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c-*fos* Sequences Necessary for Basal Expression and Induction by Epidermal Growth Factor, 12-*O*-Tetradecanoyl Phorbol-13-Acetate, and the Calcium Ionophore

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We have investigated the sequence requirements for induction of the human c-*fos* gene by epidermal growth factor (EGF), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), and the calcium ionophore A23187 by transfecting c-*fos* promoter mutants into HeLa and A431 cells. Induction by both EGF and TPA in HeLa cells required the presence of the c-*fos* enhancer located at −317 to −298 relative to the mRNA cap site. A23187, however, did not induce expression of the transfected gene, even though it strongly induced expression of the endogenous gene, suggesting that it has different requirements for induction than do EGF and TPA. We have also investigated the role of promoter sequences downstream of the enhancer in general expression and induction of c-*fos*. A sequence between −97 and −76, which includes an 8-base-pair perfect direct repeat, was needed for efficient general expression but not for induction of the gene. A factor in nuclear extracts that bound specifically to this sequence was detected by a gel mobility shift assay. A 7-base-pair sequence, located between −63 and −57 relative to the mRNA cap site and previously shown to be important for general expression of mouse c-*fos*, was also important for general expression of the human gene. In addition, this element was important for inducibility by EGF and TPA, since induction was significantly reduced when internal deletion mutants that retained the enhancer but lacked the −63 to −57 sequence element were analyzed in transfection assays.

The c-*fos* proto-oncogene is the cellular homolog of the transforming gene of FBJ osteosarcoma virus (6, 9, 12) and encodes a nuclear protein that is thought to be involved in normal cellular growth and differentiation (7, 35). In a number of different cell types, transcription of c-*fos* is rapidly and transiently activated by growth factors (1, 8, 19, 22, 25, 26, 31, 32). For example, addition of serum or various polypeptide mitogens to quiescent BALB/c-3T3 cells causes transcriptional activation of the gene within 5 min, in a manner independent of de novo protein synthesis (22). Treatment with protein synthesis inhibitors, in fact, results in superinduction of the gene (4, 20, 25, 32).

Studies with mutant genes have shown that a 20-base-pair transcriptional enhancer element, located between −317 and −298 base pairs upstream of the c-*fos* mRNA cap site, is essential for the serum inducibility of the human c-*fos* gene in serum-starved NIH 3T3 cells (10, 21, 43, 44). This 20-base-pair enhancer element contains a sequence with dyad symmetry and is strongly conserved in the mouse c-*fos* gene. Furthermore, a nuclear protein that specifically binds to the human and mouse c-*fos* gene enhancer has been identified by gel mobility shift assays in nuclear extracts of human HeLa cervical carcinoma, A431 epidermal carcinoma, and human WEHI B lymphoma cells (15, 21, 33, 44). Treatment of asynchronously growing A431 cells with the c-*fos* transcriptional activator epidermal growth factor (EGF) caused a rapid induction of enhancer-binding activity in A431 cell nuclear extracts (33). This finding suggested that EGF, like serum, stimulated c-*fos* transcription through the c-*fos* enhancer and that increased binding to the enhancer was part of the activation mechanism.

Transcription of the c-*fos* gene is also rapidly and transiently activated by a number of other agents which affect cell growth and differentiation, including the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and the calcium ionophore A23187 (1, 22, 25, 31, 33). However, in contrast to the results with EGF, neither TPA nor A23187 caused induction of enhancer-binding activity in nuclear extracts of A431 cells. This finding suggested that TPA and A23187 might be stimulating c-*fos* transcription through a mechanism at least partially distinct from that used by EGF. Because of these differences, we have undertaken an in vivo analysis of sequences which are necessary for EGF, TPA, and A23187 induction of c-*fos* expression.

For the mouse c-*fos* gene, sequence elements other than the transcriptional enhancer have been demonstrated to be important for basal-level transcription of c-*fos* following transfection into BALB/c-3T3 cells, and the gel mobility shift assay was used to identify nuclear factors binding to these elements (15). Thus deletion of a factor-binding site near −150 (relative to the cap site) caused a 25% decrease in the basal transcription level of the transfected gene, although this sequence element is not conserved in the human gene. In contrast, a 7-base-pair sequence, located at positions −68 to −62 in the mouse c-*fos* gene, is conserved, at positions −63 to −57, in the human gene. Deletion of this sequence from the mouse gene caused another 6-fold decrease in basal transcription (15). This study, however, was not designed to test for any potential cooperation between the element at −68 and the enhancer at −317. Thus, although the c-*fos* enhancer was necessary for serum induction, it was also possible that downstream elements might play a role in the transcriptional regulation by serum or other agents. We therefore analyzed mutants that retained the enhancer but contained internal deletions in other regions of the promoter

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to determine the role of other sequences in basal and induced c-*fos* expression.

MATERIALS AND METHODS

Cells and reagents. HeLa and A431 cells were grown in monolayer cultures in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. EGF (Collaborative Research, Inc.) was dissolved in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethylsulfonic acid) (pH 7.5) at 25 μg/ml. TPA (Sigma Chemical Co.) was dissolved in dimethyl sulfoxide at 1 mg/ml. The calcium ionophore A23187 (free acid; Sigma) was dissolved in dimethyl sulfoxide at 10 mg/ml.

Plasmid DNAs. pFC700 was derived from plasmid pF2, containing the human c-*fos* gene from −710 upstream of the mRNA cap site to +2164 inserted into the *Hin*dIII site of pUC19 (33), and from a second plasmid, containing the *CAT* *Bgl*II-*Bam*HI fragment from pCAT3M (27) cloned into the *Bam*HI site of pUC18. Sequence downstream of +42 in c-*fos* was removed from pF2 by digestion with *Nae*I (+42) and *Bam*HI, which cuts in the polylinker of pUC19. The *Nae*I site was filled in by using the Klenow fragment of *Escherichia coli* DNA polymerase I, and an *Xba*I synthetic DNA linker was ligated onto this site. The resulting *Xba*I-*Bam*HI fragment was ligated to an *Xba*I-*Bam*HI fragment containing the *CAT* gene and simian virus 40 poly(A)$^+$ site.

pFC2000 was derived from pFC700 and pF1, a plasmid containing the human c-*fos* gene from about position −2000 upstream of the mRNA cap site to position +2164 inserted into the *Hin*dIII site of pUC19 (33). pF1 was digested with *Xho*I and *Sst*I, generating a fragment that contained the c-*fos* promoter sequence between −710 and −2000 and the entire pUC19 plasmid, excluding polylinker sequences between the *Sst*I and *Hin*dIII sites. This fragment was ligated to a *Xho*I-to-*Sst*I fragment from pFC700 that encompassed the c-*fos* gene from −710 to +42, the *CAT* gene with its simian vius 40 poly(A)$^+$ site, and the pUC19 polylinker sequence between the *Hin*dIII and *Sst*I sites.

pFC225 and pFC99 were derived from pFC700 by digestion of pFC700 with *Apa*I (position −225) or *Bss*H2 (position −99). The *Apa*I or *Bss*H2 site was filled in with the Klenow fragment, and *Xho*I synthetic DNA linkers were ligated onto these sites. Extensive digestion with *Xho*I released excess linkers and additionally cleaved the plasmid at the *Xho*I site at −710. The resulting large fragments lacking sequences between −710 and −225 or −710 and −99 but containing the rest of the plasmid sequences were recircularized with T4 DNA ligase through their *Xho*I ends.

pFC363 was constructed by isolating a *Pst*I (−363)-to-*Apa*I (−225) fragment from pFC700. *Xho*I DNA linkers were added at the *Pst*I site. This fragment was ligated to a large fragment from pFC700 that was generated by digestion with *Xho*I and *Apa*I to remove sequence between −710 and −225. Surprisingly, the resulting construct did not contain a *Xho*I site at −363, but its identity was verified by sequence analysis (see below).

pFCΔ225/99 was derived from pFC700 by digestion with *Apa*I (−225) and *Bss*H2 (−99). These sites were filled in with the Klenow fragment and ligated to *Bgl*II synthetic DNA linkers. The small fragment containing the c-*fos* promoter sequence between −225 and −99 was removed by gel electrophoresis, and the purified plasmid was recircularized through its *Bgl*II ends.

Point mutants in the c-*fos* enhancer region were made by synthesizing the strands for three 40-base-pair oligonucleotides on an Applied Biosystems DNA synthesizer. These oligonucleotides included the c-*fos* sequence from −318 to −284 and *Xho*I and *Bgl*II cohesive ends. One of the oligonucleotides was wild type (TF1). For the mutations in TF2 and TF3, see Fig. 5. pFC700 was digested with *Xho*I and *Apa*I; the *Apa*I site was filled in with the Klenow fragment and ligated to synthetic *Bgl*II DNA linkers. Digestion with *Bgl*II and *Xho*I removed excess linkers and the c-*fos* promoter sequence between −710 and −225. The synthetic oligonucleotides were ligated to the pFC700-derived fragment through their *Xho*I and *Bgl*II ends. Analytic restriction digest of the resulting clones with *Hin*dIII and *Pst*I allowed selection of clones that contained only single copies of the oligonucleotides.

