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L-type Voltage-sensitive Ca^{2+} Channel Activation Regulates c-*fos* Transcription at Multiple Levels*

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A mechanism by which voltage-sensitive Ca^{2+} channel (VSCC) activation triggers c-*fos* transcription has been characterized. Ca^{2+} influx through VSCCs stimulates phosphorylation of the transcription factor cAMP response element-binding protein (CREB) on serine 133 leading to an increase in the formation of transcription complexes that can elongate through a transcription pause site within the c-*fos* gene. Ca^{2+}-stimulated CREB serine 133 phosphorylation is mediated by a Ca^{2+}-activated kinase and is not dependent on the cAMP-dependent protein kinase (PKA). While necessary for c-*fos* transcriptional induction following VSCC opening, CREB serine 133 phosphorylation is not sufficient for transcriptional activation. A second, PKA-dependent event is required. Following induction, c-*fos* transcription is rapidly down-regulated. Dephosphorylation of CREB serine 133 parallels and likely mediates the transcriptional shut-off event. These results suggest that the phosphorylation and dephosphorylation of CREB controls its ability to regulate transcription in membrane-depolarized cells and that multiple pathways contribute to Ca^{2+}-activated gene expression.

An important mechanism by which neurotransmitters are believed to trigger long term adaptive neuronal responses is by activating specific programs of gene expression (1, 2). Within minutes of neurotransmitter release, the expression of a family of genes termed immediate early genes (IEGs)[1] is induced in the post-synaptic neuron. Many IEGs encode transcription factors that then induce subsequent waves of late response gene expression. Late response genes encode proteins that are likely to be determinants of neuronal plasticity. These proteins may include neurotransmitter-synthesizing enzymes, neurotransmitter receptors, neurotrophins, as well as structural components of the synapse.

One critical issue that remains to be solved is how a signal that is initiated by neurotransmitter release at the synapse is propagated to the nucleus of a post-synaptic cell to activate IEG transcription. Studies of one IEG, the c-*fos* proto-oncogene, have begun to give insight into this process. A variety of neurotransmitters that trigger Ca^{2+} influx have been found to stimulate c-*fos* transcription rapidly and transiently both *in vitro* in cell culture systems (3–5) and also trans-synaptically in the intact nervous system (6–9). The c-*fos* gene encodes a transcription factor, Fos, which forms a heterodimer with members of the Jun family of transcription factors and regulates the expression of late response genes that contain Fos/Jun-binding sites (consensus 5′-TGAG/CTCA-3′) within their promoters (10).

The pheochromocytoma cell line PC12 has proven to be a useful model for elucidating the mechanisms by which neurotransmitters that trigger Ca^{2+} influx activate c-*fos* transcription. Exposure of PC12 cells to elevated levels of extracellular KCl depolarizes the plasma membrane and stimulates the opening of L-type voltage-sensitive Ca^{2+} channels (VSCCs). The subsequent rise in the level of intracellular Ca^{2+} induces c-*fos* transcription (4, 11). A Ca^{2+} response element (CaRE), located 60 nucleotides 5′ of the initiation site for c-*fos* mRNA synthesis, plays an important role in mediating the c-*fos* response to VSCC activation (12). The −60 CaRE (-TGACGTTT-) is similar in sequence to a consensus cAMP response element (CRE) (-TGACGTCA-) present within the regulatory regions of a variety of genes that become activated when cells are exposed to agents such as forskolin that activate adenylate cyclase and stimulate cAMP production (13). When the c-*fos* CaRE/CRE is inserted within the promoter of reporter genes that are normally non-responsive to forskolin or VSCC channel activators the c-*fos* CaRE/CRE confers responsiveness to these agents. The −60 CaRE functions by interacting with the cAMP response element-binding protein (CREB) which becomes newly phosphorylated at a critical regulatory site, serine 133, within minutes of exposure of PC12 cells to elevated levels of KCl or forskolin (12). Phosphorylation of serine 133 is critical for CREB-mediated transcription inasmuch as a mutation that converts serine 133 to an alanine inhibits CREB's ability to activate transcription in response to treatment with VSCC activators or forskolin (14–16).

How phosphorylation of CREB serine 133 stimulates the ability of this transcription factor to induce gene expression is not completely resolved. While phosphorylation of serine 133 may enhance the binding of CREB to CREs that might otherwise bind it with low affinity (17), the major effect of serine 133 phosphorylation is to increase CREB's ability to activate transcription once bound to the CaRE/CRE (14). Whether CREB phosphorylation regulates transcription by enhancing the rate of formation of new transcription complexes at the promoter (new initiation), or by affecting the efficiency with which transcription complexes transcribe the length of the c-*fos* gene

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[1] The abbreviations used are: IEGs, immediate early genes; VSCC, voltage-sensitive Ca^{2+} channel; CaRE, Ca^{2+} response element; CRE, cAMP response element; CREB, cAMP response element binding protein; PKA, cAMP-dependent protein kinase; CaM, Ca^{2+} calmodulin; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; SRE, serum response element; PAGE, polyacrylamide gel electrophoresis.

(elongation) remains to be determined.

Within minutes of its activation by neurotransmitters, expression of the c-*fos* gene is repressed (4). The rapid disappearance of the c-*fos* mRNA reflects the fact that this mRNA has an extremely short half-life (18) but is also a consequence of transcriptional repression. With respect to Ca^{2+} regulation of c-*fos* transcription, the mechanism of transcriptional shut-off has not been characterized. One possibility is that the −60 CaRE and its associated protein, CREB, mediate both the transcriptional activation and the subsequent shut-off event. Since the phosphorylation of CREB serine 133 triggers c-*fos* activation, the dephosphorylation of phosphoserine 133 might lead to the shut-off of c-*fos* transcription. An alternative possibility is that distinct promoter elements and transcription factors control the initial c-*fos* activation and subsequent shut-off events.

The kinase(s) that catalyzes the phosphorylation event upon activation of the L-type VSCCs has been a subject of controversy. It is well established that in forskolin-treated cells elevated levels of cAMP lead to the activation of the cAMP-dependent protein kinase (PKA) which then translocates to the nucleus where it almost certainly catalyzes the phosphorylation of CREB serine 133 (15, 19, 20). However, PKA's involvement in the Ca^{2+} signaling pathway remains unresolved. Several studies suggest that membrane depolarization of PC12 cells does not effectively stimulate cAMP accumulation, suggesting that PKA is not likely to be activated and is probably not the enzyme that triggers CREB serine 133 phosphorylation under these conditions (12, 21). In this case, a Ca^{2+}-activated enzyme, such as a Ca^{2+}-calmodulin dependent (CaM) kinase, was hypothesized to phosphorylate CREB in response to membrane depolarization. However, in another study using PKA-deficient cell lines, VSCC activation failed to effectively stimulate c-*fos* transcription, even though CaM kinases were activated (22). This finding suggested that PKA activity is important for depolarization activation of c-*fos* transcription and that it might mediate CREB serine 133 phosphorylation.

In this report, using the c-*fos* CaRE/CRE as a model, we have characterized further the signaling pathways by which VSCCs trigger gene expression. We have investigated the mechanism of transcriptional initiation and provide evidence suggesting that upon VSCC activation CREB phosphorylation leads to an increase in the rate of formation of new transcription complexes. In addition, these newly formed transcription complexes appear to have an increased efficiency of elongation through an intragenic transcription pause site. The mechanism of transcriptional shut-off that occurs subsequent to VSCC activation also has been characterized. Dephosphorylation of CREB was found to play a role in the down-regulation event. Furthermore, characterization of the signaling pathways by which VSCCs lead to CREB phosphorylation demonstrated that CREB phosphorylation is not mediated by PKA but is likely to be catalyzed by a Ca^{2+}-activated kinase. While CREB serine 133 phosphorylation is necessary for Ca^{2+}-induced CaRE/CRE-dependent transcription, it is not sufficient. In addition to CREB serine 133 phosphorylation, a second, PKA-dependent signaling pathway is also required for CREB-mediated transcriptional activation of c-*fos*.

MATERIALS AND METHODS

Plasmids—The following plasmids have all been described previously: pAF4, pAF42, and pAF42CRE (12); pAF42SRE (23); pAF42G9, GAL4CREB, and GAL4CREBΔLZ (14); GAL4(1–147) and GAL4VP16 (24); 71WTfosCAT and 71 pm3fosCAT (25); and pSVα-1 (26). The plasmids that served as templates for synthesis of RNase protection probes have also been described: pSP6-c-*fos*, (27); pSP6α133 (28); pT7α181 (28); and p149 (29).

1S, 1AS, 2S, and 2AS are sense (S) or antisense (AS) rat c-*fos* cDNA fragments from the first (1S or 1AS) or second, third, and part of the fourth (2S or 2AS) exons cloned into M13. To generate the 1S and 1AS constructs, pSP65r-fos (gift from T. Curran, Nutley, NJ) was digested with *Bgl*II and *Eco*RI and the 290-base pair fragment was then ligated into M13mp18 (for 1S) or M13mp19 (for 1AS) that had been digested with *Bam*HI and *Eco*RI. For the 2S and 2AS constructs, pSP65r-fos was digested with *Bgl*I, blunted, and then digested with *Bst*YI. The 461-base pair fragment was then ligated into either Mp13mp18 or Mp13mp19 digested with *Sma*I and *Bam*HI. M13MATA contains a 720-base pair fragment from the mouse α-tubulin inserted into M13 (23).

