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Multiple Protein-Binding Sites in the 5′-Flanking Region Regulate c-*fos* Expression

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We tested sequences flanking the mouse c-*fos* gene for the ability to form specific DNA-protein complexes with factors present in crude nuclear extracts prepared from mammalian cells. Three such complexes were detected. One complex formed in a region necessary for the induction of c-*fos* expression by serum growth factors. Two additional complexes formed at sequences that contribute to basal c-*fos* promoter activity in vivo. These complexes represent three novel sequence-specific DNA-binding activities which appear to participate in the regulation of c-*fos* transcription.

How extracellular signals are communicated to the nucleus is poorly understood. To address this problem, we chose to study the regulation of the proto-oncogene c-*fos*. The c-*fos* gene is rapidly and transiently induced in response to a diverse group of biological signals in a wide variety of cell types. It is induced in quiescent fibroblasts by polypeptide growth factors (3, 17, 19, 26), in the myeloid tumor lines HL-60 and U-937 when induced to differentiate by the phorbol ester tumor promoter TPA (24, 27), in the rat pheochromocytoma cell line PC-12 induced to differentiate by nerve growth factor (5, 15, 20), and in the B lymphoma line WEHI 231 treated with antibodies to its surface immunoglobulin (M. Z. Gilman, unpublished data). c-*fos* induction is therefore a frequent response to incoming signals, and the signalling pathway through which this induction is executed is likely to be conserved in many cell types. Constitutive expression of the c-*fos* gene morphologically transforms fibroblasts (23) and may promote differentiation of embryonal carcinoma cells (32).

Recently, *cis*-acting sequences required for induction of the human c-*fos* gene have been identified (39). As an alternative strategy for elucidating the mechanism of regulation of the c-*fos* gene, we sought nuclear proteins that specifically bind to the regulatory regions of the gene. We used a gel electrophoresis assay to identify such binding activities. This assay was originally developed to study the interaction of purified procaryotic regulatory proteins and DNA sequences (10, 11) and was extended by Strauss and Varshavsky (36) to allow detection of a eucaryotic DNA-binding protein in a crude nuclear extract. Most recently, this assay has been used with great success to detect proteins that bind to a number of cellular and viral promoters and enhancers (1, 29, 35). We used this assay to identify three sequence-specific DNA-binding activities in crude nuclear extracts that interact selectively with sequences in the 5′-flanking region of the mouse c-*fos* gene.

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MATERIALS AND METHODS

Cell culture. BALB/c 3T3 fibroblasts were obtained from A. Baldwin and P. Sharp. They were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum. WEHI 231, a B lymphoma line, was obtained from S. Pillai and was grown in RPMI 1640 medium containing 10% fetal calf serum and 50 μM β-mercaptoethanol.

Plasmid DNAs. A plasmid containing the mouse c-*fos* gene (p-cfos3 [23]) was obtained from I. Verma. The parental *fos*-chloramphenicol acetyltransferase (CAT) fusion plasmid (p146) was prepared by S. Dessain by isolating a 1.1-kilobase *Sst*I-*Acc*I fragment of p-cfos3 carrying c-*fos* sequences from −1000 to + 109, blunting the ends with T4 DNA polymerase, adding *Xba*I linkers, and inserting it into the *Xba*I site of p106CAT. The latter plasmid was created by inserting an *Xma*I-*Eco*RI (partial) fragment of pSV0CAT (14) carrying the CAT gene and downstream simian virus 40 (SV40)-derived sequences between the *Xma*I and *Eco*RI sites of pUC13.

BAL 31 deletions were prepared from p146 by cleaving it at the *Sst*II site located at −400 in the mouse c-*fos* 5′-flanking sequence and digesting with BAL 31 exonuclease (4 U/20 μg of DNA) for 1 to 2 min at 30°C. After repair of the ends with Klenow fragment DNA polymerase, *Sal*I linkers were added. Extensive digestion with *Sal*I released excess linkers and additionally cleaved the plasmid DNA at the *Sal*I site in the pUC13-derived polylinker just upstream of the inserted c-*fos* DNA, releasing c-*fos* sequences upstream of −400. The DNA fraction containing the deleted, linkered plasmids was isolated by electrophoresis through low-melting agarose and treated with T4 DNA ligase to circularize the plasmids through their *Sal*I ends. This strategy resulted in a set of 5′ deletions beginning at −400 with each endpoint joined to the *Sal*I site of the polylinker.

To create the plasmid in which binding site 2 was inserted adjacent to sequences ending at −56 (effectively deleting sequences from −110 to −57), the Δ-151 plasmid was cleaved with *Hae*II, treated with T4 DNA polymerase to blunt the *Hae*II ends, ligated to *Sal*I linkers, and cleaved with *Sal*I to release a 39-base-pair (bp) *Sal*I fragment carrying c-*fos* sequences −151 to −120. The fragment was iso-

lated from a 12% polyacrylamide gel and inserted into the *Sal*I site flanking the deletion endpoint in plasmid Δ-56.

To create the related plasmid carrying both binding sites for factor 2, the Δ-151 plasmid was cleaved with *Bss*HII which cuts at −99 and treated with the Klenow fragment of *Escherichia coli* DNA polymerase I. After the addition of *Sal*I linkers and digestion with *Sal*I, the 69-bp fragment carrying sequences from −151 to −96 was isolated and inserted into Δ-56 as above.

All constructions were verified by sequence analysis (see below). Sequencing was facilitated by the proximity of the inserts in all these constructions to the annealing site for the M13 reverse sequencing primer.

DNA sequence analysis. Nucleotide sequences were determined by the chain termination method by using supercoiled plasmid DNA templates (2). To determine the sequence of the mouse c-*fos* 5′-flanking region, we sequenced several deletion plasmids with endpoints throughout the region. Our sequence is in agreement with those previously reported (31, 39).

Preparation of extracts. Nuclear extracts from WEHI 231 cells were prepared essentially as described by Dignam et al. (7), usually from 10^9 cells. The HeLa whole-cell extract was a gift from L. Chodosh, R. Carthew, and P. Sharp and was prepared as described by Manley et al. (21).

Preparation of labeled DNA fragments. To prepare labeled restriction fragments as substrates for the binding assay, 20 μg of the appropriate deletion plasmid was cleaved with *Hin*dIII which cuts in the polylinker 19 bp upstream from the deletion endpoint. For 5′-end-labeled fragments, the digested DNA was treated with 5 U of calf intestinal alkaline phosphatase for 15 min at 37°C and was isolated by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. The dephosphorylated DNA was treated with 10 U of T4 polynucleotide kinase in a 25-μl reaction containing 200 μCi of [γ-^{32}P]ATP for 30 min at 37°C. For 3′-end-labeled fragments, the digested DNA was isolated without phosphatase treatment and was treated with 5 U of Klenow polymerase in a 50-μl solution containing 100 μCi of [α-^{32}P]dATP (5,000 Ci/mmol; New England Nuclear Corp.) and 200 μM each dCTP, dGTP, and TTP. Incubation was for 30 min at ambient temperature, followed by a 15-min chase with 500 μM dATP.

The labeled DNA was ethanol precipitated in the presence of 2 M ammonium acetate to remove much of the unincorporated nucleotide and was digested with the appropriate secondary restriction enzyme. The DNA was loaded onto an 8% polyacrylamide gel. The appropriate fragments were located by autoradiography, excised from the gel, and recovered by elution overnight at 37°C into 400 μl of a buffer containing 10 mM Tris (pH 8), 1 mM EDTA, and 400 mM NaCl. The fragments were purified by two extractions with phenol-chloroform-isoamyl alcohol, one extraction with chloroform-isoamyl alcohol, and ethanol precipitation. We typically recovered 2×10^7 to 4×10^7 dpm of fragment with a specific activity of approximately 10^8 dpm/μg.

Binding assays. Analytical binding assays were performed in 10-μl reactions. Each reaction contained approximately 10,000 dpm (0.2 ng) of probe fragment and 10 to 15 μg of extract protein. Optimum conditions differed for each complex. For complexes 1 and 2, the reactions contained 10 mM Tris (pH 7.5), 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 5% (vol/vol) glycerol, 50 mM NaCl, and 200 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia, Inc.) per ml. In addition, complex 1 assays contained 10 μg of pUC13 DNA per ml. Complex 3 assays were identical except that they contained 100 mM NaCl and 1 mM $MgCl_2$. After incubation for 15 min at ambient temperature, the reactions were loaded directly onto native polyacrylamide gels. The gels contained 4% (wt/vol) polyacrylamide (acrylamide/bis ratio of 29:1) and were run at 8 to 10 V/cm in a buffer containing 50 mM Tris, 0.38 M glycine, and 1 mM EDTA (pH 8.5). Gels were dried and exposed to Kodak XAR-5 film with intensifying screens at −70°C.

