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Oligonucleotide trapping method for transcription factor purification systematic optimization using electrophoretic mobility shift assay

Robert A. Moxley, Harry W. Jarrett *

Department of Molecular Sciences, University of Tennessee, 858 Madison Avenue, Suite G-01, Molecular Sciences Building, Memphis, TN 38163, USA

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Abstract

Oligonucleotide trapping, where a transcription factor–DNA response element complex is formed in solution and then recovered (trapped) on a column, was optimized for the purification of CAAT/enhancer binding protein (C/EBP) from rat liver nuclear extract. Electrophoretic mobility shift assays (EMSAs) with ACEP24$(GT)_5$ oligonucleotide, containing the CAAT element, was used to estimate the binding affinity and concentration of C/EBP in the nuclear extract and then low concentrations of protein and oligonucleotide, which favor specific binding, were used for all further experiments. Also using EMSA, the highest concentrations of competitors, which inhibit non-specific binding but do not inhibit oligonucleotide binding by C/EBP, were determined to be 932 nM T_{18} (single-stranded DNA), 50 ng/ml heparin (non-DNA competitor), and 50 μg/ml poly(dI:dC) (duplex DNA). Inclusion of 0.1% Tween-20 improved DNA binding. For complex formation, 110 μg nuclear extract was diluted to 0.2 nM C/EBP (apparent K_d of C/EBP) and 1.34 nM ACEP24$(GT)_5$ was added, along with Tween-20 and the competitors. After incubation, the complex was trapped by annealing the $(GT)_5$ tail of the C/EBP–[ACEP24$(GT)_5$] complex to an $(AC)_5$–Sepharose column under flow at 4 °C. The column was washed with 0.4 M NaCl and the protein eluted with 1.2 M NaCl. The purification typically resulted in two proteins of apparent molecular mass 32 000 and 38 000. The smaller one, the major product, was identified to be C/EBP-α. The yield was 2.1 μg (66 pmol) of purified C/EBP-α p32. This systematic approach to oligonucleotide trapping is generally applicable for the purification of other transcription factors.

Keywords: DNA; Proteins; Transcription factor; CAAT enhancer binding protein; Response element

1. Introduction

Transcription factors bind DNA and activate or inhibit the transcription of specific genes and precisely determine the fate of the cell. Understanding genetic regulation at a molecular level is one of the great challenges of biology. To better understand genetic regulation, the DNA elements bound by the transcription factors must be identified, and the cognate transcription factors characterized. The oligonucleotide trapping method offers a powerful tool for purification of transcription factors and other DNA-binding proteins [1].

One widely characterized family of transcription factors is the CAAT/enhancer binding proteins (C/EBPs), which bind to the CAAT consensus sequence [2]. C/EBPs are expressed in a variety of tissues including those which play a central role in energy metabolism, such as adipose and liver tissue [3]. C/EBPs are also critical for normal cellular differentiation and function, and act as master regulators of many cellular responses in a variety of other tissues [2,4]. The C/EBPs form a family of transcription factors with at least six (C/EBP-α–C/EBP-ζ) members expressed from individual genes [4], and more diversity is produced by alternative initiation sites, differential splicing, and protease cleavage [2]. C/EBPs bind to DNA as a dimer and form extensive protein–protein interactions both within the family and with proteins from outside of the C/EBP family genes [2]. C/EBPs consist of three structural components which include a C-terminal leucine–zipper dimerization domain, a basic DNA-binding region, and a N-terminal transactivation region [5]. C/EBP-α was the first

* Corresponding author. Tel.: +1 901 448 7078; fax: +1 901 448 7360.
E-mail address: hjarrett@utmem.edu (H.W. Jarrett).

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of the C/EBPs characterized and was purified from rat liver nuclear extracts using a multi-step purification procedure including DNA affinity chromatography with heterogeneous DNA fragments adsorbed to cellulose [6].

Advances in DNA affinity chromatography [7] since C/EBP-α was first purified include the use of highly specific columns made by using sequence-specific DNA element oligonucleotides that can be bound by an individual transcription factor. Several chromatographic supports are commonly used including Sepharose, cellulose, and silica and many coupling chemistries are also available for attaching DNA to these supports [8–11]. Coupling of DNA to supports can lead to modification of the oligonucleotide bases, thus potentially decreasing the ability of the DNA affinity columns to purify the protein of interest. A few methods are available for coupling of DNA to supports without modification of the oligonucleotide bases including enzymatic synthesis [12], biotinylated oligonucleotides [13–15], Teflon fiber support [16], and bromoacetyl agarose [17]. In another method, polyT coupled to agarose was used to bind a 3′ polyA tail that contained a duplex transcription factor recognition sequence 5′ of the polyA region [18].

A recent advance in DNA affinity chromatography, the oligonucleotide trapping method, yielded the *Xenopus* transcription factor, B3, to a high degree of purity in a single purification step [1]; the trapping method has been reviewed [19,20]. The oligonucleotide trapping method uses DNA affinity chromatography but is different from it. DNA trapping involves mixing in solution a specific DNA sequence, protein and competitors (molecules that diminish binding of DNA by DNA-binding proteins) at experimentally derived concentrations that optimize formation of a specific DNA–transcription factor complex and minimize binding of non-specific DNA-binding proteins. The complex is then applied to a column which will then bind (trap) it. The method [1] utilized the specific interactions between an oligonucleotide containing a specific duplex sequence element which contains a $(GT)_5$ single-stranded tail and a transcription factor, B3, to form a protein–DNA complex in solution prior to trapping, by annealing, on an $(AC)_5$–Sepharose column. Competitors that were used are T_{18} and heparin, but the concentrations and the concentrations of oligonucleotide added were all determined by a process of trial and error each time trapping the complex on a $(AC)_5$–Sepharose column [18]. Protein elution was performed by using a buffer containing high salt to disrupt the protein–oligonucleotide interaction or using moderate temperatures to melt the $(GT)_5$:$(AC)_5$ hybrid. Recently, oligonucleotide trapping was used to purify the insulin promoter RIPE3b1 activator protein for the first time and allowed it to be identified as MafA, a member of the large Maf transcription factor family [21].

In the oligonucleotide trapping method described in this paper, we systematically optimized the concentration of C/EBP, trapping oligonucleotide, and competitors necessary for specific complex formation using an electrophoretic mobility shift assay (EMSA). Experimentally estimating the concentration of C/EBP in the extract allows specific trapping oligonucleotide to be added to the trapping mix at a low concentration that would favor specific binding and be unfavorable for non-specific interactions. A 6.7-fold molar excess of trapping DNA was chosen to favor complete binding of the specific oligonucleotide by C/EBP and the concentration of C/EBP in the trapping mixture was diluted to be near the apparent K_d. By keeping the concentrations of the specific sequence oligonucleotide and C/EBP low and near the experimentally estimated K_d, non-specific binding is reduced.

Experimental determination of optimal concentrations of oligonucleotide and competitors allows for a more systematic approach that can be generally applied to discover a single-step purification of any specific transcription factor from complex protein mixtures. Here, we describe the purification of C/EBP-α from crude rat liver nuclear extract in a single step and describe this systematic approach that could be adapted to virtually any DNA-binding protein.

2. Methods

2.1. Materials

Heparin (H-3393) and dI:dC (P-4929) were from Sigma (St. Louis, MO, USA). Tween-20 (70-6531) was from Bio-Rad Laboratories (Hercules, CA, USA). Other material sources are given below or were the highest purity commercially available.

