

Rapid Communication

Multiple Human Tyrosine Hydroxylase Enzymes, Generated Through Alternative Splicing, Have Different Specific Activities in *Xenopus* Oocytes

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Abstract: A single human tyrosine hydroxylase (HTH) gene has been shown previously to generate four species of mRNA by alternative splicing. The four different HTH mRNAs were independently synthesized *in vitro*, using the SP6 transcription system. Each of these mRNA species was able to direct the synthesis of an active form of TH following injection into *Xenopus* oocytes. Quantitation of the synthesized HTH polypeptides allowed the determination of the relative specific activity of each individual HTH form. A significant difference in specific activity was found between each form, suggesting that alternative splicing may play a role in regulating HTH activity *in vivo*. **Key Words:** Tyrosine hydroxylase—Alternative splicing—*Xenopus laevis*—Translation—SP6 polymerase. Horellou P. et al. Multiple human tyrosine hydroxylase enzymes, generated through alternative splicing, have different specific activities in *Xenopus* oocytes. *J. Neurochem.* 51, 652–655 (1988).

Recent cDNA cloning and S1 mapping experiments, in conjunction with the analysis of genomic sequences, have revealed that the gene encoding human tyrosine hydroxylase (HTH), the rate-limiting enzyme in catecholamine synthesis, generates four different mRNAs through alternative splicing events (Grima et al., 1987; Kaneda et al., 1987; Le Bourdellès et al., 1988). Their corresponding sequences predict the occurrence of four polypeptides designated HTH-1, -2, -3, and -4. HTH-1 contains 497 amino acids and has a calculated molecular weight of 55,572. HTH-2 and -3 differ from HTH-1 by an insertion, at position 30, of 4 and 27 amino acids, respectively. Finally, HTH-4 is characterized by both insertions in the above order relative to the N-terminus. Because the difference between these four species is restricted to their N-terminal region, and as the catalytic site has been shown to lie in the carboxy terminal domain, the four forms were thought likely to be active. The four HTH mRNAs have been found in all the pheochromocytoma tumors that we have tested. HTH-4 mRNA appears to be characteristic of this tumor tissue,

since only HTH-1, -2, and -3 mRNAs are detected in the normal human adrenal, HTH-2 being clearly the most abundant mRNA species. Further, in human locus ceruleus and substantia nigra, only HTH-1 and -2 mRNAs have been found (Grima et al., 1987; Le Bourdellès et al., 1988).

Alternative splicing has now emerged as a widespread mechanism in the generation of developmental or tissue specific diversity (Breitbart et al., 1987). The presence of several types of HTH in the same tissue raises the possibility that alternative splicing provides an additional way of regulating catecholamine biosynthesis in various physiological and pathological situations. As a first step in these studies, we have ascertained, taking advantage of the *Xenopus* oocyte system, that all four mRNAs do encode an active enzyme and have compared the relative specific activities of the four HTH enzymes. The differences are analyzed in terms of primary structure variations in the enzyme forms. The possibility that additional diversity may result from an alternative initiation of translation is also discussed.

MATERIALS AND METHODS

Synthesis of HTH mRNAs

HTH-1, -2, and -4 cDNAs were previously isolated from a λ gt10 library (Grima et al., 1987; Le Bourdellès et al., 1988). HTH-3 cDNA was obtained from HTH-4 cDNA by deletion of 12 base pairs (bp), which correspond to four amino acid residues in HTH-4, using the Amersham site-directed mutagenesis system based on the Eckstein method (Taylor et al., 1985). The mutagenic oligonucleotide (5'-CGGGGCGCC*CATGATGGCCTCTGC-3', where * indicates the deletion site) was annealed to the single-stranded M13 HTH-4 DNA obtained after HTH-4 insertion into phage M13 vector mp18. Presumptive "mutagenized" plaques obtained were screened positively by hybridization with a radioactively labeled mutagenic oligonucleotide and negatively with another oligonucleotide

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Abbreviations used: bp, base pairs; DOPA, 3,4-dihydroxyphenylalanine; HTH, human tyrosine hydroxylase; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TH, tyrosine hydroxylase; UTR, untranslated region.

containing the 12 bp that were expected to be deleted (5'-CGGGGACTGCCCTCTTACCATGAT-3'). The effectiveness of the 12 bp deletion was checked by sequencing with the chain-termination method (Sanger et al., 1977). All four HTH cDNAs were inserted into the *EcoRI*-digested pSPT18 vector. Before transcription, plasmids containing cDNA sequences downstream from the SP6 promoter (pSP6 HTH) were linearized by *PvuI*, and filled in with T4 DNA polymerase. In vitro transcription was performed according to the method of Melton et al. (1984) except that the RNAs were capped during the transcription reaction as described by Konarska et al. (1984) with minor modifications: GTP concentration was 0.0625 mM and m7GpppG was 0.5 mM in the reaction. In vitro transcribed RNAs from pSP6 HTH plasmids contain in the 5' untranslated region (UTR), in addition to the 5' HTH mRNA UTR, 64 nucleotides due to the transcription of the vector polylinker and in the 3' UTR, downstream of the polyA tail, a 1.38-kb region of SP6 vector.

