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Identification of a Novel E1A Response Element in the Mouse c-*fos* Promoter

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Transcriptional activation of the c-*fos* gene in mouse S49 cells by the adenovirus 243-amino-acid E1A protein depends on domains of E1A that are also required for transformation and that bind the cellular protein p300. Activation additionally depends on stimulation of endogenous cyclic AMP (cAMP)-dependent protein kinase by analogs or inducers of cAMP. Transient transfection assays were used to analyze the c-*fos* promoter for sequences that confer responsiveness to E1A. Linker substitution and point mutants revealed that transcriptional activation by E1A depended on a cAMP response element (CRE) located at −67 relative to the start site of transcription and a neighboring binding site for transcription factor YY1 located at −54. A 22-bp sequence containing the −67 CRE and the −54 YY1 site was sufficient to confer responsiveness to a minimal E1B promoter and was termed the c-*fos* E1A response element (ERE). Function of the c-*fos* ERE depended on both the CRE and the YY1 site, since mutation of either site resulted in a loss of responsiveness to E1A. These results imply a specific functional interaction between CRE-binding proteins, transcription factor YY1, and E1A in the regulation of the c-*fos* gene.

Induction of cellular transformation by adenovirus involves disruption of the normal transcriptional program of the cell. This is due in large part to the action of the viral E1A proteins, which regulate transcription of cellular and viral genes. In general, the E1A proteins appear to act by way of specific protein-protein interactions with cellular regulatory factors (13, 34). In cases where these interactions have been identified, the regulatory factors targeted by E1A have been demonstrated to be important in cell cycle control (39) or in basic aspects of cellular transcription (7, 30, 32).

There are two major species of E1A protein expressed during adenovirus infection, consisting of 243 and 289 amino acid residues ($E1A_{243}$ and $E1A_{289}$, respectively). These proteins differ by an internal 46-amino-acid region present only in $E1A_{289}$ (16). The 46-amino-acid region is responsible for the ability of $E1A_{289}$ to interact physically with several transcription factors, including members of the ATF family, c-*jun*, Sp1, and USF (32). $E1A_{289}$ can also interact with the TATA-box binding protein and possesses an intrinsic transcriptional activation function encoded by the 46-amino-acid region (7, 19, 30–32). These findings support a model in which $E1A_{289}$ enters the transcription complex of certain promoters through specific protein-protein contacts, resulting in a stimulation of transcription.

Since $E1A_{243}$ and $E1A_{289}$ are both capable of inducing immortalization and transformation, it is clear that the 46-amino-acid region is dispensable for these activities (20, 33a, 49, 49a). However, $E1A_{243}$ can also regulate transcription, particularly of several cellular genes (15, 17, 26, 28, 35, 41, 44–46), and this regulation is thought to be important in the transformation process. Transformation by $E1A_{243}$ is mediated by conserved regions 1 and 2 (CR1 and CR2) as well as an N-terminal domain. Each of these domains contributes to transformation, since deletions or mutations within any of them result in loss of transformation activity (13, 34). It is becoming clear that these domains trigger several different effects on cellular transcriptional regulation.

One aspect of transcriptional regulation by $E1A_{243}$ involves the cellular transcription factor E2F. E2F is found in complexes with the retinoblastoma (Rb) tumor suppressor protein, and also the Rb-related proteins p107 and p130, along with members of the cyclin and cyclin-dependent kinase families (3–5, 8–12, 36, 43). These studies strongly suggest that E2F-containing complexes regulate cell growth-related transcriptional events, since the Rb and cyclin components are known to be important in controlling cellular proliferation (25, 47). E1A physically associates with Rb, p107, and p130, thereby dissociating E2F and altering its effects on cellular transcription (39). Indeed, binding sites for E2F are found upstream of several growth-regulatory genes (39).

A distinct transcriptional regulatory pathway triggered by $E1A_{243}$ involves the mouse c-*fos* proto-oncogene. Transcription of c-*fos* is stimulated in S49 cells by $E1A_{243}$, and this effect requires activation of cyclic AMP (cAMP)-dependent protein kinase (PK-A) by addition of analogs or inducers of cAMP (15, 37). Domains of $E1A_{243}$ required for transcriptional stimulation include CR1 and the N-terminal domain but not CR2 (17). Several deletion mutants of $E1A_{243}$ that abolish binding to Rb, p107, and p130 are fully capable of activating transcription of the c-*fos* gene (17). Therefore, regulation of c-*fos* transcription by $E1A_{243}$ involves a mechanism distinct from the Rb (p107, p130)/E2F pathway. Since the domains of $E1A_{243}$ involved in c-*fos* activation coincide with two of the domains required for transformation, these studies have suggested that the transcriptional mechanism involved is also important in the transformation process.

In an effort to understand the molecular basis for transcriptional activation of the c-*fos* gene by $E1A_{243}$ and to identify cellular regulators that might serve as its targets, we have analyzed the c-*fos* transcriptional control region for sequences conferring responsiveness to $E1A_{243}$. In this report, we demonstrate the existence of a novel E1A response element (ERE). The c-*fos* ERE contains two transcription factor binding sites, one for members of the ATF/CREB family of tran-

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scription factors and the other for transcription factor YY1. Both binding sites are required for function of the ERE. These data demonstrate that the N-terminal and CR1 domains of $E1A_{243}$ trigger activation of c-*fos* gene transcription through a functional interaction with ATF/CREB and YY1.

MATERIALS AND METHODS

Cells and viruses. S49 cells were obtained from the University of California at San Francisco Cell Culture Facility. They were grown in suspension in tissue culture dishes with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated horse serum. 293 cells were grown in DMEM supplemented with 5% calf serum. All media and sera were from GIBCO. Viruses *dl*343 (22) and *dl*520 (20) and mutant derivatives *dl*1101/520 through *dl*1108/520 (24) were constructed as previously described. All viruses were propagated in 293 cells to produce virus stocks.

Plasmids. The −356, −71, and −56 c-fosCAT plasmids and the −356 c-fosCAT mutants *pm*3, *pm*6.9, and *pm*3.6.9 were kind gifts of Michael Z. Gilman and were constructed as described previously (6). The −76/+10 construct was made by PCR amplification of plasmid −356 c-fosCAT DNA, using oligonucleotide primers RG2 (5′-ACTGAAGCTTTCCGCCCAGTGACGTA), which contained an artificial *Hin*dIII site, and RG3 (5′-CATGTCTAGACAGTCGCGGT TGGAGT), which contained an artificial *Xba*I site. The amplified fragments were digested with *Hin*dIII and *Xba*I, isolated by agarose gel electrophoresis, and ligated to the chloramphenicol acetyltransferase (CAT) vector fragment isolated from *Hin*dIII and *Xba*I-digested −56 c-fosCAT, and confirmed by sequencing.

