Identification and cell type specificity of the tyrosine hydroxylase gene promoter

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ABSTRACT

Genomic DNA encoding the rat tyrosine hydroxylase (TH) gene was isolated from a lambda phage library using a nick-translated fragment from a cDNA clone for rat TH. We have determined the initiation site for TH RNA synthesis and have sequenced 1100 bases of the primary transcript and 5' flanking region. The 5' end of the transcript is the same in several rat tissues in which TH is expressed as well as in rat pheochromocytoma cells (PC). RNA prepared from PC cells that had been stimulated with dexamethasone also mapped to the same transcription start site. Sequence upstream from the initiation site contains the canonical TATA box, but no apparent CAAT box. When a portion of the 5' flanking region of the TH gene (-773 to + 27) is fused to the chloramphenicol acetyltransferase (CAT) gene, it promotes expression of CAT in pheochromocytoma cells and GH₄ cells, but not in two neural tumour lines, RT4-D and B103, nor in several non neural cell lines. This suggests that this region of the TH gene has features that confer tissue-restricted expression on the TH promoter.

INTRODUCTION

Tyrosine hydroxylase (TH) converts tyrosine to dihydroxyphenylalanine (DOPA), the rate-limiting step in the catecholamine pathway generating the neurotransmitters dopamine, norepinephrine, and epinephrine. TH is expressed in the catecholaminergic neurons of both the peripheral and central nervous system and in chromaffin cells of the adrenal medulla which are of neural crest origin. The control of this enzyme is under complex regulation affecting both its rate of synthesis (induction) and its level of activity via post-translational phosphorylation of pre-existing enzyme molecules (activation).

In vivo, TH enzyme activity has been shown to increase during development (1) and to be modulated by factors such as glucocorticoids (2, 3), nerve growth factor (reviewed in ref. 4) and trans-synaptic neuronal activity (1, 5, 6). In cultured adrenal chromaffin cells and transformed pheochromocytoma cells (PC) derived from such cells, TH activity is regulated by derivatives of cAMP (7, 8, 9) and epidermal growth factor (10) as well as by

glucocorticoids (11, 12, 13) and nerve growth factor (7, 12, 14, 15). Recently, the regulation of TH enzyme activity has been correlated with changes in TH RNA levels under conditions of increased trans-synaptic activity in adult rats (16, 17, 18, 19) and in pheochromocytoma cells treated with dexamethasone and cAMP (20, 9).

Previously, Lewis et al. (9) reported the isolation of a partial rat cDNA clone for TH. In the present work, this clone was used to isolate rat genomic DNA that contains TH encoding sequences. Using a nearly full length cDNA clone for TH, Grima et al. (21) have determined the sequence of the presumed protein coding regions of the gene but not the start site of transcription. We now report the identification of the transcription initiation site for TH RNA and the complete sequence of the first exon, as well as the sequence of the promoter region for this gene. Transfection studies with a portion of the TH 5' flanking region and the reporter gene chloramphenicol acetyl transferase (CAT) show that this region operates as an authentic promoter in vivo and that its expression is limited to a subset of cells tested.

A few tissue specific eukaryotic genes have been found to utilize alternate promoters in different tissues or during development, e.g. mouse amylase (22,23) and Drosophila alcohol dehydrogenase (24). We have performed a partial survey of TH expressing tissues to examine the possibility that different promoters may be used for TH RNA synthesis in these tissues. The 5' end of the message for TH has also been mapped for RNA synthesized in the presence of dexamethasone which stimulates the accumulation of TH mRNA in pheochromocytoma cells (9). Our results suggest that we have identified the promoter at which the majority of TH RNA transcripts initiate in all the cell types we have investigated. Interestingly, however, the ratio of precursor RNA to mature messenger varies in the different tissues.

MATERIALS AND METHODS

Cell culture:

All cell lines used in these experiments were derived from rat tissues except for the mouse Ltk- cells.

PC12 cells (25) are a pheochromocytoma line that contains relatively high levels of tyrosine hydroxylase and responds to glucocorticoid treatment with increased TH (11). PC12 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Sterile Systems) and 5% horse serum (Sterile Systems). Glucocorticoid stimulated cells were treated

with 10⁻⁸ M dexamethasone in the above medium plus serum for 24-48 hours.

RT4-D cells (26) were grown in DMEM supplemented with 5% fetal calf serum and 5% neonate calf serum. B103 cells (27), Ltk- cells (gift of Harold Weintraub) and FR3T3 cells (28) were grown in DMEM with 10% fetal calf serum. GH₄ cells (29), a derivative of the GH₃ pituitary line, were cultured in F10 medium with 15% horse serum and 2.5% fetal calf serum.

Phage and plasmids:

The rat genomic library in lambda Charon 4A was a gift of Drs. T. Sargent, R. B. Wallace, and J. Bonner. It was constructed from a partial Hae III digest of liver DNA from a single male Sprague-Dawley rat which was ligated to Eco RI linkers and cloned in the EcoRI site of Charon 4A. Lambda recombinants containing TH DNA inserts were selected by plaque hybridization (30) with a ³²P-nick-translated, gel purified fragment of cDNA-TH (9) that contained 280 bps of DNA extending from the Pst I site to the Kpn I site. A recombinant containing TH genomic DNA was subcloned in pBR 322 and M13 vectors and several of these subclones were transferred into the riboprobe system vector pGEM 1 (Promega-Biotec).

