MOLECULAR AND CELLULAR BIOLOGY, Oct. 1989, p. 4272–4281
0270-7306/89/104272-10$02.00/0

Vol. 9, No. 10

Multiple Sequence Elements of a Single Functional Class Are Required for Cyclic AMP Responsiveness of the Mouse c-*fos* Promoter

LAURA A. BERKOWITZ, KARL T. RIABOWOL, AND MICHAEL Z. GILMAN*
Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

Received 10 April 1989/Accepted 27 June 1989

Agents that elevate the intracellular concentration of cyclic AMP (cAMP) rapidly and transiently induce expression of the c-*fos* proto-oncogene in BALB/c 3T3 cells. We show that the mouse c-*fos* promoter-enhancer region contains multiple elements that contribute to cAMP responsiveness of the promoter in transient expression assays. The most potent element was found to correspond to a previously mapped basal promoter element and protein-binding site located 65 base pairs upstream of the transcriptional initiation site. This element and two less potent sites contained a match to the cAMP response element (CRE) core sequence defined in several mammalian genes. The relative potencies of these elements corresponded with their relative affinities for cellular factors that bound to the CRE in vitro. Mutation of all three elements failed to abolish completely cAMP responsiveness of the c-*fos* promoter in the transient expression assay. However, we present evidence that this residual responsiveness may have been due to sequences present in vector DNA. Finally, we show, by using a new microinjection competition assay, that a double-stranded oligonucleotide carrying the major c-*fos* CRE is sufficient to block induction of the endogenous c-*fos* gene by cAMP. Therefore, induction of the endogenous gene requires positively acting cellular factors that interact with a single functional class of regulatory sites in the c-*fos* gene. Unrelated regulatory elements, such as the serum response element and putative AP-2 sites, are not by themselves sufficient to mediate the cAMP response.

Transcription of the c-*fos* proto-oncogene is rapidly induced by a variety of growth factors and other extracellular stimuli (for a review, see reference 5). Induction is independent of new protein synthesis, suggesting that it is a direct consequence of signals triggered by activated growth factor receptors. Previous studies have shown that induction of c-*fos* transcription by serum growth factors requires a DNA sequence element, the serum response element (SRE), located 300 base pairs (bp) upstream of the transcriptional start site (14, 16, 47, 48). The SRE appears to be the target for at least two distinct signal transduction pathways triggered by growth factors, one dependent on protein kinase C (2, 9, 12, 43, 46) and one or more independent of this kinase (12, 46). In contrast, induction of c-*fos* transcription by agents that elevate intracellular concentrations of cyclic AMP (cAMP) or calcium is independent of the SRE (2, 12, 42, 43), suggesting that there are multiple independent signaling pathways that lead to the induction of c-*fos* transcription by extracellular signals.

cAMP is an important intracellular second messenger in many tissues. Elevation of intracellular cAMP levels induces c-*fos* expression in PC12 pheochromocytoma cells (15, 25), fibroblasts (12), and macrophages (1), although in macrophages induction appears to be stable rather than transient. cAMP induces expression of many other genes, including several genes encoding neuropeptides (for a review, see reference 40). Analysis of these genes has led to the identification of a consensus cAMP response element (CRE) containing one or more copies of the core sequence TGACG (4, 7, 8, 24, 31, 43, 44, 45, 49). An unrelated sequence element, a binding site for the transcription factor AP-2, has also been shown to be cAMP responsive in vivo (22).

Sequences similar to each of these elements are present in the c-*fos* promoter-enhancer region. In this study, we have asked which elements in the murine c-*fos* promoter-enhancer are required for response to cAMP. We find that multiple elements carrying CRE core sequences contribute to the induction of c-*fos* by cAMP. The major CRE is a sequence located 65 bp upstream of the cap site that was previously identified as a basal promoter element and protein-binding site (9, 14). Furthermore, titration of cellular CRE-binding factors by microinjection of oligonucleotides carrying this sequence abolishes detectable induction of the endogenous c-*fos* gene by cAMP. This finding suggests that no unrelated elements, such as AP-2 sites, in the c-*fos* gene are able by themselves to activate c-*fos* expression in response to cAMP.

MATERIALS AND METHODS

Plasmid construction and mutagenesis. The constructions used were based on c-*fos*–chloramphenicol acetyltransferase (CAT) fusion plasmids previously described (12, 14). All fusions were transferred to the vector pBS M13-(Stratagene Cloning Systems). Site-directed mutagenesis was performed as described by Kunkel (26). Single-stranded bacteriophage DNA was prepared from *Escherichia coli* CJ236, and the products of the in vitro mutagenesis reaction was transformed into *E. coli* TG-2. Mutants were identified by DNA sequence analysis.

To construct the −71/coreA plasmids, plasmid pβ6X coreA/coreA (32; a gift from Brian Ondek) was digested with *Pst*I and *Hin*dIII, and the fragment carrying the six coreA/coreA repeats was cloned into pBS M13+. The same fragment was excised from this plasmid with *Hin*dIII and *Sal*I and cloned between the *Hin*dIII and *Sal*I sites immediately upstream of the −71 c-*fos*–CAT fusion gene.

Transient expression assay. BALB/c 3T3 cells were grown in Dulbecco modified essential medium containing 10% calf

* Corresponding author.

serum. Cells were split at a density of 1.5×10^6 cells per 100-mm-diameter dish 18 to 24 h before transfection. Transfections were performed as described previously (12). After 48 h of serum starvation, transfected cells were washed once with warm phosphate-buffered saline and refed with 3 ml of Dulbecco modified essential medium containing no additions, 10% calf serum, or a combination of 0.5 mM isobutylmethylxanthine (IBMX) and 10 μM forskolin. Stimulation was for 45 min except for the experiment shown in Fig. 2, in which stimulation was for 60 min.

Total cytoplasmic RNA was isolated and analyzed by RNase protection as described previously (12).

Mobility shift assay. The mobility shift probe was prepared by digesting 10 μg of the −71 c-*fos*–CAT fusion plasmid with *Hin*dIII and *Hae*II. The digest was treated with Klenow fragment in the presence of 25 μCi each of four α-^{32}P-labeled deoxynucleoside triphosphates for 30 min at room temperature. The 51-bp fragment carrying c-*fos* sequences from −71 to −40 plus 19 bp of polylinker DNA at the 5′ end was excised from a polyacrylamide gel and eluted into buffer containing 10 mM Tris (pH 7.5) and 1 mM EDTA. The eluate was used directly in the mobility shift assays without any further purification.

Binding assays were performed in 10-μl reaction mixtures containing 20,000 dpm of probe, 10 mM Tris (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl, 1 mM spermidine, 5% (vol/vol) glycerol, 400 ng of poly(dI-dC) · (dI-dC), and 5 μg of whole-cell extract protein prepared from H9 cells as described previously (11). All components except probe and specific competitors were assembled on ice and preincubated for 10 min at room temperature. The probe and any specific competitors were then added, and the reaction mixture was incubated for an additional 15 min. The entire reaction mixture was loaded onto a 4% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29:1) and electrophoresed at 125 V in a buffer containing 50 mM Tris, 0.38 M glycine, and 1 mM EDTA (pH 8.5).

Oligonucleotide microinjections. Complementary single-stranded oligonucleotides were annealed in buffer containing 50 mM NaCl, 1 mM $MgCl_2$, and 10 mM Tris (pH 7.5) at a concentration of 10 mg/ml. Annealed oligonucleotides were then centrifuged for 20 min at 13,000 × *g*, divided into equal portions, and stored at −20°C. Before use, oligonucleotides were quantitated by UV absorbance, diluted to the desired concentration in half-strength phosphate-buffered saline (pH 7.4), centrifuged at 13,000 × *g* for 10 min, and then used for microinjection. Needle microinjection into the cytoplasm of fibroblasts was done as described previously (39), using a 0.1-mg/ml concentration of oligonucleotide. Cell fixation, the antibodies used in fluorescence analysis, and the conditions of staining and photography have been previously outlined (39).

