Identification of the DNA-binding Domain of the FLP Recombinase*

Hua Pan‡, Donna Clary, and Paul D. Sadowski§

From the Department of Medical Genetics, Medical Sciences Building, University of Toronto,
Toronto, Ontario M5S 1A8, Canada

We have subjected the FLP protein of the 2-µm plasmid to partial proteolysis by proteinase K and have found that FLP can be digested into two major proteinase K-resistant peptides of 21 and 13 kDa, respectively. The 21-kDa peptide contains a site-specific DNA-binding domain that binds to the FLP recognition target (FRT) site with an affinity similar to that observed for the native FLP protein. This peptide can induce DNA bending upon binding to a DNA fragment containing the FRT site, but the angle of the bend (approximately 24°) is smaller in magnitude than that induced by the native FLP protein (60°). The additional DNA bending induced by the interaction between two native FLP molecules bound to the FRT site is not observed with the 21-kDa DNA-binding peptide. Amino-terminal sequencing has been used to map this peptide to an internal region of FLP that begins at residue Leu-148. It is likely that the DNA-binding peptide includes the catalytic site of the FLP protein.

The FLP recombinase catalyzes site-specific recombination between two FLP recognition target sites present in inverted orientation on the 2-µm plasmid of Saccharomyces cerevisiae (Broach and Hicks, 1980). These sites contain three 13-base pair sequences to which FLP binds in a site-specific manner (Andrews et al., 1987). To understand the DNA-protein interactions in the FLP-mediated recombination, it is important to identify the elements of the protein which are required for site-specific DNA binding.

One approach to identifying the DNA-binding domain of a protein has been to analyze truncated proteins whose synthesis is directed from deletion mutations of their genes in vitro (Hope and Struhl, 1986; Rusconi and Yamamoto, 1987; Henry et al., 1990) or in vivo (Kadonaga et al., 1987; Moskaluk and Bastia, 1988). We have used in vitro transcription and translation of mutant FLP genes in an attempt to localize the DNA-binding domain in this protein (Amin and Sadowski, 1989). However, it was found that the DNA-binding activity of FLP is dramatically sensitive to changes in the structure of the protein and this approach was not successful in the identification of a discrete DNA-binding domain.

Partial proteolysis and subsequent purification of the proteolytic peptides have also been used to identify the DNA-binding domains in some sequence-specific DNA-binding proteins (Abdel-Meguid et al., 1984; Smith et al., 1984; Marzouki et al., 1986; Huet and Sentenac, 1987; de Vargas et al., 1988; Boulanger et al., 1989). In order to probe the location of the DNA-binding domain in FLP, we have subjected this protein to limited proteolysis. Digestion of FLP with the nonspecific protease proteinase K produced an internal polypeptide of molecular mass of 21 kDa. Gel mobility shift assays showed that this fragment bound to the FRT site in a site-specific manner with an affinity similar to that of the native FLP and formed DNA-peptide complexes with greater mobilities than those produced by the intact FLP protein. The proteolytic fragment induces bends in the substrate DNA to which it binds, but the bend angles are less than those induced by the intact FLP protein.

MATERIALS AND METHODS

Proteolytic Digestion of FLP Generates a DNA-binding Peptide—Previous attempts to use deletion analysis to identify the DNA-binding domain of FLP were unsuccessful, possibly because the deletions perturbed the proper folding of the protein (Amin and Sadowski, 1989). We reasoned that digestion of the folded native protein might yield a protease-resistant domain that would preserve DNA-binding activity. To identify such a DNA-binding domain in FLP, we incubated the purified FLP protein with various amounts of proteinase K for a fixed time period (Fig. 2). After the digestions had been terminated by the addition of PMSF, a labeled DNA fragment containing the wild-type FRT site (Fig. 1) was added to the reactions and the incubations were continued. The DNA-protein complexes formed by the undigested, and proteolyzed FLP were resolved by electrophoresis on a nondenaturing acrylamide gel. The native FLP protein generates three specific complexes believed to be caused by the binding of one, two, or three molecules of FLP to the FRT site (Fig. 2, lane 12; Andrews et al., 1987; Beatty and Sadowski, 1988; Qian et al., 1990). The proteolyzed FLP sample also generated three DNA-protein complexes with the FRT site, but the mobilities of the complexes were increased relative to those formed with native FLP (Fig. 2, lanes 3 and 4). These complexes were abolished by further increasing the concentration of proteinase K in the reactions (lanes 5 and 6).

