Tissue-Specific Alternative Splicing of the First Exon Generates Two Types of mRNAs in Human Aromatic L-Amino Acid Decarboxylase†,‡

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ABSTRACT: Aromatic-L-amino-acid decarboxylase (AADC) is an enzyme that plays an essential role in synthesizing catecholamines and serotonin in neuronal and endocrine tissues. AADC has also been detected in other nonneuronal tissues including liver and kidney, although its physiological role in nonneuronal tissues has not yet been defined. Previously we have cloned a human AADC cDNA from a neuronal tissue (pheochromocytoma) [Ichinose, H., Kurosawa, Y., Titani, K., Fujita, K., & Nagatsu, T. (1989) *Biochem. Biophys. Res. Commun. 164*, 1024–1030] and the corresponding genomic DNA [Sumi-Ichinose, C., Ichinose, H., Takahashi, E., Hori, T., & Nagatsu, T. (1992) *Biochemistry 31*, 2229–2238]. Here we present isolation and characterization of AADC cDNA and genomic DNA from a nonneuronal tissue (human liver). The nonneuronal and neuronal AADC mRNAs differed only in the region corresponding to the untranslated first exon. The first exon for the nonneuronal-type mRNA was located 4.2 kilobases upstream to that for the neuronal-type mRNA and 22 kilobases from exon 2, to which it is spliced. Determination of the transcription initiation site indicated that the length of the nonneuronal-type exon 1 was 200 bp. A TATA box-like motif was located between positions −26 and −20 from the transcription initiation site. These results showed that an alternative usage of the first exon in the 5′-untranslated regions produces two types of mRNAs in AADC and suggested that alternative splicing would regulate the tissue-specific expression of AADC.

Catecholamines, i.e., dopamine, norepinephrine, and epinephrine, are synthesized from L-tyrosine through L-3,4-dihydroxyphenylalanine (L-DOPA). Indoleamines, i.e., serotonin and melatonin, are produced from L-tryptophan through L-5-hydroxytryptophan. Decarboxylating reactions from their precursor aromatic amino acids to amines are catalyzed by aromatic-L-amino-acid decarboxylase (AADC; EC 4.1.1.28; Lovenberg et al., 1962). AADC plays an essential step for production of monoamines.

Since catecholamines and indoleamines are neurotransmitters and hormones, nervous and endocrine organs such as brain and adrenal medulla have high AADC activities. AADC, however, exists in liver and kidney as well as in these amine-producing tissues (Lovenberg et al., 1962; Rahman et al., 1981). The physiological role for AADC in liver and kidney is still uncertain.

Recently cDNA cloning of AADC was successfully done from human pheochromocytoma (Ichinose et al., 1989), rat liver (Tanaka et al., 1989), and bovine adrenal (Kang & Joh, 1990). These studies showed that the primary structures of AADC are highly conserved among species.

We first succeeded in determining the entire genomic structure of mammalian AADC and proved that a single gene codes AADC (Sumi-Ichinose et al., 1992). On the other hand, Krieger et al. (1991) has found that two types of AADC mRNAs are present in rat tissues of neuronal and nonneuronal origin and that the differences in the AADC mRNAs are restricted in the 5′-untranslated region. Results of these reports strongly suggested that an alternative splicing event may occur in AADC.

Here we found the nonneuronal-type cDNA from human liver and identified another first exon different from the one that we had previously reported. This study showed that an alternative usage of the first exon produces two types of mRNAs in AADC and suggested that alternative splicing would regulate the tissue-specific expression of AADC.

