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Oligonucleotide trapping method for purification of transcription factors

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Abstract

A new oligonucleotide trapping method in which a decameric oligonucleotide $(AC)_5$ coupled to Sepharose is used to trap a complex of a transcription factor and its corresponding specific DNA element is described. The concentration of DNA element used in the trapping method was very low (50 n*M*) and hence discouraged binding of nonspecific proteins. We have shown that this method gives higher purity for green fluorescent protein CAAT enhancer binding chimeric protein (GFP-C/EBP) than the biotin–avidin method. We have also shown that the oligonucleotide trapping method has a capacity close to 95% of the theoretical capacity, which is significantly greater than the 15% capacity obtained with conventional DNA affinity columns. The purity of GFP-C/EBP obtained using a low concentration of the oligonucleotide in our trapping method is three-fold higher (3668- versus 1028-fold) than that obtained by conventional DNA affinity chromatography and the yield was also higher (36% versus 24%). Highly purified transcription factor B3 is obtained from *Xenopus* egg crude extract using the oligonucleotide trapping method as the only purification.
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1. Introduction

DNA affinity chromatography is one of the most widely used techniques for the purification of transcription factors and other DNA binding proteins [1–3]. Either heterogeneous DNA or homogeneous, specific DNA sequence affinity columns are used for these purifications. Heterogeneous columns were the first DNA affinity columns to be made and were made by coupling diverse DNA sequences, such as fragmented salmon sperm DNA, to cellulose [4]. Since then, there have been several advances in the technique. Highly specific columns made by using the footprint region, the region of DNA that is protected from cleavage upon binding of a specific DNA binding protein, have replaced heterogeneous columns for most transcription factor purifications. Various supports such as Sepharose, cellulose and silica are routinely used for coupling of DNA and several coupling chemistries are available for attaching DNA to these supports [5]. The most commonly used method is coupling of the amino groups either inherently present in DNA or introduced during oligonucleotide synthesis to cyanogen bromide-activated Sepharose [6].

Chemical coupling of the oligonucleotide can

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cause modifications of nucleotides within the DNA sequence. Such modifications can potentially affect the specific DNA–protein interaction, which in turn may lead to decreased efficiency of the DNA affinity columns in protein purification. Modification of the nucleotide bases can also lead to decreased capacity of the column for its specific protein. There are only a few methods that allow the use of DNA sequences that are not chemically coupled. The enzymatic synthesis method, which was developed previously in our laboratory [7], involves synthesis of an unmodified DNA sequence on the column by using the Klenow fragment of DNA polymerase. In another method, the highly specific biotin streptavidin interaction is used to trap DNA protein complexes [8,9]. In this method a biotinylated oligonucleotide is immobilized on a streptavidin-containing support that is then used for the affinity chromatography of DNA binding proteins. Streptavidin-coated magnetic beads are commonly used as the support for coupling of biotinylated oligonucleotide as they can be easily separated from solution with a strong magnet. In some cases a biotinylated oligonucleotide is allowed to interact with proteins in solution and the protein–DNA complex is then trapped onto a streptavidin-containing support [8,10]. In a third method, specific DNA affinity columns are made by immobilizing a footprint region containing a 3′ polyA tail onto a polyT-agarose column [11]. These columns are then used to purify DNA binding proteins. Other methods that allow coupling of unmodified DNA include direct oligonucleotide synthesis on a Teflon fiber support [12] and coupling of thiophosphorylated oligonucleotide to bromoacetyl agarose [13], but these are not routinely used for making DNA affinity columns.

Most of the methods mentioned above have certain disadvantages. The enzymatic synthesis method can lead to tail length heterogeneity of the column DNA. The heterogeneity of these columns could cause peak broadening and thus decrease the purity and yield of the protein obtained. Comparative studies between enzymatically and chemically synthesized columns have shown that there is no distinct advantage in using the enzymatic synthesis method for transcription factor purification, at least as regards the purity obtainable [14]. The streptavidin supports have a high affinity for biotinylated oligonucleotides, but such supports can also bind to several other proteins in the crude extract, especially proteins containing the biocytin group. These proteins can potentially coelute with the protein of interest and decrease purity. Newer forms of avidin, such as monomeric avidin and NeutrAvidin, show lower nonspecific interactions, but are so far untested in DNA affinity chromatography.

We have used the green fluorescent protein CAAT/enhancer binding protein chimera (GFP-C/EBP) to study a new method, which we call the oligonucleotide trapping method. CAAT enhancer binding protein (C/EBP) regulates expression of genes in mammals and certain viruses [15]. C/EBP is one of the most studied transcription factors and the DNA sequence that is bound by C/EBP has also been well characterized [16]. We have shown previously that GFP-C/EBP has DNA binding properties similar to that of C/EBP [17]. We have also applied the trapping method to purify B3, which is a developmentally regulated transcription factor and regulates TFIIIA transcription in early *Xenopus* oocytes [18]

In the oligonucleotide trapping method described in this paper, we have used the highly specific interaction between complementary strands of DNA to trap the protein–DNA complex. Similar techniques have been used to purify mRNA from crude mixtures [19] and to make specific DNA affinity columns as discussed earlier [11]. In our method a column-attached $(AC)_5$ oligonucleotide is used to trap from solution a footprint region which has a $(TG)_5$ tail on both strands. The interaction between a specific protein and its footprint element is carried out in solution and the protein–DNA complex is passed over $(AC)_5$–Sepharose. The latter is able to trap the protein–DNA complex because of highly specific annealing of $(AC)_5$ and its complement $(GT)_5$. The protein alone can then be eluted by using buffer containing high salt to weaken the protein–DNA interaction or the intact DNA–protein complex can be eluted by using moderate temperatures to melt the interaction between $(AC)_5$ and $(GT)_5$. We have shown that using this approach gives higher purity than the biotin–avidin method.

