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PURIFICATION OF FLP RECOMBINASE USING SEQUENCE-SPECIFIC DNA AFFINITY CHROMATOGRAPHY

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ABSTRACT FLP recombinase mediates site-specific recombination at two sites within the yeast 2 micron plasmid. The gene encoding FLP recombinase has been cloned and expressed in E. coli. The recombination site is well defined and consists of three 13 bp repeats with the first and second separated by an 8 bp spacer. The FLP recombinase cleaves at the boundaries of the spacer. The protein binds to all three repeats. Using this information, a sequence-specific DNA Sepharose resin was synthesized for affinity chromatography of the recombinase. The immobilized ligand consists of a duplex DNA polymer containing multiple 13 bp repeats ligated in a head-to-tail orientation. After ammonium sulfate fractionation, cation exhange chromatography, and non-specific DNA Sepharose chromatography, FLP recombinase was purified to 95% purity using the sequence-specific DNA Sepharose resin.

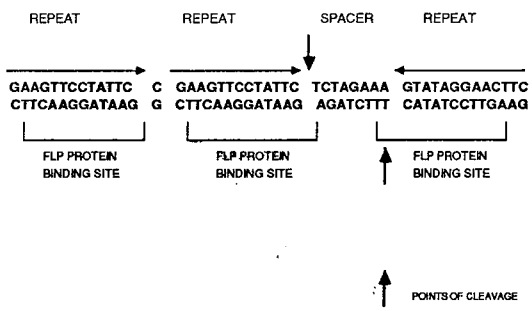
INTRODUCTION

The 2 micron plasmid is an autonomously replicating, 6318 base pair (bp) circular DNA molecule present in many strains of yeast. The plasmid encodes a site-specific recombination system that inverts unique sequences separating two 599 bp inverted repeats. The gene encoding the protein that mediates this recombination is designated FLP and is located on the 2 micron plasmid (3). This recombination event results in amplification of the plasmid copy number (1,2). The FLP system is relatively simple and provides an accessible model for studies of eukaryotic site-specific recombination.

The gene encoding FLP recombinase has been cloned and expressed in E. coli and is the only yeast protein required for recombination. Using partially purified protein, an in vitro assay was developed and used to demonstrate that the reaction promoted by FLP recombinase has very simple requirements (4,5). In vivo studies revealed that only 65 bp within the 599 bp repeat are required for recombination (6).

The prominent features of the FLP recombination site are three 13 bp repeats as illustrated in Figure 1. Two of the

Figure 1: FLP recombination site



repeats are inverted relative to one another and are separated by an 8 bp repeat containing an XbaI restriction site. A third repeat flanks the second repeat, separated from its neighbor by one base pair, and is in the same orientation as the neighboring sequence (6). FLP recombinase binds to all three repeats and cleaves at the boundaries of the spacer. However, the third repeat is not required for cleavage or recombination (7,8).

The recombination site used by FLP recombinase is well defined. However, little progress has been made in kinetic and physical analysis of the protein and its interaction with its substrate. This has been hampered, in part, by the lack of purified FLP recombinase. The most successful purification scheme yielded protein at only 50% purity (9) although this has since been revised to 5% purity (10). Since FLP protein is known to bind the third repeat, but leave the DNA uncleaved, this information was used to design an affinity resin in which the immobilized ligand consists of repeating units of the 13 bp repeat ligated in head-to-tail orientation. We now report purification of FLP

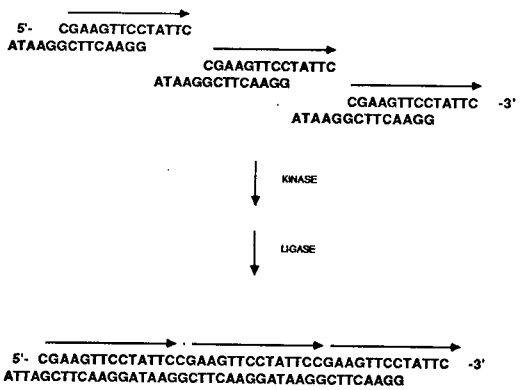
recombinase to 95% purity using this sequence-specific DNA affinity resin.

RESULTS

Design and Synthesis of Specific and Non-Specific DNA Sepharose Resins

An affinity ligand was constructed in which the 13 bp repeat was linked to a neighboring repeat and separated by a single base pair; this head-to-tail orientation is similar to that of the second and third repeats in the wild type recombination site. The construction of this ligand is illustrated in Figure 2, and details of the kinasing,

Figure 2: Design and construction of DNA affinity ligand



annealing, and ligation procedure are described elsewhere (11). The resulting polymer consisted of 5 to 20 repeating units of the 13 bp sequence. The duplex DNA ligand (5.7 mg) was coupled to cyanogen bromide (CNBr) activated Sepharose 4B (4.5 g dry weight, washed with 4 L 1 mM HCl and 1 L 10 mM sodium phosphate, pH 8.0) purchased from Pharmacia according to the procedure of Arndt-Jovin et al. (12). Approximately 2.3 mg of the DNA ligand was coupled to the resin. This was estimated by measuring the absorbance (at 260 nm (A_{260})) of the coupling solution and subsequent washes of the resin after 18 hours of reaction time at 25°C. This represents a 40% efficiency of coupling, with a concentration of 59 nmole of 13 bp site bound per gram of Sepharose. However, the

actual amount of site accessible to the recombinase is uncertain since duplex DNA binds to Sepharose with multi-point attachments (12). Thus, some of the ligand is unavailable to the protein for binding.