EXPERIMENTAL PROCEDURES

cDNA Cloning. A human liver cDNA library in λgt11 using a random primer and *Eco*RI linkers was a generous gift from Dr. Y. Ebina (The University of Tokushima, Japan). A total of 3×10^5 λ-phage plaques on *Escherichia coli* strain Y1088 lawn cells were screened with a probe of a DNA fragment (0.5 kb *Eco*RI–*Sac*I fragment) containing the 5′-terminal region of the human AADC cDNA from pheochromocytoma. The probe was purified by the glass beads method (Geneclean, Bio 101 Inc.) and labeled by a random primer method (Amersham, U.K.). The phage plaques were lifted twice onto nylon membranes (Hybond-N, Amersham, U.K.) and processed for hybridization. Prehybridization was carried out at least 2 h in 6x SSC (1x SSC = 0.15 M NaCl and 0.015 M sodium citrate) containing 5x Denhardt's solution, 0.5% SDS, and 0.1 mg/mL salmon sperm DNA at 65 °C. Hybridization followed at 65 °C for 20 h in the above-specified buffer with a labeled probe. The membranes were washed with 2x SSC containing 0.1% SDS at room temperature twice and then soaked in the same solution at 42 °C for 20 min twice. The cDNA clones with positive signal were purified and subcloned into either *Eco*RI site of pUC119 or *Sac*I–*Kpn*I site of Bluescript KS (M13+).

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DNA Sequence Analysis. All DNA sequencing was carried out bidirectionally by the dideoxy chain termination method of Sanger et al. (1977) after the restriction fragments had been subcloned into M13 vectors. Some DNA fragments around the exon L1 were treated with exonuclease III and mung bean nuclease to construct serial deletions of a desired length.

Transcription Initiation Site Mapping. Total RNA was extracted from human postmortem liver with guanidinium thiocyanate followed by centrifugation in cesium chloride solutions (Sambrook et al., 1989). The transcription initiation site was determined by both primer extension analysis (Mcknight & Kingsbury, 1982) and reverse transcription–polymerase chain reaction (RT–PCR) analysis.

In the primer extension experiment, human liver total RNA (50 μg) was annealed to approximately 3×10^5 cpm ^{32}P-end-labeled primer LA or primer 13 (see their positions in Figure 3) in 0.25 M KCl by heating at 65 °C for 1 h, followed by an incubation at room temperature for 1.5 h. The annealed primer was extended at 42 °C for 1 h in a reaction mixture containing 20 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.25 mM dNTPs, 0.1 mg/mL actinomycin D, and 175 units/mL of reverse transcriptase (originated from rous-associated virus 2). After the treatment with RNase A at 37 °C for 30 min, the sample was extracted with phenol/chloroform, precipitated by ethanol, and analyzed in a 6% acrylamide–7 M urea sequence gel. The DNA fragment containing exon L1 was sequenced with the primer LA at the same time.

For RT–PCR experiments, total RNA from human liver was reverse transcribed with random hexamer and amplified using primer 13 as an antisense primer and either primer 3, primer 2, primer 1, primer Z, or primer X as a sense primer. Their sequences and positions on genomic DNA are shown in Figure 3. The conditions for the amplification for 30 cycles were as follows: denaturation at 94 °C for 30 s, annealing, and extension at 60 °C for 1 min. The amplified DNA fragments were electrophoresed in 4% NuSieve GTG agarose (FMC Bioproduct).

Tissue Distribution of Neuronal- and Nonneuronal-Type mRNAs. Antisense primer, AADC-AS, was 5′-GCGGATC-CTCAATGCCTTCCATGTAGTT-3′, which was complementary to nucleotide 55–76 in exon 2 with an additional *Bam*HI site. Primer AADC-NS, 5′-TTCTGTGCCTCT-TAACTGTCACTG-3′, was corresponding to nucleotide −76 to −99 in the neuronal type exon N1. Primer AADC-LS, 5′-AAGGAATTCGAATTTCCAGCAT-3′, was corresponding to nucleotide −29 to −50 in the nonneuronal-type exon L1. Total RNAs from various human tissues were reverse transcribed into cDNAs by murine moloney leukemia virus reverse transcriptase (Bethesda Research Laboratories) using a random hexamer as a primer. The transcribed cDNA was amplified by the PCR using either AADC-AS and AADC-NS for amplification of neuronal-type mRNA or AADC-AS and AADC-LS for amplification of nonneuronal-type mRNA. We carried out 30 cycles of amplification using GeneAmp PCR System 9600 (Perkin-Elmer-Cetus Instruments), and the profile of amplification step was denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s. The amplified DNA fragments were electrophoresed in 4% NuSieve GTG agarose (FMC Bioproduct).

