cAMP-dependent regulation of proenkephalin by JunD and JunB: Positive and negative effects of AP-1 proteins

(opioid/protein kinase A/transcriptional regulation/second-messenger-inducible enhancer)

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ABSTRACT We demonstrate that JunD, a component of the AP-1 transcription factor complex, activates transcription of the human proenkephalin gene in a fashion that is completely dependent upon the cAMP-dependent protein kinase, protein kinase A. Activation of proenkephalin transcription by JunD is dependent upon a previously characterized cAMP-, phorbol ester-, and Ca²⁺-inducible enhancer, and JunD is shown to bind the enhancer as a homodimer. Another component of the AP-1 transcription complex, JunB, is shown to inhibit activation mediated by JunD. As a homodimer JunB is unable to bind the enhancer; however in the presence of c-Fos, high-affinity binding is observed. Furthermore, JunD is shown to activate transcription of genes linked to both cAMP and phorbol ester response elements in a protein kinase A-dependent fashion, further blurring the distinction between these response elements. These results demonstrate that the transcriptional activity of an AP-1-related protein is regulated by the cAMP-dependent second-messenger pathway and suggest that JunD and other AP-1-related proteins may play an important role in the regulation of gene expression by cAMP-dependent intracellular signaling pathways.

In the nervous system, signals transmitted across synapses are known to regulate gene expression in the postsynaptic cell (1–5). This process involves membrane depolarization and/or the binding of neurotransmitters to their cell surface receptors, and subsequent signal amplification via second-messenger pathways and protein kinases. Analysis of neural regulation of gene expression has focused primarily on two types of genes: the immediate early genes (4, 5) and genes encoding peptide neurotransmitters (2, 3). Immediate early genes are thought to function as “third messengers” transducing neural activity into long-term changes in neural functioning by activating or repressing specific target genes—for example, the genes encoding peptide neurotransmitters (4, 5). Regulation of the opioid precursor proenkephalin by neural activity and intracellular signaling pathways is well documented (6–11, 12). A 30-base-pair (bp) region of DNA closely linked to the proenkephalin promoter has been shown to act as a second-messenger-inducible DNA enhancer that regulates transcription of this gene in response to the cAMP-, phorbol ester-, and Ca²⁺-dependent intracellular signaling pathways (13–16). Located within this enhancer are two adjacent DNA elements called cAMP response elements (CRE-1 and CRE-2), which act cooperatively to regulate proenkephalin transcription in response to all three intracellular signaling pathways (14–16).

The molecular mechanisms by which transcription factors mediate cellular responsiveness to second-messenger pathways are in most cases not well understood; however, in the case of the cAMP-dependent pathway, clearly an interesting

and complex story is unfolding. The activated catalytic subunit of the cAMP-dependent protein kinase (protein kinase A, PKA) appears to translocate to the nucleus (17). Further evidence supports a model in which PKA then phosphorylates a single residue of the CRE-binding protein (CREB) (18), leading to the cAMP-dependent activation of somatostatin transcription at the CRE (TGACGTCA) (19). In addition, phosphorylation by other kinases at sites adjacent to the CREB PKA site also plays a role (20). In contrast to the somatostatin gene, where a single element is sufficient for cAMP regulation (19), analysis in our laboratory indicates that two adjacent and closely related elements are necessary for cAMP regulation of the proenkephalin gene (14). CRE-2 (TGCGTCA) is a site that most closely resembles the phorbol ester response element (tetradecanoylphorbol acetate response element, TRE) (TGACTCA) but also contains the core CGTCA element found in most CREs (14, 19). Previous studies have shown that affinity-purified AP-1 proteins bind with high affinity to CRE-2 (14, 15). This information focused our attention on proteins of the AP-1 complex (Fos, Jun, and related molecules), which were originally identified by their ability to bind and activate transcription via the TRE (3–5).