Cell Transfections and Stimulation—The PKA-deficient PC12-derived cell line (123.7) stably expresses a mutant regulatory subunit of the PKA that does not bind cAMP (30). Both wild type and mutant PC12 cells were grown on collagen-coated plates in 10% CO_2, in Dulbecco's modified Eagle's medium with 10% horse serum, 5% fetal calf serum, 0.01% penicillin, and 0.01% streptomyocin. The day before transfection, 5×10^6 cells were plated on 100-mm poly-D-lysine-coated tissue culture plates. Plasmids were transfected by the Ca^{2+} phosphate technique (27, 31) with a total of 20 μg of a c-*fos* reporter and a carrier (pUC19 or pUClacZ) plasmid, and 3 μg of pSVα-1 internal control plasmid. In the GAL4 experiments, an additional 5 μg of a GAL4 fusion plasmid was also transfected. Where indicated, PC12 cells were stimulated with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (0.3 μg/ml), forskolin (10 μM), or KCl (60 mM). Concentrations in parentheses are the final concentrations in the growth media. For addition to growth media, TPA (stock solution was 1 mg/ml in Me_2SO) was diluted to 100 times in Dulbecco's modified Eagle's medium, forskolin stock solution was 10 mM in ethanol, and KCl stock solution was 150 mM. In immunoprecipitation and immunoblot experiments, forskolin was diluted in Dulbecco's modified Eagle's medium before addition to cells.

RNA Analysis—Total cytoplasmic RNA extraction and RNase protection analysis of transcripts from the human and rat c-*fos* and human α-globin genes have been described (26). Protection of fosCAT transcripts also has been described (29). The α-globin riboprobe synthesized from the pT7α181 template was used to protect α-globin transcripts in the experiments where fosCAT constructs were transfected.

Nuclear Run-on Transcription Assay—Nuclei were isolated from PC12 cells as described (32), except that nuclei were resuspended in Nonidet P-40 lysis buffer, recentrifuged, and resuspended in glycerol storage buffer for storage at −80 °C until use. Nuclear run-on transcription reactions were performed as described (33). Nuclei were incubated at 30 °C for 30 min with nucleotides, including 10 μl of [^{32}P]UTP (800 Ci/mM). Reactions were subsequently treated with DNase I and then proteinase K. RNA was isolated by the guanidinium thiocyanate-phenol-choloroform procedure (34). RNA (equal numbers of incorporated counts/min) was hybridized for 36 h to DNA probes immobilized on nitrocellulose (23). Following hybridization, filters were washed twice for 60 min at 65 °C in 2 × SSC, once at 37 °C for 30 min in 2 × SSC, 10 μg/ml RNase A, and once again at 37 °C in 2 × SSC for 30 min. Hybridized RNA was detected by autoradiography.

Immunoprecipitations and Immunoblots—Immunoprecipitations and immunoblots were performed as described (35). Anti-CREB is rabbit antiserum directed against the first 205 amino acids of CREB fused to trpE (35). For immunoblots, a 1/5000 to 1/20000 dilution of affinity purified anti-CREB was used. Anti-phosphoCREB is a rabbit antiserum directed against the phosphorylated peptide, KRREILSRRPS(PO_4)YRK (CREBtide) (35). The IgG fraction was purified by protein A-Sepharose chromatography and further purified by application to an affinity chromatography column containing unphosphorylated CREBtide. The concentration of the IgG fraction was 0.7 mg/ml. The IgG fraction or the affinity purified anti-phosphoCREB were used at dilutions of 1/5000 to1/20000 in immunoblotting analysis, and the IgG fraction was used at a 1/200 dilution in immunoprecipitations.

RESULTS

Membrane Depolarization Induces an Increase in the Formation of New Transcription Complexes at the c-fos Promoter—The CaRE/CRE located at 60 nucleotides 5′ of the start site of c-*fos* mRNA synthesis plays an important role in mediating the effects of membrane depolarization and Ca^{2+} on c-*fos* transcription (12, 26). CREB, which binds the CaRE/CRE, becomes phosphorylated on serine 133 upon exposure of PC12 cells to elevated levels of KCl. This phosphorylation event has previously been shown to be necessary for transcriptional induction (14). It has not been determined whether CREB phosphorylation regulates transcription by affecting the rate of initiation of

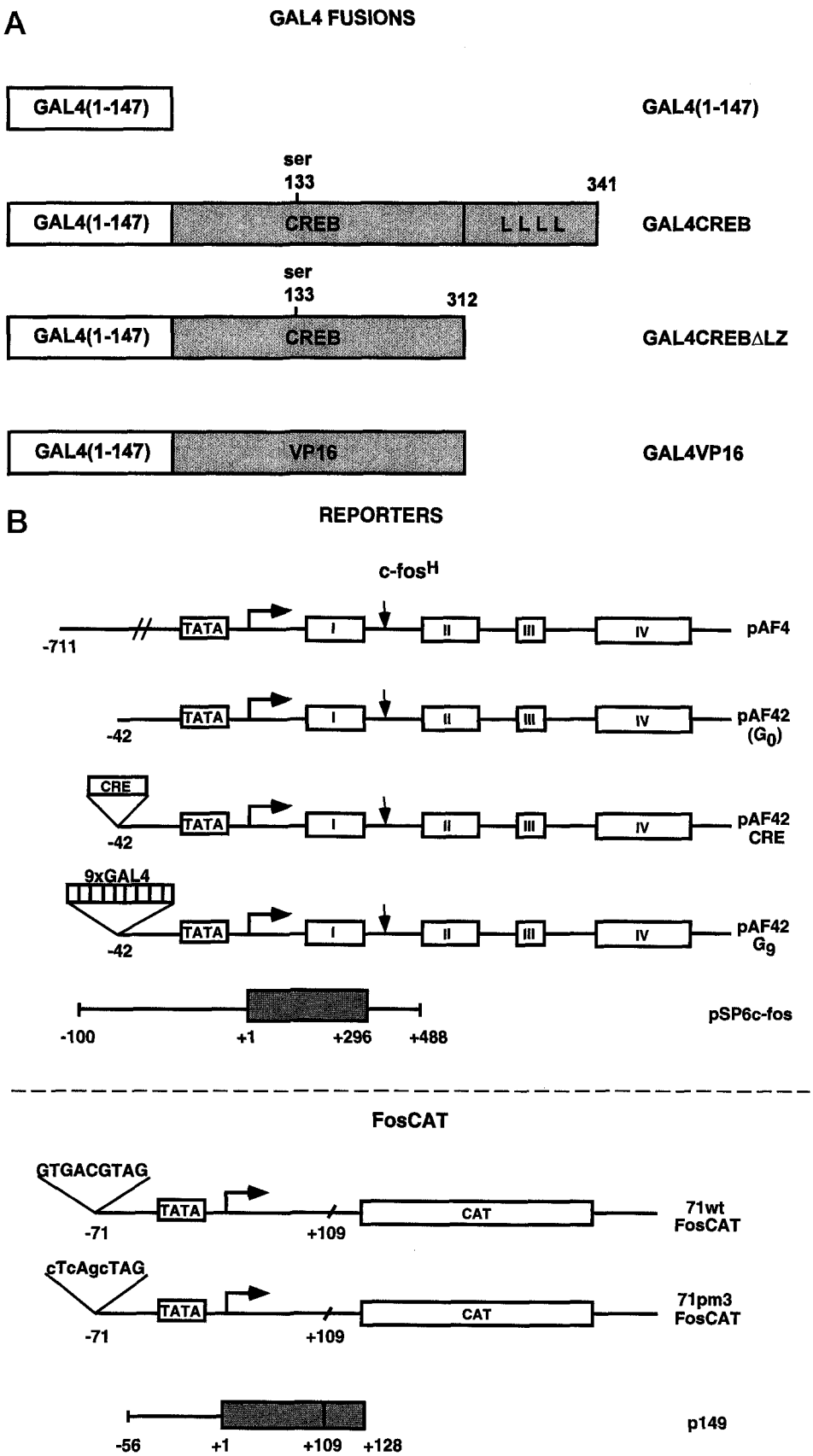

FIG. 1. *A*, structure of the GAL4 fusion constructs. *GAL4(1–147)* is the DNA-binding domain of the yeast GAL4 DNA-binding protein. *GAL4CREB* contains the complete CREB protein (341 amino acids) fused to the COOH terminus of GAL4(1–147) (40). *GAL4CREBΔLZ* lacks the COOH-terminal 29 amino acids of GAL4CREB, including the leucine repeat dimerization motif (LZ). *GAL4VP16* contains the activation domain of the herpes simplex virus transcriptional activator VP16 fused to the COOH terminus of GAL4(1–147). *B*, structure of reporter genes. *pAF4* is a human c-*fos* genomic clone that contains 711 nucleotides 5′ to the c-fos^H mRNA initiation site (26). *pAF42* (G_0) contains 42 nucleotides 5′ to the c-fos^H mRNA initiation site. *pAF42CRE* contains the consensus CRE (TGACGTCA) inserted at position −42 of pAF42, and *pAF42*G_9 contains nine GAL4-binding sites inserted at position −42. The four c-*fos* exons are indicated by *boxes*. The mRNA initiation site and the putative elongation

new transcription at the promoter, or by increasing the elongation potential of previously initiated transcripts. To distinguish between these two possibilities, a nuclear run-on transcription assay was used to examine transcription along different regions of the endogenous rat c-*fos* gene in PC12 cells left untreated or exposed to elevated levels of KCl for varying periods of time. In the nuclear run-on assay, new transcription initiation does not occur, but transcripts that have initiated prior to cell lysis are elongated. The nuclear run-on transcription assay was performed in the presence of [^{32}P]UTP, and the resulting RNA was hybridized to DNA probes bound to nitrocellulose filters. Two probes that hybridize to RNA transcribed from different regions of the c-*fos* gene were employed (Fig. 2*A*). Probe 1 consists of a rat cDNA fragment encompassing all of exon 1 and a very small (16 nucleotides) portion of exon 2. When probe 1 is used, hybridization of nuclear run-on transcripts to the 16 nucleotides of exon 2 sequences accounts for less than 10% of the total hybridization signal. Therefore, the amount of ^{32}P-labeled RNA that hybridizes to probe 1 reflects the level of transcription across c-*fos* exon 1. Probe 2 is a cDNA fragment that contains exons 2, 3, and a portion of exon 4. The amount of ^{32}P-labeled RNA that hybridizes to this probe reflects the level of transcription across these downstream exons.

