A Human Tyrosine Hydroxylase Isoform Associated with Progressive Supranuclear Palsy Shows Altered Enzymatic Activity*

(Received for publication, June 12, 1998, and in revised form, October 6, 1998)

Sylvie Bodeau-Péan†, Philippe Ravassard‡‡, Martin Neuner-Jehle‡, Baptiste Faucheux‡†, Jacques Mallet‡, and Sylvie Dumas‡‡‡

From †Laboratoire de Génétique Moléculaire de la Neurotransmission et des Processus Neurodégénératifs, CNRS UMR 9923 and ‡INSERM U289, Hôpital de la Salpêtrière, 75013 Paris, France

A novel human tyrosine hydroxylase (HTH) messenger RNA subgroup generated by alternative splicing and characterized by the absence of the third exon was recently identified. The corresponding putative protein lacks 74 amino acids including Ser31 and Ser40, two major phosphorylation sites implicated in the regulation of HTH activity. These mRNA species are detected in adrenal medulla and are overexpressed in patients suffering from progressive supranuclear palsy, a neurodegenerative disease mostly affecting catecholaminergic neurons of the basal ganglia.

In the present work, an HTH protein isoform lacking exon 3 was identified in human adrenal medulla. For this purpose, an antibody was raised against the HTH exon 3. The effect of the removal of exon 3 on the enzymatic activity of HTH was studied in vitro by comparing a purified recombinant fusion protein without exon 3 (glutathione S-transferase (GST)-HTH∆3) to the equivalent protein containing exon 3 (GST-HTH3). In initial velocity conditions, GST-HTH∆3 has 30% of the maximal velocity of GST-HTH3. Moreover, the skipping of exon 3 results in the absence of activation of GST-HTH by heparin and increases by 10-fold the retroinhibition constant for dopamine, demonstrating the involvement of exon 3 in the regulation of HTH enzymatic activity. The identification of a variably expressed HTH isoform that lacks an exon implicated in activity regulation supports the view that HTH alternative splicing contributes to the functional diversity within the catecholaminergic system and may be implicated in some neurological diseases.

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Tyrosine hydroxylase (TH)1 has been the subject of extensive investigation, largely because it catalyzes the rate-limiting step in the biosynthesis of catecholamines. The regulation of the TH level and of its enzymatic activity is thus a major mechanism for controlling the amount of these important amines in blood and synapses. TH is regulated by almost all possible mechanisms of transcriptional and post-transcriptional control including regulation of the transcription rate, alternative splicing of the premessenger, variable stability of the mRNA, translational control, and modulation of the enzymatic activity (1). Recently, it has been shown that some human TH (HTH) gene sequence variants are associated with abnormal TH enzymatic activity and may be involved in some neurodegenerative diseases. Indeed, point mutations in the coding sequence of HTH have been found that decrease the activity of the enzyme in patients with 1-DOPA-responsive dystonia (2) and inherited juvenile 1-DOPA-responsive parkinsonism (3). There is also emerging evidence that alternative splicing of TH premessenger RNA may have physiological and even pathological consequences (4, 5). Initially, four HTH mRNA variants generated by alternative splicing were cloned and characterized by (i) the differential use of two splicing donor sites within exon 1 and (ii) the differential inclusion of exon 2 (4, 6, 7). All four HTH species are present in human catecholaminergic tissues (8, 9). Interestingly, the amount of the species containing exon 2 (HTH-3 and -4) is enhanced in pheochromocytoma, a tumor of adrenal medulla (4). The four mRNAs encode proteins that differ in the N-terminal regulatory region and present different enzymatic activities (1, 10). These data suggest that alternative splicing of TH premessenger RNA is significant and prompt us to further study the diversity of HTH mRNAs. Recently, we identified previously undetected HTH mRNA species lacking exon 3 in the adrenal medulla (5). These novel variants are produced by the junction of the splicing donor sites of exons 1a-1b- or 2- directly to the acceptor site of exon 4. By RNase mapping experiments, we have shown that exon 3 was skipped in 4 to 6% of the total HTH mRNA population in normal adrenal medulla, a proportion similar to that of the previously described HTH-3 and -4 mRNA species (11, 12). These novel mRNA species were unusually abundant (5) in adrenal medulla of patients suffering from progressive supranuclear palsy (PSP), which is a severe neurodegenerative disease predominantly characterized by an alteration of the catecholaminergic neurons within the basal ganglia (13). The novel mRNA species encode putative HTH proteins lacking the 74 amino acids of the exon 3. This region includes Ser31 and Ser40, two phosphorylation sites that participate in the regulation of HTH enzymatic activity (1). Consequently, it is likely that the alternative splicing of exon 3 has consequences for the regulation of catecholamine biosynthesis.