To obtain purified restriction fragments for competition experiments, 50 μg of plasmid DNA was digested with the appropriate enzymes. After phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation, the digested DNA was loaded onto a preparative polyacrylamide gel. The appropriate bands were visualized by ethidium bromide staining, excised from the gel, and eluted at 37°C overnight into 400 μl of buffer containing 10 mM Tris (pH 8), 1 mM EDTA, 400 mM NaCl, and 25 μg of glycogen per ml. Recovery and purity of the fragments was assessed by electrophoresis adjacent to known amounts of digested plasmid DNA.

Methylation interference assays. Restriction fragments labeled at one 3′ or 5′ end were prepared as described above. A portion of this DNA was treated with dimethyl sulfate as described by Maxam and Gilbert (22). Dimethyl sulfate treatment was for 7 min at ambient temperature. The reaction was stopped as described above except that carrier tRNA was omitted from the stop buffer and replaced by 10 μg of glycogen. The methylated fragment was recovered by two precipitations with ethanol.

Preparative binding reactions were carried out scaled up 10-fold from the analytical reactions except that 300,000 dpm of methylated probe was used. Each set of reactions was loaded onto a 1.5-mm-thick 4% polyacrylamide gel and subjected to electrophoresis (as described above). The wet gel was exposed to film at 4°C overnight to locate the complexed and free DNA. The appropriate portions of the gel were excised and cast in a 1% agarose gel. The DNA was recovered from the gel slices by electrophoresis onto NA-45 membrane (Schleicher & Schuell, Inc.) and eluted from NA-45 by incubation in 20 mM Tris (pH 8)–1 mM EDTA–1 M NaCl for 45 min at 68°C. Carrier tRNA (10 μg) was added to the eluates which were then extracted twice with phenol-chloroform-isoamyl alcohol and once with chloroform-isoamyl alcohol. The DNAs were precipitated with ethanol, redissolved in 100 μl of 0.3 M sodium acetate, pH 5.2, and precipitated again with ethanol. The pellets were rinsed with absolute ethanol, dried, and redissolved in 100 μl of freshly prepared 1 M piperidine. The DNAs were incubated in piperidine for 30 min at 90°C. At this time, a portion of the original methylated DNA was also treated with piperidine to serve as a marker ladder. The piperidine was removed by lyophilization. The DNAs were redissolved in 100 μl water, frozen, and lyophilized. This cycle was repeated three times. Finally, the radioactivity in the dried samples was measured, and equal Cerenkov counts per minute of the bound and free fractions recovered from the gel, together with the same amount of the ladder, were loaded onto a 12% polyacrylamide–8 M urea sequencing gel. Gels were run at 1,000 to 1,400 V and exposed to film with intensifying screens.

Transfections. BALB/c 3T3 cells were split 1:6 to 1:12 the day before transfection and fed with 9 ml of fresh medium 2 h before transfection. For CAT assays, test plasmid DNA (20 μg) and pCH110 (*lacZ* control) DNA (10 μg) were mixed and precipitated with ethanol, dried, and redissolved in 1 ml of 250 mM $CaCl_2$. A quantity of 0.5 ml of transfection buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-

ethanesulfonic acid] [pH 7.12], 280 mM NaCl, 1.5 mM Na_2HPO_4) was gently bubbled with air while 0.5 ml of DNA-$CaCl_2$ solution was added dropwise. Bubbling was continued for 5 s, and the mixture was vortexed for 10 s. Precipitates were allowed to form for 20 min at room temperature. Duplicate transfection precipitates were formed for each test DNA. The transfection precipitates were added dropwise to the medium on the dishes, and the cells were incubated for 16 h at 37°C. The cell monolayers were washed three times with phosphate-buffered saline to remove the precipitate and refed with 10 ml of medium. After an additional 30 to 35 h, cells were harvested, and lysates were prepared as described (14).

Transfection experiments in which RNA was analyzed were performed identically, except that 2 ml of transfection precipitates were formed and distributed to two 10-cm dishes. The *lacZ* control DNA was omitted from the transfections. For serum stimulation experiments, cells were fed after transfection with DME medium containing 0.5% fetal calf serum. After 30 h, one set of dishes for each transfected plasmid was fed 10 ml of fresh DME medium containing 15% fetal calf serum. After a further 60-min incubation, cells were harvested, and total cytoplasmic RNA was extracted.

RNA analysis. Transfected cells were placed on ice and washed three times with ice-cold phosphate-buffered saline. Cells were scraped from their dishes into 1 ml of phosphate-buffered saline. At this time, cells from dishes that received the same precipitates were pooled. The cells were recovered by centrifugation and lysed by the addition of 375 μl of a buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, and 0.5% (vol/vol) Nonidet P-40. After a 5-min incubation on ice, nuclei were pelleted by centrifugation for 2 min at 4°C in a microcentrifuge. The supernatant fluid was recovered, brought to 0.2% (wt/vol) sodium dodecyl sulfate, extracted twice with phenol-chloroform-isoamyl alcohol and once with chloroform-isoamyl alcohol, brought to 0.3 M sodium acetate, pH 5.2, and precipitated with 2.5 volumes of absolute ethanol. The precipitated RNA was rinsed with 75% ethanol, dried, and redissolved in 100 μl of water. RNA concentrations were determined by measuring A_{260}.

Single-stranded end-labeled DNA probes for S1 nuclease analysis were prepared as follows. An oligonucleotide complementary to CAT RNA (a gift from R. Kingston) was end-labeled by treatment with 5 U of polynucleotide kinase in a 30-μl reaction containing 100 μCi of [γ-^{32}P]ATP (7,000 Ci/mmol; New England Nuclear). The incorporation of label into the oligonucleotide was monitored by thin-layer chromatography. The labeled oligonucleotide was recovered by precipitation with 5 volumes of ethanol. Plasmid DNA (30 μg of plasmid Δ-56) was denatured in alkali, neutralized, and precipitated with ethanol (2). Labeled oligonucleotide and denatured plasmid DNA were annealed to form a primed template for Klenow fragment DNA polymerase. Annealing was carried out for 15 min at 40°C in 79 μl of 11 mM Tris (pH 8.0)–11 mM $MgCl_2$. Unlabeled deoxynucleoside triphosphates (400 μM each) and Klenow polymerase (9 U) were added, bringing the final volume to 90 μl. Incubation was continued for 30 min at 37°C. The reaction mixture was next treated with 60 U of *Hin*dIII for 30 min at 37°C to cleave extended, double-stranded DNA at the vector *Hin*dIII site 19 bp upstream of the inserted c-*fos* DNA. After precipitation with ethanol, the DNA was denatured in formamide and loaded onto a 4% polyacrylamide–8 M urea gel. The single-stranded end-labeled fragment extending from the CAT oligonucleotide to the *Hin*dIII site was isolated as described above. The specific activity of this probe was approximately 10^8 dpm/μg.

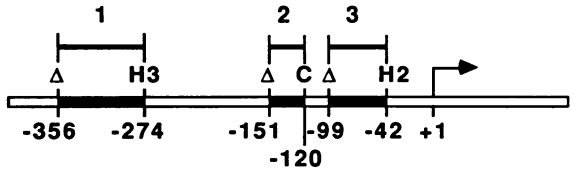


FIG. 1. Map of the mouse c-*fos* gene. Regions in black represent the fragments used in the binding assay. Each fragment contained c-*fos* sequences bounded by a BAL 31 deletion endpoint, indicated by Δ, and the restriction enzyme cleavage sites shown. In addition, all fragments contained 19 bp of pUC13 polylinker DNA extending from the deletion endpoint to the vector *Hin*dIII site that was used to excise the fragment. The start site for transcription (40) is indicated with an arrow. Abbreviations: C, *Cfo*I site; H2, *Hae*II site; H3, *Hae*III site.

For S1 nuclease analysis, 50 μg of total cytoplasmic RNA from transfected cells was precipitated with ethanol and redissolved for hybridization in 30 μl of 80% (vol/vol) formamide–40 mM PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) (pH 6.4)–0.4 M NaCl–1 mM EDTA containing 50,000 dpm of probe. Hybridization reactions were incubated at 90°C for 5 min and at 42°C for 16 h. After hybridization, 300 μl of a solution containing 300 mM NaCl, 30 mM sodium acetate, 3 mM $ZnSO_4$, 20 μg of sonicated denatured herring sperm DNA per ml, and 600 U of S1 nuclease per ml (pH 4.5) was added. The reactions were incubated for 45 min at 37°C, extracted with phenol-chloroform-isoamyl alcohol, and precipitated with ethanol. Analysis was on 8% sequencing gels.

