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Articles

Molecular Cloning of Genomic DNA and Chromosomal Assignment of the Gene for Human Aromatic L-Amino Acid Decarboxylase, the Enzyme for Catecholamine and Serotonin Biosynthesis†,‡

Chiho Sumi-Ichinose,§ Hiroshi Ichinose,‖ Ei-ichi Takahashi,⊥ Tada-aki Hori,⊥ and Toshiharu Nagatsu*,‖
Department of Pharmacology, Nagoya University School of Medicine, Nagoya 466, Japan, Institute for Comprehensive Medical Sciences, School of Medicine, Fujita Health University, Toyoake, Aichi 470-11, Japan, and Division of Genetics, National Institute of Radiological Sciences, 4-9-1 Anagawa, Chiba 260, Japan

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ABSTRACT: Aromatic L-amino acid decarboxylase (AADC) catalyzes the decarboxylation of both L-3,4-dihydroxyphenylalanine and L-5-hydroxytryptophan to dopamine and serotonin, respectively, which are major mammalian neurotransmitters and hormones belonging to catecholamines and indoleamines. This report describes the organization of the human AADC gene. We proved that the gene of human AADC consists of 15 exons spanning more than 85 kilobases and exists as a single copy in the haploid genome. The boundaries between exon and intron followed the AG/GT rule. The sizes of exons and introns ranged from 20 to 400 bp and from 1.0 to 17.7 kb, respectively, while the sizes of four introns were not determined. Untranslated regions located in the 5′ region of mRNA were encoded by two exons, exons 1 and 2. The transcriptional starting point was determined around G at position −111 by primer extension and S1 mapping. There were no typical "TATA box" and "CAAT box" within 540 bp from the transcriptional starting point. The human AADC gene was mapped to chromosome band 7p12.1-p12.3 by fluorescence in situ hybridization. This is the first report on the genomic structure and chromosomal localization of the AADC gene in mammals.

Aromatic L-amino acid decarboxylase (AADC; EC 4.1.1.28) catalyzes the decarboxylation of both L-3,4-dihydroxyphenylalanine (L-DOPA) and L-5-hydroxytryptophan (L-5HTP) to produce dopamine (DA) and serotonin (5-HT), respectively (Lovenberg et al., 1962). DA and 5-HT are neurotransmitters in the central nervous system, and DA is further converted to norepinephrine and epinephrine, which are catecholamine neurotransmitters and adrenomedullary hormones. Thus AADC is an important enzyme for production of catecholamine and indoleamine, neurotransmitters, and hormones.

The distribution of AADC in peripheral tissues is different from that of other catecholamine-synthesizing enzymes. High AADC activity is found in the liver and kidney as well as in the adrenal medulla (Lovenberg et al., 1962; Rahman et al., 1981). Recent reports suggest that AADC in the kidney is responsible for intrarenal formation of DA, which regulates sodium excretion in proximal tubule cells (Hayashi et al., 1990). The physiological role of AADC in the liver, however, is still unknown.

Furthermore, APUD (amine precursor uptake and decarboxylation) cells (Pearse, 1969) also possess high AADC activity. Small cell lung carcinoma (SCLC) is an APUD cell and has a very high AADC activity, which serves to distinguish SCLC from other lung carcinomas (Baylin et al., 1980; Nagatsu et al., 1985).

Immunohistochemical studies showed the localization of AADC-like immunoreactivity in the brain. AADC-like immunoreactivity was found not only in catecholamine- and serotonin-containing neurons but also in a group of nonmonoaminergic neurons (Jaeger et al., 1983). These neurons are classified as D-type neurons and have no monoamine neurotransmitters. Natural substrates and the functional significance of AADC in these neurons are unknown. Tissue-specific expression of AADC activity in these cells is undoubtedly controlled by certain transcriptional factors.

Completely pure enzymes from pig kidney (Christenson et al., 1970) and human pheochromocytoma (Ichinose et al., 1985) decarboxylated both L-DOPA and L-5HTP. However, the arguments still exist whether or not the decarboxylation of L-DOPA and that of L-5HTP are mediated by the same enzyme, AADC. Some reports are against the identity of DOPA decarboxylase and L-5HTP decarboxylase from the aspects of the differential affinity for pyridoxal phosphate (a cofactor for the decarboxylation) (Siow & Dakshinamurti, 1985) and differences in kinetic parameters (Bouchard & Roberge, 1979).

Recent molecular biological research has revealed characteristics of this enzyme. We isolated and characterized a full-length cDNA clone encoding human AADC from a

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* To whom correspondence should be addressed.
§ Nagoya University School of Medicine.
‖ Fujita Health University.
⊥ National Institute of Radiological Sciences.

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pheochromocytoma cDNA library (Ichinose et al., 1989), which encoded a protein of 480 amino acids with a calculated molecular mass of 54 kDa. The nucleotide sequences of rat (Tanaka et al., 1989; Krieger et al., 1991) and bovine (Kang & Joh, 1990) enzymes were also reported. These isolated clones enable us to investigate the molecular aspects of AADC.

In the present study, we characterized the structure of the human AADC gene including the 5′ flanking region and determined the transcriptional starting point, and we also assigned the human AADC gene to chromosome 7. These results clearly demonstrated that AADC is encoded by a single gene. This work is essential for studying regulatory mechanisms of expression of the AADC gene involved in tissue specificity and stage specificity. This is the first report on the genetic structure and chromosomal localization of the AADC gene in mammals.

Materials and Methods

Materials and General Methods. Standard techniques were used for the isolation and manipulation of DNA and RNA (Sambrook et al., 1989). Restriction endonucleases and nucleic acid-modification enzymes were purchased from Takara (Kyoto). Sequenase and M13, -40 primer were purchased from U.S. Biochemical Corp. Reverse primer was purchased from Pharmacia. ^{32}P-Radiolabeled nucleotides were obtained from Amersham. DNA probes were radiolabeled by a random primer method (Amersham). Oligonucleotides were synthesized by a DNA synthesizer (Applied Biosystems) at Nagoya University School of Medicine and the Center for Gene Research in Nagoya University.

Genomic Southern Analysis. Human placental DNA (10 μg) was digested with several restriction endonucleases, separated according to size in 0.8% agarose gel, and capillary blotted onto a nylon membrane (Hybond-N; Amersham). This membrane was prehybridized and hybridized with a ^{32}P-labeled full-length cDNA clone of human AADC (probe a, as indicated in Figure 2) in a solution consisting of 5 × SSPE (1 × SSPE contains 0.15 M NaCl, 0.01 M sodium phosphate, and 1 mM EDTA, pH 7.4), 5 × Denhardt's solution, 0.3% sodium dodecyl sulfate (SDS), 100 μg/mL salmon sperm DNA, and 50% formamide. After 42 °C hybridization overnight, the membrane was washed once with 2 × SSC (1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% SDS at room temperature for 5 min and twice with the same solution at 65 °C for 15 min.

