Nucleic Acids Research, Vol. 19, No. 15 4153–4159

A new cAMP response element in the transcribed region of the human c-fos gene

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Received April 25, 1991; Revised and Accepted July 3, 1991

ABSTRACT

In NIH 3T3 cells the c-fos gene is induced rapidly and transiently by cAMP. As shown by the analysis of 3T3 cells stably transfected with promoter mutants of the human c-fos gene this induction does not depend on the dyad symmetry element (position −320 to −300), but involves at least two other non-related sites: an element located around position −60 resembling the cAMP response element of the fibronectin and somatostatin genes (which has been described before), and an element located between positions +18 and +38. Destruction of one or the other element in the c-fos gene reduces cAMP inducibility. The cAMP response of c-fos promoter CAT gene constructs also depends on these elements in transient transfection assays. When cloned in front of the albumin TATA box, both elements independently mediate cAMP inducibility. These elements do not bind the same protein as shown in gel retardation analyses, suggesting that two different cAMP inducible factors mediate the activation of the c-fos gene by cAMP.

INTRODUCTION

A large variety of extracellular stimuli induce c-fos gene transcription rapidly and transiently (1–8). Most inducers as e.g. serum growth factors, phorbol esters and ultraviolet irradiation need the serum response element (also named dyad symmetry element, DSE) of the c-fos promoter for efficient induction of the gene (9–13). Only agents which increase c-fos expression through raising the cellular Ca^{2+} or cAMP level do not seem to depend on the presence of the DSE: c-fos promoter mutants possessing or lacking the DSE respond equally well to agents which increase the cellular cAMP level (11, 12, 14). In an attempt to pinpoint the site(s) which are responsible for cAMP induction of the gene, several promoter mutants of c-fos have been constructed and analysed for cAMP inducibility. The results showed that not one but several sites contribute to cAMP inducibility of the promoter. One major site is located between positions −64 and −57 (5'TGACGTTT3') relative to the start of transcription (15–17). It is homologous to the core of the cAMP response element of the somatostatin and fibronectin promoters (18, 19), it competes for protein-binding with this element (15) and binds in vitro the CRE binding protein CREB (20, 21, for review see 22). Upon destruction of this element in the context of the complete c-fos promoter, cAMP responsiveness is only barely diminished (in Balb c 3T3 cells, 17) or not affected at all (in NIH 3T3 cells, 16), suggesting that other elements may overtake its function. When cloned as single elements in front of heterologous non-responsive promoters, several sections of the c-fos promoter confer cAMP inducibility (the region from position −225 to position −99 which contains several putative AP-2 binding sites; the region from position −303 to position −281 which is homologous to the consensus binding site of the transcription factor AP-1; the region between positions −317 and −298 which contains the DSE, see ref. 16). The contributions to the cAMP response of these diverse portions of the promoter in their natural context are not known.

Since in transient transfection studies both murine and human c-fos 5' deletion promoter mutants truncated at either position −59 or position −53 respectively did not seem to respond to cAMP (16, 17), we were intrigued by our finding that a human c-fos promoter construct truncated at position −52 and stably transfected into NIH 3T3 cells, was strongly inducible by cAMP (11). We show here that this induction depends largely on a DNA element located in the transcribed but not translated region of the c-fos gene. Destruction of this element in the context of the c-fos promoter reduces cAMP inducibility. Minimal promoters carrying the isolated element respond to cAMP.

MATERIALS AND METHODS

Cell culture and transfections

NIH 3T3 cells were grown at 37°C and 6% CO_2 in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS), streptomycin (100 μg/ml) and penicillin (100 U/ml). Transfection of plasmid DNA was performed using the calcium phosphate coprecipitation technique (23). To obtain stable transfectants, NIH 3T3 cells were co-transfected with 10 μg of

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the appropriate plasmid, 10 μg of high molecular weight salmon sperm carrier DNA and 1 μg of the pSV2neo plasmid (24) per 9 cm petri dish and selected for G418 resistance in DMEM/10% FCS containing 300 μg/ml G418. About 100 G418 resistant clones per dish were pooled into mass cultures. For transient transfections 10 μg of the appropriate CAT-plasmid were used per 9 cm plate. After starvation for 36–48 h in DMEM/0.5% FCS, the cells were treated with 10 μM forskolin or 60 ng/ml TPA and cell extracts prepared after 24 hours.

Analysis of gene expression

Poly A^+-RNA was prepared 30 min after treatment of the cells by absorption to oligo dT-cellulose as described (25). S1-protection analysis was performed as described in ref. 26. The preparation of the DNA probes, detecting the human c-fos transcripts, was performed as specified in ref. 11. For CAT-assays the cells were disrupted by freezing and thawing and the CAT activity of the cell extracts was determined according to ref. 27.

