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Journal of Chromatography A, 677 (1994) 45–52

JOURNAL OF CHROMATOGRAPHY A

Novel DNA-Sepharose purification of the FadR transcription factor

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First received 14 February 1994

Abstract

A DNA sequence bound by the FadR transcription factor of *Escherichia coli* was covalently attached to Sepharose by two different approaches: by chemical coupling or by template-directed enzymatic synthesis using a DNA polymerase. The two kinds of DNA-Sepharose were packed into small columns and used for the purification of the FadR protein; chromatography was without using competitor DNA and the supports contained single-copy, non-repetitive DNA sequences. Comparison showed that the enzymatically prepared support, while having less bound DNA, bound more FadR protein than did the chemically prepared support. This probably results from the lack of detrimental DNA modification by the gentle enzymatic procedure. The chemically prepared support was of lower capacity but yielded purer FadR protein when compared under the same elution conditions. This may be explained by the simpler DNA sequence which could be coupled chemically; less contaminating proteins were bound by the simpler DNA sequence. However, the enzymatically prepared support could also yield comparable purity if the protocol was modified to include additional washes with salt containing buffers. In all cases, FadR was eluted from the DNA using high-salt (0.8 *M*) mobile phase; ligand-specific elution of FadR using a fatty acyl-coenzyme A thiol ester was ineffective. Affinity chromatography on DNA-Sepharose provided a more rapid, simple purification of FadR than conventional purification techniques and yielded biologically active protein.

1. Introduction

DNA-binding proteins serve roles as transcription factors, restriction endonucleases, DNA and RNA polymerases, and in DNA recombination and repair processes. Since many of these proteins are present in only relatively low amounts in cells, their purification can be challenging. However, due to the role of these proteins in cellular regulation, their purification and characterization is often of fundamental importance.

Affinity chromatography is a powerful protein purification technique. For DNA-binding proteins, the technique requires that DNA be stably linked to a solid support. Different strategies which have been used to produce DNA supports and their uses in purifying DNA-binding proteins have been reviewed [1,2]. DNA affinity chromatography has several advantages in the purification of DNA-binding proteins. Because affinity chromatography techniques usually give high purity and yield, they often result in simple purification schemes. In the case of DNA-binding proteins, the DNA sequence bound by the protein is also its most distinguishing feature; because of the lack of other unique characteris-

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0021-9673/94/$07.00
SSDI 0021-9673(94)00330-C

tics, classical purification techniques can only be applied empirically, while affinity chromatography using the DNA consensus binding sequence provides a rational approach.

One problem which has slowed the use of DNA affinity chromatography has involved the procedures used to couple DNA to supports. Either non-covalent attachment has been used with accompanying concerns about stability of the media to prolonged or harsh use or covalent attachment techniques which are known or suspected of modifying the DNA nucleotide bases must be used. Since these nucleotide bases provide the basis of the specific separation, their modification is a matter of concern. Recently, a new technique for producing DNA supports for affinity chromatography has been described [3]. The method involves chemically linking $(dT)_{18}$ by way of its 5′ end to a solid support using chemistry which does not modify thymidine bases. A template sequence containing a 3′ end poly(A) tail is then hybridized to the support. The template specified sequence is then copied enzymatically (using DNA polymerase) and covalently onto the 3′ end of the $(dT)_{18}$ support to make a new DNA support. Furthermore, the same recent study has shown that nucleotide bases in double helical DNA are less susceptible to at least one kind of coupling chemistry. Thus, chemical coupling of double-stranded DNA may minimize nucleotide modification over that which would occur with coupling of single strands.

Here, we extend these enzymatic methods to the synthesis of a DNA-Sepharose support which specifically binds the FadR transcription factor of *Escherichia coli*. FadR regulates many genes and operons required for fatty acid metabolism [4]. At least seven promoters whose protein products are required for growth on fatty acids as a sole carbon and energy source are negatively controlled by FadR while the *fabA* gene which is required for unsaturated fatty acid biosynthesis is activated by FadR [4,5]. FadR mediates repression and activation by binding to specific target sequences within the promoter of each of the FadR-responsive genes. DNA binding by FadR is specifically inhibited by long-chain acyl coenzyme A thiol esters. Of the FadR binding sites characterized to date that which occurs within the *fadB* promoter has the highest FadR binding affinity. This site, termed O_B, is 5′-ATCTGGTACGACCAGAT-3′ [6]. In this report, we demonstrate that FadR binds specifically and with high affinity to O_B DNA-Sepharose support prepared by either chemical or enzymatic methods. However, the DNA-Sepharoses had different properties depending upon the synthesis method used. Elution of FadR from the O_B DNA-Sepharose column resulted in a homogeneous protein fraction which retained DNA-binding activity.

