Rat Angiotensin-Converting Enzyme Promoter Regulation by β-Adrenergics and cAMP in Endothelium

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Abstract—To shed light on mechanisms of angiotensin-converting enzyme (ACE) upregulation, we used a rabbit endothelial cell model to characterize intracellular pathways of β-adrenergic stimulation. In these cells, ACE activity is increased by isoproterenol (ISO). The stably transfected 1273-bp ACE promoter is stimulated by ISO in the presence of isobutyl methylxanthine. This effect is abolished by propranolol. Promoter stimulation is mimicked by cholera toxin, forskolin, and 8BrcAMP, but not by 8BrcGMP. Promoter stimulation by ISO and isobutyl methylxanthine is blocked by protein kinase A inhibitors, indicating that β-adrenergic stimulation of the ACE gene depends on phosphorylation of protein kinase A targets. Activation by cAMP, resistance to phorbol ester, and lack of synergism between cAMP and phorbol ester suggest that promoter regulation is due to cAMP responsive element rather than to activating protein-2 sequences. Okadaic acid potentiation of 8BrcAMP induction indicated that promoter activation by cAMP is regulated by phosphatases controlling activation of typical cAMP responsive element regulated genes. In summary, β-adrenergic activation of rat ACE promoter is specific; uses G_s proteins, adenylyl cyclase, protein kinase A; and probably includes cAMP responsive element–like sequences. **(*Hypertension*. 1999;34:31-38.)**

Key Words: angiotensin-converting enzyme ■ endothelium ■ receptors, adrenergic, beta ■ cyclic AMP ■ luciferase

Angiotensin-converting enzyme (ACE) is the most important enzyme controlling the activation of angiotensin peptides and the inactivation of kinins.[1] Tissue ACE distribution is wide,[1] which suggests that control of ACE gene expression depends on the particular cell type and environment or, alternatively, that ACE gene expression is controlled by a restricted array of non–cell-specific mechanisms. In cultured cells, expression of the endothelial ACE variant is modulated by hormones,[2–4] ionophores,[5] second messengers,[5] and growth factors.[6] Cardiac hypertrophy is one of the most likely pathophysiological conditions in which tissue-specific regulation of endothelial ACE might play an important role.[7–9] Strong evidence implicates the renin-angiotensin system, and ACE in particular, in the development of hypertrophy.[10] It is known that angiotensin II acts either as a hypertrophic or growth factor agent[9]; that ACE and ACE mRNA (as well as angiotensinogen and its mRNA) are increased in early cardiac hypertrophy[7,8]; and that ACE inhibitors prevent the development, ameliorate the course, and reduce the mortality of ventricular hypertrophy.[10] Recently, a histochemical study suggested that endothelial cells are the main cell type expressing ACE in the heart.[11] However, only a few studies have been done to understand the molecular basis of renin-angiotensin system activation and, in particular, of ACE upregulation in endothelial cells.[2,4,12,13] Surprisingly, the molecular mechanisms underlying cAMP induction of ACE activity have not yet been explored. The cAMP regulated pathway is a potential modulator of ACE synthesis in the heart both in physiological as well as in pathophysiological situations such as heart failure or ventricular hypertrophy.[14,15]

To shed light on the mechanisms of upregulation of the rat ACE gene, we developed and characterized a system of stably transfected endothelial cells carrying the rat ACE promoter as well as negative and positive control promoters. This system was used to characterize receptors and intracellular pathways of activation of the rat ACE promoter by isoproterenol (ISO) and by cAMP.

Methods

Materials

Cell-culture materials and media were obtained from Gibco. ISO, isobutyl methylxanthine (IBMX), 8BrcAMP, forskolin, cholera toxin, 8BrcGMP, propranolol, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. Okadaic acid and *N*-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide, 2HCl (H89) were purchased from Calbiochem. Cell lysis buffers and the luciferase assay system were by Promega, and the β-galactosidase assay system was from Tropix.

Plasmid Constructions

Reporter plasmid constructions were made by subcloning promoter fragments into the backbone of luciferase vectors. A 1273-bp fragment of the 5′ ACE region from the Wistar-Kyoto rat (ACE

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promoter) was subcloned into the pGL2 basic vector (Promega). A positive control for cAMP responsive element (CRE) stimulation, CRE thymidine kinase luciferase (CRETKLUC) was constructed by subcloning a fragment from pTKCAT4CRE containing 4 tandem repeats of CRE consensus linked to the herpes simplex virus thymidine kinase (HSVTK) promoter LS –115/-105[16] into pGL2 basic. A negative control for cAMP stimulation (TATALUC) was constructed by subcloning a fragment from branched chain amino-transferases (BCAT)[17] containing the adenovirus *E1b* tata box into pGL2 basic. BCAT was a generous gift from Dr Trevor Williams, Yale University.

Transient Transfections

Rabbit aortic endothelial cells (REC)[18] or human renal embryonic cells (293, Invitrogen) were grown in 12-well plates on F12 medium (Coon modification) or DMEM, respectively, and supplemented with 10% FBS and antibiotics (penicillin and streptomycin). Before reaching confluence, REC or 293 were transfected (16 to 24 hours) with 3.25 μg of luciferase reporter plasmids and 0.48 μg of a control plasmid (pSV-β-galactosidase, Promega) per well by the calcium phosphate method.[19] Experiments with expression vectors for the peptide inhibitor of protein kinase A (PKA) (PKI) or for its mutated counterpart were performed with transfection of 1.78 μg of reporter plasmids and 0.48 μg of each expression vector[17] per well. Total DNA was maintained at 3.73 μg per well by manipulating the amount of pBluescript SK+ (Stratagene). Control plasmid in these experiments was LTR-β-galactosidase. Expression vectors for PKA inhibitors (RSVPKI, RSVPKI mutant) were generous gifts from Dr Richard A Maurer, Oregon Health Science University.

Stable Transfections

Stably transfected cell lines harboring the luciferase reporter constructs were made by cotransfection of 1273-bp ACE promoter CRE thymidine kinase (CRETK), SV40 promoter and enhancer (SV40+E), with pSV7-neo[20] and selection with G418. Transfection was performed in 60-mm tissue-culture dishes by the calcium phosphate method.[19] The ratio (in μg) between reporter construct and pSV7-neo was 20:1. Total DNA content was 10.2 μg per dish. Individual clones were separated with cloning rings, expanded, and frozen. To exclude functional artifacts due to uncontrolled insertion, 4 independent clones of 1273-bp ACE, 3 independent clones of CRETK, and 2 independent clones of SV40+E were used.

