The Cyclic AMP Response Element Directs Tyrosine Hydroxylase Expression in Catecholaminergic Central and Peripheral Nervous System Cell Lines from Transgenic Mice*

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Enhancer elements regulating the neuronal gene, tyrosine hydroxylase (TH), were identified in TH-expressing peripheral nervous system PATH and central nervous system CATH cell lines. Mutational analysis in which rat TH 5'-flanking sequences directed chloramphenicol acetyltransferase (CAT) reporter gene expression demonstrated that mutating the cyclic AMP response element (CRE) at −45 base pair reduced expression by 80–90%. A CRE linked to an enhancerless TH promoter fully supported expression. Cotransfection of a dominant-negative CREB protein reduced expression 50–60%, suggesting that the CRE is bound by CREB or a CREB dimerization partner. Although mutating the AP1/dyad (AD) element at −205 base pair only modestly reduced CAT levels, AD minimal enhancer constructs gave 45–80% of wild type expression when positioned at −91 or −95. However, in its native context at −205, the AD could not support expression. In contrast, a CRE, moved from its normal position at −45 to −206, gave full activity. These results indicate that the CRE is critical for TH transcription in central nervous system CATH and peripheral nervous system PATH cells, whereas the AD is less important and its enhancer activity is context- and/or position-dependent. These results represent the first attempts to map regulatory elements directing TH expression in central nervous system cell lines.

Tyrosine hydroxylase (TH) converts l-tyrosine to 3,4-dihydroxy-L-phenylalanine and is the first and rate-limiting enzyme in catecholamine synthesis (Nagatsu et al., 1964; Levitt et al., 1965). TH is expressed in specific cell types in the peripheral and central nervous systems. Sympathetic ganglia and chromaffin cells of the adrenal medulla are major sites of peripheral TH expression. In the central nervous system, TH-expressing neurons are located in the diencephalon, midbrain, brainstem, olfactory bulb, and retina (Bjorklund and Lindvall, 1984).

TH activity is regulated at the protein level and the RNA level. Activation at the protein level is short term with a time course of less than 1 h and mainly occurs via phosphorylation of preexisting protein molecules, which increases TH activity (reviewed by Zigmond et al. (1989)). Induction of TH at the mRNA level is long term, resulting in increased mRNA levels for hours to days. Long term increases of TH activity are induced by various physiological stimuli including cyclic AMP (cAMP), epidermal growth factor, glucocorticoids, nerve growth factor, transsynaptic neuronal activity, and depolarization. These inducers have been shown to increase TH mRNA levels, and several studies indicate that they increase mRNA levels by inducing transcription: cAMP (Lewis et al., 1983, 1987; Tank et al., 1986, 1990; Fader and Lewis, 1990; Carroll et al., 1991; Fassom et al., 1992; Fung et al., 1992; Kim et al., 1993), epidermal growth factor (Lewis and Chikaraishi, 1987), glucocorticoids (Lewis et al., 1983, 1987; Tank et al., 1986, 1990), nerve growth factor (Gizang-Ginsberg and Ziff, 1990), and transsynaptic neuronal activity and depolarization (Hefti et al., 1982; Black et al., 1985; Tank et al., 1985; Faucon-Biquet et al., 1989; Erlich et al., 1990; Kilbourne and Sabban, 1990, 1992; Banerjee et al., 1992). Investigation of the DNA regulatory elements that direct basal and induced TH transcription has been of interest because of the role TH has in the long term regulation of catecholamine biosynthesis.

The 5'-flanking sequence of the rat TH gene contains several sequences that bear homology to cis-acting regulatory elements, including the AP2, AP1, E box (E2A/Myo D), octamer/POU, heptamer, Sp1, cAMP response element (CRE), and TATA box found in other genes. The AP1, octamer, Sp1, and CRE sites and their relative positions from the RNA initiation site are highly conserved in the rat, human, bovine, and mouse TH genes, suggesting that they may be important for transcriptional regulation (Harrington et al., 1987; Coker et al., 1988; Kobayashi et al., 1988; Cambi et al., 1989; D'Mello et al., 1989; Iwata et al., 1992). In peripheral nervous system-derived cell lines that express TH (PC12, PC8B, and SK-N-Be(2)C), the CRE confers cAMP responsiveness (Fader and Lewis, 1990; Fung et al., 1992; Kim et al., 1993); the CRE also mediates induction by depolarization in PC12 cells (Kilbourne et al., 1992). The AP1 site partially mediates nerve growth factor induction in PC12 cells (Gizang-Ginsberg and Ziff, 1990) and may confer cAMP responsiveness in PC8B cells (Fung et al., 1992).

Previous studies of basal and induced TH transcription have been limited to these peripheral nervous system-derived cells because of the lack of TH-expressing central nervous system cell lines. However, it is also of interest to examine how central nervous system catecholaminergic cells regulate TH transcription.

Recently, a mouse catecholaminergic central nervous system
cell line, CATH.a, was derived from a TH-expressing brainstem tumor in a transgenic mouse carrying the SV40 T antigen oncogene under the transcriptional control of rat TH 5'-flanking DNA (Suri et al., 1993). CATH.a cells are morphologically undifferentiated under normal culture conditions; they grow in clumps, most are round, and most lack significant processes. Nevertheless, they exhibit differentiated neuronal characteristics. They express neurofilament proteins, the intermediate filaments specifically expressed in neurons, but do not express glial fibrillary acidic protein, characteristic of glial cells. CATH.a cells express synaptophysin, an integral membrane protein of synaptic vesicles, dopamine β-hydroxylase, and TH. They synthesize, secrete, and accumulate high levels of dopamine and norepinephrine (Suri et al., 1993). CATH.a cells express voltage-gated tetrodotoxin-sensitive sodium currents and high voltage activated calcium currents similar to those reported in other neurons (Lazaroff, et al., 1992). In total, these data suggest that CATH.a cells are immortalized derivatives of central nervous system noradrenergic neurons. Two other TH-expressing cell lines, CATH.b and PATH.2, were derived from brainstem and adrenal tumors, respectively, from different mice. CATH.b arose from the same transgenic lineage as CATH.a, while PATH.2 was derived from an independent lineage. PATH.2 cells express neurofilament proteins, synaptophysin, and TH, and they synthesize dopamine and norepinephrine (Suri et al., 1993). CATH.b cells express TH2 but otherwise have not yet been characterized.