Linker substitution mutants *pm*25, *pm*84, *pm*85, and *pm*87 were constructed in the −76/+10 c-fosCAT background by using the Altered Sites mutagenesis system (Promega) according to the manufacturer's protocol. All mutants contained the 10-nucleotide linker sequence 5′-TTCTCGAGTT-3′ (which includes an *Xho*I site) except *pm*25, in which the sequence AAAA was substituted for CCAT starting at position −54. The oligonucleotides used for the mutagenesis were as follows: *pm*25, GCGCTGTGAATGGTTTTACTTCCTACGTCACTG; *pm*84, GAAGCGCTGTGAATGAACTCGAGAACTACGTCACTGGGC; *pm*85, GCCTTTATAGAAGCAACTCGAGAAGATGGACTTCTTAC; and *pm*87, GGCGCCTCAGCTGGAACTCGAGAAGAAGCGCTGTGAAT. All mutations were confirmed by sequencing.

To make the −39/+10 c-*fos* promoter construct, mutant pm85 plasmid DNA was digested with *Hin*dIII and *Xho*I, which removed sequences upstream of the *Xho*I linker at −40. The *Hin*dIII and *Xho*I restriction site overhangs were then filled in with the Klenow fragment of *Escherichia coli* DNA polymerase as described previously (40). The blunt ends were ligated to recircularize the plasmid. The −39/+10 c-*fos* promoter was also cloned into the *Hin*dIII and *Xba*I sites of the pCAT Basic vector (Promega). The mutation was confirmed by sequencing.

The −33/+13 E1BCAT plasmid (a kind gift of David Spector) contained the −33/+13 region of the adenovirus E1B promoter inserted into the *Xba*I site of the pCAT Basic vector (Promega). In addition to the polycloning region, this construct contained a synthetic *Bgl*II site at the 5′ end of the −33/+13 fragment.

Multimerized c-*fos* sequences containing either five tandem copies of the sequence TGACGTAG (5× cAMP response element [CRE]), three tandem copies of the sequence CCCAGTGACGTAGGAAG (3× 17), or three tandem copies of the sequence CCCAGTGACGTAGGAAGTCCAT (3× 22) were synthesized as complementary oligonucleotides by the University of Virginia Biomolecular Research Facility. The ends of the oligonucleotides were synthesized such that once they were annealed, there would be a *Pst*I overhang at the 5′ end and a GATC overhang at the 3′ end. The complementary oligonucleotides were annealed and were then cloned upstream of the −33/+13 E1B promoter by digesting the E1BCAT plasmid with *Bgl*II and *Pst*I and ligating it to the c-*fos* oligonucleotides. Constructs were screened by restriction enzyme digestion and sequencing.

Transient transfections. Plasmid DNA was transfected into S49 cells with DEAE-dextran by a modification of the protocol described previously (2). Cells were grown to a density of 1×10^6 to 2×10^6 per ml and then pooled and counted with a hemacytometer. Cells were centrifuged and resuspended in STBS (25 mM Tris-Cl [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$) to a density of 10^8 cells per 20 ml; 10^8 cells were centrifuged, and the supernatant removed by aspiration. Cell pellets were then resuspended in 4 ml of STBS containing 0.2 mg of DEAE-dextran per ml plus 100 μg of the appropriate plasmid DNA and incubated at room temperature for 20 min. Dimethyl sulfoxide (400 μl) was added while swirling, and the cells were incubated for 1.5 min more at room temperature. The mixture was then diluted to 50 ml with STBS and centrifuged. The cells were washed once more with 50 ml of STBS prior to being plated out in 50 ml of DMEM supplemented with 10% horse serum. Cells were subsequently infected with the appropriate virus.

Virus infections and treatment with forskolin. At 22 to 24 h posttransfection, S49 cells were pooled and counted with a hemacytometer. Equal numbers of cells (in most cases 5×10^7 cells) were centrifuged and resuspended at a density of 5×10^6 cells per ml. Virus was added at the appropriate multiplicity (in PFU per cell) and incubated at 37°C for 1 h, with rocking. Infected cells were then diluted to 5×10^5 cells per ml with tissue culture medium and aliquoted into individual tissue culture dishes. Three hours prior to harvesting, cells were treated with forskolin (Sigma) at a final concentration of 10 μM. Cells were harvested 24 h postinfection.

CAT assays. Assays were performed essentially as described previously (2), with modifications. Cells (approximately 2.5×10^7 to 5×10^7) were resuspended in 50 μl of 250 mM Tris-Cl (pH 7.8) and then lysed by freezing (in liquid N_2) and thawing (at 37°C) three times. Lysates were incubated at 65°C for 10 min to inactivate endogenous acetylases, and then the debris was pelleted by centrifugation. All 50 μl of the lysate was assayed for CAT activity. Reactions were done in a volume of 100 μl and contained 50 μl of lysate plus 50 μl of a premix containing 200 mM Tris (pH 7.8), 0.5 mg of *n*-butyryl coenzyme A (Sigma) per ml, and 0.5 μCi of [^{14}C]chloramphenicol (Amersham). Reaction mixes were extracted with 300 μl of mixed xylenes to separate the modified (*n*-butyrylated) [^{14}C]chloramphenicol from the unmodified [^{14}C]chloramphenicol and then subjected to two back extractions with 100 μl of 250 mM Tris (pH 7.8). CAT activity was measured by liquid scintillation counting of 200 μl of the xylene extracts in 5 ml of Econofluor (NEN) scintillation fluid.

DNA band shift analysis of GST fusion proteins. The glutathione *S*-transferase (GST)-YY1 expression plasmid was a kind gift of Tom Shenk. GST fusion proteins were expressed in the *E. coli* DH5 and isolated as described previously (2). DNA band shift analysis was carried out essentially as described previously (21) except that the polyacrylamide gels were electrophoresed in 0.25× Tris-borate-EDTA buffer, without recirculation.