Drug Treatments

Stably and transiently transfected cells were cultivated in 24- and 12-well plates, respectively, with F12 supplemented with 10% FBS. Before stimulation, cells were serum-starved for 16 to 24 hours on F12 supplemented with 0.5% FBS. Antagonists and blockers were given to the cells 30 minutes before stimulation and were maintained throughout the experiments.

Enzyme Assays

Cell extracts from stably transfected cell lines were analyzed for luciferase activity with the use of an assay (Promega) and for total protein with the use of the Bradford method. Cell extracts from transient transfections were analyzed for luciferase and β-galactosidase in a luminometer according to the manufacturer's instructions (Monolight 2010, Analytical Luminescence Lab).

REC were grown to confluence on F12 supplemented with 10% FBS in 12-well plates. Cell extracts were analyzed for ACE activity with a fluorometric assay described by Santos et al,[21] and data were expressed as ACE activity in nmol His-Leu $\cdot$ mg^{-1} $\cdot$ h^{-1}.

Gel-Shift Binding Assay

Nuclear proteins of REC were extracted according to established protocols.[22] Binding of REC nuclear extracts to DNA was assayed by gel retardation analysis.[23] The DNA used in these experiments was obtained by purification of a 142-bp *Bam*HI fragment of CRETK plasmid containing 4 tandem repeats of the CREs of somatostatin. The protein-DNA complex was visualized by labeling DNA with ^{32}P by a filling-in reaction with Klenow polymerase. Nuclear proteins of REC were mixed with 15 000 cpm of labeled DNA and 2 μg of poly dI-dC (Pharmacia LKB) in a 20-μL reaction. After 20 minutes at 24°C, the mixture was loaded onto a 6.25% nondenaturing polyacrylamide gel that was previously run for 30 minutes at 200 V. To separate the protein-DNA complexes, the gel was run at 4°C for 120 to 240 minutes at 240 V.

Data Presentation

In stably transfected cell lines, luciferase activity was normalized for protein concentration. To control for differences in transfection efficiency, luciferase activity in extracts from transiently transfected cells was normalized for β-galactosidase activity. Data are expressed as mean$\pm$SEM. Unless otherwise stated, data are presented as variation from control values. Where appropriate, results were analyzed by 2-tailed *t* test or by 2-wayANOVA. Statistical significance was set at $P<0.05$.

Results

REC express mRNA for ACE (Figure 1A), display ACE activity (Figure 1B), can be transfected by the calcium phosphate precipitation method, and uptake 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated LDL (not shown). To determine whether ACE activity is regulated by β-adrenergic stimulation, REC were treated with ISO 1.0 μmol/L for 4 hours. ACE activity in lysates from cells treated with ISO increased from 2.26$\pm$0.11 to 6.37$\pm$0.6 nmol His-Leu $\cdot$ mg^{-1} $\cdot$ h^{-1}, $P<0.01$ (Figure 1B), indicating that REC is an adequate cell model to study ACE promoter regulation by the β-adrenergic pathway.

To characterize intracellular pathways of ACE promoter stimulation by β-adrenergics, we developed a panel of stably transfected REC harboring the luciferase gene. First, the 1273-bp promoter from the Wistar-Kyoto rat ACE gene[24] was stably transfected in REC. These cells displayed variable levels of luciferase activity (Figure 1C) and were able to reproduce modulation of the ACE promoter by cAMP in transient transfections (Figure 1D). Four independent clones were tested and all displayed similar behavior when stimulated by cAMP (Figure 1C). ACE promoter cell line 3 was selected for further studies. Positive control cell lines for cAMP pathway stimulation were obtained by stable transfection of REC with 4 tandem repeats of somatostatin's cAMP responsive elements fused to the thymidine kinase promoter (CRETK). Negative control cell lines were developed by stable transfection of REC with SV40 promoter plus enhancer (SV40+E). Three independent clones of CRETK and 2 independent clones of SV40+E were tested. CRETK cell line 2 and SV40+E cell line 0 were selected for further studies. As indicated in Figure 1D, stably transfected cell lines were chosen according to their ability to reproduce the behavior of transiently transfected constructs after stimulation with 8BrcAMP.

In preliminary experiments, ISO induced ACE promoter activity in a variable and inconsistent way (23.1$\pm$8.3% of increase in luciferase activity; range $-$11% to 83%; $P<0.05$), making necessary the addition of the phosphodiesterase inhibitor IBMX. Subsequently, ACE promoter, CRETK, and SV40+E stable cell lines were treated with ISO, 100 μm/L IBMX, ISO+100 μm/L IBMX, or 5.0 mmol/L 8BrcAMP for 4 hours. Figure 2A shows that neither ISO, nor 100 μm/L IBMX alone were sufficient to activate ACE promoter

