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Negative and positive regulation by transcription factor cAMP response element-binding protein is modulated by phosphorylation

(c-Jun protein/AP-1/c-Fos protein/protein kinase A/transrepression)

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ABSTRACT **We have shown that the transcriptional activity of the protooncogene *jun* (c-*jun*) promoter is repressed by a transcription factor, the cAMP response element-binding protein (CREB). This repression can be alleviated when CREB is phosphorylated by the catalytic subunit of protein kinase A. Repression cannot be alleviated by a mutant CREB deficient in the protein kinase A phosphorylation site (M1 CREB Ser-133 → Ala), suggesting that phosphorylation of CREB at this site is essential for the relief of repression. Repression by CREB requires its binding to the c-*jun* promoter. In NIH 3T3 cells stably expressing CREB, c-*jun* is no longer induced by serum, but this repression can be relieved by treatment of the cells with forskolin, an agonist of the adenylate cyclase pathway. Thus, CREB has a dual function, that of a repressor in the absence of phosphorylation and an activator when phosphorylated by protein kinase A.**

Regulation of gene expression is modulated by the interactions of positive and negative factors with specific DNA motifs (1). The AP-1 family of transcription factors serves as a good example because one of the members of the family, Jun protein, the product of protooncogene *jun*, can upregulate the transcription of its own gene, whereas a related JunB protein can suppress its transcription (2–5). Similarly, the product of the nuclear oncogene *fos* can suppress the transcription of its own promoter but activates the transcription of promoters containing AP-1 binding sites (6–9). The generic AP-1 binding site, TGACTCA, was first identified as the DNA motif responsible for induction with phorbol esters [phorbol 12-myristate 13-acetate (PMA) response element] (2, 10). The generic AP-1 binding site is very similar to the AP-1 site (TGACATCA) present in the human and mouse c-*jun* promoter (ref. 4; W.W.L., unpublished results). Surprisingly, the core consensus sequence TGACGTCA of the cAMP response element (CRE), required for induction by increases in intracellular cAMP levels, is very similar to the AP-1 binding site (11). We therefore sought to determine whether transcription factor CRE-binding protein (CREB) would act either synergistically, independently, or negatively in the presence of transcription factors, Jun or the Jun–Fos complex known to operate via the AP-1 site. Consequently we undertook experiments to determine the effect of CREB on a promoter containing an AP-1 site.

In this report we present data that shows that CREB binds to the AP-1 site present in the promoter of c-*jun* gene and, upon phosphorylation by the catalytic subunit of cAMP-dependent protein kinase A (PKA), will activate this promoter. More interestingly, however, CREB in the absence of PKA activity is a repressor of the c-*jun* promoter. The ability of CREB to repress transcription from the c-*jun* promoter extends to both serum and PMA induction. Additionally, the c-*jun* promoter can be activated by Jun or Fos–Jun proteins, but CREB can still interfere with this transactivation. Furthermore, a DNA-binding mutant of CREB that does not bind to CREs or to the c-*jun* AP-1 site does not interfere with induction of the *jun* promoter, suggesting an interference with binding of other transcription factors. We propose that transcription factor CREB may have a pleiotropic function, acting as a potent repressor of transcription, but reversing its role after phosphorylation.

MATERIALS AND METHODS

Cell Culture and Transfection. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf bovine serum. For transfection, the cells were seeded 24 hr before transfection at 5×10^5 cells per 10-cm tissue culture dish in DMEM/10% calf bovine serum. The cells were transfected by the calcium phosphate coprecipitation technique and exposed to the precipitate for 12–16 hr. After transfection, the cells were washed with phosphate-buffered saline and fed DMEM/0.5% calf bovine serum. The transfected cells were cultured for an additional 24 hr and then either harvested (serum-starved conditions) or treated with 20% fetal bovine serum/DMEM for 2 hr before harvesting the cells (serum-stimulated conditions). Chloramphenicol acetyltransferase (CAT) activity was determined as described (12).

For the production of stable cell lines, NIH 3T3 cells were transfected as described above with a 10:1 molar ratio of pBKCREB:pSV2neo. Forty-eight hours after transfection the cells were subcultured in G418 at 400 μg/ml. Two independent CREB-expressing clones were isolated; each clone had the same phenotype.