Gel retardation analyses

Gel retardation analyses were performed with nuclear extracts from growing 3T3 cells according to ref. 28. Protein concentration was determined by the method of Bradford (29). The double stranded oligonucleotides had the sequence:

c-fos CRE

```
     −73                    −54
      '                      '
5'GATCTTGAGCCCGTGACGTTTACA 3' (numbering acccording to refs 9
                                          and 11)
    3'AACTCGGGCACTGCAATGTCTAG 5'
```

+18/+38 element

```
    +16                    +37
     '                      '
5'GCGAGCAACTGAGAAGCCAAGACT 3' (numbering according to refs 9
                                          and 11)
  3'CTCGTTGACTCTTCCGTTGTGAGC 5'
```

collagenase TRE

```
      −76              −61
       '                '
5'GATCGAGCATGAGTCAGACAC 3' (numbering according to ref. 30)
    3'CTCGTACTCAGTCTGTGCTAG 5'
```

hMT-IIA AP-2

```
    −188                    −165
      '                       '
5'CGACGAACTGACCGCCCGCGGCCCGTGT 3' (numbering according to
                                          ref. 31)
  3'TGCTTGACTGGCGGGCGCCGGGCACAGC 5'
```

A polyclonal antiserum prepared from a rabbit which was immunized with an oligopeptide (aa 136–150) derived from the CREB protein (32) and the respective preimmune serum, have been purified on a protein A sepharose column and were added to the nuclear extracts 10 minutes ahead of the labeled oligonucleotide. Incubations were performed at 20°C and the native polyacrylamide gels were run in TBE buffer (90 mM Tris pH 8.3, 90 mM borat, 2.5 mM EDTA) at 20°C.

Plasmid constructs

gene constructs with the c-fos coding region. The plasmids phcfos −750, phcfos −52, phcfos Δ+12/+45 and phcfos Δ−327/−307 have been described in ref. 11. The plasmid phcfos Δ−65/−52 was constructed by combining the 5′ deletion mutant phcfos −52 with an internal deletion phcfos Δ−65/+45 (which was constructed in an analogous way to the internal deletion phcfos Δ+12/+45, ref. 11). From the plasmid phcfos Δ−65/+45 the BamHI (position −750)/XbaI (position −65) fragment was isolated, BamHI linkers were added and the fragment was inserted after BamHI digestion into BamHI digested phcfos −52. The triple mutant phcfos Δ−327/−307//−65/−52//+12/+45 was combined from the constructs phcfos Δ−327/−307//+12/+45 (11) and phcfos Δ−65/−52. A 319 bp long EcoRI/XbaI-fragment of the plasmid phcfos Δ−327/−307//+12/+45 (position −307 to +12) was subcloned in the vector pT7T3 19 (Gibco BRL). A 71 bp long NarI-fragment (position −85 to −14) was cut out of this new construct and replaced by the equivalent fragment of the plasmid phcfos Δ−65/−52, which included the internal deletion from −65 to −52. The religation of the EcoRI/XbaI-fragment and a 423 bp long EcoRI/EcoRI fragment (position −750 to −327) into EcoRI/XbaI cut phcfos Δ−327/−307//+12/+45 led to the triple mutant. The plasmid phcfos −52//Δ +12/+45 was derived from the construct phcfos Δ−327/−307//−65/−52//+12/+45 after EcoRI (position −750)/BamHI (position −52)-digestion and religation of the isolated and blunt-ended 7.3 kb fragment. Thus all the gene constructs contain promoter sequences up to position −750 and the whole fos gene with 1.1 kb 3′ flanking sequences.

fos promoter-CAT gene constructs. The construct phcfos −711/+45 CAT5 has been derived from the plasmid phcfos −711/+45 CAT3 (11) by recloning the c-fos portion into the vector pBLCAT5 (33). The constructs phcfos −52/+45 CAT5 and phcfos −52/+12 CAT5 have been derived from plasmids phcfos −52 and phcfos −52//Δ+12/+45. phcfos −52 was SmaI/XbaI digested (position −52 to +45) and the 97 bp fragment was isolated. phcfos −52//Δ+12/+45 was first digested with BamHI (position −52), blunt-ended by fill-in using Klenow-polymerase and then XbaI digested (position +12). The 64 bp fragment was also isolated. Both fragments were ligated into the vector pBLCAT5 which was HindIII digested, blunt-ended and XbaI digested.

phcfos −711/+45 Δ−65/−52 CAT5 has been derived from a Xho/XbaI digest of phcfos Δ−65/−52 and insertion of the fragment into the SalI/XbaI digested pBLCAT5.

