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Transcription factor–green fluorescent protein chimeric fusion proteins and their use in studies of DNA affinity chromatography

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Abstract

A new plasmid, pJ22, was produced by introducing the enhanced green fluorescent protein (GFP) coding sequence into the pET28 plasmid while retaining much of the multiple cloning site. This new plasmid was then used to produce a chimeric fusion protein containing the DNA-binding region of the rat liver CAAT enhancer binding protein (C/EBP) fused to the COOH-terminus of GFP. This new GFP–C/EBP fusion protein also contains $(His)_6$ to facilitate purification by Ni^{2+}–agarose and several other useful features. The plasmid and protein were developed to allow us to more rapidly investigate the DNA–Sepharose affinity chromatography of transcription factors. The GFP–C/EBP protein is virtually identical in its DNA-binding properties to a well-characterized, bacterially expressed protein called C/EBP 62 which has been shown to mimic rat wild-type C/EBP DNA-binding. GFP–C/EBP also binds to DNA–Sepharose which contains the CAAT element and is eluted by a salt gradient. Salt-dependent elution was highly temperature-dependent over the range of 4–19°C. Since temperature-dependent DNA-binding has also been reported for other DNA-binding proteins, this may also occur with other transcription factors. DNA-affinity chromatography gave higher purity than that obtained by Ni^{2+}–agarose chromatography and chromatography on the same DNA–Sepharose column at two different temperatures resulted in the greatest purification, to near homogeneity. This temperature-dependent affinity chromatography provides an important new approach to transcription factor purification.

Keywords: DNA; Proteins; Green fluorescent protein

1. Introduction

Our laboratories investigate high-performance affinity chromatography of transcription factors on DNA-supports with the aim of improving the recovery and purity obtained. It has become clear that many of the techniques used to detect and quantify transcription factors are cumbersome and time-consuming when analysis of a large number of chromatographic samples is required. For example, electrophoretic mobility shift assays (EMSA) require that each individual sample (e.g., column fraction) be mixed with radiolabeled DNA, electrophoresed, and autoradiographed [1,2]. This technique requires days to complete on even a limited number of samples and the results are at best semi-quantitative in routine use. Filter binding assays [3] would improve this situation somewhat but unfortunately this technique has never been successfully applied to several of the transcription factors we investigate. Immunological techniques such as enzyme-linked immunosorbent assay (ELISA) require high quality antibodies which

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are simply not available for some transcription factors. Because of these limitations, we have developed a chimeric fusion protein containing the DNA-binding sequence of the rat nuclear CAAT binding protein (C/EBP) at the COOH-terminus of the green fluorescent protein to learn if such fusions retain DNA binding activity and facilitate detection. The green fluorescent protein originated from the bioluminescent jellyfish, *Aequorea victoria* [4]. The variant we used is actually an artificial gene coding for a variant of this protein with enhanced fluorescence occuring at higher excitation wavelengths than the wild-type [5]. Rat liver nuclei C/EBP binds the CAAT regulatory element present in some viruses and in the 5′ untranslated region of many mammalian genes. It is the archetypal member of the basic leucine zipper motif DNA-binding proteins [6,7].

The plasmid we developed, pJ22, was produced using the pET28a plasmid which adds a $(His)_6$ sequence for easy purification by Ni^{2+}-affinity chromatography, the T7 antigen for easy immunological detection, and utilizes T7 RNA polymerase for high level expression. The pJ22 plasmid was prepared in such a way that chimeras with other proteins can be produced easily.

These techniques proved successful and led to the discovery of potentially useful effects of temperature on the elution of C/EBP from DNA–Sepharose.

2. Methods

2.1. Plasmid construction

Plasmid pEGFP-N1 was from Clonetech (Palo Alto, CA, USA) and pET28a from Novagen (Madison, WI, USA). Thermostable *pfu* DNA polymerase was from Stratagene (La Jolla, CA, USA).

2.1.1. pJ22

A 40-ng portion of pEGFP-N1, 50 pmol each of GFP5′ (**G CCG GGA TCC** ATG GTG AGC AAG GGC GAG) and GFP3′ (**GG GAA TTC** CTT GTA CAG CTC GTC CAT GCC) oligonucleotides, and ten units of *pfu* DNA polymerase were used to prime a 100 μl polymerase chain reaction (25 cycles, 95°C, 1 min 15 s, 50°C, 45 s, 72°C, 3 min). Sequences shown in bold were used to generate unique *Bam*HI and *Eco*RI restriction sites, respectively. The 717 base pair (bp) DNA, coding for full-length enhanced green fluorescent protein (E-GFP), was gel purified, digested with *Bam*HI and *Eco*RI, repurified, and ligated into a pET28a vector also restricted with the same enzymes and gel purified. The ligation mixture was then used to transform strain BL21 (DE3) *Escherichia coli*. Screening was by restriction mapping of miniprep DNA; however, positive clones also had a distinctive green color. A single positive clone was streaked on a kanamycin containing LB agar plate and a single colony was used to prepare cell stocks and purified plasmid. The plasmid is referred to as pJ22.