BAL 31 deletion mutants were prepared from pFC99 by cleaving it at the *Xho*I site at −99 and digesting it with BAL 31 exonuclease (10 U/50 μg of DNA per ml) for 1.5 to 3.5 min at 21°C. The ends were filled in with the Klenow fragment, and *Bgl*II linkers were added. Digestion with *Bgl*II and *Sst*I released excess *Bgl*II linkers and cleaved the pUC19 plasmid at the *Sst*I site just downstream of the *CAT* poly(A)$^+$ site. The resulting fragments containing c-*fos* and *CAT* sequences were isolated by gel electrophoresis and electroelution. A vector to receive these fragments was prepared from a derivative of pFC700 that contained a synthetic *Bgl*II linker inserted at the *Bss*H2 site at −99 in the c-*fos* 5' flanking sequence (pFC700Bg). This plasmid was digested with *Bgl*II and *Sst*I, and the fragment containing pUC19 fused to the c-*fos* 5' flanking sequence from −700 to −94 was isolated by gel electrophoresis and electroelution. The vector and deleted fragments were then ligated together, and the deletion endpoints of resulting clones were verified by DNA sequence analysis.

pRSVCAT, kindly provided by Filippo Cavalieri, contains the long terminal repeat of Rous sarcoma virus fused to the *CAT* gene (17).

DNA sequencing. Sequences were determined by the chain termination method (36) by using supercoiled plasmid DNA templates prepared from minipreps as described previously (3). As a primer, we used a 15-base-pair single-stranded oligonucleotide, kindly provided by Sarah Hanly, that was complementary to the *CAT* coding strand near the AUG start site.

Transfections. HeLa or A431 cells were plated at 5×10^5 or 7.5×10^5 cells per 10-cm plate, respectively, 24 h before transfection by the calcium phosphate or DEAE-dextran coprecipitation methods (29, 42, 45). The cells were refed with fresh medi 4 h before transfection. Typically, 20 μg of *fosCAT* and 5 μg of pRSVCAT reference plasmid were used per 10-cm plate. The cells were exposed to transfection precipitate for 4 h (calcium phosphate method) or 30 min (DEAE-dextran method), treated for 2 min with serum-free medi containing 15% glycerol, washed two or three times with phosphate-buffered saline to remove any remaining precipitate, and refed with medi containing 10% fetal calf serum. At 44 h after transfection, transfected cells were treated with either 100 ng of EGF per ml, 100 ng of TPA per ml, or 10 μg of A23187 per ml for 30 min. The cells were then washed twice with ice-cold phosphate-buffered saline and lysed with 2.5 ml of urea lysis buffer (7 M urea, 2% sarcosyl, 350 mM NaCl, 10 mM Tris [pH 7.9], 1 mM EDTA). The lysed cells were homogenized by 20 strokes of a glass Dounce homogenizer (type B pestle), and total cellular RNA was isolated by centrifugation of the homogenized lysates in CsCl gradients (16). RNA pellets were suspended in water and stored as ethanol precipitates.

RNA analysis. Mapping of transfected *fosCAT* fusion gene transcripts by S1 nuclease analysis was performed essentially as described previously (39), except that 4.5 mM $ZnCl_2$ was used in the S1 digestion buffer. The S1 probe was an approximately 400-base-pair fragment derived from pFC700 that spanned −99 to +42 in c-*fos* and the first 250 base pairs of coding sequence from the *CAT* gene, along with a small intervening segment of pUC polylinker. Hybridization was performed overnight (> 12 h) at 44°C. S1 digestion was performed for 30 min at 37°C with 25 U of S1 nuclease (Boehringer Mannheim Biochemicals) per 200-μl reaction. Nuclease-resistant fragments were fractionated on 5% polyacrylamide–7 M urea gels. As size markers, ^{32}P-labeled *Msp*I fragments of pBR322 were run on all analytical gels.

Transcript mapping by RNase protection was performed as described by Zinn et al. (46). A 385-base-pair complementary-strand RNA probe was generated from plasmid pGH cleaved with *Bss*H2. Plasmid pGH contained a 1-kilobase-pair *Xho*I-to-*Hin*cII (−710 to +286) c-*fos* gene fragment inserted at the *Hin*dIII site of pGEM1 (Promega Biotec). In the RNase protection assay this probe generated a 286-base-pair fragment characteristic of properly initiated c-*fos* mRNA. Nuclease-resistant fragments were fractionated on 5% polyacrylamide-7 M urea gels.

Gel mobility shift assay. Nuclear extracts were prepared essentially as described by Dignam et al. (11) as modified by Prywes and Roeder (33), except that the extracts were not dialyzed.

The gel mobility shift assay was performed essentially as described previously (13, 14, 40). In the gel shift assay to detect the −60 factor, nuclear extract (2 μg of protein from A431 cells and 5 μg of protein from HeLa cells) was incubated in 20 μl of binding buffer (10 mM Tris hydrochloride [pH 7.5], 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol) containing either 500 ng of sonicated salmon sperm DNA or 2 μg of poly)(dI-dC) · poly(dI-dC) and 50 ng of various competitor DNA fragments, as indicated in the figure legends. In the gel shift assay to detect the direct-repeat binding factor, 3 μg of poly(dI-dC) · poly(dI-dC) and 50 ng of competitor DNA fragment were used. The ^{32}P-labeled probe (0.1 to 0.8 ng) was added last, and the mixture was incubated for 30 min at room temperature, loaded directly onto a 4% polyacrylamide (acrylamide-bisacrylamide, 30:0.8) gel in 0.5 × TBE (25 mM Trizma base [Sigma], 25 mM boric acid, 1 mM EDTA), and electrophoresed at 150 V for 1.5 h at room temperature. The gel was then dried and analyzed by autoradiography.

We used four different probes in the gel mobility shift assay. To detect binding to the c-*fos* enhancer, we used a 138-base-pair probe including the c-*fos* sequence from −363 to −225 relative to the mRNA cap site. This probe was labeled with T4 polynucleotide kinase and (γ-^{32}P]ATP at a *Bgl*II site at −225 created by a synthetic DNA linker in plasmid pF9 (33a). To detect binding to the direct repeats between −97 and −76, we used an approximately 235-base-pair probe including the c-*fos* sequence between −225 and +10. This probe was labeled by filling in with Klenow enzyme and [α-^{32}P]dCTP at a *Bgl*II site at −225 created by a synthetic DNA linker in plasmid pFC225 and was subsequently digested with *Pst*I. To detect binding to the c-*fos* sequence between −63 and −57, we used two probes. One was an approximately 70-base-pair fragment, derived from deletion mutant pFCΔ94/73, spanning sequence from −73 to −10. This fragment was labeled at a synthetic *Bgl*II site at −73 by using Klenow enzyme and [α-^{32}P]dATP and was subsequently digested with *Nar*I. The second probe was a 32-base-pair double-stranded synthetic oligonucleotide (RP2) that spanned c-*fos* nucleotides −71 to −48 and included a 4-base-pair single-stranded tail (GATC) on either end. This oligonucleotide was labeled by using Klenow enzyme and [α-^{32}P]dATP. The three fragment probes described above were separated from unincorporated nucleotides by electrophoresis through 5% polyacrylamide gels. The oligonucleotide probe was separated from unincorporated nucleotides by passage over a 1-ml Sephadex G50-80 column.

Competitor fragments spanning segments of various adenovirus type 2 (Ad2) early-gene promoters were derived as follows. A 353-base-pair fragment spanning the E1a promoter from −500 to −147 and a 198-base-pair fragment spanning the E1b gene from −141 to +57 were derived from pUC19 subclones containing these adenovirus promoter fragments between the *Eco*RI and *Hin*dIII sites of the polylinker. These plasmids were kindly provided by Imri Kovesdi. A 247-base-pair fragment spanning −147 to +100 in the E1a gene was derived from pHindIIIG, which contained the Ad2 *Hin*dIII G fragment (from near 0 to 7.9 map units), as described previously (28). A 258-base-pair fragment spanning −233 to +25 in the E3 gene was derived from pHindIIIH, which contained the Ad2 *Hin*dIII H fragment (from 73.6 to 79.9 map units [28]). pHindIIIG and pHindIIIH were kindly provided by Warren Hoeffler. A 250-base-pair fragment spanning −230 to +20 in the Ad2 E4 gene was kindly provided by Pradip Raychaudhuri. A 15-base-pair oligonucleotide which spanned −80 to −70 in the E2a promoter and which had been polymerized to an average chain length of 150 to 200 base pairs was kindly provided by Amy Yee. A nonspecific competitor fragment spanning positions −119 to −42 in the *Xenopus* histone H4 promoter was kindly provided by Gerald Thomsen.

