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Distinct promoters direct neuronal and nonneuronal expression of rat aromatic L-amino acid decarboxylase

(genomic structure/alternative splicing/tissue specificity)

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ABSTRACT **Aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) catalyzes the decarboxylation of L-dopa to dopamine in catecholamine cells and 5-hydroxytryptophan to serotonin in serotonin-producing neurons. This enzyme is also expressed in relatively large quantities in nonneuronal tissues such as liver and kidney, where its function is unknown. Neuronal and nonneuronal tissues express AADC mRNAs with distinct 5′ untranslated regions. To understand how this is accomplished at the genomic level, we have isolated rat genomic DNA encoding AADC. The organization of the AADC gene suggests that there are two separate promoters specific for the transcription of neuronal and nonneuronal forms of the AADC message. A small exon containing 68 bases of the neuronal-specific 5′ end is located ≈9.5 kilobases upstream of the translation start site, which is contained in the third exon. Approximately 7 kilobases upstream from the neuron-specific promoter is another small exon containing 71 bases of the 5′ end of the nonneuronal AADC message. These data suggest that transcription initiating at distinct promoters, followed by alternative splicing, is responsible for the expression of the neuronal and nonneuronal forms of the AADC message.**

Aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) participates in two neurotransmitter biosynthetic pathways by catalyzing the decarboxylation of L-dopa to dopamine and 5-hydroxytryptophan to serotonin (1, 2). AADC is localized in the central nervous system in catecholamine- and serotonin-producing neurons and in chromaffin cells of the adrenal medulla, where the substrates L-dopa and 5-hydroxytryptophan are produced. It is also expressed in relatively high levels in nonneuronal tissues such as liver and kidney, which do not contain these primary substrates (3–6). As its name implies, AADC has a broader substrate specificity and is capable of catalyzing the decarboxylation of aromatic amino acids, including tyrosine, phenylalanine, and tryptophan (2). This activity is presumed to occur in nonneuronal tissues, although the function of AADC in these cells is unknown.

AADC has been purified from hog kidney (1), rat liver (7), rat kidney (8), bovine adrenal medulla (9), rat pheochromocytoma (10), and human pheochromocytoma (11). cDNAs encoding rat liver (12), bovine adrenal (9), and human pheochromocytoma AADC (11) have been cloned. Rat and human AADC mRNAs are ≈2.2 kilobases (kb) in length, coding for a 480-amino acid protein of 54 kDa. The human AADC gene has been isolated (13) and shown to be a large gene consisting of 15 exons and spanning >85 kb. A portion of the rat AADC gene has also been cloned (14).

In *Drosophila*, the enzyme is referred to as dopa decarboxylase (DDC) and its gene has been characterized at the molecular level in great detail. The *Drosophila Ddc* gene has been shown to encode different isoforms of the enzyme through alternate splicing (15). Two mRNAs are produced, which are differentially expressed in neuronal cells and in the hypoderm.

The broad tissue distribution of mammalian AADC has led investigators to look for tissue-specific differences in the enzyme (9, 16, 17). We have previously shown that bovine AADC is encoded by a single gene and were unable to detect tissue-specific differences in the rat or bovine enzyme (9). Recently, Krieger *et al.* (17) have found that, although the coding sequences of rat cDNAs isolated from different tissues are identical, the mRNAs encoding rat AADC contain tissue-specific 5′ untranslated regions. In cells of neuronal origin, which include monoamine neurons and chromaffin cells, AADC mRNA contains a short 5′ untranslated sequence that differs from that of AADC mRNA expressed in nonneuronal tissues such as liver and kidney.

To investigate the basis for these differences at the genomic level, we have isolated genomic clones encoding rat AADC.† In this manuscript we show that the AADC gene contains distinct neuron- and nonneuron-specific promoters. Regulated transcription from these two promoters, followed by splicing to a common exon containing the translation start site, results in the expression of tissue-specific 5′ untranslated sequences.