2. Methods

2.1. DNA-Sepharose preparation

For FadR purification two different approaches were used. One uses $(dT)_{18}$-Sepharose and a poly(dA) tailed template with DNA polymerase to copy the template specified sequence onto the 3′ end of $(dT)_{18}$ enzymatically using procedures described by Solomon *et al.* [3] for DNA-silica. The other uses a double-stranded DNA in which one strand contains a 5′ amino group. This double-stranded DNA is then coupled to CNBr-activated Sepharose.

2.2. Enzymatic synthesis

$(dT)_{18}$-Sepharose was prepared by a modification of the method of Arnt-Jovin *et al.* [7]. A 10-g amount (wet mass) of Sepharose 4B was washed thoroughly with water; 2 g cyanogen bromide were added while stirring, and the mixture was maintained at pH 11 by the addition of 5 *M* NaOH until reaction slowed (*ca.* 15 min). The activated Sepharose was then rapidly washed under vacuum on a coarse-sintered glass funnel with 100 ml of ice-cold water and then with 100 ml of cold 0.1 *M* boric acid–NaOH, pH 8. After reconstitution to 20 ml with this last buffer, 200 nmol of 5′-amino-$(dT)_{18}$, prepared with the Amino Link reagent as previously described [8] was added and the mixture was stirred overnight. The next day 0.5 *M* glycine in

the borate buffer was added and stirring was continued for 24 h. The resulting $(dT)_{18}$-Sepharose was thoroughly washed and stored at 4°C in TE (10 m*M* Tris, 1 m*M* EDTA, pH 7.5) containing 10 m*M* NaN_3. The $(dT)_{18}$ content, measured by spectroscopy from the difference of the $(dT)_{18}$ added and that which did not couple, was 11.5 nmol/g Sepharose.

A 2-g amount of the $(dT)_{18}$-Sepharose was washed three times with 2 ml of buffer E [50 m*M* Tris, 150 m*M* NaCl, 10 m*M* $MgSO_4$, 0.1 m*M* dithiothreitol (DTT), 5 μg/ml bovine serum albumin (BSA), pH 7.4]. Then, 31 nmol of O_B-$(dA)_{18}$ oligonucleotide (5′-CGACTCATCTGGTACGACCAGATCACCTAA-$(dA)_{18}$) in 2 ml of buffer E was added to the resin and incubated at 65°C for 5 min. After cooling for 15 min, the resin was centrifuged and unbound oligonucleotide was removed. The resin was washed 3 times with 1 ml of buffer E containing 1 m*M* of each deoxyribose nucleotide triphosphates (dNTP) and then 50 units of *E. coli* DNA polymerase I, Klenow large fragment was added, and the mixture was incubated at 37°C for 2 h. Control experiments in which 5′-^{32}P end-labeled oligonucleotide O_B-$(dA)_{18}$ was used demonstrated that under these conditions, 1.6 nmol of double-stranded DNA were produced per g. of Sepharose. The DNA-Sepharose was then washed thoroughly in buffer C (10 m*M* Tris, 1 m*M* EDTA, 100 m*M* NaCl, 0.1 m*M* DTT, 10 m*M* NaN_3) and stored at 4°C until needed.