RESULTS

Induction of c-*fos* in HeLa cells by EGF, TPA, and A23187. As stated above, it has been shown that a 20-base-pair enhancerlike sequence, located in the c-*fos* promoter at positions −317 to −298 relative to the mRNA cap site, is necessary for induction by serum of c-*fos* transcription in serum-starved NIH cells (43, 44). We tested whether this or other sequences are required for c-*fos* transcriptional induction in vivo by EGF, TPA, and A23187. Various c-*fos* promoter mutants were transfected and analyzed for induction in transient-expression assays. In addition to investigating the response in A431 cells, we wanted to test the response in HeLa cells, since they appeared to be more amenable to subsequent in vitro studies. The A431 cell nuclear extracts tested so far have not been transcriptionally active under our standard in vitro assay conditions (R. Prywes, unpublished data), whereas HeLa nuclear extracts are transcriptionally active. We found previously that in HeLa cells grown in spinner culture, c-*fos* was not significantly induced by EGF (33). In contrast, in HeLa cells grown in monolayer culture, which we planned to use for our in vivo studies, c-*fos* mRNA was induced within 30 min of treatment with EGF as well as with TPA and A23187 (Fig. 1). The level of induction (about 20- to 50-fold in different experiments) was similar to that observed in A431 cells (data not shown). We had previously found, using a gel mobility shift assay, that EGF (but not TPA or A23187) induced the level of enhancer-binding activity in A431 cell nuclear extracts (33). We similarly determined the amount of enhancer-binding activity in nuclear extracts from HeLa monolayer

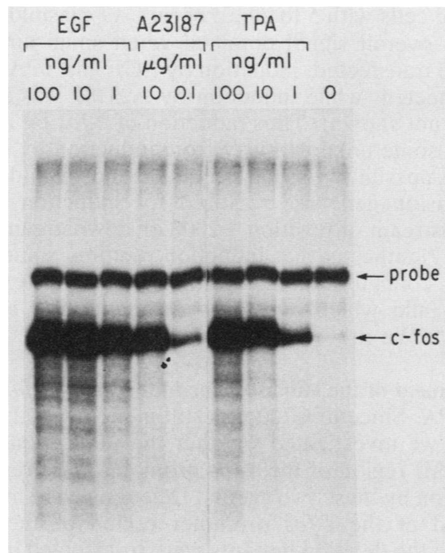

FIG. 1. Induction of c-*fos* mRNA in HeLa cells by EGF, TPA, and A23187. Asynchronously growing HeLa cells in monolayer culture were either untreated (lane 0) or treated for 30 minutes with various concentrations of EGF, calcium ionophore A23187, or TPA, as indicated. Total cellular RNA (20 μg) was analyzed for c-fos^H transcripts by RNase protection mapping as described in Materials and Methods. Undigested probe and the c-fos^H-specific protected fragments are indicated by arrows.

cells that were uninduced or treated for 30 min with EGF, TPA, or A23187. The level of enhancer-binding activity in untreated cells was high, being comparable to that found in EGF-treated A431 cells, and did not change after treatment with any of the inducers (Fig. 2). This is consistent with the results of Treisman (44), who found no change in c-*fos* enhancer-binding activity upon serum stimulation of serum-starved HeLa spinner culture cells. The different patterns of enhancer-binding activity upon induction of c-*fos* transcription in HeLa versus A431 cells suggested different mechanisms for c-*fos* regulation in these two cell types. For this reason, we transfected c-*fos* promoter mutants into both HeLa monolayer and A431 cells to determine which sequences are involved in EGF-, TPA-, and A23187-induced transcriptional regulation in these cells.

A23187 has different requirements for induction of c-*fos* than do TPA and EGF in HeLa cells. To determine the sequences which mediate induction of the human c-*fos* gene by EGF, TPA, and the calcium ionophore A23187, we fused parts of the c-*fos* gene, extending from variable 5′ sites to position +42, to the coding region of the bacterial *CAT* gene (Fig. 3C). These *fosCAT* constructs were introduced into HeLa monolayer cells by the calcium phosphate precipitation method; 44 h later the transfected cells were treated for 30 min with the various inducers, and total cellular RNA was isolated from the cells. We included an internal control plasmid, pRSVCAT, in every transfection so that we could normalize for transfection efficiency in the subsequent analyses. We chose pRSVCAT because expression of the Rous sarcoma virus promoter was not affected by TPA (24) and we have not observed any reproducible variation in pRSVCAT expression as a result of treatment with EGF, TPA, or A23187. In S1 nuclease analyses of RNA harvested from the transfected cells, transcripts derived from pRSVCAT could be distinguished on the basis of size from the *fosCAT* transcripts by use of a single S1 probe made from the coding and 5′ untranslated region of the *fosCAT* hybrid gene (Fig. 3C). A 300-nucleotide fragment extending to the 5′ end of the *fosCAT* transcript was protected from S1 digestion, while only a 250 nucleotide fragment corresponding to the *CAT* portion of the pRSVCAT transcript was protected.

The results of typical S1 analyses of the total cellular RNA harvested from transiently transfected cells are shown in Fig. 3A. A construct including 700 base pairs of promoter sequence upstream of the cap site (pFC700) was induced by both EGF and TPA. Induction of the wild-type fusion gene by EGF and TPA ranged from 6- to 10-fold, normalized to the pRSVCAT signal, as determined by densitometric analysis. This induction was lower than that of the endogenous gene (Fig. 3B), possibly because of an increased apparent basal level of expression that may have resulted from greater stability of *CAT* mRNA relative to c-*fos* mRNA (leading to a greater level of accumulation during the 44-h period preceding the induction). The rapid turnover of c-*fos* mRNA has been documented previously (2, 43). Alternatively, sequences playing a role in induction may lie downstream of position +42 in c-*fos* and would therefore have been missing in the fusion gene.

In contrast to EGF and TPA, A23187 did not induce pFC700 at concentrations that maximally stimulated the endogenous gene (Fig. 3A); furthermore, a *fosCAT* fusion gene containing about 2,000 base pairs upstream of the cap site (pFC2000) was also not induced by A23187. The endogenous gene in cells from these transfections was always induced just as well by A23187 as by EGF or TPA (Fig. 3B), and the level of induction was comparable to that obtained in untransfected control cells. This finding rules out the possibility that the calcium phosphate precipitation technique itself was abolishing A23187 induction. Furthermore, similar results were obtained when the cells were transfected by the

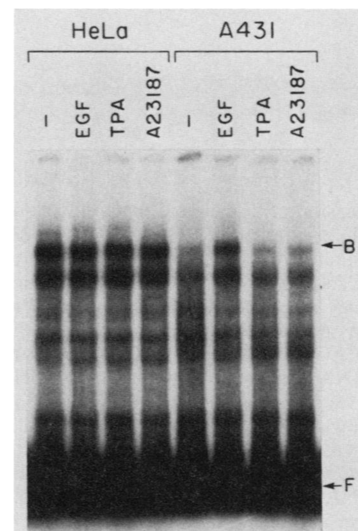

FIG. 2. c-*fos* enhancer-binding activity in EGF-, TPA- and A23187-treated HeLa and A431 cells. Nuclear extracts were prepared from HeLa and A431 cells either untreated (lanes −) or treated for 15 min with 100 ng of EGF per ml, 100 ng of TPA per ml, or 10 μg of A23187 per ml. The gel mobility shift assay was performed with 2 μg of each extract, 0.5 ng of ^{32}P-labeled probe (positions −363 to −225), 2 μg of salmon sperm DNA, and 500 ng of pUC19 DNA. The positions of migration of the specific DNA-protein complex (B) and freely migrating probe (F) are indicated (33).

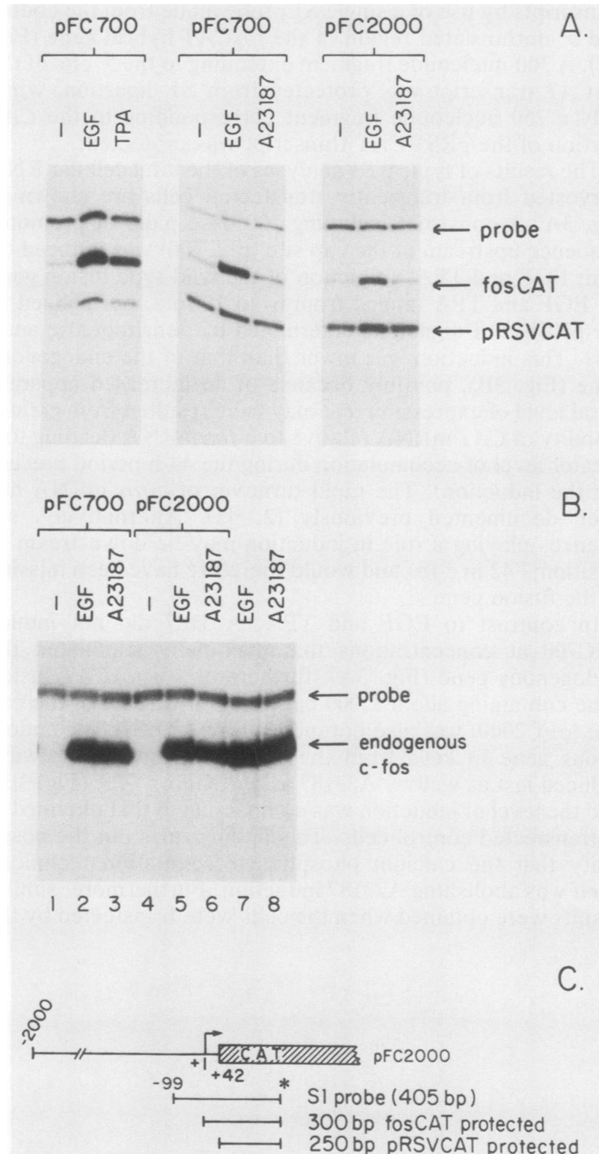

FIG. 3. Expression of transfected *fosCAT* fusion genes in transfected HeLa cells treated with EGF, TPA, or A23187. (A) HeLa cells were transfected with deletion mutants possessing approximately 700 (pFC700) or 2,000 (pFC2000) base pairs of 5' flanking sequence, together with pRSVCAT as an internal reference. Total cellular RNA was prepared either before (lanes −) or 30 min after stimulation with EGF, TPA, or A23187 and analyzed by S1 mapping; 20 μg of RNA was used in each reaction. Self-hybridized S1 probe, *fosCAT*, and pRSVCAT-specific protected fragments are indicated by arrows. (B) Induction by EGF and A23187 of endogenous c-*fos*^{H} mRNA in HeLa cells that were transfected with PFC700 (lanes 1 to 3), pFC2000 (lanes 4 to 6) or untransfected (lanes 7 and 8). Total cellular RNA (20 μg) was analyzed for c-*fos*^{H} transcripts by RNase protection mapping. (C) Diagram of the pFC2000 *fosCAT* fusion gene and strategy for differential S1 mapping of *fosCAT* and pRSVCAT transcripts. The endpoints of the S1 probe and of fragments protected specifically by *fosCAT* or pRSVCAT transcripts are indicated.

