DNA Structure Determines Protein Binding and Transcriptional Efficiency of the Proenkephalin cAMP-responsive Enhancer*

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Two precisely arranged proenkephalin cAMP response elements (CREs) behave as a single protein binding site. The experiments described support a model in which a secondary structural change creates a new binding site, which is made up of sequences from both of the CREs. The CRE-binding protein (CREB) binds CRE-1, but binding there is entirely dependent on the presence of CRE-2. Electron spectroscopic images show that a CREB dimer occupies twice as much DNA in the proenkephalin gene as in the prodynorphin gene. The enhancer region is sensitive to P1 nuclease in a CREB concentration-dependent manner, and sensitivity is strand-specific, indicating protein-stabilized structural change. DNase I analysis shows that in the native proenkephalin gene, CREB binds both CRE-1 and CRE-2. In vivo, both CREs are occupied in the transcriptionally active proenkephalin gene, while neither is in the silent gene. Whereas CREB can bind CRE-2, mutation or elimination of either proenkephalin CRE alters response to second messengers and transcription factors. Thus, binding to CRE-2 alone is not sufficient. Specific and efficient transcription of the proenkephalin gene requires the presence of both CREs, precisely arranged to allow them to form a single protein binding site.

A limited number of sequence-specific DNA-binding proteins is sufficient for the precise and complex transcriptional regulation in the brain. Within each gene, the number, type, and arrangement of binding sites allow the assembly of a gene-specific transcriptional machinery (1). Studies of the genes that encode precursors to endogenous opioid receptor ligands have clarified aspects of transcriptional control and its contribution to the overall regulation of these peptides. Opioid peptides are neuromodulators and circulating hormones that play a role in nociception, adaptation, learning, and development (2). The gene that encodes preproenkephalin is transcriptionally responsive to a variety of stimuli, including growth factors, neurotransmitters, cell depolarization, and hormones; remarkably, the several discrete pathways that carry the messages of the cell surface events converge at a 27-base pair region comprising two cAMP-responsive enhancers (3, 4). The two CREs are not, however, equivalent. CRE-2 appears to be a typical enhancer. Its sequence is similar to that of phorbol ester and cAMP response elements, and it binds factors that mediate the transcriptional response to second messengers (4–7). Tandem copies of CRE-2 can respond to stimuli that mimic receptor occupation, but by itself CRE-2 is not able to support efficient transcription or receptor response (5, 7–9). In contrast, CRE-1 is apparently not a binding site for transcription factors known to respond to second messengers nor are multiple copies of CRE-1 sufficient to confer a response to cAMP (7). Yet point mutations in or deletion of CRE-1 alters the transcriptional response to cell surface ligands, to second messengers, and to CRE-binding proteins (3, 4, 9). Comparison of sequences from several species, including human (10), rat (11), hamster (12), and guinea pig (13), reveals that the 27-base pair region containing the CREs is identical. The arrangement of the CREs with respect to each other as well as the context of each CRE must therefore be critical. Altering the spacing between the two proenkephalin CREs alters transcriptional regulation (4). That context is significant is further emphasized by the observation that the 8-bp CTGCGTCA sequence of CRE-2 is exactly that of a critical transcription factor-binding element of another opioid peptide gene, prodynorphin; yet the two elements mediate different responses to identical stimuli and transcription factors (14, 15).