DNA preparation:

Lambda DNA was prepared as described by Kao et al. (31). Plasmid DNA was isolated from cleared lysates by cesium chloride-ethidium bromide centrifugation or from miniprep lysates by the procedure of Birnboim and Doly (32). Single-strand M13 DNA was prepared from phage supernatants as previously described (33).

Dot hybridization with labeled nascent RNA from isolated nuclei:

Confluent PC12 cells were rinsed twice in cold phosphate buffered saline and removed from tissue culture plates by scraping in 1 ml cold 0.22 M sucrose, 50 mM Tris pH 7.6, 10 mM NaF, 1 mM MgCl₂, and 2 mM β-mercaptoethanol per 90 mm plate. The cells were pelleted at 4°C by centrifugation at 3000 rpm in a Sorval centrifuge and resuspended at 2x10⁷ cells/ml in the same sucrose buffer. NP40 and Triton X-100 were each added to 0.05% and the cells homogenized with 20 strokes in a glass homogenizer to lyse plasma membranes. The lysate (in 0.8 ml aliquots) was layered over one-half volume of 0.35 M sucrose, 50 mM Tris pH 7.6, 10 mM NaF, 1 mM MgCl₂, and 2 mM β-mercaptoethanol and the nuclei pelleted by centrifugation in a Beckman microfuge 11 at 8000 rpm (4200xg) for 4 minutes at 4°C. The nuclear pellet was resuspended at 4x10⁸ nuclei/ml in five volumes of 20 mM Tris pH 7.9, 50 mM (NH₄)₂SO₄, 5 mM MgCl₂, 2 mM MnCl₂, 6 mM β-mercaptoethanol, 6 mM NaF, 1 mg/ml heparin, 0.25 mM aurintricarboxylic acid (ATA), 600 μM each of ATP, CTP, GTP, and 20 μM

[α - 32 P]UTP (800 Ci/mmol, New England Nuclear). The nuclei were incubated for 30 minutes at 22°C to elongate nascent RNA chains, after which an equal volume of 10 mM Tris pH 7.5, 10 mM NaCl, 10 mM MgCl₂, 0.5 mg/ml *E. coli* tRNA and 20 ug/ml of affinity purified (34) DNase I (Worthington) was added. The lysate was digested for 15 minutes at 37°C to reduce its viscosity. The DNase I digestion was terminated by addition of two volumes of 200 mM NaCl, 40 mM Tris pH 7.5, 5 mM EDTA, 0.1% NaDodSO₄, 0.25 mM ATA and 375 ug/ml of pre-digested proteinase K (SIGMA). The proteinase K digestion mixture was incubated at room temperature for 20 minutes and then extracted twice with phenol:chloroform:isoamylalcohol (24:24:1) and ethanol precipitated. The precipitate was dissolved in 10 mM NaCl, 10 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM ATA and 10 ug/ml DNase I and incubated for 60 minutes at 25°C to digest DNA to acid soluble fragments. The lysate was treated again with proteinase K, extracted twice with phenol:chloroform:isoamylalcohol (24:24:1) and precipitated with trichloroacetic acid as described by McKnight and Palmiter (35). The incorporation ranged from between 0.3-1 cpm/cell.

Single-stranded M13 DNAs containing 0.2 ug of TH sequences from various regions of the TH gene (0.6 to 3 ug total DNA) were denatured by boiling in 100 ul of 0.1 M NH₄OH and neutralized in 500 ul of 1M ammonium acetate and applied to Nytran membranes (Schleicher and Schuell) using a Hybridot manifold (BRL). The origin of the M13 DNAs from the lambda genomic clone is as shown in Figure 1A, with the exception of EH3.2 which contains the original cDNA and was isolated from an overlapping genomic clone containing the 3' end of the gene. After baking and prehybridization, the filter was annealed to labeled RNA at 4×10^6 cpm/ml for 40 hours at 65°C in 0.9 M NaCl, 50 mM NaPO₄, pH 7.7, 5 mM EDTA 0.2% NaDodSO₄, 300 ug/ml heparin and 200 ug/ml *E. coli* tRNA. (Heparin was used in place of Denhardt's solution and carrier DNA according to the procedure of Singh and Jones (36).) The filter was washed once for 15 minutes at room temperature in 2xSSC, 0.1% NaDodSO₄ (1xSSC = 0.15 M NaCl, 0.015 M Na citrate pH 7.0) and once at 65°C in 2xSSC, 1xSSC, 0.5xSSC, 0.2xSSC, 0.1xSSC each containing 0.1% NaDodSO₄.

RNA preparation:

Total RNA was prepared using the lithium chloride-urea precipitation method (37). Tissue culture cells were rinsed twice in phosphate buffered saline (PBS) and then scraped from the plates in 2 mls PBS using a rubber policeman. Cells were pelleted by centrifugation and resuspended in 3M LiCl, 6M urea, 10 mM vanadyl ribonucleoside (BRL or Life Sciences) at a concentration of approximately 10^7 cells/ml. To reduce viscosity, samples

were sonicated briefly using a Branson sonifier with a microtip. The lysed cell suspensions were held on ice 24 hours and then centrifuged for 10 minutes at top speed (11,600 xg) in a Beckman microfuge 11 at 4° C. Supernatants were removed and the pellets were resuspended in 10 mM Tris-HCl, pH 7.5, 10 mM EDTA and 0.2% SDS. After three extractions with phenol:chloroform:isoamylalcohol (24:24:1) plus 0.1% hydroxyquinoline, the RNA samples were precipitated with 2.5 volumes absolute ethanol in the presence of 0.1M sodium acetate. The ethanol precipitated pellets were resuspended in 10mM Tris-HCl, pH 7.5, or H₂O and concentrations were determined by OD₂₆₀ absorbance. RNA samples were stored at -20° C. In some cases, total cellular RNA was prepared by lysing cells in 6M guanidinium thiocyanate and pelleting RNA through a 5.7M CsCl solution (38).

