Tissue-specific Expression of the Nonneuronal Promoter of the Aromatic L-Amino Acid Decarboxylase Gene Is Regulated by Hepatocyte Nuclear Factor 1*

(Received for publication, August 2, 1995, and in revised form, December 18, 1995)

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The rat aromatic L-amino acid decarboxylase (AADC) gene contains alternative promoters which direct expression of neuronal and nonneuronal mRNAs that differ only in their 5′-untranslated regions (UTRs). We have analyzed the expression of the nonneuronal promoter of the rat AADC gene in the kidney epithelial cell line LLC-PK1 and in cells which do not express the nonneuronal form of AADC by transient transfection. These studies revealed that the first 1.1 kilobases of the nonneuronal promoter, including the nonneuronal-specific 5′-UTR (Exon 1), contains sufficient information to direct tissue-specific expression. Serial deletions of this promoter localized the cis-active element to a region between −52 and −28 base pairs upstream of the nonneuronal transcription start site. An A/T-rich sequence, within this region which we have termed KL-1, was found to bind a kidney and liver-specific factor by DNase footprint analysis and was capable of directing tissue-specific expression from a heterologous promoter. Moreover, when the KL-1 sequence was mutated in the context of the entire promoter sequence, all transcriptional activity was abolished. DNA sequence comparison revealed that the KL-1 fragment is highly homologous to the binding site for hepatocyte nuclear factor-1 (HNF-1). Mobility shift studies utilizing an antibody to HNF-1 demonstrated binding of HNF-1 to the KL-1 fragment and cotransfection of HNF-1 cDNA into cells which do not express the nonneuronal form of AADC resulted in activation of transfected AADC nonneuronal promoter constructs. These results strongly suggest that the transcription factor which regulates the tissue-specific expression of the nonneuronal form of AADC mRNA is HNF-1.

Aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) catalyzes the decarboxylation of L-dopa to dopamine and 5-hydroxytryptophan to serotonin, as well as the decarboxylation of the aromatic amino acids tyrosine, tryptophan, and phenylalanine to the corresponding amines (1, 2). AADC is expressed in neuronal cells, where it participates in the synthesis of neurotransmitters, and in nonneuronal cells, including liver, kidney, lung, spleen, and endothelial cells, where its function is less clearly understood (3–6).

Rat (7), human (8), and as we show in this report, porcine AADC mRNA exist in two different forms. While the coding region is identical in both forms, liver and kidney AADC mRNA contains a short 5′-untranslated region (UTR) which is entirely unrelated to the 5′-UTR found in AADC mRNA expressed in tissues of neuronal origin. We have previously shown that, within the rat AADC gene, dual promoters direct the expression of these tissue-specific forms of AADC mRNA resulting in the alternative use of two untranslated exons (9). This has also been shown by others for the rat (10) and human (11–13) AADC gene. In liver and kidney, transcription initiates at the upstream, nonneuronal promoter, which results in mRNA with exon 1 as its untranslated sequence. In tissues of neuronal origin, such as brain and adrenal medulla, transcription initiates at the downstream, neuronal promoter, incorporating exon 2 as the 5′-UTR.

To investigate the mechanisms which control differential expression from these two promoters, we have initiated analyses of transcriptional regulation. We have previously shown that a region of the neuronal AADC promoter, containing 2.4 kilobases (kb) upstream of the transcription start site and including the untranslated exon 2, was functional in all cell lines tested, including those which do not express AADC endogenously (14). These studies identified several cis-active elements within the neuronal promoter which controlled the activity of this promoter, but which appeared to be binding sites for ubiquitously expressed transcription factors. The 5′-UTR itself was also found to be required for optimal expression from the neuronal promoter. Since these elements did not appear to contribute to the tissue-specific regulation of the neuronal promoter, additional regulatory mechanisms must restrict this activity to appropriate tissues. To search for additional sources of tissue-specific regulation, we have extended our transcriptional analyses to the nonneuronal promoter of the rat AADC gene.

The nonneuronal form of the AADC message has been shown to be expressed in cells of the liver and kidney (7, 9, 10, 11, 15). Although little is known about the function of AADC in the liver, a large body of evidence has accumulated which suggests that the function of AADC in the kidney is to produce dopamine from circulating L-dopa (6, 16–20). Dopamine plays an important role in the regulation of renal electrolyte and water balance (21). AADC has been localized to the proximal convoluted and proximal straight tubules in the kidney and appears to be responsible for the synthesis of the majority of the dopamine excreted by the kidney (4, 17–19, 22). Because of the noradrenergic innervation of the kidney, it has been difficult to demonstrate that dopamine is produced endogenously in the kidney.
However, the isolation of the renal epithelial cell line LLC-PK₁, which is devoid of neural input and tyrosine hydroxylase activity, and does not metabolize dopamine, yet expresses high levels of AADC, has provided a model system which has been used to study renal physiology and the regulation of dopamine synthesis and release (16, 23).

