Switching of DNA Secondary Structure in Proenkephalin Transcriptional Regulation*

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Craig Spiro and Cynthia T. McMurray‡
From the Department of Pharmacology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Proper transcriptional regulation of the proenkephalin gene requires a switch between distinct factor binding sites that cannot exist at the same time. Each of the sites is formed from a nearly palindromic region that contains two functionally defined cAMP response elements. The region can switch between cruciform and linear duplex. Formation of the cruciform creates an alternative binding site for mediators of second messenger-directed transcription and abolishes the site present in the native duplex form. Use of the cruciform site has been shown to correlate with activated transcription. Analysis of DNA structure, protein binding, and gene expression from plasmids with mutant enhancers shows, however, that both sites are required for regulation of transcription. The two distinct structures form within the same enhancer. Shifting the balance between the two alters transcriptional response.

Transcription of the proenkephalin gene, which encodes the precursor to endogenous opiate-receptor ligands, is regulated by signals from growth factors, neurotransmitters, hormones, and cell depolarization. The pathways converge on an evolutionarily conserved, nearly palindromic region that contains two functionally defined cAMP response elements (CREs)¹ (1, 2). Only CRE-2 can independently bind transcription factors. However, point mutations in either CRE have quantitatively similar effects on transcription (2), and the response to CRE-binding proteins depends on the presence of CRE-1 as well as CRE-2 (3). Because the 23-base pair region containing the CREs is nearly palindromic, it can convert to a cruciform structure in which a transcription factor-binding site is formed from the sequences of both CREs on the same strand (3–5; Fig. 1a). The switch to the alternative site eliminates the native CRE-2, substituting a site with the same sequence but with two mismatched GT base pairs (3–5). The structural change, therefore, creates a single site comprising both CRE-1 and CRE-2 (Fig. 1a).

Use of the site made from both CREs correlates with stimulated transcription, according to several types of analysis. In vivo, the site made from CRE-1 and CRE-2, but not the native duplex CRE-2 site, is occupied specifically during transcription (6). Dimethyl sulfate treatment of cells actively transcribing proenkephalin specifically modifies adenines that form non-Watson-Crick AC base pairs in the cruciform structure, indicating unusual charge distribution at those bases only during active transcription (6). Electron spectroscopic images show that a CREB dimer occupies twice as much DNA in the proenkephalin gene as in the prodynorphin gene, consistent with binding to a cruciform in the proenkephalin gene (6). The prodynorphin CRE is identical in sequence to proenkephalin CRE-2 but is in a region unable to form alternative structure (6). Finally, point mutations that do not alter transcription factor binding but that could modify hairpin stability modulate response to receptor signaling (2, 4).

Within the proenkephalin gene, therefore, CRE-1 and CRE-2 form a single binding site that is essential for transcriptional response to extracellular signals. The native CRE-2 is a relatively low-affinity binding site for CREB (3, 7), but GT base pairs in the stem of the hairpin made from CRE-1 and CRE-2 enhance CREB binding (3). The higher affinity site on the hairpin arm could correlate with high activity transcription because it is better able to recruit enhancer-binding proteins.

To understand the mechanism of proenkephalin enhancer action and to test the hypothesis that enhanced protein affinity for the altered site allows more efficient transcription, we have analyzed gene expression, protein binding, and DNA structure of reporter plasmids with point mutations in the region of the CREs. Binding to the CREs is necessary for transcriptional response, but our analysis demonstrates that efficiency of binding of CREB alone does not correlate with transcription. CREB preference for the alternative site cannot by itself explain the enhanced activity of the hairpin versus linear duplex binding site. The relative availability of the alternative sites does, however, affect transcription. Mutants in which strong binding to the linear duplex CRE-2 is favored, relative to the native gene, are less responsive to cAMP. Mutants with greater preference for hairpin rather than linear duplex CRE-2 site are more responsive to cAMP. Thus, proper regulation of transcription mediated by the native enhancer requires switching between both sites.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Cell Culture, Expression Analysis—Construction of mutant proenkephalin enhancer region-CAT reporter plasmids by overlap polymerase chain reaction (11) has been described previously (6). The mutations are described under “Results” (see Fig. 1b). SK-N-MC neuroblastoma cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Proenkephalin-CAT reporter plasmids were introduced by DEAE-dextran-mediated transformation (250 µg/ml DEAE dextran + DNA for 2–2.5 h followed by 10% dimethyl sulfoxide for 2 min). Forskolin (20 µM final) or dimethyl sulfoxide carrier (“control”) was added 15 h after 10% dimethyl sulfoxide treatment. Cells (10 cm dish) were harvested 24 h after forskolin treatment into 75 ml of enzyme extract; 15 µl was used for the CAT assay (6, 12). Phosphor storage screen was exposed to dried chromatograms and then scanned by PhosphorImager 425 (Molecular Dynamics). Acetylated and unacetylated spots were quantified from digital images by IP Lab Gel (Signal Analytics), using supplied macros (“profile plot,” “auto peak find,” and...
"analyze peaks"). Percent acetylation and standard deviation of replicate plates were calculated in Excel (Microsoft), and data were plotted using KaleidaGraph (Abelbeck).

