Regulation of Somatostatin Gene Transcription by Cyclic Adenosine Monophosphate

M. Montminy, P. Brindle, J. Arias, K. Ferreri, and R. Armstrong

Cyclic adenosine monophosphate (cAMP) stimulates transcription of somatostatin and other target genes with burst-attenuation kinetics. The kinetics of protein kinase (PK-A)–dependent cAMP response element binding protein (CREB) phosphorylation closely parallel the changes in transcription of cAMP-responsive genes by run-on assay. Nuclear translocation of PK-A, visualized by microinjection of fluorescently labeled PK-A holoenzyme, appears to represent the rate-limiting step in CREB phosphorylation and transcriptional activation. We and others have recently characterized a CREB-binding protein (CBP), which specifically recognizes sequences within the Ser133 phosphorylated form of CREB. CBP does not regulate the DNA binding, dimerization, or nuclear targeting properties of CREB, but binds selectively to the kinase-inducible 60 amino acid *trans*-activation domain (KID) of CREB, critical for PK-A–inducible transcription. We developed an antiserum directed against amino acid 634-648 within the CREB-binding domain of CBP. We detected a 265-kd polypeptide by Western blot as predicted from the cDNA, which coincided with the predominant phospho-CREB–binding activity in Hela nuclear extracts by "Far Western" blot assay. An identical phospho-CREB–binding activity was also found in NIH-3T3 cells. This phospho-CREB–binding protein appeared to be specific for Ser133-phosphorylated CREB, because no such band was detected with CREB labeled to the same specific activity at a nonregulatory phosphoacceptor site (Ser156) by casein kinase II (CKII). Following microinjection into nuclei of NIH-3T3 cells, a cAMP response element (CRE)-lacZ reporter was markedly induced by treatment with 8-Br cAMP plus isobutyl methyl xanthine (IBMX). Coinjection of CBP antiserum with the CRE-lacZ plasmid inhibited cAMP-dependent activity in a dose-dependent manner, but control immunoglobulin G (IgG) had no effect on this response. We can now begin reconstituting PK-A–dependent transcription in vitro, using well-characterized proteins such as CREB, TAF 110, and CBP. The assembly of such factors on cAMP-regulated promoters like somatostatin may enable responsiveness to a variety of hormonal stimuli that employ cAMP as their second messenger.

PREVIOUS STUDIES in our[1] and other[2,3] laboratories have identified a consensus cyclic adenosine monophosphate (cAMP) response element (CRE), usually positioned within 100 nucleotides of the transcriptional initiation site, which mediates transcriptional induction of somatostatin and other cAMP-responsive genes. Characterized by a core motif that often contains the palindromic sequence 5′-TGACGTCA-3′, the CRE can confer cAMP inducibility when placed upstream of a heterologous promoter and can function in both distance- and orientation-independent contexts. Remarkably, mutant cell lines deficient in protein kinase (PK-A) cannot support cAMP-responsive transcription from a CRE reporter gene,[1] suggesting that this kinase might phosphorylate proteins that bind to the CRE and thereby activate transcription of cAMP-responsive genes. To this end, we purified a 43-kd CRE-binding protein (CREB) from PC12 and brain nuclear extracts using CRE-oligonucleotide affinity chromatography.[4,5] The purified CREB was highly phosphorylated by PK-A in vitro, and primary sequencing of a CREB tryptic phosphopeptide revealed a consensus PK-A site: (R)R-P-S-Y-R. Using primary sequence information provided by other tryptic fragments of CREB, we obtained cDNAs encoding a 341 amino acid protein, which contained the PK-A phospho-acceptor site at Ser133.[6] cDNAs for CREB were also characterized independently by Hoeffler et al.[7]

Previous work showing that most, if not all, of the biological effects of cAMP are mediated by the cAMP-dependent PK-A prompted us to test whether CREB activity in response to cAMP might be dependent on phosphorylation by PK-A. We found that CREB is indeed phosphorylated by PK-A at a single serine phosphoacceptor site, Ser133,[8] and this phosphorylation is induced by hormonal stimuli that signal through the cAMP pathway. Remarkably, mutant cell lines that are deficient in PK-A activity cannot stimulate CREB phosphorylation at Ser133, nor can they induce CRE-dependent transcription. To determine whether PK-A phosphorylation of CREB at Ser133 was critical for cAMP-responsive transcription in vivo, we compared wild-type and mutant forms of CREB containing substitutions at the PK-A phosphoacceptor site.[8] When transfected into F9 teratocarcinoma cells, a wild-type CREB expression plasmid can stimulate CRE-chloramphenical acetyl transferase (CAT) reporter activity greater than 200-fold in the presence of PK-A. By contrast, a mutant CREB plasmid containing a Ser133-to-Ala133 substitution was completely unable to respond to PK-A induction. Acidic substitution mutants (Ser133 to Asp133 or Glu133) were also inactive, suggesting that the negative charge provided by phosphorylation is not sufficient to stimulate transcription.

