Localization of Preangiotensinogen Messenger RNA Sequences in the Rat Brain

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SUMMARY Angiotensinogen (renin substrate) and its messenger RNA are known to accumulate in the rat brain. We have cloned rat preangiotensinogen cDNAs and used them as probes to measure the accumulation of preangiotensinogen messenger RNA sequences in eight regions of rat brain, as well as in liver and kidney. The brain regions examined were the cerebral cortex, hippocampus, striatum, cerebellum, diencephalon (including basal forebrain structures), midbrain, brainstem, and pituitary. On a tissue weight basis, the accumulation of preangiotensinogen RNA sequences was greatest in the liver, midbrain, and brainstem. The relative concentrations of messenger RNA were ranked as follows: liver, brainstem, midbrain > cerebellum, diencephalon > hippocampus > cortex, striatum, kidney > pituitary. Relative RNA concentrations from liver to kidney varied over a 16-fold range. Liver and brain preangiotensinogen RNA sequences were indistinguishable in size as measured by gel electrophoresis; however, the kidney sequences appeared some 100 nucleotides larger. Our data agree with previous measurements of angiotensinogen in the rat brain as assayed by renin-catalyzed angiotensin I release. (Hypertension 8: 540-543, 1986)

KEY WORDS • complementary DNA • renin substrate • hybridization • dot blot

THERE are several lines of evidence suggesting the existence of angiotensinogen messenger (m) RNA in the central nervous system. Most germane to the present study are three reports describing the identification of angiotensinogen mRNA in RNA extracted from whole rat brains. Campbell et al.1 assayed functional angiotensinogen mRNA extracted from brain and liver by programming a cell-free translation system with these RNAs and recovering newly synthesized angiotensinogen by immunoprecipitation with monospecific antisera. Two forms were recovered (M, 52.5 and 55.7 kDa), both of which were shown to be renin substrates. Nakanishi’s group2,3 used a rat preangiotensinogen complementary (c) DNA to detect the homologous RNA sequence in several rat tissues including brain and liver. The RNA sequences from brain and liver were indistinguishable in size (1900 nucleotides) as measured by denaturing gel electrophoresis. Although this evidence clearly establishes that angiotensinogen is synthesized somewhere in the rat brain, the use of whole brain extracts precluded determination of the exact site of synthesis.

To better understand the possible roles of angiotensins in brain physiology, we used a preangiotensinogen cDNA probe to measure the accumulation of preangiotensinogen mRNA sequences in eight different regions of rat brain as well as from liver and kidney. This analysis specifically delineates those brain regions that synthesize angiotensinogen.

Materials and Methods

Isolation of Preangiotensinogen Complementary DNA

A cDNA library was constructed by synthesizing double-stranded cDNA4,5 on a Lewis rat liver poly(A)+ RNA template, digesting with the restriction endonuclease Bam H1 and ligating the cDNA into the Bam H1 site of the plasmid pBR322. One colony (of 300 screened) exhibited a strong signal on hybridization to a 40 residue oligonucleotide (complementary to nucleotides 1044–1083); this colony harbored a plasmid with a 530 base pair (bp) Bam H1 insert. Nucleotide sequence determinations and restriction mapping
revealed that the 530 bp fragment is identical to a predicted 716 bp Bam HI fragment for a contiguous stretch of 500 base pairs; the remaining 30 base pairs, which lie at the 5' end of the 530 bp fragment, bear no significant homology to any region of the published rat preangiotensinogen cDNA sequence. We do not know the reason for this polymorphism; perhaps this cDNA represents an alternative form of preangiotensinogen mRNA.

To obtain longer preangiotensinogen cDNAs, we constructed larger cDNA libraries from rat liver or kidney poly(A)+ RNA templates using the bacteriophage lambda gt10 vector system. A 1650 bp cDNA, which was isolated from the kidney library by hybridization to the 530 bp cDNA, was shown by partial nucleotide sequencing and restriction mapping to correspond to nucleotides -45 to 1589 of the sequence reported by Ohkubo et al. This cDNA, which is essentially full length, is indistinguishable from that previously described, except that an alternate polyadenylation site (113 base pairs upstream) is predicted by our cDNA.

Dissection of Rat Brains and RNA Extraction

Twelve male Sprague-Dawley rats (weight, 175–225 g) were killed by decapitation, and the following brain areas were isolated: brainstem (medulla plus pons), midbrain, cerebellum, cerebral cortex (excluding most of the portion below rhinal fissure), striatum (caudate putamen and globus pallidus), hippocampus, diencephalon plus basal forebrain (hypothalamus, thalamus, and several forebrain structures such as the bed nucleus of the stria terminalis, ventral pallidum, portions of the amygdala), and pituitary. As each section was isolated, it was snap-frozen in liquid nitrogen and stored at -140°C until use. The use of animals in this project was previewed and approved by the University of Virginia Medical Center Animal Research Committee.

The RNA was extracted from frozen brain sections or from fresh liver or kidney as described by Chirgwin et al., and poly(A)+ RNA was isolated by affinity chromatography on oligodeoxythymidylic acid cellulose (grade T3, Collaborative Research, Waltham, MA, USA). The RNA was dissolved in sterile water and stored at -70°C until use. The RNA concentrations were measured spectrophometrically.