We have exploited the LLC-PK₁ cell line, which expresses the nonneuronal form of AADC mRNA, to analyze transcriptional activation of the nonneuronal promoter of the AADC gene. In contrast to our findings with the neuronal promoter, we demonstrate that the first 1.1 kb of the nonneuronal promoter, including the nonneuronal-specific 5′-UTR, is capable of directing tissue-specific expression. Transfection experiments have localized the tissue-specific enhancer element to an A/T-rich sequence, which we have termed KL-1, located between −49 and −35 bp upstream of the transcription start site, which appears to be essential for the tissue-specific expression of the AADC nonneuronal promoter.

HEPATOCELLULAR NUCLEAR FACTOR-1 (HNF-1; also known as LFB-1) (24–27) is a homeodomain transcription factor, which regulates the transcription of genes expressed predominantly in the liver, kidney, stomach, and intestine (28–32). Sequence analysis revealed a near perfect homology between the tissue-specific KL-1 element and the HNF-1 consensus binding sequence (25, 27, 30, 31, 33–35). In this study, we demonstrate that the KL-1 element is a binding site for HNF-1 and suggest that HNF-1 is the transcription factor primarily responsible for the tissue-specific expression of the nonneuronal promoter of the AADC gene.

MATERIALS AND METHODS

All restriction enzymes, media, and cell culture reagents were purchased from Life Technologies, Inc. (use of trade names does not imply endorsement).

Cell Culture—All sera was heated at 56°C for 1 h to inactivate the complement. All cells were grown in 225-cm² Corning flasks and maintained in a humidified incubator at 37°C in 5% CO₂. LLC-PK₁, cells were grown in DMEM high glucose supplemented with 10% fetal bovine serum. Approximately 50% of the medium was replaced three times a week, and the cells were removed from the flask by trypsin treatment and subcultured every 7 days at a dilution of 1:3. PCT-12 cells (36) were grown in RPMI 1640, supplemented with 10% donor horse serum and 1% antibiotic/antimycotic. Approximately 50% of the medium was replaced every 2 days, and the cells were removed from the flask by trypsin treatment and subcultured twice a week at a dilution of 1:5. All sera was heated at 56°C for 1 h to inactivate the complement.

Construction of Transfection Plasmids—All constructs made for transfection analysis were cloned into the Promega GeneLight pGL2 basic vector previously digested with the appropriate restriction enzymes. To create the 1.1-kb luciferase construct containing the 5′-UTR, which created an XhoI site at −28 bp, was used. The PCR products were digested with XbaI, located within the luciferase gene and the appropriate 5′-end nucleotide, and cloned into the pGL2 basic vector digested with the appropriate restriction enzymes. To create the −1.1-kb luciferase constructs, an XhoI–NsiI fragment, spanning the region from −1111 to −4 for the subcloned HindII genomic fragment was cloned into the −28-bp luciferase constructs, previously digested with XhoI and NsiI. All plasmid constructs were verified by DNA sequencing using the Applied Biosystems 373A automated DNA sequencer.

The KL-1 mut and U1 mut plasmids were constructed by using PCR primers to create a Sall site or PstI site, respectively, within the KL-1 or U1 promoter region. The KL-1 mut luciferase construct, the KL-1 mut antisense primer: 5′-AAATGTCGACTTCACCAGAAAACAAGGTTT-GATCTGCAAACTACAGCTTGG-3′, which created an XhoI site at −28 bp, was used. The PCR products were digested with XbaI, located within the luciferase gene and the appropriate 5′-end nucleotide, and cloned into the pGL2 basic vector digested with the appropriate restriction enzymes. To create the −1.1-kb luciferase constructs, an XhoI–NsiI fragment, spanning the region from −1111 to −4 for the subcloned HindII genomic fragment was cloned into the −28-bp luciferase constructs, previously digested with XhoI and NsiI. All plasmid constructs were verified by DNA sequencing using the Applied Biosystems 373A automated DNA sequencer.