Genomic Libraries. Two commonly available genomic libraries were used for this study. One (TM library) was donated by Dr. Tom Maniatis (Harvard University). The other (LI 018 library), which was produced by Drs. S. Tomatsu and Y. Sakaki (Research Laboratory for Genetic Information, Kyushu University), was obtained from the Japanese Cancer Research Resources Bank.

In addition, we constructed three genomic libraries. Human placental DNA was completely digested with *Eco*RI and size-fractionated by 0.8% agarose gel electrophoresis. DNA fragments around 4 and 2 kb in size were recovered from the gel by the glass beads method (GENECLEAN, BIO 101 Inc.), ligated into a λgt11 DNA, and packaged using Gigapack II Gold packaging extracts (Stratagene). The produced libraries were designated as LE4 and LE2, respectively. In another genomic library (LB15 library) human placental DNA was completely digested with *Bam*HI and separated by 0.8% low-melting agarose gel electrophoresis. DNA fragments around 15 kb were purified from the gel by phenol/chloroform extraction (Sambrook et al., 1989), ligated to EMBL3 arms, and packaged into phage particles in vitro.

Isolation of Genomic Clones. Phages containing the AADC locus were isolated from genomic libraries by plaque hybridization using various cDNA or genomic probes as shown in Figures 2 and 3 and described under Results. The full-length AADC cDNA probe (probe a) was used to isolate the phages gTMDC7, gTMDC5, gTMDC1, and gTMDC8 from the TM library. The genomic clone gTMDC6 was isolated from the same library with the *Kpn*I fragment of the cDNA (probe b) used as a hybridization probe, which contains 181 base pairs of the 5′-terminal region of the cDNA. gTMDC13 was isolated by use of the *Bam*HI fragment of cDNA (probe d) containing 700 bp of the 3′-terminal region of the cDNA. Genomic phage gLDC1 was isolated by use of the *Eco*RI–*Bam*HI fragment of gTMDC6 (probe c) from the LI 018 library. Recombinant phages were isolated as follows: phages gEDC2 and gEDC3 were isolated from LE4 and LE2 libraries with the *Pst*I fragment of the cDNA (probe f) as a probe. Phage gBDC2 was isolated from the LB15 library by use of the *Eco*RI fragment of gEDC3 (probe g). DNA from hybridizing phages was isolated, and appropriate genomic fragments were cloned into the plasmid vectors, Bluescript (Stratagene) or pUC119, and characterized by restriction endonuclease mapping and Southern hybridization analysis.

DNA Sequence Analysis. Appropriate restriction endonuclease fragments of isolated phage clones were subcloned into Bluescript vectors or pUC119 vectors and sequenced according to Sanger et al. (1977) using Sequenase version 2.0 (U.S. Biochemical Corp.). Some DNA fragments in a plasmid vector were treated with exonuclease III and mung bean nuclease to construct nested deletions of a desired length. Some DNA fragments were sequenced by use of synthetic primers corresponding to nucleotide sequences of the human AADC cDNA or genomic DNA. Others were sequenced from the sites of restriction endonucleases near the exons. The sequencing strategy is summarized in Figure 3 and Table I. The nucleotide sequences of the 5′ flanking region and exon 1 were sequenced on both identical and complementary strands.

Primer Extension Analyses. Total RNA was extracted from human adrenal medulla with guanidinium thiocyanate followed by centrifugation in cesium chloride solutions (Sambrook et al., 1989). Synthetic oligonucleotide 5′-GAGTGACTTGCTCTCTGTCCTCT-3′, which is complementary to the sequence in exon 1 (primer A, as indicated in Figure 6), was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Two hundred micrograms of total RNA from human adrenal medulla was suspended in 20 μL of hybridization buffer [40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, 80% formamide] as described in Sambrook et al. (1989). Primer A was added to the buffer, and the mixture was heated at 80 °C for 5 min, cooled to 45 °C, and annealed at the same temperature overnight. Then the sample was precipitated by ethanol, rinsed, and resuspended with the transcriptase buffer [50 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, 1 mM dNTPs, 1 mM DTT, 1 unit/μL placental RNase inhibitor] and 25 units of reverse transcriptase derived from Rous-associated virus 2 (Takara). Complementary DNA was synthesized for 1 h at 42 °C, followed by degradation of RNA with RNase A at the same temperature for 1 h. Then the sample was extracted with phenol/chloroform and chloroform, precipitated by ethanol, and analyzed in a 6% acrylamide–7 M urea sequence gel. The DNA fragment of gLDC1 was sequenced with the primer A at the same time.

S1 Nuclease Mapping. The probe for S1 nuclease mapping was synthesized by the polymerase chain reaction (PCR) with

Table I: Strategy for Sequencing of the Intron–Exon Boundaries[a]

exon	fragments (original clones)	primers
1 and 5′ flanking region	*Eco*RI, 2.3 kb (gLDC1), and deletion clones	reverse primer (5′-AACAGCTATGACCATG-3′) primer A (5′-GAGTGACTTGCTCTCTGTCCTCT-3′) primer O (5′-GGCTCCACTCCAAGGGT-3′) primer W (5′-ACCCTTGGAGTGGAGCC-3′) primer 4 (5′-ATGGTGGGAGGAGAGAC-3′) primer 5 (5′-GCGGCTCAACACCTAAT-3′) primer 6 (5′-GCCAGTCCCAGCAGGCC-3′)
2	*Eco*RI, 3.8 kb (gTMDC7)	M13, −40 primer (5′-GTTTCCCAGTCACGAC-3′)
	*Eco*RI, 6.9 kb (gTMDC7)	M13, −40 primer
3	*Bam*HI, 1.2 kb (gTMDC7)	M13, −40 primer
4	*Bam*HI–*Sac*I, 2.1 kb (gTMDC7)	M13, −40 primer primer Y (5′-CAAATTCTGCAATGCCTG-3′)
5	*Sac*I, 2.4 kb (gTMDC5)	reverse primer
	*Sac*I–*Eco*RI, 2.9 kb (gTMDC5)	reverse primer
6	*Pst*I, 0.7 kb (gTMDC5)	M13, −40 primer
	*Pst*I, 1.5 kb (gTMDC5)	reverse primer
7	*Xba*I, 0.68 kb (gTMDC1)	reverse primer
	*Eco*RI–*Xba*I, 0.45 kb (gTMDC1)	M13, −40 primer
8	*Eco*RI, 1.6 kb (gTMDC8), deletion clones	reverse primer
9	*Hin*dIII–*Sma*I, 2.5 kb (gTMDC8), deletion clones	M13, −40 primer
10	*Eco*RI, 1.9 kb (gEDC3)	primer F (5′-GCTCCCGTTAAGTCTGTTCTC-3′) primer G (5′-ACTGGACCCCACTTACCTGAA-3′)
11	*Sal*I–*Hin*dIII, 1.8 kb (gBDC2)	M13, −40 primer
12	*Eco*RI, 3.8 kb (gEDC2)	primer H (5′-ATCCTAAATACAAACCACATT-3′) primer I (5′-AAAGGACTGCAGGCTTATAT-3′) primer 3 (5′-AAGTCCCGAAAGACCTCCG-3′)
13	*Pst*I–*Bam*HI, 0.7 kb (gTMDC13)	M13, −40 primer
	*Bam*HI–*Pst*I, 1.2 kb (gTMDC13)	M13, −40 primer
14	*Bam*HI–*Pst*I, 0.2 kb (gTMDC13)	reverse primer
	*Pst*I–*Bam*HI, 3.2 kb (gTMDC13)	reverse primer
15	*Pst*I–*Bam*HI, 1.5 kb (gTMDC13), deletion clones	reverse primer primer J (5′-TTCATGGAACTCAGCTG-3′) primer K (5′-GCTGAATAAATGAATGCC-3′)