We also discuss a theory for why the trapping method works so well. In conventional DNA affinity chromatography the concentration of DNA that is

coupled is very high. While high DNA concentrations probably contribute to high yields, having a high DNA concentration is a major disadvantage. Many proteins bind to DNA with low affinity and would bind to the columns because of these high DNA concentrations, contaminating the protein of interest. Trapping allows binding to occur at quite low DNA concentrations while using sufficient DNA amounts to preserve yield.

2. Methods

2.1. Coupling of DNA to Sepharose

All the oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). EP24 (NH_2-GCTGCAGATTGCGCAATCTGCAGC), $(AC)_5$ (NH_2-ACACACACAC) and E3 (5′-NH_2-TGTGGTTACTAGGTTACAAATTACCCTAGCAACCATG) were coupled to CNBr-preactivated Sepharose 4B (Sigma, St. Louis, MO, USA). Coupling and end capping were carried out according to the protocol provided by the manufacturer. The E3 column was made double stranded by adding the corresponding complementary strand αE3 (5′-CATGGTTGCTAGGGTAATTTGTAACCTAGTAACCACA). The mixture was then heated to 95 °C and allowed to cool slowly to room temperature. EP24 is self-complementary and does not require the addition of a complementary strand. The amount of DNA coupled was determined by the difference in the UV absorption of DNA added and recovered after coupling. Approximately 20 nmol of both EP24 and $(AC)_5$ oligonucleotides and 36 nmol of E3 were coupled per gram of Sepharose.

2.2. Production of proteins

GFP-C/EBP was produced in *Escherichia coli* strain BL21 containing plasmid pJ22-GFP-C/EBP as described previously [17].

Xenopus laevis oocyte extract used for B3 purification was made from stage I–II *Xenopus* oocytes as described in Ref. [18].

2.3. Chromatography

All supports were packed in 1 ml bed volume syringe columns (obtained from Alltech, Deerfield, IL, USA) initially equilibrated in TE0.4 buffer (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 0.4 *M* NaCl). Details of elution and the gradient used are given in the figure legends.

For the oligonucleotide trapping method EP24$(TG)_5$ (GCTGCAGATTGCGCAATCTGCAGCGTGTGTGTGT), Bi-EP24 (Bi-GCTGCAGATTGCGCAATCTGCAGC), where ''Bi'' represents biotin introduced during oligonucleotide synthesis, or E3$(TG)_5$/αE3$(TG)_5$ (5′-NH_2-TGTGGTTACTAGGTTACAAATTACCCTAGCAACCATGTGTGTGTGTG/5′-CATGGTTGCTAGGGTAATTTGTAACCTAGTAACCACATGTGTGTGTG) were incubated with either purified or crude GFP-C/EBP or *Xenopus* oocyte extract and passed over the appropriate column as specified in the figure legends. Heparin, salmon sperm DNA (both obtained from Sigma), T_{18}(TTTTTTTTTTTTTTTTTT), and μE3 (TGTAACAACTAAACAACAAATTGTTCTAGCTGTTAATGCATTG/ACATTGTTGATTTGTTGTTAATCAAGATCCACAATACGTAAC) were used in some experiments as competitors. The details are given in the figure legends. NeutrAvidin and monomeric avidin–agarose were obtained from Pierce (Rockford, IL, USA) and packed in 1 ml syringe columns.

2.4. Assay of GFP-C/EBP

GFP-C/EBP was assayed fluorometrically as described previously [17].

2.5. Protein assay

Protein concentrations were determined by the Bradford method [20] using bovine serum albumin as the standard.

2.6. Polyacrylamide gel electrophoresis

All the samples were concentrated using Centriplus centrifugal filter devices supplied by Millipore (Bedford, MA, USA). One-fourth of each sample was applied to a sodium dodecylsulfate poly-

acrylamide 4–15% Bio-Rad precast gradient gel using the method of Laemmli [21] and stained with silver using the Bio-Rad Labs. kit (Richmond, CA, USA).

2.7. Western blot analysis

Gels were electro-blotted onto nitrocellulose filters as described [22]. A 1:5000 dilution of rabbit polyserum generated against purified B3 (HTI Bio-products) was used as a primary antibody for detection of B3. Immunoreactive proteins were visualized by using 1:3000 diluted rabbit secondary antibody–alkaline phosphatase or –horseradish peroxidase conjugates and stained by using nitroblue tetrazolium or the enhanced chemiluminescence (ECL) method, respectively. For ECL, solution A (15 ml of 0.4 m*M* coumaric acid, 2.5 m*M* luminol, 0.1 *M* Tris–HCl, pH 8.5), and solution B (7.2 μl of 30% H_2O_2 in 15 ml of 0.1 *M* Tris–HCl, pH 8.5) were mixed in the dark room. The membranes were soaked in this mixture for 3–5 min and dried by blotting with Whatman filter paper. The blots were then exposed for various time intervals to X-ray films (Kodak) and developed using an automatic developer.

2.8. Mathematical apparatus

To understand the effect of DNA concentration on transcription factor binding, we constructed a model in Microsoft Excel. In this model, 100 ml of a solution containing 1 pmol each of two proteins is assumed. One protein is considered to bind the DNA used specifically with a dissociation constant (K_d) of $4\cdot10^{-12}$ *M*. The other protein is assumed to not have specific binding for this DNA, but to be able to bind it nonspecifically with a K_d of $4\cdot10^{-5}$ *M*. These values were chosen as representative of the binding of lac repressor to specific (operator) and nonspecific sequences [23,24]. To model typical affinity chromatography, 20 nmol of DNA is considered to be coupled to a 1 ml column (i.e., [DNA]=20 μ*M*), accurately reflecting the amounts of DNA present in the columns used here. For trapping, 50 nmol DNA is added to the 100 ml of protein (0.5 μ*M*) and then recovered quantitatively on a 1 ml trapping column. Since DNA exceeds protein by a large amount (50 nmol versus 1 pmol), the concentration of DNA was assumed to be free (uncomplexed). However, in the case of trapping, the amount of DNA on the column changes as the sample passes through the column and the DNA is trapped. The model calculates how much DNA is on the column as each milliliter flows through and is trapped and this is the concentration of DNA (free) used in these binding calculations. The concentration of protein free (not complexed to DNA) can be quite small under these conditions and so its conservation was incorporated into the equation used. Binding to DNA is treated as a simple binding isotherm, which under these restrictions is