All mutant fos promoters were also cloned into the vector pBLCAT51 which was derived from pBLCAT5 by the destruction of a putative vector CRE at position 4079: 5′TG-ACGTCTA3′. This sequence contains an AatII recognition site. After AatII digestion the vector was blunt-ended by T4-DNA-Polymerase and religated.

Oligonucleotide TATA/CAT gene constructs. The constructs p(DSE)TATA-CAT and p(DSE′-296′)TATA-CAT have been described in ref. 34, the construct 5×TRE-TATA/CAT in ref. 33 (designated there 5×TRE TATAAAA). The plasmids fosCRE-TATA/CAT, +18/+38-TATA/CAT and 3×+18/+38-TATA/CAT have been constructed by inserting the oligonucleotides (see above) into the multiple cloning site of the vector pTATACAT. (Because the foot-print borders were determined previously to be +18/+38 (ref. 11), we stick to the designation +18/+38 element, although the oligonucleotide used in gene constructs and gel retardation assays encompasses the bases +16 to +37.) The vector pTATACAT was derived from the plasmid PL-TG (35) by replacement of an 896 bp XmnI/BamHI fragment by a 629 bp XmnI/BamHI fragment of pBLCAT4 (33) and subsequent deletion of the G-cassette by digestion with XhoI and

religation. By destruction of the vector CRE (see above) all the constructs were changed into the CAT61 version.

All gene constructs have been confirmed by sequencing.

RESULTS

A human c-fos gene truncated at position −52 is strongly inducible by cAMP

In NIH 3T3 cells agonists of both adenylate cyclase (forskolin) and of protein kinase C (phorbol esters) rapidly and transiently increase the level of c-fos RNA. Moreover transcription from a human c-fos gene (containing 750 bases of the promoter, phcfos-750, see Fig. 1 for gene constructs and ref. 11) stably transfected into NIH 3T3 cells, is rapidly and transiently induced by both types of agents, demonstrating that the cis-acting sequences necessary for activation and subsequent repression of transcription are contained in this gene construct (11). Fig. 2 shows the RNA levels transcribed from the transfected human gene at 30 min after treatment with either 12-O-tetradecanoyl-phorbol-13-acetate (TPA, T) or forskolin (F). In an attempt to

