Expression of tyrosine hydroxylase (TH) is limited to catecholamine-producing neurons and neuroendocrine cells in a cell type-specific manner and is inducible by the cAMP-regulated signaling pathway. Previous results indicated that the cAMP response element (CRE) residing at −45 to −38 base pairs upstream of the transcription initiation site is essential for both basal and cAMP-inducible promoter activity of the 2.4-kilobase or shorter upstream sequence of the TH gene (Kim, K. S., Lee, M. K., Carroll, J., and Joh, T. H. (1993) J. Biol. Chem. 268, 15689–15695; Lazaroff, M., Patankar, S., Yoon, S. O., and Chikaraishi, D. M. (1995) J. Biol. Chem. 270, 21579–21589). Here, we further report that the CRE is critical for the promoter activity of the 5.6- or 9.0-kilobase upstream sequences of the rat TH gene, which had been shown to direct the cell-specific TH expression in vitro. To define the structure/function relationship of the CRE in transcriptional activation of the TH gene, we performed saturated mutational analyses of 12 nucleotides encompassing the CRE. Mutation of any nucleotide within the octamer motif results in a significant decrease of both basal and cAMP-inducible transcriptional activity of the TH reporter gene construct. Among the four nucleotides adjacent to the CRE (two 5′ and two 3′), only the G residue at the immediate 3′ position is important for full transcriptional activity. DNase I footprint analysis indicates a positive correlation between in vivo promoter activity and in vitro interaction between the CRE motif and its cognate protein factor(s). Reconstruction experiments using a TH promoter in which the native CRE was rendered inactive show that the CRE can transactivate transcription in either orientation through a window of approximately 200 base pairs upstream of the transcription initiation site, suggesting that CRE supports transcriptional activation of the TH gene in a distance-dependent manner. Finally, when the distance between the CRE and TATA box was changed by inserting an additional 5 or 10 bases, it was observed that both insertion mutations increased activity by approximately 3-fold. The cAMP inducibility was as intact as the wild type construct. Together, these results are consistent with a model in which transcriptional activation of the TH gene by the CRE requires that it be located within a certain proximity of the CAP site but does not depend on a stringent stereospecific alignment in relationship to the TATA element.

Neuronal plasticity, essential for the adaptive function of the nervous system, depends on the capacity of cells to alter expression of target genes in response to environmental stimuli. Many stimuli alter cellular activity via receptors coupled to adenylate cyclase. The resultant increases of the intracellular cAMP levels stimulate cAMP-dependent protein kinase, resulting in phosphorylation of the target molecules such as cAMP response element binding protein (CREB), which is an essential step in transcriptional activation by this protein factor (3, 4). In numerous cAMP-inducible eukaryotic genes, an octamer DNA motif with the nucleotide sequence 5′-T(AG)T(AG)-3′, termed cAMP response element (CRE), mediates transcriptional induction by the cAMP-regulated signaling pathway (5, 6). Recent evidence strongly suggests that the cAMP-regulated signaling pathway plays essential roles in adaptive functions of the nervous system in that CREB-dependent gene transcription is critically involved in learning and memory (reviewed in Ref. 7) as well as in behavioral manifestations of opiate dependence (8).

Tyrosine hydroxylase (EC 1.14.16.2; tyrosine 3-monoxygenase; TH) is an important brain enzyme because it catalyzes the conversion of l-tyrosine to 3,4-dihydroxy-l-phenylalanine, which is the first and rate-limiting step of catecholamine biosynthesis (9). TH is selectively expressed in catecholamine-synthesizing and secreting cells, including dopaminergic, noradrenergic, and adrenergic neurons in the central nervous system and sympathetic ganglia and adrenal chromaffin cells in the periphery. The 5′-flanking sequence of the rat TH gene contains a consensus CRE motif located at −38 to −45 bp upstream of the transcription initiation site (10). Earlier studies of the TH promoter activity by Chikaraishi and colleagues (11–13) using the PC6b cell line as the positive system showed that the API motif and an overlapping E box/dyad, located at −205 and −182 bp, is the most important basal and cell-specific promoter element. In these studies, the CRE was suggested to be unimportant for TH transcription. However, more recent evidence from several laboratories using other TH-positive cell lines supports the idea that the cAMP-regulated signaling pathway, via the CRE, importantly regulates transcription of the TH gene. First, transient expression assays in TH-expressing cell lines such as the human neuroblastoma SK-N-MC cells, as well as in neuronal cell lines, demonstrated cAMP-dependent TH induction, and cAMP-dependent induction was dependent on the CRE (14, 15).

