Acta Neurobiol. Exp. 2000, 60: 557-567

# Effect of opioids on $Ca^{2+}$/cAMP responsive element binding protein

**Wiktor Bilecki** and **Ryszard Przewłocki**

Department of Molecular Neuropharmacology, Institute of Pharmacology, Polish Academy of Sciences, 12 Smętna St., 31-343 Cracow, Poland

**Abstract.** $Ca^{2+}$/cAMP response element binding protein (CREB) is an important factor linking the opioid-regulated secondary messenger systems to alterations in gene expression. Opioids regulate CREB level, its phosphorylation and binding to its corresponding response element in the promoters of several genes implicated in drug addiction. CREB mediates the action of opioids on the expression of several genes in brain regions responsible for drug-seeking behavior and manifestation of signs of dependence. Moreover, alterations in CREB level can affect the rewarding properties of morphine and regulate the self-administration of cocaine. At the cellular level CREB acts as convergence point for different cellular pathways. Opioids affect two different intracellular mediator systems: inhibitory - connected with cAMP, and stimulatory - involving calcium and the PKC pathway. Both can affect CREB but in different phases of opiate action. The presence of this biphasic mechanism can explain the phenomenon of the induction of some CRE-controlled genes after both acute and chronic morphine administration. Cellular studies also highlight the relevance of other ATF/CREB family members which can affect $Ca^{2+}$/cAMP response element (CRE) controlled transcription as well as other transcription factors which make the opioid induction longer lasting.

Correspondence should be addressed to R. Przewłocki, Email: nfprzewl@kinga.cyf-kr.edu.pl

**Key words:** CREB(cAMP responsive element binding protein), opioid receptors, morphine, antisense, oligonucleotides, neuroblastoma, protein kinase, CRE binding activity

## INTRODUCTION

Considerable interest has focused on molecular alterations which may contribute to pathophysiological states that occur following treatment with opiates. It is well known that opiates acting on opioid receptors (coupled to $G_i/G_o$ classes of the G proteins) acutely inhibit cyclic AMP (cAMP) formation, $Ca^{2+}$ conductance and activate potassium conductance, leading to hyperpolarization of the cell (Nestler 1992). The hyperpolarization resolves with time and cAMP levels are normalized and subsequently raised above baseline. The opiate-induced changes in the activation of protein kinase A lead to alterations in the phosphorylation of proteins relevant to opioid signaling and to changes in the expression of the CREB (calcium/cAMP response element binding protein), which may be important in the development and expression of opioid dependence (Nestler et al. 1993, Widnell et al. 1994, Maldonado et al. 1996). CREB exists as three alternatively spliced isoforms: CREB-α and CREB-δ which differ by a 14 amino acid segment, and CREB-β. The CREB-δ isoform comprises 80-90% of total CREB protein in the brain (Yamamoto et al. 1990). CREB-β is expressed ubiquitously and this isoform may compensate partly for the loss of the CREB-α and CREB-δ in CREB-knockout mice (Blendy et al. 1996, Glazewski et al. 1999). CREB activity is regulated by phosphorylation in response to diverse signals. Increases in calcium or cAMP result in phosphorylation of the serine residue within the kinase-inducible domain (KID) at position 133 ($Ser^{133}$) (Gonzalez and Montminy 1989). Recent data suggest that CREB phosphorylation alone does not alter the secondary structure of CREB; rather phosphorylation creates a high-affinity binding site for other proteins (Richards et al. 1996). In particular, phosphorylation of $Ser^{133}$ promotes interaction of CREB with the KIX domains of the coactivators CREB-binding protein (CBP) and P300 (Chrivia et al. 1993). The binding of CBP causes CREB to interact with the basal transcription factor TFIIB (Kwok et al. 1994) and TFIID (Horikoshi et al. 1988) and thereby initiates induction of CRE-dependent genes.

CREB-mediated transcription is thought to be critical for a variety of adaptive neuronal responses. It has been shown to be important for memory and learning in a number of systems (Frank and Greenberg 1994, Martin and Kandel 1996, Silva et al. 1998). Generation of mutant mice provided a molecular tool to study the relevance of CREB *in vivo* (Hummler et al. 1994). Mice completely lacking CREB are not viable (Rudolph et al. 1998). Mice lacking the CREB-α and CREB-δ isoforms are viable and show no impairment of development although they exhibit profound long-term memory deficits (Bourtchuladze et al. 1994, Kogan et al. 1997). In mice lacking the CREB-α and CREB-δ isoforms there is a strong reduction in naloxone-induced withdrawal behaviors and a small decrement in tolerance, but no alterations in pain sensitivity and the density of opiate receptors (Maldonado et al. 1996). However, there are several mechanisms compensating for this mutation (e.g. the increase in CREM, CREB-β and ATF-2). Moreover, this deficiency in CREB occurs throughout all tissues making precise evaluation of CREB function difficult.

More detailed studies involved analysis of CREB expression in brain regions implicated in opiate dependence and addiction. A role for CREB mediation of gene expression in response to opioids has been proposed (Nestler et al. 1993, 1996) based on the observation that administration of opioids affects components of the cAMP pathway. Support for this scheme comes from recent studies of rats dependent on cocaine in which upregulation of CREB-mediated transcription in the nucleus accumbens (NAc) counteracted the rewarding effects of cocaine and, conversely, overexpression of a dominant -negative mutant CREB in NAc increased the rewarding effects of cocaine (Carlezon et al. 1998).

