A Novel Calcium Signaling Pathway Targets the c-fos Intragenic Transcriptional Pausing Site

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In many cell types, increased intracellular calcium gives rise to a robust induction of c-fos gene expression. Here we show that in mouse Ltk⁻ fibroblasts, calcium ionophore acts in synergy with either cAMP or PMA to strongly induce the endogenous c-fos gene. Run-on analysis shows that this corresponds to a substantial increase in active polymerases on downstream gene sequences, i.e. relief of an elongation block by calcium. Correspondingly a chimeric gene, in which the human metallothionein promoter is fused to the fos gene, is strongly induced by ionophore alone, unlike a c-fos promoter/β-globin coding unit chimeric construct. Internal deletions in the hMT-fos reporter localize the intragenic calcium regulatory element to the 5' portion of intron 1, thereby confirming and extending previous in vitro mapping data. Ionophore induced cAMP response element-binding protein phosphorylation on Ser133 without affecting the extracellular signal-regulated kinase cascade. Surprisingly induction involved neither CaM-Ks nor calcineurin, while the calmodulin antagonist W7 activated c-fos transcription on its own. These data suggest that a novel calcium signaling pathway mediates intragenic regulation of c-fos expression via suppression of a transcriptional pause site.

The proto-oncogene c-fos represents the prototype for the family of immediate early genes. Its activation follows stimulation of the cell by a wide range of extracellular stimuli but is independent of protein neosynthesis (1–3). c-fos expression is regulated at multiple levels by intracellular signaling events acting in synergy (for reviews, see Refs. 4–7). The majority of studies on how signal transduction cascades modulate c-fos gene expression have focused on its upstream promoter sequences. Several cis-acting elements present in this region have been characterized as targets for numerous stimuli (8–11): the v-sis inducible element (12, 13), the serum response element (SRE) (7, 14–16), the Fos AP1-like site (16) and the cAMP response element (CRE) (17–21).

In several cell types calcium mobilization plays a central role in the modulation of c-fos gene expression; however, the mechanisms involved are still not fully understood. Calcium ions act as intracellular secondary messengers either after entering cells through various ion channels and/or upon release from internal stores. Ca²⁺ differentially activates cellular processes, and immediate early genes such as c-fos gene provide important targets to characterize how the calcium signal is transduced to the nucleus to activate various transcription programs (22, 23). Based on results from mutagenesis and transient transfection analyses, calcium has been proposed to activate a variety of pathways targeting different promoter elements (8, 21). Some are mediated by SRE-dependent processes. In some instances this occurs via the well characterized Ras-Raf-Erk-Elk-1 signaling module (23–26). In other situations increased levels of intracellular calcium induced by membrane depolarization with elevated levels of KCl or exposure to the calcium ionophore ionomycin have been shown to activate the c-fos promoter in PC12 pheochromocytoma cells. These have been linked to SRF independently of Elk-1 (24). SRF-driven activation did not involve Ras but did appear to involve calcium/calcium-dependent kinases (25). The mechanism is still uncertain, since mutation of the major phosphorylation site in SRF showed continued activity in this study. Finally some evidence implies that calcium signals to the Fos AP1-like element immediately downstream of the SRE-binding site (26), even though no transcription factor has been directly implicated in control via the Fos AP1-like site alone.

Other pathways activate c-fos transcription independently of the SRE, primarily via the CRE located at position −65 (reviewed in Refs. 27 and 28). This element is sufficient to mediate calcium-dependent reporter gene activation in some cell contexts, while in other cells additional cryptic CREs in the upstream promoter contribute to reporter gene activity (19). To further complicate the role of the CRE, intracellular calcium fluxes can also activate kinases downstream of Ras and ERK that phosphorylate CREB at serine 133 and thus potentially modulate transcription through the CRE (14, 29).

