Vol. 173, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1990 Pages 323-330

DUAL PROMOTERS AND TISSUE-SPECIFIC EXPRESSION OF RAT TRANSTHYRETIN GENE

Kiyoto Motojima* and Sataro Goto

Department of Biochemistry, School of Pharmaceutical Sciences, Toho University, Funabashi, Chiba 274, Japan

Received October 17, 1990

The rat chromosomal gene for transthyretin was cloned and its dual promoter region was analyzed by primer extension. Semi-quantitative studies by primer extension analysis to determine the transcriptional start sites and their usage in the three tissues have suggested that the rat transthyetin gene has dual promoters: a major proximal promoter which is used in the liver and in the brain but not at all in the kidney, and a minor distal promoter which is totally used in the kidney and significantly in the brain.

Transthyretin (prealbumin), consisting of four identical subunits each having a molecular weight of approximately 14,000, binds thyroid hormones and also binds vitamin A via the retinol binding protein, playing an important role in their transport process [1]. In addition to its well characterized three dimensional structure, the cDNA and genome structures for transthyretin of several species have been determined [2-7]. The molecular and genetic basis for accumulation of a variant transthyretin in a heredofamilial amyloidotic polyneuropathy has also been studied [8,9].

In rodents, transthyretin mRNA was detected in the liver, the kidney, and the brain [2,3]. In the brain, the mRNA was detected only in the choroid plexus at much higher levels than in the liver on a per g tissue basis [10]. The mRNA was described as being expressed from the same start site of the same gene in the three tissues [5,11]. Multiple

* Correspondence should be addressed to Department of Biochemistry, School of Pharmaceutical Science, Toho University, Miyama 2-2-1, Funabashi, Chiba 274, Japan.

0006-291X/90 $1.50

nuclear factors and the proximal regulatory regions of the mouse gene functioning in liver-specific expression have been reported [11]. However, the mechanism by which the kidney and the choroid plexus selectively express the gene but not other liver-specific genes is not known.

Reexamination of the idea that the same mRNA is transcribed from the same start site in the three tissues by cloning rat transthyretin cDNAs from a brain cDNA library has suggested the presence of another promoter which is active in the brain but not all or minimally in the liver [12]. In this study, we analyzed the structure of the rat transthyretin gene and the two promoter regions, and the tissue-specific usage of the two promoters by primer extension.

MATERIALS AND METHODS

Materials---Restriction enzymes, DNA polymerase I, Klenow fragment of DNA polymerase I, T4 DNA ligase, and T4 polynucleotide kinase were obtained from Toyobo (Osaka, Japan). Reverse transcriptase was from Seikagaku-kogyo (Tokyo, Japan). Normal and reverse sequence primers were from Takara shuzo (Kyoto, Japan). The synthetic oligonucleotide for the primer extension experiment was purchased from Milligen Japan (Tokyo, Japan). [α-^{32}P]dCTP (700 Ci/mmol) and [γ-^{32}P] ATP (crude) were from ICN (CA, USA).

Isolation of Gene Clones from a Genomic Library---- About one million independent phage clones of a Sprague Dawley rat genomic library (the gift of Dr.H.Andersen, UCLA, USA) was screened under high-stringency hybridization conditions [13] with the ^{32}P-labeled insert of a cDNA clone B1 [12].

Subcloning and Sequencing----Phage DNA of the positive genomic clones was prepared according to the published method [14]. The restriction map of the overlapping genomic fragments was constructed by digestion of DNA with various restriction enzymes followed by Southern blot analysis with cDNA probes. The subfragments which hybridized were cloned into pUC18 and sequence analyses were done by the dideoxy chain termination method [15] using denatured plasmid DNAs as templates [16]. The DNASIS program of Hitachi Software Engineering (Yokohama, Japan) was used for computer-assisted alignments of the sequences.

Primer Extension Analysis----A synthetic 24mer oligonucleotide, 5'-GGAACAGGCGAAGGGAAGCCATCC-3' complementary to 5'-GGAUGGCUUCCCUUCGCCUGUUCC-3'(the initiator methionine codon is underlined) was end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase [17] and used as a primer. Total RNA was extracted by SDS-phenol-chloroform method [18] from

various tissues. Fifty µg of total RNA was directly used for the primer extension experiment as described [19]. After treatment with RNaseA, the samples were analyzed on a sequence gel with the sequence ladders of M13mp18 DNA as size markers.