[a] Sequenced fragments are indicated with original genomic clones. The *Xba*I fragment (0.68 kb) was subcloned into the pUC119 vector, and other fragments were all subcloned into Bluescript vectors. The M13, −40 primer and reverse primer are homologous and complementary to the sequences in multicloning sites of the vectors. Other primers were all synthesized. Primers A, F, and H are complementary to the sequences of human AADC cDNA. Primers G and J are homologous to those of AADC cDNA. Primers O, Y, K, and 3 are complementary to the sequences of human AADC genomic clones. Primers W, 4, 5, and 6 are homologous to those of AADC genomic clones.

primer A described previously and primer S, 5′-ATGGCTCTCTCCCCCTGGG-3′, which correspond to the sequence from −243 to −225 in the 5′ flanking region (see Figure 6). The conditions for the amplification for 25 cycles were as follows: denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min, and extension at 72 °C for 3 min. The probe was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Two hundred micrograms of total RNA from human adrenal medulla was resuspended in 30 μL of hybridization buffer. The probe (approximately 200 ng) was added to the buffer, and the mixture was then heated at 85 °C for 10 min, cooled to 59 °C, and annealed at the same temperature overnight. Then to the sample was added 300 μL of ice-cold S1 mapping buffer [0.28 M NaCl, 0.005 M sodium acetate (pH 4.5), 4.5 mM $ZnSO_4$, 20 mg/mL salmon sperm DNA] containing 190 units of S1 nuclease. The reaction was performed at 37 °C for 2 h and stopped by addition of 80 μL of S1 nuclease stop mixture (4 M ammonium acetate, 50 mM EDTA, 50 mg/mL tRNA). After phenol extraction and ethanol precipitation, the resulting solution was evaporated, resuspended in 10 μL of formamide dye, and analyzed on a 6% acrylamide–7 M urea sequence gel (Sambrook et al., 1989). The *Eco*RI 2.3-kb fragment of gLDC1 was sequenced with primer A at the same time.

Chromosomal Localization of the AADC Gene by Fluorescence in Situ Hybridization. Two *Eco*RI fragments of overlapping genomic clones, probe h of gTMDC7 and probe i of gTMDC5, were used as DNA probes in the present study. Probe h (6.9 kb) covers exons 2–4 and probe i (8.7 kb) covers exons 5 and 6 (see Figure 3). A direct mapping system, which is based on fluorescence in situ hybridization (FISH) combined with replicated prometaphase R-bands (Takahashi et al., 1990a, 1991), was applied. The procedures of FISH carried out with the biotin–avidin system have been described elsewhere (Takahashi et al., 1989, 1990b, 1991; Hori et al., 1990). A suppression hybridization procedure with total human DNA (Hori et al., 1990) was applied for the elimination of repetitive sequences in probe i. In this case, a 25-fold excess amount of total human placental DNA was added. The concentration of the probe DNA was 250 ng/20 μL of hybridization mixture per slide of both probes. The procedures of hybridization, rinsing, and detection were performed in a routine manner. Ektachrome film (ASA 100; Kodak) was used for microphotography (filter combination, B-2A; Nikon).

Results

Genomic Southern Analysis of the Human AADC Gene. Genomic Southern hybridization was performed as described under Materials and Methods against *Xba*I-, *Eco*RV-, *Hin*dIII-, *Bam*HI-, *Eco*RI- and *Sac*I-digested human placental DNA with the full-length human AADC cDNA fragment (Ichinose et al., 1989) used as a probe (probe a). The result in Figure 1 shows a multiple-band pattern for all restriction nucleases used.

Isolation and Characterization of the Human AADC Genomic Clones. The structure of the human AADC gene is depicted in Figure 3. We isolated six phage clones, gTMDC6, gTMDC7, gTMDC5, gTMDC1, gTMDC8, and gTMDC13, from a TM library as described under Materials and Methods. gTMDC6 (16.1 kb) contained exon 2; gTMDC7 (16.7 kb), exons 2–4; and gTMDC5 (13.6 kb), exons 5 and 6. These clones overlapped. gTMDC1 (13.5 kb) contained exons 7–9

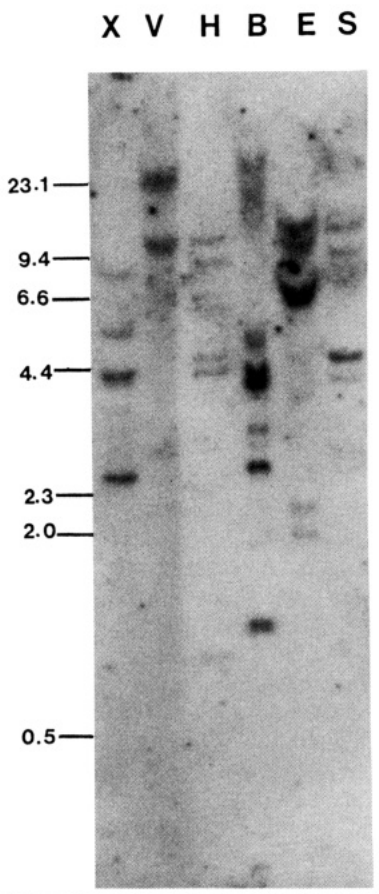

FIGURE 1: Southern hybridization analysis of genomic DNA. Ten micrograms of human genomic DNA purified from human placenta was digested with various restriction enzymes, indicated by single letters at the top of lanes: X, *Xba*I; V, *Eco*RV; H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; S, *Sac*I. After electrophoresis on a 0.8% agarose gel, each digest was transferred to a nylon membrane and hybridized with a ^{32}P-radiolabeled full-length human AADC cDNA fragment as a probe (probe a, as shown in Figure 2). Molecular weight size markers are shown at the left (kb).

