Both the Basal and Inducible Transcription of the Tyrosine Hydroxylase Gene Are Dependent upon a cAMP Response Element*

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The cAMP response element (CRE) mediates cAMP responsiveness in many eukaryotic genes (Rosler, W. J.; Vandenbark, G. R., and Hansen, R. W. (1988) J. Biol. Chem. 263, 9063–9066). The tyrosine hydroxylase gene (TH) contains a single copy of a consensus CRE at −45 to −38 base pair (bp) upstream of the transcription initiation site. Deletional and mutational analyses of the upstream 2400-base pair region of the rat TH gene using transient transfection assay demonstrated that the CRE was essential for both cAMP-mediated induction and basal transcription of the TH gene. Another domain between −365 and −151 bp, containing the AP1 site, contributed to transcription to a smaller degree. Thus, the CRE appears to play an important dual role as a basal promoter element and an inducible enhancer for TH transcription. Interactions between the DNA binding factors in nuclear extract and CRE-containing oligonucleotides were investigated by gel retardation and competition assays. Oligonucleotides corresponding to the CRE regions of the TH or somatostatin gene gave rise to a pair of distinct protein-DNA complexes with identical mobilities in the gel retardation assay, suggesting that similar nuclear factor(s) might bind to the CREs of the TH and somatostatin genes. This study emphasizes a fundamental role of the CRE in transcriptional activation of the TH gene in catecholaminergic cells.

Eukaryotic gene transcription is controlled by interactions between sequence-specific transcription factors (trans-acting elements) and specific DNA sequences (cis-acting elements) that serve as their binding sites (Ptashne, 1988; Mitchell and Tjian, 1989; Struhl, 1991; He and Rosenfeld, 1991). In neurons, interactions between transcription factors and cis-acting elements mediate changes in gene expression ultimately capable of regulating important synaptic functions such as long term potentiation (Dash et al., 1990) or neurotransmitter biosynthesis (Black et al., 1987). In catecholaminergic neurons, tyrosine hydroxylase (TH) mediates the first and rate-limiting step in the biosynthetic pathways for dopamine, norepinephrine, and epinephrine by catalyzing the conversion of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (Nagatsu et al., 1964). A variety of trans-synaptic and hormonal stimuli, acting via different signal transduction pathways, can produce acute (minutes to hours) increases in TH activity or somewhat delayed (one to several days) elevations in levels of TH (Thoenen et al., 1989; Black et al., 1987; Zigmond et al., 1989). The latter changes correlate with elevations in mRNA for TH, indicating that transcriptional regulation of TH gene expression plays an important role in the long term regulation of catecholaminergic transmission.

Among the important regulators of TH, cAMP has been shown to stimulate increases in TH activity by promoting phosphorylation of TH enzyme by cAMP-dependent protein kinase (PKA) (Joh et al., 1978). More recently, cAMP has also been shown to mediate increased transcription of mRNA for TH (Lewis et al., 1987). cAMP induces the transcription of a variety of genes via a consensus octamer, 5'-TGACGTCA-3', the cAMP response element (CRE), found in the 5'-upstream regions of genes regulated by cAMP (Rosler et al., 1988; Goodman, 1990). The protein that binds the CRE of the somatostatin gene has been characterized and cloned (Montminy and Bilezikjian, 1987; Gonzalez et al., 1989). This protein, known as CRE-binding protein (CREB), activates the transcription of responsive genes after it is phosphorylated by PKA (Yamamoto et al., 1988; Gonzalez and Montminy, 1989; Lee et al., 1990). Inspection of the TH gene and functional analysis of fusion gene constructs have identified a putative CRE at −45 to −38 bp upstream of the transcription initiation site (Harrington et al., 1987; Lewis et al., 1987; Fader and Lewis, 1990; Carroll et al., 1991). However, the function of this element in the context of the TH upstream region has been questioned. Indeed, some investigators have suggested that the CRE plays a relatively minor role in TH gene transcription (Cambi et al., 1988; Fung et al., 1992).