Enzymes assays. CAT enzyme assays were carried out as described (14), except that 0.2 μCi of [^{14}C]chloramphenicol was used in each assay. Assays contained 75 to 150 μg of extract protein and were done for 45 min at 37°C. After analysis by thin-layer chromatography, the acetylated and unreacted forms of chloramphenicol were located, cut out, and counted. CAT activity was calculated as percent chloramphenicol converted to acetylated forms.

β-Galactosidase assays were carried out as described by Hall et al. (18). Assays contained 75 to 150 μg of protein.

To calculate relative CAT activity, the specific CAT activity in each lysate was divided by the specific β-galactosidase activity in that lysate. Within each experiment, the values obtained from duplicate transfections were averaged. The values shown in Fig. 7 represent the mean and standard deviations of the indicated numbers of experiments. All assays were performed within their linear ranges.

RESULTS

Identification of sequence-specific DNA-protein complexes. We used the gel electrophoresis assay to assess the ability of various fragments of the mouse c-*fos* gene to form specific DNA-protein complexes when incubated with crude nuclear extracts. Under appropriate conditions, such complexes may be stable during electrophoresis through nondenaturing polyacrylamide gels (10, 11, 36). The complexes formed between nuclear factors and DNA fragments migrate more slowly through the gel than do the corresponding uncomplexed DNA fragments and are readily resolved as bands of lower mobility. In this way, subfemtomole amounts of sequence-specific DNA-binding proteins can be detected by their ability to retard the migration of short, highly labeled DNA fragments (35).

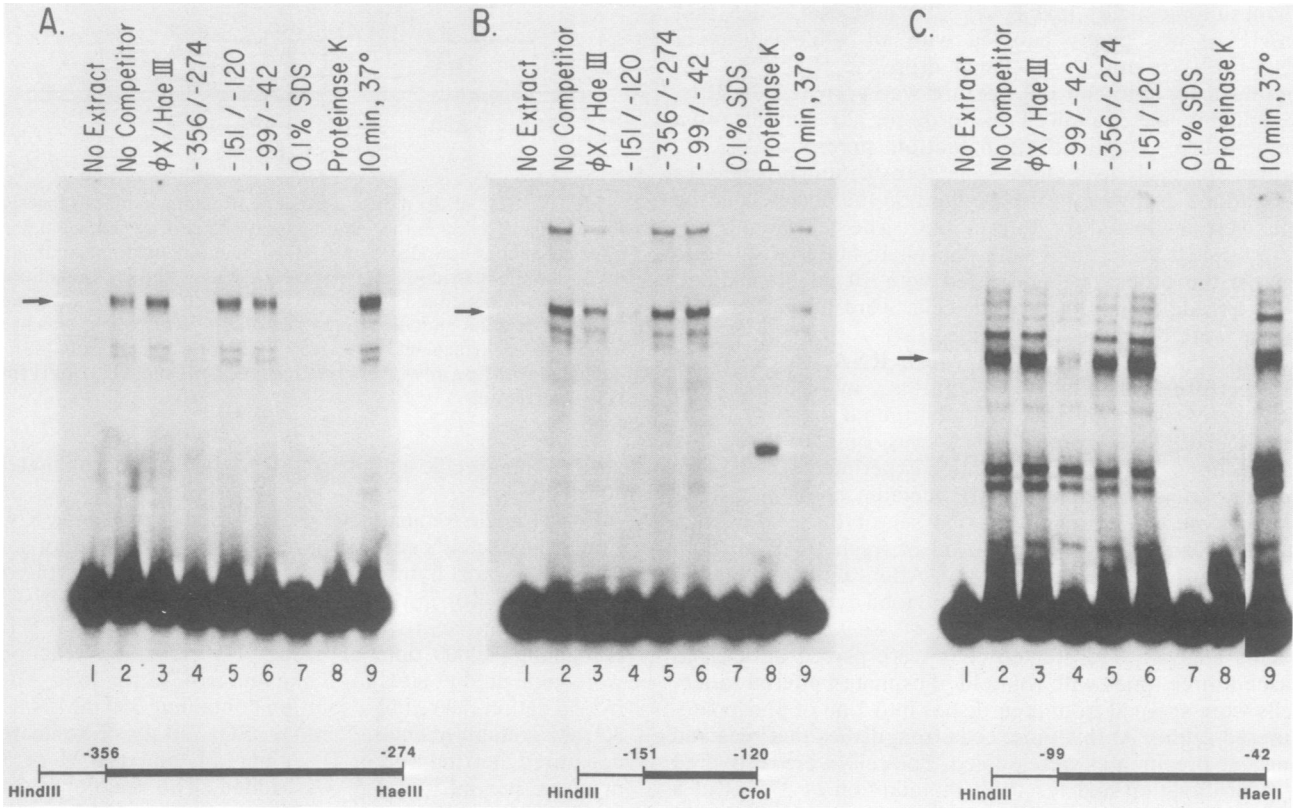


FIG. 2. DNA binding assays with three c-*fos* fragments. All binding assays were performed as described in the text. WEHI 231 nuclear extract (10 μg per reaction) was used. In all experiments the lower band represents unbound DNA and the bands indicated by arrows represent the major complexes formed in all extracts (see the text). Below each gel is diagrammed the probe fragment used in that experiment. c-*fos* DNA is indicated by the thick line, vector-derived DNA by the thin line. (A) Binding to −356 to −274 fragment. (B) Binding to −151 to −120 fragment. (C) Binding to −99 to −42 fragment. Lanes: 1, no added nuclear extract; 2, no additional competitor DNA besides the standard constituents described in the text; 3, 20 ng of φX174 replicative-form DNA cleaved with *Hae*III added to the reaction; 4, 20 ng of purified unlabeled restriction fragment identical to probe; 5 and 6, 20 ng of the indicated unlabeled restriction fragments; 7, 0.1% sodium dodecyl sulfate added to the reaction; 8, reaction with WEHI 231 nuclear extract which had been treated with 100 μg of proteinase K per ml for 10 min at 37°C; 9, control extract incubated for 10 min at 37°C without protease. In panel C, lane 9 was from a separate experiment.

The present work surveys the ability of DNA sequences immediately upstream of the mouse c-*fos* gene to form such complexes. To facilitate our analysis, we used BAL 31 exonuclease to construct plasmid derivatives of the c-*fos* gene carrying varying amounts of DNA from the c-*fos* 5′-flanking region. From several of these plasmids, covering approximately 400 bp of the 5′-flanking sequence, we prepared a series of 50 to 100-bp end-labeled DNA fragments for use as substrates in DNA binding assays. Each fragment carried 19 bp of vector-derived DNA and sequences from various portions of the mouse c-*fos* upstream region.

Nuclear extracts were prepared (7) from the murine B lymphoma cell line WEHI 231 (30, 41). This cell line was chosen because it could be readily grown in large amounts and because c-*fos* expression is induced in these cells upon treatment with antibodies to their surface immunoglobulin (M. Z. Gilman, unpublished data). As a representative human cell-derived extract, we used a whole-cell transcription extract prepared from HeLa cells (21). We detected significant complex formation with the three fragments shown on the c-*fos* map (Fig. 1). Fragments from other regions of the gene gave little or no complex formation. Each of the three positive fragments formed several complexes of various intensities. Some complexes were formed weakly and irreproducibly or without sequence specificity; these complexes were not analyzed further. The major sequence-specific complexes formed in both the mouse and human extracts, and the extracts were used interchangeably.

One complex was formed with a DNA fragment extending from position −356 to −274, relative to the transcriptional initiation site (Fig. 2A, lane 2, arrow). Formation of this complex was not affected by including in the binding reaction a 100-fold mass excess of bacteriophage DNA (Fig. 2A, lane 3). However, the addition of a 100-fold excess of a purified unlabeled restriction fragment carrying the same c-*fos* sequences as those present in the labeled probe abolished detectable complex formation (Fig. 2A, lane 4). No competition was seen with 100-fold excesses of purified restriction fragments derived from two other regions upstream of the c-*fos* gene (Fig. 2A, lanes 5 and 6). These competition experiments established that the nuclear factor involved in this complex binds with much greater affinity to this short c-*fos* DNA sequence than to other DNAs. We conclude that the complex we detected with this assay is due to a nuclear factor that binds selectively to sequences between −356 and −274. The addition of 0.1% sodium dodecyl sulfate to the binding reaction or treatment of the extract with protease abolished complex formation (Fig. 2A, lanes 7 and 8). Thus, we further conclude that complex formation involves noncovalent interaction between the

DNA fragment and a protein or proteins in the nuclear extract.