2.3. Chemical synthesis

Two complementary oligonucleotides, 5′-NH_2-et-O_B (5′-NH_2-et-CGACTCATCTGGTACGACCAGATCACCTAA) and αO_B (5′-TTAGGTGATCTGGTCGTACCAGATGAGTCG) were used where NH_2-et denotes the aminoethyl added at the 5′ end using the Amino Link reagent. A double-stranded DNA was made by mixing 30 nmol of each in 0.3 ml of TE containing 0.3 *M* sodium acetate, heating to 65°C, and cooling slowly to room temperature. Three volumes of ethanol were added and the precipitated double-stranded oligonucleotide was collected by centrifugation and dried. The DNA was then dissolved in 2.5 ml of the borate buffer and added to 2.5 g (wet mass) CNBr-activated Sepharose, prepared as described above. The mixture was stirred overnight. The next day, the DNA-Sepharose was washed with the borate buffer and with 0.5 *M* glycine in the same buffer. The amount of DNA coupled, 10 nmol per g of Sepharose, was determined by spectroscopy of the uncoupled DNA. Stirring was continued in 15 ml of the glycine–borate buffer for 24 h. The DNA-Sepharose was then washed thoroughly in buffer C and stored at 4°C until needed.

2.4. Production of FadR protein

High-level expression of FadR was induced from BL21(λDE3) carrying plasmids pCD129 and pLysS as previously described [6]. Approximately 2.5 g of cell paste were suspended in 50 ml buffer containing 20 m*M* Tris pH 7.5, 100 m*M* NaCl, 1 m*M* EDTA and 1 m*M* DTT. The cells were lysed by sonication. Unlysed cells and cellular debris were removed by centrifugation at 23 000 rpm in a Beckman SW28 rotor. Ammonium sulfate was added to the supernatant to a final concentration of 60% of saturation. The sample was incubated at 4°C overnight. Precipitated proteins were sedimented by centrifugation at 10 000 rpm in a Beckman JA21 rotor and then resuspended in buffer D (10 m*M* Tris, 1 m*M* EDTA, 0.1 m*M* DTT, pH 7.5), dialyzed against the same, and finally glycerol was added to a final concentration of 50%. This crude preparation of FadR was stored at −85°C until needed.

For comparison, FadR was also purified by using DEAE-cellulose and CM- Bio-Gel A resins as previously detailed [6].

2.5. Electrophoretic mobility shift assays

Activity of FadR in the final preparations was measured using the gel shift assay as previously detailed [6]. The DNA fragment used for these experiments was the 377 base pair HindIII-EcoRI fragment isolated from pCD154 [6]. This fragment carries a portion of the *fadB* gene containing the promoter for the *fadBA* operon

and O_B. The DNA was labeled with [^{32}P]dATP and the Klenow fragment of *E. coli* DNA polymerase I.

3. Results

The chemistries used to couple DNA to chromatography supports can modify nucleotide bases. Naturally occurring and synthetic DNAs all couple to CNBr-activated Sepharose, presumably via reaction with the amino moiety of the adenine, cytosine and guanine bases [1,2,7]. Since the affinity chromatography of DNA-binding proteins relies upon interactions with these nucleotide bases for its specificity, techniques which avoid base modification are preferable.

Previously, we have shown two feasible ways of avoiding this modification. These approaches are diagrammed in Fig. 1. One way is to enzymatically copy DNA sequences onto a support using a template DNA and DNA polymerase. Using this procedure, no modification of the DNA attached to the support is anticipated. The other approach provides an alkylamino moiety on the DNA chain as a preferred site for coupling and utilizes the observation that nucleotide bases are most resistant to modification in double-stranded DNA [3]. While the nucleotide bases are less reactive under these conditions and reaction with the aminoethyl group should predominate, some reaction (coupling) with nucleotide bases would also be expected. Here, we have compared these two basic strategies to prepare columns for purification of the transcription factor FadR.

DNA-Sepharose was prepared enzymatically using the Klenow large fragment of *E. coli* DNA polymerase I. In this procedure, a $(dT)_{18}$-Sepharose is prepared using 5′-aminoethyl-$(dT)_{18}$ and CNBr-activated Sepharose. Since thymidine bases contain no chemical groups which should be capable of coupling, coupling is expected to occur via the 5′-aminoethyl moiety. The resulting $(dT)_{18}$-Sepharose has a 3′-hydroxyl and can be used to prime template-directed DNA synthesis by DNA polymerases. To prepare the Sepharose needed for FadR protein purification, a template containing the desired complementary sequence and also containing a 3′-$(dA)_{18}$ tail is hybridized to $(dT)_{18}$-Sepharose and Klenow large fragment added. The result is that the complement of the template sequence is copied covalently onto the 3′-OH end of the $(dT)_{18}$ and the template remains on the column by hybridization with the newly produced sequence. Control experiments have demonstrated that when Klenow large fragment is added, the template-