DEAE-dextran method (data not shown). To determine whether induction was sensitive to DNA concentration, as might be expected if there were competition among transfected templates for limiting transcription factors, we transfected cells with 5 to 20 μg of *fosCAT* plasmid. Despite a reduced overall signal obtained when small amounts of DNA were transfected, induction by EGF and TPA could be clearly detected, while induction by A23187 was undetectable (data not shown). Thus induction of c-*fos* by A23187 is distinct in some unknown way from induction by EGF and TPA. One possibility is that sequences not included in the *fosCAT* fusion gene are required for induction, i.e., sequence upstream of position −2000 or downstream of position +42. Another is that induction requires some specific chromatin configuration that is not present in exogenous templates (and which may or may not involve additional sequences). We are currently investigating these possibilities.

Requirement of the enhancer for induction of c-*fos* in HeLa cells by TPA. Since pFC700 was still inducible by both EGF and TPA, we investigated whether the same sequence elements in this region of the c-*fos* promoter were responsible for induction by these two agents. Deletions were made from the 5' end of the c-*fos* promoter region of the *fosCAT* constructs; the deletion mutants were transfected into HeLa cells and RNA was isolated 44 h later, after a 30-min

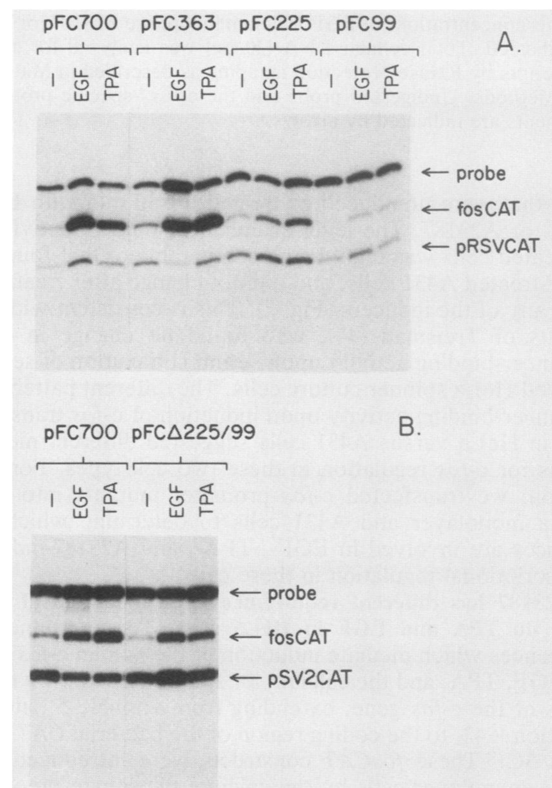

FIG. 4. Induction of *fosCAT* deletion mutants by EGF and TPA in HeLa cells. (A) Cells were transfected with 5' deletion mutants containing 700 base pairs (pFC700), 363 base pairs (pFC363), 225 base pairs (pFC225) or 99 base pairs (pFC99) of 5' flanking sequence, together with pRSVCAT. Total cellular RNA was prepared either before (lanes −) or 30 min after stimulation with EGF or TPA and analyzed by S1 mapping; 20 μg of RNA was analyzed per reaction. (B) HeLa cells were transfected with 5' deletion mutant pFC700 or internal deletion mutant pFCΔ225/99, a deletion derivative of pFC700 lacking sequence between nucleotides −225 to −99. pSV2CAT was cotransfected as the internal control in this experiment. Total cellular RNA was prepared and analyzed as described above.

induction, as described above. An S1 analysis of RNA harvested from cells transfected with the 5' deletion mutants is shown in Fig. 4A. A construct, pFC363, deleted to −363 (relative to the mRNA cap site) was still inducible by EGF and TPA, while constructs deleted to −225 or −99 were not inducible. When the data were quantitated by densitometric analysis and normalized for transfection efficiency with the pRSVCAT internal control, the induction was estimated to be 10-fold for the wild-type plasmids (pFC700 and pFC363) and a maximum of 2-fold for pFC225 and pFC99. In other experiments with pFC225 and pFC99, induction was often not apparent (data not shown). It is also notable that the basal level of expression (when normalized to the pRSVCAT control) is not significantly decreased by removal of the enhancer at −317 or of sequences between −225 and −99, in agreement with the results of Treisman (43). Further evidence for this point was provided by an internal deletion mutant lacking sequences between −225 and −99, which was similar to its parent plasmid pFC700, both in its basal level of expression and in its induction by EGF and TPA (Fig. 4B; pSV2CAT [18] was used as the internal control in this experiment). In this respect, human c-*fos* behaves somewhat differently from mouse c-*fos*, for in the latter gene a sequence located around position −150 was shown to be involved in elevating the basal level of expression of the gene (15). The mouse −150 sequence, however, is not conserved in the human gene.

Thus our studies indicate that the region from −363 to −225, spanning the c-*fos* enhancer, is critical for induction by TPA as well as EGF. While the c-*fos* enhancer has been defined as a 20-base-pair region from −317 to −298, there is a strong conservation of sequence between the mouse and human sequences from −363 to −250 (34, 43). In addition, the region −298 to −287, adjacent to the c-*fos* enhancer, bears significant homology (11 of 12 base pairs) to a sequence in the preproenkephalin gene that is necessary for induction of that gene by phorbol esters and cyclic AMP (5; Fig. 5B). To test the possible involvement of this sequence in induction of c-*fos* by TPA, we constructed a series of *fosCAT* mutants by fusing a synthetic oligonucleotide spanning both this putative element and the c-*fos* enhancer (−318 to −284) to position −225 of the remaining portion of the c-*fos* promoter (Fig. 5B). The clone pTF1 was wild type with respect to region −318 to −284, whereas pTF2 contained four single-base-pair substitutions (two in each inverted repeat of the enhancer) and pTF3 contained two single-base-pair substitutions in a conserved region of the preproenkephalin homology (Fig. 5B). Upon transfecting these constructs into HeLa cells, we found that expression of pTF1 was induced normally by both EGF and TPA, whereas mutations in the enhancer (pTF2) abolished induction of the gene by both agents (Fig. 5A). In contrast, mutation of the preproenkephalin homology (pTF3), at least of the two conserved base pairs that we chose, had no effect on induction by either TPA or EGF. Thus the c-*fos* enhancer at −317 to −298 is necessary for induction of the gene by both TPA and EGF in HeLa cells and appears to be the only element in this region that is required. The ability to activate transcription in vivo correlated with the ability of the oligonucleotides to compete for binding by the c-*fos* enhancer-binding factor in vitro in a gel mobility shift assay (data not shown). Oligonucleotides TF1 and TF3 competed effectively, while TF2 did not compete.

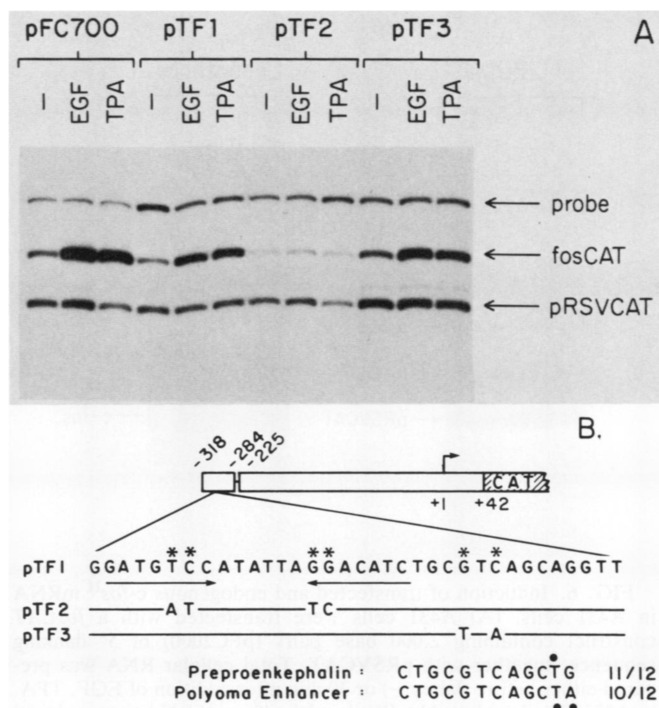

FIG. 5. Induction of transfected *fosCAT* enhancer region point mutants by EGF and TPA. (A) Cells were transfected with enhancer region point mutants that had been created by fusing 40-base-pair oligonucleotides, spanning positions −318 to −284 relative to the c-*fos* mRNA cap site, to position −225 of the *fosCAT* fusion gene, as described in the text. pFC700, pTF1 (containing no substitutions in the enhancer region), pTF2 (containing four single-base-pair changes in the dyad symmetry enhancer element) and pTF3 (containing two single-base-pair substitutions in the preproenkephalin sequence homology region) were transfected together with pRSVCAT. Total cellular RNA was prepared either before (lanes −) or 30 min after stimulation with EGF or TPA and analyzed by S1 mapping; 20 μg of RNA was used in each reaction. (B) Diagram of *fosCAT* enhancer region point mutants. The oligonucleotide spanning positions −318 to −284 is shown in detail. The dyad symmetry element is indicated by inverted arrows. The sequences in the human preproenkephalin (positions −93 to −81) and polyomavirus (positions 5130 to 5119) promoters are shown directly below the homologous c-*fos* sequences, with differences indicated by dots. Base pairs that were mutated in oligonucleotides TF2 and TF3 are marked by asterisks, and specific base pair changes made in TF2 and TF3 are indicated.

