Demonstration of Transthyretin mRNA in the Brain and Other Extrahepatic Tissues in the Rat*

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Dianne Robert Soprano‡, Joseph Herbert§¶, Kenneth J. Soprano¶, Eric A. Schon‡, and DeWitt S. Goodman‡

From the Departments of Medicine and Neurology, Columbia University, College of Physicians & Surgeons, New York, New York 10032 and the Department of Microbiology & Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Studies were conducted to ascertain if transthyretin mRNA was present in extrahepatic tissues of the rat. A transthyretin cDNA clone was isolated from a λ gt11 human liver cDNA library by antibody screening and its identity was confirmed by nucleotide sequence analysis. This transthyretin cDNA clone was used to survey poly(A+) RNA isolated from 12 different rat tissues for transthyretin mRNA by Northern blot analysis. The liver contained the highest level of transthyretin mRNA and this level was not altered by the vitamin A status of the rat. A significant amount of transthyretin mRNA was found in the brain (30% of the level of the liver) which was localized in specific regions of the brain. In addition, detectable levels of transthyretin mRNA (1% to 2% of that of the liver) were observed in the stomach, heart, skeletal muscle, and spleen. Translation of brain poly(A+) RNA in rabbit reticulocyte lysates and immunoprecipitation of the translation products with anti-transthyretin antiserum resulted in a protein band of the same size as liver pre-transthyretin. Liver pre-transthyretin was processed by the posttranslational addition of dog pancreas microsomal membranes to a protein that migrated coincidentally as a protein band of the same size as liver pre-transthyretin. Transthyretin mRNA was present in extrahepatic tissues of the rat. A significant amount of transthyretin mRNA was found in the brain and the cerebrospinal fluid results from de novo synthesis and that transthyretin may play a significant physiological function, as yet unknown, within the nervous system.

Transthyretin has been found to be associated with the amyloid deposits of patients with heredofamilial amyloidosis (6, 7). Recent studies have demonstrated that patients with the Portuguese (7), Japanese (8), and Swedish (9) type of familial amyloidotic polyneuropathy synthesize an abnormal transthyretin molecule with a methionine for valine substitution at residue 30 of the transthyretin monomeric subunit. This mutation could result from a single nucleotide change in the transthyretin gene, as supported by the nucleotide sequence of human transthyretin cDNA reported by Mita et al. (10) and by Sasaki et al. (11).

We now report the cloning of human transthyretin cDNA and the use of this cDNA clone to survey 12 different rat tissues for transthyretin mRNA. We demonstrate that together with the liver, the brain contains a significant amount of transthyretin mRNA which is localized in specific regions of the brain. Smaller quantities of transthyretin mRNA were detected in heart, skeletal muscle, stomach, and spleen. These data suggest that transthyretin may be synthesized in several extrahepatic organs.

EXPERIMENTAL PROCEDURES

Isolation of Transthyretin Clones—A library of human adult liver cDNA inserted into the expressing bacteriophage vector λ gt11 (12, 13) was obtained as a gift from G. Ricco, Meloy Laboratories, Springfield, VA. This library was screened using rabbit anti-human transthyretin as the primary antibody (14), horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) as the secondary antibody, and 4-chloronaphthol (Bio-Rad) as the color development reagent. Putative positive clones were identified as dark purple plaques.

To confirm the identity of anti-transthyretin positive plaques, a 42-base oligonucleotide probe was synthesized on an Applied Biosystems 380A DNA synthesizer, based on the 14-amino acid sequence of human transthyretin comprising amino acid residues 86 through 99 (1). Codon wobble positions were included in the derived oligonucleotide probe at six consecutive amino acid positions, to give 128 separate molecular species, at least 1 of which would contain a perfect sequence match of at least 20 bases. The oligonucleotide was synthesized in the anti-message sense (R = A or G; Y = T or C; N = A, C, G, or T) (see Scheme 1). DNA was isolated from antibody-positive plaque fields by the plate lysate method of Maniatis et al. (15). After digestion with EcoRI (Bethesda Research Laboratories, Gaithersburg, MD), the clones were analyzed by agarose gel electrophoresis and transferred to nitrocellulose filters (16). The oligonucleotide was kinased (17) using [γ-32P]ATP (3000 Ci/mmol, New England Nuclear, Boston, MA) and T4 polynucleotide kinase (Bethesda Research Laboratories). Hybridization of kinased probe to putative transthyretin-positive λ clones was performed at 42 °C overnight in 750 mM NaCl, 150 mM Tris, pH 7.5, 10 mM EDTA, 100 mM Na2 phosphate buffer, 10 mM Dnethride's (1 × -0.02% each of Ficol, polynynyprylo- didone, bovine serum albumin), 0.1% SDS, and 0.1% Na2 pyrophos-

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†‡§¶ Fellow of the Muscular Dystrophy Association.