What mechanism requires that the arrangement of CRE-1 and CRE-2 be precisely maintained? A direct role for CRE-1 in the action of CRE-binding proteins is suggested by the observation that response to CREB protein is altered when CRE-2 is intact but CRE-1 is deleted (9). Recent studies show CREB to be a mediator of the proenkephalin transcriptional response in the brain. The use of a fusion transgene implicates CREB in enhancing proenkephalin expression in response to stress within the mouse hypothalamus (16). Within rat striatum, CREB is the likely factor that acts via the proenkephalin enhancer to effect transcription both in control and in haloperidol-treated animals (6) and in response to amphetamines (17). Proenkephalin transcription is sensitive to point mutations in or loss of either CRE, even though CRE-1 does not by itself bind CRE-binding proteins such as CREB/ATF or AP-1. Although CRE-2 is known to bind such transcription factors, CRE-2 cannot function efficiently in the absence of CRE-1 (3, 4, 9). What makes the CREs effective together? We have previously studied the nearly palindromic region that contains the CREs. By intranuclear hydrogen bonding, an alternative CRE can be created from the nearly complementary sequences of CRE-1 and CRE-2 (9, 18, 19). The hairpin containing the site is stable under physiological conditions (18), and its structure has been analyzed by NMR spectroscopy (9, 19). The alternative}{**
binding site differs from that of CRE-2 by the presence of two GT mismatched base pairs (9, 18, 19), and it binds CREB protein with higher affinity than does the native duplex site (9). The presence of the T in one of the GT base pairs appears critical to the increase in binding affinity (9). Studies on the structure of this region have relied entirely on synthetic oligonucleotides and have been limited to the 23-base pair region.

To test the biological relevance of the alternative site, we have studied protein-proenkephalin complexes in very large molecules of DNA. The formation of a site consisting of both CRE-1 and CRE-2 would require an alteration in the DNA structure. In the alternative binding site, both CRE-1 and CRE-2 would directly interact with CREB protein. Furthermore, both CREs should bind protein during the formation of active transcription complexes. Quantitative electron microscopy shows that the DNA in CREB-proenkephalin complexes does adopt a different conformation from that in CREB-prodynorphin complexes. In the native gene, the CRE footprint spans both proenkephalin CREs, whereas in a mutant lacking CRE-1 the footprint is limited to the region of CRE-2. In vivo analysis shows that both CREs are occupied in the actively transcribed gene but that neither is occupied in the silent gene. Studies on the structure of this region have relied entirely on synthetic oligonucleotides and have been limited to the 23-base pair region.

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MATERIALS AND METHODS

Electron Microscopy—Proenkephalin and prodynorphin fragments were from Ncol-AflIII double digests of pENKAT-12 (3) and pCAT2.0dyn (14), respectively. The digestions yield a 1322-bp proenkephalin inserts in pCAT2.0dyn (14), respectively. The digestions yield a 1322-bp proenkephalin duplex (23 bp), and proenkephalin GT (23-mer) probes were made by hybridizing the GT (23-mer) probe with 15% horse serum and 2.5% fetal calf serum. Calcium phosphate-DNA precipitation was carried out as described in Fig. 3a; duplexes were prepared as described previously (9).

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bromphenol blue, 0.3% xylene cyanol FF, 0.37% Na₂EDTA in deionized formamide) (28). 3 μl of the stopped reaction was heated to 90°C for 5 min and then placed on ice before separation on 40-cm, 8% sequencing gels (SequaGel-8, National Diagnostics). Gels were fixed without floating off the plate (5–10 min; 5% acetic acid, 5% methanol) and then dried; and products were visualized by exposing Biomax MR (Kodak) film (without screen). Sequence markers were synthesized by 32P-end-labeled primer extension (20 cycles; times and temperatures as above) along undigested template plasmid in 6.2-μl reactions containing formamide, Triton X-100, enzyme as above, 20 ng plasmid, 3 μl of a dNTP-dideoxyNTP (A, C, G, or T) mix at the concentrations recommended by the manufacturer for use with Deep Vent (exo⁻) DNA polymerase (package insert, New England Biolabs). Reactions were stopped and processed as described above.

