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Purification of the c-*fos* Enhancer-Binding Protein

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We have purified the c-*fos* enhancer-binding protein from HeLa cell nuclear extracts. The key purification steps involved chromatography on a nonspecific DNA affinity column, from which binding activity and other proteins were eluted at low salt concentrations, followed by chromatography on a specific oligonucleotide affinity column, from which the enhancer binding activity was specifically eluted at high salt concentrations. The purified protein had a molecular weight of approximately 62,000 and contained the binding activity, as demonstrated by the renaturation of the activity from a sodium dodecyl sulfate-polyacrylamide gel slice. The purified protein had a strong affinity for the c-*fos* enhancer dyad symmetry sequence, with an equilibrium dissociation constant of 3.3×10^{-11} M. This affinity was at least 50,000-fold stronger than that found for nonspecific DNA sequences.

Expression of the c-*fos* proto-oncogene is induced rapidly by a number of mitogenic agents including serum, polypeptide growth factors, phorbol ester tumor promoters, diacylglycerol, and the calcium ionophore A23187 (2, 7, 9, 14, 15, 19, 20). Induction of the c-*fos* gene therefore provides a model for understanding how these agents transmit their signals from either the cell surface or the cytoplasm to the nucleus.

Sequences that control the transcriptional induction of c-*fos* have been defined by mutagenesis and transfection experiments (4, 26, 27). These studies have defined an enhancerlike element 300 base pairs (bp) upstream of the transcriptional initiation site. This element is necessary for serum induction (26), as well as induction by epidermal growth factor and the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (5a). The enhancer element was also sufficient for serum induction, albeit at a lower maximal level, when fused to the human β-globin promoter (27).

Other c-*fos* promoter elements are also important for efficient expression of the gene. In the mouse c-*fos* gene, elements affecting the basal level of expression are located at −60, −100, and −145 bp relative to the transcriptional initiation site (6). In the human gene, sequence elements at −60 and −90 are necessary for the strong expression of the gene (5a). Although these elements in the human and mouse genes may play some role in the transcriptional regulation of c-*fos*, as opposed to serving simply as basal promoter elements, there is not yet sufficient evidence to support this possibility.

Gel mobility shift and footprint assays have led to the identification of factors which bind to each of the c-*fos* promoter and enhancer elements (6, 8, 22, 27). In addition, a factor binding to a sequence at −346 has been identified in BALB/c-3T3 cells that were treated with conditioned medium from v-*sis*-transformed cells (11).

The c-*fos* enhancer-binding protein (f-EBP) binds to a 20-base-pair sequence with dyad symmetry from −298 to −317 (6, 8, 22, 27). The level of binding activity to this sequence in derived nuclear extracts was significantly elevated in A431 epidermal carcinoma cells which had been treated with EGF to induce c-*fos* transcription (22). The binding activity, however, was not elevated during TPA or A23187 induction of c-*fos* in A431 cells. Although the increase in binding activity induced by EGF was concomitant with increased transcription, both reaching maximal values within 15 min, binding did not decrease during the subsequent rapid decline in transcription (within 2 h) (22). Furthermore, although the treatment of HeLa cells with either serum or epidermal growth factor induced transcription, neither of these agents caused a change in the binding activity, which remained elevated regardless of the inducing agent (22, 27; Fisch et al., submitted). These results suggest that c-*fos* activation via the enhancer may involve not only changes in the level of the binding activity, but also mechanisms (e.g., catalytic or stoichiometric interactions with other factors) which affect the transcriptional activity of the protein independently of its site-specific binding activity. Alternatively or in addition, there may be regulation through other sequence elements such that the enhancer and its associated factor are necessary but not sufficient for full induction. The exact mechanism of regulation could also vary for different inducing agents and in different cell types.

To better understand the regulation of the c-*fos* gene through its enhancer, we purified f-EBP from HeLa cell nuclear extracts by using a specific DNA affinity chromatography method similar to that developed by other groups (12, 13, 21, 23; C. Wu et al., submitted for publication). The method we used relied on (i) the binding of f-EBP to a nonspecific double-stranded DNA affinity column and its elution (along with other DNA-binding proteins) at low salt concentrations and (ii) tight binding of f-EBP to a specific oligonucleotide affinity column and its specific elution at high salt concentrations.

MATERIALS AND METHODS

DNA probes. The 138-bp probe used in the gel mobility shift assay extended from a *Pst*I site at −363 to an *Apa*I site at −225 (nucleotides relative to the site of transcription initiation). The fragment was derived from plasmid pF9 in which *Xho*I and *Bgl*II linker DNA had been placed at the *Pst*I and *Apa*I restriction sites, respectively. The 138-bp probe was labeled with the Klenow fragment of *Escherichia coli* polymerase I, [α-^{32}P]dATP, and [α-^{32}P]dCTP. The labeled fragment was purified on an 8% polyacrylamide gel.

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For the DNase I footprinting experiment the fragment was labeled at either the *Xho*I or *Bgl*II site with T4 polynucleotide kinase and [γ-^{32}P]ATP.

Complementary strands of the oligonucleotide RP1 (see Fig. 2C) were synthesized in an Applied Biosystems DNA synthesizer in the laboratory of Peter Model at Rockefeller University and annealed. This oligonucleotide was used both for coupling to CNBr-activated Sepharose 4B (Pharmacia, Inc.) and as either competitor or probe in the gel mobility shift assay. When used as a probe the oligonucleotide was ^{32}P labeled with the Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P]dATP. The labeled oligonucleotide was separated from free [α-^{32}P]dATP on a 1-ml Sephadex G50-80 column.