† The abbreviations used are: SDS, sodium dodecyl sulfate; RBP, retinol-binding protein.
phate. Washing of filters was at room temperature in 100 mM total salt, 0.1% SDS, and filters were exposed to Kodak XAR film for 18 h at -70 °C in the presence of an intensifying screen (DuPont Cronex). The largest transthyretin positive insert was isolated by electroelution and subcloned into PUC9 (Clone pHTT1) and M13mp9 (18), both digested with EcoRI. The insert in M13mp9 was sequenced in both orientations by the deoxychain termination method of Sanger et al. (19).

Animals—All rats were of the Sprague-Dawley strain obtained from Camm Research Laboratories, Wayne, NJ. The rats were housed individually in an air-conditioned room with a mean temperature of 22 °C and had free access to both food and water. For the tissue distribution and brain region studies, normal male rats weighing 250-300 g were anesthetized with ether and decapitated, and the tissues were removed for the preparation of RNA. Rat brains were dissected in the following manner. Coronal sections were placed at the levels of the optic chiasm, the median eminence, and the pontomesencephalic junction. The cerebellum was dissected free from the brainstem by sectioning the cerebellar peduncles. The brains were thus divided into five regions: (I) anterior to the optic chiasm; (II) between the optic chiasma and median eminence; (III) between the median eminence and the pons; (IV) pons-medulla; and (V) cerebellum.

For the studies involving vitamin A status, male rats were obtained as weanlings and immediately placed on a semipurified vitamin A-deficient diet supplemented with 4 mg/kg diet with vitamin A (supplied as retinyl esters). Serum vitamin A levels below 3 pg/ml of poly(A+) RNA was denatured with 10 mM methylmercury chloride for 1 h at 50 °C, and the labeled RNA was purified away from the pUC9 vector after EcoRI digestion of pHTT1. The insert of pHTT1 was sequenced by the deoxychain termination method of Sanger et al. (19) after subcloning it into M13mp9 (18) digested with EcoRI.

The results of the EcoRI insert are shown in Fig. 1. It agrees completely with the recently published sequences of Mita et al. (10) and Sasaki et al. (11) except for the indicated positions in the 3′ untranslated region. Plasmid pHTT1 extends from a Pro at position 11 to the exact site of poly(A) addition, a distance of 500 base pairs. Moreover, in agreement with others, we found the poly(A) addition to be 14 nucleotides long.

RNA Isolation and Analysis—Total RNA was prepared by the method of Tushinski et al. (21). RNA enriched in poly(A)+ RNA was obtained by oligo(dT)-cellulose affinity chromatography as described by Aviv and Leder (22).

The size and the relative amount of transthyretin mRNA was analyzed by Northern blot analysis. Unless otherwise indicated, 10 µg of poly(A)+ RNA was denatured with 10 mM methylmercury hydroxide and electrophoresed in a 1% agarose gel as described by Bailey and Davidson (23). RNA was transferred to nitrocellulose paper essentially as described by Thomas (24). The 500-bp human transthyretin cDNA purified away from the pUC9 vector after EcoRI digestion of pHTT1 was nick-translated with [32P]ATP and [3H]dGTP (3000 Ci/mmol, Amberson, Amsterdam Heights, IL) to a specific activity of approximately 1×106 cpm/µg (25). Nitrocellulose filters were hybridized at 42 °C in 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.1 M Na+ citrate, pH 7.5), 1 × Denhardt's, 50 mM Na+ phosphate, pH 6.5, 250 µg/ml salmon sperm DNA, 5% dextran sulfate, and 5 × 105 cpm/ml of [32P]labeled transthyretin cDNA. Filters were washed three times with 10 min each at room temperature with 2 × SSC and 2 times for 20 min each at 51 °C with 0.1 × SSC and 0.1% SDS. Filters were exposed to Kodak SB5 x-ray film at -70 °C with intensifying screens and the autoradiograms were quantitated by Image Analysis Densitometry (Image Analysis Corp.).

