Annu. Rev. Nutr. 1998. 18:353–83

CYCLIC AMP SIGNALING AND GENE REGULATION

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KEY WORDS: cAMP response element binding protein, cAMP response element modulator, nuclear factor κB, nuclear factors, spermatogenesis

ABSTRACT

Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger produced in cells in response to hormones and nutrients. The production of cAMP is dependent upon the actions of many different proteins that affect its synthesis and degradation. An important function of cAMP is to activate the phosphorylating enzyme, protein kinase A. The key roles of cAMP and protein kinase A in the phosphorylation and regulation of enzyme substrates involved in intermediary metabolism are well known. A newly discovered role for protein kinase A is in the phosphorylation and activation of transcription factors that are critical for the control of the transcription of genes in response to elevated levels of cAMP.

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0199-9885/98/0715-0353$08.00

INTRODUCTION

Eukaryotes have developed elaborate signal transduction systems by which information provided externally in the form of hormones, ions, and other signaling molecules is converted into intracellular information that regulates the internal workings of the cell, such as the transcription of specific genes. The mechanisms involved in the transduction of extracellular signals to gene transcription are multiple and complex. They include (*a*) phosphorylation cascades, in which successive kinases are activated by phosphorylation and/or dephosphorylation; (*b*) proteolytic cascades, in which active molecules are cleaved from inactive precursors; and (*c*) systems that rely on non-protein second messengers, such as phospholipids, calcium, and cyclic nucleotides.

From events occurring at the cell membrane to changes in gene transcription, the cyclic AMP (cAMP)-dependent signaling pathway is a paradigm of signal transduction. Most components of the cAMP-dependent signaling pathway are well characterized. The binding of ligands (hormones, neurotransmitters, growth factors) to their specific receptors located in the plasma membranes of cells activates GTP-binding proteins (G-proteins) that are coupled to the receptors. The G_s protein stimulates the activation of the enzyme adenylyl cyclase, which converts ATP to cAMP. The cAMP so formed activates protein kinase A (PKA), which phosphorylates numerous proteins.

The role of cAMP and PKA in the regulation of nutrient metabolism and energy balance was first noted in the control of glycogen breakdown within liver and muscle tissue (reviewed in 72). Phosphorylations by PKA were found to initiate successive cascades of additional protein kinases resulting in the activation of glycogen synthase. More recently, a role has emerged for PKA in the regulation of gene transcription. In addition to the actions of PKA to either directly or indirectly phosphorylate liver and muscle enzymes involved in nutrient metabolism, PKA phosphorylates and thereby activates cAMP-responsive transcription factors that control the transcription of genes encoding the enzymes. For example, during fasting the peptide hormone glucagon increases cellular levels of cAMP and stimulates the transcription of genes within the liver that are involved in gluconeogenesis, such as phosphoenolpyruvate carboxykinase (PEPCK) (171).

Activation of the cAMP response element binding protein (CREB) is one of the best-studied links between the activation of PKA and gene expression.

CREB is expressed in most, if not all, tissues of the body. However, the multiplicity and complexity of tissue-specific responses to cAMP requires the involvement not only of CREB, but of additional transcription factors, some of which are also regulated by PKA.

Typically, the cAMP signal is transitory. Ligand-associated receptors are rapidly down-regulated by homologous and heterologous desensitization (reviewed in 26, 118), and the cAMP signal is reduced further by phosphodiesterases, inhibitors of PKA, and phosphatases. The components of the cAMP signaling pathway, including activating and inhibitory factors, are discussed in the first half of this review. In the second half, the cAMP-responsive transcription factors are described and discussed.

COMPONENTS OF THE cAMP SIGNALING PATHWAY

The cAMP-dependent signal transduction pathway involves several enzymatic reactions. The properties of the key enzymes, adenylyl cyclase, phosphodiesterases, PKA, and phosphatases, are discussed in turn.

Adenylyl Cyclases

Currently, nine isoforms of adenylate cyclase are known, each of which is a product of a distinct gene (types 1–9). Additional isoforms of types 5, 6, and 8 arise from the translation of alternatively spliced mRNAs. At least two subfamilies of the adenylyl cyclases (ACs) are distinguished on the basis of their amino acid sequence similarities (88).

The structures of the ACs are highly conserved and feature two membrane-anchoring regions, each with six membrane-spanning helices. The catalytic and regulatory functions of the enzyme isoforms are located in intracellular domains within the C terminus of the proteins and between the two membrane-anchoring regions (reviewed in 82).

All the AC are activated by $G_{\alpha s}$ subunits, which are released from inactive heterotrimeric G-protein complexes after the agonist binds to the receptor. However, AC can also integrate signals from intracellular calcium, from other G-protein subunits, such as $\beta\gamma$ and the inhibitory G_1 and G_0, and from protein kinase C. The effects of the signaling may be positive, negative, or neutral, depending on which AC subtype the G-proteins interact with (reviewed in 76, 82). Feedback inhibition of AC by PKA has also been observed and may represent an important early step in the desensitization of the cAMP-signaling pathway (87, 161).

The tissue-specific expression of the various isoforms of AC may influence the relative amounts of cAMP synthesized in these tissues in response to specific stimuli. The tissue distribution and developmental expression of AC have

been studied extensively (reviewed in 76, 88), but only in two systems has information been obtained about how the expression of AC genes may be regulated. In brown adipose tissue, the AC3 gene is specifically stimulated in response to β-adrenergic agents, whereas other AC subtypes expressed in the same tissue are not affected (66). The promoter of the AC3 gene contains a putative cAMP response element at position $-237/-244$ (220). Messenger RNA levels for AC1 in the pineal gland rise and fall in a circadian manner, partly in response to light (201).

Phosphodiesterases

Phosphodiesterases (PDEs) degrade intracellular cAMP. These enzymes consist of a diverse group of proteins, the products of at least 15 genes (32), including isoforms derived from splice variants in the mRNAs that encode them. Over 40 isoforms of PDEs exist (20).

