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Molecular Mechanisms of cAMP-Regulated Gene Expression

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Contents

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Abstract

The ability of many genes to be induced by cAMP is dependent on the presence of enhancers located in the regions of DNA upstream of the start sites to the genes. The two best characterized enhancers are the CRE (5'-TGACGTCA-3') and the AP-2 site (5'-CCCCAGGC-3'). The activity of the CRE is modulated by sequences adjacent to the consensus sequence as well as by promoter context and cell type. The complex control of the CRE is reflected in the large number of cloned CRE binding proteins that arise both from unique genes and from splice variants. These factors are leucine zipper proteins that must dimerize before binding to DNA. Although all of the factors isolated can form active homodimers, many are also able to form heterodimers. The amino termini of these proteins contain consensus phosphorylation sites through which these factors *trans*-activate their cognate promoters. The diversity of the *trans*-acting factors and their *cis*-acting sequences reflects the precise control that cells require in the modulation of gene expression by cAMP.

Index Entries: CRE; AP-2; CRE-binding proteins; CREB; cAMP; gene expression.

Introduction

Since the discovery of adenosine 3',5' monophosphate (cAMP) by Sutherland and Rall in 1958 and the elucidation of its role in glycogen metabolism, cAMP has shown itself to play a significant role in many aspects of cellular function. It has pleiotropic effects on cell morphology, differentiation, and cell–cell communication. In slime molds, cAMP functions as the extracellular chemoattractant necessary for its switch from unicellular, undifferentiated amoebae to a multicellular, differentiated fruiting body. In mammalian endocrine organs, cAMP affects the synthesis and release of peptide hormones. Through its activation of protein kinase A, cAMP regulates the transcription of a select number of genes. The mechanism by which these genes were targeted remained undetermined until their promoters began to be isolated and analyzed for sequences capable of mediating this ability.

Information on the mechanism by which cAMP stimulates gene transcription has greatly expanded since the first cAMP-responsive enhancers were identified (Short et al., 1986; Comb et al., 1986). These sequences, found in the regions upstream of the start sites in several cAMP-sensitive genes, have been shown to be both necessary and sufficient for full induction by cAMP. These enhancers fall into three general categories:

1. Sequences with similarity to the CRE (cAMP-responsive element), which has a consensus sequence of 5'-TGACGTCA-3', and which is found in the majority of cAMP-responsive genes;
2. Sequences with similarity to the AP-2 binding element, 5'-CCCCAGGC-3'; and
3. Those few elements that have no obvious sequence similarity to either of these sites, yet appear to confer cAMP sensitivity.

The identification and analysis of these sites has been followed by the isolation and cloning of the factors that act upon them. Since the original cloning of CREB (cAMP-responsive element binding protein), a factor that can bind to the somatostatin CRE and *trans*-activate through this site (Hoeffler et al.,1988; Gonzalez et al.,1989), several factors that bind to the CRE sequence have been isolated. These factors appear to arise both from unique genes and from alternative splicing. Below, we describe two aspects of the cAMP-mediated induction of gene transcription in detail, that of the *cis*-acting sequences that confer this sensitivity and the *trans*-acting factors that bind to them.

The CRE Consensus Sequence

The enhancer sequences that have been identified as the CRE were first located in the genes for phosphoenolpynuvate carboxykinase (Wynshaw-Boris et al., 1984; Short et al., 1986) and the neuropeptides proenkephalin (Comb et al., 1986) and somatostatin (Montminy et al., 1986). The delineation of the minimal sequences involved was achieved by determination of which regions upstream of the TATA box conferred induction by cAMP onto a heterologous promoter. The promoters of a wide variety of eukaryotic (Table 1) and viral genes have since been identified as containing the consensus CRE sequence. The CRE is generally located within 150 bp of the TATA box and fulfills the requirements of a classical enhancer, in that it is also active when placed in a reverse orientation or is relocated downstream of the gene. The CRE also acts as a basal level activator: deletion of the CRE from the somatostatin promoter decreases unstimulated expression tenfold (Andrisani et al., 1987).