In the present work, we analyzed the expression of this novel HTH isoform lacking exon 3 (HTH∆3) in human adrenals by Western blotting experiments. For that purpose, we raised an antibody against the HTH exon 3. We also tested the effect of exon 3 skipping on the in vitro enzymatic activity of HTH by the use of purified recombinant HTH proteins in fusion with glutathione-S-transferase (GST). It is clearly established that
TH activity is positively regulated by phosphorylation and by binding to any of several polyamines such as heparin, polyglutamates, and phosphatidylserine, which change the conformational state of the enzyme (14). Interestingly, the heparin binding site on rat TH (RTH) has been described as being precisely localized within an exon corresponding to RTH exon 3 (15). We therefore tested the effect of exon 3 skipping on RTH activation by heparin and show that GST·RTHΔ3 enzymatic activity is insensitive to this molecule. Feedback inhibition by catecholamines is one of the major mechanisms for modulating HTH enzymatic activity, and the N-terminal part of the enzyme plays an important role in retroinhibition by dopamine (16). Thus, we tested the effect of dopamine on GST·RTHΔ3 activity and found that HTH exon 3 is required for feedback inhibition.

EXPERIMENTAL PROCEDURES

Construction of the Expression Vectors—The complete coding sequence of HTH3 was excised from PET3a (Stratagene) (10) by Ndel-BamHI digestion. The fragment was bluntended with Klone polymerase and transferred into the Smal restriction site of PGEX-2T (Amersham Pharmacia Biotech). This plasmid permits the production of HTH3 fused to Schistosoma japonicum GST. A sequence encoding the corresponding isoform of HTH lacking exon 3 (RTHΔ3) was obtained by deletion of exon 3 from PGEX-2T-HTH3 as described in Lanie & ce (17). Briefly, two amplification products of the sequences corresponding to HTH exons 1 + 2 and exons 4 + 5 were joined by polymerase chain reaction, and the final amplification product was used to replace the HTH3 sequence between the BamHI and Xhol sites in PGEX-2T-HTH3.

FIG. 2

The primer used were (i°) ccataat ATGCCCACCCCCGACGCC (sense) and CCACCCCAAG–ACGTTTGAAGC (antisense) and (ii°) CCAACCCCAAG–ACGTTTGAAGC (junction exon2–exon 4, sense) and CCGGGTGGTCCAAGTGGAAGAGCAG, purified, digested, and introduced between the BamHI and EcoRI restriction sites of pGEX-2T (Amersham Pharmacia Biotech). The amplified products were checked by sequencing. The GST·RTHΔ3 fusion protein was produced in AX90 cells and purified according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The fusion protein was dia lyzed against 20 mM Tris, pH 7.5, 0.1 mM NaCl, 0.2 mM EDTA, 1 mM diithiothreitol and sent to Eurogentec (Ougrée, Belgium) for antisemur production in rabbits. The anti-HTH3 antisemur was purified by affinity using the GST·RTHΔ3 antigen immobilized on Immobilon-P membranes (Millipore). The immunoactivity of this antibody was checked by Western blotting experiments with total lysate from BL21 bacteria (Stratagene) transformed with PET3a-HTH1 and PET3a-HTHΔ3.

Western Blotting Experiments—Frozen post-mortem human adrenal glands from controls and PSP patients were obtained from the brain bank of Inserm U 286 (Hôpital de la Pitié-Salpêtrière, Paris) and from Dr. P. P. Plouin (Hôpital Broussais, Paris). PSP was clinically characterized, and the diagnosis was confirmed as described in Dumas et al. (5). Tissues were lyzed in 10 mM Tris, pH 7.5, 5 mM EDTA supplemented with 1 mM phenylmethylsulfonyl fluoride; Sigma) and centrifuged for 10 min at 4 °C. The total protein concentration was estimated by the Bradford method. Western blotting experiments were performed with standard procedures with an anti-HTH3 polyclonal antibody provided by Dr. J. F. Reinhard (19) and 125I-protein A. The membrane was stripped in 0.1 M glycine, pH 2.8, 0.05% Tween 20 at 80 °C and reprobed with the anti-HTH3·GST-purified antisemur. The amount of the HTHΔ3 isoform in the adrenal samples was estimated with the Image 1.59 software.