Similar results were obtained when FLP was incubated with the end-labeled DNA fragment before digestion with the protease K (Fig. 2, lanes 7–11). The FLP protein in the complexes was somewhat more resistant to proteinase K than the free FLP. This may be due to a conformational change of the protein upon binding or to the protection of FLP by the DNA.

RESULTS

*This work was supported by a grant from the Medical Research Council of Canada. 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.

‡ Held a Special Entrance Fellowship and an Open Fellowship from the University of Toronto.

§ To whom correspondence should be addressed.

1 Portions of this paper (including "Materials and Methods," Table 1, and Figs. 8 and 9) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
The FRT site is characterized by three 13-bp symmetry elements that are indicated by heavy arrows (labeled a, b, and c). Elements a and b are in inverted orientation and separated by an 8-bp core region (open box). Elements b and c are in direct orientation.

DNA substrate (compare lanes 3–6 and lanes 8–11). A control experiment (Fig. 2, lanes 12–16) indicated that proteinase K was completely inhibited by PMSF. The electric charge of a DNA protein complex in a neutral nondenaturing gel is carried mainly by the DNA component in the complex, whereas the charge on the protein does not have a great effect on the mobility (Boulanger et al., 1989). Therefore, the much faster migration of the DNA-proteolyzed FLP complexes was most likely caused by a substantial reduction of the protein mass of the FLP protein.

Products of Proteinase K Digestion of FLP—To identify the peptide(s) of FLP that retained the ability to bind to DNA, we analyzed the products of proteolyzed FLP by SDS-PAGE (Laemmli, 1970). Ten minutes of digestion at 0 °C led to the disappearance of the FLP protein (~45 kDa) and to the appearance of three major peptide bands of approximately 32 (P32), 27 (P27), and 13 (P13) kDa, respectively (Fig. 3A). Determination of the amino-terminal sequences of these peptides indicated that P27 was derived from the amino-terminal end of FLP and that the amino-terminal end of FLP was shown to be proline instead of methionine, probably because the carboxyl terminus of each peptide segment is estimated from the size of each peptide on SDS-PAGE. Two regions (I and II) indicated at the top are highly conserved (more than 60% match at the nucleotide sequence level) among six FLP proteins from six 2-μm-like plasmids (Utatsu et al., 1987). The residue numbers indicated are amino acids of FLP from the 2-μm plasmid of yeast.

The abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; AMV, avian myeloblastosis virus; bp, base pair(s); DTT, dithiothreitol; FPLC, fast protein liquid chromatography; FRT, FLP recombination target; IPTG, isopropylthiogalactoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
sequence of one (P13a) corresponded to that of mature FLP whereas the amino-terminal sequence of the other corresponded to amino acid 148 of FLP (Fig. 3B). However, P13a was found to be 5-10-fold more abundant than P13b in molar concentration (data not shown).

With increasing digestion time, new peptides of approximately 21 (P21) and 11 kDa (P11) appeared, and the amounts of bands P32 and P27 declined (Fig. 3A). The amino-terminal sequence of P21 was shown to be identical to the internal FLP sequence beginning at Leu-148, suggesting that P21 might be derived from P32 (Fig. 3B). After a 150-min digestion at 0 °C, three major bands were observed on an SDS-polyacrylamide gel, P21, P13, and P11 (Fig. 3A, lane 7). Two different peptides were present in the 11-kDa band with their amino-terminal sequences mapping to proline 2 and leucine 148, respectively (not shown). These results indicate that the FLP protein can be digested into at least two major non-overlapping peptides (P21 and P13a) by proteinase K. These results are summarized in Fig. 3C.