2.2. Coupling of DNA to Sepharose

All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). $(AC)_5$ (5′-NH_2-ACACACAC-3′) was 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 Pharmacia (New York, NY, USA). "5′-NH_2" in the oligonucleotide sequence represents an aminohexyl group added on the last synthesis cycle. The amount of DNA coupled was determined by the difference in the UV absorption of DNA added and recovered after coupling; 50 nmol of $(AC)_5$ oligonucleotide was added to 0.3 g of CNBr-preactivated Sepharose 4B and 30 nmol of $(AC)_5$ oligonucleotide coupled. The resulting support is called $(AC)_5$–Sepharose.

2.3. Production of proteins

GFP-C/EBP-α was produced in *Escherichia coli* strain BL21 containing plasmid pJ22-GFP-C/EBP-α as described previously [22] and was used as a marker and positive control.

Rat liver nuclear extracts were prepared by the method described in [23]. Briefly, 10–15 g of rat liver was minced and homogenized. The homogenate was layered over cushions of

homogenization buffer (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (pH 7.6), 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol) and centrifuged at 76 000 × *g* for 30 min at 4 °C to pellet the nuclei. The nuclear pellet was resuspended in a 9:1 (v/v) mixture of homogenization buffer and glycerol. This homogenate was layered over a cushion of the same 9:1 mixture and centrifuged as described above. The nuclear pellet was resuspended in nuclear lysis buffer (10 mM HEPES (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonylfluoride (PMSF), 10% glycerol). The nuclear suspension was diluted to 10 A_{260} units/ml, and a 1/10 volume of 4 M $(NH_4)_2SO_4$ (pH 7.9) was added. Centrifugation of the lysate was at 96 000 × *g* for 25 min at 4 °C. Solid $(NH_4)_2SO_4$ was added to the supernatant to 0.3 g/ml and left on ice for 45 min. Centrifugation was repeated at 96 000 × *g* for 25 min at 4 °C. The protein pellet was resuspended in dialysis buffer (25 mM HEPES (pH 7.6), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) at 1 ml per 20 A_{260} units of nuclear lysate. The protein extract was dialyzed twice for 2 h at 4 °C. We normally obtained 10 mg of nuclear extract per adult rat liver. The protein extract was stored in aliquots under liquid nitrogen.

2.4. Protein assay

Protein concentrations were determined by the Bradford method [24] using bovine serum albumin as the standard.

2.5. Chromatography

The $(AC)_5$–Sepharose support was packed in 1 ml bed volume syringe columns initially equilibrated in TE0.4 buffer (10 mM Tris (pH 7.5), 1 mM EDTA, 0.4 M NaCl).

For the oligonucleotide trapping method, ACEP24-$(GT)_5$ (5′-GCTGCAG**ATTG**CG**CAAT**CTGCAGCGTGT-GTGTGT-3′) was converted nearly quantitatively to the duplex by annealing. Annealing was by heating to 95 °C and allowing to cool over 1 h to 4 °C in a thermal cycler. ACEP24$(GT)_5$ is self-complementary and does not require the addition of a second strand.

Crude rat liver nuclear extract was diluted approximately 100-fold (typically adding 110 μg rat liver nuclear extract) in a final volume of 50 ml TE0.4 (10 mM Tris (pH 7.5), 1 mM EDTA, 0.4 M NaCl) containing 0.1% Tween-20 and competitors. The exact dilution was calculated to give a final concentration of C/EBP of 0.2 nM, near the experimentally determined apparent dissociation constant, K_d. Crude rat liver nuclear extract was incubated for 10 min at 4 °C with heparin, T_{18} (5′-TTTTTTTTTTTTTTTTTT-3′), and poly(dI:dC) as competitors at their experimentally determined concentrations. The annealed ACEP24$(GT)_5$ was then added to 1.34 nM, and incubated for 30 min at 4 °C. The trapping mixture was applied to the 1 ml $(AC)_5$–Sepharose, washed with TE0.4 for 20 column volumes, and then eluted with 15 column volumes of TE1.2 (10 mM Tris (pH 7.5), 1 mM EDTA, 1.2 M NaCl).

SDS polyacrylamide gel electrophoresis: Samples were concentrated using lyophilization following dialysis in 50 mM NH_4HCO_3. Lyophilized samples were applied to a 10% SDS polyacrylamide gel using the method of Laemmli [25] and stained with silver using the Bio-Rad Laboratory kit (Richmond, CA, USA), or used in immunoblot analysis.

2.6. Western blot analysis

Gels were electroblotted onto 0.2 μm pore nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) as previously described [26]. A 1:100 dilution of goat serum generated against purified C/EBP-α (14AA, cat. SC-61, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a less specific antibody cross-reactive with C/EBP family members including C/EBP-α and C/EBP-β (C/EBP-β Δ-198, cat. SC-746, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary antibodies for identification of purified proteins. Immunoreactive proteins were visualized by using 1:1200 diluted rabbit anti-goat secondary antibody–HRP conjugate (Southern Biotechnology Associates, Birmingham, AL, USA) and detected by chemiluminescence as previously described [27]. For autoradiography, the Molecular Dynamics Typhoon phosphorimager and software was used.

2.7. Oligonucleotide labeling

Oligonucleotides were 5′-end labeled with [^{32}P] by mixing 50 pmol ACEP24$(GT)_5$, 10 μCi [γ-^{32}P]-ATP (ICN Biomedicals Inc., Irvine, CA, USA), and 1 ul (10 units) T4 polynucleotide kinase New England Biolabs (Beverly, MA, USA) in a final concentration of 10 mM Tris–Cl (pH 8.0), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol in a final volume of 10 μl and incubating at 37 °C for 1 h. Reactions were stopped by addition of EDTA (pH 8.0) to 20 mM and placing in ice slurry. Unincorporated [γ-^{32}P]-ATP was removed by desalting on a 1 ml BioGel P-6 spin column in TE (10 mM Tris (pH 7.5), 1 mM EDTA).

2.8. Electrophoretic mobility shift assay

To assess DNA-binding properties, 5 μl of protein (typically 2.5 μg GFP-C/EBP containing bacterial extract or 1–2 μg rat liver nuclear extract) was mixed in a total volume of 25 μl with 40 fmol (1.6 nM final concentration) of annealed 5′-[^{32}P]-ACEP24$(GT)_5$ (see Sections 2.5 and 2.7) in an incubation buffer TE0.1 (final concentration: 10 mM Tris (pH 7.5), 1 mM EDTA, 0.1 M NaCl) containing 4% glycerol and was used as indicated unless stated otherwise. For some experiments, duplex EP18 (5′-GCAGATTGCGCAATCTGC-3′), AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′), and c-myc element (5′-CCCCAACACCTGCTGCCTGAG-3′) were also included. Other components, poly(dI:dC), T_{18}, and heparin were added to assess the effect on DNA binding as

described in the text. After 30 min at room temperature, 2.5 μl of 50% glycerol, 0.01% bromophenol blue was added. Samples were loaded on a non-denaturing 7.5% polyacrylamide mini-gel (8 cm × 10 cm) containing 0.25× TBE buffer (final concentration: 1.25 mM boric acid, 12.5 mM Tris, 0.25 mM EDTA), unless otherwise stated. Running buffer was 0.25× TBE, and 130 V was applied for 45 min at room temperature unless otherwise stated. Gels were dried and exposed to phosphorscreen (Molecular Dynamics) overnight for autoradiography. Densitometry was performed using Scion Image (Scion Corporation, Frederick, MD, USA) or the phosphorimager software.