RESULTS AND DISCUSSION

Cloning of the Rat Transthyretin Gene----Five rat transthyretin genomic clones were isolated by screening a genomic library with ^{32}P-labeled cDNA for rat transthyretin [12]. The results of restriction mapping of the overlapping genomic fragments and Southern blot hybridization are summarized in Fig. 1. The rat transthyretin gene is a single copy gene consisting of four exons and two introns. The restriction map and these conclusions were essentially the same as those reported previously [7]. In addition to exon 1 and exon 4 [7], the nucleotide sequences of and around all four exons were determined and are summarized in Table I. All the intron/exon junctions obey the AG/GT rule [20]. The number of exons, the sites of intron/exon junctions, and the sizes of the coding regions for the transthyretin are all in complete agreement with those of the mouse and the human transthyretin genes [5,6]. The structure of the transthyretin gene is highly conserved among these three species.

The Transcription Start Sites in Various Tissues---- Transthyretin mRNA has reportedly been detected in the liver, in extremely high levels in the choroid plexus of the

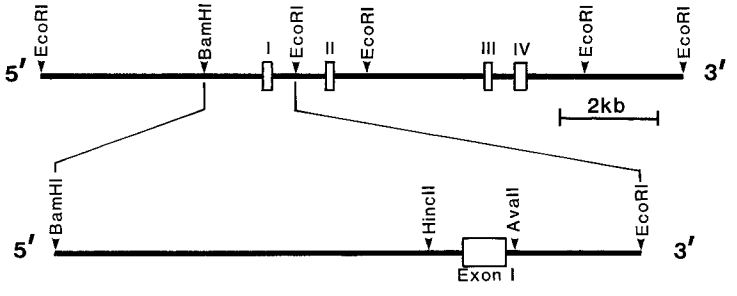

Fig.1. Organization of the rat transthyretin gene. The structure of the gene is shown as a thick bar with four boxes representing exons. The structure of exon I and its flanking regions are also shown in an extended scale. Only the relevant restriction sites are given.

Table I

Summary of intron-exon junction sequences

Intron	5'		3'	
	24		25	
	Gly Pro Gly		*Gly Ala Gly*	
I	GGC CCT GGG	gtgagtggt.....tatgcccag	GGT GCT GGA	
	67		68	
	Ala Ser Gl		*y Lys Thr*	
II	T GCC TCT GG	gtaagctta.....tgccctcag	G AAG ACC G	
	112		113	
	Tyr Ala Glu		*Val Val Phe*	
III	TAC GCA GAG	gtaagtggg.....ctctcctag	GTG GTT TTC	

brain, and in low levels in the kidney [1,3,10]. In rodents, it was described as being expressed from the same start site of the same gene in all three tissues [5,7].

Our previous result obtained by cloning rat transthyretin cDNA from a brain cDNA library, however, suggested that the brain has an additional transcriptional start site for the mRNA [12]. Dot hybridization analysis of total RNA from various tissues suggested that this site was used in the brain at a significant level, but little or not at all in the liver [12]. To determine the precise start sites for transcription and to quantitate the levels of the usage of the two sites, primer extension analysis was carried out. A synthetic 24mer-oligonucleotide complementary to the stretch around the translational start site (see Fig.3) was synthesized, end-labeled, and used as a primer. Total RNAs from the liver, the total brain, the occipital region, and the kidney of an adult rat were used (Fig.2). Several major reverse transcripts around 49 bases long were synthesized from the liver and the brain RNAs but not from the kidney RNA. Major initiation sites for transcription of the transthyretin gene determined by these transcripts closely correspond to those previously reported [7]. In addition to the major bands, doublets 129 and 130 bases long were detected in the transcripts from all three tissue RNAs. These longer primer-extended transcripts had been thought to be present from the result of cDNA cloning from the brain

[12], but their presence in all three tissues is noteworthy. The longer transcripts in the three tissues are comparable in amount, while the major one reported earlier is not; being extremely high in the liver, high in the brain, and minimal in the kidney. Althouth exact amounts of upstream and dounstream initiation may not be quantitated by primer extension analysis alone, the approximate ratio of usage of the two sites as determined by densitometric quantitation varies among three tissues: That of the longer transcript to the major one in the liver is less than 0.01, that in the brain is about 0.1. Considering the ealier observation that the brain transthyretin is expressed only in the choroid plexus and the mRNA level is at least 100 times higher than that in the liver per g tissue [10], the usage of the upstream transcriptional start site in the choroid plexus is significant though it is not brain specific or dominant. The importance of the distal transcriptional start site and its promoter is suggested by the higher degree of conservation of its nucleotide sequence than that of the proximal region among three species, i.e. rat, mouse and human [5-7].