Proteolyzed Products of FLP Bind to the FRT Site—We then tested these proteolyzed products of FLP for their ability to bind to an FRT-containing DNA fragment (Fig. 4). Even the most extensively digested FLP sample retained DNA binding activity, forming complexes FI, FI1, and FI11 (Fig. 4, lanes 2-4). The amount of complex FI11 formed was less than that obtained with the less proteolyzed sample of FLP (Fig. 4, lanes 2-4). These observations indicated that a FLP-derived peptide of 21 kDa or less retained the ability to bind to the FRT site. At the beginning time points of the digestion, some intermediate complexes were observed (lanes 2 and 3); however, only three complexes (FI, FI1, and FI11) with the increased rate of migration were found after 30 min of digestion (lanes 4-6).

Purification of the Peptides Resulting from Partial Proteolysis of FLP—To determine which peptide(s) was actually the DNA-binding species, we subjected extensively digested FLP to ion exchange chromatography (see “Materials and Methods”) (Fig. 5A). Three UV-absorbing peaks (I, II, and III) were observed. Peak I consisted of a 13 kDa peptide and a smear of lower molecular weight (Fig. 5B, lane 1), and peak II contained a protein with a molecular mass greater than FLP that was likely a contaminant (Fig. 5B, lane II).
peaks I nor II contained DNA binding activity (Fig. 5C, lanes 3 and 4). Peak III contained a major 21-kDa band and a minor 19-kDa band and had the same DNA-binding activity as the unfractonated proteolysed FLP (Fig. 2, lane 3, and Fig. 5C, lane 2).

Determination of the amino-terminal sequence of the peptides present in peaks I and III indicated that the 13-kDa peptide represented P13a and that the amino termini of both the 21- and 19-kDa peptides mapped to Leu-148 of FLP. These results indicate that FLP contains an internal 21-kDa proteinase K-resistant domain that retains DNA binding activity.

The DNA Binding Specificity and Affinity of the DNA-binding Peptide—To compare the specificity and affinity of the DNA binding of the 21-kDa peptide with that of native FLP, the purified P21 or intact FLP was incubated with a FRT-containing DNA fragment or a non-FRT-containing DNA fragment. Both the native FLP and P21 formed three complexes with the FRT-containing fragment (Fig. 6A, lanes 3 and 5), although the peptide formed less complex III (lane 5) than did native FLP. We also found that both of the proteins formed a small amount of a DNA-protein complex with a non-FRT-containing fragment (lanes 4 and 6). The amount of complex produced by the native FLP was several-fold less than that produced by P21, suggesting that the DNA-binding domain contained in P21 might have less stringent specificity than the native FLP.

A competition experiment was carried out to confirm the site specificity of the P21 DNA binding peptide. A linearized FRT site-containing plasmid or nonspecific plasmid was used as competitor. The specific complexes formed by the native FLP and P21 were competed efficiently with an excess of the specific plasmid (pBL112) (Fig. 6B, lanes 2–5 and lanes 10–13). In contrast, a nonspecific plasmid (pUC19) was much less efficient in competing for the formation of the specific complexes (lanes 6–9 and 14–17). The band labeled “RP” in Fig. 6B is a recombination product between the labeled FRT fragment and the specific competitor plasmid.