2.1.2. pJ22–CEBP

A pT5 plasmid containing full-length rat nuclear C/EBP-α was the generous gift of Drs. Steve McKnight (Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, USA) and Jon Shuman (Laboratory of Molecular Structure, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, USA). A 40-ng portion of plasmid, 50 pmol each of CEBP5′ (**GGA CAA GCT TCT** CGC ACC GGC GGC GGC GGC) and CEBP3′ (**C GGC CTC GAG** TCA CGC GCA GTT GCC CAT GGC GAC) oligonucleotides, and ten units of *pfu* DNA polymerase were used to prime a 100 μl polymerase chain reaction (25 cycles, 95°C, 1 min 15 s, 50°C, 45 s, 72°C, 3 min). The bold sequence in CEBP5′ produces a unique *Hind*III site used for cloning while the CEBP3′ bold sequence were used for purposes unrelated to this report. The resulting 300 bp insert DNA (coding for C/EBP′s COOH-terminal 99 amino acids) was digested with *Hind*III and gel purified. The vector was prepared by digesting pJ22 with *Eag*I, filling the overhang with Klenow large fragment DNA polymerase, digesting with *Hind*III, and was then gel purified. Following ligation, and transformation of *E. coli* BL21 (DE3), miniprep DNA was screened by restriction mapping. Authenticity was confirmed by DNA sequencing.

2.2. Electrophoretic mobility shift assay

To assess DNA-binding properties, protein (typically 2.5 μg of purified GFP–C/EBP or GFP or 9

μg crude bacterial extract containing C/EBP 62) was mixed in a total volume of 25 μl with 20 000 cpm (typically, 20–60 pmol) of 5′-^{32}P-EP24 oligonucleotide (5′-^{32}P-GCTGCAGATTGCGCAATCTGCAGC), 40 μg/ml poly (dI–dC) DNA, 10 m*M* Tris (pH 7.5), 1 m*M* EDTA, 40 m*M* NaCl, 4% glycerol, and 1 m*M* 2-mercaptoethanol. After 20 min at room temperature, 2 μl of 50% glycerol, 0.01% bromophenol blue was added and the samples loaded on a nondenaturing 10% polyacrylamide gel in 0.25× TBE buffer (1.25 m*M* boric acid, 12.5 m*M* Tris, 0.25 m*M* EDTA) and 150 V was applied for 45 min. Gels were then dried and exposed to Kodak X-OMAT film for 1–4 h for autoradiography.

2.3. Chromatography

DNA–Sepharose was prepared by mixing 37.5 nmol EP18 oligonucleotide (5′NH_2-ethyl-GCAGATTGCGCAATCTGC) with 0.5 g of preactivated CNBr–Sepharose (Pharmacia Biotech, Piscataway, NJ, USA) in a total volume of 4.0 ml of 0.1 *M* $NaHCO_3$, 0.5 *M* NaCl (pH 8.3) at room temperature for 24 h on a rotating wheel type mixer. Remaining reactive groups were end-capped by reaction with 0.1 *M* Tris, 0.5 *M* NaCl (pH 8) for 2 h. A 1-ml portion of the resulting DNA–Sepharose (~1.8 ml total) was packed into a 1-ml syringe column for testing. Columns when not in use were stored in 0.1 *M* NaCl in TE (10 m*M* Tris, 1 m*M* EDTA, pH 7.5) containing 10 m*M* NaN_3. Similarly, a variant of EP24 (see above) containing a 5′NH_2-ethyl moiety was also coupled and used for some experiments.

Columns were equilibrated in 0.1 *M* NaCl in TE and then 0.1 ml of protein (e.g., 0.5 mg/ml GFP–C/EBP) was applied. The column was eluted with a gradient from 0.1 *M* NaCl to 1.2 *M* NaCl in TE. Details of the gradient are given in the figure legends. One ml fractions were collected and their fluorescence was determined (λ_{ex}=395 nm, λ_{em}=515 nm, band pass 16 nm for each) on a fluorimeter (SLM Aminco Bowman Series 2) set so that 50 μg/ml GFP–C/EBP would give a reading of six volts.