Plasmids. Standard molecular biology techniques (13) were used to generate the murine c-*jun*–CAT reporter plasmid pJC6. Briefly, a 365-base-pair (bp) fragment from −217 to +148 (numbering from the major start site in the human c-*jun* promoter; ref. 4) of the murine c-*jun* promoter was cloned into the CAT expression vector pBLCAT3 (14). The other plasmids used for transfection have been described: pBK28 encodes the human c-*fos* cDNA expressed by the Finkel–Biskis–Jinkins murine sarcoma virus long terminal repeat (6); pBKCREB encodes the rat CREB cDNA expressed from the Finkel–Biskis–Jinkins murine sarcoma virus long terminal repeat (15); pM1CREB encodes a mutant CREB cDNA in which Ser-133 was converted to alanine expressed from a Rous sarcoma virus long terminal repeat (16); CREB mutant

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Abbreviations: PKA, protein kinase A; CRE, cAMP response element; CREB, CRE-binding protein; CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate.

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K304E was made in the pBKCREB background (15); pSV-c-*jun* encodes the murine c-*jun* cDNA expressed from the simian virus 40 early promoter (17); and pSKG4 encodes the catalytic subunit of PKA expressed from the simian virus 40 early promoter (18). For competition experiments with the CRE site, a synthetic double-stranded deoxyoligonucleotide, TAAATATCCCTGACGTCTGCGCTGACGCAG, was cloned into pGEM4.

Constructs for the production of cRNA probes used in the RNA-protection assays include the following: pIcj409, in which a 415-bp *Bss*HI–*Bss*HI fragment (corresponding to positions 544 and 959 in the murine c-*jun* cDNA; ref. 17) was cloned into pIBI30; pCAT250 in which the 250-bp *Eco*RI–*Hin*dIII fragment of pBLCAT3 was subcloned into pGEM4; and pSPT672 (20) contains the murine β_2-microglobulin second exon cDNA.

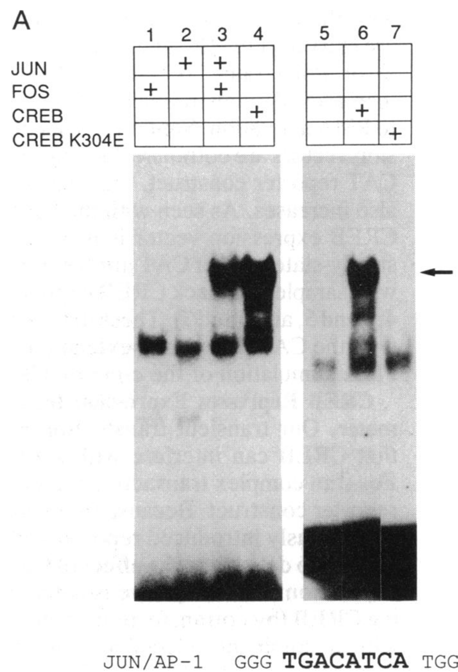
Gel Retardation Assays. Gel retardation assays were done as described (6) by using a synthetic double-stranded deoxyoligonucleotide containing the c-*jun* AP-1 site. The sequence of the murine c-*jun* AP-1 site is as follows:

CCTCGGG**GTGACATCAT**GGGCTA
GGAGCCC**CACTGTAGTA**CCCGAT

The complete sequence of the murine c-*jun* promoter including 217 nucleotides upstream of the major start site will be published elsewhere (W.W.L., unpublished results). The murine c-*jun* promoter shares 94% nucleotide sequence identity with the human c-*jun* promoter. *In vitro* translation of proteins from constructs have been described (21). RNA-protection assays were done as described (22, 23). Approximate sizes of the protected fragments are as follows: Jun, 400 bp; CAT, 250 bp; β_2-microglobulin, 180 bp.

RESULTS

Repression of Transcription from an AP-1 Site by CREB. We first determined whether CREB can bind to an AP-1 site. A double-stranded deoxyoligonucleotide corresponding to the region containing the c-*jun* AP-1 site was synthesized and used for gel-retardation assays to determine the extent of binding of various proteins to this deoxyoligonucleotide. The data show (Fig. 1*A*) that *in vitro*-translated Jun protein binds to the AP-1 site (lane 2) and, in accordance with the previously published results (21), binding with Fos–Jun complex is very efficient (lane 3). When *in vitro*-translated CREB was used in the gel-retardation assay, we found that it bound to the AP-1 site even more efficiently (lanes 4 and 6), suggesting that CREB will bind to the c-*jun* AP-1 site. In a second experiment a DNA-binding mutant of CREB (K304E) that is unable to bind to a CRE (15) is also unable to bind to the AP-1 site (lane 7).