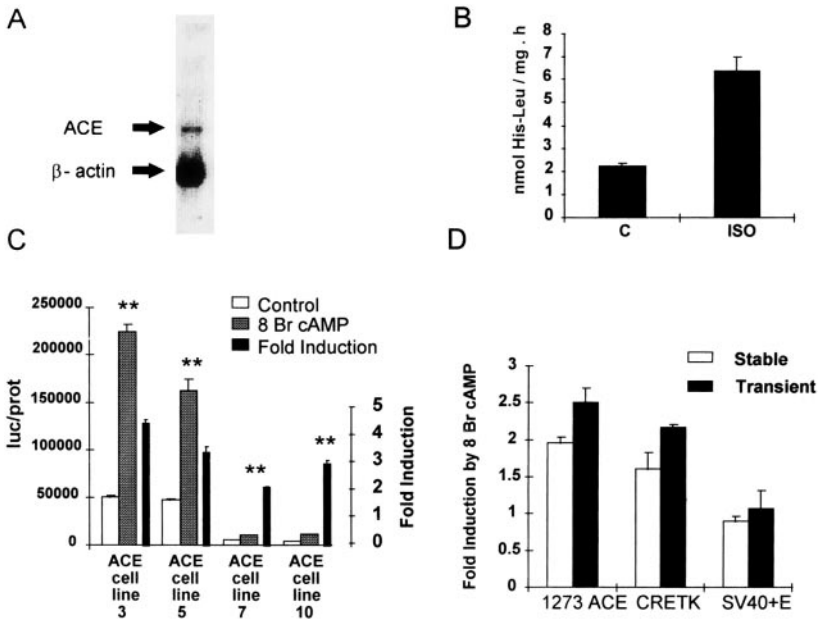

Figure 1. REC as a model to study ACE promoter regulation by β-adrenergics and cAMP. A, Northern blot containing 20 μg of total RNA extracted from confluent REC cultures. The blot was subsequently probed with rat ACE cDNA and β-actin cDNA. B, Confluent REC cultures were stimulated for 4 hours with ISO 1.0 μmol/L. Cell extracts were analyzed for ACE activity using a fluorometric assay [21] and data were expressed as ACE activity in nmol His-Leu · mg^{-1} · h^{-1}. C, Four independent stably transfected ACE promoter clones were grown to confluence and stimulated with 5.0 mmol/L 8BrcAMP for 4 hours. Data are expressed as luciferase activity per mg of protein in cell extracts and as fold induction by 8BrcAMP. Results are mean±SEM from a representative experiment (n=4). **indicates $P<0.01$ vs control. D, 1273 ACE promoter cell line 3, CRETK cell line 2, and SV40+E cell line 0 were grown to confluence. REC was grown to subconfluent levels and transiently transfected (16 to 24 hours) with 3.25 μg of 1273 ACE, CRETK, SV40+E luciferase reporter plasmids and 0.48 μg of a control plasmid (pSV-β-galactosidase, Promega) per well by the calcium phosphate method. Stably transfected cell clones and transiently transfected REC were stimulated with 5.0 mmol/L 8BrcAMP for 4 hours. Cell extracts from stably transfected cell lines were analyzed for luciferase activity and for total protein. Cell extracts from transient transfections were analyzed for luciferase and β-galactosidase activity. Data are expressed as fold induction over control levels.

(103±2.6%; 92.7±5.5% of control, respectively). However, the combination of both agents increased luciferase activity to 228.5±9.0% of control, ($P<0.01$), a stimulation similar to that obtained with 5.0 mmol/L 8Br cAMP (217±16% of control, $P<0.01$). As positive controls for cAMP stimulation, CRETK cell lines displayed levels of induction which were similar to that obtained with ACE promoter cell lines. Figure 2A shows that ISO, 100 μm/L IBMX, ISO+100 μm/L IBMX, and 5.0 mmol/L 8BrcAMP increased luciferase activity by 103.3±3.1%; 106.7±4.5%; 188.6±16.0%, 151.0±19.0% of control, respectively ($P<0.01$ for ISO+IBMX and 8BrcAMP). Negative control SV40+E cell lines were not affected by the same treatments.

To assess whether β-adrenergic receptors were responsible for activation of ACE promoter in the presence of IBMX, ACE promoter cell line 3 was treated with ISO, 100 μm/L IBMX, and ISO+100 μm/L IBMX, in the presence or absence of 1.0 μm/L propranolol. As shown in Figure 2B, propranolol 1.0 μm/L completely blocked ISO-induced potentiation of 100 μm/L IBMX. This result indicates that β-adrenergic receptors are the only receptors participating in the stimulatory effect of ISO in the presence of IBMX.

Next, we used a pharmacological approach to test the involvement of downstream elements of the β-adrenergic signaling pathway in the regulation of the ACE promoter. In stably transfected REC, cholera toxin 10 mU/L increased activity of the ACE promoter by 2.55-fold, a level of induction that is not different from that obtained with ISO +100 μm/L IBMX (2.19-fold; Figure 3A). Direct stimulation of adenylyl cyclase by 10.0 μmol/L forskolin reproduced ACE promoter stimulation (1.62-fold), albeit at levels lower than those obtained with cholera toxin. As demonstrated in Figure 2A, 5.0 mmol/L 8BrcAMP stimulated ACE promoter (2.42-fold) to levels similar to those achieved with ISO+100 μm/L IBMX or with cholera toxin (Figure 3A). Figure 3A also shows that PMA up to 100 nmol/L neither induces the ACE promoter nor potentiates the response to cAMP stimulation.

ACE promoter stimulation by drugs acting on the cAMP pathway suggests that protein phosphorylation by PKA is specifically required. To further clarify this point, we tested whether the effect of ISO+100 μm/L IBMX could be blocked by PKA inhibitors. As shown in Figure 3A, H89, an inhibitor of cyclic nucleotide protein kinases, completely blocked stimulation by ISO+IBMX. Although H89 is ≥10 times more effective as a PKA inhibitor than as a protein kinase G (PKG) inhibitor,[25] selectivity may be compromised at the concentration we used. However, ISO-induced stimulation of the ACE promoter is mimicked by 5.0 mmol/L 8BrcAMP but not by 5.0 mmol/L 8BrcGMP (Figure 3A). In addition, cotransfection of an expression vector for the peptide inhibitor of PKA (PKI) completely abolished stimulation of the ACE promoter by ISO+IBMX, whereas cotransfection of a mutated PKI was not effective (Figure 3B). These results provide compelling evidence that β-receptor stimulation in REC activates the ACE promoter solely through the cAMP/PKA pathway. Treatment with cAMP results in a concentration-dependent activation of the ACE promoter; peak levels were attained after 4 hours of stimulation (Figure 4A and 4B, respectively). These characteristics place rat ACE-responsive elements in the rapid category of cAMP regulating sequences.[26] Rapid cAMP inducible genes have 1 or sometimes 2 different classes of cAMP-responsive elements represented by CRE and activating protein-2 (AP2) consensus sequences.[26] To test whether typical CRE mechanisms operate in REC, we stimulated ACE promoter cell lines with 5.0 mmol/L 8BrcAMP and 500 μm/L IBMX, alone or in combination, in the presence and absence of okadaic acid.