and gTMDC8 (15.0 kb) contained exons 8 and 9. These were also overlapping clones. gTMDC13 (13.2 kb) contained exons 13–15. These six clones did not cover the entire region of the AADC gene. Exons 1 and 10–12 were missing in these clones. To obtain a clone containing exon 1, we screened the LI018 genomic library with a DNA fragment (probe c) from gTMDC6 as shown in Figure 3. By this means we isolated a clone, gLDC1 (13.5 kb), which contained exon 1 and the 5′ flanking region. For isolating clones containing exons 10–12, we synthesized three new genomic libraries, LE4, LE2, and LB15, containing approximately 4-kb and 2-kb *Eco*RI fragments and a 15-kb *Bam*HI fragment, respectively, for we had known that exons 10–12 are involved in DNA fragments located around these regions from genomic Southern analysis with an *Ava*II–*Pst*I fragment of cDNA (probe e) covering 140 bp between positions 985 and 1125. We screened the LE2 and LE4 libraries with a *Pst*I fragment of AADC cDNA (probe f) covering 457 bp between positions 669 and 1125 and isolated two clones, gEDC2 (3.8 kb), which contained exon 12, and gEDC3 (1.9 kb), which contained exon 10 but not exon 11. We screened the LB15 library with the *Eco*RI fragment of gEDC3 (probe g) and isolated a clone, gBDC2 (14.2 kb), which was found to contain exons 10 and 11.

Structure of the Human AADC Gene. The human AADC gene is organized into 15 exons spanning more than 85 kb, as shown in Figure 3. The sizes of the exons ranged from 20 bp (exon 11) to 400 bp (exon 15), and the sizes of the introns varied from 1.0 kb (intron 5) to 17.7 kb (intron 1), while those of introns 6, 9, 11, and 12 were not determined. As shown in Table II, the exon–intron joint sequences conform to the AG-GT rule (Breathnach & Chambon, 1981). The 5′ untranslated region was encoded by two exons, exon 1 and exon 2. ATG (+1 to +3) as a translational starting codon was found in exon 2. TAG as a stop codon is located in exon 14, at position 1441. The sequence AATAAA, as a polyadenylation signal (Breathnach & Chambon, 1981), occurred in exon 15. The 3′-mRNA processing signal, or "GT cluster" (Birnstiel et al., 1985), resided in the region about 10–40 bp downstream of the poly(A)$^+$ addition site. The nucleotide sequence in the exons was perfectly identical with that of the cDNA except for the sequence of the 3′ untranslated region in exon 15. Several differences occurred within the 3′ untranslated region. The sequence at positions 1574–1575 was TT, which had been mistakenly (due to a typing error) described as CC in the human cDNA sequence (Ichinose et al., 1989). The sequence at positions 1669–1735 in the cDNA sequence involves several sequencing errors (Ichinose et al., 1989). The entire sequence of exon 15 is shown in Figure 4.

Determination of the Transcriptional Starting Site. Primer extension and S1 nuclease mapping were performed to determine the transcriptional starting site, and the results are shown in Figure 5. For primer extension, a labeled primer (primer A indicated in Figure 6) complementary to the 5′ untranslated region in exon 1 was hybridized to total RNA from human adrenal medulla and extended by reverse transcriptase. Two major bands were shown at G of position −111 and C of position −105, and three minor bands were given at A residues of positions −115 to −113. For S1 mapping, the PCR product between primer A and primer S (as indicated in Figure 6) was used as a probe after purification to be hybridized with the total RNA purified from human adrenal

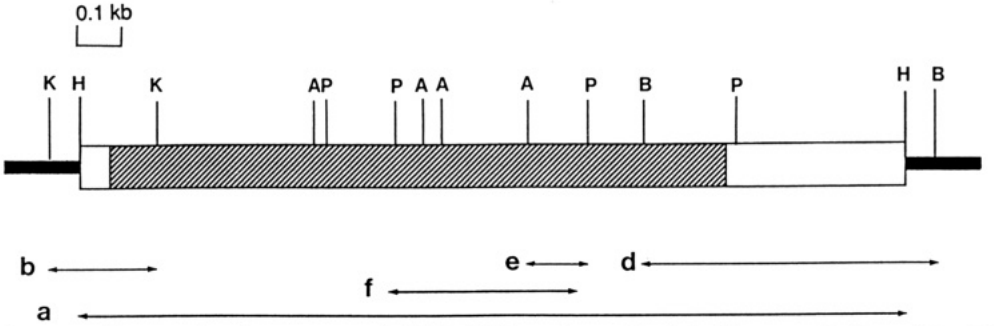

FIGURE 2: Restriction map of human pheochromocytome AADC cDNA used as probes to isolate genomic clones. The full length of human pheochromocytoma AADC cDNA was ligated to a Bluescript vector with *Hin*dIII linkers (Sumi et al., 1990). The coding region is indicated by the hatched area and the untranslated region by open areas. Restriction sites used to cut out probes are indicated as follows: H, *Hin*dIII; K, *Kpn*I; A, *Ava*II; P, *Pst*I; B, *Bam*HI. Probes a, b, d, e, and f, used to isolate genomic clones, are indicated by bilateral arrows.