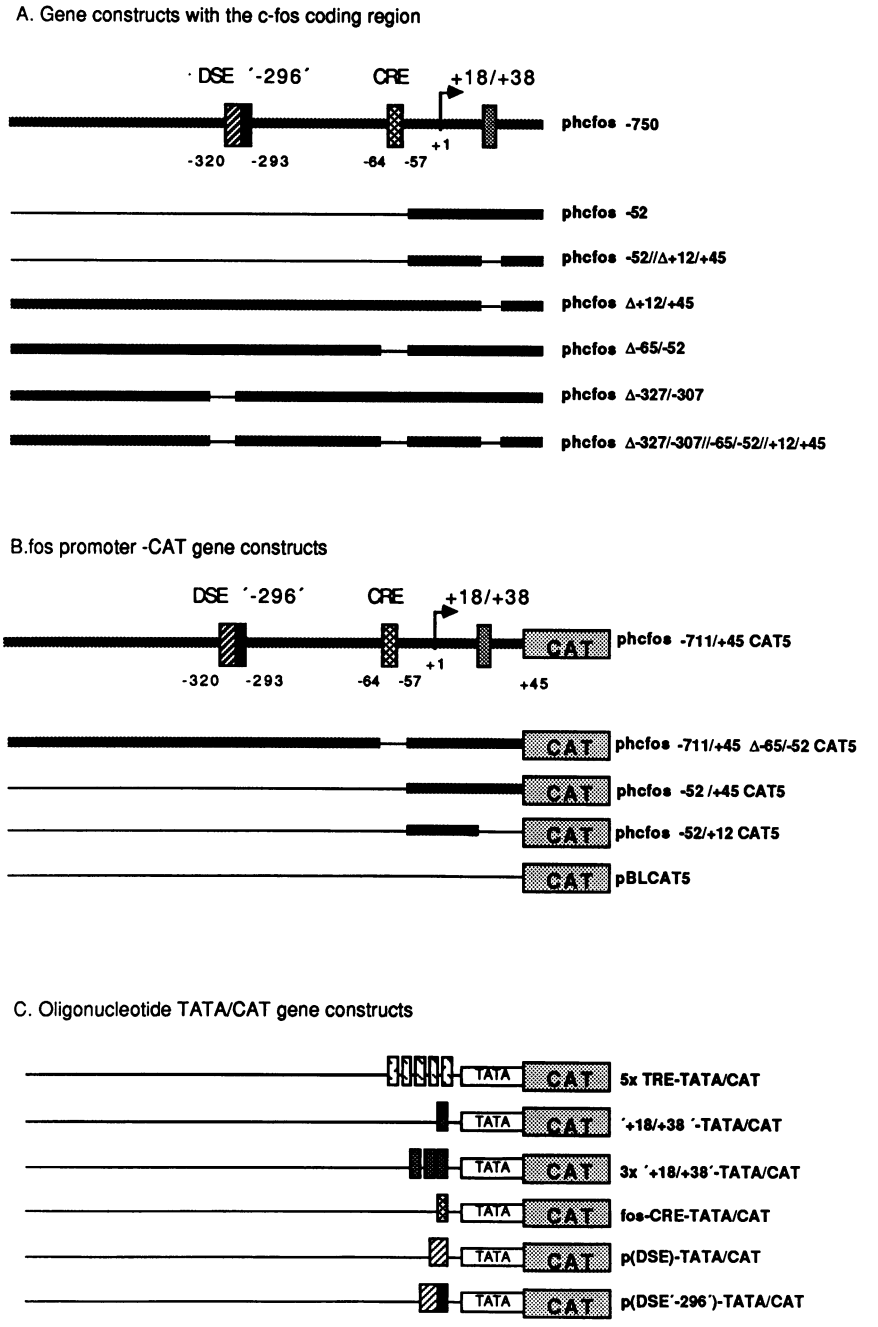

Figure 1. Structure of the c-fos gene constructs. The bold black bars represent c-fos promoter and coding sequences, CAT stands for CAT coding sequences, TATA denotes the Xenopus albumin gene TATA box. The DSE is the serum response element (position −320 to −299), '−296' designates the adjacent AP-1 like binding site (position −299 to −293) and CRE the cAMP response element at position −60 (position −64 to −57). +18/+38 is the cAMP response element described here. All positions are counted from the start site of transcription +1, which is marked by an arrow.

define regulatory sequences that mediate a cAMP response we have deleted most of the 5′ flanking sequences of the promoter. Deletion to position −52 obliterates phorbol ester inducibility almost completely. cAMP inducibility, however, is fully retained or even enhanced in comparison to the phcfos −750 gene construct (Figs. 2, 3; the 168 nucleotides long fragment indicated by the upper arrow is derived from the correctly initiated c-fos transcript; we do not know the origin of the faster migrating fragment with a length of around 120 nucleotides seen in the phcfos 52 and pfcfos −750 tracks. In the densitometric evaluation of Fig. 3, only the correctly initiated transcripts have been examined). This indicates that the cAMP responsive element (CRE) located around position −60 is not the only cAMP response element in the c-fos gene and that, at least in NIH 3T3 cells, an element 3′ of position −52 responds to cAMP. Earlier evidence had suggested the existance of a protein binding site located between positions +18 and +38, and contribution of this site to the cAMP response (11). We have, therefore, constructed several mutants of the c-fos promoter in which the CRE at −60 or the +18/+38 site have been destroyed. After stable transfection into NIH 3T3 cells, pooled clones were analysed for cAMP inducibility of the transfected gene. The results of this analysis are summarized in Fig. 3. Gene constructs containing the complete promoter (phcfos −750) are inducible about tenfold. Destruction of the dyad symmetry element at position −300 (phcfos Δ−327/−307) does not affect inducibility. Deletion of either the CRE at position −60 (phcfos Δ−65/−52) or of the element at +18/+38 (phcfos Δ+12/+45) reduces cAMP inducibility to 4.3 fold or 6.1 fold, respectively. A triple deletion mutant (phcfos Δ−327/−307//−65/−52//+12/+45) is still inducible 3.6 fold by cAMP. Thus, within the complete promoter, the CRE at position −60 and the element +18/+38 both contribute to cAMP inducibility of the promoter. The residual activity in their absence must be mediated by still another cAMP response element. In the phcfos −52 mutant, the +18/+38 element mediates the largest portion of the cAMP response. Deletion of positions +12 to +45 (phcfos −52//Δ+12/+45) reduces induction from about 20 fold to 3 fold (Figs. 2, 3).