DNA-binding assays. The gel mobility shift assay was used essentially as described previously (22). Nuclear extracts or chromatographic fractions were incubated typically for 30 min at room temperature with 0.5 ng of DNA ^{32}P-labeled probe and 2 μg of salmon sperm DNA in 20 μl of binding buffer (10 mM Tris hydrochloride [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 0.05% Nonidet P-40 [NP-40], 100 μg of bovine serum albumin per ml, 5% glycerol). The reaction mixture was then loaded directly onto a 4% polyacrylamide gel in 0.25× TBE (25 mM Trizma base [Sigma Chemical Co.], 25 mM boric acid, 1 mM EDTA) and electrophoresed at 150 V for 1.5 h at room temperature. The gel was then dried and analyzed by autoradiography.

Incubations for the nitrocellulose filter-binding assay were as described above, except for the omission of salmon sperm DNA. The reaction mixtures were diluted with 400 μl of ice-cold wash buffer (10 mM Tris hydrochloride [pH 7.5], 50 mM KCl, 5% glycerol) and applied immediately to a 13-mm-diameter nitrocellulose filter with vacuum suction. The filter was then washed twice with 400 μl of wash buffer and subsequently analyzed for radioactivity in scintillation fluid. Each incubation was carried out in duplicate, and background radioactivity was subtracted. The latter was determined from duplicate incubations under identical conditions but without added purification fraction. The oligonucleotide affinity-purified fraction was diluted in BC100 (20% glycerol, 20 mM Tris hydrochloride [pH 7.9], 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol) with 0.05% NP-40 and 100 μg of bovine serum albumin per ml.

The binding curve obtained with various amounts of the 138-bp probe was plotted by the method of Scatchard (25). A best-fit line was determined for the data by a linear regression analysis. The equilibrium dissociation constant was determined from the slope of this line, and the margin of error was determined as the standard deviation of the results of four experiments. Poly(dG-dC) · (dG-dC) and poly(dA-dT) · (dA-dT) were obtained from Pharmacia, Inc.

Chromatography. Nuclear extracts from HeLa cells grown in spinner culture were prepared as previously described (5) and dialyzed in BC100 with 0.5 mM phenylmethylsulfonyl fluoride (PMSF). All chromatography was performed at 4°C. The phosphocellulose column was run in BC100 with 0.5 mM PMSF (except for the variations in KCl concentrations indicated [millimolar] in the names of the buffers). All subsequent columns were run in BC100-PMSF and 0.1% NP-40. Protein concentrations were determined by the method of Bradford (1). The salt concentrations were determined by measuring the conductivity relative to known standards. The activity of each fraction was determined by the gel mobility shift assay.

Nuclear extract (120 ml) was applied to a 60-ml phosphocellulose (P11) column at 1 column volume/h, washed with 1 column volume of buffer, and step eluted at a rate of 2 column volumes/h with 2 column volumes each of buffer containing 0.3 M, 0.5 M, and 1.0 M KCl. Peak protein fractions were pooled from each step elution.

The 0.3 M KCl step fractions from several P11 columns (80 ml; equivalent to a starting material of 183 ml of nuclear extract derived from 46 liters of HeLa cells at 10^6 cells per ml) were pooled, dialyzed for 4 h against BC100–PMSF–NP-40, and centrifuged at 10,000 × *g* for 10 min at 4°C to remove precipitated material. The resulting 0.3 M step fraction was loaded onto a DEAE-cellulose (DE52) column (30 ml) at 1.5 column volumes/h, washed with 30 ml of buffer, and step eluted with buffer containing 1.0 M KCl.

The DE52 flowthrough fraction (90 ml) was loaded onto the double-stranded DNA (dsDNA)-Sepharose 4B column (11 ml) at 5 column volumes/h, washed with 15 ml of buffer, and eluted with a 60-ml gradient of buffer containing 0.1 to 0.5 M KCl at 5 column volumes/h. Fractions were assayed for activity, and those with salt concentrations of roughly 0.1 to 0.3 M KCl were pooled. The pooled fractions were dialyzed against buffer without KCl (BC0–PMSF–NP-40) for 30 min until a conductivity equivalent to 0.1 M KCl was reached. This material (18 ml) was centrifuged at 10,000 × *g* for 10 min at 4°C (to remove precipitated material) and reloaded onto the dsDNA-Sepharose 4B column as described above, but step eluted with 2 column volumes each of buffer containing 0.3 and 1.0 M KCl.

The 0.3 M step fraction (6 ml) from the second dsDNA column was dialyzed against BC0–PMSF–NP-40 for 20 min, centrifuged as described above, and loaded onto a 0.4-ml oligonucleotide-Sepharose 4B column at 10 column volumes/h. The column was washed with 2 column volumes of buffer (BC100–PMSF–NP-40), 8 column volumes of BC300–PMSF–NP-40, and finally with BC1000–PMSF–NP-40, all at 6 column volumes/h.

The dsDNA-Sepharose 4B resin was generated by coupling 30 mg of sheared salmon sperm DNA to 10.5 ml of CNBr-activated Sepharose 4B as directed by the manufacturer. The oligonucleotide-Sepharose 4B resin was similarly prepared by coupling 1 mg of oligonucleotide RP1 to 3.5 ml of CNBr-activated Sepharose 4B.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (16). Low-molecular-weight protein markers (Bio-Rad Laboratories) were used, and the gel was silver stained by using a kit from Bio-Rad.