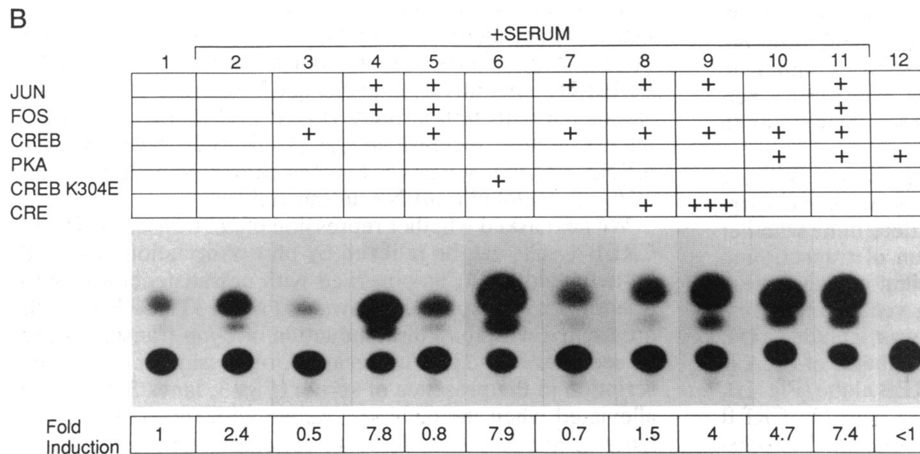


FIG. 1. (*A*) DNA-binding properties of Jun, Fos, Fos–Jun, CREB, and mutant CREB K304E proteins on the c-*jun* AP-1 site by gel-retardation analysis. Unlabeled *in vitro* translated proteins (as indicated) were incubated with the c-*jun* AP-1 site (lanes 1–7) before gel electrophoresis. The specific retarded band is indicated by the arrow; the other major band shift is caused by some protein in the rabbit reticulocyte extract (lane 5). The core consensus sequence for the c-*jun* AP-1 site is given below. Lanes: 1, Fos protein; 2, Jun protein; 3, Jun–Fos protein; 4, CREB; 5, rabbit reticulocyte extract; 6, CREB; 7, mutant CREB unable to bind DNA. (*B*) Transactivation and repression of the c-*jun* promoter–CAT reporter construct. Transient transfection assays in NIH 3T3 cells. The transfected cells were starved (lane 1) or stimulated with serum (lanes 2–11). The plasmids transfected in each lane are indicated by pluses. The c-*jun* promoter CAT reporter (pJC6) (4 μg) was transfected in all samples. The amount of each plasmid transfected is as follows: Jun, 10 μg; Fos, 6 μg; CREB or CREB K304E, 6 μg except lanes 7–9, where 2 μg of CREB wild type is used; PKA (the catalytic subunit), 4 μg; the competitor CRE site, 2 μg (lane 8) and 6 μg (lane 9). Percent converted was determined by scintillation counting of the modified and unmodified forms of chloramphenicol and is shown as fold induction relative to unstimulated sample (lane 1).

We next wanted to determine whether CREB has any functional effect on the transcription from a promoter containing an AP-1 site. When the reporter construct pJC6 is transfected into NIH 3T3 cells and the cells are starved for serum, there is a low basal level of CAT expression in these cells (Fig. 1*B*, lane 1). This low basal level of expression is consistent with the low basal level of endogenous c-*jun* expression in serum-starved NIH 3T3 cells (17, 24, 25). When the transfected cells are starved and then stimulated with serum (Fig. 1*B*, lane 2) there is a 2- to 3-fold increase in CAT activity (in the experiment shown it is 2.4-fold), demonstrating that the reporter construct responds to serum stimulation. Surprisingly, when the CREB expression vector (pBKCREB) (15) is cotransfected with the reporter pJC6, the c-*jun* promoter CAT construct is unable to respond to serum stimulation (compare Fig. 1*B*, lane 2 with lane 3). In fact, when the CREB expression vector is included in the transfection assay, the CAT activity in these cells is consistently decreased to a level below that of the serum-starved basal level of the reporter (compare lanes 1 and 3). Because CREB expression had a negative effect on both the serum-stimulated and basal level of the c-*jun* promoter CAT vector, we wanted to determine the effect CREB had on the transactivation of pJC6 by the Fos–Jun complex (lane 4). As with serum stimulation, CREB expression abolishes the ability of the Fos–Jun complex (compare lanes 4 and 5) to transactivate the c-*jun* promoter CAT construct. Again, CREB expression is able to decrease the level of CAT expression from pJC6 to a level equal to or below the serum-starved basal level of the reporter construct (compare lanes 1 and 5).