## CREB INVOLVEMENT IN THE ACTION OF OPIATES IN THE BRAIN

### Nucleus accumbens

The brain region implicated in the reinforcing properties of opiates and possibly for the long-term motivational changes associated with opiate addiction is the NAc (Koob 1992). Morphine increases dopamine release in NAc (Di Chiara and North 1992). It has been proposed that morphine acts on inhibitory μ opioid receptors located on GABA interneurons (which are inhibitory as well) in substantia nigra pars reticulata (SNr) and ventral tegmantal area (VTA) (Di Chiara and North 1992). This would disinhibit substantia nigra pars compacta (SNc) and VTA dopamine neurons, increase dopamine neuronal firing, and induce dopamine release in striatum and NAc (Bontempi and Sharp 1997). There are no data showing directly the effects of acute opiate administration on neural activity or cAMP system in the NAc. Total CREB protein level in the NAc was not af-

fected by acute opioids. However, acute morphine--μ-opioid receptors - induces c-Fos proteins and AP-1 binding in striatum and NAc (Liu et al. 1994). The c-Fos promoter is known to have the CRE and SRE elements, responsive to cAMP and $Ca^{2+}$ and coactivation of both elements is required for Fos induction in single striatal cells (Robertson et al. 1995, Konradi et al. 1996). This is in agreement with the observation that CREB phosphorylation level rather than CREB levels is responsible for CREB activity (Faentzke et al. 1998). Functional relevance of CREB was demonstrated by the abolition of the ability of an acute exposure to cocaine to induce c-Fos by antisense oligonucleotide administration in this brain region. c-Fos induction after morphine was shown to be mediated by both dopamine and glutamate inputs to striatum/NAc from the SNc/VTA and cortex, respectively (Bontempi and Sharp 1997). Chronic morphine treatment also leads to a significant induction of some components of cAMP pathway (adenylyl cyclase level and PKA activity, c-Fos level, AP-1 DNA binding activity) but decreases other ($G_{\alpha i}$ levels) in the NAc (Terwilliger et al. 1991, Noble and Cox 1996). Given the observed upregulation of the PKA pathway in the Nac one should have expected CREB to be regulated by chronic morphine treatment as well. Indeed, it was, but levels of total CREB immunoreactivity were found to be decreased in the NAc (Widnell et al. 1996). This is a striking observation but the chronic morphine-reduced total CREB level could be considered as the compensatory mechanism to the increase in the CREB phosphorylation caused by upregulation of PKA pathway. Withdrawal from morphine elicited a dramatic increase in c-Fos and AP-1 binding (Nye and Nestler 1995) and given the decreased total CREB level this rapid induction apparently depended on increased CREB activity. It is another puzzle that c-Fos is induced in the NAc by acute and chronic morphine treatment as well as during morphine withdrawal. One possible explanation is that the observed c-Fos induction occurs within different neuronal populations, another possibility is that the induction is mediated *via* different mechanisms. The latter possibility could involve activation of CREB in each case: after acute or chronic morphine administration as well as during morphine withdrawal.

### Locus coeruleus

Opioid regulation of CREB was also thoroughly studied in the locus coeruleus - major noradrenergic nucleus in the brain implicated in controlling attention and activity of the autonomic nervous system. Locus coeruleus (LC) is also important in physical opiate dependence and withdrawal. Acutely, opiates inhibit LC neuronal firing in part *via* inhibition of adenylate cyclase and cAMP-dependent protein phosphorylation (Aghajanian and Wang 1987, Duman et al. 1988, Alreja and Aghajanian 1991). Acute opiates also decrease the phosphorylation state of CREB without affecting its total protein level (Guitart et al. 1992). Consequently, levels of *c-fos* expression were decreased in LC from rats treated acutely with morphine. In contrast, chronic opiates up-regulate the cAMP system in these neurons, with increases observed in levels of G protein subunits, adenylyl cyclase, adenylyl cyclase activity, PKA and tyrosine hydroxylase levels (Duman et al. 1988, Lane-Ladd et al. 1997). The spontaneous firing rate of LC neurons in brain slices is also increased by previous chronic administration of morphine (Kogan et al. 1992). Chronic morphine administration was found to increase total levels of CREB immunoreactivity in LC. This CREB regulation in the LC was surprising because CREB expression, particularly in the brain, is generally considered to be constitutive. The increase in CREB immunoreactivity was associated with an equivalent increase in CRE binding (Widnell et al. 1994). However, in the chronic morphine-treated state, despite the up-regulated cAMP system, CREB phosphorylation was reduced down to control levels probably due to the inhibition of the system by persistent exposure to opiates (Guitart et al. 1992). Consequently, c-Fos levels in rats treated chronically with morphine were decreased along with CREB phosphorylation. It is interesting to note that among adenylyl cyclase only types VIII and I are upregulated by chronic morphine, whereas several other types are not affected (Lane-Ladd et al. 1997). These forms of adenylyl cyclase are activated by $Ca^{2+}$ and are only mildly activated by $G_{\alpha s}$ (Cooper et al. 1995). After precipitation of the opioid withdrawal the up-regulated cAMP system contributes to the dramatic increase of LC firing rates, an increase in CREB phosphorylation and a two- to three-fold increase in levels of mRNA and protein for *c-fos* (Hayward et al. 1990, Holmes et al. 1995).

### Functional relevance of CREB in drug addiction: an antisense oligonucleotide approach

Due to the lack of specific inhibitors it has proven difficult to determine the role of a constitutive transcription

factor *in vivo*. The use of an antisense oligonucleotide strategy to specifically decrease levels of CREB surmounts this problem to some degree. Functional consequences of the morphine-induced CREB in LC were studied by injections of antisense oligonucleotides against CREB mRNA. In drug-naive rats CREB antisense oligonucleotide infusions reduced CREB immunoreactivity in the injected LC compared with the uninjected control side (Lane-Ladd et al. 1997). Such infusions also elicited reduction in basal levels of types VIII and I of adenylyl cyclase, the catalytic and type II regulatory subunits of PKA, and tyrosine hydroxylase, having no effect on types III and IV of adenylyl cyclase and $G_{\alpha i}$. Thus the application of CREB antisense oligonucleotides reduced basal level of some, but not all, components of the PKA pathway. This pathway is up-regulated in the LC following chronic morphine treatment (see above). However, of these genes CREB was shown to act at a CRE within promoter of tyrosine hydroxylase gene only (Lazaroff et al. 1995). Promoters of the other regulated genes have not yet been isolated (type VIII adenylyl cyclase) or lack functional CREs (PKA, type I adenylyl cyclase). Nevertheless, CREB antisense oligonucleotide blocked the morphine-induced upregulation of type VIII adenylyl cyclase and tyrosine hydroxylase and also completely prevented the morphine-induced increase in spontaneous firing rates of LC neurons. On the other hand type I adenylyl cyclase and PKA upregulation were not affected by CREB antisense oligonucleotide in morphine-treated animals despite significant reduction of their basal levels in naive animals.