Gel Electrophoresis and Quantitation of RNA

Poly(A)+ RNA was denatured by heating for 10 minutes at 65°C in 2.2 M formaldehyde/50% formaldehde and resolved on a 1.0% agarose gel containing 2.2 M formaldehyde. The RNA was transferred to a positively charged nylon 66 membrane (Zetabind, AMF Cuno, Meriden, CT, USA) by capillary action (Northern blot). The bound RNA was heated in vacuo for 2 hours, exposed to ultraviolet light from an ultraviolet transilluminator (254 nm, 500 /uW/cm²) for 1 minute, dried, and stored until use.

Dot blots were prepared by dissolving 0.01 to 1.00 /uM total RNA in 0.5 ml of sterile 25 mM NaPO₄, pH 7.2, and applying this RNA to a Zetabind membrane preequilibrated with the same buffer. The membrane was exposed to ultraviolet light as has been described above and immediately placed in the hybridization solution.

Hybridization and Labeling of Complementary DNA

Northern blots were prehybridized (4 hours) and hybridized (48 hours) at 65°C in a solution consisting of 5 x SSPE (1 x is 180 mM NaCl, 10 mM NaPO₄, 2 mM ethylenediaminetetraacetic acid, pH 7.7), 10 x Denhardt's bovine serum albumin (1 x is 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% basal serum albumin), 0.5% sodium dodecyl sulfate (SDS), 0.05% sodium pyrophosphate, and 100 /uM/ml denatured, sheared herring sperm DNA. Following hybridization, the membranes were washed for 10 min/cycle in 250 ml of the following solutions: 2 x SSPE/0.05% NaPPi (2 cycles at 22°C), 0.2 x SSPE/0.05% NaPPi/0.05% SDS (1 cycle at 22°C, then 2 cycles at 50°C), and 0.1 x SSPE/0.01% NaPPi/0.01% SDS (2 cycles at 65°C). Dot blots were hybridized and washed precisely as previously described by Church and Gilbert.

The cDNAs (100 ng) were labeled to specific activities of 1 to 2 x 10⁶ cpm/µg by nick-translation. Signals were detected by autoradiography in the presence of an image intensifying screen.

Results

The 530 bp angiotensinogen cDNA was used as a probe to detect preangiotensinogen mRNA sequences in poly(A)+ RNA isolated from eight distinct regions of rat brain as well as liver and kidney. The resulting autoradiogram (Figure 1) demonstrates that preangiotensinogen mRNA sequences accumulated in all areas of the rat brain with the possible exception of the pituitary; however, a 10-fold longer exposure revealed a slight signal in pituitary poly(A)+ RNA (data not shown). Since this cDNA does not include the angiotensinogen sequences encoding angiotensin I, we probed an identical blot with a 42 residue synthetic oligonucleotide complementary to the region encoding the amino terminal tetradecapeptide angiotensin I-Leu-Tyr-Tyr-Ser. The intensity and size of the hybridization bands were identical to those depicted in Figure 1 (data not shown). An identical pattern was also observed when a duplicate blot was probed with the full-length (1650 bp) cDNA (data not shown).

The preangiotensinogen mRNA in liver and all brain regions dissected were indistinguishable by size. The size of this RNA was estimated to be 1900 nucleotides. This estimate agrees well with that of Nakanishi's group, obtained by gel electrophoresis as well as with the size of rat preangiotensinogen mRNA (1763 nucleotides) predicted by the sequence of a full-length cDNA. The preangiotensinogen mRNA detected in kidney, however, migrated more slowly than that in the liver or brain. We estimate this size difference to be about 100 nucleotides. This difference may simply be due to alternative polyadenylation sites in preangiotensinogen mRNA, or it may indicate that an isofrom
Angiotensinogen mRNA Distribution

FIGURE 1. Autoradiographic detection of preangiotensinogen mRNA sequences in different regions of rat brain. Poly(A)\(^+\) RNA (1.0–1.4 \(\mu\)g) isolated from the indicated rat tissues was resolved on an agarose gel under denaturing conditions and transferred to a membrane. Lane 1, kidney (KID); Lane 2, whole pituitary (PIT); Lane 3, cortex (CTX); Lane 4, cerebellum (CBM); Lane 5, hippocampus (HIP); Lane 6, striatum (STR); Lane 7, brainstem (BSM), including medulla and pons; Lane 8, midbrain (MID); Lane 9, diencephalon (DIE), including hypothalamus, thalamus, and basal forebrain structures; Lane 10, liver (LIV). Arrow indicates the migration distance of rat 18S ribosomal RNA (2000 nucleotides). The RNA sequences were visualized by autoradiography following hybridization to 100 ng of \(^{32}\)P-labeled 530 base pair cDNA (specific activity, \(1.0 \times 10^6\) cpm/\(\mu\)g); exposure time was 14 hours.

of preangiotensinogen mRNA accumulates in the rat kidney.