$$[\text{DNA–Protein}] = \frac{[\text{DNA}]_f[\text{Protein}]_0}{K_d + [\text{DNA}]_f}$$

where $[DNA]_f$ is the total concentration of DNA in the column at that point in the chromatography, $[Protein]_0$ is the total concentration of protein (10 p*M*), [DNA–Protein] is the amount of the complex present on the column, and K_d is the relevant dissociation constant ($4\cdot10^{-12}$ or $4\cdot10^{-5}$ *M*). The binding isotherm is solved for each milliliter of sample as it is applied to the column, for both trapping and non-trapping, and is solved for each of the two proteins to measure recovery. Whether the DNA is coupled or trapped, the model shows that essentially all of the specific-binding protein will be retained by the column (data not shown). However, the results for the nonspecific binding are quite different for the two approaches and are presented in Fig. 5.

3. Results

Fig. 1 shows a schematic of the oligonucleotide trapping method. In this method, a footprint region, symbolized as NNNNNN, bound specifically by the protein of interest, is extended with a single-stranded $(TG)_5$ sequence on each strand. For our studies with GFP-C/EBP we have used EP24 extended with $(TG)_5$ (EP24$(TG)_5$). EP24 contains the consensus sequence for binding of C/EBP. This EP24$(TG)_5$ sequence is incubated with extracts containing GFP-C/EBP for 30 min and then passed over $(AC)_5$–Sepharose. $(AC)_5$–Sepharose is able to trap the

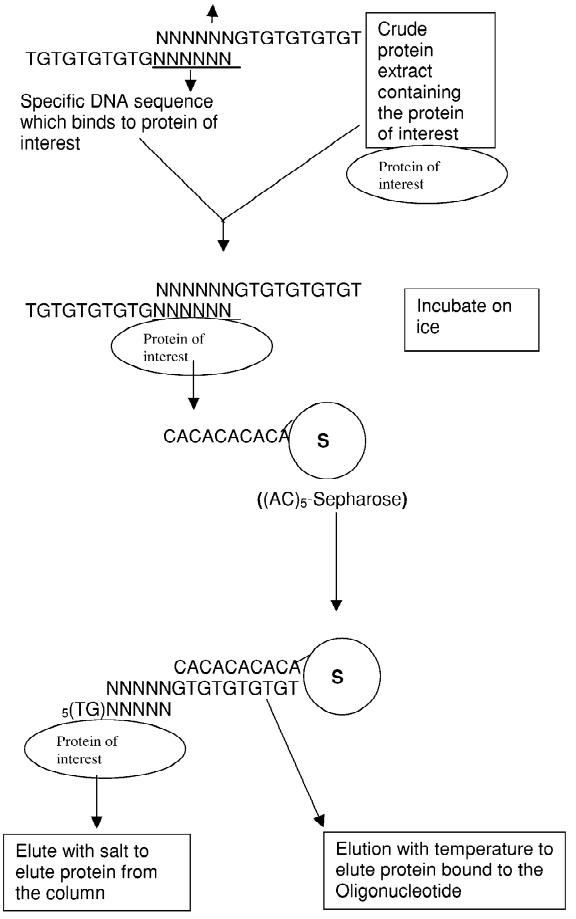

Fig. 1. Schematic of the oligonucleotide trapping method. The strategy used in the oligonucleotide trapping method is shown diagrammatically. The circled S represents the chromatographic support, in this case Sepharose. First, 5′-aminoethyl-$(AC)_5$ oligonucleotide is chemically coupled to Sepharose. A footprint region having a $(TG)_5$ extension on both strands is incubated with extract containing the protein of interest. This mixture is then passed over the $(AC)_5$–Sepharose column. The protein alone can then be eluted by using buffer containing high salt or the DNA–protein complex can be eluted using moderate temperature.

DNA–protein complex because of the specific hybrid formation between the $(TG)_5$ region of EP24$(TG)_5$ and the $(AC)_5$ region bound to the Sepharose. Elution can be achieved using high salt to disrupt the DNA–protein interaction or by using moderate temperatures and low salt to melt the hybrid between $(AC)_5$ and $(TG)_5$. When high salt is used, GFP-C/EBP alone, free of DNA, is eluted. When temperature is used, GFP-C/EBP that is bound to EP24$(TG)_5$ is eluted. A similar protocol was followed for the purification of B3 from *Xenopus* oocyte extract except element 3 DNA E3$(TG)_5$/αE3$(TG)_5$ was used in the place of EP24$(TG)_5$

Two different variants of the oligonucleotide trapping experiment performed with GFP-C/EBP are shown in Fig. 2. It can be seen from the figure that sharp peaks containing GFP-C/EBP are obtained upon elution with either salt or temperature. The peaks obtained with the two different elution schemes are similar in peak height and width. GFP-C/EBP alone, in the absence of EP24$(TG)_5$, did not bind to $(AC)_5$–Sepharose (data not shown) and, hence, both peaks observed are due to the specific interaction between GFP-C/EBP and EP24.