RESULTS

Effect of $E1A_{243}$ on a transfected *fos*-CAT reporter plasmid. It was important to establish a transfection system that mimicked our previously reported regulation of the endogenous c-*fos* gene by cAMP and E1A. S49 cells were transiently transfected with a reporter plasmid that contained sequences from −356 to +109 of the mouse c-*fos* gene, placed upstream of the bacterial CAT gene (−356 c-fosCAT) (6). Twenty-four hours later, the transfected cell population was divided, and the cells were either mock infected or infected with virus *dl*520, which expresses $E1A_{243}$. The infection was allowed to proceed for 24 h. Three hours prior to harvesting, the cultures were again divided and either treated with forskolin (an inducer of cAMP) or left untreated. The final concentration of forskolin was 10 μM. The cells were then harvested and assayed for CAT activity. As shown in Fig. 1B, mock-infected cells expressed a low level of CAT activity in the absence of forskolin and a significantly higher level upon forskolin treatment. Expression of $E1A_{243}$ (in *dl*520-infected cells) had no effect on CAT levels in the absence of forskolin. However, it had a strong effect in forskolin-treated cells, fourfold greater than occurred following forskolin treatment of mock-infected cells. This cAMP-dependent action of $E1A_{243}$ on transcription of the −356 fos CAT reporter was identical to that shown previously for the endogenous c-*fos* gene (15, 17).

The validity of the transfection system was probed further with viruses that produce mutant E1A proteins. These viruses were used previously to define domains of $E1A_{243}$ that are necessary for activation of endogenous c-*fos* transcription (17). Viruses *dl*1101/520, *dl*1104/520, *dl*1106/520, and *dl*1108/520 are derivatives of *dl*520 that produce $E1A_{243}$ proteins with in-frame deletions in various regions of the first exon (24), as depicted in Fig. 1A. Virus *dl*343 produces a nonfunctional protein, the result of an out-of-frame deletion in the first exon (22). The deletion in *dl*1101/520 defines an N-terminal domain that is necessary for activation of the endogenous c-*fos* gene as well as for induction of transformation (17, 27). Mutant *dl*1104/520 is representative of a set of mutants containing deletions in CR1, which is required for transformation and c-*fos* gene activation (17). The deletion in *dl*1106/520 lies outside all known functional domains of $E1A_{243}$. The *dl*1108/520 deletion is within CR2. This mutant behaves like wild-type virus with respect to transcriptional activation of the endogenous c-*fos* gene but is defective for transformation (17, 27).

These viruses were used to infect S49 cells that had been

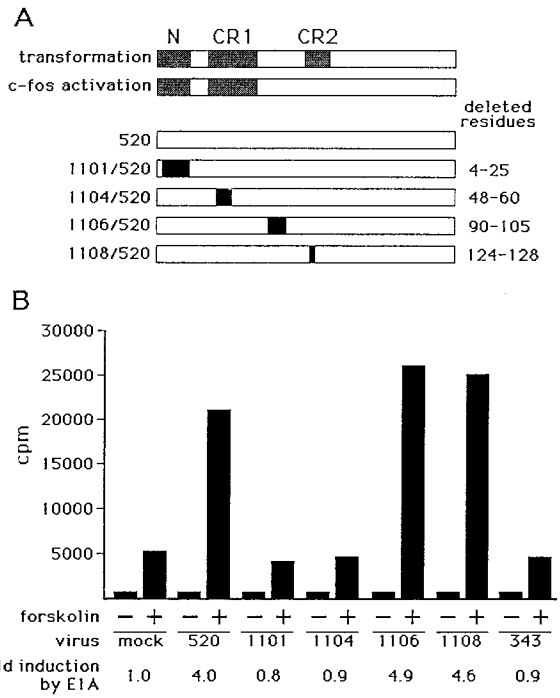

FIG. 1. Transcriptional activation of a transfected c-fosCAT reporter plasmid by E1A. S49 cells were transfected with −356 fosCAT (6) by the DEAE-dextran method. Twenty-four hours later, the cells were mock infected or infected with the indicated viruses and harvested after an additional 24 h, following a 3-h exposure to 10 μM forskolin. CAT activity was determined by the CAT liquid scintillation assay as described in Materials and Methods. (A) Functional domains of $E1A_{243}$ and structures of E1A proteins produced by the indicated mutant viruses. Gray regions represent domains required for transformation and c-*fos* transcriptional activation (17, 27, 48). Black regions represent deleted amino acid residues. N, N-terminal domain. (B) Induction of CAT activity by wild-type and mutant E1A proteins. Background of the assay (approximately 150 cpm) was determined from extracts of untransfected cells and subtracted from the data to produce the values shown. Fold induction by E1A was calculated as CAT activity (in counts per minute) from infected, forskolin-treated cells divided by CAT activity from forskolin-treated, mock-infected cells. The cells used for mock infection and infection were derived from a single original transfected cell population. In panel B, mutant viruses are indicated without the "520" designation.

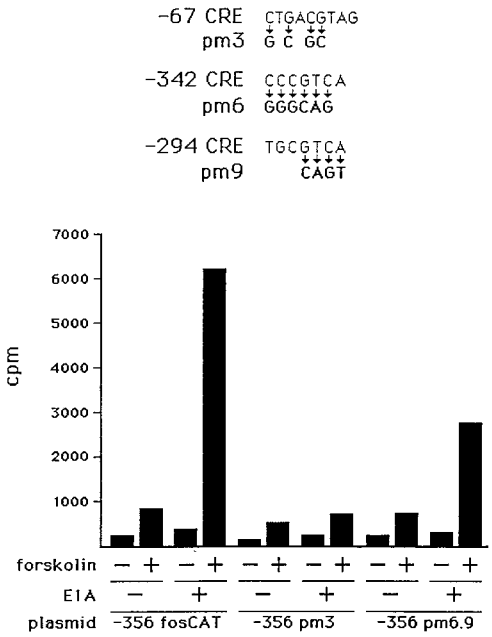

FIG. 2. Effect of c-*fos* CRE mutations on transcriptional activation by E1A. Cells were transfected with the indicated CAT reporter plasmids (6) and either mock infected or infected with *dl*520 to express $E1A_{243}$, indicated as − or + E1A, respectively. The cells were treated with 10 μM forskolin for 3 h and assayed for CAT activity as described in the legend to Fig. 1. For each plasmid, a single population of cells was transfected and subsequently divided for the infection and forskolin treatment steps. Sequences and nucleotide positions of the three c-*fos* CREs are shown at the top. Below each sequence in boldface are the altered nucleotides in the mutants tested. Data presented are representative of four independent experiments. Average fold inductions by $E1A_{243}$ for each mutant are noted in the text.