The present article presents data characterizing the upstream promoter region of the rat TH gene using the highly transfectable human neuroblastoma SK-N-BE(2)C cell line (Ross et al., 1981) and the less transfectable rat PC12 cell line (Green and Tischler, 1976). Both cell lines express high levels of TH endogenously. Functional analysis of fusion gene constructs, containing deletion mutations of the 5'-upstream region of TH, localizes the minimal promoter elements to within 60 bp of the transcription initiation site (CAP), a region which includes the CRE. Deletion of the 5'-region to −39, which removes 6 bp of the CRE, results in a drastic loss of transcriptional activity. Furthermore, site-directed mutational analyses indicate that the CRE, in the proximal region of the TH upstream sequence (−45 to −38), is not only responsible for cAMP-mediated gene induction but is essential for the basal transcription of the TH gene in these catecholaminergic cell lines. In vitro binding studies detect

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1 The abbreviations used are: TH, tyrosine hydroxylase; CRE, cAMP response element; CREB, cAMP response element-binding protein; PKA, cAMP-dependent protein kinase; CAT, chloramphenicol acetyltransferase; I MX, 3-isobutyryl-1-methylxanthine; kb, kilobase(s); bp, base pair(s).
two nuclear protein-DNA complexes of similar size using the CREs of the TH gene and somatostatin gene. In addition, the interaction of nuclear factors is analyzed further by competition assay using wild type and mutant oligonucleotides.

EXPERIMENTAL PROCEDURES

Cell Culture—Human neuroblastoma SK-N-BE(2)C cells were passaged in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. PC12 cells were grown in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal calf serum. Each serum was used after heat inactivation. All culture media contained 100 U/ml penicillin and 100 µg/ml streptomycin.

Construction of Reporter Plasmids—TH(-503/+27)CAT plasmid was constructed in the pOCAT vector (Carroll et al., 1991). This construct contains 503 bp of the 5′-flanking sequence of the rat TH gene, the transcription initiation site, and the first 27 bases of untranslated sequence of the TH transcript. To facilitate further constructions, the plasmid backbone was replaced with that of pBLCAT3 (Lucow and Schutz, 1987) by ligating the 4.0-kb BamHI-EcoRI fragment of pBLCAT3 with the 800-bp BamHI-EcoRI fragment of TH(-503/+27)CAT plasmid. Then, BglII-BamHI genomic fragment ranging from -2400 to -503 was inserted into the BamHI site of pBLCAT3 to give TH503CAT. A series of reporter constructs were made utilizing the unique PstI site in the pBLCAT3 and appropriate restriction sites in the upstream region of the TH gene. The junctions between TH upstream region and CAT gene of the fusion constructs were confirmed by sequencing analysis. To introduce mutations into the TH CRE, oligonucleotide-directed site-directed mutagenesis was performed. An M13 mp19 subclone containing the 700-nucleotide BamHI-EcoRI fragment of TH2400CAT plasmid was used as a template. Two 23-mer oligonucleotides of the sequence 5′ AGGCCAGGCTGACCTCAAAGCCCCTCTG 3′ and 5′ GCCAGGCTGACCTCAAAGCCCCTCTG 3′ were utilized as described to produce deletion and point substitution mutations (Nakamaye and Ekstein, 1986). The presence of mutations was confirmed by sequencing analysis.

