Selective Heterodimerization of CREB and Related Transcription Factors

by

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Table of Contents

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Abstract

This thesis focuses on understanding how dimerization contributes to the function of basic/leucine zipper (bZIP) transcription factors. Dimerization of factors in this class is mediated through a leucine zipper motif. The highly conservered nature of the leucine zipper permits homo- and heterodimerization among related bZIP transcription factors. While this greatly increases the diversity of complexes that can interact with a single genetic element (and thereby provides a mechanism for integrating information from various signaling pathways), it becomes difficult to ascertain the functional and biochemical properties of individual heterodimer complexes. The prototypic bZIP protein, CREB, provides an excellent model for the study of cAMP-mediated transcriptional activation. This protein must bind to a palindromic DNA sequence termed the CRE (cAMP-responsive element) as a dimer to induce a basal level of transcription. CREB achieves full transcriptional activity only after phosphorylation by protein kinase A (PKA). To simplify the functional analysis of CREB, I have substituted residues within the leucine zipper region to generate proteins that dimerize in selected combinations. These zippers permit the formation of specific, physiologically relevent complexes that can be studied *in vivo*. Using this approach, I show that hemiphosphorylated CREB complexes are half as active as fully phosphorylated complexes. This result has several implications for second messenger regulated gene activation. First, they demonstrate that the process of dimerization contibutes to the level of transcriptional activition. Second, the distinct responses to unphosphorylated, hemiphosphorylated and fully phosphorylated CREB dimers suggest that cAMP-directed transcriptional signals can be modulated according to the degree of CREB phosphorylation. Finally, the observation that hemiphosphorylated CREB maintains the ability to respond to PKA supports the concept that heterodimerization may allow the targeting of distinct second messenger-mediated signals to a single genetic element.

The half-maximal activity af hemiphosphorylated CERB complexes may result from a decreased association with other proteins required for transcriptional activation. Alternatively, complexes phosphorylated on a single subunit may function directionally. Because the CRE sequence is symmetric, the hemiphosphorylated dimer may bind in two different orientations. It is possible that only one of these orientations is functional. Directional binding of transcription factors would provide additional diversity in gene regulation. To test this model, I develop a method for directionally binding CREB dimers to asymmetric CRE sequences. Using this approach, I show that hemiphosphorylated CREB complexes are equally active bound in either orientation. This result indicates that both activation domains contribute additively to gene activation.

Finally, I use the mutant leucine zipper strategy to study the mechanism of action of negative regulators of transcription. CREMα, a bZIP transcription factor that blocks CREB mediated gene activation, may function in two ways: inactive CREMα homodimers may compete for CREB binding sites, or CREMα may combine with CREB to form inactive heterodimers. The latter model provides a mechanism for greatly increasing the efficiency and specificity of these negative regulators. Suprisingly, CREMα:CREB heterodimers are transcriptionally active indicating that CREMα can contibute to PKA-mediated gene activation. Futhermore, CREMα:CREB heterodimers are active only when both subunits can be phosphorylated by PKA.

Introduction

Catecholamine and peptide hormones regulate gene expression through the activation of second messenger signalling pathways. The signal transduction events leading to the induction of cAMP-responsive genes are, perhaps, the best understood. Regulation of genes via this pathway occurs through short, frequently palindromic DNA elements termed the cAMP-responsive enhancers (CREs). The proteins that bind to this element, such as the CRE-binding protein (CREB), stimulate transcription when the cAMP second messenger signalling pathway is activated.

The activation of CREB and related proteins occurs as the result of several steps. Hormones that elevate cellular levels of cAMP are the first messengers in the signalling pathway. Unable to enter the cell directly, these hormones recognize a receptor molecule which serves as a link between the exterior and the interior of the cell. Once bound to the appropriate ligand, hormone receptors activate GTP-binding proteins (G proteins) by catalyzing the exchange of GTP for GDP on G protein α (G_α) subunits (reviewed in Casey, 1988). Activated G_α subunits may then activate adenylate cyclase or phospholipase C which are responsible for the production of the second messengers cAMP and diacylglycerol, respectively. Elevated levels of cAMP modify the activity of cAMP-dependent protein kinases. For example, cAMP molecules associate with the regulatory subunit of protein kinase A (PKA). The catalytic subunit of PKA then separates from the regulatory subunit and is free to migrate into the nucleus (Meinkoth, 1990). Once in the nucleus, protein kinases regulate the activities of transcription factors that bind to specific DNA elements (Hunter, 1992). One recently characterized factor in this pathway is CREB. Unphosphorylated CREB dimers bind to CRE sequences and activate a low or basal level of transcription (Gonzalez, 1991). CREB stimulates a much higher level of transcription after phosphorylation by PKA (Gonzalez, 1989b).

The presence of this multistep signalling cascade may serve several functions. First, signalling cascades transmit and amplify signals initiated by circulating hormones. The effector molecules activated at each step can, in turn, activate several downstream effector molecules. As a result, a small number of hormone molecules can have profound effects on the metabolic processes of the cell. Second, each intermediate in the signalling pathway may interact with more than one downstream target. cAMP and protein kinases, for example, play many roles in the regulation of cellular metabolism. Finally, each step can provide a point of interaction or "cross-talk" between different signalling pathways.

Of all the steps in the cAMP signalling cascade, one of the least understood is how CREB activates gene expression. A key component of CREB mediated gene activation, however, is dimerization. CREB contains a highly conserved DNA binding/leucine zipper region that mediates homodimerization and heterodimerization with the related proteins CREM and ATF-1. The relative availabilities and concentrations of proteins with which CREB can dimerize may, to a large degree, confer tissue specific responses to general hormonal signals. Because individual heterodimer complexes are difficult to isolate, their contribution to gene activation is not understood. The work presented in this thesis focuses on developing techniques to isolate specific heterodimer complexes and then determining their contribution to cAMP-mediated gene activation.

cAMP and PKA

The role of cAMP as a second messenger was first established in the work of Sutherland and Rall (Sutherland, 1958). Subsequently, many investigators looked for a connection between cAMP and increased biosynthetic activity. Byuss and Russell (Byuss, 1975), for example, hypothesized that cAMP mediates the increase in ornithine decarboxylase (ODC) activity that results from the administration of hormones that affect growth processes. Their results indicated that a hormone, most likely adrenocorticotropic

hormone (ACTH), rapidly elevated cAMP levels and subsequently ODC levels in rat adrenal medulla following exposure to cold. Furthermore, they predicted that cAMP-dependent protein kinases were most likely phosphorylating nuclear proteins which increased ODC mRNA synthesis. This prediction was supported by the findings that both cyclohexamide, an inhibitor of protein synthesis, and actinomycin D, an inhibitor of RNA synthesis, blocked the increase in ODC activity.

Additional evidence for the role of cAMP in gene regulation came after the development of PC12 cells, a clonal line derived from a pheochromocytoma tumor in rat adrenal medulla (Greene 1976). When these cells were exposed to nerve growth factor (NGF), they underwent an alteration in phenotype that resembled a change from chromaffin cells to sympathetic neurons. Among the many biochemical changes that occured, NGF was shown to increase intracellular levels of cAMP (Schubert 1977) and to increase levels of the enzyme ornithine decarboxylase (ODC) (Greene 1978). Other laboratories (Hatanaka,1978; van Buskirk,1985), however, were unable to see increased levels of cAMP in response to NGF treatment in PC12 cells. Though discrepancies in the relationship between NGF and cAMP remained unresolved, a link was found between cAMP and ODC activity. Guroff *et al.* (Guroff,1981), using adenosine analogs to activate adenylate cyclase, were able to show a marked increase in ODC activity. Similarly, several laboratories were able to show an increase in tyrosine 3-monooxygenase (tyrosine hydroxylase) activity, the rate-limiting enzyme in catecholamine synthesis, by increasing cAMP levels with cholera toxin (Vacarro,1980) and adenosine analogs (Erny,1981).

The comparison of mRNA levels between uninduced and induced genes provided conclusive evidence that cAMP could mediate gene expression. After cloning the vasoactive intestinal polypeptide (VIP) gene, Hayakawa *et al.* (Hayakawa, 1984) used a radiolabled fragment of the VIP gene and a filter hybridization assay to quantitate VIP-

encoding mRNA. Neuroblastoma cells treated with a cAMP analog showed an eleven-fold stimulation in VIP gene transcription.

The role of cAMP in the regulation of gene transcription implicated cAMP-dependent protein kinase (PKA), which is activated by elevated levels of cAMP, as having a key role in cAMP-mediated gene activation. Several models were described to explain the function of PKA. PKA could, for example, phosphorylate ribosomal proteins allowing for the translation of stored mRNAs (Appleman, 1973). Alternatively, PKA could phosphorylate nuclear proteins which would allow transcription from specific genes (Langan, 1971). The results of Byuss and Russell, described previously, supported the latter model. Several additional lines of evidence also supported that model. First, microinjection of PKA catalytic subunit into rat hepatoma cells using red blood cell ghosts as vehicles caused an induction of tyrosine aminotransferase activity similar to that induced by cAMP analogs (Boney,1983). Direct microinjection of PKA catalytic subunit into rat glioma cells also increased activity from the cAMP-responsive VIP promoter (Riabowol, 1988). Second, cAMP regulated gene induction does not occur in PC12 cell lines that are deficient in cAMP dependent protein kinases. For example, van Buskirk *et al.* found that PC12 cells deficient in PKA activity were still able to induce ODC activity in response to NGF treatment but not in response to adenosine agonists (van Buskirk, 1985). These results suggested that PKA was activated specifically by cAMP and that ODC gene activation induced by NGF probably occurred through a separate pathway. In addition, Montminy *et al.* (Montminy,1986) showed that the regulatory region of the cAMP-responsive somatostatin gene was not responsive to cAMP when transfected into PKA deficient PC12 cells . Third, cotransfection of a plasmid encoding an inhibitor of PKA activity, PKI, along with a cAMP-inducible reporter plasmid prevented cAMP induction of the reporter (Grove, 1987). And finally, Nakagawa *et al.* found that the catalytic subunit of PKA could activate transcription from

the cAMP-responsive gene urokinase-type plasminogen activator in kidney tissue extracts (Nakagawa, 1988).

Identification of the CRE

To identify PKA-phosphorylated nuclear proteins which could induce gene transcription, researchers first examined the promoter-regulatory regions of several cAMP-responsive genes. Short DNA sequences similar to those in bacterial gene promoters were hypothesized to mediate the binding of potential transcriptional regulators. Once the regulatory DNA sequences were identified, the proteins which bound to them could be determined. To this end, Short *et al.* examined the regulatory regions of the phosphoenolpyruvate carboxykinase (PEPCK) gene (Short, 1986). A series of deletions in the promoter-regulatory region of the PEPCK gene revealed that a short DNA sequence approximately one hundred base-pairs upstream from the promoter conferred cAMP responsiveness to that gene. The regulatory element within that sequence was also sufficient to confer cAMP inducibility to the Herpes simplex virus thymidine kinase gene, a gene not normally responsive to cAMP. Comparison of this sequence to the regulatory regions of four other cAMP responsive genes indicated that the core sequence 5'-CTTACGTCAGAG-3' contains the cAMP-responsive element or CRE. Similar experiments were used to identify CRE's in the somatostatin gene (Montminy,1986), the proenkephalin gene (Comb,1986), and the VIP gene (Tsukada, 1987) (Table 1). The consensus CRE sequence based on those experiments was 5'-TGACGTCA-3'. Andrisani *et al.* found that in addition to simulating transcription in response to cAMP, the CRE could also induce a basal level of transcription in unstimulated cells (Andrisani, 1987). Removal of the CRE from the somatostatin gene promoter, for example, decreased unstimulated expression ten-fold.

Though there is no apparent sequence homology immediately outside of the consensus CRE, the activity of the CRE depends on its context. For example, Montminy *et al.* (Montminy, 1986) found that the ten base-pair somatostatin CRE was not sufficient to allow responsiveness to cAMP when inserted 135 base-pairs upstream from the SV40 promoter. In contrast, the seventeen base-pair VIP CRE was able to confer cAMP responsiveness when placed upstream of the Rous sarcoma virus (RSV) promoter (Tsukada, 1987). The length of the inserted sequence, the nature of the surrounding DNA, and the type of promoter may all contribute to the effectiveness of the CRE. To determine whether the DNA sequence surrounding a CRE could contribute to gene activation, CREs and adjacent sequences were placed upstream from a choriogonadotropin promoter coupled to a chloramphenicol acyltransferase (CAT) reporter (Deutsch, 1988). While an eight base pair consensus CRE was able to promote basal and cAMP induced transcription in this system, identical CREs and the surrounding ten to fifteen base pairs from the rat glucagon and the bovine parathyroid hormone genes were much less active. A slightly longer glucagon CRE was completely inactive. On the other hand, CREs and surrounding sequences from highly cAMP-responsive genes such as the somatostatin, VIP, and collagenase genes were all highly and equally active in these experiments. Thus, these experiments suggested that the sequences immediately surrounding a CRE could play a key role in determining its transcriptional activity.

Jameson *et al.* provided evidence that the transcriptional activity of a CRE could be dependent upon promoter and cell type (Jameson, 1989). The glycoprotein hormone α-subunit CRE, for example, activated basal expression from an α-subunit promoter/CAT construct in human placental JEG-3 cells but not in the rat islet cell line INR1-G9. However, the α-subunit CRE was active when placed upstream of the somatostatin promoter in INR1-G9 cells. Cell specific expression of CRE binding proteins and associated coactivator molecules may explain these differences in CRE activity.

The activity of the CRE, however, is independent of its position and orientation. The cAMP responsive region of the human enkephalin gene was able to induce transcription from an enkephalin promoter when placed either 247 or 494 bases upstream (Comb, 1986). In addition, the enkephalin CRE was active when placed 1.1 kilobases downstream from the same promoter. In all three positions, the CRE was active when inserted in either orientation. Similar experiments demonstrated that the VIP CRE activity was also position and orientation independent when measured from an RSV promoter (Fink, 1988). These results indicate that the CRE can behave in much the same way as viral enhancers.

Other cAMP-responsive elements have been discovered as well. The AP-2 site, first thought to contribute only to basal transcription of the metallothionine IIA gene, was found to be a cAMP-inducible enhancer when reiterated and placed downstream of the β-globin gene (Imagawa, 1987). The transcription factor AP-2 can also be activated by protein kinase C indicating that several distinct signalling pathways can function through this protein. Recently, the nuclear protein C/EBPβ, which binds to the consensus sequence 5'-TTATGCAAT-3', has been implicated in cAMP-mediated regulation of the prostaglandin endoperoxide synthase 2 (PGS-2) gene (Sirois, 1993). Removal or mutation of the C/EBPβ binding site diminished forskolin inducibility of a PGS-2/CAT reporter construct transfected into granulosa cells. A series of cAMP-responsive sequences (CRSs) distinct from the CRE has also been shown to regulate transcription from bovine steroid hydroxylase genes in the adrenal cortex (reviewed in Waterman, 1994). The promoter regions of two genes, mitochondrial steroid hydroxylase CYP11A and adrenodoxin, contain CRSs that are binding sites for Sp1 (Ahlgren, 1990; Momoi, 1992). Because Sp1 is a general transcription factor that is not cAMP-responsive, a second, PKA-activated protein may associate with Sp1 to activate transcription. An uncharacterized protein that may fulfill this role has been shown to bind to the adrenodoxin CRS (Waterman, 1994). The microsomal steroid hydroxylase gene, CYP21,

contains a CRS that binds Sp1 and a recently discovered protein termed ASP (Kagawa, 1994). ASP is required for cAMP-inducibiltiy of this gene. And finally, a second microsomal steroid hydroxylase gene, CYP17, contains two cAMP-responsive sequences (Lund, 1990). The first binds several proteins including COUP-TF (Waterman, 1994), while the second binds four nuclear proteins (Kagawa, 1994). Two of these are PBX-related homeobox proteins that contain potential PKA phosphorylation sites. These unique cAMP-responsive systems may allow differential expression of steroid hydroxlase genes during development (Lund, 1988).

The cloning of the CRE-binding protein CREB and related proteins

Two methods were used to identify proteins which bind to the CRE. Montminy and Bilezikjian (Mont, 1987) purified CRE-binding proteins from PC-12 cells with a sequence-specific DNA affinity column. The prominent protein isolated by this method, CREB, had a molecular weight of 43 kD and could be phosphorylated by the catalytic subunit of PKA *in vitro*. In addition, CREB isolated from PC-12 cells labeled with ^{32}P-orthophosphate was 3-4 times more phosphorylated in cells incubated with forskolin than in untreated cells. These results indicated that CREB was phosphorylated in response to activation of the cAMP-signalling pathway. The cDNA which encoded CREB was isolated from a rat cerebral cortex library using degenerate DNA primers and polymerase chain reaction (Gonzalez, 1989 Nature). Partial amino-acid sequences were used to design the oligonucleotide primers.

The cDNA for CREB was also cloned using a second approach. Hoeffler *et al.* attempted to identify CRE-binding proteins by screening a human placental cDNA expression library with a radiolabeled CRE sequence (Hoeffler, 1988). Using this technique, a cDNA encoding a 38 kD isoform of CREB was isolated. This form of CREB was identical to the larger isoform isolated from rat cortex except that it lacked a short amino-acid sequence termed the α-peptide. This approach proved to be effective

for identifying many CRE-binding proteins. Hai et al., for example, identified eight additional CREB-related proteins termed ATF-1 through ATF-8 (Hai, 1989). Of these, one of the best characterized is ATF-1. Like CREB, ATF-1 binds to CRE sequences and activates transcription after phosphorylation by PKA (Rehfuss, 1991; Liu, 1993). Though CREB and ATF-1 are structurally similar, CREB:ATF-1 heterodimers have been reported to be transcriptionally inactive (Ellis, 1995). These inactive heterodimers may explain the inability of undifferentiated F9 cells to respond to cAMP signaling pathways. In undifferentiated F9 cells, CREB exists primarily as a heterodimer with ATF-1. After differentiation, ATF-1 protein levels drop ten-fold allowing the formation of transcriptionally competent CREB homodimers (Ellis, 1995).

Because the homology between CREB-related transcription factors extends to the DNA level, additional CREB-related proteins have been isolated through DNA hybridization protocols. By screening a mouse pituitary library at moderate stringency with oligonucleotides complementary to the carboxy-terminal region of CREB, Foulkes *et al.* identified two isoforms of the CRE-modulator protein termed CREMα and CREMβ (Foulkes, 1991). The DNA probes used to identify these isofoms of CREM bound to two regions of a single cDNA species, each of which encoded a basic DNA-binding and leucine zipper (bZIP) region highly homologous to that of CREB. CREMα and CREMβ differed from CREB, however, in that they lacked two glutamine-rich regions which have been proposed to be essential for gene activation. These CREM isoforms were subsequently found to be negative regulators of gene transcription (Foulkes, 1991).

Subsequent use of various DNA hybridization protocols has revealed a large family of CREM proteins that serve as both negative and positive regulators of gene transcription. Foulkes *et al.*, for example, found that developmentally and functionally distinct isoforms of CREM are produced during spermatogenesis (Foulkes, 1992). In premiotic germ cells, small amounts of antagonistic forms of CREM are produced. However, from the pachytene spermatocyte stage onward, large amounts of an activator

isoform, CREMτ, accumulate. The developmental switch in CREM expression correlates with the use of a 5' polyadenylation site that stabilizes the CREMτ transcript. The use of this site is mediated by follicle-stimulating hormone (Foulkes, 1993). In mouse brain, however, the use of an internal translation start codon in the CREMτ transcript generates the negative regulator S-CREM (Delmas, 1992). Thus, both alternative splicing and alternative translation contribute to the diversity of CREM isoforms. A third mechanism, alternative transcription from an intronic promoter in the CREM gene, generates the ICER family of CREM isoforms (Stehle, 1993). Unlike the promoter for other CREM isoforms, the ICER promoter is regulated by the cAMP signal transduction pathway (Molina, 1993). Activation of this pathway in the pineal gland is regulated by diurnal adrenergic signals from the suprachiasmatic nucleus.

Comparison of CREB with the related proteins ATF-1 and CREM reveals a high degree of amino-acid sequence homology. ATF-1, for example, has a 75% overall amino-acid similarity with CREB and shares its structural organization (Hai, 1989). The bZIP regions of CREMα and CREMβ are 95% and 75% homologous to those of CREB, respectively (Foulkes, 1991). These three proteins also share regions of amino acid homology indicating that this gene family may have developed through gene duplication. The amino terminal portion of these proteins contains a transcriptional activation domain while the carboxy-terminal region contains a highly conserved basic DNA-binding and leucine zipper region. The leucine zipper (Landshultz, 1988) mediates bZIP transcription factor dimerization and is required for gene activation. A second gene family consisting of CRE-BP1 and ATF-a (Gaire, 1990) may also be the result of gene duplication. Transcription factors in this family contain a more centrally located DNA binding region and leucine zipper indicating that many of the functional components of bZIP proteins may function in a modular fashion that is independent of position.

The potential for the bZIP regions of CREB-related proteins to heterodimerize led to the development of an additional method to clone bZIP proteins. To identify potential

dimerization partners for CREB-related transcription factors, cDNA expression libraries have been screened with portions of radiolabled bZIP proteins (Macgregor, 1990; Hoeffler, 1991). C/EBP-homologous protein (CHOP-10), for example, was isolated from an adipocyte expression library using a ^{32}P-labeled DNA-binding/leucine zipper region from the liver-enriched transcriptional activator protein (LAP) (Ron, 1992). CHOP-10 was found to selectively heterodimerize with C/EBP and LAP. However, these heterodimers were unable to bind to C/EBP DNA sites. Consequently, CHOP was predicted to function as a dominant-negative regulator of gene activation by C/EBP family members (Ron, 1992). Interestingly, recent evidence indicates that CHOP-10 may play a role in preventing cellular proliferation. First, Fornace *et al*. found that the CHOP-10 gene is induced by cellular stresses such as DNA damage (Fornace, 1989). Second, Barone *et al*. found that CHOP-10:C/EBP heterodimers induce growth arrest when microinjected into murine fibroblastic NIH-3T3 cells (Barone, 1994). Growth arrest is dependent on the presence of the basic region of CHOP indicating that CHOP-10:C/EBP heterodimers bind DNA. Finally, a chromosomal translocation that fuses the bZIP region of CHOP to the 5' end of an RNA-binding protein generates the potent oncogene TLS-CHOP. TLS-CHOP, which may block CHOP induced growth arrest, is present in myxoid liposarcomas (Crozat, 1993) and can transform NIH-3T3 cells (Zinszner, 1994).

The structure of CREB

Of the many CRE-binding proteins that have been described, most studies have focused on CREB, which is believed to mediate transcriptional signals directed by multiple second messenger pathways. Sequence analysis indicated that CREB contained a consensus phosphorylation site for several protein kinases at serine 133 (Gonzalez, 1989). This prediction was in agreement with results showing the *in vitro* phosphorylation of CREB by PKA and protein kinase C (Yamamoto, 1988). An additional casein kinase II (CK II) phosphorylation site was predicted to exist at serine

152 (Yamamoto, 1988). These potential sites were found within a portion of CREB's activator region termed the kinase inducible domain (KID) (Gonzalez, 1989). Subsequent studies determined that a critical phosphorylation site was serine 133 which could be phosphorylated by PKA and calcium/calmodulin kinase II and IV (Gonzalez, 1989, Cell; Dash, 1991; Sheng, 1991; Sun, 1994; Enslen, 1994). Mutation of this serine to acidic residues or to alanine destroyed the ability of CREB to mediate transcriptional activation (Gonzalez, 1989). Though PKA phosphorylation at serine 133 was shown to activate CREB, additional phosphorylations were found to be necessary for activation. Glycogen synthase kinase-3, for example, has been shown to phosphorylate serine 129 only after phosphorylation of serine 133 (Fiol, 1994). Mutation of the GSK-3 phosphorylation site reduced CREB activity by 70%. Phosphorylation, then, of serine 133 may facilitate these additional and subsequent phosphorylations indicating that several signaling pathways may need to simultaneously contribute to CREB activation (Roach, 1991).

Recent evidence indicates that an additional kinase can activate CREB through phosphorylation of serine 133. The nerve growth factor receptor (Trk), a receptor tyrosine kinase, has been shown to activate CREB through activation of a Ras-dependent protein kinase (Ginty, 1994). This result explains earlier findings that NGF could activate transcription from cAMP-responsive genes, such as the *c-fos* gene (Greenberg, 1985), without raising intracellular levels of cAMP. Several distinct signalling pathways, then, can increase transcription of cAMP- responsive genes through phosphorylation of CREB at serine 133.

Three additional regions contribute to the activation of CREB. The short amino acid sequence DLSSD in the KID region of CREB is critical for transcriptional activity. CREB proteins lacking this motif are completely unable to enhance transcription from a CRE-CAT reporter plasmid in F9 cells (Gonzalez, 1991). The negative charge of the first aspartic acid residue and the spacing of this motif from serine 133 were found to be

critical for CREB activation. Two glutamine-rich regions, Q1 and Q2, surrounding the KID are also important for CREB function. Glutamine-rich regions, such as those in SP-1 (Courey, 1988), Oct-1 and Oct-2 (Tanaka, 1990), have been shown to mediate transcriptional activation. The contribution of glutamine-rich regions to CREB activity was demonstrated in experiments where deletion of the amino-terminal glutamine-rich region of CREB reduced both basal and PKA stimulated activity by more than 70% (Gonzalez, 1991). Because the Q2 region of CREB can function as a constitutive activator when fused to a GAL4 DNA-binding domain and can be replaced with the constitutive activation domain of GCN4 with no loss in basal or PKA induced activity, Brindle *et al.* have proposed that the KID region regulates the activity of the glutamine-rich regions (Brindle, 1993). The transcriptional contribution of this domain also can be inferred from proteins that are highly homologous to CREB that have portions of or no glutamine-rich regions. ATF-1, for example, is virtually identical to CREB except that it lacks a portion of the amino-terminal glutamine-rich region, Q1. ATF-1 is readily activated by PKA phosphorylation, however, it does not have basal activity suggesting that the missing portion of the activator domain may mediate basal activity (Rehfuss, 1991; Liu, 1993). CREMα which lacks both glutamine-rich regions is transcriptionally inactive (Foulkes, 1991 Cell) while CREMτ which contains both glutamine-rich regions is at least as active as CREB (Foulkes, 1992 Nature). Studies on two isoforms of CREMτ, CREMτ1 and CREMτ2, which contain one or the other of the glutamine-rich regions suggest that the two activation domains may work additively (Laoide, 1993). The transcriptional activity of CREB, then, correlates with the amount of glutamine-rich sequence it contains. This hypothesis is supported by the observation that deletions in the Q2 region reduce CREB activity in a size dependent manner (Brindle, 1993).

The function of an additional amino-terminal region, the α-peptide, has yet to be determined. The α-peptide is predicted to form a fourteen amino-acid amphipathic α-helix that may mediate protein:protein interactions (Yamamoto, 1990). It is present

amino-terminal to the KID of CREB341 but not in the splice variant CREB327. Consistent with their hypothesis that the α-peptide may be involved in transcriptional activation, Yamamoto *et al.* found that CREB341 had a ten-fold higher transcriptional activity than CREB327 (Yamamoto, 1990). Three other laboratories, however, found that both isoforms of CREB had identical transcriptional activities (Berkowitz, 1990; Ruppert, 1992; Quinn, 1993).

The carboxy-terminal portion of CREB contains a highly conserved basic DNA-binding/leucine zipper region. The basic region of CREB is part of a continuous α-helix that includes a leucine zipper. This region of CREB may serve several functions. First, Waeber *et al.* have found that a CREB mutant lacking the bZIP region fails to translocate to the nucleus. Deletion analysis revealed that nine basic amino-acids within the DNA-binding domain (RRKKKEYVK) serve as a nuclear localization signal (Waeber, 1991). Second, the basic region mediates sequence specific DNA binding. Each basic region in a CREB dimer interacts specifically with one half of the palindromic CRE sequence. Vinson *et al.* first described the interaction of the bZIP dimer with DNA as a "scissors grip" (Vinson, 1989). In this model, the leucine zipper regions form a parallel coiled-coil that aligns itself perpendicular to the DNA at the center of the CRE. The α-helices gradually diverge at their DNA-binding domains and follow the major grove of the DNA over each CRE half-site. The basic region consists of two small α-helices angled to follow the helical path of the DNA. The entire complex would have the appearance of the letter Y. The crystal structure of the yeast CRE-binding protein GCN4 complexed with DNA, however, confirmed the "induced helical fork" model (O'Neil, 1990) where there is no break in the basic region α-helix (Konig, 1993; Ellenberger, 1992). The binding of GCN4 to the CRE is mediated by basic residues that contact numerous DNA bases and phosphate oxygens. These amino-acids are conserved in virtually all CRE-binding transcription factors and are responsible, at least in part, for the specificity of the interaction (Chapter 3, Figure 1). Mutation of any of these residues can prevent DNA

binding. For example, based on the GCN4 crystal structure, arginine 287 in CREB341 forms a specific hydrogen bond with the central guanosine in the DNA strand complementary to the half site 5'-TGAC-3'. Mutation of this arginine to leucine eliminates this contact and prevents CREB binding (Walton, 1992). Other mutations also alter the specificity of binding. Substitution of three residues in GCN4 for those in C/EBP can alter the specificity of binding from a CRE to a C/EBP binding site (Suckow, 1993a). These residues, in positions -14, -16 and -17, are conserved between GCN4 and CREB. The alanine in position -14 participates in a van der Waals interaction with the thymine methyl group near the center of the CRE. This specific interaction is predicted in the GCN4/DNA crystal structure. Accordingly, Suckow *et al.* subsequently determined that mutation of this residue was sufficient to alter GCN4 binding specificity (Suckow, 1993b).

Several additional characteristics of the bZIP region of CREB contribute to DNA binding specificity. For example, GCN4 mutants that alter DNA-binding specificity typically have larger hydrophobic residues in place of GCN4 residues that contact base pairs (Kim, 1993). Molecular modeling indicated that many of these substitutions could not be accommodated in the wild-type crystal structures. Kim *et al.* hypothesized that small shifts in position and orientation or local changes in the shape of the α-helical bZIP region could contribute to binding specificity. These small differences could confer different binding specificities to an otherwise highly conserved bZIP region. The spacing of the leucine zipper from the basic region may also contribute to binding specificity (Pu, 1991). Insertion of one to six amino acids between the leucine zipper and the basic region eliminates DNA binding. Insertion of seven amino acids restores DNA binding indicating that one full turn of the α-helix again properly orients the basic region with the DNA. The leucine zippers orient the basic regions with respect to the DNA. The length or flexibility of the α-helix between the two regions may dictate the allowable spacing between DNA half-sites. The activator regions of bZIP proteins may also contribute to

binding specificity. Phosphorylated CREB, for example, has been shown to bind asymmetric or low affinity CREs better than unphosphorylated CREB (Nichols, 1992). A phosphorylation induced conformational change in CREB may be responsible for the altered binding affinity. Finally, adaptability in the DNA conformation has been shown to be important in bZIP protein/DNA interactions. GCN4, for example, binds to both TRE (TGACTCA) and CRE DNA sequences. The crystal structure of the GCN4 bZIP region/CRE complex shows that both the protein (between the leucine zipper and the basic region) and the DNA are able to bend to accommodate the additional base-pair (Konig, 1993). The DNA-binding specificities of bZIP proteins, then, are mediated by several factors. Specific protein/DNA contacts, small conformational differences, other protein domains, and protein and DNA adaptability all influence the specificity of DNA-binding.