Mechanism of c-*fos* induction in A431 cells. Since TPA, in contrast to EGF, did not induce the level of c-*fos* enhancer-binding activity in A431 cell nuclear extracts (33), and since there is a relatively high level of enhancer-binding activity in HeLa cells even before induction with any agent (Fig. 2), we wanted to find whether the mechanism of induction of the gene by TPA was different in A431 from that in HeLa cells. We thus transfected various *fosCAT* fusion constructs into A431 cells and checked for induction. As with HeLa cells, the exogenous *fosCAT* gene was not induced by A23187, but in this case it was also not induced by TPA, even when pFC2000 was transfected; in contrast, pFC2000 was induced approximately fivefold by EGF (Fig. 6A). By densitometric analysis, normalizing to the pRSVCAT internal control, we found that expression was induced 4.5-fold by EGF, 1.3-fold by TPA, and 1.0-fold by A23187. We then analyzed total cellular RNA from the same transfected cells for induction of the endogenous transcript by EGF or TPA and observed, in fact, that TPA treatment led to a much smaller rise in mRNA than did EGF treatment (Fig. 6B). The level of TPA-induced

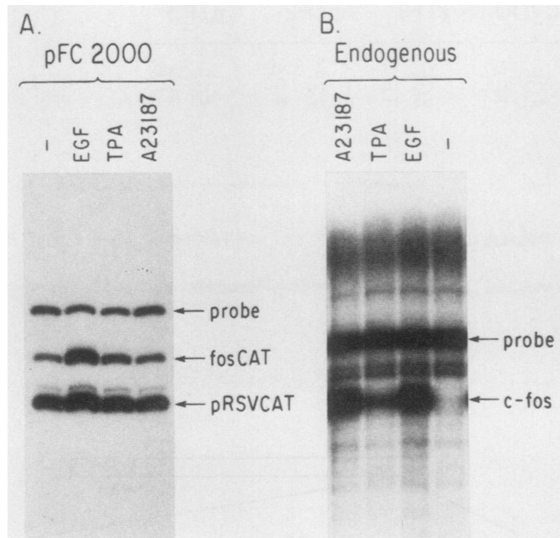

FIG. 6. Induction of transfected and endogenous c-*fos*^{H} mRNA in A431 cells. (A) A431 cells were transfected with a *fosCAT* construct containing 2,000 base pairs (pFC2000) of 5′ flanking sequence, together with pRSVCAT. Total cellular RNA was prepared either before (lanes –) or 30 min after addition of EGF, TPA, or A23187 and analyzed by S1 mapping; 40 μg of RNA was analyzed in each reaction. (B) Levels of endogenous c-*fos*^{H} mRNA in transfected A431 cells. Total cellular RNA from the experiment described in panel A was analyzed for endogenous c-*fos*^{H} mRNA by RNase protection mapping. Undigested probe and the c-*fos*^{H}-specific protected fragment are indicated by arrows.

c-*fos* mRNA was about 14% of the level of EGF-induced mRNA, as judged by densitometry. The same was true for untransfected cells (data not shown) and is consistent with the observations of Bravo et al. (1). This result contrasts with our observations in nuclear run-on experiments that transcriptional induction by a 30-min treatment with TPA was about two-thirds that observed with EGF treatment (33; data not shown). The basis for the difference in induction at the transcriptional and mRNA levels is presently unclear, but may suggest a higher rate of c-*fos* mRNA turnover after TPA treatment of A431 cells.

There are two possible explanations for our inability to detect induction of pFC2000 by TPA in A431 cells. The lower level of mRNA induction expected from TPA treatment, as seen with endogenous c-*fos* mRNA, might simply not have been detectable for the exogenous transcript in our system. Indeed, in our transfection assay, even the induction by EGF was much lower than that of the endogenous gene (Fig. 6A and B), possibly because there is a higher apparent basal level of expression as a result of greater stability of *CAT* versus c-*fos* mRNA (discussed above). On the other hand, the *fosCAT* constructs should reflect only transcriptional changes, since the c-*fos* mRNA sequences downstream of +42 have been removed. It is unlikely that TPA treatment leads to a general destabilization of mRNA in A431 cells, because there is no apparent destabilization of mRNA generated from our pRSVCAT internal control (Fig. 6B). Our previous results indicated that the level of c-*fos* transcription induced by TPA was relatively high and comparable to the level induced by EGF (34). Therefore the failure of TPA to induce pFC2000 (Fig. 6A) may indicate that TPA requires sequence elements outside of −2000 to +42 to induce c-*fos* transcription in A431 cells. In that case, the mechanism of induction of c-*fos* by TPA would be different in HeLa than in A431 cells.

Other sequences involved in c-*fos* gene expression. Our deletion mutant studies in HeLa cells failed to reveal the presence of functional sequences between positions −298 and −99. It has been shown by Gilman et al. (15), however, using progressive 5′ deletion mutants, that a 7-base-pair sequence extending from −68 to −62 in the mouse c-*fos* gene is important for basal expression of that gene. This sequence is conserved in the human gene at positions −63 to −57. To assay for the influence of this sequence on basal expression and to test for a possible role in induction, we made constructs which contained internal deletions in the region around −60 but which retained the enhancer. Thus starting with the wild-type plasmid pFC700, we used BAL 31 exonuclease to construct a series of internal deletion mutants that lacked sequences between position −94 and various downstream sites (Fig. 7B). A *Bgl*II synthetic DNA linker was inserted at the deletion site to facilitate cloning. Insertion of the *Bgl*II linker did not in itself alter the

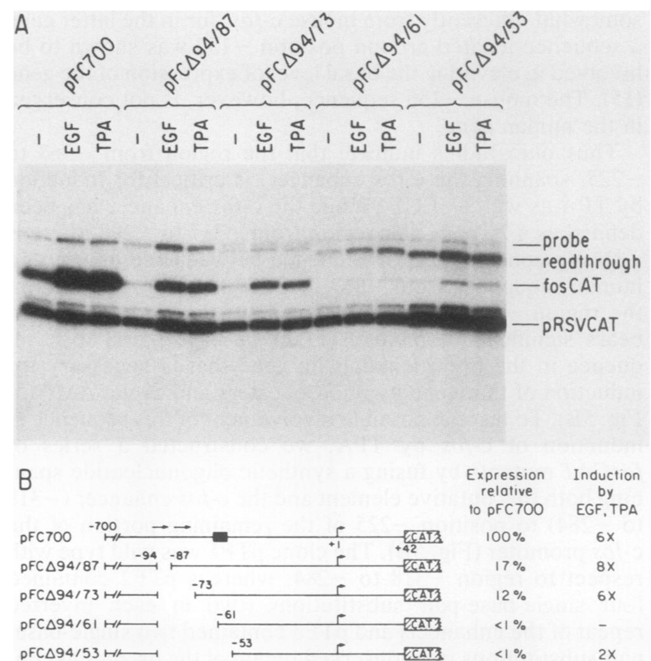

FIG. 7. Induction of transfected *fosCAT* internal deletion mutants by EGF and TPA. (A) HeLa cells were transfected with pFC700 or with internal deletion derivatives of pFC700 lacking sequence between −94 and −87 (pFCΔ94/87), −94 and −73 (pFCΔ94/73), −94 and −61 (pFCΔ94/61), or −94 and −53 (pFCΔ94/53). pRSVCAT was cotransfected as an internal control. Total cellular RNA was prepared either before (lanes –) or 30 min after addition of EGF or TPA; 20 μg of RNA was analyzed in each S1 reaction. Bands corresponding to self-hybridized probe and to fragments protected specifically by *fosCAT*, pRSVCAT, and readthrough transcripts initiated upstream of the deletion site are indicated. Fragments protected by readthrough transcripts become distinguishable by size from self-hybridized probe with plasmids containing internal deletions of sequence covered by the probe. (B) The structure of the deletion mutants analyzed in panel A, the efficiency of their induction by EGF and TPA, and the level of expression of the internal deletion mutants relative to pFC700 are indicated. The intensity of the bands in panel A was quantitated by densitometric analysis. The signal obtained from uninduced cells transfected with pFCΔ94/61 was too low in this experiment to allow accurate quantitation and thus an estimate of induction efficiency.