Antisense oligonucleotide infusions also significantly attenuated the signs of certain withdrawal behaviors (teeth chattering, wet dog shakes, ptosis, irritability, vacuous chewing) but not others (piloerection, lacrimation, salivation, diarrhea, stereotypy, weight loss). Interestingly, greater attenuation of these behaviors was seen 15-30 min. after precipitation of withdrawal than during the first quarter when the withdrawal syndrome is the most pronounced (Lane-Ladd et al. 1997). This suggests that CREB may be responsible for a secondary neuroadaptation to sustained activity of cAMP pathway in LC during withdrawal.

In NAc antisense oligonucleotides against CREB down-regulated CREB protein levels to an extent similar to that of chronic morphine. Also $G_{\alpha i}$ and levels of the catalytic subunit of PKA were reduced by administration of antisense oligonucleotides into the NAc. Although the antisense-induced reduction of $G_{\alpha i}$ resembled the action of chronic morphine on CREB, their effects on the PKA were in opposition. This is important because PKA phosphorylates CREB on $Ser^{133}$ (Gonzalez and Montminy 1989).

The above data indicate that although antisense oligonucleotides against CREB reduced the CREB protein level in both NAc and LC, their specific action on claimed CREB targets is rather unclear. Firstly, antisense oligonucleotides against CREB decreased the basal but not morphine-induced levels of adenylyl cyclase type I and catalytic and regulatory subunits of PKA whose gene promoters lack functional CRE elements. Secondly, antisense oligonucleotides against CREB reduced the level of $G_{\alpha i}$ in NAc but not in LC, and the reason for this is unclear. Lastly, in the unphosphorylated state, CREB can bind DNA but does not activate transcription. The mutation of CREB molecule by a $Ser^{133}$-to-$Ala^{133}$ substitution functions as a potent dominant-negative repressor of CREB-dependent gene expression both *in vitro* and *in vivo* (Faentzke et al. 1998). CREB activity is regulated by phosphorylation, and unphosphorylated CREB under certain circumstances can even act as a repressor of gene transcription (Vallejo 1995). The effects of CREB antisense oligonucleotides on the factors regulated by chronic morphine treatment are gathered in Table I.

Functional relevance of CREB antisense oligonucleotides administration was demonstrated by the abolition of the ability of acute exposure to cocaine to induce c-Fos in the NAc and LC. Furthermore, intra-NAc injections of CREB antisense oligonucleotides produced a transient decrease in cocaine self-administration. Similarly, overexpression of CREB in this region decreases while, conversely, overexpression of a dominant-negative mutant of CREB increases the rewarding effects of cocaine. CREB regulates dynorphin expression in the NAc. Altered transcription of dynorphin likely contributes to the CREB level regulation of the reinforcing properties of cocaine (Carlezon et al. 1998). However, this conception remains in disagreement with the reported reduction of CREB level in NAc after chronic morphine treatment with concurrent elevation of prodynorphin, its peptide α-neoendorphin and *c-fos* (i.e. CREB regulated genes) (Nye and Nestler 1995, Przewłocka et al. 1996, Nylander et al. 1997). One possible explanation is that changes in CREB level can not be simply attributed to its phosphorylation, although phosphorylation is crucial for controlling other genes

Table I

The effect of CREB antisense oligonucleotide on the levels of factors regulated by chronic morphine treatment in the nucleus accumbens (Nac) and locus coeruleus (LC)

| | Adenylyl cyclase | | Protein kinase A subunits | | Tyrosine hydroxylase | $G_{\alpha i}$ | FIRING | CREB |
|---|---|---|---|---|---|---|---|---|
| | I | VIII | Catalytic | Regulatory type II | | | | |
| Chronic morphine in LC | ⇑ | ⇑ | ⇑ | ⇑ | ⇑ | ⇑ | ⇑ | ⇑ |
| CREB antisense oligonucleotides in LC | ⇓ | ⇓ | ⇓ | ⇓ | ⇓ | no effect | ⇓ | ⇓ |
| Chronic morphine + CREB antisense oligonucleotides in LC | no effect | ⇓ | no effect | no effect | ⇓ | no effect | ⇓ | ⇓ |
| CREB antisense oligonucleotides in NAc | ? | ? | ⇓ | ? | - | ⇓ | ? | ⇓ |
| Chronic morphine in NAc | ⇑ | ⇑ | ⇑ | ⇑ | - | ⇓ | ⇑ | ⇓ |

and can be independent from CREB level. Another likely explanation is that CREB as well as CRE link opioid receptors through general ATF/CRE transcription factors to alteration in target gene expression.

## CELLULAR STUDIES

Activation of CREB in the brain depends largely on the innervation of the structures studied (Liu and Graybiel 1998). NAc receives inputs from several limbic structures including the amygdala, the hippocampus and the limbic prefrontal cortex. These limbic-associated circuits as well as the presence of different neuronal populations make the evaluation of the relevance of CREB very complicated. Moreover, studies on CREB phosphorylation in the brain require the development of special techniques which do not directly measure CREB phosphorylation (Guitart et al. 1992). The direct effect of opioids on the regulation of CREB and its phosphorylation has been studied in NG108-15 neuro- blastoma x glioma cells (Bilecki et al. 2000). This cell line was established as a cellular model for studying opioid effects and several phenomena of opioid action in the brain. The molecular mechanism of tolerance, dependence and withdrawal on the basis of the action of opioids on cAMP pathway components was first shown in these cells. Acute morphine, *via* δ-opioid receptors, exerts a stimulatory effect on CREB phosphorylation but not total CREB level in these cells. This stimulation required $Ca^{2+}$/calmodulin and activation of protein kinase C but did not involve the morphine-suppressed cAMP pathway. Increased phosphorylated CREB level was accompanied by induction in c-Fos levels, an effect blocked by $Ca^{2+}$/calmodulin protein kinase C inhibitors, suggesting that the CREB mediates the observed effect (Fig. 1). Moreover, opioid-stimulated c-Fos levels remained elevated for longer time periods (up to 6 h) than phosphorylated CREB levels (up to 3 h). This suggests that c-Fos may make the acute opioid-stimulation even longer-lasting. Prolonged (up to 5 days) treatment with morphine reduced phosphorylated CREB and c-Fos levels down to control levels. In contrast, precipitated withdrawal induced profoundly phosphorylated CREB and c-Fos levels in these cells. This indicates that opioids