The activities of PDEs are particularly important in the modulation of cAMP signaling in tissues in which high basal activity of AC occurs (32, 82). The PDEs have been grouped into seven families (PDE1–PDE7), based on their specificity for, and modulation by, cyclic nucleotides. Two of these families (PDE4, comprising four genes, and PDE7, with one known gene) have a higher specificity for activation by cAMP than cGMP (reviewed in 32). However, other PDE isoforms (PDE1, PDE2, and PDE3) also contribute to the hydrolysis of cAMP (32, 82).

Certain PDEs increase their activities in response to cAMP (47, 183), and evidence indicates direct phosphorylation of the PDE4 isoform by PKA (45, 125, 182). In addition to activating existing PDE enzymes, cAMP stimulates the synthesis of new PDE mRNA (47, 210), although the molecular basis of this regulation has not been explored.

Subunits and Structure of Protein Kinase A

The PKA holoenzyme is a tetrameric complex containing two catalytic subunits (C) bound to a homodimer of two regulatory subunits (R). On binding cAMP, active catalytic subunits are released and activated. PKA kinase activity is specific for the phosphorylation of serine and threonine contained within the motif RRX (S/T) (94). Phosphorylation by PKA regulates a variety of intracellular proteins, including ion channels, cytoskeletal elements, and enzymes (reviewed in 72). The phosphorylation of membrane receptors by PKA and other protein kinases is a key event in dampening cAMP signaling, a process known as heterologous desensitization (reviewed in 26, 118).

Transcription factors are important substrates for PKA activity, often at promoter-bound transcriptional complexes within the nucleus. To phosphorylate nuclear transcription factors, C must translocate to the nucleus following

stimulation by cAMP (146). Fluorescently labeled PKA subunits (1) have been used to examine the nuclear translocation of C in PC12 cells. Dissociation of the PKA subunits occurs within 2 min after cAMP stimulation, and nuclear translocation of C begins in 3–5 min, peaks by 30 min, and returns to basal levels after 50 min. The lag time required for translocation suggests that nuclear entry is rate limiting for subsequent nuclear phosphorylation events.

The direct link of second-messenger signaling to gene transcription is modulated in several ways and includes the participation of multiple R and C isoforms. Initially, two isotypes of PKA were identified by DEAE elution profiles (100). These isotypes consist of two forms of the regulatory subunit, RI and RII. The RI and RII types were further resolved at the genetic level into four separate gene products: RIα (113), RIβ (27), RIIα (179), and RIIβ (89). Additional isotypes of RIα arise from splice variants in the mRNA encoding the subunits (189), and a unique shortened mRNA for RIIα is expressed postmeiotically in germ cells (52, 154). Similarly, multiple genes exist for the catalytic subunits Cα (202), Cβ (203), and Cγ (11), and mRNA-derived splice variants have been reported for Cα (198) and Cβ (162, 224). The various isoforms of the R and C subunits display differences in their tissue distribution. RIβ and Cβ are expressed at higher levels in brain relative to other tissues (2, 27, 173, 188, 203), and the Cγ isoform is expressed specifically in the testis (11). That there are differences in the tissue distribution of the subunits implies specificity of their function, and some specialization is seen in the range of the cAMP sensitivity shown by combinations of R and C subunits (21, 58). Functionally, the levels of the less-responsive RIIβ-containing holoenzyme correlate with the decreased sensitivity to cAMP-induced holoenzyme dissociation in different regions of the central nervous system (209). More striking biochemical differences have also been noted. The Cγ subunit differs markedly from its homologs by not interacting with PKA-specific inhibitor proteins (12), and RII subunits differ from RI subunits in that they associate with A-kinase anchoring proteins (AKAPs) (12).

AKAPs are a large family of proteins, the full extent of which has not been defined (reviewed in 178). These proteins influence subcellular localization of PKA by interacting with RII subunits. AKAPs may facilitate activation of PKA by bringing it near the site of stimulation, as is the case in dendritic spines (23, 63), or they may limit activation by colocalizing with enzymes that degrade cAMP (29). Localization of PKA within the nucleus by AKAP-95 may contribute to rapid cAMP-driven changes in transcription (28).

Despite a number of functional differences, PKA subunits show redundancy in gene knockout models. Mice lacking either RIβ or the Cβ splice variant Cβ_1 are viable and fertile, with no obvious behavioral abnormalities (17, 83, 162). However, detailed investigations of hippocampal synaptic pathways reveal deficiencies associated with such cAMP-dependent processes as long-term

potentiation of the mossy fiber system, long-term depression, depotentiation, and late-phase long-term potentiation (17, 83). Remarkably, these deficiencies occur in Cβ partial knockouts, which still express two splice variants of Cβ at significant levels (162).

Mice lacking RIIβ have markedly reduced deposits of white fat and are resistant to diet-induced obesity (33). The underlying cause for this phenotype appears to be a compensatory increase of RIα in the brown adipose tissue. This increase in RIα was previously noted in experiments involving overexpression of the Cα and Cβ subunits (204) and results from increased subunit stability within the holoenzyme rather than from an increase in levels of RIα mRNA (4). The RIα holoenzymes are more cAMP-responsive than the RIIβ are (they more readily dissociate from the catalytic subunit in response to cAMP), leading to increased activation of triglyceride-depleting enzymes and synthesis of the uncoupling protein UCP1. As a consequence of the increased levels of RIα, thermogenesis in brown adipose tissue is markedly increased (121).

At least three R subunits—RIα, RIIα, and RIIβ—and one C subunit—Cα—are transcriptionally stimulated by cAMP in Sertoli cells (109, 153, 197), with responses that vary in rapidity and level of induction. RIIβ mRNA shows the greatest induction (greater than 50-fold), although the RIIβ promoter contains no sequence resembling a cAMP responsive motif (96, 102, 123). Consistent with these findings, only RIα induction is resistant to cycloheximide. This suggests that the levels of other PKA subunits depend on the synthesis of new proteins such as additional transcription factors (109). The relative cAMP-responsiveness may be tissue specific: RIα can be induced over 50-fold in a B lymphoid cell line (189). Two promoters, resulting in two alternative first exons, control the transcription of RIα. Both promoters contain binding sites for cAMP responsive transcription factors, but the RIα_1 promoter shows the strongest induction by cAMP. The increase in R and C subunit levels following cAMP stimulation may serve to reset the system following increased degradation of released subunits. However, overexpression of R subunits relative to C, or expression of less-sensitive RIIβ subunits, may dampen cAMP responses, promoting a refractory environment until R/C equilibrium is restored. Such mechanisms are likely responsible for the extended refractory period (3–5 days) for cAMP signaling observed in FRTL-5 cells following their exposure to thyroid-stimulating hormone. The FRTL-5 cells are derived from follicular cells of the thyroid gland and are regulated by thyroid-stimulating hormone through the cAMP second messenger pathway (7).