2.9. Determination of the apparent C/EBP dissociation constant and C/EBP concentration in liver extract

Dilution of [^{32}P]-labeled ACEP24(GT)$_5$ at constant nuclear extract concentration (22 ng/μl) was used in an EMSA experiment. The results were analyzed and quantified by phosphorimaging and the apparent dissociation constant, K_d, was estimated by a Scatchard plot. The result was the apparent $K_d \approx 0.17$ nM. To estimate the concentration of C/EBP binding activity, a constant concentration of [^{32}P]-ACEP24(GT)$_5$ (1.6 nM) was then used with different dilutions of nuclear extract. The amount of extract giving a 50% shift of the DNA under these conditions was then taken as $K_{0.5}$. The apparent concentration of C/EBP in the extract was then derived from the relationship [28,29]:

$$K_{0.5} = K_d + \tfrac{1}{2}[\text{C/EBP}]_{tot}$$

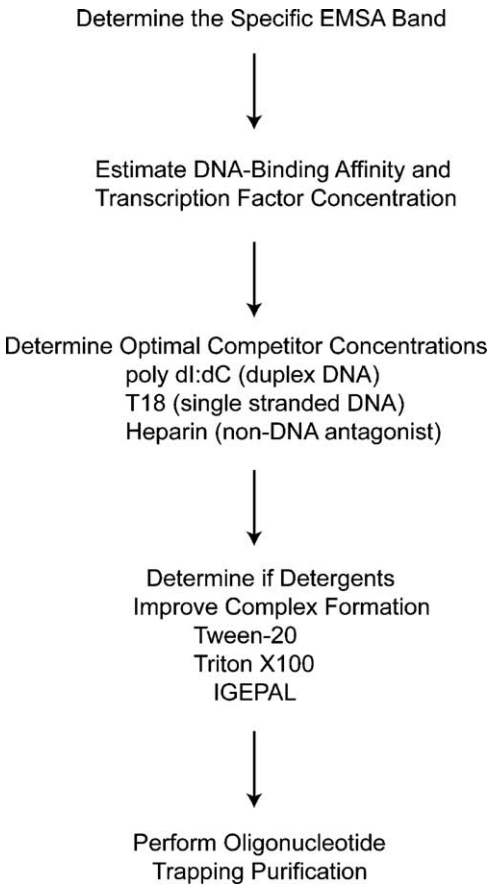

Fig. 1. Flow chart depicting the experimental approach to determination of optimal conditions for the oligonucleotide trapping method. The chart diagrams a generally applicable scheme for oligonucleotide trapping as follows: competition assay, concentration of transcription factor in extract, competitor concentrations, and detergent effects.

3. Results

3.1. Systematic optimization strategy

Fig. 1 outlines the approach taken to use EMSA as a tool to optimize oligonucleotide trapping. The first step is determination of the EMSA band representing the specific complex. Estimating the DNA-binding affinity (apparent K_d) of C/EBP for ACEP24(GT)$_5$ follows. The amount of C/EBP in the nuclear extract is next determined (see Section 2). The effect of various competitor concentrations is performed next and as each competitor is optimized, it is added to the mix and used for further testing so that the complete combination of all competitors is refined in the final mixture. The identification of detergents that improve specific band shift follows. Once all of these parameters are optimized using EMSA, large-scale transcription factor purification is performed.

3.2. Determine the specific EMSA band

Fig. 2 identifies the shifted band of the specific C/EBP–ACEP24(GT)$_5$ complex. EMSA is performed using 5′-end labeled ACEP24(GT)$_5$ and a crude rat liver nuclear extract which will eventually be used as the source material for purification. In Fig. 2, a 100- and 200-fold molar excess of cold (unlabeled) ACEP24(GT)$_5$ competed away the complex (C) band present in lane NC (no cold), while a 10-fold excess had a smaller effect. In contrast, excess AP-1, an unrelated duplex DNA, was unable to compete away this band at any tested concentration, indicating that ACEP24(GT)$_5$ is specifically bound. A band of slower migration rate than the C/EBP complex that appears in some rat liver nuclear extract preparations is labeled "*". Notice that it migrates near a band found with a bacterial extract containing a GFP fusion protein previously described [22] which contains C/EBP sequences (GC) and also binds the ACEP24(GT)$_5$ oligonucleotide; this fusion protein is also used in other experiments as a marker and positive control. Similar results were observed in other experiments and when using the EP18 (an oligonucleotide also containing the CAAT element) and c-myc oligonucleotides as specific and non-specific competitors, respectively (data not shown).

Also, because the incubation buffer commonly used in EMSA differs from the buffer (TE0.1) used in oligonucleotide trapping, a comparison of the effect of both buffers

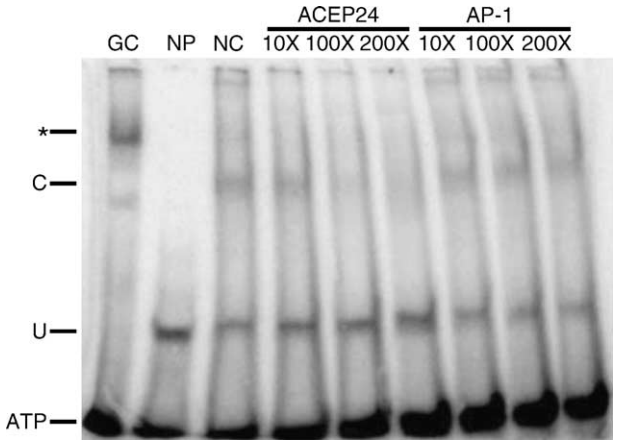

Fig. 2. Electrophoretic mobility shift competition assay identifies the specific complex. 1.6 nM labeled ACEP24(GT)$_5$ was used. Cold specific competitor ACEP24(GT)$_5$ and cold non-specific competitor AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) were added individually at 10-, 100- and 200-fold molar excess over labeled ACEP24(GT)$_5$. Poly(dI:dC) was constant at 40 μg/ml. The figure shows ACEP24(GT)$_5$ but not AP-1 competes C/EBP complex formation. NP, no protein; GC, GFP-C/EBP-α bacterial extract; NC, no cold competitor; C, specific shift complex; U, unshifted double-stranded DNA. The position of residual ATP is also indicated. A band of higher migration rate than C/EBP that appears in some rat liver nuclear extract preparations but not in all is labeled "*". The incubation buffer in this experiment was (final concentration): 10 mM Tris (pH 7.5), 1 mM EDTA, 40 mM NaCl, 4% glycerol, and 1 mM 2-mercaptomethanol).

on the C/EBP–ACEP24(GT)$_5$ complex formation in EMSAs was done. Using TE0.1 in place of the EMSA incubation had no deleterious effects on specific band formation (data not shown). All subsequent EMSA experiments used TE0.1.

