MOLECULAR AND CELLULAR BIOLOGY, May 1992, p. 2432–2443
0270-7306/92/052432-12$02.00/0

Vol. 12, No. 5

Expression Cloning of a Novel Zinc Finger Protein That Binds to the c-*fos* Serum Response Element

RICARDO M. ATTAR AND MICHAEL Z. GILMAN*

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

Received 19 December 1991/Accepted 25 February 1992

Induction of c-*fos* transcription by serum growth factors requires the serum response element (SRE). The SRE is a multifunctional element which responds to several positively and negatively acting signals. To identify cellular proteins that might mediate functions of the SRE, we screened a human cDNA expression library with an SRE probe. We report the isolation and characterization of SRE-ZBP, a previously unidentified SRE-binding protein. SRE-ZBP is a member of the C_2H_2 zinc finger family of proteins exemplified by TFIIIA and the *Drosophila* Krüppel protein. The seven tandemly repeated zinc finger motifs in SRE-ZBP are sufficient for high-affinity binding to the SRE. We show that SRE-ZBP is a nuclear protein and identify a candidate cellular protein encoded by the SRE-ZBP gene. Because we cannot detect any DNA-binding activity attributable to the endogenous protein, we propose that SRE-ZBP activity may be subject to posttranslational regulation. Like c-*fos* mRNA, SRE-ZBP mRNA is serum inducible in HeLa cells, but with slower kinetics. The role of SRE-ZBP in the regulation of c-*fos* transcription remains unestablished, but this protein binds to a region of the SRE where mutations lead to derepression.

The events that end in the regulation of cellular proliferation and differentiation start with a signal transduction mechanism in which an extracellular signal is turned into a precise adjustment of the genetic program of that cell. The first class of genes whose transcription is modified by this flow of signals is the "immediate-early" genes (2). Many of these genes encode transcription factors, including the proto-oncogenes c-*fos*, c-*jun*, c-*myc*, and members of the *zif/krox* family of zinc finger genes, which are presumed to control subsequent waves of gene transcription.

The best studied of the immediate-early genes is the c-*fos* proto-oncogene. Transcription of the c-*fos* gene can be triggered by several signal transduction pathways, each of which can independently activate transcription of the gene (for a review, see reference 17). Many of these signals act through a sequence located in the 5′ flanking region of the c-*fos* gene, termed the serum response element (SRE). Several other immediate-early gene promoters contain SRE-like sequences, and in some cases SRE activity has been demonstrated (for a review, see reference 74). The SRE is required for response to activators of protein kinase C and for growth factor-induced signals independent of protein kinase C (7, 21, 23, 26, 60, 62, 67). SRE oligonucleotides restore serum responsiveness to both deleted c-*fos* and heterologous promoters (23, 28, 44, 52, 60, 73). In addition, microinjection of SRE oligonucleotides blocks induction of c-*fos* expression by serum (5, 24, 37). These observations argue that the SRE operates in vivo at least in part as a positively acting element. On the other hand, the serum response conferred by SRE oligonucleotides on heterologous promoters is both transient and superinducible with cycloheximide (62, 69), suggesting that the SRE is also a target for transcriptional repression following serum stimulation. Moreover, cotransfection experiments suggest that the SRE is subject to negative regulation by the Fos protein itself (35, 56, 58, 78). Thus, the SRE is a multifunctional element that may involve the action of multiple SRE-binding proteins.

One of these proteins is the serum response factor (SRF), which binds in a symmetrical fashion to the SRE dyad (25, 28, 46, 49, 73). The precise function of SRF in the regulation of c-*fos* transcription is not yet clear. SRE mutagenesis experiments suggest that SRF is required for all SRE functions in vivo, but there is no detectable change in any property of SRF isolated from stimulated cells (25, 28, 49, 60, 67, 73). Moreover, the SRE remains constitutively bound by protein in cells regardless of the state of transcription of the gene (30, 34).

Two additional proteins bind to the SRE in vitro. One, $p62^{TCF}$ (ternary complex factor), recognizes the SRE-SRF complex to form a ternary complex (59). SRE mutants that fail to form this ternary complex but still bind SRF lose response to some but not all signals acting at the SRE (26, 59). An additional protein, $p62^{DBF}$ (direct binding factor), binds directly and asymmetrically to the 5′ side of the SRE (55, 76). There are no data yet about the function of $p62^{DBF}$ in the regulation of c-*fos* transcription.

In an attempt to clone $p62^{DBF}$ or other previously unidentified SRE-binding proteins, we screened a HeLa cell cDNA expression library with SRE oligonucleotides by the in situ DNA filter detection method (63, 65, 75). We report here the isolation of a human cDNA clone encoding a novel SRE-binding protein (distinct from $p62^{DBF}$) that is a member of the Krüppel family of zinc finger proteins.

MATERIALS AND METHODS

Isolation of λA_2. An oligo(dT)-primed HeLa cell cDNA expression library constructed in the bacteriophage λgt11 was generously provided by Paula Henthorn and Tom Kadesch (29). Bacteriophage infection and plating of infected cells was carried out as described elsewhere (79). Screening of the library followed published procedures (75) with some modifications.

Approximately 30,000 PFU per 150-mm dish were plated and incubated for 3 h at 42°C. Each plate was overlaid with

* Corresponding author.

a dry nitrocellulose filter impregnated with isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for 6 h at 37°C. The plates were cooled at 4°C for 1 h, and the filters were removed. A second IPTG-impregnated filter was overlaid on each plate and incubated for 2 h more at 37°C. All subsequent procedures were carried out at 4°C. The filters were submerged in 250 ml of binding buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.9], 1 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol [DTT]) supplemented with 6 M guanidine hydrochloride. After gentle shaking for 5 min, the solution was changed to an identical buffer containing 3 M guanidine hydrochloride, and the filters were shaken for 5 min. The filters were incubated in four subsequent serial twofold dilutions of guanidine hydrochloride, after which the filters were washed twice for 5 min with binding buffer lacking guanidine hydrochloride. The filters were transferred to a baking dish containing binding buffer supplemented with 5% Carnation nonfat dry milk (43) and gently shaken for 30 min. The filters were then washed twice for 5 min in binding buffer supplemented with 0.25% dry milk. Exposure of filters to the probe was carried out in 100 ml of binding buffer plus 0.25% dry milk with 10^6 cpm of radiolabeled probe per ml and 5 μg of salmon sperm DNA per ml for 6 h with gentle shaking. The filters were washed three times for 5 min with 500 ml of the binding buffer plus 0.25% dry milk. The filters were blotted on Whatman 3MM filter paper, allowed to air dry, and exposed to Kodak X-Omat AR film overnight at −70°C with the aid of a Dupont Lightning-Plus intensifying screen.

Plasmid constructions. The pBSM13$^+$/SRE-ZBP/Eco2 plasmid carries the entire cDNA insert from the λA_2 phage. It was obtained by partial digestion of phage DNA with *Eco*RI. For the construction of pCGN/SRE-ZBP, an *Spe*I site was inserted at position 1 of the coding sequence of the SRE-ZBP gene by site-directed mutagenesis (36) of the pBSM13$^+$/SRE-ZBP/Eco2 plasmid. The SRE-ZBP-encoding fragment was excised from the mutant plasmid with *Spe*I and *Bam*HI and subcloned in the eukaryotic expression vector pCGN (71) previously digested with *Xba*I and *Bam*HI.

The pET11c-GST/Znd plasmid was constructed by a procedure devised in reference 3. An *Xba*I (position 626)-to-*Bam*HI fragment of pBSM13$^+$/SRE-ZBP/Eco2 was isolated and ligated together with pET11c (66) digested with *Nde*I and *Bam*HI and an *Nde*I-*Xba*I fragment encoding glutathione S transferase (GST) (64).

Production of β-galactosidase fusion protein and Southwestern (DNA-protein) blotting. Large scale infection of *Escherichia coli* Y1089 with the λgt11 and λA_2 phages and Southwestern blotting were carried out as described previously (68). β-Galactosidase fusion protein extracts for mobility shift assays were prepared by washing the pellet from 50 ml of infected bacteria with 1 ml of ice-cold 50 mM Tris-HCl (pH 7.5)–5 mM EDTA, and then by resuspension in 1 ml of a solution containing 50 mM HEPES-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% (vol/vol) Nonidet P-40 (NP-40), 10% glycerol, 5 M urea, and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed by sonication. Insoluble material was removed by centrifugation, and the supernatant was dialyzed against 25 mM HEPES-HCl (pH 7.6), 1 mM $MgCl_2$, 1 mM DTT, 20% glycerol, 0.1% NP-40, 40 mM KCl, and 1 mM phenylmethylsulfonyl fluoride to allow renaturation of the DNA binding activity.

