NF- κ B proteins from brain interact with the proenkephalin cAMP inducible enhancer

Hung-Ming Chu, Wolfgang H.Fischer¹, Timothy F.Osborne² and Michael J.Comb*

Laboratory of Molecular Neurobiology, Massachusetts General Hospital, The Program in Neuroscience, Harvard Medical School, Boston, MA 02114, ¹The Salk Institute for Biological Studies, La Jolla, CA 92037 and ²Department of Molecular Biology & Biochemistry, University of California, Irvine, CA 92715, USA.

Received January 23, 1991; Revised and Accepted April 15, 1991

ABSTRACT

A short region of the human proenkephalin promoter has been shown previously to mediate transcriptional regulation in response to activation of the cAMP, TPA, and Ca⁺⁺ dependent intracellular signalling pathways. Two adjacent DNA elements, CRE-1 and CRE-2, are essential for this regulation although neither element alone is sufficient for inducible expression. The CRE-2 element consists of overlapping binding sites for the transcription factors AP-1 and AP-4. The CRE-1 element has been shown to interact with a DNA binding factor called ENKTF-1. Here we characterize proteins from bovine brain which bind the CRE-1 element of the human proenkephalin gene. Interactions between proteins binding the CRE-1 and CRE-2 elements are characterized *in vitro* using affinity purified DNA binding proteins. We demonstrate that CRE-1 binding proteins from bovine brain consist of three different polypeptides each belonging to the NF- κ B family of transcription factors. Point mutation analysis of the contacts of these proteins with the CRE-1 element indicate that NF- κ B proteins contact the inducible enhancer at the sequence CTGGCxxxxxCCT which overlaps the CRE-1 element (underlined) defined by *in vivo* point mutation analysis. Cotransfection of one of the three NF- κ B proteins purified from bovine brain, NF- κ B/Red1, together with a proenkephalin/bacterial chloramphenicol acetyl transferase (CAT) fusion gene repressed protein kinase A or forskolin stimulated CAT expression.

INTRODUCTION

In the nervous system the biosynthesis of the opioid peptides, Met- and Leu-enkephalin is largely controlled by signals transmitted across synapses. Over the past decade it has become clear that neurotransmitters, membrane depolarization, and electrical activity regulate the biosynthesis of proenkephalin, the precursor to Met- and Leu-enkephalin, by turning on and off the

synthesis of the mRNA encoding the precursor. Analysis of proenkephalin gene expression in primary cultures of bovine adrenal medullary chromaffin cells has demonstrated that the gene can be activated by stimulation of each of the three major intracellular signaling pathways; the cAMP dependent pathway (1,2), the phorbol ester or protein kinase C dependent pathway (3), and the Ca⁺⁺/depolarization dependent pathway (4). A short region of the proenkephalin promoter has been shown to be both necessary and sufficient to receive signals transmitted through the cAMP, TPA, and Ca⁺⁺ dependent intracellular signaling pathways (5,6). Sequences located between -110 and -65 have been shown to act in a distance and orientation independent fashion to induce transcription of the proenkephalin gene in response to cAMP and TPA. This region is active when placed upstream or downstream of heterologous promoters and multiple copies of this region result in superinducible promoters (7). Point mutational analysis of this region has shown that the cAMP and TPA inducible enhancer consists of two different DNA elements, CRE-1 and CRE-2, which are separated by one helical turn. Mutations within either of these elements eliminates inducible enhancer function indicating that these two elements and hence their respective DNA binding proteins interact in a highly synergistic fashion to mediate second messenger regulation of proenkephalin transcription (7).

In order to characterize signal transduction from the cell surface to the proenkephalin transcription complex we have purified and characterized proteins which interact with the DNA sequences of both CRE-1 and CRE-2 (7,8,9). The CRE-2 element consists of overlapping binding sites for the transcription factors AP-1 and AP-4. The CRE-1 element binds a protein present in HeLa cell nuclear extracts called ENKTF-1 (7). Several different point mutations within the CRE-1 element which strongly diminish cAMP and TPA inducible expression of the proenkephalin gene also block the binding of ENKTF-1. This suggests that ENKTF-1 might mediate second messenger-regulated transcription of the proenkephalin gene through binding to the CRE-1 element *in vivo* (7).

