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Different mRNAs code for dopa decarboxylase in tissues of neuronal and nonneuronal origin

(mRNA sequence/PCR amplification/alternative splicing/rat pheochromocytoma/tissue specificity)

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ABSTRACT **A cDNA clone for dopa decarboxylase (EC 4.1.1.28) has been isolated from a rat pheochromocytoma cDNA library and the cDNA sequence has been determined. It corresponds to an mRNA of 2094 nucleotides. The length of the mRNA was measured by primer-extension of rat pheochromocytoma RNA and the 5′ end of the sequence of the mRNA was confirmed by the PCR. A probe spanning the translation initiation site of the mRNA was used to hybridize with mRNAs from various organs of the rat. S1 nuclease digestion of the mRNAs annealed with this probe revealed two classes of mRNAs. The comparison of the cDNA sequence and published sequences for rat liver, human pheochromocytoma, and *Drosophila* dopa decarboxylase supported the conclusion that two mRNAs are produced: one is specific for tissue of neuronal origin and the other is specific for tissues of nonneuronal (mesodermal or endodermal) origin. The neuronal mRNA contains a 5′ untranslated sequence that is highly conserved between human and rat pheochromocytoma including a GA stretch. The coding sequence and the 3′ untranslated sequence of mRNAs from rat liver and pheochromocytoma are identical. The rat mRNA differs only in the 5′ untranslated region. Thus a unique gene codes for dopa decarboxylase and this gene gives rise to at least two transcripts presumably in response to different signals during development.**

Dopa decarboxylase (AADC; EC 4.1.1.28) is an enzyme that decarboxylates the aromatic L-amino acids and for this reason was named aromatic L-amino acid decarboxylase or AADC (1). Because of its enzymatic activity, AADC is mainly implicated in two essential metabolic pathways: the synthesis of serotonin and dopamine (2).

AADC has been localized in various areas of the mammalian brain, the sympathetic nervous system, and the adrenal and pineal glands (3). AADC is also present in the diffuse neuroendocrine system, which is constituted by the amine precursor uptake decarboxylation (APUD) cells (4). The essential function of AADC is to provide the organism with neurohormones and specific neurotransmitters. AADC has also been found in peripheral organs of various embryologic origins (1), such as kidney (5) and liver and pancreas (6) in which its physiological function is not yet well known. In insects, AADC functions in the synthesis of biogenic amines (7) and is involved in the synthesis of dopamine metabolites that are cross-linked during an essential step in cuticle sclerotization (8).

AADC is not regulated by mechanisms equivalent to those that activate the catecholamine metabolic pathway (9, 10)—i.e., stimulation the synthesis of tyrosine hydroxylase, the rate-limiting enzyme of the metabolic chain (11).

AADC was purified first from hog kidney (12, 13) and, subsequently, from rat liver (14), rat kidney (15), rat pheochromocytoma (16), and human pheochromocytoma (17). AADC originating from kidney is a homodimeric 52-kDa protein that binds one molecule of pyridoxal phosphate per molecule of dimer (18, 19). The amino acid sequence of the binding site for this cofactor has been determined (20).

The study of AADC from *Drosophila* thermo-conditional mutants has permitted the cloning (21, 22) of the AADC gene and led to the demonstration that different forms of the enzyme are produced by different mRNAs in *Drosophila*, depending on the specific tissues examined (23). It was established that the AADC mRNAs are derived by tissue-specific splicing mechanisms from the transcript of a unique gene; for example, the AADC mRNA of the hypodermis is not the same as the AADC mRNA found in neurons or glial cells.

Subsequently, Albert *et al.* (24) demonstrated that AADC from bovine adrenal medulla was the product of a single gene and they found a unique mRNA. A simple mRNA was also identified by Tanaka *et al.* (25) and by Ichinose *et al.* (26) in their studies on AADC cDNA from rat liver and human pheochromocytoma, respectively. Data from these latter laboratories have indicated that the AADC sequence is very well conserved during evolution.