Expression of GST fusion proteins in *E. coli*. The *E. coli* strain BL21 (DE3) was transformed with the plasmid pET11c-GST/Znd. A single colony was grown in 5 ml of M9ZB at room temperature. At an A_{600} of 0.3 to 0.5, IPTG was added to a final concentration of 0.5 mM, and the culture was incubated for 5 more h at room temperature. The bacteria were centrifuged, resuspended in ice-cold phosphate-buffered saline (PBS) (1/5 of the original volume), and incubated for 15 min at 30°C in the presence of 100 μg of lysozyme per ml. After the addition of NP-40 to a final concentration of 0.2%, the cells were lysed by 3 cycles of freeze-thawing and sonication. The insoluble material was removed by centrifugation, and 1/10 volume of 50% glutathione-agarose (Sigma) slurry in PBS was added to the supernatant. After 10 min of incubation at room temperature with rocking, the beads were washed three times with PBS-0.1% NP-40 and eluted with 2 pellet volumes of 50 mM HCl-Tris (pH 8.0)–5 mM glutathione. One volume of 50% glycerol was added to the eluted protein, which was stored at −70°C.

Generation of rabbit anti-SRE-ZBP antisera. λA_2 fusion protein expressed in strain Y1089 as described above was purified for use as antigen by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the total cell lysate on 4% gels. The 160-kDa β-galactosidase-SRE-ZBP fusion protein was visualized by briefly staining the gel with 0.05% Coomassie brillant blue R-250 and then extensive destaining with water. The protein was electroeluted from the gel and washed with PBS by centrifugation in Centricon 30 concentrators (Amicon). Two New Zealand White rabbits were injected in the popliteal lymph nodes every 2 weeks with 6 μg of the purified fusion protein. Preimmune sera were obtained prior to the first injections.

Preparation of DNA probes. All oligonucleotides used in this work were gel purified prior to use. To generate probes for library screening, the oligonucleotides were annealed in a volume of 170 μl containing 6 nmol of each strand, 50 mM Tris-HCl (pH 7.5), and 10 mM $MgCl_2$. The mix was heated at 85°C for 2 min and then incubated at 65°C for 10 min, at 37°C for 10 min, and at room temperature for 5 min. The annealed oligonucleotides (35 pmol) were phosphorylated in a reaction mixture containing 5 mM DTT, 0.5 mM spermidine, 0.58 mCi of [γ-^{32}P]ATP (specific activity, 7,000 Ci/mmol), and 10 U of T4 polynucleotide kinase, for 2 h at 37°C. ATP (final concentration, 1.5 mM) and 10 U of T4 DNA ligase were added, and the reaction mixture was incubated overnight at 15°C. Radiolabeled ligated probe was separated from free nucleotides by chromatography on Bio-Gel P60.

Probes for mobility shift assays were prepared by two different methods. For the experiments shown in Fig. 2, 5 pmol of double-stranded oligonucleotides was incubated with 10 μCi of each [α-^{32}P]-labeled deoxynucleoside triphosphate and 2 U of *E. coli* DNA polymerase I Klenow fragment for 30 min at room temperature. After phenol extraction, the probes were ethanol precipitated and purified on a native 4% polyacrylamide gel. The excised bands were eluted in water overnight at 4°C.

For the experiments shown in Fig. 4 and 5, probes were prepared by polymerase chain reaction (PCR) by using one primer complementary to the sense strand of the mouse c-*fos* promoter from position −207 (5′) to position −226 (3′) and one primer identical to the sense strand of the promoter from position −354 (5′) to position −335 (3′). The PCR templates were derivatives of the plasmid p301-356 (24, 26), which contains the mouse c-*fos* promoter (either the wild-type promoter or promoters carrying mutations in the SRE). The amplification reaction was carried out with primers labeled with T4 polynucleotide kinase. All probes were prepared simultaneously with the same labeled primers, ensuring that they were of identical specific activities. For the preparation

of probes labeled at one end only for dimethyl sulfate (DMS) interference assays, only one of the primers was labeled in each reaction. After PCR, the probes were gel purified as described above.

Mobility shift assays. Mobility shift assays were carried out with a 10-μl reaction mixture containing 10 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.5 mM $MgCl_2$, 5 mM NaCl, 1 μg of poly(dI-dC) · poly(dI-dC), 10 μM $ZnCl_2$, 0.1 mM EDTA, 5% glycerol, and 5 to 10 μg of protein. The mixture was preincubated for 10 min at room temperature. Then, 20,000 cpm (0.2 ng) of probe was added, and after 10 min of incubation at room temperature, the samples were loaded onto a 4% native polyacrylamide gel (acrylamide/bis ratio of 29:1). The gels were run at 8 to 10 V/cm in a buffer containing 45 mM Tris-borate and 1 mM EDTA.

DMS interference. DMS interference assays were done as detailed elsewhere (25, 55), except that the probes were prepared by PCR as described above and bound and free DNA was electroeluted onto Hybond M&G paper (Amersham). The paper was rinsed twice in water and once in ethanol and incubated in 1 M piperidine for 30 min at 90°C. The paper was discarded, and the cleaved DNA was purified by repeated lyophilization and phenol-chloroform-isoamyl alcohol extraction, followed by ethanol precipitation in the presence of 10 μg of glycogen.

COS transfection and preparation of extracts. Plates of COS cells that were 70% confluent were trypsinized, counted, and resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5 mM BES {*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid} (Sigma) at 2×10^7 cells per ml. A 250-μl volume of cell suspension was electroporated (180 V; 960 μF) in the presence of 1 μg of pCGN/SRE-ZBP and 5 μg of salmon sperm DNA. The cells were allowed to recover for 10 min at room temperature, rinsed in 10 ml of DMEM supplemented with 5% FCS–5 mM BES, and plated in one 10-cm plate. After 48 h of incubation, nuclear extracts were prepared essentially as described elsewhere (19).

Immunofluorescence. Transfected COS cells were fixed by sequential immersion for 10 min at room temperature in PBS containing 3.7% formaldehyde, 0.5% Triton X-100, and 0.05% Tween 20. Rabbit anti-SRE-ZBP serum was used as primary antibody at a dilution of 1:100 in PBS containing 1% bovine serum albumin. The secondary antibody used was biotinylated donkey anti-rabbit immunoglobulin G, which was followed by streptavidin-conjugated Texas-Red. Both secondary and tertiary stages were at 1:150 dilutions and were obtained from Amersham. Cells on coverslips were mounted in a solution of 90% glycerol and 10% PBS containing 3% (wt/vol) *N*-propyl gallate (as an antiquenching agent) at pH 7.5. Photomicroscopy was done as described previously (51) by using a Zeiss III RS fluorescence microscope.

Immunoprecipitation. Transfected COS cells were plated in DMEM supplemented with 5% FCS. After 48 h, the cells were washed twice with DMEM minus methionine (DMEM-Met) and incubated for 4 h at 37°C in DMEM-Met containing 0.3 mCi of [^{35}S]methionine per ml. Following incubation, the cells were washed with ice-cold PBS and harvested on ice by the addition of ice-cold RIPA buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM KCl, 1 mM EDTA, 0.25% [wt/vol] deoxycholate, 0.25% [vol/vol] NP-40, and 0.25% [vol/vol] Tween 20). The lysates were briefly sonicated, cleared by centrifugation for 2 min at 4°C, and preadsorbed with protein A-Sepharose for 30 min at 4°C. Aliquots of each lysate were assayed by scintillation counting, and identical numbers of counts were incubated with an excess of preimmune serum, anti-SRE-ZBP, or 12CA5 monoclonal antibody (20) for 2 h at 4°C with rocking. Antibody-antigen complexes were recovered on protein A-Sepharose beads, which were washed five times in ice-cold RIPA buffer and boiled in Laemmli sample buffer prior to gel electrophoresis (22).

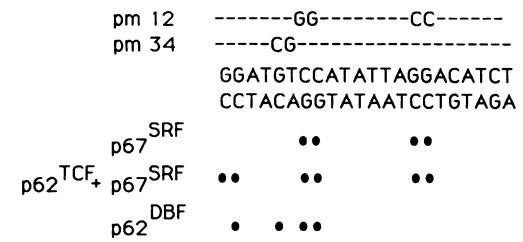

FIG. 1. SRE oligonucleotides used for library screening. The sequence shown is the 22-bp c-*fos* SRE. Below the sequence is a summary of methylation interference data for each of the known SRE-binding proteins (25, 55, 59, 73). The dots indicate guanine residues (on either strand) at which methylation interferes with protein binding. Above the sequence are the two oligonucleotides used for screening of the HeLa cDNA expression library. pm12 contains four substitutions which reduce the binding affinity of all the proteins represented in the figure. The two substitutions present in pm34 confer to it a higher affinity for $p62^{DBF}$ than the wild-type SRE.

RNA analysis. HeLa cells were grown in DMEM containing 10% FCS. At 70% confluence, the cells were starved for 48 h in DMEM containing 0.5% FCS. For the serum stimulation experiment, the medium was changed to DMEM plus 10% FCS at various times. All cultures were harvested at the same time. Total cytoplasmic RNA was isolated and analyzed by RNase protection assay as described previously (23).

The SRE-ZBP probe was prepared from a plasmid carrying SRE-ZBP sequences from nucleotides 929 to 1465. After linearization of the template with *Eco*RV and in vitro transcription with T3 RNA polymerase, an antisense probe of 282 nucleotides was obtained. Protection of this probe by SRE-ZBP mRNA yields a protected fragment of 257 nucleotides.

The pBSKS$^+$*oct*-1BS 231 plasmid carrying a 231-bp fragment of Oct-1B cDNA (18) was digested with *Bam*HI and transcribed with T7 RNA polymerase, generating a 311-nucleotide antisense probe.

