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Biochemistry

Calcium/calmodulin-dependent protein kinase mediates a pathway for transcriptional regulation

(Rous sarcoma virus/long terminal repeat/CAAT/enhancer binding protein/prolactin gene)

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ABSTRACT **Calcium influx in response to extracellular signals can modulate gene transcription. A constitutive, calcium/calmodulin-independent mutant of type II calcium/calmodulin-dependent protein kinase was capable of increasing the transcription rate of specific genes independently of protein kinase C activation. This increase was mediated by transferrable cis-active elements capable of binding the transcription factor CAAT/enhancer binding protein. Therefore, the activation of type II calcium/calmodulin-dependent protein kinase in response to stimuli that increase intracellular calcium is proposed to represent a distinct second messenger pathway in calcium-mediated regulation of gene transcription.**

Second messengers such as cAMP and calcium ions can activate serine/threonine protein kinases that modulate gene transcription (1, 2). In contrast to cAMP, calcium seems capable of stimulating multiple signaling pathways (3, 4), including protein kinase C (5, 6). Brain-specific type II calcium/calmodulin-dependent protein kinase (CaM-kinase) is a member of a family of evolutionarily conserved, widely expressed isozymes (7) implicated in exocytosis, long-term potentiation, and induction of oocyte maturation (8–10). Because calmodulin antagonists can affect the rate of transcription of the prolactin, *fos*, and proopiomelanocortin genes, a calmodulin-dependent protein kinase may regulate gene transcription (5, 6, 11–13). To investigate a role for CaM-kinase in transcriptional regulation, constitutively active CaM-kinase mutants were engineered to isolate the potential effects of CaM-kinase from other calcium-dependent events. Calcium/calmodulin-independent forms of the brain CaM-kinase α subunit (CaMKα) specifically activated the rat prolactin promoter and the Rous sarcoma virus long terminal repeat (RSV LTR) promoter in cotransfection assays with cultured GC rat pituitary tumor cells. A CaM-kinase response element (CaMRE) is described that is capable of selectively conferring induction of transcription by activated CaM-kinase.

METHODS

Bacterial Expression of CaMKα. The complete cDNA for CaMKα was constructed by fusing overlapping C3 and C31 clones encoding partial rat sequences (14). After introduction of a *Nde* I site overlapping the codon for the initiator methionine by site-directed mutagenesis, the bacterial expression vectors for CaMKα were constructed by insertion of the coding sequences into plasmid pT7-7 (15). pT7-CaMKα was expressed as described (16), and induced cells were either lysed into SDS/PAGE sample buffer or were used to prepare soluble extracts (17). Binding of antibody and calmodulin to nitrocellulose blots of SDS/PAGE size-fractionated whole-cell protein was performed as described (17). CaMKα activity in bacterial soluble extracts was assayed by using microtubule-associated protein 2 (MAP-2) as a substrate (18). MAP-2 was separated from other radiolabeled proteins by SDS/PAGE.

Transfectional Analyses. RSV CaMKα mutants were constructed by substituting the appropriate sequence into a RSV-neo^r expression vector (19, 20). Rat prolactin [−3 kilobase pairs (kbp) or −422 bp to +36 bp] and growth hormone (−316 to +7 bp) promoter reporter plasmids were described (21, 22). The mouse primase p49 promoter reporter plasmid was the generous gift of B. Tseng and C. Prussak (University of California San Diego, La Jolla, CA). One copy of the cAMP response element (CRE) from the α-chorionic gonadotropin gene (−208 to −185 bp) (1) or two copies of a phorbol ester response element (PRE) (23) were inserted into a −36- to +34-bp rat prolactin reporter plasmid (22).