Renatured activity. The oligonucleotide affinity-purified fraction was heated to 95°C for 5 min in SDS-PAGE sample buffer (67 mM Tris hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 5 mM EDTA, 0.3% β-mercaptoethanol) and electrophoresed on an SDS–10% polyacrylamide gel. Protein from the SDS-PAGE gel slices was eluted and renatured as described previously (10), except that 0.1% NP-40 was included in the dilution buffer. Oligonucleotide affinity-purified fraction (2 μg) was loaded onto an SDS–10% polyacrylamide gel for this experiment. Of the renatured material (1 ml) from each gel slice, 5 μl was assayed in a 20-μl reaction mixture for the gel mobility shift assay with 1 ng of ^{32}P-labeled oligonucleotide RP1 and no added salmon sperm DNA. To demonstrate the specificity of binding, 50 ng of either specific (TF1) or nonspecific (TF2) oligonucleotide was included in the binding reaction mixture. TF1 is a 40-bp oligonucleotide spanning c-*fos* enhancer sequences, −318 to −283; TF2 is identical to TF1, except for four point mutations, two in each half of the dyad symmetry element (5a).

DNase I footprint. The single-end-labeled 138-bp probes (1

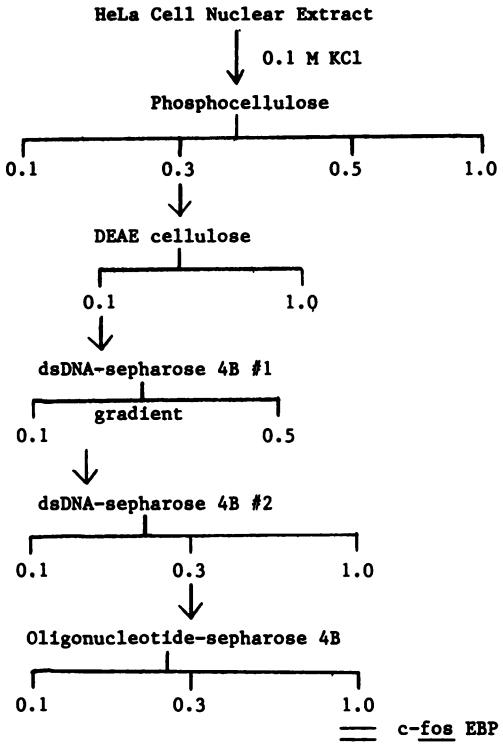

FIG. 1. Purification scheme for f-EBP. Each column was loaded at 0.1 M KCl. The molarity of KCl used to elute each fraction is indicated. Sheared salmon sperm DNA was used to generate the dsDNA-Sepharose 4B column, and the enhancer-specific oligonucleotide RP1 was used for the oligonucleotide-Sepharose 4B column.

ng), as described above, and 100 ng of salmon sperm DNA were incubated with various amounts of the oligonucleotide affinity-purified fraction under our standard conditions (20 μl; 30 min at room temperature). DNase I (1 μl of 10 μg/ml) and $MgCl_2$ (1 μl of 40 mM) were then added for 60 s at room temperature, and the reaction was stopped by the addition of 80 μl of stop buffer (7 mM EDTA, 66 mM sodium acetate, 0.3% SDS, 300 μg of yeast tRNA per ml). The DNA was isolated by phenol-chloroform and chloroform extractions followed by ethanol precipitation. The DNA was then analyzed on a 7 M urea–8% polyacrylamide sequencing gel. A G reaction of each probe was performed by the method of Maxam and Gilbert (18).

RESULTS

The f-EBP was purified from HeLa nuclear extracts by following the scheme in Fig. 1. The activity of f-EBP was monitored by a gel mobility shift assay (22), which involved the use of a 52-bp oligonucleotide (Fig. 2C) as the specific DNA probe. The key steps of the procedure were a nonspecific dsDNA affinity column, on which f-EBP eluted at low salt concentrations, and a specific oligonucleotide column, on which the activity eluted at high salt concentrations. A representative assay of the active fractions after each purification step is shown in Fig. 2A, and details of the entire purification procedure are presented in Table 1. The activity of each fraction was expressed in relative units, where 1 U was defined as the activity present in 1 ml of HeLa nuclear extract.

Chromatographic steps. Nuclear extract was applied to a phosphocellulose column at 0.1 M KCl and step eluted at 0.3, 0.5, and 1.0 M KCl. The majority of the activity eluted in the 0.3 M KCl step, although a significant amount of activity (20 to 30%) eluted at 0.5 M KCl and accounted for some of the losses on this column. The phosphocellulose 0.3 M KCl fraction was dialyzed to 0.1 M KCl and applied to a DEAE-cellulose column, which did not retain the activity. This column gave little purification, but appeared to reduce the levels of some minor contaminants in the final preparation.

The DEAE-cellulose flowthrough fraction was applied to a nonspecific dsDNA column. This column was generated by coupling sheared salmon sperm DNA to CNBr-activated Sepharose 4B. Protein was eluted from the column with a gradient of 0.1 to 0.5 M KCl. Active fractions, in which the salt concentration varied from approximately 0.1 to 0.3 M KCl, were pooled. This material was dialyzed and applied to a second dsDNA-Sepharose 4B column at 0.1 M KCl. The activity was then step eluted with 0.3 M KCl. The purpose of the second passage through this column was to more completely rid the preparation of high-salt-resistant DNA-binding proteins that might otherwise contaminate the high-salt eluate from the specific oligonucleotide column. The second passage was not absolutely necessary but did significantly reduce a number of minor contaminants in the final material (data not shown).