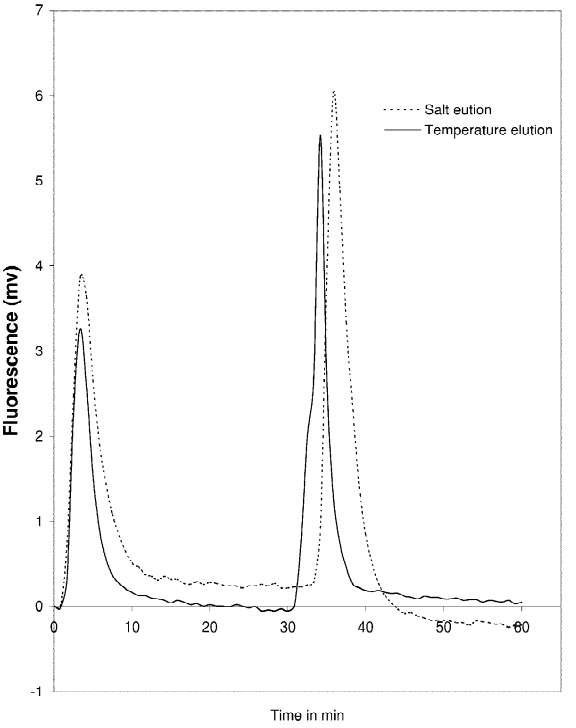

Fig. 2. Oligonucleotide trapping method for GFP-C/EBP. Purified GFP-C/EBP (20 μl) was incubated with 5 nmol of EP24$(TG)_5$ on ice for 30 min in TE0.4 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 0.4 *M* NaCl). This mixture was then passed over a 1 ml $(AC)_5$–Sepharose column. The column was washed with 15 ml of TE0.4 at 4 °C. The protein was either eluted with TE1.2 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 1.2 *M* NaCl) at 4 °C for salt elution or with TE (10 m*M* Tris, pH 7.5, 1 m*M* EDTA) at 37 °C for temperature elution. The flow-rate was maintained at 0.5 ml/min throughout the experiment.

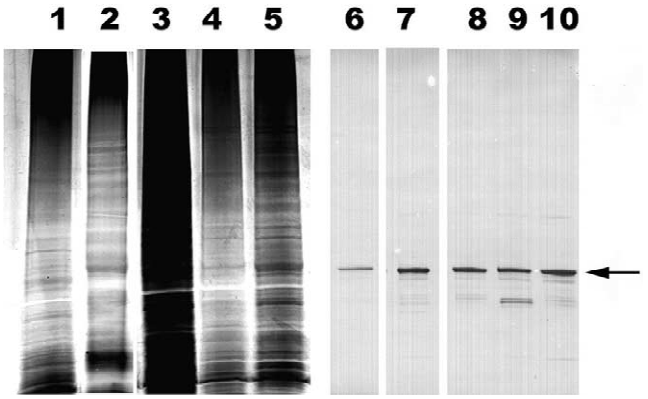

Fig. 3. Comparison of the oligonucleotide trapping method and the avidin–biotin trapping method. A crude bacterial extract (100 μl) containing GFP-C/EBP was mixed with either BiEP24 (where Bi stands for Biotin group introduced at the 5′ end during synthesis) or EP24$(TG)_5$ and incubated on ice for 30 min. The mixture containing BiEP24 was passed over a 500 μl NeutrAvidin column (lane 1) or a 500 μl monomeric avidin column (lanes 2 and 3). The mixture containing EP24$(TG)_5$ was passed over 1 ml $(AC)_5$–Sepharose (lanes 4 and 5). All the columns were washed with PBS0.4 (0.1 *M* KH_2PO_4, pH 7.5, and 0.4 *M* NaCl). The NeutrAvidin column (lane 1), monomeric avidin column (lane 3) and $(AC)_5$–Sepharose (lane 5) were eluted with PBS1.2 (0.1 *M* KH_2PO_4, pH 7.5, and 1.2 *M* NaCl). The monomeric avidin column (lane 2) was eluted with PBS0.4 containing 2 m*M* biotin, while $(AC)_5$–Sepharose (lane 4) was eluted with PBS (0.1 *M* KH_2PO_4, pH 7.5, and 0.1 *M* NaCl) at 37 °C. Lanes 6–10 are Western blots of proteins in lanes 1–5, respectively.

We compared our oligonucleotide trapping method with the biotin–avidin method. Fig. 3 shows the purity of GFP-C/EBP obtained. Lanes 1, 2 and 3 represent proteins obtained from the NeutrAvidin column with salt elution, the monomeric avidin column with biotin elution, and the monomeric avidin column with salt elution, respectively. NeutrAvidin and monomeric avidin are genetically modified forms of avidin that show less nonspecific interactions. The GFP-C/EBP obtained from NeutrAvidin–Agarose (lane 1) and monomeric avidin with biotin elution (lane 2) is significantly purer than that obtained from the monomeric avidin column with salt elution (lane 3), since the Western blot shows that each contain similar amounts of GFP-C/EBP, but lane 3 obviously contains much more contaminating protein from the intensity of the protein stain. Lanes 4 and 5 represent samples obtained with the oligonucleotide trapping method using temperature and salt elution, respectively. The Western blot shows that both have about the same amount of GFP-C/EBP, but temperature elution contains less overall protein. Thus, the oligonucleotide trapping method with temperature elution (lane 4) yields the purest GFP-C/EBP obtained. From the silver stained gel it appears that more GFP-CEBP is obtained with salt elution in the oligonucleotide trapping method than with temperature elution, but the Western blots of the same samples (lanes 9 and 10) show that similar amounts of GFP-C/EBP are present in both. Hence, the larger size of the band for GFP-C/EBP seen in lane 5 is probably because of the comigration of some contaminant protein along with GFP-C/EBP. The same is also true for the biotin eluted sample in lane 2.