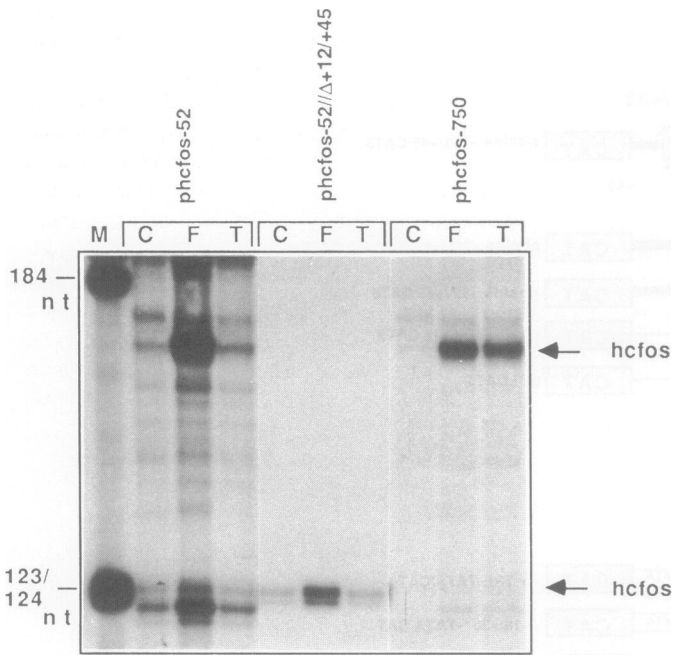

Figure 2. Analysis of c-fos promoter mutants for inducibility by forskolin (F) and TPA (T). Mass cultures of NIH 3T3 cells stably transfected with the indicated c-fos promoter mutants were starved for 40 h in DMEM/ 0.5% FCS and then treated for 30 min with 10 μM forskolin or 60 ng/ml TPA. Poly A^+-RNA was isolated and the amount of c-fos RNA transcribed from the transfected gene constructs was determined by S1-protection analysis. The marker line (M) shows the separation of HaeIII digested pBR322. RNA transcribed from phcfos −52 and phcfos −750 generates a signal at 168 nucleotides (upper arrow, hcfos), RNA transcribed from the internal deletion mutant phcfos −52//Δ+12/45 a signal at 123 nucleotides (lower arrow, hcfos). We do not know the provenance of the 120 nucleotide fragment seen in the phcfos −750 and phcfos −52 tracks.

In transient transfection experiments the CRE at position −60 and the +18/+38 element mediate cAMP inducibility

To investigate whether the response elements depended on the chromatin structure of the integrated stable transfectant, we have recloned several of the c-fos promoter mutants in front of the chloramphenicol acetyl transferase gene (CAT, Fig. 1) and have analysed these constructs in transient transfection assays. The c-fos promoter fragments were ligated at position +45 into the vector pBLCAT5. This vector is derived from pBLCAT3 (36) by the destruction of a putative TPA responsive element (33). Upon computer assisted inspection of this vector we found a second element at position 4079 with strong homology to a CRE: 5′TGACGTCTA3′. To exclude that this site contributed to cAMP inducibility of the c-fos-CAT constructs, it was destroyed and the resulting vector was renamed pBLCAT51. All c-fos promoter constructs were analysed in both vectors, with no significant difference in the outcome of analysis (Table 1).

In transient transfection assays the complete c-fos promoter (phcfos −711/+45CAT5) was inducible 3 fold by cAMP. Most if not all of this induction was due to the presence of the cAMP response element at position −60. When this element was destroyed, the promoter (phcfos −711/+45 Δ−65/−52 CAT5) was not inducible by cAMP. In these experiments, the CRE at position −60 was more decisive than in the stable transfectants. In consistency with the stable transfectants, a 5′ deletion mutant lacking the CRE at position −60 (phcfos −52/+45CAT5), although showing a much lower basal level of expression as compared to the longer constructs (consistent with the situation with stable transfectants, ref. 11) is still strongly activated by cAMP, even more so than the wild type. This induction depends largely on the sequence +18/+38. Upon destruction of this element (phcfos −52/+12CAT5) inducibility drops to 1.7 fold (Table 1). This experiment defines two CREs by functional analysis, the CRE at position −60 and the +18/+38 element.

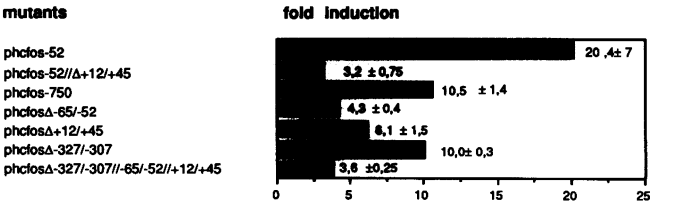

Figure 3. Forskolin inducibility of human c-fos mutant constructs stably transfected into NIH 3T3 cells. The diagram represents the results of three independent S1-protection analyses. Induction factors have been calculated after densitometric scanning of autoradiographic exposures as shown in Fig. 2. Only the correctly initated RNA's have been considered. Deviations indicated are standard deviations.