RESULTS

cAMP rapidly and transiently induces c-*fos* expression in murine fibroblasts. Our studies of c-*fos* regulation have relied on transient expression assays performed in BALB/c 3T3 fibroblasts (12, 14). Plasmids carrying mouse c-*fos* sequences fused at position +109 to the bacterial CAT gene were cotransfected into BALB/c 3T3 cells with a plasmid carrying the constitutively expressed human α-globin gene to provide an internal control for transfection efficiency and RNA recovery. Transfected cells were incubated in medium containing 0.5% calf serum for 48 h and were subsequently stimulated with different agents for various amounts of time.

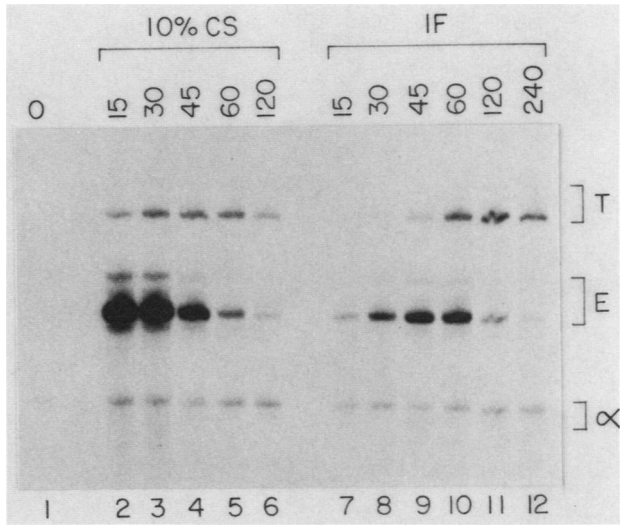

FIG. 1. RNase protection analysis of transient expression assay showing time course of c-*fos* stimulation by calf serum and by IBMX and forskolin. BALB/c 3T3 cells were transfected with the −356 c-*fos*–CAT fusion plasmid and an α-globin internal control plasmid as described in Materials and Methods. After a 48-h incubation in medium containing 0.5% calf serum, cells were exposed for the indicated times to medium containing 10% calf serum (CS) or 0.5 mM IBMX and 10 μM forskolin (IF). Total cytoplasmic RNA was harvested and analyzed by RNase protection assay, using probes specific for c-*fos* and α-globin mRNA. T, Probe fragments protected by RNA from the transfected c-*fos* plasmid; E, fragments protected by endogenous c-*fos* mRNA; α, fragments protected by RNA from the α-globin internal control plasmid. Lanes: 1, unstimulated cells; 2 to 6, cells stimulated for the indicated times with 10% calf serum; 7 to 12, cells stimulated for the indicated times with IBMX and forskolin.

Total cytoplasmic RNA was prepared from the transfected cells and analyzed by RNase protection as described previously (12). Two probes were used, one that hybridizes to RNA from both the transfected and endogenous c-*fos* genes and one that hybridizes to transcripts of the α-globin internal control.

Figure 1 shows the results of a transient expression assay using a c-*fos*–CAT fusion plasmid carrying c-*fos* sequences from −356 through +109 relative to the site of transcriptional initiation. After transfection, cells were stimulated for 15 to 240 min with 10% calf serum or with a mixture of 0.5 mM IBMX (an inhibitor of cyclic nucleotide phosphodiesterases) and 10 μM forskolin (an activator of adenylate cyclase). Refeeding cells with medium containing 10% calf serum rapidly induced the accumulation of endogenous c-*fos* mRNA in the transfected cultures, with peak levels occurring 15 to 30 min after stimulation, followed by a rapid decline toward prestimulation levels (Fig. 1, lanes 2 to 6). Induction by IBMX and forskolin was also rapid and transient (Fig. 1, lanes 7 to 12). However, peak levels of endogenous c-*fos* mRNA were lower and occurred later (45 to 60 min) than after serum induction. Expression of the transfected c-*fos*–CAT fusion genes was also induced by both stimuli. As with the endogenous gene, induction of the transfected gene by IBMX and forskolin was slower than induction by serum. In addition, maximal induction of the transfected gene occurred later than induction of the endogenous gene after both stimuli, presumably because of the increased stability of the c-*fos*–CAT RNA relative to the natural c-*fos* mRNA (36, 47, 50).

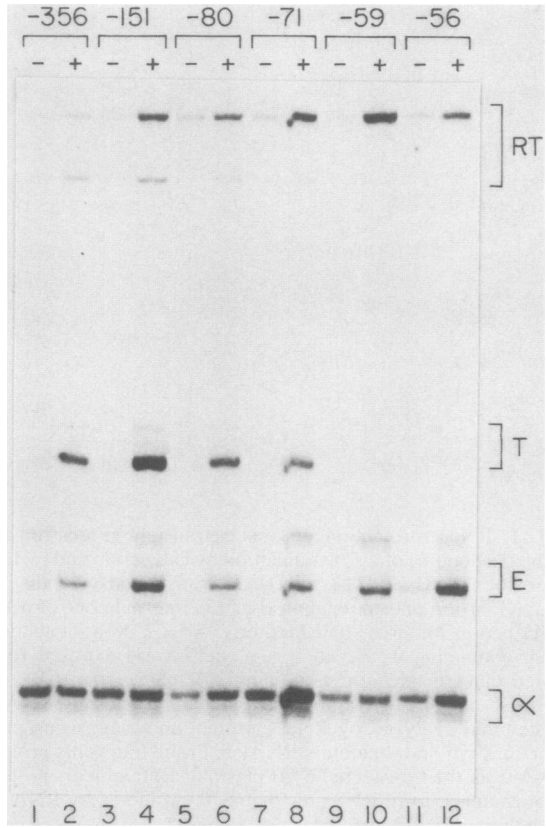

FIG. 2. RNase protection analysis of c-*fos*–CAT 5′ deletions. BALB/c 3T3 cells were transfected and treated as described for Fig. 1 except that stimulation with IBMX and forskolin was for 60 min. T, E, and α mark the positions of fusion gene RNA, endogenous c-*fos* RNA, and α-globin RNA, respectively; RT marks the position of probe fragments protected by RNA reading through c-*fos* promoter sequences from vector DNA upstream. Lanes contained the following deletion plasmids: 1 and 2, −356; 3 and 4, −151; 5 and 6, −80; 7 and 8, −71; 9 and 10, −59; 11 and 12, −56. Cells were either untreated (−) or treated with IBMX and forskolin for 60 min (+).

The c-*fos* promoter carries a CRE at −65. We showed previously that a c-*fos*–CAT fusion gene carrying c-*fos* sequences from −151 to +109 responded to cAMP when transfected into BALB/c 3T3 cells (12). This observation showed that c-*fos* induction by cAMP does not require the SRE and that sequences responsive to cAMP lay downstream of position −151. To define better the cAMP-responsive sequences in the c-*fos* promoter, we tested a series of 5′ promoter deletions (14) by the transient expression assay. Fusion genes carrying c-*fos* sequences through −356, −151, −80, and −71 all responded to cAMP, whereas genes deleted to positions −59 and −56 failed to respond (Fig. 2). Therefore, the region between −71 and −59 contains sequences required for response to cAMP. This region, previously defined as a basal promoter element and a protein-binding site in both the mouse and human c-*fos* genes (9, 14), contains a sequence related to the consensus CRE found in other cAMP-responsive genes (40). These observations suggest that this sequence element in the c-*fos* promoter functions as a CRE.

To examine further whether the previously defined protein-binding site in the c-*fos* promoter was the element required for induction by cAMP, we used site-directed mutagenesis to change the four nucleotides defined as contact sites for the cellular proteins that bind there (14). The mutations, which we term pm3, are shown in Fig. 3A. Figure 3B shows results of a mobility shift assay performed with a radiolabeled wild-type c-*fos* DNA fragment carrying sequences from −71 to −40. Using this probe, we observe at least two specific DNA-protein complexes (bracketed in Fig. 3B, lane 2) in extracts of many cell types. The extracts used in this experiment were prepared from H9 cells, a human T lymphoblast line rich in these factors, but identical results were obtained with extracts from HeLa and BALB/c 3T3 cells. Presumably, one or more of these complexes resulted from the activity of the proteins termed ATF (17, 18, 20, 23, 27, 28) or CREB (30, 51) that bind to CREs. Addition of large molar excesses of a purified unlabeled restriction fragment carrying wild-type c-*fos* DNA from −71 to +109 abolished protein binding to the labeled probe (Fig. 3B, lanes 3 to 5). In contrast, addition of a fragment identical save for the four substitutions shown in Fig. 3A failed to reduce significantly binding to the wild-type probe at up to 100-fold molar excess. Thus, these mutations substantially reduce the affinity of this site for these cellular proteins in vitro.