As shown in Fig. 2*A*, when the run-on assay was carried out using nuclei from unstimulated cells, the ^{32}P-labeled transcripts hybridized to probe 1 (*1AS*), but not to probe 2 (*2AS*). This suggests that in unstimulated PC12 cells a detectable level of c-*fos* transcription was initiated at the promoter and transcribed through exon 1. However, no detectable transcription complexes successfully transcribed through downstream exon sequences indicating a pause site (block) in transcriptional elongation at a position 5′ to exon 2. Exposure of PC12 cells to elevated levels of KCl (final concentration, 60 mM) for 15 min resulted in a 4.1-fold increase in hybridization to probe 1 and in the appearance of hybridization to probe 2. The increased hybridization was specific to the antisense (*AS*) c-*fos* probes and occurred to only a very minor extent with the c-*fos* sense (*S*) probes. Hybridization to both the exon 1 and exon 2–4 probes decreased at later time points (3 h) to the levels seen in unstimulated cells. Taken together these results suggest that membrane depolarization leads to an induction of c-*fos* transcription by increasing the rate of initiation of complexes at the promoter (reflected by the increase in ^{32}P-labeled transcripts that hybridize to exon 1), and by increasing the ability of transcription complexes to proceed through 3′ exons (reflected by an increase in KCl-treated cells in the level of ^{32}P-labeled transcripts that hybridize to exon 2). It seems likely that the elongation block site is located within the first c-*fos* intron since analogous elongation pause sites have been identified in nonneuronal cells within the first introns of the murine and human c-*fos* genes (36–38).

We next asked if the −60 c-*fos* CaRE/CRE mediates transcription in KCl-treated cells by stimulating an increase in the initiation of transcription or by relieving the block to elongation. To test whether the CaRE/CRE might mediate the initiation of transcription, the depolarization induction of a heterologous gene construct which consists of the c-*fos* promoter fused to a reporter gene was assayed. This reporter gene consists of 71 nucleotides of the mouse c-*fos* promoter, including the CaRE/CRE, the TATA box, and the c-*fos* initiation site, fused to a CAT gene (*71wtfosCAT*) (25) (Fig. 1*B*). This construct lacks the previously characterized c-*fos* elongation pause site. PC12 cells were transfected with 71wtfosCAT and then depolarized by exposure to elevated levels of extracellular KCl. Cytoplasmic mRNA was analyzed by RNase protection. Fig. 2*B* shows that depolarization leads to an increase in correctly initiated fosCAT reporter transcripts within 30 min. When a similar construct (*71pm3fosCAT*) containing a mutation within the CaRE/CRE was tested, the level of fosCAT transcripts was barely detectable, indicating the necessity for an intact CaRE/CRE for membrane depolarization induction of reporter gene expression. Because the 71wtfosCAT construct contains the c-*fos* promoter and initiation site but not the c-*fos* elongation pause site, these results suggest that the presence of an intact CaRE/CRE stimulates an increase in the formation of initiation complexes in membrane depolarized cells and indicate that the c-*fos* elongation pause sequences are not required for transcriptional activation by membrane depolarization.

While VSCC activation induces c-*fos* transcription by regulating the CaRE-dependent initiation of transcription at the promoter, depolarization-induced changes in transcription elongation also occur. The control of elongation could be due to a second Ca^{2+}-mediated regulatory event that occurs at the elongation pause site. Alternatively, the control of elongation may be linked to events that occur at the promoter. To begin to distinguish between these possibilities, we analyzed the effects of membrane depolarization on the expression of a c-*fos* gene whose transcriptional initiation was not controlled by a Ca^{2+}-responsive element, but rather by the constitutive viral transcriptional activator, herpes simplex virus VP16 (39). Because VP16 is a constitutive activator, depolarization would not be expected to affect transcriptional initiation, and any changes in expression should be due soley to effects on elongation through c-*fos* sequences. To place c-*fos* expression under the control of VP16, the yeast GAL4 fusion and reporter system was employed (40). The VP16 transcriptional activation domain was directed to the human c-*fos* reporter (GAL4-fos) promoter by fusing it to the DNA binding portion of the yeast GAL4 protein. The GAL4 portion of this fusion protein, GAL4VP16, mediates binding to GAL4-binding sites that were inserted at position −42 in the human c-*fos* reporter (GAL4-fos or G_9, Fig. 1*B*). Expression of the GAL4-fos reporter in the presence of the GAL4VP16 activator was compared to expression in the presence of GAL4CREB, which is known to be regulated by Ca^{2+}. The c-*fos* elongation pause sequences that are present within the GAL4-fos gene would be likely to function as a pause site when this gene is transiently transfected into PC12 cells, since these elongation pause sequences have been shown previously to function in an *in vitro* transcription system (41), and since elongation pause sites have been shown to function when genes are introduced into cells by transient transfection (42). As shown previously, when cells were co-transfected with GAL4-fos and the GAL4CREB fusion construct, and mRNA levels were monitored by RNase protection, human c-*fos* transcripts

pause site are indicated by the *horizontal* and *vertical arrows*, respectively. *pSP6c-fos* represents the c-*fos*^{H} sequences in the RNA probe transcribed from the pSP6c-fos plasmid. The *thin line* indicates probe sequences that are not protected in the RNase protection assays, while the *box* indicates a 296 nucleotide sequence that is protected by c-*fos*^{H} mRNA. Endogenous rodent c-*fos* mRNA has been reported to protect a 65 nucleotide sequence in this probe (26). *71wtFosCAT* is a mouse c-*fos* CAT fusion reporter that contains 71 nucleotides 5′ to the c-*fos*^{M} initiation site and 109 nucleotides 3′. The wild type mouse CaRE/CRE is indicated in *capital letters*. *71pm3FosCAT* is identical to 71wtFosCAT except for the changes in the CaRE/CRE that are indicated in *lowercase letters*. *p149* represents the c-*fos*^{M} and linker sequences in the RNA probe transcribed from the p149 plasmid. The *thin line* indicates probe sequences that are unprotected. The *box* indicates a 128 nucleotide sequence protected by fosCAT mRNA transcribed from the transfected fosCAT reporter plasmid and a 109 nucleotide sequence protected by the mRNA transcribed from the endogenous rat c-*fos*^{R} gene.