An RSV LTR luciferase reporter plasmid was constructed for expression studies. After conversion to *Xho* I of the *Nde* I site of RSV-chloramphenicol acetyltransferase (24), the *Xho* I to *Hin*dIII fragment (RSV LTR included) was subcloned into pBluescript. After conversion to *Not* I of the *Aat* II site in a pSV2ALΔ5′luciferase derivative (25), the complete luciferase cDNA and simian virus 40 (SV40) termination sequences were transferred to pBluescript RSV LTR as a *Hin*dIII/*Not* I fragment. Sequencing showed that the LTR fragment was of the nearly identical Schmidt-Ruppin strain A. The deletion series of the RSV LTR was designed as follows: a unique *Bgl* II restriction site was introduced at −59, −111, −169, −285, and −488 bp with subsequent transfer of the appropriate *Xho* I/*Bgl* II and *Bgl* II/*Hin*dIII fragments to form the complete set of 10 deletion mutants. All deletions affected baseline activity, in agreement with published data (26). Double-stranded oligonucleotides (see Fig. 3*C*) corresponding to RSV strain D were inserted into the −36- to +34-bp prolactin promoter (22) and/or the *Bgl* II site of the −169-bp RSV LTR reporter plasmids.

GC cells were transfected with DEAE-dextran as described by using 10 μg of plasmid DNA per 3×10^6 cells (20). After transfection, cells were incubated for 2 days in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were induced with dibutyryl cAMP (1 mM final concentration) or phorbol 12-myristate 13-acetate

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Abbreviations: CaM-kinase, type II calcium/calmodulin-dependent protein kinase; CaMKα, CaM-kinase brain α subunit; C/EBP, CAAT/enhancer binding protein; RSV LTR, Rous sarcoma virus long terminal repeat; CaMRE, CaM-kinase response element; PMA, phorbol 12-myristate 13-acetate; MAP-2, microtubule-associated protein 2; CRE, cAMP response element; PRE, phorbol ester response element; SV40, simian virus 40.

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(PMA; 1 μM final concentration) when appropriate. Harvesting and luciferase assays were performed as described (25).

DNA Binding Protein Assays. A cDNA encoding CAAT/enhancer binding protein (C/EBP) (27) was isolated from a GC library and expressed in bacteria as described above (16). GC nuclear extracts were prepared by the method of Sealey and Chalkley (28), and gel-shift mobility assays were performed as described (29). For competition assays, $(NH_4)_2SO_4$-fractionated 530 mM nuclear extract (29) was bound to 70 pM end-labeled double-stranded oligonucleotide containing the RSV LTR sequence from −231 to −193 bp, in the presence of the indicated concentration of unlabeled competitor oligonucleotide. DNase I footprinting (29) of the RSV LTR from −285 to −52 bp was performed with excess $(NH_4)_2SO_4$-fractionated 530 mM nuclear extract.

RESULTS

Analysis of CaMKα Mutants. Bacterially expressed mutants of the rat brain CaMKα (Fig. 1*A*) truncated prior to or following the putative calmodulin-binding domain (14) were characterized by using a specific antibody or biotinylated calmodulin (Fig. 1*B*). All mutant proteins migrated at the expected molecular weight; an additional band at M_r 32,000 (lanes WT and M42), resulting from a bacteria-specific truncation (31), was not detected with *in vitro* translation products (F. Cruzalegui and M.S.K., unpublished data). Of the four mutations assayed, only truncation at residue 290 abolished calmodulin binding (Fig. 1*B*, lane 13). Calcium-dependent phosphorylation activity of the wild-type and mutant proteins was assayed with the substrate MAP-2. As shown in Fig. 1*C*, truncation mutants at residues 326 and 316 displayed wild-type activity, while truncation at residue 290 resulted in constitutive calcium-independent MAP-2 phosphorylation. Catalytic function was eliminated by the mutation of lysine residue 42 to methionine (M42) in the consensus ATP binding site (32).