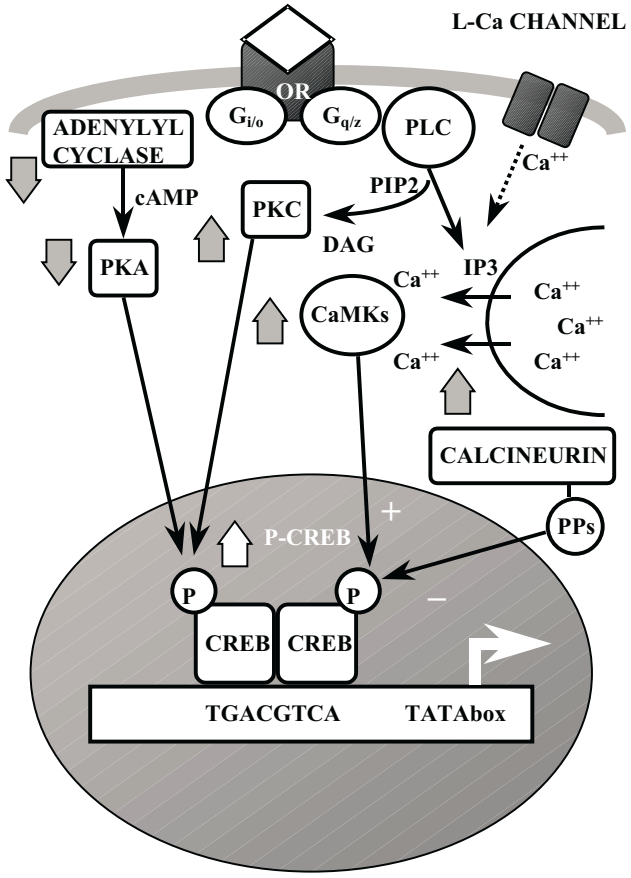


Fig. 1. Schematic representation of the signal transduction pathways leading to increased CREB phosphorylation following opiate stimulation of cells expressing μ- or δ-opioid receptors. Opioids acting through opioid receptors (OR) inhibit cAMP pathway but are able to release $Ca^{2+}$ from intracellular stores *via* inositol 1,4,5-triphosphate (IP3) production. Opioids also activate protein kinase C (PKC). Rise in [$Ca^{2+}$] activate $Ca^{2+}$/calmodulin dependent protein kinases (CaMKs) which together with PKC phosphorylate CREB bound to promoters of target genes (e.g. *c-fos*). Following the initial stimulation CREB is dephosphorylated by nuclear protein phosphatases (PPs). CREB activated by opioids in turn induces the expression of other genes.

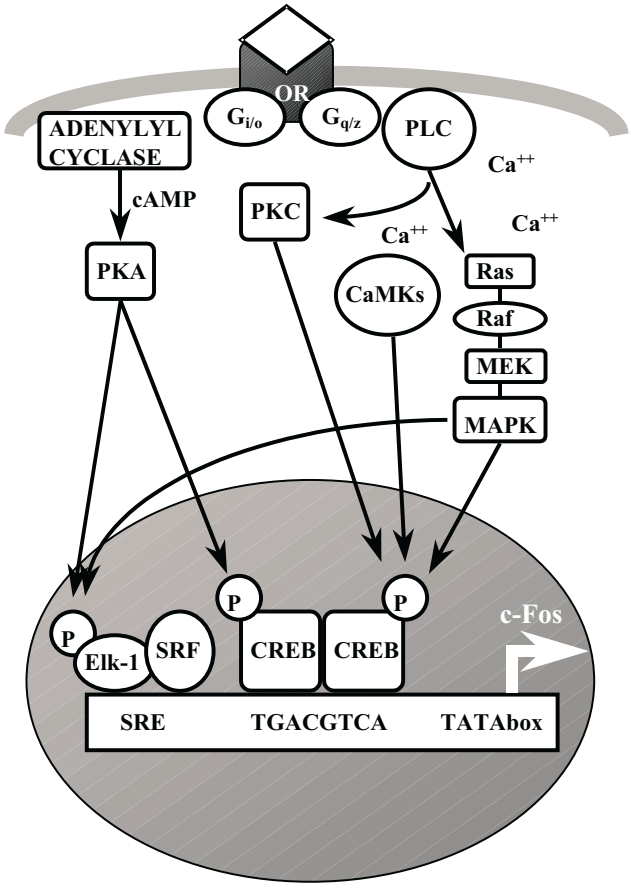


Fig. 2. Schematic illustration of c-Fos induction mediated by CREB and mitogen-activated protein kinases (MAPKs) following opioid administration. After activation of opioid receptors (OR) CREB (bound to its corresponding response element in the *c-fos* promoter) is phosphorylated by protein kinase C (PKC), $Ca^{2+}$/calmodulin dependent protein kinases (CaMKs) and MAPKs and stimulates c-Fos expression. MAPKs also phosphorylate the transcription factor Elk-1, which together with serum response factor (SRE) binds to serum response element in the *c-fos* promoter leading to enhnanced c-Fos synthesis. Protein kinase A (PKA), inhibited by opioids, is not implicated in c-Fos synthesis after acute morphine administration. In contrast, during withdrawal upregulated PKA pathway inhibits MAPKs phosphorylation but also induce c-Fos synthesis through increase in CREB phosphorylation.

could affect two different intracellular mediator systems: inhibitory - connected with cAMP, and stimulatory - involving calcium and the PKC pathway, and affect CREB in different phases of opiate action. Similar to growth factor signals, $Ca^{2+}$ can also activate the mitogen activated protein kinase (MAPK) pathway (Finkbeiner and Greenberg 1996) which has been recently shown to mediate CREB phosphorylation in response to $Ca^{2+}$ influx and PKC activation in oligodendrocyte progenitor cells (Pende et al. 1997). We have found that in NG108-15 cells the mitogen-activated protein kinases (MAPKs) pathway is activated on stimulation of δ-opioid receptors. This increase of phosphorylated MAPKs levels was short-lasting (5-20 min.) and similar to CREB phosphorylation involved PKC and neither cAMP nor cGMP de-

pendent protein kinase pathway. Therefore the MAPKs could also contribute to the induction of CREB phosphorylation following opioid treatment as well as to the induction of c-Fos synthesis (Fig. 2). Moreover their stimulation by acute opioids confirms the existence of an activatory intracellular system coupled to δ-opioid receptors and involving $Ca^{2+}$. Recent data from cultured native CNS neurons also show that the activation of opioids receptors can augment several components of neuronal $Ca^{2+}$ signaling pathways and, as a consequence, enhance intracellular $Ca^{2+}$ signals (Przewłocki et al. 1999).

