

Sequence of Two mRNAs Encoding Active Rat Tryptophan Hydroxylase

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Abstract: Two full-length cDNA clones that encode functional rat tryptophan hydroxylase (EC 1.14.16.4), the key enzyme in serotonin synthesis, have been isolated from a rat pineal gland library. These two clones correspond to the 1.8- and 4-kilobase mRNA species, respectively. They contain the same coding sequence corresponding to a 51,010-

dalton protein and differ in the length of their 3' untranslated regions. **Key Words:** Tryptophan hydroxylase—Aromatic amino acid hydroxylases—Pineal gland—Molecular cloning. **Darmon M. C. et al.** Sequence of two mRNAs encoding active rat tryptophan hydroxylase. *J. Neurochem.* **51**, 312–316 (1988).

The study of the regulation and biochemical characterization of tryptophan hydroxylase (TPH; EC 1.14.16.4), the key enzyme in serotonin synthesis, has been hampered by its limited availability and extreme instability (Deguchi and Barchas, 1973). As a first step in the study of TPH, we reported recently (Darmon et al., 1986) the identification of an incomplete putative rat TPH cDNA clone (P7) that is homologous to tyrosine hydroxylase (TH) and phenylalanine hydroxylase (PH). This probe recognizes two mRNA species of 1.8 and 4 kilobases (kb) in the pineal gland RNA (Darmon et al., 1986). A similar cDNA clone that recognizes a single major RNA species has also been isolated from a rabbit pineal gland library by Grenett et al. (1987). However, whether these clones encode functional TPH has not been established. In this study, we report that two full-length cDNA clones corresponding to the 1.8- and 4-kb mRNA species encode active rat TPH in a rabbit reticulocyte system. Analysis of the two nucleotide sequences reveals a diversity in the size of the 3' untranslated region (UTR).

MATERIALS AND METHODS

Isolation and sequencing of TPH cDNA clones

Screening of the library was performed under high stringency, according to the methods of Grima et al. (1985).

Positive clones were purified, and restriction fragments were isolated and subcloned into the M13mp8 or M13mp18 vector. The M13 subclones were subjected to DNA sequence analysis according to the method of Sanger et al. (1977).

Affinity-purified TPH antibody

The P7 insert was subcloned in a pEX vector (Stanley and Luzio, 1984), and the resulting TPH- β -galactosidase fusion protein (2 mg) was subjected to polyacrylamide gel electrophoresis, electroeluted according to the method of Hunkapiller et al. (1983), and injected into a rabbit. Two milliliters of immune serum was adsorbed on the fusion protein fixed on an immunoblot (1 mg), and ~100 μ g of specific immunoglobulin was eluted as described by Lamouroux et al. (1987).

In vitro synthesis of active TPH

TPH-1 and TPH-2 inserts were subcloned in the pSPT18 vector, and capped-sense RNA was synthesized with T7 RNA polymerase and m⁷GpppG (Tabor and Richardson, 1985). Approximately 1.6 μ g of RNA from TPH-1, TPH-2, and choline acetyltransferase (Berrard et al., 1987), as a control, were translated in 200 μ l of rabbit reticulocyte lysate (Amersham). Translation products were immunoprecipitated with either 20 μ l of affinity-purified TPH antibody (0.2 μ g/ μ l) or 10 μ g of nonimmune rabbit immunoglobulin and 100 μ l of protein A-Sepharose. The pellets were washed three times with 1 ml of 50 mM Tris-acetate (pH 7.5) containing 1 mM dithiothreitol and 10% catalase and sus-

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Abbreviations used: bp, base pairs; kb, kilobases; PH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; UTR, untranslated region.

pended in 30 μ l of 50 mM Tris-acetate. The reaction was carried out according to the procedure of Yamaguchi et al. (1981), except that 2 mM NSD 1015 was used as the decarboxylase inhibitor. The extracts were injected onto a reverse-phase column (10 μ m C18 μ Bondapak, 300 \times 3.9 mm; Millipore, Waters) and the mobile phase was composed of 5% methanol in 50 mM potassium monophosphate buffer adjusted to pH 4.5. The 5-hydroxytryptophan synthesized was quantified by fluorometric detection.

Northern blot analysis

Total RNA from pineal glands was prepared according to the technique of Civelli et al. (1982). Northern blotting was performed as described by Faucon Biguet et al. (1986).