Within the framework of this motif, deletion analysis has shown that sequence differences among the CREs are significant with regard to cAMP induction. The glycoprotein α-subunit CRE has a duplicated iteration of the 8-bp CRE sequence (Table 1). The single 5'-TGACGTCA-3' sequence, however, can also be viewed as inverted overlapping repeats of the most conserved nucleotide sequence, 5'-CGTCA-3'. Point mutations that alter identical sites in each of the CGTCA repeats can have differential effects. Both mutant sequences 5'-AGACGTCA-3' and 5'-TGACGTCT-3' cause reduced basal activities, but the former still elicits a cAMP response, whereas in the latter the cAMP response is totally obliterated (Deutsch et al., 1988a). Thus, these inverted repeats are not viewed as equivalent by the DNA binding protein(s).

The differences between inverted repeats can be further elucidated by examination of the CRE from the gene for vasoactive intestinal peptide (VIP). In this CRE, the CGTCA inverted repeat sequence is separated by five nucleotides (Tsukada et al., 1987). As with the glycoprotein α-subunit CRE, point mutations of identical sites in the inverted repeats have a differential effect. Mutation of the upstream motif to 5'-CGTCT-3' has no effect on cAMP stimulation, but changing the downstream motif from 5'-TGACG-3' to 5'-AGACG-3' reduces cAMP induction by 60% (Fink et al., 1988; Hyman et al., 1988). Thus, identical mutations in the motifs have opposite effects in the VIP and α -subunit CREs. However, it is the downstream motifs in both that have the greater effects on cAMP induction.

The AP-2 Consensus Sequence

In addition to the CRE site, a second sequence has been determined as being able to confer induction by cAMP. The AP-2 site was first identified in the SV40 and human metallothionein IIA promoters, and was initially believed to be involved solely in basal transcription (Haslinger and Karin, 1985; Karin et al., 1987; Mitchell et al., 1987). Identification as a cAMP-inducible enhancer came when five copies of the AP-2 site from the metallothionein promoter were placed downstream of the human β-globin gene and sensitivity to forskolin was assayed (Imagawa et al., 1987). Forskolin, which increases levels of cAMP in cells by activating adenylate cyclase directly, stimulated expression of β-globin message tenfold. The AP-2 site also confers sensitivity to phorbol esters, which activate protein kinase C, to a similar extent. In comparison, although the CRE from the VIP gene can stimulate transcription in the presence of phorbol esters, it is not clear if this ability is widespread among the various CRE enhancers (Fink et al, 1991).

AP-2 sites are present in a number of other genes, including human growth hormone, human c-*myc*, and human proenkephalin (Table 2) (Comb et al., 1986; Imagawa et al., 1987). Unlike CRE-dependent activity, which has a broad tissue distribution, AP-2 activity may be much more limited. For example, footprinting

Table 1
CRE Sequences Identified in Eukaryotic Promoters[a]

GENE	SEQUENCE	REFERENCE
Proenkephalin, human -94 to -82	CCTGCGTCAGCTG GGACGCAGTCGAC	Comb et al., 1986
Somatostatin, rat, -52 to -37	TGGC**TGACGTCA**GAGA ACCG**ACTGCAGT**CTCT	Montminy et al., 1986
Glycoprotein α-subunit, human, -146 to -130, -129 to -111	AAT**TGACGTCA**TGGTAA TTA**ACTGCAGT**ACCATT	Silver et al 1987
Fibronectin, human, -176 to -161 rat, -164 to -159	CCCG**TGACGTCA**CCCG GGGC**ACTGCAGT**GGGC	Dean et al, 1988
Vasoactive intestinal peptide, human, -90 to -68	TGGCCGTCATACTGTGACGTCTT ACCGGCAGTATGACACTGCAGAA	Tsukada et al., 1987
Tyrosine hydroxylase, rat, -39 to -24	GCTT**TGACGTCA**GCCT CGAA**ACTGCAGT**CGGA	Lewis et al., 1987
c-fos, mouse, -70 to -58	CCAGTGACGTAGG GGTCACTGCATCC	Gilman et al., 1986
p-enolpyruvate carboxykinase, rat, -91 to -79	CTTACGTCAGAGC GAATGCAGTCTCG	Short et al., 1986
Glucagon, rat, -302 to -287	CATT**TGACGTCA**AAAT GTAA**ACTGCAGT**TTTA	Philippe et al, 1988
Parathyroid hormone, bovine, -79 to -64	GGAG**TGACGTCA**TCTG CCTC**ACTGCAGT**AGAC	Weaver et al., 1984

[a] All CRE sequences shown include the adjacent 4 bp, except in the case of glycoprotein α-subunit, in which the entire 18-bp repeat is shown. The 8-bp palindrome, when present, is shown in bold, and each CGTCA motif is indicated by an arrow.