Landshultz *et al.* first identified the leucine zipper after comparison of homologous regions from five potential regulators of transcription (Landshultz, 1988). The transcriptional regulators GCN4 and C/EBP and the transforming proteins Myc, Fos and Jun all contained a hypothetical amphipathic α-helix that displayed a heptad repeat of leucines over at least four helical turns. They proposed that the leucines would form a hydrophobic face on one side of the α-helix. Leucines on two antiparallel helices could interdigitate and lead to dimerization. The results of cross-linking experiments and two-dimensional NMR studies, however, indicated that leucine zippers have the characteristics of parallel coiled-coils in GCN4 homodimers (O'Shea, 1989a; Oas, 1990). A new model to explain the role of leucines in dimerization was based on the structure of coiled-coils originally proposed by Francis Crick (Crick, 1953). He predicted that the α-helices in a coiled-coil would wrap around each other with a slight left-handed twist. The structure could be stabilized by hydrophobic residues every four and then three residues apart which would allow the packing of "knobs" formed by hydrophobic residues in one helix into the "holes" between hydrophobic residues in the second helix. Consistent with

this model, the crystal structure of dimerized GCN4 leucine zippers shows that the leucines do not interdigitate (O'Shea, 1991). Instead, they interact above and below (in long axis of the helices) with the alternate, non-leucine, hydrophobic residue in the opposing helix. Leucines in adjacent helices make side-to-side contacts. End and side views of dimerized leucine zippers illustrate the interaction of residues in the hydrophobic face (Chapter 1, Fig. 1). In this diagram, where the seven positions in each helical turn are labeled **a** through **g**, leucines typically occupy **d** positions while other hydrophobic residues occupy the **a** positions four residues downstream. Polar residues in the **a** positions are thought to destabilize bZIP dimerization and allow for reversible binding. They may also serve to align the helices (O'Shea, 1991). Both GCN4 and CREB contain a highly conserved aspargine at the **a3** position, for example. The destabilizing effect of this polar residue, in part, may be offset by its ability to form a hydrogen-bond with the **a3** asparagine in the adjacent helix. While a number of single substitutions in the **a** and **d** positions of GCN4 are permissive for dimerization (Hu, 1990; Zhou,1992), additional substitutions may prevent dimerization. For example, the Fos leucine zipper, which contains two polar residues in **a** positions, is unable to dimerize at physiological concentrations or pH (O'Shea, 1989b; Smeal, 1989).

Electrostatic interactions that occur outside of the hydrophobic face may also determine dimer stability. The Fos leucine zipper, for example, can dimerize at low pH indicating that ionized acidic residues in the **e** and **g** positions may contribute to dimer destabilization (O'Shea, 1989b; Schuermann, 1991). Indeed, Nicklin *et al.* found that substitution of a lysine for a glutamate in the **e2** position of the Fos leucine zipper permitted homodimerization, presumably by replacing a repulsive electrostatic interaction between glutamate residues in the **g1** and **e2** positions with a favorable glutamate/lysine interaction (Nicklin, 1991). Favorable electrostatic interactions may stabilize leucine zipper dimer formation. The existence of these stabilizing salt bridges was confirmed in the crystal structure of GCN4 leucine zipper (O'Shea, 1991). The

distances between charged side chains of adjacent dimerized leucine zippers suggested that three interhelical ion pairs could form. These ion pairs were found between position **g** of one heptad and position **e** of the following heptad in the adjacent helix (**e'**). Similar salt bridges presumably stabilize CREB leucine zipper dimers. Electrostatic interactions can also explain the stability of Jun:Fos heterodimers relative to Fos or Jun homodimers. O'Shea *et al.* have shown that the repulsive electrostatic interactions that would occur in Fos homodimers provides the driving force for the formation of Fos:Jun heterodimers which are stabilized by four salt bridges (O'Shea, 1992).

In addition to stabilizing or destabilizing leucine zipper dimers, electrostatic interactions also mediate dimerization specificity. Homodimerization of bZIP proteins generally leads to the formation of favorable electrostatic interactions between residues in the **g** and **e'** positions. Though the residues in the **a** and **d** positions of bZIP protein leucine zippers are highly conserved, considerable variability exists in the **e** and **g** positions. The ability of bZIP proteins to heterodimerize would depend on the nature of the electrostatic interactions that would form. Starting with this hypothesis, Vinson *et al.* developed an interhelical salt bridge rule to predict and design dimerization partners (Vinson, 1993). By examining the residues in the **e** and **g** positions of various C/EBP related proteins, they were able to predict that ATF4 and IGEBP1 could form heterodimers. In addition, they constructed mutant C/EBP leucine zippers that could form preferential heterodimers with wild-type C/EBP but could not homodimerize. Subsequently, mutant leucine zippers have been used to examine the *in vivo* function of Myc:Max heterodimers (Amati, 1993).

Models of gene activation by CREB

Two models explain CREB activation by PKA. The model proposed by Gonzalez *et al.* suggests that phosphorylation of serine 133 can induce an allosteric change that would allow the glutamine-rich regions of CREB to interact with proteins in the RNA

polymerase II complex (Gonzalez, 1991). Three lines of evidence support this model. First, the pattern of protein fragments generated by digestion with trypsin is different for phosphorylated CREB than for unphosphorylated CREB. The two trypsin cleavage sites are located near serine 133. Presumably, the structural change induced by phosphorylation alters the accessibility of these sites to trypsin. Second, phosphorylation of CREB increases its binding to low affinity CREs. Weih *et al.*, for example, found that increases in intracellular concentrations of cAMP led to an increase in protein binding to the tyrosine aminotransferase (TAT) CRE (Weih, 1990). In addition, overexpression of the PKA regulatory subunit, R1, appeared to eliminate this binding activity (Boshart, 1991). Nichols *et al.* identified CREB as the TAT CRE-binding protein and found, using *in vivo* footprinting experiments, that phosphorylation could increase the binding of CREB to weak, nonconsensus CRE sites (Nichols, 1992). The change in affinity may reflect a change in CREB structure induced by phosphorylation. Finally, phosphorylation-induced activation of CREB is dependent upon the presence of the adjacent glutamine-rich region Q2. The importance of this domain is illustrated in the repressor of cAMP-mediated gene expression, CREMα, which contains a kinase inducible domain but not a Q2 region. Presumably, CREMα lacks the key element in its activation domain that mediates the effects of PKA phosphorylation. Consistent with ths hypothesis, Ferreri *et al.* have shown that the Q2 region of CREB associates with the TATA-associated factor isolated from Drosophila, TAF 110 (Ferreri, 1994). The interaction between the Q2 region and TAF 110 is not phosphorylation dependent, however, and therefore it does not directly support the model that phosphorylation induces an allosteric change in CREB. The following points also weaken the validity of this model. First, the argument that phosphorylation of CREB increases its affinity for nonconsensus CREs may be incorrect. Binding constants determined at equilibrium using fluorescence anisotropy show that phosphorylation does not increase the affinity of CREB for the TAT CRE (Richards, Thesis). And second, no direct biochemical evidence

for a conformational change in CREB currently exists. Circular dichroism experiments and sedimentation studies, which can detect changes in secondary and tertiary structure respectively, do not find structural differences between phosphorylated and nonphosphorylated CREB (Richards, Thesis).

As an alternative model, Chrivia *et al.* proposed that phosphorylation of CREB allows a specific interaction with a co-activator protein (Chrivia, 1993). Consistent with this model, a 265 kD CREB-binding protein (CBP) was isolated from a lambda cDNA-expression library using phosphorylated CREB as a probe. CBP shares structural motifs with other transcriptionally active proteins, including three putative zinc finger sequences, a bromodomain, and a carboxy-terminal glutamine-rich region. The third zinc-finger motif, which may be involved in protein:protein interactions, is homologous to a region of the yeast protein ADA-2, a proposed co-activator for VP-16 (Berger, 1992). Glutamine-rich regions are characteristic of many transcriptional activators and TAFs (Mitchell, 1989). Though the function of the bromodomain remains unknown, it is present in several transcriptional co-activators (Haynes, 1992). These include SWI2 (Abrams,1986), a yeast protein that augments the function of several transcriptional activators, brahma (Tamkun, 1992), a Drosophila regulator of homeotic genes, and TAF250 (Hisataki, 1993), a component of the TFIID complex. The results of binding and functional studies also suggest that CBP is a transcriptional co-activator. First, CBP fused to a GAL4 DNA-binding domain is able to activate expression from a GAL-CAT reporter plasmid (Chrivia, 1993). In addition, F9 cells transfected with vectors encoding CBP, CREB, and the catalytic subunit of PKA show higher levels of transcription from a CRE-CAT reporter than cells transfected with CREB and PKA encoding vectors alone (Kwok, 1994). Second, Arias *et al.* have shown that microinjection of antibodies directed against the CREB-binding domain of CBP prevents cAMP-mediated activation of a CRE-reporter (Arias, 1994). Finally, Kwok *et al.* have shown that the third zinc-finger region of CBP can bind directly to the general transcription factor TFIIB (Kwok,

1994). CBP, then, may function by specifically linking phosphorylated CREB to the RNA polymerase II complex.

Viral proteins that modulate CREB activity

Viral promoters typically contain enhancer sequences that are recognized by cellular transcription factors. Though several of these factors are activated by second messenger signalling pathways, a number of viral proteins can also activate the transcription factors that bind to viral enhancer sequences. Thus, many viral promoters are inducible by second messenger signalling pathways and by certain viral proteins. The ATF/CRE sites in adenovirus promoters, for example, are activated by both cAMP and the adenoviral protein E1A (Sassone-Corsi, 1988). Several laboratories have shown that 13S E1A can activate the ATF/CRE binding protein ATF-2 (Lillie, 1989; Liu, 1990; Maekawa, 1991). The 12S E1A protein can also activate transcription from *c-jun* by interaction with ATF-2:c-Jun heterodimers (van Dam, 1993). Liu *et al.* have found that E1A can bind directly to the ATF-2 DNA-binding domain (Liu, 1994). In addition, E1A fused to a GAL-4 DNA binding domain is a strong activator of transcription (Lillie, 1989). E1A localizes its activation domain to viral promoters by binding to the DNA-binding domain of ATF-2 in ATF-2 homodimers and ATF-2:cJun heterodimers.

The HTLV-1 transactivator protein, Tax, may activate transcription from CRE sequences by different mechanisms. Like E1A, Tax is unable to bind DNA but is able to activate transcription from viral and cellular genes containing CRE enhancer sequences. Rather than simply tethering its activation domain to DNA-binding transcription factors, Tax may activate transcription by binding to and enhancing dimerization of several bZIP proteins including CREB (Wagner, 1993). Wagner *et al.* propose that a protein that can enhance dimerization, the rate limiting step in DNA binding of bZIP transcription factors, increases the number of complexes available to bind DNA and activate transcription. Alternatively, Paca-Uccaralertkun *et al.* have found that Tax alters the DNA-binding

specificity of CREB so that it preferentially binds to HTLV-1 CRE sequences (Paca-Uccaralertkun, 1994). A third viral protein from hepatitis B virus (HBV), pX, also alters the DNA binding specificity of CREB. CREB and ATF-2 are unable to bind an HBV CRE-like sequence *in vitro* (Maguire, 1991). However, the presence of the activator protein pX allowed both CREB and ATF-2 to efficiently bind the HBV CRE. By altering the DNA-binding specificity of cellular transcription factors, Tax and pX may activate selective expression of viral and cellular genes.

Deactivation of CREB by protein phosphatases

In most tissues, the CREB gene is expressed at a constitutive, basal level (Waeber,1991). Gene activation in response to cAMP is not blocked by inhibitors of protein synthesis (Sasaki, 1984), and, predictably, the levels of CREB protein remain invariant for up to twelve hours after forskolin induction (Gonzalez, 1989). As a result of these observations, Hagiwara *et al.* hypothesized that the attenuation of CREB activity following phosphorylation does not occur through proteolysis of CREB protein but through dephosphorylation by serine/threonine phosphatases (Hagiwara, 1992). In support of this hypothesis, they found that, after the initial phosphorylation of CREB following forskolin treatment, CREB was steadily dephosphorylated at serine 133 over a period of eight hours. In addition, the levels of CREB phosphorylation correlated with the relative rates of transcription from the somatostatin gene. Dephosphorylation of CREB was inhibited by okadaic acid indicating that two serine/threonine phosphatases could deactivate CREB, PP-1 and PP-2A. Only PP-1, however, was able to block transcription from a CRE-CAT reporter when transfected or microinjected into RAT2 cells, a fibroblast cell line, and WRT cells, a rat thyroid follicular cell line. In addition, protein inhibitors of PP-1 activity augmented cAMP induced transcription.

A second line of evidence, however, suggests that PP-2A may be the primary phosphatase responsible for CREB deactivation. Wadinski *et al.* found that the CREB

phosphatase activity copurified with PP-2A in anion-exchange chromotography (Wadinski, 1993). In addition, the phosphatase activity in nuclear extracts was unaffected by a specific inhibitor of PP-1 activity. PP-2A was also more efficient in dephosphorylating CREB, and only PP-2A treated phospho-CREB did not stimulate transcription from the PEPCK promoter *in vitro*. Furthermore, Wheat *et al.* found that SV40 small tumor antigen could inhibit dephosphorylation of PKA-phosphorylated CREB (Wheat, 1994). SV40 small tumor antigen binds to and inactivates PP-2A but not PP-1 (Yang, 1991). As a result, SV40 small tumor antigen can potentiate cAMP-induced gene activation.

The conflicting evidence presented in favor of each model may indicate that both PP-2A an PP-1 can contribute to CREB deactivation and that the mechanism of CREB deactivation may be cell specific.

Life without CREB

To understand the function of CREB in a living animal, the CREB gene has been altered or deleted in strains of transgenic mice. Struthers *et al.*, for example, developed a strain of mice that expresses a nonphosphorylatable form of CREB, CREB M1, specifically in anterior pituitary somatotrophic cells (Struthers, 1991). Because cAMP is a mitogenic signal in these cells (Billestrup, 1986), expression of an inactive CREB may compete with endogenous CREB and block cellular proliferation. Mice that expressed the mutant CREB, in fact, exhibited a dwarf phenotype and a marked deficiency of somatotrophic cells in their pituitary glands.

In contrast, mice that were thought to lack CREB altogether did not show any impairment in growth or development (Hummler, 1994). Hummler *et al.* predicted that proteins closely related to CREB, such as ATF-1 and CREM, could replace CREB as mediators of cAMP-induced gene activation. Examination of CREM and ATF-1 mRNA and protein levels showed that both activator and repressor forms of CREM were

upregulated but levels of ATF-1 did not change. Bourtchuladze et al., however, found that the CREB deficient mice developed by Hummler still express a third activating isoform of CREB (Bourtchuladze, 1994). This isoform may, in part, compensate for deficiencies in the other CREB isoforms. Though mice lacking the prominent activating isoforms of CREB showed no overt abnormalities, they were found to be deficient in long-term memory (Bourtchuladze, 1994). CREB mediated protein synthesis has been linked to the development of long term memory (Dash, 1990). Apparently, CREM, ATF-1, and the remaining CREB isoform are unable to replace CREB in this regard.

The role of dimerization

While the leucine zipper mediates homodimerization of bZIP transcription factors, it also allows the formation of specific heterodimer complexes. Heterodimerization may serve several functions. First, heterodimerization can increase the diversity of protein complexes that can interact with a single enhancer element. This diversity can occur within a bZIP family. Hai *et al.*, for example, found that the highly conserved leucine zippers of the CRE-binding ATF transcription factors allowed specific heterodimer complexes to form between ATF-2 and ATF-3 (Hai, 1989). CREB, CREM, and ATF-1 also have been found to heterodimerize (Foulkes, 1991 Cell; Rehfuss, 1991). Because there are many isoforms of CREB and CREM (Hoeffler, 1990; Foulkes, 1992), a large number of heterodimer complexes could form within this family. Heterodimerization is not promiscuous, however. CREB has not been found to heterodimerize with Fos, Jun, or the C/EBP and CRE/BP-1 related transcription factors. Because the activator regions of transcription factors within a family can vary greatly and can have strikingly different functional properties, heterodimer complexes may have unique transcriptional activities. For example, although several CREM isoforms contain consensus protein kinase A phosphorylation sites, these factors appear to function as negative regulators of transcription. Heterodimers of CREB and CREM, therefore, may

generate complexes with transcriptional activities that are very different from the homodimer conformations of either of these factors alone. Consistent with this idea, Ellis *et al.* have recently shown that CREB:ATF-1 heterodimers are transcriptionally inactive (Ellis, 1995). This result is surprising because CREB and ATF-1 homodimers are both strong activators of cAMP-mediated gene expression. The diversity introduced by heterodimerization may also allow the integration of information from distinct signal transduction pathways. That is, specific heterodimer complexes may form between distinct bZIP families activated by different signalling pathways. ATF-2, a protein which activates transcription from CRE sequences, readily forms heterodimers with Jun, for example (Macgregor, 1990). The ATF-2:Jun complex, then, may allow "cross-talk" between PKA and PKC signalling pathways at CRE sequences.

Heterodimerization can also increase the specificity of DNA-binding. CRE/BP1:Jun heterodimers preferentially bind to CRE sites. In this complex, Jun is switched from a TRE to a CRE binding protein (Benbrook, 1990; Macgregor, 1990). The ATF4:C/EBPβ heterodimer also shows DNA-binding specificities distinct from those of either homodimer. This complex binds preferentially to an asymmetric CRE sequence found in the promoter of the proenkephalin gene (Vinson, 1993).

Specific Aims

Though many heterodimer complexes are able to form within bZIP transcription factor families, the functional properties of these complexes are unknown. It is unclear, for example, whether the negatively-acting CREM transcription factors block transcriptional activation by forming non-functional heterodimers with the positive activators CREB and ATF-1, or by forming homodimers that prevent the activators from binding to the CRE. The former model provides a mechanism that should maximize the efficiency and specificity of the negative regulators, while the latter mechanism, while

less efficient, could additionally impinge upon genes that are regulated by proteins that do not dimerize with CREM, such as ATF-2.

A major problem in the functional analysis of bZIP transcription factors is their propensity to associate in both homodimeric and heterodimeric complexes. For example, a mixture of two proteins in this class can generate three distinct dimer combinations -- two homodimer forms and one heterodimer. As a result, it becomes difficult to ascertain the functional properties of individual heterodimers. In this thesis, I develop a method to generate specific heterodimer complexes. The transcriptional activities of these complexes are then determined to understand how dimerization impacts on the function of bZIP transcription factors. The four specific aims of this proposal are designed to study the role of dimerization in a prototypical bZIP protein, CREB. The specific aims are to:

1. *Engineer the leucine zipper of CREB to permit the formation of selected heterodimer complexes.* To simplify the functional analysis of CREB, I will substitute residues within the leucine zipper region to generate proteins that dimerize in selected combinations. These mutant leucine zippers can be used to determine the transcriptional activities of specific heterodimer complexes.

2. *Determine the activity of hemiphosphorylated CREB complexes.* Heterodimerization may serve several functions in CREB mediated gene activation. Dimerization may allow the activation domain in each CREB molecule to play a role in transcription regulation. That is, each activation domain may contribute to the total activity of a CREB dimer. Using the mutant leucine zippers, I will determine the contribution of each phosphorylated activation domain in the CREB dimer to the level the transcriptional activation.

3. *Determine the activity of CREM α:CREB heterodimers.* Dimerization may also allow CREB to heterodimerize with related factors to form complexes with unique transcriptional activities. I will use the mutant leucine zipper strategy to study the

mechanism of action of negative regulators of transcription such as CREM. Specifically, I will assess the transcriptional activity of selectively formed CREMα:CREB heterodimers.

4. *Determine whether the activity of hemiphosphorylated CREB complexes is orientation dependent.* Because the CRE sequence is symmetric, the hemiphosphorylated dimer may bind in two orientations. Only one of these orientations may be functional. To test this possibility, I will use molecular modeling and site-directed mutagenesis to generate an asymmetric CRE that will directionally bind heterodimeric CREB complexes containing mutant and wild-type DNA-binding domains.

TABLE I

Examples of CRE Sequences

Gene	Sequence	Reference
somatostatin, rat	TGGC**TGACGTCA**GAGA	Montminy, 1986
glycoprotein α- subunit, human	AAT**TGACGTCA**TGGGTAA	Delegeane, 1987
tyrosine hydroxylase, rat	GCTT**TGACGTCA**GCCT	Lewis, 1987
glucagon, rat	CATT**TGACGTCA**AAAT	Philippe, 1988
vasoactive intestinal peptide, human	TGGC**CGTCA**TACTG**TGACG**TCTT	Tsukada, 1987
tyrosine aminotransferase	TGCAGCTTCTG**CGTCA**GCGCCAGTAT	Boshart, 1990
c-fos, mouse	CCAG**TGACG**TAGG	Gilman, 1986
proenkephalin	GGCCTG**CGTCA**GCTG	Comb, 1986
p-enolpyruvate carboxykinase, rat	CTTA**CGTCA**GAGC	Short, 1986

Kindly provided by Jane Richards (Richards, Thesis)

Chapter 1

Engineered Leucine Zippers Show that Hemiphosphorylated CREB Complexes are Transcriptionally Active

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Abbreviations: (CRE) cAMP responsive element; (CREB) CRE-binding protein; (CAT) chloramphenicol acetyltransferase; (PKA) protein kinase A; (bZIP) basic/leucine zipper

Abstract

The ability of bZIP transcription factors to form homo- and heterodimers potentially increases the diversity of signaling pathways that can impinge upon a single genetic element. The capacity of these proteins to dimerize in various combinations complicates the analysis of their functional properties, however. To simplify the functional analysis of CREB dimers, we mutated selected residues within the leucine zipper region to generate proteins that could only heterodimerize. These mutants allowed us to determine if phosphorylation of both CREB subunits was necessary for transcriptional activation. Our results reveal that hemiphosphorylated CREB dimers are half as active as fully phosphorylated dimers. It is possible, therefore, that the degree of phosphorylation of CREB complexes could modulate the transcriptional responses of specific genes to cAMP.

Introduction

Transcription factors typically contain distinct DNA-binding and activation domains. In many cases, the biological activities of these factors depend on their ability to dimerize, and the various classes of transcriptional activators frequently contain discrete subdomains that participate in this process. The utility of dimerization is two-fold: in addition to increasing the specificity of DNA-binding, this process increases the diversity of protein complexes that can interact with a single genetic element. This diversity is likely to be important for integrating information from distinct signal transduction pathways. Our studies were directed toward understanding the functional properties of heterodimeric complexes that bind to the cAMP-regulated enhancer (CRE; 1,2).

Although several CRE-binding proteins have been described (3), most studies have focused on CREB (4,5), which is believed to mediate transcriptional signals directed by the cAMP and calcium second messenger pathways (6-8). Within its activator domain, CREB contains consensus phosphorylation sites for several protein kinases. The most critical of these sites is serine 133 which can be phosphorylated by both protein kinase A (PKA) and calcium/calmodulin kinase II (8,9). Mutation of this serine to alanine destroys the ability of CREB to mediate transcriptional activation (9).

CREB homo- and heterodimerizes through its leucine zipper, an amphipathic α-helix containing a heptad repeat of leucine residues at the hydrophobic dimerization interface (10). Leucines or other non-polar residues occupy most of the **a** and **d** positions of the interface (Fig. 1). As in other basic/leucine zipper (bZIP) proteins, conserved polar residues within this region of CREB probably affect zipper alignment and stability, while neighboring charged residues affect dimerization specificity. The asparagine residue, which occupies the **a3** position in the leucine zipper of many b/ZIP proteins, is predicted to destabilize the dimer complex (11). Charged residues in the **g** position of one helix

generally form complementary or repulsive electrostatic interactions with those in the **e** position of the adjacent helix (11). These electrostatic interactions have been shown to be important determinants of the dimerization potential of proteins in the bZIP class (12).

In vitro studies have shown that the various CRE-binding proteins heterodimerize only in specific combinations (13). Although it is predicted that the formation of such heterodimers generates complexes with novel binding or transcriptional activities (14,15), there are relatively few well-documented examples of this mode of regulation in intact cells. For example, CREB heterodimerizes with related proteins such as ATF-1 and CREM (16,17), but the functional properties of these dimer combinations are unknown. It is unclear, therefore, whether the negatively-acting CREM transcription factors block transcriptional activation by forming non-functional heterodimers with the positive activators CREB and ATF-1, or by forming homodimers that prevent the activators from binding to the CRE. The former model provides a mechanism that should maximize the efficiency and specificity of the negative regulators, while the latter mechanism, while less efficient, could additionally impinge upon genes that are regulated by proteins that do not dimerize with CREM, such as ATF-2.

A major problem in the functional analysis of bZIP transcription factors is their propensity to associate in both homodimeric and heterodimeric complexes. For example, a mixture of two proteins in this class can generate three distinct dimer combinations -- two homodimer forms and one heterodimer. We addressed this problem by designing CREB molecules that could only form heterodimers. These molecules could then be used to determine the functional properties of specific heterodimeric transcription factor complexes. As a test of this strategy, we asked whether PKA-mediated transcriptional activity required that both subunits in the CREB dimer were phosphorylated. Surprisingly, we found that CREB complexes phosphorylated on only one subunit were precisely half as active as complexes containing two intact PKA phosphorylation sites.

Materials and Methods

Plasmid construction and expression - A CREB 327 cDNA was isolated from a λgt11 human hypothalamic library and was subcloned into the Hind 3/Xba 1 site of pALTER (Promega) for *in vitro* mutagenesis. Mutagenesis was carried out as described in the Altered Sites *in vitro* Mutagenesis System protocol (Promega). Kpn 1/Sma 1 restriction fragments encoding the DNA-binding domain and the mutant leucine zipper region of CREB were then subcloned into a pET-11d expression plasmid (Novagen) containing a full-length CREB 327 insert (provided by T. Usui, Vollum Institute). A pET-11d plasmid containing a truncated form of wild-type or mutant CREB (designated short-form) was also constructed to allow visualization of the protein heterodimers in gel mobility shift assays. These constructions, which encoded a protein with a 60 amino acid deletion, were generated by removing a Pvu 2/Stu 1 restriction fragment that included nucleotides between 351 and 531. pET-11d plasmids were resequenced using T7 DNA polymerase (Sequenase, US Biochemical) to confirm that each contained the correct mutations. The full-length CREB 341 expression vector used in *in vivo* transfection experiments was produced by cloning a rat CREB cDNA (obtained from M. Montminy, Salk Institute) into the expression vector pRL/RSV (Invitrogen). Mutations were introduced into this vector by replacing the CREB 341 Kpn 1/Sma 1 fragment (encoding the DNA-binding domain/leucine zipper region) with the Kpn 1/Sma 1 mutant fragments generated in the CREB 327 cDNA. A form of CREB 341 that could not be phosphorylated by PKA (9) was generated by mutating serine 133 to alanine. The plasmids RSV-luciferase (18) and RSV-PKA (19) were obtained from M. Wilkinson (Oregon Health Sciences University) and R. Maurer (University of Iowa), respectively. The plasmid SS-CAT, containing sequences of the rat SS gene from -71 to +53 placed 5' to the coding region of chloramphenicol acetyltransferase (CAT) was constructed previously (1).

Bacterial expression of recombinant CREB proteins - pET-11d plasmids encoding wild-type, mutant, and CREB short-forms were transformed into the BL21(DE3) strain of *E. coli*. Bacterial cultures were grown to an OD600 = 0.6 in Luria broth containing 100 ug/ml ampicillin. Cells were induced to express recombinant proteins by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. After 3 hours, 7.5 ml of cells were harvested and lysed by sonication in 300 ul of a solution containing 50 mM dithiothreitol and 50 mM EDTA in phosphate buffered saline. Bacterial debris was cleared by centrifugation after the addition of 30 ul of 10% Triton X-100. The cleared supernatant was heated to 72°C for 2 minutes, and the precipitated proteins were again cleared by centrifugation. The supernatant was used in gel mobility shift assays. The concentration of recombinant CREB in each extract was determined by densitometric analysis of samples electrophoresed on SDS-PAGE gels stained with Fast Stain (Zoion Research, Inc.).

Gel mobility shift assays - Mixtures of extracts were heated to 72°C, added to a 20 ul reaction buffer (10 mM Tris (pH 7.5), 50 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 1 ug poly d(IC) and 3.9 ug bovine serum albumin) and incubated at 37°C with 15 fmol of end-labeled somatostatin CRE oligonucleotide. Reactions were loaded onto a 6% non-denaturing acrylamide gel and were electrophoresed at 200 volts for 10 hours. Gels were dried and autoradiographed using Kodak XAR film.

Molecular modeling - Mutant and wild-type CREB leucine zippers were modeled into the GCN4 leucine zipper using a Silicon Graphics work station and MIDASplus software. (The coordinates were kindly provided by T. Alber, University of Utah.)

Cell culture and transfection assays - F9 teratocarcinoma cells (provided by M. Montminy, Salk Institute) were grown on gelatin-coated plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells (3.5×10^5) were plated on 10 cm dishes 18 hours before transfection. DNA was transfected using the TRANSFINITY Calcium Phosphate Transfection System (BRL) in the following amounts: 5 ug SS-CAT, 2 ug RSV-luciferase, and the indicated amounts of RSV-PKA, and RSV wild-type and mutant CREBs. The total DNA in each transfection was 20 ug, the balance consisting of pRC-RSV. Fresh medium was added 24 hours after transfection and cells were collected for assay 24 hours later. CAT activity was determined by the method of Seed and Sheen (20) and was normalized to luciferase activity (18) as a control for transfection efficiency.

Results

The **a3** asparagine of CREB and related proteins is thought to function by destabilizing the hydrophobic interface of the b/ZIP dimer (11). Thus, we initially targeted this particular site for mutation. We reasoned that this position could be made even more destabilizing in the homodimer form if it was substituted with a residue with a formal positive or negative charge. By site-directed mutagenesis, the wild-type asparagine was replaced with either aspartic acid, lysine, arginine, or histidine. The aspartic acid substitution was made to introduce a formal negative charge, and the lysine and arginine mutations were made to introduce formal positive charges. The histidine mutation introduced an amino acid that could be charged or uncharged, depending upon the pH. We proposed that destabilization of the mutant homodimers could occur by charge-repulsion or by steric hindrance. We additionally hypothesized that the asparagine:histidine heterodimer could be stabilized by a hydrogen bond between the histidine and the asparagine and by van der Waal contacts with residues surrounding the **a3** positions.