It was not clear from the mammalian data whether the situation in *Drosophila*, where there is a differential expression of distinct mRNAs from a unique AADC gene in a tissue-specific pattern, also applied to mammals. In particular, it was not clear whether there were distinct AADC transcripts in tissues of neuronal and nonneuronal origin. In this paper, as a result of the isolation of an AADC cDNA probe and comparison of mRNAs in rat neuronal and nonneuronal tissues, we demonstrate the presence of two species of AADC mRNA differing in their 5′ untranslated region.

MATERIAL AND METHODS

Animals, Tumors, and Tissues. Rats of the New England Deaconness Hospital strain were injected subcutaneously with 5×10^6 PC12 cells (27). Three weeks later, tumors were collected from CO_2-asphyxiated animals. Organs (liver, kidney, adrenal, and midbrain) were dissected from nontumor-bearing animals of the same species.

RNA Preparation. Total RNA was extracted from rat tissues by the guanidine thiocyanate/cesium chloride procedure described by Kaplan *et al.* (28). The poly(A)$^+$ mRNA fraction was separated by oligo(dT)-cellulose chromatography.

Construction of a Rat Pheochromocytoma Library. After centrifugation as described (29), a sucrose gradient fraction of rat pheochromocytoma poly(A)$^+$ mRNA (30) was selected on the basis of its ability to be translated into AADC, using an *in vitro* reticulocyte lysate system. AADC was identified by immunoprecipitation with two specific anti-AADC sera (31).

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Abbreviation: AADC, aromatic L-amino acid decarboxylase or dopa decarboxylase.

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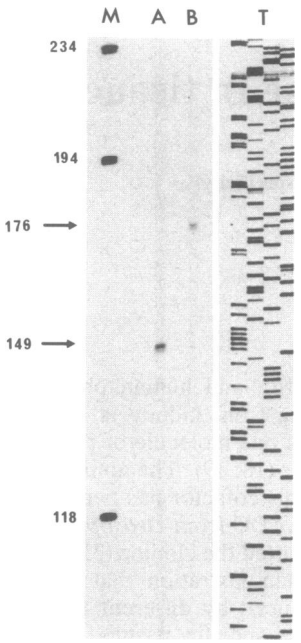

FIG. 1. Starting point determination of the rat pheochromocytoma AADC mRNA as shown by autoradiography of the sequencing gel. Lanes: M, molecular mass markers (in bases), ϕX174 replicative form *Hae* III fragments ^{32}P-labeled by polynucleotide kinase; A, primer-extension products of oligonucleotide A giving a 149-base cDNA; B, primer-extension products of oligonucleotide B giving a 176-base cDNA; T, sequence of a part of cDNA AADC to calibrate gel migration.

This fraction, enriched 10-fold in AADC mRNA, was used to prepare a cDNA library (32) in λgt11 phage according to Young and Davis (33).

Primer Extension. Two 21-mer oligonucleotides complementary to nucleotide positions +52 to +72 (oligonucleotide A) or positions +79 to +99 (oligonucleotide B) (see Fig. 2) of the AADC mRNA were used to prime reverse transcription of mRNA. Each one was end-labeled with [γ-^{32}P]ATP by using polynucleotide kinase (34). The labeled primer was annealed to 10 μg of the poly(A)$^+$ rat pheochromocytoma mRNA. Primer extension was performed in reverse transcriptase buffer with all four dNTPs (each at 1 mM), 21 units of RNase inhibitor (Appligène, Illkirch, France), and 20 units of reverse transcriptase (Appligène) for 45 min at 42°C (35). Extended products were analyzed by electrophoresis in a 6% polyacrylamide gel containing 7 M urea.