In this study we investigated transcriptional regulation of the rat TH gene in the CATH.a, CATH.b, and PATH.2 cell lines by examining expression of a transiently transfected chloramphenicol acetyltransferase (CAT) reporter gene under the transcriptional control of TH 5'-flanking DNA. Deletion analysis suggests that upstream regions between -4.8 and -0.272 kibap are not necessary for expression in these cells. Mutational analysis of various conserved sites within 0.272 kbp indicates that the CRE is critical for TH transcription in both the central nervous system-derived and the peripheral nervous system-derived cells. Expression of a dominant negative mutant CREB protein, KCREB, decreased CAT expression, indicating that a CRE-binding protein (CREB, ATF-1, and/or CREM) promotes transcription. The AP1 (A) and an overlapping dyad (D) symmetry element whose core is an E box site, both of which are also located within 0.272 kbp, appear to have a less important role in CATH and PATH cells. The primacy of the CRE is further supported by experiments in which minimal enhancer constructs containing the CRE, AP1, and/or dyad/E box elements directed reporter expression. The CRE supported full expression, comparable to that of the intact -0.272-kbp region. These findings contrast our previous work in PC8b cells (a TH-expressing PC12 pheochromocytoma subcloned; Tank et al., 1990), where the AP1 and dyad/E box sites are primarily responsible for TH transcription and the CRE has a less important role (Cambi et al., 1989; Fung et al., 1992; Yoon and Chikaraishi, 1992). Therefore, TH transcription may require the CRE, AP1, and dyad/E box elements to varying extents in different cells. MATERIALS AND METHODS

Cell Culture—CATH.a, CATH.b, and PATH.2 cells were grown at 37°C and in 5% CO2 on tissue culture plastic (Falcon, Becton Dickson, Lincoln Park, N.J.) in RPMI 1640 medium supplemented with 8% horse serum (Life Technologies, Inc.), 4% fetal bovine serum (HyClone Laboratories, Logan, UT), and 1% penicillin-streptomycin (100% stocks were 10,000 units/ml penicillin G and 10,000 μg/ml streptomycin sulfate; Life Technologies, Inc.).

DNA Constructs—The TH-CAT constructs used for deletion analysis contained varying lengths of the rat TH 5'-flanking region up to +27 base pairs (bp) linked to the CAT gene; most have been previously described (Harrington et al., 1987; Lewis et al., 1987; Cambi et al., 1989). The -4.8 THCAT constructs with internal deletions from -0.272 to -2.2 kbp (Δ -0.272/-2.2 THCAT) and from -0.773 to -2.6 kbp (Δ -0.773/-2.6 THCAT) were derived from the -4.8 THCAT construct with an SstI and XhoI digest and re-ligated respectively. The enhancerless -44 THCAT construct (labeled as THCAT in all figures), containing -44 to +27 bp of rat TH genomic DNA preceded by a polylinker, has been previously described (Fung et al., 1992). Site-specific mutant AP1, AP1*, dyad 3E box, dyad 4E box, octamer, tetramer, Sp1, and CRE -0.272 THCAT constructs (Fig. 2A) have been previously described (Yoon and Chikaraishi, 1992). The AP1 and AP1* mutants differ in that all 7 bp of the AP1 are replaced in the AP1 mutant (which disrupts the first 3 bp of the dyad), whereas only the left half of the AP1 is mutated in AP1*, preserving the dyad site. The dyad 3E box and dyad 4E box mutants replace either the left or the right half of the dyad symmetry element box, respectively. The -0.272 A-C construct was generated by mutating the AP1 (5'-TGATCCA-3') at -256 to -199 bp to a CRE (5'-TGACCTACA-3') at -206 to -199 bp. This was done in a -0.272 THCAT construct lacking a CRE at -45 to -38 bp. Minimal enhancer constructs (Fig. 4A) were generated by inserting oligonucleotides containing the CRE, AP1, and/or dyad/E box into the XhoI and SacI sites present in the polylinker of enhancerless -44 THCAT construct. A parallel set of minimal enhancer constructs was generated (Sp1-Δ) in which the partial CRE at -0.272 THCAT construct lacking a CRE at -0.272 THCAT construct was mutated to 5'-GACAAT-3' (Fig. 4A) to give -38 THCAT and to remove the partial CRE site. The minimal -0.095/-0.272 AD THCAT (Fig. 5A) construct, generated by inserting 70 bp of TH sequence from -0.230 to -0.160 kbp into the 5'ml site in THCAT, has been described previously (Fung et al., 1992); the partial CRE was similarly mutated in this construct. RSV3gigal and SV2gigal plasmids, which contain the Escherichia coli β-galactosidase gene (lac2) driven by strong, ubiquitous RSV and SV40 early promoter/enhancers, respectively, were obtained from Dr. S. Hyman (Massachusetts General Hospital, Boston, MA) and Dr. Van Cherington (New England Medical Center, Boston, MA), respectively. The RSV-KCREB plasmid, which contains the CREB gene driven by the RSV early enhancer (Walton et al., 1992), was obtained from Dr. Richard Goodman (Vollum Institute, Portland, OR). All plasmid DNAs were purified on QIAGEN columns (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions, followed by ethanol precipitation.

