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Expression of four types of human tyrosine hydroxylase in COS cells

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Alternative splicing from a single gene produces four kinds of human tyrosine hydroxylase (types 1–4), which have structural diversity only in the N-terminal region. We attempted expression of the type 1–4 enzymes in COS cells and performed kinetic analyses. All had enzymatic activities. The K_m values of the four types for L-tyrosine and 6-methyl-5,6,7,8-tetrahydropteridine were similar, although their relative homospecific activities were clearly different. The type 1 enzyme displayed the highest activity.

Tyrosine hydroxylase; Catecholamine synthesis; Transient expression; Alternative splicing; (Human, COS cell)

1. INTRODUCTION

Tyrosine hydroxylase (TH, EC 1.14.16.2) is a monooxygenase that catalyzes the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), requiring pteridine as a cofactor. TH is the first and rate-limiting enzyme in the pathway of catecholamine biosynthesis, its enzyme activity being regulated by protein phosphorylation [1,2] and by some organic compounds [3–5]. Recently, four types of human TH (types 1–4) have been found by cDNA cloning [6–8]. This protein diversity is generated through alternative splicing from a single gene [9–11]. This alternative splicing causes insertion/deletion of the short amino acid sequences in the N-terminal region of TH. The type 2 and 3 enzymes have sequences of 4 and 27 amino acids, respectively, between amino acids 30 and 31 of the type 1 enzyme. The type 4 enzyme has both the 4 and 27 amino acid sequences at the same position. Tissue- and stage-specific expression of different TH molecules has been reported [6,10], suggesting that alternative splicing plays an important role in functioning of TH. The functional differences among these TH types should be of interest. We attempted the transient expression of these four types of human TH, using a mammalian cell line, to compare their enzymatic properties.

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Abbreviations: TH, tyrosine hydroxylase; ECO-MOL, ecotropic Moloney virus; LTR, long terminal repeat; PAGE, polyacrylamide gel electrophoresis; 6-MPH$_4$, 6-methyl-5,6,7,8-tetrahydropteridine

2. MATERIALS AND METHODS

2.1. *Vector construction*

The full-length cDNA clones encoding human TH types 1–4 were isolated from human pheochromocytoma cDNA library as described in our previous papers [7,8]. pAGE145 [12] and pL1 [13] have been described. Expression vectors pAS-TH 1–4, having human TH type 1–4 cDNAs, respectively, were constructed as follows: the *Hin*dIII-*Bgl*II 4.1 kb fragment from pAGE145 (the *Bgl*II site was modified with the *Eco*RI linker) was connected with the *Hin*dIII-*Pst*I 0.5 kb fragment from pL1 (the *Pst*I site was modified with the *Eco*RI linker). The resulting plasmid, pAS, has the SV40 origin and promoter downstream

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of the ecotropic Moloney virus (ECO-MOL) long terminal repeat (LTR). The *Eco*RI-*Eco*RI fragments containing the four types of cDNA were inserted into the *Eco*RI site of pAS.

2.2. *DNA transfection*

2×10^6 COS cells (COS 1), freshly grown in 10 ml Dulbecco's modified Eagle's medium/10 cm dish, were transfected with 10 μg plasmid DNA by the calcium phosphate coprecipitation method [14]. Glycerol shock [15] was performed for 2 min, 4 h after transfection. The cells were harvested after 3 days.

2.3. *Preparation of cell extracts*

Cell extracts were prepared from 10^7 cells (5 dishes), by sonication in 500 μl of 50 mM potassium phosphate buffer (pH 7.3), containing 320 mM sucrose, 1 mM EDTA, 1 mM DTT, leupeptin 1 mg/ml, pepstatin 1 mg/ml, and 0.2 mM phenylmethylsulfonyl fluoride, followed by centrifugation for 10 min at $10000 \times g$. Protein concentration was determined using a Bio-Rad protein assay kit with bovine serum albumin as a standard [16].