Similar results were obtained with a fragment containing c-*fos* sequences from −151 to −120 (Fig. 2B). Complex formation was not affected by the addition of 100-fold mass excesses of phage DNA or DNA from the −356 to −274 and −99 to −42 regions of the c-*fos* gene. Effective competition was seen with the homologous unlabeled fragment. Treatment of the nuclear extract with protease abolished formation of the complex, but a new band of higher mobility appeared which may represent a partial proteolysis product. The addition of sodium dodecyl sulfate also blocked complex formation. These results show that a second sequence-specific DNA-protein complex forms between −151 and −120.

We detected a third complex forming on a fragment carrying sequences from −99 to −42 (Fig. 2C). Competition experiments once again established that this interaction was sequence specific, and treatment with protease or detergent prevented complex formation. Two additional complexes were formed with this fragment which were not eliminated by the addition of a large excess of homologous competitor (Fig. 2C, lane 4). These complexes may represent very abundant nonspecific DNA-binding proteins in the extract or proteins recognizing a non-sequence-specific feature of the probe such as an end or a nick. They were not studied further.

This assay allowed us to detect three sequence-specific DNA-protein complexes which form in vitro with sequences in the mouse c-*fos* 5′-flanking region. Each DNA-binding activity manifested a marked preference for its target sequence over other DNAs. Based on competitor titration experiments, we estimated the differences in affinities for specific versus nonspecific DNA as approximately 10^4 (data not shown). The formation of each complex was unaffected by the addition of large molar excesses of the DNA fragments which form the other two complexes. These complexes therefore represent three DNA-binding factors of distinct specificity.

Initial localization of binding sites. To localize within each fragment the binding sites involved in complex formation, we allowed complexes to form in the presence of unlabeled competing fragments terminating at various sites within each region. Thus, we found that for complex 1 (−356 to −274), unlabeled restriction fragments extending from +109 to endpoints at −356, −342, and −319 effectively blocked complex formation, while fragments with endpoints at −293 and −282 failed to compete (Fig. 3A). These competitions showed that the 5′ boundary of the site bound in complex 1 lies between −319 and −293.

Similarly, the analysis of complex 3 (−99 to −42) showed that unlabeled fragments with endpoints at −90, −80, −73, and −71 competed for complex formation, while fragments ending at −59 and −56 did not compete (Fig. 3D). These competitions localized the 5′ most recognition elements for this complex to between −71 and −59.

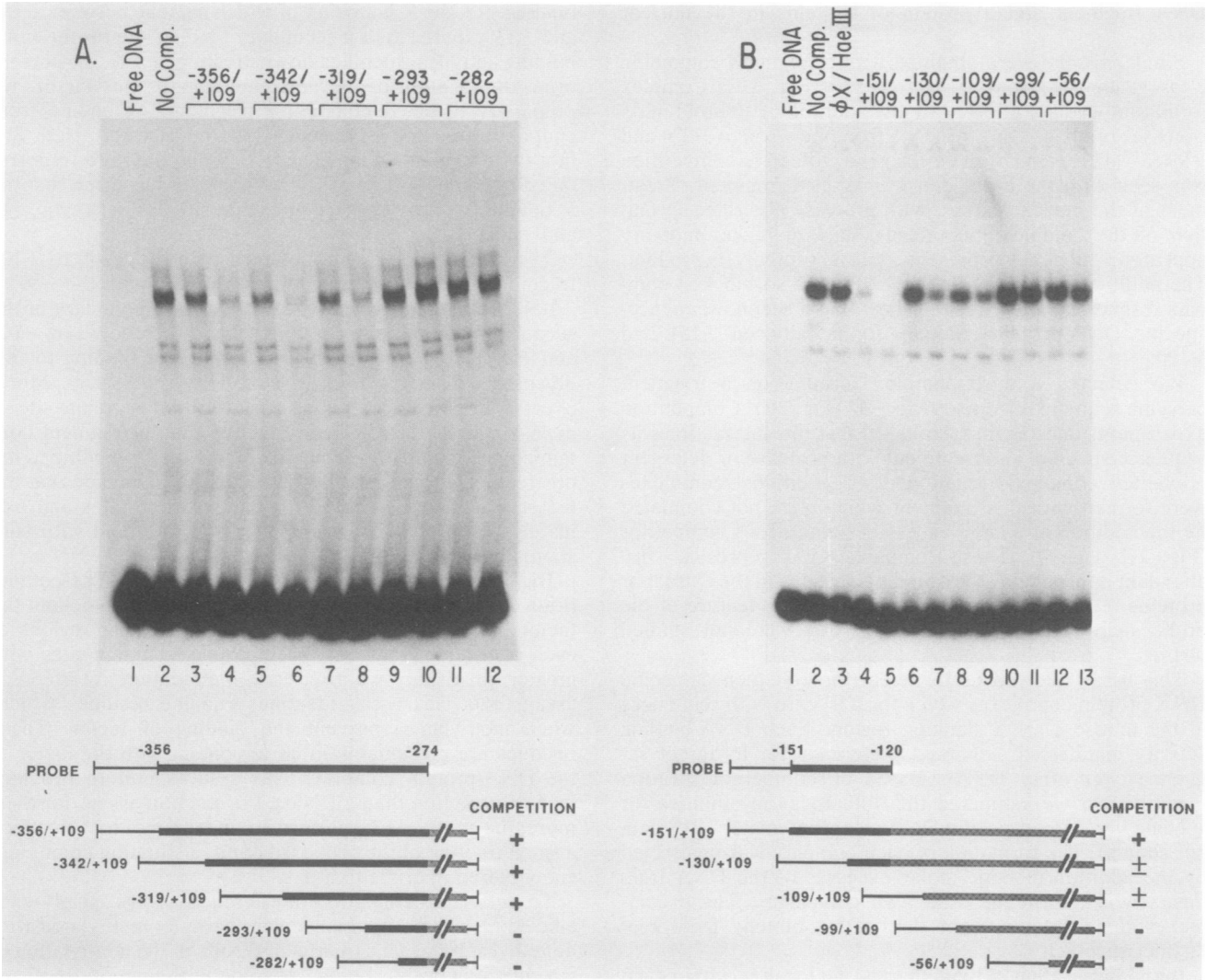
A more complicated pattern of competition emerged upon analysis of complex 2. An unlabeled fragment extending from −151 to +109 competed well for complex formation with the labeled probe, while fragments extending from −99 to +109 and from −56 to +109 failed to compete (Fig. 3B). Unlabeled fragments of intermediate size with 5′ endpoints at −130 and −109 elicited partial competition. Surprisingly, the latter fragment competed, although it contained none of the sequences present in the labeled probe. We infer from this pattern of competition that in addition to the primary binding site, the 5′ boundary of which must lie between −151 and −130, there exists a secondary binding site for the same binding activity which lies downstream of −109. To corroborate the location of the upstream site for this factor, we prepared unlabeled competitor fragments extending from endpoints at −151, −147, and −130 to nucleotide −120. The first two fragments competed well, while the third fragment failed to compete (Fig. 3C). This analysis suggested that the 5′ boundary of the primary upstream site lies between −147 and −130.

The partial competition obtained with the −109 to +109 fragment and lack of any competition seen with the −99 to +109 fragment suggested that the upstream boundary of the secondary binding site for this activity lies between −109 and −99. We were unable to detect direct binding to this downstream site by using the mobility-shift assay. Moreover, a fragment containing just this downstream site is approximately 100-fold less effective than a fragment containing just the upstream site in competing for binding to the upstream site (data not shown). Thus, we conclude that the factor which forms complex 2 binds to two sites approximately 40 bp apart. The upstream site is bound with substantially greater affinity.

High-resolution mapping of the binding sites. The competition experiments described above allowed us to delimit the factor binding sites in each complex to smaller regions of each restriction fragment. To map these binding sites with greater precision, we used the methylation interference assay (34). This assay identifies guanine residues which, when methylated, prevent the binding of factor. These residues are presumably in close contact with the factor in the DNA-protein complex. This assay therefore provides higher resolution than a DNase I protection assay. Furthermore, the assay can be performed under conditions in which a small fraction of the probe is bound, a situation commonly encountered in crude extracts.

To perform methylation interference assays, singly end-labeled restriction fragments were partially methylated with dimethyl sulfate. This treatment results in the methylation of guanine moieties at the N-7 position in the major groove of the double helix (22). The partially methylated fragments were used as substrates in scaled-up binding assays. The binding reactions were loaded onto preparative polyacrylamide gels to resolve complexed DNA from free DNA. DNA from the bound and free fractions was prepared separately, treated with piperidine to cleave the phosphate backbone adjacent to the methylated guanines, and displayed on a sequencing gel. If methylation of a particular guanine residue prevents the binding of factor, DNA molecules carrying this methylated guanine will be excluded from the bound fraction. Therefore, upon analysis of the bound DNA on a sequencing gel, the band in the sequencing ladder corresponding to molecules methylated at that site will be missing.