Total RNA from rat tissues was prepared from Sprague-Dawley rats killed by ether treatment. Tissues were removed from freshly killed animals and placed in 3M LiCl, 6M urea, 10mM vanadyl ribonucleoside. After either manual or motor driven homogenization, samples were sonicated and RNA was prepared as described above.

Nuclease protection mapping:

Single-stranded M13 DNA was digested with Sau 3A, end-labeled, hybridized with RNA and treated with S1 nuclease as described (39).

Transcription mapping with labeled cRNA was performed by modification of the procedure of Melton et al. (40). Recombinant plasmid pGEM SS.610 was cleaved with Pvu II which cuts downstream of the insert DNA. Approximately 1 ug of linearized DNA was added to a solution containing 40mM Tris-HCl, pH 7.9, 6mM MgCl₂, 2mM spermidine, 4 U/ul RNAsin (Promega Biotec), 10mM NaCl, 10mM dithiothreitol, 500uM ATP, 500uM CTP, 500uM GTP, 13-18uM [α -³²P]UTP (600-800 Ci/mMole, NEN) and 5-10 units SP6 polymerase (NEN) in a total volume of 5-10 ul. The transcription reactions were incubated at 42° C for 1 hour. Then 1 ul 5mM ATA, 1 ul RNAsin (40 U/ul), and 1 ul DNase I (1 U/ul, Promega Biotec) were added and the reactions were incubated an additional 20 minutes at 37° C. 10 ul 90% formamide containing 0.1% xylene cyanol and 0.1% bromophenol blue were added, samples were boiled for 3 minutes and chromatographed by electrophoresis through an 8M urea - 8% polyacrylamide gel at 45°-50° C. The region containing the labeled cRNA transcript was determined by autoradiography and the cRNA removed from the gel by passive elution overnight at 37° C in 0.5M ammonium acetate, 0.1% NaDodSO₄, 1mM EDTA. Approximately two thirds of the RNA could be eluted by this procedure. Following extraction with phenol:chloroform:isoamyl alcohol, the ³²P-RNA was

ethanol precipitated from the ammonium acetate solution in the presence of carrier tRNA and additional sodium salt. As calculated from the specific activity of the [α - 32 P]UTP, the labeled RNA had a final specific activity of approximately 10^9 dpm/ug. In some cases, cRNA was synthesized in the presence of a 10 fold excess of cold UTP reducing the specific activity to 10^8 dpm/ug.

Labeled cRNA (.24 or .48 ng) and the indicated amount of unlabeled RNA were mixed and ethanol precipitated (carrier tRNA was added when necessary). The pellets were redissolved in 20 ul 72% formamide, 40mM PIPES, pH 6.8, 0.4M NaCl, 1mM EDTA and overlaid with mineral oil. Samples were heated at 85 $^{\circ}$ -90 $^{\circ}$ C for 5 minutes and then incubated at 50 $^{\circ}$ C 12-18 hours. After hybridization, 200 ul of 0.3M NaCl containing either 20 ug/ml RNase A (Boehringer Mannheim) or 300 U/ml RNase T1 (Boehringer Mannheim) were added, samples were centrifuged, transferred to fresh microfuge tubes and incubated at room temperature for 2 hours. Following nuclease digestion, hybridization mixtures were extracted twice with saturated phenol (or phenol:chloroform:isoamyl alcohol, 24:24:1) and precipitated with 2.5 volumes ethanol in the presence of 10-15 ug carrier tRNA. The size of the nuclease resistant cRNA was determined by electrophoresis through 8M urea - 6% polyacrylamide gels.

DNA sequencing:

Single-strand DNA from M13 recombinants was used as template for sequencing reactions using the dideoxy-chain terminating method as previously described (33). A 15 base oligonucleotide (BRL) homologous to the M13 region immediately preceding the restriction sites used for cloning was used as primer for the reaction. An 18 base primer (described below) was used for sequencing reactions in which the 5' end of the RNA transcript was determined. Sequencing products were analyzed on 0.4 mm thick 8M urea - 8% polyacrylamide gels. Gels were run at 45-50 $^{\circ}$ C, fixed in 8% methanol - 8% acetic acid, dried onto filter paper and exposed to Kodak XL-1 film.

Primer extension:

An 18 base oligonucleotide complementary to the initiation site region for protein translation was synthesized from known sequence information (21) at the DNA synthesis facility, Institute for Molecular and Cellular Biology, Indiana University, using an Applied Biosystems automated DNA synthesizer and purified by polyacrylamide gel electrophoresis. The primer was end-labeled by incubation with [γ - 32 P]ATP (7000Ci/mmol, ICN) and polynucleotide kinase (IBI) at 42 $^{\circ}$ for 30-45 minutes. Unincorporated [γ - 32 P]ATP was removed by

multiple precipitations with 3 volumes absolute ethanol in the presence of carrier tRNA plus 60mM Na acetate, and 2.5mM MgCl₂ (final concentration). Specific activity of the primer ranged from 0.5 to 1×10^9 cpm/ug.