Figure 3C shows the results of a transient expression assay of the −71 c-*fos*–CAT fusion carrying the pm3 mutations. Whereas a wild-type −71 deletion was strongly induced by cAMP but not by serum (lanes 1 to 3), the mutant gene was induced by neither cAMP nor serum (lanes 4 to 6). Thus, destruction of the protein-binding site located at −65 abolished cAMP responsiveness of the −71 deletion. However, these mutations as well as deletion of the site (Fig. 2) also reduced basal promoter activity to below the level of detection of our RNase protection assay. Therefore, we could not formally rule out the possibility that the −65 element was purely a basal promoter element and not itself cAMP responsive. Therefore, we inserted a strong synthetic heterologous enhancer upstream of position −71 to boost basal promoter activity. The heterologous enhancer element inserted into these constructs was the 6XcoreA/coreA enhancer (32), which contains six tandem repeats of an enhancer element that is itself a tandem repeat of the simian virus 40 coreA motif. The synthetic enhancer fragment considerably elevated overall promoter activity (Fig. 3; compare lanes 1 to 3 with lanes 7 to 9). Responsiveness to cAMP was preserved, though the magnitude of induction was smaller. Surprisingly, this gene also responded strongly to serum stimulation, suggesting that the 6XcoreA/coreA enhancer is responsive to serum. When the pm3 mutations were introduced into this fusion gene, overall promoter activity declined substantially relative to that of the wild-type construct (compare lanes 10 to 12 with lanes 7 to 9) but remained at a detectable level. However, the gene was no longer induced by cAMP (lanes 10 and 11). Therefore, in addition to being a basal promoter element as reported previously (9, 14), the −65 protein-binding site also functions as a CRE in the c-*fos* promoter.

The c-*fos* promoter contains multiple CREs. The previous experiments demonstrated that the −65 element in the c-*fos* promoter is the sole CRE lying between −71 and +109. To determine whether there were additional sequences flanking the c-*fos* gene that conferred responsiveness to cAMP, we introduced the same pm3 mutations into a c-*fos*–CAT fusion gene carrying c-*fos* sequences through −356. In contrast to what we observed when these mutations were introduced into the −71 deletion, the −356 deletion carrying the pm3 mutations still responded to cAMP (Fig. 4; compare lanes 3 and 4 with lanes 1 and 2). Additional mutations that inactivate the SRE (pm12 [12]) did not abolish the remaining

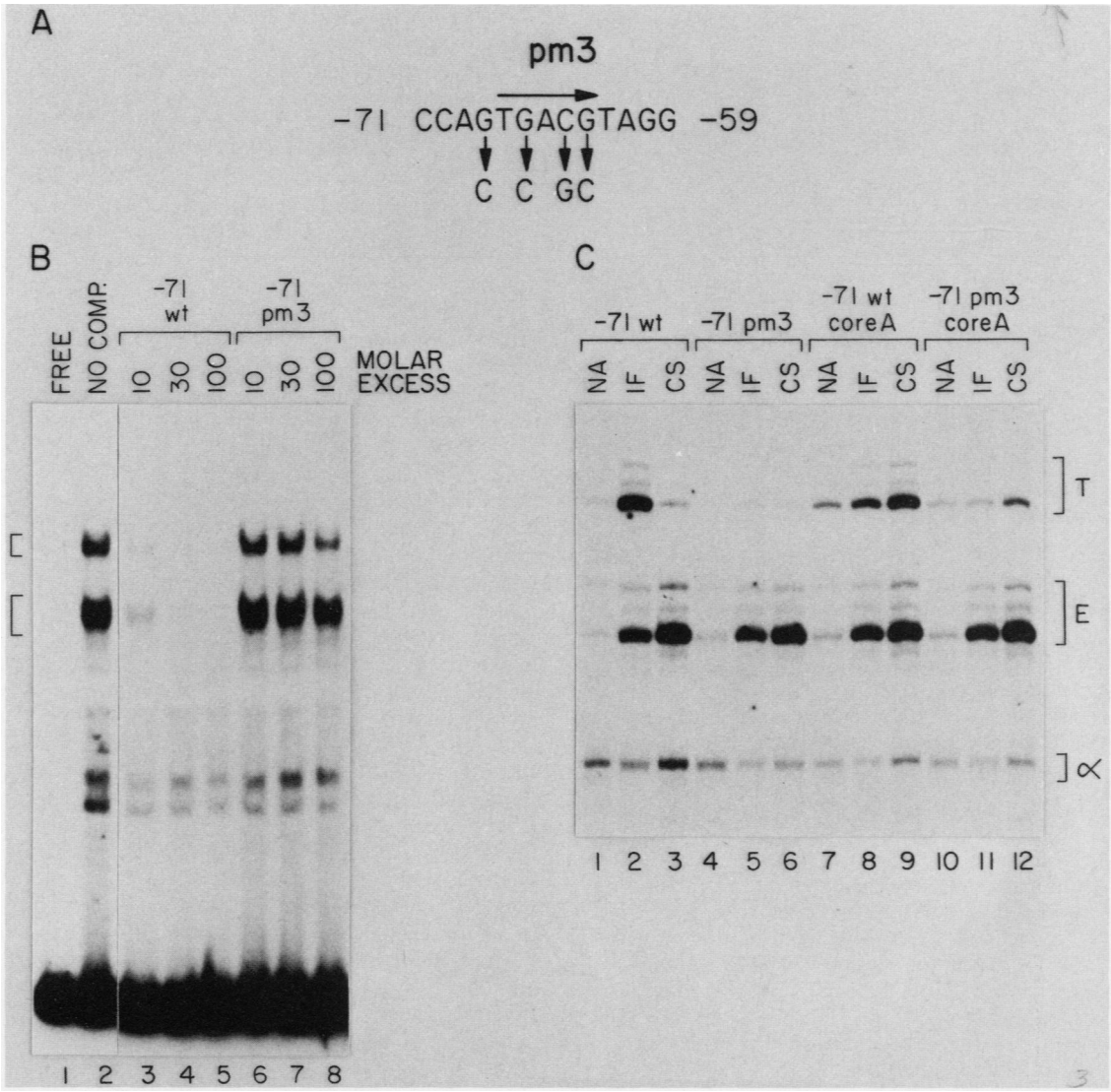

FIG. 3. (A) c-*fos* DNA sequence between positions −71 and −59. Symbols: →, match to the CRE core; ↓, positions that were altered in mutant pm3. (B) Mobility shift competition assay. The assay was carried out as described in Materials and Methods, using a wild-type c-*fos* probe carrying the −65 CRE. Purified restriction fragments carrying either wild-type or pm3 sequences were added as competitors in the molar excess indicated. Specific complexes are bracketed. These complexes failed to form on a labeled probe bearing the pm3 mutations (data not shown). Lanes: 1, no added extract; 2, standard binding reaction with no added competitor; 3 to 5, reactions containing the indicated molar excess of a purified restriction fragment comprising wild-type-c-*fos* sequences from −71 to +109; 6 to 8, reactions containing the indicated molar excess of a purified restriction fragment comprising c-*fos* sequences from −71 to +109 bearing the pm3 mutations. (C) RNase protection analysis of c-*fos*–CAT fusion plasmids carrying the pm3 mutations. Cells were transfected and treated as described in Materials and Methods. Lanes contained the following transfected plasmids: 1 to 3, −71 wild type (wt); 4 to 6, −71 pm3; 7 to 9, −71 wild type carrying six copies of the coreA/coreA enhancer element (see Materials and Methods); 10 to 12, −71 pm3 carrying the same coreA/coreA insert. Treatment were as follows: lanes 1, 4, 7, and 10, no addition; lanes 2, 5, 8, and 11, IBMX and forskolin; lanes 3, 6, 9, and 12, 10% calf serum.

cAMP responsiveness of the −356 pm3 gene (Fig. 4, lanes 7 and 8), showing that the SRE was not required for this response. Thus, cAMP responsiveness appears to be encoded redundantly in the c-*fos* promoter-enhancer.