To construct the transfection plasmids KL-1/dopamine β-hydroxylase (DBH) and KL-1 opposite orientation/DBH, sense and antisense oligonucleotides were synthesized which included a SacII site at 257 bp, was used. To construct the 1.1-kb luciferase construct containing the 5′-UTR as a template (mutated bases are underlined). The KL-1 mut sense PCR product was digested with XbaI and XhoI, the KL-1 mut antisense PCR product was digested with XbaI and XhoI. The two fragments were ligated into the pGL2 basic vector previously digested with XhoI and XbaI. Similarly, for the U1 mut, the PCR primer U1 mut sense primer, 5′-GATCTGAAATATGCTGCTGAGCCCCACCATGC-3′, and the pGL1 primer 5′-GTATCTTTATGTTACTGTACTGAGCTAAATAATACCACTGAG-3′, which combines the DBH luciferase (DBH) and KL-1 opposite orientation/DBH, sense and antisense oligonucleotides sense, 5′-CCAGATCTTAAATATATTTTTAACCAGGATCCTGAGCC⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻{-
transfected with 2 μg of a plasmid containing the human cytomegalovirus (CMV) promoter driving the β-galactosidase gene (CMV-β-gal, Clonetech) to control for transfection efficiency. The reporter plasmid containing the CMV promoter fused to the firefly luciferase gene, (CMV-luciferase, created by replacing the β-gal sequences of CMV-β-gal with the luciferase gene), and the Promega vector pGL2-Promoter containing firefly luciferase fused to the AADC cDNA at the 3′ end of each reporter plasmid were prepared as positive controls. The promoterless pGL2 basic vector was used as a negative control. Cells were grown in the appropriate media for two days after subculturing onto 6-well tissue culture plates to a density of 1 × 10^4 cells/well. Cells were assayed for luciferase activity using the Promega luciferase assay kit and the analytical luminometer Microlight Luminometer (Boehringer Mannheim). PCR products were then purified by elution from a 20% denaturing polyacrylamide gel, dissolved in 10 mM sodium phosphate, pH 7.3, 2 mM MgSO_4_, and 40 μM β-mercaptoethanol. The solution was incubated at 37°C in the dark, and activity was determined spectrophotometrically in the Molecular Devices Tech Max plate reader at 560 nm. The ratio of luciferase activity to β-galactosidase activity was determined, and the average of duplicate readings was expressed as fold expression over background (activity of the promoterless pGL2 basic vector).

RNA Isolation—Total RNA was isolated using the RNeasy method (Qiagen) and was prepared as described above.

RT-PCR Analysis—Total RNA (10 μg) was reverse-transcribed with T7 or T3 polymerase (respectively) in the presence of [γ-32P]ATP (spec activity 6,000 Ci/mmol, Amersham Pharmacia Biotech), and poly(A) selection reaction products were then cloned into the PCR-Script vector (Stratagene) to control for transfection efficiency. The reporter plasmid was used as a negative control. Cells were grown in the appropriate media for two days after subculturing onto 6-well tissue culture plates to a density of 1 × 10^4 cells/well. Cells were assayed for luciferase activity using the Promega luciferase assay kit and the analytical luminometer Microlight Luminometer (Boehringer Mannheim). PCR products were then purified by elution from a 20% denaturing polyacrylamide gel, dissolved in 10 mM sodium phosphate, pH 7.3, 2 mM MgSO_4_, and 40 μM β-mercaptoethanol. The solution was incubated at 37°C in the dark, and activity was determined spectrophotometrically in the Molecular Devices Tech Max plate reader at 560 nm. The ratio of luciferase activity to β-galactosidase activity was determined, and the average of duplicate readings was expressed as fold expression over background (activity of the promoterless pGL2 basic vector).

DNA I Footprint Analysis—Nuclear extracts were prepared from LLC-PK_1, PC12, CA77, and NRK cells as described (40). Nuclei were isolated from rat liver and kidney as described in Current Protocols in Molecular Biology (41). Extracts were then prepared from these nuclei as described above for cell nuclear extracts. Footprinting probes were prepared by PCR using the –510-bp luciferase construct with the 3′-UTR as a template. The 5′ primer was pGL1. The sequence of the 3′ primer, located at the start of the luciferase gene, was 5′-GGCCTCT-TCCATTTTACCAAGTACC-3′. To synthesize probes which labeled the sense strand, the 5′ primer was radioactively labeled with T4 polynucleotide kinase and [γ-32P]ATP. To label the antisense strand, the 3′ primer was radioactively labeled in the same way. PCR reactions were performed as described above, using 100 pmol each of the labeled and unlabeled primers. Probes were then purified by elution from polyacrylamide gels. Footprinting reactions were performed as described above for cell nuclear extracts. A 100-fold molar excess of cold oligonucleotide was added to the labeled probe prior to incubation with the extract mixture. For antibody experiments, 2 μl of HNF-1α antibody (generous gift of Dr. Gerald Crabtree) was combined with labeled probe prior to incubation with the extract mixture.