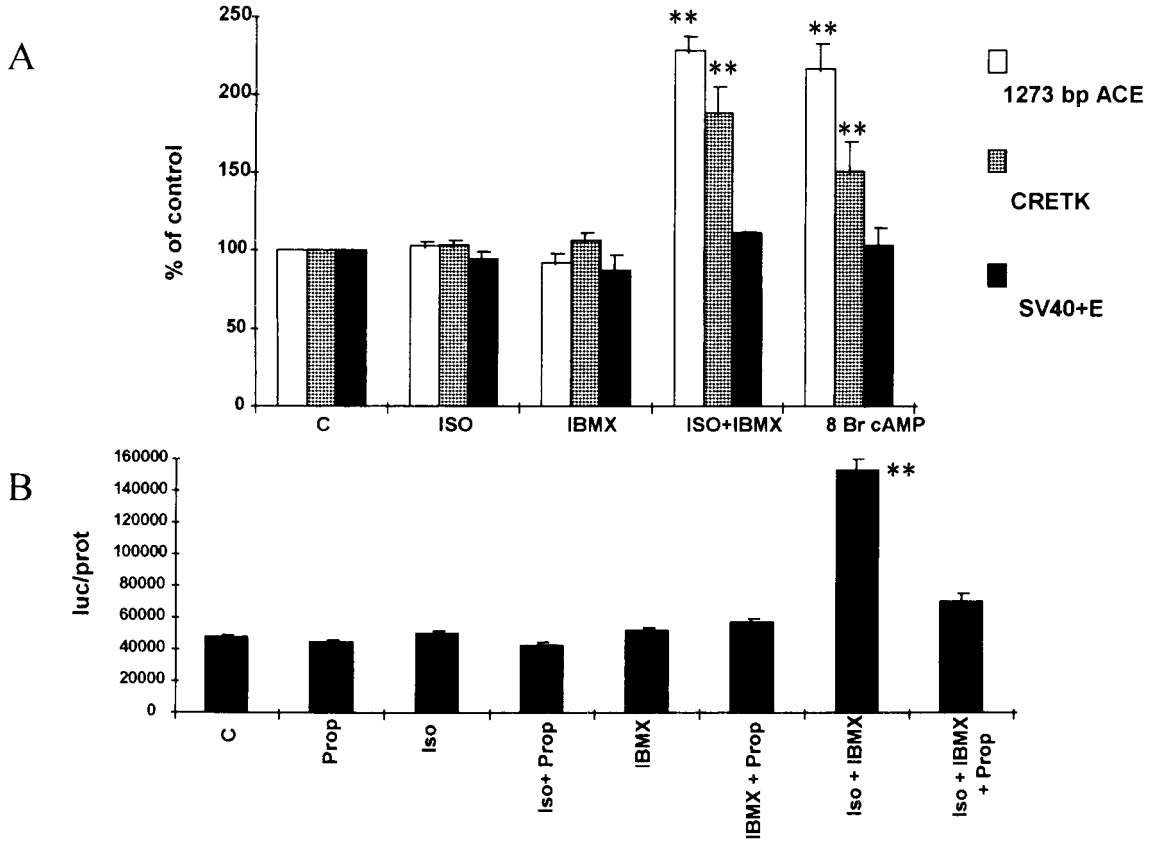

Figure 2. β-adrenergic and cAMP induction of ACE promoter activity in stably transfected cell lines. A, Confluent cultures of 1273 bp ACE promoter cell line 3, CRETK cell line 2, and SV40+E cell line 0 were stimulated for 4 hours with 1.0 μmol/LISO, 100 μmol/L IBMX, 1.0 μmol/L ISO+100 μmol/L IBMX, and 5.0 mmol/L 8BrcAMP. Luciferase activity in cell extracts was normalized for protein concentration. Data are expressed as variation from control activity. Results are mean±SEM from 4 independent experiments (n=4 in each). B, ACE promoter cell line 3 was grown to confluence and stimulated for 4 hours with 1.0 μmol/L ISO, 100 μmol/L IBMX, 1.0 μmol/L ISO+100 μmol/L IBMX in the presence and absence of 1.0 μmol/L propranolol. Luciferase activity in cell extracts was normalized for protein concentration. Data are expressed as luciferase activity per mg of protein in cell extracts. Results are mean±SEM from a representative experiment (n=4). ** $P<0.01$ significantly different from control. Prop indicates propranolol.

Okadaic acid is an inhibitor of PP1 phosphatases that have been shown to control the extent of activation of CRE regulated genes by dephosphorylation of CRE binding protein (CREB).[27] Figure 5 shows that okadaic acid (1.0 μmol/L) influenced neither basal nor 500 μm/L IBMX-induced activation of the ACE promoter. However, 5.0 mmol/L 8BrcAMP induction was slightly potentiated (19%), and the induction by 5.0 mmol/L 8BrcAMP plus 500 μm/L IBMX was increased by 63.6%. These results suggest that ACE promoter activation by cAMP is controlled by phosphatases acting on the CRE pathway. Additional support for the presence of a functional CRE pathway in REC is provided by Figure 6. As can be seen, a ^{32}P-labeled 142-bp *Bam*HI fragment containing 4 tandem repeats of the CRE of somatostatin forms at least 2 complexes with nuclear proteins of REC, which clearly supports the presence of CRE-binding proteins in REC.

To investigate whether ACE promoter activity is stimulated by cAMP pathways outside the context of REC, we used human renal embryonic cells 293, which show ACE activity and express ACE mRNA (not shown). The cells were transiently transfected with ACE promoter and CRETK. The table indicates that 293 cells support ACE promoter activity and upregulation by 10.0 μmol/L forskolin. The CRE positive control CRETK is also upregulated by forskolin, indicating that cAMP pathway induction of ACE and of CRE positive controls coexist in the 293 cells. This result shows that ACE promoter is also induced by cAMP pathway stimulation in other cell types expressing ACE.

Discussion

This work describes the development of an endothelial system to study ACE promoter regulation using luciferase as a reporter gene. New findings include (1) evidence for a transcriptional role of cAMP on ACE gene expression, (2) description of an intracellular signal transduction pathway linking β-adrenergic stimulation to ACE promoter induction, and (3) evidence for conserved mechanisms of ACE promoter regulation by cAMP.

It is generally accepted that primary cultures of endothelium display poor growth characteristics,[28] an inability to maintain expression of endothelial markers, and resistance to transfection.[29] Therefore, endothelial cells of heterologous origin have been used to study regulation of endothelium-specific promoters.[29,30] This approach can be criticized because promoter regulation can be specific in each species.