3.3. Estimate DNA-binding affinity and concentration of transcription factor

To make the purification most selective, low DNA concentrations should be used, but the amount must be sufficient to bind all of the transcription factor present in the extract. Therefore, the DNA affinity and the abundance of the transcription factor must be determined. This is shown in Fig. 3. In panel A, is a representative experiment where nuclear extract at a final dilution of 70-fold was used with different radiolabeled DNA concentrations to measure binding in a gel shift. In panel B is shown Scatchard analysis of the gel shift. While the data show some scatter, they yield an estimate of $K_d = 0.17$ nM ACEP24(GT)$_5$ in this experiment. Three separate determinations gave $K_d = 0.17 \pm 0.01$ nM (mean ± standard deviation). Using different sequences that contain the same element, others reported $K_d = 0.14$ nM for C/EBP-α [30], in good agreement with our results. These estimates of apparent K_d are not thermodynamically correct values but are sufficient for our purpose. Fig. 3B also provides an estimate of the C/EBP concentration. The maximum binding (B_{max}) of 0.089 nM for a 70-fold dilution provides an estimate of 6.2 nM C/EBP in the undiluted nuclear extract.

Using an ACEP24(GT)$_5$ concentration that is greater than the K_d allows for an independent estimation of the C/EBP protein concentration in nuclear extract (see Section 2). When the concentration of DNA is much greater than K_d, binding becomes essentially stoichiometric with protein concentration and provides a measure of it. Shown in Fig. 3C, is a typical experiment where 5′-end labeled ACEP24(GT)$_5$ is held constant at 1.6 nM, approximately 10-fold greater than the apparent K_d, and is titrated with a 2-fold serial dilution of nuclear extract. The densitometry results show the complete data from four separate experiments (Fig. 3D). A 50% shift occurred at a 6.2-fold dilution. This shows that a 6.2-fold diluted nuclear extract is approximately 0.8 nM C/EBP dimer (i.e., 50% of the 1.6 nM DNA is shifted), and the initial estimate is that the extract is about 4.9 nM C/EBP. Correcting for the K_d (0.17 nM) effect on this determination (using the equation in Section 2.8) yields a refined estimate of 6.2 nM C/EBP dimer in this nuclear extract. This is the same nuclear extract used for Fig. 3A and B which gave an estimate for C/EBP dimer which was also 6.2 nM based on Scatchard analysis. Thus, either method gives a sufficiently accurate measure of the concentration of C/EBP for our experiments. Furthermore, the concentration of ACEP24(GT)$_5$ was kept constant at 1.6 nM for all subsequent experiments and is near the concentration that will ultimately be used for the C/EBP purification.

One caveat is that the binding experiments in Fig. 3 would measure the concentration of any binder that gave a shift at the same position as the complex marked on the gels and there is no assurance that only C/EBP binding is being measured. However, the experiments shown below will strongly suggest this is the case.

The concentration of C/EBP was determined using the graphical method described above when discussing Fig. 3D for five different nuclear extracts prepared in our laboratory. These values ranged from 6.2 to 28 nM (16.1 ± 7.8 nM). Thus, there is an approximately four-fold range in these values. We adapted our purification for these differences as will be described further.

3.4. Determine optimal competitor concentration

Non-specific binding proteins may bind ACEP24(GT)$_5$ during trapping chromatography and result in decreased yield and purity. Addition of competitors, substances that can bind instead of ACEP24(GT)$_5$, can reduce the amount of non-specific binding. We also used competitors in Section 3.1 and Fig. 2 to identify the specific complex. Some of these, such as the AP-1 or c-myc oligonucleotides could certainly be used as effective competitors but we decided instead to employ other substances which can be generally used for any transcription factor. Since the ACEP24(GT)$_5$ has a double-stranded element and a single-stranded tail, we began with generic double- and single-stranded competitors, poly(dI:dC) and T$_{18}$, respectively. The effects of adding different competitors to the trapping mix were tested using EMSA. At some

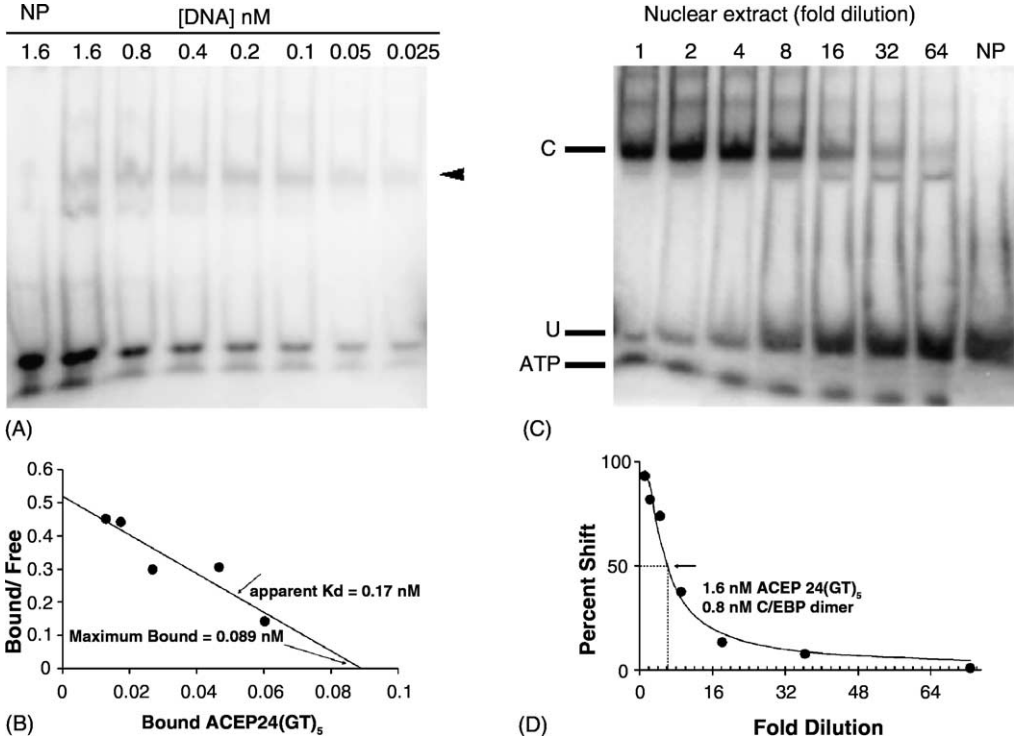

Fig. 3. Electrophoretic mobility shift assay allows estimation of the C/EBP concentration. Shown are two different methods. In panel A, no protein (NP) or an amount of nuclear extract equivalent to a 70-fold total dilution was mixed with 1.6 nM radiolabeled ACEP24(GT)$_5$ or a 2-fold serial dilution of the same. After 30 min at room temperature, the mixtures were applied to a 7.5% acrylamide gel for electrophoresis. The arrowhead shows the position of the specifically shifted band. The density of the specifically shifted band (C), the unshifted DNA band (U), and the total density for each lane (T) was used to calculate the concentration bound (=[ACEP24(GT)$_5$] × C/T) and the free DNA concentration ([ACEP24(GT)$_5$] × U/T). In panel B, the bound and free concentrations are plotted. The slope is equal to $-1/K_d$ and the abscissa intercept is the maximal binding for this nuclear extract dilution. In panel C, a two-fold serial dilution of the same rat liver nuclear extract (220 ng/μl stock) was incubated with 1.6 nM radiolabeled ACEP24(GT)$_5$. The fold dilution is shown above the gel. Poly(dI:dC) was constant at 40 μg/ml. This figure demonstrates the estimation of C/EBP concentration in rat liver nuclear extract. C, specific shift complex; U, unshifted double-stranded DNA; NP, no protein added to shift. In panel D, the percent shift for each dilution (here the dilutions are corrected for the further dilution of 22 μl of diluted extract in the 25 μl gel shift mixture) is plotted (data points are the mean, and error bars indicate standard deviation for each point, $n = 4$). The line shown was fit iteratively to the solution of the equation: $K_d = (D \times P)/\mathrm{DP}$, where D and P are the free (unbound) concentrations of DNA and C/EBP, respectively, and DP is the concentration of the complex. Inserting the conservation equations, this becomes: $K_d = (D_0 - \mathrm{DP})(P_0 - \mathrm{DP})/\mathrm{DP}$, where the subscripted zero denotes the total concentrations. Solving this for DP yields a quadratic equation whose solution is: $\mathrm{DP} = 0.5 \times \{(K_d + P_0 + D_0) - \sqrt{(-K_d - P_0 - D_0)^2 - 4P_0D_0}\}$. This latter equation was the one fit iteratively, setting $K_d = 0.17$ nM and varying P_0 until the best fit was obtained. The result is given in the text.