RESULTS

Characterization of full-length TPH cDNAs

To obtain full-length clones, the size-selected λ gt11 library generated from rat pineal gland was screened with the 1.2-kb P7 insert previously isolated (Darmon et al., 1986). Among 600 positive plaques in the 90,000 recombinants that were screened, 24 were purified and analyzed by restriction mapping. Two clones, designated pTPH-1 and pTPH-2, were selected. They were 1,646 and 3,877 base pairs (bp) long, respectively, and both contained a poly-A tail. The restriction map of TPH-1 fully matches that of the 5' portion of TPH-2. Both cDNAs extend \sim 600 bp further than P7 in the 5' region, which suggested that they encode the entire enzyme.

To rule out the hypothesis that TPH-2 might be a double insert, a Northern blot was performed on pineal gland RNA and hybridized with a TPH-2-specific probe (a 1-kb *Eco*RI fragment corresponding to the 3' UTR of TPH-2) and with a probe contained within both clones (a 1.2-kb *Eco*RI fragment corresponding to the 3' end of TPH-1). The specific probe recognized only the 4-kb mRNA, whereas both the 1.8- and 4-kb bands were visualized with the common probe (Fig. 1).

Synthesis of active TPH

To examine whether pTPH-1 and pTPH-2 encode functional TPH enzymes, we first subcloned TPH-1 and TPH-2 inserts in a pSPT18 vector and used T7 RNA polymerase to produce the corresponding RNAs (designated TPH-1 and TPH-2 RNAs, respectively). When translated in a rabbit reticulocyte lysate, both RNAs produced a protein of 51,000 daltons (data not shown).

Preliminary experiments indicated that the lysate inhibits TPH activity associated with a rat pineal gland extract. Therefore, TPH was tested on material immunoprecipitated from the translation products using an antibody raised against a fusion protein generated from a β -galactosidase-P7 hybrid DNA, as described in Materials and Methods. The affinity-purified antibody recognized a single protein of 51,000 daltons on an immunoblot of pineal gland extract, which was identical in size to the protein immunoprecipitated from the in vitro translation products of pineal mRNA, as well as from TPH-1 and TPH-2 RNAs (data not shown). Table 1 shows that both TPH-1 and TPH-2 RNA generate an obvious TPH enzymatic activity. The values obtained in the absence of exogenous RNA and with choline acetyltransferase RNA are similar to that of the blank. Hence, the use of reticulocyte lysate firmly establishes that both TPH-1 and TPH-2 encode functional enzymes. It is interesting that in two independent experiments, TPH-2 RNA yielded an enzymatic activity higher than that of TPH-1 RNA. This may be the result of the better in vitro translation efficiency of TPH-2.

Complete sequences of rat TPH cDNAs

The complete nucleotide sequences of both TPH-1 and TPH-2, as well as the predicted amino acid sequence, are presented in Fig. 2. These clones are nearly full-length, because they both encode an active protein in vitro and they contain a poly-A tail at the 3' end. The 1,332-bp open reading frame, specifying a 444-amino acid protein, is identical in both clones. The initiation codon is preceded by a CACC sequence compatible with the consensus sequence of Kozak (1984). The 5' and 3' UTRs of TPH-1 are 70 and 244 bp long, respectively, whereas those of TPH-2 are 120 and 2,422 bp, respectively. At the 3' end, TPH-2 contains a typical polyadenylation signal (Proudfoot and Brownlee, 1976), located 18 bases upstream from the poly-A tail, whereas no polyadenylation signal exists in TPH-1. TPH-2 has a longer 5' UTR than TPH-1. Whether these two lengths correspond to incomplete cDNAs or to different initiation sites awaits further investigations of the 5' region of the TPH gene.

The predicted molecular mass of rat TPH is 51,010 daltons, assuming that translation starts from the first AUG codon. This value is compatible with those of 59,000 and 55,000 daltons reported by Nakata and Fujisawa (1982) and Cash et al. (1985) for purified rat TPH. The predicted amino acid sequence of rat TPH exhibits 88.7% identity with the rabbit one reported by Grenett et al. (1987), without any gap (Fig. 3). Nine cysteines of 10 are conserved in the two sequences. No homology is observed in the 5' UTR, whereas the 3' UTR exhibits 60% identity in the first 240 nucleotides downstream from the stop codon.

The amino acid sequences of rat TPH, rat PH

FIG. 1. Northern blot analysis of TPH RNA. One microgram of total RNA from rat pineal gland was hybridized with nick-translated inserts: lane 1, *Hind*III- and *Eco*RI-digested λ DNA; lane 2, hybridization with a 1-kb *Eco*RI fragment specific for the 3' UTR of TPH-2; and lane 3, hybridization with a 1.2-kb *Eco*RI fragment common to TPH-1 and TPH-2. Exposure time was 4 h.