10 ug total PC12 RNA was hybridized with $2-3 \times 10^5$ cpm primer in 50mM NaCl, 50mM Tris, pH 8.4, 6mM MgCl₂. Mixtures were heated at 80° C for 4 minutes and slow cooled to 42° C. Dithiothreitol was added to a final concentration of 10mM and deoxyribonucleoside triphosphates to 400 uM each. Four to five units of avian myeloblastosis reverse transcriptase (Life Sciences) were added and the samples were incubated at 42° C for 30 minutes. The reactions were terminated by the addition of 2 volumes of 94% formamide, 5mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue. Samples were heated at 80-85° C for 3 minutes immediately prior to loading on sequencing gels.

Cell Transfection and CAT assays

An 800 bp fragment of λ -TH extending from the XhoI site at -773 to an AluI site at +27 was cloned into the polylinker site of pUC-CAT (gift of Dr. Richard Morimoto) as described (E. Lewis et al., in press). The resulting construct, designated p5'TH-CAT, places the AluI site of TH just upstream from the endogenous transcription initiation site of the CAT gene. pUC-CAT itself carries no promoter or enhancer elements.

All cell lines, except for GH₄ cells, were transfected with 5 ug of p5'TH-CAT or pUC-CAT or 2 ug of RSV-CAT (41) per 100 mm plate by the calcium phosphate co-precipitation method (42). GH₄ cells were transfected with identical amounts of the plasmid DNAs using the DEAE-dextran sulfate method as described by Camper et al. (43).

Two days following transfection, CAT enzyme levels in the cell cultures were determined according to the method of Gorman et al. (44).

RESULTS

Selection and restriction mapping of a TH genomic clone:

The isolation of a 400 bp partial cDNA clone for TH in our laboratory has previously been described (9). A 280 bp fragment of this clone was gel isolated, nick-translated and used to screen a lambda library containing Sprague-Dawley rat genomic DNA. The restriction map of a TH positive clone containing approximately 12 kb of genomic DNA is shown in Figure 1A. The cDNA used to select this recombinant maps to the 3' end of the insert as indicated by the stippled boxes and appears to contain partial sequences from 2 exons (unpublished results). Restriction fragments of the λ -TH recombinant were subcloned in pBR 322, M13 and pGEM I vectors. A recombinant is designated by the terminal restriction sites of the insert and its size. The

Figure 1. (A) Restriction map of TH genomic DNA in lambda recombinant selected from the Hae III library. The position of DNA sequences contained in M13 recombinants is shown below the map (S = SstI); EH3.2 was cloned from a second genomic recombinant (not shown) which overlaps the 3' end of the lambda clone diagrammed and extends further downstream. Stippled boxes denote sequences coding for cDNA-TH; solid boxes represent lambda DNA. (B) DNA dot hybridization. M13 recombinants containing sequential stretches of TH DNA were adsorbed to Nytran and hybridized with 32 P-labeled nascent RNA elongated from isolated PC12 nuclei. The M13 dot contains 3 ug of nonrecombinant M13 mp11 DNA; the other samples contain the (+) or (-) strands of the M13 clones shown in A. (C) Restriction map of subcloned genomic DNA indicated (see text). The position of M13 recombinants is shown below map (S = Sau 3A). Wavy line denotes RNA. (D) Transcription mapping of end-labeled SS.610. Approx. 2 ng of single-stranded DNA were annealed with 5 ug of total RNA at 65°C overnight and digested with S1 nuclease followed by separation on a denaturing polyacrylamide-urea gel and band detection by autoradiography. Lane M, end-labeled SV40-Eco RII fragments; lane 1, undigested SS.610 probe; lane 2, *E. coli* rRNA; lane 3, RNA from control PC12 cells; lane 4, RNA from dexamethasone-treated PC12 cells.

strand orientation of M13 recombinants is indicated as (+) for the protein coding strand and (-) for the strand that hybridized to mRNA (anti-sense).

Determination of the initiation site for TH RNA synthesis:

The portion of the TH genomic clone that is transcribed into TH RNA was roughly determined by annealing labeled nascent RNA to DNA dots containing different regions of the recombinant DNA. The RNA was labeled by incubating isolated pheochromocytoma nuclei in the presence of radioactive

ribonucleoside precursors. The labeled "run-on" RNA should hybridize with equal stoichiometry to all portions of the genomic DNA transcribed into TH precursor RNA. Figure 1B shows a dot blot of M13 recombinants hybridized with 32 P-labeled, nascent PC12 cell RNA. The EH3.2 recombinant which encodes the original 0.4 kb cDNA shows a positive signal as do the (-) strands of the adjacent 5' fragments SS3.1 and SS2.5. However, the next fragment upstream, SS1.9 fails to anneal to "run-on" RNA in either orientation. This suggests that little or none of this recombinant is transcribed, in which case, the 5' boundary of TH precursor falls somewhere near the 3' end of SS1.9. To investigate this region further, the 1.5 kb Bam HI fragment (BB1.5) containing the 3' end of SS1.9 and the presumptive initiation site for TH RNA synthesis was cut with Sau 3A and recloned in M13 mp 7 at the Bam HI site. Four of these recombinant phage and the positions to which they map are indicated in Figure 1C.