PCR. Oligonucleotide A (positions +52 to +72, antisense) and oligonucleotide C (see Fig. 4), a DNA copy of the mRNA sequence between positions −72 to −52 (sense), flanked in the 5′ position by a *Bam*HI restriction site were used. The amplification between these two primers was carried out by the protocol of Saiki *et al.* (36). Poly(A)$^+$ RNA (10 ng, 100 ng, or 1 μg) was preannealed with oligonucleotide A and reverse-transcribed. RNA·cDNA hybrid or clone λAADC9 (10^7 plaque-forming units) was amplified between these two primers. Ten microliters of the two amplified fragments was analyzed on a horizontal 1.5% agarose gel; they measured 144 base pairs. The remainder was purified on a gel, treated with the Klenow fragment of *Escherichia coli* DNA polymerase I (Klenow polymerase; 5 units) in the presence of all four dNTPs (each at 25 μM) and then submitted to *Bam*HI digestion for subcloning in M13mp18.

S1 Nuclease Analysis. The PCR AADC restriction fragment described above was cloned into M13mp18, replicative form. The DNA was extracted and purified according to Sanger *et al.* (37). Single-stranded recombinant M13 DNA (1 μg) was annealed with 2.8 ng of M13 universal sequencing primer, and the labeled complementary strand was synthesized with the Klenow polymerase (2 units) in the presence of 10 pmol of [α-^{32}P] dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) (38). The DNA was then digested with *Bam*HI, and the probe was purified by electrophoresis on a 6% denaturing polyacrylamide gel. The probe (5×10^5 cpm) was hybridized to mRNAs in 80% (vol/vol) formamide/40 mM Pipes, pH 6.4/400 mM NaCl/1 mM EDTA at 45°C for 15 hr. The hybrid was then digested with S1 nuclease (600 μl; 40 units; Appligène) at 25°C for 2 hr. Samples were extracted with phenol, precipitated, and loaded onto a 6% denaturing polyacrylamide gel. After fixation in ethanol/acetic acid/H_2O, 1:1:8 (vol/vol), the gel was dried and autoradiographed.

RESULTS

Cloning of AADC cDNA. A rat pheochromocytoma cDNA library was constructed in a λgt11 vector and probed with two anti-AADC sera (33). Of 15 immunopositive plaques, 10, designated λAADC1 to -10, were shown to contain cross-hybridizing recombinant inserts. The longest (λAADC9) contained an insert of 2094 base pairs and the others contained only the 3′ part of the mRNA.

Sequence and Structure of the AADC cDNA Clone. By using six endonucleases, the restriction map of the 2094-base-pair insert was established. Resulting fragments were purified on agarose gel, eluted, and subcloned in M13mp18 bacteriophages. Sequencing was performed on both strands (39).

The nucleotide sequence indicates a continuous reading frame from the β-galactosidase *Eco*RI cloning site of the λ vector to the termination codon TAA (positions 1441–1443). The 480-amino acid sequence deduced from the cDNA sequence includes that from the proteolytic peptides from rat pheochromocytoma AADC (16), as well as both the pyridoxal phosphate binding site (20) and the sequence of the COOH-