Recombinant CREB proteins were expressed in bacteria and purified by heating. This procedure removes most of the bacterial proteins, which precipitate at 72°C. Recombinant CREB is extremely heat-stable and returns to its native form, as analyzed by circular dichroism, after cooling to room temperature (J. Richards, Vollum Institute, unpublished observations). Samples of each extract were electrophoresed on SDS-PAGE gels to assess the quantity and purity of recombinant protein in each extract. Recombinant CREB represented greater than 90% of the protein in each preparation (See Appendix) and differences in protein expression among individual extracts were less than 10%.

The ability of the recombinant proteins to dimerize *in vitro* was analyzed by using gel mobility shift assays. In these assays, full-length proteins were mixed with a

truncated form of CREB, 60 amino acids shorter, to allow visualization of heterodimer complexes. Except for the aspartic acid substitution, each of the mutations decreased the amount of homodimer and increased the amount of heterodimer formed (See Appendix). The greatest effect was seen after mutating the **a3** position to histidine, but proteins containing this substitution still did not completely prevent formation of homodimers containing the wild-type asparagine.

To decrease homodimerization of CREB molecules containing the **a3** asparagine further, we additionally mutated amino acids in the **e** and **g** positions (Fig. 2A). The resultant CREB proteins, designated pZIP3 and pZIP12, combined only as heterodimers when equimolar amounts of the full-length and deleted forms were mixed (Fig. 2B). In mixtures containing a large excess of pZIP3, a small amount of homodimer was also seen. This form presumably resulted from retention of the wild-type **a3** asparagine. No homodimerization was detected in mutant proteins that contained a histidine residue in the **a3** position.

The biological activities of the mutated proteins were assessed by transient transfection assays in F9 teratocarcinoma cells. These cells were chosen because they have low levels of functional CREB and PKA (9). Cells were transfected with a somatostatin-chloramphenicol acetyltransferase (CAT) reporter gene and plasmids encoding PKA, RSV-luciferase (to control for transfection efficiency) and various amounts of CREB. Reporter activity depended on the amount of wild-type CREB expression vector used and, in each instance, increased after addition of PKA (Fig. 3). Activity of the CREB form containing the pZIP12 leucine zipper (341_{ZIP12}) was negligible, even in the presence of PKA, at all amounts tested. The slightly higher activity of 341_{ZIP3} is consistent with the results of the binding studies, and indicate that homodimerization of the mutant proteins can be forced to occur at high expression levels if the complementary CREB mutant is omitted. When the complementary CREB mutants were combined, transcriptional activities of the mixtures were indistinguishable

from those of wild-type CREB. To minimize background activity from the homodimer forms, 1.5 ug of each CREB plasmid was used in subsequent studies.

The leucine zipper mutants were next used to determine the biological activities of CREB dimers that could be phosphorylated on only a single subunit. For these studies, serine 133 in each of the CREB leucine zipper mutants was substituted with alanine. This mutation, designated M1, has been used previously to establish the role of PKA phosphorylation in CREB activation (9). In the absence of PKA, Ml-mutated CREB containing a wild-type leucine zipper has the same activity as native CREB (Fig. 4; see also 21), suggesting that the serine to alanine substitution does not alter general aspects of CREB structure. The same level of PKA-independent activity was directed by wild-type:M1 heterodimers (data not shown). PKA slightly decreased the transcriptional activity of the M1 homodimers containing either wild-type or mutated leucine zippers. This apparent decrease in activity is due, at least in part, to the low level of PKA stimulation of the RSV-luciferase plasmid that is used for normalization. The activities of heterodimers that contained only a single intact PKA phosphorylation site were precisely half as high as complexes containing two phosphorylation sites. Thus, transcriptional activation mediated by PKA does not require phosphorylation of both subunits of the CREB dimer.

Discussion

Our strategy for engineering the CREB leucine zipper domains was guided by several previously reported studies of related bZIP proteins. Two observations in particular suggested that substitutions at the **a3** position might alter the dimerization properties of these factors. First, the amino acid that typically occupies this position is a unique polar residue in an otherwise hydrophobic dimerization interface (Fig. 1). An asparagine is conserved at this position in the leucine zippers of C/EBP-1, c-Jun, GCN4 and CREB, suggesting that this particular residue has an important function in dimerization specificity (11). By interrupting the continuity of the hydrophobic interface, this asparagine is thought to destabilize the leucine zipper and may thereby permit reversible dimerization. This process may be important for allowing factors to exchange partners under different physiological conditions. Second, although *in vivo* studies examining the dimerization properties of GCN4 leucine zipper mutants showed that the **a3** position was relatively tolerant of amino acid substitution, an aspartic acid substitution, in particular, was able to disrupt dimer formation (22). This finding suggested that charge repulsion at this position might be particularly effective in destabilizing homodimer formation. We proposed that mutations that introduce larger polar or charged residues at this position would further destabilize homodimer formation. At the same time, it was important to identify an amino acid that would allow dimerization with CREB molecules containing the wild-type **a3** asparagine.

Of the four mutations tested, the **a3** asparagine to histidine substitution was most effective in reducing homodimer formation. To elucidate how this mutation affected stability of the CREB dimers, we modeled the histidine substitution into the crystal structure of the GCN4 leucine zipper (11) using a Silicon Graphics work station and MIDAS plus molecular modeling software. In the histidine homodimer, the best modeled histidine:histidine interaction is a hydrogen bond that is 3.3 A long and non-planar. It

was not possible to maintain this hydrogen bond and still make favorable van der Waals contacts with neighboring residues, however. Thus, we predict that unfavorable hydrogen bond geometry may destabilize homodimers containing the histidine mutation.

Molecular modeling also suggested the basis for the stability of the asparagine:histidine heterodimer. The asparagine:histidine contact can be modeled to have a 2.8 A hydrogen bond between the carbonyl oxygen of asparagine and the histidine Nε. This bond length is optimal and the proton donor and acceptor are nearly linear. van der Waals contacts between the histidine Nδ and an isoleucine within the same zipper helix, as well as between the histidine Cδ and a nearby leucine on the opposite helix, is predicted to stabilize the heterodimer form.

Amino acid substitutions were also made in the **e** and **g** positions of the CREB leucine zipper. Stabilizing electrostatic interactions between the corresponding residues have been observed in the GCN4 crystal structure (11). In addition, repulsive electrostatic interactions between amino acids in these positions are thought to prevent homodimerization of c-Fos and stabilizing interactions to direct the preferential heterodimerization of c-Fos with c-Jun (12). Nicklin et al. (23) have confirmed this model by showing that a single amino acid substitution in the **g1** position, which converts the repulsive electrostatic interaction between residues in the **e** and **g** positions to an attractive one, allows c-Fos to homodimerize. A strategy similar to the one reported in this manuscript was used recently to direct heterodimerization of Myc and Max proteins (24). In that case, mutant proteins with reciprocally modified **e** and **g** residues in their leucine zippers were transformation-defective when transfected individually into mammalian cells, but transformation-competent when combined. In our studies, mutations in the **e** and **g** positions alone were not sufficient to completely prevent homodimer formation. Only the mutant leucine zipper pZIP12, which contained a histidine substitution at the **a3** position, was completely inactive in binding and

transcription assays. Thus, the combination of **a3**, **e**, and **g** substitutions was required to direct the formation of CREB heterodimers exclusively.

The results of our transfection studies have several general implications for second messenger regulated gene expression. First, they provide evidence that the process of dimerization in bZIP proteins contributes to determining the level of transcriptional activation by juxtaposing two activation domains. These conclusions differ from those of Oliviero et al. (25) who found that heterodimers containing a GCN4 activation domain had nearly equivalent transcriptional properties whether they contained one or two activation domains. In their system, the degree of activation depended primarily on the number of binding sites upstream from the promoter. The difference between our results and those of Oliviero et al. may reflect differences in the mechanisms of PKA-inducible and acidic activator transcription factors.

The observation that hemiphosphorylated CREB complexes maintain the ability to respond to PKA, albeit at a half-maximal level, supports the concept that heterodimerization may allow the targeting of distinct second messenger-mediated signals to a single genetic element. If two phosphorylated CREB subunits were required for transcriptional responsiveness, heterodimers containing distinct subunits that respond to different second messenger pathways would not be capable of activating transcription. The approach utilized here should be useful for determining the biological functions of some of these other heterodimer complexes. Finally, the distinct activities of unphosphorylated, hemiphosphorylated, and fully phosphorylated CREB complexes raises the possibility that cAMP-directed transcriptional signals may be modulated according to the degree of CREB phosphorylation. Three possible mechanisms could generate CREB dimers that are hemiphosphorylated -- a submaximal response to PKA or protein phosphatase I (26) or heterodimerization of CREB with another transcription factor that does not participate in PKA signaling.

Although the model that hemiphosphorylated CREB complexes are half as active as fully-phosphorylated dimers is appealing physiologically, there was no reason a priori to expect this result. Additionally, the mechanism for the half-maximal level of activity of the hemiphosphorylated CREB complexes is unknown. It is possible that the hemiphosphorylated complexes may associate less efficiently with other proteins that are required for transcriptional activation. For example, the binding of a co-activator or general transcription factor could be enhanced by the presence of a second phosphorylated CREB subunit. This mechanism would not necessarily lead to a doubling of the transcriptional activity however. A second possibility is that complexes that are phosphorylated on a single subunit may function directionally. Because the CREB binding domain sequence is symmetrical, the hemiphosphorylated dimer can bind to the CRE in two different orientations. It is possible that only one of these orientations is functional. In this instance, phosphorylation of the second CREB subunit would eliminate the inherent polarity of the hemiphosphorylated complex. Experiments are currently being performed to discriminate between these two models.

Acknowledgements

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References

1. Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G., Goodman R.H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6682-6686.
2. Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E., Goodman H.M. (1986) *Nature* **323**, 353-356.
3. Hai, T., Liu F., Coukos, W. J., Green, M.R. (1989) *Genes Dev.* **3**, 2083-2090.
4. Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L., Habener J.F. (1988) *Science* **242**, 1428
5. Gonzalez, G.A., Yamamoto, K.K., Fischer, W.H., Karr, D., Menzel, P., Biggs III, W., Vale, W.W., Montminy, M.R. (1989) *Nature* **337**, 749-752.
6. Yamamoto, K.K., Gonzalez, G.A., Biggs III, W.H., Montminy, M.R. (1988) *Nature* **334**, 494-498.
7. Dash, P.K., Karl, K.A., Colicos, M.A., Prywes, R., Kandel, E.R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5061-5065.
8. Sheng, M., Thomson, M.A., Greenberg, M.E. (1991) *Science* **252**, 1427-1430.
9. Gonzalez, G.A., Montminy, M.R. (1989) *Cell* **59**, 675-680.
10. Landschulz, W.H., Johnson, P.F., McKnight, S.L. (1988) *Science* **240**, 1759-1764.
11. O'Shea, E.K., Klemm, J.D., Kim, P.S., Albler, T. (1991) *Science* **254**, 539-544.
12. O'Shea, E.K., Rutkowski, R., Kim, P.S. (1992) *Cell* **68**, 699-708.
13. Hai, T., Curran, T. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3720-3724.
14. Ivashkiv, L.B., Liou, H., Kara, C.J., Lamph, W.W., Verma, I.M., Glimcher, L.H. (1990) *Mol. Cel. Biol.* **10**, 1609-1621.
15. Jones, N. (1990) *Cell* **61**, 9-11.
16. Rehfuss, R.P., Walton, K.M., Loriaux, M.M. and Goodman, R.H. (1991) *J. Biol. Chem.* **266**, 18431-18434.
17. Foulkes, N.S., Borelli, E., Sassoni-Corsi, P. (1991) *Cell* **64**, 739-749.

18. de Wet, J.R., Wood, K.V., De Luca, M., Helinski, D.R., Subramani, S. (1987) *Mol. Cell Biol.* **7**, 725-737.

19. Maurer, R.A. (1989) *J. Biol. Chem.* **264**, 6870-6873.

20. Seed, B., Sheen, J-Y. (1988) *Gene* **67**, 271-277.

21. Yamamoto, K.K., Gonzalez, G. A., Menzel, P., Rivier, J., Montminy, M.R. (1990) *Cell* **60**, 611-617.

22. Hu, J.C., O'Shea, E.K., Kim, P.S., Sauer, R.T. (1990) *Science* **250**, 1400-1403.

23. Nicklin, M.J.H., Casari, G. (1991) *Oncogene* **6**, 173-179.

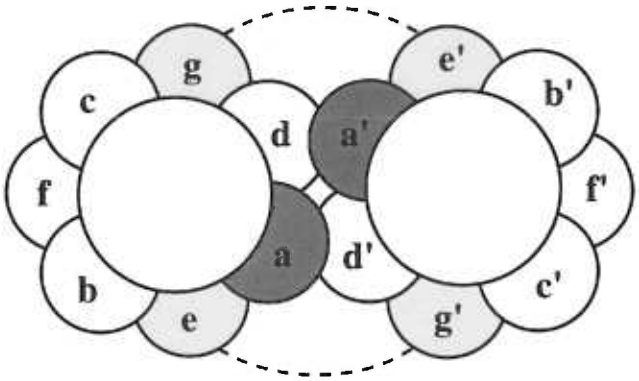
24. Amati, B., Brooks, M.W., Levy, N., Littlewood, T.D., Evan, G.I., Land, H. (1993) *Cell* **72**, 233-245.

25. Oliviero, S., Struhl, K. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 224-228.

26. Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., Montminy, M. (1992) *Cell* **70**, 105-113.

Figure 1

A)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e'
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a'
f
f'
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d'
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B)

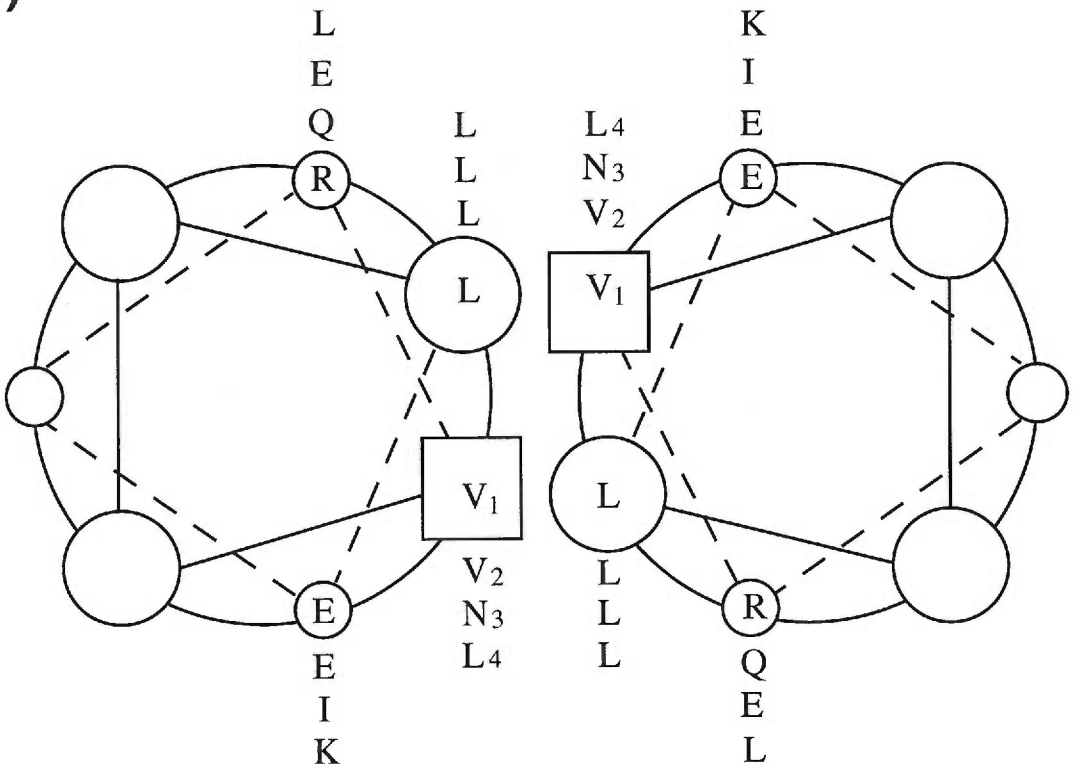
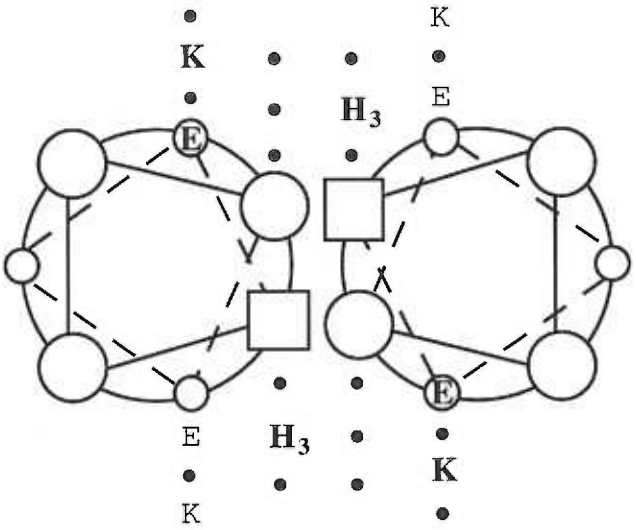

L
E
Q
R
L
L
L
L
V1
V2
N3
L4
E
E
I
K
L4
N3
V2
V1
L
L
L
L
K
I
E
E
R
Q
E
L

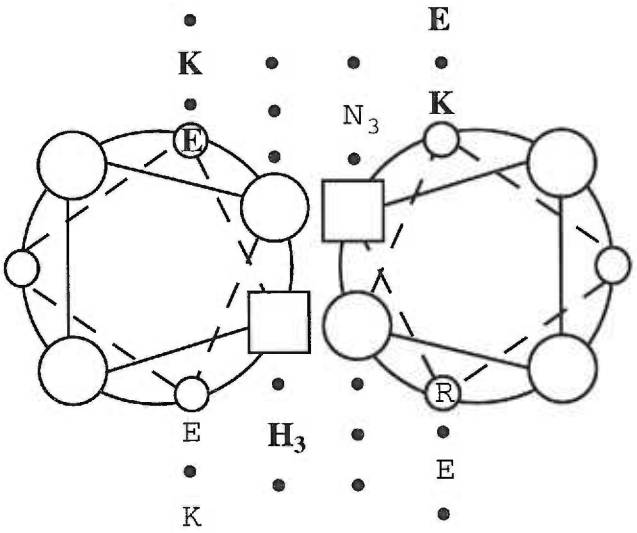
Fig. 1 End-view of parallel α-helices in a dimerized leucine zipper. A) Residues in the **a** and **d** positions form the hydrophobic dimerization interface. Electrostatic interactions (dashed lines) occur between charged residues in the **e** and **g** positions. B) Helical-wheel representation of the CREB leucine zipper. Substitutions were made for the asparagine residue (N3) within the dimerization interface and for selected residues in the **e** and **g** positions.

Figure 2A

pZIP12 leucine zipper
Long form homodimer



pZIP12 + pZIP3 leucine zippers
Heterodimer



pZIP3 leucine zipper
Short form homodimer

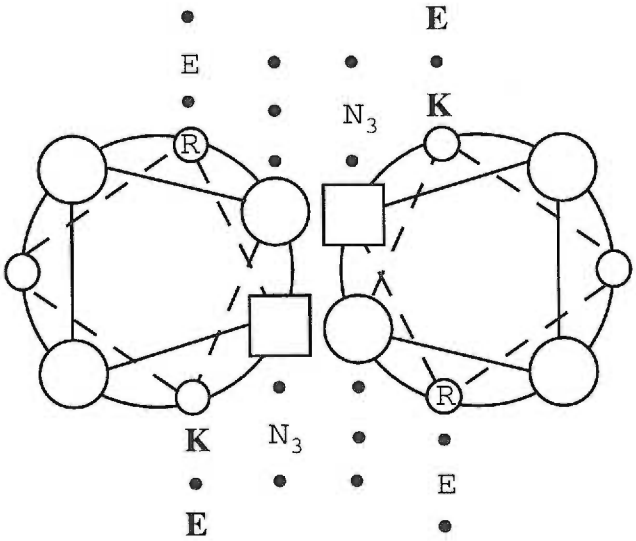


Figure 2B

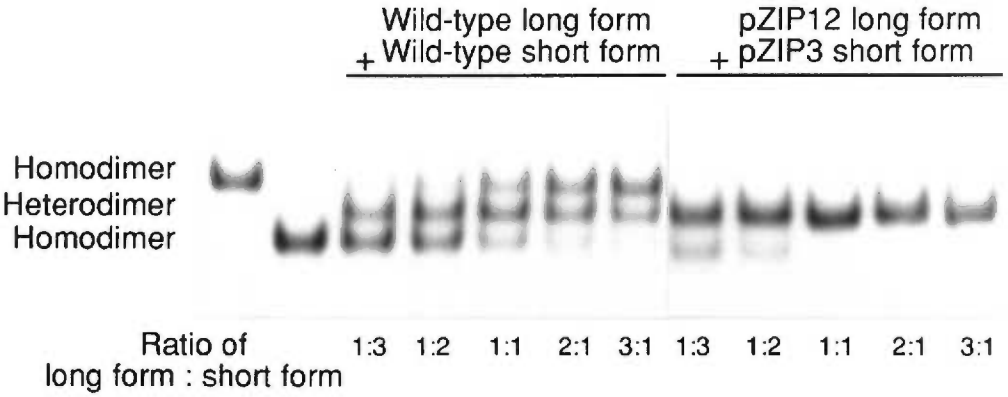

Wild-type long form
+ Wild-type short form
pZIP12 long form
+ pZIP3 short form
Homodimer
Heterodimer
Homodimer
Ratio of
long form : short form
1:3
1:2
1:1
2:1
3:1
1:3
1:2
1:1
2:1
3:1

Fig. 2 Binding properties of mutant CREB proteins. A) Helical wheel diagrams showing the mutated amino acid residues (bold letters). In the pZIP12 leucine zipper homodimer (top), a negatively charged glutamate (E) replaces the arginine (R) at the **g1** position, and a lysine (K) replaces the glutamate at the **g3** position. These mutations should result in charge-charge repulsions in the homodimer forms. This homodimer should also be destabilized by the histidine (H) substitution at the **a3** position. In pZIP3 (bottom), a lysine substitution at the **e2** position and a glutamate substitution at the **e4** position destabilize the homodimer form. pZIP3:pZIP12 heterodimers (middle) regenerate the favorable electrostatic interactions of the wild-type proteins and contain an additional stabilizing asparagine (N)-histidine configuration at the **a3** position. Thus, this combination should be favored over either homodimer form. B) Gel mobility shift assays. To visualize the homodimer and heterodimer complexes, a 60 amino acid fragment was deleted from the activator domains of the wild-type CREB and pZIP3 isoforms. Proteins were mixed in the ratios shown and analyzed on 6% non-denaturing polyacrylamide gels. When equal amounts of the full-length and deleted wild-type CREB proteins were mixed, homodimers and heterodimers formed in a 1:2:1 ratio. In contrast, the mutated CREB proteins combined only as heterodimers when mixed in equimolar concentrations.

Figure 3

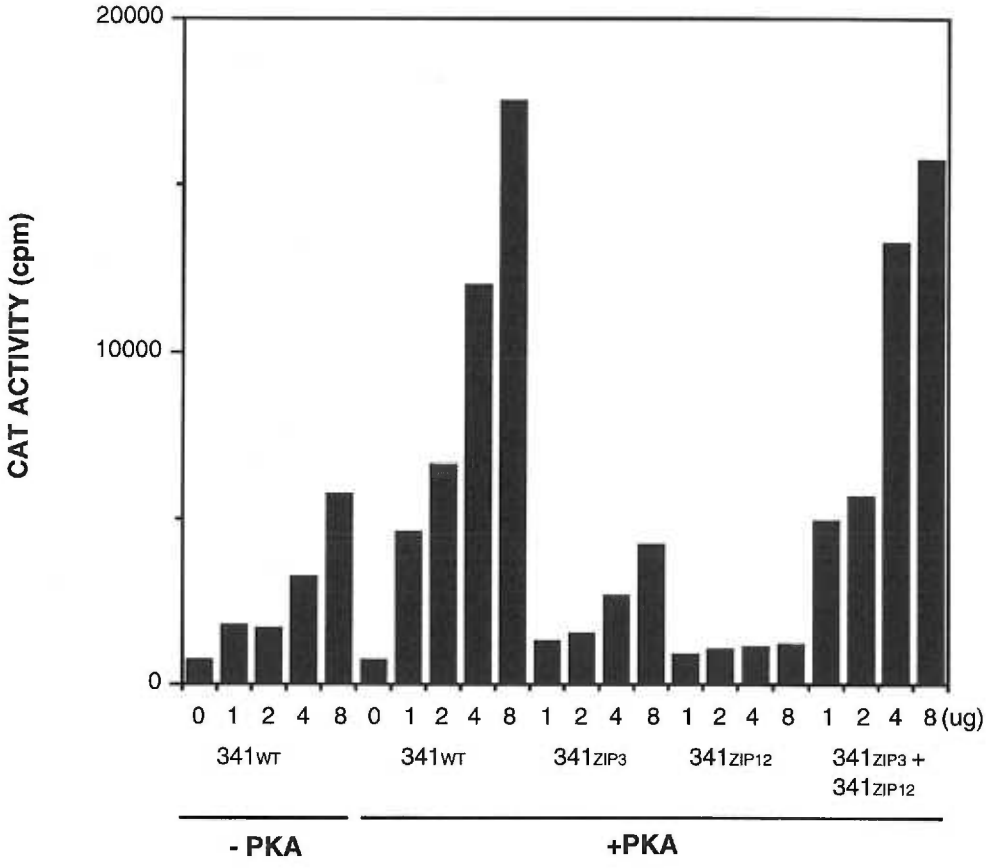

20000
10000
0
CAT ACTIVITY (cpm)
0 1 2 4 8 0 1 2 4 8 1 2 4 8 1 2 4 8 1 2 4 8 (ug)
341WT
341WT
341ZIP3
341ZIP12
341ZIP3 + 341ZIP12
- PKA
+PKA

Fig. 3 Biological activities of CREB leucine zipper mutants. CREB 341 plasmids containing wild-type, ZIP3, or ZIP12 leucine zippers were introduced into F9 teratocarcinoma cells along with 5 ug somatostatin-CAT reporter and 5 ug PKA expression vector as indicated. CAT activity is normalized to luciferase expression. Numbers refer to the total amount of CREB plasmid (in micrograms) in each transfection. Mixtures of CREB vectors containing the complementary leucine zipper mutations reconstitute wild-type activity.

Figure 4

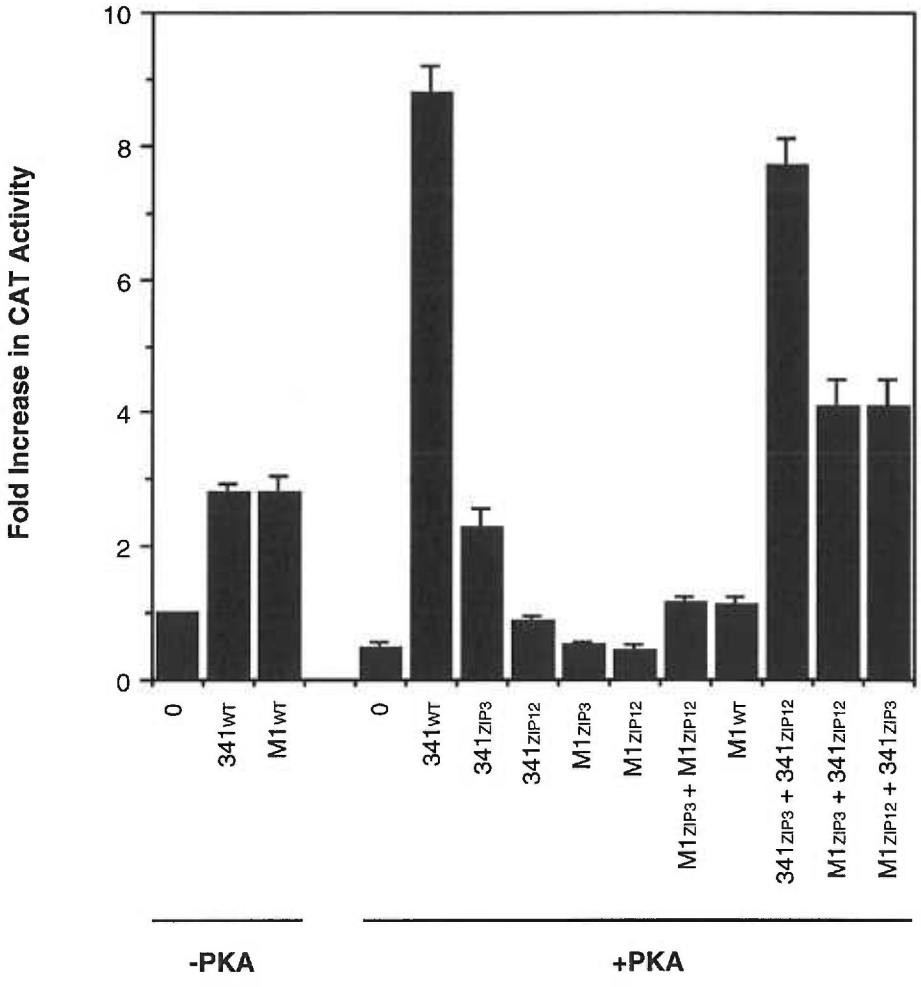

Fold Increase in CAT Activity
10
8
6
4
2
0
0
341WT
M1WT
0
341WT
341ZIP3
341ZIP12
M1ZIP3
M1ZIP12
M1ZIP3 + M1ZIP12
M1WT
341ZIP3 + 341ZIP12
M1ZIP3 + 341ZIP12
M1ZIP12 + 341ZIP3
-PKA
+PKA

Fig. 4 Transcriptional activity of hemiphosphorylated CREB complexes. F9 cells were transiently transfected with a somatostatin-CAT reporter and a mixture of CREB expression vectors. 341 and M1 refer to CREB isoforms containing either a wild-type serine or mutant alanine at the PKA site within the activator domain. Subscripts WT, ZIP3, and ZIP12 refer to the wild-type or mutant leucine zipper domains described above. A total of 3 ug of CREB expression vector was used in all experiments except those denoted 0, in which the CREB vector was omitted. Experiments were performed in the absence or presence of 5 ug PKA expression vector as indicated and results were normalized to luciferase activity. Complexes containing only one intact PKA phosphorylation site were half as active as those containing two phosphorylation sites. Values (from five experiments) represent means $\pm$ S.E.