Nucleotide sequence accession number. The sequences described in this paper can be found in the GenBank and EMBL data bases under sequence numbers M88579 and Z11773, respectively.

RESULTS

Isolation of λA_2: a λgt11 human cDNA recombinant encoding a β-galactosidase-SRE-binding fusion protein. To isolate cDNA clones encoding SRE-binding proteins, we probed a HeLa cell cDNA λgt11 expression library (29) for SRE-specific DNA-binding proteins by the in situ filter detection method (63, 65), using denaturation and renaturation of filter-bound proteins to enhance the signal (75). As probes we used the two double-stranded oligonucleotides shown in Fig. 1. One, pm34, contains two substitutions in the 5′ side of the SRE dyad that confer to this oligonucleotide higher affinity for $p62^{DBF}$ than the wild-type SRE in mobility shift

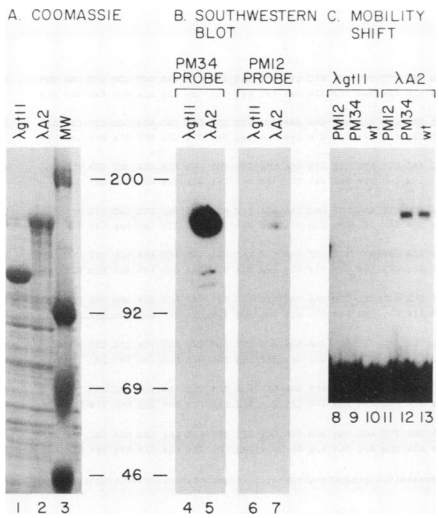

FIG. 2. The λA_2 phage encodes an SRE-binding protein. (A) One milliliter of log-phase *E. coli* Y1089 infected with λgt11 or with λA_2 at 5 PFU per cell was heat shocked and subsequently treated with 10 mM IPTG for 1 h. The cells were collected by centrifugation, and 20% of the total-cell protein was fractionated on an SDS-7% polyacrylamide gel. The fractionated proteins were visualized by Coomassie blue staining. Lanes: 1, λgt11-infected cells; 2, λA_2-infected cells; 3, markers. (B) Southwestern blot analysis was performed on lanes from the gel in panel A. Following electrotransfer to nitrocellulose, the filters were denatured and renatured with guanidine hydrochloride (75) and probed with the radiolabeled multimerized pm34 (lanes 4 and 5) or pm12 (lanes 6 and 7) probe. (C) Mobility shift assays of the λgt11 (lanes 8, 9, and 10)- and λA_2 (lanes 11, 12, and 13)-infected cell extracts (0.2 μl each). The probes were pm12 (lanes 8 and 11), pm34 (lanes 9 and 12), and wild-type (wt) SRE (lanes 10 and 13).

assays (27). In contrast, the pm12 oligonucleotide is a mutant containing four substitutions that abolish binding of SRF, $p62^{TCF}$, and $p62^{DBF}$ and that inactivate the SRE in vivo (25, 55).

We screened 4×10^5 plaques in duplicate with a radiolabeled multimerized pm34 probe. In the first round of screening, we identified four positive phages. These phages were screened a second time in duplicate with the radiolabeled, multimerized pm34 probe and with the pm12 mutant probe. Two of the four bound specifically to the pm34 probe. After three rounds of plaque purification and screening, these two phages were isolated. For one of them, we were never able to demonstrate specific DNA-binding activity for either the phage-encoded fusion protein or the protein produced by in vitro transcription and translation of the insert, so we have not characterized this phage further. We show below that the other positive phage, which we called λA_2, encoded a protein that bound the pm34 and but not the pm12 oligonucleotides.

DNA-binding specificity of the λA_2 fusion protein. To establish the authenticity of the SRE-specific DNA-binding activity of the λA_2-encoded fusion protein, we produced the protein in *E. coli* by large-scale infection with the recombinant phage (Fig. 2A) and we tested its activity by Southwestern blot (Fig. 2B) and mobility shift assay (Fig. 2C).

Figure 2A shows a Coomassie blue-stained portion of an SDS-polyacrylamide gel of bacterial extracts infected with λgt11 (lane 1) or λA_2 phage (lane 2). Whereas λgt11-infected cells produced a wild-type 116-kDa β-galactosidase protein, λA_2-infected cells produced a fusion protein of approximately 160 kDa. Replicas of these gel lanes were transferred to nitrocellulose and probed with the pm34 and pm12 oligonucleotides. The result is shown in Fig. 2B. Cells infected with λgt11 contained no proteins with significant affinity for either probe (lanes 4 and 6). In contrast, λA_2-infected cells contained a protein with mobility corresponding to that of the β-galactosidase fusion protein that bound strongly to the pm34 probe (lane 5) but only very weakly to the pm12 probe (lane 7). This experiment suggests that the SRE-specific DNA-binding activity detected in the plaque assay was encoded by the cDNA insert carried in λA_2.

To confirm by a second assay that λA_2 encoded an SRE-specific DNA-binding protein, we tested the activity of infected bacterial extracts in a mobility shift assay. As shown in Fig. 2C, λA_2-infected cells contained an activity that bound to the pm34 and wild-type SRE probes but not to the pm12 mutant SRE (lanes 11 to 13). This activity was not observed in the λgt11-infected cells (lanes 8 to 10). These results show that the recombinant λA_2 phage contained a cDNA encoding a DNA-binding protein that specifically recognizes the SRE.

Sequence analysis of the λA_2 cDNA. Figure 3A shows the sequence of a 1,664-bp portion of the 2.2-kb λA_2 insert. The nucleotide sequence of the λA_2 insert revealed an open reading frame of 410 amino acids in frame with the β-galactosidase protein. This open reading frame encodes a polypeptide with a predicted M_r of 47,623. Neither a candidate for an initiating ATG initiation codon nor poly(A) sequences were found in this cDNA, suggesting that it is incomplete at both ends. This is consistent with our observation that the cDNA hybridizes to an mRNA of approximately 3.5 kb (data not shown).

Comparison of the nucleotide sequence with those in computer data bases revealed that this cDNA encodes a previously unreported gene. However, it contains a domain in which it is closely related to a number of other genes. These genes, typified by the *Drosophila* gap gene Krüppel, carry sequence motifs believed to encode zinc fingers of the C_2H_2 class (4, 13, 42, 48, 54). As shown in Fig. 3B, the λA_2-encoded protein contains seven of these zinc finger motifs, and the invariant amino acids within the finger loops, Cys-X_2-Cys-X_3-Phe-X_5-Leu-X_2-His-X_3-His (see reference 42 for details), are completely conserved in this protein. Moreover, the zinc fingers in this protein family are linked by another conserved protein sequence, Thr-Gly-Glu-Lys-Pro-Phe or Tyr-X, called the "H/C-link" (57). This sequence is completely conserved in the A_2-encoded protein fingers II and V and partially conserved in fingers I (six of seven amino acids) and VI (five of seven amino acids). Because of the structural features of this cDNA, we have named the λA_2-encoded protein SRE-ZBP (zinc finger domain-binding protein).

Figure 3C compares the sequence of the SRE-ZBP zinc finger domain with those of the five most closely related sequences in the GenBank and EMBL data bases. The similarity of these proteins to SRE-ZBP ranges from 46 to 56% in the zinc finger domain, but outside this domain these proteins are not closely related to SRE-ZBP.

Contacts between SRE-ZBP and SRE DNA. To investigate further the DNA-binding properties of the SRE-ZBP protein, we produced the putative DNA-binding domain of SRE-ZBP in *E. coli* as a fusion protein with GST (Fig. 4A). This protein, composed only of the seven zinc finger motifs, binds DNA with the same specificity as the longer protein encoded by the 2.2-kb cDNA but with substantially higher affinity