We show here that JunD, in the presence of the catalytic subunit of PKA, transactivates the proenkephalin enhancer at the CRE-2 element. JunB is shown to antagonize activation mediated by JunD. DNA binding studies indicate that JunB alone does not bind CRE-2 but can bind this element in the presence of c-Fos. Together these findings suggest that AP-1 complexes involving JunD and JunB may act to induce or repress proenkephalin transcription in response to cAMP. In addition, our data suggest that cAMP regulates gene expression through at least two pathways: one that involves previously described CREB/ATF proteins acting at classic CRE sequences (18–21) and a second pathway involving AP-1 proteins acting at both CRE- and TRE-like sequences.

METHODS AND MATERIALS

Cell Culture. C6 glioma cells and C6-D2 (16), a line stably transfected with pENKAT-12, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. F9 embryonal carcinoma cells were grown in DMEM supplemented with 10% calf serum.

Northern Blot Analysis. For the analysis of RNA in C6-D2 cells stimulated with forskolin, cells were grown in low-serum medium (0.5%) for 14–16 hr, treated with 25 μ M forskolin and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and harvested at various times after treatment from 15 min to 14 hr. In some experiments the protein synthesis inhibitor

anisomycin (10 μ M) or cycloheximide (100 μ M) was added 30 min prior to forskolin stimulation. Total cytoplasmic RNA was isolated as described (14). The *junD* probe (bp 58–577) and *junB* probe (bp 33–427) each included 5' untranslated region and coding sequence. The *c-fos* probe (26) was a 1.5-kilobase (kb) fragment that includes the entire *c-fos* coding region.

Plasmid Constructions. To construct the RSVJunD expression vector, a 1.7-kb *Sac* I–*Eco*RV fragment containing the entire *junD* coding sequence and untranslated flanking sequences was isolated from a *junD* cDNA-containing plasmid (24) and cloned into RSV-SG (18) cut with *Bgl* II (filled in) and *Sac* I. The RSVJunB vector was constructed by isolating the *junB* cDNA from p465.20 (25) after a complete *Xho* I (filled in) digestion and a partial *Sac* I digestion. The 1.5-kb fragment containing the entire *junB* coding sequence and 5' untranslated sequences was then cloned into the RSV-SG backbone. The proenkephalin multicopy element plasmids (pENKCRE-2, pENKCRE-1, pENKAP-4) were constructed using the oligonucleotides shown in Fig. 1 as described (16). pENKCRE-2 contains five tandem copies of CRE-2 (16). pENKCRE-1 and pENKAP-4 contain five to six copies of the CRE-1 and AP-4 sites, respectively.

Transient Transfections and Chloramphenicol Acetyltransferase (CAT) Assays. F9 cells and C6 glioma cells (5×10^5 cells per 10-cm plate) were transiently cotransfected by the calcium phosphate method with a CAT reporter plasmid and the following plasmids in various combinations as indicated in the text and figure legends: pRSVJunD; pRSVJunB; pMTCaneo (27); pRSV β -galactosidase (β Gal; ref. 28). After 3.5 hr the cells were glycerol-shocked for 3 min and then harvested 24 hr later for protein extracts. CAT and β Gal activities were measured as described (13) with the exception that lysates were heat-inactivated at 65°C for 10 min (only for CAT assays).

In Vitro Synthesis of Proteins. Fifty to 100 ng of RNA was transcribed *in vitro* from pGEM-2 plasmids containing the complete coding sequences for either *junB* (25), *junD* (29), or *c-fos* (gift of J. G. Belasco and M. E. Greenberg, Harvard Medical School) and then was translated in rabbit reticulocyte lysates as described by the manufacturer (Promega). [³⁵S]Methionine-labeled translation products were analyzed by SDS/PAGE. Prestained protein standards (M_r 15,000–110,000; Bio-Rad) were used to determine molecular weight.