Due to the fact that morphine analgesia and dependence are connected predominantly with μ receptors, a model containing μ receptors (Neuro2a cells, transfected with the cloned μ receptor Neuro2a MOR) was also studied. Morphine, acting *via* μ-opioid receptors, acutely induced CREB phosphorylation in Neuro2a MOR cells but this effect was shorter (15-45 min.) and weaker than stimulation observed in NG108-15 cells. Acute administration of opiates (morphine, endomorphin-1, DAMGO) also increased binding to consensus CRE and AP-1 elements in Neuro2a MOR cells. Total CREB protein level was not affected significantly by morphine and because CREB binds CREs irrespective of its state of dimerization (Waeber and Habener 1991, Richards et al. 1996) this suggests involvement other factors - possibly ATF-2: ATF-2 or ATF-2:c-Jun dimers. The binding of these factors to various CRE motifs is similar to, or stronger than that of CREB, and the strength of their DNA-binding *in vitro* correlates with their capacity for transactivation (Benbrook and Jones 1990). A concomitantly increase in CRE DNA binding activity was observed 40-60 min. after morphine treatment, indicating a

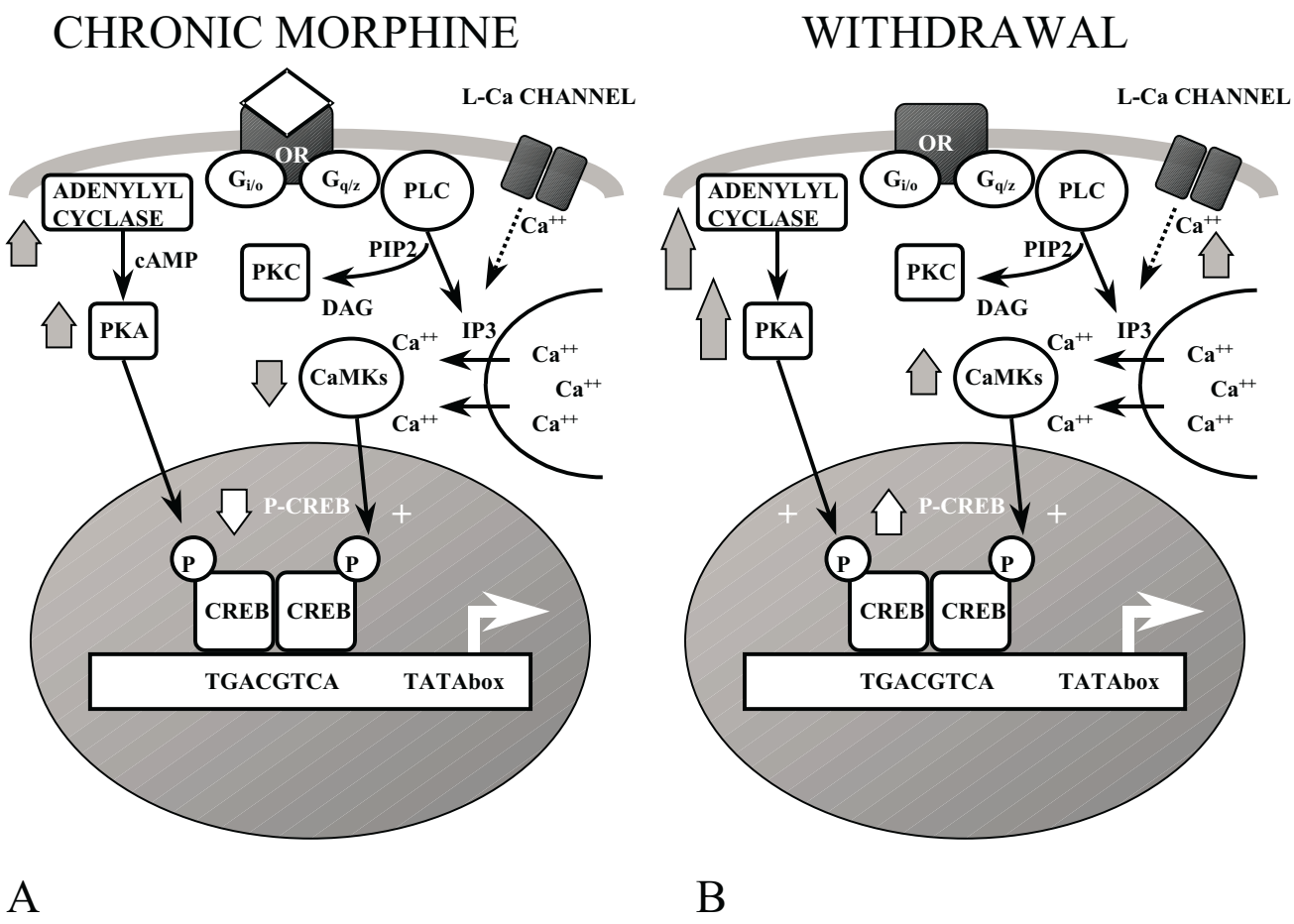


Fig. 3. Schematic representation of the mechanisms involved in chronic opiate action on CREB phosphorylation at the cellular level. A, chronic opiate treatment leads to a compensatory upregulation of the cAMP pathway, however CREB phosphorylation remains unchanged possibly due to the activation of nuclear protein phosphatases. B, withdrawal profoundly activates cAMP pathway and induces $Ca^{2+}$ influx which in turn promote CREB phosphorylation and activation of target genes (e.g. c-Fos).

secondary adaptation, possibly dependent on newly synthesized factors. In contrast, increase in AP-1 DNA binding activity displayed a fast onset reaching its maximum at 15 min and declined slowly over several hours to the basal level. Opioid activation of μ-opioid receptors induced also transient (5-10 min) phosphorylation of MAPKs pathway which may not only contribute to CREB phosphorylation but also lead to *c-fos* induction and thus contribute to increase in AP-1 DNA binding activity (Fig. 1).