The Purified DNA-binding Peptide Bends DNA—We have found previously that the FLP protein bends the DNA of the FRT site upon binding (Schwartz and Sadowski, 1989). We have measured the angles of the bends induced by FLP in the FRT site and found that the binding of the native FLP to the target site with two inverted symmetry elements (a and b) separated by an 8-bp core region causes two types of DNA bending: the type I bend occurs when one FLP molecule binds to a single symmetry element; the type II bend occurs when two FLP molecules bind to the two symmetry elements on opposite sides of the core region (Schwartz and Sadowski, 1990). The type I bend angle is approximately 60°, and the type II bend is greater than 140°. The fact that the type II bend angle is much greater than the sum of two type I bends is believed to be the result of protein-protein interactions (Schwartz and Sadowski, 1990).

To investigate whether the isolated DNA-binding peptide induces bending on binding to FRT-containing DNA, the bending assay was carried out using fragments that contained a partial FRT site in the middle or at the end of the fragment. DNA bending is indicated when the mobility of the fragment with the FRT site in the middle is slower than the mobility of the DNA with the FRT site at the end. The bending angles were measured as described by Schwartz and Sadowski (1990) by comparing the mobility of the bent DNA fragments with that of a series of DNA standard fragments that contain sequence-directed bends of known magnitude (Thompson and Landy, 1988). We found that the DNA of complexes FI and FII induced by P21 contained a bend (compare the positions of complexes FI and FII in Fig. 7, lane 3 versus 4). However, the bend angles for the DNA complexes FI and FII were much smaller.
FLP DNA-binding Domain

11351

**DISCUSSION**

The initial step in the FLP recombination reaction is the site-specific binding of the FLP protein to the FRT site. To further understand the interaction between FLP and the FRT site, it is important to identify the DNA-binding domain in FLP. So far, there is no evidence that FLP contains any of the previously defined DNA binding motifs, e.g. helix-turn-helix, helix-loop-helix (Murre *et al.*, 1989), leucine zipper (Buech and Sassone-Corsi, 1990), or zinc finger (Evans and Hollenberg, 1988).

Our previous attempts to identify the DNA-binding domain of FLP using an *in vitro* transcription and translation system were unsuccessful, possibly because the deletion or insertion mutations used may have disturbed the proper folding or thermostability of the protein (Amin and Sadowski, 1989).

In this paper, we report the use of partial proteolysis by proteinase K to search for a structural domain that preserves the DNA binding specificity of the FLP protein. We found that FLP could be digested into two major peptides which may represent distinct functional domains. A 21-kDa peptide was purified and was shown to contain a DNA-binding domain. A function for P13 has not yet been identified.

The P21 peptide is able to interact with the full FRT site to form three specific complexes. The affinity of this peptide for the FRT site is comparable with the intact FLP protein (Fig. 6B), although this peptide does not form complex III as well as intact FLP (Fig. 6B). However, we have also found that the specificity of this isolated DNA-binding domain may be less stringent than that of native FLP (Fig. 6A). Although we have not observed a DNase I footprint produced by the 21-kDa peptide, it should be pointed out that even the binding of intact FLP to a single 13-bp symmetry element of the FRT site (formation of complex I) does not give a prominent DNase I footprint (Andrews *et al.*, 1987). Apparently, the production of an identifiable DNase I footprint by intact FLP requires protein-protein interactions in complexes II and III. These interactions may not be possible for peptide P21.

The DNA-bending assay has shown that the 21-kDa peptide induces a DNA bend upon binding to the FRT site, although the bending angle induced by P21 is less than that caused by native FLP. The extreme bend which is thought to be the result of the interactions between two FLP molecules bound to the same substrate (type II bend, Schwartz and Sadowski, 1990) is not induced by P21. We hypothesize that some regions of the FLP protein that are necessary for contact between the protein and DNA are absent from P21. These contacts are apparently necessary for bending although not specificity or even high affinity. It is possible that the regions required for protein-protein interactions needed for the type II bend are also absent. Alternatively, these regions may be present but are not close enough together to interact properly in the FII complex.