Gel Shift Analysis—Nuclear extracts were prepared as described above. Oligonucleotides of sequences of gel shift probes were: rat β-fibrinogen, 5′-GATCTGCAATTAATACCTAGGG-3′ (30); KL-1, 5′-AAAT-TAATGTTTTAACCAAGACA-3′; KL-1M, 5′-AAATTCGGGGTTGACGAGCAGAGGCA-5′; KL-1M, 5′-GACATCCTGGACCTTACATGACTAATATTAACTAAAGGGG-3′; U1, 5′-GACTTCTGGACCTTACATGACTAATATTAACTAAAGGGG-3′; U1M, 5′-GACTTCTGGACCTTACATGACTAATATTAACTAAAGGGG-3′. Mutated bases are underlined. All oligonucleotides were purified on a 20% denaturing polyacrylamide gel, eluted overnight in 0.5 mM ammonium acetate, 10 mM magnesium acetate, and 0.1% SDS, and precipitated with ethanol. Both sense and antisense oligonucleotides were radioactively end-labeled with [γ-32P]ATP and then annealed by heating to 70°C followed by slow cooling to room temperature. 0.01 nmol of labeled probe (10,000–30,000 cpm) was combined with 400 ng of nuclear extract from LLC-PK_1, PC12, CA77, and NRK cells. Extracts were then prepared from these nuclei as described above for cell nuclear extracts. Footprinting reactions were performed as described above for cell nuclear extracts. A 100-fold molar excess of cold oligonucleotide was combined with the labeled probe prior to incubation with the extract mixture. For antibody experiments, 2 μl of HNF-1α antibody (generous gift of Dr. Gerald Crabtree) was combined with labeled probe prior to incubation with the extract mixture.

RESULTS

LNC-PK_1 Cells Express the Nonneuronal Form of AADC mRNA—The porcine kidney epithelial cell line, LLC-PK_1, like the kidney from which it is derived, exhibits robust endogenous AADC activity and was therefore used to study the expression of the nonneuronal AADC promoter. RT-PCR analyses were performed to establish the presence of the nonneuronal form of the AADC mRNA in LLC-PK_1 cells. RT-PCR was performed on poly(A)−-selected RNA isolated from LLC-PK_1 cells and porcine adrenal glands. A primer specific for the common coding region was used in conjunction with primers specific for either the neuronal- or nonneuronal-specific 5′-UTR. The resulting PCR fragments were separated on a 2% agarose gel and are shown in Fig. 1. The 264-bp fragment specific for the nonneuronal form of porcine AADC cDNA was observed only in LLC-PK_1 cDNA (Fig. 1B). Conversely, the 255-bp fragment specific for the neuronal form of the porcine AADC cDNA was observed only in porcine adrenal cDNA (Fig. 1A). No fragment is observed when either RNA is used as a negative control. These results demonstrate that LLC-PK_1 cells express only the nonneuronal form of AADC message.

The AADC Nonneuronal Promoter Directs Tissue-specific Expression of the Luciferase Reporter Gene—We have previously identified the location of the nonneuronal transcription start

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site of the rat AADC gene (9). To identify cis-acting elements in the nonneuronal AADC promoter, 1111 bp of the region 5’ of this transcription start site were placed upstream of the firefly luciferase reporter gene in the pGL2 basic vector expression (Fig. 2A). Two constructs were made which either included or excluded the nonneuronal 5’-UTR (Exon 1) to assess the role of the 5’-UTR in regulation of expression. These constructs were introduced into a variety of cell lines by transient transfection to determine whether sequences within the nonneuronal promoter could direct expression of the luciferase gene in a tissue-specific manner. Transfections were performed in PC12 cells, a rat adrenomedullary tumor cell line which expresses the neuronal form of AADC mRNA, CA77 cells, a rat thyroid C cell line which also expresses the neuronal form of AADC mRNA, NRK cells, a rat kidney epithelial cell line which does not express AADC (14), and in LLC-PK1 cells. These experiments (Fig. 2B) showed expression from the nonneuronal promoter exclusively in LLC-PK1 cells. In the absence of the 5’-UTR, 1.1 kb of the nonneuronal promoter directed expression of luciferase to levels which were 5-fold greater than the expression of the vector alone. Inclusion of the 5’-UTR resulted in a 10-fold increase in expression levels. No expression was observed in PC12, CA77, or NRK cells in the presence or absence of the 5’-UTR. Thus, the first 1111 bp of the nonneuronal promoter contain sufficient information to direct tissue-specific expression of a reporter gene.

To delineate regions within this promoter which were responsible for this tissue-specific expression, a series of deletion constructs were made (Fig. 3A). For each deletion, two constructs were made which either included or excluded the 5’-UTR. When these constructs were introduced into LLC-PK1 cells, as shown in Fig. 3B, a similar pattern of expression was observed when the 5’-UTR was included (+5’-UTR) or excluded (−5’-UTR). However, expression levels were 3-10-fold greater in the presence of the 5’-UTR. While the higher levels of expression observed with the −510-bp construct suggest the presence of both positive and negative elements within this 1.1-kb region, the data show that removal of all sequences upstream of −70 bp results in levels of expression similar to those observed with the entire 1.1-kb promoter sequence. Deletion of sequences between −70 and −28 bp abolished this expression, suggesting that sequences between −70 and −28 bp contain a positive cis-active element. Introduction of these deletion constructs into CA77, PC12, or NRK cells resulted in no appreciable levels of expression (data not shown).

Fig. 3C shows the use of the correct transcription start site for transfected plasmids. RNase protection analyses of total RNA isolated from LLC-PK1 cells transfected with the −70-bp luciferase constructs with or without the 5’-UTR, show the appropriate protected fragments of 101 and 68 nucleotides, respectively.