Transfections—CATH.a, CATH.b, and PATH.2 cells were plated at 2.3×105 cells/100-mm diameter plate 48 h before transfection. Cell lines were transfected with calcium phosphate/DNA precipitates. For each 100-mm plate, 5 μg of THCAT plasmid DNA, coprecipitated with 2 μg of RSVβgal plasmid DNA for CATH.a cells and 5 μg of RSVβgal plasmid DNA for CATH.b and PATH.2 cells, were used. For experiments with KCREB, each 100-mm plate was transfected with 0, 3, or 6 μg of RSV-KCREB, 2 μg of -4.8 THCAT, 2 μg of SV2βgal, and 6, 3, or 0 μg of the SV2M1 plasmid DNA to bring the total amount of DNA to 10 μg. The RSVβgal and SV2βgal DNA served as internal controls for transfection efficiency. In all experiments, DNAs were tested in duplicate. For multiple cultures transfected with the same test plasmid, calcium phosphate/DNA precipitates were pooled before distribution to plates. Cells were incubated at 37°C with the precipitate for 4 h; afterward, the medium was removed and 15% glycerol in HEPES-buffered saline was added to the cells for 3 min; the glycerol was removed by washing twice with phosphate-buffered saline, new medium was added, and cells were incubated at 37°C. 48 h after transfection, cells were washed twice with phosphate-buffered saline and harvested in phosphate-buffered saline with a rubber policeman. Harvested cells were centrifuged and resuspended in 3 ml of 1X PBS (pH 8.0). The cells were lysed by three freeze-thaw cycles, and the cellular debris was removed by centrifugation. Each lysate was assayed for amount of protein, CAT activity, and β-galactosidase activity. For cyclic AMP induction, dibutyryl cAMP (Sigma), which was stored as a stock solution of 200 mM in distilled water, was added to cultures at a final concentration of 1 mM 12–24 h before harvesting for CAT assays. 

β-Galactosidase Assays—For CATH.a cells, 50–100 μg of protein lysate were assayed for CAT activity using 0.5 μCi of [3H]chloramphenicol (Du Pont NEN or ICN); incubations were 8–12 h at 37°C. In experiments where cells were treated with dibutyryl cAMP, less protein was used and incubations were shorter to keep the assay in the linear range; 15–25 μg of protein were assayed and incubations were 4–5.5 h. For CATH.b and PATH.2 cells, 50–100 μg of protein lysate were used and incubations were 12–18 h.

β-Galactosidase activity was determined by incubating 50 μg of

2 M. Lazaroff and D. M. Chikaraishi, unpublished observations.
The CRE Directs Tyrosine Hydroxylase Expression in Cell Lines

protein lysate in 0.1 M sodium phosphate (pH 7.0), 10 mM KCl, 1 mM MgSO4, 83 mM β-mercaptoethanol, and 2.2 mM o-nitrophenyl-β-d-galactopyranoside (Sigma) at 22°C. The colorimetric reaction was measured (A405) after 1.5–5 h for CATH.a cells and 6–24 h for CATH.b and PATH.2 cells when RSVVgpl served as the internal control for transfection efficiency. The colorimetric reaction was measured after 20–24 h for CATH.c cells and SV2gpl served as the internal control for transfection efficiency.

For each transfected plate of cells, CAT activity was expressed as the percentage of [14C]chloramphenicol converted to acetylated forms per μg of protein lysate per hour, divided by the yield of β-galactosidase activity (A420) per μg of protein lysate per hour to normalize for differences in transfection efficiency. Activities have been corrected for the molarity of the given construct, since the length of the S′ region used varied. In all figures normalized CAT activities are expressed as a percentage of that obtained with −0.272 THCAT from the same experiment except for Fig. 7, where normalization was to −4.8 THCAT.

For all experiments, each DNA precipitate was tested in duplicate plates. At least two different DNA preparations of each construct were tested except where noted; different DNA preparations of any one construct gave similar results.

Different DNA preparations of the same construct gave essentially the same percent conversion in a given experiment. However, there were differences between the absolute values of normalized CAT activities from different experiments, which probably reflect differences in the state of the cells at the time of transfections. For example, in one particular experiment with CATH.a cells, four different preparations of −0.272 THCAT DNA gave CAT activities of 27.1 ± 2.8%. In a second experiment, two of the same DNA preparations were transfected and gave CAT activities of 56.5 ± 1%. The reason for such variability is unclear but may be due to unidentified microenvironmental cues and/or variable culture conditions. Despite this variability, the relative ratios of CAT expression among the various THCAT constructs and the enhancerless negative control THCAT construct were the same in all experiments.

RNase Protection Assay—Total cellular RNA was harvested 48 h after transfection using RNAzol B (Biotechex Laboratories, Inc., Houston, TX). Total RNA extractions were performed as described by Fung et al. (1991, 1992) with some minor modifications. The RNase protection probe was constructed as follows. The XbaI/EcoRI (−364 to −274 bp) fragment of −0.773 THCAT was subcloned into pGEM-1. This construct was linearized at −109 bp with Bsu36I. A 397-nucleotide (nt) antisense riboprobe product was synthesized from the T7 promoter. Gel-purified probe (approximately 124,000 cpm) was coprecipitated with 150 μg of RNA from transfected cells. The RNA pellets were resuspended in 40 μl of hybridization solution (72% formamide, 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl), overlaid with mineral oil, heated in boiling water for 3 min, and hybridized for 16–20 h at 50°C. RNase digestion was performed for 2 h at room temperature in 300 μl RNase digestion buffer (0.3 M NaCl, 20 mM Tris (pH 7.4), 2 mM EDTA) containing 2 μg/ml RNase A (Sankyo Co. Ltd., Japan) and 40 μg/ml RNase T1 (Sankyo Co. Ltd., Japan) and 40 μg/ml RNase A (Sigma). RNase digestion was terminated by the addition of protease K (10 μl of 10 mg/ml; Sigma) and SDS (20 μl of 10% SDS) for 30 min at 37°C, followed by two extractions with 350 μl of phenol-chloroform and one extraction with 350 μl of chloroform. The RNA was then ethanol-pelleted in the presence of carrier E. coli tRNA and resuspended in 10 μl of a solution containing 95% formamide, 20 mM EDTA, 0.03% bromphenol blue, and 0.05% xylene cyanol. The nucleic-resistase RNA species were electrophoresed on 5% polyacrylamide, 8 M urea denaturing gels run in 0.09 M Tris borate (pH 8.3) at 450 V at 22°C. Autoradiographic exposures were for 24–96 h.