Nuclease protection mapping with these recombinants was used to further localize the initiation site for TH RNA synthesis. Single strand M13 DNAs, containing the individual Sau 3A fragments, were digested with Sau 3A, end-labeled, hybridized with total PC12 RNA, digested with S1 nuclease and the protected fragments analyzed on denaturing urea-acrylamide gels. TH fragments downstream of SS.610 were fully protected suggesting that they were transcribed in their entirety, whereas end-labeled SS.610 was only partially protected (Figure 1D). RNA from both control and dexamethasone induced PC12 cells protects bands at approximately 444 and 250 bases (Figure 1D, lanes 3 and 4). No full length protection of end-labeled SS.610 DNA was detected. These results indicate that the probable start site for TH RNA synthesis falls approximately 444 nucleotides from the 3' end of the SS.610 fragment. Although the 250 nucleotide fragment could represent a second initiation site, the data below do not support this interpretation.

Sequence and primer extension mapping of the 5' end of TH RNA:

M13 recombinants containing the Sau 3A fragments of BB1.5 protected by PC12 cell RNA were sequenced in both orientations as described in Materials and Methods. The results are shown in Figure 2.

Hybridization of biosynthetically-labeled genomic TH DNA to pheochromocytoma RNA had demonstrated the presence of a single 125 b exon in the 1.5 kb Bam HI fragment which mapped to the SS.610 region (unpublished data, also see Figure 4B). The coding portion of this exon was precisely located by comparison of our sequence with that of the TH cDNA sequence published by Grima et al. (21) indicating that translation presumably begins

GATC

at the A at position +36 in our sequence. An 18 base oligonucleotide complementary to the region surrounding the translation initiation site (underlined bases in Figure 2) was synthesized and used for primer extension assays to determine the precise site of transcription initiation. Labeled primer was annealed to total RNA from PC12 cells and extended with reverse transcriptase at 42° C. The products from this reaction are shown in Figure 3 alongside a DNA sequence ladder generated by using the same 18 b primer. The results of this experiment indicate that the transcription of TH DNA begins 35 bases upstream from the translation initiation site at the T residue marked +1 in Figure 2. This site corresponds to the +1 position of the estimated 444 nucleotide band protected by S1 nuclease in Figure 1D. Primer extension assays were also performed at 52° C to further destabilize any RNA secondary structures that may have contributed to artificial stops. In these experiments reverse transcription terminated at the same T residue (data not shown). We realize that it is unusual for transcription to initiate at a T residue; it is possible that termination at the T residue is

Figure 3. Primer extension mapping of 5' end of TH-RNA from PC12 cells. An 18 base oligonucleotide complementary to the TH RNA translation initiation site was annealed to PC12 RNA and incubated with reverse transcriptase as described in Materials and Methods. The primer extended samples were denatured and run on 0.4 mm thick 8 M urea-8% polyacrylamide gels alongside the sequencing products of M13-BB1.5+ DNA using the same 18 base primer. Lanes A, C, G, and T are the sequencing products of BB1.5+; lanes 1 and 2 contain 0.2 ul and 0.4 ul, respectively, of the primer extension reaction. The base sequence on the left is that of the template strand (anti-sense) for TH RNA synthesis which corresponds to the sequencing ladder that can be read in the figure.

an artifact of reverse transcription on capped RNA and that transcription actually begins at the G residue at +2 or, if reverse transcription is blocked by the cap, the actual start site may be at -1 or -2.

Tissue distribution of TH 5' ends:

To determine whether there is a single, primary transcription start site for TH RNA synthesis, in vitro labeled cRNA (40) from the SS.610 fragment cloned in pGEM I was used for transcription mapping RNA from a variety of TH expressing tissues as described in Materials and Methods. The use of high specific activity cRNA provided a sensitive method for simultaneously

detecting precursor and mRNA (exon). The design of the experiment and our interpretation of the results, which we discuss below, are diagrammed in Figure 4A. Figure 4B shows the results of these experiments for total RNA from a set of rat tissues known to express TH, as well as, a control tissue, liver, that does not contain TH. In all TH containing tissues a TH RNA exon band at 125 bases is observed. Neither this band nor any others are seen with liver RNA (lane 3). A protected band at 444 bases is seen in RNAs from the adrenal, olfactory bulb, midbrain tegmentum and superior cervical ganglion (lanes 5-8, respectively). This band is faint in the olfactory bulb and tegmentum lanes; the inset below the figure shows a longer exposure in which the 444 band is more visible for these two tissues. Differences in the ratio of the 444 b precursor band to the 125 b exon band may be due to different steady state levels of precursor RNA relative to processed mRNA in the various tissues. The lack of a detectable 444 base band for brain total RNA (lane 4) is presumably due to the low overall signal for TH RNA as indicated by the faintness of the 125 base exon band. The lack of a 444 base protected fragment for PC12 RNA (Figure 4B, lane 9) and the presence of two strong bands at 250 bases and 190 bases required further analysis.