transfected with the −356 fosCAT reporter. The cells were treated with forskolin for 3 h prior to harvesting and assayed for CAT activity. As shown in Fig. 1B, forskolin treatment of cells infected with either *dl*1106/520 or *dl*1108/520 resulted in levels of CAT activity similar to those of forskolin-treated, wild-type (*dl*520)-infected cells. However, cells infected with viruses *dl*1101/520, *dl*1104/520, and *dl*343 failed to stimulate forskolin-dependent CAT activity above that in mock-infected cells. Equivalent results were obtained when the cells were treated with the cAMP analog dibutyryl cAMP (data not shown). These results are identical to those reported earlier for the endogenous gene (17). Taken together, they demonstrate that the transfected −356 fosCAT plasmid was transcriptionally activated by the synergistic action of cAMP and $E1A_{243}$ and that domains of $E1A_{243}$ required for this activation (the N terminus and CR1) were identical to those required for activation of the endogenous gene.

The CRE at −67 is required for transcriptional activation by $E1A_{243}$. The transcriptional control region of the c-*fos* gene contains binding sites for a number of cellular transcription factors, including three functional CREs between −356 and the +1 start site of transcription (6). The CREs are located at positions −342, −294, and −67 (6). Since activation of c-*fos* transcription by $E1A_{243}$ requires cAMP, it seemed possible that one or more of these elements would be necessary for responsiveness. To test this, cells were transfected with the wild-type −356 fosCAT reporter or −356 fosCAT plasmids containing specific CRE mutations (6). The cells were then infected with virus *dl*520 to express $E1A_{243}$, treated with forskolin, and harvested for CAT assays as described above. The sequences of each of the CREs and the altered nucleotides are shown in Fig. 2 along with the results of the CAT assays.

The *pm*3 construct (6) contains a mutant −67 CRE but normal −342 and −294 sequences. Transcription from this plasmid was induced by forskolin in mock and *dl*520-infected cells, but there was little or no effect of $E1A_{243}$, even in the presence of forskolin. The average fold induction of *pm*3 by $E1A_{243}$ was 1.3 ± 0.3. This demonstrated that the −67 CRE was necessary for $E1A_{243}$-dependent transcriptional activation. The cAMP inducibility of *pm*3 was apparently due to the presence of the upstream −342 and −294 CRE sequences, but these sequences were not capable of mediating a response to $E1A_{243}$ in *pm*3. In contrast, the double mutant *pm*6.9 (6), which contains mutations in the −342 and −294 CREs but has a normal −67 CRE, was clearly activated by $E1A_{243}$ in a forskolin-dependent manner. The average fold induction of *pm*6.9 by $E1A_{243}$ was 4.8 ± 0.8. This result showed that the −342 and −294 CREs were not necessary for transcriptional activation by $E1A_{243}$. Mutant *pm*3.6.9 (6) contains mutations in all three CREs. Transcription from this construct was induced poorly by forskolin, and there was no effect of $E1A_{243}$ on transcription (not shown).

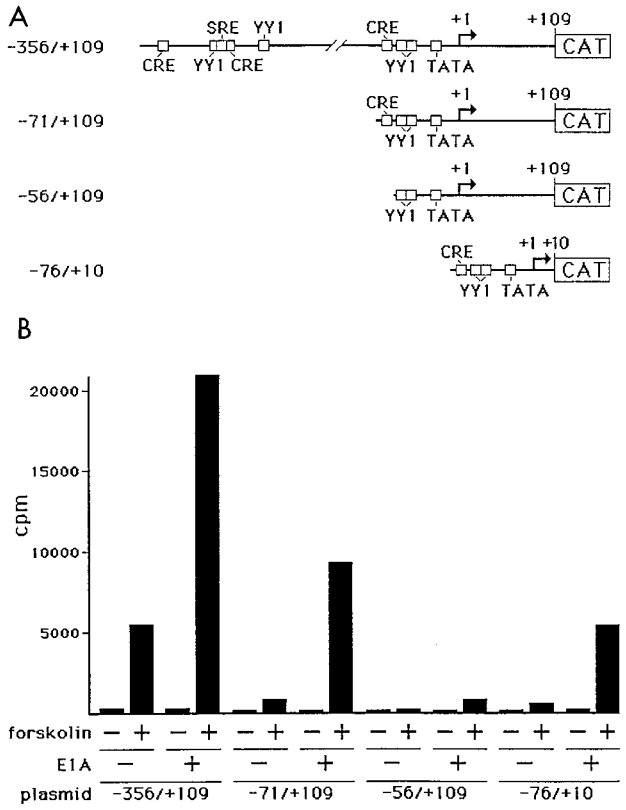

FIG. 3. Deletion analysis of the c-*fos* transcriptional control region. (A) Structures of CAT reporter plasmids. Shown are the positions of several known regulatory elements in the −356 to +1 region (6, 23, 38). SRE, serum response element; YY1, binding site for YY1; TATA, binding site for TATA-box-binding protein. (B) Cells were transfected with the indicated CAT reporter plasmids and either mock infected or infected with *dl*520 to express $E1A_{243}$, indicated as − or + E1A, respectively. The cells were treated with 10 μM forskolin for 3 h and assayed for CAT activity as described in the legend to Fig. 1. For each plasmid, a single population of cells was transfected and subsequently divided for the infection and forskolin treatment steps. Data presented are representative of three independent experiments. Average fold inductions by $E1A_{243}$ for each mutant are noted in the text.

Two points can be made from these experiments. First, the −67 CRE is unique in that it is the only CRE in −356 fosCAT that was absolutely necessary for the transcriptional response to $E1A_{243}$. Second, the fact that the *pm*3 construct was noticeably inducible by forskolin alone demonstrates that cAMP responsiveness per se is not sufficient to confer E1A responsiveness.