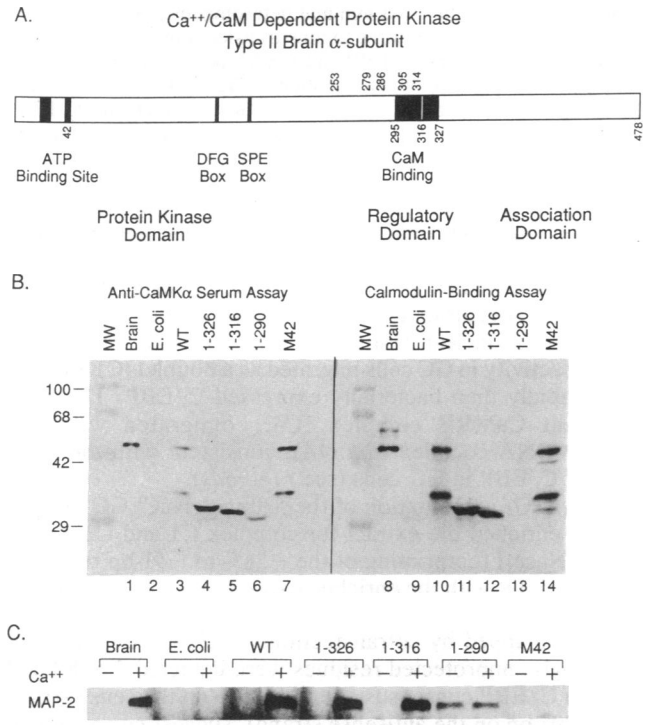

FIG. 1. Preparation of a constitutive CaMKα. (*A*) CaMKα domain structure is shown with autophosphorylation sites (7, 17, 30) indicated above and motif positions indicated below the bar. (*B*) Bacterially expressed CaMKα proteins assayed for ability to bind a specific CaMKα antibody and calmodulin (17). The M_r 54,000 bacterial CaMKα, wild-type (lanes 3 and 10) and M42 substitution (lanes 7 and 14), comigrated with purified rat brain CaMKα (lanes 1 and 8). MW, molecular weight markers. (*C*) Bacterially expressed soluble protein was assayed in the presence (lanes +) or absence (lanes −) of calcium for calmodulin-dependent MAP-2 kinase activity (18). Truncation to residue 290 abolished calmodulin binding and calcium-dependent catalytic function.

CaMKα 1–290 Specifically Activates Gene Transcription. The potential for CaM-kinase to modulate gene transcription was investigated by cotransfection analyses in GC rat pituitary tumor cells (Fig. 2). Expression of rat prolactin and RSV LTR fusion genes was induced 2.7- and 14-fold, respectively, by cotransfection with the constitutive CaMKα 1–290 truncation protein (Fig. 2*A*). The expression of the rat growth hormone and mouse primase promoters was not altered. This induction was dependent on the presence of active CaM-kinase, since transcriptional effects were not observed when cells were cotransfected with either unstimulated, wild-type enzyme or an inactivated CaMKα 1–290 (lysine to methionine at residue 42) mutant [1.1 ± 0.1- and 1.2 ± 0.3-fold induction of RSV LTR, respectively (n = 3)]. RSV LTR inductions were also obtained in cotransfections by using two full-length mutant forms of CaMKα containing constitutive activity (M.S.K., unpublished data).

Reporter plasmids containing either CREs (1) or PREs (23) were not regulated by the addition of constitutive CaMKα. These results suggest that CaMKα does not regulate specific gene induction via activation of protein kinase A or protein kinase C, even though bacterially expressed CRE binding protein is an effective *in vitro* CaM-kinase substrate (M.S.K., unpublished observations). Consistent with distinct functions of protein kinase C and CaM-kinase, addition of the phorbol ester PMA to cells cotransfected with CaMKα 1–290 activated the RSV LTR reporter plasmid approximately