low concentration, competitors will have little effect, while at higher concentrations, competitors may have deleterious effects on specific binding. For the subsequent purification, we chose the highest concentration of each competitor that has a positive effect or does not have a deleterious effect on specific binding in EMSAs.

3.4.1. Poly(dI:dC)

Poly(dI:dC) shows a slight detrimental effect in the range 100–400 μg/ml but little effect from 6 to 50 μg/ml in a two-fold dilution series (Fig. 4). This and other results are independent of the amount of DNA shifted in EMSA (data not shown). In Fig. 4, sufficient nuclear extract was added to shift nearly 100% of the DNA. In other experiments with less nuclear extract and a 50% shift, similar results were obtained (data not shown). The amount of poly(dI:dC) that was chosen for trapping was 50 μg/ml, as this was the highest concentration that resulted in a band as dark or darker than that found with no dI:dC (ND) in Fig. 4. This concentration (50 μg/ml)

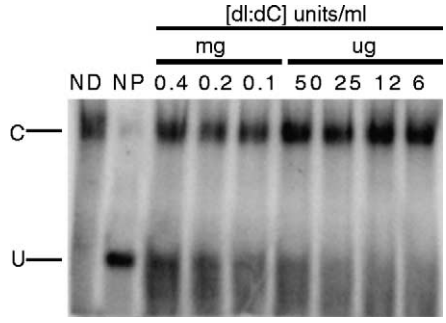

Fig. 4. Electrophoretic mobility shift assay with poly(dI:dC) displays no detrimental effects over a wide concentration range. A two-fold serial dilution of poly(dI:dC) from 400 to 6 μg/ml shows the highest concentration of poly(dI:dC) that does not interfere with complex formation is 50 μg/ml. C, specific shift complex; U, unshifted double-stranded DNA; ND, no poly(dI:dC); NP, no protein. These samples were run on a 10% polyacrylamide gel.

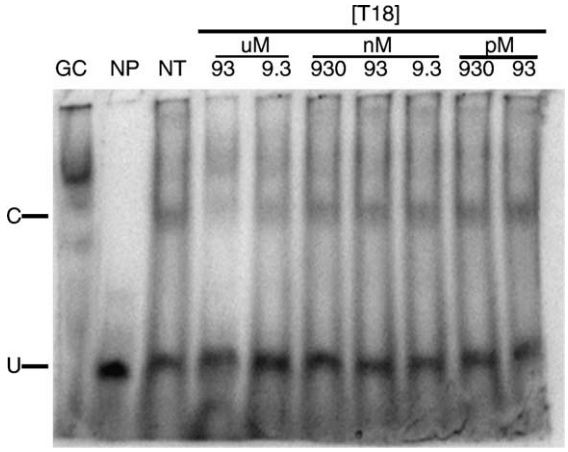

Fig. 5. Electrophoretic mobility shift assay indicates the highest concentration of T_{18} that does not interfere with specific band shift. A 10-fold serial dilution of T_{18} from 93 μM to 93 pM with poly(dI:dC) constant at 50 μg/ml. This figure shows the highest non-interfering concentration of T_{18} is 930 nM. C, specific shift complex. U, unshifted double-stranded DNA; GC, GFP-C/EBP-α bacterial extract; NP, no protein added to shift; NT, no T_{18}.

was then included in all subsequent EMSA experiments and eventually used for trapping purification.

3.4.2. T_{18}

Another competitor tested was the single-stranded DNA, T_{18}, from 93 μM to 93 pM in a 10-fold dilution series (Fig. 5). Poly(dI:dC) was present at a constant concentration of 50 μg/ml throughout. The range from 930 nM to 93 pM did not significantly inhibit complex formation as compared to the lane with no T_{18} (NT). However, 93 and 9.3 μM T_{18} had an inhibitory effect. Similar results were observed in another experiment (data not shown). The amount of T_{18} that was chosen for trapping was 930 nM, as this is the highest concentration that does not significantly interfere with shifting.

3.4.3. Heparin

We also tested the non-DNA, anionic polysaccharide heparin as a competitor. Heparin has previously been shown to directly compete with DNA for transcription factor binding [31]. Over the range 500 μg/ml to 500 pg/ml in a 10-fold dilution series (Fig. 6), poly(dI:dC) and T_{18} were present at constant concentrations of 50 μg/ml and 930 nM, respectively. The complex was slightly diminished by 500 ng/ml (Fig. 6) as compared to no heparin (NH) and other experiments show that even higher concentrations of heparin further diminished the complex (data not shown). The range 50 ng/ml to 5 pg/ml had little or no deleterious effects on shifting. Similar results were observed in three other experiments (data not shown). The amount of heparin that was chosen for trapping was 50 ng/ml, since this is the highest concentration that does not significantly interfere with shifting and this concentration was included in all subsequent experiments.

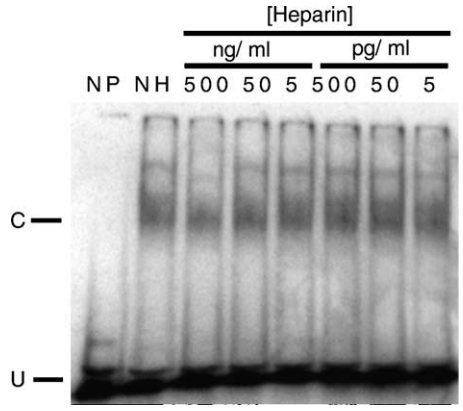

Fig. 6. Electrophoretic mobility shift assay with heparin shows the highest concentration of heparin that does not interfere. A 10-fold serial dilution of heparin from 500 ng/ml to 5 pg/ml with poly(dI:dC) constant at 50 μg/ml and T_{18} constant at 930 nM. Shown in this figure, the highest non-interfering concentration of heparin is 50 ng/ml. C, specific shift complex; U, unshifted double-stranded DNA; NH, no heparin; NP, no protein.

3.5. Detergents

In DNA affinity chromatography, detergents improve the yield of C/EBP [32] and have been shown to dramatically affect complex formation for many different transcription factors reviewed in [33]. We tested the effect of some detergents on the C/EBP–ACEP24$(GT)_5$ complex formation in the presence of the optimized dI:dC, T_{18}, and heparin competitors. Shown in Fig. 7 is the plotted densitometry results of tested detergents. Tween-20 (TW), Triton-X (TX) and IGEPAL (IG) increase the amount of the specifically shifted band the greatest of the detergents tested. However, deoxycholic acid (DC) decreased the amount of specifically shifted band. Finally, to show the effect of an interfering detergent, SDS was used and significantly reduced the amount of specifically shifted band. Tween-20 was chosen for trapping experiments. Thus, we now have a double-stranded (dI:dC, 50 μg/ml) and single-stranded DNA (T_{18}, 930 nM) and non-DNA polyanionic sugar (heparin, 50 ng/ml) which can be used to lessen non-specific binding and a detergent (0.1% Tween-20) which improves DNA binding.