A Binding Site for a Kidney- and Liver-specific Factor Is Identified within the AADC Nonneuronal Promoter—DNase I footprint analyses were performed to identify potential transcription factor binding sites within the nonneuronal promoter. A DNA fragment containing the first 510 bp of promoter sequence was labeled on the antisense strand and incubated with nuclear extracts prepared from rat liver and kidney, and from PC12, CA77, NRK, and LLC-PK1 cells. As shown in Fig. 4A, three protected regions were observed between −105 and −135 bp, between −55 and −75 bp, and between −24 and −49 bp. Footprints were observed in the same regions when the DNA
was labeled on the sense strand (data not shown). No footprints were observed in the region of the 5'-UTR. Although two protected regions were observed in all extracts, the protected region between -24 and -49 bp was observed only in extracts from liver, kidney, and LLC-PK1 cells.

Fig. 4B shows a comparison of sequences around the transcription start sites of the rat and human AADC nonneuronal promoters. The greatest degree of homology is observed within the first 80 bp upstream of the transcription start site of the rat promoter. This region includes both the non-tissue-specific footprint (-75 and -55 bp), which we have termed U1, and the kidney/liver-specific binding site (-49 to -25 bp). Within the kidney/liver-specific binding site is an A/T-rich sequence, AATTAATGTTTAAC, which we have termed KL-1, which is 100% homologous to the human AADC sequence. Interestingly, little if any homology is observed within the 5'-UTR itself.

KL-1 Controls Tissue-specific Expression—To assess the roles of the KL-1 and the U1 regions in the tissue-specific expression from the AADC nonneuronal promoter, an additional deletion construct, -52 bp including the 5'-UTR, was designed which includes only the KL-1 element. Constructs were also made which mutated the KL-1 or U1 regions within the context of the -1.1-kb nonneuronal promoter construct (Fig. 5A). Introduction of the -52-bp construct into LLC-PK1 cells resulted in levels of expression which were similar to those obtained with the -1.1-kb construct, although less than levels obtained with the -70-bp construct. Mutation of the U1 fragment within the context of the -1.1-kb construct (U1 mut) also resulted in slightly reduced levels of expression. However, introduction of the mutated KL-1 construct (KL-L mut) into LLC-PK1 cells resulted in a total loss of expression of the luciferase reporter gene. Transfection of all of these constructs into CA77 cells yielded no appreciable expression. These data suggest that while the U1 region may play a minor role in regulating promoter expression levels, it is the KL-1 element which is essential for the tissue-specific expression of the AADC nonneuronal promoter.

To further test whether the KL-1 or the U1 sequence could act as a tissue-specific enhancer element, DNA fragments containing these elements were placed upstream of the first 29 bp of the DBH promoter (-29 DBH), in either normal or opposite orientation (Fig. 5B). The -29 DBH construct has previously been shown to direct no appreciable levels of luciferase expression in all cells lines tested (38). Plasmids containing mutated versions of these fragments were constructed in the same way. These KL-1/DBH and U1/DBH constructs were then assayed for their ability to direct tissue-specific expression of the luciferase reporter gene. Introduction of KL-1/DBH constructs into LLC-PK1 cells resulted in luciferase expression levels which were 20–30-fold greater than -29 DBH expression levels regardless of orientation. However, mutation of the KL-1 element totally abolished expression from this reporter gene. On the contrary, the normal U1/DBH constructs directed barely detectable expression of the luciferase reporter gene in LLC-PK1 cells, regardless of orientation, which was abolished by the mutation. Introduction of these constructs into CA77 cells resulted in no detectable levels of luciferase expression above background. These results demonstrate that KL-1 acts as a tissue-specific enhancer by directing tissue-specific expression from a heterologous promoter regardless of orientation. Fur-
thermore, since the U1 fragment has little effect on the activity of a heterologous promoter and a minimal effect on the activity of its own promoter in the absence of functional KL-1, these data confirm that KL-1 is solely responsible for controlling the cell-specific expression of the AADC nonneuronal promoter.