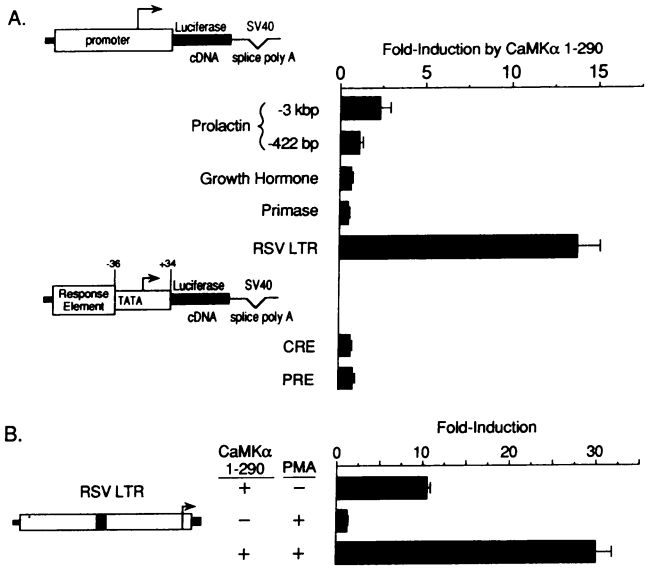

FIG. 2. (*A*) GC pituitary cells were cotransfected with a luciferase reporter plasmid under control of a test promoter with a RSV-neo^r or RSV-CaMKα expression plasmid (see *Methods*). Data are the average inductions (-fold) ± SEM for 3–24 experiments. Promoters were fused to the identical luciferase coding and SV40 termination sequences. Copies of the CRE and PRE were inserted upstream of the −36- to +34-bp prolactin promoter, which contains only a functional TATA box and cap site (22). (*B*) The effect of CaMKα 1–290 on RSV LTR promoter function was evaluated in the absence (−) or presence (+) of 1 μM phorbol ester (PMA). Data are the ratios determined under each condition to that observed using control plasmid and no PMA and represent the average of two experiments. Experiments repeated under stripped serum and serum-free conditions provided similar results.

3-fold. PMA treatment of cells transfected with the RSV LTR reporter alone exerted little or no effect (1.2 ± 0.1-fold; Fig. 2*B*), suggesting that the effects of protein kinase C and CaM-kinase may be synergistic.

To determine the specificity of the CaM-kinase induction of the RSV LTR, several additional cell lines were examined by cotransfection assays. Experiments in P19 teratocarcinoma, HeLa ovarian carcinoma, and MB231N mammary carcinoma cells showed no induction of the RSV LTR by cotransfection with CaMKα 1–290. Cotransfection of Rat-1 fibroblasts, however, resulted in a 6.6 ± 1.1-fold induction (n = 4) by CaMKα 1–290 of the RSV LTR. This induction was similar to a 5.3 ± 0.9-fold induction (n = 2) of RSV LTR activity observed in Rat-1 cells by the addition to the medium of 50 mM KCl and 300 nM Bay K8644, a dihydropyridine Ca^{2+}-channel modulator (33). In contrast, treatment of GC cells with KCL and Bay K8644 did not modulate the RSV LTR even when cells were cotransfected with a transcription unit expressing wild-type CaMKα. This result is consistent with the inefficacy of Bay K8644 to induce intracellular calcium sufficiently high for effective calmodulin binding in endocrine cells (33, 34). The basal level of RSV LTR activity in transfected GC cells was not consistently inhibited (<2-fold) by exposure to 2 mM EGTA, 200 nM nimodipine (dihydropyridine Ca^{2+}-channel antagonist), or 1 μM calmidazolium (phenothiazine calmodulin antagonist) in the presence of serum, suggesting that low levels of calmodulin-stimulated transcriptional activity are present in these cells.

Mapping of a CaMRE. The RSV LTR was further studied in GC cells by using a series of deletions to define a discrete cis-active element that conferred CaM-kinase activation. Deletion of the region RSV LTR between −285 and −169 bp specifically abolished CaMKα inducibility (Fig. 3*A*). Because the −285- to −169-bp region had been shown to include sequences required for DNA binding activities in avian and mammalian extracts by DNase I footprinting (28, 35), a double-stranded oligonucleotide comprising the protected sequence (−231 to −193 bp) was tested for the ability to confer CaM-kinase regulation. Insertion of this element upstream of the −169-bp RSV LTR deletion restored CaMKα 1–290 inducibility (WT/−169; Fig. 3*A*). In addition, this sequence transferred CaMKα-dependent activation to a heterologous promoter, −36- to +34-bp prolactin (Fig. 3*B*).