The abbreviations used are: CREB, cAMP response element-binding protein; CRE, cAMP response element; TH, tyrosine hydroxylase; CAT, chloramphenicol acetyltransferase; bp, base pair(s); kb, kilobase(s).
BE(2)C and rat PC12 (1) or mouse CATH.a and PATH.a (2) show that the CRE is critical for both basal and cAMP-inducible transcription of the rat TH gene. Secondly, TH gene expression is significantly attenuated at the transcriptional level in several cAMP-dependent protein kinase-deficient PC12 cell lines (14). Finally, coexpression of the catalytic subunit of cAMP-dependent protein kinase dramatically increases the transcriptional activity of the rat TH gene in a dose-dependent manner, whereas coexpression of the specific cAMP-dependent protein kinase inhibitor blocks cAMP-stimulated induction and reduces basal transcriptional activity (15).

To understand TH gene regulation by the CRE in greater detail, we investigated the structure/function relationship of the CRE in transcriptional activation. To define individual nucleotides that are important for transcriptional activation function, we performed saturated mutagenesis of the 12-bp region of the rat TH gene that encompasses the CRE and examined the effect of individual mutation on basal and cAMP-induced transcription by transient transfection assay using TH-expressing SK-N-NE(2)C cell line. In vivo promoter activity was correlated with in vitro interaction between the CRE and transcription factors by comparing the DNase I footprint patterns of the wild type and CRE-mutated upstream sequences using nuclear extracts of the SK-N-NE(2)C as well as purified CREB protein. We also determined whether the position of the CRE relative to the TATA box can affect its transcriptional activation function. Surprisingly, this study indicates that transcriptional activation by the CRE is distance-dependent but does not require a stringent stereospecific alignment in relation to the TATA element.

EXPERIMENTAL PROCEDURES

Cell Culture—Human neuroblastoma SK-N-NE(2)C cells (16) were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.), containing 10% heat-inactivated fetal calf serum (Hyclone Lab.), 100 units/ml of penicillin (Life Technologies, Inc.), and 100 μg/ml of streptomycin (Life Technologies, Inc.). CATH.a cells (17) were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% horse serum (Hyclone Lab.), 5% fetal bovine serum, and 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cells were maintained in a humidified 5% CO2 incubator at 37 °C.

TH-CAT Fusion Constructs—TH2400CAT fusion construct contained 2.4-kb upstream sequences of the rat TH gene fused to the bacterial CAT gene as described (1). Longer reporter gene constructs, TH9000CAT and TH5600CAT, containing 9.0- and 5.6-kb upstream sequences, respectively, were also generated. TH9000CAT was constructed by replacing the 1.6-kb HindIII-XhoI DNA fragment of TH2400CAT by an 8.3-kb HindIII-XhoI DNA fragment of pTH9.0 (18). A 3.4-kb SphiI DNA fragment was then deleted from TH9000CAT, resulting in TH5600CAT. To make CRE mutant forms of these longer constructs, TH2400(14C04G)CAT, which contains a double mutation within the CRE (15), was used in place of TH2400CAT in the above procedure.

Site-directed mutagenesis was performed based on the procedure of Nakamaye and Ekstein (19) as described previously (1). To generate saturated mutations in the TH CRE, the present procedure employed a degenerate 24-mer oligonucleotide, 5′-GGCCAGGCAGCTGAGCCTG-3′ and 5′-CGCCGGTTGCGTGTCACGCAC-3′, by which the restriction sites generate bases with 5% substitution of each of the other three nucleotides (Bio-Synthesis, Inc., Denton, TX). Among 136 individual clones isolated and sequenced, 56 were found to be mutants. Of these mutant clones, 19 single substitution mutants and 11 double mutants were further analyzed in this study. Other mutants were constructed to examine the position dependence of the CRE in transcriptional activation function. Oligonucleotides with sequences 5′-AGGGGCGTTTGCGTACAGCTG-3′ and 5′-GGCCAGGCAGCTGAGCCTG-3′, representing both strands of the CRE region at −54 to −52 bp of the rat TH gene, were annealed and inserted at BglII (−168 bp), BamHI (−503 bp), XhoI (−773 bp), PstI (−2400 bp), or Smal (−1700 bp) sites of the 42A38g mutant construct that contain a double substitution mutation (see Fig. 2). The copy number and orientation of the inserted DNA were confirmed by sequence analysis. In addition, oligonucleotides of the sequences 5′-GCGCTCTTCTTTAAATGACGCTAAAGGC-3′, 5′-TCTTTA-