As discussed before, chemical coupling of DNA to Sepharose can cause modification within the DNA that could affect the efficiency of the column. Our method, on the other hand, allows the use of unmodified DNA, which could lead to a higher capacity for proteins. Furthermore, since binding occurs in solution, steric crowding is not a concern. Fig. 4 shows that the oligonucleotide trapping method has a high capacity and is able to bind to greater amounts of GFP-C/EBP than a conventional chemically coupled DNA affinity column having a comparable amount of DNA to that used in the trapping experiments. The maximum amount of GFP-C/EBP bound in the trapping experiments is 28 000 fluorescence units, which is equivalent to 0.19 mg of GFP-C/EBP (data not shown). The 5 nmol of EP24 used for this trapping experiment can theoretically bind 0.2 mg of GFP-C/EBP assuming that C/EBP binds to its DNA element as a dimer. Hence, around

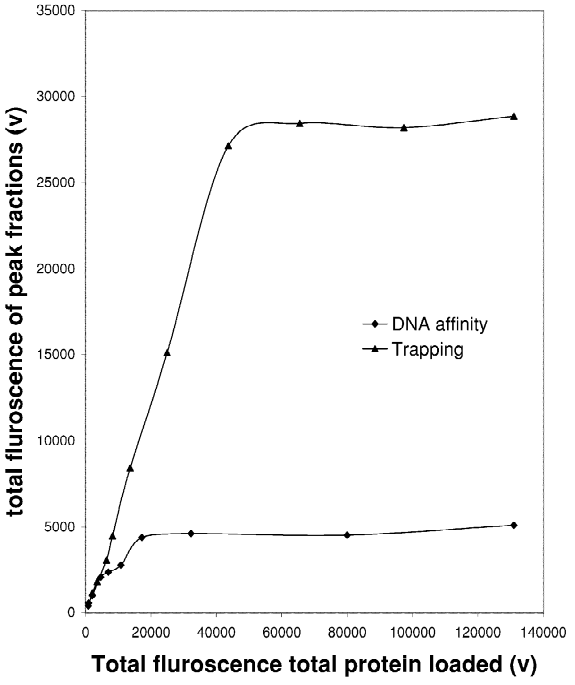

Fig. 4. Capacity of oligonucleotide trapping and DNA affinity chromatography. 5 nmol of EP24$(TG)_5$ was mixed with different amounts of purified GFP-C/EBP. The mixture was incubated on ice for 30 min and loaded onto a 1 ml $(AC)_5$–Sepharose column. The column was washed with 10 ml TE0.4 and eluted with TE1.2. Different concentrations of GFP-C/EBP alone were loaded onto a 250 μl (5 nmol DNA) EP24–Sepharose column, the column was then washed and eluted as described for the $(AC)_5$–Sepharose column. The fluorescence for GFP-C/EBP was monitored continuously using a Shimadzu fluorescence spectromonitor RF-530 and the peak areas calculated using Gilson Unipoint software. All chromatography was at 4 °C and the flow-rate was 0.5 ml/min throughout.

95% of the theoretical capacity can be obtained in the oligonucleotide trapping method. The DNA affinity column containing 5 nmol of EP24 can maximally bind only 5000 fluorescence units of GFP-C/EBP, which corresponds to around 0.03 mg of GFP-C/EBP, and hence only 15% of the theoretical capacity was achieved by conventional DNA affinity chromatography.

DNA affinity columns used routinely have a high concentration of coupled DNA, ranging from 15 to 200 μ*M*. These high concentrations may be desirable to obtain high yields in small columns. This approach has a major drawback: the high concentration of DNA on the column can encourage nonspecific binding of proteins that have a low affinity for DNA. In a crude extract there are typically several DNA binding proteins and most of these proteins would bind to the DNA on the column. The lac repressor protein, which is the most widely studied transcription factor, has $K_d = 0.7–11 \cdot 10^{-5}$ for nonspecific DNA sequences [23] and $K_d = 3.5 \cdot 10^{-10}–1.7 \cdot 10^{-12}$ for a specific operator sequence [24]. Because of this difference in specific and nonspecific affinities of DNA binding proteins, a specific protein alone is supposed to bind with a very high affinity to the DNA affinity column. However, the high DNA concentration on the column drives the binding of even low affinity binding proteins. Separation of multiple nonspecific proteins from the protein of interest with a suitable elution scheme would be challenging. This contamination could be minimized by keeping the concentration of the column DNA low. Although this could be achieved in conventional DNA affinity columns, it would require low DNA concentrations in large columns to allow reasonable capacity. The trapping method can be more efficiently utilized for this purpose.

Fig. 5 illustrates how the binding equilibria would affect the binding of a contaminant protein having a K_d of $4 \cdot 10^{-5}$ *M* for the DNA footprint used in conventional DNA affinity chromatography and the oligonucleotide trapping method. Since the binding constants of C/EBP or B3 for nonspecific DNA are not known we chose to use the known constants for the lac repressor protein. In this model the concentration of DNA in the conventional DNA affinity column is assumed to be 20 μ*M*, which is about the average DNA concentration of most of our DNA affinity columns. The DNA concentration in the oligonucleotide trapping method is assumed to be 50 n*M*, as it should be low enough to discourage binding of nonspecific proteins but high enough that specific binding will be nearly quantitative. The model also assumes that, in either case, the DNA is in excess and all of the DNA binding proteins in the extract could be bound. Furthermore, it assumes the binding stoichiometry is 1:1 and that equilibrium is obtained. It can be seen from the figure that the conventional DNA affinity column retains at least three-fold more contaminant protein than the oligonucleotide trapping method. Both methods would trap close to 100% of a protein of interest having an