In vivo synthesis of active HTH forms

Mature oocytes from *Xenopus laevis* were microinjected with 50 nl of water containing 50 ng of HTH RNA as described by Horellou et al. (1986). To label TH proteins radioactively, they were incubated for 48 h immediately following injection in the presence of [³⁵S]methionine (Amersham). Oocytes injected with the same mRNA were pooled. As both TH protein level and TH activity were determined in the same sample, protein extraction was performed with a buffer that preserved TH enzyme activity. Typically, 25 oocytes were sonicated for 30 s in 300 μ l of ice-cold 5 mM potassium phosphate buffer, pH 6.0, containing 0.2% Triton X-100 and centrifuged at 10,000 *g* for 10 min. Quadruplicate samples (50 μ l) were used for TH enzymatic assays. Incubation was performed as described by Horellou et al. (1986). Fifty microliters were used for HTH protein quantitation performed after immunoprecipitation. Three serial dilutions corresponding to 1, 2, and 4 μ l of the original oocyte lysate were incubated 12 h at 4°C in the presence of antibody raised against rat TH (P. Kahn, in preparation). This antibody was diluted 1:100 in 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 0.1% Nonidet P-40, 10 μ g/ml of soybean trypsin inhibitor, and 0.5 mM phenylmethylsulfonyl fluoride. The incubation was continued in the presence of Protein A-Sepharose for 2 h at room temperature. Material bound to the Protein A-Sepharose immunoglobulin complex was washed three times in the same buffer. It was then eluted as described by Dobberstein et al. (1979). The radioactivity associated with an aliquot of eluted samples was counted and that remaining was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) followed by autoradiography using an Amersham Beta-max film. The absorbance of the film was measured using a densitometric scanner. To determine the relative abundance of the various HTH proteins, a standard curve was first obtained using a serial dilution of a ³⁵S-labeled HTH-1 polypeptide electrophoresed on SDS-PAGE. This polypeptide was obtained by immunoprecipitation after in vitro synthesis in rabbit reticulocyte lysate (Amersham) in the presence of [³⁵S]methionine.

RESULTS

All four HTH mRNAs were independently synthesized in vitro from the appropriate cDNAs subcloned in the SP6

system. HTH-1, -2, and -4 cDNAs that contain the complete coding sequence of the corresponding mRNAs have been previously isolated from a human λ gt10 pheochromocytoma library. The entire coding sequence of HTH-3 was obtained by deleting the 12 nucleotide second exon of HTH-4 as described in Materials and Methods.

The ability of each HTH mRNA to direct the synthesis of an active enzyme was tested by injection into *Xenopus* oocytes as previously described for rat TH (Horellou et al., 1986). As shown in Table 1 each mRNA does encode an active enzyme.

Labeling of proteins with [³⁵S]methionine allowed us to visualize the corresponding polypeptides after autoradiography of SDS gels of immunoprecipitates obtained with a specific anti-TH antibody; HTH-1 and -2 polypeptides are 62.3 kilodaltons (kDa), whereas HTH-3 and -4 are 67.6 kDa; a minor band of slightly lower molecular mass is also detectable in the case of HTH-3 and -4 (Fig. 1B). An antibody dilution of 1:100 was saturating for each HTH form for the amounts of oocyte lysate assayed (Fig. 2). To estimate the relative specific activity of the HTH enzymes, the relative abundance of the HTH polypeptides contained in the gel was determined after measuring the intensity of the signals on the autoradiogram. Oocytes from the same batch incubated with identical concentrations of [³⁵S]methionine yield polypeptides whose specific radioactivity depends solely on the number of methionines in their sequences. Since this number is the same, the relative abundance of the various HTH may then be correlated to the intensities of the autoradiographic signals, thus allowing the relative specific activities of the enzymes to be compared. The deduced relative specific activities of the four HTH enzymes are reported in Table 1. HTH-1 is the most active form, with HTH-3 and -4 60% and HTH-2 35% as active, respectively.