**DNase I Analysis**—Bacterially expressed CREB protein was expressed and purified as described previously (6). Protein-DNA complexes formed on the same plasmids used for expression studies (overnight at 4 °C) were analyzed by DNase I digestion and primer extension as described previously (6). After separation of fragments on 8% sequencing gels, the gels were dried for exposure of phosphor storage screen. ImageQuant software was used to convert the data from the phosphor storage screen to images of gels and to numbers used to quantify band strength. Scale of scanned images was adjusted to reduce variation in signal between lanes. Comparison of bands was as described previously (6); equivalent regions of each lane were selected and quantified (ImageQuant); data were exported to KaleidaGraph; curves were normalized to correct for uneven loading; and relative intensities of individual bands were estimated by peak-to-valley measurement.

**Electrophoretic Mobility Shift Assay**—Oligonucleotide probes are described under “Results” (Fig. 5). Crude extract from bacteria expressing recombinant CREB protein (6) was incubated for 15 min at room temperature in the presence of 1.5 µg of sonicated salmon sperm DNA and 0.1 pmol of 32P-end-labeled oligonucleotide probe. Complexes were analyzed on 5%, 0.5 Tris glycine gel as described previously (3, 6).

### RESULTS

**Design and Construction of Mutants for Analysis of Proenkephalin Enhancer Region**—The ability of specific mutations to either enhance or reduce transcription contributed to an understanding of the effect of secondary structure in proenkephalin gene expression (4). Certain mutations eliminate protein binding, which can explain their deleterious effect on transcription (e.g., enk−88C,−89A; Refs. 3 and 6 and Figs. 1b and 3a). In contrast, other mutations do not abolish binding yet have a significant effect on transcription (e.g., enk subCRE1, Refs. 3, 4,
and 6; and Figs. 1b and 3a). To understand the relationships among binding efficiency, structure, and gene expression, we have produced and analyzed mutant proenkephalin-CAT reporter plasmids (Fig. 1b). Binding and structural analyses were carried out on intact plasmids, identical to those used for transcriptional analysis. The plasmids analyzed included the previously described mutants lacking a functional CRE-2 (enk $-88C,-89A$) or a functional CRE-1 (enk sub CRE1) as well as mutants with single or two-base changes designed to alter the cruciform. Because CRE-2 is essential for binding to both the linear duplex and the hairpin site (6), modification of CRE-2 was avoided in this latter group of mutant plasmids. These mutations have been chosen for their ability to alter stability of the cruciform.

The mutants designated enk $-98T$ and enk $-105G,-106A$ have a less stable cruciform structure because of reduced hydrogen bonding in the stem (Fig. 1b; Refs. 4, 5, 17, and 18). The mutants designated enk $-91A$ and enk $-102A$ and the double mutant enk $-91A,-102A$ have either one or both of the mismatched base pairs in the stem corrected and, consequently, a more stable stem-loop structure (Fig. 1b). Alterations in the loop have been shown previously to modify hairpin stability (18). Substitution of an A or T for the G at position $-95$ creates a more stable hairpin, as determined by thermal melting analysis (17, 18). Reporter plasmids with the desired mutations were constructed for use in the analyses (Fig. 1b; and see “Experimental Procedures”).