2.4. Protein purification

For most experiments, purified GFP or GFP–C/EBP was prepared by growing and inducing (with IPTG) expression from *E. coli* BL21 (DE3) containing either the pJ22 or pJ22–C/EBP plasmid, respectively, using Ni^{2+}–agarose (Novagen His-Bind Resin) and the procedures recommended by Novagen except with the following modifications: induction was with 1 m*M* IPTG for 2–3 h. The bacteria were recovered by centrifugation, washed, and suspended in 20 ml 'bind buffer' (5 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 7.9 containing 1 m*M* phenylmethylsulfonyl fluoride) for each 500 ml of culture. Bacteria were homogenized by sonication in an ice–water slurry (three times of 30 s sonication alternated with 30 s cooling). After centrifugation, the extract was mixed on a tube rotator for 30 min with 5 ml of Ni^{2+} charged His-Bind resin. The resin was recovered by centrifugation (1000 *g*, 2 min) and washed three times with 20 ml portions of bind buffer. After the final wash, the resin was resuspended in bind buffer, packed into a column, and further washed and eluted with imidazole as suggested by Novagen. This batch absorption was necessary to abate unwanted proteolysis of the fusion proteins.

Protein concentration was determined by the method of Bradford [8] with bovine serum albumin as the standard.

3. Results

In Fig. 1A is shown the region of the pJ22–C/EBP plasmid which produces the GFP–C/EBP fusion protein. In Fig. 1B is shown a region of the pJ22 multiple cloning site before it was modified with the C/EBP DNA; pJ22 contained seven restriction sites useful for producing fusion proteins in different reading frames. Since pJ22 is derived from the pET28a plasmid, it contains a T7 RNA polymerase promoter and terminator and a lac operator to allow high level expression in response to IPTG induction. The plasmid also confers kanamycin resistance and has an f1 origin for single strand production. pJ22 is a 6086 bp plasmid which yielded over 100 μg plasmid DNA from 200 ml of an overnight culture in rich broth. The bacteria have a distinctive green color. In Fig. 1C is shown a schematic of the GFP–C/EBP fusion protein.