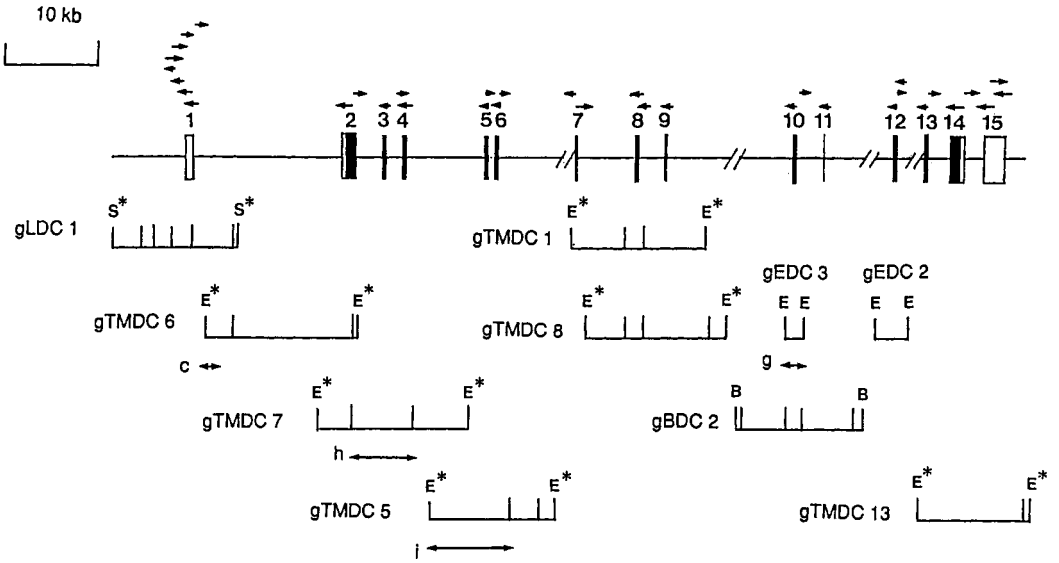

FIGURE 3: Structure of the human AADC gene and the restriction endonuclease mapping of isolated genomic clones. The structure of the human AADC gene is depicted at the top of the figure. Coding regions are indicated by closed boxes and untranslated regions by open ones. Introns are represented by the thin horizontal line. The direction and length of each sequencing are indicated above the genomic structure by small arrows. Ten isolated clones covering 97 kb of the human AADC gene are depicted below the structure of the gene. gLDC1 (13.5 kb) contains exon 1; gTMDC6 (16.1 kb), exon 2; gTMDC7 (16.7 kb), exons 2–4; gTMDC5 (13.6 kb), exons 5 and 6; gTMDC1 (13.5 kb), exons 7–9; gTMDC8 (15.0 kb), exons 8 and 9; gEDC3 (1.9 kb), exon 10; gEDC3 (3.8 kb), exon 12; gBDC2 (14.2 kb), exons 10 and 11; and gTMDC13 (13.2 kb), exons 13–15. Restriction sites of both ends of all clones are indicated by E (*Eco*RI), S (*Sal*I), and B (*Bam*HI), and artificial sites for ligation to vectors are indicated by asterisks (*). All other vertical bars indicate other *Eco*RI sites in the clones. Small bilateral arrows below gTMDC6, gTMDC7, gTMDC5, and gEDC3 indicate probs c, h, i, and g used to isolate genomic clones or to determine the chromosomal localization as described under Materials and Methods.

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                                                1500
GAATCAAAAATTGAAGAGAGATATATCTGAAAACTGGAATAAGAAGCAAATAAATATCATCCTGCCTTCA

          1550                                                      1600
TGGAACTCAGCTGTCTGTGGCTTCCCATGTCTTTCTCCAAAGTTATCCAGAGGGTTGTGATTTTGTCTGC

                                          1650
TTAGTATCTCATCAACAAAGAAATATTATTTGCTAATTAAAAAGTTAATCTTCATGGCCATAGCTTTTAT

                    1700
TCATTAGCTGTGATTTTTGTTGATTAAAACATTATAGATTTTCATGTTCTTGCAGTCATCAGAAGTGGTA

     1750                                                     1800
GGAAAGCCTCACTGATATATTTTCCAGGGCAATCAATGTTCACGCAACTTGAAATTATATCTGTGGTCTT

                          1850
CAAATTGTCTTTTGTCATGTGGCTAAATGCCTAATAAACAATTCAAGTGA
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FIGURE 4: The entire sequence of exon 15. Nucleotides are numbered above the DNA sequence with the first base of the ATG initiation codon designated at +1.

medulla and digested with S1 nuclease. In S1 nuclease mapping, protection bands were shown between G of position −111 and C of position −105, while a few nucleotides downstream of them were mapped by primer extension, probably due to "nibbling" of the S1 nuclease at the end of the protected fragments. On the basis of these two results the transcriptional starting site of the human AADC mRNA was designated as the region around G of position −111.

DNA Sequence Analysis of the 5′ Flanking Region of the Human AADC Gene. The nucleotide sequence of the 5′ flanking region was determined to about 540 bp upstream from the putative transcriptional starting site and is shown in Figure 6. The AT-rich motif (Breathnach & Chambon, 1981) was present at position −118. The typical CAAT box (Breathnach & Chambon, 1981) and Sp1 binding site (Dynan et al., 1985) were not present, while some parts of this region showed a high GC content; 77% (−233 to −187 and −512 to −487) and 83% (−361 to −344). We searched the sequence in the 5′ promoter region with the aid of a computer search program (DNASIS, Hitachi), and putative regulatory elements are summarized in Figure 6.

Chromosomal Mapping of the Human AADC Gene. We examined 100 (pro)metaphase plates showing typical R-bands for both probe h of gTMDC7 and probe i of gTMDC5 (shown in Figure 3). The hybridization efficiency of probe h was higher than that of probe i. For probe h, 28% of the plates exhibited complete double spots on both homologues. The other metaphase plates showed incomplete single and/or double spots on either or both homologues (58%) or no spots (13%). The signals of probe h were localized to the band p12.1-p12.3 of the short arm of chromosome 7, as were those of probe i. No signals were observed on other chromosomes. Thus the AADC gene could be assigned to band 7p12.1-p12.3 (Figure 7).

Discussion

The human AADC gene is composed of 15 exons interrupted by 14 introns and spans more than 85 kb. The genomic size of the AADC gene is the largest among genes of human catecholamine-synthesizing enzymes, for the genomic sizes for tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), and phenylethanolamine *N*-methyltransferase (PNMT) are

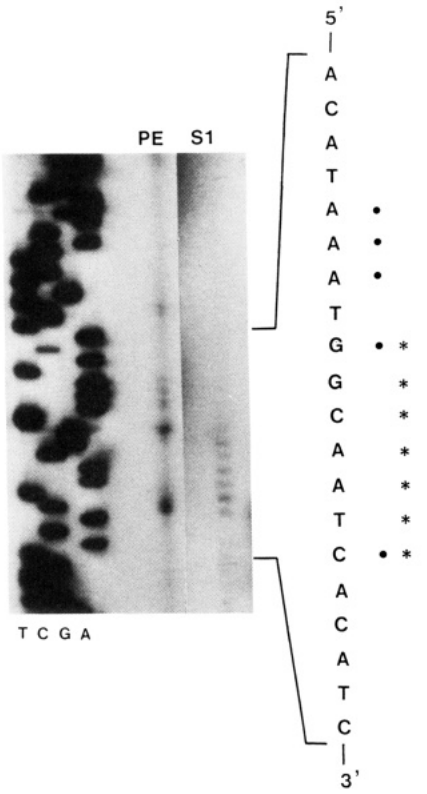

FIGURE 5: Primer extension analysis and S1 nuclease mapping. To determine the transcriptional starting site, primer extension analysis and S1 mapping were performed. The results of primer extension (PE) and S1 mapping (S1) are shown with sequences of the 5′ flanking region. For primer extension, a ^{32}P-radiolabeled primer (primer A, 5′-GAGTGACTTGCTCTCTGTCCTCT-3′), which is complementary to the 5′ untranslated region in exon 1 as shown in Figure 6, was hybridized to total RNA purified from human adrenal medulla and extended by reverse transcriptase. Two major bands were given at G of position −111 and C of position −105 accompanied by minor bands at A's of positions −115 to −113 as shown by the closed circles (●) at the right side of the sequence. For S1 mapping, the PCR product between primer A and primer S (5′-ATGGCTCTCTCCCCCTGGG-3′, which is homologous to the sequence in the 5′ flanking region as indicated in Figure 6) was used as a probe to be hybridized to the same total RNA and digested by S1 nuclease. The protected bands were given between G of position −111 and C or position −105 as shown by asterisks (*) at the right side of the sequence.