Transcriptional Response of Mutant CREs—Transcription was analyzed by transient transfection of proenkephalin-CAT reporter plasmids into the SK-N-MC human neuroblastoma cell line, in which the endogenous proenkephalin gene is expressed. CAT activity of mutant plasmids was compared with that of the native plasmid (Fig. 2). Mutations that eliminate either CRE-1 (enk sub CRE1) or CRE-2 (enk $-88C,-89A$) reduce transcriptional efficiency (Fig. 2), as has been shown previously (6). The strongest inhibition of transcriptional response is caused by the mutation that alters the conserved CGTCA in CRE-2 (Fig. 2, enk $-88C,-89A$), and, thereby, eliminates binding to the enhancer region (Fig. 3a; Ref. 6). Modification of CRE-1 (enk sub CRE1) does not alter CRE-2, but it does destroy the palindromic character of the region, precluding formation of a cruciform (Refs. 3 and 6; and Fig. 1). Two changes (enk $-98T$ and enk $-105G,-106A$) cause reduced transcriptional response very much like that caused by the mutations that abolish CRE-1 in the mutant enk sub CRE1 (Fig. 2). Both modifications (in enk $-98T$ and in enk $-105G,-106A$) destabilize hairpins. Enk $-98T$ has a single-base change in CRE-1, which introduces another mismatched base pair into each stem (Fig. 1b), and enk $-105G,-106A$ has two mutations which substitute mismatched base pairs at the base and reduce the stem to 8-base pairs (Fig. 1b). Neither of these plasmids has a mutation in or close to CRE-2 in the linear duplex form (Fig. 1b, left side), and one of them (enk $-105G,-106A$) modifies neither CRE at all, yet they both show an effect similar to the complete elimination of CRE-1 (Fig. 2).

Mutations that correct mismatched base pairs in the stem, by substituting an A for the G at either $-91$ or $-102$ or at both, stabilize the cruciform (Ref. 4; Fig. 1b). Either or both of these changes enhance transcriptional response (Fig. 2). We do note, however, that each of these mismatch-correcting mutations complicates direct comparison with the native. The mutation at $-102$ creates within CRE-1 the conserved CTGTA of CREs, so that transcriptional response could be due in part to the creation of a different binding site. The mutation at $-91$ modifies CRE-2 so that the binding site is not identical to the CRE-2 in the native. Because of these ambiguities, we do not consider these mutant plasmids in our structural analysis (see below).

The final two mutant plasmids were altered at base $-95$, which is between the two CREs in the linear duplex and forms the middle base in the loop (Fig. 1b). Changes at the middle position of the loop (insertion of GT base pairs into a more stable cruciform structure, even at the expense of eliminating the mismatched base pairs, can also enhance receptor responses, while destabilization correlates with reduced response.

Binding of CREB Protein to Mutant Enhancer Regions—In short synthetic enhancers, insertion of GT base pairs into a duplex site increases CREB protein binding, indicating that GT base pairs enhance binding when the structure is otherwise held constant (3). The cruciform site, which has mismatched GT base pairs (Fig. 1a; Ref. 5), can, therefore, have an advantage over the linear duplex site in recruiting transcription factors such as CREB, which mediates proenkephalin expression in vivo via the CREs (8–10). Conversely, stabilizing the structure, even at the expense of eliminating the mismatched base pairs, can also enhance receptor response (Fig. 2, enk $-91A$ and/or enk $-102A$).

CREB binds to the enhancer in the plasmid with the substituted CRE-1 even at the lowest concentration of protein (Figs. 1b and 3a, enk sub CRE1; and Ref. 6). The enk sub CRE1 enhancer, however, cannot support efficient transcription (Fig. 2; Ref. 6), and the response to CREB itself is abrogated in plasmids lacking a functional CRE-1 (3). In plasmid enk sub CRE1, protection is limited to the region around CRE-2; it does not extend into CRE-1 (Fig. 3a). The plasmid designed to abolish CRE-2 (enk $-88C,-89A$) shows little protection even at the highest concentration of protein (Fig. 3a). Thus CRE-2 alone is sufficient for binding and is necessary for CREB binding to either CRE. Binding, however, does not correlate with efficient transcription. Other mutants, which differ less from the native, are likely to be more informative as to the importance of individual bases in the action of the CREs. Thus, we have analyzed for binding those plasmids with least disruption of the CREs.