RESULTS

Deletion Analysis—To define DNA elements that regulate TH transcription, CATH.a, CATH.b, and PATH.2 cells were transiently transfected with CAT reporter genes under the transcriptional control of various lengths of TH 5′-flanking DNA (Fig. 1A). −44 THCAT (labeled as THCAT in all figures), a construct containing the TH promoter (−44 to +27 bp) but lacking enhancer elements, served as the negative control. Various 5′ deletions from −4.8 to −0.272 kb gave approximately the same level of CAT activity (Fig. 1, B–D), although expression from the −0.773 THCAT construct was slightly less than from the other constructs, particularly in the CATH.b and PATH.2 cells. These results suggest that regions between −4.8 and −0.272 kb are not essential for TH expression in these cell lines. Therefore, DNA elements critical for TH transcription reside within 0.272 kb of TH 5′-flanking DNA for the CATH.a, CATH.b, and PATH.2 cells.

The amount of CAT activity obtained from CATH.a cells was usually about 5–50-fold higher than from CATH.b and PATH.2 cells. These differences correlate with higher endogenous TH levels expressed in CATH.a cells compared to CATH.b and PATH.2 cells. Western blots and immunohistochemistry indicate that CATH.a cells express much more TH than do CATH.b and PATH.2 cells. In addition, TH activity is 5-fold higher in CATH.a cells than in PATH.2 cells, and Northern analysis of TH mRNA indicates that CATH.a cells express more TH than do PATH.2 cells (Suri et al., 1993).

Site-directed Mutation Analysis—Within 0.272 kb of the rat, human, bovine, and mouse TH 5′-flanking DNA, consensus sites for a number of transcription factors have been reported (Harrington et al., 1987; Coker et al., 1988; Kobayashi et al., 1988; Cambi et al., 1989; D’Mello et al., 1989; Iwata et al., 1992). These sites include AP2, AP1, E box (E2A/Myo D), octamer/POU, heptamer, Sp1, and CRE elements and the TATA box (Cambi et al., 1989).

−0.272 THCAT constructs bearing mutations at the AP1, dyad/E box (includes the E box and the 20-bp dyad symmetry element in which it lies), octamer/POU, heptamer, Sp1, and CRE sites (Fig. 2A) were transfected into CATH.a, CATH.b, and PATH.2 cells. In all three lines, mutation of the CRE diminished transcriptional activity 80–90% (Fig. 2, B–D). In addition, a −4.8 THCAT construct in which the CRE was mutated, −4.8 CRE −1, diminished CAT activity to near background levels in all three lines (CATH.a data shown in Fig. 2E; CATH.b and PATH.2 data not shown). These results demonstrate that the CRE is the crucial site mediating expression in the CATH.a, CATH.b, and PATH.2 cell lines. In contrast, mutations of the AP1 and dyad 4/E box (right half of the dyad/E box) only modestly reduced CAT activity (20–40%), while dyad 3/E box mutations (left half of the dyad/E box) had no effect (Fig. 2, B–D). Therefore, the AP1 and dyad/E box sites have significantly less enhancer activity than the CRE under basal conditions (with no inducers in the culture medium). This is in contrast to the situation in PC8b cells where the AP1 and dyad/E box are critical for expression and the CRE is less important; CAT expression was reduced 95% by mutation of the AP1 and 65–85% by mutation of the dyad, whereas CAT expression was reduced 50% by mutation of the CRE in PC8b cells (Yoon and Chikaraishi, 1992).

Mutations of the octamer and Sp1 sites in the −0.272 THCAT construct gave 35–45% less CAT expression than did wild type −0.272 THCAT in the CATH.b and PATH.2 cells (Fig. 2, C and D). These decreases in expression were not further investigated. It is possible that these sites, as well as the AP1 and dyad/E box sites, have a more prominent role than seen here in regulating TH expression in response to various physiological stimuli or during certain stages of development.

In CATH.b and PATH.2 cells, mutation of the heptamer site in the −0.272 THCAT construct gave more CAT expression than did wild type −0.272 THCAT in the CATH.b and PATH.2 cells (Fig. 2, C and D). These decreases in expression were not further investigated. It is possible that these sites, as well as the AP1 and dyad/E box sites, have a more prominent role than seen here in regulating TH expression in response to various physiological stimuli or during certain stages of development.

In CATH.b and PATH.2 cells, mutation of the heptamer site in the −0.272 THCAT construct gave more CAT expression than did wild type −0.272 THCAT (Fig. 2, C and D), suggesting that in some neuronal cells, the heptamer may repress expression, perhaps via Oct-2 (see Dawson et al. (1994)). A similar result was obtained in PC8b cells (Yoon and Chikaraishi, 1992).