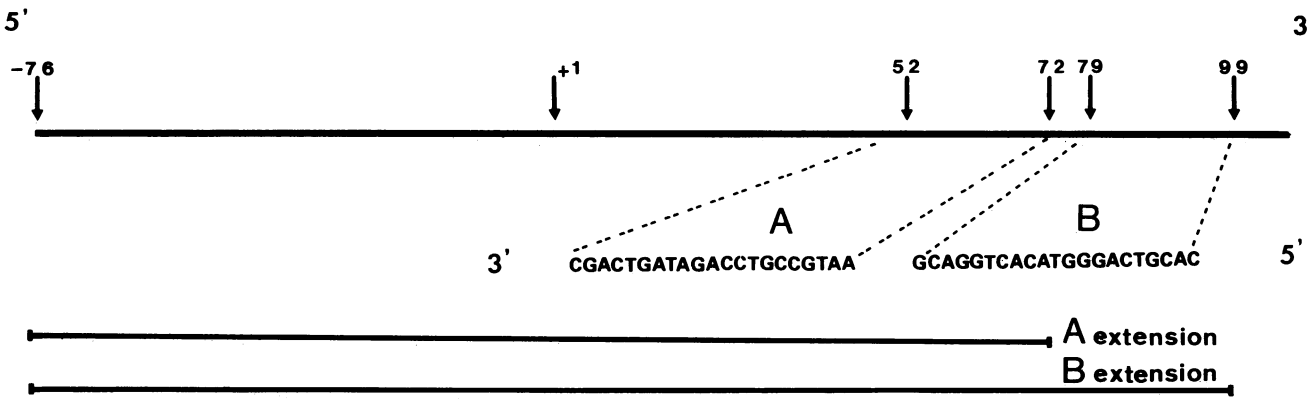

FIG. 2. Schematic drawing of the position of oligonucleotides A and B and of the two extended fragments.

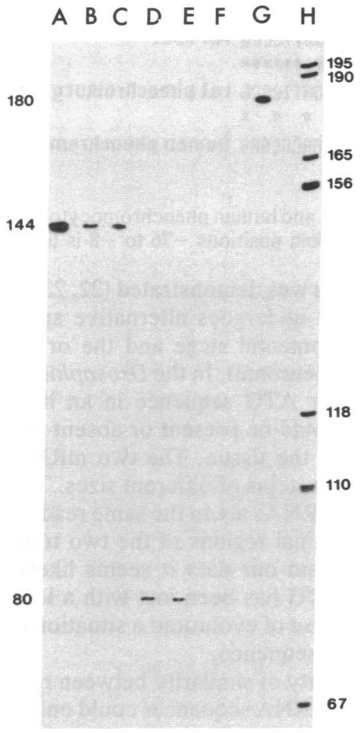

FIG. 3. S1 nuclease mapping analysis of the 5′ region of AADC poly(A)$^+$ RNAs of various rat tissues. Lanes: A, pheochromocytoma (5 μg); B, midbrain (50 μg); C, adrenal medulla (50 μg); D, liver (50 μg); E, kidney (50 μg). RNAs were hybridized with the 180-nucleotide single-strand DNA probe (including 36 nucleotides from M13 vector). The hybrids were then digested with 40 units of S1 nuclease. Band 180 corresponds to entire cDNA probe, band 144 corresponds to cDNA probe minus nucleotides from M13, and band 80 corresponds to homologue region of pheochromocytoma and liver sequence (25). Controls with yeast transfer RNA (50 μg) were performed using the same cDNA probe either in the presence (lane F) or in the absence (lane G) of S1 nuclease. Lane H corresponds to size markers; positions of markers are indicated in bases.

terminal moiety reported in pig kidney AADC (40). The first ATG codon in the open reading frame was numbered +1. From nucleotide −76 to nucleotide −8, the sequence was 5′-TTAACTGTCACCAAGGAGAGAGAGAGAGCAAGAGAGCGAATAGAGAGGAGGCGACTCCAGCTGCCT-3′. From nucleotide −8 to nucleotide +1879, it was found to be identical to the sequence for rat liver AADC mRNA (25).

To confirm the identity of the starting point of the mRNA, two oligonucleotides were used in primer-extensions experiments. cDNAs of 149 and 176 nucleotides were obtained when the first (oligonucleotide A) and second (oligonucleotide B) primers were used, respectively (Fig. 1). From these results we deduced that the 5′ end of AADC mRNA was at position −76 (Fig. 2).

Since the 5′ sequence of λAADC9 was different from that found by Tanaka *et al.* (25) for the liver AADC mRNA, the possibility of an artifactual recombination in the 5′ part of the cDNA was tested. A cDNA fragment, obtained in the mRNA primer-extension experiments, with antisense oligonucleotide A, was amplified using the sense oligonucleotide C by PCR. The amplified fragment was digested at a specific *Bam*HI restriction site that had been added to primer C (see Fig. 4). The resulting DNA molecules were cloned and sequenced. Examination of the sequence confirmed that the amplified sequence corresponded to the 5′ untranslated portion of the AADC mRNA.