Gel Retardation Assay. A double-stranded oligonucleotide spanning –110 to –80 of the proenkephalin promoter (Fig. 1) was labeled using Moloney murine leukemia virus reverse transcriptase (New England Biolabs) and [α -³²P]dCTP (3000

Ci/mmol; 1 Ci = 37 GBq). Binding was carried out in 15 μ l of 10 mM Hepes, pH 8.0/15% (vol/vol) glycerol/0.2 mM EDTA/50 mM KCl/2 mM MgCl₂/2 mM dithiothreitol containing 1 μ g of poly(dI-dC). Translation products (1 μ l) were incubated in the above reaction buffer with 1–2 ng of labeled probe for 20 min on ice. Reaction mixtures were then analyzed by PAGE as described (29).

RESULTS

Expression and Regulation of Proenkephalin, JunD, JunB, and c-Fos in C6 Glioma Cells. To determine the time course and protein synthesis dependence of proenkephalin activation by cAMP we analyzed the kinetics of a CAT mRNA (Fig. 2A) encoded by a stably expressed proenkephalin–CAT fusion gene in C6 glioma cells stimulated by forskolin, an activator of adenylate cyclase, and IBMX, an inhibitor of phosphodiesterase. Induction of cytoplasmic RNA was observed within 15 min of forskolin addition, peaked between 1 and 2 hr, and was significantly reduced by 8 hr. Induction was not blocked by pretreatment with cycloheximide (data not shown) or anisomycin (Fig. 2A); however, the decline in message levels observed between 3 and 8 hr was completely blocked by these protein synthesis inhibitors (Fig. 2A). Protein synthesis inhibitors in the absence of forskolin and IBMX had no effect on proenkephalin expression (data not shown). Endogenous proenkephalin transcripts show a similar pattern of expression although the time course is less informative due to the high level of basal proenkephalin mRNA and its long half-life compared with CAT mRNA (0.5 day versus several hours) (11).

As a first step in determining what role different AP-1 components may play in the forskolin-induced stimulation of proenkephalin transcription, we analyzed the expression of mRNAs encoding three AP-1 factors—JunD, JunB, and c-Fos—before and after treatment of C6 glioma cells with forskolin and IBMX. Significant levels of *junD* RNA were constitutively expressed in the C6 glioma cells (Fig. 2B). One hour after treatment with forskolin and IBMX, *junD* RNA levels decreased severalfold and remained lowered up to 14 hr later. RNAs encoding JunB and c-Fos were not detectable

FIG. 1. Human proenkephalin second-messenger-inducible enhancer. Sequence spanning bp –117 to –74 of the proenkephalin gene is shown. The CRE-1 and CRE-2 elements are boxed and the AP-4 and AP-2 elements are highlighted by stippling. Oligonucleotides used in gel shift assays and to construct multicopy element plasmids are shown and identified by the enhancer element that they contain. Bases within CRE-2 that are mutated in oligonucleotides used in gel shift and cotransfection assays are indicated by arrows.

FIG. 2. A C6 glioma cell line (C6-D2) stably transfected with pENKAT-12 was treated for various intervals (as indicated in hours above each lane) with 25 μ M forskolin and 500 μ M IBMX. Total cytoplasmic RNA was isolated and 20- μ g samples were analyzed by Northern blot analysis. (A) Activity of the proenkephalin enhancer was measured using a cDNA probe specific for CAT (derived from pENKAT-12). In some cases C6-D2 cells were treated with 10 μ M anisomycin (ANISO.) 30 min prior to forskolin stimulation and the resultant RNA was hybridized with the CAT cDNA probe. (B) RNA isolated from C6-D2 cells treated with forskolin and IBMX also was hybridized with cDNA probes specific for JunD, JunB, and c-Fos.

in untreated control C6 glioma cells but were rapidly and transiently induced by forskolin and IBMX (Fig. 2B).