Inhibitors of Protein Kinase A

Protein kinase inhibitors (PKI) are a class of small proteins (71–76 amino acids) that inhibit the function of C. Three different genes give rise to the α, β, and γ isoforms of PKI (31, 151, 152, 207, 208). For PKIβ, an alternative

translation start site (177) and different posttranslational modifications (101) have also been reported. There are differences in tissue distribution: PKIβ is most prevalent in the germ cells of the testis (206, 207), and PKIα and PKIβ show distinct patterns of localization within the brain (180, 207).

The three different PKIs share a homologous region near their amino terminus. This region contains a pseudosubstrate motif through which PKI interacts with and inhibits C (31). The association of PKI with C promotes nuclear export of C (48). This is an active transport phenomenon, apparently triggered by a nuclear export signal located in the amino acid sequence of PKI (221, 222). The inhibitory and nuclear export functions of the PKIs are distinct; a subfragment of PKI inhibits the kinase activity of PKA without preventing nuclear localization (48).

The exact physiological role of the PKIs in different tissues, and the control of specific genes, has not been thoroughly investigated. However, high endogenous levels of PKI expression in an insulinoma cell line (RIN1027-B2) renders CREB unresponsive to stimulation by cAMP even when additional PKA activity is introduced by cotransfection and expression of the C-subunit (205). PKIs may also play an important role in desensitization following cAMP stimulation. Follicle stimulating hormone induces PKIα and PKIβ expression in the Sertoli and germ cells of the testis (206). Because cAMP accumulation is a feature of the spermatogenic cycle, driven in part by follicle stimulating hormone acting on Sertoli cells, PKI levels may contribute to the periodicity of the spermatogenic cycle.

Phosphatases

Cellular phosphatases are grouped into four major classes on the basis of their biochemical activities (reviewed in 30). Two particular activities (PP1 and PP2A) account for the majority of serine/threonine phosphatase activity in most cells. Controversy exists regarding the identity of the primary phosphatase that acts on the transcription factor CREB responsive to PKA signaling. Some evidence favors PP1 (3, 74), whereas other evidence points toward PP2A (167, 212, 223) in the dephosphorylation of CREB. Other pathways affecting the activity of CREB may operate through the modulation of phosphatase activity. Calcineurin (PP2B) may control the activity of PP1, and decreased levels of calcineurin can extend the activity of phosphorylated CREB (15). The increase in cAMP levels and CREB activity in adipocytes in response to insulin may be mediated through inhibition of PP2A activity (167).

TRANSCRIPTION FACTORS

Several transcription factors are regulated by and are responsive to the activation of the cAMP signaling pathway. These factors include cAMP response element

binding protein (CREB), cAMP response element modulator (CREM), activating transcription factor-1, NF-κB, and nuclear receptors and are discussed below.

CREB

The cAMP response element (CRE) is defined as a DNA sequence that imparts cAMP responsiveness to genes when present within the gene's promoter. The consensus CRE has the nucleotide sequence (TGACGTCA) or variants thereof as found in the well-characterized somatostatin promoter (134). The cDNA for CREB protein was cloned from a human placental cDNA library, with oligonucleotides containing CREs used as probes (81). Related clones were generated by polymerase chain reaction and hybridization strategies on cDNA from rats, bovines, and mice (65, 174, 225). CREB has been extensively studied for its role in cAMP signal transduction and is the subject of several recent reviews (73, 133).

STRUCTURE, FUNCTIONS, AND PHOSPHORYLATION CREB belongs to a group of transcription factors that contain basic region leucine zippers, termed bZIPs (in reference to a region of basic residues required for DNA recognition and binding that is flanked by a leucine zipper region mediating homo- and heterodimerization among family members) (110) (Figure 1). The bZIP family includes Jun, Fos, CAAT/enhancer binding protein, the activating transcription factors, (ATFs), and yeast GCN4 (reviewed in 128). The cAMP-responsive bZIP transcription factors, including CREM and ATF-1 (128), form a distinct subclass. CREB, CREM, and ATF-1 share a conserved structure, with a carboxyl terminal bZIP region, an amino proximal terminal kinase–inducible domain (KID) also known as the phosphorylation box (P-box), and glutamine-rich transactivation domains flanking the KID/P-box. Central to the role of cAMP-responsive transactivation, the KID contains potential phosphorylation sites for several different kinases. However, it is the phosphorylation of serine 133 in CREB 347, or 119 in CREB 327 (formed by deletion of a 14-residue exon), that is critical for the transactivation properties of CREB (64).

Serine 133 is also a target for phosphorylation by kinases other than PKA. One group of these kinases are the Ca^{2+}-calmodulin–dependent kinases(CaM kinases) activated by intracellular calcium (35, 184) (Figure 1). The phosphorylation of serine 133 of CREB by Cam kinases I and IV potentiates transcriptional transactivation by CREB (100), whereas Cam kinase II phosphorylates both serine 133 and the nearby serine 142, resulting in a net inhibition of CREB transactivation (192, 193). In addition to cAMP and calcium, the phosphorylation of CREB serine 133 also occurs through Ras-dependent signaling pathways (37, 61). A member of the ribosomal S6 kinase family, RSK2, is a CREB kinase