8.5 kb (Kobayashi et al., 1988), 23 kb (Kobayashi et al., 1989) and 1.6 kb (Baetge et al., 1988), respectively.

Deduced from restriction maps of the genomic clones, the sizes of *Eco*RI fragments in genomic DNA that contained AADC exons were 2.3, 13.0, 6.9, 11.3, 1.6, 7.3, 1.9, 5.4, and 3.8 kb, and the sizes of *Eco*RI fragments containing exon 7 and exons 13–15 were not determined, though the former was more than 6.0 kb and the latter was more than 11.6 kb. These fragments correspond well to the result of the multiple-band pattern seen by genomic Southern hybridization with a full-length AADC cDNA probe. Furthermore, the human AADC gene was mapped in the p12.1-p12.3 region of chromosome 7. These results prove that there is a single copy of the AADC gene per haploid.

This observation of multiple bands shown in the genomic Southern hybridization contradicts the report of Albert et al. (1987). Their Southern blot analysis with a cDNA of bovine AADC showed a single band in *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I digests within approximately 2–3 kb, though they used bovine cDNA as a probe instead of a human cDNA we used.

Decarboxylation of L-DOPA to DA and of L-5HTP to 5-HT is considered to be catalyzed by the same enzyme, AADC, for the purified enzyme (Christenson et al., 1970; Ichinose et al., 1985), and the enzyme expressed in COS cells by human AADC cDNA (Sumi et al., 1990) decarboxylated both L-DOPA and L-5HTP to produce DA and 5-HT, respectively. However, some controversies still exist as to whether a single enzyme catalyzes the decarboxylation of both L-DOPA and L-5HTP. First, the ratio of decarboxylase activity for L-DOPA to that for L-5HTP in crude preparations was different in various tissues (Siow & Dakshinamurti, 1985; Rahman et al., 1982). Second, the 5-HT content in the brain of the pyridoxine-deficient growing rats was significantly decreased, whereas the brain level of catecholamines was not altered (Dakshinamurti et al., 1976). Third, dialysis of the rat brain homogenate caused nearly total loss of decarboxylase activity for L-5HTP but not for L-DOPA (Siow & Dakshinamurti, 1985). These results suggest the presence of two similar but distinct enzymes in catecholaminergic and serotonergic neurons, but the evidence is all indirect. The question whether or not a single enzyme catalyzes the decarboxylation of these two substrates in vivo has not clearly been answered yet. In this work we proved that the AADC gene exists as a single copy, and extra bands that hybridize to AADC cDNA were not detected in the genomic Southern hybridization. Thus no other enzymes showing high homology to AADC exist. Considering our previous report that recombinant human AADC cDNA expressed in COS cells catalyzed the decarboxylation of both L-DOPA and L-5HTP to DA and 5-HT, respectively (Sumi et al., 1990), the present results suggest that AADC is a single enzyme that catalyzes the decarboxylation of both L-DOPA and L-5HTP in vivo. However, minor modifications such as an alternative splicing or a posttranslational modification could cause different characteristics of this enzyme in catecholaminergic neurons, serotonergic neurons, or other nonneuronal tissues such as liver and kidney.

Primer extension and S1 nuclease mapping analyses indicated that the transcriptional starting site of human AADC mRNA was located around G of position −111. The transcriptional starting site of rat AADC mRNA in PC12 cells was reported at position −76 by Krieger et al. (1991). The 5′ untranslated region of the human AADC is longer than that of rat, since the human gene has 20 extra bases next to the first ATG.

The 5′ untranslated region was separated into two exons, exons 1 and 2, in the human AADC gene. The presence of exons in the 5′ untranslated region is known in several genes, such as those of c-myc (Battey et al., 1983), D_2 DA receptor (O'Malley et al., 1990), and liver/bone/kidney alkaline phosphatase (EC 3.1.3.1) (Weiss et al., 1988; Matsuura et al., 1990). In the c-myc gene, exon 1 in the untranslated region is though to have some functional significance, for the exon containing four potential hairpin loops was closely conserved between mouse and human, and its transcription is regulated by two alternative TATA boxes (Battey et al., 1983). There may be a possibility that exon 1 in the 5′ untranslated region of the AADC gene has also some functional meaning.

In the 5′ flanking region within 540 bp from the transcriptional starting site, an AT-rich motif was present at position −118. However, this position is just upstream of the putative transcriptional starting site, so the motif may not be a typical TATA box, which is usually located between positions −34 and −26 from the mRNA starting site (Breathnach & Chambon, 1981). There was no CAAT box or Sp1 binding site (Dynan et al., 1985) in the 5′ flanking region, though some parts of this region showed a high GC content. These characteristics of the AADC promoter region resemble the

```
                                                    -600
ATGGTGGGAGGAGAGACTGAGCCCAAACCATGTTTTAGATGCTGATAGGCTTAAGGGTAACAGCACAGGA
              ERE               C2
                               -550
GTTTGAGATGCATGCGGCTCAACACCTAATCTACATCTCACTTCACTTTCTCATCTGGGGAAGTGGGCTT
                          Pit-1/GHF-1                          E4TF1
  -500                                                   -450
GGGACCCTGAGCCTCCCGGGTATCACAGGGTCCTAATAGTCCCTCACAGAAGGAGCAGACCCAGAGTGAG
          HSE
                                    -400
CACTCCCCAAATGCCACGCCGTCCCTTCCTCACCCTTGGAGTGGAGCCTGGTCGTTCTCAAAGTTGTGGG
                         MRE AP-3 CACCC

              -350                                                 -300
AGAGTCCCAGGAGCCCTGGCCCCAAATCTGCATCCTACACAGTGCCTGGGAACACAGGGCCCATTTTTTC
 NFKB          MRE          Oct-1,2
                                                    -250
CTTGCCTCTCGCCAGTCCCAGCAGGCCCTGATGCTCCTCTCCATCCTGCTAGGATGGCTCTCTCCCCCTG
                                                                Primer S
                               -200
GGGGCAGAGTGGGGGCAGGAGGTGGTGGGAGGGTGGAGGGATGCTGCTCAGTAAATAATGCAGAGCCGGC
-->
  -150                                           ••• •.    • -100
AGCTCTGATTGGCTTCGGGGAGGCAGACACTCTGTCTACATAAATGGCAATCACATCTTCTGTGCCTCTT
         NFY                              AT-rich*******
                                    -50                 -29
AACTGTCACTGTGGAGAGGAGAGAGAGAGGACAGAGAGCAAGTCACTCCCGGCTGCCTgtgagtactgct
                       <---------------------
                                    Primer A
                                                             -28
ctttgtcctcat-----Intron 1 (17.7 kb)-----cactgaatcattttctttctgcagTTTTCA

                    +1
CCTCTGACAGAGCCCAGACACCATGAACGCAAGTGAATTC
                        MetAsnAlaSerGluPhe
```