Mutations in four plasmids (enk $-98T$, enk $-105G,-106A$, enk $-95T$, and enk $-95A$) do not modify CRE-2 and do not create possible new CREB-binding sites in CRE-1. Two of these four plasmids show diminished response (Fig. 2, enk $-98T$ and enk $-105G,-106A$), while two of them are more responsive transcriptionally (Fig. 2, enk $-95T$ and enk $-95A$). Only one of
this group (enk −98T) has a change in either of the CREs (Fig. 1b). The plasmids were incubated with CREB protein over a 30-fold range and then analyzed for protection from DNase I digestion. Binding was detected at all of the enhancers, but the depth and shape of the protected area along the top strand differed among these mutant plasmids and in comparison to the native (Fig. 3b). There was no greater protection at low levels of CREB in those mutants that were more active transcriptionally (enk −95T and enk −95A) as compared with those that were less active (enk −98T and enk −105G, −106A). On the contrary, at the lowest level of protein (second lane from left in each set), enk −105G, −106A showed greater protection around CRE-2 than the native, enk −95T or enk −95A (lower rectangles along the gels in Fig. 3b). The protected area in mutant plasmid enk subCRE1 (Fig. 3a) is a useful reference for protection of CRE-2, since enk subCRE1 lacks CRE-1 and cannot form a stem-loop structure (Fig. 1b). Thus the area from −78 through −95 can be protected along the top strand by binding to CRE-2 (Fig. 3a). The footprint in plasmid enk −98T is similar to that in plasmid enk subCRE1. That is, there is protection of CRE-2 but little within CRE-1 (upper rectangle along enk −98T in Fig. 3b), even at the highest levels of protein. In mutant plasmid enk −105G, −106A, which has a diminished response to forskolin as compared with native, there is a footprint over CRE-1 (along upper rectangles in Fig. 3b). In summary, more efficient binding of CREB protein alone does not correlate with stronger transcriptional response.

Analysis of the Structure of the Mutants—Switching between structures cannot be detected in the absence of protein (Fig. 3b, 0 protein lane). Because CREB can bind to the site in the cruciform enhancer and protect from DNase I digestion, it is a probe for the cruciform. CREB binds to the linear duplex site as well, but the pattern of protection differs between the two sites (Fig. 4). Binding to the linear duplex site should protect bases only in CRE-2 on both strands (Fig. 4, left side); binding to the cruciform should protect bases within both CRE-1 and CRE-2 but only on the top strand (Fig. 4, right side). Switching creates one site as it eliminates the other (Fig. 1a, Fig. 4, top). We have designed mutations to alter the stability of the cruciform, but the mutations have little to no influence on duplex stability. A
CRE-2 is the binding site for CREB protein, while in the cruciform, CREB binds to a site made of both CREs along the top strand (3, 6). Arrows along CREs show the 5′ to 3′ direction (solid = CRE-1; hatched = CRE-2); hatched box represents CAT coding region. For analysis, plasmid-CREB binding reactions are treated with DNase I, and the plasmid DNA is purified. Protection is determined by primer extension through the region of the CREs with visualization on gels. Model images for the gels are shown below the cartoons of plasmid-protein complexes and depict a pattern of protection for a pure population. Actual binding reactions can contain some molecules with linear duplex enhancer and others with cruciform enhancer (see text). The simulated gel pattern shows expected protection within the CREs for each type of complex. Note that in the diagram the bases are evenly spaced; in contrast, in the gel, spacing between bases at the top (area of CRE-1 for top strand; CRE-2 for bottom strand) is compressed.