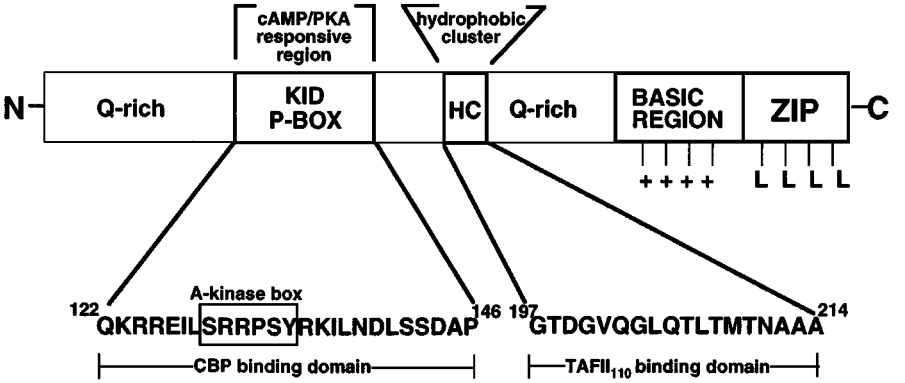

Figure 1 Schematic representation of the structure of the cyclic AMP (cAMP) response element binding (CREB) protein. Depicted are the glutamine-rich (Q-rich) regions located at either side of the kinase inducible domain (KID) or phosphorylation box (P-box), the basic region that contains the DNA binding domain, and the leucine zipper domain (ZIP) that mediates protein dimerization. The KID (amino acids 122–146) contains the cAMP-dependent protein kinase A (PKA) phosphorylation site (A-kinase box) and is also the region that binds to CREB binding protein (CBP). The amino acid sequence of the hydrophobic cluster that interacts with the TATA box associated factor $TAF_{II}110$ is also shown (adapted from Reference 73).

(231). Protein kinase C also phosphorylates CREB in vitro (234), but its role in vivo is uncertain; the phosphorylation of CREB in response to agents that activate protein kinase C may occur through the activation of mitogen-activated protein kinases (MAPK/RSK) (158).

Unphosphorylated CREB is predominantly located in the nucleus in vivo; its translocation is mediated by nuclear localization sequences located within the bZIP region (213). Whereas CREB forms dimers and binds DNA in the absence of phosphorylation, phosphorylation at serine 133 may increase the strength of the binding of CREB to CREs found in certain promoters, notably those with imperfect (asymmetric) CREs (144). However, other studies have failed to detect any effects of phosphorylation on the dimerization and/or binding of CREB to DNA (168 and references therein).

Additional sites (serines and threonines) for phosphorylation exist within the KID of CREB. Several of these sites are phosphorylated by the processive kinases, glycogen synthase kinase 3 (GSK3), and casein kinase II (CKII). Phosphorylations by both of these secondary kinases are dependent on the phosphorylation of serine 133 by PKA as a primary kinase (169). The GSK3 site (serine 129) is necessary for mediating the full transcriptional responses from Gal4-CREB fusion proteins expressed in PC12 cells. Further, transfection of GSK3 into undifferentiated F9 cells elicited a 60-fold increase in cAMP

responsiveness (50). The CKII sites have not been shown to contribute to the transactivation capabilities of CREB, although they do so in the related transcription factor, CREMτ (38).

CREB dimers do not require phosphorylation at both serine 133 sites to transactivate. Use of CREB proteins with bZIP domains engineered to preclude homodimerization and mutant CREB with a nonphosphorylatable alanine substitution at serine-133 (120) showed that hemiphosphorylated CREB dimers transactivate at half the strength of fully phosphorylated dimers. This finding suggests a direct relationship between the extent of phosphorylation and the transactivation potential of CREB.

CREB-BINDING PROTEIN The major effect of the phosphorylation of CREB within the KID domain is on the association of transcriptional coactivators. A 256-kDa nuclear factor termed CBP (CREB binding protein) was isolated as a candidate coactivator for CREB (25) (Figure 2). CBP associates specifically with CREB when phosphorylated at serine 133 and augments cAMP-induced transcription (104). CREB-directed transcription is suppressed by microinjecting CBP antisera, which presumably interferes with the interactions between CBP and CREB (6). The domain within CBP that distinguishes between phosphorylated and unphosphorylated serine 133 is termed KIX (156), and the interaction relies upon an arginine residue located adjacent to the serine-133. Other regions of CREB are also important for CBP interaction: KID-derived phosphopeptides have greatly reduced affinities for CBP (25, 156). A CREB-related

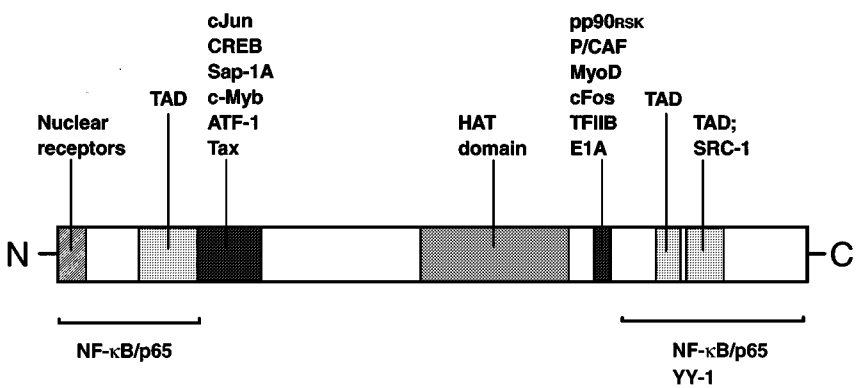

Figure 2 Functional domains of CBP/p300. A schematic of the binding protein (CBP) of the cyclic AMP response element binding protein (CREB) and the homologous p300 detailing regions with transactivational [TAD (transcription activating domains)] and histone acetyltransferase (HAT) activity, as well as sites mediating interaction with other proteins such as transcription factors (CREB, cFos) and kinases ($pp90_{RSK}$).

transcription factor, ATF-4, interacts with CBP at multiple domains, including the KIX-interactive domain, the SRC-1 and E1A interacting domains, and the histone acetyltransferase (HAT) domain (115). A closely related but distinct nuclear factor, p300, can substitute for CBP. Both CBP and p300 are also targets for the adenovirus E1A protein, which represses the transactivation functions of CREB (5, 122).

CBP/p300 integrates signals from a variety of other transcription factors including AP-1, basic helix-loop-helix factors, NF-κB, nuclear receptors, and signal transduction activating transcription (STATs) (70, 149, 199) (Figure 2). CBP has transcription activating domains that function in Gal4 chimeras, one of which is located within the amino terminus and interacts with TATA binding protein (195).