The 250 base fragment is seen in transcription mapping experiments with PC12 RNA using both end-labeled and internally-labeled probe. The intensity of the 250 band relative to the 444 band, however, varied in experiments with end-labeled DNA (unpublished observations) and was quantitatively very different in experiments with end-labeled DNA and S1 nuclease as compared to internally-labeled cRNA and RNase A (Figure 1D vs. Figure 4B). A 250 base

Figure 4. Transcription mapping with internally labeled cRNA-TH. Samples were hybridized and digested as described in Materials and Methods. A. Schematic diagram of nuclease protection mapping using cRNA from pGEM SS.610. Open boxes represent exon RNA and lines correspond to intron RNA. Asterisks indicate internally labeled cRNA probe. The loop and stem structure shown represents a possible interpretation of our results and is discussed in the text. B. Transcription mapping with RNaseA. RNA:cRNA hybrids were digested with 20 ug/ml RNase A and analyzed on denaturing polyacrylamide gels. Lane 1, undigested cRNA probe from pGEM SS.610; lane 2, 50 ug tRNA; lane 3, 50 ug liver RNA; lane 4, 50 ug total brain RNA; lane 5, 25 ug adrenal RNA; lane 6, 25 ug olfactory bulb RNA; lane 7, 25 ug midbrain tegmentum RNA; lane 8, 2 ug superior cervical ganglion RNA; lane 9, 1.25 ug PC12 cell RNA. C. Transcription mapping with RNase A vs. RNase T1. All samples contain 5 ug tRNA in addition to dexamethasone treated PC12 RNA and cRNA probe. Hybrids were digested with either 20 ug/ml RNase A or 300 u/ml RNase T1. Lane 1, tRNA alone digested with RNase T1; lane 2, tRNA alone digested with RNase A; lane 3, 0.25 ug PC12 RNA digested with RNase T1; lane 4, 1.25 ug PC12 RNA digested with RNase T1; lane 5, 1.25 ug PC12 RNA digested with RNaseA. Sizes shown were determined from SV40-Eco RII fragments run on the same gel (not shown) whose positions are indicated at right.

RNA that protects the end of the *Sau* 3A site at +444 would extend to approximately base +194 in the intron. Examination of the DNA sequence in this region revealed a potential hairpin structure between +182 and +203, containing the bases UAAGAG in the loop. The inverted repeat that would form the 8 base-paired stem of this structure is indicated by inverted arrows in Figure 2. It is possible that the variation in transcription mapping may be explained by the presence of a hairpin structure whose stability varies with experimental conditions. Cleavage of the 444 b precursor RNA fragment in the loop region would produce two bands of approximately 250 bases and 190 bases (Figure 4A) which corresponds to the 190 b and 250 b RNA fragments seen in lane 9 of Figure 4B. *S1* nuclease cleavage in this sequence after hybridization with end-labeled DNA would generate only the 250 base fragment, as is observed, but not the 190 base fragment, which would be unlabeled.

The complement of this possible loop sequence in the labeled cRNA (CUCUUA) contains no G residues and, thus, should be resistant to degradation with RNase T1 which cleaves only after guanosine residues (45). RNase A, however, which is normally used for our nuclease digestions would cleave in this sequence at the pyrimidine linkages (46). This hypothesis was tested by annealing labeled cRNA from pGEM SS.610 with total PC12 RNA and digesting separately with RNase A and RNase T1. When the hybridized samples were digested with RNase T1, a major band is seen at 444 bases (Figure 4C, lanes 3 and 4) whereas the same mixtures digested with RNase A again produce two bands at 190 b and 250 b (lane 5). This suggests that in the absence of an enzyme that can cleave at one of the bases in the proposed loop sequence the entire 5' end of the precursor RNA is protected and does, indeed, extend to the +1 base indicated in Figure 2. (The dominant 141 b band after RNase T1 digestion presumably reflects the fact that the first G residue in the cRNA outside of the exon complementary region does not occur until +140. Cleavage at the G/C bp at -1 would generate the other boundary of the exon hybridizing region, thus producing a fragment 16 bases longer than the exon).

TH DNA sequence analysis:

A 6 base sequence corresponding to the canonical TATA box for polymerase II genes (47) is found at -29 to -24. There is no apparent CCAAT related sequence in the -80 region. Recent evidence has suggested that short, repeated sequences in the polymerase II promoter area of some genes may be involved in regulating gene expression, e.g. SV40 (48, 49), herpesvirus thymidine kinase (50), and rabbit β globin (51). In light of this we have noted several repeated sequences in the promoter region of TH. There is a

tandem, 7 bp direct repeat starting at -78, AGGAGGT, that is indicated by dashed underlining in Figure 2. The sequence between -107 and -94 contains two repeats of the pentanucleotide CAGGC. The TATA box region is flanked by a 7 bp repeat, GCCTGGC at -37 to -31 and -13 to -7, and the sequence GAGGGC is found at -23 to -18 and -6 to -1. There is also a 12 bp duplication of the sequence coding for the first 4 amino acids of TH at -127 to -113, ATGCCCACCCCC.

Nucleotides surrounding the ATG at +36, in particular the A at +33, (3 bases upstream from the translation initiation site) indicates that this is probably a strong translation initiation site as defined by Kozak (52,53).