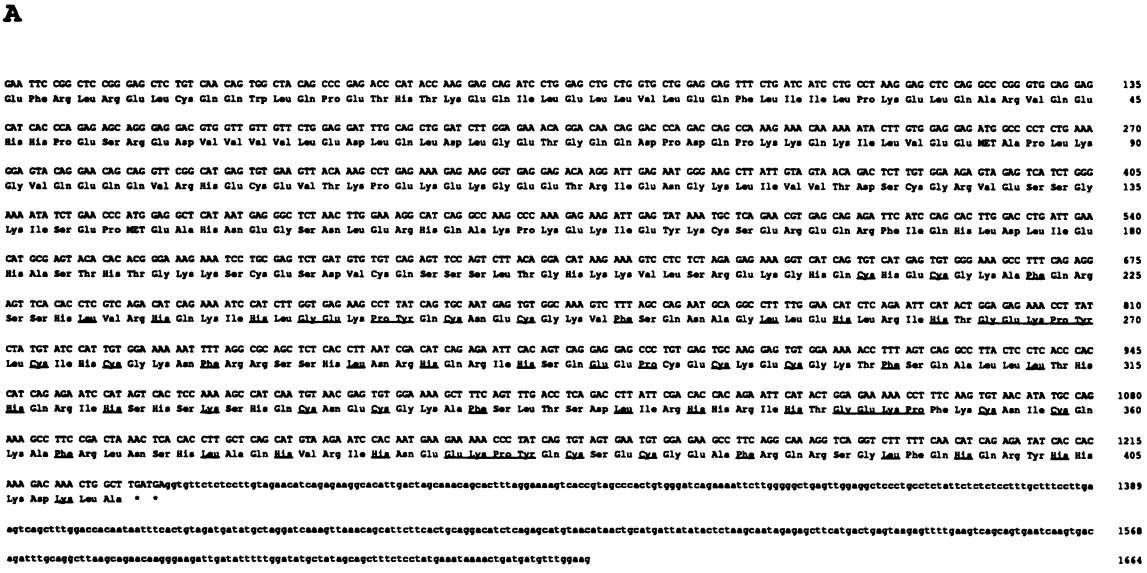

FIG. 3. Nucleotide and deduced amino acid sequences of SRE-ZBP. (A) The DNA sequence and deduced amino acid sequence of the SRE-ZBP cDNA derived from the λA_2 clone. The numbering system shown at the right of the sequence begins at the *Eco*RI site at the 5′ end of the cDNA. The conserved amino acids forming the putative zinc fingers are underlined. (B) The amino acid sequences of the seven SRE-ZBP zinc fingers aligned and compared with the C_2H_2-Krüppel family zinc finger consensus sequence (4, 13, 42, 57). (C) The primary structure of the SRE-ZBP zinc finger domain (amino acids 207 to 410) is aligned with those of the zinc finger domains of the most closely related proteins in the GenBank and EMBL data bases. These are MUSMKR2 (amino acids 148 to 347) (12), MUSZFP35 (amino acids 153 to 356) (16), MUS2NF5MK (amino acids 244 to 447) (13), HSZNF2 (amino acids 152 to 355, numbered from the first in-frame ATG) (53), and HUMHPLK (amino acids 186 to 389) (32). The underlined amino acids are the conserved residues in the seven C_2H_2 zinc fingers of SRE-ZBP.

(data not shown). Thus, the DNA-binding domain of SRE-ZBP resides within the zinc finger domain. To identify SRE-ZBP contacts with the SRE DNA probe, we performed interference assays. For these assays, a wild-type SRE probe 5′ end labeled on either the coding or the noncoding strand was partially methylated with DMS and used in preparative mobility shift assays. The DNA in the shifted complexes was eluted, cleaved with piperidine at the position of modified guanines, and analyzed on a sequencing gel. The interference pattern obtained with the purified GST-SRE-ZBP zinc finger domain is shown in Fig. 4B (lanes 3 and 6) and summarized in Fig. 4C. Interference was observed on one guanine residue in each strand. Both residues lie on the 3′ side of the SRE dyad. One of the guanines forms part of

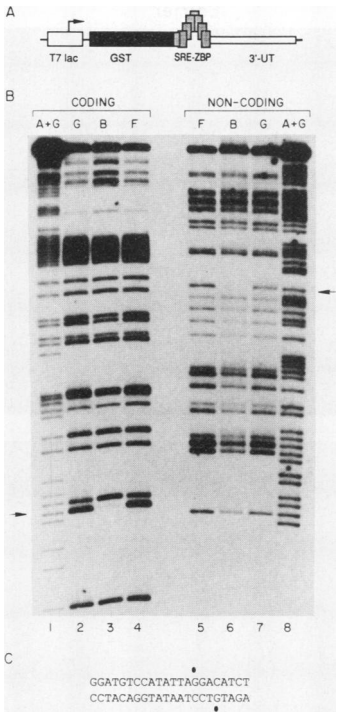

FIG. 4. DMS interference assay. (A) Schematic representation of the vector used to produce the GST-SRE-ZBP zinc finger domain fusion protein utilized in the assay. (B) DMS interference assay. The wild-type SRE fragment was 5′ end labeled on the coding strand (lanes 1 to 4) or on the noncoding strand (lanes 5 to 7). The purine and guanidine ladders are shown in lanes 1 and 8 and lanes 2 and 7, respectively. Bound fragments are shown in lanes 3 and 6. Free fragments are shown in lanes 4 and 5. Guanine bands suppressed in the bound lane and are indicated by arrows. (C) Summary of the DMS interference results.

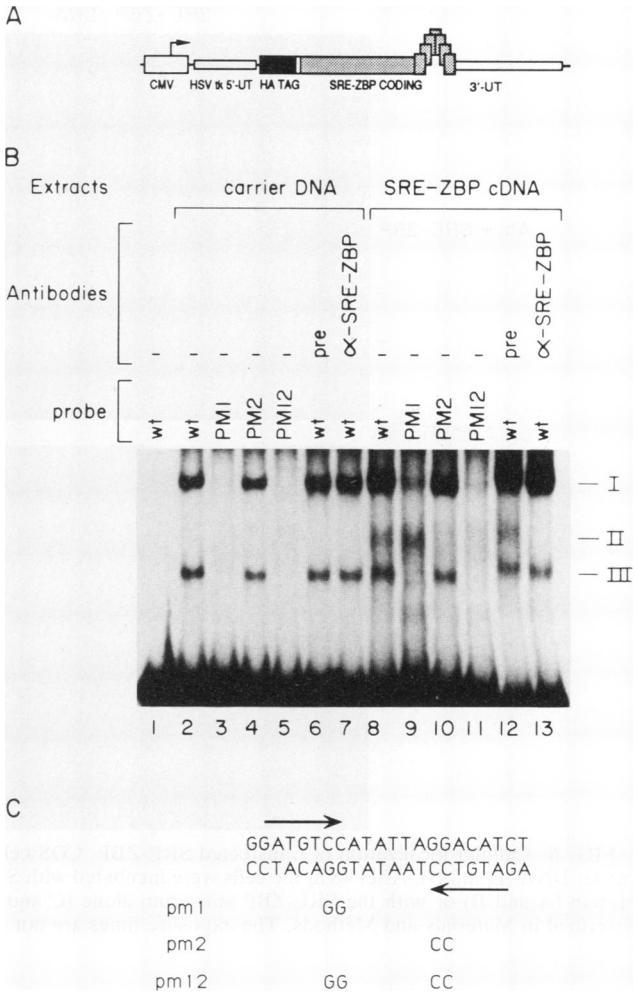

FIG. 5. Mobility shift assays of the SRE-ZBP protein produced in COS cells. (A) Structure of the pCGN/SRE-ZBP expression vector with, from left to right, the cytomegalovirus (CMV) promoter-enhancer, herpes simplex virus (HSV) thymidine kinase (tk) gene 5′ untranslated leader and initiation codon, an epitope tag derived from the influenza virus hemagglutinin (HA) gene (20), the SRE-ZBP cDNA including the entire coding sequence shown in Fig. 3A, and 600 bp of 3′ untranslated (UT) sequence. (B) Mobility shift assays of nuclear extracts prepared from COS cells transfected with salmon sperm DNA (lanes 2 to 7) or pCGN/SRE-ZBP (lanes 8 to 13). The assays were performed as described in Materials and Methods, using probes of identical specific activities. The reaction mixtures in lanes 6 and 12 contained preimmune rabbit serum, while those in lanes 7 and 13 contained rabbit antiserum against SRE-ZBP. Complex I is due to $p62^{DBF}$. Complex II is the putative SRE-ZBP complex. The identity of complex III is unknown but may be a degradation product of $p62^{DBF}$. wt, wild type. (C) Sequences of the wild-type and mutant SRE probes used in these assays.

the core sequence found in all SREs (74). This result suggests that SRE-ZBP makes asymmetric contacts with the SRE confined to the 3′ side of the dyad. This pattern of interference is different from that observed with $p67^{SRF}$ or $p62^{DBF}$ (25, 55, 73), suggesting that this cDNA encodes a novel SRE-binding protein distinct from those previously characterized.

To confirm this conclusion, we produced SRE-ZBP by transient expression in COS cells using the vector illustrated in Fig. 5A. We performed mobility shift assays on nuclear extracts prepared from control COS cells (Fig. 5B, lanes 2 to 7) or cells transfected with the SRE-ZBP expression vector (lanes 8 to 13). These assays used either a wild-type SRE probe or mutant probes shown in Fig. 5C.

Control extracts contained two SRE-specific DNA-binding activities, labeled I and III in Fig. 5B. Complex I corresponds to $p62^{DBF}$, as determined by its mobility and DMS interference pattern (data not shown). The identity of complex III is not certain, but it may be a degradation product of $p62^{DBF}$, since it shows the same pattern of binding to mutant SREs. As predicted from its previously reported DMS interference pattern, $p62^{DBF}$ bound to the wild-type probe (Fig. 5B, lanes 2 and 8) and to a mutant probe carrying substitutions in the 3′ side of the dyad (pm2; lanes 4 and 10). It did not, however, bind to probes carrying substitutions in the 5′ side of the SRE (pm1 and pm12; lanes 3, 5, 9, and 11).