Recently, interest has been focused on the role of histone acetylation in the control of gene transcription (reviewed in 69, 157). CBP and p300 increase acetylation of individual histones and core particles by intrinsic HAT activity (9, 149), and through the HAT activity of factors associated with CBP and p300 such as P/CAF (238). The acetyltransferase activity of CBP and p300 may also target proteins other than histones. CBP acetylates several general transcription factors (86), as well as the transcription factor p53 in vitro (70). Under the same conditions, CREB itself is not acetylated.

Is the phosphorylation of CREB at serine 133 sufficient to mediate transactivation? Although phospho-CREB microinjected into cells transactivates without the requirement for stimulation by PKA (3), other model systems exhibit constraints in the absence of PKA. Stimulation of the T-cell receptor in Jurkat cells leads to the phosphorylation of CREB at serine 133, but not in the recruitment of CBP or in activation of transcription unless a background of cAMP signaling is present (19). Similarly, the phosphorylation of CREB induced by ionomycin in PC12 cells that lack PKA does not activate the expression of cAMP-responsive immediate early genes (61). Notably, other workers have observed an enhancement of CBP coactivator functions in response to PKA (25, 195), and a site for phosphorylation by PKA exists in the C-terminal region of CBP (25). Furthermore, transactivation of transcription by a C-terminal CBP fragment is stimulated in the presence of activated PKA (90). However, PKA also stimulates CREB-dependent coactivation by an amino terminal CBP fragment lacking the PKA site. This fragment of CBP also lacks the HAT domain and the P/CAF-binding region, which suggests that acetylation is not the sole coactivating function of CBP (25).

The interaction of CBP with CREB in and by itself is not sufficient to elicit the transactivation of gene promoters. The additional phosphorylation of serine-142 of CREB by CaM kinase II (192) compromises transactivation by CREB without preventing the formation of a CREB-CBP complex (193). Unlike serine 133,

substitution of an acidic amino acid in place of serine 142 mimics the effects of phosphorylation. This exquisite sensitivity to modifications of amino acids within the KID domain may also account for the positive effects of phosphorylation at the GSK3 site on the transactivational functions of CREB.

The glutamine-rich domains of CREB are also required for transactivation (139) and can mediate associations with TFIID and other components of the general transcriptional apparatus, such as $dTF_{II}110$ and hTAF(II)130 (49, 139). Furthermore, these domains can promote strong, phosphorylation-independent transactivation (18, 99, 233). For unknown reasons, the state of phosphorylation does not appear to affect the activity of CREB in in vitro transcription assays (3).

The existence of these potent transactivation domains in CREB raises the question regarding whether unphosphorylated CREB may cause basal transactivation. Previously, unphosphorylated CREB has been regarded as a repressor (reviewed in 73), in part because overexpression of CREB can block serum-induced expression of genes such as *c-fos* (148) and *c-jun* (108).

In many cotransfection experiments, overexpression of CREB fails to stimulate promoters that contain CREs. This failure to stimulate is relieved by cotransfection and expression of PKA catalytic subunit (25, 64, 205). These findings suggest that unphosphorylated CREB has a limited potential to stimulate basal levels of transcription. In addressing the question of basal transactivation it is important to differentiate between transactivation by unphosphorylated CREB and activity due to stoichiometrically low levels of phosphorylation. The use of PKI reduces both basal and cAMP-stimulated expression from the promoters of the rat tyrosine hydroxylase, human proenkephalin, and human metallothionein promoters (36, 68, 95), whereas PKI does not diminish basal transcription from the PEPCK promoter (232).

In studies of CREB activation of the PEPCK gene, CREB amino acids 165–252 were found to contain a basal activation domain that works in concert with amino acids 41–68 to stimulate transcription in the absence of PKA stimulation (163). These data argue that CREB may play a role in regulating basal transcription. This work establishes that CREB contains distinct basal and PKA-activated domains, that they operate independently for both loss of function and gain of function, and that they work on different promoters in different cell types (163). Currently, the question of the ability of CREB to regulate basal transcriptional activity remains unclear, but if it does occur it may be defined by several factors, including the cell phenotype, the context in which the CRE resides in the promoter, and the cellular environment (signaling pathways). The fact that the basal activation of transcription by unphosphorylated CREB is not readily apparent in most cotransfection experiments strongly suggests that productive interactions with CBP exert a controlling influence on the glutamine-rich domains of CREB. A well-studied model of the participation of CREB in transcription in a phosphorylation-independent manner is its actions

on the HTLV-1 promoter. The HTLV-1-derived Tax protein is capable of associating with CREB through the bZIP region (145). Tax may then act to link CREB to CBP, which Tax is also able to associate with (103, 112).

ALTERNATIVE SPLICING OF CREB TRANSCRIPTS CREB mRNA can be spliced into a number of different transcripts, each having specific regulatory consequences (Figure 3). It is worth noting that the nomenclature describing the structure of the CREB gene is not universally employed (reviewed in 73), and