These results suggest that the −231- to −193-bp region of the RSV LTR (Fig. 3*C*) contains a CaM-kinase response element. This sequence consists of a 13-bp direct repeat, which overlaps two 9-bp C/EBP-binding site consensus sequences (35) and shares sequence homology with a non-responsive binding element (−156 to −122 bp) (28). Although the sequence comprising the −156- to −122-bp RSV LTR region increased the basal level of transcription of the minimal promoter, it failed to provide CaMKα inducibility (Fig. 3*B*). The SV40 enhancer core element, known to confer regulation by C/EBP (35), was likewise uninducible and enhanced the basal expression of the minimal promoter less than either RSV element (Fig. 3*B*). Because the SV40 enhancer bound bacterially expressed C/EBP with 50-fold lower affinity than the −231- to −193-bp RSV element (data not shown), it remained necessary to determine the relative significance of the 13-bp repeat and C/EBP motifs by mutagenesis. Mutating the direct repeats (M1) did not prevent either basal activation or CaMKα inducibility of −169-bp RSV LTR (Fig. 3*A*). In contrast, altering the C/EBP consensus (M2) completely abolished enhancer activity, both for baseline and CaMKα inducibility (Fig. 3*A*). Similar enhancement functions of the M1 or M2 sequences were observed in context of the heterologous −36- to +34-bp prolactin promoter.

CaMRE Binding Proteins. Although information necessary for C/EBP regulation of the RSV LTR was essential for a functional CaMRE, C/EBP was not phosphorylated by CaM-kinase *in vitro* and, therefore, might not represent the regulated factor(s). At least four different specific complexes could be detected by gel mobility shift assay using the −231- to −193-bp site, although the significance of the weak C_3 and C_4 bands is unclear (Fig. 4*A*). The majority of CaMRE binding activity in GC cells migrated as a doublet (C1 and C2) more rapidly than bacterially expressed C/EBP. The least abundant CaMRE complex, C4, comigrated with the C/EBP–DNA complex (Fig. 4*A*), consistent with the presence of C/EBP in GC cells (see *Methods*).

$(NH_4)_2SO_4$ precipitation of the 530 mM NaCl GC nuclear extract enriched the extract for complex C1 and C2 formation. DNase I footprinting of the −285- to −51-bp region of the RSV LTR with the enriched extract revealed a protected region from about −225 to −188 bp, corresponding to the CaMRE mapped by cotransfectional analysis (Fig. 4*B*). Interestingly, unprotected residues were detected 7 or 8 bp 3′ of each C/EBP consensus site (−204 bp on the sense strand and −207 bp on the antisense strand), suggesting a symmetrical occupancy of adjacent sites. Bacterially expressed C/EBP gave similar footprints over the identical region (data not shown) as described (35). Competition of C1 and C2 complexes with the −231- to −193-bp site by the addition of unlabeled oligonucleotide in the gel mobility shift assay in both complexes showed high affinity binding (>10^9 M^{-1}) to the inducible WT and M1 sites and low or undetectable affinity for the nonregulated SV40 and M2 sites (Fig. 4*C*). Similar C1 and C2 complexes were detected in gel mobility shift assays using Rat-1 extracts, cells in which the CaM-kinase induction in cotransfection assays was also abolished