Chapter 2

Modulatory Function of CREB:CREMα Heterodimers Depends Upon CREMα Phosphorylation

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The abbreviations used are:
(CRE) cAMP-responsive element; (CREB) CRE-binding protein; (PKA) protein kinase A; (bZIP) basic/leucine zipper; (CREM) cAMP-responsive element modulator; (ATF-1) activating transcription factor-1; (CaM kinase) calcium/calmodulin-dependent kinase; (RSV) Rous sarcoma virus

Summary

The cAMP-responsive element (CRE) modulator protein CREMα has been proposed to be a negative regulator of the CRE-binding protein (CREB). Precisely how CREMα inhibits CREB function is unclear, however. CREMα and CREB have highly related structures, and both proteins bind to consensus CRE sequences with similar affinities. Furthermore, both proteins can be phosphorylated by the cAMP-dependent protein kinase A (PKA). Two models have been proposed to explain how CREMα could prevent the activation of genes by PKA-phosphorylated CREB: inhibitory CREMα homodimers could prevent occupancy of the CRE by CREB, or CREMα could block gene activation by forming nonfunctional CREB:CREMα heterodimers. To determine whether CREB:CREMα heterodimers are indeed nonfunctional, we engineered the leucine zipper regions of the two proteins to direct the pattern of dimerization. We then tested the biological activities of the phosphorylated and non-phosphorylated complexes in *in vivo* transcription assays. Our results indicate that CREMα can contribute to PKA-mediated gene activation when selectively heterodimerized with CREB. Furthermore, this transcriptional activity depends upon the ability of the complexes to be phosphorylated by PKA.

Introduction

Of the numerous CRE-binding proteins, CREB, CREM, and ATF-1 appear to belong to a distinct subclass. Isoforms of these three transcription factors can be activated by protein kinase A (PKA) and potentially by calcium/calmodulin-dependent kinases such as calmodulin kinases II and IV (1-5). In CREB, ATF-1, and some isoforms of CREM, the phosphorylation sites are flanked by one or two glutamine-rich regions, termed Q1 and Q2, which have been proposed to be essential for gene activation (6). CREMα, on the other hand, is thought to regulate the CRE in a negative manner, and this inhibitory function has been attributed to the fact that it lacks either of the glutamine-rich regions (7). CREB, CREM, and ATF-1 can dimerize with each other through a highly conserved leucine zipper region. Thus, in addition to CREB, CREM, and ATF-1 homodimers, evidence exists for CREB:CREM and CREB:ATF-1 heterodimers as well (8,9). The finding that targeted mutations in the CREB gene produce no obvious phenotypic effects in transgenic mice supports the hypothesis that some of the properties of this class of CRE-binding proteins are redundant (10). The up-regulation of CREM mRNA in these mice suggests that this factor may be able to subserve functions normally fulfilled by CREB.

Our studies addressed the modulation of the activating transcription factor CREB by the negative factor CREMα. It has been proposed that CREMα inhibits CREB function by forming inactive CREB:CREM heterodimers (9,11). A significant problem in examining the properties of such heterodimers, however, is that each of these individual proteins readily homodimerizes. Thus, it is difficult to determine the biological activity of the heterodimeric complex alone. We have previously designed a set of specific leucine zipper mutations in the CREB molecule that selectively direct heterodimer formation (12). Here, we use a similar strategy to direct the formation of CREB:CREMα heterodimers. This approach allowed us to test how CREMα could

modulate the activity of CREB:CREM heterodimeric complexes. Our results show that CREB:CREMα heterodimers can be transcriptionally active dependent upon the ability of CREMα to be phosphorylated.

Materials and Methods

Plasmid construction and expression - To minimize potential differences in protein translation, CREMα and CREB expression plasmids both contained the 5'- and 3'-untranslated sequences from the CREB 341 cDNA (13). In addition, the b/ZIP regions of both proteins were encoded by the CREB cDNA sequence to minimize potential differences in protein stability. To construct the CREM expression plasmids, the Sma1 site in the DNA-binding region of the CREMα coding sequence (kindly provided by P. Sassone-Corsi, INSERM) was mutated to an Xho1 site using site-directed mutagenesis. Mutagenesis was carried out as described (altered Sites *in vitro* mutagenesis system protocol, Promega). An Nco1/Xho1 fragment encoding the amino terminal portion of CREMα was then subcloned into an Rc-RSV mammalian expression vector (Invitrogen) containing the 5'-untranslated portion of the CREB 341 cDNA and mutant or wild-type CREB leucine zipper sequence. The construction of the CREB expression plasmids and mutant leucine zippers has been described previously (12). To construct epitope tagged expression vectors, the oligomer 5'-CATGGACTACAAAGACGATGACGATAAAGG-3' and its complementary strand 5'CATGCCTTTATCGTCATCGTCTTTGTAGTC-3' were annealed and ligated into the 5'-Nco1 site in the CREMα and CREB protein coding sequences. This sequence encodes the polypeptide MetAspTyrLysAspAspAspAspLys which is recognized by the anti-FLAG M2 monoclonal antibody (IBI) (14). The plasmids RSV-luciferase (15) and RSV-PKA (16) were obtained from M. Wilkinson and R. Maurer (Oregon Health Sciences University), respectively. The plasmid SS-CAT, containing sequences of the rat somatostatin gene from -71 to +53 placed 5' to the coding region of chloramphenicol acetyltransferase, was constructed previously (17).

Bacterial expression of recombinant CREB proteins - pET-11d plasmids encoding wild-type and mutant forms of CREB and CREMα were transformed into the BL21(DE3) strain of *Escherichia coli.* Protein extracts were prepared as described previously (12). The concentration of recombinant CREB or CREMα in each extract was determined by densitometric analysis of samples electrophoresed on SDS-PAGE gels stained with Fast Stain (Zoion Research, Inc.).

Gel mobility shift assays - Mixtures of extracts were incubated at 37°C for 15 minutes, added to a 20 ul reaction buffer (10 mM Tris (pH 7.5), 50 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 1 ug poly(di-dC) and 3.9 ug bovine serum albumin), and incubated at 37°C with 15 fmol of end-labeled somatostatin CRE oligonucleotide. Reactions were loaded onto a 6% non-denaturing acrylamide gel and were electrophoresed at 200 volts for 10 hours. Gels were dried and autoradiographed using Kodak XAR film.

Cell culture and transfection assays - The transfection of F9 teratocarcinoma cells has been described previously (12). Briefly, DNA was transfected using the TRANSFINITY calcium phosphate transfection system (Life Technologies, Inc.) in the following amounts: 5 ug SS-CAT, 2 ug RSV-luciferase, and the indicated amounts of RSV-PKA, and RSV wild-type and mutant CREB vectors. The total DNA in each transfection was 20 ug, the balance consisting of pRc-RSV. CAT activity was determined by the method of Seed and Sheen (18) and was normalized to luciferase activity (15) as a control for transfection efficiency.

Western blot analysis - $1.0x10^6$ F9 embryocarcinoma cells were plated onto 10-cm dishes 18 hours before transfection. The cells were then transfected with a total of 30 ug of CREB and/or CREMα expression plasmid DNA. Cells from five plates were

pooled in 1 ml of phosphate buffered saline, pelleted, and resuspended in 100 ul of a solution containing 50 mM dithiothreitol and 50 mM EDTA in phosphate buffered saline. Cells were lysed by sonication and debris was cleared by centrifugation after the addition of 10 ul of 10% Triton X-100. The cleared supernatant was heated to 72°C for 2 minutes, and the precipitated proteins were again cleared by centrifugation. The proteins in 100 ul of each extract were separated on 12% SDS-polyacrylamide gels and electroblotted onto nitrocellulose filter paper (Schleicher & Schuell). The filters were blocked, incubated in a 1:300 dilution of the anti-FLAG M2 monoclonal antibody, washed, and incubated with a 1:10,000 dilution of alkaline phosphatase conjugated goat anti-mouse monoclonal antibody (Novagen). Immune complexes were visualized using the alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate (Zymed Laboratories, Inc.), as described in the manufacturer's protocol.

Results and Discussion

We have shown previously that the leucine zipper region of CREB can be mutated to generate proteins that preferentially associate as heterodimers (12). A similar strategy was used to construct the chimeric proteins utilized in the current experiments. Mutations in the **a3**, **e2**, **e4**, **g1**, and **g3** positions of the CREB leucine zipper were introduced to promote specific patterns of heterodimerization (Fig. 1A). The histidine mutation introduced into the **a3** position is believed to destabilize the formation of homodimers by charge repulsion or steric hindrance (12). Additionally, the histidine/asparagine heterodimer may be stablized by additional hydrogen bond and van der Waals contacts. Mutations in the **e** and **g** positions promote heterodimer formation by allowing complementary electrostatic interactions (12). Fragments containing these mutations were fused onto the CREB and CREM activation domains. The binding properties of the mutated proteins were confirmed by analyzing bacterially expressed products in electrophoretic gel mobility shift assays. The distinct sizes of the CREB and CREMα proteins allow the homo- and heterodimeric complexes to be distinguished easily in these assays. As indicated in Fig. 1B, wild-type CREB and CREMα produce two homodimeric complexes and one intermediate-sized heterodimer when mixed in a 1:1 ratio. CREMα and CREB containing the mutant leucine zippers, $CREM_{ZIP12}$ and $CREB_{ZIP3}$ respectively, combine exclusively as heterodimers when equimolar amounts of the two proteins are mixed. A small amount of the homodimeric forms of CREB or CREMα can be seen only when an excess of one or the other protein is added.

Functional analysis of the chimeric CREB and CREM proteins was performed by introducing expression vectors encoding these factors into F9 teratocarcinoma cells along with a somatostatin CRE-chloramphenicol acetyl transferase reporter gene and an expression vector encoding the catalytic subunit of PKA. These cells were chosen because they contain only low levels of functionally active PKA and CREB (19). Full

activity of the reporter gene in F9 cells requires the addition of both CREB and PKA, as reported previously (1, 19) and shown in Fig. 2A. The transcriptional activities of CREB constructs containing either of the two CREB leucine zipper mutants, $CREB_{ZIP3}$ or $CREB_{ZIP12}$, were negligible when expression vectors encoding these proteins were introduced independently into F9 cells, even in the presence of PKA (Fig. 2A). These studies indicate that neither of the non-dimerizing subunits are transcriptionally active alone, confirming our previous results (12). Mixing the $CREB_{ZIP3}$ and $CREB_{ZIP12}$ vectors restored wild-type activity, as would be expected if the complementary leucine zipper mutations allow approximately normal levels of dimerization. CREM expression vectors containing either wild-type or mutated leucine zippers did not promote PKA-mediated transcription of the reporter (Fig 2B), as shown previously (9,11).

Subsequent experiments used the same DNA-binding domain and leucine zipper mutations to examine the transcriptional properties of CREB:CREMα heterodimers (Fig. 2C). These experiments indicated that CREB:CREMα heterodimers had about one-third the activity of CREB homodimers, but were clearly more active than the CREM homodimeric forms (Fig. 2B). To insure that the two proteins were expressed equally in these assays, epitope-tagged forms of CREMα and CREB were constructed. Expression vectors encoding the two epitope-tagged proteins were mixed, introduced into F9 cells by calcium phosphate precipitation, and the gene products were quantitated by Western blotting. Fig. 3 shows the relative amounts of CREMα and CREB in cells transfected simultaneously with expression vectors encoding each protein. The levels of epitope-tagged CREB and CREMα expressed were approximately equal, although there was a slight excess in this experiment of CREB over CREM.

To confirm that the activities of the CREB:CREMα heterodimers did not reflect the substoichiometric expression of the CREMα component, we performed a CREMα titration experiment. Various amounts of expression plasmid encoding $CREM_{ZIP3}$ or $CREM_{ZIP12}$ were cotransfected with 1.5 ug of expression plasmid encoding the

complementary CREB form (Fig. 4). The levels of activation did not change when the amount of CREMα vector was doubled, indicating that a 1:1 ratio of the two vectors produces a maximal response. Presumably, the activity does not decrease when 3.0 ug of CREMα expression vector is transfected because the mutant leucine zippers do not allow the excess CREM proteins to homodimerize.

To determine how phosphorylation affected the activities of the transcription factor complexes, the PKA sites were mutated in selected CREB and CREM vectors. This mutation (designated M1) has been shown previously to block the PKA-dependent transcriptional activation mediated by CREB (1). As shown in Fig. 5, the M1-CREB homodimer was completely inactive in stimulating expression of the CRE-reporter. M1-CREM was also transcriptionally inactive. The hemiphosphorylated $CREB_{ZIP3}$:M1-$CREB_{ZIP12}$ and $CREB_{ZIP12}$:M1-$CREB_{ZIP3}$ complexes were half as active as the doubly-phosphorylated CREB complexes, as noted previously (12). Activities of the hemiphosphorylated CREB:CREMα dimers were negligible, regardless of which subunit, CREMα or CREB, contained an intact PKA site.

In summary, the ability to direct the pattern of association of specific CREB leucine zipper mutants has allowed us to analyze the biological properties of particular CREB family member heterodimers. As outlined in Fig. 6, the modulation of cAMP-regulated gene expression by CREB and CREMα is complex and depends, in part, on whether CREMα is capable of being phosphorylated by PKA. Our data indicate that the phosphorylation of at least one CREB subunit is required to mediate PKA-induction of a CRE reporter gene. Complexes containing one phosphorylatable CREB subunit have variable activities, however, and the level of activity appears to be determined by the identity and phosphorylation status of the dimerizing component. For example, the doubly-phosphorylated CREB:CREMα heterodimer is considerably more active than the CREB:M1-CREM dimer and slightly less active than the hemiphosphorylated CREB homodimer. Thus, phosphorylatable CREMα is almost

equivalent to non-phosphorylatable CREB when paired with a wild-type CREB subunit, and non-phosphorylatable CREMα is a dominant negative. These findings indicate that the patterns of heterodimerization and phosphorylation of just two CRE-binding proteins can provide multiple levels of transcriptional control.

A further level of transcriptional control, not addressed in the current studies, could be provided by modulating the amount of CREMα in the cell. Certain CREM mRNA levels are themselves regulated by cAMP (20), so the ratios of CREB and CREM are likely to be dynamic. Because even fully phosphorylated CREMα homodimers do not induce reporter expression, such homodimers could block gene activation by preventing the binding of CREB to the CRE.

Two models can be proposed to explain the mechanism of CREB transcriptional activation by PKA. One idea is that phosphorylation-induced intramolecular changes activate CREB by exposing a glutamine-rich region (6). Accordingly, phosphorylation of one CREB molecule of a dimer should be sufficient to induce activation. Our finding that CREB:M1-CREM heterodimers lack activity argues against this model. The other model involves domains from both monomers of the CREB dimer, *i.e.* an intermolecular activation, whereby the phosphorylation of serine 133 of one CREB subunit results, directly or indirectly, in the availability of the glutamine-rich region(s) of the other subunit. This model can explain the ability of CREMα to to contribute to gene activation and is consistent with our observation that only the fully phosphorylated CREB:CREMα heterodimer is transcriptionally active. In such heterodimers, CREM may donate the phosphorylation domain, while CREB donates the glutamine-rich region. That M1-CREB:CREMα only minimally activates transcription can be ascribed to the different architectures of the 341-amino acid CREB and 289-amino acid CREM proteins. Clearly, these proteins may not present the same molecular faces to a phospho-CREB-binding protein. The mechanism of CREMα modulation of CREB function may be clarified by

testing how the various CREB:CREM heterodimers interact with the putative CREB co-activator CBP (21,22).

Acknowledgements

We thank R. Maurer and members of the Goodman laboratory for helpful discussions.

References

1. Gonzalez, G.A. and Montminy, M.R. (1989) *Cell* **59**, 675-680.
2. Sheng, M., Thompson, M.A. and Greenberg, M.E. (1991) *Science* **252**, 1427-1430.
3. Dash, P.K., Karl, K.A., Colicos, M.A., Prywes, R. and Kandel, E.R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5061-5065.
4. de Groot, R.P., den Hertog, J., Vandenheed, J.R., Goris, J. and Sassone-Corsi, P. (1993) *EMBO* **12**, 3903-3911.
5. Enslen, H., Sun, P., Brickey, D., Soderling, H.S., Klamo, E., Soderling, T.R. (1994) *J. Biol. Chem.* **269**, 15520-15527
6. Gonzalez, G.A., Menzel, P., Leonard, J., Fischer, W. and Montminy, M.R. (1991) *Mol. Cell. Biol.* **11**, 1306-1312.
7. Foulkes, N.S., Mellstrom, B., Benusiglio, E. and Sassone-Corsi, P. (1992) *Nature* **355**, 80-84.
8. Foulkes, N.S., Borelli, E. and Sassone-Corsi, P. (1991) *Cell* **64**, 739-749.
9. Rehfuss, R.P., Walton, K.M., Loriaux, M.M. and Goodman, R.H. (1991) *J. Biol. Chem.* **266**, 18431-18434.
10. Hummler, E., Cole, T.J., Blendy, J.A., Ganss, R., Aguzzi, A., Schmid, W., Beermann, F. and Schutz, G. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 5647-5651.
11. Foulkes, N.S., Laoide, B.M., Schlotter, F. and Sassoni-Corsi,P. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5448-5452.
12. Loriaux, M.M., Rehfuss, R.P., Brennan, R.G. and Goodman, R.H. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9046-9050.
13. Gonzalez, G.A., Yamamoto, K.K., Fisher, W.H., Karr, D., Menzel, P., Biggs III, W., Vale, W.W. and Montminy, M.R. (1989) *Nature* **337**, 749-752.
14. Hopp, T.P., Prickett, K.S., Price, V., Libby, R.T., March, L.J., Cerretti, P., Urdal, D.L., Conlon, P.J. (1988) *Bio/Technology* **6**, 1205-1210

15. de Wet, J.R., Wood, K.V., De Luca, M., Helinski, D.R., Subramani, S. (1987) *Mol. Cell. Biol.* **7**, 725-737.

16. Maurer, R.A. (1989) *J. Biol. Chem.* **264**, 6870-6873.

17. Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. and Goodman, R.H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6682-6686.

18. Seed, B. and Sheen, J-Y. (1988) *Gene* **67**, 271-277.

19. Masson, N., Ellis, M., Goodbourn, S. and Lee, K.A.W. (1992) *Mol. Cell. Biol.* **12**, 1096-1106.

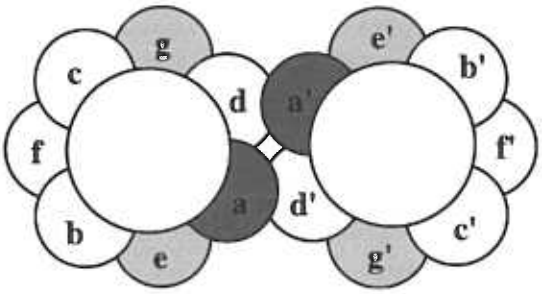
20. Molina, C.A., Foulkes, N.S., Lalli, E. and Sassoni-Corsi, P. (1991) *Cell* **75**, 875-886.

21. Chrivia, J.C., Kwok, R.P.S., Lamb, N., Hagiwara, M., Montminy, M.R. and Goodman, R.H. (1993) *Nature* **365**, 855-859.

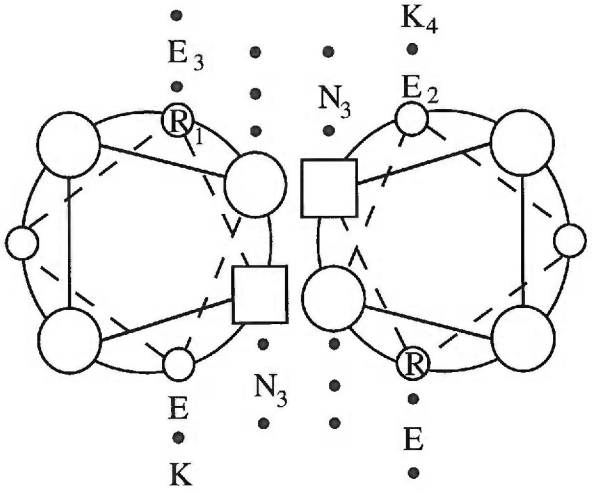
22. Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R. and Goodman, R.H. (1994) *Nature* , in press.

23. O'Shea, E.K., Klemm, J.D., Kim, P.S. and Alber, T. (1991) *Science* **254**, 539-544.

Figure 1A



Wild-type CREB homodimer



pZIP12 +pZIP3 leucine zipper heterodimer

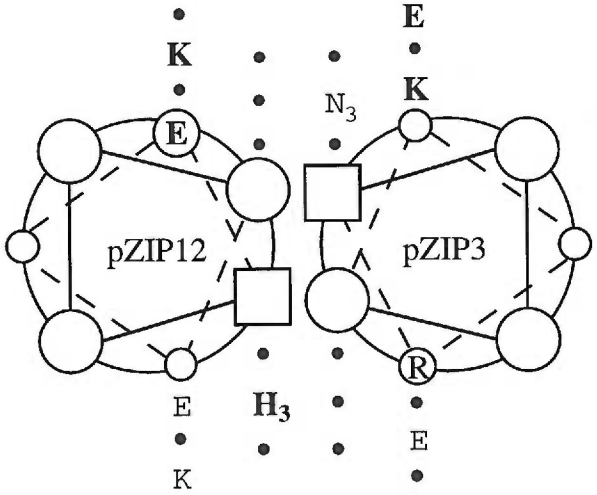


Figure 1B

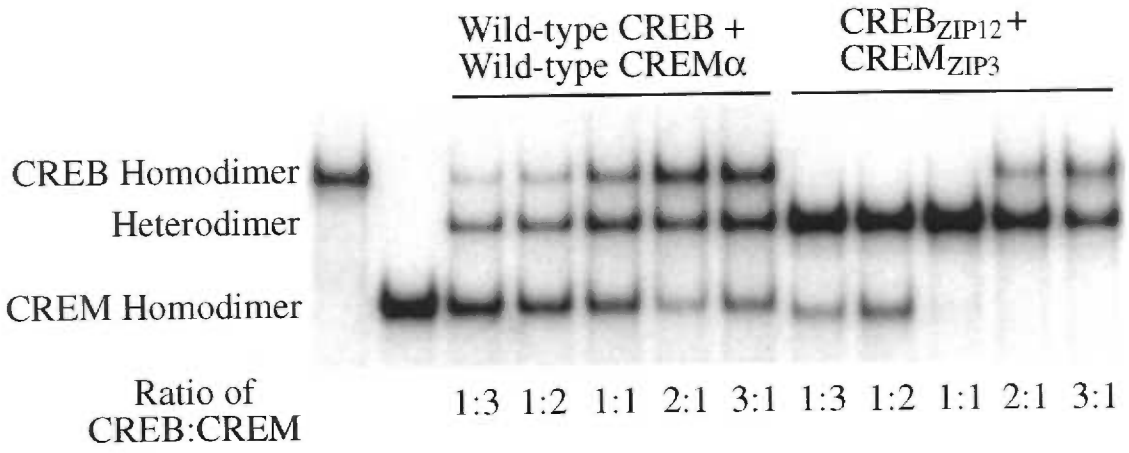

Wild-type CREB +
Wild-type CREMα
CREB$_{ZIP12}$+
CREM$_{ZIP3}$
CREB Homodimer
Heterodimer
CREM Homodimer
Ratio of
CREB:CREM
1:3 1:2 1:1 2:1 3:1 1:3 1:2 1:1 2:1 3:1

Figure 1

(*A*) (**Top**) End-view of parallel α-helixes in a dimerized leucine zipper. Residues in the **a** and **d** positions form the hydrophobic dimerization interface. (**Middle**) Helical-wheel representation of a wild-type CREB leucine zipper dimer. Electrostatic interactions between oppositely charged residues in the **g1** and **e2** positions and between residues in the **g3** and **e4** positions are predicted to stabilize the leucine zippper dimer (23). The asparagine residue (N3) within the dimerization interface may serve to destabilize and align the leucine zippers (23). The residues indicated were mutated to form two leucine zippers, each of which is unable to homodimerize but which can selectively heterodimerize. (**Bottom**) Helical wheel diagrams showing the substituted amino acid residues (boldface letters). In the pZIP12 leucine zipper, a negatively charged glutamate (E) replaces the arginine (R) at the **g1** position, and a lysine (K) replaces the glutamate at the **g3** position. These mutations should result in charge-charge repulsions in the homodimer forms. Homodimers should also be destabilized by the histidine (H) substitution at the **a3** position. In pZIP3, a lysine substitution at the **e2** position and a glutamate substitution at the **e4** position destabilize the homodimer form. pZIP3:pZIP12 heterodimers regenerate the favorable electrostatic interactions of the wild-type proteins and contain an additional stabilizing asparagine (N)-histidine configuration at the **a3** position.

(*B*) Gel mobility shift assay showing the relative amounts of CREB and CREMα homodimers and CREB:CREMα heterodimers in mixtures of the two proteins. The size difference between CREB and CREMα permits visualization of the intermediate-sized heterodimer complexes. The first and second lanes show wild-type CREB and wild-type CREMα homodimers, respectively. The next five lanes show various ratios of CREB and CREMα. Both CREB and CREMα contain the leucine zipper region of wild0type CREB to allow direct comparison with proteins containing mutant leucine zippers. The

last five lanes show varios ratios of $CREB_{ZIP12}$ and $CREM_{ZIP3}$. When equal amounts of wild-type CREB and CREMα are mixed, both homo- and heterodimers formed. In contrast, only heterodimer complexes form in equimolar mixtures of $CREB_{ZIP12}$ and $CREM_{ZIP3}$.

Figure 2

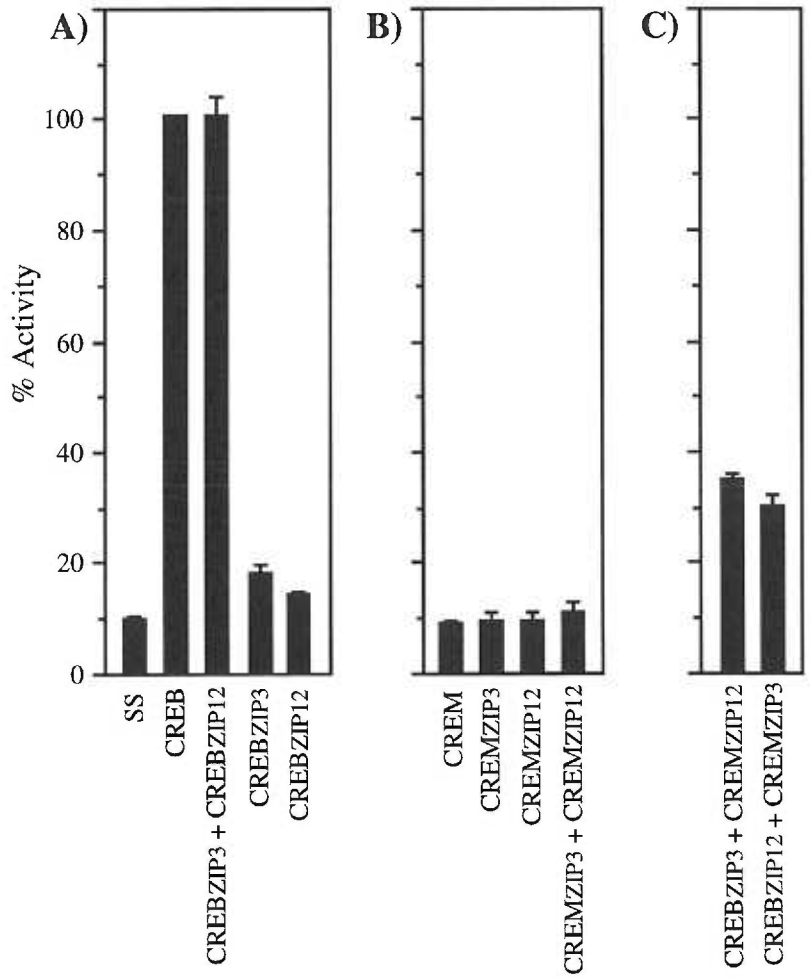

A)
B)
C)
% Activity
0
20
40
60
80
100
SS
CREB
CREBZIP3 + CREBZIP12
CREBZIP3
CREBZIP12
CREM
CREMZIP3
CREMZIP12
CREMZIP3 + CREMZIP12
CREBZIP3 + CREMZIP12
CREBZIP12 + CREMZIP3

Figure 2

Transfection experiments comparing the activities of CREB and CREMα homodimers and heterodimers. In each experiment, F9 cells were transfected with 5 ug of somatostatin CRE-CAT reporter, 5 ug PKA expression vector, and a total of 4 ug of CREB and/or CREMα expression vector as indicated. (A) Mixtures of $CREB_{ZIP12}$ and $CREB_{ZIP3}$ expression vectors reconstitute the transcriptional activty of CREB 341 expression vector alone. The $CREB_{ZIP12}$ expression vector transfected individually have little transcriptional activity. $CREB_{ZIP3}$ has a low level of activity that probably reflects residual homodimer formation (12). (B) Transfections of wild-type and mutant CREM expression vectors show background levels of activity. (C) Mixtures of CREB and CREM vectors containing complementary leucine zipper mutations have 30-40% of the transcriptional activity of wild-type CREB complexes. Values (from 5 experiments) represent means ± S.E.

Figure 3

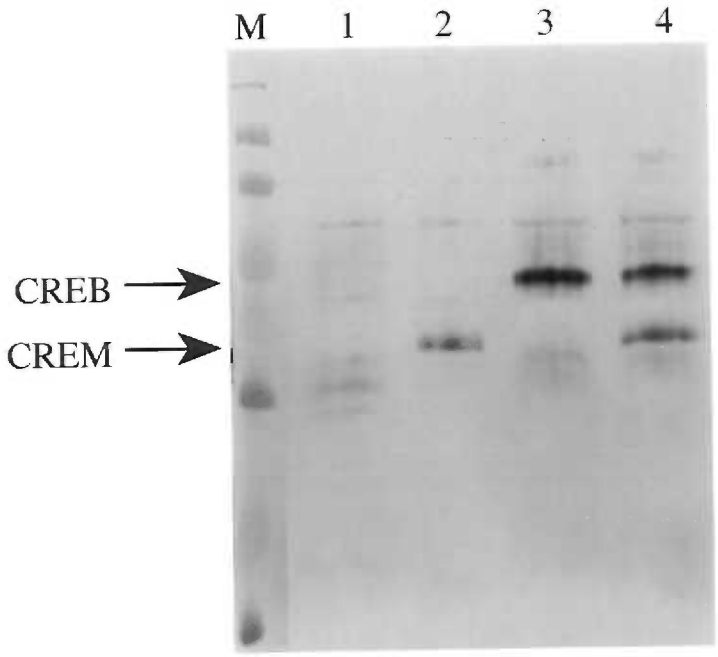


Figure 3

Western blot analysis showing the relative amounts of epitope-tagged CREB and CREM α in transiently transfected cells. Lane 1, extract from untransfected F9 cells. Lanes 2 and 3, extracts from cells transfected with 30 ug of CREMα and CREB expression vectors, respectively. Lane 4, relative amounts of CREMα and CREB expressed in cells transfected with 15 ug of each vector. Lane M, molecular weight markers.