FLP has two regions that are highly conserved among six FLP proteins from six 2-μm-like plasmids of various yeast strains (Utatsu *et al.*, 1987). They cover amino acid residues 185–203 and 295–313 of 2-μm plasmid FLP. It is likely that P21 includes both conserved regions (Fig. 3C) and the residues in these conserved regions play an important role in forming the DNA binding domain. We have found that several mutations in these two conserved regions give a binding-deficient and bending-deficient phenotype. Three residues (His-305, Arg-308, and Tyr-343) are absolutely conserved among the integrase family of recombinases (e.g. Int, Cre, and FLP) (Argos *et al.*, 1986). It has been shown that Tyr-343 is directly involved in DNA cleavage, Arg-308 may help this step and His-305 is essential for the strand exchange and religation reactions (Parsons *et al.*, 1988). Conserved region II contains His-305 and Arg-308 (Fig. 3C). We do not know whether P21 contains Tyr-343 as well, but we have not observed any DNA cleavage activity from P21.

**Acknowledgments**—We thank Jackie Segall, Marvin Gold, Tony Amin, and Helena Friesen for critical reading of the manuscript and Ann Bemrose-Fetter, Lisa Petro, and Suze Detombe for help in plasmid constructions. We also thank Linda McBroome for her gift of the MATa DNA fragment and Linda Beatty for her help in typing.

**REFERENCES**

Abdel-Meguid, S. S., Grindle, N. D. F., Templeton, N. S., and Steitz, T. A. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 2001–2005

Amin, A. A., and Sadowski, P. D. (1989) *Mol. Cell. Biol.* 9, 1987–1995

Andrews, B. J., Proteau, G. A., Beatty, L. G., and Sadowski, P. D. (1985) *Cell* 40, 705–803

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-Ljunquist, E., Hoess, R. H., Kahn, M. U., Kalionis, B., Narayana, S. V. L.,

H. Pan, unpublished data.

H. Friesen and J. Kulpa, unpublished data.
Supplemental Material to Identification of the DNA-Binding Domain of the FLP Monomeric Protein

Kia Pho, Donna Clazy and Paul D. Sadowski

**Materials and Methods**

**Plasmids and strains**

*Escherichia coli* strain HB101 (F-, hsdR17 [rK- mK-]), ara-lac, proC, lacZ, galK, gpdA, xylA2, mtlA, nfo, auxC2, mcrA, rpsL, tRNA^Lys^, tRNA^Glu^, tRNA^Glu^ (777, boyer's *Roulland-Dussoix*, 1969) was used for construction and propagation of plasmids.

The construction of the subclone plasmids pF1L12 and pF1S12 has been described previously (Andrews et al., 1985; Lea et al., 1989; Schwartz & Sadowski, 1990). Plasmid pF1056, pF1056, pF1057, and pF1058 were prepared as described previously (Babineau et al., 1985).

**Flp** plasmid pG03 contains the Flp coding sequence joined to the ribosome binding site of the T7 gene 10 promoter. This plasmid was constructed in three steps (see Figure 8). (a) The entire Flp coding sequence contained on a 1.4 kb fragment was excised from plasmid pG013 with BglII and ligated into the BglII site of plasmid pBD10 (from F.W. Studier). This plasmid contains the CM 10 promoter, the translation initiation site for the T7 gene 10 protein, and a BglII cloning site followed by the transcription terminator for T7 RNA polymerase. Cloning of the Flp gene fragment from pG013 gives a coding sequence consisting of the first 11 codons from the gene 10 protein, four extraneous codons followed by the entire Flp coding sequence. This plasmid was called pG013 (Figure 8a). Although this construct produced a large amount of a protein that was the correct size to be the Flp fusion product (not shown), the protein was apparently unipositive and inactive. Plasmid pG021 was therefore modified to remove the extraneous amino acid codons in front of the Flp coding region. (b) Plasmid pG021 was linearized with EcoRI, which cuts in the ATG initiation code of the Flp gene. The 3' protecting ends were made blunt with T4 DNA polymerase and deoxynucleoside triphosphates and a double phosphorylated blunt-end linker (Bacterial cloning kit) was ligated to the blunt-ended (termini). After spontaneous precipitation to remove excess linker and heating to 80°C for 5 minutes, the plasmid (termini were allowed to anneal) and the resultant plasmids were transformed into *E. coli*. This procedure resulted in the replacement of the SalI site in the ATG of the Flp gene with an NdeI site. The resulting plasmid was called pG04 (Figure 8b). (c) A 1.2 kb fragment from pG04 was excised with NdeI and XhoI and transferred to plasmid pG021 that had been cut with XhoI. The resulting plasmid, pG05, has therefore lost the 15 extra codons that were originally present in pG013 (Figure 8b). The DNA sequence of the junction region was verified by DNA sequence analysis. The plasmid was then transferred into strain B62 (8063) pSG6 B (obtained from F.W. Studier, Brookhaven National Laboratory). This strain contains a Lambda prophage with the gene for T7 RNA polymerase under control of the lac UV5 promoter as well as a plasmid (pSG6) that constitutively expresses the gene encoding T7 lysozyme. This enzyme binds to and inhibits any basal amount of T7 polymerase activity that is made from the lac UV5 promoter (Studier et al., 1985).