METHODS

Screening of Genomic Library. Total mRNA was isolated from rat brain and used as a template for oligo(dT)-primed cDNA (18). Two hundred nanograms of cDNA was used in a polymerase chain reaction (PCR) using pyrostase (Molecular Genetics, Minnetonka, MN) for 30 cycles of 1 min at 94°C, 2 min at 59°C, and 3 min at 72°C. The sequence of the 5′ oligonucleotide used was 5′-AGTCGAGCTCTTAACTGTCACCAAGGAGAG-3′ and was obtained from the published sequence of the rat neuronal AADC 5′ untranslated region (17) with an *Sst* I site added for cloning. The sequence of the 3′ oligonucleotide was 5′-GCGACAAGCTTTTCCATGAGAGC-3′, obtained from published sequences of rat liver AADC cDNA (12), which included an internal *Hin*dIII site. A 632-base-pair (bp) fragment was obtained, which was cloned into the pBKSII+ vector (Stratagene) and sequenced (19). This 632-bp fragment and other DNA fragments were labeled with [^{32}P]dCTP by random priming (Prime-it, Stratagene) and used to screen a rat genomic library in EMBL3 SP6/T7 purchased from Clontech. Each time 2×10^6 phage plaques were screened as described (18).

Analysis of Phage Clones. Genomic DNA was isolated from phage clones as described (18), and a restriction map was generated. The locations of AADC coding sequences and 5′

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Abbreviations: AADC, aromatic L-amino acid decarboxylase; DDC, dopa decarboxylase; nt, nucleotide(s).

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L03415, L03416, and L03417).

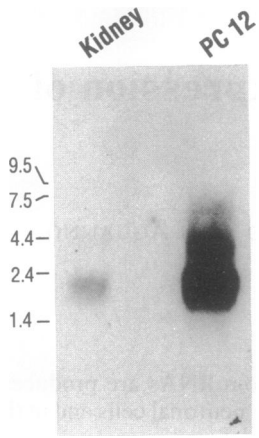

FIG. 1. Northern blot of total rat kidney and PC12 cell RNA, using the 632-bp rat AADC cDNA probe. Twenty micrograms of total RNA was loaded in each lane. The sizes of RNA markers are indicated in kb.

untranslated regions were determined by hybridization of genomic DNA to oligonucleotides derived from published AADC sequences (12, 17). Fragments of interest were subcloned into the pBKSII+ vector for sequencing. DNA sequence analysis and sequence comparisons were done with the MACVECTOR sequence analysis program (International Biotechnologies).

Northern Blot Analysis. Total RNA was isolated from rat kidney and rat pheochromocytoma (PC12) cells (20) as described above. Northern analysis using 20 μg of total RNA and the 632-bp AADC cDNA probe was performed as described (18) using a GeneScreen*Plus* membrane (DuPont). The blot was hybridized for 16 hr at 42°C and washed with 0.2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) and 0.1% SDS at 65°C.

Primer Extension. Total RNA was isolated from rat PC12 cells and rat liver as described above. Primer extension was performed using 50 μg of either total liver or PC12 RNA as described (21). The oligonucleotides used were primer a, 5'-TCTTCTCCGGAATTCACGGG, and primer b, 5'-AATGCCGTCCAGATAGTCAGC. Extension products were separated on a 6% acrylamide/urea gel, along with a sequencing reaction for calibration.

RNase Protection. A 780-bp *Sau*3A–*Stu* I fragment of the rat AADC gene that included 360 bp 5' and 350 bp 3' to exon 1 was subcloned into pBSKII+ (Stratagene) digested with *Bam*HI and *Sma* I. After linearization of the plasmid with *Xba* I, a probe of ≈840 nucleotides (nt) was synthesized with T7 polymerase and [^{32}P]UTP. For exon 2, a 990-bp *Xba* I–*Pst* I fragment of the AADC gene that included 565 bp 5' and 355 bp 3' to exon 2 was subcloned into pBKSII+ (Stratagene). A 1030-nt probe was synthesized with T3 polymerase and [^{32}P]UTP after linearization with *Xba* I. RNase protection experiments were performed using the RPA II kit (Ambion, Austin, TX) using 50 μg of total rat kidney, liver, PC12, or yeast RNA. Protected fragments were separated on a 6% acrylamide/urea gel along with a sequencing reaction for calibration of the gel.