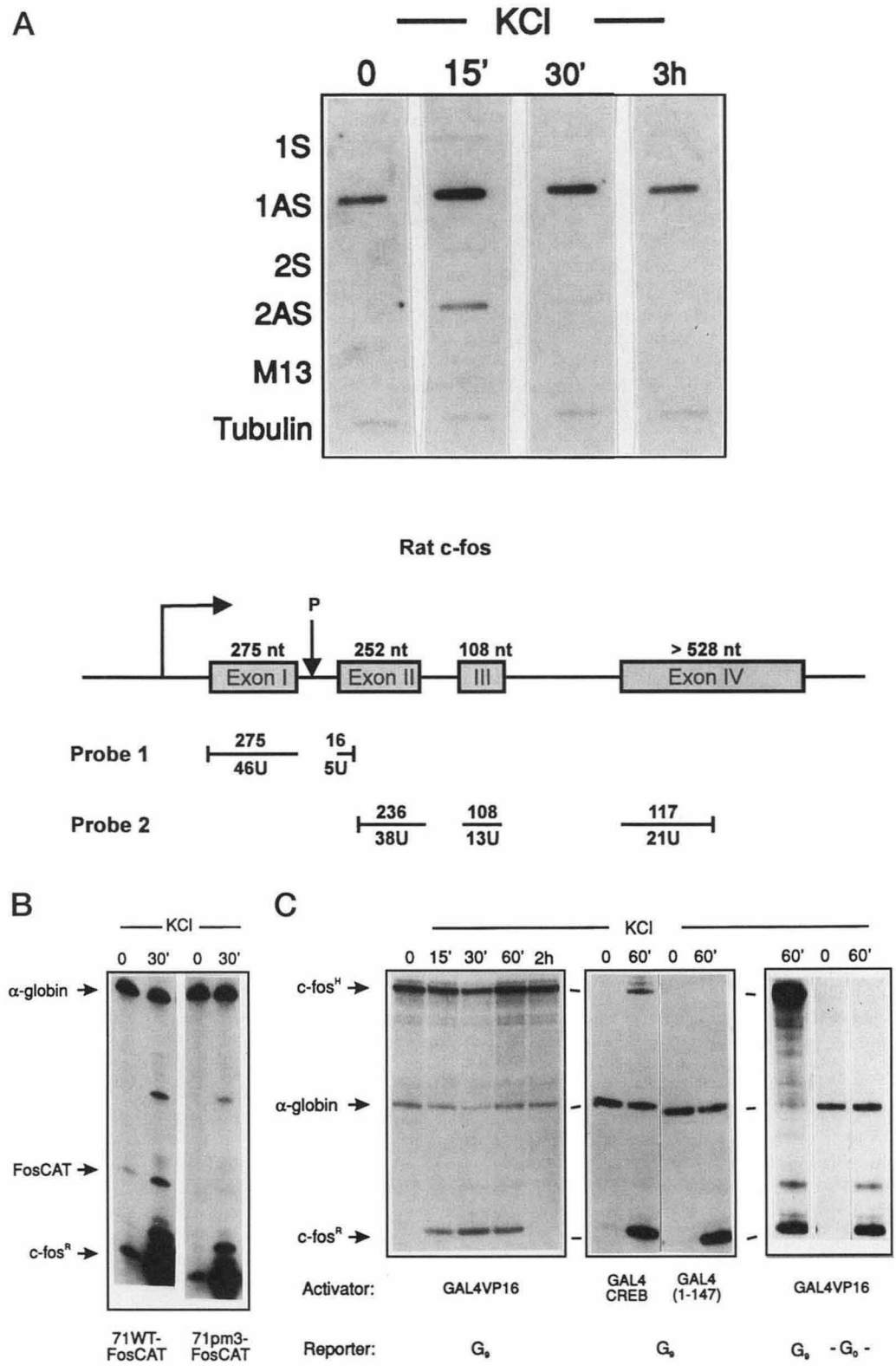

FIG. 2. *A*, transcription through c-*fos* exon 1 and exon 2 in membrane-depolarized PC12 cells. Nuclei were isolated from PC12 cells that were untreated or treated for the indicated time (minutes) with depolarization solution (60 mM KCl). Nascent RNA transcripts were labeled in an *in vitro* nuclear run-on transcription assay, and equal numbers of incorporated counts/minute were hybridized to single strand probes. *1S*, *1AS*, *2S*, and *2AS* are the sense (*S*) or antisense (*AS*) strands of probes 1 and 2 cloned into M13. *M13* is M13mp18. *Tubulin* is mouse tubulin in M13. Tubulin serves as an internal control for the total number of incorporated counts/minute added to each hybridization, as the transcription rate of the tubulin gene does not change in membrane depolarized cells (62). Diagram of single-stranded probes to rat c-*fos* exons 1 and 2. The number of nucleotides as well as the number of radiolabeled uracil residues in each probe is indicated. *Probe 1* contains the entire first c-*fos* exon (275 nucleotides (*nt*)) and 16 nucleotides of exon 2. The 16 nucleotides of exon 2 do not contribute significantly to the probe 1 signal in stimulated cells, since they are located at the end of the first intron. The RNA synthesized during the nuclear run-on reaction contains intron sequences which are not contained in the cDNA probe sequences bound to the filter. Therefore, the 16 nucleotides in the synthesized RNA follow a long stretch of RNA that cannot hybridize to the filter. This short 16 nucleotide sequence is not expected to hybridize to the filter well and is expected to be digested during the subsequent RNase wash step. Nevertheless, in calculating the fold induction of the probe 1 signal, 10% of the total probe 1 signal in stimulated

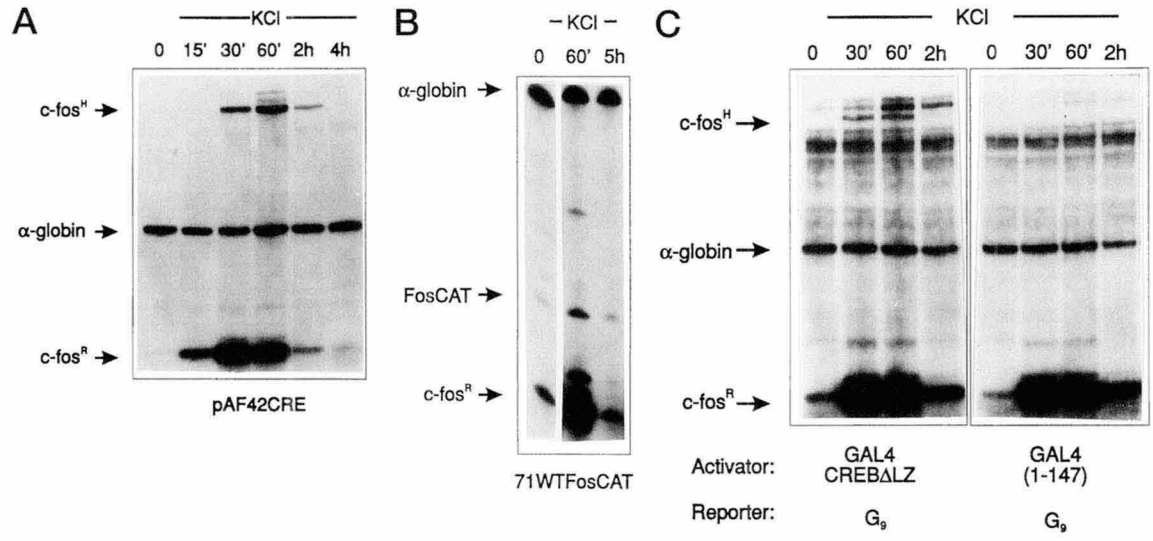

FIG. 3. *A* and *B*, the c-*fos* CaRE/CRE can mediate transcriptional shut-off following membrane depolarization induction. PC12 cells were co-transfected with pAF42CRE (*A*) or 71wtFosCAT (*B*) and pSVα-1. Cells were stimulated by membrane depolarization for the indicated times, and cytoplasmic mRNA was assayed by RNase protection with either antisense c-*fos*^{H} or antisense FosCAT and antisense α-globin riboprobes. The correctly initiated c-*fos*^{H}, FosCAT, α-globin, and endogenous rat c-*fos*^{R} transcripts are indicated with *arrows*. *C*, CREB can mediate transcriptional shut-off following membrane depolarization induction. PC12 cells were co-transfected with GAL4CREBΔLZ or GAL4(1–147) fusion constructs, pAF42G$_9$, and pSVα-1. Cells were stimulated for the indicated times by membrane depolarization, and cytoplasmic mRNA was assayed by RNase protection. The correctly initiated c-*fos*^{H} mRNA is indicated with an *arrow*. The doublet of bands migrating above the correct band are transcripts that are incorrectly initiated from the transfected reporter gene.

were detected in cells that had been exposed to elevated levels of KCl, but were not detected in unstimulated cells (Fig. 2*C*, *panel 2*) (14). In contrast to GAL4CREB, when GAL4VP16 was expressed in PC12 cells, high levels of reporter gene expression were detected both before and after membrane depolarization (Fig. 2C, *panel 1*). Transcription was dependent on the fused activators, as GAL4 alone (*GAL4(1–147)*) did not activate transcription (*panel 2*, *lanes 3* and *4*). The presence of a GAL4-binding site was also required since no transcripts were synthesized when the reporter gene pAF42 (also termed G$_0$), which lacks GAL4-binding sites, was cotransfected with GAL4VP16 (*panel 3*, *lanes 2* and *3*). If the c-*fos* transcriptional elongation block was effectively regulated by Ca^{2+} by a mechanism that is completely independent of transcriptional initiation, then no detectable cytoplasmic c-*fos* mRNA would be expected to accumulate in unstimulated cells as a result of transcription from the GAL4-fos reporter in the presence of GAL4VP16. The simplest explanation of these results is that for the wild type c-*fos* gene the pause in the elongation of transcription can be affected by the transcription complex that forms at the promoter, and regulation at the elongation pause site may be dependent on events occurring at the promoter. However, proof of this hypothesis requires that it be demonstrated that the c-*fos* elongation pause sequences still function effectively in transiently transfected genes.

Down-regulation of c-fos Transcription Is Mediated by the CaRE and CREB—Fig. 2*A* shows that the Ca^{2+}-dependent increase in transcriptional initiation is rapidly down-regulated following its activation by membrane depolarization. While Ca^{2+}-activated transcription is known to involve CREB bound to the CaRE/CRE, the mechanisms controlling the shut-off of Ca^{2+}-activated transcription are not understood. To investigate the possibility that the CaRE/CRE might mediate both the transcriptional induction and the subsequent shut-off event, the expression of a CaRE/CRE driven c-*fos* reporter (pAF42CRE) was examined at various times after stimulation. Because c-*fos* mRNA is very unstable, with a half-life of only 15 min in the cytoplasm (18), it is possible to measure the shut off of pAF42CRE transcription by monitoring the disappearance of c-*fos*^{H} mRNA. Fig. 3*A* shows that following its induction in KCl-treated PC12 cells, c-*fos* mRNA transcribed from the transfected pAF42CRE gene and the endogenous rat c-*fos* gene declined over time and was nearly undetectable within 4 h. Likewise a truncated human c-*fos* gene containing the −60 c-*fos* CaRE/CRE (-TGACGTTT-) element inserted at position −42 was transiently transcribed in membrane-depolarized cells.[2] Expression of 71WTfosCAT mRNA was also found to decline rapidly following its activation, suggesting that c-*fos* intragenic sequences are not required for transcriptional down-regulation (Fig. 3*B*). Taken together these findings suggest that the presence of the CaRE/CRE confers not only the transcriptional activation in response to Ca^{2+}, but also the subsequent shut off of transcription.

We next investigated the possibility that CREB mediates transcriptional shut-off as well as activation. The expression of

[2] M. A. Thompson and M. E. Greenberg, unpublished observation.

cells was subtracted to eliminate the maximum potential contribution of the 16 nucleotides based on the number of uracil residues. *Probe 2* contains all exon 2 (236 nucleotides) and 3 (108 nucleotides) sequences and 117 nucleotides of exon 4. *P* indicates site of putative elongation pause sequences. *B*, membrane depolarization activation of transcription through the c-*fos* CaRE/CRE is not dependent on c-*fos* intragenic sequences. PC12 cells were transfected with 71wtFosCAT, or 71 pm3FosCAT, and pSVα-1 as an internal control for transfection efficiency. Cells were stimulated for 30 min by membrane depolarization with KCl. Cytoplasmic mRNA was isolated and assayed by RNase protection. mRNA transcribed from the transfected FosCAT, and α-globin genes and the endogenous rat c-*fos*^{R} gene are indicated with *arrows*. The *FosCAT arrow* indicates correctly initiated transcripts. The higher molecular weight band represents incorrectly initiated transcripts from the transfected FosCAT reporter. *C*, c-*fos*^{H} intragenic sequences cannot mediate membrane depolarization regulation of transcription initiated by GAL4VP16. PC12 cells were transfected with the indicated GAL4 fusion and reporter constructs and with pSVα-1. Cells were stimulated for the indicated times by membrane depolarization, and mRNA was isolated and assayed by RNase protection. Bands representing transcription of the transfected human c-*fos* reporter (c-*fos*^{H}), α-globin, and the endogenous rat c-*fos* (c-*fos*^{R}) are indicated with *arrows*.