An explanation for the multiplicity of the effects mediated by calcium on gene expression has recently been provided by elegant microinjection experiments aimed at unraveling how spatially distinct calcium signals generate diverse transcriptional responses (30). Nuclear injection of a non-diffusible calcium chelator blocked increases in nuclear, but not cytoplasmic, calcium concentrations following activation of L-type voltage-gated Ca²⁺ channels; DRE, downstream regulatory element; IBMX, isobutylmethylxanthine; PMA, phorbol 12-myristate 13-acetate; 8-Br-cAMP, 8-bromo-cAMP; ERK, extracellular signal-regulated kinase; CaM, calmodulin; CaM-K, CaM-activated kinase family; SRF, serum response factor; MOPS, 4-morpholinepropanesulfonic acid; MTLIIa, human metallothionein IIa promoter; gapdh, glyceraldehyde-3-phosphate dehydrogenase.
gated calcium channels in a mouse pituitary cell line. Using reporters driven by different c-fos promoter regions, Hardingham et al. (30) showed that increases in nuclear calcium control CRE-mediated transcription, whereas a rise of cytoplasmic calcium activated SRE-driven transcription. In fact, this suggests that the mode of calcium entry and the cell type determine which upstream promoter element is required for the activation of a transiently introduced reporter gene. Accordingly the CRE alone can mediate activation by calcium signals triggered by membrane depolarization of PC12 pheochromocytoma cells (21, 22, 31), an effect that is not reproduced in HeLa cells (8).

More recently, a new calcium-sensitive transcriptional repressor has been proposed to bind a downstream regulatory element (DRE) present within the human prodynorphin gene (32). Upon stimulation by calcium this repressor, named DREAM for DRE-antagonist modulator, is no longer able to bind the DRE. In addition to prodynorphin promoter, DREAM represses also transcription from the c-fos gene in a transient transfection assay. However, whether this is true for the endogenous gene remains to be established.

A close inspection of the c-fos transcription unit through high resolution run-on analysis has also suggested the involvement of intragenic regulatory elements as important targets of c-fos regulation by calcium (33–36). In cultured macrophages, c-fos transcription is stimulated by multiple pathways requiring the mobilization of calcium from internal sources (34, 37). A strong block to transcriptional elongation, mapping beyond c-fos exon 1, was observed when freshly isolated peritoneal macrophages were put into primary culture (34). Calcium-dependent relief of this block strongly increased c-fos mRNA levels. In T cells, elevated cytoplasmic calcium is a critical mediator of activation upon stimulation of the antigen receptor. The synergistic action of calcium ionophore and agonists of protein kinase C mimics authentic antigen treatment in some T cell hybridomas (38). In the latter case, the principal effect of calcium was shown to be on the elongation of c-fos transcripts (35).

Using nuclear extracts from Ltk− cells, we had previously mapped an in vitro arrest site within the murine c-fos gene (39). In this work we confirm and extend these results in vivo. However, because most previous studies on c-fos transcription have dealt with transient transfection experiments, we sought to use permanent cell lines carrying integrated reporter genes. We find that a sequence within c-fos intron 1, while barely active on its own, can strongly augment a calcium ionophore-driven transcriptional response together with its homologous or a heterologous upstream promoter. Even though this is correlated with CREB phosphorylation on Ser133, it is mediated or a heterologous upstream promoter. Even though this is

Cell Culture—Mouse Ltk− fibroblasts were grown at 37 °C in a 5% CO2 atmosphere containing in the presence of 10% fetal calf serum in Dulbecco's modified Eagle's medium. When indicated, cells were serum starved for 24 h and stimulated by refeeding with 10% serum for the indicated times. 3–5 × 106 exponentially growing cells were treated with either 100 nM PMA, 100 nM IBMX, 5 μM 8-BrcAMP, 0.1–10 μM ryanodine or cyclopiazonic A, 10 μM KN62 or KN93, 2.5–250 μM W5 or W7, 20 μM PD98059, or 10 mg/ml of the calcium ionophore A23187 alone, or a combination of these agents as indicated in the legends of the figures. All media were supplemented with streptomycin, penicillin, and l-glutamine. Cells were transfected with the various constructs indicated in the legends using calcium phosphate and pools of G418 resistant cells were used throughout this work.

pHOOK-2 Transient Transfection and Selection—6 μg of pHOOK-2-Lac Z (control), CaM-KII-HOOK, CaM-KIIα-HOOK, or CaM-KIVα-HOOK were transfected in 3–5 × 106 Ltk− p191 cells by the calcium phosphate technique. 16 h later, cells were washed with HS buffer (25 mM Hepes, 140 mM NaCl, pH 7.4), fresh Dulbecco's modified Eagle's medium containing 10% fetal calf serum was added and after 2 h A23187 stimulation was performed, where indicated, for 1 h. Cells were then detached with 6 mM EDTA, spun down, and resuspended in 5 ml of Dulbecco's modified Eagle's medium, 10% fetal calf serum, also containing A23187 where indicated. 50 μl of magnetic beads (Invitrogen Capture-Tec system) were added and tubes were gently stirred at 37 °C for 15 min. After magnetic stand and cell extraction while the other one was treated with Laemmli's polyacrylamide gel electrophoresis-SDS sample buffer.