Table 2
AP-2 Sequences Identified in Eukaryotic Promoters[a]

GENE		SEQUENCE	REFERENCE
Proenkephalin, human,	-81 to -66	CAGC**CCGCCGGC**GATT GTC**GGGCGGCCG**CTAA	Comb et al., 1986
Metallothionein IIA, human,	-221 to -206	GCGT**CCCCGAGG**CGCA CGCA**GGGGCTCC**GCGT	Imagawa et al., 1987
	-182 to -167	ACCG**CCCGCGGC**CCGT TGGC**GGGCGCCG**GGCA	
	-124 to -109	GTTT**CGCCTGGA**GCCG CAAA**GCGGACCT**CGGC	
Growth hormone, human,	-288 to -273	GTAC**CGGACGCC**GGTC CATG**GCCTGCGG**CCAG	Ibid
	-166 to -151	TGTT**CGGTCCCC**CGTA ACAA**GCCAGGGG**GCAT	
c-myc, human,	606 to 621	CCGC**CCACCGGC**CCTT GGCG**GGTGGCCG**GGAA	Ibid
	648 to 663	GGAC**CCCCGAGC**TGTG CCTG**GGGGCTCG**ACAC	

[a]All AP-2 sequences shown include the adjacent 4 bp, and either bind purified AP-2 or, when mutated, attenuate cAMP responsiveness. The 8-bp sequence that denotes the binding site is shown in bold, with an arrow indicating direction.

activity, transactivation, and AP-2 message are readily apparent in HeLa cells, but are not observed in the hepatoma-derived HepG2 cell line (Williams et al., 1988; Rickles et al., 1989).

Promoters Without Identified cAMP-Responsive Enhancer Sequences

The vast majority of cAMP-inducible genes appear to have either the CRE or AP-2 consensus sites. However, a small number of genes fall outside these categories.

The steroid hydroxylase genes are a class of genes in which some of the members are cAMP-inducible, but the upstream sequences that confer this sensitivity do not always fall into the CRE or AP-2 families. The region of DNA from the human steroid 21-hydroxylase ($P\text{-}450_{C21}$) gene that confers cAMP induction (Kagawa and Waterman, 1990) contains a consensusequence for the basal transcription factor SP1 (G/TGGG CGGG/AG/AC/T) (Kadonaga et al., 1986). However, SP1 has not been shown to confer sensitivity to cAMP. Likewise, the gene for bovine steroid 17 α-hydroxylase has a region of DNA that confers cAMP induction, but it does not have obvious sequence similarity to the CRE or AP-2 sites (Lund et al., 1990). Analysis by southwestern assay indicates that this sequence has a protein selectivity that is different from a consensus CRE. Furthermore, although the CRE can com-

pete with the steroid sequence for protein binding in a gel shift assay, it has an affinity tenfold lower than the steroid sequence itself.

The gene for chorionic gonadotropin β-subunit (CGβ) is also stimulated by cAMP, but the induction is delayed relative to the glycoprotein α-subunit gene, which has a consensus CRE (Jameson and Lindell, 1988; Milstead et al., 1987). Transient transfection assays with the promoters of these two genes placed upstream to the reporter gene chloramphenicol acetyltransferase (CAT) indicate that the induction of activity from theCGβ construct lags behind that of the α-subunit construct by 6–8 h. Induction through CRE or AP-2 sites is rapid (within 30 min) after the addition of cAMP, and is cyclohexamide-insensitive, indicating the recruitment of preexisting factors. The induction of CGβ by cAMP is also cyclohexamide-insensitive, but the differential kinetics combined with the lack of an identified consensus site for cAMP sensitivity suggest that here, too, the activity is mediated by a *cis*-acting sequence other than the CRE or AP-2 enhancers.