Analysis of the 5′ End of AADC mRNA from Various Rat Tissues. The DNA extracted from the M13mp18 AADC phage containing positions −72 to +72 of the AADC mRNA sequence served as a template for Klenow polymerase to produce a radioactive probe with high specific activity. This radioactive single-stranded DNA was hybridized with various poly(A)$^+$ mRNAs extracted from rat pheochromocytoma, adrenal medulla, midbrain, liver, and kidney. Then, hybrids were submitted to S1 nuclease digestion. As shown in Fig. 3, the protected fragment was 144 nucleotides long when annealing was done with mRNA from pheochromocytoma, adrenal medulla, and midbrain, but only 80 nucleotides long when liver and kidney mRNAs were annealed. The difference in the length of the fragment protected from S1 digestion indicates that at least two families of AADC mRNA exist depending on the organs examined (Fig. 4).

Comparison of AADC mRNA Sequences. The nucleotide sequence of AADC mRNA from nucleotides −76 to +1876 was compared with AADC mRNA sequences from rat liver (25), human pheochromocytoma (26), plant tryptophan decarboxylase (41), and the sequence deduced from the AADC *Drosophila* gene (22) by using a diagon comparison of C.I.T.I. 2 (Centre Inter Universitaire d'Informatique à Orientation Biomédicale). Rat pheochromocytoma and rat liver AADC mRNAs possessed exactly the same sequence from nucleotide −8 to the polyadenylylated 3′ end; a difference appeared in the region lying upstream of nucleotide −8 (Fig. 5). Table 1 summarizes the percent homology found with other species.

Homologies between all these sequences from positions −76 to +1876 of the rat pheochromocytoma AADC nucleotide sequence indicate a very high degree of conservation throughout evolution. A remarkable observation is the partial

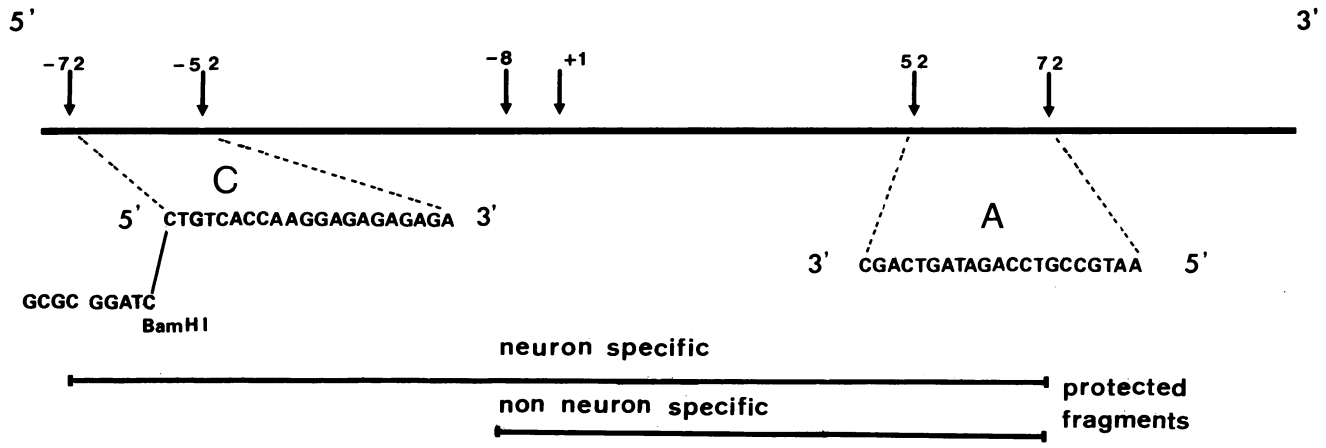

FIG. 4. Schematic drawing of the common and different sequences of neuron-specific and nonneuron-specific fragments of the mRNAs.