RNase protections confirmed that transcription in CATH.a cells was initiated at the correct start site for the wild type, mutant AP1, and mutant dyad/E box −0.272 THCAT constructs. Consistent with CAT activity measurements, no correctly initiated CAT RNA could be detected from the mutant CRE −0.272 THCAT construct (Fig. 3, A and B). RNA from
transfected cells was hybridized to a 397-nt probe corresponding to 109 to 274 nt of THCAT plus 14 nt of vector sequence. Hybridization of correctly initiated transcripts generates a 274-nt fragment, which was observed with RNA from cells transfected with wild type, mutant AP1, and mutant dyad/E box –0.272 THCAT constructs. In contrast, a 274-nt protected fragment was not obtained with RNA from cells transfected with the mutant CRE construct (even at longer exposure times; data not shown), indicating that with this construct, transcription was not correctly initiated. These results support the contention that the CRE is critical for TH transcription, but the AP1 and dyad/E box sites are not.

Additional protected fragments are likely due to read-through transcripts that originated from incorrect transcription start sites, some of which may be in the vector, as suggested by Gizang-Ginsberg and Ziff (1990) and Fung et al., (1992). This is supported by the fact that protected fragment *1* is approximately 383 nt (Fig. 3B), the size predicted from hybridization of the 397-nt probe to a transcript that initiates from an incorrect transcription start site located upstream of 109. Since the 397-nt probe contains 14 nt of vector sequence that do not hybridize to the transcript, this leaves 383 nt of THCAT sequence in the 383-nt protected fragment.

Protected fragment *2* is 311 nt (Fig. 3B); this is the size predicted to result from hybridization of the probe to a transcript that initiates from an incorrect transcription start site located upstream of 109 and that contains a mutated CRE. Since the CRE located at 45 to 38 nt is mutated and does not hybridize to the probe, this region is digested and two smaller read-through fragments (311 and 63 nt) are generated rather than the larger 383-nt protected fragment. Only the 311-nt protected fragment is detected in Fig. 3B, because the 63-nt protected fragment was run off the gel. The origin of the 290–300-nt band is uncertain; it may be due to incomplete RNase digest of correctly initiated transcripts hybridized to the probe.

Similar read-through transcripts were detected by Fung et al. (1992) in PC8b cells transfected with various THCAT constructs. It is likely that the larger read-through transcripts are not translated into functional CAT protein because of translational stop signals upstream of the +1 site. Evidence for this is provided by Fung et al. (1992), where it was shown that the enhancerless –44 THCAT construct generates similar read-through products that do not give high levels of CAT activity in transfected PC8b cells.

Minimal Enhancer Construct Analysis—As an alternative
Fig. 2. The CRE directs tyrosine hydroxylase expression in CATH.a, CATH.b, and PATH.2 cells. A, schematic diagram showing the upstream region of the rat TH gene and the positions of enhancer motifs. Site-specific mutants of AP1, dyad/E box, octamer, heptamer, Sp1, and CRE sites within the -0.272 THCAT construct were generated previously (Yoon and Chikaraishi, 1992). The wild type and mutated sequences (underlined, bold) are indicated. Normalized CAT activity in transfected CATH.a cells (B), CATH.b cells (C), and PATH.2 cells (D) is expressed as described in Fig. 1. E, normalized CAT activity in CATH.a cells transfected with wild type -0.272 THCAT and -4.8 THCAT and their corresponding mutant CRE constructs, -0.272 CRE- and -4.8 CRE-. For all experiments, each DNA precipitate was tested in duplicate plates. For CATH.a cells, at least two different DNA preparations of each construct were tested except Sp1 and -4.8 CRE-. Different DNA preparations of any one construct gave similar results. The number of transfected plates (n) is indicated above each bar. The normalized values for -0.272 THCAT were as follows: CATH.a cells in panel B, 23.9 ± 1.0 (n = 6), 30.4 ± 2.9 (n = 6), 69.4 ± 8.8 (n = 2), 59.0 ± 4.0 (n = 4), and 43.4 ± 3.2 (n = 4) in five different experiments; CATH.a cells in panel E, 55.0 ± 0.3 (n = 2), 69.4 ± 0.8 (n = 2), 59.0 ± 4.0 (n = 4), and 43.4 ± 3.2 (n = 4) in four different experiments; CATH.b cells in panel C, 5.8 ± 1.2 (n = 6) and 6.0 ± 2.0 (n = 4) in two different experiments; and PATH.2 cells in panel D, 0.6 ± 0.1 (n = 4) and 5.4 ± 1.2 (n = 4) in two different experiments.
The CRE Directs Tyrosine Hydroxylase Expression in Cell Lines

Fig. 3. Correctly initiated transcription is directed by wild type, transfect with these constructs gave similar the partial CRE (Fig. 4) A, schematic diagram of the probe, correctly initiated RNA, and read-through RNA. Thin lines below represent the size of the RNA probe protected by hybridization and correspond to the bands seen on the gel. B, RNase protections with RNA from CATH.a cells transfected with the wild type and mutant AP1, dyad/E box, and CRE −0.272 THCAT constructs. The 274-nt protected band marked by an arrow indicates correctly initiated RNA. The other bands marked by asterisks represent predicted read-through transcription shown in A. Negative control lanes include RNA from CATH.a cells not transfected and E. coli RNA. This experiment was repeated twice with different preparations of transfected cell RNA.

way to assess the importance of individual sites regulating TH transcription, synthetic enhancer constructs were made in which a single copy of the CRE (C), AP1 (A), or dyad (D) site was inserted into the enhancerless THCAT construct alone or in various combinations (i.e. C THCAT, A THCAT, D THCAT, AD THCAT, AC THCAT, DC THCAT, ADC THCAT; Fig. 4A). In CATH.a cells, C THCAT gave full expression (100–120% of −0.272 THCAT). D THCAT activity did not exceed that of the enhancerless THCAT construct. Whereas A THCAT partially increased CAT activity (to 35–20% of −0.272 THCAT), AD THCAT resulted in a synergistic increase to about 45%. However, when the AP1 and dyad/E box elements were combined with the CRE, CAT expression did not significantly exceed that obtained with the CRE alone (Fig. 4B).