To locate additional regions that confer cAMP responsiveness, we examined the sequence of the c-*fos* promoter-enhancer for sequences that matched the CRE consensus. Two matches were found in regions of the enhancer that are highly conserved between the mouse and human genes. One is located at position −340, upstream of the SRE, and overlaps a site that binds a protein factor induced in BALB/c 3T3 cells stimulated with v-*sis*-conditioned medium (19). The second is located at position −290, immediately downstream of the SRE, within a sequence that binds the AP-1 complex, including the Fos and Jun proteins (33; B. R. Franza and M. Z. Gilman, unpublished data). This site contains a high degree of similarity to the element in the human proenkephalin gene that confers cAMP and phorbol ester responsiveness (3, 4). Using site-directed mutagenesis, we mutated these sites to the sequences shown in Fig. 5A. For the −290 AP-1 site, these mutations abolish in vitro binding of the Fos-Jun complexes (Franza and Gilman, unpublished data).

We assayed −356 promoter deletions carrying various combinations of these mutations to determine whether these

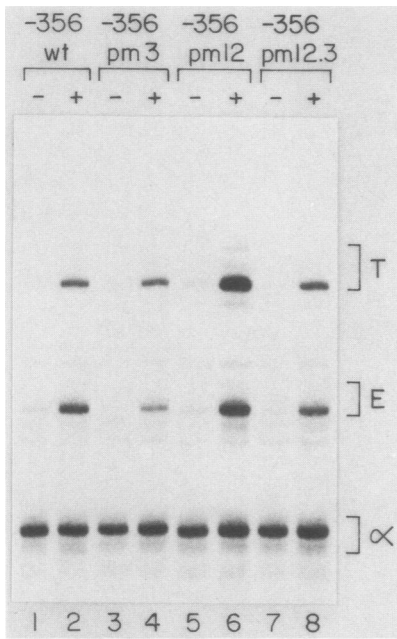

FIG. 4. RNase protection analysis of −356 deletions carrying the pm3 mutations or additional mutations (pm12) in the SRE. Cells were transfected and treated as described in Materials and Methods. Lanes contained the following transfected plasmids: 1 and 2, −356 wild type (wt); 3 and 4, −356 carrying the pm3 mutations in the CRE; 5 and 6, −356 carrying the pm12 mutations in the SRE; 7 and 8, −356 carrying both the pm3 and pm12 mutations.

conserved CRE homologies were responsible for the remaining cAMP responsiveness of the −356 pm3 mutant. The wild-type gene responded strongly to cAMP (Fig. 5B, lanes 1 and 2). Mutation of the −65 site alone had a moderate effect on cAMP induction (pm3; lanes 4 and 5). Mutation of either the −290 or the −340 site alone had little effect (data not shown), but mutation of both sites together detectably reduced the cAMP response to a small degree (pm6.9; lanes 13 and 14). Combining mutations in the −290 site with mutations at −65 suppressed the cAMP response to a much greater extent than did either mutation alone (pm3.9; lanes 7 and 8). Combining mutations at −340 with the −65 mutations had little effect compared with the −65 mutations alone (pm3.6; lanes 11 and 12). A construct carrying mutations in all three sites showed the lowest response to cAMP (pm3.6.9; lanes 17 and 18), but response was not completely lost. We found one further match to the CRE core consensus at position −120 in the mouse c-*fos* promoter that is not conserved in the human promoter. Adding mutations in this site (pm13; Fig. 5A) to the pm3.6.9 triple mutant had no further effect on the cAMP response (Fig. 5C). Mutations in the CRE core consensus sequences also affected the response of the transfected gene to serum, a phenomenon also reported by others (9). For serum, in contrast to cAMP, mutations in the −340 site had a stronger effect than did mutations at −290.

Thus, the c-*fos* promoter carries multiple CREs. The site at −65 appears to be the most potent CRE because mutations in this site had the strongest effect on cAMP responsiveness. Two additional sites, located at −290 and −340, functioned more weakly as CREs, with the −290 site being more potent than the −340 site. Mutations in these weaker sites detectably altered cAMP responsiveness only in the absence of stronger sites.

Affinities of CREs for factors in vitro correlate with their transcriptional activities in vivo. Although mutation of all recognizable matches to the CRE core consensus substantially reduced the response of transfected c-*fos*–CAT fusion genes to cAMP, residual responsiveness remained in these mutants. To determine whether this response was due to c-*fos* CREs that do not match the consensus, we performed a series of in vitro DNA-binding assays. Restriction fragments purified from each of the mutant c-*fos*–CAT fusion plasmids were tested for the ability to compete for binding of factors to the strong CRE at −65. We used a mobility shift assay identical to that shown in Fig. 3B. The major specific protein-DNA complex was excised from each gel lane and quantitated as described in the legend to Fig. 6. The results of this experiment (Fig. 6A) showed that fragments carrying wild-type sequence at −65 (wild type and pm6.9) competed best for these factors. Fragments carrying mutations at −65 but wild-type sequence at −290 (pm3 and pm3.6) competed at an intermediate level. Fragments carrying mutations at both sites (pm3.9 and pm3.6.9) competed indistinguishably from nonspecific calf thymus DNA. This experiment shows that the order of avidity of these sites for CRE-binding proteins (−65 > −290 > −340) was identical to their order of activity as CREs in the transient expression assay, suggesting that their activity in vivo reflects their ability to bind similar cellular factors. Moreover, it shows that the multiple mutant pm3.6.9 lacks sites that effectively bind these factors, suggesting that the mutant c-*fos* fragments lack functional CREs of this class.

This observation raised the possibility that the residual responsiveness of the pm3.6.9 mutant to cAMP could be due to fortuitous CRE-like sequences in vector DNA. This possibility was further supported by our observation that readthrough transcription of transfected plasmids originating in vector DNA was highly inducible by cAMP (bands marked RT in Fig. 2). To test this idea, we performed an additional in vitro competition assay, using intact plasmids as competitors. Vector DNA competed for CRE-binding activity significantly more avidly than did calf thymus DNA (Fig. 6B). In fact, competition by vector DNA was not significantly different from competition by a plasmid bearing a c-*fos* DNA insert with mutations at the −65 CRE. Thus, vector DNA contains DNA sequences able to bind CRE factors in vitro, whereas c-*fos* DNA with CRE mutations lacks such sequences. These observations support the notion that the residual cAMP responsiveness of the c-*fos* CRE mutants in the transient expression assay resulted from the effects of vector-derived CREs on transcription from the c-*fos* promoter.

CRE-binding factors are required for cAMP responsiveness of the endogenous c-*fos* gene. The results of the transient expression assays suggest that a sequence element located at −65 in the mouse c-*fos* promoter is the most potent CRE in the promoter and that secondary CREs appear to bind the same protein factors in vitro although less strongly. To determine whether the −65 site and related sequences are functional CREs for the endogenous c-*fos* gene in its natural chromatin configuration and whether the gene carries other elements able to function as CREs, we performed the experiment shown in Fig. 7. Quiescent cultures of BALB/c 3T3 cells were microinjected with double-stranded oligonucleotides corresponding to either the c-*fos* CRE at −65, the same site carrying the pm3 mutations, or the SRE located at −300. The injected cells were stimulated with either IBMX and forskolin or with calf serum. After 90 min, the cells were