**Genomic Footprinting**—Genomic footprinting was essentially according to the protocol of Mueller et al. (29). Forskolin (Calbiochem; 20 μM from 20 mM stock in Me₂SO) or Me₂SO carrier was added to cultured cells. 1 h after the addition of forskolin, cells were removed from the incubator, treated for 10 min with 0.1% dimethylsulfate (DMS) in PBS or with PBS alone, washed with PBS, and then lysed in the presence of SDS and proteinase K. DNA was then purified, cleaved in hot piperidine, and analyzed by ligation-mediated polymerase chain reaction (29–31). “Naked” DNA (purified from cells treated with PBS) was modified in vitro by DMS (32), deaved, and analyzed by ligation-mediated polymerase chain reaction. Gene-specific primers, based on the rat genomic sequence (11), were −217 to −198, −197 to −173, and −194 to −167 for analysis of the bottom strand, and 111 to 92, 84 to 60, and 83 to 56 for analysis of the top strand. Because of the GC content of the region, the reactions included deaza-dGTP and dGTP in a 3:1 molar ratio in place of dGTP alone (33). Products were resolved on a 6% 62-cm gel using an electrolyte gradient as described by Sheen and Seed (34). The dried gel was exposed to a phosphor storage screen and scanned by PhosphorImager 425 (Molecular Dynamics). Equivalent regions of each lane were selected and quantified (ImageQuant), and data were exported to KaleidaGraph (Abelbeck). Baselines were smoothed and curves were normalized to correct for uneven loading. Relative intensities of individual bands were estimated by peak-to-valley measurement. Images for figure were from exposure of Biomax MR film (Kodak).

**RESULTS**

CREB Dimer Occupies Different Mass of DNA in Two Opioid Peptide Genes—Electron spectroscopic imaging has been used previously to examine the interaction of proteins with nucleic acids (20, 21). Electron spectroscopic imaging of phosphorus shows the distribution of DNA within a protein-DNA complex and, consequently, provides information on the structure and mass of the DNA occupied by the protein (see “Materials and Methods”). The proenkephalin DNA (Fig. 1a) is a 1.3-kilobasepair fragment from the enkat12 reporter plasmid (3); it contains the enhancer-promoter region from the human proenkephalin gene (Fig. 1e). The use of fragments in which CREs are asymmetrically located allows measurement of electron micrographs to determine whether protein is binding in the expected region. Under our experimental conditions, about 90% of proenkephalin molecules were free of bound protein, whereas approximately 10% contained a single complex in a position consistent with binding at the CREs (Fig. 1, b and e). Parallel studies were carried out using CREB with another DNA fragment (prodynorphin) and the proenkephalin fragment with another DNA-binding protein (TATAA-binding protein, TBP). CREB was bound to a
2.9-kilobase-pair fragment from a rat prodynorphin-CAT fusion construct (Fig. 1, d and f); it contains four CREs of differing affinities (14, 15). One of the rat prodynorphin CREs (CRE-3; Fig. 1f) is identical in sequence to that of proenkephalin CRE-2 but not located within a region able to form a stable alternative structure (14, 15). As with the proenkephalin fragment, about 10% of the prodynorphin molecules contained a single complex in a position consistent with the two highest affinity sites (CRE-1 or CRE-3) (Refs. 14 and 15 and Fig. 1, b and f). The TBP-proenkephalin interaction was used to examine the proenkephalin region in the presence of a DNA-binding protein that binds in a region other than the CRE.

Structural information is derived from phosphorus mapping (Fig. 2). For each complex, reference electron microscopic images (Fig. 2, left panels) and net phosphorus images (Fig. 2, center panels) are generated. The phosphorus image shows only the DNA within an intact protein-DNA complex. Superposition of the phosphorus (DNA) image on the mass image (Fig. 2, right panels) creates a picture of RNA within the complex and allows measurement of the mass of DNA within the complex. With respect to the CREB-proenkephalin and CREB-prodynorphin complexes, this approach allows direct comparison of the DNA within two different complexes in which the protein component is the same. Binding of CREB to the proenkephalin fragment alters the path of the DNA (Fig. 2, a, b, and c). The DNA appears throughout the complex (Fig. 2, a, b, and c, right panel). Mass analysis indicates that the CREB-proenkephalin complex contains about 60 bp of DNA and 87 kDa of protein (Table I). The protein mass is close to the predicted 80-kDa mass of a CREB dimer (26). Binding of TBP monomers to the same proenkephalin DNA fragment shows the alteration in DNA to be characteristic of the CREB-proenkephalin complex rather than characteristic of the DNA fragment itself (Fig. 2e). TBP monomer induces bending in the DNA fragment at the TATAA box, but the DNA folds neatly along the edge of the protein DNA complex, without the distortion observed in the CREB-proenkephalin DNA complex (Fig. 2e). TBP monomer-proenkephalin DNA complexes contain about 17 bp of DNA (Table I) and about 25.5 kDa of protein (Table I and Ref. 35). TBP binds both as monomer and dimer (not shown), and the degree of bending is proportional to the number of bound TBP molecules.