Transactivation by JunD Is Dependent Upon PKA and CRE-2. To test the functional activity of different AP-1 components at the proenkephalin enhancer, we introduced full-length cDNAs encoding JunD and JunB into expression vectors utilizing a Rous sarcoma virus (RSV) promoter/enhancer and the simian virus 40 polyadenylation site. These expression vectors (pRSVJunD and pRSVJunB) were then used to drive constitutive expression in a number of different cell lines after transfection and transient expression. Expression was first investigated in undifferentiated F9 cells, as these cells express low or undetectable levels of AP-1 DNA-binding activity (30). The proenkephalin-CAT fusion gene, pENKAT-12 (13), was expressed in these cells at very low levels in transient transfection assays (Fig. 3A, bar a). Cotransfection with pRSVJunD had no effect on CAT expression driven by the proenkephalin promoter, whereas cotransfection with a plasmid expressing the catalytic subunit of PKA, pMTCaneo (27), produced a reproducible 2- to 5-fold increase in CAT expression (Fig. 3A, bars b and c). Cotransfection of pENKAT-12 together with JunD- and PKA-expressing plasmids produced a 10- to 30-fold increase in CAT expression (Fig. 3A, bar d), indicating that transactivation of the proenkephalin promoter by JunD is dependent upon expression of an active PKA catalytic subunit. Similar observations (Fig. 3B) have been made in C6 glioma cells which express the proenkephalin gene and regulate it in a forskolin dependent fashion. However, because transfections with PKA alone produce such large effects in these cells, it was necessary to reduce the amount of pMTCaneo in order to observe the effect of JunD.

To map the elements essential for transactivation by JunD, we analyzed the effects of pRSVJunD and pMTCaneo on plasmids that contained multiple copies of each element (CRE-2, CRE-1, AP-4; see Fig. 1) cloned into a plasmid, pENKAT-Δ80, that has all enhancer elements removed and no longer responds to the activation of second-messenger pathways. Multiple copies of the CRE-2 element but not the CRE-1 or AP-4 element reconstitute wild-type enhancer transactivation by JunD and PKA in transiently transfected F9 cells (Fig. 3C). Furthermore, point mutations within CRE-2 that block second-messenger regulation *in vivo* (14) also block transactivation by JunD (Fig. 3C). All CAT activities shown are normalized to βGal activities measured from the same extracts as described previously (13).

FIG. 3. JunD transactivation of proenkephalin is PKA-dependent and maps to the CRE-2. (A) F9 cells were cotransfected with 5 μ g of pENKAT-12 and pRSVβGal in various combinations with pRSVJunD and pMTCaneo as follows: bar a, pENKAT-12 alone; bar b, pENKAT-12 plus 5 μ g of pRSVJunD; bar c, pENKAT-12 plus 10 μ g of pMTCaneo; bar d, pENKAT-12 plus 5 μ g of pRSVJunD and 10 μ g of pMTCaneo. The total amount of DNA was maintained at 40 μ g with pGEM-3. (B) C6 cells were cotransfected as described above with two exceptions, the amount of pMTCaneo was reduced to 2 μ g and the total amount of DNA was maintained at 25 μ g with pGEM-3. (C) F9 cells were transfected (solid bars) with 5 μ g of the following: pENKAT-12; pENKAT-Δ80; pENKCRE-2; pENKCRE-1; pENKAP-4; or derivatives of pENKAT-12 in which single bases of CRE-2 were mutated, -86 (A to T), -87 (C to T), or -88 (T to A). CAT-expressing plasmids also were cotransfected with 5 μ g of pRSVJunD and 10 μ g of pMTCaneo (striped bars). All CAT activities were normalized for expression of pRSVβGal. These results were repeated at least three times.

JunB Inhibits PKA-Dependent Transactivation by JunD. To gain insight into the functional effects of JunB we carried out cotransfection experiments with pRSVJunB. In F9 cells, JunB alone or together with PKA had no effect on transcription from the proenkephalin-CAT fusion gene (Fig. 4). The small, 3-fold effect seen together with PKA was indistinguishable from the effect seen with PKA alone. However, when a JunB plasmid was cotransfected with JunD- and PKA-expressing plasmids, a dose-dependent reduction in JunD transactivation was observed (Fig. 4). When cotransfected with JunD at a 4-fold molar excess, JunB dramatically inhibited the transactivation observed with JunD and PKA. Neither JunB nor JunD had any effect on the pRSVβGal plasmid, which was cotransfected as an internal control. This observation makes it unlikely that inhibition by JunB is due to the titration of limiting factors required by the Rous sarcoma virus promoter.