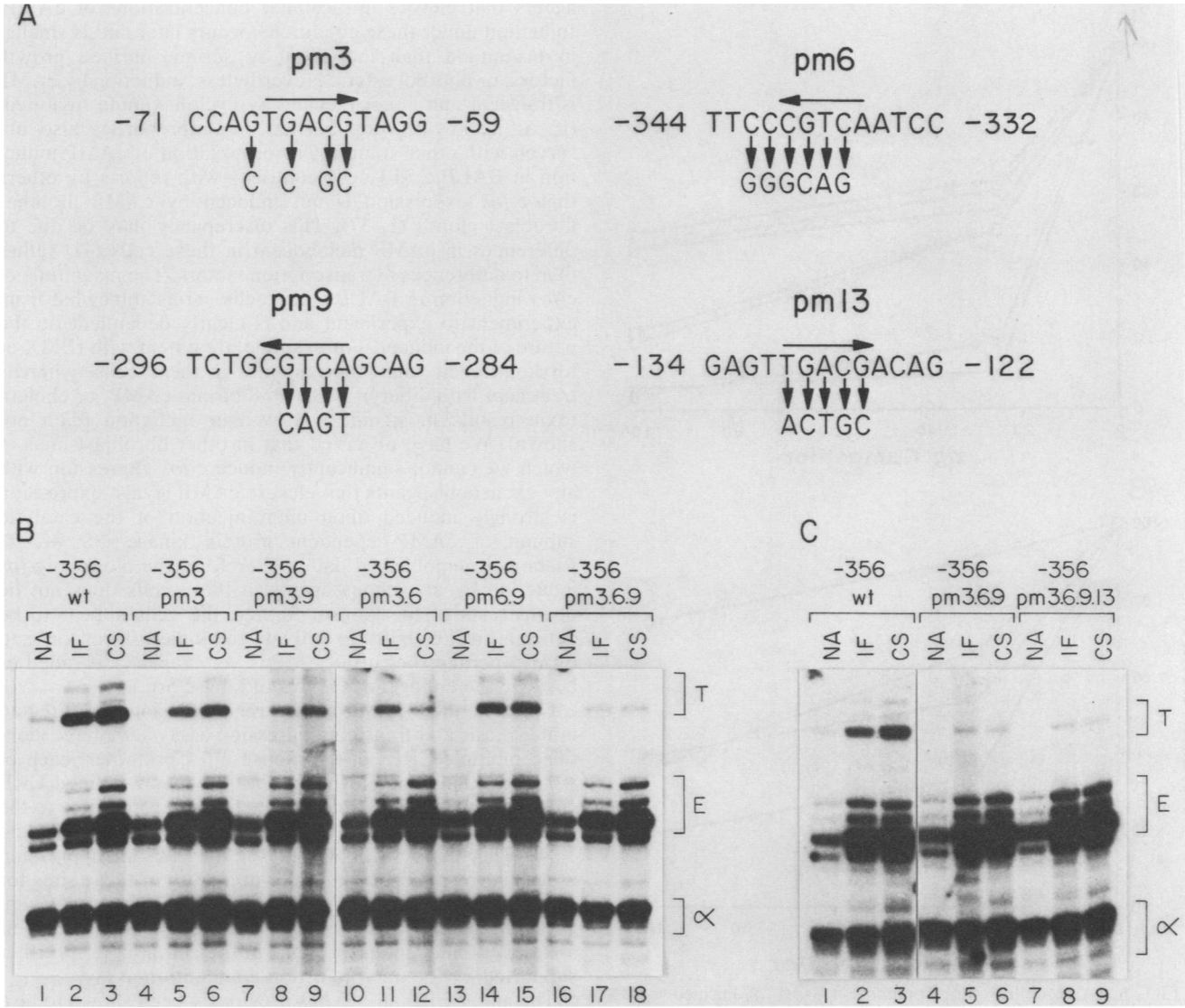

FIG. 5. (A) Sequences of the four matches to the CRE core found in the murine c-*fos* promoter. Symbols: →, ←, position and relative orientation of the core; ↓, mutations introduced into these sites by oligonucleotide-directed mutagenesis. (B) RNase protection analysis of −356 deletions carrying various combinations of CRE core mutations. Lanes contained the following transfected plasmids: 1 to 3, −356 wild type (wt); 4 to 6, −356 carrying the pm3 mutations; 7 to 9, −356 carrying both the pm3 and pm9 mutations; 10 to 12, −356 carrying both the pm3 and pm6 mutations; 13 to 15, −356 carrying both the pm6 and pm9 mutations; 16 to 18, −356 carrying the pm3, pm6, and pm9 mutations. Treatments were as follows: lanes 1, 4, 7, 10, 13, and 16, no addition (NA); lanes 2, 5, 8, 11, 14, and 17, IBMX and forskolin (IF); lanes 3, 6, 9, 12, 15, and 18, 10% calf serum (CS). (C) RNase protection analysis of −356 deletions carrying the pm3, pm6, pm9, and pm13 mutations. Lanes: 1 to 3, wild type; 4 to 6, pm3.6.9 mutant; 7 to 9, pm3.6.9 mutant carrying in addition the pm13 mutations. Treatments were as described above.

fixed and stained for Fos protein by indirect immunofluorescence, using affinity-purified antibodies against Fos (39).

In uninjected serum-starved BALB/c 3T3 cells fixed and stained for Fos protein, little fluorescence was apparent (Fig. 7C). In uninjected cells stimulated with calf serum or with IBMX and forskolin for 90 min and stained for Fos protein, nuclear fluorescence characteristic of Fos protein (39) was observed (Fig. 7A and B). Fluorescence was stronger in serum-stimulated cells, consistent with the difference in the level of c-*fos* mRNA induced in these cells by these treatments (Fig. 1). Injection of the CRE oligonucleotide blocked induction of Fos protein by cAMP (Fig. 7E) but only slightly affected induction by serum (Fig. 7D). Injection of the mutant CRE oligonucleotide had no detectable effect on Fos protein induction by either stimulus (Fig. 7F and data not shown). In contrast, injection of the SRE oligonucleotide blocked induction of Fos protein by serum (Fig. 7G), as we have shown previously (13), but had little detectable effect on Fos protein induction by cAMP (Fig. 7H).

This experiment shows that cellular proteins that bind either directly or indirectly to the c-*fos*-65 CRE are required for the response of the endogenous c-*fos* gene to cAMP and that these factors are positive activators of transcription. If there are unrelated sequence elements within the c-*fos* gene that independently mediate the response to cAMP, either their contributions are minor or they share some common cellular factor with proteins that bind to the CRE. Thus, this experiment strongly suggests that the CRE at −65 and

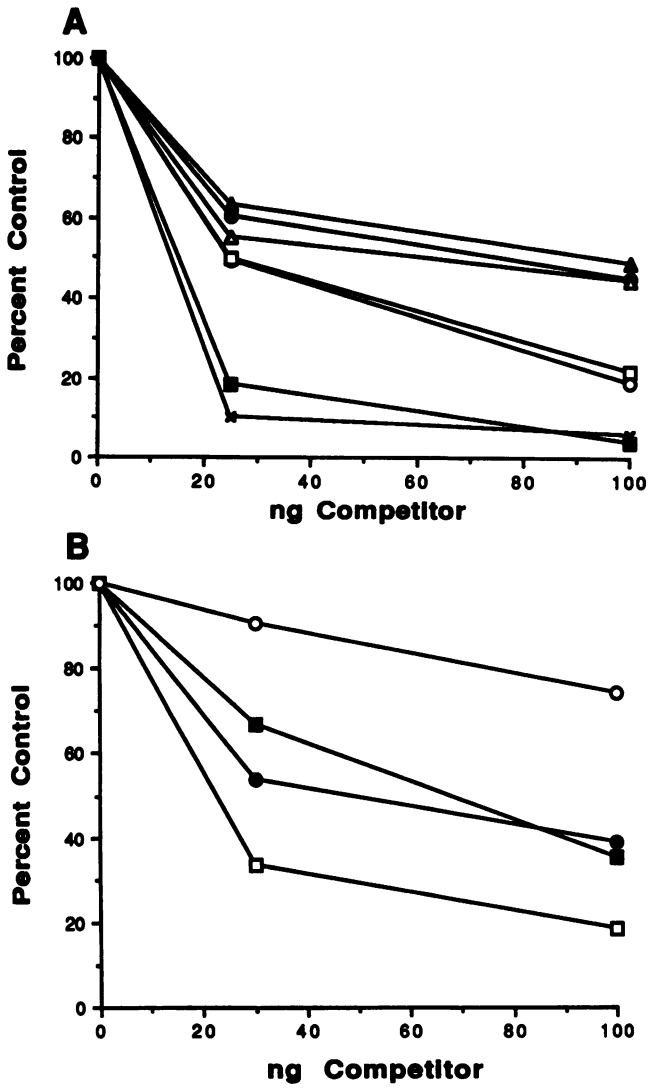

FIG. 6. (A) Mobility shift competition assay of purified c-*fos* DNA fragments carrying wild-type and mutant CRE cores. Assays were performed as described in Materials and Methods. For each reaction, the major (faster-migrating) complex (see Fig. 2B) was excised from the dried gel and quantitated by liquid scintillation counting. After subtraction of background, the amount of complex formed was expressed as a percentage of the control reaction with no added competitor. The results shown are from a representative experiment. The competitors were purified restriction fragments carrying c-*fos* sequences from −356 to +109, excised directly from the plasmids used in the transient expression assays. Competitor DNAs: ×, wild type; ○, pm3; ●, pm3.9; □, pm3.6; ■, pm6.9; △, pm3.6.9; ▲, calf thymus DNA. (B) Mobility shift competition assay of purified plasmid DNAs. Competition was calculated as described above. A representative experiment is shown. Competitors: ○, calf thymus DNA; ●, CAT vector; □, −356 wild type; ■, −356 pm3.

functionally related elements in the c-*fos* gene are the major contributors to cAMP responsiveness for the endogenous gene in its natural chromatin configuration.