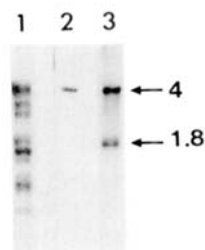


TABLE 1. Expression of active TPH in rabbit reticulocyte lysate

	TPH activity in immunoprecipitation pellets	
	Anti-TPH antibody	Nonimmune immunoglobulin
Experiment 1		
No RNA	2.11 (2)	—
ChAT RNA	1.88 (2)	—
TPH-1 RNA	14.81 (3)	3.38 (2)
TPH-2 RNA	43.81 (4)	3.43 (2)
Experiment 2		
No RNA	1.98 (2)	—
TPH-1 RNA	14.58 (2)	4.10 (2)
TPH-2 RNA	106.28 (2)	7.34 (2)

Data are pmol of 5-hydroxytryptophan formed/h/per assay (no. of assays). The blank value of the assay was 1.8 pmol/h. ChAT, choline acetyltransferase.

(Dahl and Mercer, 1986), and rat TH (Grima et al., 1985) can be aligned by introducing one, four, and six gaps in the TPH, PH, and TH sequences, respectively. It is interesting that these gaps are all located in the N-terminal regions of these proteins (Fig. 3). The overall identity is 55.7% between rat TPH and PH and 50.1% between rat TPH and TH. The central region (amino acids 187–324), which exhibits the greatest identity (73.3% with PH and 71.8% with TH), contains the five conserved cysteines.

DISCUSSION

In this study, we have established that TPH activity is encoded by at least two mRNAs that share the same coding sequence and that differ in their 3' UTR.

The characteristic features of the TPH amino acid sequence are best discussed in the context of homology to the TH and PH sequences, considering the broad functional similarities among the three aro-

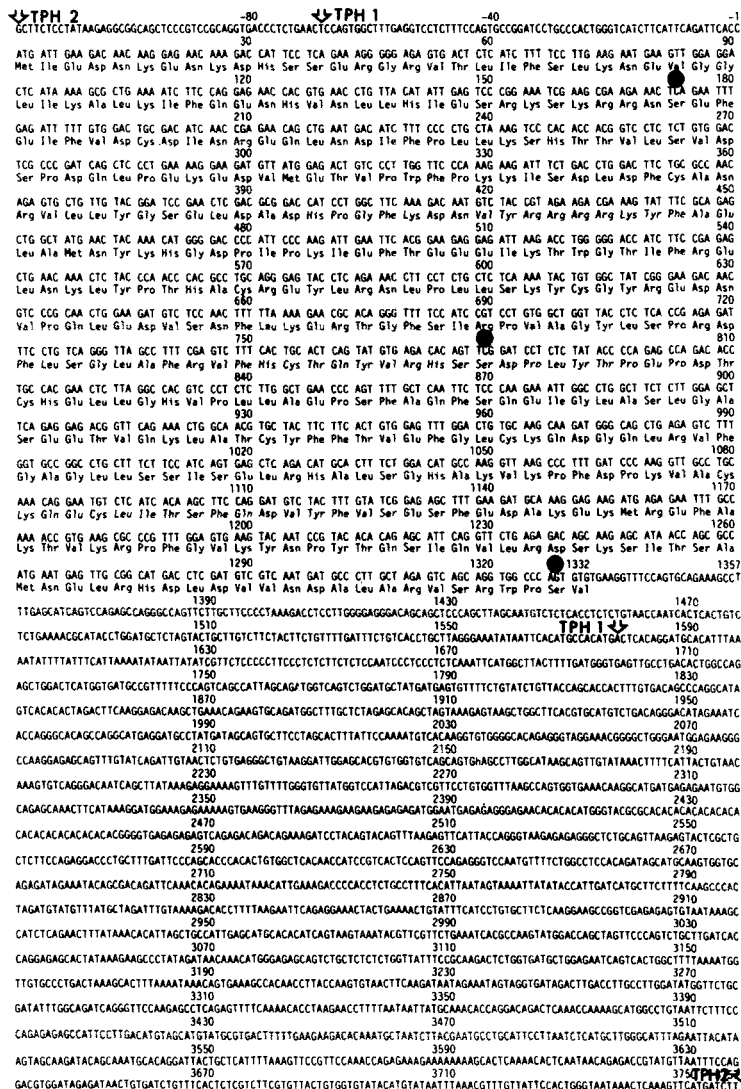


FIG. 2. Nucleotide and deduced amino acid sequence of rat TPH. The coding sequence is numbered from nucleotide 1, and the 5' UTR is indicated by negative numbers. Two open arrows indicate the 5' end and the poly-A tail of TPH-1 and TPH-2, respectively. The three potentially phosphorylated serines are labeled with black dots (Ser 58, 260, and 423).