Figure 4

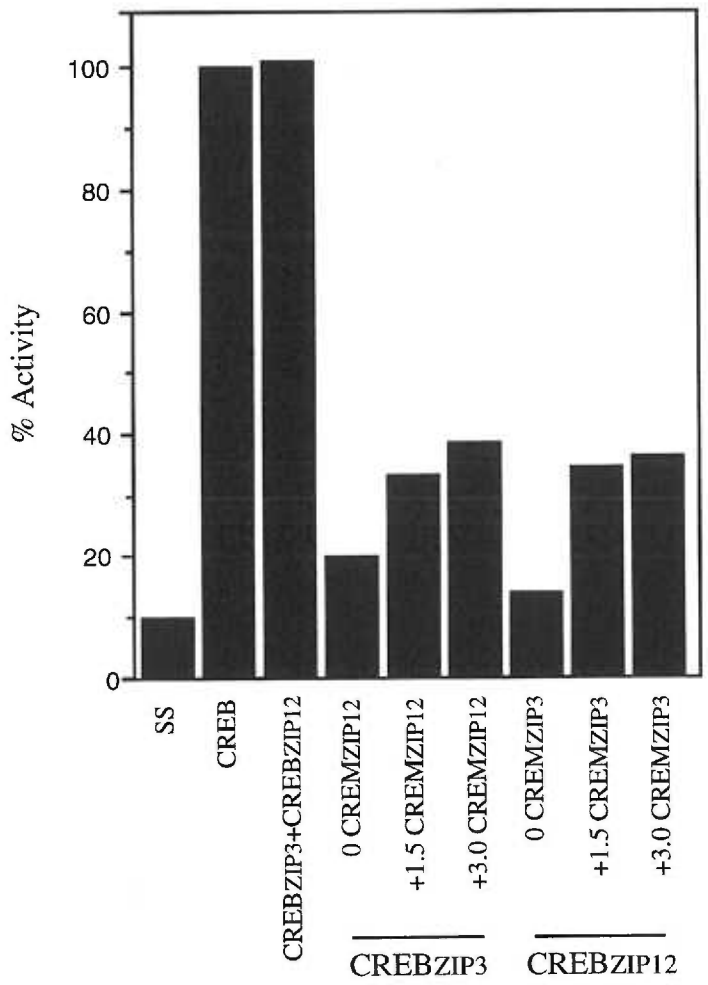

% Activity
100
80
60
40
20
0
SS
CREB
CREBZIP3+CREBZIP12
0 CREMZIP12
+1.5 CREMZIP12
+3.0 CREMZIP12
0 CREMZIP3
+1.5 CREMZIP3
+3.0 CREMZIP3
CREBZIP3
CREBZIP12

Figure 4

Titration of CREB:CREMα heterodimers. Increasing amounts of expression vector encoding $CREM_{ZIP3}$ or $CREM_{ZIP12}$ were transfected with 1.5 ug of vector encoding the indicated CREB expression vector. A maximal response occurs in transfections containing equal amounts CREMα and CREB vectors. Each transfection contains 5 ug PKA expression vector and 5 ug of somatostatin CRE-CAT reporter. The transfections labeled CREB and $CREB_{ZIP3}$+$CREB_{ZIP12}$ contain a total of 3 ug of CREB expression plasmid. These results repesent the averages of duplicate experiments.

Figure 5

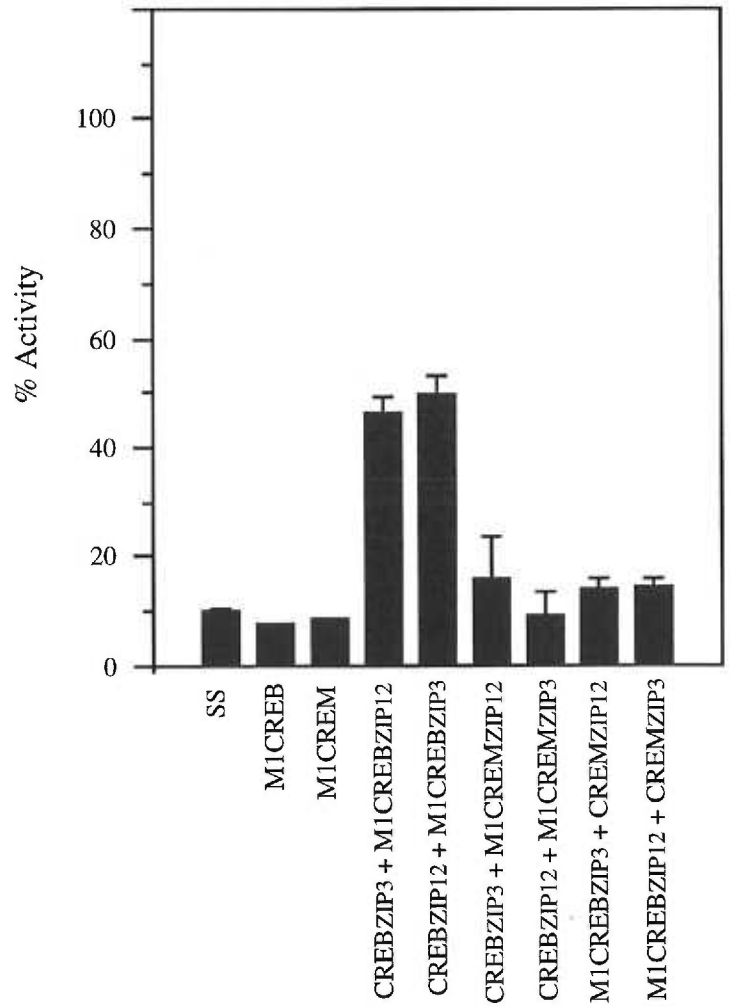

% Activity
0
20
40
60
80
100
SS
M1CREB
M1CREM
CREBZIP3 + M1CREBZIP12
CREBZIP12 + M1CREBZIP3
CREBZIP3 + M1CREMZIP12
CREBZIP12 + M1CREMZIP3
M1CREBZIP3 + CREMZIP12
M1CREBZIP12 + CREMZIP3

Figure 5

Transfection experiments comparing the activities of hemiphosphorylated CREB homodimers and CREB:CREMα heterodimers. 100% represents the activity of phosphorylated wild-type CREB homodimers. M1-CREB and M1-CREM isoforms contain a serine to alanine substitution within the PKA phosphorylation site. Each transfection contains 5 ug PKA expression vector, 5 ug of somatostatin CRE-CAT reporter, and a total of 4 ug of CREB and/or CREM expression vector. Values (from 5 experiments) represent means ± S.E.

Figure 6

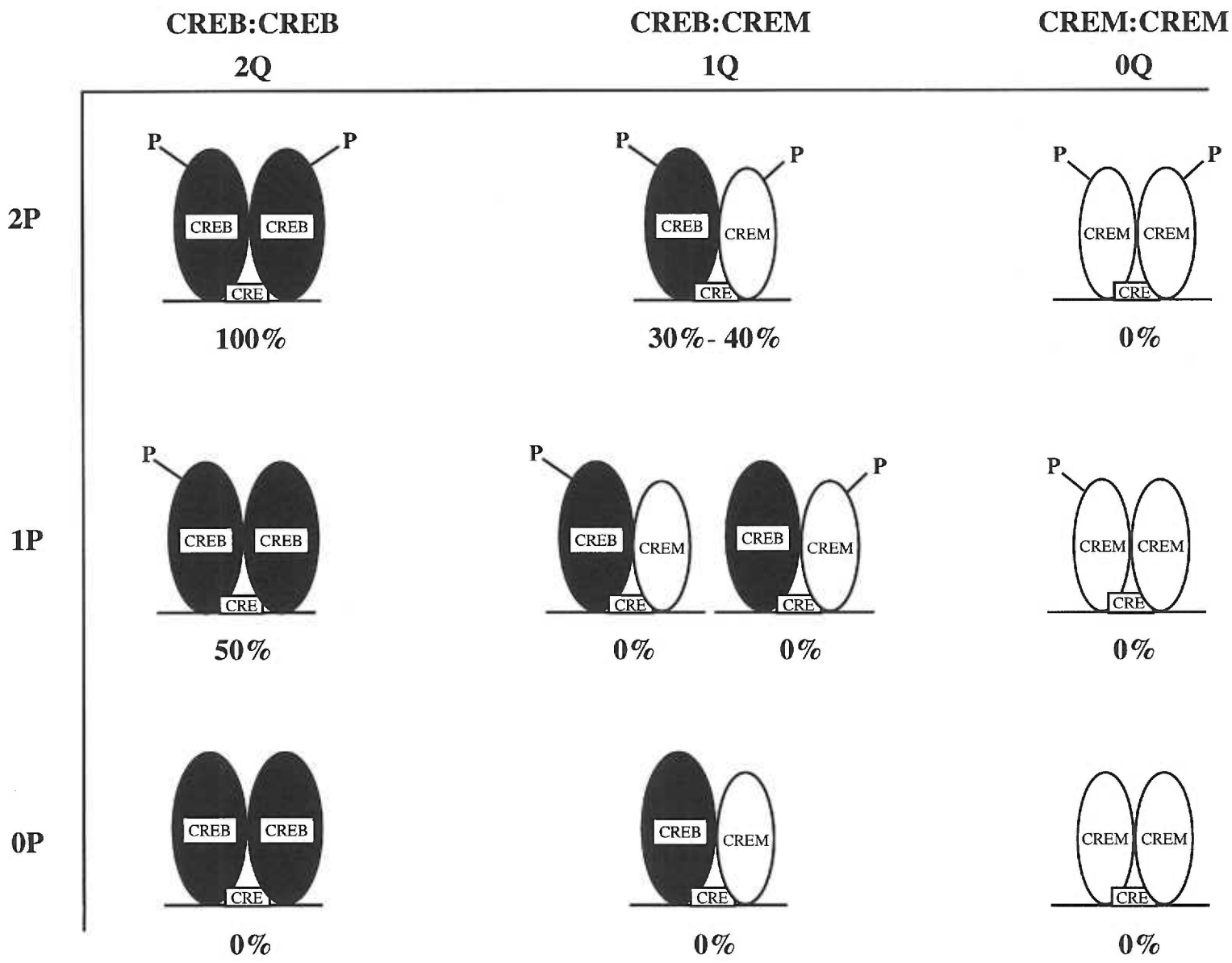

CREB:CREB
2Q
CREB:CREM
1Q
CREM:CREM
0Q
2P
1P
0P
P
CREB
CREM
CRE
100%
30%- 40%
0%
50%
0%
0%
0%
0%
0%

Figure 6

Relative activities of CREB and CREMα homodimers and heterodimers. The number of potential PKA phosphorylation sites (P) in each dimer is indicated on the left. The number of Q2 glutamine-rich regions (Q) is indicated across the top.

Chapter 3

Transcription Activation by Hemiphosphorylated CREB Dimers Bound to DNA is Independent of Binding Orientation

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The abbreviations used are:

(CRE) cAMP-responsive element; (CREB) CRE-binding protein; (PKA) protein kinase A; (bZIP) basic/leucine zipper; (CREM) cAMP-responsive element modulator; (ATF-1) activating transcription factor-1; (RSV) Rous sarcoma virus

Summary

Viral and cellular enhancers are thought to function in an orientation and position independent manner. However, heterodimeric transcription factor complexes that bind to certain enhancer sequences provide distinct activation domains which may interact differentially with other proteins required for gene transcription. Depending on the orientation in which these complexes bind DNA, different activation domains may be accessible to the general transcription machinery. Recently, transcriptional activation by a number of bacterial and eukaryotic transcription factor complexes has been shown to be orientation dependent. We have previously shown that CREB dimers phosphorylated on a single subunit are half as active as fully phosphorylated complexes (Loriaux, 1993). Because CREB binds to a symmetrical cAMP-responsive element (CRE), hemiphosphorylated complexes can bind in two orientations. Only one of these orientations may be functional. To test this hypothesis, we have developed a strategy for directionally orienting hemiphosphorylated CREB complexes bound to DNA. Our results show that hemiphosphorylated CREB heterodimers are able to activate transcription in either orientation.

Introduction

Short, *cis*-acting DNA sequences increase basal and regulated transcription from cellular and viral promoters. Termed enhancers, these sequences are thought to function in an orientation and position independent manner (Banerji, 1981; Goodbourn, 1985; Treisman, 1985). Two 72 base pair repeats in the SV40 late gene promoter, for example, function equally well, in either orientation, 1400 base pairs upstream or 3300 base pairs downstream from a heterologous β-globin promoter (Banerji, 1981). The enhancer sequences in SV40 were subsequently shown to consist of overlapping and inverted DNA repeats (5'-TGACTCA-3') (Angel, 1987; Lee, 1987). AP-1, the heterodimeric transcription factor complex that binds to this sequence (Lee, 1987), consists of the oncogene products Jun and Fos. Each subunit binds to one repeat or half-site, and there is no preferential orientation of the heterodimer on the AP-1 DNA site (Glover, 1995). The Fos:Jun heterodimer, however, is asymmetric and provides distinct activation domains which may interact differentially with other proteins involved in transcription. The orientation of the Fos:Jun heterodimer may be determined by the interactions with these proteins.

Recent evidence indicates that the orientation of dimeric transcription factor complexes that bind to enhancer sequences can be an important determinant in the activation of transcription. A bacterial protein, the catabolite gene activator protein (CAP), can activate transcription in an orientation dependent manner (Zhou, 1993). Heterodimers consisting of active and inactive CAP subunits activate transcription only when the intact activation domain is oriented toward the promoter. The transcriptional activity of properly oriented heterodimers is identical to that of wild-type dimers. Transcriptional activation by CAP, then, requires only one activation domain. A second bacterial activator, AraC, also activates transcription from only one orientation (Reeder,

1993). Unlike the CAP DNA binding site, AraC dimers bind to two direct repeats. Reversal of the promoter-proximal binding site does not allow gene transcription.

Orientation dependence is not limited to bacterial transcription factor/DNA complexes. Several mammalian transcription factors bind to enhancer sequences in an orientation dependent fashion. These proteins recognize direct repeats of the hormone-response element core half-site and include receptors for vitamin D3, thyroid hormone (TR), and retinoic acid (RAR) (Umesono, 1991). TR and RAR preferentially form heterodimers with the retinoid X receptor (RXR), and the interaction of the two receptors always places RXR in the 5' position on the direct DNA repeats (Perlman, 1993).

We have shown previously that hemiphosphorylated CREB complexes are half as transcriptionally active as fully-phosphorylated dimers (Loriaux, 1993). Two models may explain the half-maximal level of activity of the hemiphosphorylated CREB complexes. First, hemiphosphorylated complexes may associate less efficiently with other proteins that are required for transcriptional activation. For example, the binding of a co-activator or general transcription factor could be enhanced by the presence of a second phosphorylated CREB subunit. Second, complexes that are phosphorylated on a single subunit may function directionally. Because the CRE sequence is symmetrical, the hemiphosphorylated dimer can bind in two different orientations. It is possible that only one of these orientations is functional. Directional binding of transcription factors would provide additional diversity in gene regulation. To test the latter model, we have developed a strategy for directionally orienting hemiphosphorylated CREB complexes bound to DNA. Our results show that hemiphosphorylated CREB heterodimers are able to activate transcription in either orientation.

Materials and Methods

The construction of the CREB expression plasmids and mutant leucine zippers has been described previously (Loriaux, 1993). Plasmids encoding $CREB_{ZIP3}$ and $CREB_{ZIP12}$ proteins containing mutant DNA-binding domains were constructed using site-directed mutagenesis. Mutagenesis was carried out as described (altered Sites *in vitro* mutagenesis system protocol, Promega). We used the oligonucleotides 5'-CACTCTCGA GTTGTTTCCCTGTCCTTCATTAGAC-3', 5'-CGACACTCTCGAACTGCAATATTG TTCTTCTC TAGACGGACCCTATACTTTCGTGCTGC-3', and 5'-CGACACTCTC GATTTGCTTCCC-3' to generate the mutant DNA-binding domains DBD-1, DBD-2, and DBD-3, respectively. For gel shift analyses, CRE sequences specific for each DNA-binding domain were substituted for the somatostatin CRE in the oligonucleotide 5'-GA TCCTTGGCTGACGTCAGAGAGAGA-3' and its complementary strand 5'-GATCTCTC TCTCTGACGTCAGCCAAG-3'. Similar substitutions were made in the plasmid SS-CAT (Montminy, 1986) for transfection experiments. We replaced one or both half-sites of the somatostatin CRE with DBD-3 specific CRE half-sites using site-directed mutagenesis. The plasmids RSV-luciferase (de Wet, 1987) and RSV-PKA (Maurer, 1989) were obtained from M. Wilkinson and R. Maurer (Oregon Health Sciences University), respectively.

Bacterial expression of recombinant CREB proteins - pET-11d plasmids encoding wild-type and mutant forms of CREB were transformed into the BL21(DE3) strain of *Escherichia coli*. Protein extracts were prepared as described previously (Loriaux, 1993). The concentration of recombinant CREB in each extract was determined by densitometric analysis of samples electrophoresed on SDS-PAGE gels stained with Fast Stain (Zoion Research, Inc.).

Gel mobility shift assays - Mixtures of extracts were incubated at 37°C for 15 minutes, added to a 20 ul reaction buffer (10 mM Tris (pH 7.5), 50 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 1 ug poly(di-dC) and 3.9 ug bovine serum albumin), and incubated at 37°C with 15 fmol of end-labeled somatostatin or mutant CRE oligonucleotide. Reactions were loaded onto a 6% non-denaturing acrylamide gel and were electrophoresed at 200 volts for 10 hours. Gels were dried and autoradiographed using Kodak XAR film.

Cell culture and transfection assays - The transfection of F9 teratocarcinoma cells has been described previously (Loriaux,1993). Briefly, DNA was transfected using the TRANSFINITY calcium phosphate transfection system (Life Technologies, Inc.) in the following amounts: 5 ug SS-CAT, 2 ug RSV-luciferase, and the indicated amounts of RSV-PKA, and RSV wild-type and mutant CREB vectors. The total DNA in each transfection was 20 ug, the balance consisting of pRc-RSV. CAT activity was determined by the method of Seed and Sheen (Seed, 1988) and was normalized to luciferase activity (de Wet, 1987) as a control for transfection efficiency.

Results and Discussion

The strategy for directionally binding hemiphosphorylated CREB dimers on a CRE sequence is straightforward. CREB monomers with different DNA binding specificities are selectively heterodimerized using the mutant leucine zippers pZIP3 and pZIP12 (Loriaux, 1993). One CREB subunit is unaltered and specifically recognizes a wild-type CRE half-site. The other CREB monomer specifically recognizes a mutant CRE half-site. An asymmetric CRE which consists of one wild-type and one mutant half-site should bind to this heterodimer in a specific orientation.

We used two strategies for altering the DNA-binding specificity of CREB. First, we predicted that the DNA-binding domain of CREB may make many of the same protein-DNA contacts as GCN4. The crystal structure of the GCN4 DNA-binding domain/CRE complex shows that the residues that contact the DNA are highly conserved among all bZIP transcription factors (O'Shea, 1992 and Fig. 1). Fos:Jun heterodimers, for example, make similar DNA contacts (Glover, 1995). We reasoned that substitution of several conserved amino acids might allow the DNA-binding domain of CREB to make specific contacts with a different DNA sequence (Fig. 2). These substitutions were incorporated into a mutant CREB341 DNA-binding domain termed DBD-1. In DBD-1, we predicted that the van der Waals contact between the methylene carbon of alanine 297 (position -14 in Fig. 1) and the thymidine in the 2' position of the CRE half-site could be replaced by a hydrogen bond between a threonine 297 substitution and a cytidine at the 2' position. Similarly, asparagine 293-to-aspartate (position -18) and alanine 296-to-threonine (position -15) substitutions could generate a new hydrogen bond with a cytidine in position 4. The net effect of these substitutions would be to change the binding specificity of CREB from a 5'-TGAC-3' half-site to a 5'-CGGC-3' half-site.

Second, we predicted that substitutions that alter the binding specificity of GCN4 may also alter the DNA-binding specificity of CREB. Several DNA-binding domain substitutions in GCN4 alter its binding specificity. Suckow *et al.*, for example,

systematically replaced residues in the DNA-binding domain of GCN4 with those in the DNA-binding domain of C/EBP (Suckow, 1993a). Replacement of residues in the -17, -16 and -14 positions (Fig. 1) with those in C/EBP allowed GCN4 to bind specifically to the C/EBP binding site (5'-ATTGCGCAAT-3'). We introduced the same substitutions into the DNA-binding domain of CREB to alter its DNA-binding specificity. This mutant basic region was termed DBD-2. We also tested a single amino acid substitution, alanine 297-to-asparagine (position -14), that alters the binding specificity of GCN4 so that each subunit recognizes only a 5'-TCAC-3' half-site (Suckow, 1993b). This mutant basic region was termed DBD-3.

CREB proteins containing the mutant DNA-binding domains were electrophoresed with radiolabeled mutant and wild-type CREs on nondenaturing polyacrylamide gels. In these gel mobility shift experiments, CREB containing DBD-1 failed to bind both the wild-type CRE and the mutant CRE, 5'-CGGCGCCG-3' (results not shown). Either the predicted contacts were unable to form, or small changes in protein shape, which are predicted to contribute to determining binding specificity (Kim, 1993), were incompatible with these enhancer sequences. However, both mutant DNA-binding domains, DBD-2 and DBD-3, show altered DNA-binding specificities (Fig. 3). Figure 3A shows that CREB containing DBD-2 ($CREB_{DBD-2}$) can bind equally well to the wild-type CRE (lane 3) and to the C/EBP binding site (lane 4). This result is in agreement with previous experiments which have shown that C/EBP can bind to CRE sequences (Park, 1990; Kageyama, 1991; Liu, 1991; Vallejo, 1993). However, wild-type CREB not only binds to the somatostatin CRE (lane 1) but, at a lower affinity, to the C/EBP binding site (lane 2). This result was unexpected because previous reports have indicated that neither GCN4 (Suckow, 1993) nor CREB (Benbrook,1994) could bind to this sequence. Figure 3B shows a similar experiment except that CREB containing DBD-3 ($CREB_{DBD-3}$) and oligonucleotides containing the mutant CRE, 5'-TCACGTGA-3', are used. Lanes 3 and 4 show that the effect of the alanine-to-asparagine substitution at

position -14 is to broaden the DNA-binding specificity to include the mutant CRE. In this case, however, wild-type CREB is unable to bind to the mutant CRE (lane 2).

We then performed an additional gel-shift experiment to determine whether wild-type CREB and $CREB_{DBD-3}$ could form heterodimers on a hybrid CRE containing one consensus CRE half-site and one mutant half-site (5'-TGACGTGA-3') (Fig. 4). To visualize heterodimer complexes, wild-type full-length CREB was mixed with a truncated form of CREB containing either mutant or wild-type DNA-binding domains. Lane 1 shows an equimolar mixture of long and short forms of wild-type CREB incubated with a somatostatin CRE. Lanes 2 through 4 contain equimolar mixtures of wild-type CREB and truncated $CREB_{DBD-3}$ incubated with various CREs. A small amount of $CREB_{DBD-3}$ homodimer binds to the somatostatin CRE (lane 2) while wild-type CREB homodimers are completely unable to bind the mutant CRE (lane 3). Only a small amount of heterodimer is visible in these lanes presumably because only one subunit can bind with a high affinity to its complementary half-site. In contrast, heterodimers form preferentially on the hybrid CRE (lane 4). Two arguments suggest that heterodimers bind the hybrid CRE in a specific orientation. First, only one orientation allows each subunit to bind with a relatively high affinity to its complementary half-site. Second, wild-type CREB cannot bind to the mutant CRE.

We next determined the biological activities of directionally oriented hemiphosphorylated CREB dimers. The strategy for orienting the phosphate in a hemiphosphorylated complex with respect to the promoter can be divided into three steps. First, a nonphosphorylatable form of CREB, M1-CREB, was constructed by substituting serine 133 with alanine. This mutation has been used previously to establish the role of PKA phosphorylation in CREB activation (Gonzalez, 1989). Second, phosphorylatable and nonphosphorylatable forms of CREB are selectively heterodimerized using the mutant leucine zippers pZIP3 and pZIP12 described previously (Loriaux, 1993). Finally, the DNA-binding domain of one subunit of the heterodimer is

replaced with the mutant DNA-binding domain, DBD-3. The result is a hemiphosphorylated CREB complex that can bind to hybrid CRE in an orientation specific manner. F9 embryocarcinoma cells were transiently transfected with plasmids encoding the various mutant forms of CREB, the catalytic subunit of PKA, and RSV-luciferase (to control for transfection efficiency). To measure the transcriptional activity of CREB complexes, cells were also transfected with a somatostatin CRE-CAT reporter plasmid. The somatostatin CRE, however, was replaced with an asymmetric hybrid CRE inserted in each orientation. The results of this experiment are summarized in Figure 5. Four doubly phosphorylated complexes were formed by reversing the orientation of the hybrid CRE and by placing DBD-3 in $CREB_{ZIP3}$ or $CREB_{ZIP12}$. The reduced transcriptional activity of these complexes compared to that of wild-type CREB bound to the somatostatin CRE probably reflects the somewhat lower affinity of DBD-3 for its complementary half-site. Nevertheless, both possible hemiphosphorylated complexes were transcriptionally half as active as the corresponding doubly phosphorylated complex. For example, when $CREB_{ZIP12}$ containing DBD-3 is oriented toward the promoter, the doubly phosphorylated complex induces a 5.5 fold increase in transcription. The corresponding hemiphosphorylated complexes induce 2.5 and 2.7 fold increases in transcription with the phosphate oriented away from and toward the promoter, respectively.

These results support several conclusions. First, CREB may make many of the same DNA contacts as other bZIP proteins which bind to CRE-like sequences (O'Shea, 1992; Glover, 1995). Accordingly, amino-acid substitutions that alter the DNA-binding specificity of one member of this family will function similarly in another member. Differences in DNA-binding specificity within the family may reflect differences in protein flexibility or subtle differences in protein shape (Konig, 1993; Kim, 1993). Second, they confirm our original findings that hemiphosphorylated complexes are half as active as fully phosphorylated complexes (Loriaux, 1993). Third, these results

indicate that both activated CREB subunits contribute additively to gene activation. In this context, heterodimerization can serve to form complexes of intermediate activity. For example, hemiphosphorylated complexes may be generated by graded responses to phosphorylation by PKA or dephosphorylation by protein phosphatases. Finally, the orientation of hemiphosphorylated CREB complexes is unlikely to play a role in gene activation. Gene activation from the somatostatin CRE is similar to that defined for many cellular and viral enhancers.

These results differ from those described for several bacterial transcription factors. Only a single promoter-proximal activation domain is sufficient for gene activation by AraC and CAP dimers, for example. Directionality may be imposed by the close association of these transcription factors with RNA polymerase. Both AraC and CAP bind to DNA sites that are directly adjacent to or overlap the core promoter. Increasing the spacing between these two DNA regions reduces or eliminates gene activation . As a result, AraC and CAP are predicted to make direct protein:protein contacts with RNA polymerase (Zhou, 1993b; Reeder, 1993). In contrast, CRE sequences can function in virtually any position relative to the promoter. DNA flexibility could allow either subunit to interact with the RNA polymerase II complex either directly or indirectly through a coactivator protein. Alternatively, flexibility in the activation domain of CREB (Richards, Thesis) may permit the intact activation domain to move into a functional position.

A current model suggests that phosphorylated CREB activates transcription though interaction with the coactivator protein, CBP (Chrivia, 1993). The results presented here indicate that the half-maximal activity of hemiphosphorylated CREB complexes most likely results from a less efficient association with proteins that are required for transcriptional activation. A less efficient association with CBP may result through two mechanisms. First, because nonphosphorylated CREB cannot bind to CBP, hemiphosphorylated heterodimers may associate half as efficiently as fully

phosphorylated complexes. Alternatively, hemiphosphorylated CREB dimers may recruit fewer molecules of CBP. Recent experiments in our laboratory (Lundblad, personal communication) indicate that phosphorylated CREB binds noncooperatively to CBP both in the absence and presence of DNA. One possible interpretation of this result is that phospho-CREB can bind to CBP as a monomer. Conceivably, fully phosphorylated CREB dimers could recruit two CBP molecules where each CREB subunit binds to a single CBP molecule. The submaximal activity of hemiphosphorylated complexes, then, would result from the recruitment of only a single CBP molecule. Experiments that examine the stoichiometry of the CBP-CREB interaction may help to resolve this question.

Figure 1

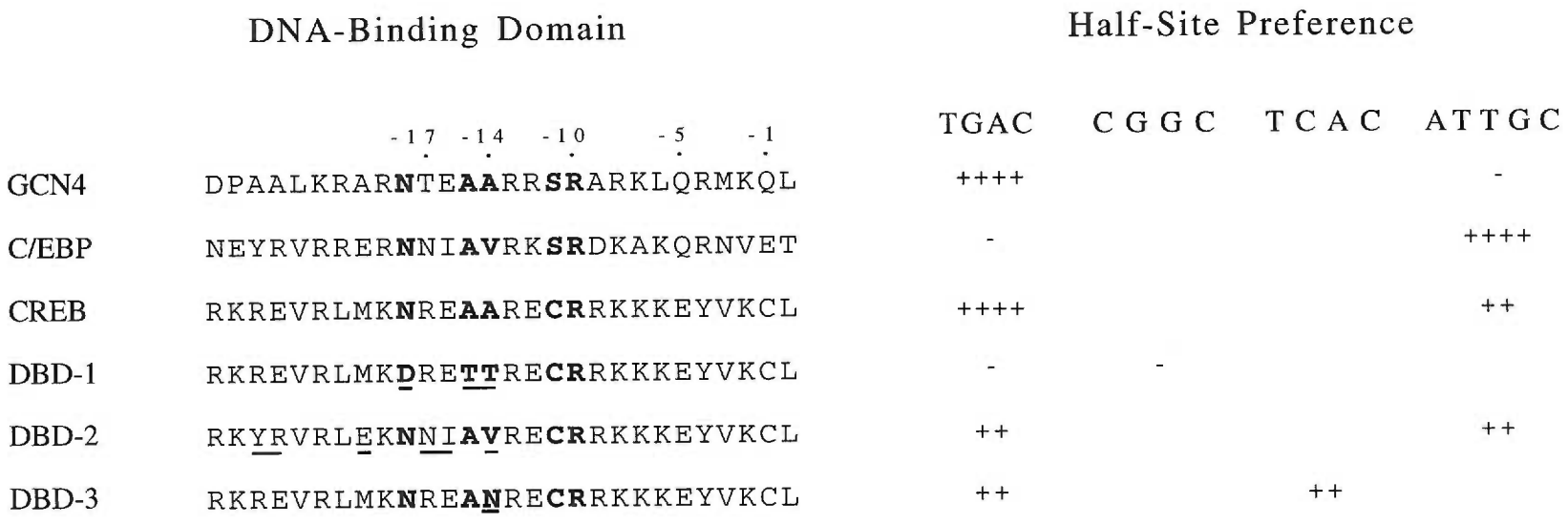
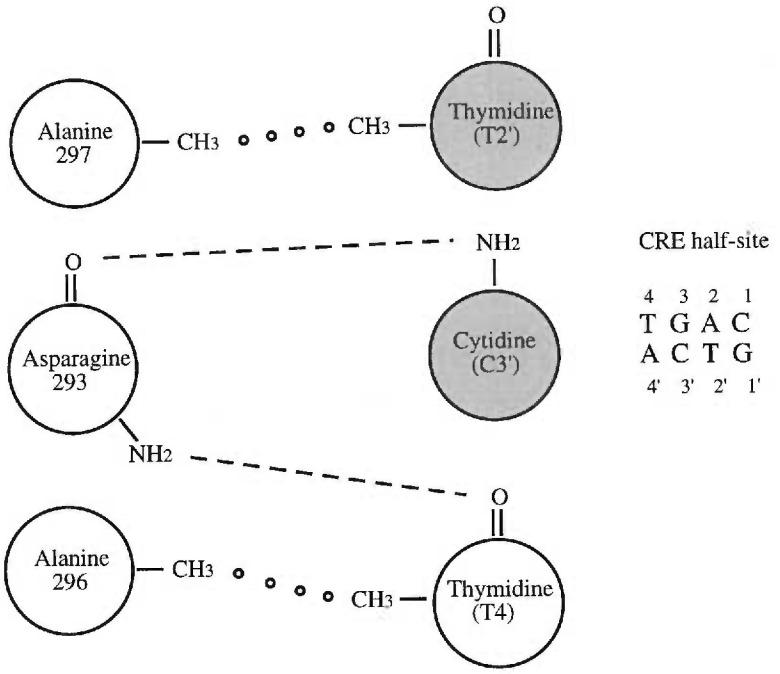

DNA-Binding Domain
Half-Site Preference
-17 -14 -10 -5 -1
TGAC CGGC TCAC ATTGC
GCN4 DPAALKRARNTEAARRSRARKLQRMKQL ++++ -
C/EBP NEYRVRRERNNIAVRKSRDKAKQRNVET - ++++
CREB RKREVRLMKNREAARECRRKKKEYVKCL ++++ ++
DBD-1 RKREVRLMKDRETTRECRRKKKEYVKCL - -
DBD-2 RKYRVRLEKNNIAVRECRRKKKEYVKCL ++ ++
DBD-3 RKREVRLMKNREANRECRRKKKEYVKCL ++ ++

Figure 1

Sequence alignment of wild-type and mutant basic regions and their DNA-binding specificities. Residues that contact DNA are shown in bold, and those that represent substitutions are underlined. The numbering indicates the amino-acid position before the first leucine in the leucine zipper region. Pluses indicate the relative intensities of shifted probe in gel mobility shift analyses. The DNA-binding specificities for GCN4 and C/EBP have been described previously (Suckow, 1993a).