The synthetic enhancer constructs used in Fig. 4B contained a partial CRE at −44 to −38 as part of the −44 to +27 THCAT promoter region. The partial CRE, 5′-GACGTCG-3′, is shown underlined in Fig. 4A; it contains 7 out of 8 bp of the consensus CRE, 5′-TGAGCTC-3′. Given the low level of CAT expressed by the enhancerless THCAT construct, the partial CRE contributes little enhancing activity by itself under basal conditions (Fig. 4B). In fact, CAT activity obtained with THCAT was less than that obtained with puCCAT, an enhancerless and promoterless construct (data not shown). In addition, the THCAT construct containing the partial CRE is not cAMP-responsive (Fig. 6, A and B). Nevertheless, it was of concern that this partial CRE might interact or synergize with synthetic enhancer sites. Hence a second set of minimal enhancer constructs was prepared in which the partial CRE was mutated to give −38 THCAT (see base pairs in parentheses in Fig. 4A). CATH.a cells transfected with these constructs gave similar results (Fig. 4C) to those obtained with constructs containing the partial CRE (Fig. 4B). The CRE increased expression to 77% of −0.272 THCAT. Interestingly, AD THCAT, lacking the partial CRE, gave about 65% activity, which was statistically similar to the C THCAT value. As in PC8b cells, the AP1 and dyad/E box sites work synergistically to direct transcription, since the AP1 and dyad/E box alone had little or no enhancing activity.

Distance Analysis—The finding that the synthetic AD THCAT can support 45–65% of the expression observed with −0.272 THCAT was surprising, since the AP1 and dyad/E box elements in their native positions (−205 to −182 bp) directed less than 20% CAT activity in the absence of the CRE site (Fig. 2, B–E). In AD THCAT, the AP1 element resides at −91 to −68 bp, suggesting that context and/or position of the AP1 and dyad/E box sites may influence expression. To investigate this further, another minimal AD construct was prepared in which the position of the site (−95 to −72 bp) was essentially the same, but sequences flanking the AD site corresponded to native TH sequences, rather than polylinker sites. This construct, −0.095/−0.072 AD THCAT, which has 25 bp of TH sequence 5′ to the AD site and 22 bp of TH sequence 3′ to the AD site, was even better at restoring expression, supporting 80% of the −0.272 THCAT level (Fig. 5, A and B). Together, these results suggest that the AP1 and dyad/E box sites work synergistically to direct THCAT transcription when positioned at −91 or −95 but cannot efficiently direct expression from their native position at −205 bp. One possibility to explain these results is that TH DNA sequences between −160 and −46 bp, which are present in the −0.272 CRE− and −4.8 CRE− but not in the AD THCAT minimal enhancer constructs, contain repressor elements that prevent AD function in the CATH.a cells. In PC8b cells, however, the AD is able to direct transcription when located at −205 bp as well as when close to the TH promoter (Fung et al., 1992; Yoon and Chikaraishi,
suggesting that such putative repressor elements do not function in PC8b cells.

Alternatively, it is possible that in CATH.a cells, only enhancers that are relatively close to the TH promoter (e.g. within 100 bp) function well. In this case, the native CRE site at −45 bp would be fortuitously positioned within this region. To test this possibility, the AP1 site at −205 was mutated to a CRE

site and the native CRE site at −45 was eliminated, essentially moving the CRE from −45 to −206 bp. This construct (−0.272 A>C) gave 90% the level of wild type −0.272 THCAT (Fig. 5, A and B), demonstrating that the CRE can effectively function at a more distant position, whereas the AP1 element cannot.

CAMP Induction—TH transcription is induced by cAMP through the CRE site in several TH-expressing cell lines including PC8b, PC12, and SK-N-BE(2)C cells (Lewis et al., 1983, 1987; Fader and Lewis, 1990; Carroll et al., 1991; Huang et al., 1991; Fung et al., 1992; Kim et al., 1993a, 1993b). In CATH.a
cells, 1 mM dibutyryl cAMP induced expression of −0.109 THCAT, −0.187 THCAT, and −0.272 THCAT constructs by approximately 50–150% (Fig. 6A). Since the smallest deletion construct, −0.109 THCAT, contains the CRE, it is likely that the CRE is, at least in part, able to mediate cAMP responsiveness. This is directly demonstrated by the fact that the CRE minimal enhancer construct (C THCAT) supported the same level of cAMP induction as did the intact −0.272 THCAT plasmid and mutation of the CRE site in −0.272 THCAT abolished induction, whereas mutations of the AP1 or dyad/E box did not (Fig. 6B). Together, these results suggest that the CRE is critical for both basal and cAMP-induced TH transcription.

**Dominant Negative Mutant CREB Analysis**—Transcription mediated by CRE and CRE-like sites involves the binding of transcription factors including those belonging to the CREB (CRE-binding protein), ATF (activating transcription factor), and CREM (cAMP response element modulator) families. CREB, ATF, and CREM proteins have a basic DNA binding domain adjacent to a leucine zipper dimerization domain and belong to a larger family of proteins, the bZip proteins. The bZip proteins bind to DNA as obligate homodimers or het-

**Fig. 5.** The AP1 and dyad/E box can direct THCAT transcription when located close to the TH promoter but not when located at −205/−182 bp; the CRE can direct THCAT transcription when located close to the promoter and when it is moved to the AP1 site. A, diagram of the AD THCAT minimal enhancer construct, −0.095/−0.072 AD THCAT, −0.272 CRE (CRE mutant −0.272 THCAT), −0.272 A/C, and wild type −0.272 THCAT constructs. Distances from the TH promoter of the AP1, dyad/E box, and CRE sites are indicated underneath in parentheses. Boxed regions represent authentic TH sequences, and dashed lines represent polylinker sequences. B, normalized CAT activity in CATH.a cells transiently transfected with constructs shown in A is expressed as in previous figures. −0.272 CRE − data is taken from Fig. 2B. The normalized value for −0.272 THCAT in CATH.a cells was 125.9 ± 5.3 (n = 4).
decreased basal and cAMP-induced 4.8 THCAT expression by 50–60%, suggesting that CREB, ATF-1, and/or CREM proteins contribute to basal and cAMP-induced TH transcription.