RESULTS

Isolation of AADC Genomic Clones. A portion of the cDNA encoding rat neuronal AADC was obtained from rat brain cDNA using the PCR with oligonucleotides derived from published sequences (12, 17). This fragment, containing the first 632 bp of rat AADC coding sequence, hybridized predominantly to a 2.2-kb message on Northern blots of total rat kidney and pheochromocytoma (PC12) cell mRNA (Fig. 1). The 632-bp AADC cDNA fragment was used to screen a rat genomic library in EMBL3 Sp6/T7. Three overlapping clones were obtained (rDDC4, rDDC8, and rDDC9, Fig. 2), which encompassed ≈28 kb of DNA. Included in this 28 kb were the translation start site and AADC coding sequences contained within the cDNA probe.

To obtain additional 5' genomic DNA, a 500-bp *Hin*dIII fragment from the 5' end of rDDC8 was used to rescreen the rat genomic library. Three additional clones were obtained (rDDCH1, rDDCH4, rDDCH6, Fig. 2), encompassing an additional 12 kb of DNA. Two of these clones, rDDCH1 and rDDCH6, contained the 5' untranslated region of the neuronal form of AADC mRNA. This small exon was located ≈9.5 kb upstream from the exon containing the translation start site.

An *Eco*RI fragment from the 5' end of rDDCH1 was used to screen the library a third time. A single clone (rDDCRI1) of ≈15 kb was isolated, which contained within it the nonneuronal AADC 5' untranslated sequences and an additional 2 kb of 5' flanking DNA. This exon was located ≈7 kb upstream from the exon containing the neuronal 5' untranslated region.

A restriction map of the portion of the AADC gene encompassed by these seven clones, showing the location of the first six exons, is diagramed in Fig. 2. The locations of exons 5 and 6 have been previously reported by Hahn *et al.* (14).

Determination of Transcription Initiation Sites. Primer extension analysis was performed to determine the site of transcription initiation for neuronal and nonneuronal AADC mRNA (Fig. 3). Two oligonucleotide primers, a and b, complementary to AADC coding sequences in exon 3 were used (see Fig. 5 and *Methods*).

Using total PC12 RNA, an extension product of 103 nt was observed with primer a, and an extension product of 148 nt was observed with primer b (Fig. 3), indicating that neuronal transcription initiates at the T labeled +1 in exon 2 (see Fig. 5). A minor extension product 7 nt longer, observed with both primers, suggests that another transcription start site is used occasionally.

Using total liver RNA, major extension products of 111 and 156 nt were observed with primers a and b, respectively (Fig. 3), suggesting that the nonneuronal transcription start site is located at the T labeled +1 in exon 1 (see Fig. 5). Larger extension products were also observed for both primers. These bands appeared to be due to artifactual hybridization

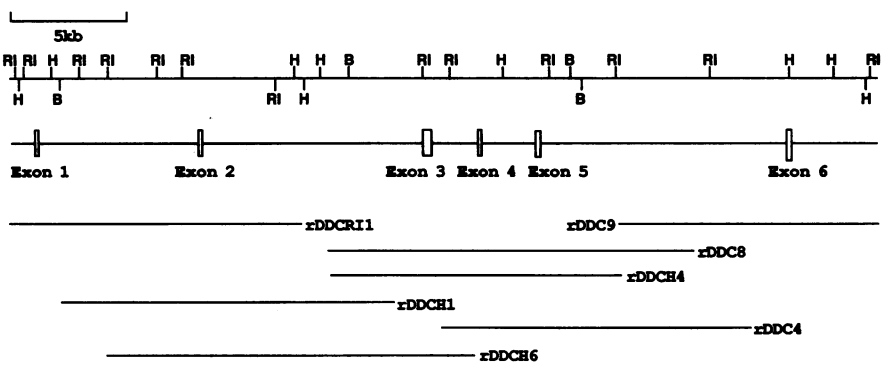

FIG. 2. Restriction map of 40 kb of the rat AADC gene showing the location of exons 1–6. H, *Hin*dIII; RI, *Eco*RI; B, *Bam*HI. The locations of the seven overlapping clones isolated from the genomic library are shown underneath. Exons 1 and 2 are noncoding exons and are 71 and 68 bp in length, respectively; exon 3 is 224 bp in length; exon 4 is 113 bp; and exons 5 and 6 are 119 and 134 bp, respectively (14).