**Assay of Flp Activity**

Flp activity in crude extracts was assayed using the inversion plasmid CV5, which contains the A form of the 2 micron plasmid. One unit of activity is the amount of protein required to invert 50 per cent of the substrate to the B form of the 2 micron plasmid (Schwartz et al., 1985). The activity of Flp was assayed following the purification procedure using the linear excision substrate, plasmid pF1054. A unit of activity is the amount of Flp required to excise 50 per cent of the substrate (Schwartz et al., 1985). The composition of various fractions was assessed by SDS polyacrylamide gel (15 per cent separating gel), cast in Bio-Rad mini-apparatus, followed by staining with Coomassie blue. Protein concentrations were determined by the Bradford colorimetric method (Bradford, 1976).

**Purification of Flp Protein**

Induction of Flp expression from T7 promoter. We previously induced Flp expression from the T7 promoter by the addition of IPTG to the medium (Schwartz et al., 1985). When we attempted to produce Flp from the T7 promoter using the same protocol, we were unable to detect any activity at levels of protein where Flp activity was readily detectable from the lac construct. It was possible that the Flp protein was being highly expressed from the T7 promoter, but that the protein was inactive in the E. coli cell (Marston, 1986). This hypothesis was confirmed by the finding of a large amount of a band that comigrated with authentic Flp protein when cells containing the plasmid pG03 were induced with IPTG, and analyzed by SDS-PAGE followed by Coomassie blue staining (not shown). This band was present one hour after induction and its intensity increased markedly after three hours of induction. It therefore seemed that a large amount of inactive Flp protein was being expressed from the T7 promoter. We therefore sought methods which might facilitate the synthesis of enzymatically competent protein and found that induction of Flp expression at low temperature led to the synthesis of substantial amounts of active protein. When we induced Flp expression at 25°C we obtained about twofold more Flp activity in crude cell extracts than we previously detected in cell extracts where Flp was expressed from the T7 promoter. However, we were unable to detect any activity at levels of protein where Flp activity was readily detectable from the lac construct. We therefore sought methods which might facilitate the synthesis of enzymatically competent protein and found that induction of Flp expression at low temperature led to the synthesis of substantial amounts of active protein. When we induced Flp expression at 25°C we obtained about twofold more Flp activity in crude cell extracts than we previously detected in cell extracts where Flp was expressed from the T7 promoter. However, we were unable to detect any activity at levels of protein where Flp activity was readily detectable from the lac construct.