The +18/+38 element confers cAMP inducibility to minimal promoters

The CRE at position −60 has been shown previously to suffice in conferring cAMP inducibility to a non-responsive promoter (15, 16). We now examine whether the new element also possesses this property. We have cloned both sequences, the CRE and the +18/+38 element, as single or multimerized oligonucleotides, in front of the TATA box of the albumin promoter linked to the CAT gene (Fig. 1), and examined inducibility by cAMP in transient transfection assays (Table 2). We included in this analysis two constructs which had been shown previously to be inducible by cAMP (16), the dyad symmetry element at position −300 and a gene construct which contains the dyad symmetry element and the adjacent putative AP-1 binding site (DSE'-296').

A control construct carrying the 5 fold oligomerized TPA responsive element of the collagenase gene (5×TRE-TATA/CAT), and a gene construct containing the dyad symmetry element of the c-fos gene (p(DSE)-TATA/CAT) are not inducible above the level of the vector pTATACAT (Table 2). Also the construct with the dyad symmetry element plus the adjacent AP-1 binding site (p(DSE'-296')-TATA/CAT) is only barely inducible. As expected the cAMP response element of the c-fos gene (position −60, fos-CRE-TATA/CAT) clearly confers inducibility by cAMP. The new +18/+38 element (+18/+38'-TATA/CAT) turned out to be at least as effective as the CRE (at position −60): as single element the cAMP induction was 2 to 6 fold, as trimerized element it was 3 to 9 fold (Table 2).

Different proteins bind to the cAMP response element at position −60 and to the +18/+38 element

The two elements show no obvious sequence similarity. Since examples have been described that one protein may bind to DNA elements of completely different sequence (for instance in the SV40 enhancer, ref. 37), we examined whether the same or different proteins bind to the two elements. To this end we competed gel shift incubations with an excess of one or the other element. Both oligonucleotides form a major retarded band, when incubated with nuclear extracts prepared from growing NIH 3T3 cells. The shifted bands run to different positions (Fig. 4, arrows), suggesting that different proteins bind to these oligonucleotides. This suggestion is substantiated in competition experiments. While the binding to the two oligonucleotides is completely abolished upon incubation with an 100 fold excess of the non-labeled homologous oligonucleotide (Fig. 4A, compare lines 1 and 2, and lines 6 and 8), there is no competition with protein binding to the +18/+38 element by an 100 fold excess of the −60 element (compare lines 1 and 3), or with protein binding to the −60 element by an 100 fold excess of the +18/+38 element (compare lines 6 and 7). These experiments suggest that different proteins bind to the c-fos CRE and to the +18/+38 element. Also the TPA response element of the collagenase gene (which does not respond to cAMP, Table 2) does not compete for binding to either site (lines 4 and 9). Sequence comparisions and competition experiments (lines 5 and 10) showed that another cAMP response DNA element, the AP-2 binding site (38), is also unrelated to the +18/+38 element.

In order to examine whether a factor which is immunologically related to the CRE binding protein CREB (21) binds to the +18/+38 element, we preincubated nuclear extracts of 3T3 cells with CREB specific antibodies before performing the gel shift assays. When the labeled CRE oligonucleotide is incubated with a nuclear extract in the presence of CREB specific antibodies, the retarded band generated without antibodies disappears and a band that migrates more slowly is formed (Fig. 4B, compare lines 7 and 8) suggesting an interaction of the antibody with the factor binding to the CRE. In contrast incubation with preimmune serum (line 11) does not change the mobility of the retarded band and neither the immune serum (line 9) nor the preimmune serum (line 12) incubated with the labeled CRE (without nuclear extract) form a retarded band. When a nuclear extract containing CREB specific antibodies is incubated with the labeled +18/+38 element, no change in migration or intensity of the retarded band as compared to an extract containing preimmune serum can be detected (compare lines 1, 2 and 5) suggesting that the protein binding to the +18/+38 element is not immunologically related to CREB.