DISCUSSION

Rapid and transient induction of the c-*fos* gene by cAMP in fibroblasts. We have shown that c-*fos* expression is rapidly and transiently induced in BALB/c 3T3 cells treated with agents that elevate intracellular concentrations of cAMP. Induction under these conditions occurs later and is smaller in magnitude than induction by serum, purified growth factors, or phorbol esters. Nevertheless, induction by cAMP is transient and is not blocked by cycloheximide treatment (L. A. Berkowitz, unpublished data), properties also observed with other stimuli. Our observation of cAMP induction in BALB/c 3T3 cells contrasts with reports by others that c-*fos* expression is not induced by cAMP in other fibroblast clones (1, 37). This discrepancy may be due to differences in cAMP metabolism in these cells (41) rather than to differences in transcription factors. The magnitude of c-*fos* induction in BALB/c 3T3 cells varies somewhat from experiment to experiment and is clearly dependent on the nature of the inducer. For example, treatment with IBMX or forskolin elicits maximal response in these cells, whereas treatment with dibutyryl-cAMP, 8-bromo-cAMP, or cholera toxin results in significantly weaker induction (data not shown). We have observed that in other fibroblast lines in which we cannot significantly induce c-*fos* expression with any exogenous agents that elevate cAMP levels, expression is strongly induced upon microinjection of the catalytic subunit of cAMP-dependent protein kinase (38; K. T. Riabowol, unpublished data). Therefore, the block to c-*fos* induction by exogenous agents in these cells does not lie downstream of the kinase. Rather, the cells appear to be deficient in the ability to activate the kinase in response to cAMP, perhaps because cAMP is very rapidly degraded or because the endogenous levels of kinase are low.

CREs in the c-*fos* promoter region. Using site-directed mutagenesis and transient expression assays, we have identified multiple CREs in the mouse c-*fos* promoter, each of which contains homologies to the previously defined CRE core consensus. The strongest site lies at −65 relative to the transcriptional start site. Two weaker sites lie at −290 and −340. This hierarchy of activities in the transient expression assays corresponds to the order of avidity of these sites for cellular CRE-binding factors in vitro. This observation suggests that the in vivo activities of these elements reflect their abilities to bind similar proteins in the cell, a notion further supported by the microinjection competition assay.

The major CRE at −65 has previously been shown to be a basal promoter element and protein-binding site (9, 14). It contains a match to the CRE core consensus (40), it binds both purified CREB (42, 51) and CREB translated in vitro from a cDNA clone (Berkowitz, unpublished data), and it confers cAMP responsiveness to a heterologous promoter (10, 42). Here we showed that in addition to functioning as a major basal promoter element, this site is also an active CRE within its natural promoter context. Other CREs have also been reported to function as basal promoter elements (6, 35, 44). Whether these two activities are due to similar or different cellular DNA-binding proteins is not yet clear.

The secondary CRE at −290 falls within a sequence shown to bind the AP-1 complex, including the Fos and Jun proteins (32; Franza and Gilman, unpublished data). Besides binding the AP-1 complex, we have shown that this site also competes weakly for the CREB or ATF factors that bind to the −65 CRE. The −290 site also competes weakly for crude and purified factors that bind to other CREs (21). Whether the −290 element in c-*fos* functions as a CRE by virtue of its affinity for AP-1 complex proteins or its affinity for CREB or ATF is not certain. However, our observation that the strength of this site in the transient expression assay correlated with the strength of its binding to factors with the properties of CREB or ATF supports the latter model. Like

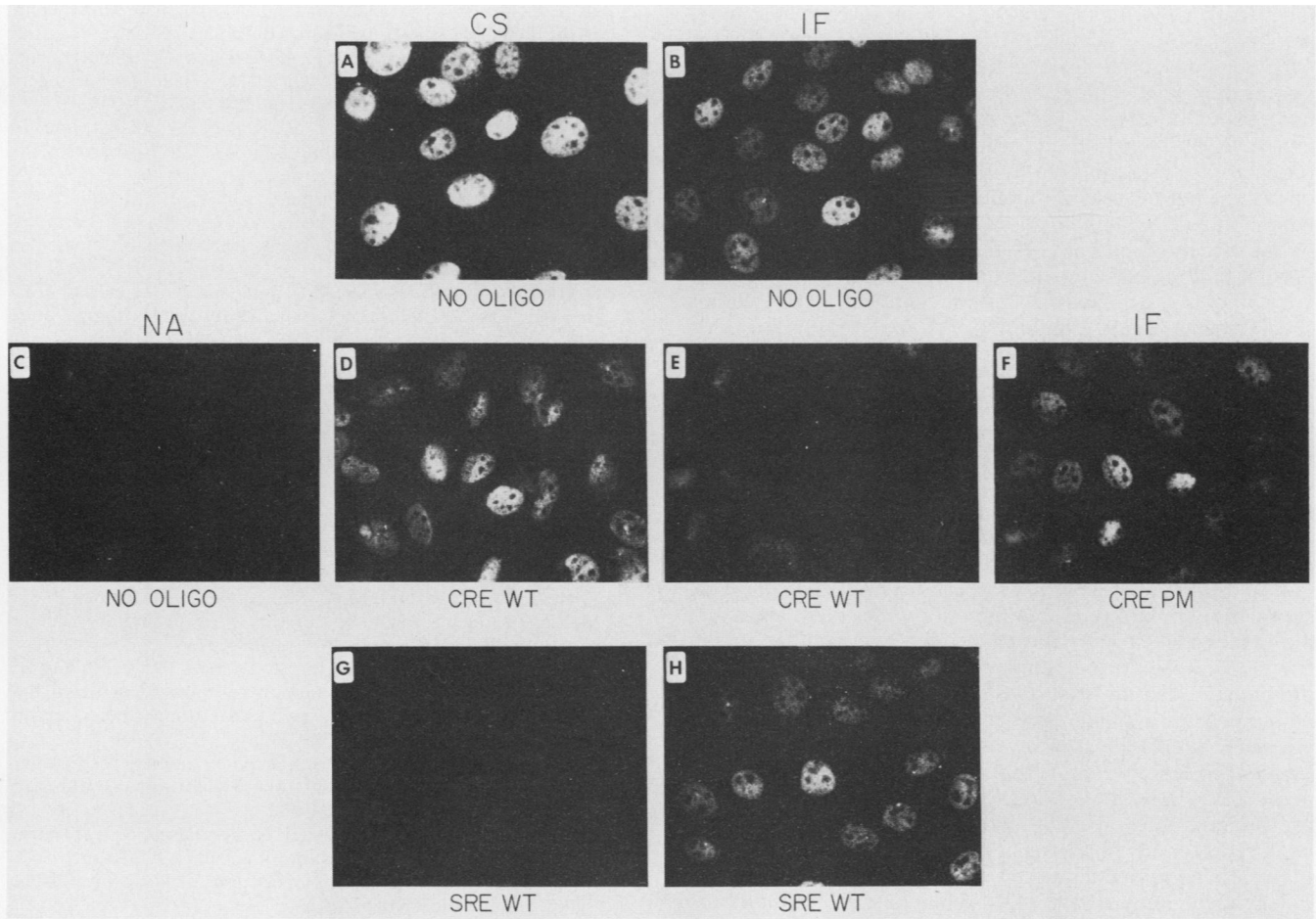

FIG. 7. Demonstration that injection of CRE oligonucleotides blocks cAMP-induced endogenous c-*fos* expression. Quiescent BALB/c 3T3 fibroblasts (C) or cells stimulated for 90 min with 10% calf serum (CS) or with IBMX and forskolin (IF) were processed to detect c-*fos* immunofluorescence. At 1 h before stimulation, cells were microinjected with double-stranded oligonucleotides as described in Materials and Methods. Cells received no oligonucleotide (no oligo), the wild-type CRE oligonucleotide (CRE WT), the mutant (pm3) CRE oligonucleotide (CRE PM), or the wild-type SRE oligonucleotide (SRE WT), as indicated. Fields of cells were photographed and processed identically to allow direct comparison among panels.

the CRE at −65, this element has also been shown to function as a strong CRE on heterologous promoters (21) and at position −53 in the human c-*fos* promoter (10). Yet our data suggest that in its natural position, it functions only weakly as a CRE. A possible explanation for this discrepancy is the position of this site in the c-*fos* promoter immediately adjacent to the SRE. We noted that mutations in the SRE reproducibly elevated the response of c-*fos*–CAT fusion genes to cAMP (Fig. 4). Because the SRE is a binding site for cellular proteins (13–15, 29, 34, 48), occupation of the SRE could block access of factors to the neighboring CRE. In the absence of SRE factors, this CRE may be more potent.