**FIG. 4. Model of expected protection from DNase I digestion of native proenkephalin-CAT plasmid in DNA-CREB complexes**

The region containing CRE-1 and CRE-2 is nearly palindromic and can form stable cruciform (right side). In the native, linear duplex (left side) CRE-2 is the binding site for CREB protein, while in the cruciform, CREB binds to a site made of both CREs along the top strand (3, 6). Arrows along CREs show the 5′ to 3′ direction (solid = CRE-1; hatched = CRE-2); hatched box represents CAT coding region. For analysis, plasmid-CREB binding reactions are treated with DNase I, and the plasmid DNA is purified. Protection is determined by primer extension through the region of the CREs with visualization on gels. Model images for the gels are shown below the cartoons of plasmid-protein complexes and depict a pattern of protection for a pure population. Actual binding reactions can contain some molecules with linear duplex enhancer and others with cruciform enhancer (see text). The simulated gel pattern shows expected protection within the CREs for each type of complex. Note that in the diagram the bases are evenly spaced; in contrast, in the gel, spacing between bases at the top (area of CRE-1 for top strand; CRE-2 for bottom strand) is compressed.

single binding reaction might, therefore, contain some molecules with linear duplex enhancer and others with cruciform enhancer. Because protection in CRE-1 results only from binding to the cruciform site, increasing protection there indicates switching to the cruciform (Fig. 4, predicted patterns of protection).

The pattern of protection in the mutant enk sub<sub>C</sub>B<sub>1</sub> (Fig. 3a) can serve as a reference. The removal of the palindromic character of the region makes formation of the cruciform site impossible, but CRE-2 is intact (Fig. 1b). Protection from DNase I is limited to CRE-2 (Fig. 3a). In contrast, the native and the mutants with single or two base changes show some protection in CRE-1, indicating that there is switching to the alternative site (Fig. 3b).

Two of the plasmids (enk <−98T and enk <−105G, −106A) contain cruciform destabilizing mutations; two (enk <−935T and enk <−95A) contain loop mutations that stabilize cruciform. All four mutant plasmids and the native share an identical CRE-2 with the enk sub<sub>C</sub>B<sub>1</sub> mutant (Fig. 1b). Consequently, they show protection of CRE-2 but differ in protection at CRE-1 (Fig. 3). Differences in relative amount of protection in CRE-1 and CRE-2 can indicate changes in the balance of availability of the sites.

In the native plasmid, there is protection throughout CRE-1 (native, Fig. 3b). At the lowest concentration of protein, however, CRE-1 is not protected, but CRE-2 is (Fig. 3b). This is
consistent with the prediction that CRE-2 would be protected on the top strand in both linear duplex and cruciform (that is, all molecules) (Fig. 4, left side). CRE-1, in contrast, would be protected only in those molecules in which CREB protein stabilized the alternative (cruciform) binding site (Fig. 4, right side).

Direct comparison of the native with each of the mutants shows that mutations affect protection of CRE-2 and CRE-1. Plasmid enk −98T shows little protection in CRE-1 as compared with the other plasmids (Fig. 3b), but at 1 μl (lowest amount of added protein) protection of CRE-2 is similar to the native (Fig. 3b; see also Table I). Thus, this mutation, which adds another mismatched base pair near the hairpin loop (Fig. 1b), shifts the balance of protection toward the linear duplex site as compared with native. The plasmid enk −105G, −106A is modified in neither CRE, so that both linear duplex and hairpin binding sites are identical to the native (Fig. 1b). Base changes upstream from CRE-1 (Fig. 1b, left side) destabilize hairpin structure by shortening the stem (Fig. 1b, right side). There is in enk −105G, −106A protection in CRE-1, so that a cruciform site is present. At the lowest concentration of protein there is greater protection throughout CRE-2, as compared with the native.

Both mutations at −95 (in plasmids enk −95T and enk −95A) enhance the transcriptional response (Fig. 2) and within synthetic enhancers can increase the stability of hairpin structures (17, 18). Plasmids with each of these mutations show protection within CRE-2 that is similar to the native (compare 1 μl, Fig. 3b, second lane, and Table I) but show greater protection in CRE-1 (which is protected only when protein is bound to the cruciform site) than the native (Fig. 3b, Table I, 7.5 μl). Thus, within the plasmids, the single change at −95 increases the availability of the hairpin binding site and increases the response to cAMP. This is consistent with previous observations that availability of the cruciform site correlates with enhanced expression (3, 4, 6).