**Bacterial growth**

A 200 ml volume of M9 broth (10 g Trizion, 5 g yeast extract, 5 g NaCl, 0.1 ml of 10 N NaOH per 1) was inoculated with a 2 liter overnight culture of strain D23 (2212/2212), pSG6, pSG3 that had been grown at 37°C. Both cultures contained 20 microns per ml of ampicillin and 20 microns per ml of chloramphenicol. The 200 l culture was grown at 37°C with an air flow of 100 liters per minute, and an agitation speed of 200 rpm in a New Brunswick fermentor. After the O.D.600 reached 0.7, the temperature was raised to 37°C and solid IPTG was added to a concentration of 50 microns. Growth was continued for six hours at 37°C; the cells were collected by centrifugation and stored at 70°C.
FLP DNA-binding Domain

Preparation of crude extract. All the procedures were carried out at 4-8°C unless otherwise indicated. One hundred and fifty grams of frozen cells (wet weight) were thawed with gentle stirring on ice for 45 min in 2 M ammonium sulfate. The mixture was centrifuged at 20,000 g for 30 min at 4°C, and the supernatant was recovered and dialyzed against 20 mM sodium phosphate, pH 7.4, 50% (w/v) sucrose, 1 mM EDTA, 0.5 mM PMSF. The mixture was sonicated for 3 min to suspend the cells and then for 20 periods of 1 min, interspersed with 45 sec of cooling in an ice-salt bath. The sonicated extract (45 l) was dialyzed against a Bio-Rex buffer containing 300 mM NaCl and finally with a Bio-Rex buffer containing 50 mM NaCl. The two fractions (150 ml each) were stored as the FPLC pool, and will withstand three cycles of freezing and thawing. The FPLC pool was stored at -70°C and collected with 200 ml of Bio-Rex buffer containing 300 mM NaCl.

Preparation of crude extract. The proteolysis was done in two different ways. a. The purified FLP (3.5 mg) was treated with 5-40 ng of proteinase K in a 50 ml reaction containing 50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 150 mM NaCl (binding assay buffer) and 4 ng of the end-labeled DNA substrate fragment to form DNA-protein complexes and was then partially digested with 5-40 ng of proteinase K in a binding assay buffer at 15°C (see legend to Fig. 1). b. The reaction was terminated with 3% glycerol as above. In both methods, a loading dye was added to all the reactions which were then analyzed on a 5% acrylamide gel (Andrews, 1987).

Preparation and sequencing of the proteolyzed peptides. The proteolysis was carried out at 1°C for various periods of time at a fixed ammonium sulfate fraction (1900, proteinase K: FLP:water) of digestion in the binding assay buffer for 20 min at 26°C. The digestion was terminated by addition of 400 pg of PMSF. A 10% (v/w) column 5050 ml. The column was pre-equilibrated with buffer D (150 mM sodium phosphate buffer, 0.1% Tween 20, 1 mM EDTA) using a Pharmacia FPLC system. The proteolyzed FLP protein sample was loaded onto the column at a flow rate of 0.5-0.9 ml/min and the chroctogram was developed using a 12.5 ml linear NaCl gradient (6.3-7.5) ml in buffer D. The column fractions (3.5 ml) were analyzed on SDS-PAGE and assayed for DNA-binding activity. Peptide sequencing was done as described above.

Specificity and competition experiment. The native FLP or the purified 21KDa peptide (peak 112 in Table 1, A and B) were incubated with a 21KDa site-containing DNA fragment or a non-FRT-containing fragment in triplicate (100 pg, 314.2 pg of DNA) and the reactions were analyzed on a native acrylamide gel. In the competition experiment, the reactions were incubated with several concentrations of either a linear FRT site-containing plasmid (pBu1211) or a non-specific plasmid (pUC19) for 20 min at 15°C. The end-labeled DNA fragment was then added to the samples and the reactions were incubated for an additional 20 min. The reactions were analyzed on a 5% acrylameg gel (Andrews, 1987).

Protein-induced DNA bending assay. The plasmid pCD30 was used for preparation of the substrate in the bending assay of the purified 21 KDa peptide. The preparation of the substrate, the bending assay, and the estimation of the magnitudes of the protein-induced bends have been described elsewhere (Trowbridge & Radman, 1990; Trowbridge & Lody, 1990).