3.6. Perform oligonucleotide trapping purification

At this point, all conditions are known for the purification. Since the apparent K_d is 0.17 nM, 1.34 nM ACEP24$(GT)_5$ was chosen for trapping since this 7.9-fold excess would ensure approximately 90% binding. The nuclear extract used contains 22 nM C/EBP dimer and was diluted to 0.2 nM for trapping to ensure that ACEP24$(GT)_5$ is in excess over C/EBP subunits and to also dilute C/EBP to near its apparent K_d (0.17 nM). A volume of 50 ml was chosen for trapping since this volume can be loaded onto a 1 ml column in 2 h or less. Typically, this corresponds to 110 μg nuclear extract per 50 ml. Since we had previously shown that binding of C/EBP to DNA occurs at 0.4 M NaCl but not at 1.2 M [1], TE0.4

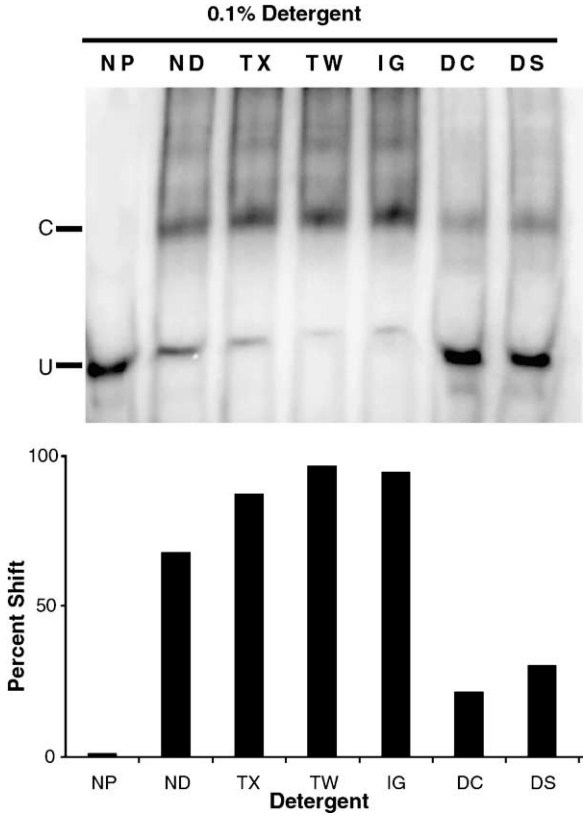

Fig. 7. Densitometry of electrophoretic mobility shift assay of detergents indicates (TW) Tween-20, (TX) Triton-X100, and (IG) IGEPAL increase the specific shift band. C, specific shift complex; U, unshifted double-stranded DNA; NP, no protein; ND, no detergent; DC, deoxycholic acid; DS, sodium dodecylsulfate. Poly(dI:dC), T_{18}, and heparin constant at 50 μg/ml, 930 nM, and 50 ng/ml, respectively. Percent maximal shift is plotted as the mean, and error bars indicate standard deviation, $n = 4$.

(10 mM Tris (pH 7.5), 1 mM EDTA, 0.4 M NaCl) was used for the trapping incubation buffer and column washing while TE1.2 (10 mM Tris (pH 7.5), 1 mM EDTA, 1.2 M NaCl) was used for elution. The 50 ml trapping mixture contained the previously defined competitor and detergent concentrations (i.e., 50 μg/ml poly(dI:dC), 50 ng/ml heparin, 930 nM T_{18}, 0.1% Tween-20) while washing and elution were with TE0.4 and TE1.2, respectively, containing 0.1% Tween-20 but no competitors. Under these conditions, oligonucleotide trapping was performed. In Fig. 8, EMSA (with no competitors) was performed to monitor the chromatography. To clearly observe the complex (C), electrophoresis was prolonged. In the flow through (unretained) fraction (FT), two faint bands are observed which are also found in the rat liver nuclear extract and one of these has the same mobility as the specific complex. Thus, some small fraction of C/EBP is probably not retained under these conditions. The wash fraction (W) also shows a faint band with a mobility similar to the C/EBP complex, however, Western blotting with a specific antibody against C/EBP-α (see further) failed to detect this protein (data not shown) and it may be an unrelated protein. Fractions eluted in TE1.2 (F1–F3) resulted in one shifted band

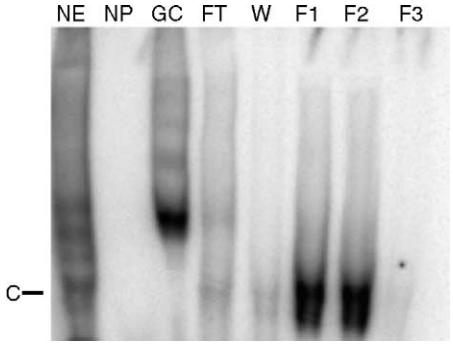

Fig. 8. Electrophoretic mobility shift assay of C/EBP activity during purification. The 50 ml trapping mix in TE0.4 was applied to a 1 ml $(AC)_5$–Sepharose column, and the column washed with 20 ml of TE0.4, 0.1% Tween-20. TE1.2, 0.1% Tween-20 was applied to elute the column. The first 5 ml of flow through and wash were collected. The TE1.2 elution fractions were collected in three 5 ml fractions. A 500 ul of each fraction was removed and concentrated using Microcon centrifugal filter devices (Millipore Corporation, Bedford, USA) for use in EMSA. No competitors were used, and EMSA incubation was performed in TE0.1. Samples were loaded on a large (16 cm × 14 cm) 5% gel for EMSA. This figure demonstrates DNA binding activity in TE1.2 elution fractions of similar relative molecular weight as C/EBP complex in rat liver nuclear extract. C, specific shifted complex. NE, rat liver nuclear extract; NP, no protein; GC, His-tag purified GFP-C/EBP-α; FT, flow through fraction; W, wash fraction; F1–F3, elution fractions in TE1.2.

at the mobility corresponding to the specific C/EBP complex and clearly contains the majority of the C/EBP activity.

In Fig. 9, a silver stained gel and Western blot with a specific C/EBP-α antibody are shown of elution fraction 1 (F1). Two bands of relative molecular mass between 31 000 and 42 000 appear on the silver stain gel. Also, some higher molecular mass bands are present and data suggest these are gel artifacts (see Section 4). The lowest band was estimated by a log molecular mass versus R_f plot to have relative molecular mass of 32 000. The second band from the bottom was estimated to have a relative molecular mass of 38 000 (data not shown). The molecular masses of the C/EBP-α isoforms are 42 000 and 30 000, [34,35] and the molecular masses of C/EBP-β isoforms are 38 000, 35 000, and 21 000 [36]. The two lower molecular mass bands are thus in the molecular weight range of C/EBP-α and the C/EBP-β isoforms. The purified proteins were subjected to Western blotting. In Fig. 9, antibody 14AA, an antibody that recognizes both C/EBP-α p42 and C/EBP-α p30 was used to stain the eluted fraction, F1. C/EBP-α was present in the eluted fractions and is the lower band seen in the silver stained gel. The fraction tested for the presence of C/EBP-α stained with 14AA at a molecular mass of approximately 32 000, thus, suggesting that C/EBP-α p30 was purified. Neither the 14AA C/EBP-α antibody nor the C/EBP-β Δ-198 antibody ever stained the 38 000 molecular mass band, suggesting it is not C/EBP (data not shown). Applying the trapping mixture to a column of $(AC)_5$–Sepharose in the absence of ACEP24$(GT)_5$ and collecting the flow through (pre-clearing) prior to applying the trapping mixture in the presence of ACEP24$(GT)_5$ to $(AC)_5$–Sepharose did not improve purity (data not shown).