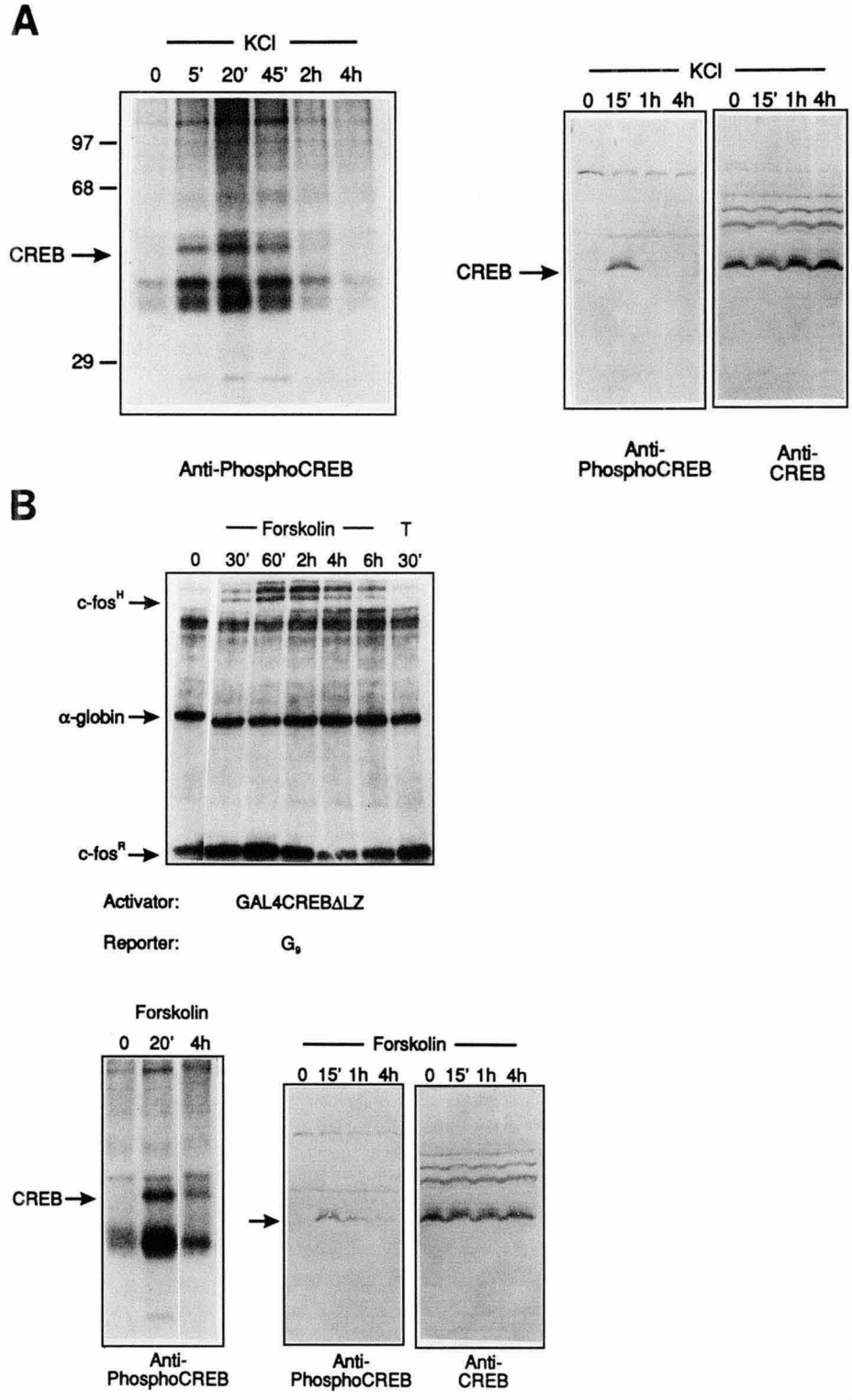

FIG. 4. *A*, CREB serine 133 is transiently phosphorylated in membrane-depolarized PC12 cells. *Left panel*, PC12 cells were labeled with [^{32}P]orthophosphate and stimulated with KCl for the indicated time. Lysates were collected in boiling SDS and immunoprecipitated with anti-phosphoCREB. The *arrow* indicates CREB. The lower molecular weight bands are believed to represent CREB-related proteins that share amino acid homology with the peptide used to generate anti-phosphoCREB. *Right panels*, PC12 cells were depolarized for the indicated times and then collected in boiling SDS lysis buffer. Samples were run on SDS-PAGE, transferred to nitrocellulose, incubated with anti-phosphoCREB or anti-CREB, and antibody-antigen complexes were detected with alkaline phosphatase as described under "Materials and Methods." The *arrow* indicates CREB. *B*, forskolin treatment of PC12 cells leads to both prolonged CREB-dependent transcription and prolonged phosphorylation of CREB serine 133. *Top panel*, PC12 cells were transfected with GAL4CREBΔLZ, pAF42G$_9$, and pSVα-1. Cells were treated for the indicated times with forskolin or TPA (*T*), and mRNA was assayed by RNase protection. The correct bands representing transcription of the transfected and endogenous genes are indicated with *arrows*. *Bottom left panel*, PC12 cells were labeled with [^{32}P]orthophosphate and stimulated with forskolin for the indicated time. Lysates were collected in boiling SDS and immunoprecipitated with anti-phosphoCREB. *Bottom right panel*, PC12 cells were treated with forskolin for the indicated times and then collected in boiling SDS lysis buffer. Samples were run on SDS-PAGE and transferred to nitrocellulose. Filters were incubated with anti-phosphoCREB or anti-CREB antibodies. The *arrow* indicates CREB.

the GAL4-fos reporter in the presence of a GAL4CREB fusion protein that has a deletion of the CREB leucine zipper (*GAL4CREBΔLZ*, Fig. 1*A*) was examined at various times before and after exposure of PC12 cells to elevated levels of KCl. Deletion of the leucine zipper prevents potential heterodimerization with endogneous CREB family members that might affect the biological activity of the fusion protein (43). Fig. 3*C* shows that as with pAF42CRE, in the presence of GAL4CREBΔLZ, the GAL4-fos reporter was transiently induced by depolarization. This induction was dependent on the CREB sequences fused to GAL4 as GAL4 alone (GAL4(1–147)) did not mediate the induction event. These findings suggest that CREB bound near the c-*fos* TATA box contributes to both transcriptional activation and shut off in membrane-depolarized PC12 cells.

Dephosphorylation of CREB Correlates with Transcription Shut-Off—To determine if the disappearance of phosphoCREB might mediate the shut-off event, an antibody that specifically recognizes CREB phosphorylated on serine 133 (anti-phosphoCREB) (35) was used to monitor serine 133 phosphorylation in cells depolarized for varying periods of times. As shown in Fig. 4*A* (*left panel*), anti-phosphoCREB immunoprecipitated phos-

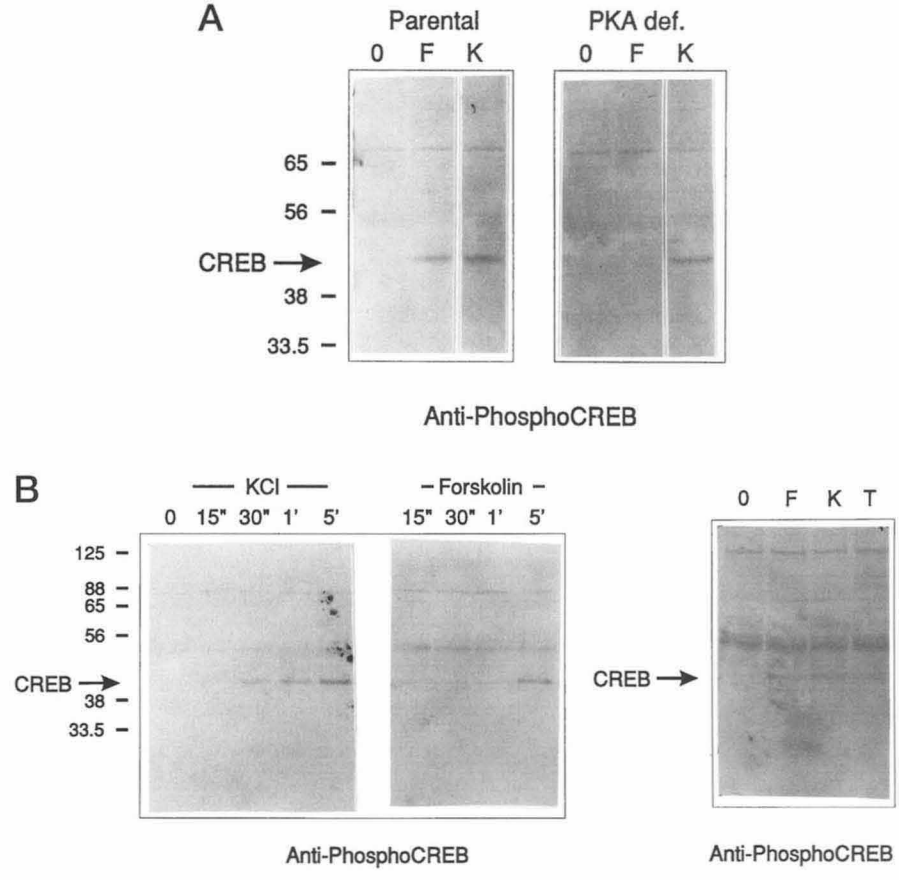

FIG. 5. *A*, the activity of PKA is not required for CREB serine 133 phosphorylation in membrane-depolarized PC12 cells. The PC12 wild type (*parental*) and the PKA-deficient (*PKA def.*) 123.7 cell line were either untreated (*O*) or treated with forskolin (*F*) or with KCl (*K*) for 10 min. Cell lysates were collected in boiling SDS sample buffer, separated by SDS-PAGE, and filters were incubated with anti-phosphoCREB antibodies. The *arrow* indicates CREB. *B*, CREB serine 133 phosphorylation occurs within 30 s of membrane depolarization. TPA can also induce CREB serine 133 phosphorylation. *Left panels*, PC12 cells were stimulated with either KCl or forskolin for the indicated times. Cell lysates were collected in boiling SDS sample buffer, separated by SDS-PAGE, and filters were incubated with anti-phosphoCREB antibodies. The *arrow* indicates CREB. *Right panels*, PC12 cells were treated with either forskolin (*F*), KCl (*K*), or TPA (*T*) for 10 min. Lysates were analyzed as above. The *arrow* indicates CREB.

phoCREB from cells stimulated for 5, 20, and 45 min, but not from unstimulated cells. Within 4 h of stimulation with KCl, phosphoCREB was nearly undetectable in PC12 cell extracts. Therefore, like GAL4CREBΔLZ-mediated transcription, the presence of phosphoCREB was transient in depolarized cells. This is consistent with the idea that the disappearance of phosphoCREB contributes to transcriptional down-regulation.