In Vitro Translation of Liver and Brain mRNA—One µg of liver and 3 µg of brain poly(A)+ RNA were translated in the rabbit reticulocyte lysate protein-synthesizing system in the presence and absence of dog pancreas microsomal membranes (gift of P. Feigelson, Columbia University) as previously described (26) except that the reticulocytes were obtained from Pronega Biotech, Madison, WI. Transthyretin was specifically immunoprecipitated with rabbit antitransthyretin anti-serum (27) in the same manner as previously described for RBP (26). The immunoprecipitated products were electrophoresed on SDS-17.5% polyacrylamide slab gels and fluorographs were prepared as previously described (26).

RESULTS

Isolation and Characterization of a Transthyretin cDNA Clone—Sixty thousand plaques of human adult liver cDNA inserted into λ gt11 were screened with a polyclonal rabbit antiserum raised against purified human transthyretin. Three clones yielded a positive colorimetric reaction upon repetitive screening to plaque purity. DNA from these three clones was prepared by the plate lyse method (15) digested with EcoRI, electrophoresed, transferred to nitrocellulose (16), and hybridized to a kinased 42-nucleotide synthetic oligonucleotide probe based on 14 amino acids of the human transthyretin protein sequence (1). All three inserts hybridized strongly to this probe, implying that they were indeed cDNAs encoding transthyretin. Accordingly, the largest of the three, about 500 base pairs in size, was subcloned into PUC9 (18) digested with EcoRI (clone pHTT1). The insert of pHTT1 was sequenced by the deoxychain termination method (19) after subcloning it into M13mp9 (18) digested with EcoRI.

The size of the EcoRI insert is shown in Fig. 1. It extends from a Pro at position 11 to the exact site of poly(A) addition, a distance of 500 base pairs. Moreover, in agreement with others, we found the poly(A) addition to be 14 nucleotides long.
with the data of Mita et al. (10) and Sasaki et al. (11), we find that the amino acid sequence assignment at positions 61–63, which had been reported to be Glx-Glx-Gln (1) is actually Glu-Glu-Glu.

**Transthyretin mRNA Levels in Tissues**—The relative amount of transthyretin mRNA in 12 rat tissues was examined by Northern blot hybridization of poly(A)⁺ RNA using the human transthyretin cDNA probe. Comparison of the amino acid sequence of human (1) and rat (40) transthyretin reveals that they are 86% homologous; thus, human transthyretin cDNA and rat transthyretin mRNA will cross-hybridize under stringent conditions. The Northern blots (Fig. 2) revealed that rat transthyretin mRNA migrated as a single band of approximately 700 bases in all tissues displaying hybridization. As expected, transthyretin mRNA was most abundant in the liver. A significant amount of transthyretin mRNA was detected in the brain, which was quantitated by Image Analysis to be approximately 30% of that of the liver. Low levels, 1% to 2% of that of the liver, were observed in the spleen, stomach, heart, and skeletal muscle (skeletal muscle not shown). We were unable to detect transthyretin mRNA sequences in the lungs, large intestines, small intestines, testes, and pancreas. Data on kidney were inconsistent in that two of seven rats studied were positive for transthyretin mRNA and the remaining five kidneys had undetectable levels.

**Transthyretin mRNA in Brain Sections**—To further investigate transthyretin mRNA levels in the brain, poly(A)⁺ RNA was isolated from 5 brain regions: (I) anterior to the optic chiasm; (II) between the optic chiasm and the median eminence; (III) between the median eminence and the pons; (IV) pons-medulla; and (V) cerebellum. Northern blot analysis of these poly(A)⁺ RNA samples, shown in Fig. 3, revealed that Region II and Region IV were greatly enriched in transthyretin mRNA. These two regions contained 34% and 54% of the brain transthyretin mRNA, respectively. Regions III and V contained low levels of transthyretin mRNA ranging from 4% to 8% of hybridizable mRNA in the whole brain. Transthyretin mRNA was undetectable in Region I even after 30 times longer exposure of the blot shown in Fig. 3.