Figure 2

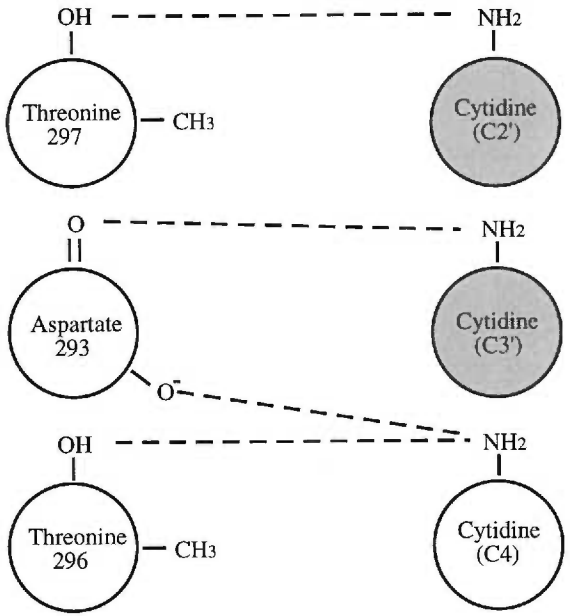
A. Wild-type DNA-binding domain and CRE half-site



CRE half-site

4	3	2	1
T	G	A	C
A	C	T	G
4'	3'	2'	1'

B. Mutant DNA-binding domain and complementary half-site



Mutant CRE half-site

4	3	2	1
C	G	G	C
G	C	C	G
4'	3'	2'	1'

Figure 2

Diagram depicting the wild-type and the mutant DNA-binding domain, DBD-1, and their complementary half-sites. Hydrogen bonds and van der Waals contacts are indicated by dashed lines and circles, respectively. Nucleotides that are complementary to the CRE sense strand are shaded. The hydrogen bonds in the mutant DNA-binding domain and CRE half-site should induce binding specificity.

Figure 3

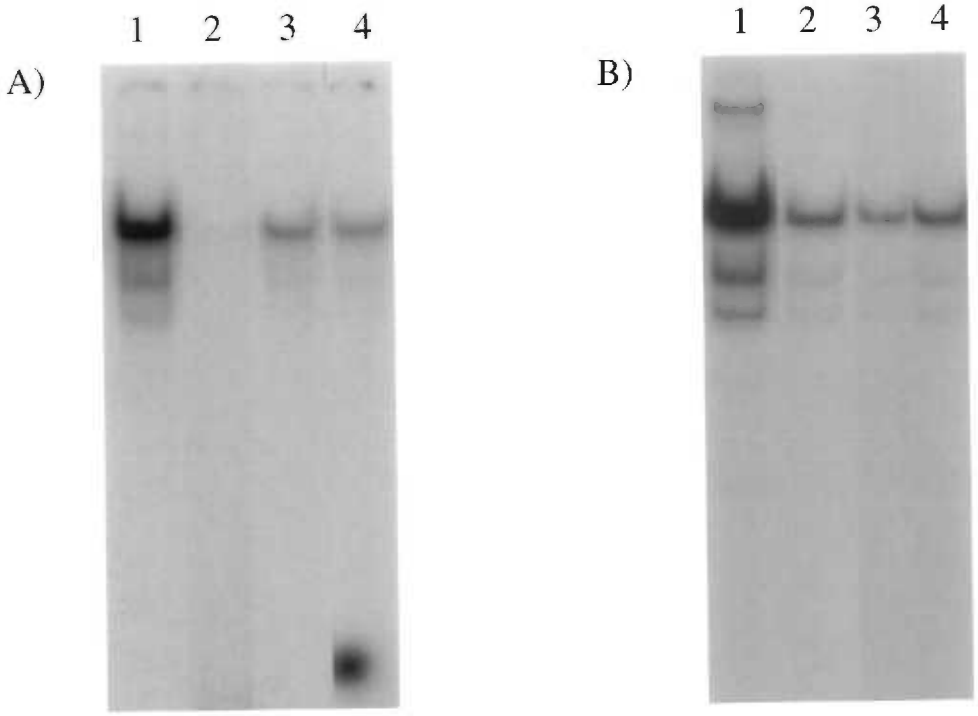


Figure 3

Gel mobility shift assays showing the relative abilities of CREB containing wild-type and mutant DNA-binding domains to bind to various CRE sequences. A) Wild-type CREB binds to the somatostatin CRE (lane 1) but not to the mutant CRE sequence 5'-TCACGTGA-3' (lane 2). In contrast, $CREB_{DBD-3}$ binds equally well, but at a lower affinity, to both the somatostatin CRE and the mutant CRE in lanes 3 and 4, respectively. B) Wild-type CREB binds to both the somatostatin CRE and the C/EBP binding site in lanes 1 and 2, respectively. Though CREB containing a C\EBP DNA-binding domain ($CREB_{DBD-2}$), binds with a higher affinity to the C/EBP binding site (lane 4), it also binds to the somatostatin CRE (lane 3).

Figure 4

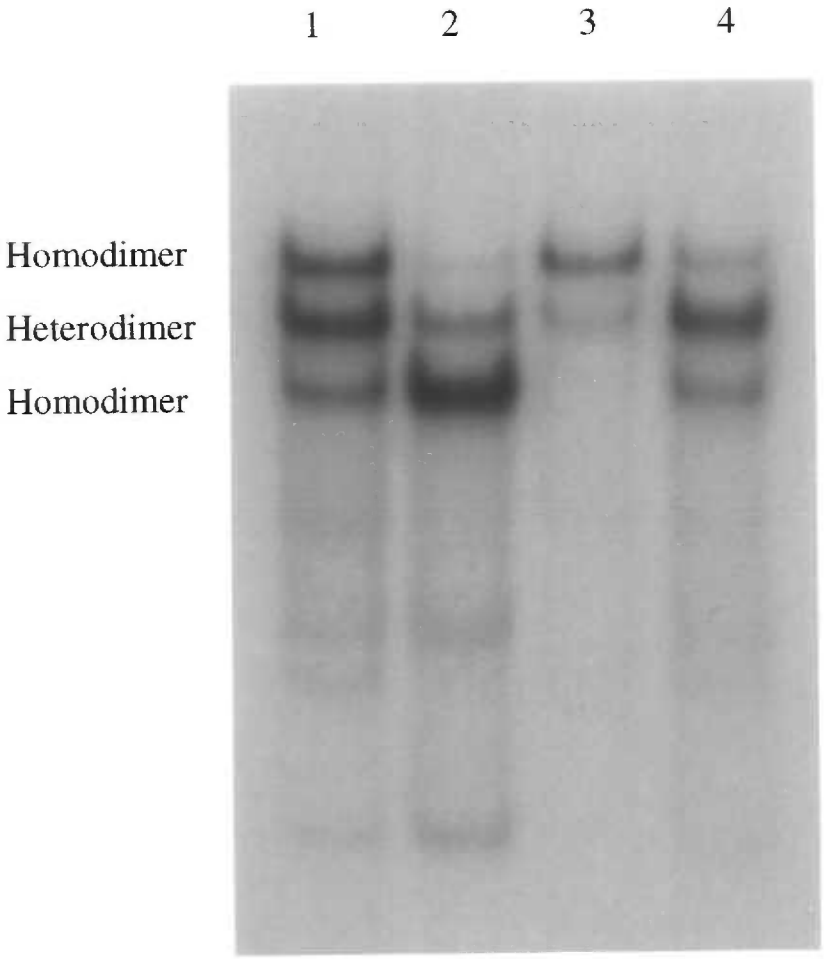


Figure 4

$CREB_{WT}$:$CREB_{DBD-3}$ heterodimers preferentially bind to a hybid CRE. In these gel mobility shift assays, long forms of $CREB_{WT}$ or $CREB_{DBD-3}$ are mixed in a 1-to-1 ratio with a short form of CREBWT and incubated with various CRE sequences. The differences in size permit a direct comparison of the binding affinities of homodimer and heterodimer complexes for each CRE sequence. Lane 1 contains a mixture of long and short forms of wild-type CREB incubated with the somatostatin CRE. In lanes 2-4, a long form of $CREB_{DBD-3}$ is mixed with an equal amount of the short form of $CREB_{WT}$. When this mixture is incubated with the somatostatin CRE, wild-type CREB homodimers predominate (lane 2). A small amount of $CREB_{DBD-3}$ homodimers is also visible. However, only $CREB_{DBD-3}$ homodimers shift the mobility of the mutant CRE (lane-3). While only a small amount of heterdimer is visible in lanes 2 and 3, $CREB_{WT}$:$CREB_{DBD-3}$ heterodimers preferentially bind to a hybid CRE containing one consensus half-site and one 5'-TCAC-3' half-site (lane 4).

Figure 5

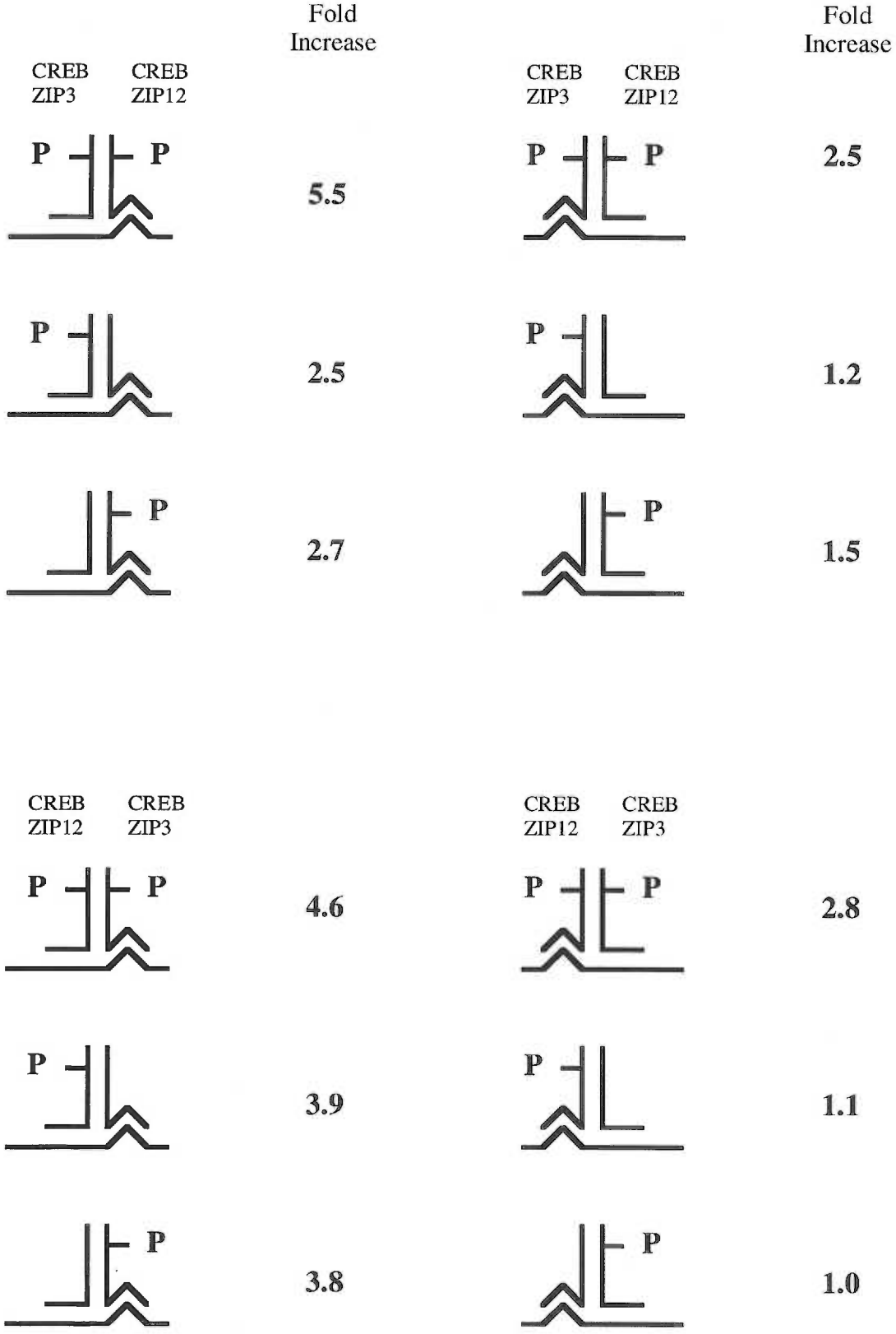

CREB ZIP3
CREB ZIP12
Fold Increase
P
P
5.5
2.5
2.7
CREB ZIP3
CREB ZIP12
Fold Increase
P
P
2.5
1.2
1.5
CREB ZIP12
CREB ZIP3
P
P
4.6
3.9
3.8
CREB ZIP12
CREB ZIP3
P
P
2.8
1.1
1.0

Figure 5

Transfection experiments testing the orientation dependence of hemiphosphorylated CREB-mediated gene activation. In these four experiments the transcriptional activities of oriented hemiphosphorylated CREB complexes are compared to the corresponding fully phosphorylated complexes. In each case the orientation of the phosphate does not affect the activity of hemiphosphorylated complexes. In this figure, CREB subunits are represented by an "L". A "P" indicates a phosphorylated subunit. The mutant DNA-binding domain, DBD-3, is indicated by an angled line at the bottom of each L-shaped CREB subunit.

Discussion

The primary goal of this thesis is to gain a better understanding of the role of dimerization in CREB-mediated gene activation. The experiments described in chapters I through III address possible functions for dimerization. First, dimerization may allow each activation domain in a CREB dimer to contribute to gene activation. Second, dimerization may allow the formation of heterodimeric complexes with unique transcriptional activities. And finally, dimerization may allow transcription factors with different DNA-binding specificities to form complexes that bind directionally to asymmetric enhancer sequences. The orientation in which these complexes bind DNA may determine their transcriptional activity.

The highly conserved basic/leucine zipper (bZIP) region of CREB and related transcription factors makes these hypotheses difficult to address, however. The leucine zipper not only allows CREB to homodimerize but to heterodimerize with other leucine zipper proteins. As a result, it becomes difficult to ascertain the functional properties of individual heterodimers. To overcome this obstacle, I have developed a method to generate specific heterodimer complexes. The transcriptional activities of these complexes can then be determined to understand how dimerization impacts on the function of basic/leucine zipper (bZIP) transcription factors.

To generate transcription factors which are unable to homodimerize but which can selectively heterodimerize, I mutated residues within the hydrophobic face of the leucine zipper and residues in the adjacent **e** and **g** positions which may form stabilizing electrostatic interactions. Two observations in particular suggested that substitutions at the **a3** position might alter the dimerization properties of these factors. First, the conserved asparagine that occupies this position is a unique polar residue in an otherwise hydrophobic dimerization interface. By interrupting the continuity of the hydrophobic interface, this asparagine is thought to destabilize the leucine zipper and permit reversible

dimerization. The introduction of larger polar or charged residues at this position may further destabilize homodimer formation. Second, although *in vivo* studies examining the dimerization properties of GCN4 leucine zipper mutants showed that the **a3** position was relatively tolerant of amino acid substitution, an aspartic acid substitution was able to disrupt dimer formation (Hu, 1990). This finding suggested that charge repulsion at this position might be particularly effective in destabilizing homodimer formation. Accordingly, I introduced four larger polar or charged residues at the **a3** position. Of these, an asparagine to histidine substitution was most effective in reducing homodimer formation. Surprisingly, the results of these experiments indicated that a histidine at the **a3** position not only disrupted homodimer formation but lead to preferential formation of histidine mutant:wild-type heterodimers. However, in mixtures of wild-type and mutant proteins, homodimers of each component protein were able to form.

To eliminate homodimer formation, amino acid substitutions were also made in the **e** and **g** positions of the CREB leucine zipper. Stabilizing electrostatic interactions between the corresponding residues have been observed in the GCN4 and Jun:Fos leucine zipper crystal structures (O'Shea, 1991). In addition, repulsive electrostatic interactions between amino acids in these positions are thought to prevent homodimerization of Fos and stabilizing interactions to direct the preferential heterodimerization of Fos with Jun (O'Shea, 1992; Nicklin, 1991). Helical-wheel diagrams show that four stabilizing electrostatic interactions can form between dimerized CREB leucine zippers. To destabilize CREB homodimer formation, I reciprocally substituted residues that participate in the formation of favorable electrostatic interactions to generate the mutant leucine zippers pZIP3 and pZIP12. These leucine zippers were designed so that repulsive interactions would disrupt both homodimer forms while stabilizing interactions would exist only in the heterodimer. As was the case with substitutions at the **a3** position, mutations in the **e** and **g** positions alone were not sufficient to completely prevent homodimer formation. Only the mutant leucine zipper pZIP12, which also contained a

histidine substitution at the **a3** position, was completely inactive in transcription assays. Thus, the combination of **a3**, **e**, and **g** substitutions was required to direct the formation of CREB heterodimers exclusively.

These results differ form those of Amati *et al.* who found that reciprocal substitution of residues that form electrostatic interactions was sufficient to direct heterodimerization of Myc and Max proteins (Amati, 1993). In that case, mutant proteins with reciprocally modified **e** and **g** residues in their leucine zippers were transformation-defective when transfected individually into mammalian cells, but transformation-competent when combined. The leucine zippers of Myc and Max contain residues that can contribute to the formation of two additional electrostatic interactions. The six repulsive interactions that would occur in the mutant Myc and Max homodimers (in contrast to the four that would occur in bZIP3 and bZIP12 homodimers) may prevent homodimer formation.

The ability to engineer the leucine zipper of CREB by modeling its structure into the GCN4 leucine zipper crystal structure indicates that their bZIP regions are structurally similar. Though this similarity probably extends to many bZIP proteins, transcription factors in this family can form only certain homodimer and heterodimer combinations. The results of these experiments indicate that residues in several positions of the leucine zipper mediate dimerization specificity. First, these results provide evidence that the polar residue in the **a3** position of CREB can contribute to dimerization specificity. However, an asparagine invariably occurs at this position in bZIP transcription factors, and, as a result, the polar residue in this position probably only serves to destabilize dimer formation and/or align the leucine zippers. Second, these results show that residues in the **e** and **g** positions not only contribute to dimer stability as has been shown in the crystal structures of GCN4 and Jun:Fos (Glover, 1995), but they are in agreement with recent work which indicates that these residues determine dimerization specificity (Nicklin, 1991; Schuermann, 1991; O'Shea, 1992; Amati, 1993; Vinson, 1993). Krylov

et al. have carried this observation further and determined the relative stabilities of electrostatic interactions generated by different amino acid pairs (Krylov, 1994). They have also determined that certain interactions contribute more to dimerization specificity than others. Their results have lead to "design rules" that can be used to generate bZIP proteins with novel dimerization properties.

To determine whether each activation domain in a CREB dimer contributes to gene regulation, the mutant leucine zippers pZIP3 and pZIP12 were next used to determine the transcriptional activity of hemiphosphorylated CREB complexes *in vivo*. The results of these experiments indicated that CREB complexes which could be phosphorylated on a single subunit were half as active as fully phosphorylatable complexes. In these heterodimers each activation domain contributes additively to transcriptional activation. These results also provide evidence that the process of dimerization contributes to determining the level of transcriptional activation by juxtaposing two activation domains. With each activation domain contributing to gene activation, cAMP-directed signals may be modulated according to the degree of CREB phosphorylation. Three possible mechanisms could generate CREB dimers that are hemiphosphorylated -- a submaximal response to PKA or a protein phosphatase (Hagiwara, 1992; Wadinski, 1993) or heterodimerization of CREB with another transcription factor that does not participate in PKA signaling. The latter mechanism suggests that heterodimerization may allow the targeting of distinct second-messenger signals to a single genetic element.

In a similar manner, I used the mutant leucine zipper strategy to determine whether dimerization could lead to the formation of heterodimeric transcription factor complexes with transcriptional activities that are unique from those of homodimers of either component protein. Specifically, I determined the transcriptional activity of CREMα:CREB heterodimer complexes *in vivo*. CREMα is thought to be a negative regulator of cAMP-mediated gene activation. Though the true mechanism is currently

unknown, two models may explain how CREMα modulates transcriptional activity. CREMα homodimers may block PKA mediated gene activation by competing for CREB DNA-binding sites, or CREMα may form inactive heterodimers with CREB. The former model provides a mechanism for greatly increasing the efficiency and specificity of these negative regulators, while the latter could be less specific, thereby increasing the number of genes that might be influenced.

The results of these experiments indicate that fully phosphorylated CREB:CREM α heterodimers have about one-third the activity of CREB homodimers, but are clearly more active than the CREMα homodimeric forms. Furthermore, the activities of hemiphosphorylated CREB:CREMα dimers are negligible, regardless of which subunit, CREMα or CREB, contained an intact PKA site. These results indicate that the modulation of cAMP-regulated gene expression by CREMα is complex and depends, in part, on whether CREMα is capable of being phosphorylated by PKA. These data also indicate that the phosphorylation of at least one CREB subunit is required to mediate PKA-induction of a CRE reporter gene. Complexes containing one phosphorylatable CREB subunit have variable activities, however, and the level of activity appears to be determined by the identity and phosphorylation status of the dimerizing component. These findings suggest that the patterns of heterodimerization and phosphorylation of just two CRE-binding proteins can provide multiple levels of transcriptional control.

In a cell, an additional level of transcriptional control could be provided by modulating the amount of CREMα. Certain CREM mRNA levels are regulated by cAMP (Molina, 1993), so the ratios of CREB and CREM are likely to be dynamic. Increasing the amount of CREM may serve to attenuate transcription in response to prolonged activation of the cAMP signaling pathway. Recently, CREB activity has been shown to be modulated by concentrations of another dimerization partner, ATF-1 (Ellis, 1995). High levels of ATF-1 in undifferentiated F9 cells prevent CREB-mediated gene activation through the formation of inactive ATF-1:CREB heterodimers. In

differentiated F9 cells, ATF-1 levels are lower, and transcriptionally active CREB homodimers are able to form.

The final goal of this thesis is to determine which of two models accounts for the half-maximal activity of hemiphosphorylated CREB complexes. In one model, hemiphosphorylated complexes associate less efficiently with other proteins that are required for transcriptional activation. Fully phosphorylated complexes may have a higher affinity for a single protein involved in transcriptional activation or may be capable of recruiting more of these proteins. The latter alternative would be more likely to explain the half-maximal activity of the hemiphosphorylated complexes. In the second model, hemiphosphorylated CREB dimers function directionally. The CRE sequence is symmetrical and, therefore, the hemiphosphorylated dimer can bind to it in two different orientations. Only one of these orientations may be functional. The former model would support the concept that each activation domain in a CREB dimer contributes to transcriptional activation. The second model would provide a mechanism to increase diversity in gene regulation and would challenge previous reports that enhancers function in an orientation independent manner.

To orient the phosphorylated activation domain in a hemiphosphorylated CREB dimer with respect to the promoter, CREB subunits with different DNA-binding specificities were selectively heterodimerized using the mutant leucine zippers pZIP3 and pZIP12. The hemiphosphorylated heterodimer was then bound to an asymmetric CRE consisting of a somatostatin CRE half-site and the mutant half-site TCAC. The wild-type CREB subunit specifically recognized the somatostatin CRE half-site while the altered CREB subunit, $CREB_{DBD-3}$ preferentially bound to the mutant CRE half-site.

Amino acid substitutions that could alter the DNA-binding specificity of CREB were modeled on those that have been previously found to alter the DNA-binding specificity of GCN4. Of the substitutions that were tested, only an alanine to asparagine substitution at position 297 significantly altered the DNA-binding specificity of CREB.

This substitution allowed the mutant DNA-binding domain to bind to the CRE sequence 5'-TCACGTGA-3' -- a sequence that could not be bound by wild-type CREB. Because substitutions that alter the DNA-binding specificity of GCN4 also alter the binding specificity of CREB, the similarities between these two transcription factors extend to their basic regions.

The asymmetric CRE was then used to direct the orientation of hemiphosphorylated complexes bound upstream from a somatostatin promoter/CAT reporter plasmid. Hemiphosphorylated CREB complexes had equal transcriptional activity when bound in either orientation. These results indicate that orientation is not a factor in determining the transcriptional activity of hemiphosphorylated CREB complexes. Each activation domain in a CREB dimer, then, contributes additively to the total level of transcriptional activity, and dimerization contributes to gene activation by juxtaposing these activation domains. These results are consistent with those of Krajewski *et al*. who have shown that both activation domains must be present for a CREB dimer to have transcriptional activity (Krajewski, 1994). The activation domain of CREB fused to the DNA-binding domain of a protein that binds DNA as a monomer is unable to mediate basal or cAMP-mediated gene expression. Similarly, both activation domains contribute to gene regulation in other transcription factor complexes which function in an orientation and position independent manner. For example, Abate *et al*. have shown that the activation domains of Jun and Fos function cooperatively to activate gene transcription (Abate, 1990). Deletion analysis of JunD has also shown that, in a JunD:Fos dimer, both proteins contribute to gene activation (Hirai, 1990). Interestingly, experiments by Arias et al. suggest that CREB and Jun:Fos heterodimers may activate transcription through interaction with the same coactivator protein CBP (Arias, 1994).

For a number of other activator proteins, however, a single activation domain is sufficient to induce high levels of gene transcription. For example, Fos:Jun DNA-binding domain heterodimers fused to a single GCN4 activation domain are as active as

similar constructs containing two activation domains (Oliviero, 1991). The difference between my results and those found for GCN4 may reflect differences in the mechanisms by which these transcription factors regulate gene expression. A single activation domain is also sufficient for transcriptional activity from several dimeric, bacterial transcription factors. Only a single promoter-proximal activation domain is sufficient for gene activation by AraC and CAP dimers, for example. In these systems, directionality may be imposed by the close association of these transcription factors with RNA polymerase. Both AraC and CAP bind to DNA sites that are directly adjacent to or overlap the core promoter. Increasing the spacing between these two DNA regions reduces or eliminates gene activation. As a result, AraC and CAP are predicted to make direct protein:protein contacts with RNA polymerase (Zhou, 1993b; Reeder, 1993).

In addition to providing support for the potential roles of dimerization in gene activation, the results of these experiments lend insight into the mechanism of PKA-mediated CREB activation. One idea is that phosphorylation-induced intramolecular changes activate CREB by exposing a glutamine-rich region (Gonzalez, 1991). If this model is correct, the phosphorylated subunit in a hemiphosphorylated CREB dimer alone would be responsible for the activity of that complex. However, as mentioned previously, monomeric CREB is transcriptionally inactive (Krajewski, 1994). In addition, the finding that CREB:M1-CREMα heterodimers are inactive indicates that a single phosphorylated CREB subunit is incapable of stimulating transcription. A second model that is more consistent with these observations suggests that domains from both monomers of the CREB dimer interact to activate transcription. In this model, the phosphorylation of serine 133 of one CREB subunit results, directly or indirectly, in the availability of the glutamine-rich region(s) of the other subunit. This model can explain the ability of CREMα to contribute to gene activation and is consistent with our observation that only the fully phosphorylated CREB:CREMα heterodimer is transcriptionally active. In such heterodimers, CREMα may donate the phosphorylation

domain, while CREB donates the glutamine-rich region. That M1-CREB:CREMα only minimally activates transcription can be ascribed to the different architectures of the 341-amino acid CREB and 289-amino acid CREM proteins.

The transcriptional activities of hemiphosphorylated CREB and the various CREB:CREMα heterodimers may reflect differences in the abilities of these complexes to interact with a coactivator molecule or other component of the RNA polymerase complex. A current model suggests that phosphorylated CREB activates transcription though interaction with the coactivator protein, CBP (Chrivia, 1993; Kwok, 1994). As discussed in chapter III, the results of the "orientation" experiments indicate that the half-maximal activity of hemiphosphorylated CREB complexes most likely results from a less efficient association with proteins that are required for transcriptional activation. One possibility is that hemiphosphorylated complexes interact less efficiently with CBP. In this model, hemiphosphorylated heterodimers may associate half as efficiently as fully phosphorylated complexes, or hemiphosphorylated CREB dimers may recruit fewer molecules of CBP. Alternatively, hemiphosphorylated complexes may associate less efficiently with an additional protein required for gene activation. I have previously suggested that activation of CREB may involve an intermolecular interaction in which the KID region of one subunit cooperates with the glutamine-rich region of the second subunit. Only one such interaction can occur in a hemiphosphorylated complex. This may result in a less efficient interaction with a component of the general transcription machinery such as human homolog of Drosophila TAF110 (Ferreri, 1994).