In this paper we further characterize proteins which bind the proenkephalin CRE-1 element. Bovine brain was shown to be

* To whom correspondence should be addressed

a rich source of CRE-1 binding proteins, which by footprinting analysis are indistinguishable from the HeLa cell protein, ENKTF-1. DNA recognition site affinity chromatography was used to purify and obtain sequence information from three different CRE-1 binding proteins. We show that each of the three CRE-1 binding proteins are different members of the nuclear factor I (NF-I) family of transcription/DNA replication factors (10,11,12,13,14). These proteins are shown to bind a site which overlaps the CRE-1 element. NF-I molecules bind together with AP-4 and AP-2 to form a stable complex at the proenkephalin inducible enhancer. In contrast, NF-I proteins cannot simultaneously bind together with AP-1 suggesting that these factors may act at the enhancer in a mutually exclusive fashion *in vivo*. Overexpression of one of the purified NF-I molecules, NF-I/Red1 (12), results in repression of protein kinase A or forskolin stimulated expression of the proenkephalin gene, suggesting the NF-I/Red1 molecule may play a negative role in regulation at the CRE-1 element.

MATERIALS AND METHODS

Materials

All restriction and DNA modifying enzymes used in construction of plasmids and footprinting probes were from New England Biolabs. Phosphocellulose P11 resin was purchased from Whatman and DEAE CL-6B from Pharmacia. [³H]Chloramphenicol was supplied by Amersham and Butyryl-CoA by Pharmacia.

Cell Culture

HeLa S3 cells were grown in spinner culture using Joklik modified Eagle's medium supplemented with 7.5% horse serum. CV-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 2.5% fetal calf serum and 7.5% horse serum. All sera were from Sigma.

Nuclear Extracts

Large scale nuclear extracts were prepared from HeLa S3 cells as described (7,15). Bovine brain nuclear extracts are prepared as described (16) except the following modifications. Homogenization was done with 10 strokes of loose B type Dounce glass tissue grinder (40 ml, Wheaton) followed by 10 strokes of tight A type grinder. Nuclear pellet was extracted with exactly 1.9 volume of Buffer C (20 mM Hepes, pH=7.9; 25% Glycerol; 0.42 M NaCl; 1.5 mM MgCl₂; 0.2 mM EDTA; 0.5 mM DTT, and 0.5 mM PMSF). The extracted nuclear protein was precipitated with 0.33 gm/ml (NH₄)₂SO₄.

Ion Exchange and Sequence Specific Affinity Chromatography

HeLa cell nuclear extracts were fractionated by Sephacryl S-300 chromatography as described (17). Bovine brain nuclear extracts prepared from 2.5 kg of brain were dialyzed against Buffer D (20 mM Hepes, pH=7.9; 10% Glycerol; 0.2 mM EDTA, pH=8.0; 1 mM DTT, and 0.5mM PMSF) containing 100 mM KCl (BD[0.1]) and loaded onto 100 ml P11 phosphocellulose column pre-equilibrated with BD[0.1]. The column was eluted with a 500 ml KCl gradient from 0.1 M to 1 M in Buffer D at the rate of 0.5 ml/min. The P11 fractions containing peak footprint activity were pooled and adjusted to 0.05 M KCl and applied to a 100 ml DEAE CL-6B column pre-equilibrated with 0.05 M KCl in Buffer D. The column was eluted with 500 ml

KCl gradient from 0.05 M to 1 M in Buffer D at the rate of 0.5 ml/min. Fractions containing footprint activity were pooled for further DNA affinity chromatography. Preparation of DNA affinity columns and affinity purification were carried out as described (18). The oligonucleotide sequences used for affinity chromatography were as follows:

CRE-1+2 5' - GCGGCGCGGGGCTGGCGTAGGGCTGCGTCAGCTGCA 3'
ACGTCGCCGCGCCCCGACCGCATCCCCGACGACGTCG
CRE-1 5' - GATCGCGGGGCTGGCGTAGGG 3'
CGCCCCGACCGATCCCCCTAG
CRE-2 5' - GATCGGGCTGCGTCAGGG 3'
CCCCGACGACGTCGCCCTAG
AP-1 5' - GATCGGCTGAGTCAGGG 3'
CCGACTCAGTCCCCCTAG
AP-4 5' - GATCGTCAGCTGCAGGG 3'
CAGTCGACGTCGCCCTAG
AP-2 5' - GATCCAGCCCGCGGCGATTG 3'
GTCGGGCGGCCGCTAACCTAG

Peptide Microsequencing

Affinity purified ENKTF-1 (60 µg) was fractionated by 10% SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Schleicher & Schuell, BA85). Staining of the membrane with Amido Black B10 revealed three bands (62, 50, and 35 kDa) which were excised and treated with trypsin (sequencing grade, Boehringer Mannheim) (19,20). Peptide fragments released into the supernatant were resolved by reversed phase HPLC and subjected to Edman degradation in a gas phase sequencer (Applied Biosystems 470A) as described (20). Initial yields ranged from 14.4 pmol for glutamic acid in peptide A2 to 3.9 pmol for threonine in peptide B2.