**In Vitro Translation of Liver and Brain mRNA**—To investigate whether the transthyretin mRNA within the brain detected by Northern blot analysis was capable of being translated into transthyretin protein, poly(A)⁺ RNA from the brain and liver was translated in rabbit reticulocyte lysates and the translation products were immunoprecipitated with rabbit anti-rat transthyretin antiserum. As shown in Fig. 4, a protein of approximately 16,000 to 17,000 Da was immunoprecipitated with anti-transthyretin antisemur from both the liver and brain translation products. The nature of the additional band at 35,000 Da in the brain sample is unknown. It is possible that this band represents a brain-specific product which cross-reacts with our antibody or that this band is a dimeric or trimeric form of transthyretin. Dickson et al. (28) have previously observed a similar band, which they termed trimeric transthyretin, when rat transthyretin was electrophoresed in SDS-polyacrylamide gels without boiling. Regardless of the nature of this 35,000 Da band, the experiment demonstrates that mRNA isolated from brain is capable of coding for a protein which is recognized by anti-transthyretin serum and migrates to the same position as liver transthyretin.

The size of the immunoprecipitated transthyretin was not surprising since the nucleotide sequence of transthyretin reported by Mita et al. (10) and Sasaki et al. (11) predicts that this protein, like many secreted proteins, is synthesized as a larger molecular weight precursor. To demonstrate this, liver poly(A)⁺ RNA was (a) translated with no microsomal membranes, (b) translated in the presence of dog pancreas micro-
Fig. 4. Translation of liver and brain mRNA and immunoprecipitation of transthyretin. One µg of poly(A+)* RNA from the liver and 3 µg of poly(A+)* RNA from the brain was translated in the rabbit reticulocyte lysate protein-synthesizing system. Transthyretin was immunoprecipitated with rabbit anti-rat transthyretin serum. Immuno precipitated products were analyzed on SDS-17.5% polyacrylamide slab gels and fluorographed. Molecular weight markers used were insulin (3,000), bovine trypsin inhibitor (6,200), lysozyme (14,300), β-lactoglobulin (18,400), carbonic anhydrase (30,000), and ovalbumin (43,000).

somal membranes, and (c) translated and dog pancreas microsomal membranes were added post-translationally. Fig. 5 shows that the co-translational addition of dog pancreas microsomal membranes results in a smaller molecular weight protein (Lane C) which co-migrates with monomeric transthyretin isolated from rat serum. Post-translational addition of the dog pancreas microsomal membranes (Lane D) did not result in the processing of the pre-transthyretin to transthryretin.

Transthyretin mRNA Levels and Vitamin A Status of Rats—Since transthyretin functions in the binding of RBP, the specific transport protein for retinol, we wished to ascertain if the nutritional vitamin A status of the rat altered the transthyretin mRNA levels in the liver. Fig. 6 is a representative Northern blot of liver poly(A+) RNA isolated from a rat of normal vitamin A status and a rat which was vitamin A-deficient. This figure and more quantitative analysis by Image Analysis demonstrates that normal and vitamin A-deficient rat livers have the same accumulated level of transthyretin mRNA, indicating that vitamin A does not control the levels of transthyretin mRNA in the liver.

DISCUSSION

These studies were undertaken to determine if any extrahepatic tissues contain transthyretin mRNA and hence synthesize transthyretin. Navab et al. (27) previously demonstrated by radioimmunoassay the presence of immunoreactive transthyretin in a large number of tissues; however, it was not possible in that study to distinguish transthyretin synthetized de novo in a given tissue from transthyretin which was taken up by the tissue from the plasma (but was originally synthesized in the liver). In order to address this question directly, we prepared a cDNA which could be used as a probe for transthyretin mRNA levels. We obtained a nearly full length transthyretin cDNA clone from a human liver λ gt11 library. Sequence analysis of this clone revealed the identical sequence recently reported by Mita et al. (10) and Sasaki et al. (11) with the exception of 8 nucleotides in the 3' untranslated region. While these may be sequencing errors or printing errors, they may also be naturally occurring polymorphisms. Clones from other individuals would have to be sequenced to prove this.