Characterization of JunD and JunB Binding at the Proenkephalin Enhancer. To examine the binding of JunD and JunB to CRE-2, we transcribed RNA encoding JunD, JunB, and c-Fos and then translated these individually and in combinations, as described below, in rabbit reticulocyte lysates. Fig. 5A shows *in vitro* translated, [³⁵S]methionine-labeled JunD, JunB, and c-Fos proteins. Lysates used in the DNA binding assays described below were translated in parallel under identical conditions except for the replacement of [³⁵S]methionine with nonradioactive methionine. Control lysates showed no specific binding to a 30-bp oligonucleotide spanning the proenkephalin enhancer from -110 to -80 (Fig. 5B, lane 1). However, lysates programmed with JunD RNA produced a shifted band (arrowhead, Fig. 5B, lane 2) whose formation was specifically inhibited by unlabeled proenkephalin enhancer oligonucleotide (lanes 3 and 4) but not by AP-2 or AP-4 oligonucleotides and only very weakly by a CRE-1 oligonucleotide (lanes 5-7). Both TRE and CRE oligonucleotides competed effectively for JunD DNA binding (lanes 8-11).

We next attempted to correlate binding of JunD with the *in vivo* functional activity of a series of single base substitution mutations spanning CRE-2. In these experiments a wild-type CRE-2 oligonucleotide (-93 to -85 bp, see Fig. 2) was used as nonradioactive competitor (Fig. 5B, lanes 12 and 13) and this competition was compared with that of CRE-2 oligonucleotides containing the indicated single base substitution mutation (lanes 14-16). Mutations within the 3' end of CRE-2, which have the most profound effect on second-messenger regulation of proenkephalin transcription (14),

FIG. 4. JunB represses transactivation of proenkephalin by JunD. F9 cells were cotransfected with 5 μ g of pENKAT-12, 5 μ g of pRSVJunD, 10 μ g of pMTCaneo, and 5, 10, or 20 μ g of pRSVJunB as indicated. Five nanograms of pRSV β Gal also was included in each transfection as an internal control for normalization of CAT activity. These results were repeated at least three times.

produced the most severe effect on JunD binding, leading to a lack of competition. Although the CRE-2 oligonucleotide competed for JunD binding it did not compete as well as oligonucleotides that included both CRE-1 and CRE-2 and flanking sequences (compare lanes 3 and 4 with 12 and 13).

As shown in Fig. 5C, binding of *in vitro* translated JunB to the enhancer was not observed (lane 2). Because JunB is known to dimerize with JunD (29) and c-Fos (29, 31), we also analyzed the interaction of these complexes with CRE-2. As shown in lane 3, lysates programmed with equal amounts of *junB* and *junD* RNAs bound the proenkephalin enhancer probe with an affinity similar to that of *junD*-programmed lysates (compare lanes 1 and 3). Consistent with previous reports using AP-1 oligonucleotides (12, 29, 31), binding to CRE-2 was observed when c-Fos and JunB were cotranslated (Fig. 5C, lane 6), whereas JunB or c-Fos alone did not bind (lanes 2 and 4). Also note that c-Fos stimulated the binding of JunD to the proenkephalin enhancer (lane 5).

Classic CRE and TRE also Mediate PKA-Dependent Transcription via JunD. Because the proenkephalin enhancer contains a hybrid TRE/CRE-like element, CRE-2, we tested the ability of JunD to transactivate "classic" TRE and CRE elements in a PKA-dependent fashion by transiently transfecting F9 cells with plasmids containing the somatostatin CRE, p Δ (-71)CAT (32), or the collagenase TRE, p-73Col-TRE (30), together with pRSVJunD and pMTCaneo. As shown in Fig. 6, JunD transactivated the somatostatin CRE $\approx$ 10-fold and the collagenase TRE $\approx$ 4-fold. These results

indicate that JunD can act at both CREs and TREs to stimulate transcription in a PKA-dependent fashion.