DNase 1 Footprinting

DNase I footprinting was carried out as described (21). Typically 10 fmol of ³²P-labeled DNA probe extending from the human proenkephalin CAP site +1 to -440 was end-labeled on the coding strand at position +1 using the 'filling in' reaction with [α -³²P]dCTP. The probe was incubated on ice for 15 minutes with crude nuclear extract in the presence of 1 µg poly(dA/dT) or with purified proteins in the absence of competitor DNA. Samples were then treated with DNase I (Worthington) for 1 minute at 25°C. Samples were phenol/chloroform extracted, isopropanol precipitated and analyzed by 7.8% polyacrylamide-urea gel electrophoresis.

Glycerol Gradient Analysis

Affinity purified ENKTF-1 was concentrated with an Amicon 30 microcentrator and adjusted to 50 mM KCl in buffer Z (25 mM Hepes, pH=7.9; 12.5 mM MgCl₂; 10% Glycerol; 0.1% NP-40, and 1 mM DTT). Two hundred microliters of protein sample was layered onto 4.2 ml 10–35% glycerol gradient and ultracentrifuged with the Beckman SW60i rotor at 45K rpm, 4°C, for 20 hours. Protein standards (cytochrome C; alcohol dehydrogenase; bovine serum albumin; aldolase) were run in parallel. Serial 250 µl fractions were collected for SDS-PAGE and footprinting analysis.

Transient Analysis of Gene Expression

Transfection of CV-1 cells was performed as previously described (7). For transient expression pENKAT-12 plasmids (5 µg) were co-transfected with 10 µg pRSVβGAL (22), 5 µg pMTCα Neo

(gift from M. Uhler unpublished, see also reference 23) and various amounts of a plasmid that contains the entire coding sequence of NF1/Red1 inserted downstream of the CMV promoter, pCMVNFI/RED1 (see also reference 24). The total amount of DNA was adjusted to 25 to 30 μ g with pGEM3. Following transfection with CaPO_4 precipitated DNA and glycerol shock, the cells (3×10^5 /10cm plate) were incubated for 24 hours in media containing 2.5% fetal calf serum and 7.5% horse serum. In the case of induction by regulators, forskolin (10 μ M) and IBMX (0.5 mM) were added 6 hours prior to harvesting cells and extract preparation. CAT activity was measured by a modification of the standard assay as described (6). Butyryl-CoA (0.25 mM) was used and the reaction products were extracted with 400 μ l of a 2:1 mixture of pristane/xylene. Under these conditions butyrylated chloramphenicol was preferentially extracted into the organic phase and 300 μ l was counted directly in scintillation fluid. β -Galactosidase activity was determined as previously described (6) and used to normalize CAT activity within a given experiment.

RESULTS

Binding interactions between ENKTF-1 and AP-1, AP-2, and AP-4

We have previously identified 4 different DNA binding factors which interact with the proenkephalin inducible enhancer (Figure 1), ENKTF-1, AP-1, AP-4, and AP-2 (7). Each of these proteins has been purified from HeLa cell nuclear extracts using multimerized proenkephalin CRE-1, CRE-2, AP-4, and AP-2 oligonucleotides covalently linked to sepharose as an affinity matrix to purify DNA binding proteins (18). As shown in Figure 2, each affinity purified preparation results in a single footprint over the proenkephalin inducible enhancer. The DNA binding interactions between ENKTF-1 and proteins which bind at the CRE-2, AP-4, and AP-2 elements have been examined and are shown in Figure 2. ENKTF-1 can bind to the CRE-1 element in the presence of AP-4 or AP-2 as the sum of the two individual footprints are observed. Careful analysis of the 5'- and 3'-borders of the footprint shown in Figure 2 (repeated several times) indicates that ENKTF-1 and AP-1 do not bind simultaneously to the CRE-1 and CRE-2 elements as the AP-1 footprint (also see reference 7) and not the ENKTF-1 footprint is always observed when these two factors are allowed to bind together.

Purification of CRE-1 binding proteins from bovine brain

Attempts to purify amounts of the CRE-1 binding protein, ENKTF-1, sufficient for microsequencing from HeLa cells were not successful. Footprinting experiments (see Figure 3A)

Figure 1. Sequence of the human proenkephalin second messenger-inducible enhancer. Footprints of the HeLa cell nuclear factors, ENKTF-1, AP-1, AP-2, and AP-4 are shown above the sequence.

indicated that nuclear proteins isolated from bovine brain contained a CRE-1 binding activity indistinguishable from the HeLa cell protein. Using standard ion exchange and DNA recognition site affinity chromatography we have purified nuclear proteins from bovine brain which bind the CRE-1 element. As shown in Figure 3B and 3C, after DEAE and phosphocellulose chromatography and sequential chromatography on CRE-1+2 (containing both CRE-1 and CRE-2 sequences) followed by CRE-1 DNA affinity columns, three major bands of 62, 50, and 35 kDa are detected by SDS gel electrophoresis and silver staining (see Figure 3C). These three bands are present in several different CRE-1 binding protein preparations from bovine brain. Attempts to purify further CRE-1 binding activity resulted in either a loss of DNA binding or copurification of the three major bands. As shown in Table 1 the specific DNA binding activity of these three proteins after two rounds of affinity chromatography was enriched approximately 9,000 fold.