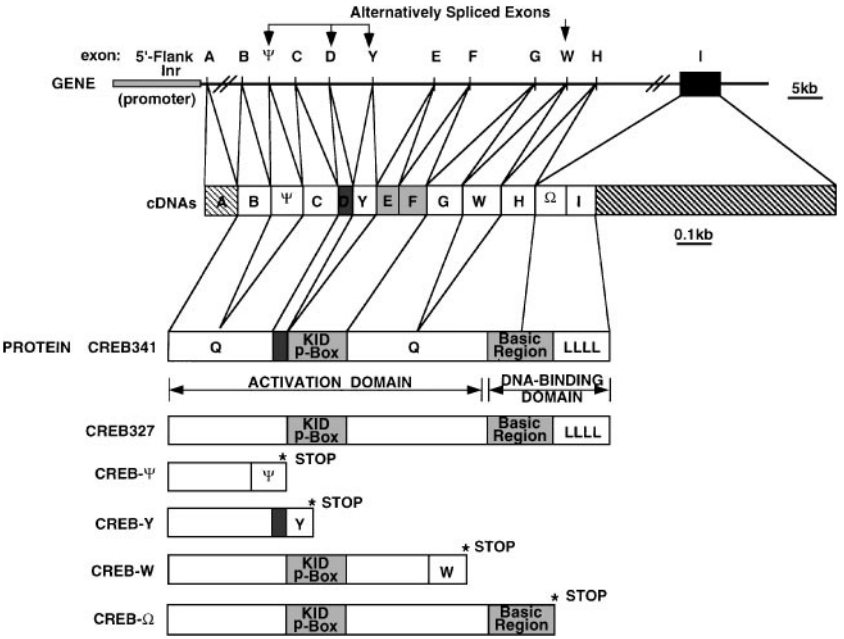

Figure 3 Diagrams of the exonic organization of the gene, mRNA, and encoded proteins for the cyclic AMP response element binding protein (CREB). The CREB gene contains at least 12 exons spanning >80 kb of DNA. The introns between exons A and B and H and I are large (>25 kb). The exons comprise functional modules in CREB. Exons E and F encode the phosphorylated domain [kinase inducible domain (KID) and phosphorylation box (P-box)], and exons B, C, and G encode the glutamine-rich domains (Q), important for transcriptional transactivation. Exons H and I encode the bZIP domain involved in dimerization and DNA binding. Notably, exons Ψ, Y, D, and W are alternatively spliced in tissue- and developmental-specific patterns. The Ω sequence is not an alternatively spliced exon but rather intron slippage caused by use of an alternative acceptor splice. The alternative splicing of exon D retains the open reading frame in CREB mRNAs, resulting in the formation of the two CREB isoforms, CREB327 and CREB341, which differ by 14 amino acids. However, exons Ψ, Y, and W and the alternative Ω splice result in the premature termination of translation of the CREB mRNA. These isoforms of CREB are truncated at the C-terminal end and are devoid of the DNA-binding bZIP domain and the encoded nuclear translocation signal to interrupt a positive autofeedback regulation of CREB on its own promoter.

that the system used here (see Figure 2) is from Kennelly & Krebs (94). One of the first variations noted in CREB was the skipping of exon D, resulting in the translation of CREB 327 (80). All tissues appear to express both CREB 341, containing the D exon, and CREB 327, in which exon D is absent (13, 174). This exon contributes to the amino terminal glutamine-rich transactivation domain, and a positive influence on transactivation has been observed in undifferentiated F9 cells (235). However, this positive influence of exon D has not been universally observed, even in the same cell type (13, 174).

A number of splice variants of CREB mRNAs cause premature termination of translation (Figure 3). The cDNAs for three such isoforms were isolated from mouse testis cDNA libraries (174). One isoform results from a miss-splicing of exon H (Creb W or H′). The remaining two isoforms occur by inclusion of additional exons either 5′ or 3′ of exon C (exons ψ and γ, respectively, with γ equivalent to exon Y described below).

Some splice variants of CREB are expressed in a tissue-specific manner. Exons Y and W (see Figure 2) are both expressed primarily in the germ cells of the rat testis (214, 215). Additionally, exon W shows evidence of developmental stage-specific expression. The cycle of rat spermatogenesis is divided into 14 stages (designated I to XIV) defined by patterns of cellular associations within the seminiferous tubule (112a). During development, which takes approximately 45 days, a committed germ cell passes through three and a half cycles, driven by the alternating influences of cAMP and androgenic hormones (156a). The highest levels of the exon W–containing CREB-W isoform is expressed in Sertoli cells during stages VI–VII of the spermatogenic cycle (215), and in spermatocytes during the pachytene stage of meiosis. In humans, an additional exon, exon Z, is co-spliced with exon W (62). Exons Y, W, and Z share the property of introducing stop codons into the reading frame of CREB, resulting in the formation of truncated CREB isoforms defective in the activation of transcription. Studies of the CREB-W and CREB-WZ have revealed that reinitiation of translation can occur downstream from the stop codons. Two reinitiation sites have been observed, one within exon W and another in exon H (217). Products from these reinitiation events are dominant-negative bZIP DNA-binding proteins, termed I-CREBs, and are capable of competing with a full-length activator CREB protein for CRE binding sites on the promoters of genes.

The CREB promoter contains several CRE elements that bind CREB and thereby control cAMP-induced transcription (129, 216). In the testis, the stage-specific changes in CREB activator and I-CREB repressor levels offer a mechanism to regulate CREB gene expression. Specifically, in spermatocytes, the peak in CREB-W expression and I-CREB production corresponds with the observed fall in the levels of CREB expressed at stages VII–XIV. In Sertoli

cells, the CREM-derived inducible cAMP early repressor protein transiently represses cAMP-mediated induction of CREB in stages XIII–I, after which CREB induces transcription from the CREB promoter at stages II through VI (WH Walker, PB Daniel, and JF Habener, submitted for publication). Alternative translation occurs from another site in the CREB mRNA, near the amino terminus. A shortened form of CREB, CREBβ, is produced at low levels from a translational start site in exon C. Levels of CREBβ are increased in targeted gene deletions of CREB exons A and B (16). Recently, a CREB splice variant missing exons D and G was described from mice (237). The tissue distribution of this variant assessed by reverse transcriptase–polymerase chain reaction showed a strong specificity for expression in the thymus. This new variant may, therefore, play a role in thymocyte development. Loss of the G exon while retaining the reading frame compromises the second glutamine-rich (Q-rich) transactivation domain (Q2 domain). The transactivation potential of this variant has not been investigated.