RESULTS

To study TH gene regulation, rat TH genomic clones containing 5′-flanking sequences were isolated (Carroll et al., 1991). Potential cis-acting motifs such as AP1, AP2, POU/OCT, SP1, and CRE are located in the 5′-proximal region of the rat (Harrington et al., 1987; Carroll et al., 1991) and the human genes (Kobayashi et al., 1988) (Fig. 1A). Both the nucleotide sequences of these motifs (under boxes) and their relative distance (numbers above boxes) from the CAP site are highly conserved in both species. The 5′-proximal sequence contains a single copy of the palindromic consensus CRE (5′-TGACGTC-3′); located only 7 and 8 base pairs upstream of the TATA box in the human and rat genes, respectively (Fig. 1A). To localize important cis-acting elements, a series of plasmids were constructed containing different lengths of the rat TH upstream region fused to the bacterial CAT gene as a reporter gene (Fig. 1B). SK-N-BE(2)C cell line expresses levels of TH message similar to the human adrenal medulla, as assessed by ribonuclease protection analysis (Carroll et al., 1991). The CaPO4, co-precipitation method reproducibly provided a high transfection efficiency in the SK-N-BE(2)C cell line as quantitated by the β-galactosidase histochemistry (>20%; data not shown). Although PC12 cells were transfected with a much lower efficiency (1–2%) than SK-N-BE(2)C, this was still significantly higher

Fig. 1. Rat TH-CAT fusion genes and determination of transcription initiation site in the fusion gene construct. A, schematic diagram of putative cis-acting elements residing in the upstream region of the rat and human TH genes. Nucleotide sequences and the relative position of the proximal nucleotide in each motif in relation to the transcription initiation site are shown below and above each box, respectively. B, CAT fusion constructs containing different length of TH 5′-upstream region. Restriction enzymes which were used for deletion construction are indicated above. Arrow represents the transcription initiation site.
than the transfection efficiency reported previously (0.1%) in PC12 cells (Gandelman et al., 1990). Primer extension analysis using poly(A) RNA prepared from SK-N-BE(2)C cells transfected with TH2400CAT fusion construct verified that the CAT activity measured in our transient transfection assay resulted from proper transcription initiation (data not shown; Fig. 1A).

Deletional Analysis of 5′-Flanking Sequence Reveals Two Potentially Important Regions for TH Gene Transcription—The expression of different fusion constructs was compared using the transient transfection method in SK-N-BE(2)C cells. Deletions from the −2400-bp region to the −773-, −503-, or to −365-bp region did not influence CAT activity (data not shown). Thus, upstream sequences ranging from −2400 to −365 bp exerted no significant influence on basal transcription as measured by this transient transfection assay. When the upstream sequence was trimmed down further to −151 bp, a 30–40% drop in CAT activity was observed, suggesting the presence of a positive regulatory element or elements in the region spanning −365 to −151 bp. As depicted in Fig. 1A, several putative cis-acting elements such as AP2, AP1, and POU/OCT are located here. Further deletion to −108 or to −60 bp had little effect on residual transcriptional activity. Thus, the TH60 CAT construct, containing only 60 bp of proximal sequence, retained approximately 60% of the activity observed with the 2400-base pair construct, localizing the minimal upstream promoter to within −60 bp of the CAP site. Further deletion of 21 bp to −39 bp, however, abolished all remaining transcriptional activity, indicating that important basal promoter element(s) reside between −60 and −39 bp.

The response of these fusion constructs to forskolin treatment was tested in the presence of the phosphodiesterase inhibitor, IMX. In the SK-N-BE(2)C cell line, all fusion constructs except TH39CAT demonstrated approximately 3-fold stimulation of CAT activity, localizing CAMP responsiveness to the proximal 60 bp (Fig. 2). TH39 CAT and the promoterless plasmid, pBLCAT3 (Luckow and Schütz, 1987), showed negligible basal expression and minimally responded to forskolin treatment. These data strongly indicate that the CRE, located at −45 to −38 bp, is necessary and sufficient for forskolin induction.

It is possible that differences across species could influence the expression of the rat upstream region in the human cell line. To address this issue, the promoter analysis was replicated using rat PC12 cells as the host. Overall, a very similar profile of CAT activities was observed using the same series of deletional constructs in PC12 cell line. Again, removal of the −365 to −151 upstream regions decreased expression by 30–40%. TH60CAT expressed approximately 60% of TH2400CAT activity, and further deletion to −39 bp decreased the CAT activity to the level of pBLCAT3 (data not shown). Also, forskolin treatment stimulated the transcriptional activity of all fusion constructs, with an exception of TH39CAT, by 3–4-fold (data not shown).