The reduced activity of CREMα:CREB heterodimers most likely reflects a less efficient interaction with a component of the transcription machinery other than CBP. Several lines of evidence support this conclusion. First, phosphorylated CREB and CREMα homodimers bind to CBP with equal affinities (Lundblad, personal communication). Second, though incompatibilities in the architectures of CREMα and CREB may prevent the efficient interaction of heterodimers with CBP, the recent finding

that ATF-1:CREB heterodimers are transcriptionally inactive (Ellis, 1995) suggests that an alternative mechanism could be responsible for the reduced activity. Like CREMα, phosphorylated ATF-1 homodimers bind to CBP with a high affinity (Lundblad, personal communication). Unlike CREMα, however, ATF-1 contains the glutamine-rich region, Q2, and parts of the amino-terminal glutamine-rich region, Q1. Because CREB and ATF-1 have similar architectures, it is unlikely that ATF-1 is inhibiting the ability of the phosphorylated CREB subunit to interact with CBP. In contrast, both CREMα and ATF-1 lack all or part of the of the amino-terminal glutamine-rich region. This region of CREB may form contacts with the transcriptional machinery that mediate basal activation (Rehfuss, 1993). The inability of CREM and ATF-1 to contribute to this interaction in heterodimers with CREB may reduce the activity of these complexes. These results indicate that CREB and related proteins may make necessary contacts with several proteins required for gene transcription.

Conclusions

1) The bZIP regions of CREB and GCN4 are functionally and structurally similar.

2) Residues in the **a3**, **e**, and **g** positions of the CREB leucine zipper contribute to dimerization specificity.

3) Hemiphosphorylated CREB complexes are half as active as fully phosphorylated complexes.

4) The activation domains of both subunits in a CREB dimer contribute to gene activation.

5) These results support the concept that heterodimerization allows distinct signalling pathways to regulate transcription simultaneously from a single genetic element.

6) Hemiphosphorylated CREB dimers function in an orientation independent manner.

7) CREMα can contribute to PKA-mediated gene activation.

8) The transcriptional activity of CREMα:CREB heterodimers is phosphorylation dependent.

Appendix

Leucine zipper mutations at the **a3** position of transcription factor CREB induce selective patterns of heterodimerization

Results

As discussed in Chapter 1, substitutions were introduced at the **a3** position of the leucine zipper of CREB to destabilize homodimer formation. By site-directed mutagenesis, the wild-type asparagine was replaced with either aspartic acid, lysine, arginine, or histidine. The aspartic acid substitution was made to introduce a formal negative charge, and the lysine and arginine mutations were made to introduce formal positive charges at physiological pH. The histidine mutation introduced an amino acid that could be charged or uncharged, depending upon the pH. Destabilization of these mutant homodimers could occur by charge-repulsion or by steric hinderance.

Recombinant CREB proteins were expressed in bacteria and purified by heating. This procedure removes most of the bacterial proteins, which precipitate at 72^{o}C. Recombinant CREB is extremely heat-stable and returns to its native form, as analyzed by circular dichroism, after cooling to room temperature (J. Richards, Thesis). Samples of each extract were electrophoresed on SDS-PAGE gels to assess the quantity and purity of recombinant protein expression in each extract. Recombinant CREB represents greater than 90% of the protein in each preparation (Figure 1). Extracts containing full-length proteins were shown to contain approximately 0.8 ug/ul of recombinant protein. Differences in protein expression among individual extracts as less than 5%. The extract containing the deleted-CREB was shown to contain approximately 1.0 ug/ul of recombinant protein.

The ability of the recombinant proteins to dimerize was analyzed by using gel mobility shift assays. In these assays, full-length proteins were mixed with a truncated

form of CREB (deleted-CREB), which is 60 amino acids shorter, to allow visualization of heterodimer complexes. Figure 2 depicts titrations of deleted-CREB, containing the wild-type asparagine residue in the **a3** position, with varying concentrations of wild-type or mutant full-length CREB. Lane 1 in each series of titrations shows the full-length (long) forms of wild-type or mutant CREB analyzed individually. Lanes containing only deleted-CREB are labeled dC. Lanes 2 through 6 in each series show mixtures of extracts containing full-length and deleted (short) forms of CREB. In lanes labeled 2, the ratio full-length to deleted-CREB extract is 1:10; in lanes 3, 1:3; in lanes 4, 1:1; in lanes 5, 3:1; and in lanes 6, 10:1. Homodimers of long and short forms as well as long:short heterodimers are indicated. Surprisingly, the mutant CREB proteins appear to form homodimers as well as the wild-type protein when analyzed in isolation. However, when mixed with wild-type deleted-CREB, the lysine, arginine, and histidine mutations appear to shift the pattern of dimerization towards the formation of heterodimers. For example, lane 4 in the lysine titration shows a decrease in homodimer formation and an increase in heterodimer formation when compared to lane 4 of the wild-type titration. The arginine mutation similarly enhances heterodimer formation. The greatest effect was seen with the histidine mutation. Lane 4 in the histidine titration shows that the mutant homodimers are completely eliminated, the wild- type homodimes are decreased and the heterodimers are increased as compared to the control. In mixtures containing higher ratios of lysine-, arginine- and histidine-mutated proteins (lanes 5 and 6), the amount of heterodimer is also increased relative to the amount of asparagine:asparagine heterodimer. In contrast, the aspartic acid mutation does not appear to inhibit homodimer formation.

To gain insight into the structural mechanisms underlying the instability of the histidine homodimer and the apparent increase in stability of the histidine:asparagine heterodimer, we modeled the histidine mutation into the recently solved crystal structure of the GCN4 leucine zipper (O'Shea, 1991). Figure 3 shows the predicted homodimer

and heterodimer structures of the wild-type and mutant histidine leucine zippers. In these models, amino acids of the CREB leucine zipper replace the equivalent residues of the GCN4 leucine zipper. These minor alterations do not perturb the interface and are unlikely to affect the structure of the dimerization region significantly. As in GCN4, the wild-type CREB dimer is stabilized by a 2.6 angstrom hydrogen bond between the carbonyl oxygen and the amide nitrogen of the opposing asparagine residues. The hydrogen bond is represented by an orange, dotted line in Figure 3b. The geometry of this hydrogen bond, i.e., the rather short bond length and less than ideal angle between the hydrogen donor and acceptor, suggests that this bond is somewhat strained. The best hydrogen bond that can be modeled in the mutant histidine homodimer is shown in Figure 3c. Simple replacement of the asparagine residues with histidine using the same X_1 and X_2 values of the GCN4 asparagines is not possible due to steric clash between these larger side chains. The modeled histidine:histidine hydrogen bond is 3.3 angstroms long and non-planar. However, it was not possible to model this hydrogen bond and still make favorable van der Waals contacts with neighboring residues. Thus, a relatively unfavorable hydrogen bond geometry appears to destabilize the histidine homodimer. The asparagine:histidine heterodimer depicted in Figure 3d can readily be modeled to have a 2.8 angstrom hydrogen bond between the carbonyl oxygen of asparagine and the Nε of histidine. This bond length is optimal and the proton donor and acceptor are nearly linear. Equally important, van der Waals contacts between the Nδ of histidine and an isoleucine located at a position four residues carboxy-terminal in the same zipper helix, as well as between the Cδ of the histidine and a nearby leucine at position 19 of the opposite helix, should stabilize the heterodimer form further. These contacts may account for the increased stability of the heterodimer relative to the homodimer configurations. The tight packing of the **a3** residues is also apparent from the computer generated CPK model (Figure 3e) which better depicts van der Waals contacts in the heterodimer.

Discussion

We expected that each of our mutations would increase the amount of mutant:wild-type heterodimer and decrease the amount of each homodimer. Surprisingly, the aspartic acid mutation did not effectively alter the pattern of homo- and heterodimer formation. Our observation that aspartic acid homodimers form readily contrasts with the *in vivo* leucine zipper-λ repressor fusion protein experiments in which aspartic acid mutations at the **a3** position were nonpermissive, implying that these zippers were unable to dimerize (Hu, 1990). The most likely explanation for the differences between our results and those of Hu et al. is that the aspartic acid mutant homodimers that are apparent in our gel mobility shift assays may not form under the conditions of their *in vivo* assay. Two obvious differences between the two assay systems are the concentrations of recombinant proteins and the temperature of the dimerization reactions. It is also possible that the *in vivo* analysis is not as sensitive as the mobility shift assay. The stability of mutant aspartic acid homodimers in our assay may result from the formation of an ionic interaction with a divalent cation, effectively neutralizing the negatively charged aspartic acid residues, or the sharing of a proton between the two aspartic acids.

The arginine and lysine mutations altered the dimerization patterns from those of the wild-type protein only slightly; a small enhancement of heterodimer formation was evident. These effects were less pronounced than expected because the long side chains of these amino acids and their positive charges were envisioned to interfere with dimerization due to both electrostatic and steric effects. Our results suggest that the tendancy of these mutations is to destabilize homodimer formation, however, and they support the hypothesis that the lysine at the **a3** position in the c-Fos leucine zipper may contribute to the inability of this factor to homodimerize. It is plausible that charge-

charge destabilization is mitigated by the methylene carbons in the side chains of these amino acid residues which may, in turn, maintain a stable hydrophobic interface.

In contrast to the lysine and arginine mutations, the relative size and inflexibility of the histidine imidazole ring would be expected to promote unfavorable contacts in a mutant histidine homodimer that would not be present in an asparagine:histidine heterodimer. Our experimental data supported this prediction; histidine homodimers were considerably less favored than histidine:asparagine heterodimers. It additionally appeared that the histidine:asparagine heterodimer was stablized relative to homodimers of wild-type **a3** asparagine CREB. Molecular modeling predicts that this shift in the dimerization may result from improved hydrogen bond geometry in the heterodimer. Equally important, van der Waals contacts in the asparagine:histidine heterodimer that are absent in both the wild-type and the mutant homodimer forms may increase the stability of the heterodimer relative to the homodimers.

These results point to an approach for controlling the specificity of transcription factor heterodimerization. Mutants that favor the formation of selected heterodimers can now be used as tools to study the *in vivo* function of CREB and related transcription factors.

Figure 1

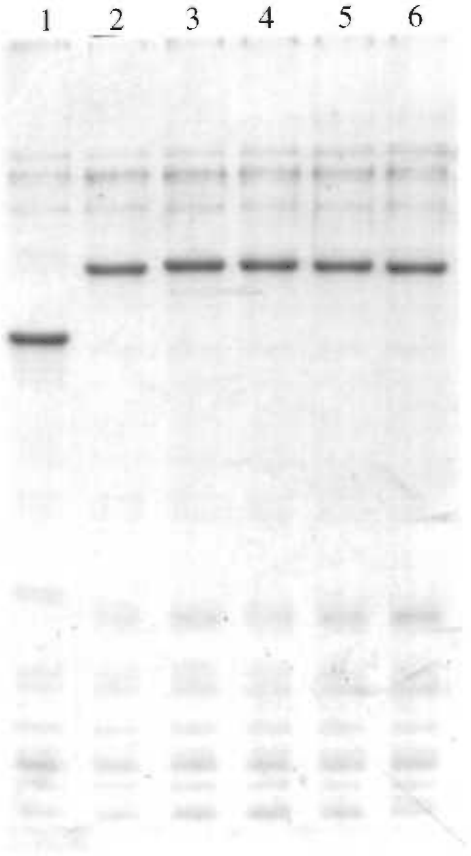


Fig. 1 Samples of heat-treated recombinant CREB extracts electrophoresed on an SDS-PAGE gel. Lane 1 contains 2 ul of deleted-CREB extract. Lanes 2-6 contain samples of full-length CREB extracts. Lane 2 contains wild-type CREB-327; lane 3, the arginine mutant; lane 4, the lysine mutant; lane 5, the histidine mutant; and lane 6, the aspartic acid mutant.

Figure 2

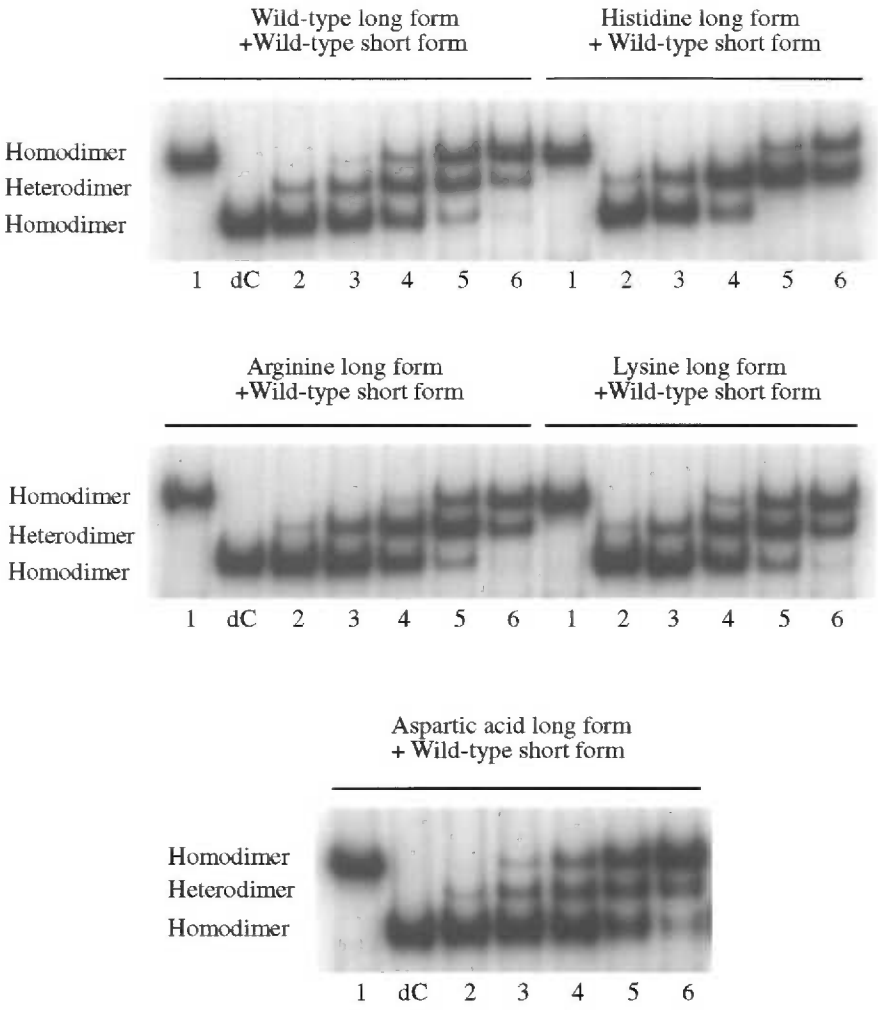
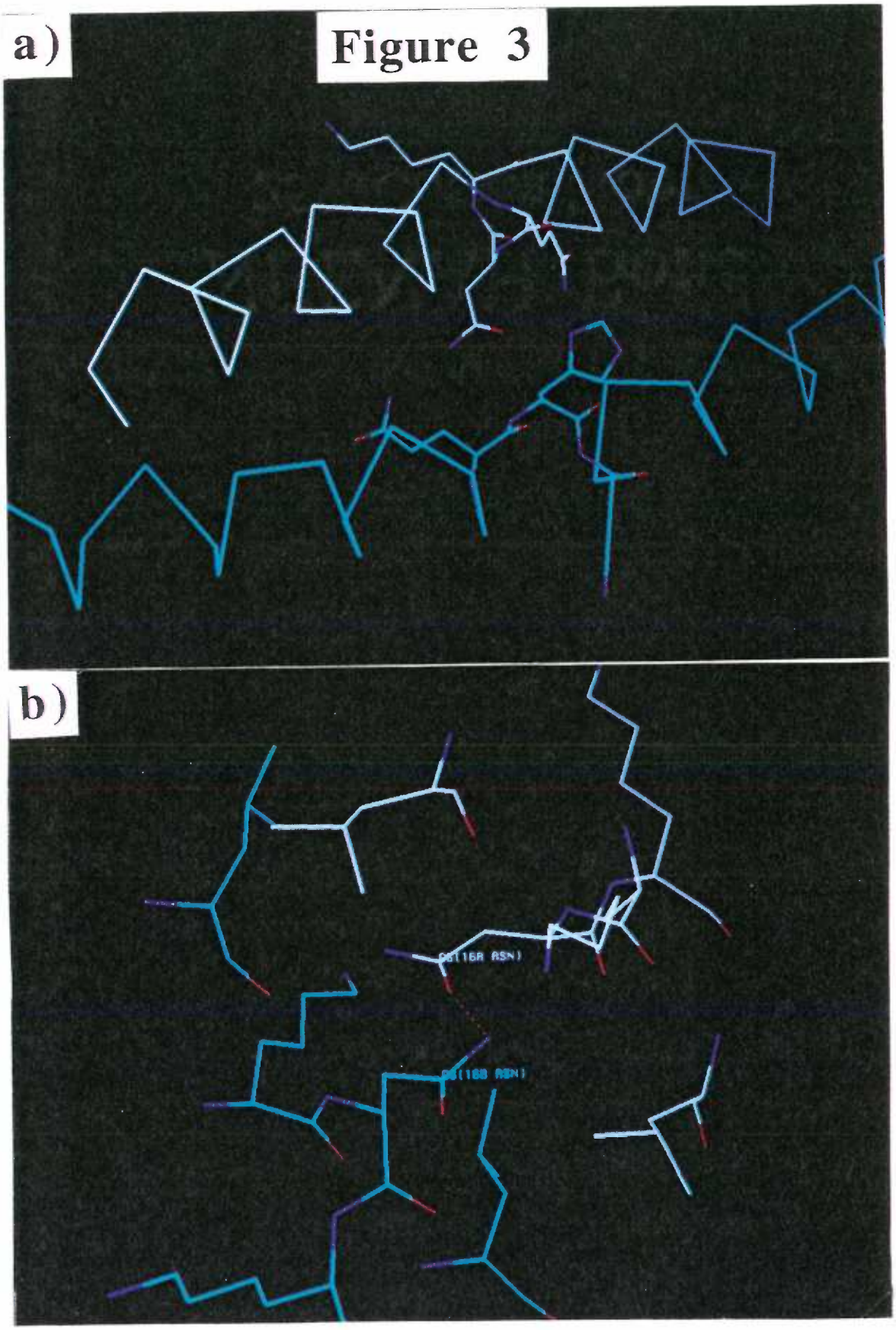
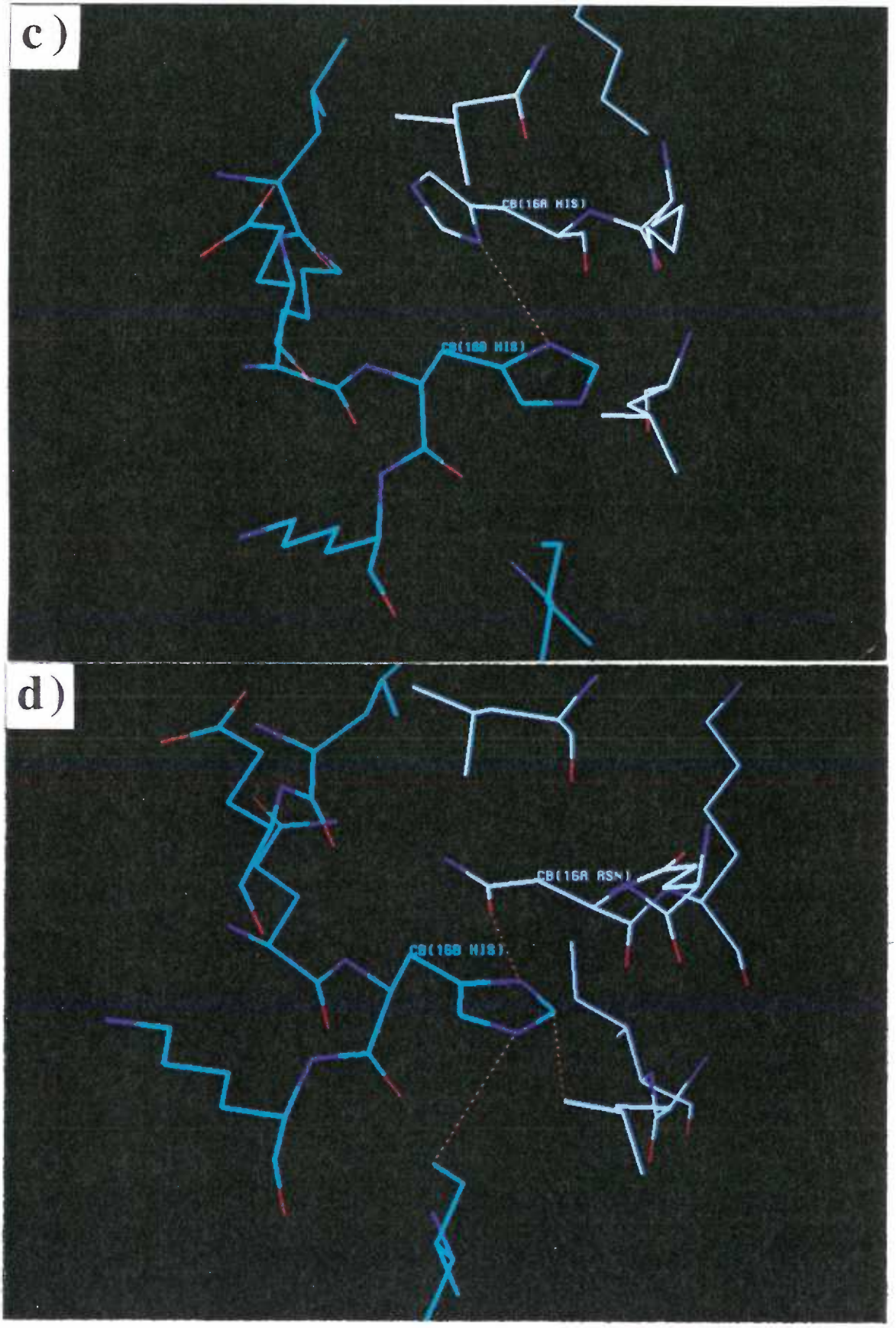

Wild-type long form
+Wild-type short form
Histidine long form
+ Wild-type short form
Homodimer
Heterodimer
Homodimer
1 dC 2 3 4 5 6 1 2 3 4 5 6
Arginine long form
+Wild-type short form
Lysine long form
+Wild-type short form
Homodimer
Heterodimer
Homodimer
1 dC 2 3 4 5 6 1 2 3 4 5 6
Aspartic acid long form
+ Wild-type short form
Homodimer
Heterodimer
Homodimer
1 dC 2 3 4 5 6

Fig. 2 Gel mobility shift assays in which a full-length wild-type or mutant CREB is mixed with truncated wild-type CREB (deleted-CREB). Lane 1 in each titration shows the full-length form shifted individually. Lanes containing only deleted-CREB are labeled dC. Lanes 2 through 6 of each titration show extracts of deleted-CREB (short form) mixed in varying ratios with extracts containing full-length CREB (long form). In lanes labeled 2, the the ratio of the amount of extract containing full-length CREB to the amount of extract containing deleted CREB is 1:10; in lanes 3, 1:3; in lanes 4, 1:1; in lanes 5, 3:1; and in lanes 6, 10:1. Homodimers of long and short forms as well as long:short heterodimers are indicated. Although mutant homodimers are able to form in the absence of wild-type protein, titrations of proteins containing lysine, arginine and histidine mutations with deleted-CREB show an increase in the amount of heterodimer formation when compared to the wild-type titration. The aspartic acid mutation, in contrast, shows little effect.

Figure 3




c)
CB(16A HIS)
CB(16B HIS)
d)
CB(16A ASN)
CB(16B HIS)

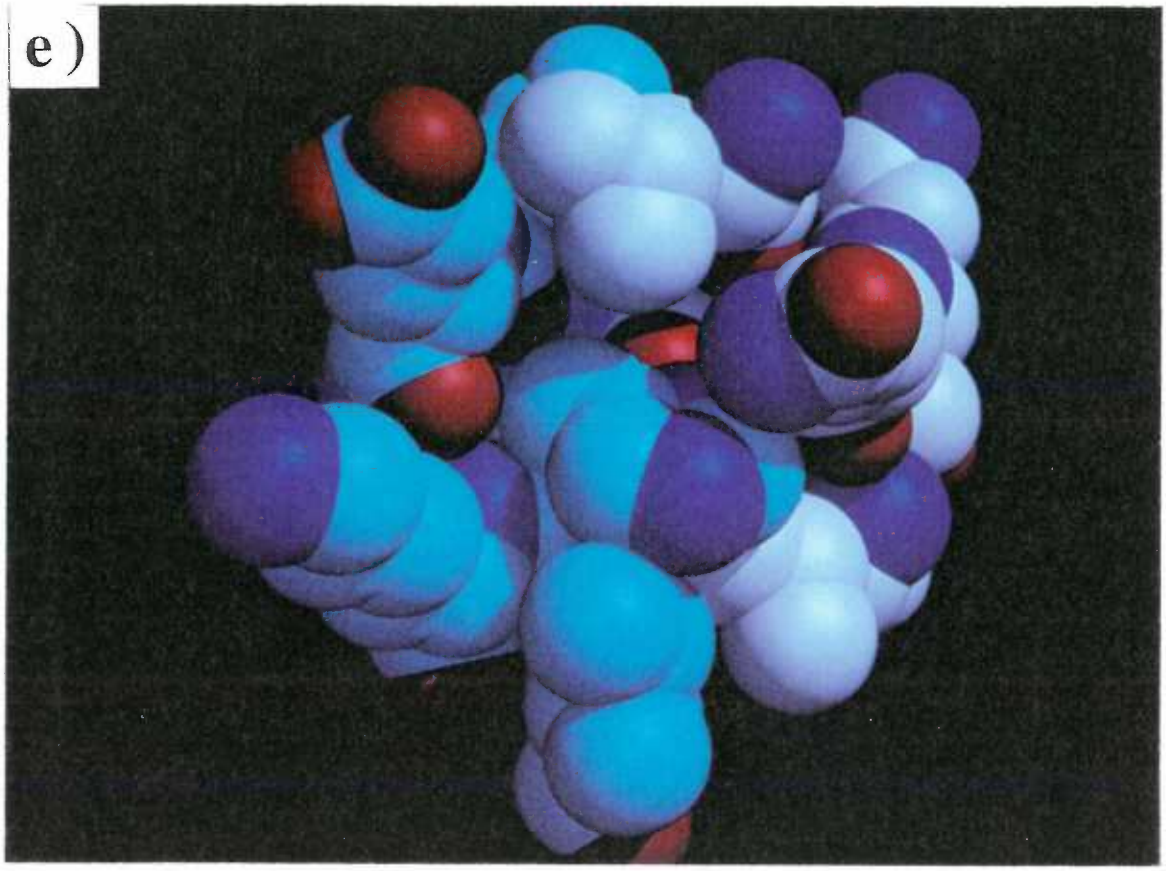

e)

Fig. 3 Molecular models of wild-type and mutant CREB leucine zipper structures. In these models, oxygen atoms are represented in red, nitrogen atoms in dark blue, and carbon atoms in white or light blue. Hydorgen bonds and van der Waals contacts are represented as orange dashed lines. a) The α-helical backbones of adjacent wild-type (white helix) and mutant (blue helix) leucine zippers are shown. Structures are modeled according to the crystal structure of the GCN4 leucine zipper, with CREB amino-acid substitutions introduced as appropriate. In the mutant helix shown, a histidine replaces the wild-type asparagine at the **a3** position. Adjacent residues are also shown. b) The asparagine residues in parallel wild-type α-helices line up in asymmetrical conformations, and a hydrogen bond between the amide and carbonyl groups is formed. This is identical to the GCN4 asparagine:asparagine hydrogen bond. c) In the histidine:histidine homodimer, no configuration could be generated that would allow formation of an optimal hydrogen bond and van der Waals contacts. The best possible hydrogen bond is depicted. d) In the asparagine:histidine heterodimer, a hydrogen bond is shown between the carbonyl oxygen of asparagine and the histidine Nδ. This orientation also permits van der Waals contacts between the histidine and adjacent residues of both helices. d) A computer generated CPK model shows the potential van der Waals contacts in a histidine:asparagine heterodimer.

References

Abate, C., Luk, D., Gagne, E., Roeder, R.G., Curran, T. (1990) Fos and Jun cooperate in transcriptional regulation via heterologous activation domains. *Mol. Cell. Biol.* **10**, 5532-5535

Abrams, E., Neigeborn, L., Carlson, M. (1986) Molecular analysis of *SNF2* and *SNF5* genes required for expression of glucose-repressible genes in *S. cerevisiae*. *Mol. Cell. Biol.* **6**, 3643-3651

Ahlgren, R., Simpson, E.R., Waterman, M.R., Lund-J. (1990) Characterization of the promoter/regulatory region of the bovine CYP11A (P-450scc) gene. Basal and cAMP-dependent expression. *J. Biol. Chem.* **265**, 3313-3319

Amati, B., Brooks, M.W., Levy, N., Littlewood, T.D., Evan, G.I., Land, H. (1993) Oncogemic activity of the c-Myc protein requires dimerization with Max. *Cell* **72**, 233-245.

Andrisani, O.M., Hayes, T.E., Roos, B., Dixon, J.E. (1987) Identification of the promoter sequences involved in the cell specific expression of the rat somatostatin gene. *Nucleic Acids Res.* **15**, 5715-5728

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Ramsdorf, H.J., Jonat, C., Herrlich, P., Karin, M. (1987) Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* **49**, 729-739

Appleman, M.M., Thompson, W.J., Russell, T.R. (1973) Cyclic nucleotide phosphodiesterases. *Adv. Cyclic Nucleotide Res.* **3**, 65

Arias, J., Alberts, A.S., Brindle, P., Claret, F.X., Smeal, T., Karin, M., Feramisco, J., Montminy, M.R. (1994) Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature* **370**, 226-229

Banerji, J., Rusconi, S., Schaffner, W. (1981) Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. *Cell* **27**, 299-308

Barone, M.V., Crozat, A., Tabee, A., Philipson, L., Ron, D. (1994) CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G1/S arrest. *Genes Dev.* **8**, 453-464

Benbrook, D.M., Jones, N.C. (1990) Heterodimer formation between CREB and JUN proteins. *Oncogene* **5**, 295-302

Benbrook, D.M., Jones, N.C. (1994) Different binding specificities and transactivation of variant CRE's by CREB complexes. *Nucleic Acids Res.* **22**, 1463-1469

Berger, S.L., Pina, B., Silverman, N., Marcus, G.A., Agapite, J., Regier, J.L., Triezenberg, S.J., Guarente, L. (1992) Genetic isolation of ADA-2: a potential transcriptional adaptor required for function of certain acidic activation domains. *Cell* **70**, 251-265

Berkowitz, L.A., Gilman, M.Z. (1990) Two distinct forms of active transcription factor CREB. *Proc. Natl. Acad. Sci. USA* **87**, 5258-5262

Billestrup, N., Swanson, L.W., Vale, W. (1986) Growth hormone-releasing factor stimulates proliferation of somatotrophs in vitro. *Proc. Natl. Acad. Sci. USA.* **83**, 6854-6857

Boney, C., Fink, D., Schlichter, D., Carr, K., Wicks, W.D. (1983) Direct evidence that the protein kinase catalytic subunit mediates the effects of cAMP on tyrosine aminotransferase synthesis. *J. Biol. Chem.* **258**, 4911-4918

Boshart, M., Weih, F., Nichols, M., Schutz, G. (1991) *Cell* **66**, 849-859

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., Silva, A.J. (1994) Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell* **79**, 59-68

Brindle, P., Linke, S., Montminy, M. (1993) Protein-kinase-A-dependent activator in transcription factor CREB reveals new role for CREM repressors. *Nature* **364**, 821-824

Byuss, C.V. and Russell, D.H. (1975) Ornithine decarboxylase activity: control by cyclic nucleotides. *Science* **187**, 650-652

Cao, Z., Umek, R.M., McKnight, S.L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes and Development* **5**, 1530-1552

Casey, P.J., Gilman, A.G. (1988) G protein involvement in receptor-effector coupling. *J. Biol. Chem.* **263**, 2577-2580

Chrivia, J.C., Kwok, R.P.S., Lamb, N., Hagiwara, M., Montminy, M.R., Goodman, R.H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855-859

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E., Goodman H.M. (1986) A cyclic AMP- and phorbol ester-inducible DNA element. *Nature* **323**, 353-356.