CRE Activity Depends on Context

The environment of the CRE can be defined by three parameters: The sequences adjacent to the CRE, the promoter to which the CRE is attached, and the cell type in which the activity is being assayed. Thus, an examination of CREs from a number of different genes on an identical promoter and in a single cell type can reveal significant functional differences. Consensus CRE sites and their adjacent sequences from the glucagon (-290 to -268), parathyroid hormone (-79 to -62), glycoprotein α-subunit (-128 to -111) and somatostatin (-56 to -33) genes were placed upstream of the glycoprotein α-subunit promoter deleted of all upstream regulatory sequences (Deutsch et al., 1988b). Although each of these CREs have the identical 8-bp consensus sequence, the basal activities conferred by each varied markedly. The glucagon and parathyroid constructs increased basal activity by only 1.5-fold; the α-subunit and somatostatin CREs produced activities of seven- and tenfold higher, respectively. Furthermore, these differences extended to the cAMP response. The glucagon and parathyroid hormone sequences each produced a cAMP induction of eightfold, whereas induction by the glycoprotein α-subunit and somatostatin sequences mediated a 20-fold increase. Extension of the sequence normally adjacent to the glucagon CRE from 9 to 14 bp eliminated both the basal and stimulated responses. Indeed, this element has not been shown to confer any cAMP response to the glucagon gene itself. Thus, the activity of a CRE sequence can be greatly affected by the sequences located adjacent to it.

Cell-specific effects were detailed by Jameson et al. (1989b) when the basal activity of the α-subunit CRE placed upstream to the enhancerless somatostatin promoter was examined in two different cell lines. In JEG-3 cells (a human placental cell line) this construction shows a 2.5-fold increase in activity above the enhancerless promoter, whereas in INRI-G9 cells (a rat islet cell line) it produces a 36-fold increase. These activities, however, are dependent on the promoter to which the CRE is attached; placement of the CRE on a different promoter can yield significantly different results. The α-subunit CRE increases the basal activity of the enhancerless α-subunit promoter by tenfold in JEG-3 cells, yet it has no effect on activity in INRI-G9. These differences may reflect the ability of factors to bind to the various CREs or the binding of different factors to each of the CREs, which may be controlled by cell-specific expression of the CRE-binding proteins.

Interactions with Other Enhancer Sequences

In addition to the interactions between cAMP-responsive elements and their promoters as described above, another level of complexity is added by the interaction between CREs and

adjacent enhancer sequences. Several examples are described below.

The CRE present in the fibronectin gene is expressed in a tissue-specific manner. Although the promoter is cAMP-inducible in JEG-3 cells (Dean et al., 1988) it is inhibited by cAMP in granulosa cells (Dorrington and Skinner, 1986; Bernath et al., 1990). This cell-specific inhibition is attributable to *cis*-acting sequences found upstream (within 300 bp) of the CRE. However, the sequences involved have yet to be fully elucidated.

In the proenkephalin gene, there are two CRE-like sequences identified as ENKCRE-1 and ENKCRE-2 (Hyman et al., 1988; Comb et al., 1988). Although it has been shown that the same factor that binds ENKCRE-2 will bind the VIP CRE, a novel factor, ENKTF-1, binds the distal ENKCRE-1 site. Deletion of the upstream ENKCRE-1 site leaves the promoter with a greatly diminished (about tenfold) cAMP response. Deletions that remove both of the ENKCRE sites virtually eliminate all basal activity as well as the cAMP response. In addition, all point mutations within the 5'-CGTCA-3' motif of ENKCRE-2 eliminate the basal and cAMP-stimulated activity as well. The spacing between these two sites is critical: they are normally five bases apart, i.e., one half turn of the helix. Adding five bases, which increases this distance to a full turn, decreases the cAMP response to 33% of control. Adding another five bases, which returns the two sites to their original orientation, increases the activity to 66% of control. This indicates that the relative orientation of the proteins that bind to these two sites is critical.

There is also an AP-2 site proximal to ENKCRE-2 (Hyman et al., 1988; Comb et al., 1988). Mutation of this sequence inhibits basal activity by 50% and decreases cAMP responsiveness by fourfold (Hyman et al., 1989). In addition, the factors AP-1 and AP-4 can bind to sequences in and around ENKCRE-2, indicating that this element is likely to be involved not only with the cAMP response of this gene, but also with its responsiveness to phorbol esters.