Attempts to footprint each of the three bands renatured from SDS gels individually or in various combinations were not successful. To demonstrate that footprinting activity correlated with the 62, 50, and 35 kDa bands, protein fractions shown in Figure 3C were fractionated by sedimentation through a glycerol gradient. As shown in Figure 4A, all three proteins migrated through the glycerol gradient at the same position. DNase I

Figure 2. DNase I footprinting analysis of DNA binding interactions between ENKTF-1 and AP-1, AP-2, and AP-4. Footprinting probe is a 440 base pair Apa I/Xma I fragment labeled at the Xma I site (+1 or CAP site). Left lane shows A+G sequence ladder of the footprinting probe. 'TF-1' represents ENKTF-1. The 5' boundary of the AP-1 footprint in the NF-1/AP-1 mixing experiment is clearly defined by the band positioned at the junction of the two black bars at right indicating AP-1 footprints. Since the highly purified factors still reveal multiple bands on the SDS gel, the volume (μ l) of each sample is shown to indicate the relative amount of footprint activity added in the reaction.

Figure 3. Characterization and purification of ENKTF-1 from bovine brain. A) Comparison of footprints observed using affinity purified ENKTF-1 from HeLa cells and bovine brain. B) Purification of ENKTF-1 from bovine brain with ion exchange and CRE-1 affinity chromatography of bovine brain nuclear protein ('FPU' = footprint unit, 1 FPU equals the amount of protein required to completely protect 10 fmol of footprint probe). C) Analysis of affinity purified CRE-1 binding proteins from bovine brain by silver staining and 10% SDS polyacrylamide gel electrophoresis. E1 and E2 indicate the first and second elution from the DNA affinity column. Molecular weight standards are: bovine serum albumin, 66 kDa; β -amylase, 50 kDa; alcohol dehydrogenase, 39 kDa; carbonic anhydrase, 29 kDa.

footprinting across the gradient (Figure 4B) indicates that DNA binding activity comigrates with the 62, 50, and 35 kDa molecules.

CRE-1 binding proteins belong to the NF-I family of transcription factors

Approximately 60 micrograms of the CRE-1 binding proteins (20 micrograms each of the 62, 50, and 35 kDa proteins) from bovine brain were size fractionated on a SDS polyacrylamide gel, transferred to a nitrocellulose membrane, stained with amido black, and the 62, 50 and 35 kDa bands cut from the membrane. Each of the three proteins immobilized on nitrocellulose was treated with trypsin (19) and tryptic peptides eluted and purified by HPLC chromatography as described (20). Well resolved peptides were subjected to microsequencing. Figure 5A shows the sequences obtained from 6 different tryptic fragments derived from the 62 kDa protein and 2 different peptide sequences obtained from each of the 50 and 35 kDa proteins. A computer search revealed that each of the six peptide sequences derived from the 62 kDa protein could be aligned perfectly with the amino acid sequence of the protein nuclear factor-I/L, (NF-I/L) (13). Although the predicted amino acid sequence was derived from a rat cDNA clone and our peptide sequences were from bovine brain, the tryptic fragments A1-A6 were found to match the predicted rat sequence at 100% of the amino acid positions shown in Figure 5B. This suggests that the 62 kDa CRE-1 binding

Table 1. Purification of CRE-1 binding proteins from bovine brain.

	Total Protein (mg)	Total Activity (FPU)	Specific Activity (FPU/mg)	Purification (Fold)	Yield (%)
Nuclear Extract	8,400	4,000,000	500	1	100
Phospho-Cellulose	140	3,000,000	21,000	42	75
DEAE-Sepharose	27.0	2,000,000	74,000	148	50
CRE1+2 Affinity	1.90	1,100,000	580,000	1,160	28
CRE1 Affinity	0.090	400,000	4,400,000	8,800	10

protein we isolated is the bovine homologue of the rat NF-I/L protein. The peptides sequenced from the 50 and 35 kDa proteins were not found within the NF-I/L molecule. As shown in Figure 5B, the two tryptic peptide sequences derived from the 50 kDa protein, B1 and B2, and the two derived from the 35 kDa protein, C1 and C2, are found within two hamster NF-I proteins, NF-I/RED-1 and NF-I/X, respectively (12). In all cases the bovine tryptic peptides, B1, B2, C1, and C2 perfectly match the hamster NF-I sequences suggesting that the 50 kDa protein is the bovine NF-I/Red1 homologue, and the 35 kDa protein is the bovine NF-I/X homologue.