The sizes of the HTH polypeptides obtained in oocytes were compared with those generated in the reticulocyte lysate and a similar pattern was obtained (Fig. 1A). A higher resolution SDS-PAGE of translation products from rabbit reticulocyte lysate allowed us to separate HTH-1 and -2 forms of the enzyme, and to detect the presence of a minor band for each form of HTH.

TABLE 1. Relative specific activity of each TH form in oocyte lysates

RNA type	TH activity (pmol of DOPA formed/h)	Protein estimation (optical density in arbitrary units)	Relative specific activity (percent of HTH-1)
HTH-1 RNA	527 \pm 17	18.5 \pm 0.9	100.0 \pm 6.9
HTH-2 RNA	157 \pm 2	15.6 \pm 0.2	34.8 \pm 1.7
HTH-3 RNA	1,680 \pm 19	99.2 \pm 5.6	58.0 \pm 5.8
HTH-4 RNA	413 \pm 9	24.2 \pm 2	59.2 \pm 3.2

Oocytes were incubated 48 h in the presence of [³⁵S]methionine just immediately following their injection. TH activity was assayed by measuring the amount of tyrosine converted to DOPA in 15 min at 37°C in pH 5.8 reaction medium containing 100 mM potassium phosphate, 0.5 mM NADPH, 1 mM 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine, 2,600 U catalase, 1 mM FeSO₄, and 40 μ M L-tyrosine. The blank background value was 2 pmol of DOPA formed/h. All results are expressed as mean \pm SEM values and represent quadruplicate measurements.

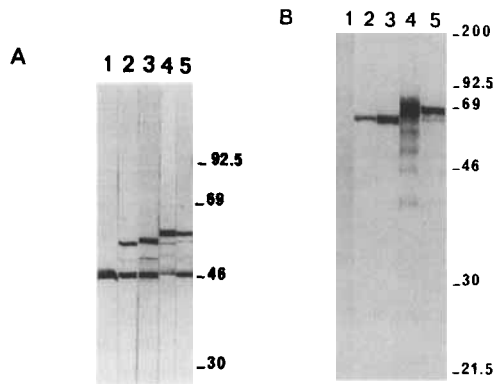


FIG. 1. Fluorographs from SDS-PAGE of ^{35}S -labeled TH antigen. **A:** In vitro translation products from HTH RNAs generated in rabbit reticulocyte lysates were analyzed on slab gel consisting of 5–12% linear gradient of polyacrylamide containing 5 M urea. **B:** Immunoprecipitated HTH antigen from injected oocytes were analyzed on a 12% slab gel. A and B: Lane 1, no RNA; lane 2, HTH-1 mRNA; lane 3, HTH-2 mRNA; lane 4, HTH-3 mRNA (a higher translation yield of this mRNA resulted, in the case of injected oocytes, in a better visualization of degradation products); lane 5, HTH-4 mRNA.

DISCUSSION

The results presented here confirm our prediction that the four different HTH mRNAs that have been characterized encode active HTH enzymes (Le Bourdellès et al., 1988). More importantly, their relative specific activities, as tested after translation in the oocyte system, differ quite significantly, suggesting that differential splicing may play an important role in the physiology of catecholaminergic cells.

The *Xenopus* oocyte represents a convenient way of expressing exogenous mRNAs. Recently, we have used this system to establish that a single mRNA species contains all the necessary information to generate the active rat TH enzyme. The efficiency of this system is however rather low, and to visualize exogenous polypeptides, oocytes are incubated in the presence of [^{35}S]methionine. The HTH enzymes were immunoprecipitated and their apparent molecular weights estimated on SDS-PAGE. Each of the mRNAs yielded a major polypeptide; HTH-3 and -4 products exhibit a lower mobility than HTH-1 and -2, in accordance with their difference in molecular weight. No difference in apparent molecular weight was observed between HTH-1 and -2 or between HTH-3 and -4. A similar pattern was obtained when the polypeptides were produced in the rabbit reticulocyte translation system. However, in this instance, the use of a higher resolution gel system allows discrimination between HTH-1 and -2, although the calculated difference in molecular mass is only 440 daltons.