The CRE located in the glycoprotein α-subunit interacts with defined *cis*-acting sequences both upstream and downstream of the enhancer. This CRE consists of two identical 18-bp repeats in which reside a perfect 8-bp CRE. Although a single copy can provide a cAMP response, basal activity is about fourfold lower (Deutsch et al., 1987). Immediately upstream of these CREs lies a tissue-specific enhancer (URE), which acts to increase both basal and stimulated activity by three- to fivefold and is dependent on the CRE for activity (Jameson et al., 1989a; Delegeane et al., 1987). Distance requirements between these elements have yet to be determined.

Mutational analysis of the region immediately downstream of the α-subunit CREs identified another activity (Kennedy et al., 1990), which, when mutated, decreased basal transcription by tenfold. This element has been shown to bind a unique factor, which is somewhat tissue-specific (Anderson et al., 1990). In addition, this promoter can be repressed by activated glucocorticoid receptor. It has been suggested that the mechanism of repression is through direct binding of the receptor to sequences that overlap the CRE, thus blocking activity of the CRE-binding proteins (Akerbloom et al., 1988). However, conflicting evidence indicates that the mechanism may be direct interaction of the glucocorticoid receptor with the CRE-binding proteins, not with the DNA elements (Chatterjee et al., 1991).

Multiple CRE-Binding Proteins with Similar Structural Elements

An explanation for the complex pattern of responses among the individual CREs is that a variety of different CRE-binding proteins exist, each with their own unique affinities and activities. The initial isolation of some of these proteins has confirmed this hypothesis and has demonstrated the existence of a complex super-family of genes that encode CRE-binding proteins (Table 3). All of these factors isolated to date share the common structural elements of a carboxy-ter-

Table 3
List of Cloned Vertebrate CRE-Binding Proteins[a]

FACTOR	MW	SOURCE	REFERENCE	COMMENTS
ATF-a	52	HeLa	Gaire et al., 1990	N & C terminus similar to CRE-BP1
ATF-aΔ	50	HeLa	Gaire et al., 1990	splice varient of ATF-a
ATF 1	?	MG63	Hai et al., 1989	70% similar to CREB
ATF 2	?	MG63	Hai et al., 1989	identical to CRE-BP1
ATF 3	?	HeLa	Hai et al., 1989	dimerizes with CRE-BP1
ATF 4	?	MG63	Hai et al., 1989	
ATF 5	?	HeLa	Hai et al., 1989	zipper identical to c-fos
ATF 6	?	HeLa	Hai et al., 1989	
ATF 7, 8	?	MG63	Hai et al., 1989	
CREB 341	37	rat brain	Gonzalez et al., 1989	activated by kinase A
CREB 327	35	human placenta	Hoeffler et al., 1988	splice varient of CREB 341
CRE-BP1	54.5	human fetal brain	Maekawa et al., 1989	forms CRE binding heterodimer with c-jun
HB 16	?	human B-cell	Kara et al., 1990	truncated CRE-BP1
mXBP/CRE-BP2	?	mouse spleen	Ivashkiv et al., 1990	splice varient of CRE-BP1
TREB 5	28.7	HUT 102	Yoshimura et al., 1990	basic region homology only
TREB 36	29.2	HUT 102	Yoshimura et al., 1990	identical to ATF-1
TREB 7	54.5	HUT 102	Yoshimura et al., 1990	identical to CRE-BP1
CREM α,β,γ	26	mouse pitutary	Foulkes et al., 1991	supressors of CREB activity

[a]Question marks indicate incomplete cDNA-coding sequence. MG63 is a human osteosarcoma; HUT 102 is a human T-cell line; HeLa is a human epithelial carcinoma.

minal basic DNA-binding region and a leucine-zipper motif (Landschultz et al., 1988). Dimerization of the CRE-binding proteins is mediated by the leucine zipper, which consists of 4–5 leucines separated from each other by seven amino acids. The zippers appear to interact with each other by forming a coiled-coil structure that stabilizes the dimer (O'Shea et al., 1989). The primary sequence of amino acids in the basic DNA-binding domain varies slightly among the different CRE-binding proteins, with >50% of the residues present being either lysine or arginine. This region has been proposed to form an alpha helix that, together with a second alpha helix from the other half of the dimer, recognizes DNA in a "scissors-grip" in which each helix recognizes half of the pseudosymmetrical CRE sequence (Vinson et al., 1989). These factors are capable of binding DNA only as dimers. In addition to a DNA-binding region and a leucine zipper, all of the CRE-binding proteins have amino-terminal domains that, although poorly conserved among the class as a whole, seem to have consensus phosphorylation sites for a number of different protein kinases. These domains are presumed to be involved in transcriptional activation.