Nuclear extracts prepared from COS cells transfected with the SRE-ZBP cDNA expression vector contained an additional SRE-binding activity, labeled complex II in Fig. 5B (visible in lanes 8, 9, and 12). In contrast to $p62^{DBF}$, binding of this protein was abolished by substitutions in the 3′ side of the SRE (pm2 and pm12; lanes 10 and 11) and unaffected by substitutions in the 5′ side (pm1; lane 9). This pattern of binding is consistent with the DMS interference pattern obtained with the *E. coli*-expressed GST-SRE-ZBP

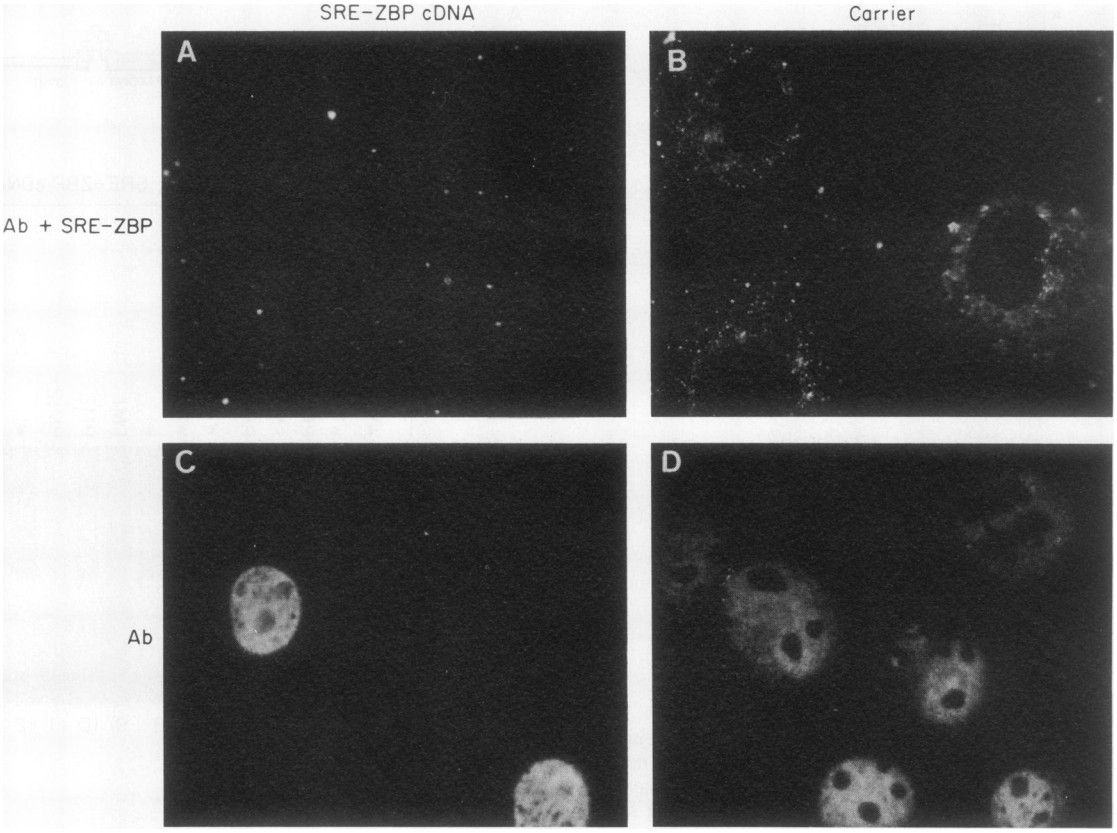

FIG. 6. Cellular localization of transfected SRE-ZBP. COS cells were transfected with the pCGN/SRE-ZBP plasmid (A and C) or with carrier DNA (B and D). After 48 h, the cells were incubated with SRE-ZBP antiserum preadsorbed with the β-galactosidase-SRE-ZBP fusion protein (A and B) or with the SRE-ZBP antiserum alone (C and D). Then, the cells were processed for indirect immunofluorescence as described in Materials and Methods. The exposure times are not identical for each panel. Ab, SRE-ZBP antiserum.

zinc finger domain, in which all contacts were confined to the 3′ side of the SRE.

Further evidence that complex II is due to the protein encoded by the SRE-ZBP cDNA is shown in lanes 12 and 13 of Fig. 5B. We raised a polyclonal antiserum against SRE-ZBP. Preincubation of the antiserum with nuclear extract from COS cells expressing the SRE-ZBP cDNA resulted in loss of complex II (lane 13). Preimmune serum from the same rabbit had no effect on complex II (lane 12). Thus, we conclude that this activity is encoded by the SRE-ZBP cDNA and therefore represents an N-terminal truncation of the natural protein. We also conclude that SRE-ZBP binds directly to the 3′ side of the SRE.

Two additional observations deserve comment. First, the SRE-ZBP antiserum does not affect formation of the $p62^{DBF}$ complex (Fig. 5B, lanes 7 and 13), confirming that this cDNA does not encode this protein. Second, none of the DNA-binding activities in control COS extracts (or in extracts of HeLa, BALB/c3T3, and HepG2 cells [data not shown]) have the binding specificity of SRE-ZBP, nor are any endogenous activities reactive with SRE-ZBP antibodies. Thus, there is no constitutive DNA-binding activity attributable to SRE-ZBP in these cells, even though, as we demonstrate below, they contain immunologically related protein.

Cellular localization of SRE-ZBP. To determine the intracellular localization of SRE-ZBP, in situ immunofluorescence assays were performed on COS cells transfected with the SRE-ZBP cDNA. The transfected cells were maintained for 48 h in the presence of DMEM supplemented with 5% FCS, fixed, and incubated with anti-SRE-ZBP serum (Fig. 6C and D) or serum that had been preincubated with purified β-galactosidase-SRE-ZBP fusion protein (Fig. 6A and B). The anti-SRE-ZBP serum strongly stained the nuclei of a fraction of the cells in the COS cultures transfected with the cDNA (Fig. 6C). This signal was specific because it was abolished by preincubation of the serum with the purified β-galactosidase fusion protein (Fig. 6A). A similar number of nuclei on parallel coverslips were stained with a monoclonal antibody against an epitope tag incorporated into SRE-ZBP (Fig. 5A); control nuclei were not stained by this antibody (data not shown). Together, these data suggest that SRE-ZBP protein expressed from the cDNA localizes to the nucleus, as expected for a DNA-binding protein.

With longer exposure of the photomicrographs, fluorescence in all nuclei of control COS cell cultures was also observed (Fig. 6D), suggesting the presence of an endogenous protein in COS cells immunologically related to SRE-ZBP. This staining was also specific because it was abolished by preincubation of the antiserum with the β-galactosidase-SRE-ZBP fusion protein (Fig. 6B).

Identification of putative cellular SRE-ZBP. To visualize directly the proteins recognized by the anti-SRE-ZBP serum in transfected and control COS cell cultures, an immunoprecipitation experiment was performed. COS cells transfected with the SRE-ZBP cDNA or with carrier DNA were metabolically labeled with [^{35}S]methionine, and the cell extracts

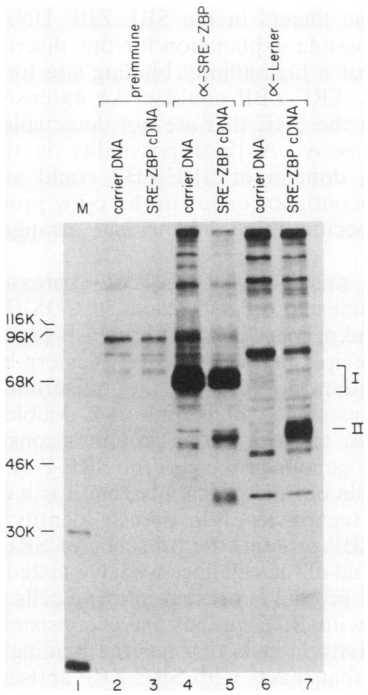

FIG. 7. Immunoprecipitation of transfected COS cell extracts with SRE-ZBP antiserum and 12CA5 monoclonal antibody. COS cells were transfected with carrier DNA (lanes 2, 4, and 6) or with pCGN/SRE-ZBP (lanes 3, 5, and 7). After 48 h, the cells were metabolically labeled for 4 h with [^{35}S]methionine. Extracts were prepared and immunoprecipitated with preimmune rabbit serum (lanes 2 and 3), SRE-ZBP antiserum (lanes 4 and 5), or 12CA5 monoclonal antibody against the epitope tag (lanes 6 and 7). Lane 1 shows molecular markers (M). Complex I is putative endogenous SRE-ZBP protein. Complex II is epitope-tagged SRE-ZBP produced from the expression vector.