Prolonged, up to 5 days morphine treatment normalized back to basal the levels of c-Fos, AP-1 and CRE DNA binding activity and slightly decreased the levels of phosphorylated CREB and MAPKs. The attenuation of the opioid effects on the above factors may underlie the molecular basis for tolerance (Fig. 3A). Washing out morphine or application of the opioid antagonists induced withdrawal in both NG108-15 and Neuro2a MOR cells. Withdrawal from morphine elicited increase in c-Fos and phosphorylated CREB levels and induced CRE and AP-1 DNA binding activity with higher potency but with kinetics similar to acute opioid action in these cells (Fig. 3B). On the other hand, decreased phosphorylated MAPKs levels caused by chronic morphine were further lowered during withdrawal. Interestingly, the kinetics of the withdrawal-elicited decrease in the phosphorylation of MAPKs and the withdrawal--elicited increase in the CREB phosphorylation were similar suggesting the involvement of one common mechanism in these two opposite effects. The upregulation of the cAMP pathway during withdrawal is the most likely and obvious mechanism because the induction of PKA pathways is frequently a negative regulator of the MAPKs cascade (Neary 1997) and forskolin almost completely blocked the phosphorylation of MAPKs in these cells. Meaningfully, chronic morphine treatment and withdrawal increase the phosphorylation of the stress-activated protein kinases (SAPKs) also known as Jun N-terminal kinases. Their major targets are transcription factors c-Jun and ATF-2 which can form heterodimers and bind to CRE elements. In addition the phosphorylation of c-Jun in the AP-1 complex by SAPKs can increase AP-1 DNA binding activity (Fig. 4).

The impact of opioids on the CRE- and AP-1- regulated expression of target genes was also investigated in Neuro2a MOR cells transiently transfected with luciferase genes under control of CRE and AP-1 elements. Acute administration of μ opioid agonist DAMGO increased both CRE and AP-1 controlled expression of re-

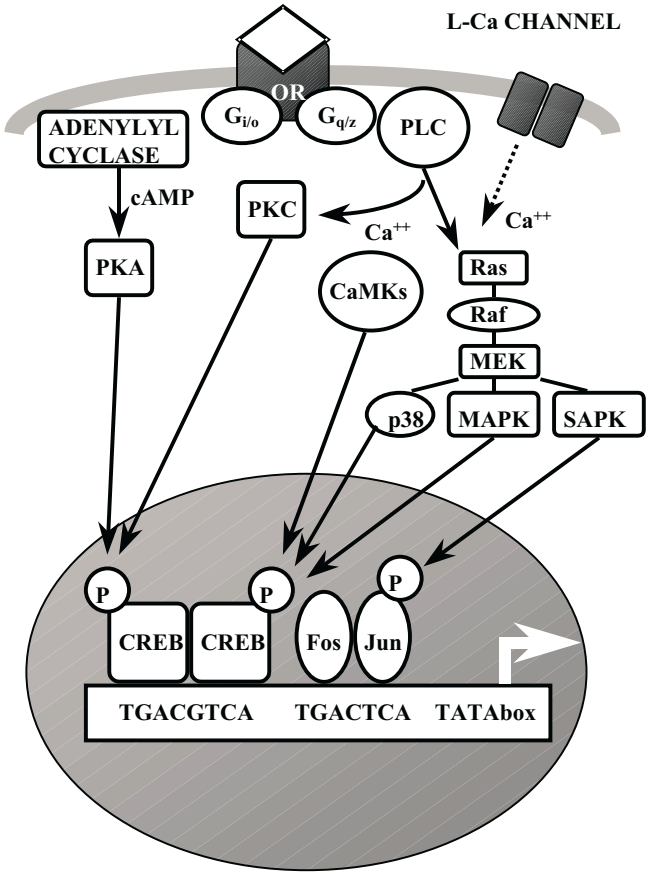


Fig. 4. The involvement of mitogen-activated protein kinases (MAPKs) and stress-activated protein kinases (SAPKs) in opioid signaling in cells expressing μ- or δ-opioid receptors. Opioids acting through opioid receptors (OR) activate MAPKs, contributing to increased CREB phosphorylation, and together with activated CREB induce c-Fos synthesis, facilitating activator protein-1 (AP-1) complex formation. CREB and AP-1 activation in turn stimulate expression of subsequent genes which may be involved in the development of tolerance and dependence. Chronic morphine treatment decreases the levels of phosphorylated MAPKs possibly due to the upregulated protein kinase A (PKA) pathway which acts as a negative regulator of the MAPKs cascade. Withdrawal from morphine further lowers the decreased levels of phosphorylated MAPKs due to the augmented activation of PKA in this state. In contrast, withdrawal from morphine induces SAPKs phosphorylation which can phosphorylate AP-1 and ATF-2 transcription factors. This mechanism may be responsible for increased AP-1 and CRE DNA binding activity observed during withdrawal. Acute and chronic opioid treatments regulate MAPKs and SAPKs activity in an opposite manner, however, they could serve as convergence points for CRE and AP-1 controlled transcription of target genes.

porter gene, an effect totally attenuated after chronic DAMGO treatment. The application of opioid antagonist naloxone precipitated withdrawal in Neuro2a MOR cells and again induced CRE- and AP-1-controlled expression of the reporter gene. The above findings clearly indicate that on the cellular level the main mediators of opioid action on gene expression are $Ca^{2+}$ and cAMP. Depending on the time of the exposure to opioids or opioid-withdrawal, both pathways are able to induce CREB phosphorylation, CRE DNA binding activity and CRE-controlled expression of reporter genes, as well as other transcription factors and the genes influenced by them. This cascade of events creates the basis for general and long-lasting cellular adaptations to opioid action. Interestingly, cells treated with morphine and subsequently cultured for several days in the absence of any opioids maintain some sort of tolerance to morphine action on the level of CREB phosphorylation, highlighting the role of CREB in this process. Recent studies of CREB activation in organotypic cultures of striatum have shown that dynamics of CREB phosphorylation is brain region-dependent, raising an intriguing possibility that different levels of CREB activation might serve to accommodate different neuronal information processing in these different brain regions (Liu and Graybiel 1998). The data derived from cell cultures may also explain the *in vivo* phenomenon of opioid-induced expression of genes like *c-fos* or dynorphin whether during acute or chronic treatment as well as during withdrawal.