Physical contact between ATF/CREB factors and TFIID has been reported (36, 37). In preliminary studies of complexes containing both CREB and TBP, we have not observed interaction between the two (data not shown). Thus, interaction of CREB with TFIID would involve a protein other than TBP, as has been suggested (37).

Analysis of CREB binding to the prodynorphin fragment (Fig. 2d and Table I) indicates that the CREB-prodynorphin

FIG. 2. Electron spectroscopic images of protein-DNA complexes. a–c, three independent CREB complexes on the proenkephalin gene fragment. d, CREB complex on the prodynorphin gene fragment. e, TBP complex on the proenkephalin gene fragment. For each complex, reference (mass) images are shown on the left, net phosphorus images in the center, and the superposition of the mass and net phosphorus images are shown on the right. The bar represents 16 nm (a–c), 19 nm (d), and 21 nm (e).

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DNA complex contains an intermediate amount of DNA as compared with CREB-proenkephalin DNA and TBP monomer-proenkephalin DNA complexes (Table I). Most of the DNA in the prodynorphin complex appears to be along the side of the complex (e.g. Fig. 2d, right panel). The protein mass in both prodynorphin and proenkephalin (83 and 87 kDa, respectively; Table I) is consistent with the binding of a CREB dimer (26). However, only about half as much DNA (33 versus 59 bp) is contained in the prodynorphin complexes with CREB dimer as compared with proenkephalin (Table I). Thus, although a CREB dimer binds within each enhancer, the DNA within the complexes is in different conformation in prodynorphin versus proenkephalin.

Role for Each Proenkephalin CRE in Protein Binding—We have previously reported that CREB binds to an alternative CRE, which is formed by intrastrand hydrogen bonding within a synthetic enkephalin enhancer (9). This alternative site is similar to CRE-2, but it contains two GT mismatches (Fig. 3A, GT). Purified CREB protein binds this CRE with higher affinity than the native duplex when analyzed by gel shift (Fig. 3B, compare lanes NAT and GT; Ref. 9). As would be expected, altering CRE-2 by insertion of point mutations within the critical CGTCA region (Fig. 3A, −88C, −89A) reduces CREB binding substantially (Fig. 3B, −88C, −89A). However, altering CRE-1 while leaving the native CRE-2 intact (Fig. 3A, subCRE2) also reduces CREB binding substantially, as indicated by gel shift analysis (Fig. 3B, subCRE2). This CRE-1 mutation consists of substitution of several bases within and upstream of CRE-1 (Fig. 3A); these substitutions eliminate the possibility of formation of a hairpin structure by the top strand in the duplex. They do not, however, alter the sequence of CRE-2 or the bases that immediately surround it. The gel shift analysis thus indicates that within the 23-bp probe used for gel shift analysis, both CREs contribute to CREB-DNA interaction. In order to better understand how such mutations might affect CREB-DNA complexes within large pieces of DNA, we have constructed proenkephalin-CAT fusion plasmids containing the mutations (see “Materials and Methods”). Either mutation reduced expression to about half of the native level (Fig. 3C), consistent with previous studies of deletion of proenkephalin CREs (3). CAT activity was measured after introduction of plasmid DNA into the C6 rat glioma cell line, in which the endogenous proenkephalin gene is expressed (Fig. 3D) (38) and in which phosphorylated CREB protein is present (Fig. 3E).