expression of the gene, since a construct that included a *Bgl*II linker at position −99 but was otherwise exactly analagous to pFC700 was expressed and induced as well as pFC700 (data not shown). The results of a typical S1 analysis of RNA from HeLa cells transfected with the BAL 31 deletion mutants are shown in Fig. 7A. Both the basal and induced levels of mRNA obtained from the first mutant in the series, pFCΔ94/87 (Fig. 7B), missing sequence between positions −94 and −87 upstream of the cap site, were reduced to 17% of the respective mRNA levels obtained from the wild-type gene (pFC700). The basal and induced levels of mRNA obtained from the next mutant, pFCΔ94/73, were further reduced to about 12% of the respective wild-type levels. Although the absolute amounts of mRNA produced by these two deletion mutants after induction by EGF and TPA were lower than those produced by pFC700, the overall level of stimulation of mRNA expression by EGF and TPA was as large for pFCΔ94/87 and pFCΔ94/73 as for pFC700 (six- to eightfold; Fig. 7B). The sequence removed in these constructs contains an 8-base-pair perfect direct repeat (GCGCCACC) that spans positions −97 to −76; pFCΔ94/87 is missing the first repeat, and pFCΔ94/73 is missing both repeats. These repeats are not conserved in the mouse gene. It appears that the direct repeats constitute a binding site for a transcription factor which amplifies the general expression of the gene, but which appears not to be a site through which a regulatory factor acts directly. Because the construct retaining one of the repeats was expressed somewhat better than the construct missing both repeats, it is possible that a single repeat retains some binding activity.

Deletion to −61 further depressed basal expression of the gene (by more than 90%, as judged by densitometry; Fig. 7). This mutant, pFCΔ94/61, is lacking two base pairs of the element homologous to the mouse c-*fos* sequences around position -60, and thus the decrease in expression is most probably due to removal of this element. A deletion mutant which lacked sequences between −94 and −66, and therefore contained the −60 element, was phenotypically like the Δ94/73 mutant (data not shown). A small induction by EGF and TPA was still evident in the construct pFCΔ94/61, but the low levels of expression observed made it difficult to quantitate the data. Further deletion to −53 (pFCΔ94/53; Fig. 7) and to −40 (data not shown) resulted in similarly poor expression, with a small remaining induction (about twofold) by EGF and TPA. Similar results were obtained when this series of deletion mutants was transfected into A431 cells (data not shown). Thus the murine sequence at −68 to −62, whose function was identified by Gilman et al. (15), appears also to be important for expression of the human gene. Furthermore, in our deletion mutants, which retained the enhancer element, deletion of the −60 element resulted in a significant loss of inducibility (twofold versus six- to eightfold in constructs including the −60 element).

Specific factors in nuclear extracts bind to the direct repeat and −60 sequences. By using a gel mobility shift assay, we were able to detect a protein in HeLa cell extracts that binds in the region of the 8-base-pair direct repeats. We constructed an end-labeled 235-base-pair probe that spanned positions −225 to +10 in the c-*fos* promoter and 5′ untranslated region (Fig. 8). This probe included not only the direct repeats but also the putative factor-binding site at around −60. To detect a shift caused specifically by a potential direct repeat-binding protein, we performed the analyses in the presence and absence of a 50-fold molar excess of two unlabeled competitor DNA fragments (Fig. 8). One competitor fragment included sequence from −225 to +42 and thus should have competed for binding of both the direct repeat factor and the −60 factor; the other competitor fragment spanned the same sequence, but was derived from plasmid pFCΔ94/73 and was thus missing the direct repeats, although it still included the putative binding site around −60. The latter fragment was thus capable of competing for all factors binding specifically to the probe except those binding to the direct repeat region. In such a gel shift assay, we were able to detect a band (B1) that was diminished in intensity when the complete fragment was used as a competitor (Fig. 8, second to fourth lanes) but not when the deleted competitor fragment missing the direct repeat sequence was used as a competitor (first lane). Although 50 ng of the deleted competitor fragment did not effectively compete for binding, 10 ng of the intact fragment greatly diminished the intensity of this band. The analysis also revealed two lower bands (B2 and B3) that were sensitive to a lesser extent than B1 to competition by the intact fragment. These bands migrated within a region of a number of nonspecific complexes such that is was difficult to clearly analyze their specificity. The sequence of each direct repeat is 5′-GCGCCACC-3′. We have not yet found a transcriptional element with a similar sequence defined in the literature.

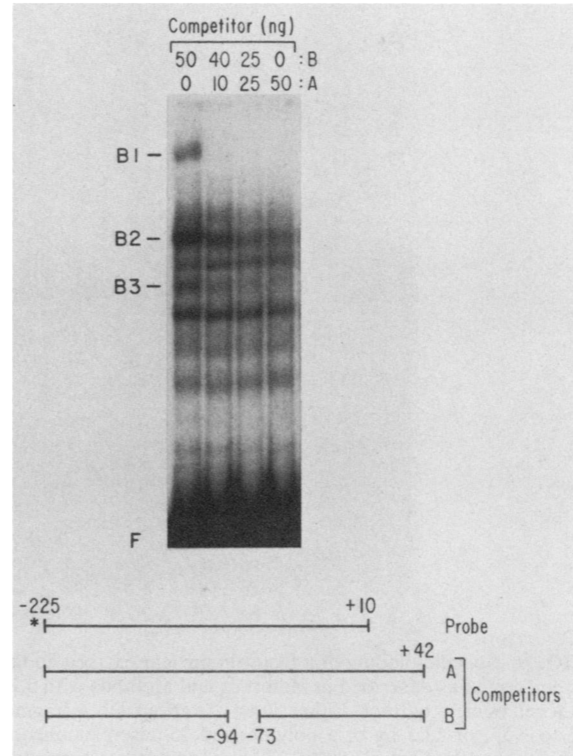

FIG. 8. Specific binding of a factor in nuclear extracts to the c-*fos* direct repeat region. The gel mobility shift assay was performed as described in Materials and Methods with 0.8 ng of ^{32}P-labeled probe, 3 μg of poly(dI-dC) · poly(dI-dC), 5 μg of HeLa cell nuclear extract, and 50 ng of fragment competitor. The probe, containing positions −225 to +10 of *fos* 5′ flanking sequence, is diagrammed, as well as specific (A) and nonspecific (B) competitors. Competitor B was derived from deletion mutant pFCΔ94/73 and was thus lacking the direct repeat sequence. The positions of three potentially specific bands (B1, B2, and B3) and the freely migrating probe (F) are indicated. The sequence of the direct repeat region is (−97)-GCGCCACCCCTCTGGCGCCACC-(−76).

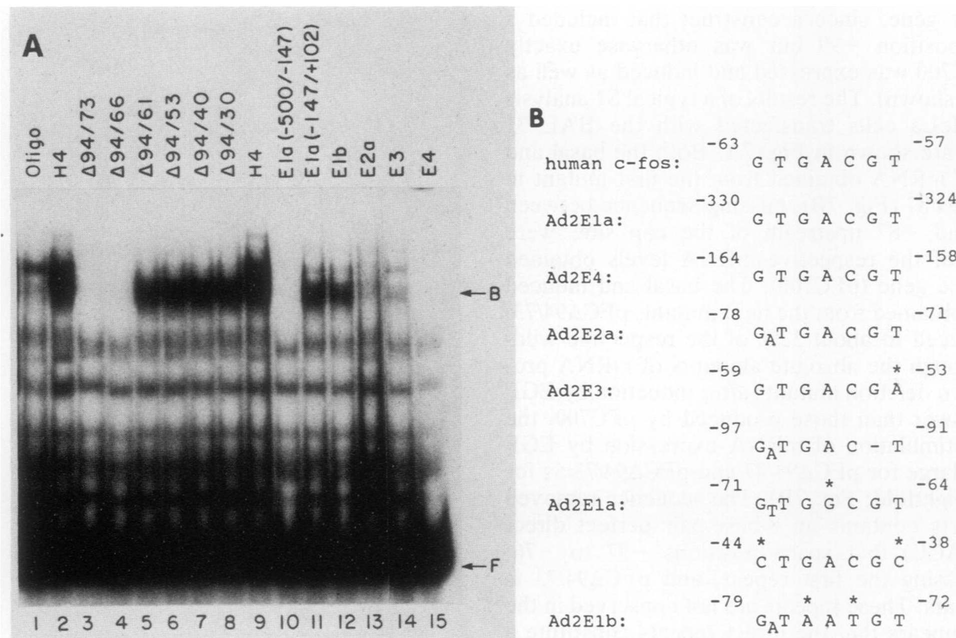

FIG. 9. Specific binding of a factor in nuclear extracts to the c-*fos* promoter region around position −60. (A) The gel mobility shift assay was performed as described in Materials and Methods with 0.2 ng of ^{32}P-labeled probe, 500 ng of sonicated salmon sperm DNA, and 5 μg of HeLa cell nuclear extract. Either 50 ng of various DNA fragments, 25 ng of a 32-base-pair oligonucleotide including the c-*fos* sequence from −63 to −57, or 12.5 ng of a polymerized 15-base-pair oligonucleotide (average chain length, 150 to 200 base pairs) containing Ad2 E2a promoter sequence from −80 to −70 was included as competitor in each reaction. The positions of a specific protein-DNA complex (B) and the freely migrating probe (F) are indicated. Competitor fragments or oligonucleotides were included as follows: lane 1, 32-base-pair oligonucleotide spanning the *fos* sequence from −71 to −48; lanes 2 and 9, fragment including the *Xenopus* histone H4 promoter from −119 to −42; lanes 3 to 8, fragments derived from *fosCAT* internal deletion mutants (the fragments spanned c-*fos* 5′ flanking sequence from −225 to +42 but were missing the internal sequences indicated); lane 10, fragment spanning −500 to −147 in the Ad5 Ela promoter; lane 11, fragment spanning −147 to +100 in the Ad2 Ela gene; lane 12, fragment spanning −141 to +57 in the Elb gene; lane 13, polymerized 15-base-pair Ad2 E2a oligonucleotide; lane 14, fragment spanning −233 to +25 in the Ad2 E3 gene; lane 15, fragment spanning −230 to +20 in the Ad2 E4 promoter. (B) Diagram of sequences in various Ad2 promoters homologous to the c-*fos* sequence from −63 to −57. Ad2 and Ad5 are exactly homologous in the region spanning −331 to −324 upstream of the Ela mRNA cap site.