We considered the possibility that the loop mutants (enk −95T and enk −95A) might show different pattern of protection due to use of a different site. However, for these mutants, as for the native, binding depends on the presence of a functional CRE-2 (Fig. 5). For gel shift analysis, probes were synthesized as 74-base oligos containing complementary proenkephalin sequence (−78 to −112) connected by a 4-T loop (Fig. 5a). One oligonucleotide contained the mutations that abolish CRE-1 to force formation of a linear duplex (CRE-2-only) probe (enk subCRE1, Fig. 1b, Fig. 5a). One probe contained an insert of (TA)11T in the portion complementary to the 23-base pair palindrome to force hairpin formation and permit only the alternative binding site (forced hairpin, Fig. 5a). Binding to four of the probes is similar, but elimination of CRE-2 abolishes binding (Fig. 5b, enk −95A/−88C, −89A), consistent with what is seen in enk −88C, −89A plasmid (Fig. 3a).

Protection in CRE-1 on the top strand indicates presence of the cruciform enhancer (Fig. 4, right side), and change in shape of the top strand footprint is direct evidence that the mutations affect enhancer structure (Fig. 3b). Protection along the bottom strand provides additional evidence about structural switching. Bottom strand protection (Fig. 6) is a result of CREB's binding to molecules with the linear duplex enhancer (Fig. 4, left side). Protection of the bottom strand is limited to CRE-2 in native as well as in hypersensitive (enk −98T) or hyperresponsive (enk −95A) mutants (Fig. 6). This is consistent with bottom strand protection resulting only from binding to the linear duplex site (Fig. 4, left side) and with the formation of the cruciform site from sequences on the top strand only (Fig. 4, right side). Stabilizing cruciform (enk −95A) or destabilizing cruciform (enk −98T) changes the shape of the top strand but not of the bottom strand footprint.

Thus, in these plasmids, two distinct, double-stranded binding sites can be detected, one in the linear duplex and the other within a cruciform. Changing the relative availability of the sites by mutation modifies transcriptional response.

**DISCUSSION**

In the brain, transcriptional control of the proenkephalin gene depends on CREB interaction with CRE-1 and CRE-2...
of protein (Figs. 2 and 3b, Table I). Although the hyporesponsive plasmids (enk −98T and enk −105G, −106A) can respond to forskolin, their response is reduced compared with the native (Fig. 2). Both sites, with bound proteins, may enhance transcription in response to second messengers but at different efficiencies.

In vivo analysis of the actively transcribed proenkephalin gene has shown that only the cruciform site is detected during active transcription in C6 glioma cells (6). Thus factors other than CREB protein must be available to provide energy and stabilize the hairpin site, so that hairpin formation is greatly favored over linear duplex (Figs. 1a and 4, right side would be favored). Affinity and gel shift analysis of nuclear extracts show that hairpin probes bind sequence and structure-specific factors not bound by linear duplex probes. The loop mutations described above (enk −95T and enk −95A) allow a DNA-directed shift toward availability of the hairpin site, and these mutations greatly enhance the receptor response (Fig. 2). The effect of the loop mutations indicates that both sites must be used for proper regulation of transcription.

CRE-1 alone cannot independently bind transcription factors, and even several copies of CRE-1 cannot respond to receptor signals (23), but CRE-1 is an essential part of the high activity binding site created by the alternative structure. Furthermore, response to CREB depends on the presence of CRE-1 (3). We have previously hypothesized (3–6) that occupancy and activity of the hairpin site was due at least in part to its higher affinity for the transcription factor CREB. The structure may, however, affect transcription in other ways as well.

We have demonstrated that by mutation the availability of the sites and the transcriptional response can be altered. Binding of CREB protein by itself does not account for the properties of the two sites within the palindromic region. The DNA may play an allosteric role: orientation of bound transcription factors would be different at the two sites. Polarity has previously been shown to alter selection of co-factors and, as a consequence, the direction of transcriptional response mediated by nuclear receptors (24). In addition, the cruciform may create new binding sites. Binding of factors to these sites could stabilize the cruciform and/or contribute to recruiting and stabilizing transcriptional machinery. Footprint analysis with nuclear extracts indicates that the pattern of protection distant from the CRE is modified by mutation or competitors for binding at the CREs. This is consistent with the structure of the binding sites playing a critical role in assembly of the transcriptional complex. The data presented here demonstrate that DNA structure plays a regulatory role in gene expression. Two distinct sites are formed by structural switching within the same 23-base pair region, and both must be used for proper transcriptional regulation of the proenkephalin gene.

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