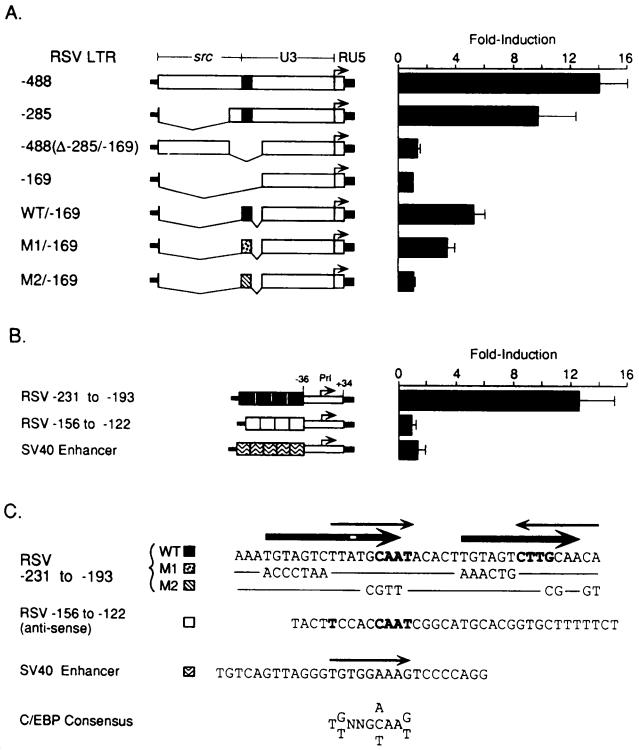

FIG. 3. Definition of the CaMRE in the RSV LTR. (*A*) A deletion series of the RSV LTR was tested for CaMKα 1–290 inducibility in 4–13 experiments. (*B*) Elements were tested for the ability to transfer CaM-kinase inducibility to a minimal heterologous −36- to +34-bp prolactin promoter (22) in three to nine transfections. (*C*) Alignment of sequences shows the C/EBP and CAAT box homologies in the CaM-kinase responsive oligonucleotides. The thick arrows overlay the 13-bp direct repeat, and the thinner arrows align the C/EBP consensus binding sites (35). CAAT sequences are indicated by boldface type.

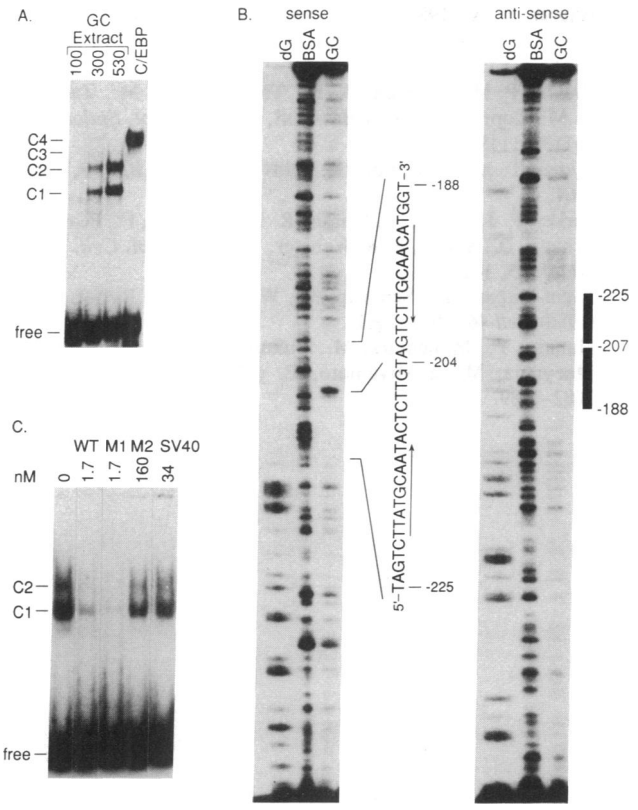

FIG. 4. (*A*) Extracts of GC cell nuclei prepared with increasing salt concentrations were analyzed for binding activity to −231- to −193-bp RSV LTR oligonucleotide by gel mobility shift assay. Four specific complexes (C1–C4) were detected. (*B*) DNase I footprinting of the −285- to −53-bp region of the RSV LTR was performed with $(NH_4)_2SO_4$-fractionated 530 mM NaCl GC nuclear extract. The dG Maxam–Gilbert sequencing ladder and DNase I digestions in the presence of albumin (BSA) or extract are shown for sense (*Left*) and antisense (*Right*) strands. The sequence shown is for strain SR-A. Compare Fig. 3*C*; the C/EBP consensus sequences are overlined by arrows. (*C*) Relative site affinity of C1 and C2 complexes was analyzed by competition with the indicated concentration of unlabeled oligonucleotide of binding to the −231- to −193-bp WT site by $(NH_4)_2SO_4$-fractionated 530 mM NaCl GC nuclear extract.