Courey, A.J., Tijan, R. (1988) Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* **55**, 887-898

Crick, F.H.C. (1953) The packing of α-helices: simple coiled-coils. *Acta Crystallogr.* **6**, 689-697

Crozat, A., Aman, P., Mandahl, N., Ron, D. (1993) Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. *Nature* **363**, 640-644

Dash, P.K., Karl, K.A., Colicos, M.A., Prywes, R., Kandel, E.R. (1991) cAMP response element-binding protein is activated by Ca+/calmodulin- as well as cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **88**, 5061-5065.

de Wet, J.R., Wood, K.V., De Luca, M., Helinski, D.R., Subramani, S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**, 725-737.

Dean, D. C., Newby, R. F., & Bourgeois, S. (1988) Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor ß, and cAMP in human cell lines. *J. Cell. Biol.* **106**, 2159-2170

Delegeane, A. M., Ferland, L. H., & Mellon, P. L. (1987) Tissue-specific enhancer of the human glycoprotein hormone α-subunit gene: dependance on cyclic AMP-inducible elements. *Mol. Cell. Biol.* **7**, 3994-4002

Delmas, V., Laoide, D.M., de Groot, R.P., Foulkes, N.S., Sassone-Corsi, P. (1992) Alternative usage of initiation codons in mRNA encoding the cAMP-responsive-element modulator generates regulators with opposite functions. *Proc. Natl. Acad. Sci. USA* **89**, 4226-4230

Deutsch, P.J., Hoeffler, J.P., Jameson, J.L., Lin, J.C., Habener, J.F. (1988) Structural determinants for transcriptional activation by cAMP-responsive genes. *J. Biol. Chem.* **263**, 18466-18472

Ellenberger, T.E., Brandl, C.J., Struhl, K., Harrison, S.C. (1992) The GCN4 basic region leucine zipper binds as a dimer of uninterrupted α helices: crystal structure of the protein-DNA complex. *Cell* **71**, 1223-1237

Ellis, M.J., Lindon, A.C., Flint, K.J., Jones, N.C., Goodbourn, S. (1995) Activating transcription factor-1 is a specific antagonist of the cyclic adenosine 3',5'-monophosphate (cAMP) response element-binding protein-1-mediated response to cAMP. *Mol. Endo*. **2**, 255-265

Enslen, H., Sun, P., Brickey, D., Soderling, H.S., Klamo, E., Soderling, T.R. (1994) Characterization of Ca+/Calmodulin-dependent protein kinase IV. *J. Biol. Chem.* **269**, 15520-15527

Erny, E.R., Berezo, M.W., Perlman, R.L. (1981) Activation of tyrosine 3-monooxygenase in pheochromocytoma cells by adenosine. *J. Biol. Chem.* **256**, 1335-1339

Ferreri, K., Gill, G., Montminy, M. (1994) The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex. *Proc. Natl. Acad. Sci. USA* **91**, 1210-1213

Fink, J.S., Verhave, M., Kasper, S., Tsukada, T., Mandel, G., Goodman, R.H. (1988) The CGTCA sequence motif is essential for the biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer. *Proc. Natl. Acad. Sci. USA* **85**, 6662-6666

Fiol, C.J., Williams, J.S., Chou, C-H., Wang, Q.M., Roach, P.J., Andrisani, O.M. (1994) A secondary phosphorylation of CREB341 at ser129 is required for the cAMP-mediated control of gene expression. *J. Biol. Chem.* **269**, 32187-32193

Fornace, A.J.Jr., Nebert, D.W., Hollander, M.C., Luethy, J.D., Papathanasiou, M. Fargnoli, J., Holbrook, N.J. (1989) Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. *Mol. Cell. Biol.* **9**, 4196-4203

Foulkes, N.S., Borelli, E., Sassoni-Corsi, P. (1991) CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* **64**, 739-749.

Foulkes, N.S., Mellstrom, B., Benusiglio, E., Sassone-Corsi, P. (1992) Developmental switch of CREM function during spermatogenesis: from antagonist to activator. *Nature* **355**, 80-84

Foulkes, N.S., Schlotter, F., Pevet, P., Sassone-Corsi, P. (1993) Pituitary hormone FSH directs the CREM functional switch during spermatogenesis. *Nature* **362**, 264-267

Gaire, M., Chatton, B., Kedinger, C. (1990) Isolation and characterization of two novel, closely related ATF cDNA clones from Hela cells. *Nucleic Acids Res.* **18**, 3467-3477

Gentz, R., Rauscher, F.J.III, Abate, C., Curran, T. (1989) Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. *Science* **243**, 1695-1699

Ginty, D.D., Bonni, A., Greenberg, M.E. (1994) Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-*fos* transcirption via phophorylation of CREB. *Cell* **77**, 713-725

Glover, J.N.M., Harrison, S.C. (1995) Crystal structure of the heterodimeric bZIP transcription factor c-Fos-C-Jun bound to DNA. *Nature* **373**, 257-261

Gonzalez, G.A., Menzel, P., Leonard, J., Fischer, W.H., Montminy, M.R. (1991) Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. *Mol. Cell. Biol.* **11**, 1306-1312

Gonzalez, G.A., Yamamoto, K.K., Fischer, W.H., Karr, D., Menzel, P., Biggs III, W., Vale, W.W., Montminy, M.R. (1989a) A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *Nature* **337**, 749-752

Gonzalez, G.A., Montminy, M.R. (1989b) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675-680.

Goodbourn, S., Zinn, K., Maniatis, T. (1985) Human beta-interferon gene expression is regulated by an inducible enhancer element. *Cell* **41**, 509-520

Greenberg, M.E., Greene, L.A., Ziff, E.B. (1985) Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC-12 cells. *J. Biol. Chem.* **260**, 14101-14110

Greene, L.A. and Tishler, A.S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Nat. Acad. Sci. USA* **73**, 2424-2428

Greene, L.A. and McGuire, J.C. (1978) Induction of ornithine decarboxylase by nerve growth factor dissociated from effects on survival and neurite outgrowth. *Nature* **276**, 191-193

Grove, J.R., Price, D.J., Goodman, H.M., Avruch, J. (1987) Recombinant fragment of protein kinase inhibitor blocks cyclic AMP-dependent gene transcription. *Science* **238**, 530-533

Guarente, L. (1988) UASs and enhancers: common mechanism of transcriptional activation in yeast and mammals. *Cell* **52**, 302-305

Guroff, G., Dickens, G., End, D., Londos, C. (1981) The action of adenosine analogs on PC-12 cells. *J. Neurochem.* **37**, 1431-1439

Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., Montminy, M. (1992) Transcriptional attenuation following cAMP induction requires PP-1 mediated dephoshorylation of CREB. *Cell* **70**, 105-113

Hai, T., Liu F., Coukos, W. J., Green, M.R. (1989) Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* **3**, 2083-2090.

Hai, T., Curran, T. (1991) Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA-binding specificity. *Proc. Natl. Acad. Sci. USA* **88**, 3720-3724.

Hatanaka, H., Otten, U., Thoenen, H. (1978) Nerve growth factor-mediated selective induction of ornithine decarboxylase in rat pheochromocytoma; a cyclic AMP-independent process. *FEBS Lett.* **92**, 313

Hayakawa, Y., Obata, K., Itoh, N., Yanaihara, N., Okamoto, H. (1984) Cyclic AMP regulation of pro-vasoactive intestinal polypeptide/PHM-27 synthesis if human neuroblastoma cells. *J. Biol. Chem.* **259**, 9207-9211

Haynes, S.R., Dollard, C., Winston, F., Beck, S., Trowsdale, J., Dawid, I.B. (1992) The bromodomain: a conserved sequence found in human, Drosophila and yeast proteins. *Nucleic Acids Res.* **20**, 2603

Hirai, S., Bourachot, B., Yaniv, M. (1990) Both Jun and Fos contribute to transcription activation by the heterodimer. *Oncogene* **5**, 39-46

Hisatake, K., Hasegawa, S., Takada, R., Nakatani, Y., Horikoshi, M., Roeder, R.G. (1993) The p250 subunit of native TATA box-binding factor TFIID is the cell-cycle regulatory protein CCG1. *Nature* **362**, 179-181

Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L., Habener J.F. (1988) Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA, *Science* **242**, 1430-1433

Hoeffler, J.P., Lustbader, J.W., Chen, C.Y. (1991) Identification of multiple nuclear factors that interact with cyclic adenosine 3',5'-monophosphate response element-binding protein and activating transcription factor-2 by protein-protein interactions. *Mol. Endo.* **5**, 256-266

Hoey, T., Weinzierl, R.O., Gill, G., Chen, J.L., Dynlacht, B,D., Tjian, R. (1993) Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. *Cell* **72**, 247-260

Hu, J.C., O'Shea, E.K., Kim, P.S., Sauer, R.T. (1990) Sequence requirements for coiled-coils: analysis with λ repressor-GCN4 leucine zipper fusions. *Science* **250**, 1400-1403.

Hummler, E., Cole, T.J., Blendy, J.A., Ganss, R., Aguzzi, W.S., Beermann, F., Schutz, G. (1994) Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. *Proc. Natl. Acad. Sci. USA* **91**, 5647-5651

Hunter, T., Karin, M. (1992) The regulation of transcription by phosphorylation. *Cell* **70**, 375-387

Imagawa, M., Chiu, R., Karin, M. (1987) Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell* **51**, 251-260

Ivashkiv, L.B., Liou, H., Kara, C.J., Lamph, W.W., Verma, I.M., Glimcher, L.H. (1990) mXBP/CRE-BP2 and cJun form a complex which binds to the cyclic AMP, but not to the 12-*O*-tetradecanoylphorbol-13-acetate response element. *Mol. Cel. Biol.* **10**, 1609-1621.

Jameson, J.L., Powers, A.C., Gallagher, G.D., Habener, J.F. (1989) Enhancer and promoter element interactions dictate cyclic adenosine monophosphate mediated and cell-specific expression of the glycoprotein hormone α-gene. *Mol. Endocrinol.* **3**, 763-772

Jones, N. (1990) Transcriptional regulation by dimerization: two sides to an incestuous relationship. *Cell* **61**, 9-11.

Kagawa, N., Ogo, A., Takahashi, Y., Iwamatsu, A., Waterman, M.R. (1994) *J. Biol. Chem.* **269**, 18716-18719

Kageyama, R., Sasai, Y., Nakanishi, S. (1991) Molecular characterization of transcription factors that bind to the cAMP responsive region of the substance P precursor gene. cDNA cloning of a novel C/EBP-related factor. *J. Biol. Chem.* **266**, 15525-15531

Kim, J., Tzamarias, D., Ellenberger, T., Harrison, S.C., Strulh, K. (1993) Adaptability at the protein-DNA interface is an important aspect of sequence recognition by bZIP proteins. *Proc. Nat. Acad. Sci. USA* **90**, 4513-4517

Konig, P., Richmond, T.J. (1993) The X-ray structure of the GCN4-bZIP bound to ATF-CREB site DNA shows the complex depends on DNA flexibility. *J. Mol. Biol.* **233**, 139-154

Krawjewski, W., Lee, K.A.W. (1994) A monomeric derivative of the cellular transcription factor CREB functions as a constitutive activator. *Mol. Cel. Biol.* **14**, 7204-7210

Krylov, D., Mikhailenko, I., Vinson, C. (1994) A thermodynamic scale for leucine zipper stability and dimerization specificity: e and g interhelical interactions. *EMBO* **13**, 2849-2861

Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R., Goodman, R.H. (1994) CBP serves as a co-activator for the transcription factor CREB. *Nature* **370**, 223-226

Landschulz, W.H., Johnson, P.F., McKnight, S.L. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **240**, 1759-1764

Langan, T.A. (1971) Cyclic AMP and histone phosphorylation. *Ann. N. Y. Acad. Sci.* **185**, 166

Laoide, B.M., Foulkes, N.S., Schlotter, F., Sassone-Corsi, P. (1993) The functional versitility of CREM is determined by its modular structure. *EMBO* **12**, 1179-1191

Lee, C.Q., Yun, Y., Hoeffler, J.P., Habener, J.F. (1990) Cyclic-AMP-responsive transcriptional activation of CREB-327 involves interdependent phosphorylated subdomains. *EMBO* **9**, 4455-4465

Lee, M., Mitchell, P., Tijan, R. (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **49**, 741-752

Lewis, E.J., Tank, A.W., Weiner, N., Chikaraishi, D.M. (1983) Regulation of tyrosine hydroxylase mRNA by glucocorticoid and cyclic AMP in a rat pheochromocytoma cell line. *J. Biol. Chem.* **258**,14632-14637

Lewis, E. J., Harrington, C. A., Chikaraishi, D. M. (1987) Transcriptional regulation of the tyrosine hydroxylase gene by glucocorticoid and cyclic AMP. *Proc. Natl. Acad. Sci. USA.* **84**, 3550-3554

Lillie, J.W., Green, M.R. (1989) Transcription activation by the adenovirus E1a protein. *Nature* **338**, 39-44

Liu, F., Thompson, M.A., Wagner, S., Greenberg, M.E., Green, M.R. (1993) Activating transcription factor-1 can mediate Ca2+- and cAMP-inducible transcriptional activation. *J. Biol. Chem.* **268**, 6714-6720

Liu, F., Green, M.R. (1990) A specific member of the ATF transcription factor family can mediate transciption activation by the adenovirus E1A protein. *Cell* **61**, 1217-1224

Liu, J.S., Park, E.A., Gurney, A.L., Roesler, W.J., Hanson, R.W. (1991) Cyclic AMP induction of phosphoenolpyruvate carboxykinase (GTP) gene transcription is mediated by multiple promoter elements. *J. Biol. Chem.* **266**, 19095-19102

Loriaux, M.M., Rehfuss, R.P., Brennan, R.G., Goodman, R.H. (1993) Engineered leucine zippers show that hemiphosphorylated CREB complexes are transcriptionally active. *Proc. Nat. Acad. Sci. USA* **90**, 9046-9050

Loriaux, M.M., Brennan, R.G., Goodman, R.H. (1994) Modulatory function of CREB:CREMα heterodimers depends upon CREMα phosphorylation. *J. Biol. Chem.* **46**, 28839-28843

Lund, J., Ahlgren, R., Wu, D.H., Kagimoto, M., Simpson, E.R., Waterman, M.R. (1990) Transcriptional regulation of the bovine CYP17 (P-450(17)alpha) gene. Identification of two cAMP regulatory regions lacking the consensus cAMP-responsive element (CRE). *J. Biol. Chem.* **265**, 3304-312

Lund, J., Faucher, D.J., Ford, S.P., Porter, J.C., Waterman, M.R., Mason, J.I. (1988) Developmental expression of bovine adrenocortical steroid hydroxylases. Regulation of P-450(17 alpha) expression leads to episodic fetal cortisol production. *J-Biol-Chem.* **263**, 16195-16201

Macgregor, P.F., Abate, C., Curran, T., (1990) Direct cloning of leucine zipper proteins: Jun binds cooperatively to the CRE with CRE-BP1. *Oncogene* **5**, 451-458

Maekawa, T., Matsuda, S., Fujisawa,J., Yoshida, M., Ishii-S. (1991) Cyclic AMP response element-binding protein, CRE-BP1, mediates the E1A-induced but not the Tax-induced trans-activation. *Oncogene* **6**, 627-632

Maguire, H.F., Hoeffler, J.P., Siddiqui, A. (1991) HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein-protein interactions. *Science* **252**, 842-844

Maurer, R.A. (1989) Both isoforms of the cAMP-dependent protein kinase catalytic subunit can activate transcription of the prolactin gene. *J. Biol. Chem.* **264**, 6870-6873.

Meinkoth, J.L., Ji, Y., Taylor, S.S., Feramisco, J.R. (1990) Dynamics of the distribution of cyclic AMP-dependent protein kinase in living cells. *Proc. Nat. Acad. Sci. USA* **87**, 9595-9599

Mitchell, P.J., Tjian, R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **17**, 371-378

Molina, C.A., Foulkes, N.S., Lalli, E., Sassone-Corsi, P. (1993) Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. *Cell* **75**, 875-866

Momoi, K., Waterman, M.R., Simpson, E.R., Zanger, U.M. (1992) 3',5'-cyclic adenosine monophosphate-dependent transcription of the CYP11A (cholesterol side chain cleavage cytochrome P450) gene involves a DNA response element containing a putative binding site for transcription factor Sp1. *Mol. Endo.* **6**, 1682-90.

Montminy, M.R., Bilezikjian, L.M. (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature* **328**, 175-178

Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G., Goodman R.H. (1986) Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **83**, 6682-6686.

Nakabeppu, Y., Ryder, K., Nathans, D. (1988) *Cell* **55**, 907-915

Nakagawa, J., von der Ahe, D., Pearson, D., Hemmings, B.A., Shibahara, S., Nagamine, Y. (1988) Transcriptional regulation of a plasminogen activator gene by cyclic AMP in a homologous cell-free system. Involvement of cyclic AMP-dependent protein kinase in transcriptional control. *J. Biol. Chem.* **263**, 2460-2468

Nichols, M., Weih, F., Schmid, W., DeVack, C., Kowenz-Leutz, E., Luckow, B., Boshart, M., Schutz, G. (1992) Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. *EMBO* **11**, 3337-3346

Nicklin, M.J.H., Casari, G. (1991) A single site mutation in a truncated Fos protein allows it to interact with the TRE *in vitro*. *Oncogene* **6**, 173-179.

O'Neil, K.T., Hoess, R.H., DeGrado, W.F. (1990) Design of DNA-binding peptides based on the leucine zipper motif. *Science* **249**, 774-778

O'Shea, E.K., Rutkowski, R., Kim, P.S. (1989a) Evidence that the leucine zipper is a coiled coil. *Science* **243**, 538-542

O'Shea, E.K., Rutkowski, R., Stafford III, W.F., Kim, P.S. (1989b) Preferential heterodimer formation by isolated leucine zippers from Fos and Jun. *Science* **245**, 646-648

O'Shea, E.K., Klemm, J.D., Kim, P.S., Albler, T. (1991) X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science* **254**, 539-544.

O'Shea, E.K., Rutkowski, R., Kim, P.S. (1992) Mechanism of specificity in the Fos-Jun oncoprotein heterodimer. *Cell* **68**, 699-708.

Oas, T.G., McIntosh, L.P., O'Shea, E.K., Dahlquist, E.K., Kim, P.S. (1990) Secondary structure of a leucine zipper determined by nuclear magnetic resonance spectroscopy. *Biochemistry* **29**, 2891-2894

Oliviero, S., Struhl, K. (1991) Synergistic transcriptional enhancement does not depend on the number of acidic activation domains bound to the promoter. *Proc. Natl. Acad. Sci. USA* **88**, 224-228

Paca-Uccaralertkun, S., Zhao, L-J., Adya, N., Cross, J.V., Cullen, B.R., Boros, I.M., Giam, C-Z. (1994) In vitro selection of DNA elements highly responsive to the Human T-Cell Lymphotrophic Virus type 1 transcriptional activator, Tax. *Mol. Cell. Biol.* **14**, 456-462

Park, E.A., Roesler, W.J., Liu, J., Klemm, D.J., Gurney, A.L., Thatcher, J.D. Shuman, J., Friedman, A., Hanson, R.W. (1990) The role of the CCAAT/enhancer-binding protein *Mol. Cell. Biol.* **10**, 6264-6272

Perlmann. T., Rangarajan, P.N., Umesono, K., Evans, R.M. (1993) Determinants for selective RAR and TR recognition of direct repeat HREs. *Genes Dev.* **7**, 1411-1422

Philippe, J., Drucker, D. J., Knepel, W., Jepeal, L., Misulovin, Z., Habener, J. F. (1988) Alpha-cell-specific expression of the glucagon gene is conferred to the glucagon promoter element by the interactions of DNA-binding proteins. *Mol. Cell. Biol.* **8**, 4877-4888

Pu, W.T., Struhl, K. (1991) The leucine zipper symmetrically positions the adjacent basic regions for specific DNA binding. *Proc. Nat. Acad. Sci. USA* **88**, 6901-6905

Quinn, P.G. (1993) Distinct activation domains within cAMP response element binding protein (CREB) mediate basal and cAMP stimulated transcription. *J. Biol. Chem.* **268**, 16999-17009

Reeder, T., Schleif, R. (1993) AraC can activate transcription from only one position and when pointed in only one direction. *J. Mol. Biol.* **231**, 205-218

Rehfuss, R.P., Walton, K.M., Loriaux, M.M. and Goodman, R.H. (1991) The CRE-binding protein ATF-1 activates transcription in response to cAMP-dependent protein kinase A. *J. Biol. Chem.* **266**, 18431-18434.

Riabowol, K.T., Fink, J.S., Gilman, M.Z., Walsh, D.A., Goodman, R.H., Feramisco, J.R. (1988) The catalytic subunit of cAMP-dependent protein kinase induces expression of genes containing cAMP-responsive enhancer elements. *Nature* **336**, 83-86

Roach, P.J. (1991) Multisite and hierarchal protien phosphorylation. *J. Biol. Chem.* **266**, 14139-14142

Ron, D., Habener, J.F. (1992) CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transrciption. *Genes Dev.* **6**, 439-453

Ruppert, S., Cole, T.J., Boshart, M., Schnid, E., Schutz, G. (1992) Multiple mRNA isoforms of the transcription activator protein CREB: generation by alternative splicing and expression in primary spermatocytes. *EMBO* **11**, 1503-1512

Santiago-Rivera, Z.I., Williams, J.S., Gorenstein, D.G., Andrisani, O.M. (1993) Bacterial expression and characterization of the CREB bZIP module: circular dichroism and 2D 1H-NMR studies. *Protein Science* **2**, 1461-1471

Sasaki, K., Cripe, T.P., Koch, S.R., Andreone, T.L., Peterson, D.D., Beale, E.B., Granner, K.K. (1984) Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. *J. Biol. Chem.* **259**, 15242-15251

Sassone-Corsi, P. (1988) Cyclic AMP induction of early adenovirus promoters involves sequences required for E1A trans-activation. *Proc. Natl. Acad. Sci. USA* **85**, 7192-7196

Schubert, D. and Whitlock, C. (1977) Alteration of cellular adhesion by nerve growth factor. *Proc. Nat. Acad. Sci. USA* **74**, 4055-4058

Schuermann, M., Hunter, J.B., Henning, G., Muller, R. (1991) Non-leucine residues in the leucine repeats of Fos and Jun contribute to the stability and determine the specificity of dimerization. *Nucl. Acids Res.* **19**, 739-746

Seed, B., Sheen, J-Y. (1988) A simple phase-extraction assay for chloramphenicol acyltransferase activity. *Gene* **67**, 271-277.

Sheng, M., Thomson, M.A., Greenberg, M.E. (1991) CREB: a Ca2+-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252**, 1427-1430.

Short, J.M., Wynshaw-Boris, A., Short, H.P., Hanson, R.W. (1986) Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. *J. Biol. Chem.* **261**, 9721-9726

Sirois, J., Richards, J.S. (1993) Transcriptional regulation of the rat prostaglandin endoperoxidase synthase 2 gene in granulosa cells. *J. Biol. Chem.* **268**, 21931-21938

Smeal, T., Angel, P., Meek, J., Karin, M. (1989) Different requirements for formation of Jun: Jun and Jun: Fos complexes. *Genes Dev.* **3**, 2091-2100

Stehle, J.H., Foulkes, N.S., Molina, C.A., Simonneaux, V., Pevet, P., Sassone-Corsi, P. (1993) Adrenergic signals direct rhythmic expresssion of transciptional repressor CREM in the pineal gland. *Nature* **365**, 314-320

Struthers, R.S., Vale, W.W., Arias, C., Sawchenko, P.E., Montminy, M.R. (1991) Somatotroph hypoplasia and dwarfism in transgenic mice expressing a non-phosphorylatable CREB mutant. *Nature* **350**, 622-624

Suckow, M., von Wilcken-Bergmann, B., Muller-Hill, B. (1993a) Identification of three residues in the basic regions of the bZIP proteins GCN4, C/EBP and TAF-1 that are involved in specific DNA binding. *EMBO* **12**, 1193-1200

Suckow, M., von Wilcken-Bergmann, B., Muller-Hill, B. (1993b) The DNA binding specificity of the basic region of the yeast transcriptional activator GCN4 can be changed by the substitution of a single amino acid. *Nucleic Acids Res.* **21**, 2081-2086

Sun, P., Enslen, H., Myung, P.S., Maurer, R.A. (1994) Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev.* **8**, 2527-2539

Sutherland, E.W. and Rall, T.W. (1958) *J. Biol. Chem.* **232**, 1077-1091

Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C., Kennison, J.A. (1992) brahma, a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell* **68**, 561-572

Tanaka, K.K., Herr, W. (1990) Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. *Cell* **60**, 375-386

Treisman, R., Maniatis, T. (1985) Simian virus 40 enhancer increases number of RNA polymerase II molecules on linked DNA. *Nature* **315**, 73-5

Tsukada, T., Fink, J.S., Mandel, G., Goodman, R.H. (1987) Identification of a region in the vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. *J. Biol. Chem.* **262**, 8742-8747

Umesono, K., Murakami, K.K., Thompson, C.C., Evans, R.M. (1991) Direct repeats as selective elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* **65**, 1255-1266

Vaccaro, K.K., Liang, B.T., Perelle, B.A., Perlman, R.L. (1980) Tyrosine 3-monooxygenase regulates catecholamine synthesis in pheochromocytoma cells. *J. Biol. Chem.* **255**, 539-6541

Vallejo, M., Ron, D., Miller, C.P., Habener, J.F. (1993) C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to cAMP response elements. *Proc. Natl. Acad. Sci. USA*. **90**, 4679-4683

van Buskirk, R., Corcoran, T., Wagner, J.A. (1985) Clonal variants of PC12 pheochromocytoma cells with defects in cAMP-dependent protein kinase induce ornithine decarboxylase in response to nerve growth factor but not to adenosine agonists. *Mol. Cell. Biol.* **5**, 1984-1992

van Dam, H., Duyndam, M., Rottier, R., Bosch, A., de Vries-Smits, L., Herrlich, P., Zantema, A., Angel, P., van der Eb, A.J. (1993) Heterodimer formation of c-Jun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. *EMBO* **12**, 479-487

Vinson, C.R., Sigler, P.B., McKnight, S.L. (1989) Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* **246**, 911-916

Vinson, C.R., Hai, T., Boyd, S.M. (1993) Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. *Genes Dev.* **7**, 1047-1058

Waeber, G., Habener, J.F. (1991) Nuclear translocation and DNA recognition signals colocalized within the bZIP domain of cyclic adenosine 3',5'-monophosphate response element-binding protein CREB. *Mol. Endocrinol.* **5**, 1431-1438

Wadzinski, B. E., Wheat, W. H., Jasper, S., Peruski, L. F., Jr., Lickteig, R. L., Johnson, G. L., Klemm, D. J. (1993) Nuclear protein phosphatase 2A dephosphorylates Protein Kinase A phosphorylated CREB and regulates CREB transcriptional stimulation. *Mol.Cell. Biol.* **13**, 2822-2834

Wagner, S., Green, M.R. (1993) HTLV-1 Tax protein stimulation of DNA binding of bZIP proteins by enhancing dimerization. *Science* **262** 395-399

Walton, K.M., Rehfuss, R.P., Chrivia, J.C., Lochner, J.E., Goodman, R.H. (1992) A dominant repressor of cyclic adenosine 3',5'-monophosphate (cAMP)-regulated enhancer-binding protein activity inhibits the cAMP-mediated induction of the somatostatin promoter *in vivo*. *Mol. Endocrinol.* **6**, 647-655

Waterman, M.R. (1994) Biochemical diversity of cAMP-dependent transcription of steroid hydroxylase genes in the adrenal cortex. *J. Biol. Chem.* **45**, 27783-27786

Weih, F., Stewart, A.F., Boshart, M., Nitsch, D., Schutz, G. (1990) In vivo monitoring of a cAMP-stimulated DNA-binding activity. *Genes Dev.* **4**, 1437-1449

Wheat, W. H., Roesler, W. J., Klemm, D. J. (1994) Simian virus 40 small tumor antigen inhibits dephosphorylation of protein kinase A phosphorylated CREB and regulates CREB transcriptional stimulation. *Mol.Cell. Biol.* **14**, 5881-5890

Yamamoto, K.K., Gonzalez, G.A., Biggs III, W.H., Montminy, M.R. (1988) Phosphorylation induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* **334**, 494-498.

Yamamoto, K.K., Gonzalez, G. A., Menzel, P., Rivier, J., Montminy, M.R. (1990) Characterization of a bipartite activator domain in transcription factor CREB. *Cell* **60**, 611-617.

Yang, S.I., Lickteig, R.L., Estes, R., Rundell, K., Walter, G., Mumby, M.C. (1991) Control of protein phosphatase 2A by simian virus 40 small-t antigen. *Mol. Cell. Biol.* **11**, 1988-1995

Zhou, N.E., Kay, C.M., Hodges, R.S. (1992) Synthetic model proteins. *J. Biol. Chem.* **267**, 2664-2670

Zhou, Y., Busby, S., Ebright, R.H. (1993a) Identification of the functional subunit of a dimeric transcription activator protein by use of oriented heterodimers. *Cell* **73**, 375-379

Zhou, Y., Zhang, X., Ebright, R.H. (1993b) Identification of the activating region of catabolite gene activator protein (CAP): isolation and characterization of mutants of CAP specifically defective in transcription activation. *Proc. Nat. Acad. Sci. USA* **90**, 6081-6085

Zinszner, H., Albalat, R., Ron, D. (1994) A novel effector domain from RNA-binding protein TLS or EWS is required for oncogenic transformation by CHOP. *Genes Dev.* **8**, 2513-2526