The most striking finding of this study is the lower specific activities of HTH-2, -3, and -4 compared with HTH-1. In each less active form of HTH, an arginine residue is present in nearly the same position relative to the C-terminal region within the four amino acid residues in HTH-2 and the C-terminal portion of the 27 amino acid segment of HTH-3 and HTH-4. The lower specific activities of HTH-2, -3, and -4 relative to HTH-1 may result from this additional positive charge. Analyses of rat TH as well as HTH sequences, together with biochemical data (Vigny

and Henry, 1981; Grima et al., 1985) indicate that the enzyme contains two main domains. The N-terminal part which is positively charged could constitute a regulatory domain whereas the central and C-terminal part which are negatively charged contain the catalytic site. Electrostatic interactions between the two domains are likely to play an important role in the regulation of TH activity and indeed polyanions such as heparin have been shown to increase activity. The presence of this positive charge in HTH-2, -3, and -4 could thus be responsible for the inhibitory effect on TH activity. Two forms of HTH with high and low specific activity and separable by DEAE Sephacel chromatography have been found in human adrenal by Mogi et al. (1984). The enzyme of low specific activity appears to be most abundant in the human adrenal. This enzyme could correspond to HTH-2 since HTH-2 mRNA was shown to be predominant in this tissue (Grima et al., 1987) and generates in oocytes the least active HTH.

In addition to this theory based on structural analysis, the possibility also exists that activators or inhibitors are present in the oocyte extracts that differentially affect the activity of the multiple HTH enzymes. In particular TH has been shown to be activated on phosphorylation in vivo and in vitro by various kinases including cyclic AMP-dependent protein kinase (Joh et al., 1978). Kinase A has been shown to exist in the *Xenopus* oocyte, suggesting that HTH enzymes synthesized in this system may be at least partially phosphorylated in the oocyte. Therefore the results may reflect differences in the primary structure as well

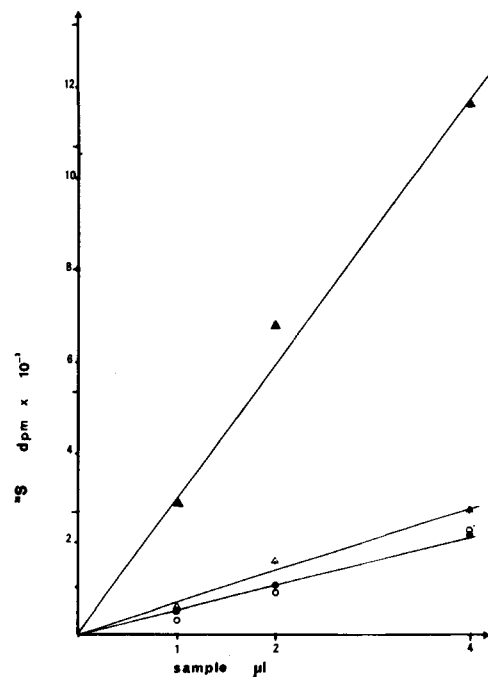


FIG. 2. Linearity of immunoprecipitation of HTH species. Three serial dilutions of HTH-injected oocyte lysate (corresponding to 1, 2, and 4 μl) were incubated in the presence of 1:100 anti-TH antibody; water-injected oocytes were used to determine the blank background value. A fraction (1:20) of ^{35}S -eluted material was assayed by liquid scintillation counting at 61% efficiency. The results are from quadruplicate measurements and are expressed as ^{35}S dpm after blank background value subtraction. HTH-I (\bullet), HTH-II (\circ), HTH-III (\blacktriangle), HTH-IV (\triangle).

as in the phosphorylation state of the various HTH enzymes. Synthesis of the enzyme in exogenous systems which are sufficiently efficient that each individual form may be purified to homogeneity is required before the generality of the oocyte experiment can be ascertained. More particularly, expression of *E. coli* would represent a convenient means by which to analyze the kinetic parameters of the enzyme in various states of phosphorylation that could then easily be controlled in vitro.

Finally, we should note that the four HTH mRNA species generate in the rabbit reticulocyte, in addition to the major polypeptide, a minor one of slightly lower molecular weight. Inspection of the HTH sequences reveals, 90 bp downstream of the first AUG, the presence of an internal in-frame AUG flanked by a Kozak consensus sequence (Kozak, 1986) which could serve as an alternative translation initiation site. These additional polypeptides could then originate from translation initiation at this second site. Multiple initiations of translation have been observed in a few instances (Strubin et al., 1986) and could provide further molecular diversity of the HTH enzyme. Synthesis of HTH mRNAs from which the first AUG has been deleted should clarify this issue. It should be noted that the second AUG, preceded by the same consensus sequence, is also present in the quail (Fauquet et al., 1988) and in the bovine (Saadat et al., 1988) TH mRNAs but not in the rat (Grima et al., 1985).

Recent results show that TH molecular diversity originating from differential splicing does not occur in rat (C. Boni, in preparation). The functional significance of this difference, as well as the role played by TH diversity in human normal and pathological situations, await further studies. Clearly, a subtle balance in the control of differential splicing stands as an alternative mechanism to control the biosynthesis of catecholamines.

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