The final step in the purification procedure involved the

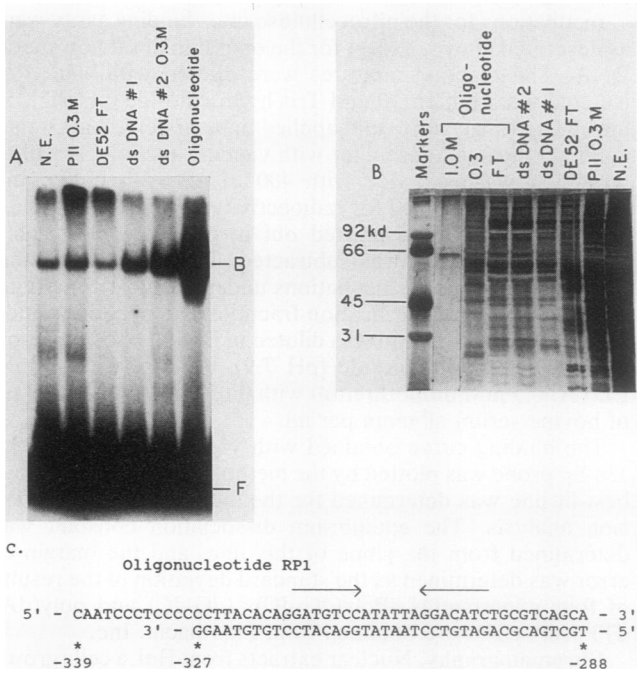

FIG. 2. Analysis of chromatography fractions. (A) Gel mobility shift assay of f-EBP active fractions. Each of the indicated fractions (0.5 μl) was incubated with 1 ng of ^{32}P-labeled oligonucleotide RP1 and 2 μg of sheared salmon sperm DNA. The positions of migration of the specific DNA-protein complex (B) and free DNA (F) are indicated. Abbreviations: N.E., nuclear extract; P11, phosphocellulose; DE52, DEAE-cellulose; dsDNA, dsDNA-Sepharose 4B; oligonucleotide, oligonucleotide RP1-Sepharose 4B; FT, flow-through fraction; 0.3M, fraction step eluted with 0.3 M KCl. (B) SDS-PAGE. Each of the indicated fractions was analyzed on an SDS–10% polyacrylamide gel. The equivalent of 1.0 μl of each fraction was loaded on the gel, except for the oligonucleotide affinity column fraction, for which 2.0 μl was loaded. (C) DNA sequence of synthetic oligonucleotide RP1 is indicated. The position of the dyad symmetry sequence is shown by the inverted arrows.

TABLE 1. Purification of f-EBP

Fraction	Vol (ml)	Total protein (mg)	Activity[a]		% Yield		Fold purification	
			Total (U)	Specific (U/mg)	Per step	Overall	Per step	Overall
Nuclear extract[b]	183	1,830	183	0.10				
Phosphocellulose (0.3 M step)	73	402	110	0.27	60	60	2.7	2.7
DEAE-cellulose (0.1 M)	90	248	68	0.27	61	37	1.0	2.7
dsDNA-Sepharose 4B no. 1 (0.1 to 0.3 M gradient)	18	30.6	54	1.76	79	30	6.4	18
dsDNA-Sepharose 4B no. 2 (0.3 M step)	6.3	20.8	50	2.40	93	27	1.4	24
Oligonucleotide-Sepharose 4B (1.0 M step)	0.4	0.021	19	905	38	11	377	9,000

[a] Relative activity units are shown with 1 U equal to the binding level in 1 ml of HeLa cell nuclear extract.
[b] The nuclear extract was derived from 4.6 liters of HeLa cells at 10^6 cells per ml.

specific binding of f-EBP to a c-*fos* enhancer-containing oligonucleotide column. The synthetic oligonucleotide RP1 spanned the f-EBP binding site and contained 10 bp on each side of the dyad symmetry region of the binding site (Fig. 2C). Oligonucleotide RP1 also contained a 12-nucleotide single-stranded region on its upstream side to facilitate coupling to CNBr-activated Sepharose 4B. The binding site within the c-*fos* enhancer has been previously determined by dimethylsulfate interference, dimethylsulfate protection, and DNase I footprinting studies with material from crude extracts (6, 22, 27).

The dsDNA-Sepharose 4B 0.3 M KCl fraction was dialyzed and applied to the oligonucleotide column at 0.1 M KCl. The column was step eluted with 0.3 M KCl to remove nonspecific DNA-binding proteins and then washed extensively with 6 column volumes of the 0.3 M KCl buffer. This wash reduced the amount of minor contaminants in the final material. Finally, the column was washed with 1.0 M KCl, which eluted the specific binding protein(s).

The entire procedure resulted in a purification of roughly 9,000-fold and a yield of 11% of the activity in the original nuclear extract (Table 1). The overall yield could be improved somewhat by eliminating the DEAE-cellulose and second dsDNA-Sepharose 4B steps, although this led to a higher level of minor contaminants in the final preparation (data not shown).