RESULTS

cDNA Cloning of AADC from Human Liver. The *Eco*RI-*Sac*I fragment (nucleotide number 13–522) of human AADC cDNA from pheochromocytoma was labeled and used for screening through plaque hybridization of 3×10^5 recombinants from a human liver cDNA library. Five strongly hybridized clones were isolated. One clone, λhLDC-1, which had the longest 5′-region, was selected by the PCR analysis. Clone λhLDC-1 was subcloned into the *Eco*RI site or the *Sac*I–*Kpn*I site of a Bluescript vector because there is an internal *Eco*RI site in the AADC cDNA (see Figure 1A). Sequence analysis of clone λhLDC-1 revealed that it consisted of 1.2 kb ranging from nucleotide −47 to +1180 compared with the cDNA from human pheochromocytoma and that it had a 19-base sequence different from the sequence we previously reported (Ichinose et al., 1989) in a region corresponding to the first exon (Figure 1B). The sequence was 5′-GAATTCGAATTTCCAGCAT-3′. This cDNA library was made using an *Eco*RI linker consisting of 8-mers, 5′-GGAATTCC-3′. Since the clone was terminated at the sequence of 5′-GAATTCGAA-3′ but not 5′-GAATTCC-GAA-3′, the terminal *Eco*RI site of the clone seemed to be the second internal *Eco*RI site of the cDNA.

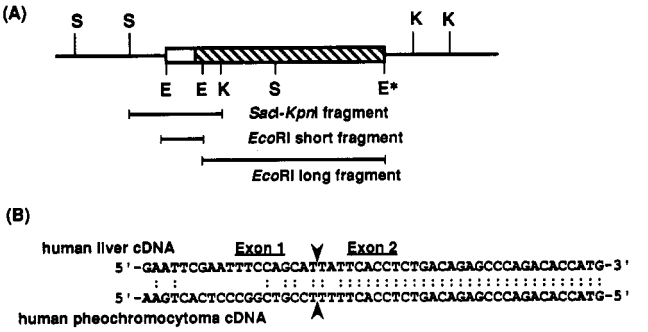

FIGURE 1: (A) Restriction endonuclease map of λhLDC-1. The box indicates the cDNA insert, and the hatched box depicts the coding region. The vector DNA of λgt11 is indicated by a thick line. Restriction sites: E, *Eco*RI; K, *Kpn*I; S, *Sac*I. An asterisk means the artificial site for ligation to vectors. (B) Partial nucleotide sequences of AADC cDNAs from human pheochromocytoma and human liver. Arrow heads indicate the breakpoint between exon 1 and exon 2. Identical nucleotides between human liver cDNA and human pheochromocytoma cDNA are indicated by the dotted lines.

The other difference in the sequence of λhLDC-1 was the position −27 in the untranslated region of exon 2. The sequence of λhLDC-1 was A instead of T (Figure 1B). Because the sequences of the cDNA clone from human pheochromocytoma and of the human genomic DNA clone were T, this difference could be explained by a polymorphism.

Determination of Position of Liver-Specific Exon. The result of cDNA cloning from human liver strongly suggested the presence of the nonneuronal-type first exon. We had isolated several human genomic clones (Sumi-Ichinose et al., 1992). One clone, gLDC1, contained the neuronal-type first exon (designated as exon N1). As it turned out that the nonneuronal-type exon (designated as exon L1) contained an internal *Eco*RI site, subcloned *Eco*RI fragments of gLDC1 were sequenced from both ends. Fortunately, we could find the exon L1 located 4.2 kb upstream from the exon N1 (Figure 2). We sequenced the regions flanking this exon (Figure 3). Sequencing of fragments subcloned into an *Eco*RI or *Hin*dIII site was performed with plasmids containing serial deletions of various lengths. The junction of exon L1 and the intron, CAT ! GTAAGT, matched well with the consensus sequence of the splicing donor site (Breathnach & Chambon, 1981).