C. CREB binds both CREs in wild-type proenkephalin gene—We have used a modification of the standard DNase I footprinting technique (39) to analyze CREB-proenkephalin complexes in the identical fusion plasmids described above (Fig. 3C). Rather than using a short fragment, we used as substrate the entire closed, circular plasmid to more closely mimic chromosomal DNA. After CREB protein was allowed to bind, the reactions were treated with DNase I. The DNase I-treated DNA was purified and used as template for extension of a 32P-labeled primer complementary to the sequence about 100 bases from the region of the CREs. CREB protein did, as expected, protect the region of the CREs from DNase I digestion (Fig. 4A, native). As indicated by the sequence, the binding extended over both CREs (Fig. 4A, native). Because the region protected in DNase I digestion generally extends beyond the bases at which protein binds, this merely confirms that CREB binds around the CREs. The footprint could be due to binding at CRE-2 alone or at both CREs. To further characterize the site of binding, we used the mutant plasmids containing mod-

![DNA Structure and Transcriptional Efficiency](http://www.jbc.org/)

**FIG. 3.** Protein binding and expression with mutant CREs. A, sequences of native and mutant proenkephalin duplexes and of GT hairpin. GT hairpin is formed from the top strand of native duplex (9, 18, 19). The numbering is for the human proenkephalin gene (9). The boxes in duplex sequences mark locations of CREs; in the GT hairpin, the boxes indicate the non-Watson-Crick GT base pairs (9, 18, 19), and the horizontal arrows mark the CREs. The vertical arrows indicate altered base pairs. B, gel shift analysis of binding of purified 6-histidine CREB to oligonucleotide probes. Only the top portion of the gel, with the shifted complexes, is shown. SOM, somatostatin; NAT, native proenkephalin. The other labels are as in A. C, fractional acetylation of chloramphenicol for the native and mutant proenkephalin plasmids as determined by transient transfection analysis. Numbers are the average for three dishes. D, Northern analysis of total RNA from C6 and R2C cultured cells. The positions of 28S and 18S RNA were determined by ethidium bromide staining. The arrow indicates the position of 1.4-kilobase proenkephalin mRNA. E, immunoblot analysis of C6 cell extract with anti-phosphorylated-CREB serum. Molecular weight positions were based on migration of pre-stained standards.
ifications in one or the other CRE (Fig. 3A). To determine whether the footprint over CRE-1 was due to independent binding in CRE-1, we analyzed binding to the plasmid with mutations in CRE-2 (Fig. 3A, -88C, -89A). Changing CGTCA of CRE-2 to CACCA virtually eliminates protein binding, as indicated by loss of the footprint (Fig. 4A, -88C, -89A). This suggests that the footprint on CRE-1 is entirely dependent on binding at CRE-2. If CRE-1 is an independent binding site, then binding there becomes likely only after binding at CRE-2.

The mutant plasmid with the seven bases substituted in the region of CRE-1 (Fig. 3A, subCRE1) was used to evaluate the contribution of CRE-1 to CREB binding. The substitutions both change the sequence in the area of CRE-1 and reduce the palindromic nature of the region so that CRE-1 and CRE-2 are not able to form a CREB site of the type formed by the top strand of the native duplex (the GT hairpin) (Fig. 3A). This substitution altered the pattern of the footprint as compared with the wild type (compare Fig. 4A, subCRE1 and native). There is clearly still protection of CRE-2, indicating that CREB still binds, but the protected region does not extend upstream toward CRE-1. Whereas in the native gene the region of CRE-1 is protected from DNase I digestion (along upper rectangle, Fig. 4A, native), in the plasmid without CRE-1 there is no protection of CRE-1 (along upper rectangle, Fig. 4A, subCRE1). In fact there is not protection even of the several bases 3' to CRE-1, that is, between the CREs. As discussed above, the core sequence at CRE-2 is identical to that of the high affinity CRE located at -1541 in the rat prodynorphin gene (14, 15). DNase I analysis