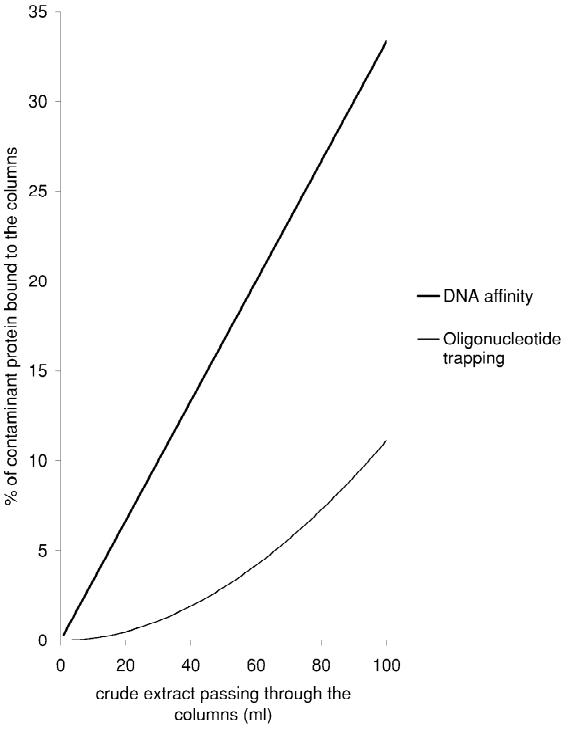

Fig. 5. Theoretical model for nonspecific binding of protein in the oligonucleotide trapping method and DNA affinity chromatography. Nonspecific binding of a protein having an affinity of $4 \cdot 10^{-5}$ *M* for the footprint region in conventional DNA affinity (thick line) and the oligonucleotide trapping method (thin line). The concentration of DNA in DNA affinity chromatography and the oligonucleotide trapping method is assumed to be 20 μ*M* and 50 n*M*, respectively. The binding constant of proteins for the small $(AC)_5$ sequence on the column is considered to be negligible. The model accounts for each milliliter of the crude extract that passes through each column.

affinity of $4 \cdot 10^{-12}$ for the DNA on the column or in solution (data not shown). Moreover, the figure illustrates the retention of a contaminant protein with respect to the amount of crude extract passing through the columns and the two methods behave differently. The same fraction of the contaminant protein is retained throughout DNA affinity chromatography while in the trapping method only a negligible fraction of the contaminant protein is retained as the first 50 ml of crude extract passes through the column. The major fraction of the total contaminant protein is retained in the trapping method only when the last few milliliters pass through the column. This is because, in the oligonucleotide trapping method, the initial concentration of the footprint element on the column is very low and builds up only towards the end of the experiment. In conventional DNA affinity columns the concentration of the DNA footprint remains constantly high throughout the run.

We tried to apply this model to the actual purification of crude GFP-C/EBP. The amount of GFP-C/EBP was adjusted so that it was comparable to the low amounts present naturally in cells. The concentration of DNA used in the oligonucleotide trapping method and DNA affinity chromatography were similar to those in the model experiment. It can be seen from Table 1 that the GFP-C/EBP obtained with the oligonucleotide trapping method is significantly purer than that obtained with conventional DNA affinity chromatography. Table 1 shows threefold higher purity; this is reasonable considering the higher purity obvious in Fig. 6. It can also be seen that the yield obtained with the oligonucleotide trapping method is comparable to that obtained with DNA affinity chromatography, although almost 10-fold more DNA is used in the latter method. It should also be pointed out that elution here was with salt instead of moderate temperature to prevent the

Table 1
Balance sheet for purification of GFP-C/EBP with DNA affinity and the oligonucleotide trapping method

	Fold purification[a]	Yield (%)
Trapping	3668±491	36±16
DNA affinity	1028±226	24±2

The probability (*P*) that the fold purification obtained by the two methods is not different is 0.00054. The probability that the yield is not different is 0.146.

[a] The results of three experiments were averaged ($n=3$) and averages are reported for both the columns. For DNA affinity chromatography, 100 μl of purified GFP-C/EBP was mixed with 10 ml of crude bacterial extract (lacking the fusion protein) and the volume was adjusted to 50 ml with TE0.4 and loaded onto a 1 ml EP24–Sepharose (containing 20 nmol EP24) column. For the oligonucleotide trapping experiment, 2.5 nmol of $EP24(TG)_5$ was added to the same extract, incubated on ice for 30 min and then loaded onto a 1 ml $(AC)_5$–Sepharose column. Both columns were washed with 25 ml of TE0.4 and then eluted with TE1.2 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 1.2 *M* NaCl). Active fractions were pooled for assay. The flow-rate was maintained at 0.5 ml/min and all experiments were performed at 4 °C.

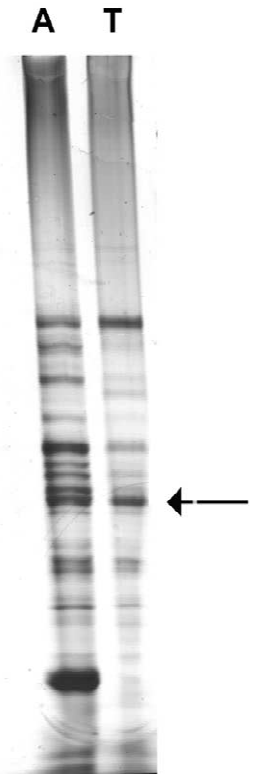

Fig. 6. Purity of GFP-C/EBP obtained with conventional DNA affinity chromatography and the oligonucleotide trapping method. The active fractions from the balance sheet in Table 1 were concentrated and applied to a polyacrylamide gel and stained with silver. Lanes ''C'' and ''T'' show proteins purified with DNA affinity chromatography and oligonucleotide trapping, respectively.

interference of DNA with the fluorescence measurements. Elution with moderate temperatures would be expected to give higher purity (see Fig. 3).

Fig. 6 shows sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the purified proteins from Table 1. It can be seen from the figure that the GFP-C/EBP obtained (shown by an arrow) from the oligonucleotide trapping method is significantly purer than that obtained with conventional DNA affinity chromatography. Several contaminant protein bands that are present in samples obtained from DNA affinity chromatography (lane ''A'') are either completely absent or are highly reduced in samples obtained with the oligonucleotide trapping method (lane ''T'').

Purification of transcription factor B3 was carried out from a *Xenopus* oocyte extract by trapping and conventional DNA affinity chromatography. It can be seen from Fig. 7A that B3 obtained with the trapping method (shown by an arrow, lane T) is purer than that obtained with DNA affinity chromatography (lane A) and several contaminant bands present in lane A are either absent or greatly reduced in B3 obtained from the trapping approach.