2.4. *Western blotting*

After SDS-PAGE [17], proteins were transferred to a nitrocellulose membrane (Bio-Rad). The membrane was incubated with anti-rat TH antibody (1:100 dilution). Immunoreactive proteins were detected using a Bio-Rad immunostaining kit with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or using ^{125}I-labeled anti-rabbit IgG (Amersham).

2.5. *Assay of TH activity*

TH activity was determined based on the measurement of DOPA formed from L-tyrosine by HPLC with electrochemical detection as in [18]. The cell extract was incubated in 200 μl of the standard reaction mixture: 0.2 M sodium acetate buffer (pH 6.0), containing 0.1 M 2-mercaptoethanol, 0.2 mg/ml catalase, 1 mM 6-methyl-5,6,7,8-tetrahydropteridine (6-MPH$_4$) and 0.2 mM L-tyrosine. Incubation was carried out for 10 min at 37°C. For kinetic analysis, TH activities were assayed at various concentrations of either L-tyrosine or 6-MPH$_4$. Kinetic constants were determined by Lineweaver-Burk analysis. The relative homospecific activities (activity per enzyme protein [19]) were estimated as follows: to determine the relative amounts of the four enzymes in cell extracts, Western blotting was carried out with anti-rat TH antibody and ^{125}I-labeled anti-rabbit IgG. The intensities of TH bands on the autoradiogram were determined using a densitometer (Shimadzu). The activities of the type 1–4 enzymes were divided by the relative amounts of the proteins.

3. RESULTS AND DISCUSSION

Fig.1 illustrates the expression vector. After DNA transfection, COS cell extracts were prepared and analyzed by Western blotting (fig.2). Cells transfected with each of pAS-TH 1–4 gave major immunoreactive bands at 61, 61, 65 and 65 kDa, respectively. The pBR322-transfected cells did not produce these bands. Thus, these bands

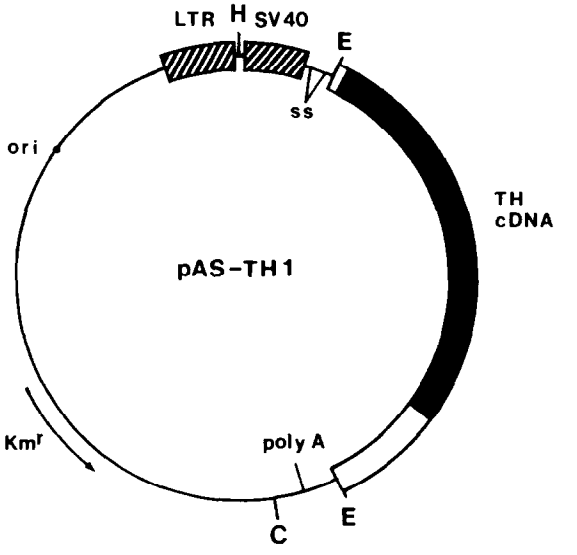

Fig.1. Structure of pAS-TH 1. The ECO-MOL LTR (LTR) and the SV40 origin/promoter (SV40) are indicated by the hatched areas. The SV40 polyadenylation signal (poly A), splice donor/acceptor sites (ss), pBR322 replication origin (ori) and kanamycin-resistance gene (Km^r) are shown. Unfilled areas indicate the 5′- and 3′-untranslated regions of human TH type 1 cDNA; the filled area represents its coding region. In pAS-TH 2–4, the *Eco*RI-*Eco*RI fragment containing the type 1 cDNA is exchanged by the corresponding cDNA fragments. E, *Eco*RI; H, *Hin*dIII; C, *Cla*I.

correspond to the type 1–4 enzymes, respectively. The molecular masses estimated from SDS-PAGE were similar to those predicted from the cDNA sequences [6–8]. We assayed TH activities in cell extracts. As shown in table 1, cells transfected with each of pAS-TH 1–4 showed TH activity whereas pBR322-transfected cells exhibited none. This indicates that all four types of human TH have enzymatic activity.