FIGURE 6: The 5′ flanking sequence of the human AADC gene. The nucleotide sequence is numbered with the first base of the initiation ATG codon designated as +1. Nucleotides within intron 1 are not numbered. The 5′ flanking region, exon 1, and exon 2 are written in upper-case letters, while intron 1 is written in lower-case letters. The bands shown in primer extension analysis are indicated by closed circles (●) at the top of the letters, and bands shown in S1 mapping are indicated by asterisks (*) at the bottom of the letters. The potential regulatory sequence of CACCC (Dierks et al., 1983), HSE (heat shock response element; Pelham, 1982), MRE (mineral response element; Stuart et al., 1985), ERE (estrogen response element; Klock et al., 1987), binding sites of Pit-1/GHF-1 (Ingraham et al., 1988; Bodner et al., 1988), Oct-1,2 (Wirth et al., 1987; Sturm et al., 1988, Clerc et al., 1987), E4TF1 (Watanabe et al., 1988), NFY (Dorn et al., 1987), NFkB (Sen & Baltimore, 1986), AP-3 (Chiu et al., 1987), and C2 (Peterson & Calame, 1989) are underlined. An AT-rich motif (Breathnach & Chambon, 1981) is also underlined. The degree of homology of those sequences to the consensus is higher than 80%. The positions of primers A and S used for both primer extension analysis and S1 mapping are indicated by arrows.

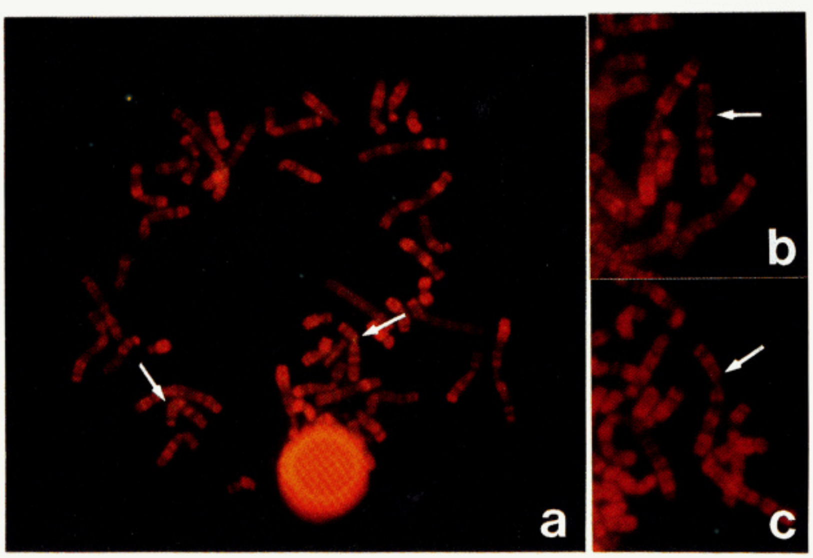

FIGURE 7: Chromosomal localization of the AADC gene by fluorescence in situ hybridization. R-banded (pro)metaphase hybridized with the biotinylated AADC gene (probe h): a, whole field; b and c, partial fields. Arrows indicate signals on 7p12.1-p12.3.

structure found in a group of housekeeping genes, which lack the TATA box, often having GC-rich sequences and multiple transcriptional starting sites.

The size of intron 1 (approximately 17.7 kb) of the AADC gene was extremely larger than that of the other introns. In some genes, such as human apolipoprotein B (Brooks et al., 1991), β-globin (Collins et al., 1990), and dihydrofolate reductase (EC 1.5.1.3) (Schmidt et al., 1990), functional regulatory elements are present in the first and second introns. Analysis of such introns may be needed to study the regulatory mechanism of AADC.

We searched for consensus sequences of known regulatory elements in the sequence of the 5′ flanking region of AADC. AATCTACAT at position −549 (8 out of 9 positions matched

Table II: Intron–Exon Organization of the Human AADC Gene[a]

Exon	Size (b)	Sequence	Intron	Size (kb)	Sequence
		-29			-28
1	83	CCGGCTGCCT gtgagtactg	1	17.7	ctttctgcag TTTTCACCTC
		201			202
2	229	C ATG CCT GGG gtaagtgtgt	2	3.8	tcccatgcag GTG ACG CAC T
		Met Pro Gly			Val Thr His
		315			316
3	114	C TTC TCC TGG gtgagggtcc	3	1.8	cccacgacag GCG GCA AGC C
		Phe Ser Trp			Ala Ala Ser
		435			436
4	120	A GTG ATC CAG gtaaggtggg	4	9.3	ttgaatttag GGA AGT GCC A
		Val Ile Gln			Gly Ser Ala
		570			571
5	135	A TCC GAT CAG gtgagtgtgt	5	1.0	ttctttccag GCA CAC TCC T
		Ser Asp Gln			Ala His Ser
		714			715
6	144	T CCT TTC TTT gtaagttcgg	6	>7.6	cattatctag ATG GTT GCC A
		Pro Phe Phe			Met Val Ala
		781			782
7	67	GGT CCT ATC T gtaagtatcc	7	6.6	tgaactttag GC AAC AAG GA
		Gly Pro Ile			Cys Asn Lys
		876			877
8	95	T GGA GTG GAG gtaggtgcac	8	3.0	tctttggcag TTT GCA GAT T
		Gly Val Glu			Phe Ala Asp
		944			945
9	68	CT GCC ATG TG gtaagtttcc	9	>12.8	ctaaaaccag G GTG AAA AAG
		Ala Met Trp			Val Lys Lys
		1021			1022
10	77	CAG GAT TCA G gtaagctgtg	10	3.4	ttttctccag GG CTT ATC AC
		Gln Asp Ser			Gly Leu Ile
		1041			1042
11	20	T GAC TAC CGG gtaagtgaga	11	>6.8	ttcatttcag CAT TGG CAG A
		Asp Tyr Arg			His Trp Gln
		1140			1141
12	99	T ATC CGC AAG gtgactttgt	12	>2.5	ctctttgaag CAT GTC CAG C
		Ile Arg Lys			His Val Gln
		1242			1243
13	102	T CGG CTA AAG gtttgtctac	13	3.3	aatcctccag GGT TCC AAC A
		Arg Leu Lys			Gly Ser Asn
		1461			1462
14	219	CCAGCTGCAG gtaggctgcc	14	4.1	ttccctgtag GAATCAAAAA
		1861			
15	400	AATAAACAATTCAAGTGA aatactaattgtgtcagatgatcatcttgttctatgttgtatc			