Nuclear Run-on Transcription—Extraction of nuclei, run-on transcript labeling and hybridization were carried out as described (39). Preparations of crude nuclei were split into aliquots containing 5 × 106 nuclei which were frozen in liquid nitrogen and thawed immediately prior to the labeling reaction. Incubations were carried out at 30 °C for 30 min in the presence of 100 μCi of [α-32P]UTP (400 Ci/mmol, 10 μCi/μl). Labeled run-on transcripts were purified and hybridized to nitrocellulose filters containing equimolar amounts of the plasmids indicated. Hybridization was carried out for 48 h at 42 °C. Filters were washed twice at 65 °C in 0.2× SSC and at 25 °C in 2× SSC containing 2 μg/ml DNA-free RNAse A. Signals were corrected for the thymidine content of each hybridizing DNA strand and standardized to those obtained with the gapdh CDNA probe.

RNA Blots and RNase Protection Assays—Total RNA was run using a standard 5 μg guanidinium thiocyanate-phenol procedure at pH 5. RNA blots were sequentially hybridized to a mouse c-fos genomic cDNA probes labeled by random priming with [α-32P]UTP (3000 Ci/mmol). The RNA probe was prepared from PM37.37 (containing a mouse genomic c-fos DNA spanning nucleotides −599 to +251, cloned into pBluescript) linearized with BssHII, uniformly labeled with [α-32P]UTP (400 Ci/mmol), and purified by polycrylamide gel electrophoresis. 20 μg of RNA for each sample was hybridized, processed for degradation by RNAse A, and the resulting labeled bands analyzed by polyacrylamide gel electrophoresis in 5% polyacrylamide sequencing gels as described (40).

Plasmid Constructs—For c-fos constructs the starting construct was pI91, which contains a 4-kilobase mouse c-fos (21, 22, 31), an effect that is not reproduced in HeLa cells (8). Large deletions were generated using unique or a combination of these agents as indicated in the legends of the figures and pools of G418 resistant cells were used throughout this work.

**EXPERIMENTAL PROCEDURES**

Materials and Reagents—Tissue culture medium, penicillin, streptomycin, glutamine, G418, Random primers DNA labeling system, and TRIZOL were obtained from Life Technologies, Inc. (Cergy Pontoise, France). Phorbol myristate acetate (PMA), 3-isobutyl-1-methylxanthine (IBMX), 8-bromo cyclic adenosine monophosphate (8-Br-cAMP), A23187, W5, W7, KN62, KN93, and secondary antibodies were purchased from Upstate Biotechnology Inc. (EUROMEDEX, France). Bradford protein assay kit was from Bio-Rad (Ivy Sur Seine, France) and Immobilon polyvinylidene difluoride membranes from Millipore. Radioactive signals were revealed by autoradiography using intensifying screens at −80 °C and quantitated by PhosphorImager technology.
Calcium ionophore is required for robust, sustained induction of \textit{c-fos} by PMA or cAMP in \textit{Ltk}\textsuperscript{-} cells. A, exponentially growing \textit{Ltk}\textsuperscript{-} cells were left uninduced (lane 1) or stimulated for 1 (lanes 2–6), 2 (lane 7), or 4 (lane 8) h with PMA (lane 2), IBMX + 8-Br-cAMP (lane 3), A23187 (lane 4), or a combination of A23187 and PMA (lane 5) or IBMX + 8-Br-cAMP (lanes 6–8). 20 \textmu M of total RNA was fractionated by electrophoresis through a formaldehyde-agarose gel, transferred onto nylon membranes and hybridized successively to \textit{c-fos} (top panels) and \textit{gapdh} (bottom panels) probes. B, transient expression of \textit{c-fos} mRNA induced by serum restimulation of \textit{Ltk}\textsuperscript{-} cells starved for 24 h. Total RNA was prepared from either serum-starved cells (lane 1) or cells restimulated for 30 (lane 2), 60 (lane 3), or 90 min (lane 4) and analyzed as above.

assay. 5 \mu g of whole cell extracts were fractionated by electrophoresis through 8.5% SDS gels and transferred onto polyvinylidene difluoride membranes. After a quick dip into methanol, membranes were saturated for 1 h at room temperature in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20) containing 5% bovine serum albumin (fraction V). Primary antibodies (anti-phospho-Thr\textsuperscript{183}/Tyr\textsuperscript{185} ERK or anti-phospho-Ser\textsuperscript{133} CREB) were incubated overnight at 4 \textdegree C in the same medium at a 1/1000 dilution. After 3 washes with TBST, an anti-rabbit IgG antibody was added at a 1/5000 dilution for 1 h at room temperature. Antibody complexes were revealed by enhanced chemiluminescence (ECL kit) after 6 washes 5 min each with TBST.