RESULTS

Identification of an HTH Isoform Lacking Exon 3 in Human Adrenals—To test human tissues for the presence of an HTH protein isoform lacking exon 3, we raised and purified an antisemur against the third exon of HTH. We ascertained the immunoactivity and specificity of affinity-purified anti-HTHΔ3 antibodies by Western blotting experiments with HTHΔ3 (containing exons 1a,3,4–14) and HTH1 (containing exons 1a,3,4–14) produced in E. coli by the PET-3a expression system. As expected, this anti-HTH3 antibody only recognized the HTH1 isoform (Fig. 1B), whereas a polyclonal anti-RTH antibody (19) recognized both HTH isoforms (Fig. 1A), attesting to the specificity of the anti-HTHΔ3 antibody.

Then, to determine the expression profile of the HTHΔ3 isoform in human tissues, total protein extracts from control and PSP adrenal medullas were probed with both antibodies by Western blotting experiments. Recombinant HTH proteins expressed from the PET-3A system in E. coli were used as size controls. With the polyclonal anti-RTH antibody (19), several bands were detected in these tissue samples (Fig. 2A). The two slower migrating bands correspond, respectively, to RTHΔ3 and 4 and HTH 1 and 2, as described previously (20). The faster band (of approximately 55 kDa) was found in all tissue samples; they were also detected in a noncatecholaminergic rat cell line (ST14A) stably transfected with HTH1-cDNA, showing that they were not the result of alternative splicing.

Interestingly, as seen on Fig. 2A, the polyclonal anti-RTH antibody detected a novel protein of the same size as recombinant HTHΔ3 in the PSP adrenal medulla sample. To confirm

To analyze the regulation of GST·HTH3 and -RTHΔ3 by polyamines, heparin (Calciparine Sanofi, France) was added to the assay mix (5 to 15 IU) before the addition of BH4 (500 μM). Similarly, final product retroinhibition was tested by the addition of dopamine (3-hydroxytyramine; Sigma) (10 to 100 μM) to the reaction before the addition of BH4 (500 μM). The inhibition constant for dopamine (Km) was determined graphically for both isoforms by plotting 1/V as a function of dopamine concentration for 3 different BH4 concentrations (250, 500, and 1000 μM). The point of intersection of the 3 lines gives K, directly (Dixon method, see Ref. 18). Synthesis of anti-HTH-exon 3 Antibody—The nucleotide sequence corresponding to the 59 first amino acids of the third exon of HTH was amplified by polymerase chain reaction with oligonucleotides cattactccccggatccCTACTTGCGCGAGCAG and cattactccccgcttggggGGAGGCGAG, purified, digested, and introduced between the BamHI and EcoRI restriction sites of pGEX-2T (Amersham Pharmacia Biotech). The amplified products were checked by sequencing. The GST·RTHΔ3 fusion protein was produced in AX90 cells and purified according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The fusion protein was dia lysed against 20 mM Tris, pH 7.5, 0.1 mM NaCl, 0.2 mM EDTA, 1 mM diithiothreitol and sent to Eurogentec (Ougrée, Belgium) for antisemur production in rabbits. The anti-HTH3 antisemur was purified by affinity using the GST·RTHΔ3 antigen immobilized on Immobilon-P membranes (Millipore). The immunoactivity of this antibody was checked by Western blotting experiments with total lysate from BL21 bacteria (Stratagene) transformed with PET3a-HTH1 and PET3a-HTHΔ3.

The primers used were (i°) ccataat ATGCCCACCCCCGACGCC (sense) and CCACCCCAAG–ACGTTTGAAGC (antisense) and (ii°) CCAACCCCAAG–ACGTTTGAAGC (junction exon2–exon 4, sense) and CCGGGTGGTCCAAGTGGAAGAGCAG (within exon 5). The construction was obtained from PET3a-HTH3 by deletion of the third exon using the same method. All amplified sequences were checked by sequencing.