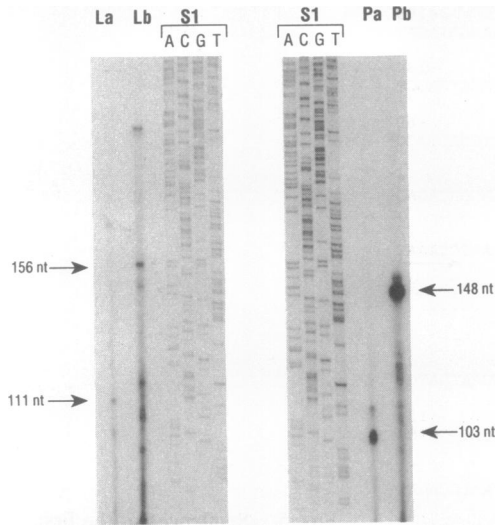

FIG. 3. Primer extension analysis of AADC mRNA in liver and PC12 cells. La and Lb, extension products using total rat liver RNA with primer a and primer b, respectively; Pa and Pb, extension products using total PC12 RNA with primer a and primer b, respectively. The sizes of the extension products are indicated in nt. S1, sequence of a portion of exon 2 for calibration. The sequences of primers a and b and the sequencing primer (seq. primer-1) are underlined in Fig. 5.

of the primers because they appeared in all RNAs tested and did not correspond to the same end point. To be sure that this was the case, RNase protection experiments were performed.

An 840-nt probe, which encompassed exon 1 and surrounding sequences (see *Methods* and Fig. 4*C*), was hybridized to total rat kidney or liver RNA. Digestion with RNase produced a 71-nt protected fragment indicating that exon 1 is 71 bp in length (Fig. 4*A*). This is the size predicted by the primer extension results. Because total RNA was used in this experiment, a higher molecular weight protected fragment of ≈420 nt, representing hybridization to unprocessed RNA containing the first intron, is also observed.

For exon 2, a 1030-nt probe that contained exon 2 and surrounding sequences (see *Methods* and Fig. 4*C*) was used. After hybridization to total PC12 RNA and RNase digestion, a 68-nt protected fragment was observed (Fig. 4*B*), confirming the location of the neuronal transcription start site determined by primer extension. The alternate transcription start site 7 bp upstream is also detected in the RNase protection assay. The higher molecular weight species of ≈425 nt again represents hybridization to unprocessed RNA.

Structural Analysis of the Rat AADC Gene. The sequence of exon 3, the first exon containing AADC coding sequences, is presented in Fig. 5. It is 214 bp in length, initiating 12 bp upstream of the translation start site.

A comparison of the sequences of neuronal and nonneuronal AADC cDNAs to the genomic sequence shows that splicing occurs at a different acceptor for neuronal and nonneuronal mRNAs. Exon 1 is spliced to exon 3 at a site 5 nt upstream of the exon 2 acceptor site, as diagramed in Fig. 5. The neuronal site shows a perfect match to the consensus splice acceptor sequence C/TAG (22), whereas the nonneuronal site has a 1-nt mismatch (Fig. 5).

Analysis of Promoter Sequences. In the neuronal promoter the sequence CATAAAT, found 30 nt upstream from the transcription start site, could be considered to be a TATA box (22) (Fig. 5). Two possible TATA boxes were found in the nonneuronal promoter: the sequence TTTAAAT at −22 and the sequence TTTAA at −40 (Fig. 5).

The human AADC gene has been cloned (13); however, only one promoter has been found. Comparison of the human promoter sequence to the rat AADC promoters shows significant homology only to the rat neuronal promoter. This sequence comparison (Fig. 6) shows particularly high homology around the transcription start site. A highly conserved region from −52 to −63 contains a CCAAT box in reverse orientation (23), and another highly conserved region between −83 and −88 contains sequences bearing homology to an AP-1 site (24). No significant homology was found between the two rat promoters.