TH Promoter Activity in Transfected Cells:

To confirm that the TH gene region upstream of the mapped initiation site functions as an authentic promoter and to determine whether its activity is limited to TH-producing cells, a variety of cell lines were tested for expression of a hybrid gene containing TH 5' flanking sequence. An XhoI/AluI fragment containing 773 bases of 5' flanking region and 27 bases of the 5' untranslated region was excised from λ -TH and inserted in the poly-linker region of pUC-CAT adjacent to the CAT gene. This hybrid TH-promoter/CAT gene construct, p5'TH-CAT, was used to transfect cultured cells which were then assayed for CAT enzyme activity two days following transfection. Background CAT activity was determined by assaying cell cultures transfected with the original pUC-CAT vector which lacks promoter and enhancer elements. As a control for CAT expression in these experiments, cells were transfected in parallel with RSV-CAT in which CAT gene transcription is directed by the RSV promoter (41).

Pheochromocytoma cells transfected with p5'TH-CAT express the CAT gene (TABLE I). CAT activity is also present when GH₄ cells, derived from a rat pituitary tumour, are transfected with p5'TH-CAT. However, two neural tumour lines, RT4-D (peripheral) and B103 (central nervous system) did not express CAT activity when transfected with p5'TH-CAT. CAT activity was also not detected with fibroblast-like cell lines from rat, FR 3T3, or mouse, Ltk-, when the TH promoter was used. All cell lines did, however, demonstrate CAT activity after transfection with RSV-CAT. In addition, an epithelial-derived rat line, H35 hepatoma cells (54), was tested for p5'TH-CAT expression. These cells appeared to have extremely poor transfection efficiencies, such that CAT activity after transfection with RSV-CAT was very low (data not shown). In these same experiments, however, no expression of p5'TH-CAT above background could be detected.

TABLE I: Expression of p5'TH-CAT in different cell lines

Cell	Tissue of Origin	ExpI		ExpII	
		RSV-CAT ^a	TH-CAT ^a	RSV-CAT ^a	TH-CAT ^a
PC	rat adrenal medulla (endocrine)	14%	5%	0.3%	0.7%
B103	rat central nervous system	5%	0	0.6%	0
RT4-D	rat peripheral nervous system	79%	0		
GH ₄	rat pituitary (endocrine)	72%	24%		
FR3T3	rat embryo (fibroblast)			3.8%	0
Ltk-	mouse connective tissue (fibroblast)			0.5%	0

^a ¹⁴C Chloramphenicol acetyl transferase activity was quantitated by incubating ¹⁴C chloramphenicol and acetyl CoA with 50 ug of protein extract from transfected PC, B103, RT4-D FR3T3 and Ltk-cells, and 25 ug from GH₄ cells. The data are presented as percent conversion per hr and are normalized to 50 ug protein. Background conversion of ¹⁴C chloramphenicol to the acetylated form was determined with protein extracts from cells transfected with pUC-CAT and subtracted from the percent conversion numbers given above.

These results suggest that, whereas all the cell lines tested are capable of expressing the RSV-CAT vector, only PC and GH₄ cells are capable of efficiently expressing CAT when it is linked to the TH promoter. Unlike PC cells, GH₄ cells do not contain detectable levels of TH RNA as assayed by RNA dot hybridization, nor do RT4-D and B103 cells (unpublished data).

DISCUSSION:

In the present work, we have described the isolation of a lambda phage recombinant containing approximately 12 kb of rat genomic DNA of which 7.6 kb is transcribed into TH precursor RNA with the remainder of the precursor coding region extending further downstream. The initiation site for TH RNA synthesis was determined by nuclease protection mapping, using total RNA from TH expressing tissues. Protection experiments with RNA from both unstimulated and dexamethasone-induced pheochromocytoma cells, in which TH activity and TH mRNA are increased (9), indicated that the TH RNA transcription start site maps to the genomic subclone SS.610. The 5' end of TH RNA from adrenal, olfactory bulb, tegmentum, and superior cervical

ganglion also maps to this same site. Primer extension mapping with PC12 RNA and an 18 base oligonucleotide complementary to the translation initiation site region confirms and precisely locates the position of the transcription start site. We conclude, therefore, that in the tissues examined and in control and dexamethasone-stimulated PC12 cells, TH RNA initiates at (or very near) the T designated +1 in Figure 2 and utilizes a single major promoter. The transcription mapping results presented in Figure 4 suggest, however, that the ratio of unspliced to spliced TH RNA may vary in these tissues. This may indicate that the efficiency of TH-RNA processing or TH-mRNA stabilization differs among tissues. We also find that at very high RNA concentrations protected bands extending upstream of +1 are detected (unpublished data). This suggests there may be additional upstream initiations that occur at a very low frequency *in vivo*.

The analysis of PC12 RNA was complicated by the presence of protected bands at 250 b and 190 b when labeled cRNA was used in nuclease protection experiments with RNase A. However, cleavage of RNA:cRNATH hybrids with RNase T1, which only cleaves at guanosine residues, protects the entire 5' end of the precursor RNA from PC12 cells generating a 444 b fragment which corresponds to the 5' end mapped with RNA from the other rat tissues. Since the intact 444 b fragment is detected in the various Sprague-Dawley rat tissues examined but not in the PC12 RNA when RNase A is used, we speculate that minor sequence heterogeneity in the first intron of the TH gene in the +190 region may account for the differences in transcription mapping results. In this regard, PC12 cells (25) were derived from a New England Deaconess Hospital strain white rat (55) while the other tissues examined in this work were from Sprague-Dawley rats, as was the genomic library from which λ TH was selected.