Like other signaling pathways, cAMP stimulates transcription of somatostatin and other target genes with burst-attenuation kinetics: transcription usually peaks within 20 to 30 minutes of induction and gradually declines over the

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next 4 to 8 hours (Fig 1). The kinetics of PK-A–dependent CREB phosphorylation closely parallel the changes in transcription of cAMP-responsive genes by run-on assay (Fig 1). Nuclear translocation of PK-A, visualized by microinjection of fluorescently labeled PK-A holoenzyme, appears to represent the rate-limiting step in CREB phosphorylation and transcriptional activation.[9,10]

We and others have recently characterized a CREB-binding protein (CBP) that specifically recognizes sequences within the Ser133 phosphorylated form of CREB.[11] CBP does not regulate the DNA binding, dimerization, or nuclear targeting properties of CREB. Rather, CBP binds selectively to the kinase-inducible domain (KID) of CREB, a 60 amino acid *trans*-activation domain that is critical for PK-A–inducible transcription. To characterize the functional properties of CBP, we developed an antiserum directed against amino acid 634-648 within the CREB-binding domain of CBP.[12] Using this antiserum, we detected a 265-kd polypeptide by Western blot (Fig 2A, lane 1) as predicted from the cDNA,[11] which coincided with the predominant phospho-CREB–binding activity in Hela nuclear extracts by "Far Western" blot assay (Fig 2A, lane