**KCREB reduced 0.272 THCAT expression similarly (data not shown).**

**DISCUSSION**

We investigated transcriptional regulation of the rat TH gene in TH-expressing peripheral nervous system and central nervous system cell lines derived from transgenic mice bearing TH-expressing tumors. PATH.2 cells are peripheral nervous system-derived; CATH.a and CATH.b cells are central nervous system-derived, and are the first catecholamnergic central nervous system cell lines used to map TH regulatory elements. Deletional analysis performed with THCAT constructs containing various lengths of 5'-TH-flanking DNA suggests that regions within 0.272 kbp of the transcription start site are sufficient and necessary for TH expression (Fig. 1, A–D). Site-directed mutagenesis of the CRE (5'–TGACGTCA–3'), located at −245 to −238 bp, diminished CAT activity to near background levels. Site-directed mutagenesis of the AP1 (5'–TGATTCA–3') and the partially overlapping dyad/E box sites (5'–TGATTCA-GAGGCAGGTGCCTGTGA–3'), located at −205 to −182 bp, reduced CAT activity between 20 and 40% (Fig. 2, A–E). These results suggest that the CRE is critical for TH transcription in these cells, whereas the AP1 and dyad/E box sites have a less significant role. In CATH.a cells, a minimal enhancer construct, consisting of one copy of the CRE inserted in front of the TH promoter, gave 80–120% of −0.272 THCAT expression. The AP1 minimal enhancer construct increased CAT activity slightly (15–20%), but together with the dyad/E box increased CAT activity to 45–80% of wild type levels (Figs. 4, A–C, and 5, A and B). The dyad by itself had no enhancer activity. Therefore, the results of deletion analysis, site-directed mutations, and synthetic enhancer constructs demonstrate the prime importance of the CRE for TH transcription in these cells, whereas the AP1 and dyad/E box sites have a less significant role.

Kim et al. (1993a), in a previous study using PC12 (a rat pheochromocytoma line) and SK-N-BE(2)C (a human peripheral nervous system neuroblastoma line), obtained similar results. They showed that mutation of the rat TH CRE site abolished expression, whereas deletion of 5' sequences containing the AP1 and dyad/E box sites reduced expression by 40%. Thus, it appears that the CRE is essential for TH expression in both central nervous system-derived CATH.a and CATH.b cells and peripheral nervous system-derived PATH.2, PC12, and SK-N-BE(2)C cells, whereas the AP1 and dyad/E box sites are less important. Thus, the CRE was more critical than the CRE, whose mutation reduced expression by 50% (Yoon and Chikaraishi, 1992). As in the CATH and PATH lines, the AP1 and dyad elements had little or no enhancer activity by themselves and needed to work together to support transcription in PC8b cells. In summary, the same elements, AP1, dyad/E box, and CRE, seem to be important in a variety of TH+ cell lines including CATH.a, CATH.b, PATH.2, PC12, SK-N-BE(2)C, and PC8b. However,
the relative contribution of each element to TH transcriptional activation varies among lines.

A region between −503 and −578 bp has also been shown to directly regulate TH expression in another PC12 line (Gandelman et al., 1990; Wong et al., 1994). Deletions that lacked the −503/−578 bp region but retained the CRE reduced expression by 66%. A deletion construct containing only the CRE gave very low expression, suggesting that the CRE is not important or only works in conjunction with the −503/−578 bp region. Differences between these data and those of Kim et al. (1993a) may reflect differences among the PC12 cells carried in different laboratories.

In CATH.a cells, the AD (AP1 and dyad/E box) element cannot support transcription when located at its native position (−205 bp) as demonstrated by the low CAT activity obtained from the −0.272 CRE and −4.8 CRE constructs (Figs. 2, A–D, and 5, B). However, restoration (45–80%) of CAT activity was obtained with three AD minimal enhancer constructs; two constructs positioned the AD element at −91 bp surrounded by linker sequences, and the other placed the AD at −95 bp surrounded by native TH sequences (Figs. 4, A–C, and 5, A and B). This suggests two possibilities. The first is that position itself is important such that only enhancers relatively close to the TH promoter function efficiently in CATH.a cells. The fact that a single copy of the CRE gives full expression when positioned at −206 bp (Fig. 5, A and B) would argue against this, although it is possible that only the AD and not the CRE is subject to position dependence. Alternatively, TH DNA sequences between −160 and −46 bp, which are present in the −0.272 CRE and −4.8 CRE but not in the AD THCAT minimal enhancer constructs, may contain repressor elements that prevent AD function. Since the AD is able to direct transcription in PC8b cells when positioned at −205 bp, as well as when located close to the TH promoter (Yoon and Chikaraishi, 1992), the putative position or repressor effect may be cell line-specific. Importantly, the finding that the AD can efficiently support reporter expression in CATH.a cells suggests that differences between various cell lines may not simply be due to the absence of factors that can activate at the AP1, dyad/E box, or CRE sites.