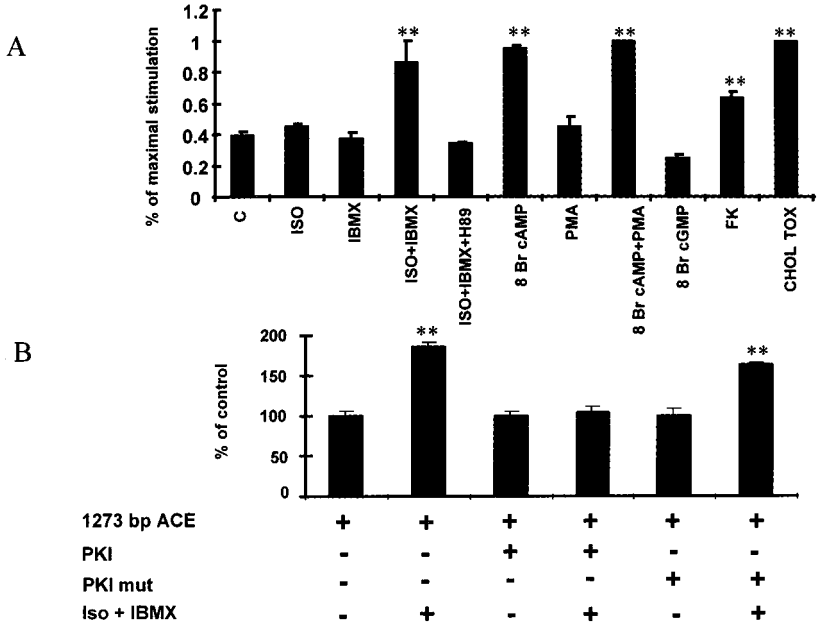

Figure 3. Regulation of ACE promoter activity. A, Confluent cultures of ACE promoter cell line 3 were stimulated for 4 hours with 1.0 μmol/LISO, 100 μmol/L IBMX, 1.0 μmol/L ISO+ 100 μmol/L IBMX, 1.0 μmol/L ISO+100 μmol/L IBMX+25 μmol/L H89, 5.0 mmol/L 8BrcAMP, 100 nmol/L PMA, 5.0 mmol/L 8BrcAMP+100 nmol/L PMA, 5.0 mmol/L 8BrcGMP, 10 μmol/L forskolin, and 10 mU/L cholera toxin. Luciferase activity in cell extracts was normalized for protein concentration. Data are expressed in relation to maximal stimulation. Results are mean±SEM from 3 independent experiments (n=4 in each). ** $P<0.01$ vs control. B, REC were grown to subconfluent levels in 12 well plates and transiently transfected by the calcium phosphate precipitation method (16 to 24 hours) with 1.78 μg of ACE promoter and 0.48 μg of expression vectors for PKI or its mutated counterpart. Control plasmid in these experiments was LTR-β-galactosidase. One day after transfection the cells were stimulated with 1.0 μmol/L ISO+100 μmol/L IBMX for 4 hours. Luciferase activity was normalized for β-galactosidase expression. Data are expressed as percent of control activity. ** $P<0.01$ vs control. FK indicates forskolin; CHOL TOX, cholera toxin; PKImut, mutated peptide inhibitor of PKA.

However, heterologous transfections of the rat endothelin-1 promoter into bovine aortic endothelial cells and of human ACE promoter into rabbit endothelial cells have been used successfully to model conserved mechanisms of gene regulation.[29,30] As shown by studies in bovine[4,12,13] and human cells,[31] cAMP-dependent regulation of somatic ACE is conserved between mammals. Therefore, we used REC[18] to establish cAMP regulation of the rat ACE promoter.

Using stable transfections, we could study ACE promoter regulation in endothelial cells at higher densities and for times longer than with transient transfections. This was important because ACE production by endothelial cells is remarkably influenced by growth and confluence.[3,32] Moreover, stably transfected cell lines carry chromosome-integrated copies of the reporter. This enabled us to study regulation of the ACE gene in the context of normal chro-

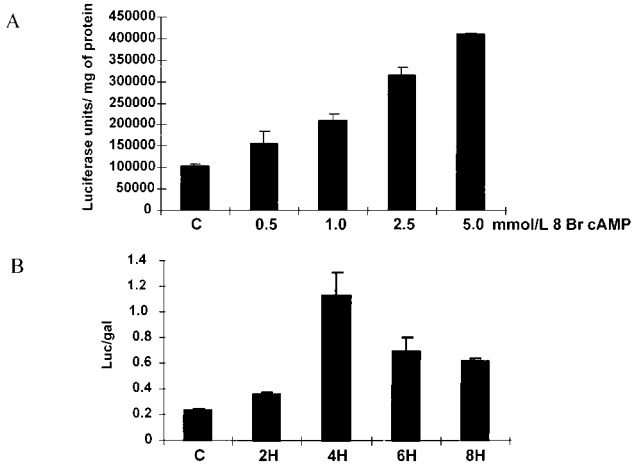

Figure 4. Concentration-response and temporal characterization of ACE promoter stimulation by 8BrcAMP. A, Confluent cultures of 1273-bp ACE promoter cell line 3 stimulated for 4 hours with increasing concentrations of 8BrcAMP (0.5, 1.0, 2.5 and 5.0 mmol/L). Data are expressed as luciferase activity per mg of protein in cell extracts. B, REC were grown to sub-confluent levels and transiently transfected by the calcium phosphate precipitation method (16 to 24 hours) with the 1273-bp ACE promoter and pSV-β-galactosidase. One day after transfection the cells were stimulated for different times (2,4,6 and 8 hours) with 5.0 mmol/L 8BrcAMP. Luciferase activity was normalized for β-galactosidase expression. Data are expressed as luciferase activity over β-galactosidase expression . Results are mean±SEM from representative experiments (n=4 in each).