**FIG. 4.** DNase I analysis of CREB-CRE complexes formed on supercoiled CAT fusion plasmids. Footprints of top strand (see also Fig. 3A) are shown. A, analysis of native and mutant proenkephalin CRE-CREB complexes. The native sequence of the region of CREs is shown at left. Sequence markers were dideoxy chain termination reactions (not shown) as described under "Materials and Methods." The arrows indicate bases mutated in one of the mutant plasmids, with specific mutations as indicated in Fig. 3A. The rectangles in the sequence enclose CRE-1 (top) and CRE-2. The positions of the CREs in the gel are shown by the two rectangles along the side. Filled circles alongside images of the gels indicate the positions of the mutated bases. For each set, the leftmost lane is 0 CREB control, with increasing concentration of protein as described under "Materials and Methods." B, DNase I analysis of native prodynorphin CRE-3 in plasmid pCAT2.0dyn (14, 15). CRE-3 contains 8-bp sequence (CTGCATCA) like that of proenkephalin CRE-2. The positions of CRE within sequence of region and on gel are indicated by rectangles.

**FIG. 5.** Nuclease P1 analysis of native proenkephalin-CREB complexes. Top and bottom strands are as shown in Fig. 3A. For each gel, the leftmost lane is 0 CREB control, with increasing CREB as described under "Materials and Methods." Sequence markers were dideoxy chain termination reactions (not shown) as described under "Materials and Methods." Positions of the CREs are shown by the two rectangles along the side.
of that prodynorphin CRE in plasmid pCAT2.0dyn (14) yields a footprint that appears the same as that at CRE-2 in the sub-
CRE1, proenkephalin plasmid (Fig. 4, compare A, sub- CRE1, with B), indicating that the 8-bp sequence they share is sufficient to produce the footprint. In the native proenkephalin gene, how-
ever, the binding site comprises CRE-1 and CRE-2. Thus, al-
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is in different conformation from the DNA in the prodynorphin
and sub-CRE1 complexes. In the native proenkephalin gene
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CREB-induced Sensitivity to Nuclease P1—A protein-in-
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in DNA and is particularly useful in identifying single-
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the effect of CREB on DNA structure in the region of the CREs.
We find that although in the area analyzed there are sites of a
wide range of sensitivity to the enzyme, indicating local struc-
tural and sequence effects, no site on the top strand appears
altered by the presence of CREB (Fig. 5, top). The access of the
enzymatic probe may be limited by bound CREB. On the bot-
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presence of added CREB (Fig. 5, bottom). Approximately 15 bp
from the region of the CREs (Fig. 5, –68) there is a site that is
cut extensively when CREB is present but only weakly when
CREB is absent (Fig. 5, bottom). Throughout the CREs there is a
CREB concentration-dependent increase in sensitivity to nuc-
lease P1, as shown by the darkened bands adjacent to both
CREs (indicated by the rectangles) in the lanes with added
CREB (Fig. 5, bottom). Thus, the formation of the CREB-CRE
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Occupation of Both CREs during Transcription in Vivo—
Binding sites for sequence-specific DNA-binding proteins can
be visualized by in vivo DMS footprinting (41). Comparison of
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which the gene is expressed, and in R2C cells, in which it is not,
indicates that there is structural alteration during transcrip-
tion (Fig. 6). Cells were treated with DMS, which methylates
N-7 of guanine (32); the DNA was purified and then cleaved in
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CREs and flanking sequence were examined for reactivity in
living cells as compared with naked DNA. Dried gels were
exposed to storage phosphor screens. The quantified images for
each lane were smoothed and normalized to correct for differ-
ences in background and signal strength (see "Materials and
Methods"). For both top and bottom strands, normalized curves

![Image](http://www.jbc.org/)
for naked DNA from C6 cells, naked DNA from R2C cells, and in vivo methylated DNA from R2C cells were essentially superimposable (not shown). In contrast, curves from cells expressing proenkephalin (Fig. 6, C6 in vivo and C6 + forsk) deviated from naked DNA at several peaks. Similarly, inspection of the images of the gels reveals several bands that differ in the lanes from expressing cells as compared with lanes from nonexpressing cells or from naked DNA (Fig. 6). In the top strand, altered sensitivity is seen at five bases, four of which are in the CREs (Fig. 6, top gel). In each CRE, one G is protected from DMS modification (−97 in CRE-1 and −86 in CRE-2), indicating occupation by a DNA-binding protein; and one G two residues from the protected G (−99 in CRE-1 and −88 in CRE-2) is hypersensitive to DMS (Fig. 6, top gel). Such enhanced reactivity may be due to a hydrophobic pocket in the binding protein, which causes an increase in local DMS concentration (33).