CaM-activated kinases were purified through a small scale quick batch binding of a whole cell extract (100 \mu g) to a calmodulin affinity resin (Stratagene). Activity was measured by incubating the immobilized kinases for 10 min at 30 \textdegree C in 50 \mu l of a mixture containing 100 \mu M auto Camtide II, 2 \mu M each of PKC and PKA inhibitor peptides, 25 mM MgCl\textsubscript{2}, 100 \mu M \gamma\textsuperscript{32P}ATP (10 \mu Ci), 20 mM MOPS, pH 7.2, 25 mM \beta-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and then actinomycin D was added for the indicated times (Fig. 3A). c-fos mRNA was still detectable 4 h after actinomycin D addition, whereas the same level was obtained with a 1-h actinomycin D chase after serum induction (Fig. 3B). The synergy between calcium ionophore and the other inducers suggests that both upstream and downstream regulatory elements are required for a full response to calcium in these cells.

RESULTS

c-fos Induction in \textit{Ltk}\textsuperscript{-} Cells Requires the Concerted Action of Calcium Ionophore and Phorbol Ester or cAMP—We initially analyzed the response of mouse \textit{Ltk}\textsuperscript{-} fibroblasts to agents that elevate intracellular levels of calcium and/or cAMP, since these pathways were shown to synergize in c-fos activation (46, 47). In \textit{Ltk}\textsuperscript{-} cells, PMA, IBMX + 8-Br-cAMP, or calcium ionophore (A23187) generated only a minor induction of \textit{c-fos} mRNA (Fig. 1A). In contrast, co-treatment with A23187 and either PMA or IBMX + 8-Br-cAMP gave rise to a much stronger induction of \textit{c-fos} (Fig. 1A). Notably the signal was still high after 4 h of stimulation by a combination of A23187 + IBMX + 8-Br-cAMP, whereas it was undetectable 90 min after serum refeeding (Fig. 1B). This strong induction was sensitive to actinomycin D (data not shown; Fig. 4), indicating that it resulted from an increase in \textit{de novo} transcription.

This was confirmed by a run-on analysis carried out on nuclei prepared from cells treated for 1 h with A23187, 8-Br-cAMP and IBMX (Fig. 2). The labeled nascent transcripts were hybridized to two DNA fragments spanning the 5'-half of the murine \textit{c-fos} gene. The first one, (A), contains the first exon and the 3'-half of the first intron. The second (B), spans the 3'-half of the first intron, the second exon and the 5'-half of the second intron. Previous work has shown that c-fos transcription can be regulated in part at the level of elongation (34, 35, 40, 48, 49), and fragment A contains the premature termination site mapped in \textit{vitro} (39). Prior to stimulation, a signal was detected on the promoter proximal fragment, whereas that from fragment B was disproportionately low, especially upon correction for the amount of uridine transcribed into RNA hybridizing to each fragment. Induction gave rise to a small increase in transcription of fragment A, together with a strongly enhanced signal from fragment B. Thus, in \textit{Ltk}\textsuperscript{-} cells the endogenous \textit{c-fos} gene is regulated at the level of transcriptional elongation.

Interestingly, A23187 also acts to stabilize \textit{c-fos} mRNA. Cells were stimulated for 1 h with A23187 + IBMX + 8-Br-cAMP, and then actinomycin D was added for the indicated times (Fig. 3A); \textit{c-fos} mRNA was still detectable 4 h after actinomycin D addition, whereas the same level was obtained with a 1-h actinomycin D chase after serum induction (Fig. 3B). The synergy between calcium ionophore and the other inducers suggests that both upstream and downstream regulatory elements are required for a full response to calcium in these cells.