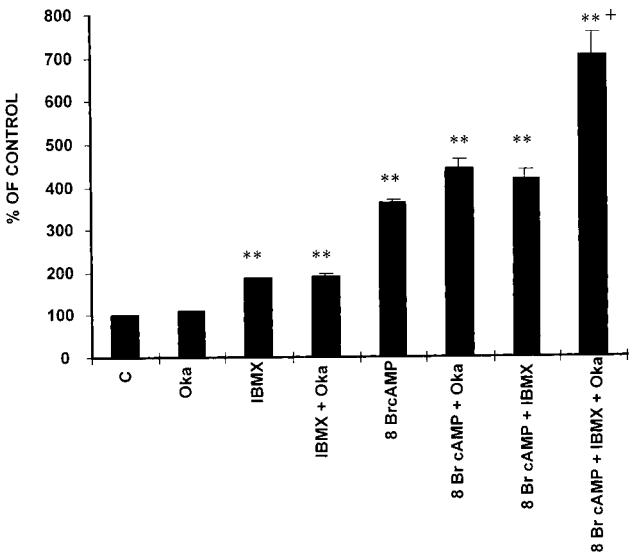

Figure 5. Okadaic acid modulates ACE promoter induction by the cAMP pathway. Confluent cultures of ACE promoter cell line 3 were stimulated for 4 hours with 5.0 mmol/L 8BrcAMP and 500 μmol/L IBMX, alone or in combination, in the presence and absence of okadaic acid, a PP1 phosphatase inhibitor. Luciferase activity in cell extracts was normalized for protein concentration. Data are expressed as percent of control activity. Results are mean±SEM from 3 independent experiments (n=4 in each). ** $P<0.01$ vs control; + $P<0.05$ vs 8BrcAMP+IBMX. Oka indicates okadaic acid.

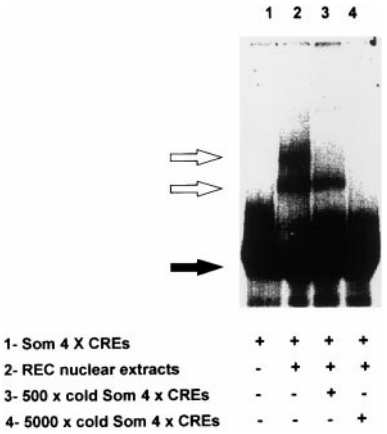

Figure 6. Binding of REC nuclear proteins to 4 tandem repeats of CREs. Nuclear proteins of REC were mixed with 15 000 cpm of a ^{32}P-labeled 142-bp *Bam*HI fragment containing 4 tandem repeats of CRE of somatostatin (Som 4× CREs), binding buffer, and 2-μg poly dI-dC (Pharmacia LKB) in a 20-μL volume reaction. Lane 1 shows labeled probe; Lane 2, labeled probe plus 5.0 μg of REC nuclear proteins; Lane 3, labeled probe plus 5.0 μg of REC nuclear proteins plus 500×) unlabeled probe; Lane 4, labeled probe plus 5.0 μg of REC nuclear proteins plus 5000× unlabeled probe. The ^{32}P-labeled probe (lanes 1, 2, 3, and 4; black arrow) containing 4 tandem repeats of CRE of somatostatin forms 2 easily identified complexes with nuclear proteins of REC (lane 2, white arrows). Binding of REC nuclear proteins to CRE of somatostatin is effectively completed by increasing quantities of unlabeled probe (lanes 3 and 4).

matin organization, which modulates both gene accessibility and the response to various activators and repressors,[33] in a more realistic scenario than that obtained with transient transfections.

Both the 1273-bp ACE promoter and a positive control for CRE (CRETK) were stimulated similarly by cAMP, but cAMP did not affect luciferase expression driven by SV40+E. These results indicate that cAMP regulation of 1273 ACE is selective and that CREs in the ACE promoter have the same inducibility of 4 tandem repeats of canonical CREs. One could argue that cAMP responsiveness is nonspecific because of cryptic CREs in the vector backbone[34] and that SV40+E resistance to cAMP induction is only apparent because the SV40 enhancer is already maximally activated in the basal state. We find this hypothesis untenable because a very low activity promoter such as the enhancer-trap adenovirus *E1b* tata box was not activated by cAMP when fused to the luciferase backbone (from 0.05±0.02 to 0.045±0.017 β-galactosidase-normalized luciferase units).

Forskolin Stimulation of the ACE Promoter in 293 Cells

	Control	10 μmol/L Forskolin	Fold Stimulation
ACE promoter	53±11	100±20*	2.08±0.22*
CRETK	1790±500	7049±2130†	3.73±0.58†

Confluent cultures of 293 cells were transiently transfected by the calcium phosphate precipitation method (16 to 24 h) with the ACE promoter and pSV-β-galactosidase. One day after transfection, the cells were stimulated for 4 h with forskolin 10 μmol/L. Data are expressed as luciferase activity over β-galactosidase expression. Results are mean±SEM from 7 independent experiments (n=4 in each).

*$P<0.01$ vs control; †$P<0.05$ vs control.

Adenovirus *E1b* tata box resistance to cAMP was not the result of luciferase activity being too low to measure, because it was 3 to 5 times greater than background (not shown). ISO treatment alone did not stimulate 1273-bp ACE promoter consistently, which suggests that β-adrenergic receptor density in REC membranes is too low to produce activation of the ACE promoter. Alternatively, ISO may act through β_2 receptors and shut down adrenergic signaling through coupling to a G_i protein in a negative feedback loop previously demonstrated in cardiac cells.[35,36] Treatment with ISO plus IBMX in sublimiar doses (100 μmol/L) was associated with levels of induction similar to those obtained with 5.0 mmol/L 8BrcAMP, which suggests that, when cyclic nucleotide degradation is blocked by phosphodiesterase inhibitors such as IBMX, stimulation of β-adrenergic receptors by ISO raises intracellular cAMP concentration to a level sufficient for ACE promoter activation. This view is supported by propranolol blockade of ISO + IBMX, which indicates that REC possesses functional β-adrenergic receptors. The need to use IBMX to obtain ACE promoter activation by ISO raises the issue of physiological significance. However, as noted above, the patterns of ACE promoter activation by β-adrenergic stimulation can be complex. In addition, our results clearly show that integral links exist between surface β-adrenergic receptor stimulation and ACE promoter activity in REC and that stimulation of every step in the pathway leads to promoter activation. Moreover, evidence exists that β-adrenergic and cAMP regulation of the rat ACE promoter described here in vitro can have pathophysiological relevance in vivo. In rats, selective β-adrenergic stimulation by ISO induces hypertrophy[15] and increases ACE activity.[37] Transfections of the 1273 bp rat ACE promoter into hearts of living rats showed that the construct is activated by ISO in doses that consistently induce cardiac hypertrophy.[37] These data indicate that the 1273 bp rat ACE promoter is activated by ISO either in vitro or in vivo.