In the bottom strand, no residue is protected but several show enhanced reactivity (Fig. 6, bottom gel). Only one G in the immediate vicinity of the CREs, that at −102, shows altered sensitivity. Gs at −106 and −73 are also more reactive in cells expressing proenkephalin (ratio of about 1:3.1 at −106 and about 1:8.1 at −73 for both C6 in vivo and C6 + forsk when compared with C6 naked). Within each CRE, however, there is an unusual A band only in the DNA from proenkephalin-expressing cells (C6 in vivo and C6 + forsk) (Fig. 6; −96 in CRE-1 and −85 in CRE-2). There is also a hypersensitive A at −112 that is specific to the cells expressing proenkephalin (Fig. 6). In C6 cells expressing the proenkephalin gene, these A bands are of approximately equal strength to the weakest of the Gs (see G at −84 and G at −111, Fig. 6, bottom gel). The environment of individual G residues, due to sequence and stacking effects, leads to varied sensitivity of Gs in the essentially G-specific DMS hot piperidine protocol (32) (e.g. G ladders from naked DNA, Fig. 6). The mechanism for the appearance of the A bands is not clear, but change in conformation and/or charge distribution can cause unusual methylation on A. Hot piperidine does not cleave N1- or N3-methylated As (32, 42), but N-7-methyl A can be cleaved by the same mechanism as N-7-methyl G residues (42). It is possible that change in charge distribution, perhaps due to AC base pairing (18), causes some methylation at N-7 of As. In the synthetic proenkephalin enhancer, such AC base pairs are formed (18, 19). The appearance of the three A bands on the bottom strand indicates that their environment is specifically altered in the vicinity of the CREs; within each CRE, one A residue is hypersensitive.

The pattern of modification of G and A residues within the two CREs is the same (Fig. 6, diagram of binding to duplex), showing that each CRE is similarly occupied during transcription but that neither is occupied in R2C cells not expressing the gene. There are no protected residues along the bottom strand, but the enhanced signals indicate structural alteration. Representation of the 23-bp region containing the CREs as hairpins (9, 18, 19, 43) suggests a possible mechanism for the pattern of DMS sensitivity: in the top strand hairpin, the residues that are similarly modified (hypersensitive at −88 and −99; protected at −86 and −97) appear as equivalent to each other in a symmetrical site (Fig. 6, top strand hairpin). Within the CREs, the normally strong A signals appear at sites of AC base pairs (Fig. 6, bottom strand hairpin). The A bands, as well as hypersensitivity of Gs, is consistent with structural change, perhaps including unusual base pairing.

**Discussion**

The expression and binding data we have presented support the important observations of others (3, 4) that although CRE-2 can function independently as a binding site, it cannot by itself support efficient transcription. Studies on a series of mutant CREs emphasized that CRE-1 is critical in transcription (4). Data presented in this manuscript show CRE-1 to be part of the binding site for a well characterized CRE-binding protein, and in vivo analysis indicates that specific occupation of both CREs correlates with transcriptional activity (Fig. 6).

Although DNase I footprinting shows that CRE-1 does bind CREB protein, binding there is dependent on binding at CRE-2. The point mutations that altered only CRE-2 eliminated binding in both CREs (Fig. 4A, −88C, −89A). Two mechanisms could account for the DNase I footprinting results. The CREs could be separate sites, each of which binds a CREB dimer. In this case, CRE-1 would be a separate but very poor binding site for CREB protein; bound protein at CRE-2 would cause a cooperative interaction, facilitating binding at CRE-1. Alternatively, together the CREs form a single binding site occupied by a single dimer. The electron spectroscopic imaging studies support a single dimer’s use of both CREs (Table I); furthermore, the absence of binding at CRE-1, even at very high concentrations of CREB in the −88C, −89A mutant (Fig. 4A), argues against CRE-1 functioning as a separate CREB site. Therefore, the short region contains two different sites capable of binding factors. CRE-2 can bind CREB, but in the native gene CREB prefers a site composed of both CREs.