Figure 4 shows the results of such assays for the three complexes. The binding assay for complex 1 was carried out with a shorter fragment than the one used in the earlier experiments. This fragment extends from −319 to −274. It fully competed for binding with the larger fragment (Fig. 3A, lanes 7 and 8) and gave a similar pattern on mobility-shift gels (data not shown). Two bands, corresponding to adjacent guanine residues at −301 and −300 on the coding strand of the gene, are missing from the bound DNA fraction (Fig. 4A, lane 2). In addition, two bands are missing from the bound fraction of the fragment labeled on the noncoding strand (Fig. 4A, lane 5). This doublet corresponds to a pair of



guanines at positions −309 and −308. Thus, we conclude that the factor forming complex 1 contacts at least two guanine residues on each DNA strand between nucleotides −309 and −300. This location is consistent with the competition data (Fig. 3A), which mapped the 5′ border of this binding site to between nucleotides −319 and −293.

Figure 4B displays the results of a methylation interference assay of complex 2. Bands corresponding to guanines at positions −146, −141, −140, and −139 on the coding strand and at −145, −144, and −138 on the noncoding strand are missing from the bound DNA fractions (Fig. 4B, lanes 2 and 5). Thus, the factor forming complex 2 makes contact with guanine residues on both strands between nucleotides −146 and −138, consistent with the results of the competition experiments described above which implicated sequences between −147 and −130. These competition experiments had revealed a second region, downstream of the first, that bound this factor weakly. This secondary site carries sequences closely related to the primary site (see Fig. 5). Each guanine identified by methylation interference as a contact residue in the upstream site has a counterpart in the weaker downstream sequence.

Figure 4C shows a methylation interference assay of complex 3. For this assay, we used a shorter fragment, extending from −80 to −39. As before, this fragment fully competed for complex formation with the larger fragment (Fig. 3D, lanes 5 and 6). Analysis of this fragment showed that guanines at positions −68, −66, and −63 on the coding strand and −64 on the opposite strand are missing from the bound fractions (Fig. 4C, lanes 2 and 5). These data are in accord with the competitions shown in Fig. 3D, which suggested that the 5′ boundary of this binding site lay between −71 and −59.

The nucleotide sequences recognized by the nuclear factors in each complex are displayed in Fig. 5. The sequence bound in complex 1 lies within an extended palindrome, and the major guanine contacts are symmetrically related. This sequence is almost entirely conserved between the mouse and human c-*fos* genes, and the binding site falls within a small deletion in the human gene which abolishes serum inducibility (39).

Complex 2 formed over a G+C-rich region. Close contacts were made with most but not all guanine residues over a 9-bp stretch. This sequence bears some resemblance to the consensus recognition site for the cellular transcription factor Sp1 (8, 9). However, a *Bgl*I-*Sph*I restriction fragment of the simian virus 40 (SV40) promoter which carries six Sp1 binding sites (12, 13) failed to compete for the binding of

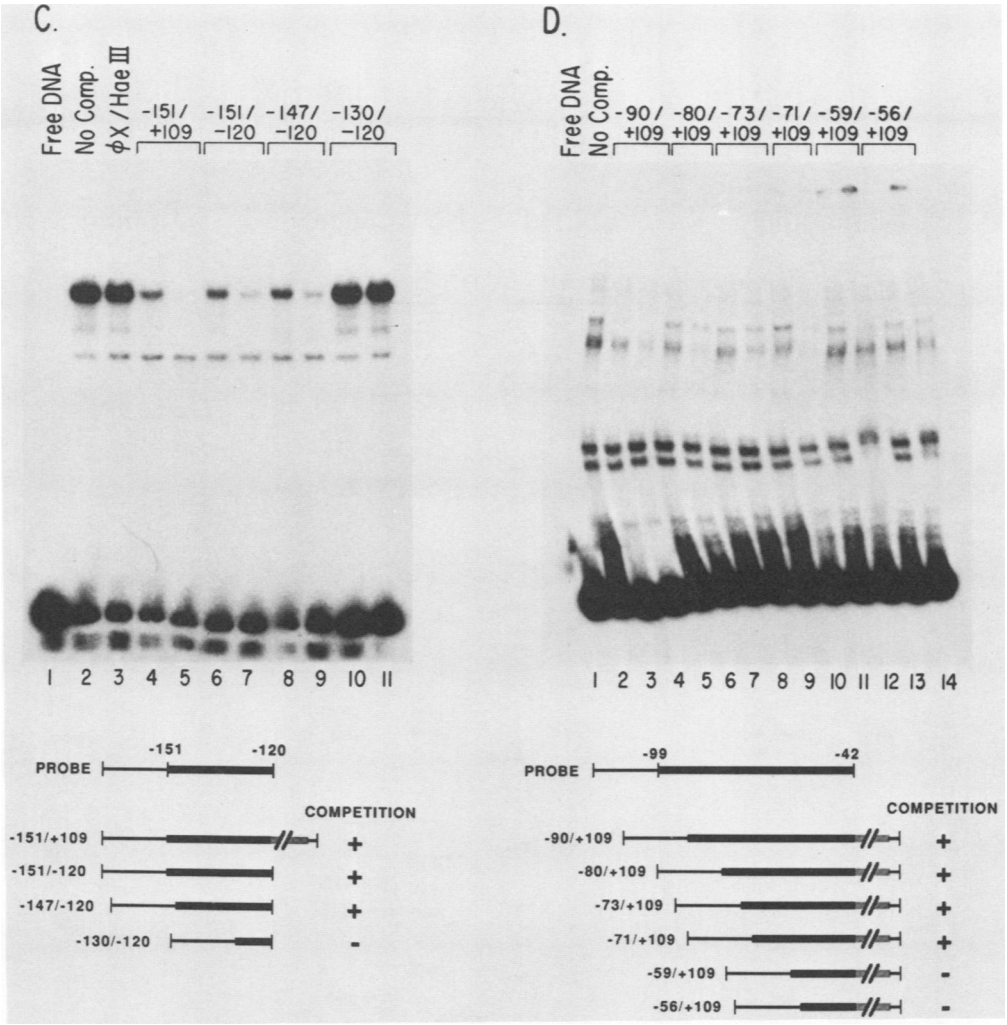


FIG. 3. Competition assays with deletion fragments. (A) Binding to −356 to −274 fragment. Each assay contained 10 μg of WEHI 231 nuclear extract. Lanes: 1, unbound DNA; 2, no added competitor DNA; 3, 5, 7, 9, and 11, 10 ng of the indicated unlabeled restriction fragments. 4, 6, 8, 10, and 12, 50 ng of unlabeled restriction fragment. (B) Binding to −151 to −120 fragment. Each assay contained 15 μg of HeLa whole-cell extract. Lanes: 1, unbound DNA; 2, no added competitor; 3, 20 ng of φX-*Hae*III DNA; 4, 6, 8, 10, and 12, 10 ng of the indicated unlabeled restriction fragments; 5, 7, 9, 11 and 13, 50 ng of restriction fragment. (C) Reactions as in panel B. (D) Binding to −99 to −42 fragment. Assays contained 10 μg of WEHI 231 nuclear extract. Lanes: 1, unbound DNA; 2, no added competitor, 3, 5, 7, 9, 11, and 13, 10 ng of the indicated unlabeled restriction fragments; 4, 6, 8, 10, 12, and 14, 50 ng of unlabeled restriction fragment.

factor 2 to c-*fos* DNA (data not shown). Therefore, this complex does not involve Sp1. The mouse and human c-*fos* promoters were not highly conserved in this region. However, two sequences closely related to this site were found in the opposite orientation in similar locations in the human promoter.

The formation of complex 3 required four guanine contacts over a 6-bp region. This sequence was fully conserved between the mouse and human c-*fos* genes. The binding site is similar in sequence to a region of the adenovirus major late promoter which is known to bind a sequence-specific cellular transcription factor (1, 25, 33). However, no competition for complex formation was seen with a fragment bearing the adenovirus promoter, nor was competition observed in the reciprocal experiment by using a partially purified preparation of this transcription factor (data not shown). Therefore, complex 3 does not involve this previously characterized transcription factor.

Immediately upstream of the factor 3 binding site is a sequence which is identical over 13 bp to a region of the SV40 promoter that binds Sp1 in vitro (12, 13). Although the fragment we used to assay the formation of complex 3 contains this sequence, the addition to the binding reaction of an SV40 restriction fragment carrying the Sp1 sites did not affect the formation of any of the complexes (data not shown). Therefore, we do not detect binding to this sequence. Nevertheless, this site may contribute to c-*fos* promoter activity (see below).