Cholera toxin, forskolin, and 8BrcAMP stimulated ACE promoter activity to levels similar to ISO+IBMX. This suggests that β-adrenergic receptor stimulation of ACE promoter proceeds from receptor occupation to G_s protein stimulation, adenylyl cyclase activation, cAMP synthesis, and possibly, PKA stimulation. Blockade of ISO+IBMX effects by H89 or by PKI indicated that PKA stimulation is necessary and sufficient for ACE promoter activation by β-adrenergics. ACE promoter stimulation by cAMP and by drugs that increase its intracellular concentration agrees well with earlier reports of ACE induction by the same drugs.[4,12,13,38] In contrast to cAMP, cGMP did not modify ACE promoter activity (Figure 3A), a finding that is consistent with the inability of cGMP to modify ACE activity in bovine pulmonary artery endothelial cells.[38]

The 1273 bp rat ACE promoter has 3 potential AP2 consensus sequences and 3 CRE-like sequences contained in its sequence. In principle, any combination of putative binding sites could be responsible for cAMP induction. Our results, however, argue against regulation of the ACE promoter by AP2 mechanisms. As shown in Figure 3A, the ACE promoter is not induced by PMA and PMA failed to potentiate ACE promoter stimulation by 8BrcAMP. These results

contrast with typical mechanisms of AP2 regulation[26,39] and suggest that ACE regulation by β-adrenergics and cAMP is most likely due to CRE rather than to AP2 sequences. Nevertheless, we cannot exclude the possibility of an atypical AP2 mechanism or a synergistic role of AP2 sequences in CRE regulation as demonstrated in human tissue plasminogen activator[40] and proenkephalin[41] promoters.

CRE and 12-*O*-tetradecanoyl-phorbol-13–acetate responsive element (TRE) consensus sequences differ by just 1 nucleotide. TRE-like sequences mediate gene induction by phorbol esters but can also bind CRE binding proteins with reduced affinity[42] and support cAMP stimulation in some promoters.[43] In this sense, cAMP modulation of the ACE promoter could be due to the presence of functional TRE-like sequences rather than to CRE-like sequences. However, as shown in Figure 3, PMA up to 100 nmol/L did not induce the ACE promoter. These results indicate that in REC, the 1273 bp ACE promoter is primarily sensitive to cAMP pathway stimulation.

As shown in Figure 5, okadaic acid increased cAMP pathway stimulation of the ACE promoter, which indicates that typical mechanisms of CRE regulated expression operate in REC.[27] Moreover, as indicated in Figure 6, REC displays nuclear proteins capable of binding to CRE consensus sequences, providing further evidence for a fully functional CRE pathway in REC. Taken together, our results establish β-adrenergic/cAMP regulation and suggest that CRE mechanisms regulate the ACE promoter.

The CREB/activating transcription factor pathway of gene regulation is a critical point in the information path from the cell surface to the nucleus.[26,44] CREB/activating transcription factor proteins are potential dimerization partners for TRE-binding proteins such as Jun, Fos, and Fra-1[42] and, when phosphorylated, bind calmodulin-binding peptide, a protein involved in the transmission of information from enhancers to the basal transcriptional machinery in the CRE, TRE, and serum-responsive element pathways,[45] providing a biochemical substrate for interaction of distinct signaling pathways. The presence of functional CRE-like sequences in the ACE promoter makes it a target for convergence of multiple signal transduction pathways, uncovering a potential pathway of ACE gene activation in endothelial cells in pathophysiological situations that involve increased sympathetic neurohumoral activation such as heart failure or β-adrenergic stimulation in ISO-induced cardiac hypertrophy.

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References

1. Erdös EG. Angiotensin I converting enzyme and the changes in our concepts through the years. *Hypertension*. 1990;16:363–370.
2. Friedland J, Setton C, Silverstein E. Angiotensin converting enzyme: induction by steroids in rabbit alveolar macrophages in culture. *Science*. 1977;197:64–65.
3. Krulewitz AH, Baur WE, Fanburg BL. Hormonal influence on endothelial cell angiotensin-converting enzyme activity. *Am J Physiol*. 1984;247: C163–C168.
4. Dasarathy Y, Lanzillo JJ, Fanburg BL. Stimulation of bovine pulmonary artery endothelial cell ACE by dexamethasone: involvement of steroid receptors. *Am J Physiol*. 1992;263:L645–H649.
5. Dasarathy Y, Fanburg BL. Calcium ionophore A23187 elevates angiotensin-converting enzyme in cultured bovine endothelial cells. *Biochim Biophys Acta*. 1989;1010:16–19.
6. Fishel RS, Thourani V, Eisenberg SJ, Shai SY, Corson MA, Nabel EG, Bernstein KE, Berk, BC. Fibroblast growth factor stimulates angiotensin converting enzyme expression in vascular smooth muscle cells: possible mediator of the response to vascular injury. *J Clin Invest*. 1995;95: 377–387.
7. Baker KM, Chernin MI, Wixson SK, Aceto JF. Renin-angiotensin system involvement in pressure-overload cardiac hypertrophy in rats. *Am J Physiol*. 1990;259:H324–H332.
8. Schunkert H, Dzau VJ, Tang SS, Hirsch AT, Apstein C, Lorell B. Increased rat cardiac angiotensin-converting enzyme activity and mRNA levels in pressure overload left ventricular hypertrophy: effects on coronary resistance, contractility and relaxation. *J Clin Invest*. 1990;86: 1913–1920.
9. Sadoshima J-I, Izumo S. Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. *Circ Res*. 1993;73:413–423.
10. Bruckschlegel G, Holmer SR, Jandeleit K, Grimm D, Muders F, Kromer EP, Riegger GAJ, Schunkert H. Blockade of the renin-angiotensin system in cardiac pressure-overload hypertrophy in rats. *Hypertension*. 1995;25: 250–259.
11. Falkenhahn M, Franke F, Bohle RM, Zhu Y-C, Strauss HM, Bachmann S, Danilov S, Unger T. Cellular distribution of angiotensin-converting enzyme after myocardial infarction. *Hypertension*. 1995;25:219–226.
12. Lloyd CJ, Cary DA, Mendelsohn FAO. Angiotensin converting enzyme induction by cyclic AMP and analogues in cultured endothelial cells. *Mol Cell Endocrinol*. 1987;52:219–225.
13. Dasarathy Y, Fanburg BL. Elevation of angiotensin converting enzyme by 3-isobutyl-1-methylxanthine in cultured endothelial cells: a possible role for calmodulin. *J Cell Physiol*. 1988;137:179–184.
14. Hirsch AT, Talsness CE, Schunkert H, Paul M, Dzau VJ. Tissue-specific activation of cardiac angiotensin converting enzyme in experimental heart failure. *Circ Res*. 1991;69:475–482.
15. Boluyt MO, Long X, Eschenhagen T, Mende U, Schmitz W, Crow MT, Lakatta EG. Isoproterenol infusion induces alterations in expression of hypertrophy-associated genes in rat heart. *Am J Physiol*. 1995;269: H638–H647.
16. Matsuda S, Maekawa T, Ishii S. Identification of the functional domains of the transcriptional regulator CRE-BP1. *J Biol Chem*. 1991;266: 18188–18193.
17. Williams T, Tjian R. Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes Dev*. 1991;5: 670–682.
18. Buonassisi V, Venter JC. Hormone and neurotransmitter receptors in an established vascular endothelial cell line. *Proc Natl Acad Sci U S A*. 1976;73:1612–1616.
19. Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*. 1973;52:456–459.
20. Armelin HA, Armelin MCS, Kelly K, Stewart T, Leder P, Cochran BH, Stiles CD. Functional role for c-myc in mitogenic response to platelet-derived growth factor. *Nature*. 1984;310:655–660.
21. Santos RAS, Krieger EM, Greene LJ. An improved fluorometric assay of rat serum and plasma converting enzyme. *Hypertension*. 1985;7: 244–252.
22. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. *Current Protocols in Molecular Biology*. New York, NY: John Wiley and Sons; 1993:12.1.1–12.1.6.
23. Ribeiro RCJ, Apriletti JW, Yen PM, Chin WW, Baxter JD. Heterodimerization and deoxyribonucleic acid-binding properties of a retinoid X receptor-related factor. *Endocrinology*. 1994;135:2076–2085.
24. Koike G, Krieger JE, Jacob HJ, Mukoyama M, Pratt RE, Dzau VJ. Angiotensin converting enzyme and genetic hypertension: cloning of rat cDNAs and characterization of the enzyme. *Biochem Biophys Res Commun*. 1994;198:380–386.
25. Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic-AMP-dependent protein kinase, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), of PC12D pheochromocytoma cells. *J Biol Chem*. 1990;265:5267–5272.