The TH CRE Is an Essential Promoter Element of TH Gene Transcription—The TH39CAT fusion construct is transcriptionally silent and unresponsive to cAMP induction. In the −60 to −39-bp region, there is no identifiable cis-acting motif besides the CRE. The fact that the TH39CAT lacks 6 out of 8 bases of the CRE motif led to the hypothesis that the CRE may not only be a CAMP-response element but also a core promoter element for TH gene transcription. To test this possibility, we constructed deletion as well as base substitution mutants in the CRE and compared them with wild type constructs using transient transfection assays (Fig. 3). Strikingly, deletion of 5 internal bases of the TH CRE (GACGTCAG out of TGACGTCACA) abolished all transcriptional activity from the rat 2400 and 503-bp upstream regions both in SK-N-BE(2)C (Fig. 3B) and PC12 cell line (data not shown). Furthermore, these mutant constructs were not responsive to forskolin treatment. Thus, the CRE appears essential both for basal transcription and induction in response to elevated cAMP. The effect of a single base change within the CRE was tested while maintaining the spatial and contextual surroundings of the TH upstream region. Based on the previous observation that two guanosines on the sense strand (−42 and −39 bp) and a third guanosine on the opposite strand (−43 bp) of TH CRE motif are protected from methylation by dimethyl sulfate through the interaction with the CRE-binding protein (ATF) (Lin and Green, 1988), we mutagenized one of these residues. TH2400(42C → G)CAT, which has a single base substitution mutation at −42 position in the context of the whole 2400 bp upstream, displayed a dramatic loss of transcriptional activity when assayed by transient transfection in SK-N-BE(2)C cell line (Fig. 3B). First, the basal transcription was profoundly decreased (>80%). Second, induction by forskolin treatment was substantially reduced when compared with wild type constructs (from 3.0 × down to 1.6 ×). In PC12, TH2400(42C → G)CAT appeared to be as inactive as pBLCAT3 (data not shown).

Binding of Nuclear Proteins to the Wild Type and Mutant CRE in the TH Gene—The interaction between the DNA binding factors in crude nuclear proteins and CRE-containing oligonucleotides was investigated by gel retardation assay. Oligonucleotides (23-mer) corresponding to the CRE of the rat TH (WT oligo) and rat somatostatin genes (SOM oligo) were synthesized (Fig. 4A). These oligonucleotides do not
share any apparent homologies except an identical octamer CRE motif. TH CRE oligonucleotides containing deletions (DEL oligo) as well as point mutations were also made (Fig. 4A). Both the wild type oligonucleotide and the somatostatin oligonucleotide formed two distinct protein-DNA complexes (I and II) with identical mobility in the gel shift assay (Fig. 4B), suggesting that the same protein factor(s) may bind to the CRE in each gene. The same pattern of complex formation was observed when the probe was labeled by kinase or by filling in reactions, indicating that these two complexes were not related to single strand DNA binding activities. Deletion of 5 out of 8 bases in the TH CRE motif substantially reduced the affinity to nuclear proteins. Intriguingly, the same complexes re-emerged when excess amounts of crude nuclear proteins were used (Fig. 4B). The relative affinity of wild type and mutant oligonucleotides was assessed by incubating with molar excess of unlabeled wild type oligonucleotide using nuclear proteins from SK-N-BE(2)C. Sequences of CRE motif are indicated in bold. SOM oligo S and A represent the sense and antisense nucleotide sequences, respectively. All other nucleotides represent the rat TH CRE region in wild type, deletion mutation, or point mutation.

The data are presented in the same manner as in Fig. 2.
tor, 2-3-fold excess was required, which represents a moderate decrease (50-70%) in the binding affinity (Fig. 4C; data not shown).