Table 1. The CRE and the +18/+38 element are necessary to mediate cAMP inducibility in transient transfection experiments.

phcfos-711/+45CAT5			phcfos-711/+45 Δ-65/-52 CAT5			phcfos-52/+45CAT5			phcfos-52/+12CAT5			pBLCAT5		
C	F	fold	C	F	fold	C	F	fold	C	F	fold	C	F	fold
1472,6	5569,7	3,8	—	—	—	384,9	1928,9	5,0	103,8	187,5	1,8	49,3	50,2	1,0
10640,0	22680,0	2,1	3530,2	3536,6	1,0	618,3	1754,5	2,8	72,8	166,6	1,6	25,6	26,0	1,0
6637,5	24430,0	3,7	2204,3	2324,1	1,0	148,4	728,0	4,7	27,6	54,6	1,9	40,4	40,4	1,0

phcfos-711/+45CAT51			phcfos-711/+45 Δ-65/-52 CAT51			phcfos-52/+45CAT51			phcfos-52/+12CAT51			pBLCAT51		
C	F	fold	C	F	fold	C	F	fold	C	F	fold	C	F	fold
732,4	3266,5	4,5	—	—	—	284,2	1990,7	7,1	65,2	105,0	1,6	55,0	65,0	1,2
10710,0	30940,0	2,8	6965,4	10507,4	1,5	217,0	542,5	4,3	45,2	113,7	1,5	42,0	51,3	1,2

NIH 3T3 cells were transfected with 10 μg of the indicated fos promoter CAT gene construct. The cells were starved for 36 h and treated with 10 μM forskolin for 24 h; CAT activity (given in pmol/mg^{-1} h^{-1}) was determined as described in ref. 27.

DISCUSSION

Our report describes a new cis-acting element of the c-fos promoter which is interesting in two respects. 1) It mediates cAMP responsiveness and differs from other examples of cAMP response elements described so far. As shown previously this

Table 2. The CRE and the +18/+38 element are sufficient to confer cAMP inducibility to a heterologous promoter.

5x TRE-TATA/CAT			'+18/+38 '-TATA/CAT			3x '+18/+38'-TATA/CAT			fos-CRE-TATA/CAT			p(DSE)-TATA/CAT			p(DSE'-296')-TATA/CAT			pTATACAT		
C	F	fold	C	F	fold	C	F	fold	C	F	fold	C	F	fold	C	F	fold	C	F	fold
622,2	1088,9	1,7	152,6	984,4	6,5	210,7	1900,3	9,2	185,9	620,5	3,2	38,5	54,3	1,4	39,6	83,2	2,1	40,5	61,3	1,5
85,8	112,2	1,3	131,1	603,0	4,3	143,8	747,8	5,2	69,7	195,2	2,8	47,7	51,5	1,1	52,3	94,2	1,8	----	----	----
499,5	519,7	1,0	133,8	348,9	2,6	398,5	1275,2	3,2	----	----	----	25,4	60,5	1,6	----	----	----	63,3	66,6	1,0

Transient transfections and the determination of CAT activity were performed as described in Table 1.