by 5′ deletion of the RSV LTR to −169 bp (data not shown). These results show that multiple C/EBP-like binding activities exist in the CaM-kinase-responsive cell types, each of which exhibits an appropriate hierarchy of binding-site affinity for a CaMRE binding protein and, therefore, may represent the relevant trans-acting factor(s).

DISCUSSION

These data indicate that a calcium/calmodulin-independent mutant of the brain α subunit of CaM-kinase can induce specific gene transcription by a mechanism distinct from protein kinase A and protein kinase C activation. Because the −231- to −193-bp region of the RSV LTR confers regulation by CaMKα 1–290, this sequence appears to include a specific CaMRE(s). CaM-kinase is activated under conditions of higher calcium influx (700–900 nM) than that required for protein kinase C stimulation (<500 nM) (36). Thus, CaM-kinase may mediate higher-threshold calcium regulation for a distinct subset of genes with C/EBP consensus sequences. In contrast to protein kinase C, CaM-kinase autophosphorylation *in vitro* results in a calcium/calmodulin-independent form with similar activity to the constitutive truncation and full-length mutants used in these studies (7, 17). This raises the intriguing possibility that long-term responses could result from transiently induced calcium currents and CaM-kinase-mediated transcriptional activation.

In addition to the CaMRE of the RSV LTR described above, the rat prolactin gene, which can be induced by CaM-kinase (Fig. 2*A*), may provide an example of a gene under calcium regulation by both calmodulin and protein kinase C-mediated pathways (5, 11). Removal of sequences distal to −422 bp eliminated responsivity to CaMKα 1–290, in agreement with a report that phorbol ester-independent calcium channel agonist regulation was conferred by the distal prolactin enhancer (−1765 to −1495 bp) (5). It will be necessary to determine the presence of C/EBP-like binding activity within this region. In addition, we have found that CaMKα activates a vitellogenin estrogen responsive element (2.2- ± 0.3-fold; $n = 8$) (37), suggesting that a pituitary form of CaM-kinase (38) could modulate prolactin gene expression via the functionally important estrogen receptor binding site in the distal prolactin enhancer (20).