Figure 4. Glycerol gradient analysis of affinity purified ENKTF-1 from bovine brain. **A)** Analysis of glycerol gradient fractions by silver staining and 7% SDS polyacrylamide gel electrophoresis. The 62 kDa doublet presents in fractions 13 to 19 is resolved into a single band under higher reducing conditions (0.5 M 2-mercaptoethanol) which were used in Figure 3C. **B)** Analysis of DNA binding activity across the glycerol gradient. Note the complete coincidence of the 62, 50, and 35 kDa proteins with DNA binding activity.

Footprinting suggests NF-I contacts CRE-1 and part of CRE-2

To analyze the interaction of the NF-I proteins with the CRE-1 element we examined the binding of highly purified NF-I proteins (see Figure 3C) to a series of single base substitution mutations spanning the proenkephalin CRE-1 and CRE-2 elements. Mutations which blocked the binding of these proteins to the CRE-1 element were found to occur at two positions spanning the CRE-1 and CRE-2 elements. Mutations within the sequence CTGGC and the sequence CCT six bases downstream were found

Figure 5. **A)** Amino acid sequence of tryptic peptides derived from the 62, 50, 35 kDa proteins and **B)** the location of each tryptic peptide within the predicted protein sequence of NF-I/L (13), NF-I/Red1 (12), and NF-I/X (12). Sequences A3 and A4 were coeluted in the HPLC separation.

to block binding of the NF-I proteins (see Figure 6A). This data suggests that NF-I molecules contact the enhancer at two sites: the CRE-1 element and an additional CCT site which overlaps the CRE-2 element (see Figure 6B). In addition, as shown in Figure 7, highly purified NF-I preparations also contact the proenkephalin promoter at a site directly spanning the TATA box at position -33 to -27 (data not shown). As shown in Figure 7, the footprint observed over the TATA box titrates with NF-I binding to the CRE-1 element.

Cotransfection with NF-I/Red1 suggests it acts as a repressor

To examine the functional activity of NF-I proteins at the CRE-1 element a plasmid which expresses NF-I/Red1 (12) under the control of the CMV promoter was cotransfected together with pENKAT-12 and pRSV β GAL into CV-1 cells. These plasmids were cotransfected in the presence or absence of a plasmid expressing the catalytic subunit of the cyclic AMP dependent protein kinase (PKA) (25). As shown in Figure 8, NF-I/Red1 had little effect on basal expression of the proenkephalin promoter, suggesting that alone NF-I/Red1 cannot activate proenkephalin transcription. However, coexpression of this plasmid together with PKA inhibited the PKA dependent induction of CAT expression in a dose dependent fashion. Cotransfection of NF-I/Red1 also inhibited CAT expression stimulated with forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX). Cotransfection with NF-I/Red1, however, had no significant effect on β -galactosidase

Figure 6. Point Mutation analysis of the NF-I binding site within the human proenkephalin second messenger-inducible enhancer. A) Footprint analysis using 1.0 footprint unit of affinity purified NF-I proteins from bovine brain and DNA footprinting probes containing the single base substitution shown above. 'NF-Is' indicates the footprint obtained with sample containing the three NF-I proteins. B) Summary of nucleotides which block (black arrows) or reduce (shaded arrows) NF-I binding *in vitro*. '*' indicates the effect is unknown due to the lack of -93 point mutant.

expression directed by the RSV promoter/enhancer, suggesting the effect is specific to the proenkephalin promoter. CAT expression shown in Figure 8 has been normalized to the β -galactosidase activities determined for each extract as previously described (6,22).