Structure of the Dual Promoter Region and Tissue-specific Expression-----The nucleotide sequence of the dual promoter region of the rat transthyretin gene and the transcriptional start sites in the brain, liver, and kidney are shown in Fig.3. In the upstream region of the major transcriptional start site, which is used in the liver and the brain but not in the kidney, there exists a "TATA-box"-like sequence, 5'-TATATAA-3' at nucleotides -30 to -24. A "CAAT-box"-like sequence, 5'-GTCAAT-3' is also found at nucleotides -101 to -96 as reported previously [7]. On the other hand, neither of these sequences was found in the upstream region of the distal transcriptional start site. The sequence 5'-AATAAT-3' at nucleotides -97 to -93, which was suggested to be a "TATA-box"-like sequence by cDNA cloning [12] may be too close to the distal transcriptional start site determined in this study, i.e. -18 to -13 nucleotides upstream, to meet the general rule of a "TATA-box" sequence [21]. However, because the factor can bind tightly to a functional TATA

element that does not match the consensus sequence as suggested [22], the possibility cannot be excluded that the distal promoter also has an element to bind TFIID. Furthermore, Smale *et at.* [23] recently suggested that mammalian TFIID may contain additional function to activate transcription from some TATA-less promoters. It will be very important to determine whether the two promoters are independent or overlapping.

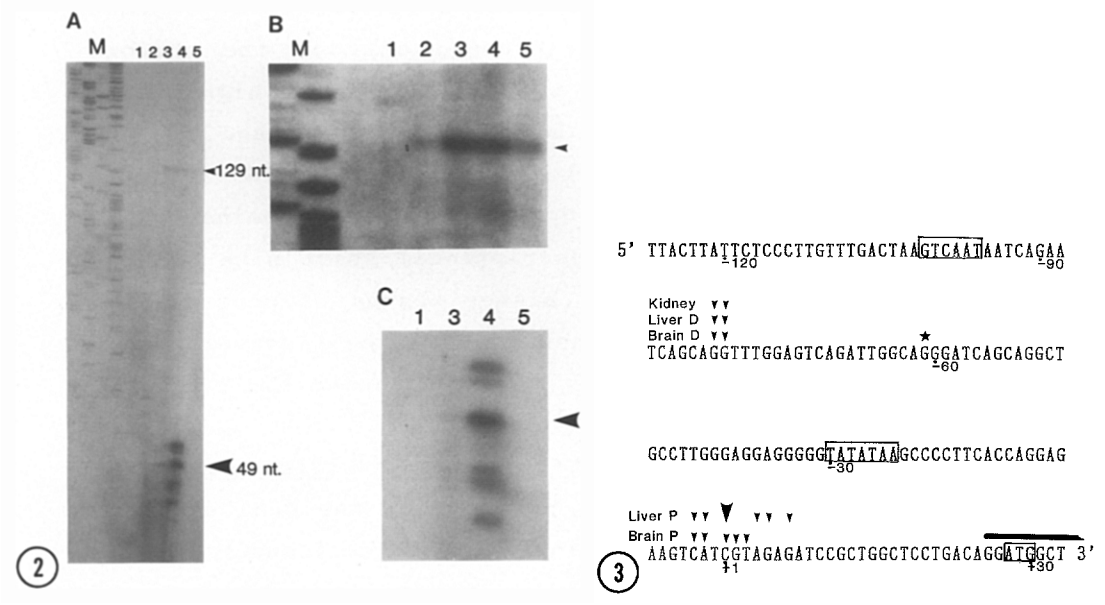

Fig.2. Primer extension analysis for determination of the initiation sites for transcription of the transthyretin gene. A synthetic 24mer oligonucleotide complementary to the stretch around the translational start site was used as a primer to anneal with tRNA (lane 1), total RNA from the occipital region (lane 2), from the total brain (lane 3), from the liver (lane 4), or from the kidney (lane 5). The region of the longer transcripts is enlarged in B. C shows the region of the major transcripts (the result of a different experiment from A). The minor DNA bands of primer-extended transcripts are indicated by small arrowheads and most major bands by large arrowheads. M indicates the sequence ladders of M13mp18 DNA for size markers.