On the basis of our results and those of Gilman et al. (15), we expected to find a factor in HeLa cell nuclear extracts that would bind the human c-*fos* sequence from −63 to −57. Using a gel shift assay, we did detect a protein in HeLa cell nuclear extracts that bound to this sequence. The assay shown in Fig. 9 involved the use of a probe that extended from −73 to −10 and revealed a specific band that was sensitive to competition only by fragments containing the sequence from −67 to −53 but not by nonspecific fragments. Fragments spanning positions −225 to +42 were prepared from various *fosCAT* internal deletion mutants and used as competitors. The fragments derived from mutants missing sequence from −94 to −73 (lane 3) and from −94 to −66 (lane 4), respectively, competed efficiently for binding of the specific protein, whereas fragments derived from internal deletion mutants missing sequence from −94 to −61 and beyond (lanes 5 to 8) did not compete for the specific binding. Similarly, an approximately 80-base-pair fragment derived from the *Xenopus* histone H4 promoter did not compete for binding to the −60 c-*fos* sequence (lanes 2 and 9). A synthetic oligonucleotide (RP2) spanning positions −71 to −48 also competed for the specific binding (lane 1). In addition to the specific band seen in Fig. 9, there was another slightly more slowly migrating band that was inhibited by all of the competitor fragments derived from the c-*fos* promoter, but not by the oligonucleotide or by the *Xenopus* H4 promoter fragment. It is thus possible that this band represents binding of a factor to the sequence between −30 and −10 in the c-*fos* promoter. This more slowly migrating band, however, was also sensitive to competition by fragments that were derived from various adenovirus promoters and that do not share any apparent homology with the c-*fos* sequence located between −30 and −10 (Fig. 9). We are unsure of the identity of the upper band.

Sequences found in a number of adenovirus early-gene promoters are homologous to the 7-base-pair sequence found in c-*fos*. Figure 9B shows these homologous sequences. Fragments containing the homologous sequences were prepared from the relevant adenovirus promoter regions for use as competitors in the gel shift assay (Fig. 9A). Two fragments from the Ela promoter, one spanning positions −500 to −147 upstream of the cap site and one spanning positions −147 to +102, were used. Fifty nanograms of unlabeled competitor fragment and approximately 0.2 ng of labeled probe were used per gel shift reaction (an approximately 60-fold excess of competitor to probe-binding site). As a competitor representing the E2a binding site, we used 12.5 ng of a 15-base-pair polymerized oligonucleotide that spanned −80 to −70 in the E2a promoter. Our competitions showed that the Ela fragment containing the binding site at −331 (lane 10) and the E4 fragment (lane 15) competed best for binding of the −60 factor to the c-*fos* promoter. The binding sites included in these two fragments are homologous in seven of seven base pairs to the c-*fos*

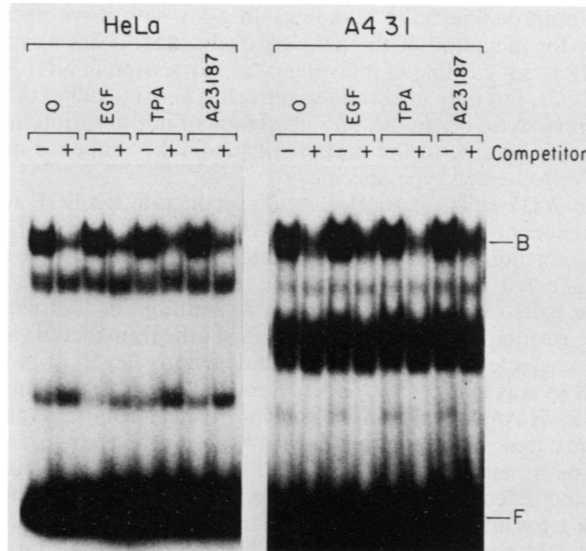

FIG. 10. −60 factor-binding activity in EGF-, TPA-, and A23187-treated HeLa and A431 cells. Nuclear extracts were prepared from A431 or HeLa cells either untreated (lanes 0) or treated for 15 min with 100 ng of EGF per ml, 100 ng of TPA per ml, or 10 μg of A23187 per ml. The gel mobility shift assay was performed with 2 μg (A431) or 5 μg (HeLa) of nuclear extract, 0.1 ng of ^{32}P-labeled probe, 500 ng of sonicated salmon sperm DNA (HeLa), or 2μg of poly(dI-dC) · (dI-dC) (A431) and 25 ng of nonspecific (−) or specific (+) oligonucleotide competitor. The probe and the specific competitor in this experiment was the 32-base-pair oligonucleotide spanning the c-*fos* sequence from −71 to −48 (RP2). The nonspecific competitor was the 40-base-pair oligonucleotide TF3 (Fig. 5B).

sequence. The E3 promoter fragment (lane 14) also competed for binding, although less effectively than the E1a and E4 fragments; its binding site contains a 1-base-pair change relative to the c-*fos* binding site (Fig. 9B). The E2a binding site (lane 13) competed for binding, although apparently less well than the E4 and E1a sites, even though a greater molar excess of E2a oligonucleotide was used in the competition; the E2a binding site includes a 1-base-pair insertion relative to the c-*fos* binding site. This less efficient competition may be due to the nature of the polymerized oligonucleotide compared with a fragment, however, because the E2a oligonucleotide competed as effectively for binding as an equimolar amount of the synthetic c-*fos* oligonucleotide (compare lanes 1 and 13). The E1a fragment extending from positions −147 to +102 (lane 11) and the E1b fragment (lane 12), containing potential binding sites that differed in two and three bases, respectively, from the c-*fos* binding site, did not compete for binding of the −60 factor to the c-*fos* promoter.

To determine whether the level of binding of the −60 factor might be regulated by the inducing agents, we analyzed nuclear extracts obtained from HeLa and A431 cells either untreated or treated with EGF, TPA, or A23187 (Fig. 10). For this assay, we made an end-labeled probe from the synthetic oligonucleotide (RP2) spanning positions −71 to −48 upstream of the cap site in the c-*fos* promoter and used either poly(dI-dC) · (dI-dC) (A431 experiment) or sonicated salmon sperm DNA (HeLa experiment) as a nonspecific competitor. The binding activity of the factor did not appear to change significantly upon induction with the various inducers in nuclear extracts made from either cell type.

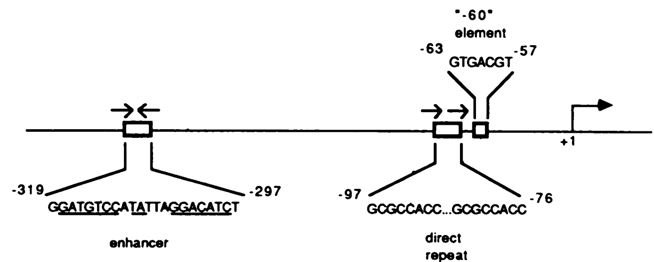

FIG. 11. Relative positions of the three sequence elements in the c-*fos* gene. The inverted repeats in the enhancer and the direct repeats from positions −97 to −76 are denoted by arrows.

DISCUSSION

We have investigated the importance of various regions of the c-*fos* gene to basal expression and induction by EGF, TPA, and the calcium ionophore A23187. Our findings are summarized in Fig. 11 and Table 1 and are discussed below.

EGF and A23187 mediate c-*fos* induction by different mechanisms. Treatment of A431 cells with EGF greatly increased the level of binding of a nuclear factor to the c-*fos* enhancer, while treatment with A23187 had no effect (33). These results suggested that EGF and A23187 use distinct mechanisms for the induction of c-*fos* transcription. In contrast, the level of binding in untreated HeLa cells was relatively high and was not elevated by any of the inducing agents. In the present studies, a transfected *fosCAT* fusion gene containing 2,000 base pairs upstream of the mRNA cap site was inducible by EGF but not by A23187 in both HeLa and A431 cells. A23187, however, induced the endogenous gene as effectively as EGF in both transfected and untransfected HeLa and A431 cells. These results are consistent with the nuclear factor binding data for A431 cells and suggest that c-*fos* induction by A23187 is mediated via different, or additional, sequence elements (e.g., sequences upstream of position −2000 or downstream of position +42). Alternatively, A23187 may be able to induce the gene only if it resides within a chromosomal context. We are investigating these different possibilities.