Determination of Transcriptional Starting Site. Two additional approaches were taken to determine the transcriptional initiation site of the nonneuronal-type exon L1. First we employed primer extension analyses using ^{32}P-end-labeled

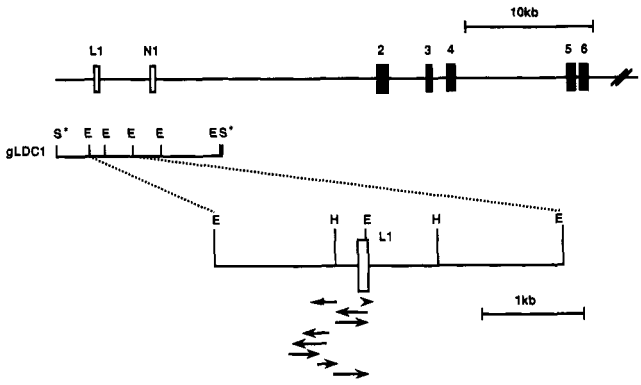

FIGURE 2: Partial structure and restriction map of the human AADC gene. The structure of the upstream portion of the human AADC gene is depicted at the top of the figure. Coding regions are indicated by solid boxes and untranslated regions by open ones. Introns are represented by the thin horizontal line. One clone, gLDC1, which contains exon L1 and exon N1, is shown in the figure. *Eco*RI sites and a part of *Hin*dIII sides of the clone are indicated by E (*Eco*RI) and H (*Hin*dIII), and the *Sal*I sites in the vector DNA are indicated by S*. The direction and length of each sequencing of gLDC1 are indicated below the restriction map by small arrows.

primer LA located in exon L1 and primer 13 located in exon 2. As shown in Figure 4, this experiment yielded a major primer-extended product of 186 nucleotides in primer LA and 240 nucleotides in primer 13. As the distance between primer LA and primer 13 was 54 nucleotides, the fragments of these length maped the unique initiation site at the "G" residue of nucleotide −228.

In order to confirm this result, we further performed RT–PCR analysis using primer 13 as an antisense primer and either primer LS3, LS2, LS1, LSZ, or LSY as a sense primer (Figure 5). Total RNA from human liver generated amplified products of the predicted size with primer LS3, LS2, and LS1, whereas primer LSZ and LSY showed no band. The result that all of these sense primers were able to amplify the plasmid DNA containing exon L1 with primer LA as an antisense primer (data not shown) indicated that PCR reaction was carried out correctly. Thus, these results suggested that the 5′-end of the mRNA in the exon L1 was present between primer LS1 and LSZ. This result agreed with those of the primer extension analyses.

5′-Flanking Region. The nucleotide sequence of the 5′-flanking region was determined to be about 600 bp upstream from exon L1 (Figure 3). A possible TATA box-like motif (5′-TATTTCT-3′) was present at −20 to −26 from the transcriptional starting site. The flanking region did not contain typical consensus elements for the binding of AP-1 (Lee et al., 1987) or SP-1 (Briggs et al., 1986). Further, we could not find the binding sequences for the known liver-specific nuclear factors (Courtois et al., 1987; Cereghini et al., 1988).

Expression of AADC mRNAs in Different Tissues. To examine the distribution of the AADC mRNAs, we performed a RT–PCR experiment with specific primer sets. A primer set, AADC-AS and -NS, amplified only neuronal-type mRNA, while a primer set, AADC-AS and -LS, did only nonneuronal-type mRNA (Figure 6A). Approximately 1 μg of total RNA