HNF-1 Binds the KL-1 Element in the AADC Nonneuronal Promoter—Comparison of the KL-1 sequence to binding sites of identified transcription factors revealed a near perfect homology to the consensus binding site for the hepatocyte transcription factor HNF-1 (Fig. 6A). To verify that the KL-1 element is an HNF-1 binding site, mobility shift studies were performed, using the KL-1 element, an HNF-1 binding site previously identified in the β-fibrinogen gene (30), and a mutated KL-1 element, which was altered at sites which have previously been shown to abolish HNF-1 binding (42). The mutated KL-1 element is the same element used in earlier transfection studies (see Fig. 5). These sequences are shown in Fig. 6A. As shown in Fig. 6B, a similar sized complex is formed with both the KL-1 and β-fibrinogen probes using nuclear extracts from LLC-PK1 cell and rat liver. No complexes are formed with either probe using nuclear extracts from CA77 cells. Binding to the KL-1 probe is competed by a 100-fold molar excess of the cold KL-1 element or the β-fibrinogen element, but not by the mutated KL-1 element. Similarly, a 100-fold molar excess of the KL-1 fragment, but not the mutated KL-1 fragment, can compete for binding to the β-fibrinogen probe. Fig. 6C shows that addition of an antibody to HNF-1α causes a supershift of the complex formed with LLC-PK1, rat liver and kidney nuclear extracts on the KL-1 element, which is identical to the supershift obtained with the β-fibrinogen probe. This antibody has been extensively characterized (24, 29, 42) and shown to be specific for HNF-1α. The increased mobility of complexes bound in liver and kidney extracts is due to protein degradation as previously reported (30). These binding experiments strongly suggest that HNF-1 is the factor which binds to the KL-1 element in liver and kidney cells.

Mobility shift studies were also performed to analyze binding to the U1 region. Fig. 6D shows that the U1 element binds poorly to a factor which is present in nuclear extracts from LLC-PK1, CA77, and PC12 cells, which is consistent with our earlier footprinting data. This binding is inhibited in the presence of 100-fold molar excess of the U1 element, but is not competed by equal amounts of a mutated U1 sequence, demonstrating that this binding is specific. The weak binding is consistent with the low levels of activity observed in transfection experiments. The sequence of the elements used in these experiments are shown in Fig. 6A. The mutated U1 sequence used in these experiments is the same sequence assayed in earlier transfection experiments.

Cotransfection of HNF-1 cDNA into CA77 Cells Activates AADC Nonneuronal Promoter Constructs—To demonstrate that HNF-1 binding activates the KL-1 fragment to control
tissue-specific expression, KL-1 was tested for its ability to direct transcription in the presence of HNF-1 in a cell line which does not normally express the nonneuronal form of AADC or HNF-1. Cotransfection experiments were performed by introducing HNF-1 α cDNA (24) along with AADC nonneuronal promoter constructs into the CA77 cell line. Fig. 7A shows that cotransfection of HNF-1 α cDNA results in expression of the −1.1-kb, −70-bp, and −52-bp AADC nonneuronal promoter constructs to levels in CA77 cells that were comparable to those levels observed in LLC-PK1 cells in the absence of cotransfected HNF-1 (see Fig. 5A). The mutated KL-1 construct exhibited no appreciable expression over background, while the U1 mutant displayed normal expression levels. Cotransfection of HNF-1 α cDNA with constructs containing the KL-1 fragment directing expression of the DBH promoter yielded high levels of expression while no appreciable levels of expression were detected from the mutated KL-1 (Fig. 7B). These analyses establish that HNF-1 can fully activate the KL-1 element both in its normal context and when associated with a heterologous promoter to direct high levels of tissue-specific expression from luciferase reporter gene in cells which do not normally express the nonneuronal promoter of AADC.

**DISCUSSION**

Transcription from alternative promoters of the AADC gene, followed by alternative splicing of untranslated exons, leads to the expression of AADC mRNAs with distinct 5′-UTRs in neuronal and nonneuronal cells. While the neuronal promoter directs expression to catecholamine and serotonin producing neurons in the central and peripheral nervous system and the adrenal medulla, the nonneuronal promoter directs high levels of expression in the liver and kidney, as well as lower levels in lung, spleen, and intestine. In this study we demonstrate that the tissue-specific expression of this promoter is directed by the hepatocyte transcription factor, HNF-1.

We have shown that 1.1 kb of the upstream, nonneuronal promoter of the rat AADC gene contains cis-active elements which can direct expression of a reporter gene only in a cell line which expresses the nonneuronal form of AADC mRNA. Transfection experiments and DNase footprint analysis identified an A/T-rich sequence, AATTAATGTTTAAC, which we have termed KL-1, as a binding site for a protein found only in liver and kidney cell nuclear extracts. Mutational analysis further demonstrated that this region is essential for nonneuronal
promoter activity, and a DNA fragment containing this element is capable of directing tissue-specific expression of a reporter gene from the heterologous DBH promoter, in an orientation-independent manner. The A/T-rich KL-1 element contains sequences similar to the TATA box sequence and shares some similarity with the A/T-rich binding sites of homeodomain proteins (43–45). A comparison of this region to binding sites for known transcription factors revealed a near perfect sequence homology to the HNF-1 consensus binding sequence (25, 27, 30, 31, 33–35). We present several lines of evidence to demonstrate that HNF-1 is the factor which binds to this sequence and is responsible for the tissue-specific activation of the nonneuronal AADC promoter. First, mobility shift experiments demonstrated that a complex of similar size binds to both the KL-1 sequence and the HNF-1 binding site located within the β-fibrinogen gene, and that both fragments can compete for binding to a factor which is present only in nuclear extracts of liver and kidney cells. Second, we show that this complex is recognized by an antibody to HNF-1α. Finally, we show that cotransfection of HNF-1α cDNA into CA77 cells, which do not express the nonneuronal AADC promoter, leads to activation of the nonneuronal promoter, and that cotransfected HNF-1α can activate transcription from a construct containing the KL-1 element placed in front of the DBH promoter.