To determine whether the disappearance of phosphoCREB was due to either dephosphorylation or degradation of phosphoCREB, parallel immunoblot analyses were performed with anti-phosphoCREB antibodies and antibodies that recognize both unphosphorylated and phosphorylated forms of CREB (*anti-CREB*). Anti-phosphoCREB antibodies detected the 43 kDa phosphorylated CREB band in extracts of cells depolarized for 15 min (Fig. 4*A*, *right panel*) but not in extracts of cells depolarized for 4 h. When the same extracts were immunoblotted with anti-CREB antibodies, the 43 kDa CREB band was detectable at both time points (Fig. 4*A*, *right panel*). Taken together with the finding that the majority of CREB molecules within a cell becomes phosphorylated on serine 133 within minutes of membrane depolarization (35), these observations suggest that the disappearance of serine 133-phosphorylated CREB in depolarized cells is not due to degradation, but rather due to dephosphorylation of serine 133.

To examine further the correlation between the level of c-*fos* transcription and the extent of CREB serine 133 phosphorylation, the time courses of transcription and CREB phosphorylation were analyzed in cells stimulated with forskolin to elevate intracellular levels of cAMP. Fig. 4*B* shows that in contrast to depolarization, stimulation with forskolin resulted in an extended c-*fos* mRNA signal. c-*fos*H mRNA expression was detected as late as 6 h after stimulation of cells transfected with pAF42CRE or GAL4fos in the presence of GAL4CREBΔLZ (Fig. 4*B*, *top panel*, and data not shown). Analysis of the phosphorylation state of CREB indicated that in forskolin-treated cells, serine 133 remains phosphorylated for an extended time, with phosphoCREB detectable as late as 6 h after stimulation (Fig. 4*B*, *bottom panels*, and data not shown). For both reporter gene transcription and the phosphorylation of CREB, peak levels were seen at early times (20–60 min) following forskolin addition with some decrease in these levels detected at later times. The observation that CaRE driven transcription and the phosphorylation state of CREB are correlated in forskolin-treated cells further suggests that the transcriptional shut-off event is linked to the dephosphorylation of CREB serine 133.

PKA Does Not Phosphorylate CREB in Response to Depolarization—PKA has been implicated in the regulation of c-*fos* transcription in forskolin-treated cells (1, 22). Whether this enzyme catalyzes CREB serine 133 phosphorylation in KCl-treated cells is unresolved. To test directly if PKA is required for CREB phosphorylation in KCl-treated cells, we examined if CREB becomes phosphorylated on serine 133 upon membrane depolarization of PKA-deficient PC12 cells. These PKA-deficient cells overexpress a dominant negative PKA regulatory subunit that cannot bind cAMP but will inactivate the PKA catalytic subunit (30). These cells are deficient in cAMP-activable PKA. Fig. 5*A* shows that forskolin fails to induce CREB serine 133 phosphorylation in these cells. However, KCl treatment of PKA-deficient cells stimulated CREB serine 133 phosphorylation normally. These observations suggest that while PKA phosphorylates CREB in response to signals that trigger an increase in intracellular cAMP, a kinase other than PKA must phosphorylate CREB in membrane-depolarized cells. This Ca^{2+}-activated kinase phosphorylates CREB serine 133 within seconds of membrane depolarization (Fig. 5*B*). In contrast, stimulation with forskolin, which activates PKA, does not stimulate the phosphorylation of CREB until 5 min after treatment (Fig. 5*B*).

A Second Depolarization-induced, PKA-dependent Event Is Required for CaRE/CRE-mediated Activation of Transcription—In the original analysis of c-*fos* transcription in PKA-deficient cells, expression of the endogenous c-*fos* gene was assessed (22), but the ability of individual promoter elements to confer a depolarization response was not determined. Since the induction of c-*fos* in these cells was only partially inhibited, it was of interest to test each promoter element independently for its efficacy as a Ca^{2+} response element. Given the observation that CREB becomes phosphorylated normally upon membrane depolarization of the PKA-deficient PC12 cells, it remained a possibility that the −60 CaRE still retained the ability to confer a Ca^{2+} response in these cells while other elements in the c-*fos* promoter were inactive.

To determine which of the Ca^{2+} response elements within the c-*fos* gene lose their ability to function in PKA-deficient cells, PKA-deficient and wild type PC12 cells were transfected with various human c-*fos* reporter constructs, and the levels of human c-*fos* mRNA transcripts were determined after membrane depolarization, forskolin treatment, or phorbol ester addition. Both the endogenous rat c-*fos* gene and the human c-*fos* gene pAF4, which contains 711 base pairs of upstream regulatory sequences including several Ca^{2+} response elements were effectively induced upon membrane depolarization of wild type PC12 cells (Fig. 6*A*, *top panel*). However, these genes were poorly induced by KCl treatment of PKA-deficient PC12 cells. Likewise, pAF42CRE, which contains only a CREB-binding site upstream of the TATA box, was activated to a much greater extent (42-fold) in wild type PC12 cells than in the PKA-deficient cells (Fig. 6*A*, *bottom panel*). The loss of a transcription response in the PKA-deficient cells was also seen when these cells were exposed to forskolin to activate adenylate cyclase (Fig. 6*A*). However, another inducer, TPA, was able to effectively induce expression of the pAF4 gene in the PKA-deficient cells, indicating that the failure of the CaRE to respond to KCl and forskolin was not a general phenomena (Fig. 6*A*). These findings, taken together with the observation that CREB serine 133 becomes phosphorylated upon KCl treatment of these cells (Fig. 5*A*), demonstrate that although CREB phosphorylation is critical, it is not sufficient for depolarization-induced CaRE/CRE-dependent transcription. A second PKA-dependent phosphorylation event is also required. This conclusion is supported by the finding that in wild type or PKA-deficient PC12 cells TPA induced CREB serine 133 phosphorylation (Fig. 5*B*, *right panel*), but did not activate GAL4CREBΔLZ-mediated transcription or transcription of pAF42CRE (Figs. 4*B*, *top panel*, *last lane*; Fig. 6*A*, *bottom panel*, respectively).

We tested the ability of a second Ca^{2+} response element, the c-*fos* serum response element (SRE) (3, 31), to function in the PKA-deficient cells. In contrast to the activation of pAF42CRE, depolarization resulted in nearly equivalent activation of pAF42SRE in both wild type and PKA-deficient PC12 cells (Fig. 6*B*). Membrane depolarization resulted in a level of transcription in the wild type cells that was only 4.5-fold greater than the level detected in the mutant cells. This was a significantly reduced difference than the 42-fold difference detected for pAF42CRE (Fig. 6*A*). Because the pAF42SRE construct contains the same minimal promoter sequences and intragenic sequences as the pAF42CRE plasmid, these findings suggest that the PKA-dependent phosphorylation event that is necessary for depolarization-activation of pAF42CRE probably involves the modification of factors that interact at the CRE rather than components of the basic transcriptional machinery or factors that regulate transcriptional elongation.

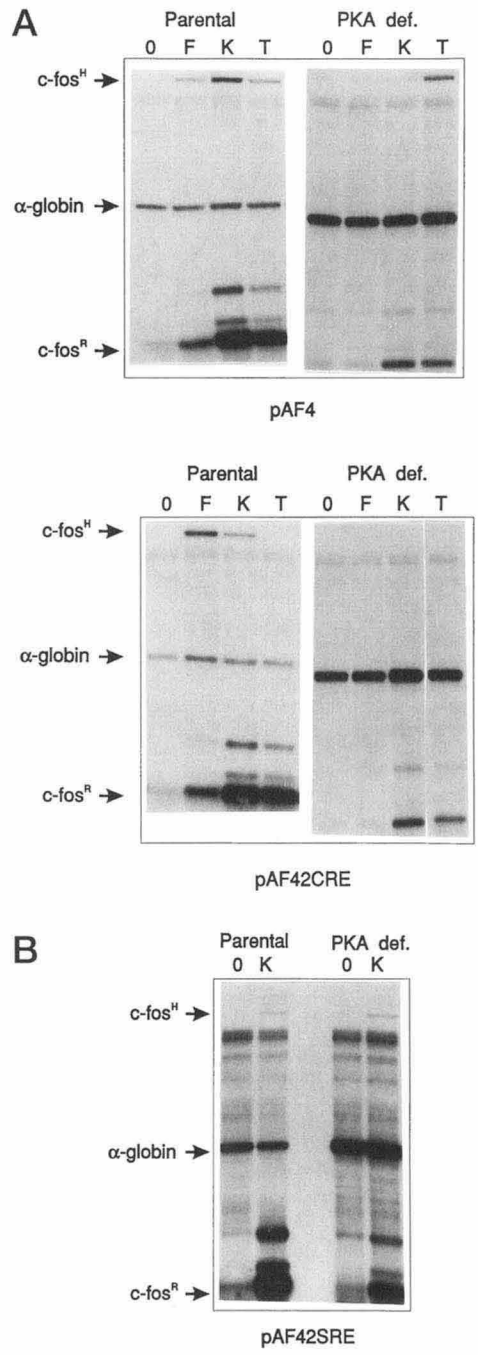

FIG. 6. *A*, the CRE cannot mediate a transcriptional response to membrane depolarization in PKA-deficient PC12 cells. Wild type (*parental*) and PKA-deficient (*PKA def.*) PC12 cells were transfected with either pAF4 (*top panel*) or pAF42CRE (*bottom panel*) and pSVα-1. Cells were stimulated for 60 min with either forskolin (*F*) or KCl (*K*) or for 30 min with TPA (*T*). Cytoplasmic RNA was analyzed by RNase protection. *Arrows* indicate protected transcripts from the transfected c-*fos*^{H} and α-globin genes and the endogenous c-*fos*^{R} gene. *B*, SRE-dependent transcriptional induction by membrane depolarization is not compromised in PKA-deficient cells. Wild type (*parental*) and PKA-deficient (*PKA def.*) PC12 cells were transfected with pAF42SRE and pSVα-1. Cells were either unstimulated (*O*) or stimulated with KCl (*K*) for 60 min. RNA was analyzed by RNase protection, and *arrows* indicate c-*fos*^{H}, α-globin, and c-*fos*^{R} protected transcripts.