What then is the nature of the binding site? One possibility is that bending accommodates a single dimer’s use of sequences in both CREs, that is, each CRE would form a half-site. Because affinity for CRE-1 by itself is so low (Fig. 4A, −88C, −89A), this model would predict that a transcription factor scanning the DNA would recognize the CTGCGTCA at CRE-2, a competent binding site (Fig. 4A, subCRE2, and Fig. 4B), bind there, and then adopt as part of its site the neighboring CRE-1. The mechanism by which the protein would straddle the region to select the low affinity CRE-1 as part of its site is unclear. Protein-induced bending might provide energy for adjustment from binding at CRE-2 alone to using each CRE as a half-site. However, if this model is correct, CRE-1 is auxiliary, a premise that is not supported by studies with mutant enhancers. Analysis of point mutations throughout the region shows that mutations in either CRE can have similar effects on transcription (4). In vivo footprinting supports the functional equivalence of the CREs, because the pattern of modification in the transcribed gene is the same in both CREs (Fig. 6). Such a pattern is unlikely for two sites of very different affinities placed side by side. Furthermore, the very low affinity for CRE-1 by itself argues against the protein adjusting its binding to use that site. Thus, the data are not consistent with this model.

A second model proposes that a structural change creates a site different from either CRE. We have previously demonstrated that the enhancer region adopts an alternative structure that can contribute to transcriptional regulation (9, 18, 19, 42). This structure creates a unique site that uses CRE-1 and CRE-2 from one strand and that includes two GT base pairs (Refs. 9, 18, 19 and Fig. 3A). The alternative site has higher affinity for CREB than does CRE-2 (Ref. 9 and Fig. 3), and in vitro analysis has demonstrated that increased affinity is dependent on a T in one of the GT base pairs (9). The higher affinity for this site suggests that a transcription factor scanning the DNA could bind there and stabilize the structure. Because formation of the alternative site depends on sequences in both CREs, point mutations in either CRE would be as likely to affect transcription, as has been shown (4). The experiments described here support this model. P1 nucleosome sensitivity of the enhancer region is CREB concentration-dependent, indicating protein-stabilized structural change, and CREB-CRE
complexes increase nuclease P1 sensitivity of the enhancer region in a strand-specific manner, suggesting differential occupation of the strands. In vivo footprinting shows not only transcription-dependent occupation of both CREs but also shows that the pattern of base modification is the same within both CREs (Fig. 6, sequence diagrams). The similarity in protection and enhancement is striking: the same bases are altered to the same extent. A shared site would depend equally on the CREs, so the protection and enhancement are consistent with protein binding to a site made up of CRE-1 and CRE-2 (Fig. 6, top strand hairpin). In addition, there are specifically enhanced A bands on the bottom strand. A structural basis for this unusual methylation could be the conformational change in the CRE region; the hypersensitive A in each CRE corresponds to a mismatched AC base pair, the environment of which alters charge distribution (18) (Fig. 6, bottom strand hairpin).

The data presented here provide the physical basis for the previously observed essential role of CRE-1 in regulating proenkephalin transcription; CRE-1 acts in concert with CRE-2 to create the transcription factor binding site. The DNA must accommodate a secondary structure that accommodates a single binding site made up of sequences from CRE-1 and CRE-2, so that they are equivalent contributors to CREB binding. Although the conformational details of the structure require further study, we have shown that both CREs have similar and specific roles as factor binding sites during transcription. The presence of alternative sites within the short region could contribute in roles as factor binding sites during transcription. The existence of three sites (CRE-1, CRE-2, and the site made up of both) allows for flexibility in binding to factors of differing affinities and sequence specificities.

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