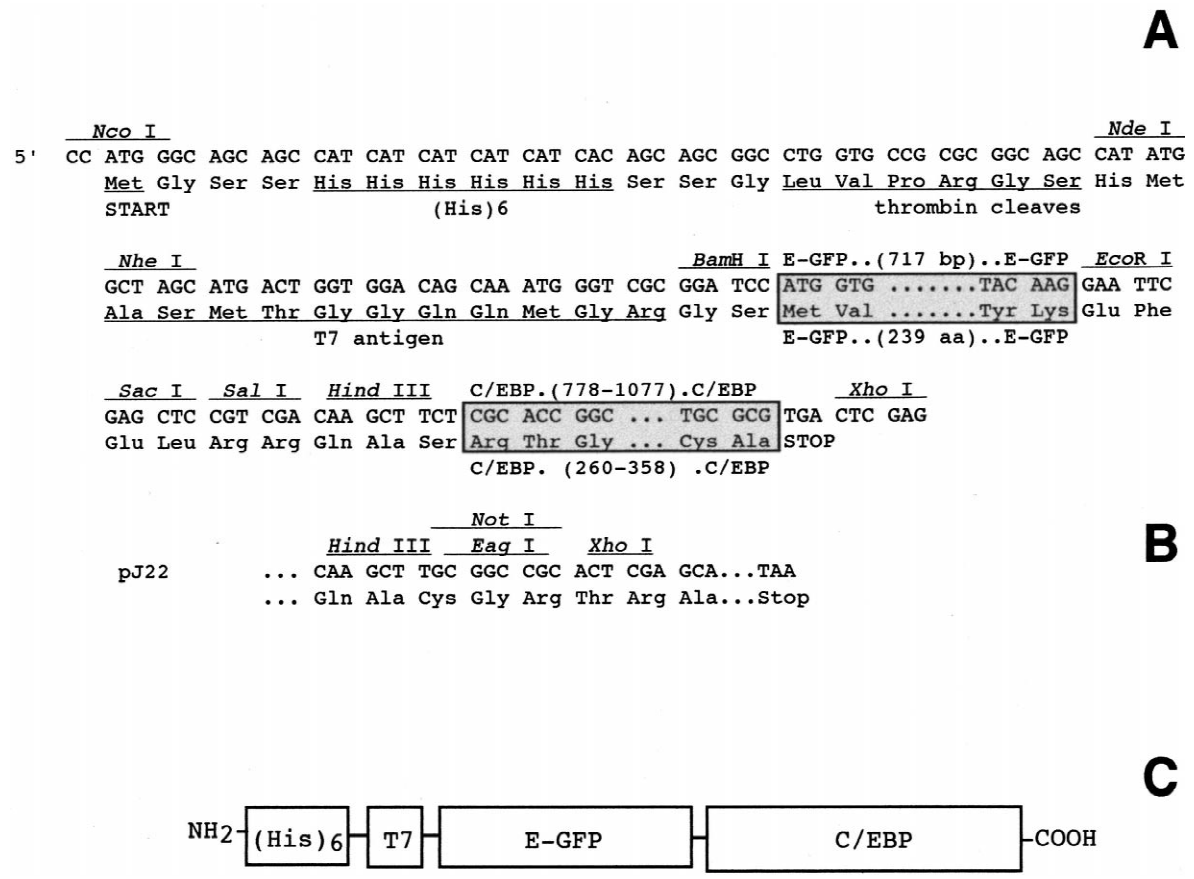

Fig. 1. pJ22 and pJ22-C/EBP plasmids. (A) The region of the pJ22-C/EBP plasmid which produces the chimeric fusion protein is diagrammed. The position of unique restriction sites and other useful regions of the DNA and protein sequence are shown. (B) The region of pJ22 is shown before modification with the C/EBP DNA insert. (C) The GFP–C/EBP fusion protein produced is shown schematically.

In Fig. 2 is shown an sodium dodecyl sulfate (SDS)-polyacrylamide gel of the GFP and GFP–C/EBP fusion protein after purification on Ni^{2+}–agarose using a batch absorption method. Per liter of culture, 4–5 mg of purified, soluble protein was obtained in either case. The molecular masses measured from the gel were 36 000 and 46 000 for the GFP and GFP–C/EBP proteins comparable to the calculated values of 33 200 and 42 600, respectively. Prior to using batch absorption (see Section 2), we had found that much of the protein was hydrolysed to a lower molecular mass form. Also, while protein purity appears high on this Coomassie stained gel, higher protein loads or using silver staining reveals a number of contaminants (data not shown but see Fig. 7 below).

To assess the DNA binding properties of the GFP–C/EBP fusion protein, it was compared by EMSA to C/EBP 62, the 62 amino acid (C/EBP Asn^{281}–Gly^{340}) COOH-terminus DNA-binding domain which was well characterized by Shuman et al. [6]. The results in Fig. 3 show that while the GFP sequences alone cause no shift, either C/EBP 62 or GFP–C/EBP bind and shift the mobility of the radiolabeled EP24 DNA. EP24 is a self-complementary oligonucleotide (as is EP18) which spontaneously forms the double-stranded DNA which C/EBP binds. When the protein binds, it shifts the electrophoretic mobility of the DNA due to the increase in mass. The differences in the shifted position observed for C/EBP 62 and GFP–C/EBP (Fig. 3) is due to differences in the protein molecular masses,

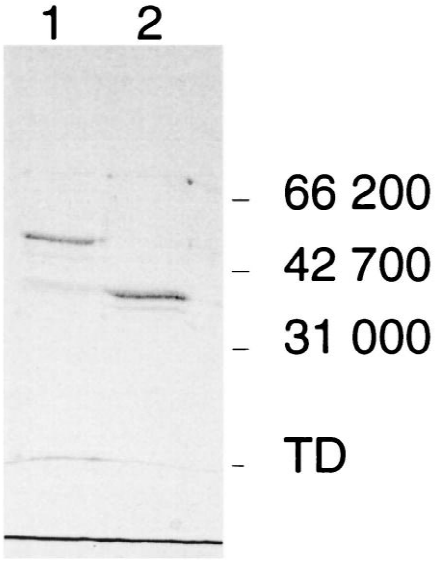

Fig. 2. Purified GFP and GFP–C/EBP are of the expected size. Shown is a 10% SDS-polyacrylamide gel [9] of 1 μg each of GFP–C/EBP (lane 1) and GFP (lane 2) after purification on Ni^{2+}–agarose using the described batch procedure. The position of molecular mass markers is also shown. The gel was stained with Coomassie Brilliant Blue.