Fig.3. Nucleotide sequence of the dual promoter region of the rat transthyretin gene. The nucleotide corresponding to the most major transcriptional start site is numbered +1. A "CAAT-box"-like sequence, a "TATA-box"-like sequence and the initiation codon ATG are boxed. The thick line indicates the 5'-portion of the sequence complementary to the synthetic oligonucleotide used in the primer extension analysis. The star identifies the 5'-end of a cDNA clone obtained from a brain cDNA library [12]. Arrowheads indicate the transcriptional start sites determined by the primer extension analysis in this study (Fig.2).

The sole use of the distal promoter in the kidney may suggest the presence of some mechanism to repress the use of the proximal promoter. Thus, in addition to several trans factors which activate transcription in a tissue-specific manner, knowledge of cis and/or trans element(s) which repress the proximal promoter are also important to understand the mechanism of the unique regulation of expression of the transthyretin gene.

Acknowledgments: We are grateful to Mr.M.Kobayashi for his technical assistance in isolating the genomic clones. Thanks are also due to Dr.H.Andersen (UCLA, USA) for his generous gift of rat genomic library.

REFERENCES

[1] Dickson,P.W., Aldred,A.R., Menting,G.T., Marley,P.D., Sawyer,W.H. and Schreiber,G. (1987) J. Biol. Chem. 262, 13907-13915.
[2] Mita,S., Maeda,S., Shimada,K. and Araki,S. (1984) Biochem. Biophys. Res. Commun. 124, 558-564.
[3] Wakasugi,S., Maeda,S., Shimada,K., Nakashima,H. and Migita,S. (1985) J. Biochem. 98, 1707-1714.
[4] Sundelin,J., Melhus,H., Das,S., Lind,P., Tragardh,L., Peterson,P.A. and Rask,L. (1985) J. Biol. Chem. 260, 6481-6487.
[5] Wakasugi,S., Maeda,S. and Shimada,K. (1986) J. Biochem. 100, 49-58.
[6] Tsuzuki,T., Mita,S., Maeda,S., Araki,S. and Shimada,K. (1985) J. Biol. Chem. 260, 12224-12227.
[7] Fung,W.P., Thoma,T., Dickson,P.W., Aldred,A.R., Milland, J., Dziadek,M., Power,B., Hudson,P. and Schreiber (1988) J. Biol. Chem. 263, 480-488.
[8] Dwulet,F.E. and Benson,M.D. (1984) Proc. Natl. Acad. Sci. USA. 81, 694-698.
[9] Yamamura,K., Wakasugi,S., Maeda,S., Inomoto,T., Iwanaga,T., Araki,K., Miyazaki,J. and Shimada,K. (1987) Dev. Genet. 8, 195-205.
[10] Dickson,P.W., Aldred,A.R.,Marley,P.D., Guo-Fen,T., Howlett,G.J. and Schreiber,G. (1985) Biochem. Biophys. Res. Commun. 127, 890-895.
[11] Costa,R.H., Grayson,D.R. and Darnell,J.E. (1989) Mol. Cell. Biol. 9, 1415-1425.
[12] Motojima,K. and Goto,S. (1989) FEBS Lett. 258, 103-105.
[13] Maniatis,T., Frisch,E.F. and Sambrook,J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[14] Manfioletti,G. and Schneider,C. (1988) Nucl.Acids Res. 16, 2873-2884.
[15] Sanger,F., Nicklen,S. and Coulson,A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
[16] Hattori,M. and Sakaki,Y. (1986) Anal. Biochem. 152, 232-238.
[17] McGraw,R.A.,III (1984) Anal. Biochem. 143, 298-303.

[18] Perry,R.P., LaTorre,J., Kelly,D.E. and Greenberg,J.R. (1972) Biochim. Biophys. Acta 262, 220-226.
[19] Kingston,R.E. (1987) in Current Protocols in Molecular Biology (Ausubel,F.M., Brent,R., Kingston,R.E., Moore,P.D., Smith,J.A., Seideman,J.G. and Struhl,K., eds.) unit 4.8, John Wiley & Sons, New York, NY.
[20] Mount,S.M. (1982) Nucl. Acid Res. 10, 459-472.
[21] Breathnach,R. and Chamborn,P. (1981) Ann. Rev. Biochem. 50, 349-383.
[22] Hahn,S., Buratowski,S., Sharp,P.A. and Guarente,L. (1989) Proc. Natl. Acad. Sci. USA 86, 5718-5722.
[23] Smale,S.T., Schmidt,M.C., Berk,A.J. and Baltimore,D. (1990) Proc. Natl. Acad. Sci. USA 87, 4509-4513.