DISCUSSION

To investigate the regulation of AADC gene expression we have isolated genomic clones encoding rat AADC. We have isolated seven overlapping clones from an EMBL3 SP6/T7 library encompassing ≈40 kb of DNA. This genomic DNA contains sequences encoding slightly more than the first 632 nt of AADC mRNA but does not include the 3′ end of the message. A partial analysis of additional portions of the AADC gene has been reported (14). Our data, together with information from this partial analysis, suggest that the rat AADC gene, like the human gene (13), is a relatively large gene spanning >70 kb. The size of this gene must have

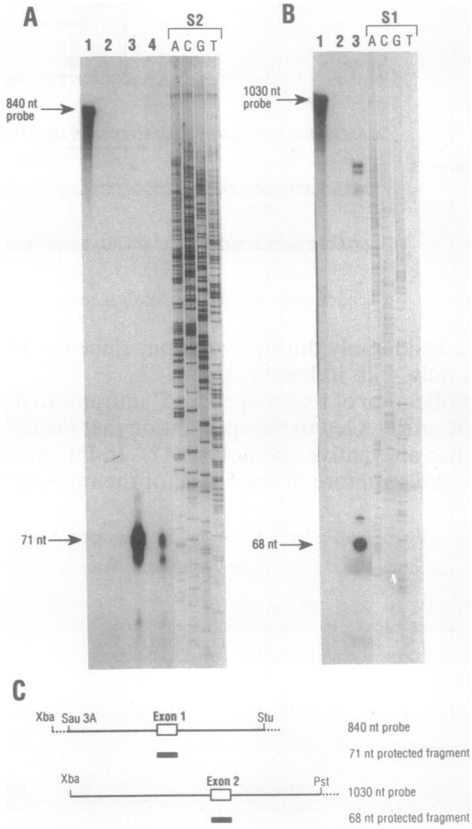

FIG. 4. (*A*) RNase protection experiment using the 840-nt probe specific for the AADC nonneuronal promoter. Lane 1, undigested probe; lane 2, 50 μg of yeast RNA control; lanes 3 and 4, protected fragments using 50 μg of rat kidney and liver RNA, respectively. S2, sequence of a portion of exon 1 for calibration. The primer used for this sequencing reaction is underlined in Fig. 5 (seq. primer-2). (*B*) RNase protection experiment using the 1030-nt probe specific for the neuronal promoter. Lane 1, undigested probe; lane 2, 50 μg of yeast RNA control; lane 3, protected fragment using 50 μg of total PC12 RNA. S1, sequence of a portion of exon 2 for calibration of the gel. The oligonucleotide used for this sequencing reaction is underlined in Fig. 5 (seq. primer-1). (*C*) Diagram showing the probes used for RNase protection experiments and the predicted protected fragments. Dotted lines represent vector sequences.