Role of binding sites in promoter activity in vivo. Having mapped the binding sites for three nuclear factors which form complexes with sequences upstream of the mouse c-*fos* gene, we next determined whether these binding sites contribute to c-*fos* expression in vivo. For these experiments, we constructed fusion genes in which sequences containing the mouse c-*fos* promoter were joined to the bacterial gene encoding CAT (14). We transfected these plasmids into BALB/c 3T3 fibroblasts, which we chose because they are efficiently transfected and because their endogenous c-*fos*

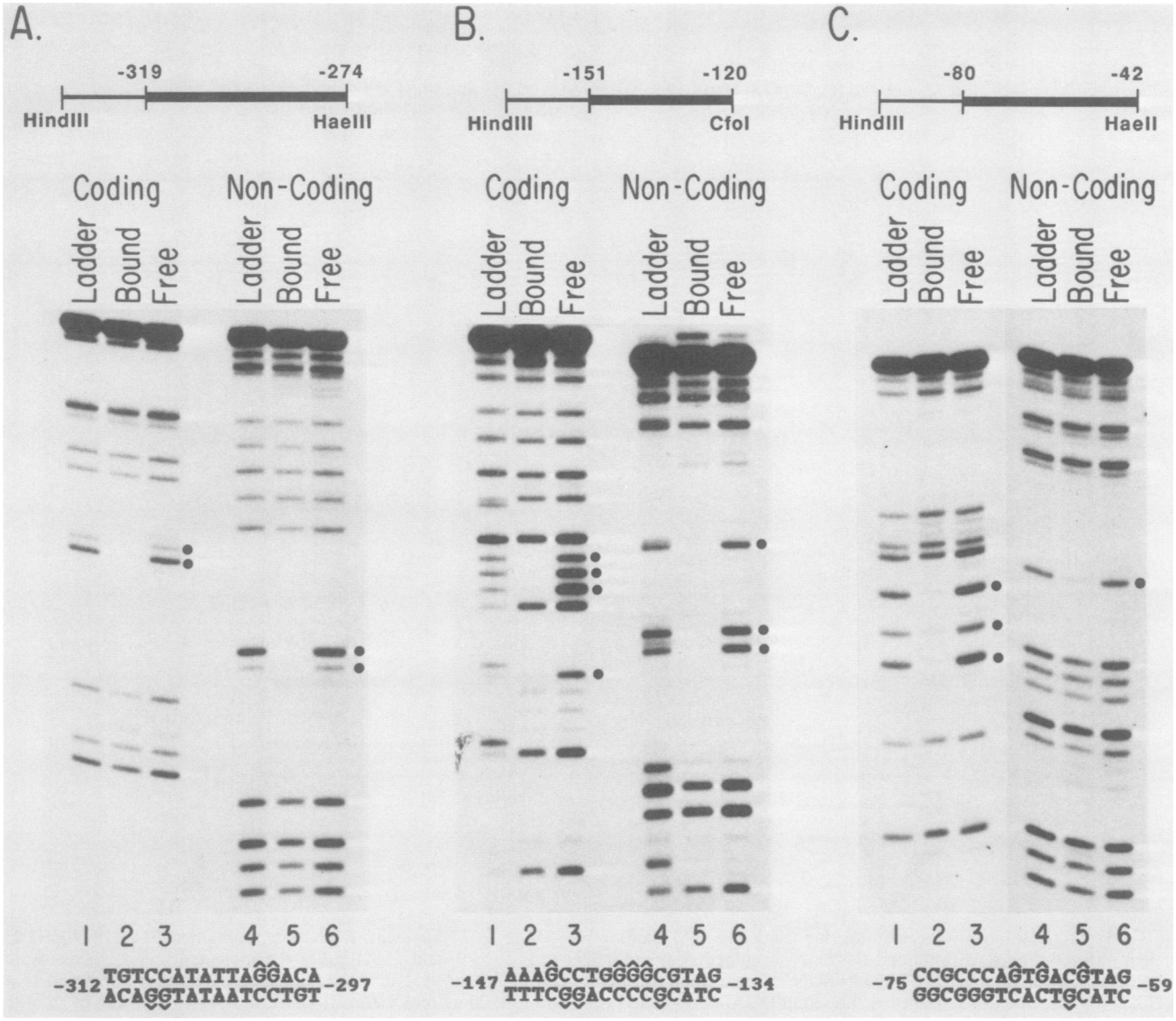


FIG. 4. Methylation interference assays. Preparative binding reactions were carried out with partially methylated DNA fragments as described in the text. (A) Complex 1. (B) Complex 2. (C) Complex 3. Lanes: 1 through 3, assays carried out with fragments 5'-end-labeled at the *Hin*dIII site; 4 through 6, the same fragments 3'-end-labeled at the *Hin*dIII site; 1 and 4, partially methylated and cleaved ladder; 2 and 5, DNA recovered from the bound fraction; 3 and 6, DNA recovered from the free fraction. Results of the assays are summarized below each set of gels. Guanine residues missing from the bound ladder are marked. Several artefactual bands are seen in the ladders (B, lanes 1 and 4). These bands were not reproduced in other experiments, and the correct ladders are those generated with the free fragments (lanes 3 and 6).

genes respond strongly to serum stimulation (3, 17). By assaying CAT enzyme activity, we were able to measure sensitively and accurately the relative activities of various promoter derivatives. In addition, we could analyze RNA from the transfected cells and distinguish transcripts from the transfected genes from any endogenous c-*fos* transcripts in the recipient cells.

The deletion of sequences between −328 and −306 in the human c-*fos* gene abolishes serum inducibility in a transient expression assay (39). The mouse c-*fos* gene is identical in sequence through this region, and the binding site for factor 1 lies within the corresponding sequence in the mouse gene (Fig. 5). To confirm that these sequences are indeed necessary for inducibility in the mouse promoter, we transfected BALB/c 3T3 cells with three fusion plasmids containing various amounts of c-*fos* upstream sequences. After transfection, the cells were incubated in medium containing 0.5% fetal calf serum for 30 h. One set of dishes for each plasmid was then exposed to medium containing 15% fetal calf serum for 1 h. Total cytoplasmic RNA was isolated and subjected to S1 nuclease analysis by using a single-stranded probe specific for *fos*-CAT fusion RNA (Fig. 6C).

Fig. 6A shows that plasmids carrying mouse c-*fos* sequences through −356 and −319 directed the serum-induced expression of a transcript initiating at the authentic mouse c-*fos* cap site (40). Deletion of the factor 1 binding site (−313 to −297) together with sequences to −275 eliminated inducibility. These results confirm those obtained with the human c-*fos* gene (39). They are consistent with a role for this binding site in induction of c-*fos* transcription.

While promoters lacking sequences around the factor 1 binding site are no longer inducible in response to serum,

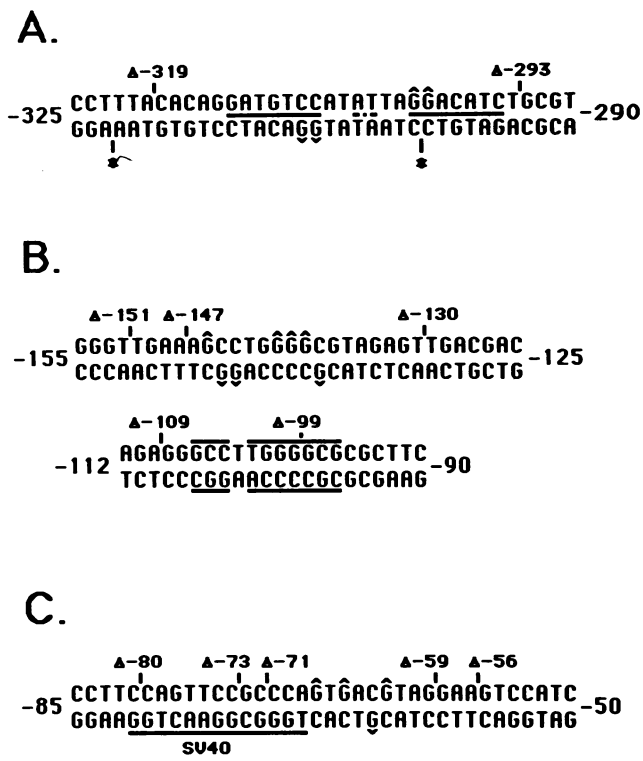


FIG. 5. Nucleotide sequences in the bound regions. Contacted guanine residues identified by methylation interference are marked. Also shown are the endpoints of relevant deletion plasmids discussed in the text. (A) A palindromic sequence is underlined. Asterisks indicate the limits of a deletion in the human c-*fos* gene which abolishes serum inducibility (39). (B) Sequences of both the primary and secondary sites are shown. One of several possible alignments of these sites is marked by over- and underlining. (C) Underlined is a sequence identical to nucleotides 51 to 63 of SV40 DNA (see the text).

they retain substantial basal promoter activity (Fig. 6) (6, 39). To assess the contributions of the binding sites for factors 2 and 3 to this activity, we transfected additional *fos*-CAT fusion plasmids into BALB/c 3T3 fibroblasts. After transfection, the cells were grown in DME medium containing 10% fetal calf serum for an additional 30 to 35 h and harvested without further feeding. This protocol allowed us to measure the activity of the c-*fos* promoter in growing cells without serum induction. Under these conditions, BALB/c 3T3 cells express little if any detectable mRNA from their endogenous c-*fos* genes.