E1A-responsive sequences lie within −71 and +10 of the c-*fos* promoter. 5′ and 3′ deletion analysis was applied to the −356 to +109 sequences of −356 fosCAT. The structures of the deletion mutants used are shown in Fig. 3. These plasmids were tested for responsiveness to $E1A_{243}$ according to the transfection protocol described above. As shown in Fig. 3, the −71/+109 (6) and −76/+10 constructs responded similarly to the wild-type −356/+109 construct (same construct as −356 c-fosCAT described above), although the overall levels of forskolin-dependent and $E1A_{243}$-dependent transcription were lower. The average fold inductions for the −356/+109, −71/+109, and −76/+10 constructs were 5.1 ± 1.5, 7.6 ± 0.7, and 4.5 ± 0.5, respectively. This result indicated that sequences between −71 and +10 were sufficient for conferring a response to $E1A_{243}$, although other sequences outside this region may serve to increase the response.

Sequences between −56 and +109 were poorly transcribed; nevertheless, there was a slight but reproducible increase in transcription in forskolin-treated cells expressing $E1A_{243}$. Because of the low overall level of transcription of the −56/+109 construct, it was not possible to calculate an accurate fold induction by E1A, however. The −56 to +109 region contains the TATA box, which in the case of the *hsp70* gene has been shown to mediate a transcriptional response to $E1A_{243}$ (29). Consistent with this observation, we found that a CAT reporter containing only the −39 to +10 region of c-*fos* functioned independently as a weak ERE (not shown). However, data presented in Fig. 2 demonstrated a lack of E1A responsiveness of mutant *pm*3, despite the presence of an intact −39 to +10 region. Therefore, in the context of the complete c-*fos* promoter (in *pm*3), the contribution of the −39 to +10 region appears insignificant.

Maximal effect of $E1A_{243}$ requires the −54 YY1 binding site. The data presented in Fig. 2 demonstrated that the −67 CRE was necessary for the response to $E1A_{243}$ and that the −342 and −294 CREs were not. There are several possible reasons for the specificity of function of the −67 CRE with respect to the action of $E1A_{243}$. The distance from the transcription start site, the exact sequence of the −67 CRE itself, and/or the sequence context of the −67 CRE might all contribute to its ability to mediate a response to $E1A_{243}$. Interestingly, immediately downstream of the −67 CRE are located two closely spaced binding sites for transcription factor YY1 (42) at −54 and −50 (38). YY1 has been implicated in transcriptional activation of the adeno-associated virus (AAV) P5 promoter by E1A (42). Therefore, it seemed possible that the presence of binding sites for YY1 adjacent to the −67 CRE might form the basis for a functional ERE. Furthermore, our laboratory has recently demonstrated that members of the ATF/CREB family of transcription factors, which recognize the −67 CRE, can physically interact with YY1 (50). Given these considerations, the roles of the −54 and −50 YY1 sites in cAMP-dependent E1A responsiveness of the c-*fos* promoter were tested.

Three deletion/insertion mutants were constructed in the context of the −76/+10 fosCAT plasmid, whose structures are shown in Fig. 4A. Mutants *pm*84 and *pm*85 carried exact 10-bp linker substitutions containing an *Xho*I site. These mutations disrupted the core YY1 sites as well as some flanking sequences. A third mutant, *pm*25, changed the CRE-proximal YY1 core CCAT to AAAA. Transfection assays were performed with these mutants as well as the parental −76/+10 fosCAT construct. As shown in Fig. 4B, removal of the CRE-proximal (−54) YY1 site in *pm*25 resulted in an increase in forskolin-dependent transcription in the absence of $E1A_{243}$. The effect of forskolin on this mutant was 2.2-fold greater than on the wild-type −76/+10 reporter, indicating that the CRE-proximal YY1 site has a negative effect on cAMP-dependent transcription. The *pm*84 mutant, which also disrupted the CRE-proximal YY1 site, had an identical effect on forskolin-dependent transcription in the absence of $E1A_{243}$ (not shown). These data are totally consistent with earlier work of Natesan and Gilman that demonstrated that the CRE-proximal YY1 site negatively regulates basal transcription of the mouse c-*fos* promoter in transfected HeLa cells (38). The mutation in *pm*85, which altered the CRE-distal YY1 site, had no effect on forskolin-dependent transcription (not shown). This result is also in line with those of Natesan and Gilman, who showed that basal transcription of a similar fosCAT reporter was unaffected by mutation of the CRE-distal YY1 site in HeLa cells (38).

The absolute levels of CAT activity produced in *dl*520-in-

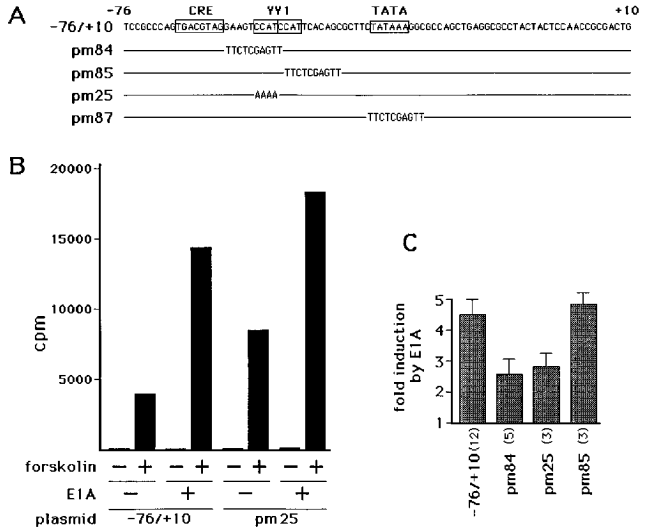

FIG. 4. Effect of YY1 site mutations on transcriptional activation by E1A. (A) Structures of CAT reporter plasmids. The nucleotide sequence of the entire wild-type −76 to +10 region is shown, along with locations of the −67 CRE, YY1 sites, and TATA element. Solid lines indicate regions of identity with the wild type. Black boxes represent positions of a 10-bp linker substitution, described in Materials and Methods. (B) Cells were transfected with the indicated CAT reporter plasmids and either mock infected or infected with *dl*520 to express $E1A_{243}$, indicated as − or + E1A, respectively. The cells were treated with 10 μM forskolin for 3 h and assayed for CAT activity as described in the legend to Fig. 1. For each plasmid, a single population of cells was transfected and subsequently divided for the infection and forskolin treatment steps. Data are representative of multiple experiments, as further presented in panel C. (C) Same experimental protocol as for panel B. The data are presented as fold induction by E1A, calculated as CAT activity (in counts per minute) from infected, forskolin-treated cells divided by CAT activity from forskolin-treated, mock-infected cells. Numbers in parentheses indicate number of experiments performed.