DISCUSSION

The regulation of gene expression in neurons is critical for their long term responses to stimulation (1, 2). A major signaling mechanism in stimulated neurons involves the membrane depolarization-activated opening of VSCCs and the resultant

increase in intracellular free Ca^{2+} (44). We have studied the mechanism by which Ca^{2+} regulates c-*fos* expression in PC12 cells and have found that this regulation is complex, involving multiple processes. In this study, we present evidence suggesting that a rise in intracellular Ca^{2+} and the subsequent phosphorylation of CREB serine 133 result in a transient increase in both the formation of new transcription complexes at the promoter and the ability of transcription complexes to elongate through 3′ exons. We also present evidence that the dephosphorylation of CREB serine 133 parallels and therefore may mediate the shut-off of c-*fos* transcription that occurs following induction. Furthermore, our findings support the idea that CREB is directly phosphorylated by a Ca^{2+}-activated kinase in response to VSCC opening. However, in addition to the Ca^{2+}-dependent phosphorylation of CREB serine 133, a second PKA-dependent function is necessary for CaRE/CRE-mediated transcriptional activation. This additional event may be the phosphorylation of a second site on CREB, the CREB-binding protein (45), or a distinct CaRE/CRE-binding protein. The PKA-dependent function appears to be activated by Ca^{2+}, but not by TPA, which nevertheless can induce CREB phosphorylation at serine 133.

Depolarization Induces New Transcription Complex Formation and Relief of a Block to Elongation—Ca^{2+} regulates c-*fos* expression by affecting both transcriptional initiation and elongation. The regulation of both of these events allows for tight control over c-*fos* expression. Thus, no transcription is apparent in unstimulated cells, while a very rapid and strong response to stimulation is ensured by the coordinate activation of both initiation and elongation. This high level of control may be important for a precise neuronal response to stimulation.

The transcriptional pause site within the rat c-*fos* gene described here is comparable to pause sites found in the first intron of the murine and human c-*fos* genes (36, 37, 41). The observation that Ca^{2+} influx through VSCCs induces the formation of transcription complexes that can elongate through the pause site in the rat gene is consistent with a previous report in non-neuronal cells that Ca^{2+} is required for elongation through the pause site in the murine and human c-*fos* genes (36, 37).

The regulation of the initiation of c-*fos* transcription most likely occurs independently of regulation of elongation. This idea is supported by the observations that membrane depolarization can induce transcription of the CaRE-driven reporter genes, CAT (Fig. 2*B*) and β-globin (12), both of which lack the c-*fos* pause sequences (Fig. 2*B*). In contrast, the regulation of c-*fos* transcriptional elongation appears to be affected by the transcription factors that regulate c-*fos* transcriptional initiation. GAL4VP16 was observed to stimulate transcription of the GAL4-fos gene even in unstimulated PC12 cells, suggesting that the function of the c-*fos* pause site can be bypassed by a strong transcriptional activator. This interpretation is based on the assumption that the c-*fos* pause sequences function in genes transiently transfected into PC12 cells, and thus remains to be demonstrated.

Several examples of transcription factor-dependent elongation have been described previously. For example in the c-*myc* gene, which contains two promoters, whether an elongation pause site is recognized by transcription complexes depends on which promoter is used to initiate transcription (46). More recent analysis of the c-*myc* gene has revealed that the processivity of RNA polymerase II along this gene is increased when transcription complexes form at the promoter in the presence of a strong activator such as VP16 (47). Taken together these findings are consistent with the possibility that transcription complexes that form in the presence of phosphorylated CREB (a strong activator) can elongate through the pause site, while those formed in the presence of unphosphorylated CREB (a weak activator) cannot.

Dephosphorylation of CREB and Transcriptional Down-regulation—A major determinant of the depolarization regulation of c-*fos* transcription is the CaRE/CRE at −60 in the promoter (12). This element is sufficient to confer depolarization inducibility to an otherwise unresponsive c-*fos* gene. We have shown that in addition to induction, CaRE/CRE contributes to transcriptional shut-off. A critical determinant of the c-*fos* response to serum, the SRE, also can mediate both the activation and shut-off of transcription (23, 48). The serum induction of SRE-mediated transcription has been shown to be down-regulated by the COOH terminus of the Fos protein, leading to the suggestion that the Fos protein may play a general role in shutting off c-*fos* transcription (48). However, Fos does not down-regulate cAMP-induced transcription through a CRE (49) and most likely does not play a role in shutting off CaRE/CRE-mediated transcription.

Our results suggest a simple model that might explain the transient nature of c-*fos* transcriptional induction that occurs when VSCCs are activated. Following the phosphorylation of CREB serine 133 and induction of transcription, the dephosphorylation of CREB serine 133 may lead to a shut-off of transcription. A different model has been proposed that is based on the observation that CREB is a member of a large family of CRE-binding proteins, some of which are repressors (49–51). These repressors, CREMs, or cAMP response element modulator proteins, can heterodimerize with CREB and inhibit CRE-mediated, cAMP-induced transcription under some circumstances (49, 52). Thus, transcriptional down-regulation has been proposed to involve the formation of a CREB:CREM heterodimer or the replacement of a CREB:CREB homodimer at the CRE with a CREM:CREM homodimer. However, our findings with GAL4CREBΔLZ in PC12 cells suggest that while CREM function may contribute to transcriptional down-regulation, it is not required. GAL4CREBΔLZ, which does not contain the CREB leucine zipper, and therefore cannot heterodimerize with endogenous leucine zipper-containing proteins, mediates both transcriptional activation and shut-off of a GAL4-fos reporter (Fig. 3*C*). This demonstrates that CREB-activated transcription can be shut-off without a contribution by other family members, such as the CREM proteins. In support of the idea that transcriptional shut-off is mediated at least in part by dephosphorylation of CREB, there was a strict correlation between the level of transcription of the CRE-fos reporter gene and the extent of CREB serine 133 phosphorylation in both depolarized and forskolin-treated cells. An alternative interpretation of these findings is that shut-off of transcription is followed by CREB dephosphorylation. However, in support of the idea that the dephosphorylation of CREB serine 133 precedes and is critical for transcriptional shut-off, we found that in KCl-treated cells the phosphatase inhibitor, okadaic acid, blocks both the dephosphorylation of CREB serine 133 and c-*fos* transcriptional shut-off.[2] Two protein phosphatases, PP1 and PP2A, have been shown to dephosphorylate CREB serine 133 *in vitro* (53, 54). Both of these phosphatases have been postulated to play a role in CREB serine 133 dephosphorylation that occurs several hours following forskolin treatment and could also play a role in CREB dephosphorylation that occurs at later time points following membrane depolarization.

The Role of PKA in Depolarization Activation of Transcription—In this study, we have also resolved a controversy in the literature regarding the role of PKA in membrane depolarization activation of IEG transcription. Several studies have demon-

strated that cAMP levels do not rise in KCl-treated PC12 cells, suggesting that PKA is not likely to be activated and is probably not the enzyme that catalyzes CREB phosphorylation in response to increased levels of intracellular Ca^{2+} (12, 21). However, in one study (22) using a mutant PC12 cell line that is deficient in PKA activity, it was found that membrane depolarization is rather ineffective at inducing c-*fos* transcription. This finding suggested the possibility that PKA might actually be important for CREB phosphorylation and c-*fos* activation in KCl-treated cells. In this report we show that CREB phosphorylation occurs normally in PKA-deficient PC12 cells exposed to elevated levels of KCl. These findings indicate that PKA is not the enzyme that catalyzes CREB phosphorylation in KCl-treated cells and are consistent with the idea that a Ca^{2+}-regulated kinase mediates the phosphorylation event.

We conclude that CREB serine 133 phosphorylation is not sufficient for CRE-mediated transcription in KCl-treated cells, since this phosphorylation event occurs normally in PKA-deficient cells, but the CRE is incapable of conferring a depolarization response. Therefore, a second PKA-dependent phosphorylation event may exist that is critical for CaRE/CRE-dependent transcription. We propose that the PKA-dependent function acts on the CaRE/CRE, rather than on c-*fos* basal promoter elements or intragenic sequences since a different reporter (SRE-fos) that also contains the c-*fos* promoter and intragenic sequences is effectively activated in the PKA-deficient cells (Fig. 6*B*). The PKA-dependent event could involve phosphorylation of CREB on a site other than serine 133. Alternatively, it may reflect involvement of CRE-binding proteins in addition to CREB or the CREB-binding protein (CBP) which has been shown to mediate cAMP-dependent transcription in non-neuronal cells (45).