DISCUSSION

Using transient transfection assay, several investigators have suggested that the upstream region of the rat TH gene can drive transcription in a cell type-specific manner (Har- rington et al., 1987; Cambi et al., 1989; Gandelman et al., 1990). Recently, Kaneda et al. (1991) showed that an 11-kb DNA isolate of the human TH gene containing the 2.5-kb upstream region, the entire exon-intron structure, as well as the 0.5-kb 3'-flanking region can direct expression of human TH mRNA in the brains and adrenal glands of transgenic mice in a tissue-specific manner. This result indicates that the 5'-upstream sequences involved in tissue specificity may largely reside within -2500 bp of the CAP site. In fact, detailed functional analyses of the 5'-flanking region of the TH gene from different laboratories have produced conflicting results, leaving this issue unresolved. Chikaraishi and her colleagues (Cambi et al., 1989; Yoon and Chikaraishi, 1992) proposed that the AP1-surrounding domain was essential for rat TH gene transcription by showing a dramatic decrease of expression (>90%) upon removal or mutation of sequences between -212 and -157 bp. However, other investigators have detected no decrease in basal expression after deletion of this AP1-surrounding region as measured by RNase protection or CAT assay (Gizang-Ginsburg and Ziff, 1990; Fader and Lewis, 1990). In addition, Gandelman et al. (1990) reported that the upstream sequence between -749 and -505 bp of the rat TH gene was also important for transcriptional activity in PC12 cells.

We examined expression driven by the 2.4-kb upstream region of the rat TH gene in both the human SK-N-BE(2)C and rat PC12 cell lines. The human neuroblastoma cell line, SK-N-BE(2)C, was reproducibly transfected with extremely high efficiency (>20%) and provided statistically reliable transfection analysis. Primer extension analysis demonstrated that CAT activity in SK-N-BE(2)C human cell line arises from correctly initiated transcription by the rat upstream sequence (data not shown). When the upstream sequence was deleted up to -365, no discernable effect on transcriptional activity was observed. Further deletion to -150 bp resulted in a 30-40% decrease in CAT activity in both the human SK-N-BE(2)C and the rat PC12 cells, suggesting the presence of positive element(s) between -365 and -150 bp. Our data are thus qualitatively consistent with those of other groups which showed that the AP1 site, located in the first 170 bp of the upstream region of their respective genes (short et al., 1986; Andrisani et al., 1987; Roesler et al., 1988). While it remains to be determined how commonly the CRE performs a dual role in basal and inducible expression, our recent study on gene regulation of dopamine β-hydroxylase, which converts dopamine to norepinephrine, indicated that the CRE plays a similar dual role in transcriptional regulation of this gene (Ishiguro et al., 1993).