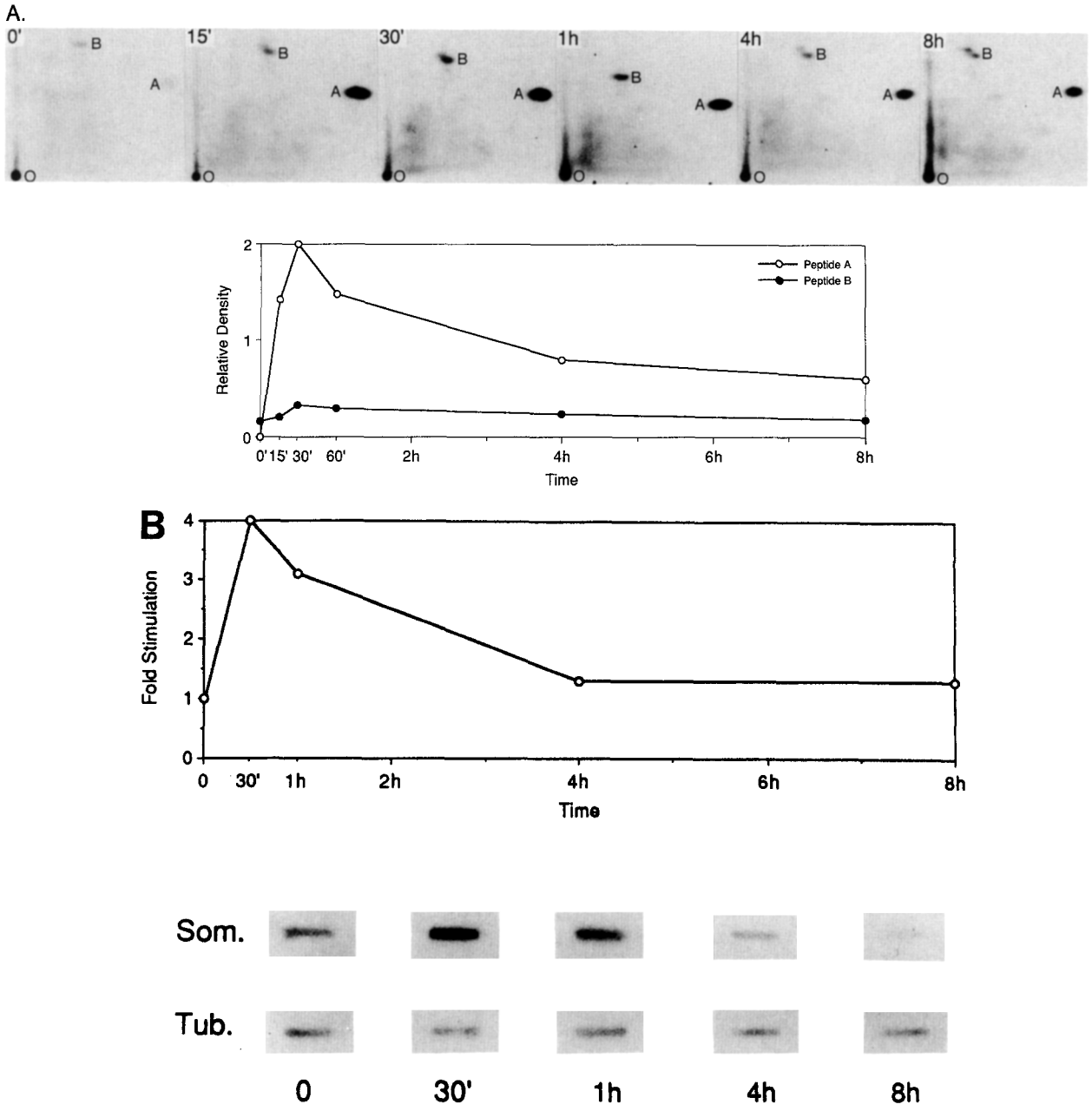

Fig 1. (A) Two-dimensional phosphotryptic mapping of ^{32}P-labeled CREB in PC12 cells after treatment with forskolin. Spot A contains PK-A phosphorylation site (Ser133); spot B contains casein kinase II (Ser156) phosphorylation site. (B) Run-on transcription of the cAMP-responsive somatostatin gene in stable lines of NIH-3T3 cells transfected with the somatostatin gene. Tubulin refers to its mRNA transcription.