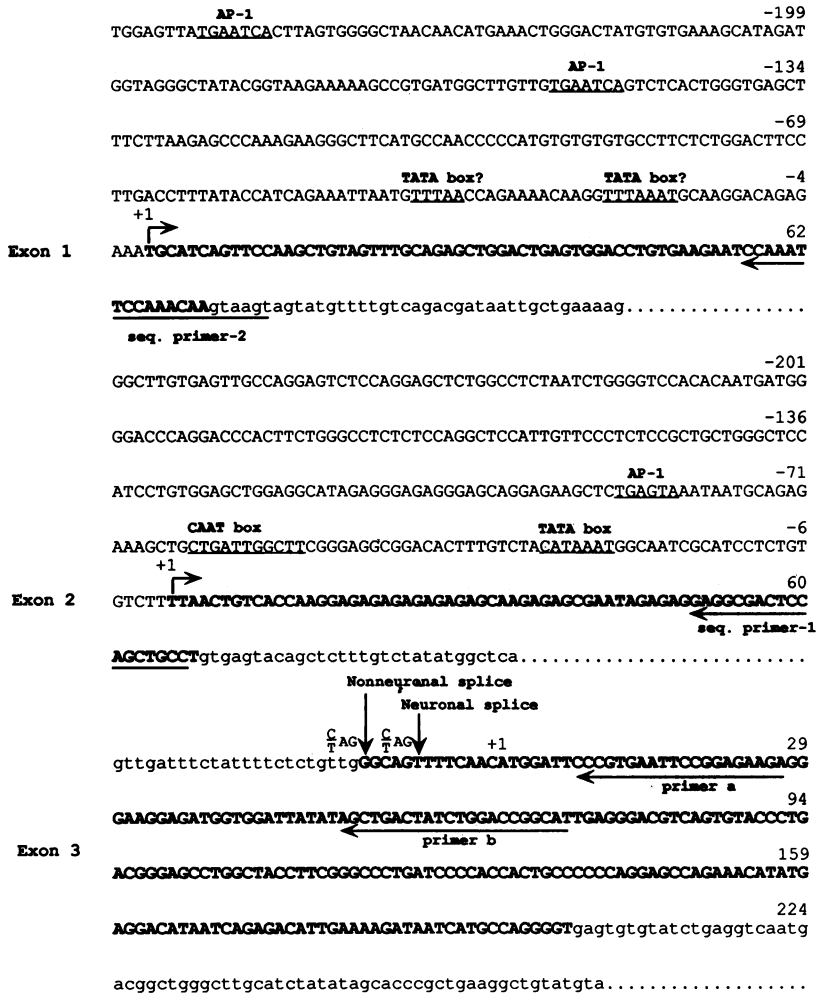

FIG. 5. Sequences of the first three exons of the rat AADC gene including some surrounding intronic sequences. Exon sequences are shown in bold type; introns are shown in lowercase letters. Each promoter is numbered according to its transcription start site. Putative transcription factor binding sites and TATA boxes are underlined. In exon 3 the translation start site is numbered +1. The neuronal and nonneuronal splice acceptors are indicated, and the consensus sequences are shown above the sequence. The oligonucleotides used for primer extension and sequencing reactions shown in Figs. 3 and 4 are indicated.

increased considerably during evolution, since the *Drosophila* gene is only 4 kb in length (15).

The identification of tissue-specific 5′ untranslated regions in rat AADC mRNA led to the speculation that the rat AADC gene may use alternative promoters (17). In this manuscript we present the structure of the 5′ end of the rat AADC gene and show that this gene does contain two alternative promoters. The most 5′ exon, which we call exon 1, contains sequences specific to the 5′ untranslated region of AADC mRNA expressed in nonneuronal tissue. This exon is separated by ≈7 kb from exon 2, which contains sequences specific to the 5′ untranslated region of neuronal AADC mRNA. A 9.5-kb intron separates this exon from exon 3, which contains the translation initiation site. This structure, along with sequence comparisons between genomic DNA and cDNA, suggests that the use of tissue-specific promoters, followed by alternative splicing, is responsible for the generation of tissue-specific AADC mRNAs. As diagramed in Fig. 7, in nonneuronal cells, transcription initiates at the most 5′ promoter, synthesizing a long transcript, which includes exons 1–3. RNA processing then results in a mature transcript in which exon 1 has been spliced to exon 3. In brain and adrenal chromaffin cells, transcription initiates at the more 3′ promoter, giving rise to a shorter transcript, which does not contain exon 1. Exon 2 is then spliced directly to exon 3.

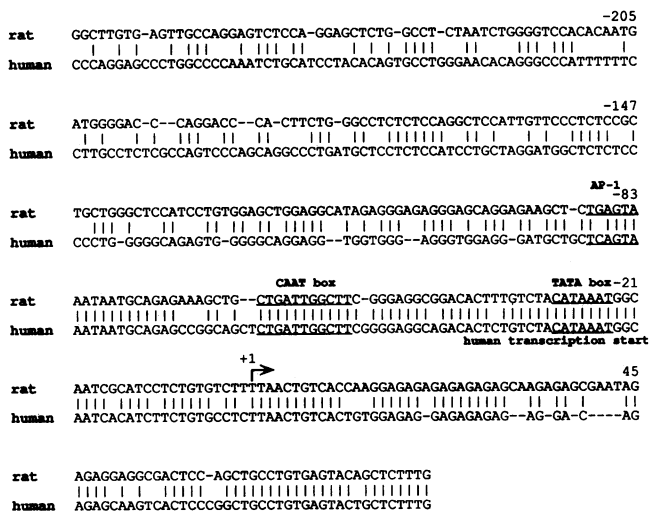

FIG. 6. Comparison of rat neuronal and human AADC promoter sequences. Human sequences are from Sumi-Ichinose *et al.* (13). Nucleotide identities are shown by vertical bars. A putative CCAAT box, AP-1 site, and a highly conserved region that is the putative rat TATA box and human transcription initiation site are indicated. The numbering is according to the rat AADC genomic sequence.