DISCUSSION

Multiple NF-I Proteins from Brain Bind the Proenkephalin Enhancer

To understand the role of the CRE-1 element in signal transduction and transcriptional regulation of the proenkephalin gene we have purified proteins from bovine brain which bind this element. Microsequencing of three different brain CRE-1 binding proteins indicate that each of the three proteins is a distinct member of the NF-I family of proteins. The molecular weight of the brain 62 kDa protein agrees well with the predicted size of the NF-I/L protein from rat (13). In contrast, the 50 and 35 kDa brain proteins are smaller than the predicted size of the hamster NF-I/Red1 (60 kDa) and NF-I/X (49 kDa) proteins based on published cDNA sequences (12). This could be due to species differences, alternative splicing as observed for the NF-I/CTF and NF-I/L (11,14,26), or protein processing or degradation as has been observed for NF-I/L (13) and NF-I/Red1 (12). Although NF-I/CTF message is expressed in mouse brain (26), we have not identified this protein in our purification procedure, which might indicate this protein does not bind the proenkephalin

Figure 7. NF-I proteins bind CRE-1 and the TATA box region of the human proenkephalin promoter. DNase I footprinting analysis of NF-I proteins purified from bovine brain showing a titration of NF-I DNA binding activity from 0.3 to 3.0 footprint units (FPU). Note that protection of the CRE-1 and TATA elements occurs at similar concentrations of NF-I proteins. The arrowheads indicate two hypersensitive cleavage sites at the 3' boundary of the footprint.

promoter. Members of the NF-I family are known to serve both as transcription factors regulating RNA polymerase II activity and as initiation factors required for replication of adenovirus DNA in infected cells (10). An NF-I binding site has been reported to mediate transcriptional activation of the mouse $\alpha 2(I)$ collagen gene to TGF- β (27), and NF-I/CTF proteins have been shown to activate the α -globin promoter *in vitro* (28). Together, this information suggests that NF-I proteins may interact with the CRE-1 element to regulate proenkephalin transcription.

Previous studies have shown both NF-I/L and NF-I/CTF bind to DNA as dimers (14,29). Furthermore, cross-linking and gel filtration studies suggest that the native NF-I configuration may be tetrameric (29). Comigration of the three brain NF-I molecules on glycerol gradients raises the possibility that the NF-I proteins we have purified bind the proenkephalin CRE-1 element as homo- or hetero- dimeric/tetrameric complexes. Clearly, further analysis of these proteins is necessary to determine the precise nature of the NF-I complex which bind the proenkephalin enhancer.

NF-I Binding Site Spans Both the Proenkephalin CRE-1 and CRE-2 Elements

The high affinity NF-I binding site found in the adenovirus replication origin consists of a palindrome containing two TGGC sequences on opposite strands separated by 5 bases (30). It has

Figure 8. Repression of protein kinase A and forskolin induced expression of a proenkephalin CAT gene by cotransfection with NF-I/Red1. See materials and methods for a description of the experimental protocol. Black bars indicate cells treated with forskolin/IBMX or transfected with a plasmid expressing the catalytic subunit of the cAMP dependent protein kinase. Normalized CAT activities represent CAT values relative to the levels of β -galactosidase activity determined from the same cell extract.

been shown, however, that NF-I can recognize promoter elements where the binding site resembles one half of the palindrome (31). Analysis of the proenkephalin NF-I binding site indicates that important contacts occur at two different sites. Point mutations within the sequences underlined CTGGCGTAGGGCCTGCG TCA block binding of NF-I proteins to the enhancer (the CRE-1 and CRE-2 elements are shown in large type). Although the NF-I recognition sequence is an imperfect palindrome (CTGGCxxx-xxGCCTG), mutations at equivalent positions within the palindrome produce very different effects on NF-I binding. This suggests that the NF-I proteins recognize the two contact sites in a nonsymmetrical fashion, perhaps as heterodimers. Although mutations within the CCT sequence in the proenkephalin enhancer block binding, as determined by DNase I footprinting, the CRE-1 column used in affinity purification of these proteins lacks the CCT element. This suggests that these proteins can still bind the CTGGC element in the absence of a downstream CCT site although with lower affinity.

The observation that highly purified NF-I proteins also contact the TATA box is novel. The fact that binding of NF-I to the TATA box site and CRE-1 element occurs at identical protein concentrations suggests that the same complex which binds the CRE-1 element can somehow interact simultaneously at the TATA box. We cannot, however, rule out the possibility that contacts at the TATA box occur via a co-purified TFIID/NF-I complex.

Protein-Protein Interactions at the Proenkephalin Enhancer

Comparison of the footprint produced by the HeLa cell CRE-1 binding protein, ENKTF-1, with bovine brain NF-I proteins indicates that the human HeLa and bovine brain proteins protect an identical region of the proenkephalin enhancer. In addition, the binding profile of the HeLa ENKTF-1 to point mutations within the CRE-1 element is identical to the binding profile determined for brain NF-I molecules (7), with the exception that the brain NF-I proteins appears less sensitive to point mutations at nucleotide -100. Although we were not able to obtain quantities of the HeLa cell CRE-1 binding protein, ENKTF-1,

sufficient for microsequence determination, the similarities described above suggest that the HeLa cell CRE-1 binding protein, ENKTF-1, is also a member of the NF-I family of transcription factors.