A third conserved CRE core consensus is found at position −340 in the mouse and human c-*fos* genes overlapping a sequence bound by a factor induced in fibroblasts treated with v-*sis*-conditioned medium (19). In our experiments, mutation of this site had little detectable effect on cAMP response in vivo, and it did not affect the ability of c-*fos* fragments to compete for CREB or ATF factors in vitro. Therefore, this site functions weakly if at all as a functional CRE in the c-*fos* promoter.

Because our analysis has been confined to the c-*fos* promoter region from −356 to +109, we cannot rule out the potential involvement of elements downstream of this position. Inspection of the sequence of the first intervening sequence of the c-*fos* gene reveals a 7-bp match to the −65 CRE located within a block of several hundred base pairs highly conserved between the mouse and human c-*fos* genes. It is possible that this sequence is also a functional CRE in the natural c-*fos* gene.

CRE-binding factors are required for induction of endogenous c-*fos* gene expression by cAMP. To determine whether the results of our transient expression assays reflected the regulatory mechanisms that activate the endogenous c-*fos* gene, we used oligonucleotide microinjection to titrate cellular CRE-binding proteins. We found that injection of wild-type CRE oligonucleotides prevented cells from activating expression of their c-*fos* genes in response to cAMP. Therefore, the CRE oligonucleotide is capable of titrating factors absolutely required in vivo for induction of the endogenous c-*fos* gene.

There are three limitations on our interpretation of this experiment. First, the indirect immunofluorescence assay used to assess Fos protein expression is only semiquantitative, so we cannot rule out small contributions of other sequence elements that are below the limit of detection of the assay. Second, reductions in the final level of Fos protein

in injected cells may reflect reductions in basal promoter activity as well as inducibility. Indeed, microinjection of the CRE oligonucleotide also resulted in a noticeable decrease in Fos protein levels after serum stimulation, consistent with our observation that CRE mutants fail to attain the same final level of RNA in the transient expression assay. Third, the factors titrated in vivo by the microinjected oligonucleotides are not necessarily identical to the factors we detect with in vitro DNA-binding assays. However, the correlation of the order of potency of the CREs in vivo with their protein-binding activities in vitro argues that even if the in vitro and in vivo factors are not identical, their binding specificities are very similar.

Despite these limitations, the microinjection competition assay strongly corroborates the results of the transient expression assay. Both assays show that (i) functional CREs are required for the cAMP response, (ii) response to cAMP is independent of the SRE, and (iii) the CRE is in part a basal element that affects the final level of c-*fos* induction by serum. Thus, conclusions based on the transient expression assays likely apply also to regulation of the endogenous c-*fos* gene in its natural chromatin configuration.

Two distinct nuclear signaling pathways activate c-*fos* transcription. Our data reinforce the notion that there are multiple independent routes through which extracellular signals travel to targets in the genome (2, 12, 42, 43). We showed previously that at least two distinct signaling pathways converged on a single c-*fos* regulatory element, the SRE, whereas the cAMP pathway was communicated to a distinct target (12). Here we show that the primary target for cAMP is a CRE located at −65 in the c-*fos* promoter and secondarily related sites at −290 and −340. Similar results have been reported for fibroblasts (2) and for PC12 cells (42, 43), in which c-*fos* induction by cAMP and calcium is also independent of the SRE (43). Whereas c-*fos* responds to multiple signaling pathways, we presume that there are genes that respond only to subsets of these pathways. The β-actin gene, for example, is induced by polypeptide growth factors in PC12 cells but not by agents that elevate cAMP and calcium concentrations (15). The existence of multiple nuclear signaling pathways suggests a basis for the differences in biological response elicited by agents that, for example, generate similar c-*fos* inductions. We suggest that these agents trigger the activation of distinct sets of genes whose products work in concert with proteins like Fos that are induced by a large variety of extracellular signals.

ACKNOWLEDGMENTS

We thank Gina Graham for technical assistance; Nouria Hernandez, Winship Herr, Ed Harlow, and David Helfman for helpful discussions and comments on the manuscript; and Ron Prywes for communicating results prior to publication.

This work was supported by Public Health Service grants CA45642, CA46370, and AI27270 from the National Institutes of Health.

LITERATURE CITED

1. **Bravo, R., M. Neuberg, J. Burckhardt, J. Almendral, R. Wallich, and R. Muller.** 1987. Involvement of common and cell type-specific pathways of c-*fos* gene control: stable induction by cAMP in macrophages. Cell **48**:251–260.
2. **Buscher, A., H. J. Rahmsdorf, M. Litfin, M. Karin, and P. Herrlich.** 1988. Activation of the c-*fos* gene by UV and phorbol ester: different signal transduction pathways converge to the same enhancer element. Oncogene **3**:301–311.
3. **Comb, M., N. Mermod, S. E. Hyman, J. Pearlberg, M. E. Ross, and H. M. Goodman.** 1988. Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. EMBO J. **7**:3793–3805.
4. **Comb, M. C., N. C. Birnberg, A. Seasholtz, E. Herbert, and H. M. Goodman.** 1986. A cyclic AMP- and phorbol ester-inducible DNA element. Nature (London) **323**:353–356.
5. **Curran, T.** 1988. *fos*. *In* E. P. Reddy, A. M. Skalka, and T. Curran (ed.), Oncogene handbook, p. 307–325. Elsevier/North-Holland Publishing Co., Amsterdam.
6. **Delegeane, A. M., L. H. Ferland, and P. L. Mellon.** 1987. Tissue-specific enhancer of the human glycoprotein hormone α-subunit gene: dependence on cyclic AMP-inducible elements. Mol. Cell. Biol. **7**:3994–4002.
7. **Deutsch, P. J., J. P. Hoeffler, J. L. Jameson, J. C. Lin, and J. F. Habener.** 1988. Structural determinants for transcriptional activation by cAMP-responsive DNA elements. J. Biol. Chem. **263**:18466–18472.
8. **Fink, J. S., M. Verhave, S. Kasper, T. Tsukada, G. Mandel, and R. H. Goodman.** 1988. The CGTCA sequence motif is essential for biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer. Proc. Natl. Acad. Sci. USA **85**: 6662–6666.
9. **Fisch, T. M., R. Prywes, and R. G. Roeder.** 1987. c-*fos* sequences necessary for basal expression and induction by epidermal growth factor, 12-*O*-tetradecanoyl phorbol-13-acetate, and the calcium ionophore. Mol. Cell. Biol. **7**:3490–3502.
10. **Fisch, T. M., R. Prywes, M. C. Simon, and R. G. Roeder.** 1989. Multiple sequence elements in the c-*fos* promoter mediate induction by cAMP. Genes Dev. **3**:198–211.
11. **Franza, B. R., S. J. Josephs, M. Z. Gilman, W. Ryan, and B. Clarkson.** 1987. Characterization of proteins recognizing the HIV enhancer using a microscale DNA-affinity precipitation assay. Nature (London) **330**:391–395.
12. **Gilman, M.** 1988. The c-*fos* serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. Genes Dev. **2**:394–402.
13. **Gilman, M. Z., L. A. Berkowitz, J. R. Feramisco, B. R. Franza, R. M. Graham, K. T. Riabowol, and W. A. Ryan.** 1989. Intracellular mediators of c-*fos* induction. Cold Spring Harbor Symp. Quant. Biol. **53**:761–767.
14. **Gilman, M. Z., R. N. Wilson, and R. A. Weinberg.** 1986. Multiple protein-binding sites in the 5′-flanking region regulate c-*fos* expression. Mol. Cell. Biol. **6**:4305–4316.
15. **Greenberg, M. E., L. A. Greene, and E. B. Ziff.** 1985. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC-12 cells. J. Biol. Chem. **260**:14101–14110.
16. **Greenberg, M. E., Z. Siegfried, and E. B. Ziff.** 1987. Mutation of the c-*fos* dyad symmetry element inhibits serum inducibility of transcription in vivo and the nuclear regulatory factor binding in vitro. Mol. Cell. Biol. **7**:1217–1225.
17. **Hai, T., F. Liu, E. A. Allegretto, M. Karin, and M. R. Green.** 1988. A family of immunologically related transcription factors that includes multiple forms of ATF and AP-1. Genes Dev. **2**:1216–1226.
18. **Hardy, S., and T. Shenk.** 1988. Adenoviral control regions activated by E1A and the cAMP response element bind to the same factor. Proc. Natl. Acad. Sci. USA **85**:4171–4175.
19. **Hayes, T. E., A. M. Kitchen, and B. H. Cochran.** 1987. Inducible binding of a factor to the c-*fos* regulatory region. Proc. Natl. Acad. Sci. USA **84**:1272–1276.
20. **Hurst, H. C., and N. C. Jones.** 1987. Identification of factors that interact with the E1A-inducible adenovirus E3 promoter. Genes Dev. **1**:1132–1146.
21. **Hyman, S. E., M. Comb, Y. Lin, J. Pearlberg, M. R. Green, and H. M. Goodman.** 1988. A common trans-acting factor is involved in transcriptional regulation of neurotransmitter genes by cyclic AMP. Mol. Cell. Biol. **8**:4225–4233.
22. **Imagawa, M., R. Chiu, and M. Karin.** 1987. Transcription factor AP-2 mediates induction by two different signal transduction pathways: protein kinase C and cAMP. Cell **51**:251–260.
23. **Jalinot, P., M. Wintzerith, M. Gaire, C. Hauss, J. M. Egly, and C. Kedinger.** 1988. Purification and functional characterization of a cellular transcription factor that binds to an enhancer