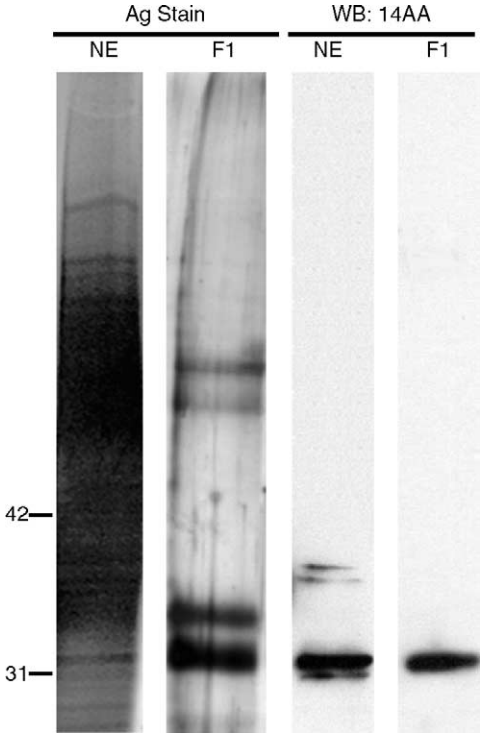

Fig. 9. Silver stain and Western blot of oligonucleotide trapping fractions. Carbonic anhydrase (31 000) and ovalbumin (42 000) molecular masses are indicated. NE, rat liver nuclear extract. Fraction F1 was collected starting as TE1.2 reached column. Silver staining of a TE1.2 elution fraction (F1) shows two highly purified and isolated proteins of similar relative molecular weight as C/EBP-α isoforms. A Western blot using 1:100 SC-61 (14AA anti-C/EBP-α antibody) demonstrates the presence of C/EBP-α in rat liver nuclear extract, and confirms the isolation of C/EBP-α using the oligonucleotide trapping method. One microgram of NE was added in both the silver stain and Western blot. Silver stain F1, 1/5 of total fraction was used. Western blot F1, 1/20 of total fraction was used.

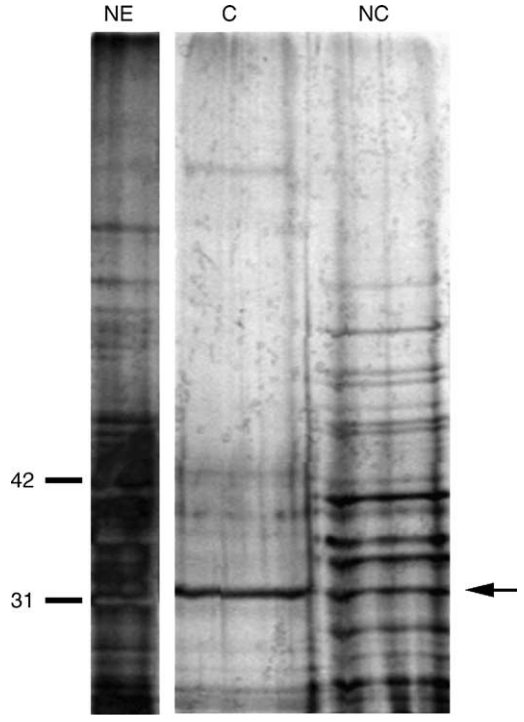

Fig. 10. Silver stain of oligonucleotide chromatographic fractions under optimized and non-optimized conditions. Carbonic anhydrase (31 000) and ovalbumin (42 000) molecular masses are indicated. NE, rat liver nuclear extract. Trapping purification under optimized conditions using competitors and detergent is shown in lane C. Purification under non-optimized conditions is shown in lane NC. For either, 1 ml rat liver nuclear extract was used. For the latter (NC), the undiluted nuclear extract and 5 nmol ACEP24$(GT)_5$ were mixed at 4 °C for 30 min and then added to the 1 ml $(AC)_5$–Sepharose column. For the optimized purification (C), the nuclear extract was diluted to 50 ml containing 1.6 nM ACEP24$(GT)_5$ and the concentrations of competitors and detergents given in the text. Other conditions are as in the legend to Fig. 8. Both lanes C and NC were collected starting as TE1.2 eluted the column. An arrow to the right of the gel shows the expected position of C/EBP-α p30.

Oligonucleotide trapping under these conditions was performed nine times with similar results. While some of these purifications gave even higher purity, the results shown in Figs. 8 and 9 are representative data. From these, we used densitometry of silver stained gels calibrated with known amount of carbonic anhydrase and ovalbumin, proteins of similar size to C/EBP, to determine the amount of C/EBP purified in the lower, C/EBP-α p30 band. The results for four different preparations was 2.1 ± 1.1 μg (66 ± 34 pmol, mean ± standard deviation). Assuming that the 50 ml trapping mixture contained 0.2 nM C/EBP dimer, it would contain 0.64 μg (20 pmol). The most likely explanation for the higher than expected yield is that our initial estimate of the concentration of C/EBP present in the nuclear extract was too low but within a factor of about three.

The optimized purification was clearly successful but the question remained whether these refinements of highly dilute DNA (1.6 nM) and protein (0.2 nM) and the addition of competitors was responsible. To answer this, 1 ml of undiluted nuclear extract (6.2 nM C/EBP dimer) was mixed with 5 nmol ACEP24$(GT)_5$ without using competitors and purified by trapping on $(AC)_5$–Sepharose. Side-by-side, the optimized procedure, also with 1 ml nuclear extract but diluted to 50 ml, with 1.6 nM DNA and competitors, was performed. The comparison is shown in Fig. 10. In this purification and a few others, the use of competitors (C) resulted in a single predominant band of the C/EBP-α p30 isoform while with no competitors (NC), at least seven strong bands and a greater number of lesser bands were found on the silver stained gel, the most abundant of which is not C/EBP-α p30. Thus, the optimized procedure leads to dramatically higher purity. For comparison, the starting material nuclear extract (NE) is shown; clearly either approach leads to purification though the optimized purification alone gives a nearly homogeneous product.

4. Discussion

Several methods have been developed for the purification of transcription factors [20]. Many of these purification methods suffer from contaminating proteins and low

yield for several reasons. Coupling DNA to supports often results in the modification of attached DNA and can affect the DNA interaction with the transcription factor of interest [1,33,37]. Some methods allow coupling of DNA to supports without DNA modification. However, many of these supports bind proteins in a non-specific manner such as in the biotin–avidin and biotin–NeutraAvidin methods [1]. Further, in methods where DNA is immobilized on the column, or carried out in solution, the DNA is often in the μM range and promotes non-specific DNA binding. This non-specific binding results in contamination by non-specific DNA-binding proteins [1].