26. Roesler WJ, Vandenbark GR, Hanson RW. Cyclic AMP and the induction of eukaryotic gene transcription. *J Biol Chem*. 1988;263:9063–9066.
27. Hagiwara M, Alberts A, Brindle P, Meinkoth J, Feramisco J, Deng T, Karin M, Shenolikar S, Montminy M. Transcriptional attenuation following cAMP induction requires PP-1 mediated dephosphorylation of CREB. *Cell*. 1992;70:105–113.
28. Lechardeur D, Schwartz B, Paulin D, Scherman D. Induction of blood-brain barrier differentiation in a rat brain-derived endothelial cell line. *Exp Cell Res*. 1995;220:161–170.
29. Testut P, Soubrier F, Corvol P, Hubert C. Functional analysis of the human somatic angiotensin I-converting enzyme gene promoter. *Biochem J*. 1993;293:843–848.
30. Paul M, Zintz M, Böcker W, Dyer M. Characterization and functional analysis of the rat endothelin-1 promoter. *Hypertension*. 1995;25(pt 2):683–687.
31. Iwai N, Matsunaga M, Kita T, Tei M, Kaway C. Regulation of angiotensin converting enzyme activity in cultured human vascular endothelial cells. *Biochem Biophys Res Commun*. 1987;149:1179–1185.
32. Shai S-Y, Fishel RS, Martin BM, Berk BC, Bernstein KE. Bovine angiotensin converting enzyme cDNA cloning and regulation: increased expression during endothelial cell growth arrest. *Circ Res*. 1992;70:1274–1281.
33. Lewin B. Chromatin and gene expression: constant questions, but changing answers. *Cell*. 1994;79:397–406.
34. Kushner PJ, Baxter JD, Duncan KG, Lopez GN, Schaufele F, Uht RM, Webb P, West, BL. Eukaryotic regulatory elements lurking in plasmid DNA: the activator protein-1 site in pUC. *Mol Endocrinol*. 1994;8:405–407.
35. Xiao RP, Ji X, Lakatta EG. Functional coupling of the β2-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. *Mol Pharmacol*. 1995;47:322–329.
36. Pepe S, Xiao RP, Hohl C, Altschuld R, Lakatta EG. "Cross talk" between opioid peptide and adrenergic receptor signaling in isolated rat heart. *Circulation*. 1997;95:2122–2129.
37. Oliveira EM, Koike MK, Junqueira ML, Oliveira VLL, Pires MD, Fortner PL, Krieger JE. Isoproterenol induced hypertrophy increases left ventricle ACE activity and ACE promoter expression in vivo. *J Hypertens Suppl*. 1996;14:S211. Abstract.
38. Krulewitz AH, Fanburg BL. Stimulation of bovine endothelial cell angiotensin-I-converting enzyme activity by cyclic AMP-related agents. *J Cell Physiol*. 1986;129:147–150.
39. Imagawa M, Chiu R, Karin M. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell*. 1987;51:251–260.
40. Medcalf R, Rüegg W, Schleuning W-D. A DNA motif related to the cAMP-responsive element and an exon-located activator protein-2 binding site in the human tissue-type plasminogen activator gene promoter cooperate in basal expression and convey activation by phorbol ester and cAMP. *J Biol Chem*. 1990;265:14618–14626.
41. Hyman SE, Comb M, Pearlberg J, Goodman HM. An AP-2 element acts synergistically with the cyclic AMP- and phorbol ester-inducible enhancer of the human proenkephalin gene. *Mol Cell Biol*. 1989;9:321–324.
42. Hai T, Curran T. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Nat Acad Sci U S A*. 1991;88:3720–3724.
43. Deutsch PJ, Hoeffler JP, Jameson JL, Habener JF. Cyclic AMP and phorbol ester-stimulated transcription mediated by similar DNA elements that bind distinct proteins. *Proc Natl Acad Sci U S A*. 1988;85:7922–7926.
44. Sheng M, Thompson MA, Greenberg ME. CREB: a Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science*. 1991;252:1427–1430.
45. Arias J, Alberts AS, Brindle P, Claret FX, Smeal T, Karin M, Feramisco J, Montminy M. Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature*. 1994;370:226–229.