The transcription factor HNF-1 was first identified as a regulator of several liver-specific genes, including albumin, α- and β-fibrinogen, α1-antitrypsin, α-fetoprotein, pyruvate kinase, transthyretin, and aldolase B (see Blumenfeld et al. (46) and references therein). Although originally thought to be liver-specific, it was later found to be expressed in kidney, stomach, intestine, spleen, and colon (28–32, 34, 46, 47). Thus
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the expression pattern of HNF-1 is highly consistent with that of the nonneuronal form of AADC. HNF-1 contains a divergent homeodomain and sequences homologous to the A box of the POU domain (27). Consistent with it being a homeodomain protein, there is also evidence that HNF-1 plays an important role in the differentiation of the hepatocyte phenotype (48), as well as a role early in embryogenesis (46, 47).

A comparison of the rat and human nonneuronal promoter sequences encompassing this region reveals a high degree of homology within the region corresponding to −85 to −30 bp of the rat promoter. Moreover, the KL-1 sequence is 100% homologous to the corresponding human AADC sequence (see Fig. 5A). Because the human transcription start site has been proposed to be located 60 bp upstream from the corresponding rat transcription start site (11), the human KL-1 element appears to be located within the first exon. It is possible that this cis-active element is located 3′ of the transcription start site in the human gene. However, the high percentage of homology exhibited between the human and rat promoters in this region, along with the transfection data presented here, suggests that it is more likely that the human transcription start site is located further downstream than previously reported.

A binding site for an apparently ubiquitously expressed protein (U1) was also identified in the AADC nonneuronal promoter, in a second region which shares a high degree of homology with the human promoter, suggesting that this region may also play an important role in the regulation of the nonneuronal promoter. Mutation of this region within the context of the entire 1.1-kb promoter led to a moderate decrease in activity, while placement of this element in front of a heterologous promoter resulted in very minimal expression only in LLC-PK1 cells. Since this element is capable of binding a protein present in all cells, and is not necessary for high level expression in LLC-PK1 cells, it does not appear to play a role in the tissue-specific expression of AADC.

The demonstration of tissue-specific regulation of the nonneuronal AADC promoter is in marked contrast to our previous analyses of the neuronal promoter of the AADC gene (14). In similar transfection experiments, 2.4 kb of the neuronal AADC promoter was found to direct expression of a reporter gene in both AADC expressing and nonexpressing cells. Taken together, these data suggest that, while tissue-specific expression of the nonneuronal promoter is regulated by binding of HNF-1 to the KL-1 element, the neuronal promoter either requires elements not included in the first 2.4 kb of 5′-flanking sequence, or some other aspect of its context within the AADC gene, to direct appropriate cell-specific expression. These observations are further supported by a recent report by Sumi-Ichinose et al. (49), in which transgenic studies of the human AADC gene demonstrated a requirement for both promoters to obtain appropriate expression patterns. Specifically, transgenes containing the neuronal promoter alone were expressed in all tissues examined. Conversely, transgenes containing the nonneuronal promoter or both promoters exhibited correct expression patterns, although higher expression levels were observed with transgenes containing both promoters.

There are numerous examples of multiple promoter systems (50). For the systems whose transcriptional regulation has been studied in great detail, several mechanisms have been identified by which expression from a proximal promoter can be inhibited by a distal one (51–53). Although this type of promoter regulation has been demonstrated for promoters separated by short distances, similar mechanisms have been proposed for alternative promoters located as far as 4 kb apart.

Fig. 7. Cotransfection of HNF-1 cDNA into CA77 cells activates AADC nonneuronal promoter constructs. Results of cotransfection experiments of HNF-1α cDNA with AADC nonneuronal promoter constructs. Five μg of each plasmid construct, co-transfected with 2 μg of the reporter plasmid CMV-β-gal, were introduced by the lipofectamine (Life Technologies, Inc.) transfection method into CA77 cells. All transfections were performed in duplicate. Values represent the ratio of luciferase activity to β-galactosidase activity, expressed as fold induction over background, which is the activity of the promoterless pGL2 basic vector. Results shown represent the average of three independent experiments. Error is expressed as S.E. A, analysis of −1.1-kb, −70-bp, and −52-bp promoter constructs containing the 5′-UTR and mutations of the KL-1 and U1 elements in the context of the native promoter. KL-1 mut, −1.1-kb nonneuronal construct with KL-1 mutation; U1 mut, −1.1-kb nonneuronal construct with U1 mutation. Blackened area approximates location of mutated region. B, analysis of plasmids containing natural and mutated versions of the KL-1 element, upstream of the heterologous DBH promoter. , natural sequence; , mutated sequence; , DBH promoter; , orientation.
(54). Our present data cannot rule out the possibility that inhibitors or silencers of neuronal promoter expression lie elsewhere in the gene. However, the demonstration of tissue-specific regulation of the upstream nonneuronal AADC promoter by HNF-1 suggests that transcriptional interference, or a similar mechanism, may control tissue-specific expression from the downstream neuronal AADC promoter.