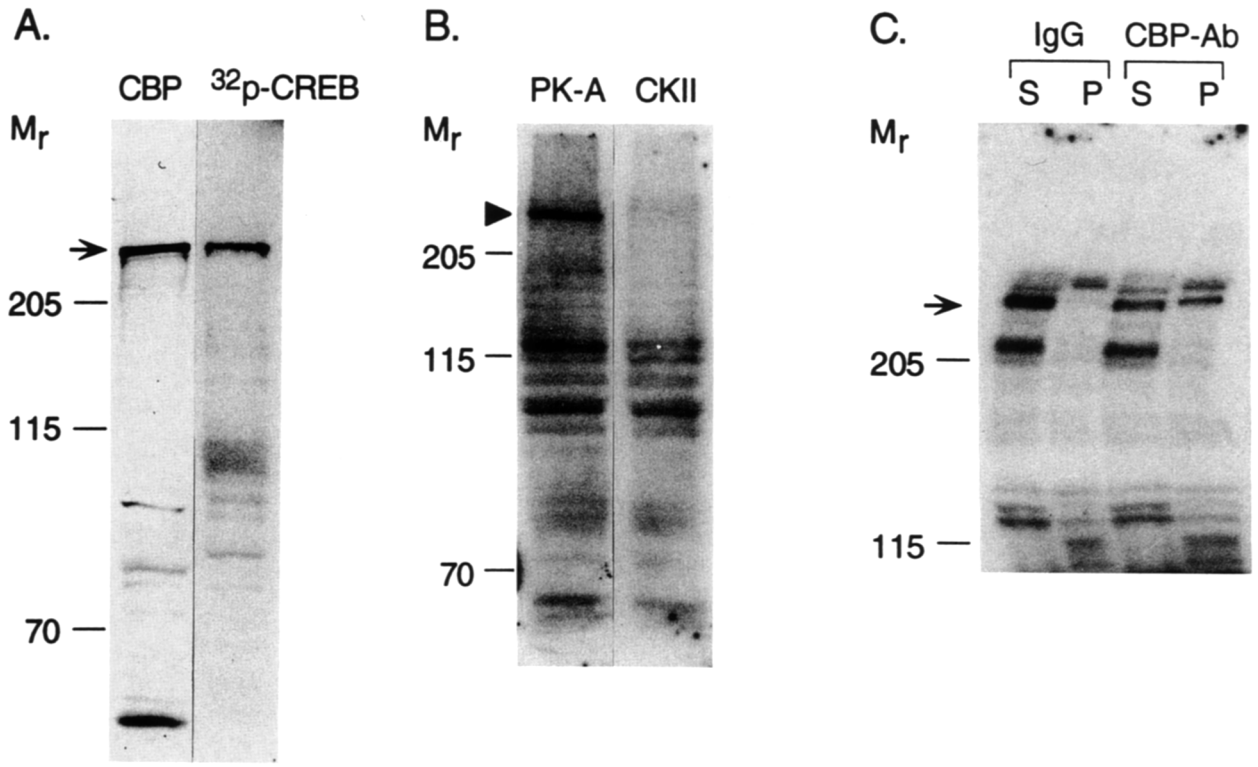

Fig 2. CBP is the predominant phospho-Ser133 CREB-binding activity in nuclear extracts. (A) Western (CBP) and Far Western (^{32}P-CREB) blot analysis of Hela nuclear extract following SDS-PAGE and transfer to nitrocellulose. (B) Far Western blot of crude Hela nuclear extract using ^{32}P-CREB phosphorylated with PK-A or casein kinase II (CKII). In A, B, and C, arrow points to major 265-kd band. Mr, relative mass in kilodaltons. (C) Far Western blot analysis of immunoprecipitates prepared from Hela nuclear extracts with control IgG (IgG) or affinity-purified CBP antiserum. CREB-binding activity was detected with ^{32}P-CREB phosphorylated with PK-A. S, P, supernatant and pellet fractions, respectively.

2). An identical phospho-CREB–binding activity was also found in NIH-3T3 cells (not shown). This phospho-CREB–binding protein appeared to be specific for Ser133-phosphorylated CREB, because no such band was detected with CREB labeled to the same specific activity at a nonregulatory phosphoacceptor site (Ser156) by casein kinase II (CKII)[9] (Fig 2B). To further demonstrate that the major phospho-CREB–binding protein in Hela and 3T3 cells is specifically bound by the anti-CBP antibody, we prepared immunoprecipitates from crude nuclear extracts using the CBP antiserum. Far Western analysis of these immunoprecipitates (Fig 2C) revealed a 265-kd band in samples incubated with CBP antiserum, but not with control Immunoglobulin G (IgG). To examine whether the phosphorylation-dependent interaction between CREB and CBP was critical for cAMP-responsive transcription, we employed a microinjection assay using CBP antiserum, which would be predicted to impair formation of a CREB-CBP complex. Following microinjection into nuclei of NIH-3T3 cells, a CRE-lacZ reporter was markedly induced by treatment with 8-Br cAMP plus isobutyl methyl xanthine (IBMX). Coinjection of CBP antiserum with the CRE-lacZ plasmid inhibited cAMP-dependent activity in a dose-dependent manner, but control IgG had no effect on this response.

With this information, we can now begin the process of reconstituting PK-A–dependent transcription in vitro, using well-characterized proteins such as CREB, TAF 110, and CBP. The assembly of such factors on cAMP-regulated promoters such as somatostatin may thereby permit responsiveness to a variety of hormonal stimuli that employ cAMP as their second messenger.

REFERENCES

1. Montminy MR, Sevarino KA, Wagner JA, et al: Identification of a cyclic-AMP responsive element within the rat somatostatin gene. Proc Natl Acad Sci USA 83:6682-6686, 1986

2. Short JM, Wynshaw-Boris A, Short HP, et al: Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. II. Identification of cAMP and glucocorticoid regulatory domains. J Biol Chem 261:9721-9726, 1986

3. Comb M, Burnberg NC, Seascholtz A, et al: A cyclic-AMP- and phorbol ester-inducible DNA element. Nature 323:353-356, 1986

4. Montminy MR, Bilezikjian LM: Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328:175-178, 1987

5. Yamamoto KK, Gonzalez GA, Biggs WH III, et al: Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature 334:494-498, 1988

6. Gonzalez GA, Yamamoto KK, Fischer WH, et al: A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature 337:749-752, 1989

7. Hoeffler JP, Meyer TE, Yun Y, et al: Cyclic-AMP-responsive

DNA-binding protein: Structure based on a cloned placental cDNA. Science 242:1430-1432, 1988

8. Gonzalez GA, Montminy MR: Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675-680, 1989

9. Hagiwara M, Alberts A, Brindle P, et al: Transcriptional attenuation following cAMP induction requires PP-1 mediated dephosphorylation of CREB. Cell 70:105-113, 1992

10. Hagiwara M, Brindle P, Harootunian A, et al: Coupling of hormonal stimulation and transcription via cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase. Mol Cell Biol 13:4852-4859, 1993

11. Chrivia JC, Kwok RP, Lamb N, et al: Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365:855-859, 1993

12. Arias J, Alberts A, Brindle P, et al: Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. Nature 370:226-228, 1994