Characterization of the affinity-purified fraction. Samples of each of the active fractions were analyzed by SDS-PAGE and by silver staining (Fig. 2B). The specific oligonucleotide affinity-purified fraction contained predominantly one species, whose molecular weight was determined to be 62,000 when compared with known molecular weight markers. Coomassie blue staining of similar gels revealed the same pattern (data not shown), indicating that artificial levels of staining by the silver technique were not a problem in this case. Although the preparation shown contained a minor contaminant with an apparent molecular weight of 68,000, this species was not present in other purified preparations. Other minor contaminants were barely visible upon silver staining. Passage of the dsDNA-Sepharose 4B 0.3 M step fraction through a nonspecific oligonucleotide affinity column did not result in the retention of any of the f-EBP activity, nor was a 62-kilodalton (kDa) protein present in significant amounts in a 1.0 M step fraction of this column (data not shown).

To determine whether the 62-kDa protein band contained the f-EBP activity, we attempted to renature the activity from slices of the SDS-polyacrylamide gel by the method of Hager and Burgess (10). A 2-μg sample of the affinity-purified material was electrophoresed, and the gel was stained with 0.25 M KCl. Both the 62- and 68-kDa bands could be visualized and excised separately. In addition, the entire lane was cut into 12 slices from the top of the gel (slice 1) to the bottom (slice 12). Slices 4 and 5 corresponded to the 68- and 62-kDa bands, respectively. Protein was eluted from the gel slices, acetone precipitated, denatured in 6 M guanidinium hydrochloride, and diluted 50-fold to renature the proteins to an active form. Samples from the renaturation of each slice were then tested for activity in the gel mobility shift assay (Fig. 3A). Only slice 5, corresponding to the 62-kDa protein, contained activity. A comparison with the activity of native protein indicated that approximately 20% of the activity was regained upon elution from the gel and renaturation (Fig. 3A). The migration of the oligonucleotide affinity-purified fraction in the gel mobility shift assay sometimes appeared to be smeared, as in Fig. 3A. We do not understand the basis for this phenomenon at present.

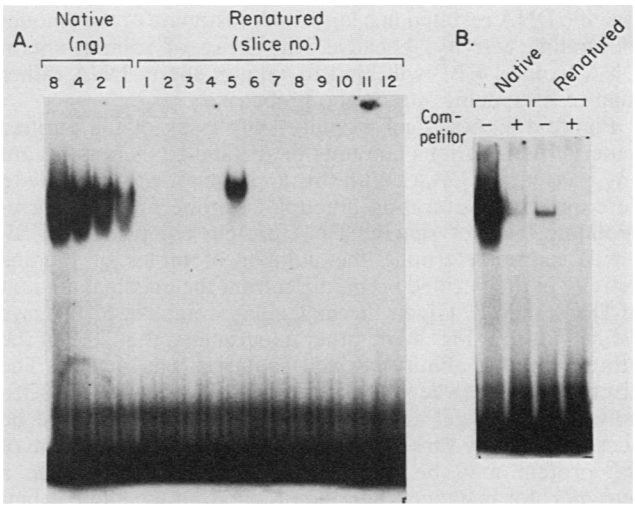

FIG. 3. Renaturation of f-EBP activity from an SDS-polyacrylamide gel. (A) The binding activity of either the oligonucleotide affinity-purified fraction (native) or the renatured protein from the indicated SDS–10% polyacrylamide gel slices was tested by the gel mobility shift assay with ^{32}P-labeled oligonucleotide RP1 in the absence of any nonspecific carrier DNA. The top of the gel corresponded to slice 1, and the bottom corresponds to slice 12. (B) The binding activity of the renatured protein was tested with 50 ng of either a nonspecific (−) or specific (+) oligonucleotide as described in Materials and Methods. A 10-ng sample of the oligonucleotide affinity-purified fraction was used as a native control.

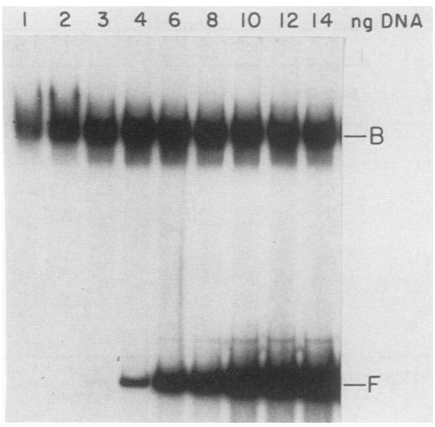

FIG. 4. Titration of binding activity in the absence of nonspecific carrier DNA. The gel mobility shift assay was performed without any salmon sperm DNA, with various amounts, as indicated, of a 138-bp c-*fos* enhancer probe (−363 to −225) and 25 ng of the oligonucleotide affinity-purified fraction. The position of migration of the specific DNA-protein complex (B) and free probe DNA (F) are indicated.

The specificity of the renatured activity from the gel slice was demonstrated by performing the gel mobility shift assay with either specific or nonspecific oligonucleotides as competitors (Fig. 3B). The renaturation experiment indicates that the f-EBP activity corresponds to a protein with a molecular weight of 62,000 ± 3,000, and not to other minor protein contaminants within the preparation.

The gel mobility shift assay (Fig. 2A) involved the use of 2 μg of salmon sperm DNA in the binding reaction mixture as nonspecific carrier. Although this was necessary when crude preparations were assayed, it was not necessary for assaying the affinity-purified fraction, which is devoid of other DNA-binding proteins. In fact, the presence of nonspecific DNA resulted in a large underestimate of the amount of binding activity, because with 2 μg of salmon sperm DNA, some f-EBP will bind to salmon sperm DNA rather than to its specific site on the probe.