We have previously shown that inclusion of the neuronal 5'-UTR (Exon 2) in transfection constructs results in increased levels of expression from the neuronal AADC promoter (14). Here we show a similar effect of the nonneuronal 5'-UTR (Exon 1). Although in the absence of the 5'-UTR expression from the nonneuronal promoter is 5-50-fold over background, the presence of the 5'-UTR increases this expression 3-10-fold. We do not believe that this effect is due to the presence of additional sequences around the transcription start site, since the constructs which did not include the 5'-UTR did contain 25 bp of sequence 3' of the transcription start site. As was the case for the neuronal 5'-UTR, our experiments cannot distinguish between a transcriptional or a posttranscriptional function for this untranslated exon. This region could represent a binding site for a cis-active transcription factor, although we did not detect binding to this region in our DNase I footprint assays, nor did we find any homology to the human nonneuronal 5'-UTR. Alternatively, this region could function at the level of the mRNA to stabilize the message or to increase translational efficiency.

The different expression patterns exhibited by the two AADC promoters raises questions regarding the function of the alternative promoters of the AADC gene. The presence of multiple promoters in genes generally allows greater flexibility in the regulation of expression, providing a mechanism for differential tissue-specific expression, developmental and hormonal regulation, or differential regulation of expression levels. Since both forms of AADC mRNA differ only in a short 5'-UTR, we can presume that differential regulation of AADC may occur at the level of the mRNA or at the level of transcription. While our data does not provide any evidence for differences in the function of the two 5'-UTRs at the mRNA level, we have identified major differences in the control of transcriptional activation of the two promoters. The presence of two differentially regulated promoters may therefore provide a mechanism for differential regulation of AADC mRNA in brain versus liver and kidney where the products of AADC activity may perform different functions. Although it has long been assumed that AADC was not a regulated enzyme, recent reports have shown that AADC is modulated at both the enzyme and mRNA levels. AADC mRNA levels are regulated in response to various agents including reserpine (55), dexamethasone (56), dopamine receptor antagonists (57), interleukin 1β and prostaglandin E2 (58), and AADC (59) and monoamine oxidase B inhibitors (60). These experiments have measured AADC mRNA levels in various brain regions, adrenal medulla, or in PC12 cells. It is not known how these agents can cause similar increases in AADC mRNA expression in liver or kidney.

Until recently, little has been known about the function of AADC in nonneuronal tissues such as liver and kidney. The recent recognition of the importance of dopamine as a renal hormone suggests that AADC may play a role in the regulation of kidney function. In the kidney, dopamine appears to regulate renal salt and water balance by modulating both Na⁺,K⁺-ATPase activity and the Na⁺/H⁺ exchanger via DA₁ and DA₂ dopamine receptors (reviewed in Lee (18)). Specifically, it has been shown that AADC activity in the kidney can be modulated in response to sodium intake (61, 62). The association of altered renal dopamine levels with several diseases including hyper-tension, diabetes and congestive heart failure (see Lee (18) and references therein) also suggests that modulation of nonneuronal AADC enzyme or mRNA levels may play a role in maintaining proper kidney function. In fact, increases in AADC activity in the kidney have been observed in experimental hypertension (63). Further analysis of AADC expression in the kidney is required to determine whether any of these effects on enzyme activity are due to alterations in levels of AADC mRNA. The ability to study this regulation in the renal epithelial cell line LLC-PK₁, which has been shown to be a valuable model for renal cellular physiology, will contribute to the understanding of renal dopamine production and function.

Identification of HNF-1 as a transcription factor essential for directing tissue-specific expression of the nonneuronal AADC promoter is an important initial step toward the understanding of the function and regulation of the AADC gene in nonneuronal cells. Because HNF-1 is a major regulator of the expression of liver-specific enzymes, its role in the regulation of the AADC nonneuronal promoter further suggests that AADC also performs important functions in the liver.

Acknowledgments—We thank Drs. Sidney Udenfriend and Paul Liebman for helpful comments on the manuscript. We are also grateful to Dr. Andrew Russo for providing CA77 cells and Dr. Sidney Udenfriend for providing LLC-PK₁ cells. We also appreciate Dr. Gerald Crabtree for generously providing the HNF-1a antibody and cDNA. Finally, we thank Mavis Lee, Andrea Gault, and Robert Wurzburger for technical assistance.

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J. Biol. Chem. 1996, 271:4528-4538.
doi: 10.1074/jbc.271.8.4528

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