It is possible that, in vivo, different populations of TH-expressing cells or cells at certain stages of development differentially rely on the CRE, AP1, and dyad/E box, or other sites for TH transcription; this would allow for finer regulation among various TH-expressing populations. An analysis of the regulation of TH transcription in various cell groups and during development requires studies in transgenic mice, similar to those performed in cultured cells. At present, transgenic studies indicate that the regulation of TH transcription is complex and probably involves multiple positive and negative elements located further upstream of those elements required in cultured cells. Transgenic studies by Suri et al. (1993), Min et al. (1994), and Liu et al. (1994) suggest that crucial DNA elements reside between −9 and −0.773 kbp of 5’-flanking region of the rat TH gene. It is likely that upstream elements which direct expression in different groups of TH-expressing cells in vivo work in conjunction with proximal elements like the CRE to direct basal transcription and mediate responses to various physiological stimuli.

CRE motifs are of major importance for transcriptional regulation and cAMP induction of several other neuronally expressed genes including somatostatin (Montminy et al., 1986; Andrisani et al., 1987; Powers et al., 1989; Leonard et al., 1992), vasoactive intestinal peptide (Tsukada et al., 1987; Fink et al., 1988; Fink et al., 1991), proenkephalin (Comb et al., 1986, 1988), and dopamine ß-hydroxylase (Ishiguro et al., 1993; Lamouroux et al., 1993; Kim et al., 1994). Transcriptional control of these genes involves the binding of transcription factors to the CRE motif (for reviews see Goodman (1990), Habener et al. (1990), Meyer and Habener (1993), and Lee and Mason (1993)). These transcription factors include related families of CREBs (Montminy and Bilezikjian 1987; Yamamoto et al., 1988; Hoeflir et al., 1988, 1990; Yamamoto et al., 1990; Ruppert et al., 1992), ATFs (Hai et al., 1989; Gaire et al., 1990; Yoshimura et al., 1990), and CREMs (Foulkes et al., 1991a, 1992; Laide et al., 1993), all of which have a basic DNA binding domain adjacent to a leucine zipper dimerization domain so that they bind to DNA as homodimers or heterodimers. ATFs are capable of selectively forming heterodimers with each other (Hai et al., 1989). In addition, ATF-3/CREM (Hurst et al., 1990; 1991; Rehfuss et al., 1991; Liu et al., 1993) and CREM/CREM (Foulkes et al., 1991a, 1991b; Laide et al., 1993; Hummeler et al., 1994) heterodimers bind to CREs.

Of the bZip proteins, a likely candidate for mediation of basal and cAMP-induced TH transcription is CREB-341, which has been purified from PC12 cells (Montminy and Bilezikjian, 1987) and rat brain (Yamamoto et al., 1988). CREB-341 has been shown to bind to the somatostatin CRE as a dimer (Montminy and Bilezikjian 1987; Yamamoto et al., 1988; Gonzalez et al., 1989). It becomes phosphorylated at serine 133 when cAMP-dependent protein kinase is activated by increased intracellular cAMP levels (Montminy and Bilezikjian, 1987; Gonzalez and Montminy, 1989); this phosphorylation increases somatostatin transcription through an associated co-activator, CBP (Yamamoto et al., 1988; Gonzalez and Montminy, 1989; Chrvia et al., 1993; Kwo et al., 1994). An alternatively spliced form of CREB-341, termed CREB-327, which is also an activator, has been described (Hoeflir et al., 1988, 1990; Yamamoto et al., 1990; Ruppert et al., 1992). It is also possible that CREB-327, ATFs, and/or CREM (a CREM activator) mediate basal and cAMP-induced TH transcription. Widnell et al. (1994) have shown that CATH.a cells contain CREB-327/341, but the presence of other factors has not been assayed.

To determine whether CREB-327/341, ATF-1, or CREM proteins direct −4.8 THCAT transcription, we compromised their function by co-expressing a dominant-negative mutant of CREB, KCREB. KCREB is identical to CREB-327 except for a point mutation which prevents binding to DNA. However, KCREB retains the ability to selectively dimerize with the endogenous activators CREB-327/341, ATF-1, and CREM. Hence, KCREB prevents these proteins from binding to the CRE and thereby prevents activation of transcription (Walton et al., 1992). CREB-327/341, ATF-1, and/or CREM (or an unknown CREB dimerizer) contribute to basal and cAMP-induced TH transcription in CATH.a cells (Fig. 7).

The lack of complete inhibition may be due to our inability to express sufficient amounts of KCREB to sequester all endogenous CREB proteins within the time frame of the experiment. Alternatively, residual expression may be mediated by CRE-binding proteins that do not dimerize with CREB (or KCREB) such as CRE-BP1 (Maekawa et al., 1989; Kara et al., 1990; Benbrook and J ones, 1990; Macgregor et al., 1990; Matsuda et al., 1991) and CRE-BP2 (Ivashkiv et al., 1990); it is possible that TH transcription is in part mediated by such proteins. Finally, it is possible that when endogenous TH CRE-binding proteins are inactivated by KCREB, there is functional compensation by other proteins that do not normally direct TH transcription.

In summary, these results represent the first attempts to map specific DNA regulatory elements that direct TH expres-
sion in central nervous system cells. The finding that the proximal region (~0.272 kb) and, in particular, the CRE site alone can support TH basal and cAMP-induced expression suggests that the CATH.a, CATH.b, and PATH.2 cell lines may be similar to some PC12 cell lines and SN-K(NE2)C(2) cells (Kim et al., 1993a) but different from PC8b cells (Cambi et al., 1989; Fung et al., 1992; Yoon and Chikaraishi, 1992) and other PC12 lines (Gandelman et al., 1990; Wong et al., 1994). These differences may be due to differences in the relative amounts or efficacy of various transcription factors in different cell lines; such differences may exist in vivo among different populations of TH-expressing cells or among TH-expressing cells at different stages of development.

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