Fig. 7A shows that trapping can be successfully used for obtaining high purity B3. By combining the trapping approach with the use of competitors, even higher purity can be obtained (Fig. 7B). It can be seen from the figure that heparin alone (lanes 2 and 7) and heparin along with T_{18} (lanes 3 and 8) could be successfully used for improving the purity of B3 and the latter approach gives highly purified B3, as seen in lane 3. Specific competitor μE3 (lanes 4 and 9) and salmon sperm DNA (lanes 5 and 10) could not be used as competitors because they greatly affect the yield of B3, as can be seen in the Western blot in lanes 9 and 10. Again, note that elution was with salt in these experiments.

When the same competitors were used with salt elution after washing the column more extensively (40 ml in Fig. 8 versus 25 ml in Fig. 7B), nearly homogenous B3 was obtained, as shown in Fig. 8. The crude extract from *Xenopus* oocytes (C) is an extremely complex sample. Purification by trapping (T), using heparin and T_{18} competitors and temperature-dependent elution, reduced this to the predominant M_r 70 000 B3 protein band detected by the antibody. B3 eluted in this way would actually be a complex of B3 with the trapping oligonucleotide, but the oligonucleotide could be easily removed by simply repeating the column chromatography and eluting with salt.

4. Discussion

Coupling of DNA to solid supports in conventional DNA affinity chromatography has allowed purification of many transcription factors. This technique suffers from two major drawbacks: DNA modification and high nonspecific binding. Coupling can lead to modification in the DNA sequence unless special coupling procedures are used. Base modification can