Binding of CREB to the AP-1 Site Is Required for Repression. To determine whether the ability of CREB to interfere with transactivation by serum or the Fos–Jun complex requires the DNA-binding of CREB, we conducted transient transfection experiments with a CREB mutant that is unable to bind to DNA (Fig. 1*A*, lane 7). Mutant CREB K304E, which cannot bind to either the somatostatin CRE site or the c-*jun* AP-1 site, is unable to interfere with Jun or serum transactivation of the c-*jun* promoter (Fig. 1*B*, lane 6). Therefore, the DNA-binding ability of CREB is required for interference with transactivation of the c-*jun* promoter CAT construct. Because DNA-binding of CREB is required for interference of transactivation of the c-*jun* promoter, we wanted to determine, using competition experiments, whether we could alleviate the repression of the c-*jun* promoter by adding increased amounts of a plasmid containing only a CRE site. In these experiments the amount of the CREB expression vector was decreased from 6 to 2 μg without compromising interference of transactivation (Fig. 1*B*, lane 7). When increased amounts of the competitor plasmid containing only CRE site (2 μg in lane 8 or 6 μg in lane 9) are included in transfection, the ability of CREB to interfere with transcription of c-*jun* promoter is decreased in a dose-dependent manner (compare Fig. 1*B*, lanes 7–9). These experiments strongly imply a direct competition between CREB and Jun or the Fos–Jun complex for binding to the c-*jun* AP-1 site. Alternatively, CREB may form a heterodimer with the Jun or Fos protein and alter their ability to activate transcription.

Phosphorylation of CREB Relieves Repression. Because phosphorylation of CREB is obligatory to activate transcription from CRE (16, 26, 27), we wanted to determine whether its phosphorylation will relieve repression of transcription from the AP-1 site. We carried out transient cotransfection experiments in NIH 3T3 cells in which a vector expressing the catalytic subunit of PKA (pSKG4) was included (18). When the vector expressing the catalytic subunit of PKA is included in the transfection with either CREB alone (Fig. 1*B*, lane 10), or CREB plus the Fos–Jun complex (lane 11), CREB loses its ability to interfere with c-*jun* promoter CAT expression. Therefore, modification of CREB by PKA activity may alter the affinity of CREB for the AP-1 site and allow Jun or the Fos–Jun complex to activate expression. Alternatively, CREB when modified by PKA activity could act as a positive regulator and activate c-*jun* expression. No effect on repression or activation was seen with PKA alone (Fig. 1*B*, lane 12).

Addition of the catalytic subunit of PKA could affect the activity of other transcriptional factors including Jun and Fos. To confirm that relief of CREB repression by PKA is a direct consequence of its phosphorylation, we used a CREB mutant (M1 CREB) deficient in the PKA phosphorylation site (Ser-133 was mutated to alanine; ref. 16). Results in Fig. 2*A* show that M1 CREB can repress AP-1-dependent promoter activity observed with Jun (data not shown) or Fos–Jun complex like wild-type CREB (compare lanes 4 and 6), but unlike the wild-type CREB (lane 5) it is unable to show any activation upon treatment with the catalytic subunit of PKA (lane 7). We therefore conclude that phosphorylation of CREB is obligatory to relieve repression and perhaps activate transcription.