CREM

CREM strongly resembles CREB and has most of the same domains: a bZIP DNA-binding and dimerization region, and a P-box flanked by two glutamine-rich transactivation domains. Functionally, however, there are important differences between the expression and functions of CREM and CREB. Most of these differences arise from the alternative splicing and promoter usage that are features of CREM. For example, the CREM gene encodes two DNA-binding domains that are alternatively selected by alternative RNA splicing. The sequences of the two domains differ: The upstream DNA-binding domain 1 (DBDI) has greater similarity to the CREB DBD than does the CREM DBD2. Differences in DNA-binding affinities for the two CREM bZIP regions have been noted (111), although no regulated splicing has been described for these exons. Exon skipping in the CREM gene gives rise to a variety of negative regulators (18, 54, 219), reviewed by Walker & Habener (218). CREM isoforms with either or both transactivation domain-encoding exons (C and G) can transactivate in a cAMP-dependent manner (111). Positively transactivating variants of CREM (CREMτ) are diffusely expressed in the brain (126) and strongly expressed in round spermatids (55). In the germ cells of the testis, high levels of CREM expression are augmented by changes in the choice of the polyadenylation site. This results in an enhanced stability of the mRNA by eliminating nucleotide sequences (AUUUA) that signal rapid degradation (56). Expression of the τ2 isoform (containing exon G) in the brain gives rise to a negative regulator, S-CREM (39). An alternative translation start site near the 3′ end of the KID can also be utilized. Although it contains the Q2 domain, it inhibits transcription mediated by CREs. CREM inhibitors in general

show a specific distribution in the brain, and S-CREM may be developmentally regulated (39, 126). Control of the major upstream CREM promoter (P1) has not been formally characterized, but it is reported not to be inducible by cAMP (131). In contrast, a second internal CREM promoter called P2 within the intron preceding exon γ has been studied extensively. The P2 promoter is strongly and rapidly induced by cAMP via four CRE-like elements (called CAREs) within its promoter (131). The result of translation from the CREM internal promoter is a short bZIP-containing negative regulator termed inducible cAMP early repressor (ICER) (131). ICER is found mainly in neuroendocrine tissues (105, 190), including the Sertoli cells of the testis (132).

The effects of ICER are typically cyclical or short lived because ICER feeds back and binds to the CAREs on the P2 promoter and thereby represses the transcription of the ICER mRNA. In this manner, ICER plays a role in the development of circadian rhythms of gene expression (53, 190) and may play additional roles in the causation of the refractory periods that may follow the cAMP stimulation of cells (106, 107, 132).

The requirement for CREM or ICER in any of these systems is not clearly determined. Complete targeted disruption of the CREM gene has been accomplished in mice. The phenotype described thus far is that of male infertility owing to an arrest of spermatid development during late stages of spermatogenesis (stages VII–VIII) (16, 140). These stages coincide with translation of CREMτ protein (55) and this altered phenotype suggests that CREMτ may control the expression of genes needed for spermatid maturation (40, 194, 241). The generation of engineered bZIP proteins that preferentially heterodimerize and allow formations of CREB/CREM heterodimers (119) show that CREB/CREMα heterodimers transactivate about half as efficiently as CREB/CREB heterodimers (119). Also, when CREB is rate limiting, increasing the amount of CREMα increases rates of gene transcription.

ATF-1

ATF-1 was originally cloned as one of an extended family of CREB homologs (75). Like CREB, it contains a PKA phosphorylation site and interacts with Tax (239). ATF-1 binds CREs and forms heterodimers with CREB (85, 166). Phosphorylation has been reported to enhance its binding to CREs (98), but the same phenomenon in CREB is debated (see 168). Despite its similarity to CREB, the role of ATF-1 in PKA-dependent transactivation has not been demonstrated unequivocally. For example, although ATF-1/CREB heterodimers transactivate (97), ATF-1 levels in cell lines negatively correlate with cAMP responsiveness (46). In Gal-4 fusion proteins, the amino-terminal transactivation domains and P-box of ATF-1 transactivated when phosphorylated by CaM kinases I and IV at serine-63. Phosphorylation by PKA at serine 63, however, did not induce

transactivation. Specific modification of coactivators may be necessary for ATF-1 activity and previous results showing PKA-inducibility of ATF-1 (166, 117) may have resulted from recruitment of endogenous CREB to CREB/ATF-1 heterodimers. This and other evidence (51) suggests that ATF-1 is only weakly activated by PKA.

AP-2

AP-2 is a 50-kDa homodimeric transcription factor expressed in a limited number of cell types and tissues, notably neural crest lineages (130). It has two transactivation domains, one proline-rich toward the amino terminus, and one acidic domain near the center of the protein (227, 228). AP-2 has been identified as a factor involved in rapid, cyclohexamide-insensitive cAMP responses (reviewed in 172, 226). AP-2 also has a strong basal transcription activity, as evidenced by its independence from PKA in transfection systems (44, 91, 226). How PKA affects AP-2 is not known but posttranslational modification has been inferred (124), and phosphorylation by PKA occurs in vivo and in vitro (155). Although a site for phosphorylation on AP-2 has not been identified firmly, a conserved PKA-like motif (RRLS) exists in mouse AP-2 and in three human AP-2 variants (135, 138, 226, 229). The physiological roles of AP-2 and CREB are probably distinct. AP-2 has a more limited tissue distribution, marked cell-specificity, and strong basal activity. It may have a role in differentiation, and the basal activity may allow it to regulate genes that have constitutive as well as cAMP-induced activity.

NF-κB

NF-κB transcription factors also may be controlled directly by cAMP. The Rel/NF-κB family (67) induces a variety of inflammatory response genes in monocytes and endothelial cells. Elevated cAMP, or overexpressed PKA, has cell-specific effects on NF-κB–regulated reporter gene expression. Stimulation of peripheral T lymphocytes with PKA impairs activation of the interleukin 2 gene through NF-κB binding sites (24, 143), and cAMP can inhibit NF-κB activation in mesenchial cells (175). Elevated cAMP also inhibited the NF-κB activation of transmembrane receptor tissue factor, E-selectin,vascular cell adhesion molecule-1 (V-CAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and tumor necrosis factor alpha genes in monocytes and endothelial cells. Products of these genes permit binding and transmigration of leukocytes into sites of inflammation (150). In contrast, the κ immunoglobulin light chain is induced by cAMP through a NF-κB binding element (186), and in the promyelocytic cell line, HL-60, transcription of a NF-κB reporter gene, was stimulated after exposure to cAMP, although the effect was diminished in more differentiated cells (181).