Variation in appearance of the 250 band relative to the 444 band in experiments with PC12 RNA and end-labeled DNA or internally-labeled cRNA probes indicated that the accessibility of the internal site for cleavage varied with experimental conditions. The intron DNA contains an inverted repeat in the region to which the 5' end of the 250 b band mapped (Figure 2). It is possible that a hairpin structure is forming at this site creating a nuclease sensitive loop (Figure 4A), containing bases +190 to +195, and that its stability varies according to the conditions of each experiment or the nature of the probe used (RNA vs DNA). A sequence difference between PC12 RNA and Sprague-Dawley RNA could contribute to a generally more stable hairpin in PC12 RNA that accounts for the different patterns of transcription

mapping between these two sources. Alternatively, the varying susceptibility of hybridized molecules to nuclease cleavage may simply be due to differences in the ability of S1 as compared to RNase A to cleave at internal mismatches that are due to sequence heterogeneity at or near position +190 in the RNAs examined. Myers et al. (56) have recently shown that RNase A has an enhanced ability to cleave at single base mismatches compared to S1 and RNase T1.

The first exon of the TH gene is 125 bases in length. Comparison of our sequence with the cDNA-TH sequence published by Grima et al. (21) places the protein coding region of this exon at +36 to +125. The left junction intron consensus sequence, $\begin{smallmatrix} A \\ C \end{smallmatrix}AG/GT\begin{smallmatrix} A \\ C \end{smallmatrix}AGT$ (47), is matched at 7 out of 9 bases in the region from +123 to +131 including the required GT at +126, +127. The 5' end of a 125 base exon would extend approximately 35 bases up from the ATG which agrees well with our transcription mapping data.

The TH promoter contains a TATA-like sequence at the appropriate location (47), but no apparent CAAT box in the -80 region (57). Regions within 110 bp of the cap site that contain short, repeated sequences have been implicated in regulation of polymerase II promoters in several systems. It may be that short, repeated sequences in the TH promoter region may have an analogous function in the regulation of TH RNA synthesis. One of the repeats in the TH gene, AGGAGGT (-78 to -65), is also found in a single copy in the major liver promoter for the mouse α -amylase gene (23) at a similar position, -69 to -63. The rat prolactin gene contains a sequence at -77 to -67 (relative to the initiation site), GAAGAGGATGC (58), that is matched at 10 out of 11 bases by the GGAGAGGATGC at -91 to -81 in the rat TH promoter. These homologous sequences may have similar functions in these rodent genes. Cooke and Baxter (58) suggest that the GAAGAGGATG sequence, which is repeated at +340 to +349 in the first intron of the prolactin gene, may be an integration hotspot at which the present day promoter for prolactin was introduced. In light of this, we find that the homologous sequence in TH is matched at 8 out of 10 bases at the 5' end of the first TH intron, +125 to +134. This may suggest that the promoter and first exon of the TH gene were also the result of DNA integration at this sequence.

Transfection studies of several rat cell lines of neural and endocrine origin have shown that TH DNA sequence from -773 to +27 is sufficient to provide promoter activity when fused to the CAT gene. However, the expression of the TH promoter was limited to PC cells and GH₄ cells; two neural tumour lines, RT4-D and B103, did not express p5'TH-CAT above background nor did several other non neural lines. This suggests that there

Figure 5. Comparison of the 5' flanking region of the promoters for rat tyrosine hydroxylase, TH; rat growth hormone, GH (62); rat phosphoenolpyruvate carboxykinase, PEPCK (63); mouse metallothionein-I, MT-1 (64); and mouse hypoxanthine phosphoribosyltransferase, HPRT (65). The TATA box is in boldface and the sequence GAGGGC (or a single base variant) that is found near the initiation site of each of these genes is underlined.

Comparison of the sequence reported here with that of the rat somatostatin gene (59) revealed an exact 9 bp homology just upstream of the TATA box. The sequence TGACGTCAG is found at -45 to -37 in the TH 5' flanking region and at -47 to -40 in the somatostatin sequence. This sequence resides within a 31 bp region (-60 to -29) of somatostatin which is sufficient for conferring cAMP inducibility on a heterologous promoter (60). A segment of the 5' flanking sequence of the TH gene which contains this sequence confers cAMP responsiveness onto a heterologous gene transfected into pheochromocytoma cells or into GH₄ cells (E. Lewis et al., manuscript in press). Comparison of cAMP regulated genes whose 5' flanking regions are known has revealed the presence of a similar sequence in many of these genes (60, 61).

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mouse hypoxanthine phosphoribosyltransferase gene (65) which does not contain a TATA box. We do not know what the potential significance of this sequence might be, but it is interesting to note that these five rodent genes containing this sequence do not have CAAT boxes. Perhaps the GAGGGC sequence serves a function in these genes related to the absence of CAAT. It may also be significant that 6 of the 7 bps found in the GCCTGGC repeat (-37 to -31 and -13 to -7) of TH are found in a 10 bp repeat, CGGAGCCGTGG, flanking the GAGGGC sequence of mouse HPRT (65).

Experiments are currently in progress in our laboratory to determine in more detail which regions of the TH gene are functionally important for the regulation of TH RNA synthesis.

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