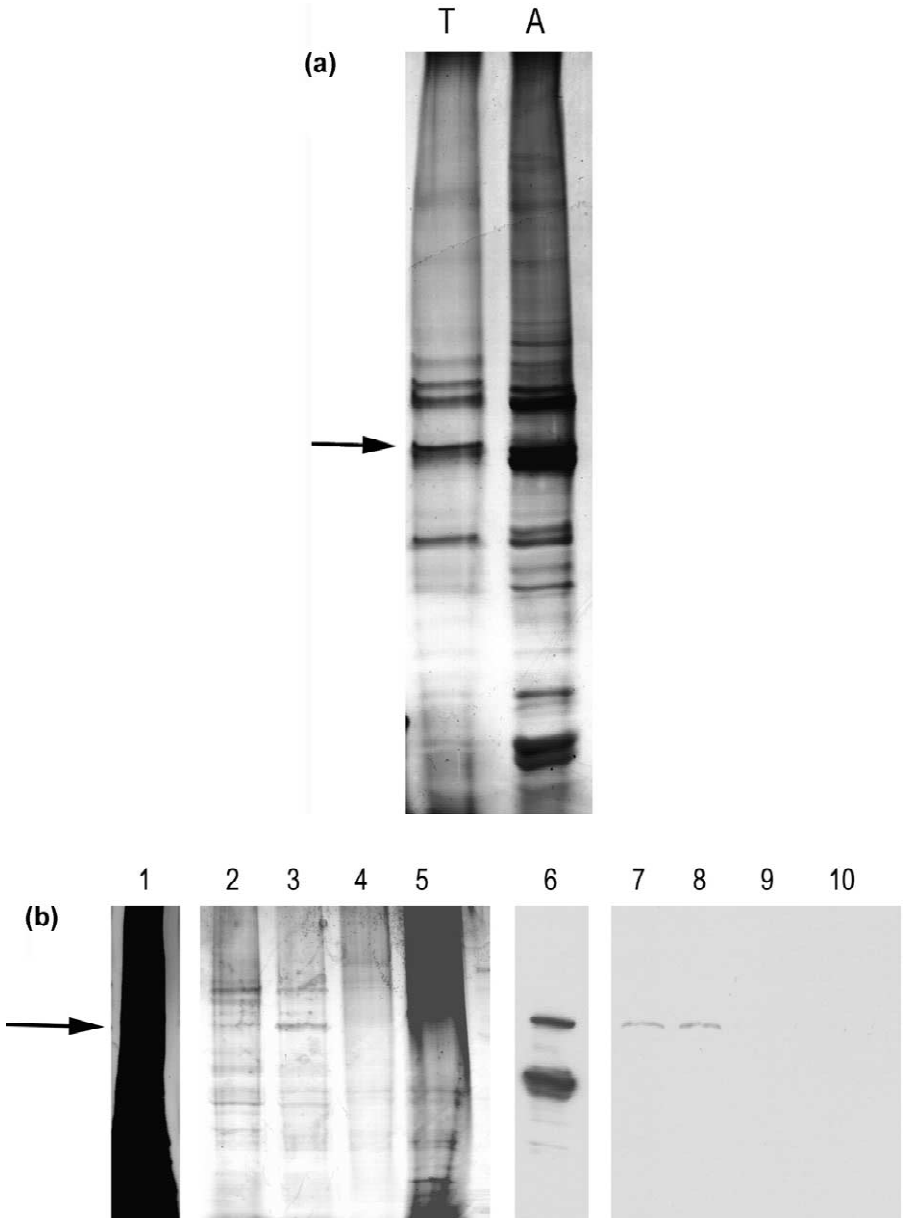

Fig. 7. Purification of B3 using the trapping approach. (A) For DNA affinity chromatography (''A''), 2.5 ml of *Xenopus* oocyte extract was diluted to 50 ml with TE0.4 and loaded onto a 1 ml E3–Sepharose column. For the oligonucleotide trapping (''T'') experiment, 2.5 nmol of $E3TG_5/\alpha E3TG_5$ was added to the same extract, incubated on ice for 30 min and then loaded onto AC_5–Sepharose. Both columns were washed with 25 ml of TE0.4 and then eluted with TE1.2. The flow-rate was maintained at 0.5 ml/min and all the experiments were carried out at 4 °C. Active fractions were concentrated, applied to an SDS polyacrylamide gel, and stained with silver. Lanes T and A show proteins purified with the oligonucleotide trapping method and conventional DNA affinity chromatography, respectively. (B) 200 μl of *Xenopus* oocyte extract was diluted to 4 ml using TE0.4 and incubated with 0.5 μ*M* of $E3TG_5/\alpha E3TG_5$ along with competitors: 4 mg/ml heparin (lanes 2 and 7), 4 mg/ml heparin and 20 μ*M* of T_{18} (lanes 3 and 8), 5 μ*M* μE3 and 20 μ*M* T_{18} (lanes 4 and 9), and 1 mg/ml salmon sperm DNA (lanes 5 and 10). The mixtures were incubated on ice for 30 min and then loaded onto AC_5–Sepharose. Both columns were washed with 25 ml of TE0.4 and then eluted with TE1.2. Active fractions were concentrated and applied to SDS polyacrylamide gel lanes 1–5; a duplicate gel was subjected to Western blotting for lanes 6–10. Lanes 1 and 6 represent 10 times diluted crude *Xenopus* oocyte extract.

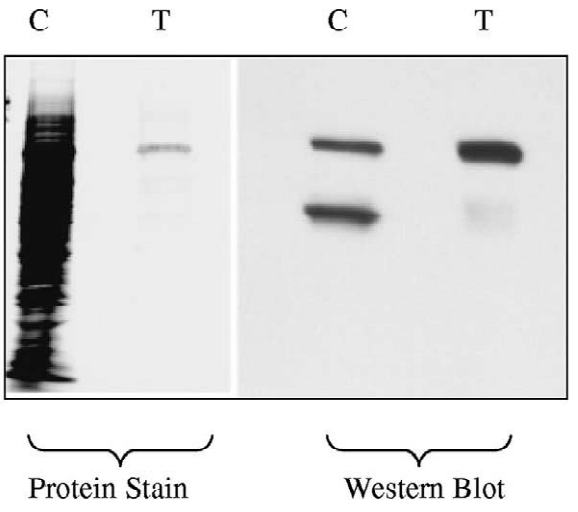

Fig. 8. Trapping eluted with salt yields nearly homogeneous B3. 2 ml of *Xenopus* oocyte extract was diluted to 40 ml using TE0.4 and incubated with 0.5 μ*M* $E3TG_5/\alpha E3TG_5$ along with competitors, 4 mg/ml heparin and 20 μ*M* T_{18}. The mixtures were incubated on ice for 30 min and then loaded onto a 5 ml $(AC)_5$–Sepharose column. The column was washed with 40 ml of TE0.4 and then eluted with TE1.2. Active fractions were concentrated, applied to SDS polyacrylamide electrophoresis, and either stained with Coomassie blue (lanes 1 and 2) or subjected to Western blotting (lanes 3 and 4) with a specific B3 antibody. Lanes 1 and 3 represent 20 μl of 10 times diluted crude (C) and lanes 2 and 4 concentrated B3 (one-fourth of the total) from trapping (T).

affect the DNA interaction with the protein of interest. Methods such as enzymatic column synthesis or biotin–avidin technologies can solve this problem to a certain extent, but each of these methods has its own limitations. From Fig. 2 it can be seen that a purer protein can be obtained with our oligonucleotide trapping method than with the biotin–avidin method, even after using modified forms of avidin such as NeutrAvidin and monomeric avidin, which have relatively low nonspecific interactions with proteins. A method similar to our method, in which a footprint extended with polyA is used, has also been described [11]. In that method, salt elution is used for obtaining the protein, and from our results it is clear that elution with moderate temperature and low salt concentration is a better approach than salt elution in terms of the purity of the GFP-C/EBP or B3 obtained.

A second drawback of DNA affinity chromatography is that the DNA immobilized on the column is not in true solution and, hence, the kinetic parameters of the binding of proteins to DNA cannot be extrapolated to column chromatography. Methods such as magnetic bead purification and the previously described polyA trapping method [11] could have been efficiently used to solve this problem. But most of the methods described so far fail to do so and employ the biotin group or oligonucleotide extensions as other means of coupling the footprint region to the solid support and then perform conventional affinity chromatography. In other cases when the binding is carried out in solution, the concentration of DNA used is very high. Such high concentrations can encourage nonspecific binding of proteins.

In our method the binding between protein and DNA occurs in solution and only then is $(AC)_5$–Sepharose used to trap the DNA–protein complex. By carrying out binding in solution we can adjust the concentrations of DNA to levels which do not favor the nonspecific binding of proteins. This can very rarely be achieved by conventional column chromatography. The advantages of using low concentrations of DNA are clear from Table 1 and Fig. 6, which show that the GFP-C/EBP obtained from our approach is significantly purer than that obtained with conventional chromatography. It is important to note that we have used high salt to elute GFP-C/EBP in the oligonucleotide trapping method in Table 1 and Fig. 6. High salt is not the best method for elution and better purity can be obtained with moderate temperature elution (Fig. 3). But since accurate measurements of fluorescence are required for the balance sheet in Table1, and the DNA that is bound to protein after temperature elution can affect its fluorescence, we chose to use salt elution. Thus, Table 1 establishes a lower limit of the extent of purification possible with trapping. The 2.5 nmol of oligonucleotide used in purifications such as those in Table 1 is usually not recovered, but is an inexpensive, expendable reagent.

Several competitors, such as heparin, specific DNA (μE3), nonspecific DNA (fragmented salmon sperm DNA), and single-stranded DNA (T_{18}), can be used together with the trapping approach. It is noteworthy that the use of single-stranded DNA along with heparin is most efficient in purifying B3. T_{18} probably competes with the $(AC)_5$-tail for

binding to single strand-binding proteins and hence prevents contamination by these proteins.

From the overall results it can be seen that our oligonucleotide trapping method is a promising alternative to conventional DNA affinity chromatography.

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