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1. Mellon, P. L., Clegg, C. H., Correll, L. A. & McKnight, G. S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4887–4892.
2. Gonzalez, G. A. & Montminy, M. R. (1989) *Cell* **59,** 675–680.
3. Greengard, P. (1978) *Science* **199,** 146–152.
4. Nishizuka, Y. (1986) *Science* **233,** 305–312.
5. Day, R. N. & Maurer, R. A. (1990) *Mol. Endocrinol.* **4,** 736–742.
6. Loeffler, J. Ph., Kley, N., Louis, J. C. & Demeneix, B. A. (1989) *J. Neurochem.* **52,** 1279–1283.
7. Schulman, H. (1988) *Adv. Second Messenger Phosphorylation Res.* **22,** 39–112.
8. Llinas, R., McGuinness, T. L., Leonard, C., Sudimori, M. & Greengard, P. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3035–3039.
9. Malinow, R., Schulman, H. & Tsien, R. H. (1989) *Science* **245,** 862–866.
10. Waldmann, R., Hanson, P. I. & Schulman, H. (1990) *Biochemistry* **29,** 1679–1684.
11. White, B. A. (1985) *J. Biol. Chem.* **260,** 1213–1217.
12. Morgan, J. L. & Curran, T. (1986) *Nature (London)* **322,** 552–555.
13. Sheng, M., McFadden, G. & Greenberg, M. E. (1990) *Neuron* **4,** 571–582.
14. Lin, C. R., Kapiloff, M. S., Durgerian, S., Tatemoto, K., Russo, R. F., Hanson, P., Schulman, H. & Rosenfeld, M. G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5962–5966.
15. Tabor, S. & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1074–1078.
16. Studier, F. W. & Moffatt, B. A. (1986) *J. Mol. Biol.* **189,** 113–130.
17. Hanson, P. I., Kapiloff, M. S., Lou, L. L., Rosenfeld, M. G. & Schulman, H. (1989) *Neuron* **3,** 59–70.
18. Schulman, H., Kuret, J., Jefferson, A. B., Nose, P. S. & Spitzer, K. H. (1985) *Biochemistry* **24,** 5320–5327.
19. Giguere, V., Hollenberg, S. M., Rosenfeld, M. G. & Evans, R. M. (1986) *Cell* **46,** 645–652.
20. Waterman, M. L., Adler, S., Nelson, C., Greene, G. L., Evans, R. M. & Rosenfeld, M. G. (1988) *Mol. Endocrinol.* **2,** 14–21.
21. Nelson, C., Albert, V. A., Elsholtz, H. P., Lu, L. I.-W. & Rosenfeld, M. G. (1988) *Science* **239,** 1400–1405.
22. Ingraham, H. A., Chen, R., Mangalam, H. J., Elsholtz, H. P., Flynn, S. E., Lin, C. R., Simmons, D. M., Swanson, L. & Rosenfeld, M. G. (1988) *Cell* **55,** 519–529.
23. Lee, W., Mitchell, P. & Tjian, R. (1987) *Cell* **49,** 741–752.

24. Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I. & Howard, B. H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6777–6781.
25. DeWet, J. R., Wood, K. V., Deluca, M., Helinski, D. R. & Subramani, S. (1987) *Mol. Cell. Biol.* **7**, 725–737.
26. Cullen, B. R., Raymond, K. & Ju, G. (1985) *Mol. Cell. Biol.* **4**, 438–447.
27. Landschulz, W. F., Johnson, P. F., Adashi, E. Y., Graves, B. J. & Mcknight, S. L. (1988) *Genes Dev.* **2**, 786–800.
28. Sealey, L. & Chalkley, R. (1987) *Mol. Cell. Biol.* **7**, 787–798.
29. Mangalam, H. J., Albert, V. R., Ingraham, H. A., Kapiloff, M. S., Wilson, L., Nelson, C., Elsholtz, H. & Rosenfeld, M. G. (1989) *Genes Dev.* **3**, 946–958.
30. Patton, B. L., Miller, S. G. & Kennedy, M. B. (1990) *J. Biol. Chem.* **265**, 11204–11212.
31. Waxham, M. N., Aronowski, J. & Kelly, P. T. (1989) *J. Biol. Chem.* **264**, 7477–7482.
32. Maurer, R. A. (1989) *J. Biol. Chem.* **264**, 6870–6873.
33. Enyart, J. J., Biagi, B. & Day, R. N. (1990) *Mol. Endocrinol.* **4**, 727–735.
34. Hinkle, P. M., Jackson, A. E., Thompson, T. M., Zavavki, A. M., Coppola, D. A. & Bancroft, C. (1988) *Mol. Endocrinol.* **2**, 1132–1138.
35. Ryden, T. A. & Beemon, K. (1989) *Mol. Cell. Biol.* **9**, 1155–1164.
36. Parker, P. J., Kour, G., Marais, R. M., Mitchell, F., Pears, C., Schaap, D., Stable, S. & Webster, C. (1989) *Mol. Cell. Endocrinol.* **65**, 1–11.
37. Klein-Hitpass, L., Schorpp, M., Wagner, U. & Ryffel, G. U. (1986) *Cell* **46**, 1053–1061.
38. Hatada, Y., Munemura, M., Fukunaga, K., Yamamoto, H., Maeyama, M. & Miyamoto, E. (1983) *J. Neurochem.* **40**, 1082–1089.