**FIG. 1.** The CRE is an essential element for the promoter activities of TH upstream sequences. The CRE is mutated by double substitution in the context of 9000 (lanes 2), 5600 (lanes 4), and 2400 bp (lanes 6) upstream sequences of the rat TH gene, and its effect on promoter activities was examined by transient expression assays in the human neuroblastoma SK-N-NE(2)C (A) and the mouse central nervous system cell line CATH.a (B). Transient expression assays were performed twice in triplicate utilizing plasmid DNAs that were independently prepared with identical results. An autoradiogram of a representative TLC is presented for each cell line. The conversion of chloramphenicol into the acetylated forms (the upper two spots) was determined by cutting the spots and scintillation counting and shown as percentages below the panel.

AGGCGCAGTGAGCTACCTCA-3′, and 5′-CTTTTAAAGGCGCAGTGAGCTACCTCA-3′ were used to generate 2400TH- (8)CAT, 2400TH(+5)CAT, and 2400TH(+10)CAT, respectively. The modified sequences of the upstream region of these mutant reporter genes were verified by DNA sequencing analysis.

**Transfection and Enzyme Assays—**Cells were transfected using the calcium phosphate coprecipitation method (20) as described (21). For SK-N-NE(2)C cells, 50% confluent 60-mm dishes were transfected with 2 μg of the reporter construct, 1 μg of pRSV-βgal, and pUC19 plasmid to a total of 5 μg of DNA. For CATH.a cells, doubled amounts of plasmids were used. For the experiments described in Fig. 1, an equimolar amount of each reporter construct was used for transfection. Transfected cells were collected 24 h after transfection, and activities of CAT and β-galactosidase were determined as described elsewhere (20, 22). To ensure that all CAT assays were performed in the linear range, the final sample volume was adjusted following preliminary assays so that chloramphenicol conversion amounted to 5–50%. The CAT activity was normalized by the β-galactosidase activity to correct for differences in transfection efficiency among different DNA precipitates.

**Nuclear Extract Preparation and DNase I Footprintting—**Crude nuclear extracts were prepared from SK-N-NE(2)C cells based on described procedures (23). To obtain a CRE area probe labeled on the noncoding strand, p2400THCAT and the 42A38g mutant construct were cut with Bsu36I and end-labeled with Klenow enzyme and [α-32P]dCTP according to procedures suggested by vendors. The probe was then digested with XhoI and purified by polyacrylamide gel electrophoresis as described previously (24). Labeled probes (50,000 cpm) were incubated with nuclear extracts or purified CREB protein, treated with DNase I, and resolved on a 6% polyacrylamide gelsequencing gel as described (21). Location of the protected area was determined by Maxam-Gilbert sequencing of labeled probes.

**RESULTS**

**The Intact CRE Is Required for the Promoter Activities of the 2.4-, 5.6-, and 9.0-kb 5′-flanking Sequences of the Rat TH Gene—**As shown in Fig. 1A, double mutation within the CRE diminished the promoter activity of the 2.4-kb sequence of the rat TH gene as well as activities of longer 5.6- and 9.0-kb upstream sequences in the human neuroblastoma SK-N-NE(2)C cell line. These longer upstream sequences were shown to confer cell type-specific expression of reporter genes in transgenic mice (18, 25). The CRE is likewise essential for the promoter activities of these upstream sequences in another TH-expressing cell line, CATH.a (17), which has been derived from the central nervous system (Fig. 1B). In addition, these CRE mutant constructs do not respond to forskolin treatment (data not shown), indicating that the CRE is required for both basal and cAMP-inducible transcriptional activities of the 5′ TH upstream sequences.