A \textit{c-fos} Gene under the Control of an Heterologous Promoter Shows a Full Response to Calcium Ionophore—To address the contribution of regions downstream from the promoter to the calcium effect, we transfected \textit{Ltk}\textsuperscript{-} cells with a construct expressing the mouse \textit{c-fos} gene under the control of the human metallothionein IIa promoter (MTLI\textsubscript{a}) (p19/1; Ref. 41), which has high basal activity. A23187 alone was sufficient to substantially induce (60-fold) the transfected gene (Fig. 4A), unlike the endogenous \textit{c-fos} gene (see above). A slightly reduced induction (30-fold) was observed with a mutant lacking the 3'-untranslated region (p19/\textsc{Ds}I-MstII) that was previously shown to enhance mRNA stability (49–52). This is consistent with the increase in stability observed above for the endogenous gene. Actinomycin D blocked the induction but had a less striking effect on basal transcription (Fig. 4A). This observation was confirmed by RNase protection using an antisense probe spanning from nucleotides –95 to +251 relative to the transcription start site (Fig. 5A). The \textit{c-fos} mRNA generated by the transfected gene lacks the first 42 nucleotides and can thus easily be distinguished from the endogenous mRNA (see "Experimental
Calcium ionophore leads to c-fos mRNA stabilization. A, Northern blot analysis of c-fos mRNA decay after treatment with A23187 + IBMX + cAMP for 1 h prior to addition of actinomycin D for the indicated times. RNA was processed as described in the legend of Fig. 1. B, quantitation of the data presented in panel A after normalization to the gapdh signals (closed bars). Also shown is the decay rate of c-fos mRNA following a 30-min serum induction (open bars).

Fig. 4. c-fos intragenic sequences confer calcium inducibility to a heterologous promoter. A, exponentially growing Ltk− cells, transfected with p19/1 (lanes 1–3) or p19/1ΔNsiI-MstII (lanes 4–6), were stimulated for 1 h with A23187, followed by actinomycin D as indicated. B, exponentially growing Ltk− cells, stably transfected with either pMT-globin (lanes 1 and 2) or pFos-globin (lanes 3 and 4), were induced for 1 h with A23187 as indicated. RNA from cells left untreated (−) or treated as indicated (+) was processed for Northern blot analysis as described in the legend of Fig. 1. A mouse c-fos cDNA probe was used in A and a rabbit genomic DNA probe was used in B. In all cases membranes were rehybridized to a rat gapdh probe for normalization. The band marked by an asterisk in the upper panels of panel B, lanes 3 and 4, represent a globin splicing intermediate (53).

Ltk− cells was completely abolished by preincubation with the elongation inhibitor DRB (data not shown).

In order to confirm the involvement of the transcriptional pause site in this effect, we generated a new series of deletions restricted to intron 1 (Fig. 7C). Stably transfected pools of Ltk− cells were established as above and used for in vitro run-on experiments (Fig. 7A). Dot blot hybridizations were performed on DNA from each pool of cells in order to ensure that the copy number of each transfected construct (which ranged from 20 to 30) was high enough to render insignificant the signal arising from the endogenous gene. This was confirmed by run-on analysis (data not shown). Although not as pronounced as for the endogenous gene (Fig. 2), the transgene still harbored a block to transcriptional elongation at the level of basal transcription (Fig. 7). Mutant Δ1 showed the same biased hybridization signal to fragment A observed with the wild type gene (Fig. 7, A and B), which is indicative of a transcriptional pause (see above). Notably the signal became less biased upon further deletion (compare white bars (fragment A) and black bars (fragment B) in Fig. 7B), suggesting the gradual alleviation of transcriptional pausing. This confirms the key role played by intron 1 sequences in c-fos regulation in Ltk− cells.

c-fos Induction by Calcium in Ltk− Cells Does Not Involve Calmodulin-activated Kinases II and IV or Calcineurin—A major mechanism by which an increase in intracellular calcium concentration regulates cellular events is through its association with calmodulin (CaM). The calcium-CaM complex binds to and modulates the activity of multiple regulatory molecules,
including the CaM-activated kinase family (CaM-K) (54). CaM-Ks appear to play a role in transcriptional activation because the calcium-dependent induction of several immediate early genes, such as c-fos, is blocked by the CaM-K inhibitors KN92 and KN93 (26, 55). This has been proposed to occur through CREB phosphorylation (44, 56, 57), SRF phosphorylation (28), and via an interaction between the CaM-K cascade and mitogen-activated protein kinase signaling pathways (58). We therefore investigated these possibilities in our experimental system.