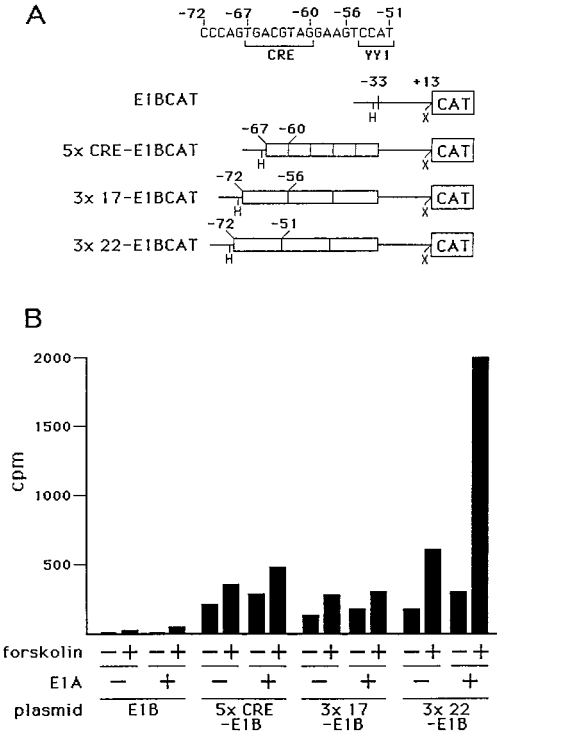

FIG. 5. Identification of a c-*fos* E1A response element. (A) Structures of CAT reporter plasmids. At the top is shown the −72 to −51 sequence, with the locations of the CRE and YY1 core binding site. Also indicated are endpoints of the CRE and 17- and 22-nucleotide elements described in the text. The minimal E1B promoter contains a TATA element that is not responsive to $E1A_{243}$. Boxes represent reiterations of the CRE and 17- and 22-nucleotide elements. H, *Hin*dIII; X, *Xba*I. (B) Cells were transfected with the indicated CAT reporter plasmids. Twenty-four hours later, they were mock infected or infected with *dl*520 to express $E1A_{243}$, indicated as − or + E1A, respectively. The cells were harvested after an additional 24 h, following a 3-h exposure to 10 μM forskolin, and assayed for CAT activity. For each plasmid, a single population of cells was transfected and subsequently divided for the infection and forskolin treatment steps. Data presented are representative of four independent experiments. Average fold inductions by $E1A_{243}$ for each mutant are noted in the text.

fected, forskolin-treated cells also increased for the *pm*84 and *pm*25 constructs compared with the control −76/+10 construct. However, the fold increase by $E1A_{243}$ over the stimulation produced by forskolin alone was reproducibly less for *pm*84 and *pm*25 than for the wild-type construct. These data are presented in Fig. 4B for *pm*25, and further experiments demonstrating this point are presented in Fig. 4C. They show a reproducible 40% decrease in responsiveness to $E1A_{243}$ in mutants lacking an intact −54 YY1 binding site. Disruption of the CRE-distal YY1 site (in *pm*85) had no effect on $E1A_{243}$-inducible transcription.

We constructed a fourth deletion/insertion mutant, *pm*87, that abolished the c-*fos* TATA element located at −34. Its structure is also shown in Fig. 4A. This construct was transcriptionally inactive under all conditions of forskolin treatment and infection (not shown). This is not surprising, given the role of the TATA element in formation of the transcription initiation complex. This result showed, however, that virtually all of the transcription observed from the −76/+10 fosCAT construct was directed by the TATA element and therefore represented properly initiated transcripts.

A 22-nucleotide sequence containing the −67 CRE and CRE-proximal YY1 site is sufficient to confer $E1A_{243}$ responsiveness to a heterologous minimal promoter. Given the involvement of the −67 CRE and the CRE-proximal YY1 site in cAMP-dependent transcriptional activation by $E1A_{243}$, it became important to determine if these elements, either alone or in combination, could confer E1A responsiveness to an otherwise unresponsive minimal promoter. Three artificial reporter plasmids were constructed; their structures are shown in Fig. 5A. One contained five tandem copies of the 8-nucleotide −67 CRE (−67 to −60) placed upstream of the minimal adenovirus E1B promoter linked to the CAT gene (5× CRE-E1BCAT). A second construct contained three copies of a 17-nucleotide sequence from c-*fos* (−72 to −56), including the −67 CRE and some flanking sequences but not including the CRE-proximal, −54 YY1 site (3× 17-E1BCAT). The third construct was composed of three copies of a 22-nucleotide sequence (−72 to −51) that was identical to the 17-nucleotide sequence at its 5′ end but extended 5 nucleotides in the 3′ direction to include the −54 YY1 site (3× 22-E1BCAT).

Transfection assays of these plasmids were conducted as before, and the results are presented in Fig. 5B. Transcription from the control E1BCAT reporter was not significantly affected by either forskolin, $E1A_{243}$, or the combination of the two. Transcription from 5× CRE-E1BCAT and 3× 17-E1BCAT was detectable in the absence of forskolin, and there was a slight induction upon treatment with forskolin. However, there was little or no induction by the combination of $E1A_{243}$ and forskolin. This result indicated that neither the −67 CRE itself nor the 17-nucleotide sequence containing the −67 CRE was sufficient to confer E1A responsiveness outside the context of other sequences in the c-*fos* transcriptional control region.