Purification of GST·HTH Fusion Proteins—PET-3T–HTH3 and RTHΔ3 constructions were introduced into electrocompetent AX90 Escherichia coli cells. Production of the fusion proteins (GST·HTH3 and GST·RTHΔ3) was induced by isopropyl-1-thio-D-galactopyranoside, and the proteins were affinity-purified on glutathione-Sepharose beads according to the manufacturer’s instructions (Amersham Pharmacia Biotech) with minor modifications. E. coli cells were harvested at 25 °C instead of 37 °C to avoid formation of inclusion bodies. After elution from the glutathione-agarose beads, fusion proteins were extensively dialyzed against phosphate-buffered saline at 4 °C, then to desalt, concentrated, and eliminate low molecular weight protein contaminants, fusion proteins were centrifuged on Microcon–100 microconcentrators (Amicon, 100-kDa molecular mass cut-off columns). Protein purity was confirmed by electrophoresis on a 9% polyacrylamide denaturing gel followed by Coomassie Blue staining. The protein quantities were first estimated with the Bradford method, then precisely measured by comparing the intensity of the Coomassie Blue-stained band to a standard curve obtained with bovine serum albumin (Image 1.59 software).

Measuring of GST·HTH Activities—GST·HTH3 and -RTHΔ3 enzymatic activities were tested by a radioenzymatic assay as described in Alterio et al. (10) with minor modifications. Briefly, the enzymatic activities were measured as the release of tritiated water from L-[3H]tyrosine at 30 °C in 1 mM Hepes, pH 7, 40 μM tyrosine, 0.5 mg/ml catalase, 0.1 mM FeSO4, 0.32 μCi of L-[3H]Tyrosine (Amersham Pharmacia Biotech) following addition of the cofactor BH4 (250 to 3000 μM) diluted in 20 mM dithiothreitol. Fusion proteins were diluted before activity assay in 0.1 mM Hepes, pH 7, containing 1 μg/ml bovine serum albumin. Each activity was measured in triplicate, and further experiments were performed with several independent enzyme batches.

We first established the initial velocity conditions in which the activity increases linearly with time and enzyme quantity. Using different BH4 concentrations (250 to 3000 μM) diluted in 20 mM dithiothreitol, Lineweaver-Burk curves were then traced for both isoforms with the Kaleidagraph software. The experimental results were fitted for the inhibition by excess of BH4 substrate (10) with the following equation: 1/V = V0/K + [BH4] + [BH4]2/K2, according to Dixon and Webb (18), where V0 was the maximal velocity of the enzyme, K1 is the apparent Michaelis constant for BH4, and K2 the inhibition constant for BH4.
FIG. 2. Presence of an HTH isoform lacking exon 3 in human adrenal medulla samples. Western blotting experiments were performed with 10 to 100 μg of total protein from PET3a-HTH1-transformed E. coli cells (HTH1), PET3a-HTHΔ3-transformed E. coli cells (HTHΔ3), HTH1-cDNA-transfected eukaryotic cells (TC), adrenal medulla from a PSP patient (P1), and adrenal medulla from two controls (C1 and C2). The blot was incubated with polyclonal antibody (19) (A), then stripped and incubated with anti-HTH exon 3 purified antiserum (B). The sizes of the different HTH isoforms are indicated. The proportion of HTHΔ3 is 2, 1.5, 12, respectively, in C1, C2, and P1.

FIG. 3. Purity and integrity of GST-HTH fusion proteins. Coomassie Blue staining of a 12% denaturing polyacrylamide gel showing protein molecular mass (M), total lysates from E. coli cells transformed with PGEX-2T-HTH3 (1) and with PGEX-2T-HTHΔ3 (2), purified GST-HTH3 protein (3), purified GST-HTHΔ3 protein (4) is shown.

Role of Exon 3 in HTH Activity Regulation

the identity of this isoform, the membrane was stripped and reincubated with the anti-HTH exon 3 antibody (Fig. 2B). The novel protein was not recognized by this antibody, evidencing that it lacks exon 3 and is certainly HTHΔ3. This isoform is present in a substantial amount in PSP adrenal medulla and is present in much lower amounts in normal adrenal medullas (Fig. 2).