Consistent with previous studies (59), treatment of p19/1 cells with calcium ionophore led to increased CREB phosphorylation, as measured by Western blotting and immunodetection with anti-phospho-CREB antibodies (data not shown). In contrast, A23187 did not affect the level of activated ERK1 and ERK2, as monitored on the same blot with antiphospho-ERK antibodies (not shown). Thus ionophore does not induce the ERK cascade in Ltk− cells. Ionophore addition did stimulate CaM-K activity, as demonstrated with a pull-down kinase assay using calmodulin-affinity resin (Fig. 8A). A 1-h treatment of cells with KN92 not only abolished ionophore-mediated increase in CaM-K activity, but led also to a 70% decrease in basal activity (Fig. 8A). However, this did not inhibit c-fos mRNA induction in p19/1 cells (Fig. 8B). This was somewhat surprising in light of previously published reports. To confirm the lack of CaM-K effect on c-fos, we transfected p19/1 cells with pHook-based vectors expressing constitutively active CaM-KII and CaM-KIV kinases. Transfected cells were enriched by immunoselection, and RNA and proteins processed for Northern and Western blot analysis. Consistent with the results above, neither CaM-KII nor CaM-KIV were able to recapitulate c-fos induction by calcium ionophore (Fig. 9A). In contrast, they were both able to increase phosphorylation of endogenous CREB (Fig. 9B). Furthermore, overexpression of inactive CaM-KII, which should compromise activation driven by CaM-KII, did not block c-fos induction by calcium ionophore (Fig. 9A) but did prevent A23187-induced CREB phosphorylation (Fig. 9B).

The calcium-CaM complex binds also to calcineurin, a calcium-dependent protein phosphatase implicated in gene regulation (38, 60). The immunosuppressant cyclosporin A is a potent calcineurin antagonist, whereas rapamycin, another immunosuppressant, acts independently of the phosphatase (60). We therefore checked whether cyclosporin A might influence A23187-mediated c-fos induction. Neither immunosuppressant had a notable effect on a wide range of concentrations (Fig. 10), therefore ruling out calcineurin in the effects we observe.

**Calcium-mediated c-fos Induction Can Be Mimicked by the Functional Inactivation of CaM.—**These data eliminated ERKs, CaM-Ks, and calcineurin as effectors of ionophore induction of c-fos. In order to evaluate the possible involvement of CaM in another pathway, we treated the cells with the anti-CaM drug W7 that has been extensively used to inhibit CaM in culture cell systems. As a control, we used W5, a drug chemically very similar to W7 but with a much lower affinity for CaM. To our surprise, exposure of cells to W7 prior to A23187 strongly induced c-fos either with or without A23187, while the control compound W5 was inactive (Fig. 11). This suggests that a novel calcium dependent pathway, antagonized by CaM, is involved in controlling transcriptional pausing in the murine c-fos locus.
Calcium-dependent Resumption of c-fos Transcription Elongation

**FIG. 7.** The transcriptional pause site targeted by calcium ionophore maps in the 5' part of c-fos intron 1. A, nuclei were prepared from exponentially growing Ltk− cells stably transfected with the various deletion mutants of p19/1. Run-ons were performed as indicated in Fig. 2 and nascent transcripts were hybridized to the c-fos Nael-Xho1 (fragment A) and Xho1-Xho1 (fragment B) probes as well as to gapdh as described in the legend to Fig. 2. Experiments have been carried out three times and only a representative example is shown here. B, ratios of the signals detected on fragments A and B. The hybridization signals in panel A were quantitated and normalized to gapdh (open bars). The theoretical ratio, based on uridine content of transcripts arising from each deletion mutant, is presented as the solid bars. C, schematic representation of the deletions introduced into intron 1 of p19/1. The symbols are described in the legend to Fig. 6. The horizontal lines below portray the overlapping deletions centered on the Xho1 site. Numbers refer to positions relative to the transcription initiation site. The white box present in intron 1 between positions +363 and +387 shows the previously mapped in vitro transcriptional pause site (39).

**FIG. 8.** CaM Kinase inhibitors do not affect c-fos mRNA induction by calcium ionophore. A, cells were pretreated with the CaM-K inhibitor KN93 (10 µM, 10 min) and then stimulated with A23187 as indicated. Whole cell lysates were prepared and passed over a calmodulin affinity resin. The immobilized proteins were tested for kinase activity toward CaM-tide, a CaM-K substrate peptide. Reactions were spotted on phosphocellulose paper, washed exhaustively, and the radioactivity measured by scintillation counting. B, cells were preincubated for 1 h with 10 µM KN93, then induced as indicated above the lanes. Total RNA extraction, Northern blotting, and hybridization were performed as described in the legend to Fig. 1.