PKA is capable of releasing and activating NF-κB sequestered in the cytoplasm by phosphorylating and initiating the degradation of IκB-β (60, 116, 187). However, some reports suggest that PKA may stabilize IκB-α and impair nuclear transport of NF-κB p65 (143). Several of the rel protein family members contain the PKA consensus motif, RRPS (136). Phosphorylation of c-Rel may enhance its nuclear localization (137). c-Rel inhibits transactivation by p50/p65 heterodimers, apparently by homodimer occupation of κB sites (43). The NF-κB subunit is inducibly phosphorylated at a PKA consensus motif during the activation of NF-κB (141) (143). Furthermore, PKA phosphorylation of p65 may increase its affinity for κB enhancer elements and stimulate NF-κB–regulated transcription (143, 240). There is also evidence of a cAMP-independent role for the catalytic subunit of PKA, which may form complexes with cytoplasmic IκB and NF-κB subunits (240). Signals that cause degradation of IκB and release of NF-κB then allow the complexed PKA to phosphorylate and further activate NF-κB p65. NF-B p65 also interacts with CBP/p300 (59, 160).

Nuclear Receptors

This family of transcription factors includes receptors for steroid and thyroid hormones, which are activated by binding of specific ligands. It also includes factors (orphan receptors) for which ligands are unknown or not required for transcriptional activation. A variety of nuclear receptors appears to respond to elevations of cAMP, but whether the response is due to phosphorylation of the receptor by PKA is not known. Overall, the role of phosphorylation in modulating the transcriptional activity of different receptors is controversial (reviewed in 10). For some nuclear receptors, stimulation by PKA may activate the receptor in the absence of ligand. Such an effect has been demonstrated for the progesterone, estrogen, vitamin D, and androgen receptors (34, 42, 142, 147). Phosphorylation may be required for receptor activation. Phosphorylation of the estrogen and progesterone receptors in vivo is stimulated by both estrogen and PKA (8, 42). However, ligand-stimulated phosphorylation occurs at three sites, all distinct from the single identified PKA phosphorylation site (41). Other receptors—those for glucocorticoid retinoic acid, and thyroid hormone—show a distinct potentiation of the ligand-induced activity on mouse mammary tumor virus (MMTV) promoters, and no cAMP-inducible activity in the absence of ligand (84, 114, 147, 165, 170). The exact role of the phosphorylation of nuclear receptors remains unclear. Cooperative interactions with other MMTV promoter binding proteins may be a factor. The observation that PKA represses rather than stimulates transcription when the MMTV promoter is stably integrated into DNA rather than in an episomal configuration indicates that the circumstances involved in determining

whether PKA phosphorylations can both activate and suppress transcription of genes are complex (159).

Several of the orphan receptors may be regulated by PKA. The liver-enriched transcription factor HNF4 and the neuronal differentiation factor NGFI-B may be negatively regulated by PKA at the level of DNA-binding; phosphorylation at the A-box, located adjacent to the DNA-binding domain, may be inhibitory (79, 211). In whole animal studies, the DNA binding activity of HNF4 from liver nuclear extracts is directly affected by nutritional status: food-deprived animals have reduced HNF4 binding compared with animals deprived of food and then fed (211). cAMP-stimulating agents further reduce HNF4 binding. HNF4 provides feedback for the fine tuning of energy metabolism as it binds to key regulatory sequences in the promoter of L-type pyruvate kinase, an enzyme involved in gluconeogenesis. Nuclear receptors also interact with CBP, both directly and through additional coactivating proteins such as NCoA1/SRC-1/p160, NCoA2/TIF2/GRIP1, and p/CIP (78, 92) (for steroid receptor coactivator references, see 199). p/CIP may also be required for CBP coactivation of gamma interferon and tetradecanoyl phorbol acetate–responsive transcription factors (199), but whether it plays any role in PKA-CREB coactivation has not been examined. Because of limiting amounts of CBP in the cells, steroid receptors may usurp CBP and affect the activity of other factors, such as CREB, that require CBP for activity.

Other Transcription Factors

The activity of the homeodomain protein, thyroid transcription factor 1, is stimulated by PKA-mediated phosphorylation of an amino-terminal threonine, a modification that may increase its DNA-binding affinity (185, 236). Another homeodomain protein, pituitary-specific Pit-1/GHF-1, contains a site that, when phosphorylated, inhibits DNA-binding (93). That this site is phosphorylated by PKA has been suggested, but the negative effect does not correlate with the action of cAMP to stimulate both prolactin gene expression and the autoregulation of Pit-1 (57, 164). The inhibitory site of Pit-1 may be phosphorylated by cell-cycle–specific kinases rather than by PKA (22).

Transcription factors involved in muscle cell differentiation may also be targets for cAMP regulation. The muscle-specific factors belong to the basic helix-loop-helix family (bHLH) and bind DNA at E-box motifs. Transactivation by Myf-5, MyoD, and MRF4 is inhibited by PKA, effectively blocking differentiation (77, 230). However, direct phosphorylation of MRF4 is not essential for inhibition (77). Other bHLH proteins, E12 and E47, are not affected by PKA (230).

Some genes show a delayed or cycloheximide-sensitive response to cAMP (172), which implies a requirement for de novo synthesis of other transcription

factors. For example, the proenkephalin gene, in bovine chromaffin cells, is responsive to cAMP indirectly through increased synthesis of c-Fos. It is known that transcription of *c-fos*, along with that of other immediate early genes, is stimulated by cAMP and that its promoter contains a functional CRE (14, 176). The gene for the CAAT/enhancer binding protein (C/EBP) β transcription factor also is controlled by CREB during liver regeneration (145). Stimulation of expression of C/EBP factors by CREB also may be important for induction of granulosa cell differentiation by luteinizing hormone (191), and for the cAMP-induced activity of acetyl coenzyme A carboxylase (196) and PEPCK promoters (171). There is also evidence that C/EBPβ is phosphorylated in response to cAMP (127, 196). However, in vitro phosphorylation of C/EBPβ by PKA at a PKA consensus site has no effect on DNA binding, whereas phosphorylation at other sites inhibits DNA binding (200).

CONCLUSIONS

CREB and CREM proteins have been a major focus of efforts to elucidate cAMP signaling at the level of gene transcription. However, many other transcription factors are also modulated by cAMP signaling. The mechanisms by which PKA-mediated phosphorylation affects transcription factors are multiple and include effects on the recruitment and association of coactivators, changes in DNA-binding affinities, and nuclear translocation. Studies of the activation of CREB by phosphorylation have led to the identification of the multifunctional coactivator proteins, CBP and P300. Much remains to be elucidated about the role of CBP and other coactivators, especially whether they are modulated themselves by PKA.

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