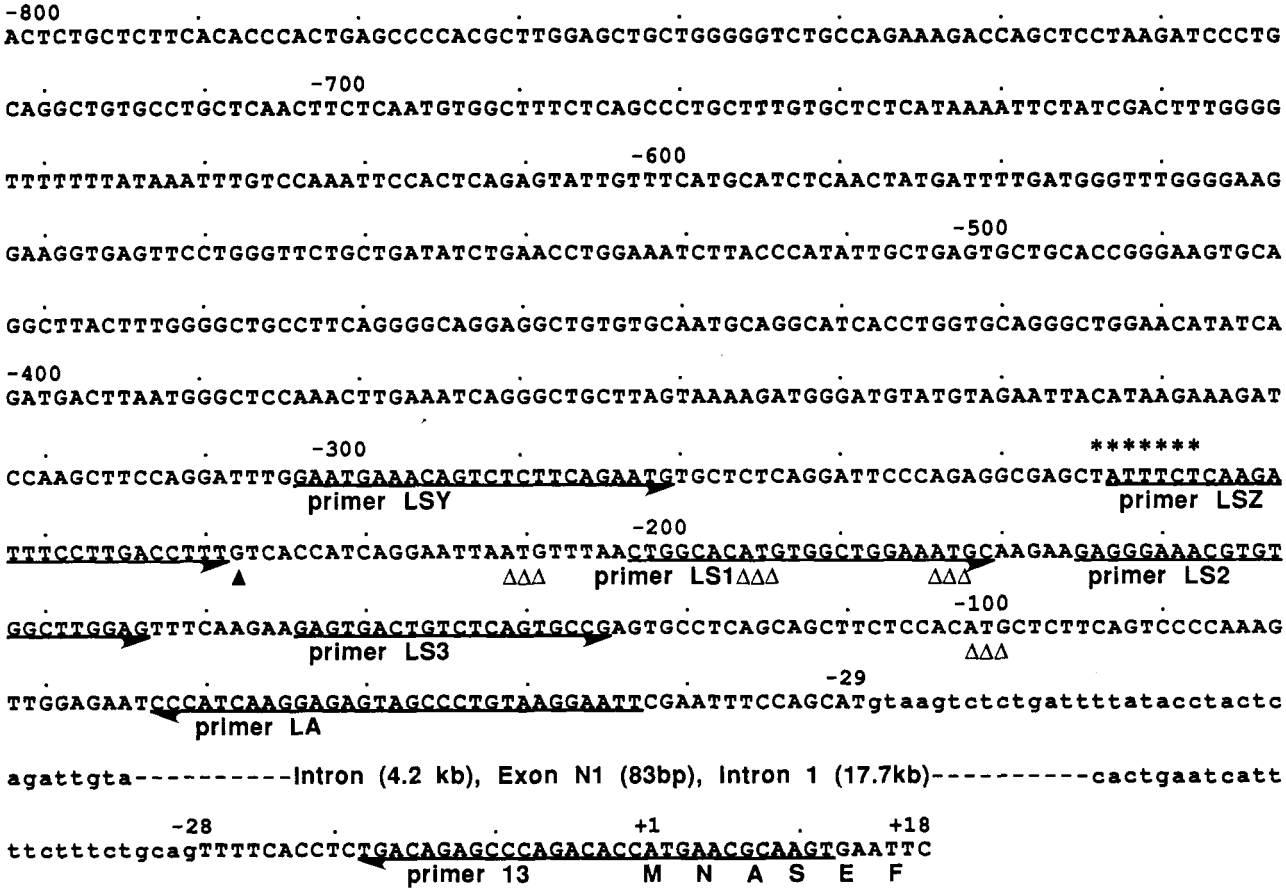

FIGURE 3: DNA sequence of the AADC alternative exon L1 and flanking sequences. The nucleotide sequence is numbered with the first base of the initiation ATG codon in exon 2 designated as +1. Nucleotides within intron are not numbered. The 5′-flanking and exon sequences are in uppercase letters, while the intron sequence is in lowercase letters. The locations of the transcription initiation site (as determined in Figure 4) are indicated by a solid triangle. Open triangles indicate the ATG sequences preceding the initiator ATG codon. A possible TATA box sequence is shown by asterisks. Primers used for primer extension and RT–PCR are designated by arrows.

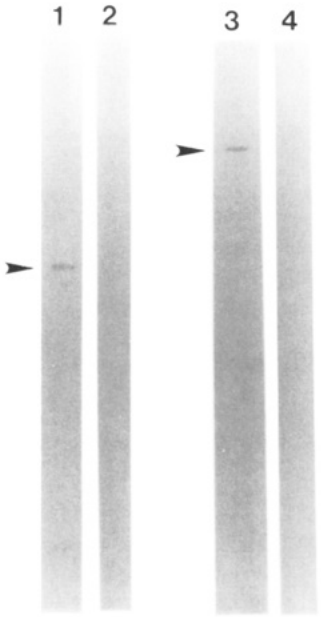

FIGURE 4: Primer extension of human AADC gene. A 5′-labeled oligonucleotide (primer LA or primer 13) complementary to the AADC mRNA was hybridized to 50 μg of total RNA from human liver and extended at 42 °C by using rous-associated virus 2 reverse transcriptase. The locations of the extension stop points were determined by parallel lanes containing a dideoxynucleotide-terminated sequence. Primer LA was used in lanes 1 and 2, and primer 13 was used in lanes 3 and 4; no reverse transcriptase was added in lanes 2 and 4.