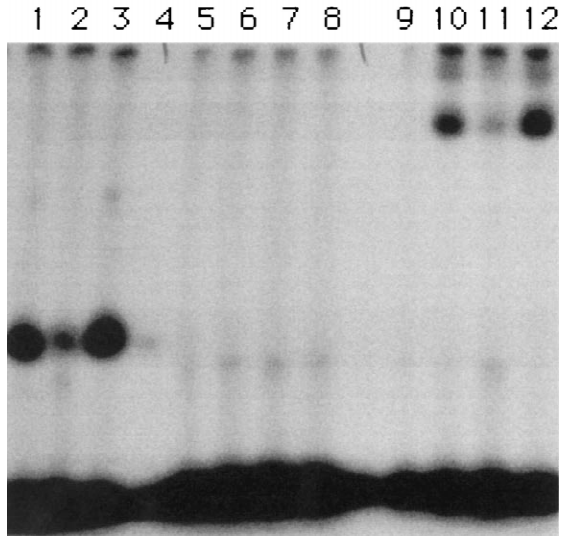

Fig. 3. Binding to the CAAT motif DNA shows similar specificity for both the fusion protein and a well characterized, recombinant C/EBP protein. Shown is the autoradiogram of an electrophoretic mobility shift assay. Radiolabeled EP24 oligonucleotide (13.5 pmol per lane) containing a high affinity CAAT element [7] is present in each lane of the nondenaturing gel and is present alone in lanes 4 and 9. Three different proteins were tested: C/EBP 62 (9 μg crude extract per lane, lanes 1–3), GFP (0.32 μg purified protein (9.6 pmol) per lane, lanes 6–8), and GFP-C/EBP (0.65 μg purified (15 pmol), lanes 10–12). In lanes 1, 5, and 10, protein was added. In lanes 2, 7, and 11, protein was added along with a 100 pmol of unlabeled EP24 (7.4-fold molar excess over the radiolabeled amount) to show specific competition for binding. In lanes 3, 8, and 12, protein was added along with a 100 pmol of an irrelevant double-stranded oligonucleotide (in this case, 5S ICR, 5′ - GAAGCCAAGCAGGGTCGGGCCTGGTAGTACTTGGATGGGAGAC, the 'internal control region' element involved in regulating 5S RNA production, annealed to the complement sequence) showing a lack of competition.

7400 vs. 42 600, respectively. Furthermore, for either C/EBP 62 or GFP–C/EBP, unlabeled EP24 oligonucleotide (containing the CAAT element) effectively competes for the radiolabeled oligonucleotide while the unrelated, double-stranded 5S ICR oligonucleotide (lacking CAAT) does not compete demonstrating the specificity of the DNA interaction.

The affinity of crude C/EBP 62 and purified GFP–C/EBP was further compared using competitive EMSA in the experiment illustrated in Fig. 4. Clearly, both proteins show similar affinities for the competitor (EP18) DNA relative to the radiolabeled probe (EP24). The dried gel was also analysed by two-dimensional radiometric scanning using the Packard Instant Imager (data not shown). The result was that half displacement of radiolabeled EP24 occurs at 5.1 μ*M* EP18 for C/EBP 62 versus 1.8 μ*M* for GFP–C/EBP. Given the different state of purity of the two proteins, this less than three-fold difference in affinity is probably too small to be relevant and we conclude that the two proteins show nearly equivalent DNA-binding properties.

That the C/EBP sequences are necessary for DNA-binding is also clear from the chromatogram in Fig. 5. In this case, the EP18 oligonucleotide (containing a 5′ aminoethyl moiety) was attached to CNBr-activated Sepharose and used for chromatography. GFP flows through this column unretained while a portion of the GFP–C/EBP is retained and is eluted by the salt gradient at fraction 30. Other experiments have demonstrated that the GFP–C/EBP protein only binds to columns which contain the CAAT element and do not bind to unrelated DNA columns such as double-stranded ICR-Sepharose (data not shown).