[a] Exon sequences are written in upper-case letters and intron sequences in lower-case letters. Nucleotides at the 5′ and 3′ ends of each exon are numbered with the first base of the initiation ATG codon designated as +1 (nucleotides within introns are not numbered). Amino acids are indicated below the nucleotide codons. Sizes of both exons and introns are indicated. Intron sizes were determined by the restriction map of genomic clones, though introns 6, 9, 11, and 12 were not determined. AATAAA as a polyadenylation signal (Breathnach & Chambon, 1981) is indicated by an underline. GT clusters as a 3′-cleavage signal (Birnstiel et al., 1985) are also underlined. Nucleotides not present in the cDNA, which are 3′ to the polyadenylation site, are shown in lower-case letters.

the consensus sequence) was homologues to the binding site of Pit-1/GHF-1 (Ingraham et al., 1988; Bodner et al., 1988). ATCTGCAT at position −341 (7/8) was homologous to the binding site of Oct-1,2 (Wirth et al., 1987; Strum et al., 1988; Clerc et al., 1988). Pit-1/GHF-1 and Oct-1,2 belong to the POU-domain family, which are expressed widely in the developing nervous system (He et al., 1989; Treacy et al., 1991), and it is known that the 5′ flanking region of the TH gene has sequence homology with the POU/Oct binding site (Cambi et al., 1989). It is possible that AADC expression may be regulated by one of the POU domain-binding proteins. Further investigations are needed to clarify whether these putative regulatory sequences are essential or not.

In the mammalian catecholamine-synthesizing system, TH is the first and rate-limiting enzyme (Nagatsu et al., 1964; Levitt et al., 1965). AADC is thought to be less regulated and rather nonspecific in relation to catecholamine biosynthesis. However, recent reports indicate that AADC activity in rat retina is modulated by α_2 adrenoceptors (Rossetti et al., 1989) and D_1 DA receptor (Rossetti et al., 1990) and that AADC is increased according to the change in the cell cycle induced by dibutyryl-cAMP in vivo in SCLC cells (Francis et al., 1983). These reports suggest that expression of the AADC gene is regulated in vivo.

Recently Krieger et al. (1991) found the existence of two types of mRNA for rat AADC with a difference only in the 5′ untranslated region. One was specifically expressed in the nervous system such as brain and in the adrenal medulla, and the other was expressed in nonneuronal tissues such as liver and kidney. Comparing the rat cDNA sequences with the human AADC genomic structure reported here, we found the differences were limited only to the first exon. Human AADC may also use an alternative first exon for tissue-specific expression. Previously we cloned an AADC cDNA from human pheochromocytoma, which is one of neuronal tissues (Ichinose et al., 1989). Though human AADC cDNA from nonneuronal tissues has not been reported yet, another first exon specific to nonneuronal tissues may also exist in the human gene.

The human AADC gene was mapped in the p12.1-p12.3 region of chromosome 7. TH, which catalyzes the conversion of tyrosine to L-DOPA, is mapped to chromosome 11p15.5 (Mckusick, 1990); DBH, which catalyzes the conversion of DA to norepinephrine, is mapped to chromosome 9q34 (Smith & Sympson, 1989); and PNMT, which catalyzes the conversion of norepinephrine to epinephrine, is mapped on chromosome 17 (Kaneda et al., 1988). Thus all genes of the catecholamine-synthesizing enzymes are mapped on different chromosomes, and their coexpression in catecholaminergic neurons would be regulated by some transactivating factors according to each different tissue and stage of development.

SCLC belongs to one category of APUD cells and has a very high AADC activity especially in the poorly differentiated type (Baylin et al., 1980; Nagatsu et al., 1985). Abnormalities in the chromosomal structure of SCLC have been found in 3p, 13q, and 17p (Naylor et al., 1987; Yokota et al., 1987; Kok et al., 1987; Weston et al., 1989; Mori et al., 1989). These loci are different from the position of AADC. Thus the high activity of AADC does not directly relate to the chromosomal abnormalities. Around the region of 7p12.1-p12.3, several genetic disorders have been assigned, Creig cephalopolysyndactyly syndrome (GCPS, 7p13), craniosynostosis (CRS, 7p21.2-p21.3), and myopathy (phosphoglycerate mutase deficiency, PGAMM, 7p13-p12) (Harper et al., 1989), and cancer break points of chromosomal rearrangements have also been reported: 7p13-p11 in acute lymphoblastic leukemia, 7p11 in myeloproliferative disorder, myelodysplastic syndrome, and acute myeloid leukemia (Trent et al., 1989). EGFR (epidermal growth factor receptor) is mapped to 7p12.1-p12.3 (Mckusick, 1990).

Recently two patients with AADC deficiency were reported (Hyland & Clayton, 1990). They were twins born to first cousin parents, and they suffered from severe hypotonia and developmental delay. Their AADC activity in plasma was only 1.7% of controls. The parents also showed low activities of AADC compared with normal controls (Hyland & Clayton, 1990). This genetic data will be useful to analyze the molecular mechanisms operating in these patients.

This is the first report on the mammalian AADC gene. In other species, only the *Drosophila* AADC gene has been reported (Eveleth et al., 1986). Though an optimal alignment of the amino acid sequence of human and *Drosophila* AADC reveals a 59% sequence identity and a 77% sequence similarity (Ichinose et al., 1989), the organization of the *Drosophila* AADC gene is entirely different from that of the human gene. The former is composed of 4 exons with a typical TATA box 28 bp upstream from the transcriptional starting site and spans over 4 kb (Eveleth et al., 1986). There are two major enhancer elements: one is located 23 bp upstream from the TATA box and named element I; the other is between 1.6 and 0.7 kb upstream from the first exon, a distal enhancer element (Bray et al., 1988; Johnson et al., 1989). The former contains a binding site for NTF-1, a DNA binding protein that regulates the expression of genes such as *ubx* and *ftz* in *Drosophila* neural cells (Dynlacht et al., 1989). The latter contains 5-HT- and DA-specific activator binding sites, and one of the activators, Cf1a, belongs to the POU-domain protein (Johnson & Hirsh, 1990). We could not find any sequences significantly homologous to element I and Cf1a binding sites within 540 bp upstream of the human AADC gene, though DA- or 5-HT-specific regulatory elements may exist in the human AADC gene elsewhere.

We described the genomic structure of human AADC gene and its chromosomal localization. Further study is needed to answer the important question whether or not mechanisms exist to coregulate the expression of the four catecholamine-synthesizing enzymes in different tissues and at different stages. Catecholamine neurons can be a good model to study the molecular mechanisms of neural development.

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