**FIG. 9.** Constitutively active CaM-Ks lead to CREB phosphorylation but fail to induce c-fos expression. Exponentially growing Ltk− cells were transfected with either the parental vector (pHOOK-2-LacZ: lanes A1, A2, and B1) or expression vectors encoding constitutively active CaM-KII (CaM-KII-HOOK: lanes A3 and B2), constitutively active CaM-KIV (CaM-KIV-HOOK: lanes A4 and B3), or inactive, dominant negative CaM-KII (CaM-KII-HOOK: lanes A5 and B4). Transfected cells were selected on magnetic beads and lysed either with TRIZOL for Northern blot analysis (panel A) or with SDS-polyacrylamide gel electrophoresis sample buffer for anti-phosphoCREB Western blotting (panel B). The membrane was stained with Ponceau Red to ensure identical loading. The lower panel presents the quantitation of the c-fos hybridization signals normalized to gapdh.

**FIG. 10.** Calcineurin does not contribute to calcium regulation of the c-fos intragenic pausing site. Exponentially growing Ltk− p19/1 cells were pretreated with various concentrations of either cyclosporin A or rapamycin for 1 h (+) and then induced where indicated (+) with A23187. Only results obtained with 1 µM of either drug are shown here. RNA isolation, Northern blotting, and hybridization were performed as described in the legend to Fig. 1. The lower panel shows the ratio of the c-fos signal to gapdh after PhosphorImager quantitation of the Northern blot.

c-fos, any combination of A23187 with the different inducers led to a strong induction of transcription. Notably upstream c-fos promoter sequences were not sufficient to confer a high level of inducibility by calcium to a reporter β-globin gene. Conversely, replacing the c-fos upstream promoter with that from the human metallothionein IIa promoter generated a chimeric gene highly inducible by calcium ionophore alone. Nuclear run-on analysis showed that mRNA accumulation was mainly due to a dramatic increase in polymerase processivity.

**DISCUSSION**

In this work, based on integrated c-fos reporter genes and not on transient transfection experiments, we show that calcium acts synergistically with other stimuli to activate c-fos transcription through both upstream and downstream regulatory elements. Whereas PMA, IBMX + cAMP, and A23187 alone were not sufficient to give rise to a significant activation of
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FIG. 11. A calmodulin antagonist disrupts regulation of the c-fos intragenic pausing site. Exponentially growing Ltk− p19/1 cells were preincubated for 1 h with various concentrations of W5 or W7, followed by induction with A23187. Only results obtained with 25 μM of either drug are shown here. RNA isolation, Northern blotting, and hybridization were performed as described in the legend to Fig. 1. The lower panel presents the quantitation of the c-fos hybridization signals normalized to gapdh.

These data suggest that intragenic regulatory elements mediate calcium effects provided that transcription initiation has taken place.

The pathway leading from calcium entry to transcription resumption was then evaluated: whereas CaM-Ks activate c-fos gene transcription through promoter proximal elements, astonishingly, their participation, as well as that of the calcium-dependent phosphatase calcineurin, was ruled out in the relief of the block to elongation. However, calmodulin is actually implicated in this phenomenon through a mechanism that remains to be characterized. Taken together, these arguments form a compelling body of data pointing to a new calcium/calmodulin-dependent pathway that targets intragenic sequences to allow transcription to proceed through the pause site. We show here that the metallothionein promoter drives barely detectable transcription through c-fos downstream sequences. The striking induction of transcription upon A23187 treatment therefore reflects a tremendous increase in transcription elongation efficiency. Importantly the same result was obtained with other constitutively active promoters, such as the adenovirus major late or rat β-actin promoters (data not shown). This is consistent with run-on experiments carried out on cells stably transfected with mutants deleting intron sequences of c-fos, which further stressed the important role played by the intragenic transcriptional pause site previously identified in vivo (40, 48, 61) and mapped in vitro (39). Thus, in living cells, the first exon plus the 5' part of the first intron were shown to be crucial for the elongation block, whose release is responsible for calcium induction of the p19/1 construct.