**Saturated Mutational Analysis of the CRE of the Rat TH Gene—**Using site-directed mutagenesis procedure with a de-
generate oligonucleotide we isolated 19 different point mutations that include at least one mutation in every position of twelve bases encompassing the CRE motif and its four proximal bases (two 5' and two 3' to the CRE octamer) (Fig. 2). The effects of these mutations on basal transcription and cAMP inducibility were examined in the context of the 2400 bp upstream sequence of the rat TH gene by the transient transfection assay using the TH-expressing SK-N-BE(2)C cells. First, mutation of any nucleotide within the CRE resulted in a significant loss of basal transcription activity, demonstrating that the octamer CRE motif of the TH gene is the functionally optimal sequence motif. Intriguingly, mutations to different nucleotides at a particular position appear to have differential effects; for instance, replacement of C residue by T at −39 bp (39T in Fig. 2C) diminished more than 90% of the basal transcription but an A substitution (39A in Fig. 2C) retained approximately half of the promoter activity. Substitution mutations of surrounding bases at −36, −46, and −47 bp show little or no effect on the promoter activity, indicating that these nucleotide sequences do not affect the CRE function. However, the G residue at −37 bp, immediately 3’ to the CRE, appears to be important because its change to an A or a T residue results in a decrease of basal transcription activity by 60–70%. The 11 double mutations all exhibited a significant decrease of pro-
CRE Structure/Function Relationship of CRE in TH Transcription

Positional Effects on Transcriptional Activation via the TH CRE—To address whether the CRE activates TH transcription in a position- and distance-dependent manner, reconstruction experiments were performed using the 42A38G mutant construct (Fig. 4A; see also Figs. 2 and 3). In this experimental paradigm, we tested the effects of the location, copy number, and orientation of the CRE motif on its ability to support the basal transcription as well as the cAMP inducibility. As shown in Fig. 4B, when located at −168 bp upstream of the transcription initiation site, the CRE supported both basal transcription and cAMP-mediated induction of the reporter gene as efficiently as the wild type construct. At the same locus, two copies of the CRE in opposite orientation drive basal CAT expression at approximately twice the level of the wild type construct. However, the level of cAMP inducibility was the same as that of the native promoter. Additional copies appear to exert no further increases in basal and cAMP-inducible transcription of the reporter gene. Strikingly, the CRE did not support basal transcription at further upstream regions, e.g., at −503, −773, and −2400 bp upstream of the transcription initiation site. In addition, the CRE at these loci no longer mediated transcriptional induction following treatment with forskolin. To determine whether multiple copies of the CRE could overcome the incompetence of CRE-mediated transcription activation at these loci, we inserted two and three copies of the CRE at −503 bp, two copies at −773 bp, and two and four copies at −2400 bp. These constructs did not significantly support basal transcription of the reporter gene. In contrast, cAMP inducibility was restored to the level of the native promoter when three and four copies of the CRE were inserted at −503 and −2400 bp, respectively. When located 3’ to the reporter gene at +1600 bp, a single copy of the CRE supported neither basal transcription nor cAMP inducibility of the reporter construct.

The TH CRE Does Not Require Stereospecific Alignment in Relation to the TATA Box for Transcriptional Activation—The CRE of the TH gene resides 8 nucleotides upstream of the TATA box, showing the closest proximity to the TATA box among known cAMP-regulated genes. This unique spatial arrangement suggests that the stereospecific alignment between the CRE and the TATA may be important for transcription activation. To test this, we examined whether alterations of the distance between the CRE and TATA affect transcriptional activity (Fig. 5). Five nucleotides were inserted between the CRE and TATA, adding a half helical turn in the reporter construct TH2400(+5)/CAT. Surprisingly, this mutant construct drove CAT expression 2.8-fold more strongly than did the wild type reporter construct. Insertion of 10 nucleotides between the CRE and the TATA, which would produce approximately a full helical turn between them, similarly increased basal CAT expression by 3-fold. The CAT expression by these two spatial mutant constructs was induced to a level comparable with that by the wild type construct following treatment with forskolin. Taken together, the CRE of the TH gene does not require stereospecific arrangement in relationship to the TATA box for its transcriptional activation activity. Furthermore, our results suggest that the native location of the CRE may contribute to determining the level of basal expression of the TH gene.