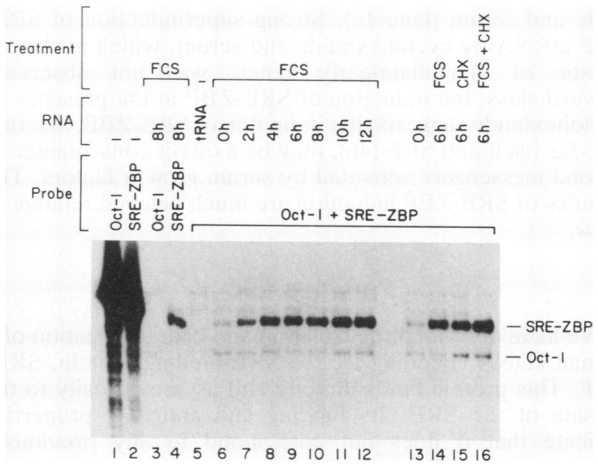

FIG. 8. RNase protection assay of serum-stimulated HeLa cells. HeLa cells were starved for 48 h in 0.5% FCS and then refed for the indicated lengths of time with DMEM containing 10% FCS, 10 μg of cycloheximide (CHX) per ml, or both. The RNase protection assay was carried out with 10 μg of total cytoplasmic RNA. Lanes: 1 and 2, the input probes; 3 and 4, RNA from serum-stimulated cells (6 h) probed with Oct-1 or SRE-ZBP probes alone; 5, control hybridization to 10 μg of tRNA; 6 to 12, RNA from cells stimulated with serum for the indicated lengths of time; 13, untreated serum-starved cells; 14 to 16, cells treated for 6 h with 10% FCS (lane 14), 10 μg of cycloheximide per ml (lane 15), and FCS and cycloheximide together (lane 16).

were immunoprecipitated with preimmune serum (Fig. 7, lanes 2 and 3), SRE-ZBP antiserum (lanes 4 and 5), and monoclonal antibody against the epitope tag incorporated into the protein (lanes 6 and 7). The antibody against the epitope tag precipitated a 48-kDa protein (labeled band II) from SRE-ZBP-transfected cells (Fig. 7, lane 7). A protein of identical size was precipitated from the same cells with the SRE-ZBP antiserum (Fig. 7, lane 5). This protein was absent from the control cells. Its size is consistent with the coding capacity of the SRE-ZBP expression plasmid (Fig. 5A). Together, these data identify this protein as the product of the SRE-ZBP expression vector.

In addition to band II, the SRE-ZBP antiserum precipitated a doublet band of approximately 70 kDa (band I). These proteins were present in control cells as well as cells transfected with the cDNA (Fig. 7, lanes 4 and 5). The proteins were not, however, precipitated by either the preimmune serum (Fig. 7, lanes 2 and 3) or the monoclonal antibody against the epitope tag (lanes 6 and 7). These observations suggest that these 70-kDa proteins may correspond to endogenous SRE-ZBP in COS cells or to proteins sharing epitopes with SRE-ZBP. Partial proteolytic mapping of these proteins with V8 protease indicated that these two bands are nearly identical in structure (1) and therefore likely derive from the same primary translation product.

Thus, the 70-kDa proteins are candidates for the endogenous full-length SRE-ZBP protein. The larger size of these proteins is consistent with the observation that the cDNA we isolated is incomplete at its 5′ end.

Analysis of the SRE-ZBP RNA. The expression of SRE-ZBP mRNA in different cell lines was studied by RNase protection assay. These experiments showed that SRE-ZBP mRNA was present in detectable but low amounts in most of the human cell lines tested, including HeLa, H9, Jurkat, 293, and BJAB. A three- to-sixfold-higher level of RNA was found in the HepG2 hepatoma and Ntera2D teratocarcinoma cell lines (data not shown). Northern (RNA) blot experiments show that the size of the SRE-ZBP mRNA is approximately 3.5 kb (data not shown).

To determine whether the SRE-ZBP mRNA was, like c-*fos* itself, subject to growth factor control, we performed an RNase protection assay on RNA from serum-stimulated HeLa cells. The cells were starved in DMEM supplemented with 0.5% FCS for 48 h and then incubated for different lengths of time in DMEM containing 10% FCS. RNA was isolated from the cells, and RNase protection assays were performed with an antisense SRE-ZBP probe spanning positions 1208 to 1465 in the SRE-ZBP cDNA. As an internal control, a probe specific for the mRNA encoding Oct-1 transcription factor was used. This protein is present in HeLa cells and is not strongly regulated by serum (18).

Figure 8 shows that the level of SRE-ZBP mRNA in serum-starved HeLa cells is low (lane 6) but rises significantly 2 h after serum stimulation (lane 7), reaching peak levels at 10 to 12 h (lanes 11 and 12). In contrast, the levels of Oct-1 RNA remained constant during serum stimulation. To determine whether induction of SRE-ZBP RNA required synthesis of new proteins, we measured RNA levels in cells stimulated with serum for 6 h in the presence of 10 μg of cycloheximide per ml (Fig. 8, lanes 13 to 16). We observed that SRE-ZBP mRNA was induced with cycloheximide alone (Fig. 8, lane 15) and by a combination of cyclohexi-

mide and serum (lane 16). Strong superinduction of SRE-ZBP mRNA by cycloheximide and serum, which is characteristic of immediate-early genes, was not observed. Nevertheless, the induction of SRE-ZBP in the presence of cycloheximide suggests that induction of SRE-ZBP, like that of c-*fos* itself and SRF (46), may be a direct consequence of second messengers activated by serum growth factors. The kinetics of SRE-ZBP induction are much delayed relative to c-*fos*.

DISCUSSION

We have described the isolation and characterization of a human cDNA encoding a c-*fos* SRE-binding protein, SRE-ZBP. This protein binds directly and asymmetrically to the 3′ side of the SRE. Its binding and antigenic properties indicate that it does not correspond to any previously characterized SRE-binding protein.

The sequence of SRE-ZBP indicates that it is a member of the C_2H_2 class of zinc finger genes, exemplified by the *Xenopus laevis* transcription factor TFIIIA and the product of the *Drosophila* gap gene Krüppel (42, 48, 54). There is a large family of such zinc finger genes in the mammalian genome, and the seven repeats found at the C terminus of SRE-ZBP match the consensus for these elements precisely (4, 13, 42). In addition, members of the Krüppel family contain a conserved protein sequence situated between adjacent fingers, called the H/C link (57). This sequence is also well conserved in SRE-ZBP.

Several members of this gene family exhibit developmental and tissue-specific expression in mammals. For example, EGR1/Krox-24/Zif268 is expressed at high levels within adult mouse brain, thymus, lung, and heart (15, 38, 70); EGR2/Krox 20 is expressed in a specific pattern in the developing mouse brain and peripheral nervous system, and in adults it is found in the thymus, spleen, and testis (10, 77). The Wilms' tumor gene, another member of this family, is highly expressed in mouse fetal kidney, and in adult mice and baboons it is limited to kidney and spleen (6, 8). The expression of two mouse Krüppel-related genes, *mkr1* and *mkr2*, decreases upon in vitro differentiation of F9 teratocarcinoma cells by retinoic acid (11). In adult mice, *mkr1* is expressed exclusively in the nervous system.

SRE-ZBP, *mkr1*, *mkr2*, and ZNF2 (53) appear to belong to a subfamily of structurally related finger proteins containing finger motifs of the Krüppel type at the C terminus (nine in ZNF2 and seven in SRE-ZBP and *mkr1*). A conserved amino acid module present in the N terminus of the ZNF2 (53) is not obvious in SRE-ZBP when both polypeptide sequences are aligned. Interestingly, both *mkr1* and SRE-ZBP contain at least one degenerate zinc finger motif; in *mkr1* it is C terminal to the seven complete fingers, and in SRE-ZBP it is N terminal. The similarity among these proteins is approximately 52 to 54% when the intact zinc fingers are compared. N terminal to the zinc finger domain, however, there is little similarity among the proteins.

By producing a fusion protein that contains only the seven zinc fingers of SRE-ZBP, we found that this portion of the protein indeed comprises the sequence-specific DNA-binding domain. Structural studies with the EGR1/Krox-24/Zif268 protein suggest that a single zinc finger makes contact with 3 bp in its cognate recognition site (47). It is therefore somewhat surprising that our DMS interference data suggest that the only guanine residues in which methylation interferes with SRE-ZBP binding are tightly clustered. This recognition would appear to require only a small number of the seven zinc fingers in the SRE-ZBP DNA-binding domain. One possible explanation for this discrepancy is that the SRE is not a high-affinity binding site for this protein. Alternatively, SRE-ZBP could make extensive redundant contacts with the SRE that are not detectable in the DMS interference assay. A third possibility is that the large DNA-binding domain of SRE-ZBP could simultaneously bind two noncontiguous sites in the c-*fos* promoter region, forming a specific three-dimensional arrangement of the DNA.

Antibodies raised against *E. coli*-expressed SRE-ZBP react with nuclear proteins present in COS (Fig. 6) and in HeLa cells and primary human fibroblasts (data not shown). In immunoprecipitation (Fig. 7) and Western blot (immunoblot) assays (data not shown), the antiserum recognizes a single major protein, which runs as a doublet of approximately 70 kDa. The size of this protein is consistent with its being the endogenous product of the SRE-ZBP gene, but we still cannot rule out the possibility that it is a cross-reacting protein. But, regardless of the precise identity of the endogenous SRE-ZBP protein, the presence of SRE-ZBP-encoding mRNA in all of the cell lines we have tested suggests that the SRE-ZBP protein is present in these cells. Yet mobility shift assays with SRE probes have consistently failed to reveal an activity in cells that has the binding specificity of SRE-ZBP or that reacts with SRE-ZBP antiserum.