Transthyretin is known to be biosynthesized in and secreted (into the plasma) by the liver. In patients with chronic liver disease, plasma transthyretin levels are decreased and the extent of decrease from normal is correlated with the extent of decrease of plasma RBP (RBP is known to be produced in the liver) and the extent of hepatic parenchymal dysfunction (29). Transthyretin has been shown to be produced and secreted by isolated liver cells in vitro; detailed studies have been carried out both with a differentiated rat hepatoma cell line (30) and with primary rat hepatocytes in culture (31).2 Also, transthyretin has been localized within rat hepatic parenchymal cells by specific immunohistochemical studies (32). As reported here, transthyretin mRNA is abundant in liver, readily translated to pre-transthyretin with an in vitro protein-synthesizing system, and transthyretin mRNA levels in liver are not altered by the vitamin A status of rats. Taken together, these various studies clearly establish the liver, and specifically the hepatic parenchymal cell, as a major site of transthyretin synthesis. It has, in fact, been concluded that

2 J. Dixon and D. S. Goodman, unpublished observation.
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the hepatocytes synthesize the majority of transthyretin in the body (31). In our studies of transthyretin mRNA levels in extrahepatic tissues, we found that the brain contained significant levels of transthyretin mRNA, which could be translated in vitro to a protein recognized by anti-transthyretin antiserum, and was the same size as liver pre-transthyretin. This transthyretin mRNA was localized in two regions of the brain, Region II and Region IV. Region II principally contains the hypothalamus, thalamus, basal ganglia, third ventricle, and the bodies of the lateral ventricles, and Region IV contains the pons, medulla, and the fourth ventricle. In addition, 4 other tissues, the heart, skeletal muscle, spleen, and stomach, of a total of 12 tissues examined, contained detectable levels of transthyretin mRNA.

The finding of substantial amounts of transthyretin mRNA in the brain was not completely unexpected. Relatively high levels of transthyretin have been reported in cerebrospinal fluid of man (33). In fact, transthyretin is 12 times more concentrated in cerebrospinal fluid than expected relative to other plasma proteins (33). The relatively high concentration of transthyretin in cerebrospinal fluid cannot be completely accounted for by passive diffusion of transthyretin from plasma, hence transthyretin must be actively concentrated or synthesized, de novo, presumably within the choroid plexus of the brain (34). Immunochemical staining of the human choroid plexus epithelium by Aleshire et al. (35) demonstrated intense positive staining with transthyretin antiserum. In addition, this staining appeared localized in the endoplasmic reticulum and Golgi apparatus of these cells. Our finding of transthyretin mRNA within the brain, localized within two regions of the brain (88% of total hybridizable brain transthyretin mRNA) which contain the lateral, third, and fourth ventricles and hence the bulk of choroid plexus epithelium, strongly supports the idea that transthyretin in cerebrospinal fluid is a result of de novo synthesis. However, this does not eliminate the possibility that other cell types in the brain besides the choroid plexus epithelium also transcribe transthyretin mRNA and synthesize transthyretin. Clear demonstration of which cells within the brain contain transthyretin mRNA awaits further studies, such as in situ hybridization of brain tissue sections with transthyretin cDNA.

As predicted by the nucleotide sequence of Mita et al. (10) and Sasaki et al. (11), liver transthyretin is synthesized as a larger molecular weight precursor, pre-transthyretin. Two lines of evidence were obtained which demonstrate that pre-transthyretin is processed to a protein identical in size with serum transthyretin, presumably during translocation of transthyretin across the endoplasmic reticulum bilayer (36–38). First, the co-translational addition of dog pancreas microsomal membranes to the reticulocyte lysate resulted in the processing of pre-transthyretin to a protein that migrated coincidentally with the monomeric subunit of purified transthyretin. Second, post-translational addition of pancreas microsomal membranes did not result in the formation of a smaller protein of the size of serum transthyretin.

Poly(A*) RNA from the brain synthesized in vitro pre-transthyretin of the same size as that of the liver. This suggests that transthyretin in the brain, like that of liver, is, I secrected from the cells in which it is synthesized. This is consistent with the histochemical localization of transthyretin in the choroid plexus epithelium within the rough endoplasmic reticulum and Golgi apparatus (35) organelles known to be involved in the secretory process.

Finally, the role of transthyretin mRNA in the brain, as well as its role in spleen, stomach, skeletal muscle, and heart is unclear at this time. Further experiments are necessary to determine if transthyretin has a function in these tissues different from its known functions concerning the transport of retinol (via its binding of RBP) and of thyroid hormone in plasma.

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REFERENCES

Fig. 6. Levels of transthyretin mRNA in normal and vitamin A-deficient rats. Ten µg of poly(A*) RNA from normal (N) and deficient (D) rats was denatured with methylmercury hydroxide, electrophoresed in a 1% agarose gel, transferred to a nitrocellulose filter, and hybridized to 32P-labeled transthyretin cDNA.
Transthyretin mRNA in Brain