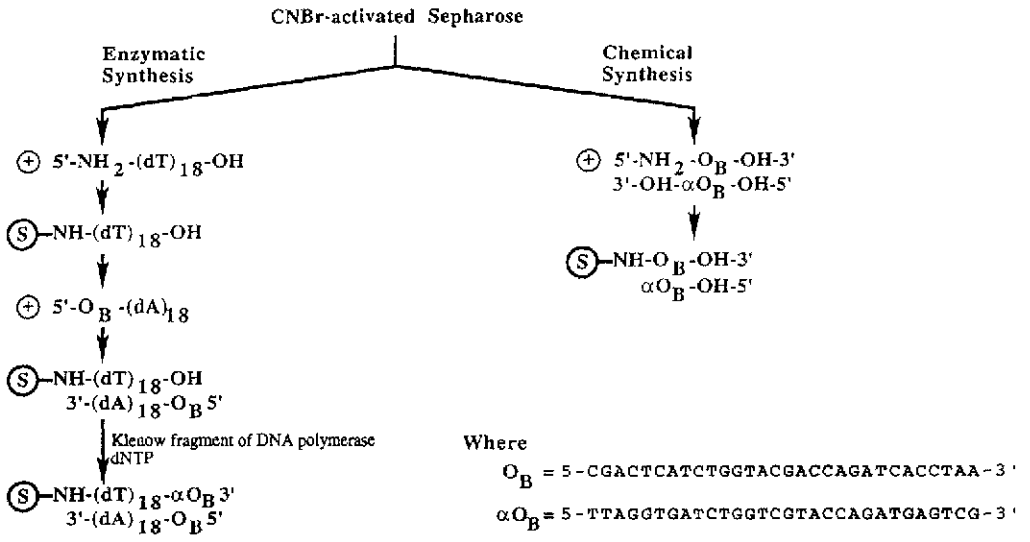

Fig. 1. Schematic presentation of the chemical and enzymatic procedures used to prepare DNA-Sepharose.

directed sequence is indeed copied onto the Sepharose and can be detected by the increase in melting temperature found for the longer DNA hybrid present after synthesis. This high-melting hybrid is not observed when the Klenow large fragment is omitted (data not shown). These procedures were originally described for DNA-silica [3] and have been adapted here to Sepharose.

To prepare a similar DNA-Sepharose chemically, two complementary oligonucleotide strands were prepared, one of which contained an aminoethyl group at its 5′ end to provide a site for coupling to CNBr-activated Sepharose. The two strands were then hybridized and coupled to the support. The enzymatically and chemically prepared supports were then compared in the binding of FadR. To make comparison easier, a 15-nmol amount of DNA was used per gram of Sepharose for both procedures and all chromatographic comparisons were made between two columns of equivalent size, to which were applied equal quantities of the same extract of FadR eluted using the same conditions.

The amount of FadR specifically eluted from DNA-Sepharose prepared by the two approaches was not equivalent as shown in Fig. 2. In this figure is shown the elution profile obtained for equivalent amounts (9 mg) of a crude FadR protein preparation from the two kinds of columns of the same size (1 ml) prepared from equal amounts of DNA and run in parallel. Both columns were then washed thoroughly with buffer D and then washed with 0.2 and 0.8 *M* NaCl to elute the FadR protein. At 0.2 *M* NaCl, most of the contaminating proteins elute from the column along with a small amount of FadR protein. Most of the FadR protein however elutes in a highly purified state with 0.8 *M* NaCl. Fig. 2 shows that the column prepared by enzymatic synthesis bound and eluted about four-fold more of the FadR protein based upon the peak in absorption which elutes at higher salt. Additional experiments confirmed that higher yields of FadR were obtained from O_B-Sepharose prepared enzymatically. When the eluted protein obtained from the two columns was