The relative amount of preangiotensinogen mRNA sequences in these tissues was estimated by the dot blot technique (Figure 2). The use of total, rather than poly(A)\(^+\), RNA in this analysis allows the direct comparison of samples on a per weight total RNA basis. To maximize signal strength, we hybridized dot blots with the full-length (1650 bp) angiotensinogen cDNA probe. The observed signal intensities suggest that the greatest amounts of preangiotensinogen mRNA sequences accumulate in the liver, brainstem, and midbrain, while lesser amounts are found in the diencephalon (with associated forebrain structures) and in the cerebellum. The hippocampus, striatum, cerebral cortex, and kidney all had substantially lower levels of this RNA sequence. The preangiotensinogen mRNA sequence levels in these tissues varied over a 16-fold range (compare liver with kidney).

FIGURE 2. Relative levels of preangiotensinogen mRNA in different rat brain regions. Total RNA (0.01–1.00 \(\mu\)g) isolated from the indicated rat tissues was applied to a membrane held in a 96-well template. Preangiotensinogen mRNA sequences were detected by hybridization to 100 ng of \(^{32}\)P-labeled 1650 base pair cDNA (specific activity, \(1.8 \times 10^6\) cpm/\(\mu\)g); exposure time was 110 hours. See Figure 1 for key to abbreviations.

Discussion

In this report, we demonstrated that preangiotensinogen mRNA sequences accumulate in all areas of the rat brain examined, although the levels in the pituitary are very low. The brain mRNA species were indistinguishable by size from that in the liver and varied in relative concentration over a 16-fold range. Equivalent relative concentrations of preangiotensinogen mRNA were found in the brainstem, midbrain, and liver; if this value is assigned 100%, then the relative concentration is 25% in both the diencephalon (including basal forebrain structures) and cerebellum, 12% in the hippocampus, and 6% in cerebral cortex, striatum, and kidney. That we were actually detecting preangiotensinogen mRNA sequences is supported by the observation that the same hybridization pattern was obtained when a synthetic oligonucleotide encoding the tetradecapeptide angiotensin I-Leu-Tyr-Tyr-Ser was used as a probe.

We also found that kidney preangiotensinogen mRNA sequences were slightly larger than those in brain or liver. Although this difference was small, it was observed consistently. Two groups have reported results that address the issue of preangiotensinogen mRNA size heterogeneity. Campbell et al.\(^1\) have shown (by in vitro translation and immunoprecipitation) that two preangiotensinogen mRNA species accumulate in rat brain and liver. A major mRNA encodes a \(M_s\) 52.5-kDa protein, while a small amount of a \(M_s\) 55.7-kDa protein is also encoded. This difference
in size would require approximately 85 additional nucleotides in the coding region of the minor mRNA, presumably at the 5' end of the molecules. Ohkubo et al.\(^3\) have also noted that a slightly larger form of preangiotensinogen mRNA accumulates in the rat kidney. They examined size heterogeneity at the 5' and 3' ends of the brain and liver mRNA by primer extension and S\(_1\) nuclease mapping, respectively. In contrast to the 5' heterogeneity predicted from the results of Campbell et al.,\(^1\) they found no evidence of multiple 5' termini in brain or liver preangiotensinogen mRNA. However, S\(_1\) nuclease mapping revealed the existence of three polyadenylation sites (at 1650, 1800, and 1840 base pairs relative to the start site of the mRNA) in addition to the one described by their original cDNA cloning (at 1763 base pairs).\(^6\) We have verified the existence of the 1650 bp polyadenylation site by direct DNA sequence analysis (C. E. Burnham and K. R. Lynch, unpublished data, 1985). These data suggest that the larger form of preangiotensinogen mRNA present in the rat kidney may reflect the predominant use of the most distal polyadenylation site in this tissue.

It is of interest to compare our global localization of preangiotensinogen mRNA sequences in the rat brain with analogous studies measuring angiotensinogen levels. Our data are most readily compared to that of Sernia and Mowchanuk,\(^13\) who divided the rat brain into five regions: telencephalon (equivalent to our cortex plus hippocampus plus striatum), diencephalon, midbrain, brainstem, and cerebellum. Angiotensinogen (as measured by renin-catalyzed release of immunologically recognizable angiotensin I) levels varied over a twofold range, with highest amounts found in the diencephalon and midbrain and least in the cortex. Other groups\(^14,\)\(^15\) have further subdivided the rat brain, often concentrating on measurements in extracts from tissue punch biopsies. Highest levels of angiotensinogen (i.e., renin substrate) were consistently found in hypothalamic, brainstem, and midbrain structures while lower levels were measured in forebrain structures. Although these observations correlate well with our measurements in terms of uneven distribution of preangiotensinogen mRNA in the rat brain, several differences were produced by the different methods. First, preangiotensinogen mRNA levels in the diencephalon were only 25% of those in either the midbrain or brainstem. Second, the preangiotensinogen mRNA levels in the cerebellum were the same as that in the diencephalon. Third, little preangiotensinogen mRNA was detected in the pituitary, although angiotensin II is readily detected\(^16\) in this area. The pituitary angiotensin II may be either synthesized in hypothalamic nuclei with projections to the posterior pituitary or derived from the bloodstream.

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