## SUMMARY

CREB is one of the most important factors linking the opioid-regulated secondary messenger systems to alterations in gene expression. Opioids regulate CREB level, its phosphorylation, and binding to promoters of several genes implicated in drug addiction. CREB controls expression of addiction-related genes in brain regions responsible for drug-seeking behavior and manifestation of signs of dependence. In cultures of cells expressing μ- and δ-opioid receptors, opioids affect CREB and *via* CREB several genes implicated in development of tolerance and addiction. On the other hand, our understanding of this process is affected by the sole presence of the CREB acting at its CRE site on DNA. Our recent finding highlights that not only CREB but also other putative factors affecting CRE DNA binding activity could be crucial in transcriptional adaptation to opioids. Recent data provide evidence that regulation of gene expression contributes importantly to development of tolerance and addiction. However, detailed knowledge of the molecular steps by which opioids induce adaptations within different cells is yet to be discovered. Knowledge of the molecular mechanisms of these processes would provide a basis for development of new strategies for treatment of addiction.

### REFERENCES


Aghajanian G. K., Wang Y.Y. (1987) Common alpha 2- and opiate effector mechanisms in the locus coeruleus: intracellular studies in brain slices. Neuropharmacology 26: 793-799.

Alreja M., Aghajanian G.K. (1991) Activation of locus coeruleus (LC) neurones by cholera toxin: mediation by cAMP-dependent protein kinase. Neurosci. Lett. 134: 113-117.

Benbrook M., Jones K. (1990) Heterodimer formation between CREB and JUN proteins. Oncogene 5: 295-302.

Bilecki W., Hollt V., Przewłocki R. (2000) Acute delta-opioid receptor activation induces CREB phosphorylation in NG108-15 cells. Eur. J. Pharmacol. 390: 1-6.

Blendy J.A., Kaestner K.H., Schmid W., Gass P., Schutz G. (1996) Targeting of the CREB gene leads to upregulation of a novel CREB mRNA isoform. EMBO J. 15: 1098-10106.

Bontempi B., Sharp F.R. (1997) Systemic morphine-induced Fos protein in the rat striatum and nucleus accumbens is regulated by miu opioid receptors in the substantia nigra and ventral tegmental area. J. Neurosci. 17: 8596-8612.

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.

Carlezon W.A. Jr, Thome J., Olson V.G., Lane-Ladd S.B., Brodkin E.S., Hiroi N., Duman R.S., Neve R.L., Nestler E.J. (1998) Regulation of cocaine reward by CREB. Science 282: 2272-2275.

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

Cooper D.M., Mons N., Karpen J.W. (1995) Adenylyl cyclases and the interaction between calcium and cAMP signalling. Nature 374: 421-424.

Di Chiara G., North R.A. (1992) Neurobiology of opiate abuse. Trends. Pharmacol. Sci. 13: 185-193.

Duman R.S., Tallman J.F., Nestler E.J. (1988) Acute and chronic opiate-regulation of adenylate cyclase in brain: specific effects in locus coeruleus. J. Pharmacol. Exp. Ther. 246: 1033-1039.

Fentzke R. C., Korcarz C. E., Lang R. M., Lin H., Leiden J. M. (1998) Dilated cardiomyopathy in transgenic mice ex-

pressing a dominant-negative CREB transcription factor in the heart. J. Clin. Invest. 101: 2415-2426.

Finkbeiner S., Greenberg M.E. (1996) Ca(2+)-dependent routes to Ras: mechanisms for neuronal survival, differentiation, and plasticity? Neuron 16: 233-236.

Frank D.A., Greenberg M.E. (1994) CREB: a mediator of long-term memory from mollusks to mammals. Cell 79: 5-8.

Glazewski S., Barth A.L., Wallace H., McKenna M., Silva A., Fox K. (1999) Impaired experience-dependent plasticity in barrel cortex of mice lacking the alpha and delta isoforms of CREB. Cereb. Cortex. 9: 249-256.

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

Guitart X., Thompson M.A., Mirante C.K., Greenberg M.E., Nestler EJ (1992) Regulation of cyclic AMP response element-binding protein (CREB) phosphorylation by acute and chronic morphine in the rat locus coeruleus. J. Neurochem. 58: 1168-1171.

Hayward M.D., Duman R.S., Nestler E.J. (1990) Induction of the c-fos proto-oncogene during opiate withdrawal in the locus coeruleus and other regions of rat brain. Brain. Res. 525: 256-266.

Holmes P.V., de Bartolomeis A., Koprivica V., Crawley J.N. (1995) Lack of effect of chronic morphine treatment and naloxone-precipitated withdrawal on tyrosine hydroxylase, galanin, and neuropeptide Y mRNA levels in the rat locus coeruleus. Synapse 19: 197-205.

Horikoshi M., Hai T., Lin Y.S., Green M.R., Roeder R.G. (1988) Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. Cell 54: 1033-1042.

Hummler E., Cole T.J., Blendy J.A., Ganss R., Aguzzi A., Schmid W., 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: 5647-51.

Kogan J.H., Nestler E.J., Aghajanian G.K., (1992) Elevated basal firing rates and enhanced responses to 8-Br-cAMP in locus coeruleus neurons in brain slices from opiate-dependent rats. Eur. J. Pharmacol. 211: 47-53.

Kogan J.H., Frankland P.W., Blendy J.A., Coblentz J., Marowitz Z., Schutz G., Silva A.J. (1997) Spaced training induces normal long-term memory in CREB mutant mice. Curr. Biol. 7: 1-11.