element within the adenovirus early EIIa promoter. Proc. Natl. Acad. Sci. USA **85**:2484–2488.

24. **Jameson, J. L., P. J. Deutsch, G. D. Gallagher, R. C. Jaffe, and J. F. Habener.** 1987. *trans*-Acting factors interact with a cyclic AMP response element to modulate expression of the human gonadotropin α gene. Mol. Cell. Biol. **7**:3032–3040.
25. **Kruijer, W., D. Schubert, and I. M. Verma.** 1985. Induction of the proto-oncogene *fos* by nerve growth factor. Proc. Natl. Acad. Sci. USA **82**:7330–7334.
26. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
27. **Leza, M. A., and P. Hearing.** 1988. Cellular transcription factor binds to adenovirus early region promoters and to a cyclic AMP response element. J. Virol. **62**:3003–3013.
28. **Lin, Y., and M. R. Green.** 1988. Interaction of a common cellular transcription factor, ATF, with regulatory elements in both E1a- and cyclic AMP-inducible promoters. Proc. Natl. Acad. Sci. USA **85**:3396–3400.
29. **Mohun, T., N. Garrett, and R. Treisman.** 1987. Xenopus cytoskeletal actin and human c-*fos* gene promoters shared a conserved protein-binding site. EMBO J. **6**:667–673.
30. **Montminy, M. R., and L. M. Bilezikjian.** 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature (London) **328**:175–178.
31. **Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman.** 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA **83**:6682–6686.
32. **Ondek, B., L. Gloss, and W. Herr.** 1988. The SV40 enhancer contains two distinct levels of organization. Nature (London) **333**:40–45.
33. **Piette, J., and M. Yaniv.** 1987. Two different factors bind to the α-domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-*fos* enhancers. EMBO J. **6**: 1331–1337.
34. **Prywes, R., and R. G. Roeder.** 1986. Inducible binding of a factor to the c-*fos* enhancer. Cell **47**:777–784.
35. **Quinn, P. G., T. Wong, M. A. Magnuson, J. B. Shabb, and D. K. Granner.** 1988. Identification of basal and cyclic AMP regulatory elements in the promoter of the phosphoenolpyruvate carboxykinase gene. Mol. Cell. Biol. **8**:3467–3475.
36. **Rahmsdorf, H. J., A. Schonthal, P. Angel, M. Litfin, U. Ruther, and P. Herrlich.** 1987. Posttranscriptional regulation of c-*fos* mRNA expression. Nucleic Acids Res. **15**:1643–1659.
37. **Ran, W., M. Dean, R. A. Levine, C. Henkle, and J. Campisi.** 1986. Induction of c-*fos* and c-*myc* mRNA by epidermal growth factor or calcium ionophore is cAMP dependent. Proc. Natl. Acad. Sci. USA **83**:8216–8220.
38. **Riabowol, K. T., J. S. Fink, M. Z. Gilman, D. A. Walsh, R. H. Goodman, and J. R. Feramisco.** 1988. The catalytic subunit of cAMP-dependent protein kinase induces expression of genes containing cAMP-responsive enhancer elements. Nature (London) **336**:83–86.
39. **Riabowol, K. T., R. J. Vosatka, E. B. Ziff, N. J. Lamb, and J. R. Feramisco.** 1988. Microinjection of *fos*-specific antibodies blocks DNA synthesis in fibroblast cells. Mol. Cell. Biol. **8**:1670–1676.
40. **Roesler, W. J., G. R. Vandenbark, and R. W. Hanson.** 1988. Cyclic AMP and the induction of eukaryotic gene transcription. J. Biol. Chem. **263**:9063–9066.
41. **Rozengurt, E., A. Legg, G. Strang, and N. Courtenay-Luck.** 1981. Cyclic AMP: a mitogenic signal for Swiss 3T3 cells. Proc. Natl. Acad. Sci. USA **78**:4392–4396.
42. **Sassone-Corsi, P., J. Visvader, L. Ferland, P. L. Mellon, and I. M. Verma.** 1988. Induction of proto-oncogene *fos* transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element. Genes Dev. **2**:1529–1538.
43. **Sheng, M., S. T. Dougan, G. McFadden, and M. E. Greenberg.** 1988. Calcium and growth factor pathways of c-*fos* transcriptional activation require distinct upstream regulatory sequences. Mol. Cell. Biol. **8**:2787–2796.
44. **Short, J. M., A. Wynshaw-Boris, H. P. Short, and R. W. Hanson.** 1986. Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. J. Biol. Chem. **261**:9721–9726.
45. **Silver, B. J., J. A. Bokar, J. B. Virgin, E. A. Vallen, A. Milsted, and J. H. Nilson.** 1987. Cyclic AMP regulation of the human glycoprotein hormone α-subunit gene is mediated by an 18-base-pair element. Proc. Natl. Acad. Sci. USA **84**:2198–2202.
46. **Stumpo, D. J., T. N. Stewart, M. Z. Gilman, and P. J. Blackshear.** 1988. Identification of c-*fos* sequences involved in induction by insulin and phorbol esters. J. Biol. Chem. **263**:1611–1614.
47. **Treisman, R.** 1985. Transient accumulation of c-*fos* RNA following serum stimulation requires a conserved 5′ element and c-*fos* 3′ sequences. Cell **42**:889–902.
48. **Treisman, R.** 1986. Identification of a protein-binding site that mediates transcriptional response of the c-*fos* gene to serum factors. Cell **46**:567–574.
49. **Tsukada, T., J. S. Fink, G. Mandel, and R. H. Goodman.** 1987. Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. J. Biol. Chem. **262**:8743–8747.
50. **Wilson, T., and R. Treisman.** 1988. Removal of poly(A) and consequent degradation of c-*fos* mRNA facilitated by 3′ AU-rich sequences. Nature (London) **336**:396–399.
51. **Yamamoto, K. K., G. A. Gonzalez, W. H. Biggs, and M. R. Montminy.** 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature (London) **334**:494–498.