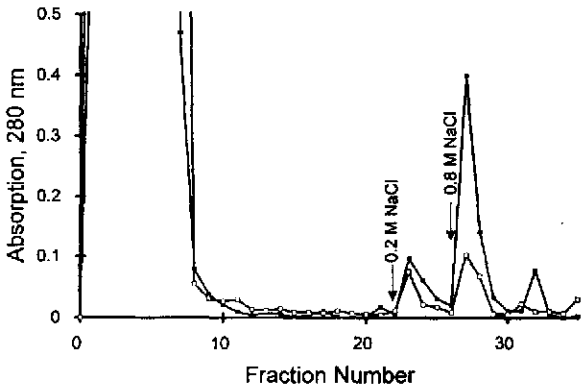

Fig. 2. The enzymatically prepared DNA-Sepharose column has higher capacity for FadR. Two 1-ml bed volume columns were prepared from DNA-Sepharose prepared by either the enzymatic procedure (■) or the chemical procedure (□). These columns contained 1.6 and 11.5 nmol of the O_B-DNA sequence, respectively. A 5-ml volume of crude, frozen FadR protein in buffer C containing 9 mg protein was applied to each column. The columns were then washed with 20 ml of buffer C (pH 7.5) and then eluted with 5-ml portions of TE containing either 0.2 or 0.8 *M* NaCl as shown in the figure. Fractions were 1.2 ml. Acrylamide gel electrophoresis demonstrates that the bulk of the FadR protein elutes with 0.8 *M* NaCl from either column (see Fig. 3).

analyzed by acrylamide gel electrophoresis, the amount of FadR protein observed on the gels was estimated to be at least three-fold higher for the enzymatic column eluted protein (data not shown). These results cannot be due to the amounts of DNA coupled to the columns. Even though the same amounts of DNA were used in column preparation, enzymatic synthesis was less efficient and resulted in about one-sixth as much attached DNA as the chemically prepared column (1.6 *versus* 11.5 nmol per gram, respectively). These results suggest that enzymatic synthesis results in columns in which more of the DNA is suitable for binding by the FadR protein, presumably because chemical coupling has rendered some of the DNA unsuitable for affinity binding by the protein.

Fig. 3 shows acrylamide gels of the various fractions obtained from the chromatography on the 1 ml O_B-DNA-Sepharose columns. In Fig. 3A, the fractions from the enzymatically prepared column are shown while in Fig. 3B results with the chemically prepared column are presented. In either case, the same amount of crude

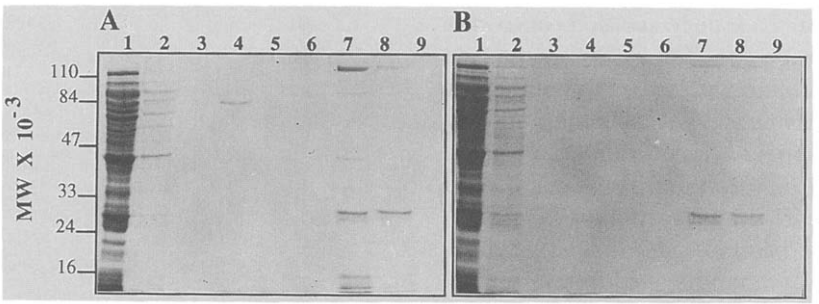

Fig. 3. Chemically synthesized DNA-Sepharose yielded higher-purity FadR. Volumes of 12.5 μl of the column fractions from Fig. 3 were diluted with sodium dodecyl sulfate (SDS) sample buffer and applied to 12% acrylamide gels [9]. Wells: 1 = crude FadR protein before chromatography; 2–9 = fraction numbers 5, 22, 23 (peak at 0.2 *M* NaCl), 25, 26, 27 (peak at 0.8 *M* NaCl), 28 and 29, respectively, from the elution profiles shown in Fig. 2. (A) Fractions from the enzymatically synthesized support; (B) for the chemical support.

FadR protein was loaded onto the column. In this figure, a volume representing about 1% of the total fraction volume was loaded onto the acrylamide gel; since this amount contained less protein in Fig. 3B, the contrast of the photograph was adjusted so that the FadR protein band is clearly visible. The results show that while the chemically prepared column binds less FadR protein (*i.e.*, about four-fold less in the experiment in Fig. 2), the protein eluted was of higher purity. This may be because of the simpler sequence of the column DNA since the chemical coupling procedure does not have or require the $(dT)_{18}$:$(dA)_{18}$ hybrid sequences. The enzymatically prepared column binds more FadR protein and also binds some other proteins as well. The higher capacity probably results from the lack of chemical modification of the DNA nucleotide bases.