In Fig. 6 is one of the discoveries we have made

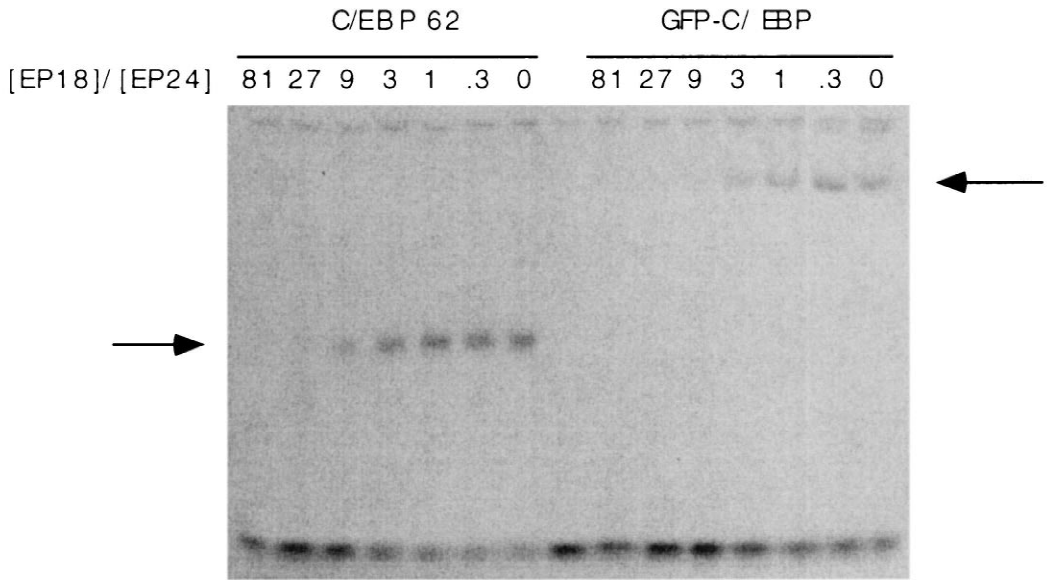

Fig. 4. Competitive EMSA shows that C/EBP 62 and GFP–C/EBP have similar affinities for the CAAT element. Each assay contained 0.72 μ*M* radiolabeled EP24 oligonucleotide (23 400 cpm) and either no competitor or the molar ratio of EP18 oligonucleotide shown. On the left side of the autoradiogram are results obtained using 5 μl of 1.8 mg/ml crude bacterial extract containing the C/EBP 62 protein and on the right 5 ml of 0.5 mg/ml purified GFP–C/EBP. Arrows indicate the position of the shifted radiolabeled oligonucleotide.

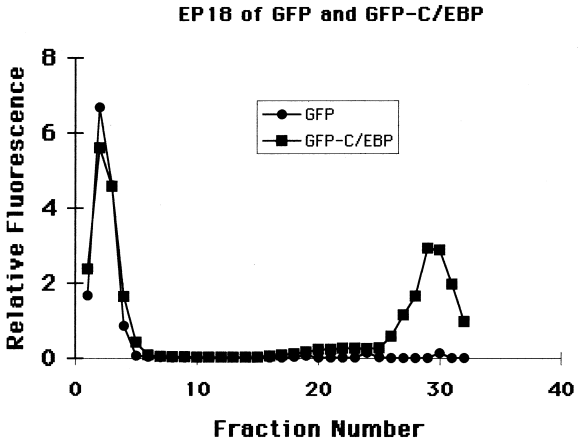

Fig. 5. Fluorescent C/EBP fusion protein also binds a specific DNA–Sepharose and elutes with increasing salt concentrations. Chromatograms under identical elution conditions are shown for the GFP (8 μg, circles) and GFP-C/EBP (32 μg, squares) applied to the EP18-Sepharose column. The column buffer was TE (10 m*M* Tris, 1 m*M* EDTA, pH 7.5) containing NaCl. Elution was by a NaCl gradient consisting of 66 min of constant 0.1 *M* NaCl, followed by a linear gradient to 1.2 *M* NaCl over the next 20 min, and finally constant 1.2 *M* NaCl for the next 64 min. The flow-rate was 0.3 ml/min throughout and 1.0 ml fractions were collected.

using this technology. The same DNA–Sepharose column was eluted using a salt gradient at two different temperatures, 19°C and 4°C, using a carefully calibrated chromatograph. Clearly, GFP–C/EBP elutes much later in the 4°C gradient. This temperature-dependence was investigated by performing chromatography at 4°C and 19°C, alternately, on seven different occasions. The results are summarized in Table 1. The fraction number and salt concentration was determined for the peak eluted fraction in each experiment and the results averaged. In all experiments, GFP–C/EBP eluted earlier at 19°C and at lower [NaCl] and the difference with 4°C is statistically different to a high confidence level ($P \leq 0.0001$).

We also purified the GFP–C/EBP protein by both Ni^{2+}- and DNA-affinity chromatography in side-by-side experiments from the same induced bacteria extract. For these experiments, the batch absorption purification was not used so that both kinds of column chromatography could be compared. The same amount of crude bacteria extract was added to the Ni^{2+} and EP24 columns and chromatography was performed at 4°C using the usual elution proto-