All of these potential drawbacks are addressed in the DNA trapping method [1]. By coupling the $(AC)_5$ oligonucleotide with a 5′ aminohexyl group to Sepharose with CNBr chemistry, attachment is primarily through the aminohexyl group and DNA modification is lessened. Furthermore, any modification is confined to the $(AC)_5$ sequence and cannot affect the DNA element (CAAT) present in the trapping oligonucleotide, ACEP24$(GT)_5$. Sepharose is also a support that has only weak interactions with proteins or DNA. In DNA trapping, binding between the transcription factor of interest and DNA occurs in solution. Concentrations of DNA can be adjusted to levels that do not favor non-specific interactions. Here we used 1.34 nM DNA. To further reduce non-specific DNA–protein interactions, competitors are added to the trapping mixture. Previous use of the DNA trapping method resulted in highly purified target protein in a single-step purification [1]. Recently, oligonucleotide trapping was used to purify the insulin promoter RIPE3b1 activator protein for the first time and allowed it to be identified as MafA, a member of the large Maf transcription factor family [21]. However, the concentrations of DNA, target transcription factor, and competitors used were determined by chromatography in a tedious trial-and-error process.

In this paper, we describe the systematic experimental determination of optimal concentrations of oligonucleotide, transcription factor, and competitors that allows for a generally applicable trapping method for the purification of transcription factors. We outlined this systematic experimental plan in a flow chart (Fig. 1). The chart diagrams a scheme for using EMSA to estimate DNA-binding affinity, transcription factor concentration, effective competitor concentrations, and detergent effects. EMSA was chosen for these experiments because all of the proteins that bind ACEP24$(GT)_5$ are visible and individually quantifiable. Thus, allowing the identification and observation of the C/EBP–ACEP24$(GT)_5$ complex and non-specific DNA binding ACEP24$(GT)_5$ complexes under variable conditions. Previously, EMSA was used to discover an appropriate competitor concentration for dI:dC [38], here, we have expanded this approach to refine protein, DNA, three competitors, and detergents to yield a very refined purification scheme.

The specific C/EBP band in the EMSA experiments was determined (Fig. 2.) Once the C/EBP band was identified, the dissociation constant, K_d, of C/EBP for ACEP24$(GT)_5$ was estimated by an oligonucleotide serial dilution to be $K_d = 0.17$ nM (Fig. 3A and B). This approach agrees with values previously reported [30] and was of sufficient accuracy for our purposes. The concentration of C/EBP in the rat liver nuclear extract was determined from these same data and confirmed by performing a protein serial dilution and identifying the extract dilution that resulted in a 50% shift of ACEP24$(GT)_5$ (see Section 2) (Fig. 3). The C/EBP concentration in the nuclear extract was estimated to be 6.2 nM by these two different methods. While we used some fairly sophisticated methods to analyze these data, this was probably unnecessary. If K_d were already known or could be estimated, a simple titration with protein of a relatively high amount of DNA (as in Fig. 3C and D) and graphically estimating the dilution giving a 50% shift should be of sufficient accuracy to design an appropriate oligonucleotide trapping purification protocol.

We then determined the optimal concentrations of poly(dI:dC), heparin, and T_{18} to be 50 μg/ml, 50 ng/ml, and 932 nM, respectively (Figs. 4–6). The optimal concentration for each was determined as the highest concentration of competitor that did not decrease the C/EBP band density compared to adding no competitor. As each competitor was optimized, it was included in all subsequent experiments. Because detergents are often used in purifications to increase yield, we tested several detergents to see if any had a positive effect on the binding of C/EBP to ACEP24$(GT)_5$. Of the detergents tested, 0.1% Tween-20 increased the C/EBP band density the most, though IGEPAL and Triton would have also been good choices (Fig. 7).

For a new transcription factor, the appropriate concentration for each of these agents may be different and could be determined by the described methods. These optimized conditions were then used for the oligonucleotide trapping chromatography experiment. Following the activity, we observed that C/EBP was eluted in TE1.2, and little was found in the flow through and wash fractions (Fig. 8). This result shows that at 0.2 nM C/EBP and 1.34 nM ACEP24$(GT)_5$, the majority of C/EBP is bound and at these concentrations there is little interference from non-specific DNA-binding proteins in the trapping experiment. Silver staining of a TE1.2 elution fraction shows two distinct, well-separated bands of 32 000 and 38 000 molecular mass (see Section 3). Both of these bands are in the range of relative molecular weights of known C/EBP-α and C/EBP-β isoforms, the two dominant C/EBP family members expressed in rat liver. To confirm that either or both of the suspect bands were C/EBP, we stained the fraction (F1) in a Western blot with 14AA, an antibody reactive with both isoforms of C/EBP-α (Fig. 9). In rat liver nuclear extract (NE) several bands developed. In F1, one band appeared that was the same molecular mass as the 32 000 molecular mass band seen in the silver stain, and reflected a band seen in the nuclear extract, confirming that C/EBP-α was purified. F1, was also probed with a different antibody cross-reactive with C/EBP family members including C/EBP-α and C/EBP-β, (the Δ-198 antibody from Santa

Cruz Biotechnology) (data not shown). The antibody reacted only poorly with nuclear extract and not at all with fraction F1 in multiple experiments. Because of the weak reaction, the results are inconclusive.

The 38 000 molecular mass band has not been identified, but some possibilities exist to explain this outcome. Another C/EBP family member might have been purified, as all C/EBP proteins are able to form heterodimers in all intrafamilial combinations and, with the exception of C/EBP-ζ, interact with an identical recognition sequence in vitro [39–50]. Also, the C/EBPs can form protein–protein interactions with other bZIP and non-bZIP factors including; NF-kB, CREB,/ATF, AP-1, glucocorticoid receptor, and the retinoblastoma protein (Rb) [51-54]. The possibilities are too large to test them all and proteomic techniques have so far failed to identify this protein (unpublished data).

As mentioned in Section 3, two other, high molecular weight bands are present on the silver stained gel (Fig. 9). These bands appear on gels stained in this way when the freshly prepared SDS-PAGE sample buffer alone is electrophoresed and cannot be proteins. They can be eliminated by eliminating 2-mercaptoethanol from the buffer and reappear if 2-mercaptoethanol is replaced by dithiothrietol. These two bands are thus gel artifacts somehow related to the presence of sulfhydryl reducing agents and were not investigated further.

Two proteins were purified using the oligonucleotide trapping method. Fig. 9 shows that the proteins are sufficiently separated for individual extraction from the gel and could be used for microsequencing. Microsequencing requires less than 1 pmol under ideal conditions and the average 66 pmol obtained by trapping should allow the successful identification of even novel proteins. It was unnecessary here since an antibody was available for positive identification of C/EBP.

5. Conclusions

EMSA can be used systematically to discover conditions necessary for specific transcription factor–DNA complex formation. By forming this complex in solution, the investigator has nearly complete control of the factors—DNA and transcription factor concentration, competitors to inhibit non-specific binding, and detergents that augment binding—which are necessary for specific binding and reduction of non-specific binding. Such precise control of conditions would be difficult if not impossible on a column in standard forms of DNA affinity chromatography and there would be no efficient way, such as EMSA, to model the effect of each component of the stationary phases. Oligonucleotide trapping uses chromatography only for binding the specific complex once formed and for effective elution. The procedures described high yield and purity in a single purification step and should be applicable to any DNA-binding protein.

Acknowledgement

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