Table 1 summarizes the results of kinetic analysis. The K_m values for L-tyrosine ranged from ~100 to ~250 μM, those for 6-MPH$_4$ being from ~180 to ~250 μM. The K_m values for L-tyrosine are similar to those obtained from the purified rat [20] and bovine [21] enzymes. The low-affinity form of TH to pteridine (K_m ~200 μM) reportedly changes to the high-affinity form (K_m ~20 μM) through protein phosphorylation [22,23]. The K_m values for 6-MPH$_4$ suggest that the four types of TH may exist as the low-affinity form in COS cells. Recently, Ginns et al. [24] have reported ex-

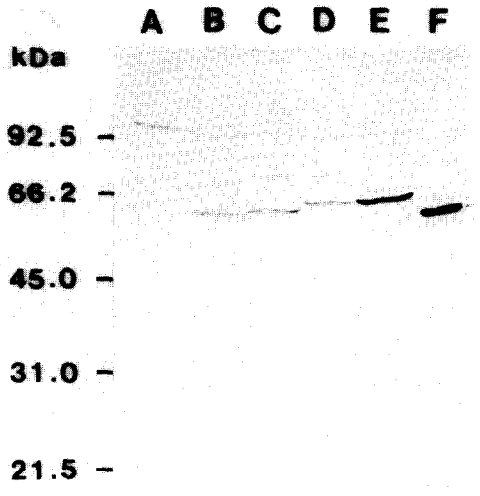

Fig.2. Western blotting of expression products. Cell extracts (~100 μg) from COS cells transfected with pBR322 (A), pAS-TH 1 (B), pAS-TH 2 (C), pAS-TH 3 (D), pAS-TH 4 (E), and purified bovine TH (F) were electrophoresed on a 10% SDS-polyacrylamide gel. Western blotting was carried out with anti-rat TH antibody and HRP-conjugated anti-rabbit IgG. Molecular mass standards: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

pression of human TH type 2 using a baculovirus vector. The results of their kinetic analysis are similar to ours.

We also estimated the relative homospecific activities (table 1). The type 1 enzyme had the highest homospecific activity, the values for the other enzymes ranging from ~0.3 to ~0.4. The insertion sequences seem to inhibit the TH activity. Mogi et al. [25] have reported the existence of the active form (form 1) and inactive or less active type (form 2) of human TH in adrenal medulla [25]. Both forms have similar molecular masses (~60 kDa), but the homospecific activity of form 2 is clearly lower than that of form 1. Comparison of the enzymatic properties of the four types suggests that types 1 and 2 may correspond to forms 1 and 2, respectively, or form 2 could be produced by some modifications and not be due to the differences between the TH types.

The N- and C-terminal half regions of TH are the regulatory and catalytic domains, respectively [26]. The major phosphorylation sites of some protein kinases have been identified in the N-terminal region of rat TH [27,28]. Cyclic AMP-dependent protein kinase mainly phosphorylates Ser 40. Ca^{2+}/calmodulin-dependent protein kinase II phosphorylates both Ser 19 and Ser 40. These Ser residues are conserved between rat TH and the type 1 enzyme of human TH. Since the insertion sequences characteristic to the type 2–4 enzymes of human TH exist near these major phosphorylation sites, it has been suggested that the sequence diversity may affect the regulatory mechanism of the enzymatic activity through protein phosphorylation. Moreover, TH is regulated by some organic compounds, such as polyanions [3,4], phospholipids [3], and catecholamines [4,5], which may be involved in the functional differences among the TH types.

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Table 1

Kinetic constants and relative homospecific activities of the four types of human TH

Enzyme type	K_m (μM)[a]		V_{max} (pmol/min per mg protein)[a]		Relative homospecific activity[b]
	L-Tyrosine	6-MPH_4	L-Tyrosine	6-MPH_4	
1	105 ± 15	253 ± 69	58.1 ± 5.1	57.6 ± 9.8	1.00
2	159 ± 43	254 ± 36	44.7 ± 4.3	26.0 ± 2.3	0.40
3	148 ± 34	182 ± 69	19.9 ± 3.3	12.4 ± 2.4	0.31
4	253 ± 71	191 ± 39	75.4 ± 2.3	44.6 ± 5.0	0.26

[a] Average ± SE

[b] Ratio vs homospecific activity of the type 1 enzyme

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