CREB also Inhibits PMA Stimulation of the c-*jun* Promoter. To confirm and to extend the CAT assay results, we isolated cytoplasmic RNA from NIH 3T3 cells transiently transfected with the reporter pJC6. Analysis by RNase protection assays using a CAT-specific cRNA probe confirmed the data obtained with the CAT assays. When the transiently transfected cells are starved (Fig. 2*B*, lane 1) and then treated with serum (lane 2) or PMA (lane 3), the steady-state level of CAT RNA increases, demonstrating that the reporter construct responds to PMA activation. Similarly, when the c-*jun* and c-*fos* expression vectors are cotransfected (lane 6) with the c-*jun* promoter CAT reporter construct, the steady-state level of CAT RNA also increases. As seen with the CAT assay results, when the CREB expression vector is included in the transfection, the steady-state level of CAT mRNA is decreased when compared with samples that lack CREB expression (compare lanes 2 and 4, 3 and 5, and 6 and 7). These data confirm the results obtained with the CAT assays and extend the results to interference of PMA stimulation of the c-*jun* by CREB.

CREB Represses Expression from Endogenous c-*jun* Promoter. Our transient transfection experiments have shown that CREB can interfere with serum induction and Jun or Fos–Jun complex transactivation of the c-*jun* promoter CAT reporter construct. Because these were transient assays with exogenously introduced reporter and expression vectors, we wanted to determine the effect of CREB on endogenous c-*jun* expression. To do this, we isolated cell lines stably expressing CREB (by cotransfection of the CREB expression vector with a dominant selectable marker, pSV2neo) and tested them for c-*jun* mRNA induction by serum. When NIH 3T3 cells are starved and then induced with serum, there is a rapid and transient increase (16-fold in this experiment) in c-*jun* mRNA (Fig. 3; lanes 1–4). In contrast, when a cell line (3T3 CREB-1) constitutively expressing CREB is starved and then stimulated with serum, there is little (<2-fold in this experiment), if any, increase in the steady-state level of c-*jun* mRNA (see Fig. 3, lanes 5–8) when compared with the levels of β_2-microglobulin mRNA in the cell.

We next asked whether repression of *jun* transcripts in 3T3 CREB-1 cells can be relieved by phosphorylation of CREB by activated PKA, as observed with cotransfection experiments (Fig. 1). Fig. 3 also shows that when 3T3 CREB-1 cells are treated with forskolin, induction of c-*jun* transcripts can be seen (lanes 9–11). Furthermore, repression of c-*jun* transcription in the presence of serum (Fig. 3, lanes 5–8) is also alleviated when the cells are treated with forskolin (Fig. 3, lanes 12 and 13). Therefore, we conclude that repression by