A non-specific DNA Sepharose resin was synthesized by coupling sonicated calf-thymus DNA to CNBr activated Sepharose 4B. The Sepharose (70 g, suction dried) was activated using the procedure of Kohn and Wilchek (13), and the DNA was immobilized on the resin using the procedure of Arndt-Jovin et al. (12). The amount of DNA immobilized on the resin was estimated by measuring the A_{260} of the coupling solution and subsequent washes. Approximately 0.45 mg of DNA was bound per gram of Sepharose, representing a 30% binding efficiency.

Purification of FLP Recombinase

The source of FLP recombinase was E. coli C600K$^-$ containing the plasmid pMMC20; the construction of this plasmid and growth of the bacteria are described in detail elsewhere (11). The early stages of purification were as follows: (1) lysis of the cells by sonication and subsequent high speed (150,000 x G) centrifugation; (2) ammonium sulfate fractionation; (3) removal of the ammonium sulfate from the sample using a desalting resin; and (4) separation of FLP recombinse activity from proteins and nucleic acids using a sodium chloride gradient on a cation exchange resin (BioRex 70 from BioRad). The details of these steps and the assay conditions of FLP recombinase activity are described elsewhere (11). Protein concentrations were determined using the method of Warburg and Christian (14).

All the following steps were carried out at 0 to 5°C. Protein in fractions containing FLP recombinase activity eluted from the cation exchange resin was precipitated by addition of ammonium sulfate to 80% saturation at 5°C. Ammonium sulfate was removed on a BioGel P6-DG column (100 ml bed volume) equilibrated in 5 column volumes of 25 mM N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid (HEPES) buffer, pH 7.0 at 5°C (14% anion), containing 1 mM EDTA and 0.1 M NaCl. This buffer henceforth will be referred to as Buffer H(0.1); the value of the number in parentheses will be varied according to the molar concentration of NaCl used at any given step. Fractions containing protein, as determined using a UV monitor with a set wavelength of 280 nm, were pooled and loaded onto the non-specific DNA

Sepharose column (70 ml bed volume) equilibrated in approximately 7 column volumes of Buffer H(0.1). The column was run using a peristaltic pump set to give a flow rate of 1 ml/min. After all of the sample was loaded the column was washed with 1 column volume of Buffer H(0.1). FLP recombinase activity was eluted with a step wash of Buffer H(0.35).

The protein sample containing activity eluted from the non-specific DNA Sepharose column was diluted with Buffer H(0) to give a sodium chloride concentration of 0.25 M as determined by conductivity measurements. This sample was then loaded onto the specific DNA Sepharose column (15 ml bed volume; equilibrated with 10 column volumes of Buffer H(0.25)). A peristaltic pump was used and set to give a flow rate of 1 ml/min. The protein sample was recycled over the resin at 1 ml/min for approximately 40 hours. Given that the volume of the sample load was 40 ml, the sample passed through the column at least 60 times during the 40 hour recycling. Next, the column was washed with 4 column volumes of Buffer H(0.4) to remove contaminating protein. FLP recombinsae activity ws then eluted with a step wash of Buffer H(1.0).

The protein concentration, units of activity, yield and purification factors for the above procedures are presented in TABLE 1. The protein components of the lysate, cation exchange chromatography pool, the non-specific DNA Sepharose 0.35 M NaCl step wash, and the specific DNA Sepharose 1.0 M NaCl step wash were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE); the results are shown in Figure 3, and details are given in the figure legend.

DISCUSSION

The procedure described above yields FLP recombinase at approximately 95% purity as judged by a densitometric scan of the SDS-PAGE gel. Recycling the protein sample over the specific DNA Sepharose column increased both purity and yield, possibly because the prolonged exposure of FLP recombinase to the resin allows the recombinase to displace non-specific DNA binding proteins from the sequence-specific DNA ligand. The entire procedure requires four to five days to complete. However, the recombinase activity appears to be stable after elution from the non-specific DNA Sepharose column. The recombinase eluted from the specific DNA

Sepharose column is stable with no loss of activity observed for 5 days at 5°C or several months at -70°C. FLP recombinase is reported here to be purified 127-fold. Similar purifications have resulted in 600 to 1000-fold purification, depending on the efficiency of cell lysis and the initial amount of ativity.