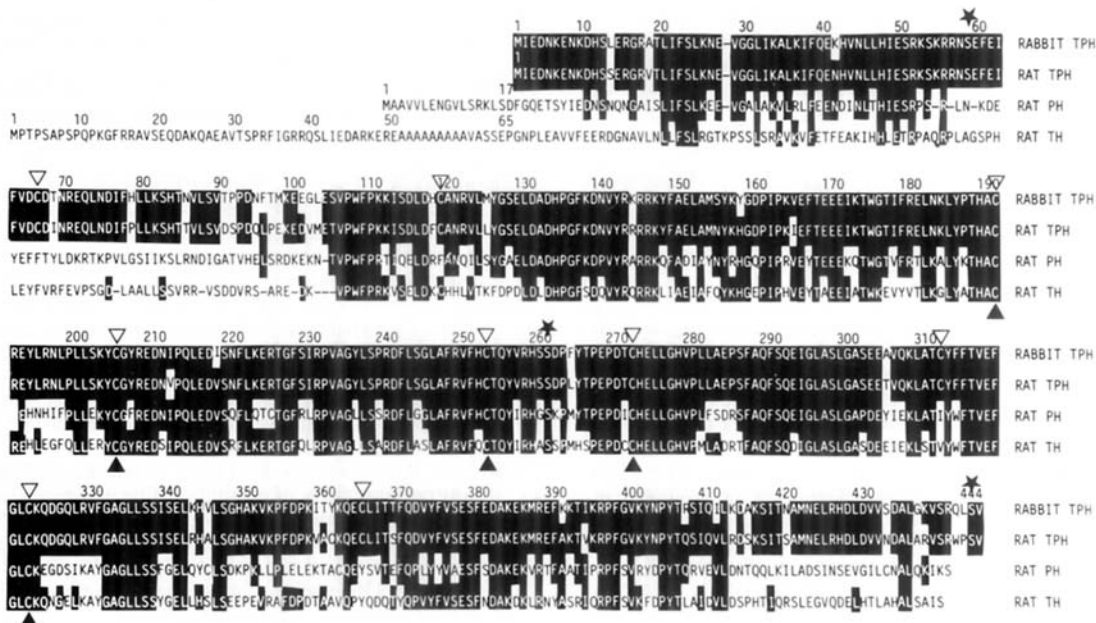


FIG. 3. Homologies among the aromatic amino acid hydroxylases. Alignment of the predicted amino acid sequences of rabbit TPH (first line; Grenett et al., 1987), rat TPH (second line), rat PH (third line; Dahl and Mercer, 1986), and rat TH (fourth line; Grima et al., 1985) is shown. The numbering refers to rat TPH. Residues that are identical to those in the two TPH enzymes are enclosed in solid boxes. The cysteines conserved between rat and rabbit TPH are shown with open triangles; those conserved between the four hydroxylases are indicated with solid triangles. Asterisks indicate the potential phosphorylated sites of the TPHs.

matic amino acid hydroxylases. Homologous sequences reside in the central and C-terminal regions of these enzymes. However, a stretch of 30 amino acids near the N-terminal region displays 70% identity between rat TPH and rat PH, which may be related to the fact that TPH can use phenylalanine as a substrate (Ichiyama et al., 1976). The central domain (amino acids 187–324), which exhibits the greatest similarity, is most likely to contain the catalytic site. It is interesting that the cysteines that are conserved in the three sequences reside in this domain, an observation suggesting a common conformation. Biochemical evidence suggests that the nonhomologous N-termini of TH and PH, which contain the phosphorylation sites (Abita et al., 1985; Campbell et al., 1986), serve as regulatory domains. In this respect, a serine at position 58 constitutes a good candidate for cyclic AMP-dependent phosphorylation (Cohen, 1985). This observation will help to reconcile the conflicting results that have been obtained by Hamon et al. (1977) and Sawada et al. (1985) concerning this mode of phosphorylation. In addition, outside the N-terminal region, two serines, at positions 260 and 443, are also potential sites for Ca²⁺-calmodulin-dependent phosphorylation (Pearson et al., 1985), a finding in accordance with the *in vitro* and *in vivo* studies of Kuhn et al. (1980) and Nagatsu et al. (1983). These three serines are conserved between rat and rabbit TPH, a finding suggesting that these sites may be phosphorylated *in vivo*.

Finally, it is noteworthy that the two TPH cDNA

clones differ in the length of their 3' UTR. The importance of the UTR has not been fully defined, but evidence suggests that such regions may play a role in the translation efficiency and stability of mRNA (Ragho, 1987). Further investigation is required for determining the functional implications of the diversity in the TPH UTR, which may play a role in regulating TPH synthesis *in vivo* and, therefore, control of the availability of serotonin.

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