These results confirm and extend the previous observation by Collart et al. (34) in macrophages and by Lee and Gilman (35) in murine T cells. In particular, we show that this calcium-sensitive block to elongation in the 5' part of the c-fos locus can act independently of the promoter but requires the whole coding unit structure to be fully active. Moreover, this phenomenon is no longer restricted to macrophages and T-cells but also applies in fibroblasts, suggesting that it reflects a mechanism with wider relevance than previously appreciated.

Interestingly, transcriptional induction was amplified by stabilization of c-fos mRNA when the endogenous gene was activated by a combination of A23187 and PMA or cAMP. c-fos transcripts generated by the various p19/1-derived constructs after stimulation by A23187 alone were stabilized to the same extent (data not shown), suggesting that calcium also modulates c-fos mRNA decay directed by sequences in the 3'-untranslated region. This region cannot function alone, as shown by the inactive deletion mutants where it was still present. Furthermore, the fact that no deletion mutant retained full calcium-induced activity is consistent with data from transgenic mice showing that full c-fos inducibility in vivo requires the entire locus (62).

We then questioned which pathway led to elongation block release in response to calcium ionophore. Increased intracellular calcium can lead to activation of PKA and PKC, two important effectors of c-fos activation in culture cells and in vivo. The fact that ionophore could synergize with forskolin and PMA, strong activators of PKA and PKC, makes it unlikely that these two kinases mediate the effects we observe. Thus CaM-dependent kinases seemed the most likely candidates, since they have been implicated in mediating c-fos induction by several different signals and mechanisms (25, 26, 55, 63, 64). Surprisingly, the CaM-K inhibitor KN93 (nor KN-62: data not shown) did not affect fos induction although it inhibited both basal and calcium-induced CaM-K activity. Similarly overexpression of dominant-negative CaM-K did not block ionophore-driven p19/1 expression, which was also not reproduced by transfection of constitutively active CaM-Ks II or IV. In contrast, the latter did lead to CREB phosphorylation. This renders any role for CaM-Ks in this process very unlikely and suggests that their previously described activation of c-fos transcription takes place at the level of initiation rather than elongation.

The calcium-activated phosphatase calcineurin regulates gene expression by activating the nuclear localization of the cytoplasmic transcription factor NF-ATc (38, 60). This may account for certain signaling events attributed to cytoplasmic but not nuclear calcium fluxes (30). Nevertheless calcineurin does not mediate increased elongation driven by A23187, since the latter was insensitive to the calcineurin inhibitor cyclosporin A (65).

Recently, DREAM, a new repressor acting through a location-dependent silencer (DRE) has been shown to lead to a calcium-dependent repression of a human c-fos reporter in transient transfection experiments (32). The same sequence is present in the mouse locus but is not present in our reporter constructs that show calcium-dependent regulation. Therefore, the phenomenon we describe here is distinct from DREAM-dependent repression.

These experiments ruled out a number of well documented pathways activated by calcium. We thus tested whether the ionophore signal involved calmodulin itself, using the calmodulin antagonist W7. This compound led to a dramatic induction of the c-fos p19/1 transgene both with and without A23187. Thus this calmodulin antagonist acts in the same direction as calcium entry, which seems paradoxical at first sight. One possible explanation is that this reflects a calcium- and antagonist-sensitive interaction between calcium-free calmodulin and a factor responsible for the block to elongation. This mechanism might resemble that described previously for neurocalmodulin (66, 67), a neurospecific protein whose function is believed to be to bind calcium-free calmodulin and concentrate it within specific regions of the neuron.

The nature of this downstream element and its regulation by calcium remain to be characterized. It might represent a down-stream component of the promoter, inactive on its own, that interacts with a bona fide transcription factor. For example, some bHLH proteins have been shown to interact directly with and be regulated by calcium/calmodulin in their ability to bind DNA. Accordingly, calcium ionophore was shown to selectively inhibit transcriptional activation by these CaM-sensitive bHLH protein in vivo (68). Alternatively this phenomenon
might reflect a constrained chromatin structure responsive to calcium. This would explain why this effect has proven refractory to analysis by transient transfection. Given the quick response to calcium, one can imagine a direct interaction between calmodulin and an elongation factor, such as TFIIIS, P-TEFb (cyclcT/cdk9), or elongin A, a chromatin remodeling system, e.g. Swi/Snf complex, or a histone acetyltransferase. Although the exact molecular mechanisms remain unclear, our data add a new, calcium-dependent pathway and promoter region to the complex signaling network that ensures the tight regulation of c-fos transcription.

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