Fig. 4 shows that greater purity can be obtained from the enzymatically prepared column by washing the column with intermediate salt concentrations. When the column is washed at 0.2, 0.4 and 0.6 *M* NaCl, contaminating proteins are eluted from the column along with some of the bound FadR. After washing, elution at 0.8 *M* NaCl results in highly purified FadR protein. No further protein elutes at 1.0 *M* NaCl. However, elution with this more complex NaCl step gradient, while increasing the purity of the protein obtained at 0.8 *M* NaCl, results in lower recovery of the purified protein.

The results presented in Fig. 4 suggested that FadR could be purified on the enzymatically prepared column by applying the crude protein, washing at 0.2 and 0.4 *M* NaCl, and collecting the purified protein which elutes with 0.8 *M* NaCl. This is the procedure we currently use and it yields FadR protein of sufficient purity for most purposes. The capacity of the enzymatically prepared O_B-DNA-Sepharose for FadR under these conditions averaged 113 μg FadR protein per ml of DNA-Sepharose (standard deviation 21 μg/ml, $n = 2$). Thus, relatively small columns in

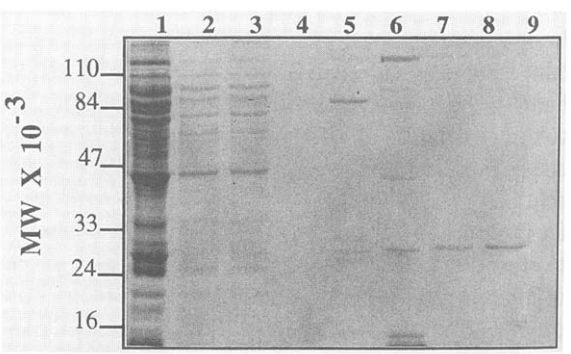

Fig. 4. The effect of different concentrations of NaCl on the elution of FadR. Crude FadR protein was loaded onto the enzymatically prepared DNA-Sepharose column, the column was washed with different buffers, and the fractions obtained examined on 12% SDS-polyacrylamide gel electrophoresis. Wells: 1 = crude FadR before chromatography; 2 = an early flow through fraction; 3 = a late flow through fraction; 4 = a late wash fraction after the column had been washed to baseline with buffer C; 5–9 = the column was washed with 0.2, 0.4, 0.6, 0.8 and 1.0 *M* NaCl made up in TE buffer. MW = Molecular mass.

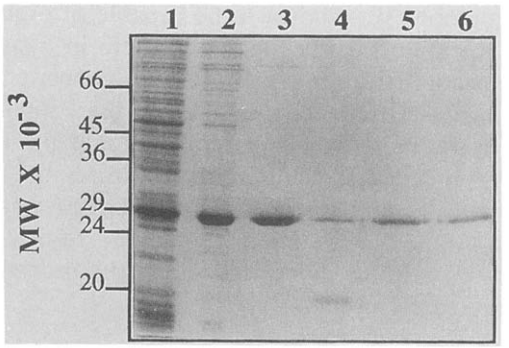

Fig. 5. Comparison of FadR purified by ion-exchange and O_B-DNA-Sepharose chromatography. A 15% SDS-polyacrylamide gel was used to monitor FadR during purification. Lanes: 1 = ammonium sulfate precipitate after dialysis (*i.e.*, crude FadR); 2 = partially purified FadR after DEAE-cellulose chromatography; 3 = FadR after CM-Bio-Gel A chromatography; 4 = FadR after O_B-DNA-Sepharose chromatography and elution with 0.4 *M* NaCl; 5 = 0.6 *M* NaCl; 6 = 0.8 *M* NaCl.

the range of 10 ml can purify mg amounts of FadR in a single chromatographic step.

The purity and activity of FadR purified using the O_B-DNA-Sepharose was compared to FadR purified by chromatography on sequential DEAE-cellulose and CM-Bio-Gel A columns. Fig. 5, presents a representative polyacrylamide gel on which FadR purified by each method is displayed. It was noted that a high-molecular-mass protein generally observed after purification by ion-exchange chromatography was not visible in protein samples purified using O_B-DNA-Sepharose. FadR purified by each method showed comparable DNA binding affinity when tested using the standard protein-DNA gel mobility shift assay (Fig. 6). Therefore, we conclude that FadR obtained by either purification procedure is of comparable activity. Since the DNA-Sepharose method is simpler and more rapidly performed, it is to be preferred.