The enhancer is necessary for induction by both EGF and TPA in HeLa cells. We have demonstrated that in asynchronously growing HeLa cells, induction of c-*fos* by both EGF

TABLE 1. Effect of c-*fos* sequence elements on basal expression and inducibility of transfected *fosCAT* plasmids

Sequence element[a]	Relative basal expression	Cell type	Level of induction by:		
			EGF	TPA	A23187
ENH, DR, −60	1	HeLa	7–10×	7–10×	1×
		A431	4–5×	1×	1×
DR, −60	1	Both	1×	1×	ND[b]
ENH, −60	0.12–0.17	HeLa	7–10×	7–10×	ND
		A431	4–5×	1×	ND
ENH	0.01	HeLa	2×	2×	ND
		A431	–[c]	1×	ND

[a] c-*fos* sequence elements in various transfected plasmids. ENH, c-*fos* enhancer from −318 to −298; DR, sequence from −97 to −76 including an 8-base-pair direct repeat; −60, 7-base-pair sequence element from −63 to −57.

[b] ND, Not done.

[c] –, Level of expression was too low to quantitate accurately.

and TPA requires an intact enhancer dyad symmetry element. A *fosCAT* fusion gene consisting of a synthetic oligonucleotide spanning positions −318 to −284 fused to the remainder of the c-*fos* promoter at position −225 was induced by both EGF and TPA in transient transfection assays. An otherwise identical construct containing two point mutations in each half of the dyad symmetry element was not induced by either EGF or TPA. Thus EGF and TPA seem to be members of a class of factors, such as those present in serum, that mediate induction of c-*fos* through the enhancer. Further in vivo studies involving the use of other purified growth factors are needed to determine whether all growth factors require the enhancer for induction of c-*fos* transcription. Indeed, recent results have indicated that induction of c-*fos* in BALB/c-3T3 cells by conditioned media from v-*sis*-transformed cells may be mediated through a sequence upstream of the enhancer. It was found that the conditioned media increased the binding of a nuclear factor to a sequence element at −346 (23).

Although it is clear that the c-*fos* enhancer is necessary for induction of the gene by both EGF and TPA in HeLa cells, it is not clear what changes, if any, occur in the enhancer-binding factor after induction to initiate a higher level of transcription. In nuclear extracts of HeLa cells that were untreated or induced with EGF, TPA, or A23187, no changes in the relatively high level of binding activity were evident. It is possible that changes in compartmentalization of the enhancer-binding factor within the nucleus might allow for differential expression of c-*fos* before and after induction without requiring a change in the total amount of active factor present in the cell. Alternatively, induction with growth factors and TPA may induce structural changes in the enhancer-binding factor that do not affect its binding activity per se, but, rather, its ability to activate transcription. Development of an in vitro transcription system to assay for the effects of the enhancer and other factors on transcription may help to elucidate these various possibilities.

Recent observations from in vivo competition experiments with transfected genes suggest that c-*fos* expression may be regulated by negatively acting factors as well as requiring a positive factor(s) (37). We have not found any evidence for a negatively acting factor from our mutational analysis; however, we would not have detected one if such a factor bound to the enhancer region and if our point mutations in the enhancer abolished binding of both the negatively and positively acting factors.

The phorbol ester TPA is known to induce expression of a number of cellular genes (5, 24, 38). Although some of the sequence elements required for induction of expression by TPA have been identified, it is not yet clear whether different genes use common or distinct elements. The fact that c-*fos*, for instance, is induced much more rapidly than other genes may indicate the latter possibility. On the other hand, the element shown to be involved in the induction of the human preproenkephalin gene by TPA is conserved in a number of other genes induced by TPA, including c-*fos* (5). In the preproenkephalin promoter, this element includes a 12-base-pair sequence at positions −93 to −81 that is also needed for induction of the gene by cyclic AMP. Although this sequence is conserved in 11 of 12 bases at a region (−298 to −287) immediately adjacent to the c-*fos* enhancer, it is apparently not used for TPA induction of c-*fos* transcription, since mutation of two base pairs in the conserved region of this element had no effect on induction of transfected *fosCAT* fusion genes by TPA. It is possible, however, that the preproenklephalin homology in c-*fos* will prove necessary for induction of the gene by cyclic AMP. Since cyclic AMP is not capable of inducing c-*fos* expression in NIH 3T3 cells (2), but only in certain other cell types, including those of monocytic origin (2), any involvement in transcription of a factor binding to the preproenkephalinlike sequence may prove to be cell type specific.

In A431 cells, as in HeLa cells, induction by EGF was dependent on the c-*fos* enhancer. However for A431 cells, it was not possible to detect induction by TPA of any of the transfected *fosCAT* constructs, even one containing 2,000 base pairs of upstream sequence. In addition, in contrast to the results with HeLa cells, in both transfected and untransfected A431 cells the endogenous level of c-*fos* mRNA was induced by TPA to a much lesser extent than by EGF. However, we have found in nuclear run-on experiments that TPA stimulated c-*fos* transcription about two-thirds as effectively as EGF did. The *fosCAT* constructs should reflect changes in transcription of c-*fos* because they lack c-*fos* mRNA sequence downstream of +42; it is unlikely that TPA causes a general destabilization of mRNA in A431 cells, since we did not see any destabilization of mRNA generated from pRSVCAT, the internal control. Thus our results suggest that TPA requires sequence elements outside of −2000 to +42 for induction of c-*fos* in A431 cells and that the mechanism of induction by TPA is different in A431 from that in HeLa cells. It is presently unclear, however, why there is a difference between transcription of the c-*fos* gene and cellular mRNA levels. We are currently investigating the basis for this difference.

Role of the −60 element. The c-*fos* promoter includes another sequence, at −63 to −57, that is homologous to a rat somatostatin gene sequence, at position −49 to −43, which is included in a portion of the promoter necessary for induction of the somatostain gene by cyclic AMP (30). We and others (15) have shown that this sequence in c-*fos* does, in fact, bind a nuclear factor that appears to promote the general expression of the gene. When this element was deleted in *fosCAT* fusion genes that still retained the enhancer, basal expression in transfection assays was reduced 10- to 15-fold. In addition, induction by EGF and TPA or fusion genes missing the −60 element was much lower than that observed for the wild-type gene (twofold versus six- to eight-fold). Further experimentation is required to clarify whether this lower induction indicates an additional regulatory role for the −60 sequence element or merely represents the inability of a generally weakened promoter to be well induced. The low levels of expression and induction do indicate, however, that the enhancer alone is not sufficient for induction of the gene, but rather that it requires the cooperation of downstream factors for optimal induction of c-*fos* expression. Thus, although in NIH cells the enhancer was sufficient for serum induction when fused to a β-globin promoter, it mediated submaximal levels of induction in this context (43). We did not find any change in the amount of −60 binding factor in nuclear extracts of either HeLa or A431 cells that had been induced by EGF, TPA, or the calcium ionophore A23187. Nevertheless, regulation could be exerted by altering the ability of the factor to activate transcription (e.g., to interact with other factors) rather than simply its ability to bind to the target sequence.

The 7-base-pair sequence found in human c-*fos* at −63 to −57 is homologous to sequences found in a variety of adenovirus early-gene promoters. By using fragments from the relevant adenovirus promoter regions as competitors in the gel shift assay for the −60 factor, we have demonstrated

a hierarchy of affinities of the different binding sites for this factor. The homologous sequences in both the E1a enhancer (around −331) and the E4 promoter (around −165) competed best for binding of the −60 factor; these sequences have seven contiguous base pair identical to the c-*fos* sequence. The homologous sequences in both the E2a and E3 promoters also competed well, albeit less effectively than the E1a and E4 sequences. E2a and E3 each contain a single-base-pair change from the c-*fos* sequence. Finally, sequences found in the E1b promoter at −79 and in the E1a promoter at −71 or −44 contain either three- or two-base-pair changes, respectively, from the c-*fos* sequence, and none of these competed effectively for binding of the −60 factor. It is likely, then, that the −60 factor is identical or at least closely related to proteins previously demonstrated to bind the homologous sequences in the E2a and E4 promoters (28a, 41).

Role of the direct repeat sequence in human c-*fos* gene expression. By using in vivo transfection assays, we have demonstrated a transcriptional function for a human c-*fos* promoter sequence whose significance was previously unrecognized. This sequence, located at positions −97 to −76 relative to the mRNA cap site, includes an 8-base-pair perfect direct repeat (5′-GCGCCACC-3′). This sequence element is apparently important in promoting the basal level of expression of the gene, but is not directly involved in induction by EGF or TPA, since its removal did not lower the degree of induction by these agents. We have demonstrated by means of a gel shift assay that a factor in HeLa cell nuclear extracts specifically bound to this sequence. The direct repeat is not conserved in the mouse c-*fos* promoter, while in turn, the mouse c-*fos* gene includes two relevant sequence elements not conserved in the human gene (15). One of these, located at around position −150 in the mouse c-*fos* promoter, has been shown to have an effect on the basal level of transcription from transfected mouse c-*fos* genes. The second is an SP1 site, located at positions −79 to −71, which has not yet been demonstrated to be important for transcription of the mouse gene. Thus, the mouse and human genes may be similar in that they require additional transcription factors binding upstream of −70 to elevate the general level of transcription; however, the specific transcription factors used at these positions by the mouse and human genes appear to be different.

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