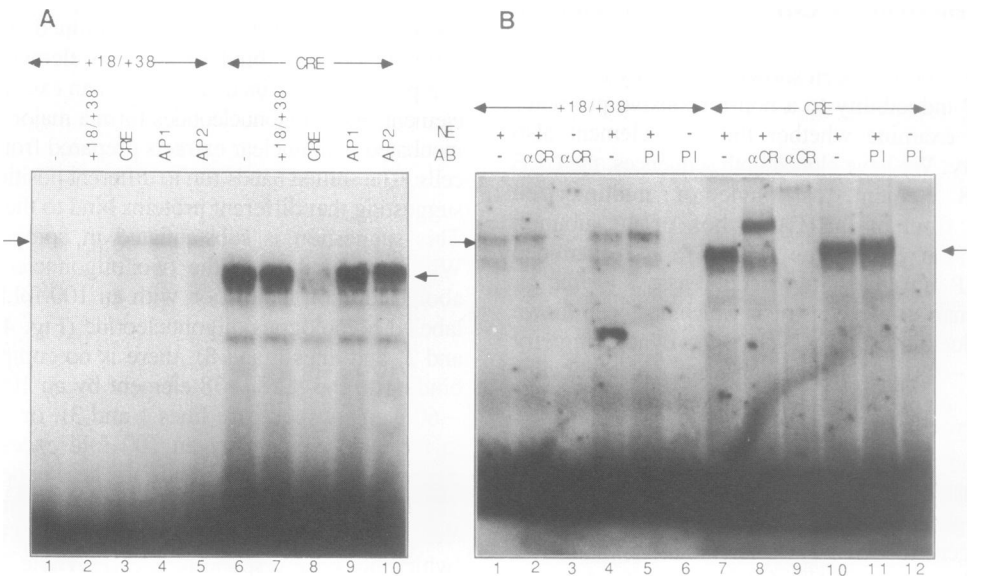

Figure 4. Different proteins bind to the +18/+38 and to the −60 (CRE) element. **A.** Nuclear extracts (4 μg) of growing 3T3 cells were incubated either with an end labeled +18/+38 oligonucleotide (lines 1 to 5) or an end labeled CRE oligonucleotide (lines 6 to 10) for 30 minutes at 20°C. Where indicated a 100 fold molar excess of unlabeled oligonucleotide (see Materials and methods) was added to the incubation mixture 15 min before the addition of the labeled oligonucleotide. After incubation free oligonucleotides were separated from protein bound oligonucleotides by native polyacrylamide gel electrophoresis in TBE buffer at 20°C. Arrows point to the major specific retarded bands. **B.** Nuclear extracts of growing 3T3 cells were incubated either with an end labeled +18/+38 oligonucleotide (lines 1 to 6) or an end labeled CRE oligonucleotide (lines 7 to 12) as above. Antibodies directed against CREB (lines 2 and 8) respectively preimmune serum (lines 5 and 11) were preincubated with nuclear extract at 20°C for 10 min before addition of the labeled oligonucleotides. Lines 3 and 9 show incubation of the oligonucleotides with CREB specific antibodies, lines 6 and 12 incubations with preimmune serum (without nuclear extract). αCR: antibodies directed against CREB; PI: preimmune serum.

element is also involved in phorbol ester and UV induction of the gene (11). 2) The element influences the rate of transcription but is still located within the transcribed region of the gene. This latter observation adds to other examples of transcriptional elements within genes that have been described previously (refs 39−45). Such gene segments may be involved in the control of transcription elongation and cannot freely be moved around. For instance elements at the first exon/first intron border control transcriptional elongation of c-fos and c-myc (39−42). Also HIV-1 and HIV-2 appear to be regulated by transcriptional elongation, mediated by the virus specific factor TAT and cellular proteins through the TAR region (43−45). With the new cAMP response element found in the transcribed region of the c-fos gene the situation seems to be different; it can be placed upstream of a TATA box and still control transcription of a reporter gene obviously controlling the initiation of transcription. This is reminiscent of the intragenic enhancers as found e.g. in the immunoglobulin genes (46−48), the collagen gene (49) and in the human Hepatitis B virus (50).

Our data are at variance, it seems, with other reports. According to ref. 16 a −53/+42 c-fos CAT gene construct (pFC53) is not cAMP inducible in NIH 3T3 cells, in transient transfection assays. We interpret this failure as being caused by the low sensitivity of the RNA assay used. The accumulation of CAT enzyme activity as measured here, increases sensitivity. Note that we see less induction in the transient as compared to the stable transfection conditions. Two other gene constructs described in ref. 16 are relevant: pFC53 to which a 650 bp fragment of the human histone 4A gene had been ligated (creating pH4FC53), and pFC53 to which the SV40 promoter/enhancer region had been linked (pSVFC53). Neither one of these constructs was cAMP inducible in transient transfection assays. The basal levels were, however, enhanced. The reason for the non-reactivity of these gene constructs to cAMP is not clear, but in view of the finding (17) that a −71 c-fos promoter construct carrying 6 copies of the core A enhancer element of SV40 is barely cAMP inducible (in contrast to a −71 c-fos promoter construct), it may well be that the sequences introduced to elevate basal level expression interfere negatively with cAMP induction.

Our data are also at variance with a second report (17): injection of an excess of the CRE oligonucleotide (position −60) into cells prevented c-fos induction by cAMP but not by serum. Since, as stated by the authors, the competing oligonucleotide does not only inhibit cAMP inducibility but also basal level expression of the gene, the contribution of the +18/+38 element may have been missed.

Why should a gene possess more than onc cis-acting element addressed by the cAMP dependent signal transduction pathway? The presence of two different elements and two different factors would make sense if they were utilized in different differentiation pathways or in different gowth conditions. We have no information on these options. The +18/+32 element is the third cAMP response element recognized, after the CRE and the AP-2 binding site (18, 38). The fact that the c-fos promoter is (increasingly) packed with regulatory elements (51) points at the key role of Fos in various physiological processes. Fos appears to represent a switch function in the nucleus (52, 53) that, through interacting with several other transcription factor subunits, participates in many processes. The novel protein binding site described here may differentially activate Fos protein synthesis in yet unknown physiologic conditions that do not involve CREB or AP-2, and thereby put Fos into another functional context.

ACKNOWLEDGEMENTS

We wish to thank Dr. G.Schütz, Heidelberg, for providing the CREB specific antibodies, Ulrike Wagner-Karch for synthesizing the oligonucleotides and Ingrid Kammerer for the preparation of the manuscript.

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