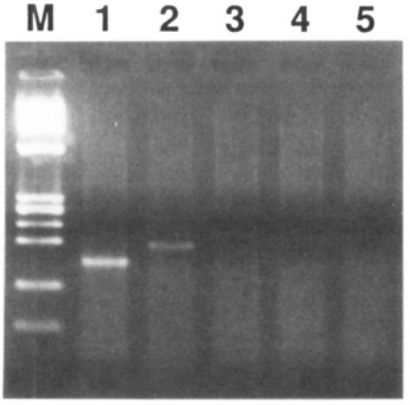

FIGURE 5: PCR analysis of 5′-region of nonneuronal-type human AADC mRNA. RNA from human liver was reverse-transcribed using random hexamer oligonucleotides. The cDNA was subjected to PCR amplification using primer 13 in exon 2 as an antisense primer and primer LS3 (lane 1); primer LS2 (lane 2); primer LS1 (lane 3); primer LSZ (lane 4); or primer LSY (lane 5) as a sense primer. Lane M contains *Hae*III-digested φX174 markers.

from human liver, kidney, adrenal, carcinoid tumor, and brain (substantia nigra) were reverse-transcribed followed by 30 cycles of the PCR.

As shown in Figure 6B, nonneuronal-type mRNA was detected in both liver and kidney, while we detected a small amount of neuronal-type mRNA in kidney. Neuronal and endocrine tissues such as adrenal, carcinoid, and brain had only the neuronal-type mRNA.

DISCUSSION

The main physiological role of AADC is the synthesis of bioactive monoamines from their precursor amino acids, for instance, dopamine from L-DOPA and serotonin from L-5-hydroxytryptophan. As these aromatic amines act as neurotransmitters and hormones, they are produced in neuronal

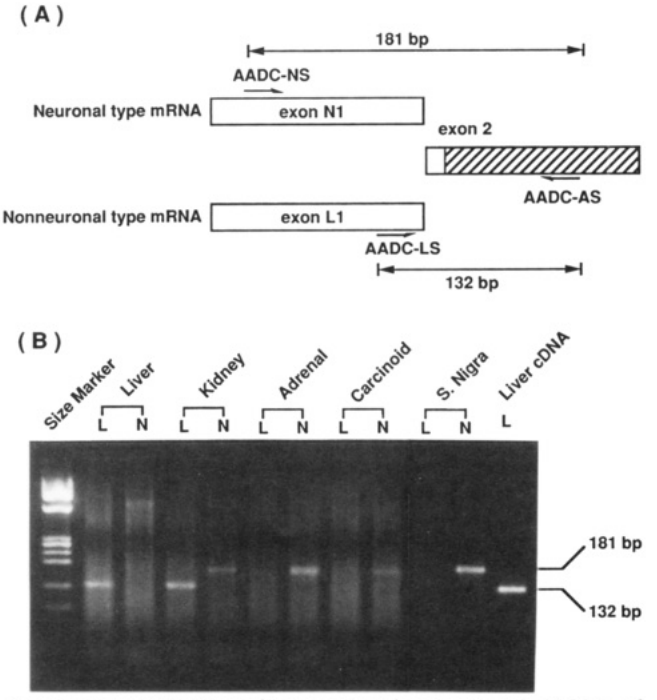

FIGURE 6: Distribution of neuronal and nonneuronal mRNAs of AADC in various tissues. (A) Schematic illustration of the position of each primer used for the PCR analysis. A hatched box represents the coding region. (B) Total RNAs from various tissues are subjected to RT–PCR analysis for the distinguishable detection of neuronal- or nonneuronal-type mRNA. Approximately 1 μg of total RNAs was reverse-transcribed into cDNAs as described under Experimental Procedures and then amplified by the PCR using each set of specific primers. PCR products were electrophoresed in 4% NuSieve GTG agarose. Lanes: L, PCR products using the primer set for the nonneuronal-type mRNA; N, PCR products using the primer set for the neuronal-type mRNA. *Hae*III-digested φX174 DNA were used as a size marker.

and endocrine tissues. High AADC activity, however, has been found in nonneuronal tissues, such as liver and kidney. The physiological function of AADC in nonneuronal tissues is still uncertain.