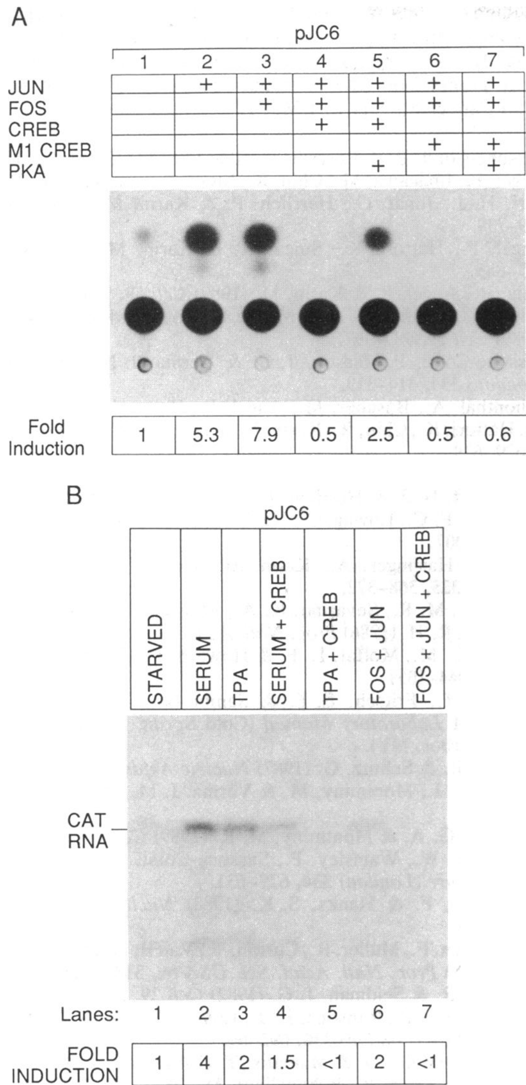


FIG. 2. (*A*) Relief of repression requires phosphorylation of CREB. The transiently transfected cells were starved for serum (lanes 1–7). CAT reporter plasmid pJC6 (4 μg) was transfected in all lanes; other plasmids transfected in each sample are indicated. The amount of each plasmid transfected was the same as described to Fig. 1*B* except M1 CREB, 6 μg. The mutant M1 CREB contains a Ser-133 → Ala mutation such that it cannot be phosphorylated by the catalytic subunit of PKA (16). (*B*) PMA stimulation of the c-*jun* promoter CAT construct is also inhibited by CREB, RNA-protection analysis. NIH 3T3 cells transiently transfected with pJC6 (lanes 1–7) were starved (lane 1) or stimulated with serum (lane 2) or PMA (TPA) (lane 3). Lanes: 4, serum plus CREB, 5, PMA plus CREB; 6, Fos–Jun; 7, Fos–Jun plus CREB. Additional plasmids transfected are as follows: CREB, 6 μg (lanes 4, 5, and 7); Fos, 6 μg (lanes 6 and 7) and Jun, 10 μg (lanes 6 and 7). To determine the amount of CAT mRNA present, total cytoplasmic RNA (20 μg) from each sample was hybridized with a CAT-specific cRNA probe and analyzed by RNA-protection analysis; the protected fragment is indicated. Fold induction was determined by scanning the autoradiogram with a densitometer and is given relative to the level of CAT mRNA under serum-starved (lane 1) conditions. Numbers represent the average of two assays.

CREB and relief after its phosphorylation seen in transiently transfected cells is also seen in the normal genomic setting.

DISCUSSION

We have studied the interaction and subsequent effects of the transcription factor CREB on the activity of a promoter containing an AP-1 site. Using transient transfection assays in NIH 3T3 cells, we have shown that CREB represses the transcription of the c-*jun* promoter and that this repression can be alleviated if CREB is phosphorylated by the catalytic subunit of PKA. Our data suggest that this interference with c-*jun* promoter activity requires a CREB that can bind to an AP-1/CRE-like site because cotransfection of a plasmid containing only a CRE site can abolish the ability of the CREB to interfere with c-*jun* promoter activation. From these data, we conclude that CREB must directly bind to the c-*jun* AP-1 site and that this binding interferes with the binding of other transcriptional factors (Fos–Jun) to the c-*jun* promoter. In addition, phosphorylation of CREB allows allosteric activation of a domain capable of transcriptional transactivation.

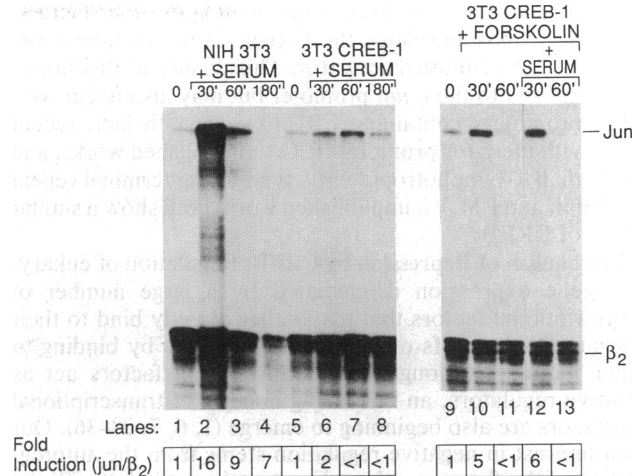


FIG. 3. CREB expression represses the endogenous c-*jun* promoter, RNA-protection analysis. NIH 3T3 cells or a cell line stably expressing CREB, 3T3 CREB-1, were starved (lanes 1 and 5) or stimulated with serum (lanes 2–4 and 6–8). Total cytoplasmic RNA was isolated at the minutes (') indicated, hybridized with both c-Jun-specific and β_2-microglobulin-specific cRNA probes and analyzed for RNA-protection analysis. The specific protected fragments are indicated for Jun and for β_2-microglobulin (β_2; RNA loading control). Fold induction was determined by densitometric scanning of the autoradiogram for both the Jun-specific fragment and the β_2-microglobulin-specific fragment. The amount of Jun-specific RNA was determined relative to the total amount of RNA present (β_2-microglobulin-specific fragment), and fold induction is given relative to serum-starved level of Jun mRNA. The CREB-expressing cell line, 3T3 CREB-1, was serum starved (lane 9) and then stimulated with either 20 μM forskolin (lanes 10 and 11) or forskolin plus serum (lanes 12 and 13). Total cytoplasmic RNA was isolated at the indicated time in minutes, hybridized with both c-Jun and β_2-microglobulin-specific cRNA probes, and RNA-protection analysis was done; the specific protected fragments are indicated. Fold induction was determined as described above. For densitometric quantification of β_2-microglobulin-protected fragment, a shorter exposure was used.