DISCUSSION

Analysis of the time course of proenkephalin-CAT RNA expression in C6 glioma cells after treatment with forskolin or with forskolin and protein synthesis inhibitors suggests that the rapid stimulation of the proenkephalin-CAT RNA by forskolin is independent of new protein synthesis and hence mediated by preexisting proteins. Several properties of JunD suggest that this molecule may play a role in the rapid increase in RNA levels observed after stimulation by cAMP and possibly other intracellular signaling pathways. First, unlike all other known members of the Fos/Jun family, the level of *junD* RNA is constitutively expressed in a wide variety of cell types (24, 33). Second, the JunD protein is a very stable component of the AP-1 complex, with a half-life of 6 hr in Swiss 3T3 fibroblasts (34). JunD transactivates the proenkephalin promoter in a cAMP-dependent fashion and specifically binds CRE-2 with high affinity as a homodimer. Finally, point mutations that eliminate inducible enhancer function *in vivo* (14) also block the binding of JunD to CRE-2.

Experiments using *in vitro* translated JunD suggest that this protein binds to CRE-2 both as a homodimer and also as a heterodimer when cotranslated with c-Fos. Although we cannot eliminate the possibility that our JunD-expressing lysates also express c-Fos, the observation that JunB binding is entirely dependent on c-Fos cotranslation suggests that the lysates contain little if any endogenous Fos. The observation that JunD binds CRE-2 as a homodimer together with the lack of c-Fos expression in undifferentiated F9 cells (30) suggest that the JunD homodimer may mediate the PKA-dependent activation of proenkephalin transcription.

How does PKA regulate the transcriptional activity of JunD? It has been shown that JunD is a phosphoprotein (34). It is unknown whether it can be phosphorylated *in vitro* or *in vivo* by PKA. Unlike CREB, JunD does not contain a PKA consensus site of the type Arg-Arg-Xaa-Ser. However, a single site of the Lys-Xaa-Xaa-Ser type is present that is not present in the closely related JunB molecule. Alternatively, PKA may regulate dimerization of JunD with itself or other Jun/Fos/CREB-like factors or may induce expression of other AP-1 proteins, which, like Fos, can heterodimerize with JunD. Significantly, cotransfection with a c-Fos-expressing plasmid has little or no effect on JunD transactivation (data not shown), suggesting that PKA probably does

FIG. 5. (A) *In vitro* translated proteins. [³⁵S]Methionine-labeled JunD, JunB, and c-Fos translated in rabbit reticulocyte lysates are shown. Migration of standards of known molecular weight are indicated by arrowheads ($M_r \times 10^{-3}$). (B) Binding specificity of the JunD homodimer. One microliter of *in vitro* translation reaction programmed either without (lane 1) or with (lanes 2–16) JunD RNA was incubated in gel mobility-shift assays with radiolabeled CRE1+2 double-stranded oligonucleotide (see Fig. 1). Unlabeled DNA competitors were added in molar excess to the CRE1+2 probe as follows: CRE1+2, 50-fold (lane 3); CRE1+2, 150-fold (lane 4); AP-4, 300-fold (lane 5); AP-2 (GATCCAGC-CCGCCCCGATTG), 300-fold (lane 6); CRE-1, 300-fold (lane 7); AP-1, ACCAGCTGACTCAGATGTCCT, 50-fold (lane 8); AP-1, 150-fold (lane 9); -17 to -27 of the rat somatostatin promoter, 50-fold (lane 10) and 150-fold (lane 11); CRE-2, 50-fold (lane 12); CRE-2, 150-fold (lane 13); CRE-2 A-to-T with substitution at -86, 150-fold (lane 14); CRE-2 with C-to-T substitution at -87, 150-fold (lane 15); CRE-2 with T-to-A substitution at -88, 150-fold (lane 16). Unless otherwise indicated, the sequences of unlabeled competitors are as indicated in Fig. 1. (C) Interactions of JunD, JunB, and c-Fos at the proenkephalin enhancer. Lysates containing JunD (lane 1), JunB (lane 2), cotranslated JunD and JunB (lane 3), c-Fos (lane 4), cotranslated JunD and c-Fos (lane 5), or cotranslated JunB and c-Fos (lane 6) were incubated in gel mobility-shift assays with the CRE1+2 oligonucleotide. Specific protein-DNA complexes are indicated by the arrowhead to the left in B.