Our previous analysis of CRE-1 function suggested that proteins bound at this element interact synergistically with proteins bound at the CRE-2 element to mediate cAMP and TPA inducible enhancer function. To analyze the *in vitro* interactions between proteins bound at the CRE-1 element and proteins bound at the CRE-2, AP-4, and AP-2 elements we chose to study proteins existing within a single cell type. HeLa cell NF-I-like proteins (ENKTF-1) were analyzed because we had previously purified AP-1, AP-4, and AP-2 proteins from this cell type (7). HeLa cell NF-I binds simultaneously with AP-2 and AP-4 to the enhancer to form a stable complex. In contrast, NF-I proteins cannot bind simultaneously with AP-1. The affinity of AP-4 and AP-2 binding to their recognition sites was not dramatically enhanced by the presence of NF-I proteins bound at the CRE-1 element. However, we reproducibly observe that 2 fold less AP-4 or AP-2 protein is required to give a complete footprint in the presence of NF-I proteins suggesting some degree of positive cooperativity in their binding (data not shown). No change in the binding affinity of NF-I was observed in the presence of AP-4 or AP-2 bound to their recognition sites. The observation that AP-1 and NF-I proteins do not simultaneously interact with the CRE-1 and CRE-2 elements can be understood in terms of the overlapping nature of NF-I and AP-1 binding sites.

Functional Role of NF-I Proteins in Second Messenger Regulation of Proenkephalin Gene Expression

Mutations within the sequence TGGCGTA (CRE-1 element) have been shown to produce a strong detrimental effect on cAMP/TPA induction of proenkephalin gene expression in CV-1 or C6-glioma cells (7). However, several observations suggest that NF-I binding *in vitro* does not completely correlate with the above *in vivo* analysis of CRE-1 function. First, two mutations (-99 and -98) within the 3'-end of the CRE-1 element strongly repress inducible enhancer function *in vivo* yet have only a weak effect on *in vitro* NF-I binding. Second, a mutation at position -94 located within the downstream contact site, CCT, blocks NF-I binding *in vitro* but does not influence inducible enhancer function *in vivo*. Such differences may be due to the heterogeneous nature of the NF-I proteins used in our DNA binding analysis which may form hetero- or homodimeric complexes and interact differentially with the CRE-1 site. For example, it is possible that the -100 and -98/99 mutations which *in vitro* only slightly reduce NF-I binding may *in vivo* block the binding of a fraction of the NF-I molecules which are critical regulators of CRE-1 function.

NF-I/Red1 inhibition of PKA inducible enhancer function could be most simply explained by the assumption that NF-I/Red1 acts as a transcriptional repressor by competing in a mutually exclusive fashion at the CRE-2 site with a transcriptionally active AP-1 complex. However, the possibility that overexpressed NF-I/Red1 outstrips and/or competes with a co-activator should also be considered. Alternatively, as some members of the AP-1 complex are known to act as negative regulators of transcription (32,33), it is possible that NF-I molecules could activate transcription by antagonizing AP-1 mediated repression.

Together, the above data suggest that NF-I proteins expressed in bovine brain bind to a site which closely overlaps the CRE-1 element defined *in vivo*. Although overexpression of NF-I/Red1 represses PKA inducible expression, the functional role of NF-I/L

and NF-I/X remain to be determined. Recently, a family of NF-I/L like molecules have been identified from mouse brain and one of these molecules has been shown to activate the myelin basic protein promoter by cotransfection experiments (26). This suggests that some members of the NF-I family may act as repressors while others may activate gene expression. Clearly, the expression and functional activities of the different NF-I proteins in cells expressing proenkephalin need to be further characterized before their role in transcriptional regulation can be determined.

ACKNOWLEDGMENTS

We thank Michael Uhler for the generous gift of the plasmid, pMTC α Neo, which expresses the catalytic subunit of the cyclic AMP-dependent protein kinase under the control of the mouse metallothionein promoter. We also thank Haim Rosen, Linda Kobierski, Aviva Symes, and Yunn-Yi Chen for critical reading of the manuscript. This work was supported by US Public Health Service grant DA 05706-01 to M.C..