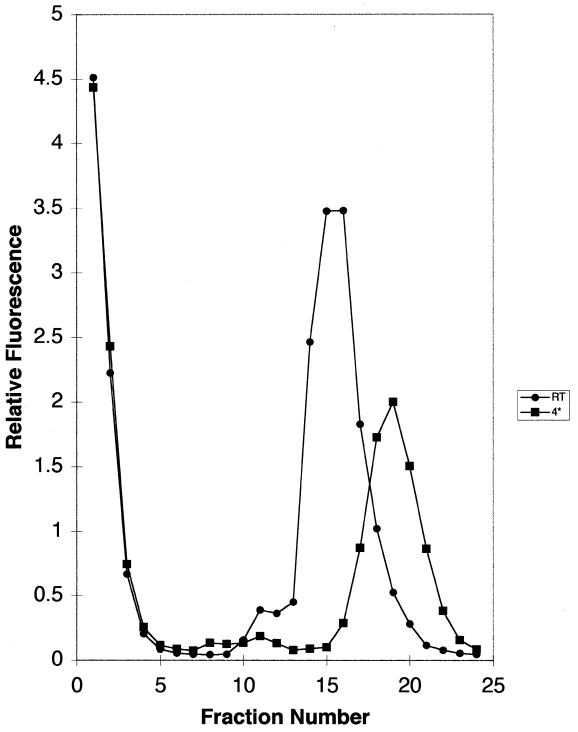

Fig. 6. Chromatography was highly temperature dependent. Before each chromatogram, the chromatograph was equilibrated at either 4°C (in the cold room, ■) or 19°C (room temperature (RT), ●) and the flow-rate carefully calibrated. The flow-rate was 0.3 ml/min and fractions were 1 ml. In each case, 0.1 ml of 0.5 mg/ml of GFP–C/EBP was loaded on the column. The gradient was a linear increase from 0.1 *M* to 1.2 *M* NaCl in TE over 66 min followed by constant 1.2 *M*.

cols for the two columns. As the results in Table 2 show, both procedures gave comparable yields but the DNA-affinity chromatography gave higher purity. These data would suggest that the Ni^{2+}–agarose purified protein can be no more than about 37% pure. The pooled fractions from the 4°C DNA–Sepharose chromatography were then applied to the same column at room temperature. Sufficient fluorescence was eluted to quantify the yield obtained (2%) but unfortunately so little protein was present that it could not be accurately quantified to determine if higher purity was obtained by this approach.

To answer this question of whether higher purity could be obtained, the purification procedure was repeated (this time using the EP18 column) and the protein obtained was applied to a 10% SDS polyacrylamide gel which was silver stained using the kit from BioRad Laboratories (Hercules, CA, USA). The result is shown in Fig. 7. The crude extract (Cr) and Ni^{2+}–agarose purified proteins are clearly overloaded on this gel and under these conditions even minor protein constituents become visible. The Ni^{2+}–agarose purified protein on Coomassie stained gels (not shown) reveals that the major species present is the proteolysed form of C/EBP indicated by the asterisk in the figure with some full-length C/EBP also present. Batch adsorption circumvents this proteolysis (Fig. 2) but was not used here so that the two kinds of chromatography could be more fairly compared. The DNA–Sepharose purified material obtained by a single passage over the column at 4°C is much purer (Fig. 7) in agreement with the balance sheet results (Table 2) but still has some impurities. It is also proteolysed about 50%. A second passage through the column at room temperature (RT) removes the impurities and results in highly purified C/EBP and its truncated form (*).

4. Discussion

To improve the purification of transcription factors by DNA affinity chromatography, we began a comparative study of several different transcription factor families. C/EBP was chosen as a representative of the basic leucine zipper motif family. While we

Table 1
Summary of salt gradient elution of EP24 DNA–Sepharose elution at two different temperatures

Temperature (°C)	Fraction number[a]	*P*	[NaCl] (*M*)[b]	*P*
4	19.7±1.2	0.00003	1.03±0.1	0.0001
19	15.0±0.8		0.77±0.03	

[a] Fraction number of the peak eluted fraction is given as the mean±the standard deviation for seven separate experiments ($n=7$).
[b] Molar [NaCl] of the peak eluted fraction determined by conductivity.
P=probability that the means for the two temperatures are not different.

Table 2
Balance sheet for the purification of GFP–C/EBP by Ni^{2+}–agarose and DNA–Sepharose

Fraction	Total[a] fluorescence (%F)	Total protein (mg)	Yield (%)	Purification fold
Crude	139.1	5.1	100	1
Ni^{2+}–agarose	47.1	0.06	34	39
1st DNA–Sepharose, 4°C	40.4	0.03	29	104
2nd DNA–Sepharose, 19°C	2.6	N.D.	2	N.D.

[a] Total fluorescence and protein are corrected for volumes saved out and resulted from purification from 5 ml of crude bacterial extract. N.D.=not determined.

could always detect the presence of C/EBP by electrophoretic mobility shift assays, when this was applied to the hundreds of fractions resulting weekly from our experiments, the assay quickly became tedious to the point of being unworkable. Since we have fluorescent detectors which can be used in-line with the chromatography, producing fluorescent chimeric fusion proteins seemed to be a very feasible approach.