Gel shift assays demonstrated that the CREs of the somatostatin (SOM oligo) or TH genes (WT oligo) incubated with crude nuclear extract of PC12 cells form similar patterns of DNA/protein complexes (Fig. 4B). These oligonucleotides do not share any sequence identities beyond the CRE and are presumably the targets of the same or similar CRE-binding proteins. Yamamoto et al. (1988) previously observed the same pattern of complex formation using the somatostatin CRE and nuclear extracts of PC12 cells. Hyman et al. (1988) suggested that several neuronaly expressed genes, i.e. somatostatin, tyrosine hydroxylase, vasoactive intestinal polypeptide, and proenkephalin genes, might be co-regulated by a common trans-acting element. At present, definite identification of the specific transcription factor involved in TH gene regulation, via the CRE, awaits further investigation. In vitro competition experiments showed that >95% of the relative affinity is lost by deletion mutation, in general agreement with the in vivo transfection result (Figs. 3 and 4). Nevertheless, it was rather surprising to observe that the deletion oligonucleotide retained some binding activity (Fig. 4B). In the deletion oligonucleotide, 5 out of the 8 bp in the CRE motif are missing. The resulting oligonucleotide contains an almost intact half-site of the palindromic octamer (3 out of 4 bp) as well as identical surrounding sequences. Yamamoto et al. (1988) showed previously that the half-site of the CRE out of the octamer CRE motif rendered the entire 2400-bp upstream sequence as silent as the promoterless plasmid both for basal and inducible transcription. Even a single base substitution (42C → G) within the TH CRE severely reduced basal transcription (>80%) and forskolin induction. Previous investigators have suggested that the TH CRE may function only as a cAMP-inducible element (Lewis et al., 1987; Fader and Lewis, 1990; Huang et al., 1991; Fung et al., 1992). Our data, for the first time, clearly implicate the CRE as an essential basal element for TH gene transcription. All fusion constructs except TH39CAT showed about 3-fold induction by forskolin treatment. Notably, deletion of the AP1 and AP2 sites did not influence the inducibility of the upstream region in response to forskolin treatment. In contrast, Fung et al. (1992) showed that either the AP1 or the CRE would confer cAMP responsiveness when placed in front of the TATA box. One possible explanation for their observation is that the AP1 motif can function as a CRE-like element under certain circumstances (Comb et al., 1990). Clearly, our results show that in the native context, the CRE suffices for cAMP responsiveness of the TH gene. Thus, our deletional and mutational analyses indicate that the CRE is crucial for both basal and cAMP-inducible transcription of the TH gene in TH-expressing cells. These data clearly contrast with previous reports by other investigators which suggested that the CRE contributes little to basal transcription of the TH gene (Cambi et al., 1989; Yoon and Chikaraishi, 1992; Fung et al., 1992).

The CRE motif has been found in the upstream regions of many genes, including neuropeptide genes, which are transcriptionally inducible by the elevation of intracellular cAMP concentration (Table I). Most CREs are located within the first 170 bp of the upstream region of their respective genes (the tyrosine aminotransferase gene being an exception), suggesting that the CRE may also function as a basal transcriptional element. Indeed, the dual role of the CRE as a basal and inducible transcription element has been suggested in several genes (Short et al., 1986; Andrisani et al., 1987; Roesler et al., 1988). While it remains to be determined how commonly the CRE performs a dual role in basal and inducible expression, our recent study on gene regulation of dopamine β-hydroxylase, which converts dopamine to norepinephrine, indicated that the CRE plays a similar dual role in transcriptional regulation of this gene (Ishiguro et al., 1993).
Nucleotide sequences surrounding the consensus CRE in different genes

Table I

| Gene                      | Sequence (5’ to 3’)                          | Distance to the CAP site | Ref.       |
|---------------------------|-----------------------------------------------|--------------------------|------------|
| Tyrosine hydroxylase      | GGGCTTTGACGTACGCTGTCGG                        | -45                      | Lewis et al. (1987) |
| Somatostatin              | CTTGGGCATGCGTACAGAGA                         | -48                      | Montminy et al. (1986) |
| Phosphoenolpyruvate kinase | GGGCCCTTTACGTCATTACGCA                       | -90                      | Short et al. (1986) |
| c-fos                     | AGACGCTGACGCCCTTAC                           | -60                      | Fisch et al. (1989) |
| Chorionic gonadotropin (α-subunit) | AAAATTCGTCAGTCGTTAA                      | -123                     | Bokar et al. (1988) |
| Vasoactive intestinal peptide | AGCCGCGTGACGTACAAC                          | -143                     | Tsukuda et al. (1987) |
| Fibronectin               | CCCCCCTACGTCACCCCCGGG                        | -173                     | Dean et al. (1989) |
| Lactate dehydrogenase (A subunit) | CCACTCCTACGTCACGGGG                      | -48                      | Short et al. (1991) |
| VGF(a2/NGF33.1)           | GAACATTGACGTCGG                              | -82                      | Hawley et al. (1992) |
| Proenkephalin             | AGGCGCTGCTCGAGCTCA                           | -91                      | Comb et al. (1986) |
| Tyrosine aminotransferase | AGCTTCTGCTGACGCCCA                          | -3650                    | Boshart et al. (1990) |

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