Major losses of activity are observed at two stages of the purification. Removal of ammonium sulfate from the

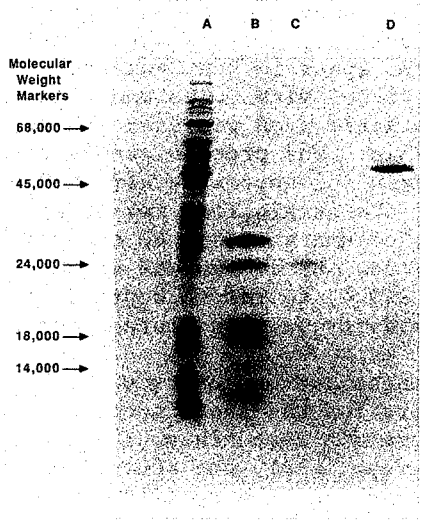


Figure 3. SDS polyacrylamide gel electrophoresis of FLP recombinase fractions

Protein in lanes A-D was denatured by heating to 100°C for 15 minutes in the presence of an SDS/reducing buffer before loading onto an SDS-polyacrylamide gel containing 11% crosslinking components in the separating gel and 6% in the stacking gel (21). The gel was stained, fixed, and destained according to published procedures (22). The molecular weight marker proteins (not shown) were bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000), beta-lactoglobulin (18,000), and lysozyme (14,000). Lane A: 50 micrograms of the crude lysate. Lane B: 30 micrograms of the protein eluted from the BioRex-70 resin. Lane C: 15 micrograms of protein from the Buffer H(0.35) wash of the non-specific DNA Sepharose column. Lane D: 21 micrograms of protein eluted from the sequence-

specific DNA Sepharose column with the Buffer H(1.0) wash; the major band with a MW of approximately 45,000 is FLP recombinase.

resuspended protein after the first ammonium sulfate fractionation (11) results in a large loss of activity. Similarly, activity is lost in the desalting step prior to the non-specific DNA Sepharose column. Use of a heparin-agarose column instead of the non-specific DNA Sepharose

Table I

Fraction	Total Protein (mg)	A(280)/A(260)	Total Units* (x1000)	Specific Activity (Units/mg x 1000)	Purification (x-fold)	Percent Recovery
Crude Extract	1670	0.5	8350	5	1	100
90% Ammonium Sulfate	960	0.5	4800	5	1	57
Biorex-70 Pool	73	1.3	1400	19	4	17
Non-Specific DNA-Sepharose Pool	19	1.3	400	21	4	5
Specific DNA-Sepharose Pool	0.63	1.3	400	63	127	5

*A unit of FLP recombiase activity is defined as the minimum amount of protein required to produce products in an agarose gel after a 60 minute reaction (see ref. 11)

resin results in improved yield of recombinase activity, but lower (85%) purity (11). Loading the protein sample eluted from the BioRex column directly onto the specific DNA Sepharose resin equilibrated in Buffer H(0.35) also results in a higher yield in activity, but with only 75 to 80% purity (15). Apparently, a step using non-specific DNA as a ligand prior to the specific DNA Sepharose column is a requirement for higher purity. Efforts are underway to improve the overall yield of pure recombinase using this information (15).

DNA binding proteins have been purified using two different strategies. The first was a procedure suggested by Alberts (17) in which the protein of interest is eluted from a non-specific cation exchange resin or a non-specific DNA affinity resin with short DNA fragments containing the binding site of the protein. This approach was used in an attempt to purify FLP recombinase. Although FLP recombinase

was purified to 99% homogeneity by elution from heparin-agarose with a DNA fragment containing half the spacer and one adjacent repeat, half of the protein was covalently bound to the eluting DNA fragment and thus inactive (16). This, coupled with the expense of the procedure, led to the search for an alternative approach.

The second strategy, affinity chromatography in which the ligand that specifically binds to an enzyme is immobilized to a support of some type, has long been used by protein chemists. Purification of some DNA binding proteins has been accomplished by using resins of non-specific DNA linked to cellulose matrices. However, the procedures used to immobilize the DNA to the cellulose matrix do not result in stable, covalent bonds (12). Furthermore, the resins do not specifically separate all DNA binding proteins. A number of DNA binding proteins, FLP recombinase among them, recognize and bind to specific sequences of DNA, much like a dehydrogenase binds to a pyridine nucleotide substrate, whether free in solution or immobilized on a resin. By covalently linking the DNA binding site to a Sepharose resin, an effective affinity resin may be synthesized which separates the protein of interest from other DNA binding proteins. This approach is cost effective since the affinity resin can be used for extended periods of time with little or no leakage of the ligand from the resin. The affinity resin designed and synthesized for the purification of FLP recombinase was successful in producing protein of high purity. While the work described herein was in progess, similar procedures using sequence-specific DNA affinity chromatography were published for the purifications of Nuclear Factor I (18) and for transcription factor SpI (19,20). The use of affinity resin with covalently linked DNA containing specific recognition sequences has the potential for purification of a variety of DNA binding proteins.

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