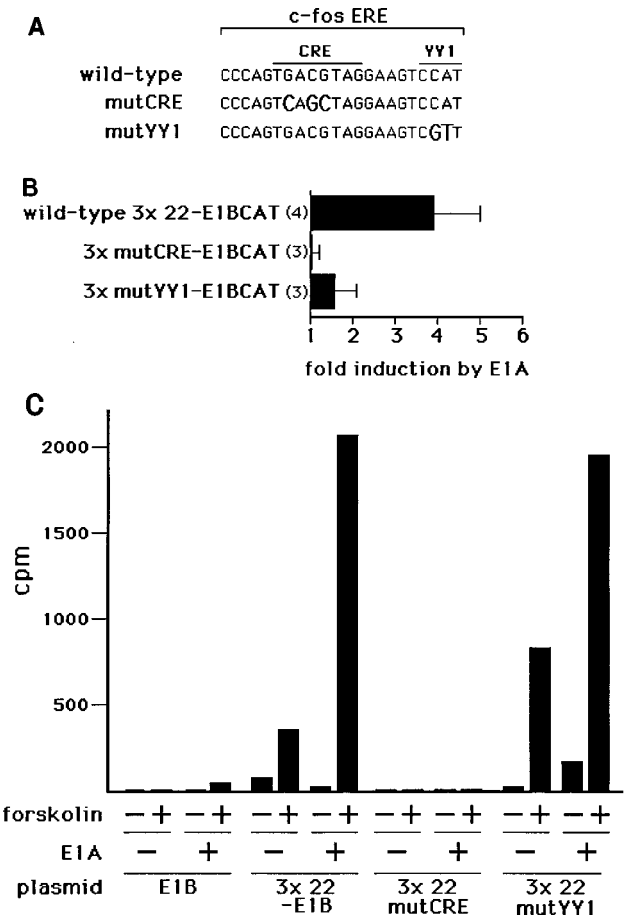

FIG. 6. Effect of CRE and YY1 mutations on function of the c-*fos* ERE. (A) Three tandem copies of the depicted wild-type and mutant c-*fos* EREs were inserted into a CAT expression vector containing the minimal −33 to +13 E1B promoter as described in Materials and Methods. (B) Cells were transfected with the indicated CAT reporter plasmids. Twenty-four hours later, they were mock infected or infected with *dl*520 to express $E1A_{243}$. The cells were harvested after an additional 24 h, following a 3-h exposure to 10 μM forskolin, and assayed for CAT activity. For each plasmid, a single population of cells was transfected and subsequently divided for the infection and forskolin treatment steps. The data are presented as fold induction by E1A, calculated as CAT activity (in counts per minute) from infected, forskolin-treated cells divided by CAT activity from mock-infected, forskolin-treated cells. Data presented are an average of at least three independent experiments (number of experiments indicated in parentheses). (C) A representative experiment, performed as described above.

In contrast, the 3× 22-E1BCAT construct, containing the −67 CRE and CRE-proximal YY1 site, was clearly induced by the combination of $E1A_{243}$ and forskolin, despite a lack of response to $E1A_{243}$ alone. The average fold induction was 3.7 ± 0.9. To confirm this result, we constructed an additional plasmid that contained a single copy of the 22-nucleotide sequence placed upstream of the minimal E1B promoter. This construct was also induced by E1A in a forskolin-dependent manner, demonstrating that the E1A responsiveness of 3× 22-E1BCAT was not a function of multimerization of the element (not shown). These data clearly demonstrate that the −72 to −51 sequence of the mouse c-*fos* transcriptional control region can confer responsiveness to $E1A_{243}$. This sequence therefore constitutes a c-*fos* ERE.

The roles of the −67 CRE and the YY1 site in the context of the ERE were explored by introduction of mutations in these sites. Figure 6 shows the sequences of the wild-type and mutant EREs. The mutCRE sequence contains three nucleotide changes in the core of the CRE (TGACG to TCAGC), including a C-to-G change at position −64 that has been shown to abolish CREB binding in vitro (33). The mutYY1 sequence contains a mutated YY1 site core sequence (CCAT to CGTT), which abolishes YY1 binding in vitro (38). For each reporter plasmid, the ERE (mutant or wild type) was present in three tandem copies immediately upstream of the minimal E1B promoter linked to the CAT gene. The results of transfection assays with these reporter constructs are also shown in Fig. 6. The CRE mutation resulted in a complete loss of responsiveness to $E1A_{243}$, and mutation of the YY1 site reproducibly resulted in a greater than 50% reduction of the response. Therefore, both sites were necessary for conferring the response to $E1A_{243}$. We have noted that in the context of the 3× 22 ERE, mutation of the YY1 site (in 3× 22 mutYY1) did not result in complete loss of responsiveness to $E1A_{243}$. This is in apparent contradiction to the results presented in Fig. 5, which show that the 3× 17 construct gave no response to E1A even though it only differed from the wild-type 3× 22 construct by the lack of the YY1 site. The most likely explanation for this difference is that the nucleotide spacing of the 3× 17 construct with respect to the E1B TATA element differs by five nucleotides from that of the 3× 22 construct, and this may result in a less responsive promoter. Consistent with our data with the 3× 22 mutYY1 construct are the data shown in Fig. 4, which show that linker disruption of the −54 YY1 site in the context of the −76/+10 promoter had the same effect on E1A responsiveness (about 50%) as in the 3× 22 mutYY1 construct.

Since extension of the 17-bp sequence to include the −54 YY1 site (in 3× 22-E1BCAT) resulted in responsiveness to $E1A_{243}$, we wanted to determine if this in fact correlated with the ability to bind YY1. Therefore, specific DNA fragments isolated from the plasmids shown in Fig. 5A were tested for the ability to bind YY1 in vitro. Each plasmid was digested with *Hin*dIII and *Xba*I to liberate a fragment containing the minimal E1B promoter and the inserted upstream sequences (see Fig. 5A for positions of the *Hin*dIII and *Xba*I sites). In addition, a fragment from the wild-type −76/+10 plasmid encompassing the entire c-*fos* promoter region was isolated. The DNA fragments were end labeled with [^{32}P]dCTP and Klenow polymerase and added to a DNA band shift assay containing bacterially expressed, affinity-purified GST-YY1. The results of the assay are shown in Fig. 7. As expected, GST-YY1 (but not the GST-alone control) bound efficiently to the wild-type −76/+10 fragment as well as to the fragment from 3× 22-E1BCAT. There was little or no binding to the minimal E1B fragment or to the fragments from 5× CRE-E1BCAT or 3× 17-E1BCAT. Fragments were also isolated from the 3× mut YY1-E1BCAT and 3× mutCRE-E1BCAT plasmids. As expected, GST-YY1 bound to the 3× 22 mutCRE fragment but not the 3× 22 mutYY1 fragment (not shown). These data demonstrate that the 22-nucleotide ERE can indeed bind to YY1 and that the ability to bind YY1 correlates with the ability of this sequence to confer a response to $E1A_{243}$ in transfection assays.