DISCUSSION

TH gene transcription is inducible by the cAMP-regulated signaling pathway (10, 26–28). Although there are some conflicting data in regard to the critical role in TH transcriptional regulation (11–13), probably due to cell line differences, the CRE, which resides at −38 to −45 bp upstream of the transcription initiation site, appears to be responsible for not only the cAMP-mediated induction but also basal transcription of
**Fig. 4.** Position-dependent transcriptional activation by the CRE in the TH promoter. A, a schematic diagram of the 42A38G mutant construct that was used for subcloning the CRE-containing oligonucleotide, in which the native CRE was rendered inactive by a double substitution mutation. The restriction sites in which the oligonucleotide was subcloned and their relative positions to the transcription initiation site are indicated. The black boxes at both ends denote the multiple cloning sites (1). B, basal and forskolin-inducible promoter activities of the wild type and mutant TH-CAT constructs. The locations at which the CRE-containing oligonucleotides were subcloned are indicated under the brackets. Oligonucleotides subcloned in opposite orientation are indicated in the parentheses. The number before CRE denotes the copy number subcloned in each location. Transient expression assays were performed as in Fig. 2. Each bar represents the mean ± S.E. from three independent samples. These assays were repeated twice more in triplicate using independently prepared plasmids and resulted in similar patterns.

**Fig. 5.** The effect of spatial alignment between the CRE and the TATA elements on the transcriptional activity of TH-CAT reporter constructs. Schematic diagrams of the wild type TH2400CAT and spatial mutants are depicted at the left side of the panel. The white and black boxes denote the CRE and the TATA elements, respectively. Numbers above each box represents the relative position of the proximal nucleotide base of the sequence motifs. +1 denotes the transcription initiation site. Transient expression assays were performed as in Fig. 2. Each bar represents the mean ± S.E. from six independent samples.

The dual role of the CRE as a basal and cAMP-inducible transcription element appears to be a common regulatory feature for many CRE-containing genes (5, 29–34). To understand TH gene regulation by the cAMP-regulated signaling pathway in greater detail, this study defined the relationship between the structure and function of the CRE in transcriptional activation of the TH-CAT reporter gene.

Mutation of the CRE in the context of 9.0-, 5.6-, and 2.4-kb upstream sequences of the rat TH gene largely diminished their transcriptional activities in TH-expressing SK-N-BE(2)C and CATH.a cell lines (Fig. 1). Previous transgenic mouse experiments demonstrated that longer upstream sequences of the rat TH gene, e.g. 4.8 or 9.0 kb, can direct expression of the reporter genes in a tissue-specific manner (18, 25). Based on our data showing that the CRE is critical for the promoter activities of the 6.6- and 9.0-kb upstream sequences in both TH positive cell lines, we conclude that the CRE may be important for in vivo TH gene transcription.

The present analysis (Fig. 2) showed that (i) base substitution of any nucleotide within the octamer motif results in a significant decrease of the promoter activity (55–90%), demonstrating that every base of the octamer motif is important for intact transcription activation function of the CRE; (ii) among the nucleotides adjacent to the TH CRE, only the G residue residing immediately 3’ to the CRE is important for full transcriptional activity; (iii) mutation of a specific nucleotide may have differential effects depending on the substituted nucleotide; and (iv) a mutation that shows a significant reduction in basal transcription no longer mediates an intact cAMP induction.

Although the upstream area at −32 to −50 bp of the wild type sequence was footprinted by both nuclear proteins of SK-N-BE(2)C cells and bacterially expressed CREB, that of the mutant 42A38G construct with minimal promoter activity was not protected at all, indicating a positive correlation between in vitro promoter activity and in vitro DNA-protein interaction (Fig. 3). In addition, this observation is consistent with the idea that CREB is the transcription factor or one of the factors that bind to and transactivate the CRE. In support of this, coincubation of nuclear extracts with anti-CREB antibody results in formation of a supershifted band in electrophoretic mobility shift assay (35). However, in view of the increasing number of identified CRE-binding proteins (reviewed in Refs. 36, 37), it is likely that additional protein factors may be involved in binding to the CRE and regulating TH transcription. Antibody coimmunoprecipitation experiments indeed suggested that additional protein factors including ATF1 and CREM may bind to the TH CRE. Further, forskolin treatment of PC12 cells and reserpine treatment of rats induced expression of the inducible cAMP early repressor, an isoform of the CREM family (38), which represses the transcriptional activity of the TH gene promoter (35).