To improve our ability to quantitate these assays, we cotransfected each test plasmid with a second plasmid that directs expression of the *E. coli lacZ* gene under control of the SV40 promoter (18). Each cell lysate was assayed for both CAT and β-galactosidase activities. The latter activity served as an internal standard for transfection efficiency. To calculate relative CAT activity, we divided the measured CAT activity by the measured β-galactosidase activity. This procedure resulted in highly reproducible assays.

The results of these transfection assays are summarized in Fig. 7. In each experiment we included as a reference a plasmid containing sequences through −151. This plasmid carries all binding sites for factors 2 and 3, and we arbitrarily designated the activity of this plasmid as 1.0. Each value for relative CAT activity was derived from three or more independent experiments, with each construct transfected in duplicate.

The deletion of sequences between −151 and −130, which removes the strong binding site for factor 2, resulted in a 25% reduction in CAT activity. Deletion from −130 to −99, which eliminates the second weaker factor 2 binding site, had little further effect. Although these differences were quantitatively reproduced in several experiments, their relatively small magnitudes makes their significance unclear. However, we believe these data suggest a modest effect on basal promoter activity arising from the deletion of the strong binding site for factor 2.

Deletions from −99 to −90, −80, and −73 had little effect on CAT activity. However, deletion to −71, 3 bp from the first contact guanine in site 3, reduced CAT activity approximately twofold, and deletions to −59 and −56, which eliminate the entire factor 3 binding site, reduced activity an additional threefold. These results suggest that sequences between −73 and −59 contribute substantially to c-*fos* promoter activity.

To further clarify the contribution of the binding sites for factor 2 to c-*fos* expression in vivo, we constructed plasmids in which these sites were joined to the weakly active −56 fusion gene. These plasmids directed expression of almost twice the CAT activity of the −56 plasmid (Fig. 7). No difference was seen when the weaker downstream site was eliminated. These results confirm that sequences carrying the strong binding site for factor 2 contribute to c-*fos* promoter activity.

To confirm that the measured CAT activities indeed reflected the levels of transcripts initiating properly at the c-*fos* cap site, we performed S1 nuclease analysis on RNA prepared from BALB/c 3T3 cells transfected with some of these plasmids. Because no serum stimulation was performed, very low levels of RNA were observed in these cells. RNA isolated from transfected cells protected a 197-bp portion of the S1 probe, corresponding to the size expected for transcripts initiated at the authentic mouse c-*fos* cap site (Fig. 6B). This experiment showed that RNA carrying translatable CAT sequences originated predominantly at the c-*fos* cap site. Furthermore, the results of this experiment are consistent with the results of our CAT assays: deletion of site 2 reduced the amount of RNA from the transfected gene (compare Fig. 6B, lanes 4 and 5), and deletion of site 3 resulted in a further reduction (compare Fig. 6B, lanes 7 and 8). Therefore, the CAT activities shown in Fig. 7 are likely an accurate representation of the levels of RNA expressed from each plasmid.

DISCUSSION

We exploited a sensitive DNA binding assay to identify sites within the mouse c-*fos* promoter region that bind factors present in mammalian nuclei. By using this assay, we found three distinct DNA binding activities that interact specifically in this region. Through the use of competition experiments and methylation interference assays, we mapped the factor binding sites at nucleotide resolution. This approach allows rapid identification of putative regulatory sequences within a gene and also provides an assay for the purification of the proteins that bind to those sequences.

One of the factor binding sites revealed in this fashion lies in a region previously shown to be necessary for the induction of c-*fos* transcription by serum growth factors (39). The deletion of these sequences from both the human (39) and mouse (Fig. 6) c-*fos* genes eliminated induction by serum.

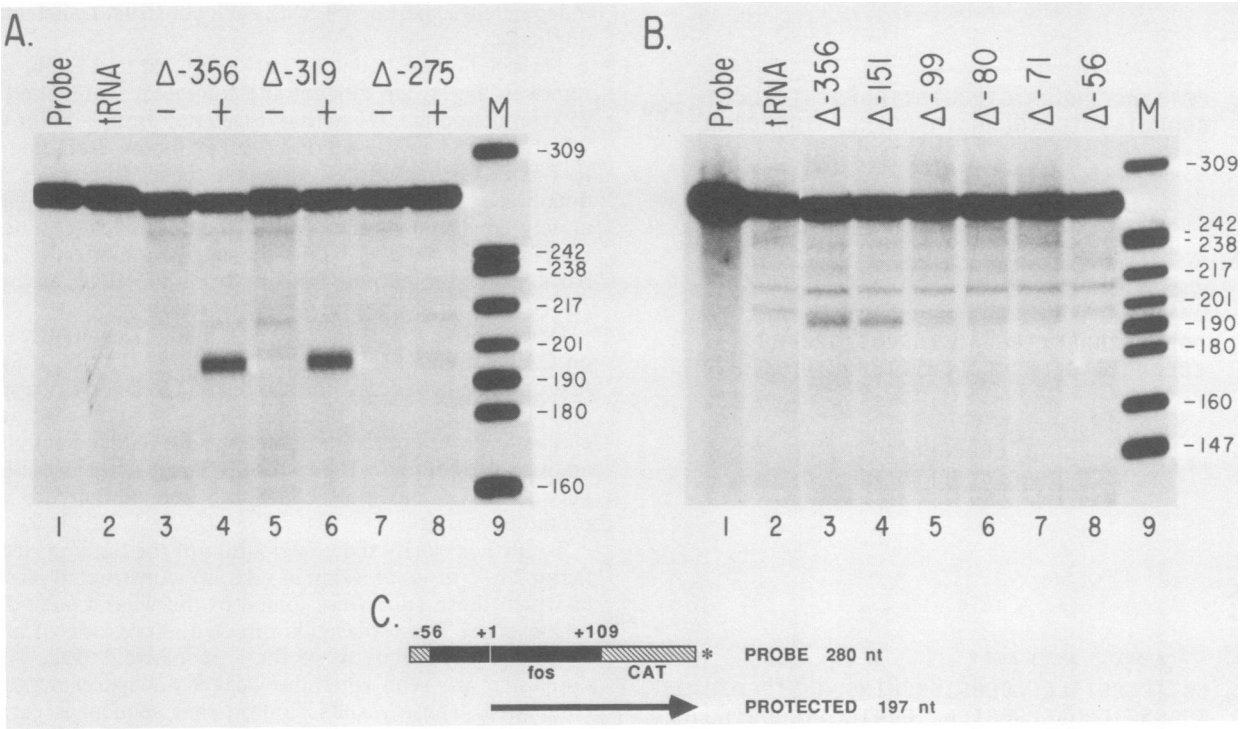


FIG. 6. S1 nuclease analysis of transient expression assays. BALB/c 3T3 cells were transfected and total cytoplasmic RNA was extracted and assayed as described in the text. (A) Serum inducibility of transfected c-*fos*-CAT fusion genes. Lanes: 1, S1 probe fragment; 2, 50 μg of tRNA hybridization control; 3, 5, and 7, 50 μg of RNA from cells transfected with the indicated plasmids and serum starved; 4, 6, and 8, duplicate transfectants stimulated for 1 h with 15% fetal calf serum; 9, *Hpa*II-cleaved pBR322 DNA. (B) Uninduced levels of RNA from several c-*fos*-CAT fusion genes. Lanes: 1, probe; 2, tRNA control; 3 through 8, 50 μg of RNA from cells transfected with the indicated plasmids; 9, *Hpa*II-cleaved pBR322 DNA. (C) Diagram of the hybridization probe. The filled box indicates c-*fos* DNA and the hatched boxes indicate vector or CAT-derived DNA. The arrow indicates the start site and direction of transcripts correctly initiated at the c-*fos* promoter. nt, Nucleotides. *, Position of the 5′-terminal phosphate label.