DISCUSSION

In this report, we have identified a novel ERE in the transcriptional control region of the c-*fos* gene. The c-fos ERE contains a CRE and a YY1 binding site, and each of these sites is required for function of the ERE. Binding of ATF/CREB and YY1 to these sites in the c-*fos* promoter has previously been documented (38), and we have also observed such binding in DNA band shift assays of S49 cell nuclear extracts (not

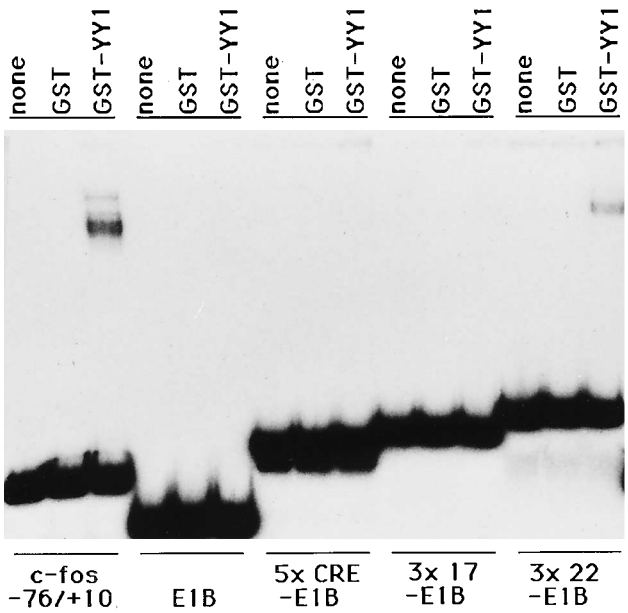

FIG. 7. Binding of GST-YY1 to c-*fos* E1B hybrid promoters in vitro. DNA band shift assays were performed with the indicated promoter fragments as ^{32}P-labeled probes. The fragments were isolated following *Hin*dIII and *Xba*I digestion of the plasmids shown in Fig. 5. The −76/+10 fragment was isolated from the −76/+10 c-fosCAT plasmid (shown in Fig. 3) , also by digestion with *Hin*dIII and *Xba*I. Each fragment was incubated with either GST-YY1, GST, or no protein, and the products of the binding reactions analyzed as described in Materials and Methods.

shown). Therefore, ATF/CREB and YY1 are likely the targets (direct or indirect) of $E1A_{243}$ in this system. Transcription factor AP-1 is also capable of binding to some CRE sequences (37). However, prior to transcriptional activation of the c-*fos* gene, which encodes a component of the AP-1 complex, S49 cells possess no detectable AP-1 DNA binding activity (37). Therefore, preexisting ATF/CREB factors, along with YY1, are probably mediating the effects of $E1A_{243}$.

In other studies in our laboratory, we have found that YY1 interacts directly with several members of the ATF/CREB family of transcription factors (CREB, ATF-2, ATFa1, ATFa2, and ATFa3) and that this interaction leads to repression of CRE-dependent transcription of the c-*fos* promoter in the absence of E1A (50). Also, $E1A_{243}$ can bind directly to YY1 in in vitro assays (18, 42). This finding strongly suggests that YY1 is a direct target of $E1A_{243}$ in the activation of c-*fos* transcription. Since YY1 normally represses CRE-dependent transcription of the c-*fos* promoter, one attractive hypothesis is that E1A acts to relieve YY1-mediated repression by binding to YY1. Such relief of repression could take place by altering the activity of YY1 or by displacing it from the promoter. Under this hypothesis, ATF/CREB need not be a direct target of $E1A_{243}$. Rather, the activity of ATF/CREB might increase simply due to a reversal of the normal effects of YY1. Alternatively, or in addition, $E1A_{243}$ may become bound to the promoter through its interaction with YY1, thereby allowing it to directly stimulate or facilitate transcription. Another possibility is that in the absence of binding sites for YY1, transcription of the c-*fos* promoter approaches a maximal level due to limiting amounts of a factor(s) whose function is unrelated to the function of E1A. This would result in a blunted response to E1A. Given the known interactions between E1A and YY1, however, this possibility seems unlikely.

Several members of the ATF/CREB family have been shown to interact with $E1A_{289}$ but not with $E1A_{243}$ (32). However, ATFa2 has been shown to interact with both $E1A_{289}$ and $E1A_{243}$ in vitro (8a). It remains to be determined if any members of the ATF/CREB family are direct targets of $E1A_{243}$ at the c-*fos* promoter. One might speculate that $E1A_{289}$ would behave somewhat differently than $E1A_{243}$ in this system, since $E1A_{289}$ is known to interact directly with both ATF factors and with YY1, whereas $E1A_{243}$ may only interact with YY1. Accordingly, we have found that $E1A_{289}$ is a more potent inducer of the c-*fos* promoter in transfection experiments (18). It will be interesting to determine if $E1A_{289}$ behaves similarly to $E1A_{243}$ with respect to the c-*fos* ERE identified here.

At the AAV P5 promoter, one of the effects of E1A involves relief of YY1-mediated repression (42). However, in that case, repression probably does not involve ATF/CREB, since there are no ATF/CREB binding sites in the AAV P5 promoter. Therefore the effects of E1A on the AAV P5 promoter and the c-*fos* promoter are probably somehow different.

Another protein that may be involved in $E1A_{243}$-dependent activation of c-*fos* is p300 (14, 48) and/or its relative CREB-binding protein (CBP) (1). We have shown previously that regions of $E1A_{243}$ that are required for activation of the c-*fos* gene are the same as, or significantly overlap with, regions involved in binding to p300 (17). Specific regions of homology between CBP and p300 have led to the prediction that CBP can bind $E1A_{243}$ and that p300 can bind CREB (1). Our identification of a CRE within the c-*fos* ERE provides further reason to speculate that CBP and/or p300 are involved in E1A-dependent transcriptional activation.

Our data indicate that the c-*fos* TATA element functions as an E1A response element only when most of the upstream sequences have been removed. This was the case for the −56/+109 and −39/+10 constructs. The weak response of these constructs to E1A suggests that the effects of E1A in the context of the complete promoter might be masked by the higher levels of basal and/or cAMP-dependent transcription that are achieved in the complete promoter. Further experiments will be required to determine how the function of the c-*fos* TATA element relates to the function of the E1A-inducible *hsp70* TATA element. For instance, it will be interesting to determine if, like the *hsp70* promoter, function of the c-*fos* TATA element as an ERE depends on a specific TATA sequence (29).

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