Diversity Can Be Generated Through Dimerization

Although there are structural restrictions limiting which leucine zippers may interact with each other, all of the CRE-binding proteins are capable of binding to DNA as homodimers and several of the proteins are able to form heterodimers. CRE-BP1/ATF-2, for instance, readily forms with heterodimers *jun* that are capable of high-affinity interaction with a CRE (Macgregor et al., 1990). This complex may represent a novel mechanism of cross-talk between the kinase C and kinase A signal-transduction pathways. CRE-BP1/ATF-2 has a wide tissue distribution and is capable of binding to a number of different CREs, including those from the long terminal repeat (LTR) of HTLV 1, the CRE from the MHC Aα gene, and the ATF/CRE site from adenovirus (Hai et al., 1989; Kara et al., 1990; Yoshimura et al., 1990). The *jun* protein is activated by the kinase C pathway and when dimerized with *fos* in the AP-1 complex displays a lower affinity for the CRE than for the TRE. However, the heterodimer formed between *jun* and CRE-BP1/ATF-2 has a greater affinity for the CRE element (Benbrook and Jones, 1990; Macgregor et al., 1990). CRE-BP1/ATF-2 is also able to form dimers with ATF-3 (Hai et al., 1989), although the physiological relevance of this heterodimer combination is unknown.

Theoretical analysis of the secondary structure of the leucine zipper (O'Shea et al., 1989) indicates that additional heterodimer combinations are possible. CREB, for instance, should be capable of dimerization with ATF-1.

Diversity Can Be Generated Through Alternative Splicing

In addition to being able to form heterodimers, many of the CRE binding proteins occur in multiple isoforms, which are generated through the use of alternative splicing pathways (Yamamoto et al., 1990; Ivashkiv et al., 1990; Gaire et al., 1990). Presumably, this structural diversity reflects a physiological mechanism to fine-tune the cAMP response on the various CREs. With the exception of CREB, the functional differences between the various isoforms have not been explored.

CREB occurs in two isoforms that differ by the presence of a 14 amino acid in the amino terminus, identified as the alpha peptide. Both CREB isoforms are expressed in a wide variety of tissues, with the smaller isoform (ΔCREB/CREB 327/CREB A) being severalfold more abundant than the larger (CREB/CREB 341/CREB B). In t ransient transfection assays using Balb/c 3T3 fibroblasts, Berkowitz and Gilman (1990) were unable to detect any difference between the two isoforms in their ability to impart cAMP responsiveness to a c-*fos* promoter. In sharp contrast, Yamamoto et al. (1990) have reported that CREB (containing the alpha peptide) was ten times more efficient than ΔCREB in *trans*-activating a CRE–CAT construct in F9 cells.

In at least one case, different DNA-binding domains may be utilized on the same amino terminus (Foulkes et al., 1991). CREM (CRE modulator) has at least two different binding domains that, together with their cognate zipper, generate the three known isoforms, α, β, and γ. The α isoform expresses binding domain II; CREM β and CREM γ both express binding domain I. CREM β differs from CREM γ by the presence of an 11 amino acid exon in the amino terminus. The amino acid sequence of binding domain I is 95% identical to that of CREB, while the amino acid sequence of binding domain II is 75% identical. All three isoforms can be found in a variety of tissues, including heart, pituitary, kidney, and brain. All the isoforms are capable of dimerization with CREB, but the binding domains of CREB and CREM are not identical. These differences may confer unique binding specificities to CREB/CREM homo- and heterodimers.