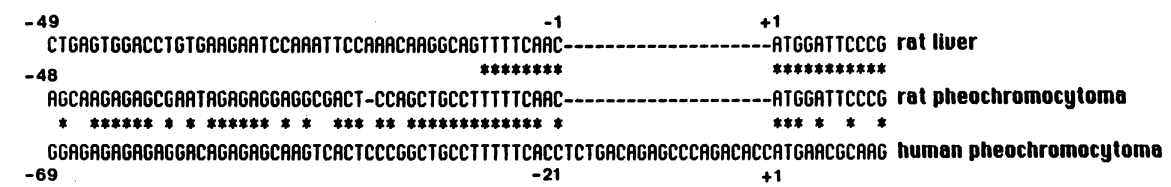

FIG. 5. Comparison of nucleotide sequence of the 5′ end of rat pheochromocytoma, rat liver, and human pheochromocytoma AADC mRNAs. Alignments are indicated by an *; deletions are indicated by a –. The entire new sequence from positions −76 to −8 is listed in the text.

homology between the 5′ untranslated end of the rat pheochromocytoma mRNA (nucleotides −47 to −1) and the homologous region of human pheochromocytoma mRNA (nucleotides −68 to −20) (Fig. 5).

DISCUSSION

We have isolated cDNA clones containing the total AADC mRNA from rat pheochromocytoma. Such a tumor represents a very rich source of AADC protein since its content in this enzyme is greater than that found in the adrenal medulla (27). From the 6×10^5 clones that have been tested by immunoblotting with our antisera, only 10 appeared to be positive. This low value approximates that found by Bruneau *et al.* (31) who calculated by an *in vitro* translation system that 5 in 10,000 mRNAs were AADC mRNA in these same tumors. In spite of the imprecision of such determinations, we can assume that this mRNA is of somewhat low abundance.

Analysis of the cDNA clones indicates a 1440-nucleotide long open reading frame having exactly the sequence observed by Tanaka *et al.* (25) for rat liver AADC. The fact that the 3′ noncoding sequences of both mRNAs are identical reinforces the similarity between rat pheochromocytoma and liver AADC mRNAs and proteins and, furthermore, indicates that posttranscriptional modification does not affect this part of the mRNAs.

Albert *et al.* (24) found that bovine AADC was produced from a unique gene. If, as seems probable, there is only one gene in the rat and since our results along with those of Tanaka *et al.* (25) demonstrate the presence of two mRNAs that, when translated, give an identical protein, we can assume that the differences observed in K_m and V_{max} of AADC in different organs (3) were probably due to the fact that kinetic measurements were done with impure enzyme preparations, that different mechanisms may regulate the activity of the enzymes, or that the enzymes may be modified by different post-translational events.

Comparison of the 5′ untranslated moiety of AADC mRNA of rat liver and pheochromocytoma reveals an abrupt change in the homology of their nucleotide sequence at position −8 from the initiation codon ATG. Such an interruption could be explained by a differential splicing taking place during the maturation of the AADC pre-mRNA in the liver, in pheochromocytoma, or in both tissues. This possibility is not unlikely, since, as was demonstrated (22, 23), the *Drosophila* AADC transcript undergoes alternative splicing depending upon the developmental stage and the origin of the tissue (neuronal or nonneuronal). In the *Drosophila* gene, there was a second initiator ATG sequence in an intron of the pre-mRNA, which could be present or absent depending on the differentiation of the tissue. The two mRNAs thus formed give rise to two proteins of different sizes. The initiator ATG codons of both mRNAs are in the same reading frame and the long COOH-terminal regions of the two translated proteins are identical. From our data it seems likely that the most distal initiator ATG has been lost with a long part of the 5′ region in the course of evolution: a situation compatible with only one coding sequence.