Why is endogenous SRE-ZBP activity not detected in these assays? One possibility is that the DNA-binding activity of the endogenous protein is subjected to regulation in vivo. That regulation may consist of biochemical modifications that affect SRE-ZBP DNA-binding affinity. In support of the idea that SRE-ZBP DNA-binding activity may be regulated, we found that a truncated SRE-ZBP protein consisting solely of the zinc finger domain binds the SRE with much higher affinity than does a longer protein containing all of the N-terminal sequence encoded in the cDNA (data not shown). This observation is consistent with the presence of an inhibitory domain in the full-length protein. Inactivation of this inhibitory domain may require covalent modification of the protein or association with an accessory subunit. We note, however, that the β-galactosidase-SRE-ZBP fusion protein produced by the original recombinant phage was quite active, suggesting that the putative inhibitory domain in SRE-ZBP is counteracted by β-galactosidase sequences. Regulation of the activity of a zinc finger protein has been reported for the *Drosophila glass* gene. The *glass* protein is expressed in all cell types in the developing eye but is active only in certain cell types (45).

What is the function, if any, of SRE-ZBP in the regulation of c-*fos* transcription? Although our data do not show that SRE-ZBP functionally associates with the SRE in vivo, genomic footprinting data in A431, NIH 3T3, and F9 cells (30, 34) indicate that an unidentified cellular protein is bound to the SRE 3′ to the SRF-binding site, in the region that we have shown is bound by SRE-ZBP in vitro. This site is also bound by rNFIL-6, whose activity is induced by treatment of PC12 cells with forskolin (41). Several mutations in this region of the SRE have been reported to result in derepression of c-*fos* transcription (39, 58). Some of these mutations affect SRF binding, whereas others affect only the sequences immediately flanking the SRF binding site. The phenotypes of these mutations, generally attributed to SRF, may be due instead to loss of binding of a protein such as SRE-ZBP with repressor activity. The possibility that SRE-ZBP functions as a repressor is consistent with the observation that Krüppel acts as a repressor in both *Drosophila* and mammalian

cells (40, 80). More recently, a human Krüppel-related protein, YY1, has been identified as a transcriptional repressor of the adeno-associated virus P5 promoter in the absence of E1A (61).

An alternative role for SRE-ZBP is in some specific developmental aspect of c-*fos* regulation. During mouse development, several tissues exhibit constitutive high-level c-*fos* expression (9). Such regulation may require disruption of the complexes that keep c-*fos* transcription under the tight control seen in most cells. A developmental role for SRE-ZBP is an attractive one, given the frequent association of zinc finger proteins with important events in development.

Although many genes encoding members of this family of DNA-binding proteins have been cloned, specific binding sites are known for only a few of these proteins (14, 31, 33, 50, 72). In many of these cases, the biological functions of the binding sites have not been established. Thus, the association of SRE-ZBP with a well-characterized regulatory element provides an important opportunity to study the structure and function of both the DNA-binding and the transcriptional regulatory domains of this type of zinc finger protein.

ACKNOWLEDGMENTS

We thank Paula Henthorn and Tom Kadesch for the cDNA library; Masafumi Tanaka, Karl Riabowol, Reggie Aurora, Gokul Das, Shushma Abraham, and Mark Ustav for reagents and technical advice; Regina Whitaker for technical assistance; members of the Gilman laboratory for advice and support; and Winship Herr and Nouria Hernandez for comments on the manuscript.

This work was supported by grants from NIH (CA45642 and CA46370).

REFERENCES

1. **Abraham, S. (Cold Spring Harbor Laboratory).** 1991. Personal communication.
2. **Almendral, J. M., D. Sommer, H. Macdonald-Bravo, J. Burckhardt, J. Perera, and R. Bravo.** 1988. Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. **8**:2140–2148.
3. **Aurora, R., and W. Herr.** 1992. Segments of the POU domain influence one another's DNA-binding specificity. Mol. Cell. Biol. **12**:455–467.
4. **Berg, J.** 1986. Potential metal-binding domains in nucleic acid binding proteins. Science **232**:485–486.
5. **Berkowitz, L. A., K. T. Riabowol, and M. Z. Gilman.** 1989. Multiple sequence elements of a single functional class are required for cyclic AMP responsiveness of the mouse c-*fos* promoter. Mol. Cell. Biol. **9**:4272–4281.
6. **Buckler, A. J., J. Pelletier, D. A. Haber, T. Glaser, and D. E. Housman.** 1991. Isolation, characterization and expression of the murine Wilms' tumor gene (WT1) during kidney development. Mol. Cell. Biol. **11**:1707–1712.
7. **Buscher, M., H. J. Rhamsdorf, M. Litfin, M. Karin, and P. Herrlich.** 1988. Activation of the c-*fos* gene by UV and phorbol ester; different signal pathways converge to the same enhancer element. Oncogene **3**:301–311.
8. **Call, K. M., T. Glaser, C. Y. Ito, A. J. Buckler, J. Pelletier, D. A. Haber, E. A. Rose, A. Kral, H. Yeger, and W. H. Lewis.** 1990. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilm's tumor locus. Cell **60**:509–520.
9. **Caubet, J. F.** 1989. c-*fos* proto-oncogene expression in the nervous system during mouse development. Mol. Cell. Biol. **9**:2269–2272.
10. **Chavrier, P., M. Zerial, P. Lemaire, J. Almendral, R. Bravo, and P. Charnay.** 1988. A gene encoding a protein with zinc fingers is activated during G_0/G_1 transition in cultured cells. EMBO J. **7**:29–35.
11. **Chowdhury, K., U. Deutsch, and P. Gruss.** 1987. A multigene family encoding several "finger" structures is present and differentially active in mammalian genomes. Cell **48**:771–778.
12. **Chowdhury, K., G. Dressler, G. Breier, U. Deutsch, and P. Gruss.** 1988. The primary structure of the murine multifinger gene mKr2 and its specific expression in developing and adult neurons. EMBO J. **7**:1345–1353.
13. **Chowdhury, K., H. Rodhewohld, and P. Gruss.** 1988. Specific and ubiquitous expression of different Zn finger protein genes in the mouse. Nucleic Acids Res. **16**:9995–10011.
14. **Christy, B., and D. Nathans.** 1989. DNA binding site of the growth factor-inducible protein Zif268. Proc. Natl. Acad. Sci. USA **86**:8737–8741.
15. **Christy, B. A., L. F. Lau, and D. Nathans.** 1988. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc. Natl. Acad. Sci. USA **85**:7857–7861.
16. **Cunliffe, V., P. Koopman, A. McLaren, and J. Trowsdale.** 1990. A mouse zinc finger gene which is transiently expressed during spermatogenesis. EMBO J. **9**:197–205.
17. **Curran, T.** 1988. *fos*, p. 307–325. *In* E. P. Reddy, A. M. Skalka, and T. Curran (ed.), Oncogene handbook. Elsevier/North-Holland Publishing Co., Amsterdam.
18. **Das, G. (Cold Spring Harbor Laboratory).** 1991. Personal communication.
19. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
20. **Field, J., J. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler.** 1988. Purification of a RAS responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. **8**:2159–2165.
21. **Fisch, T. M., R. Prywes, and R. G. Roeder.** 1987. c-*fos* sequences required for basal expression and induction by EGF, TPA and Ca^{2+}ionophore. Mol. Cell. Biol. **7**:3490–3502.
22. **Franza, B. R., Jr., L. C. Sambucetti, D. R. Cohen, and T. Curran.** 1987. Analysis of Fos protein complexes and Fos-related antigen by high resolution two-dimensional electrophoresis. Oncogene **1**:213–221.
23. **Gilman, M. Z.** 1988. The c-fos serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. Genes Dev. **2**:394–402.
24. **Gilman, M. Z., L. A. Berkowitz, J. R. Feramisco, B. R. Franza, R. M. Graham, K. T. Riabowol, and W. A. Ryan.** 1988. Intracellular mediators of c-*fos* induction. Cold Spring Harbor Symp. Quant. Biol. **53**:761–767.
25. **Gilman, M. Z., R. N. Wilson, and R. A. Weinberg.** 1986. Multiple protein-binding sites in the 5'-flanking region regulate c-*fos* expression. Mol. Cell. Biol. **6**:4305–4316.
26. **Graham, R., and M. Gilman.** 1991. Distinct protein targets for signal acting at the c-fos serum response element. Science **251**:189–192.
27. **Graham, R., and M. Z. Gilman.** Unpublished data.
28. **Greenberg, M. E., Z. Siegfried, and E. B. Ziff.** 1987. Mutation of the c-*fos* dyad symmetry element inhibits inducibility of transcription in vivo and the nuclear regulatory factor binding in vitro. Mol. Cell. Biol. **7**:1217–1225.
29. **Henthorn, P. S., M. Raducha, Y. H. Edwards, M. J. Weiss, C. Slaughter, M. A. Lafferty, and H. Harris.** 1987. Nucleotide and amino acid sequences of human intestinal alkaline phosphatase: close homology to placental alkaline phosphatase. Proc. Natl. Acad. Sci. USA **84**:1234–1238.
30. **Herrera, R. E., P. E. Shaw, and A. Nordheim.** 1989. Occupation of the c-*fos* serum response element in vivo by a multiprotein complex is unaltered by growth factor induction. Nature (London) **340**:68–70.
31. **Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian.** 1987. Isolation of the cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51**:1079–1090.
32. **Kato, N., K. Shimotohno, D. VanLeeuwen, and M. Cohen.** 1990. Human proviral mRNAs down regulated in choriocarcinoma