GST-HTHΔ3 Presents 30% of GST-HTH3 Maximal Velocity in Vitro—To analyze the involvement of exon 3 in HTH enzymatic activity, the cDNAs encoding HTHΔ3 and HTH3 (the equivalent isoform containing exon 3) were inserted into PGEX-2T (Amersham Pharmacia Biotech), allowing the synthesis of GST fusion proteins. Both GST-HTHΔ3 and GST-HTH3 fusion proteins were produced and purified from total bacterial lysate on an affinity column. The integrity and purity of both fusion proteins were ascertained by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining (Fig. 3). The activities of the recombinant GST-HTH proteins were measured by a radioenzymatic activity assay. The absence of activity presented by GST alone in this assay was ascertained (data not shown). The initial velocity conditions in which product formation increases linearly with time and enzyme concentration were determined (Fig. 4), and all further experiments were performed in the following conditions: 50 ng of fusion protein, 5 min reaction time. The GST-HTH activities were measured with various BH4 concentrations from 400 to 3000 μM, and the Lineweaver-Burk plots were traced, showing typical excess substrate inhibition (Fig. 5). The kinetic parameters were calculated according to the classical analysis of substrate inhibition kinetics, as described under “Experimental Procedures.” The maximal velocities of GST-HTH3 and -HTHΔ3 were, respectively, 1400 (±70) and 450 (±40) pmol/min/μg of enzyme, which shows that GST-HTH3 has approximately 30% GST-HTH3 maximal velocity. The absence of exon 3 did not significantly change the Michaelis constant (Km) of the enzyme for the cofactor BH4 (174 ± 24 μM for GST-HTH3, 180 ± 50 μM for GST-HTHΔ3) nor the inhibition constant (Ki) for BH4 (4100 ± 500 μM for GST-HTH3, 2800 ± 500 μM for GST-HTHΔ3).

The Exon 3 Participates in the Regulation of HTH by Dopamine and Heparin—HTH enzymatic activity is tightly controlled in vivo, subject to down-regulation by catecholamines as the end products of the biosynthetic chain, and activated by phosphorylation and polyanion binding (1).

Because retroinhibition by catecholamines is one of the major mechanisms for controlling TH activity, we tested the effect of exon 3 skipping on the regulation of HTH activity by dopamine. Both GST-HTH3 and GST-HTHΔ3 activities were analyzed with different concentrations of dopamine from 10 to 100 μM. As seen on Fig. 6B, GST-HTH3 was more strongly inhibited; its activity was reduced to 50% in the presence of 10 μM dopamine, whereas GST-HTHΔ3 was only decreased by 10%. To further characterize the kinetic parameters of the inhibition of GST-HTH proteins by dopamine, we measured GST-HTH3 and -HTHΔ3 activities at various dopamine concentrations in the presence of three different quantities of BH4 (Fig. 7). We graphically determined the inhibition constant (Ki) of both GST-HTH isoforms for dopamine according to the Dixon method (18). The calculated Ki for dopamine was increased from 1 to 10 μM by skipping exon 3, demonstrating that the exon 3 is implicated in the retroinhibition of TH by dopamine.

We also studied the effect of exon 3 skipping on the activation of HTH by heparin. GST-HTHΔ3 and -HTH3 activities were measured in the presence of a series of concentrations of heparin (Fig. 6A). GST-HTH3 activity increased gradually with heparin concentration (up to 20%), whereas no significant activation of GST-HTHΔ3 was observed, strongly implicating exon 3 in the activation of HTH by heparin.

Altogether these observations provide the first demonstration that exon 3 is implicated in the HTH overall catalytic activity and, most importantly, in the regulation of HTH activity by polyamions and by catecholamines.

**DISCUSSION**

A great diversity of mechanisms controlling TH activity has been described, and among these, alternative splicing of HTH...
pre-mRNA is thought to be significant (25). In the present study, we evidenced the presence in adrenal medulla of a novel HTH isoform, HTHD3, that lacks the third exon comprising two major phosphorylation sites, Ser31 and Ser40. This isoform corresponds to the translation of a previously unsuspected HTH mRNA generated by alternative splicing recently identified in adrenal medulla and overexpressed in adrenals of patients suffering from PSP, a neurodegenerative disease affecting predominantly catecholaminergic neurons of the basal ganglia (5). We also established that the exon 3 participates in the regulation of HTH enzymatic activity in vitro.