To produce these chimeras, we began with the very versatile pET28a plasmid and modified it by inserting the green fluorescent protein coding sequences in such a way that much of the multiple cloning site was available for incorporating transcription factor DNA-binding sequences. The new plasmid, pJ22, was then used with C/EBP to test whether such chimeras would retain fluorescence and the DNA-binding properties sought. This resulted in

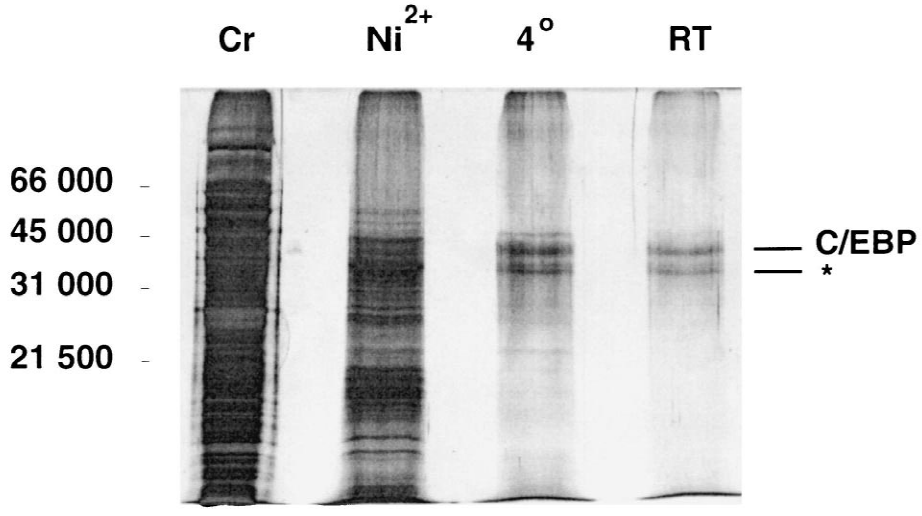

Fig. 7. GFP–C/EBP was highly purified by temperature dependent affinity chromatography. Shown is a silver stained 10% SDS polyacrylamide gel of fractions obtained from a balance sheet experiment such as that described for Table 2. For purification, 5 ml of the crude extract (Cr) was applied either to a 1 ml Ni^{2+}–agarose column (Ni^{2+}) or to a 1 ml DNA–Sepharose column at 4°C (4°C) made with the EP18 oligonucleotide. The columns were then washed in the usual way and eluted (with 1 *M* imidazole for Ni^{2+}–agarose or a NaCl gradient for DNA–Sepharose) and the eluted peak pooled. A portion of the DNA–Sepharose eluted material was then diluted with an equal amount of TE and reapplied to the same DNA–Sepharose column except this time at room temperature (RT, 19°C). From left to right on the gel: the crude bacterial extract (Cr, 4 μg), Ni^{2+}–agarose eluate (Ni^{2+}, 0.84 μg), the first DNA–Sepharose eluate obtained at 4°C (0.33 μg), and the second eluate obtained at room temperature (0.29 μg). The position of molecular mass markers is indicated to the left of the gel. To the right is indicated the position of full length GFP–C/EBP (C/EBP) and a smaller, partially proteolysed fragment derived from GFP–C/EBP (*).

the expression of milligram amounts of a highly fluorescent and specific DNA-binding protein which effectively mimics the DNA-binding properties of C/EBP.

An important observation which resulted during these experiments was to show that DNA-affinity chromatography is highly temperature dependent. This has also been observed now with the *lac* repressor protein (unpublished data) and may be generally true. To recognize specific DNA sequences, transcription factors must interact with purine and pyrimidine bases which reside in the interior of the double helix, a hydrophobic environment composed of stacked bases and no water. Thus, binding would be expected to involve hydrophobic interactions. Hydrophobic interactions are typically entropically driven and thus are temperature-dependent. This temperature-dependence of DNA-binding has been demonstrated for *lac* repressor and *Eco*R I restriction endonuclease [10] but has apparently gone unreported for C/EBP. Furthermore, the base-stacking involved in double helix-formation and the 'melting' (dissociation) of the double-stranded DNA make the state of the DNA on the column also temperature dependent. Which of these temperature dependent phenomena account for the temperature dependence of elution must await further investigation, however, the effect is potentially useful in any case.

Transcription factors and other DNA-binding proteins have typically been eluted from DNA–supports using salt gradients. It has also been common practice in transcription factor purification to repeat chromatography on DNA–supports. For example, Kadonaga [11] suggested that all DNA affinity chromatography be performed at 4°C and that samples from one round of purification be reapplied to the column. The results in Figs. 6 and 7 suggest that if chromatography is to be repeated, choosing a different temperature may greatly enhance purity. The effect of temperature on DNA-binding has only been studied in a very few cases. Temperatures 14°C apart showed nearly a ten-fold difference in binding affinity for two different proteins studied [10]. Here, chromatography performed 14°C apart showed markedly different elution behavior with the peak eluted fractions separated by four fractions and over 13 min in time. While it is not possible to translate these chromatographic measurements directly into the effect of temperature on the binding constant, clearly temperature does affect binding of C/EBP to DNA and the effect is sizable and of practical use for transcription factor purification.

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