FIG. 6. PKA-dependent transactivation of TRE and CRE DNA elements. F9 cells were transfected with 5 μ g of pENKAT-12, 5 μ g of pΔ(-71)CAT (33), or 5 μ g of p-73Col-TRE (30) under three different conditions: control, no cotransfected expression plasmid; PKA, 5 μ g of pMTCane; JunD/PKA, 5 μ g of pRSVJunD and 5 μ g of pMTCane. CAT activity was normalized to β Gal expression. These data were repeated several times.

not act simply to induce c-Fos in F9 cells. Finally, PKA may directly regulate JunD DNA binding, as has been recently reported for protein kinase C and c-Jun (35).

In addition to activating proenkephalin transcription, forskolin also rapidly activates synthesis of *junB* and *c-fos* mRNA in C6 glioma cells. JunB has been shown to repress c-Jun-dependent activation of the c-Jun promoter (30) and to inhibit the transforming activity of v-Jun (36). Our cotransfection experiments demonstrate that JunB also is a potent inhibitor of the PKA-dependent activation of proenkephalin transcription by JunD and suggest that induction of JunB may be related to the protein synthesis-dependent reduction of proenkephalin mRNA that is apparent 3–8 hr after forskolin stimulation. It has been reported in rodent hippocampal granule cells that *junD* mRNA is constitutively expressed (21) and that proenkephalin (9, 10), *junB* (21, 22), and *c-fos* (10, 21, 22) mRNAs are induced after electrical or chemical stimulation of the dentate gyrus. These data are consistent with a model in which preexisting JunD would mediate the rapid cAMP-dependent activation of the proenkephalin enhancer, while the products of immediate early genes, for example JunB or c-Fos, whose messages are induced by activation of second-messenger pathways would be involved in subsequent inactivation.

There are at least four mechanisms that can explain the inhibitory effect of JunB on the PKA-dependent activation of proenkephalin transcription by JunD. (i) JunB could compete with JunD as a homodimer for binding to CRE-2. Our inability to detect binding of JunB homodimer to CRE-2 makes this possibility unlikely. (ii) JunB may form heterodimers with JunD, inhibiting the ability of JunD to bind DNA or to activate transcription in response to PKA. It has been shown that JunD/JunB heterodimers form *in vitro* (29), and our data indicate that the JunD/JunB cotranslation product does not strongly inhibit DNA binding. (iii) JunB may complex with a protein other than JunD and compete directly with JunD homodimers for binding at CRE-2. Because *c-fos* is rapidly and transiently coinduced with *junB* in many cell types and because c-Fos stimulates binding of JunB to CRE-2, it is a good candidate. This mechanism seems unlikely to occur in F9 cells, as these cells do not express c-Fos. We cannot, however, rule out the possibility that PKA acts to induce *c-fos* in F9 cells. (iv) JunB may act to inhibit JunD-stimulated transcription by competing with JunD for a required "coactivator" that plays a critical role in the activation process.

Although much attention has been focused on the role of CREB as a mediator of cAMP-dependent transcriptional activation, our results demonstrate that members of the AP-1 complex also may play a critical role in both activation and

repression of gene expression by cAMP. Several recent studies have shown that AP-1 proteins, in addition to binding the TRE or AP-1 site, also bind the CRE with equal or higher affinity (29, 31). Other studies have indicated that TREs can regulate transcription in response to stimulation of the cAMP-dependent pathway (23). Taken together with our findings that AP-1 proteins also mediate cAMP-dependent gene regulation, these data suggest that JunD, JunB, and perhaps other components of the AP-1 complex may be important regulators of CRE as well as TRE function.

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