encode a zinc finger protein related to Krüppel. Mol. Cell. Biol. **10**:4401–4405.
33. **Kinzler, K. W., and B. Vogelstein.** 1990. The *GLI* gene encodes a nuclear protein which binds specific sequences in the human genome. Mol. Cell. Biol. **10**:634–642.
34. **König, H.** 1991. Cell-type specific multiprotein complex formation over the c-fos serum response element *in vivo*: ternary complex formation is not required for the induction of c-fos. Nucleic Acids Res. **19**:3607–3611.
35. **König, H., H. Ponta, U. Rahmsdorf, M. Büscher, A. Schönthal, H. J. Rahmsdorf, and P. Herrlich.** 1989. Autoregulation of fos: the dyad symmetry element as the major target of repression. EMBO J. **8**:2559–2566.
36. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**: 488–492.
37. **Lamb, N. J. C., A. Fernandez, N. Tourkine, P. Jeanteur, and J. M. Blanchard.** 1990. Demonstration in living cells of an intragenic negative regulatory element within the rodent c-fos gene. Cell **61**:485–496.
38. **Lemaire, P., O. Revelant, R. Bravo, and P. Charnay.** 1988. Two mouse genes encoding potential transcription factors with identical DNA-binding domains activated by growth factors in cultured cells. Proc. Natl. Acad. Sci. USA **85**:4691–4695.
39. **Leung, S., and N. G. Miyamoto.** 1989. Point mutational analysis of the human c-fos serum response factor binding site. Nucleic Acids Res. **17**:1177–1195.
40. **Licht, J. D., M. J. Grossel, J. Figge, and U. M. Hansen.** 1990. Drosophila *Krüppel* protein is a transcriptional repressor. Nature (London) **346**:76–79.
41. **Metz, R., and E. Ziff.** 1991. cAMP stimulates the C/EBP-related transcription factor rNFIL-6 to trans-locate to the nucleus and induce c-fos transcription. Genes Dev. **5**:1754–1766.
42. **Miller, J., A. D. McLachlan, and A. Klug.** 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO J. **4**:1609–1614.
43. **Miskimins, W. K., M. P. Roberts, A. McClelland, and F. H. Ruddle.** 1985. Use of a protein-blotting procedure and a specific DNA probe to identify nuclear proteins that recognize the promoter region of the transferring receptor gene. Proc. Natl. Acad. Sci. USA **82**:6741–6744.
44. **Mohun, T. J., N. Garret, and R. H. Treisman.** 1987. Xenopus cytoskeletal actin and human c-fos gene promoters share a protein binding site. EMBO J. **6**:667–673.
45. **Moses, K., and G. M. Rubin.** 1991. glass encodes a site-specific DNA-binding protein that is regulated in response to positional signals in the developing Drosophila eye. Genes Dev. **5**:583–593.
46. **Norman, C., M. Runswick, R. Pollock, and R. Treisman.** 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell **55**:989–1003.
47. **Pavletich, N. P., and C. O. Pabo.** 1991. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. Science **252**:809–817.
48. **Preiss, A., U. B. Rosenberg, A. Kienlin, E. Seifert, and H. Jäckle.** 1985. Molecular genetics of *Krüppel*, a gene required for segmentation of the Drosophila embryo. Nature (London) **313**:27–32.
49. **Prywes, R., and R. G. Roeder.** 1986. Inducible binding of a factor to the c-fos enhancer. Cell **47**:777–784.
50. **Rauscher, F. J., III, J. F. Morris, O. E. Tournay, D. M. Cook, and T. Curran.** 1990. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science **250**: 1259–1262.
51. **Riabowol, K. T., M. Z. Gilman, and J. R. Feramisco.** 1988. Microinjection of the catalytic subunit of cAMP-dependent protein kinase induces expression of the c-fos gene. Cold Spring Harbor Symp. Quant. Biol. **53**:85–90.
52. **Rivera, V. M., M. Sheng, and M. Greenberg.** 1990. The inner core of the serum response element mediates both the rapid induction and subsequent repression of c-fos transcription following serum stimulation. Genes Dev. **4**:255.
53. **Rosati, M., M. Marino, A. Franzé, A. Tramontano, and G. Grimaldi.** 1991. Members of the zinc finger protein gene family sharing a conserved N-terminal module. Nucleic Acids Res. **19**:5661–5667.
54. **Rosenberg, U. B., A. Schröeder, A. Preiss, A. Keinlin, S. Coté, I. Riede, and H. Jäckle.** 1986. Structural homology of the product of the Drosophila Krüppel gene with Xenopus transcription factor IIIA. Nature (London) **319**:336–339.
55. **Ryan, W. A., Jr., R. B. Franza, Jr., and M. Z. Gilman.** 1989. Two distinct cellular phosphoproteins bind to the c-fos serum response element. EMBO J. **8**:1785–1792.
56. **Sassonne-Corsi, P., J. C. Sisson, and I. M. Verma.** 1988. Transcriptional regulation of the c-fos proto-oncogene. Nature (London) **334**:314–319.
57. **Schuh, R., W. Aicher, U. Gaul, S. Coté, A. Preiss, D. Maier, E. Seifert, U. Nauber, C. Schröeder, and R. Kemler.** 1986. A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Krüppel, a Drosophila segmentation gene. Cell **47**:1025–1032.
58. **Shaw, P. E., S. Frasch, and A. Nordheim.** 1989. Repression of c-fos transcription is mediated through $p67^{SRF}$ bound to the SRE. EMBO J. **8**:2567–2574.
59. **Shaw, P. E., H. Schroter, and A. Nordheim.** 1989. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. Cell **56**:563–572.
60. **Sheng, M., S. T. Dougan, C. McFadden, and M. E. Greenberg.** 1988. Calcium and growth factor pathways of c-*fos* transcriptional activation require distinct upstream regulatory sequences. Mol. Cell. Biol. **8**:2787–2796.
61. **Shi, Y., E. Seto, L.-S. Chang, and T. Shenk.** 1991. Transcriptional repression by YY1, a human GLI-*Krüppel*-related protein, and relief of repression by adenovirus E1A protein. Cell **67**:377–388.
62. **Siegfried, Z., and E. B. Ziff.** 1989. Transcription activation by serum, PDGF and TPA through the c-fos DSE: cell type specific requirements for induction. Oncogene **4**:3–11.
63. **Singh, H., J. H. LeBowitz, A. S. Baldwin, and P. A. Sharp.** 1988. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a regnition site DNA. Cell **52**:415–423.
64. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene **67**:31–40.
65. **Staudt, L. M., R. G. Clerc, H. Singh, J. H. LeBowitz, P. A. Sharp, and D. Baltimore.** 1988. Cloning of a lymphoid specific cDNA encoding a protein binding the regulatory octamer DNA motif. Science **241**:577–580.
66. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185**:60–89.
67. **Stumpo, D., T. N. Stewart, M. Z. Gilman, and P. J. Blackshear.** 1988. Identification of c-fos sequences involved in induction by insulin and phorbol esters. J. Biol. Chem. **263**:1611–1616.
68. **Sturm, R. A., G. Das, and W. Herr.** 1988. The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. Genes Dev. **2**:1582–1599.
69. **Subramaniam, M., L. J. Schmidt, C. E. Crutchfield III, and M. Getz.** 1989. Negative regulation of serum-responsive enhancer elements. Nature (London) **340**:64–66.
70. **Sukhatme, V. P., X. Cao, L. C. Chang, C.-H. Tsai-Morris, D. Stamencovich, P. C. P. Ferreira, D. R. Cohen, S. A. Edwards, T. B. Shows, and T. Curran.** 1988. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell **53**:37–43.
71. **Tanaka, M., and W. Herr.** 1990. Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. Cell **60**:375–386.
72. **Treisman, J., and C. Desplan.** 1989. The products of the Drosophila gap genes hunchback and Krüppel bind to the hunchback promoters. Nature (London) **341**:335–337.
73. **Treisman, R.** 1986. Identification of a protein-binding site that mediates transcriptional response of the c-fos gene to serum

factors. Cell **46**:567–574.
74. **Treisman, R.** 1990. The SRE: a growth factor transcriptional regulator. Semin. Cancer Biol. **1**:47–58.
75. **Vinson, C. R., K. L. LaMarco, P. F. Johnson, W. H. Landschulz, and S. L. McKnight.** 1988. In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. Genes Dev. **2**:801–806.
76. **Walsh, K.** 1989. Cross-binding of factors to functionally different promoter elements in c-fos and skeletal actin genes. Mol. Cell. Biol. **9**:2191–2201.
77. **Wilkinson, D. G., S. Bhatt, P. Chavrier, R. Bravo, and P. Charnay.** 1989. Segment-specific expression of a zinc finger gene in developing nervous system of the mouse. Nature (London) **337**:461–464.
78. **Wilson, T. C., and R. Treisman.** 1988. Fos C-terminal mutations block down-regulation of c-fos transcription following serum stimulation. EMBO J. **7**:4193–4202.
79. **Young, R. A., and R. W. Davis.** 1983. Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. USA **80**:1194–1198.
80. **Zuo, P., D. Stanojevic, J. Colgan, K. Han, M. Levine, and J. L. Manley.** 1990. Activation and repression of transcription by the gap proteins hunchback and Krüppel in cultured Drosophila cells. Genes Dev. **5**:254–264.