4. Discussion

DNA affinity chromatography is usually performed while including a "competitor" DNA (*e.g.*, plpC oligomers) in the mobile phase during chromatography to diminish "non-specific" binding of proteins other than the one of interest to the column (for reviews, see refs. 1 and 2). No competitor DNA was used in the present studies since this could obscure differences between the columns. It was also found to be unnecessary; specific binding and elution of FadR could be obtained in its absence. Other affinity chromatography protocols have involved columns made from DNA concatemers containing multiple copies of the DNA target sequence [2]. This was also not done in the present study because such complex sequences may also bind other proteins as some of our results suggest (see below). By using simple sequences, sequence complexity effects can be minimized, the DNA is easier to synthesize, and the shorter sequences

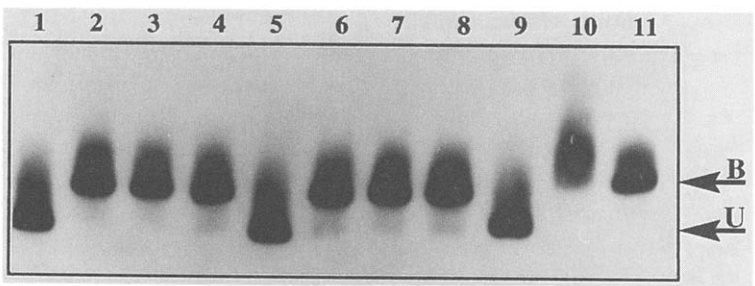

Fig. 6. Comparison of FadR-specific DNA binding activity after purification by ion-exchange and O_B-DNA-Sepharose chromatography. Electrophoretic mobility shift assays were performed as previously described [6]. U denotes migration of DNA which is unbound and B denotes migration of the labeled DNA bound by protein. Reaction mixtures contained $1 \cdot 10^{-12}$ *M* [^{32}P]*fadB* DNA alone for lanes 1, 5 and 9; for lanes 2–4, 10^{-7}, 10^{-8} and 10^{-9} *M* FadR protein, respectively, prepared using O_B-DNA-Sepharose synthesized by the enzymatic method; for lanes 6–8, 10^{-7}, 10^{-8} and 10^{-9} *M* FadR prepared using O_B-DNA-Sepharose synthesized by the chemical method; and for lanes 10 and 11, 10^{-8} and 10^{-9} *M* FadR respectively, prepared using ion-exchange chromatography.

can be used in higher molar amounts. Concatemeric sequences were also not found to be necessary for the efficient binding and chromatography of FadR.

Comparison of an enzymatic and a chemical procedure for preparing a DNA-Sepharose for the affinity chromatographic purification of FadR revealed certain advantages for each approach. The chemical procedure is simpler, yields purer FadR when used in a single-step purification, but in lower yield. The enzymatically prepared column has a higher capacity for FadR, yields larger amounts of FadR under comparable conditions but the protein obtained is of lower purity. These results cannot be explained by differences in the amount of O_B-DNA sequence on the column. The enzymatically prepared column contains less coupled DNA but binds greater amounts of FadR presumably because all of the DNA is in a native, unmodified form. However, the enzymatic procedure also requires the use of more complex DNA sequences because of the requirements that a template be hybridized prior to enzymatic copying. This more complex sequence probably accounts for the lower purity of FadR obtained since the contaminants may bind by association to these extra DNA sequences. The use of selective washing with intermediate salt concentrations removes these impurities (Fig. 4) but reduces yield.

However, these considerations about the best way to synthesize DNA-Sepharose should not detract from the clear advantages this affinity chromatography has over more conventional purification procedures. DNA-Sepharose chromatography was shown to be capable of producing apparently homogeneous protein in a single purification step and gave results of purity and biological activity comparable to that obtained by much more laborious conventional purification procedures. FadR purified using the enzymatically prepared column and 0.4 *M* washes with 0.8 *M* NaCl elution has yielded sufficiently pure protein for most uses and is the procedure we currently use.

Acknowledgements

This work was supported by the National Institutes of Health, grants GM 43609 and GM 38104. We thank Tamara Heimert for excellent technical assistance.

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