To address whether the CRE activates transcription in a distance-dependent manner, an oligonucleotide encompassing the TH CRE was inserted at different loci 5’ or 3’ to the reporter gene in the mutant 42A38G construct (Fig. 4). Strikingly, this reconstruction experiment demonstrates that the CRE can support basal and cAMP-inducible transcription in either orientation only through a window of approximately 200 bp upstream of the transcription initiation site. When three or four copies of the CRE are inserted at −503 and −2400 bp, 2 C. Yang and K.-S. Kim, unpublished data.
respectively, they restore the forskolin inducibility but still do not support basal transcription of the reporter gene.

The CRE is generally known to have properties of an enhancer (39). This notion is based on earlier studies indicating that the CRE of the proenkephalin gene can activate transcription when located in positions relatively distant from the transcription initiation site or at 3′ to the gene (40). Our results thus contrast to the general notion that the CRE can work in a distance- and position-independent manner. The structure and function of the CRE of the proenkephalin gene differ from those of the TH gene in that it is heptanucleotide with the sequence 5′-TGCCGTCA-3′ and mediates both CAMP and phorbol ester inducibility. Thus, the mechanisms underlying the function of the octamer CRE and the heptamer CRE may be significantly different. It is to be noted that most known CREs are located within the proximal 170 bp of upstream region and have the octamer structure (Ref. 1 and references therein). One obvious exception is the tyrosine aminotransferase gene, which has a functional CRE at the −3650-bp position (41). Here, the CRE has the same heptamer motif as that of the proenkephalin gene and is one of the functionally interdependent components of a hepatocyte-specific enhancer (41). In this context, one interesting possibility is that the CRE activates TH transcription synergistically with other cis-element(s), and this synergism works in a distance- and/or promoter context-dependent manner. In support of this, we recently found that mutation of a contiguous cis-regulatory element, Sp1, at −120 to −113 bp (11) diminished the basal promoter activity by 85%, suggesting a synergistic activation of TH transcription by the CRE and Sp1. This potential mechanism underlying the distance-dependent activation of TH transcription is currently being investigated.

Finally, we determined whether the location of the CRE relative to the TATA box is important for transcriptional activity (Fig. 5). For many genes that are inducible by hormone, the optimal inducibility and synergism often require stereospecific alignment (42, 43). Furthermore, stereospecific alignment of several promoter elements of eukaryotic genes relative to basic elements, e.g., TATA box, are known to be critical for full promoter activity (44–46). The CRE and the TATA elements are essential for TH gene transcription (1, 2, 11) and are separated from each other by 8 nucleotides. Insertion of either 5 or 10 nucleotides between these elements resulted in an increase of the basal promoter activity by approximately 3-fold, indicating that phasic interactions between the cognate binding proteins are not required for supporting basal transcription. This observation is thus consistent with a model that the CRE-binding protein(s) may activate TH transcription through a co-factor. CREB-binding protein, a recently identified transcriptional co-activator of CREB (47), is one such candidate molecule. CREB-binding protein, recruited to CAMP-dependent protein kinase-phosphorylated CREB by protein-protein interaction (48), may activate transcription of the TH gene, thus obviating the requirement for stereospecific alignment between the CRE and the TATA elements.

In summary, we defined the structure/function relationship of the CRE in TH transcription by performing the saturated mutagenesis, reconstruction experiments, and insertional mutation analyses and by examining their effects on the promoter activity in the context of the 2.4-kb upstream sequence. Our results for the first time show that every nucleotide of the octamer of the CRE as well as the G base immediately 3′ are important for full basal and CAMP-inducible transcriptional activity of the TH promoter. In addition, the CRE needs to be located within a certain proximity in the upstream region for exhibiting full transcriptional activation function, indicating that the CRE supports transcriptional activation of the TH gene in a distance-dependent manner. Furthermore, insertional mutation analyses suggest that the native spatial arrangement of the CRE and TATA elements may contribute to determining the level of basal TH gene expression in vivo. In support of these notions, the nucleotide sequences and the location of the CRE in relation to the TATA box are well conserved in all the TH genes identified from different species (Fig. 6). Specifically, 12 nucleotides encompassing the CRE are strictly conserved in the TH upstream sequences of all six species thus far identified.

Acknowledgments—We thank Dr. M. Montminy (The Salk Institute) for providing the bacterially expressed CREB. We also thank Dr. T. S. Nowak, Jr., in the Department of Neurology for critically reading this manuscript.

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