Krieger et al. (1991) reported the existence of two types of mRNAs in rat AADC. However, at that time, there was no information about the structure of the AADC gene in mammals. We first succeeded in determining the structure of the human AADC gene and proved that a single gene codes AADC (Sumi-Ichinose et al., 1992). The results presented in this paper proved that two types of mRNAs in human AADC were produced from a single gene through an alternative splicing event. These results strongly suggest that expression of AADC in nonneuronal tissues is regulated by a different promoter from that used in neuronal tissues. A variety of genes use an alternative-promoter mechanism for regulating tissue-specific expression, i.e., growth hormone-releasing hormone gene for the expression in anterior pituitary and placenta (González-Crespo & Boronat, 1991) and dystrophin gene for the expression in muscle and brain (Boyce et al., 1991). The proposed mechanisms in this paper are novel for the neuronal versus nonneuronal cell systems.

Exon L1 has the length of 200 bp and four ATG sequences as shown by triangles in Figure 3. These ATG sequences would not be used for the protein synthesis in vivo, for the true translational starting ATG sequence for AADC located in exon 2. In an attempt to understand a role of exon L1, a conformation was drawn for exon L1 mRNA which would be expected if the molecule had minimal free energy (data not shown). Exon L1 formed a strong stem–loop structure which

resulted in −44 kcal/mol. As the AUG codons in exon L1 are involved in this stem–loop structure, they may not function as the translational starting site.

The RT–PCR experiment clearly showed that the alternative splicing of the first exon is regulated in a tissue-specific manner. Liver has only nonneuronal-type mRNA, and brain, adrenal, and carcinoid tumor have only neuronal-type mRNA. Carcinoid tumor produces serotonin, while catecholamines are hormones in adrenal medulla. This suggests that the same mRNA species of AADC is used in both catecholamine- and serotonin-producing cells. We detected not only the nonneuronal-type but also the neuronal-type mRNA in kidney by the RT–PCR analysis, while the amount of the neuronal-type mRNA was less than that of the nonneuronal one (Figure 5). The reason for the presence of the neuronal-type mRNA in kidney cannot be explained at the present.

AADC in *Drosophila melanogaster*, which is often called DOPA decarboxylase, has been extensively studied. AADC has two identified functions in the fly. One is the production of the putative neurotransmitters, dopamine and serotonin, in the central nervous system (CNS) (Livingstone & Tempel, 1983); the other is the synthesis of dopamine in the hypoderm, which is metabolized to compounds used in cuticle hardening and pigmentation (Lunan & Mitchell, 1969). The existence of long and short mRNAs for AADC have been observed in *Drosophila* which are produced from a single gene by alternative splicing (Morgan et al., 1986; Eveleth et al., 1986). The long mRNA is expressed in the CNS, whereas the short one is expressed in the hypoderm (Morgan et al., 1986). The long mRNA encodes an AADC isoform which differs from the hypodermal form by the addition of 33–35 amino acids at the N-terminus of the protein (Morgan et al., 1986). Thus, the similar mechanism is used for the tissue-specific expression of AADC between mammals and insect, although two mRNAs in mammals encode the same protein.

We have found a putative TATA box-like motif at 26 bp upstream of the transcriptional starting site of exon L1. However, other consensus sequences for the binding of known transcriptional factors do not exist within 600 bp of the upstream region. The promoter region of neuronal first exon N1 has no TATA- and CAAT-like sequences (Sumi-Ichinose et al., 1992). Several cis-acting transcriptional elements have been characterized, that act on the promoter region of AADC in *Drosophila*. The DNA binding protein, Cf1a, is one of such factors (Johnson & Hirsh, 1990). Studies on the mechanism for regulating gene expression of AADC would be necessary to understand the tissue-specific expression of AADC. We are now planning to study cis-acting elements for the expression of AADC.

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