Figure 4 shows a gel mobility shift assay of the purified material with various amounts of specific DNA but without any nonspecific DNA. With this approach it was possible to determine the saturating amount of probe and the corresponding level of specific DNA-protein complex. This allowed us to determine the number of moles of binding activity in the purified preparation from the maximal amount of DNA shifted. Given the molecular weight of 62,000, we estimated from Fig. 4 and other experiments that 20% of the protein in the affinity-purified material was active. The discrepancy between the apparent purity on the SDS-polyacrylamide gel and the 20% level of activity could be accounted for by three possibilities. First, the active form of the protein may be a multimer. If the protein were a tetramer, for instance, then the preparation would be calculated to be 80% active. Second, the protein may have lost activity after elution from the final column. We have found that the preparation lost activity when left at 4°C, with one-half of the activity lost in approximately 6 h. Third, there may be a contaminant which comigrates with the 62-kDa protein.

DNase I footprint of f-EBP binding. To demonstrate further the specificity of binding and to define the binding site, we performed a direct footprinting experiment with the affinity-purified fraction. This experiment contrasts with the previous footprinting, which involved treatment with DNase I and isolation of the DNA-protein complex from a gel (22). In the present experiment we incubated the purified fraction with the DNA probe along with 100 ng of sonicated salmon sperm DNA, treated it with DNase I, and analyzed the DNA directly on a sequencing gel. The footprint (Fig. 5A) is similar to that previously described (22, 27) and is consistent with results of previous methylation interference experiments (6, 27). The binding region extended entirely across the region of dyad symmetry. The protected region was shifted to the left by about 4 nucleotides on the lower strand relative to the upper strand (Fig. 5B). In addition, several hypersensitive sites were apparent on the lower strand.

Equilibrium binding. The lack of additional bands in the gel mobility shift assay without nonspecific DNA indicated that there were few, if any, contaminating DNA-binding proteins in the oligonucleotide affinity-purified fraction (Fig. 4). This allowed us to use a nitrocellulose filter binding assay to measure specific binding activity. The purified material was incubated with the specific DNA probe, filtered through nitrocellulose, and washed with binding buffer, and the radioactivity of each filter was determined. This gave us a

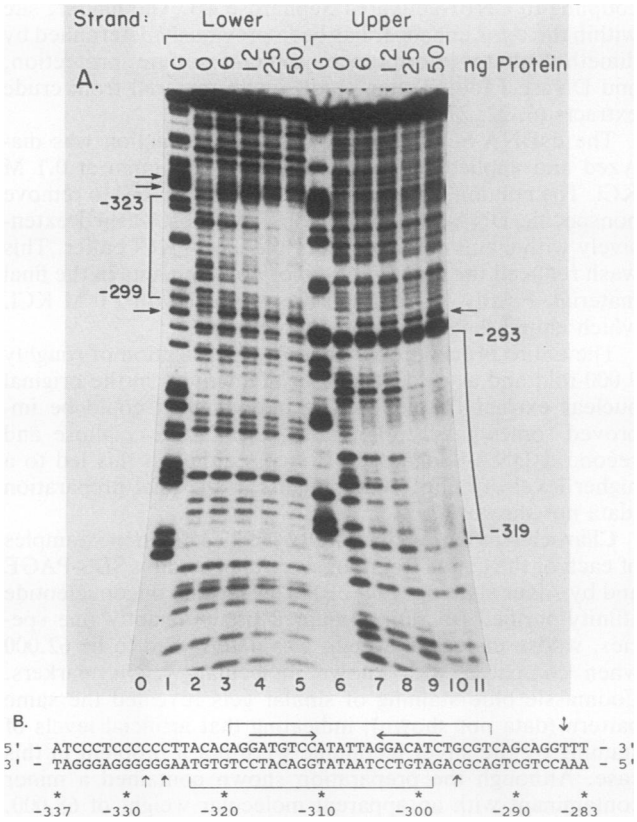

FIG. 5. DNase I footprint of f-EBP binding. (A) The indicated amounts of the oligonucleotide affinity-purified fraction analyzed in Fig. 2 were incubated with 1 ng of single-end-labeled 138-bp probe along with 100 ng of sonicated salmon sperm DNA and treated with DNase I. The DNA was isolated and analyzed on a sequencing gel. Either the upper or lower strand (as shown in panel B) was end-labeled with T4 polynucleotide kinase, and a G reaction of each probe is shown (lanes G). The region of DNase I protection and the position of hypersensitive sites are indicated. (B) The c-*fos* sequence which is protected from DNase I digestion by f-EBP is indicated by brackets, and the hypersensitive sites are indicated by vertical arrows. The horizontal arrows mark the dyad symmetry of the sequence.

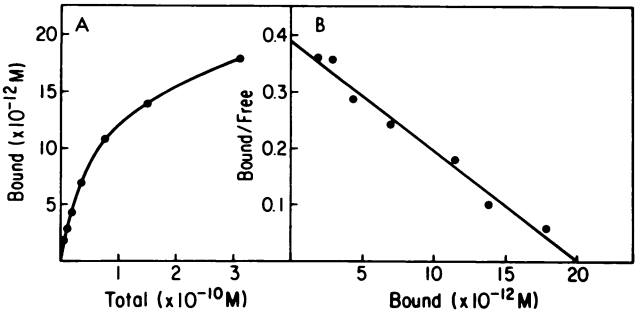

FIG. 6. Equilibrium binding of f-EBP. (A) Various amounts of the 138-bp probe were incubated in 50 μl with 0.5 ng of the oligonucleotide affinity-purified fraction analyzed in Fig. 2. The final concentration of the 138-bp probe added to each incubation is indicated (Total). The amount of probe bound was determined by the nitrocellulose filter-binding assay. Each point was determined in duplicate, and the average value is indicated. (B) The data were analyzed on a Scatchard plot. From four experiments of this type it was determined from the slope that the equilibrium dissociation constant K_D for binding of f-EBP to the 138-bp probe was (3.3 ± 1.3) × 10^{-11} M.