The discontinuity of similarity between rat liver and pheochromocytoma mRNA sequences could only be explained by posttranscriptional modifications such as splicing. Since the difference concerns the first exon, an alternative promotor usage, leading in turn to a different splicing pathway, is the most possible. Such a situation has been described for other proteins and is generally a mechanism of expression of ubiquitous versus specific proteins (42–44). To confirm this hypothesis, as nonneuronal (liver and kidney) and neuronal AADC mRNAs were about the same length (unpublished experiments), we attempted to measure the distance between 5′ ends of both RNAs. Unfortunately, since PCR extensions using different primers failed, we hypothesize that one or several intron(s) longer than 5000 nucleotides interspace(s) these extremities.

S1 nuclease resistance experiments have permitted us to identify two classes of mRNAs. The lengths of S1 nuclease-resistant hybrids corresponds to the expected values after analysis of the sequences. Therefore in rat there exist at least two classes of AADC mRNA having a common sequence from nucleotide −8 to the poly(A)$^+$ stretch but differing in their 5′ ends. The thymidine at position −8 could probably be an acceptor of a splice in peripheral organs such as kidney or liver, since the preceding nucleotides adenine and guanine found by Tanaka *et al.* (25) in the rat liver sequence are often found to function as a splice donor (45, 46). In pheochromocytoma, adrenal medulla, and brain stem, there is no consensus sequence permitting splicing at position −8 unless such a sequence is not the usual consensus.

Table 1. Comparison of protein and mRNA regions of rat pheochromocytoma AADC and various AADCs

Enzyme	Homology with rat pheochromocytoma AADC						
	mRNA						Protein
	5′ UTR		Coding region		3′ UTR		
Rat liver AADC (25)	9%	(−78 to −1)	100%	(1 to 1400)	100%	(1441 to 1925)	100%
Human pheochromocytoma AADC (26)	54%	(−69 to −1)	79%	(1 to 1440)	46%	(1441 to 1932)	88%
Drosophila AADC (23)	13%	(−212 to −1)	49%	(1 to 1509)	23%	(1510 to 1808)	59%
Plant tryptophan decarboxylase (41)	24%	(−70 to −1)	38%	(1 to 1500)	17%	(1501 to 1747)	43%

UTR, untranslated region. Results are expressed as percent homology between AADC mRNA regions from various species and the corresponding region of rat pheochromocytoma AADC mRNA [5′ UTR (−76 to −1), coding region (+1 to 1440), and 3′ UTR (1441 to 1964)]. Sequence alignments of *Drosophila* compared to rat pheochromocytoma mRNAs have been done using the sequence of the small *Drosophila* AADC mRNA described by Eveleth *et al.* (22). The low value found between 5′ UTR of rat and *Drosophila* is due to the difference in length.

The publication of the human pheochromocytoma AADC mRNA sequence (26) allowed us to determine the extent of homology between rat and human AADC mRNA (Fig. 5). The study indicates a very high level of conservation through evolution. Fine analysis of the 5′ ends of these mRNAs indicates a continuous homology of sequence for both species. Between nucleotides −8 and −47 in rat and nucleotides −21 to −68 in human, there are only seven mismatches and one deletion. These two homologous regions, however, are not colinear with the starting points of the coding region of the mRNAs. Twenty additional nucleotides (positions −1 to −20) are present in human pheochromocytoma mRNA. Perhaps a single mutation could modify a splice site and thus add these nucleotides. In any event, since the sequence of this part of human and rat AADC mRNAs is very similar and contains a repetitive GA stretch, it could play a hypothetical role in the stability or the translational control of AADC mRNA.

The pheochromocytomas are derived from adrenal medulla (47). It was therefore natural to find the same mRNA in both tissues. In view of the fact that the embryological origin of adrenal medulla is the neural crest (48–50), a neuroectodermal tissue, our data suggest that the AADC mRNA found in brain and in peripheral organs of neuroectodermal origin belong to a defined category represented by nerve-specific tissues. On the other hand, the AADC mRNA processed from peripheral organs of various embryologic origin, such as kidney mesoderm and liver endoderm (51), would correspond to a distinct form of mRNA. Therefore, the sequence upstream of nucleotide −8 could play an important regulatory function in relation to tissue specificity. Further insight concerning control of the AADC gene will have to await analysis of its structure.

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