The presence of an HTH protein isoform lacking the third exon in vivo was evidenced by Western blotting experiments. This HTH isoform, having the size of recombinant HTHD3, was not recognized by an antibody specifically raised against HTH exon 3, whereas it was clearly detected by a polyclonal antibody directed against whole rat TH recognizing all HTH isoforms. This protein isoform was clearly detected in PSP adrenal medulla and was about six times less abundant in control adrenal medullas. Substantial amounts of the novel isoform were also found in all other PSP adrenals tested (data not shown), in accordance with the higher levels of HTHD3 mRNA previously observed in PSP adrenals (5). This variable expression of the HTHD3 isoform indicate that alternative splicing of HTH exon 3 may be significant. The increased amount of HTHD3 in PSP suggests that the splicing alteration may be a cause of the disease. This schema involving a poorly expressed truncated enzyme isoform generated by alternative splicing has already been proposed by Hirano et al. (42). Indeed, some familial patients with DOPA-responsive dystonia have been shown to express a small proportion of incorrectly spliced mRNA encoding GTP-cyclohydrolase I (the limiting enzyme in the synthesis of BH4), and the amount correlates with the severity of the symptoms. Because several familial cases of PSP have been described (39), we searched by sequencing for mutations at exon 3 splice junctions within HTH gene on genomic DNA extracted from the cerebellum of 10 PSP patients. Among these, only three adrenal medullas were available, and they show an expression of the HTHD3 protein (data not shown). No mutation was found in a region of 100 bases around the donor and acceptor splice junctions of exon 3 (data not shown), indicating that the modification of the splicing of exon 3 is not because of a mutation of the HTH sequence in these patients. Alternatively, there may be a modification in the amount of

FIG. 4. Enzymatic activity of GST-HTH3 and GST-HTHΔ3 fusion proteins: initial velocity conditions. TH activity was measured with 25 to 100 ng of purified fusion protein by a radioenzymatic assay using \( L-[3H] \) tyrosine as described under “Experimental Procedures” with various incubation times. The activity is expressed as pmoles of DOPA according to the radioactive counts. A, pmoles of DOPA increase linearly with time for both isoforms; B, the slopes of the curves obtained in A were plotted against enzyme quantity, showing that pmoles of DOPA/min increases linearly with the amount of enzyme.

FIG. 5. Lineweaver-Burk plots for GST-HTH3 and GST-HTHΔ3. The enzymatic activities of both forms were measured with a series of quantities of BH4 ranging from 400 to 3000 μM. The inhibition by high BH4 concentrations described in Alterio et al. (10) was observed. The curves were fitted by Kaleidagraph software according to Dixon and Webb (18), as described under “Experimental Procedures.” micro, microgram.
splicing factors influencing the choice of splice junctions and the inclusion/exclusion of alternative exons, such as SF2/ASF and heterogeneous nuclear ribonucleoprotein A1 (40).

Interestingly, in Western blot experiments, an unidentified 55-kDa HTH isoform that was not the result of alternative splicing was detected in all tissue samples. This isoform has already been reported in previous studies (8, 20) but was interpreted as a degradation product. However, it may rather be a genuine HTH isoform. Indeed, its size corresponds to (i) the use of a second AUG initiator codon located at the end of exon 1 that matches perfectly with a translation initiation sequence (21) and/or to (ii) the result of a cleavage of HTH by calpain (Ca²⁺-activated neutral protease) (22–24). The possibility that this 55-kDa isoform is the proteolysis calpain cleavage product is of particular interest. Indeed, calpain is present in catecholaminergic tissues and activates TH in vitro (22). The 55-kDa isoform seems to be particularly abundant in PSP adrenals (Fig. 2) but further experiments are needed to confirm its identity and any link with PSP disease.

