal of as few as six amino acids from the amino- and carboxy-terminal ends of the FLP protein. DNA-binding activity is also diminished in FLP proteins with tetrapeptide insertions at amino acids 79, 203, and 286. We estimate that the mobility shift assay is sensitive enough to have detected at least a 10-fold reduction in binding affinity for the FLP recombinase.

The results may suggest that the FLP protein is sensitive to changes in its structure at certain regions such that the small deletions and insertions affect the folding or thermostability of the protein. However, this is not generally true, since three insertions, at amino acids 115, 364, and 411, preserved DNA-binding activity and one (amino acid 411) was fully recombination competent. It is possible that the amino and carboxyl termini play a critical role in the thermodynamic stability of the FLP protein, as has recently been shown for the ends of T4 lysozyme (30).

The other possibility is that the DNA-binding domain of the FLP protein responsible for FRT recognition is not

constituted from contiguous amino acids as for the helix-turn-helix motif (2, 10, 36, 48) or zinc finger domain (13, 33, 43). Rather, the domain in FLP may consist of residues from several diverse regions of the polypeptide, as in the case of the *Eco*RI enzyme (22, 32). If this is true, then the insertion-tolerant regions of FLP could identify regions between segments that form the DNA-binding domain. A genetic study (27) identified changes at amino acids 51, 258, 298, and 343 that resulted in an elevated affinity of the FLP protein for the FRT site. These results would tend to support the hypothesis that the DNA-binding domain of FLP consists of diverse regions of the polypeptide. Figure 6 summarizes all known mutations from this study and others (27, 37, 39) on the FLP gene and their effects on the sequence-specific DNA-binding function of the protein. A better understanding of the organization of the DNA-binding domain of the FLP protein will have to await further detailed physical analysis of the protein.

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