CREB Functions Like a Switch. The data presented here establish the dual function of CREB. On the one hand, it can function as a repressor of a promoter containing an AP-1 site, whereas, on the other hand, it acts as a transcriptional activator of the same promoter. The negative and positive regulation by CREB is modulated by its phosphorylation status. Transcriptional transactivation by CREB is controlled by PKA-induced phosphorylation of a specific serine residue contained within a consensus PKA phosphorylation site of CREB (16). The data with the CREB M1 mutant (Ser-133 → Ala), which is unable to be phosphorylated by PKA, support the idea that the pleiotropic effect of CREB is controlled by PKA-induced phosphorylation. Hence, it would appear that CREB can bind to promoters containing CRE-like sites but

acts as a repressor (by preventing binding of other factors) until PKA phosphorylates the CREB, at which time it becomes a transcriptional activator. This duality of function is not restricted to the c-*jun* promoter but may also occur with other promoters containing CRE-like sites. In fact, recent work with the c-*fos* promoter (R.O., unpublished work), and the human T-lymphotropic virus type I long terminal repeat (M. Fujii, and I.M.V., unpublished work) both show a similar effect of CREB.

Mechanism of Repression by CREB. Regulation of eukaryotic gene expression is mediated by a large number of transcriptional factors that may either directly bind to their cognate DNA motifs or exert their influence by binding to other factors. Although most transcription factors act as positive regulators, an increasing number of transcriptional repressors are also beginning to emerge (1, 6, 7, 28–36). Our own interest in negative regulation stems from the autoregulation of the *fos* gene, where Fos protein can "shut off" the transcription of its own promoter (6, 7). The precise mechanism of negative transcriptional regulation by eukaryotic transcriptional factors is not understood, but it could act by the following three likely modes. (*i*) A transcriptional repressor protein can interact with other DNA sequence-specific activator proteins and consequently inactivate them (30, 31). (*ii*) Transcriptional factors can bind to DNA in a sequence-specific manner, thereby influencing the binding of other activator proteins. For instance, such a mechanism anticipates the influence of a repressor at some distance from the binding site. One can imagine a transcription factor with a DNA-binding domain and an activator domain, in which the DNA-binding domain will bind in a sequence-specific manner and the activator domain can cause interference by interacting with another activator protein (36). (*iii*) The binding of the transcriptional factor to the specific DNA sequence can sterically hinder the binding of another factor. One such example would be the negative regulation by glucocorticoids where the glucocorticoid receptor binds to a region overlapping with cAMP enhancer element (32–34). We believe that CREB repression is carried out by steric interference of binding of the Fos–Jun heterodimer. Once CREB is modified by phosphorylation of a specific site, it can function in two ways, either (*i*) the phosphorylation causes some allosteric change in the protein that allows its transactivation domain to interact with other transcriptional factors, or (*ii*) the phosphorylated form of CREB has a greater binding affinity to its cognate CRE than to the AP-1 site. Currently we cannot resolve these two modes but favor the former because allosteric modification of CREB after phosphorylation can be demonstrated (M.M., unpublished results). It is interesting to note that unmodified CREB is a repressor, whereas the phosphorylated form is an activator. This contrasts with Fos protein, the phosphorylated forms of which act as a repressor (R.O., unpublished data).

Finally, it is worth noting that CREB belongs to a multigene family and may have multiple siblings because a number of CREB-related proteins have been identified from mammals to plants (37–40). Whether the binding affinities of these proteins to their cognate DNA-binding sites are different for various members of these families or whether they have differential tissue distribution is yet unknown. If, however, the CREB-related proteins interact similarly to CREB, which can be either a negative or a positive transcriptional regulator, the vast combinatorial possibilities of these transcription factors to regulate gene expression during growth, differentiation, and development can be envisaged.

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