Table 1. PURIFICATION OF FLP PROTEIN

| Fraction | Total Recovery | Specific Activity (units/mg) | Protein (mg) | DNA (ng) | Total Recovery (percent) |
|----------|----------------|-----------------------------|-------------|----------|-------------------------|
| A         | 7.600          | 1                           | 0.25        | 7.600    | 93.1                    |
| B         | 19.13          | 0.5                         | 0.35        | 9.55     | 100.0                   |
| C         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| D         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| E         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| F         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| G         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| H         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| I         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| J         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| K         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| L         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| M         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| N         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| O         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| P         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| Q         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| R         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| S         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| T         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| U         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| V         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| W         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| X         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| Y         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |
| Z         | 7.600          | 0.25                        | 0.25        | 7.600    | 100.0                   |

Since FLP activity could not be assayed in crude extracts using the electrophoresis method, we define the total units of the ammonium sulfate fraction (1900, proteinase K: FLP:water) as 1 unit. The specific activity of the crude extract to be used for the bend assay was determined by the FPLC system after step 2 (Habib et al. 1988). The proteinase K digestion was loaded onto the Sephacryl S-300 column. The proteinase K digestion was loaded onto the Mono Q column.
Figure 8 Construction of FLP-containing expression plasmid, pD3. (A). A 1.4 kb BglII fragment containing the FLP coding sequence was isolated from plasmid pGEM (Baltimore et al., 1988) and ligated into the BglII cloning site of pUC18 at the promoter. Two boxes are 5' and 3' to the FLP-coding sequence (filled arrow) respectively. N, BamHI linker; P, T7 promoter and first 15 codons from 43S rRNA and J, transcription terminators. (B). Plasmid pGEM was linearized with EcoRI and a double Nhel linker was ligated into the blunt-ended termini and the plasmid was religated. The resulting plasmid (pD3) had the T7 site replaced by an NheI site (open box) and the FLP coding sequence (open arrow). P, T7 promoter and first 15 codons from 43S rRNA; N, Nhel linker; A, 5' and 3' sequences from the FLP coding sequence. (C) A 1.4 kb BglII fragment from plasmid pGEM was inserted into pUC18 plasmid that had been digested with NheI. An NheI site is located between the promoter sequence and the first 15 codons from the T7 gene10. Therefore, the resulting plasmid pD3 had lost all extraneous codons upstream of FLP coding sequence. P, T7 promoter sequence; N, NheI site; T, transcription terminators.

Figure 9 SDS-PAGE of different fractions during purification. (A). Fractions from Sephacryl S-300 were stained with Coomassie Blue. All fractions were analysed by SDS-PAGE. Lane 1 contains protein standard markers with molecular masses (kb) indicated by the numbers. Lanes 2 to 7 show the protein present in the peak fractions. The amount of protein in each lane: lane 2, 2.4 µg; lane 3, 5.8 µg; lane 4, 6.6 µg; lane 5, 10.6 µg; lane 6, 10.5 µg; lane 7, 10.4 µg. (B). Fractions from FPLC Mono S column stained with Coomassie Blue. Lane 1 contains protein standard markers with molecular masses (kb) indicated by the numbers. Lanes 2 to 7 show the protein present in the peak fractions. The amount of protein in each lane: lane 2, 1.1 µg; lane 3, 1.0 µg; lane 4, 3.1 µg; lane 5, 3.2 µg; lane 6, 1.7 µg; lane 7, 1.5 µg. Lanes 8 to 10 show the purity of fractions by staining with Coomassie Blue (C) and silver (D). Lanes 1, 2, 3, 6, 7, 8, 9, fraction 16; lane 4, fraction 18; lane 5, fraction 20. Molecular weight markers are in lane 1. Arrows indicate the FLP protein.