To evaluate the role of exon 3 specifically, the enzymatic activity of the purified fusion proteins GST-HTH3 and GST-HTHΔ3, which differ only by the presence of exon 3, were compared. This work was carefully performed in conditions in which such a comparison is meaningful, e.g. the two fusion proteins were (i) purified in the same way and (ii) tested precisely in initial velocity conditions with exactly the same protocol. In these conditions, the GST-HTH proteins displayed some of the enzymatic properties characteristic of TH, in particular inhibition by excess BH4, heparin activation, and dopamine retroinhibition. Moreover, the two fusion proteins displayed kinetic parameters (Kₘ and Kᵢ for BH4) of the same magnitude. However, because of the possible influence of GST on HTH activity, the kinetic parameters obtained can only be used to compare GST-HTH3 and GST-HTHΔ3 proteins and thereby to evaluate the role of exon 3. Our results show that GST-HTHΔ3 presents in vitro 30% of the maximal velocity of GST-HTH3, the equivalent isoform containing exon 3. Interestingly, this lower GST-HTHΔ3 maximal velocity is not associated with a modification of the Michaelis constant (Kₘ) for the cofactor BH4 nor of the inhibition constant (Kᵢ) for BH4. Thus, exon 3 may influence the accessibility of some other components such as tyrosine and/or ferrous iron, possibly by modifying the conformation of the enzyme. It is unlikely that the skipping of exon 3 simply affects the catalytic process itself, as exon 3 is not localized within the catalytic part of the enzyme.

The third exon of HTH, absent from the novel isoform, is located in the N-terminal regulatory part of the enzyme (26).
This N-terminal region has been implicated in the action of numerous allosteric regulatory substances, including polyanions like heparin. Little is known about possible direct actions of heparin on enzymatic activities within the cell. However, it has been hypothesized that a direct interaction can occur between intracellular proteins and heparin in the Golgi complex or that heparin might mimic the effects of intracellular heparin-like factors (27). Concerning TH, it has been shown that heparin reversibly activates the enzyme probably by changing its conformation (14, 28). Here, we show that exon 3 is involved in the activation of HTH by heparin because GST-HTH3 is activated by heparin, whereas GST-HTHΔ3 is not. This result is consistent with other studies evidencing that in rat TH, the heparin binding site is precisely localized within an exon homologous to HTH exon 3 (15). As the interaction of the HTH N-terminal part with heparin involves a similar mechanism as several polyanions (1), it is probable that the absence of exon 3 also affects the activation of HTH by other physiological polyanions such as phospholipids, polyglutamic acid, and nucleic acids (14, 28).

Feedback inhibition of TH enzymatic activity by catecholamines is considered as the primary mechanism by which this amine biosynthesis is regulated (29). The regulation of TH activity by dopamine has been extensively studied (1, 30, 31) and shown to involve a portion of the N-terminal part of the enzyme close to Ser40 (16). Thus, we tested if HTH exon 3 played a role in the retroinhibition process. We found that the skipping of the HTH third exon increases the inhibition constant (K_i) of the GST-HTH protein for dopamine by approximately 10-fold, demonstrating that exon 3 is implicated in the inhibition of HTH activity by dopamine. Generally, phosphorylation of TH and retroinhibition by catecholamines act in coordination to regulate TH activity according to the cellular requirement. For example, phosphorylation of Ser40, mediated by most second messenger systems, activates TH and partially activates protein kinases (ERK1 and 2) has been shown to present specific regulatory properties after cAMP-dependent protein kinase phosphorylation (10).

It is clear that exon 3 plays a role in the regulation of HTH enzymatic activity. Interestingly, this part of TH may also have a role in other species. Indeed, the region homologous to the HTH exon 3 is also alternatively spliced in rat (17) and in Drosophila (41) (Fig. 8). Moreover, in Drosophila, the TH isoform that differs in the region corresponding to exon 3 is specifically expressed in the hypoderm and has been proposed to be hyperactive and contribute to the synthesis of the large amounts of dopamine within the cuticle.

Finally, the identification of a variably expressed HTH isoform that lacks an exon implicated in enzymatic activity regulation supports the view that HTH alternative splicing may be involved in some neurological diseases and/or in the functional diversity of the catecholaminergic system.

Acknowledgements—We are grateful to Dr. J. F. Reinhard for providing the anti-TH antibody. Thanks to O. Corti for providing the HTH1-transfected cell line. Special thanks to V. Berthelier for technical advice and helpful discussions.