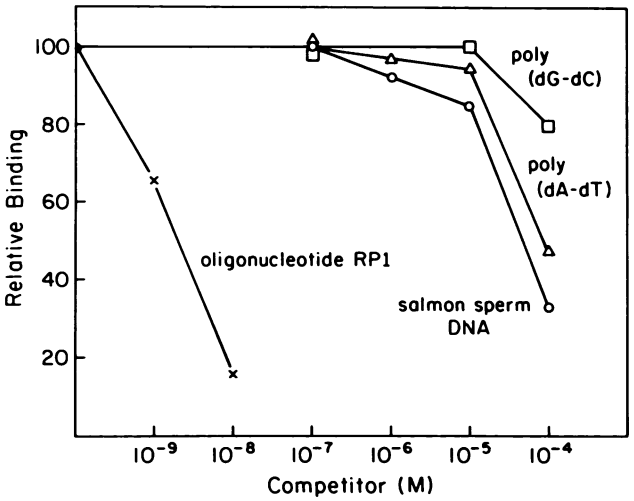

FIG. 7. Nonspecific binding by f-EBP. The indicated concentrations of the various competitors and 10^{-9} M of the 138-bp probe were incubated with 1 ng of the oligonucleotide affinity-purified fraction in 20 μl. The amount of probe bound was determined by the nitrocellulose filter-binding assay. Each point was determined in duplicate, and the average value is indicated. Both poly(dA-dT) and poly(dG-dC) are double-stranded alternating copolymers. The concentrations of the nonspecific competitors were calculated by assuming that each nucleotide starts a potential binding site.

rapid assay to measure the variables affecting binding. The binding could be completely inhibited by a 100-fold excess of cold oligonucleotide RP1 but was unaltered by a comparable amount of nonspecific oligonucleotide or salmon sperm DNA (see Fig. 7; data not shown). Equilibrium binding was achieved within 15 min of incubation at room temperature and was not changed for up to 2 h (data not shown).

To determine the equilibrium dissociation constant K_D of f-EBP binding to its specific site, we generated a binding curve by conducting the filter-binding experiment with a small amount of f-EBP (near the K_D) and titrating the amount of specific DNA probe added (Fig. 6A). These data were analyzed by using a Scatchard plot (Fig. 6B), in which the slope of the line is equal to $-1/K_D$. The linearity of the data by itself indicates that there is a protein with only one binding affinity present in the purified material. If there were more than one affinity a curvilinear plot would be expected. From four experiments of this type we determined the K_D to be (3.3 ± 1.3) × 10^{-11} M.

The relative binding to specific versus nonspecific DNA sites was determined by incubating f-EBP with a specific 138-bp probe at 10^{-9} M and increasing amounts of either specific oligonucleotide RP1 or nonspecific salmon sperm DNA, poly(dA-dT) · (dA-dT), or poly(dG-dC) · (dG-dC) (Fig. 7). The ratio of the competitor to probe concentrations required to inhibit binding by 50% is proportional to the relative equilibrium dissociation constants for these DNAs (17). Approximately twice the amount of oligonucleotide RP1 relative to the amount of 138-bp probe was required to reduce the binding by 50%, indicating that f-EBP has a slightly poorer affinity for the 40-bp oligonucleotide than for the 138-bp fragment. In contrast, a 50,000-fold excess of salmon sperm DNA, a 100,000-fold excess of poly(dA-dT), and a 500,000-fold excess of poly(dG-dC) were required to reduce binding by 50% (Fig. 7; data not shown). This corresponds to K_Ds of approximately 1.7 × 10^{-6} M for salmon sperm DNA, 3.4 × 10^{-6} M for poly(dA-dT), and 1.7 × 10^{-5} M for poly(dG-dC) relative to a K_D for the 138-bp fragment of 3.3 × 10^{-11} M. It should be noted that the concentrations of sites in the nonspecific DNAs were calculated by assuming that each nucleotide starts a site.

The dependency of binding on KCl and $MgCl_2$ concentrations was also determined by filter binding. Low protein and DNA concentrations, near the K_D, were used to observe differences due to altered affinity as well as altered maximal activity. Figure 8 shows titrations of both KCl and $MgCl_2$ in the binding reaction. Optimal binding was observed at 50 to 75 mM KCl and in the absence of $MgCl_2$. Binding was particularly sensitive to the presence of low concentrations of magnesium. The latter will be of particular significance for in vitro transcription reactions, which typically involve the use of 6 mM $MgCl_2$, although the sensitivity to $MgCl_2$ might be compensated for by cooperative binding with other transcription factors (24).