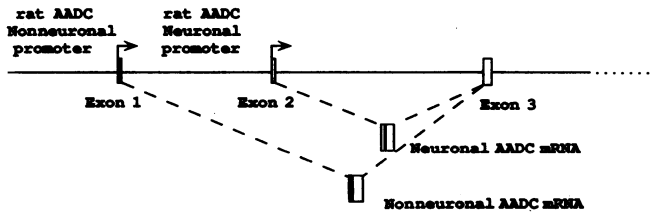

FIG. 7. Diagram of the structure of the first three exons of the rat AADC gene, showing how alternative promoters are utilized to generate AADC mRNAs with different 5′ untranslated sequences in neuronal and nonneuronal cells.

The exon 3 splice acceptor site for neuronal AADC is located 5 nt downstream from that for nonneuronal AADC. Both acceptor sites have homology to the consensus acceptor sites (22), although the neuronal site shows a better homology (Fig. 5). The existence of alternative splice acceptor sites could suggest that tissue-specific splicing factors might bind at slightly different sites and, in one case, block the more preferred acceptor site.

In *Drosophila*, the *Ddc* gene is also alternatively spliced to give rise to tissue-specific mRNAs (15). In this case, however, an internal exon is spliced out of the nonneuronal, but not the neuronal, message, resulting in two proteins differing by 33–35 amino acids. In the rat, the coding sequences of AADC are identical in all tissues examined, and thus the differences in the 5′ end of the mRNA sequence most likely reflect differential regulation of AADC protein expression rather than differences in the protein itself.

The use of alternative promoters to direct gene expression to different tissues has been observed for numerous other genes (25–27). Like AADC, dystrophin (28), α-tropomyosin (29), and the prepro gastrin-releasing peptide (30) have also been shown to utilize alternative promoters to regulate neuronal and nonneuronal expression. By analogy to these other examples, we expect that the differential expression of AADC will involve cell-specific transcription factors that will recognize different cis-active elements in the two promoters.

Primer extension and RNase protection experiments with PC12 mRNA showed the neuronal transcription initiation site to be identical to that determined by Krieger *et al.* (17). Approximately 30 bp upstream from this transcription start site is the sequence CATAAAT, which could serve as a TATA box. As shown in Fig. 6, there is perfect homology between rat and human sequences in this region. Interestingly, Sumi-Ichinose *et al.* (13) have identified multiple transcription start sites for human AADC in this highly conserved CATAAAT sequence. It is possible that the two species have different transcription start sites, although this seems unusual because of the perfect homology in this region.

Because of the high degree of homology between the human and rat cDNAs (17) and the rat neuronal and human promoters as well as the fact that tissue-specific AADC mRNAs are found in species as evolutionarily distant as *Drosophila*, it is likely that a nonneuronal promoter is also present further upstream in the human AADC gene, as suggested by Sumi-Ichinose *et al.* (13).

Examination of the sequences of the 5′ flanking regions of the two promoters showed homology with some known transcription factor binding sites. In the neuronal promoter, possible binding sites for an AP-1 site and a CAAT box were found. Although homology to a binding site cannot be taken as evidence for transcription factor binding or activity, these sites are located in areas of high homology between the human and rat promoter sequences, suggesting that these sites might play a role in transcriptional regulation. In the nonneuronal promoter, two possible AP-1 sites were found, but no significant homology was found between the two promoters, consistent with the assumption that transcription from the two promoters will be regulated by different, tissue-specific factors.

We have elucidated the structure of the 5′ end of the rat AADC gene and have shown that the rat AADC gene contains neuron- and nonneuron-specific promoters. The ability to analyze and compare transcriptional regulation of the two AADC promoters should lead to important insights into mechanisms of transcriptional regulation in neuronal and nonneuronal cells.

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