REFERENCES

- Eiden, L.E., Giraud, P., Affolter, H.-U., Herbert, E. and Hotchkiss, A.J. (1984) *Proc. Natl. Acad. Sci. USA* 81, 3949–3953.
- Quach, T.T., Kageyama, H., Mocchetti, I., Guidotti, A., Meek, J.L., Costa, E. and Schwartz, J.P. (1984) *Mol. Pharmacol.* 26, 255–260.
- Kley, N. (1988) *J. Biol. Chem.* 263(4), 2003–2008.
- Kley, N., Loeffler, J.P., Pittius, C.W. and Holtt, V. (1986) *EMBO J.* 5(5), 967–970.
- Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E. and Goodman, H.M. (1986) *Nature* 323, 353–356.
- Nguyen, T.V., Kobierski, L., Comb, M. and Hyman S.E. (1990) *J. Neurosci.* 10(8), 2825–2833.
- Comb, M., Mermod, N., Hyman S.E., Pearlberg, J., Ross, M.E. and Goodman, H.M. (1988) *EMBO J.* 7(12), 3793–3805.
- Hyman, S.E., Comb, M., Lin, Y.-S., Pearlberg, J., Green, M.R. and Goodman, H.M. (1988) *Mol. Cell. Biol.* 8(10), 4225–4233.
- Hyman, S.E., Comb, M., Pearlberg, J. and Goodman, H.M. (1989) *Mol. Cell. Biol.* 9(1), 321–324.
- Rosenfeld, P.J. and Kelly, T.J. (1986) *J. Biol. Chem.* 261(3), 1398–1408.
- Santoro, C., Mermod, N., Andrews, P.C. and Tjian, R. (1988) *Nature* 334: 218–224.
- Gil, G., Smith, J.R., Goldstein, J.L., Slaughter, C.A., Orth, K., Brown, M.S. and Osborne, T.F. (1988) *Proc. Natl. Acad. Sci. USA* 85, 8963–8967.
- Paonessa, G., Gounari, F., Frank, R. and Cortese, R. (1988) *EMBO J.* 7(10), 3115–3123.
- Mermod, N., O'Neill, E.A., Kelly, T.J. and Tjian, R. (1989) *Cell* 58, 741–753.
- Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) *Nucl. Acids Res.* 11, 1475–1489.
- Yamamoto, K.K., Gonzalez, G.A., Biggs III, W.H. and Montminy, M.R. (1988) *Nature* 334, 494–498.
- Briggs, M.R., Kadonaga, J.T., Bell, S.P. and Tjian, R. (1986) *Science* 234, 47–52.
- Kadonaga, J.T. and Tjian, R. (1986) *Proc. Natl. Acad. Sci. USA* 83, 5889–5893.
- Aebbersold, R.H., Leavitt, J., Saavedra, R.R., Hood, L.E. and Kent, S.B. (1987) *Proc. Natl. Acad. Sci. USA* 84, 6970–6974.
- Fischer, W.H., Karr, D., Jackson, B., Park, M., and Vale, W. (1990) *Methods Neurosci.* 6, (in press)
- Lee, K.A.W., Hai, T.Y., SivaRaman, L., Thimmappaya, B., Hurst, H.C., Jones, N.C. and Green, M.R. (1987) *Proc. Natl. Acad. Sci. USA* 84, 8355–8359.
- Edlund, T., Walker, M.D., Barr, P.J. and Rutter, W.J. (1985) *Science* 230, 912–916.
- Uhler, M.D. and McKnight, G.S. (1987) *J. Biol. Chem.* 262(31), 15202–15207.
- Andersson S., Davis, D.L., Dahlback, H., Jornvall, H. and Russell, D.W. (1989) *J. Biol. Chem.* 264(14), 8222–8229.
- Uhler, M.D., Carmichael, D.F., Lee, D.C., Chrivia, J.C., Krebs, E.G. and McKnight, S.G. (1986) *Proc. Natl. Acad. Sci. USA* 83, 1300–1304.
- Inoue, T., Tamura, T., Furuichi, T. and Mikoshiba, K. (1990) *J. Biol. Chem.* 265(31), 19065–19070.
- Rossi, P., Karsenty, G., Roberts, A.B., Roche, N.S., Sporn, M.B. and de Crombrughe, B. (1988) *Cell* 52, 405–414.
- Jones, K.A., Kadonaga, J.T., Rosenfeld, P.J., Kelly, T.J. and Tjian, R. (1987) *Cell* 48, 79–89.
- Gounari, F., De Francesco, R., Schmitt, J. Van der Vliet, P.C., Cortese, R. and Stunnenberg, H. (1990) *EMBO J.* 9(2), 559–566.
- De Vries, E., van Driel, W., van den Heuvel, S.J.L. and van der Vliet, P.C. (1987) *EMBO J.* 6(1), 161–168.
- Lichtsteiner, S., Wuarin, J. and Schibler, U. (1987) *Cell* 51, 963–973.
- Sassone-Corsi, P., Sisson, J.C. and Verma, I.M. (1988) *Nature* 334, 314–319.
- Chiu, R., Angel, P. and Karin, M. (1989) *Cell* 59, 979–986.