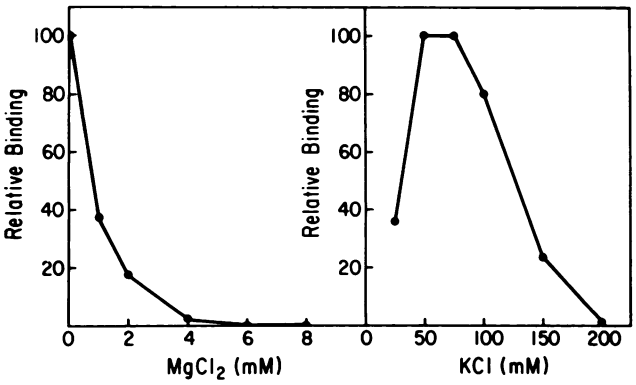

FIG. 8. Variables affecting equilibrium binding. Equilibrium binding was determined by the nitrocellulose binding assay under standard conditions, except that the concentration of either $MgCl_2$ (left) or KCl (right) was varied. The oligonucleotide affinity-purified fraction (0.3 ng/50 μl) was incubated with 3 × 10^{-11} M 138-bp probe in all cases. Each point was determined in duplicate, and the average value is indicated.

DISCUSSION

We have purified the f-EBP from HeLa cell nuclear extracts. The method we used relied primarily on two columns. A nonspecific dsDNA affinity column, from which f-EBP was eluted at low salt concentrations (0.3 M KCl), was used to rid the preparation of nonspecific DNA-binding proteins eluting at high salt concentrations. The next column was a specific oligonucleotide affinity column. The remaining nonspecific DNA-binding proteins eluted from this column at low salt concentrations, while the specific DNA binding protein, f-EBP, eluted at high salt concentrations (greater than 0.3 M KCl), resulting in a huge purification.

Our purification yielded predominantly a single protein of molecular weight 62,000. It was demonstrated that this protein band contained the f-EBP activity by the renaturation of the functional binding activity of this protein after excision from an SDS-polyacrylamide gel. In addition, neither the binding activity nor the 62-kDa protein was detectable if a nonspecific oligonucleotide column was substituted for the specific column (data not shown).

We were able to determine the stoichiometry of binding by assaying the purified material in the absence of any nonspecific DNA carrier. Assuming a monomer molecular weight of 62,000, 20% of the protein in the final preparation was capable of binding the DNA probe. However, it is possible that f-EBP is a dimer or greater, since the binding site contains a dyad symmetry (inverted repeat). It is interesting that mutations in only one-half of the binding site completely abolish binding (8). Another possible reason for the low stoichiometry of binding is that the protein has lost some activity after elution from the affinity column. We have noticed a slow decay in activity, with a half-life of about 6 h, when the protein is left at 4°C. Finally, we cannot rule out the possibility that there is a contaminant which comigrates at 62 kDa on the SDS-polyacrylamide gel.

In vitro analysis of binding properties of f-EBP have indicated a very high affinity for the enhancer site, with a K_D of about 3.3×10^{-11} M. This affinity is similar to those found for nuclear factor I in binding to its recognition site in adenovirus DNA (23) and for the binding of a specific factor (major late transcription factor) to the adenovirus major late promoter (3; M. Sawadago, unpublished data). The affinity of f-EBP for nonspecific DNA, as represented by salmon sperm DNA, was approximately 50,000-fold less than that for the specific site.

We determined the total amount of binding activity in the oligonucleotide affinity-purified fraction by assaying without nonspecific DNA carrier. Given the yield of activity during our purification procedure and the number of moles of binding activity in our final preparation, we calculated that there are approximately 10,000 c-*fos* enhancer-binding molecules per HeLa cell. It was not possible to measure the number per cell directly in crude extracts because of the need to assay for binding activity in the presence of salmon sperm DNA. The distribution of f-EBP between specific and nonspecific sites (on salmon sperm DNA) under these conditions would lead to an underestimate of the maximal amount of binding activity present.

The number of molecules per cell and the relative affinities for specific and nonspecific sites are important factors in determining the occupancy of f-EBP on the c-*fos* enhancer (17). There are, however, a number of other important considerations. The proportion of accessible nonspecific DNA in chromatin is not known, and the effect of other protein factors on stabilizing binding could be substantial. For instance, the factor binding to the upstream segment of the adenovirus major late promoter is stabilized by the TATA binding factor (24). Furthermore, in vivo conditions, such as salt and magnesium concentrations, may be crucial. We have utilized optimal conditions for in vitro binding, yet the free concentration of magnesium in vivo would be expected to substantially reduce binding by f-EBP in the absence of compensating factors.

Although the c-*fos* promoter directs accurate initiation in an in vitro transcription system (R. Prywes, unpublished data), we have not been able to demonstrate a sequence-dependent effect of the c-*fos* enhancer. Neither crude nuclear extracts from epidermal growth factor-induced HeLa cells nor reconstituted systems with purified f-EBP have been effective (Prywes, unpublished data). It should be noted, though, that our purified f-EBP was derived from cells not expressing the endogenous c-*fos* gene (22). We have not been able to purify f-EBP from induced cells because in HeLa cells grown in suspension c-*fos* expression was not induced by either serum or epidermal growth factor (data not shown). Other cell types we have analyzed which are inducible for c-*fos* expression grow in monolayers and are difficult to grow and extract in large amounts.

It will be interesting to examine in the future the mechanism of regulation of f-EBP. The alteration of binding affinities or levels could be important regulatory mechanisms. In addition, the putative f-EBP transcriptional-activating mechanism may be regulated independently of binding activity. The purification of f-EBP should enable us to study these questions in several ways. Further in vitro transcriptional studies will be important for mechanistic studies and, in particular, for assessing the functional significance of potentially modified forms. Amino acid sequencing may allow the cloning of the structural gene for f-EBP. Finally, the generation of antibodies to f-EBP will be extremely valuable for analysis of the protein in small amounts of induced or uninduced cells.

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