The VSCC-activated CREB Kinase—Since we have shown that VSCC-dependent phosphorylation of CREB serine 133 does not require PKA, an alternative kinase must phosphorylate CREB in response to membrane depolarization. This kinase must satisfy a number of requirements. It must be activated in membrane-depolarized cells. The activation must be rapid, as CREB is phosphorylated within 30 s. And, because immunostaining studies indicate that CREB is nuclear (15),[3] the kinase must phosphorylate CREB within the nucleus. The Ca^{2+}/calmodulin-dependent kinases are good candidates that fulfill these criteria. CaM kinase II is activated within 5–10 s of depolarization of PC12 cells (55), and this kinase, as well as the CaM kinases I and IV, can phosphorylate CREB serine 133 *in vitro* (14, 56, 57).[2] In addition, CaM kinase IV and certain isoforms of CaM kinase II have been localized to the nucleus (58–60). And finally, a constitutively active form of CaM kinase IV has been shown in transient transfection assays to enhance CRE/CaRE-mediated transcription (61, 63). Thus, it seems likely that a Ca^{2+}-activated CaM kinase may phosphorylate and activate CREB in depolarized PC12 cells. However, activation of a nuclear CaM kinase in membrane-depolarized PC12 cells and direct phosphorylation of CREB *in vivo* by such a kinase remain to be demonstrated.

We conclude that the precise control of c-*fos* expression is necessary for the conversion of extracellular stimuli to long term functional changes in neurons. This study illustrates the intricate nature of c-*fos* regulation by membrane depolarization. Membrane depolarization leads to an increase in the rate of formation of new initiation complexes, as well as an increase in the passage of transcription complexes through an elongation pause site. This Ca^{2+}-dependent activation of c-*fos* transcription not only requires CREB serine 133, which is phosphorylated in response to depolarization, but also an additional PKA-dependent function. Finally, c-*fos* transcription must shut-off. Dephosphorylation of CREB serine 133 is a likely mechanism by which this occurs.

[3] D. D. Ginty and M. E. Greenberg, unpublished observation.

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REFERENCES

1. Sheng, M., and Greenberg, M. E. (1990) *Neuron* **4,** 477–485
2. Goelet, P., Castellucci, V. F., Schacher, S., and Kandel, E. R. (1986) *Nature* **322,** 419–422
3. Bading, H., Ginty, D. D., and Greenberg, M. E. (1993) *Science* **260,** 181–186
4. Greenberg, M. E., Ziff, E. B., and Greene, L. A. (1986) *Science* **234,** 80–83
5. Curran, T., and Morgan, J. I. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8521–8524
6. Morgan, J. I., Cohen, D. R., Hempstead, J. L., and Curran, T. (1987) *Science* **237,** 192–197
7. Nguyen, T. V., Kosofsky, B. E., Birnbaum, R., Cohen, B. M., and Hymen, S. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4270–4274
8. Graybiel, A. M., Moratalla, R., and Robertson, H. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6912–6916
9. Kornhauser, J. M., Nelson, D. E., Mayo, K. E., and Takahashi, J. S. (1990) *Neuron* **5,** 127–134
10. Curran, T., and Franza, B. R. J. (1988) *Cell* **55,** 395–397
11. Morgan, J. I., and Curran, T. (1986) *Nature* **322,** 552–555
12. Sheng, M., McFadden, G., and Greenberg, M. E. (1990) *Neuron* **4,** 571–582
13. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 6682–6686
14. Sheng, M. E., Thompson, M. A., and Greenberg, M. E. (1991) *Science* **252,** 1427–1430
15. Gonzales, G. A., and Montminy, M. R. (1989) *Cell* **59,** 675–680
16. Schwaninger, M., Lux, G., Blume, R., Oetjen, E., Hidaka, H., and Knepel, W. (1993) *J. Biol. Chem.* **268,** 5168–5177
17. Nichols, M., Weih, F., Schmid, W., DeVack, C., Kowenz-Leutz, E., Luckow, B., Boshart, M., and Schutz, G. (1992) *EMBO J.* **11,** 3337–3346
18. Shyu, A.-B., Greenberg, M. E., and Belasco, J. G. (1989) *Genes & Dev.* **3,** 60–72
19. Meinkoth, J. L., Ji, Y., Taylor, S. S., and Feramisco, J. R. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9595–9599
20. Adams, S. R., Harootunian, A. T., Buechler, Y. J., Taylor, S. S., and Tsien, R. Y. (1991) *Nature* **349,** 694–697
21. Nguyen, T. V., Kobierski, L., Comb, M., and Hyman, S. E. (1990) *J. Neurosci.* **10,** 2825–2833
22. Ginty, D. D., Glowacka, D., Bader, D. S., Hidaka, H., and Wagner, J. A. (1991) *J. Biol. Chem.* **266,** 17454–17458
23. Rivera, V. M., Sheng, M., and Greenberg, M. E. (1990) *Genes & Dev.* **4,** 255–268
24. Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988) *Nature* **335,** 563–564
25. Berkowitz, L. A., Riabowol, K. T., and Gilman, M. Z. (1989) *Mol. Cell. Biol.* **9,** 4272–4281
26. Sheng, M., Dougan, S. T., McFadden, G., and Greenberg, M. E. (1988) *Mol. Cell Biol.* **8,** 2787–2796
27. Treisman, R. (1985) *Cell* **42,** 889–902
28. Charney, P., Treisman, R., Mellon, M., Chao, R., Axel, R., and Maniatis, T. (1984) *Cell* **38,** 251–263
29. Gilman, M. Z., Wilson, R. N., and Weinberg, R. A. (1986) *Mol. Cell Biol.* **6,** 4305–4315
30. Ginty, D. D., Glowacka, D., DeFranco, C., and Wagner, J. A. (1991) *J. Biol. Chem.* **266,** 15325–15333
31. Misra, R. P., Bonni, A., Miranti, C. K., Rivera, V. M., Sheng, M., and Greenberg, M. E. (1994) *J. Biol. Chem.* **269,** 25483–25493
32. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, pp. 4.10.1–4.10.5, Green Publishing Associates, New York
33. Celano, P., Berchtold, C., and Casero, R. A. (1989) *BioTechniques* **7,** 942–944
34. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
35. Ginty, D. D., Kornhauser, J. M., Thompson, M. A., Bading, H., Mayo, K. E., Takahashi, J. S., and Greenberg, M. E. (1993) *Science* **260,** 238–241
36. Werlen, G., Belin, D., Conne, B., Roche, E., Lew, D. P., and Prentki, M. (1993) *J. Biol. Chem.* **268,** 16596–16601
37. Collart, M. A., Tourkine, N., Belin, D., Vassalli, P., Jeanteur, P., and Blanchard, J.-M. (1991) *Mol. Cell. Biol.* **11,** 2826–2831
38. Lamb, N. J. C., Fernandez, A., Toukine, N., Jeanteur, P., and Blanchard, J.-M. (1990) *Cell* **61,** 485–496
39. Triezenberg, S. J., LaMarco, K. L., and McKnight, S. L. (1988) *Genes & Dev.* **2,** 730–742
40. Sadowski, I., and Ptashne, M. (1989) *Nucleic Acids Res.* **17,** 7539
41. Mechti, N., Piechaczyk, M., Blanchard, J.-M., Jeanteur, P., and LeBleu, B. (1991) *Mol. Cell. Biol.* **11,** 2832–2841
42. Wright, S., and Bishop, J. M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 505–509
43. Dwarki, V. J., Montminy, M., and Verma, I. M. (1990) *EMBO J.* **9,** 225–232
44. Murphy, T. H., Worley, P. F., and Baraban, J. M. (1991) *Neuron* **7,** 625–635
45. Chrivia, J. C., Kwok, R. P. S., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) *Nature* **365,** 855–859
46. Spencer, C. A., and Groudine, M. (1990) *Oncogene* **5,** 777–785
47. Yankulov, K., Blau, J., Purton, T., Roberts, S., and Bentley, D. L. (1994) *Cell* **77,** 749–759
48. Konig, H., Ponta, H., Rahmsdorf, U., Buscher, M., Schonthal, A., Rahmsdorf, H. J., and Herrlich, P. (1989) *EMBO J.* **8,** 2559–2566

49. Foulkes, N. S., Laoide, B. M., Schlotter, F., and Sassone-Corsi, P. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5448–5452
50. Mellstrom, B., Naranjo, J. R., Foulkes, N. S., Lafarga, M., and Sassone-Corsi, P. (1993) *Neuron* **10,** 655–665
51. Habener, J. F. (1990) *Mol. Endocrinol.* **4,** 1087–1094
52. Foulkes, N. S., Borrelli, E., and Sassone-Corsi, P. (1991) *Cell* **64,** 739–749
53. Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., and Montminy, M. (1992) *Cell* **70,** 105–113
54. Wadzinski, B. E., Wheat, W. H., Jaspers, S., Peruski, L. F., Jr., Lickteig, R. L., Johnson, G. L., and Klemm, D. J. (1993) *Mol. Cell. Biol.* **13,** 2822–2834
55. MacNicol, M., Jefferson, A. B., and Schulman, H. (1990) *J. Biol. Chem.* **265,** 18055–18058
56. Dash, P. K., Karl, K. A., Colicos, M. A., Prywes, R., and Kandel, E. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.,* **88,** 5061–5065
57. Enslen, H., Sun, P., Brickey, D., Soderling, S. H., Klamo, E., and Soderling, T. R. (1994) *J. Biol. Chem.* **269,** 15520–15527
58. Ohmstede, C. A., Jensen, K. F., and Sahyoun, N. E. (1989) *J. Biol. Chem.* **264,** 5866–5875
59. Jensen, K. F., Ohmstede, C.-A., Fisher, R. S., and Sayhoun, N. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2850–2853
60. Srinivasan, M., Edman, C. F., and Schulman, H. (1994) *J. Cell Biol.* **26(4),** 839–852
61. Matthews, R. P., Guthrie, C. R., Wailes, L. M., Zhao, X., Means, A. R., and McKnight, G. S. (1994) *Mol. Cell. Biol.* **14,** 6107–6116
62. Cho, K. C., Scarnes, W. S., Minsk, B., Palmieri, S., Jackson-Grusby, L., and Wagner, J. A. (1989) *Mol. Cell Biol.* **9,** 135–143
63. Sun, P., Enslen, H., Myung, P. S., and Maurer, R. A. (1994) *Genes & Dev.* **8,** 2527–2539