Transcriptional Activity

The transcriptional activity of CREB is clearly the most extensively studied of all the CRE-binding proteins, and forms the basis for our understanding of the mechanism of transactivation by these proteins. Phosphorylation of CREB by protein kinase A is necessary for complete transcriptional activity. Substitution of the serine in the kinase A site (Ser 119/133) with alanine, glutamic acid, or aspartic acid produces CREB proteins that are severely impaired in their ability to activate transcription (Gonzalez and Montminy, 1989; Lee et al., 1990). The phosphorylation of CREB by kinase A is, however, apparently not sufficient to produce a fully active transcription factor. In examining several deletion mutants of the smaller isoform of CREB fused to the binding domain of the yeast transcription factor GAL 4, Lee et al. (1990) were able to establish that additional serine-rich sequences near the kinase A site were also necessary for activity. Phosphopeptide mapping of mutant CREB proteins expressed in JEG-3 cells indicates that these "phosphorylation boxes" are modified subsequent to the primary phosphorylation at serine 119/133 and, further, that two of these elements (PDE 1 and PDE 2) are absolutely required for complete activity. At present it is not understood how these subsequent phosphorylations of CREB produce an active transcription factor. Regions of negatively charged amino acids have previously been shown to act as potent transcriptional activators (Ma and Ptashne, 1987a,b), and possibly the multiple phosphorylations of the PDE elements may serve to increase the net negative charge of this region of CREB. Alternatively, phosphorylation may serve to induce conformational changes in CREB that reveal previously hidden activator sequences, such as glutamine- or proline-rich sequences (Courey et al., 1989; Mermod et al., 1989). In either case, the observation that CREB requires multiple phosphorylations in order to be fully active in JEG-3 cells implies that several different signaling pathways acting through different kinases could control the activity of the CREB protein. It will be interesting to determine the nature of these other kinases and to correlate their activity to activation of various signaling pathways. Because the CRE binding proteins as a whole display multiple phosphorylation sites, it seems likely that all of these proteins are able to integrate inputs from a variety of pathways.

In addition to the positively acting transcription factors that bind to the CRE, there are also negatively acting CRE binding factors. All three isoforms of CREM, when expressed in both JEG-3 and F9 cells, are able to block CREB-induced transcription of a CRE–thymidine kinase CAT reporter gene. Although CREM is distinctly smaller than CREB, with a mol mass of only 29 kDa, the amino terminus contains regions that appear to be homologous to the PDE boxes found in CREB. Their role in transcriptional repression is only conjectural at this point, but it is possible that CREM mediates a phosphorylation-dependent repression of CRE activity.

It has been suggested that CREM could suppress CREB activity either by dimerizing with CREB to form transcriptionally inactive complexes or by occupying the CRE sequence, preventing CREB from activating transcription. Whatever mechanism CREM employs to block CREB function, it will be necessary to understand how this protein is regulated and how the interplay between CREB and CREM affects gene transcription.

Transcription Factor AP-2

The vast majority of information about the transcriptional proteins that mediate cAMP responses has been gathered using the CRE, but some progress has been made in understanding the other known cAMP-inducible activator, AP-2. This protein does not contain a leucine zipper and, in fact, the DNA-binding domain of AP-2 is markedly similar to the helix–loop–helix (HLH)

DNA-binding motif that has been observed in a number of other proteins, including *myc*, daughterless, and E12 (Williams et al., 1988). Curiously, the only consensus phosphorylation site for protein kinase A is located just to the amino-terminal side of the basic DNA-binding region. The role of this phosphorylation site in mediating AP-2's transcriptional or binding activity is, at present, unknown.

Conclusions

Great strides have been made in the past few years in understanding the general mechanisms involved in second-messenger signal transduction along the cAMP pathway, but much still needs to be done. The CRE and AP-2 enhancers are currently the best-studied sites of action for conferring a cAMP response to a promoter, but it is obvious that there are other enhancers that can mediate this response. Indeed, it has been shown that the classic kinase C sensitive enhancer from the metallothionein IIA gene can also respond to cAMP (Fink et al., 1991). These sites are greatly affected by neighboring sequences, as well as promoter and cell-type contexts, in an intricate manner, which is likely to reflect the complexity of the proteins that bind to the sites. Given the central role that this pathway plays in the control of metabolism and cell growth, this is perhaps not surprising. Future work must focus on gaining a better understanding of exactly how many different binding proteins are involved and on which CRE enhancers they act, how these proteins are controlled by specific kinases, and what role the ability of these proteins to form heterodimers plays in modulating activity.

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