This region is also the site of a prominent DNase I hypersensitive site in both the human and mouse genes (6, 31). Thus, it is possible that these sequences control transcriptional induction and that the factor we detect in these experiments participates in this induction. The palindromic nature of this binding site is similar to other regulatory sequences which are bound by dimers of identical subunits (28, 37). This arrangement is consistent with the reported bidirectionality of sequences in this region for both serum induction and conventional enhancer activity (6, 39).

The second binding activity we detected interacts at two sites which were not previously implicated in c-*fos* transcriptional control. The two sites are very similar in sequence. Indeed, each of the contacted guanine residues in the upstream site is conserved in the downstream site. However, the downstream site is substantially weaker. It is possible that these sites are bound cooperatively as seen with the *Drosophila melanogaster* heat shock transcription factor (38), but purified preparations of this factor will be required to test such a hypothesis. The deletion of the strong upstream site reduced c-*fos* promoter activity in vivo, suggesting that it may be a basal promoter element and that the factor that binds there may be a sequence-specific transcription factor.

The third binding site lies within a brief stretch of sequence identity between the human and mouse c-*fos* genes. The deletion of sequences in this vicinity strongly affected c-*fos* promoter activity. The removal of sequences immediately upstream of this binding site reduced promoter activity twofold. Complete removal of the site reduced activity an additional threefold. Therefore, this site may also bind a sequence-specific transcription factor.

These results show that each of the binding sites we identified in vitro has an apparent role in c-*fos* expression in vivo. The deletion of site 1 eliminated inducibility of the c-*fos* gene in response to serum and decreased moderately the uninduced activity of the promoter (data not shown) (6). The deletion of 95 bp carrying the binding sites for factors 2 and 3 destroyed 90% of the remaining activity. With one possible exception, each loss of promoter activity was associated with the loss of an identified binding site. This possible exception concerns sequences just upstream of −71, 3 bp from the first contact guanine for factor 3. The deletion of sequences between −73 and −71 resulted in a twofold drop in promoter activity (Fig. 7) without apparently affecting the binding of factor 3, as judged by the competition experiment (Fig. 3D). However, these competition experiments are not sensitive enough to detect small changes in binding affinity which may be significant in vivo. Therefore, it is unclear whether the deletion of sequences upstream of −71 reduced promoter activity by compromising the binding of factor 3 or whether sequences upstream of −71 contribute independently to promoter activity. As noted above, this sequence contains a possible binding site for the cellular transcription factor Sp1 (8, 13), but in contrast to the factor 3 binding site, the Sp1 site is not conserved between the mouse and human c-*fos* promoters.

Although it is clear that the binding sites for factors 2 and

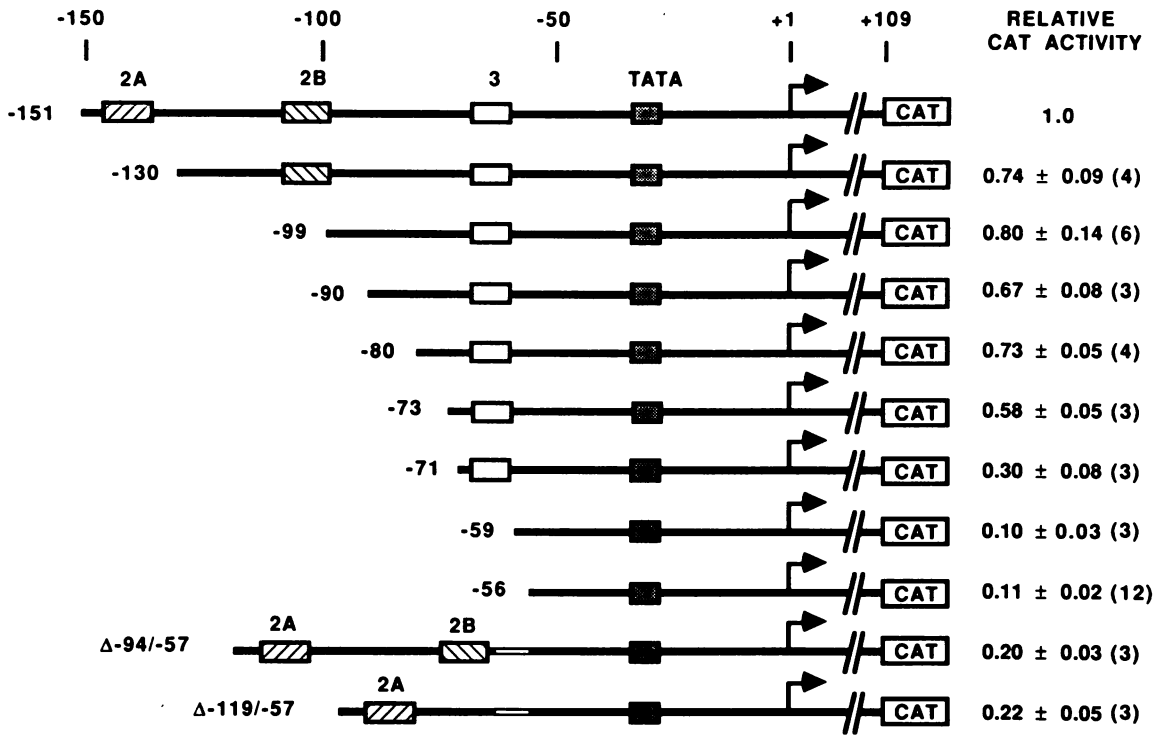


FIG. 7. CAT activities directed by transfected c-*fos*-CAT fusion plasmids. Transfections and enzyme assays were carried out as described in the text. Within each experiment, all transfections were in duplicate. Each experiment included plasmid Δ-151 (top line) as a reference. For each transfection, the specific CAT activity was calculated and divided by the specific β-galactosidase activity in the same lysate. Normalized CAT activities from duplicate transfection lysates were averaged and divided by the average normalized CAT activity of the Δ-151 reference in that experiment to arrive at relative CAT activity. Values shown represent the mean of several independent experiments (the number of experiments is indicated in parentheses). The standard deviations are also shown. c-*fos* sequences in each construct are indicated by the solid line and boxes indicate factor binding sites. In the bottom two constructs, the small open lines represent linker DNA.

3 contribute substantially to basal promoter activity, our results do not rule out an additional role for these sites in serum induction. Thus, we cannot conclude that these sites are exclusively basal promoter elements.

While our analysis of the sequences within the mouse c-*fos* gene required for serum induction is consistent with those reported for the human gene (39), our identification of basal promoter elements farther downstream is not consistent with the results of Renz et al. (31). They concluded that a mouse c-*fos* promoter containing sequences through −95 (−98 in our numbering scheme) was inactive when fused to the CAT gene and stably transfected into fibroblasts. In our hands, CAT fusions containing sequences through −73 retained substantial levels of activity, sixfold higher than a fusion containing sequences through −56. Our results therefore suggest that there are indeed important regulatory elements downstream of −98. Perhaps the difference in our conclusions arises from differences in the transfection protocols and fusion genes used.

We observed no differences in the pattern of binding to any of our sites in extracts prepared from cells induced or uninduced for c-*fos* expression (data not shown). Thus, our results do not address directly the mechanism of induction of c-*fos* transcription by serum. However, several other observations suggest that the molecular changes at the promoter accompanying induction are indeed subtle. Transcriptional induction of c-*fos* does not require protein synthesis (16), implying that all proteins required for induction are present in uninduced cells. Induction is extremely rapid, detectable within 5 min in serum-stimulated fibroblasts (17), suggesting that substantial changes in chromatin organization are unlikely. Moreover, the pattern of DNase I hypersensitive sites upstream of the mouse c-*fos* gene is unchanged upon induction (31). Therefore, we favor the hypothesis that a complete transcription complex is maintained at the c-*fos* promoter at all times and that transcriptional induction results from a modification of this complex in situ or a change in its interaction with other nuclear factors.

The experiments described here are based on an assay that has only recently been applied to mammalian DNA binding proteins (1, 4, 29, 35, 36). Therefore, the general utility of the assay and the validity of its results remain to be established. In the cases cited above, binding sites identified by using this assay correlated with functional sites first identified genetically. For the adenovirus major late promoter, a binding activity detectable with this assay copurified through several columns with an in vitro transcriptional stimulatory activity dependent on the same sequences (1). In our experiments, we were able to demonstrate that each of the binding sites we identified in vitro contributed to c-*fos* expression in vivo. However, without an independent functional assay for these binding activities, we cannot prove at this time that the activities we detect in vitro represent the proteins which functionally interact with these sites in vivo. Such proof will ultimately depend on the purification of these sequence-specific DNA-binding proteins, a task greatly facilitated by this assay.

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