Konradi C., Leveque J.C., Hyman S.E. (1996) Amphetamine and dopamine-induced immediate early gene expression in striatal neurons depends on postsynaptic NMDA receptors and calcium. J. Neurosci. 16: 4231-4229.

Koob G.F., (1992) Drugs of abuse: anatomy, pharmacology and function of reward pathways. Trends. Pharmacol. Sci. 13: 177-184.

Kwok R.P., Lundblad J.R., Chrivia J.C., Richards J.P., Bachinger H.P., Brennan R.G., Roberts S.G., Green M.R., Goodman R.H. (1994) Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature 370: 223-226.

Lane-Ladd S.B., Pineda J., Boundy V.A,. Pfeuffer T., Krupinski J., Aghajanian G.K., Nestler E.J. (1997) CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. J. Neurosci. 17: 7890-7901.

Lazaroff M., Patankar S., Yoon S.O., Chikaraishi D.M. (1995) The cyclic AMP response element directs tyrosine hydroxylase expression in catecholaminergic central and peripheral nervous system cell lines from transgenic mice. J. Biol. Chem. 270: 21579-21589.

Liu F.C., Graybiel A.M. (1998) Region-dependent dynamics of cAMP response element-binding protein phosphorylation in the basal ganglia. Proc. Natl Acad. Sci. USA 95: 4708-4713.

Liu J., Nickolenko J., Sharp F.R . (1994) Morphine induces c-fos and junB in striatum and nucleus accumbens via D1 and N-methyl-D-aspartate receptors. Proc. Natl Acad. Sci. USA 91: 8537-8541.

Maldonado R., Blendy J.A., Tzavara E., Gass P., Roques B.P., Hanoune J., Schutz G. (1996) Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. Science 273: 657-659.

Martin K.C., Kandel E.R. (1996) Cell adhesion molecules, CREB, and the formation of new synaptic connections. Neuron 17: 567-570.

Neary J.T. (1997) MAPK cascades in cell growth and death. NIPS 12: 286-293.

Nestler E.J., Hope B.T., Widnell K.L. (1993) Drug addiction: a model for the molecular basis of neural plasticity. Neuron 11: 995-1006.

Nestler E.J., Berhow M.T., Brodkin E.S. (1996) Molecular mechanisms of drug addiction: adaptations in signal transduction pathways. Mol. Psychiatry. 1: 190-199.

Nestler E.J. (1992) Molecular mechanisms of drug addiction. J. Neurosci. 12: 2439-2450.

Noble F., Cox B.M. (1996) Differential desensitization of mu- and delta- opioid receptors in selected neural pathways following chronic morphine treatment. Br. J. Pharmacol. 117: 161-169.

Nye H.E., Hope B.T., Kelz M.B., Iadarola M., Nestler E.J. (1995) Pharmacological studies of the regulation of chronic FOS-related antigen induction by cocaine in the striatum and nucleus accumbens. J. Pharmacol. Exp. Ther. 275: 1671-1680.

Nylander I., Stenfors C., Tan-No K., Mathe A.A., Terenius L. (1997) A comparison between microwave irradiation and decapitation: basal levels of dynorphin and enkephalin and the effect of chronic morphine treatment on dynorphin peptides. Neuropeptides31: 357-365.

Pende M., Fisher T.L., Simpson P.B., Russell J.T., Blenis J., Gallo V. (1997) Neurotransmitter- and growth factor-induced cAMP response element binding protein phosphorylation in glial cell progenitors: role of calcium ions, protein kinase C, and mitogen-activated protein kinase/ribosomal S6 kinase pathway. J. Neurosci. 17: 1291-1301.

Przewłocka B., Turchan J., Lason W., Przewłocki R. (1996) The effect of single and repeated morphine administration on the prodynorphin system activity in the nucleus accumbens and striatum of the rat. Neuroscience 70: 749-754.

Przewłocki R., Parsons K.L., Sweeney D.D., Trotter C., Netzeband J.G., Siggins G.R., Gruol D.L. (1999) Opioid enhancement of calcium oscillations and burst events involving NMDA receptors and L-type calcium channels in cultured hippocampal neurons. J. Neurosci. 19: 9705-9715.

Richards J.P., Bachinger H.P., Goodman R.H., Brennan R.G. (1996) Analysis of the structural properties of cAMP-responsive element-binding protein (CREB) and phosphorylated CREB. J. Biol. Chem. 271: 13716-13723

Robertson L.M., Kerppola T.K., Vendrell M., Luk D., Smeyne R.J., Bocchiaro C., Morgan J.I., Curran T. (1995) Regulation of c-fos expression in transgenic mice requires multiple interdependent transcription control elements. Neuron 14: 241-252.

Rudolph D., Tafuri A., Gass P., Hammerling G.J., Arnold B., Schutz G. (1998) Impaired fetal T cell development and perinatal lethality in mice lacking the cAMP response element binding protein. Proc. Natl Acad. Sci. USA 95: 4481-4486.

Silva A.J., Kogan J.H., Frankland P.W., Kida S. (1998) CREB and memory. Annu. Rev. Neurosci. 21: 127-148.

Terwilliger R.Z., Beitner-Johnson D., Sevarino K.A., Crain S.M., Nestler E.J. (1991) A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. Brain. Res. 548: 100-110.

Waeber G, Habener JF (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.

Widnell K.L., Russell D.S., Nestler E.J. (1994) Regulation of expression of cAMP response element-binding protein in the locus coeruleus in vivo and in a locus coeruleus-like cell line in vitro. Proc. Natl Acad. Sci. USA 91: 10947-10951.

Widnell K.L., Self D.W., Lane S.B., Russell D.S., Vaidya V.A., Miserendino M.J., Rubin C.S., Duman R.S., Nestler E.J. (1996) Regulation of CREB expression: in vivo evidence for a functional role in morphine action in the nucleus accumbens. J. Pharmacol. Exp. Ther. 1996: 306-315.

Vallejo M., Gosse M.E., Beckman W., Habener J.F. (1995) Impaired cyclic AMP-dependent phosphorylation renders CREB a repressor of C/EBP-induced transcription of the somatostatin gene in an insulinoma cell line Mol. Cell. Biol. 15: 415-424 .

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.