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The genomic structure and the expression profile of the *Xenopus laevis* transthyretin gene

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ABSTRACT

Transthyretin (TTR) is a major thyroid hormone-binding protein in the amphibian tadpole whose plasma mRNA and protein levels are altered during metamorphosis. While the temporal and spatial expression patterns and genomic structure of the *TTR* gene are well studied in higher vertebrates, detailed expression pattern in the extrahepatic tissues, the transcriptional regulation, and the genomic structure have not yet been identified in amphibians. In this study, we attempted to elucidate these mechanisms. Here, we determined the genomic structure of the *Xenopus laevis TTR* gene including 5′-flanking regions, and examined *TTR* expression patterns in several tissues. The *TTR* gene of *X. laevis* is composed of 4 exons and 3 introns, and the nucleotide sequence of intron 1 is not similar to that previously reported. This suggests that the *TTR* gene of *X. laevis* was duplicated and the gene cloned in this study was the other copy of previously reported gene. We also found that TTR was primarily transcribed in the liver of both tadpoles and adults. In the adult liver, TTR transcripts were more abundant in males than females. In higher vertebrates, the expression of TTR is controlled by several transcription factors including forkhead box A2 (FoxA2). However, in the *X. laevis* liver, FoxA2 expression patterns were not similar to TTR. We also found that exogenous FoxA2 increased the *X. laevis TTR* promoter-driven luciferase activity. These results suggest that, in amphibian, the expression of *TTR* is regulated partially by FoxA2, and that another system may exist to control *TTR* expression.

1. Introduction

Transthyretin (TTR) is a one of the major thyroid hormone-binding proteins. Recently, complete genomic sequences have been analyzed in various organisms, and the genomic structure of the *TTR* genes has been validated. However, the genomic sequences including 5′- and 3′-flanking regions, intron–exon borders, and part of introns have only been completely analyzed in 3 vertebrates (human, mouse, and rat). The *TTR* genes of these organisms are composed of 4 exons and 3 introns. The evolution of the *TTR* gene structure reveals that mutations near the N-terminal region lead to changes in length and hydropathy of the TTR proteins (Prapunpoj et al., 2006).

In general, TTRs are synthesized mainly in the liver and the choroid plexus, and then secreted into blood stream and cerebrospinal fluid, respectively. TTRs contribute to the circulation of thyroid hormones (THs) throughout the whole body. In amphibians, TTR transcripts were detected mainly in the liver of tadpoles and this expression was stage-dependent: the levels of TTR mRNA in the liver reached a maximum at prometamorphic stages and declined thereafter through the climax of metamorphosis (Prapunpoj et al., 2000).

In order to identify a distinct expression profile, it is necessary to understanding the strict mechanisms of transcriptional regulation. In the rodents, the genomic structures of *TTR* genes were validated and the proximal promoter and the distal enhancer were identified as regulatory regions (Costa et al., 1986). It has also been reported that the expression of the *TTR* gene was controlled by several transcription factors, such as forkhead box A2 (FoxA2), formerly called hepatocyte nuclear factor 3β(HNF3β), HNF6, and HNF4 in a cell- or tissue-specific manner (Costa and Grayson, 1991; Costa et al., 1986, 1989, 1990; Nagata et al., 1995; Rausa et al., 1997; Sladek et al., 1990; Tan et al., 2001; Yan et al., 1990). Among these factors, the binding of FoxA2 to approximately −100 base pairs (bp) upstream of transcription start site was required. Santos et al. reported the cDNA sequence of the teleost fish *Sparus aurata* (Santos and Power, 1999). Furthermore, they and Funkenstein et al. found the high levels of TTR transcripts in the liver and skin, and moderate expression was detected in the several other tissues (Funkenstein et al., 1999; Santos and Power, 1999). However, in the higher vertebrates, such as frogs, birds, and mammals, the liver was the one of the main tissues in which TTR was synthesized. Additionally, TTR expression is also very

Abbreviations: TTR, transthyretin; TH, thyroid hormone; Fox, forkhead box; HNF, hepatocyte nuclear factor; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; GAPDH, glyceraldehydephosphate dehydrogenase; FBS, fetal bovine serum; βGal, β-galactosidase; TR, thyroid hormone receptor; RBP, retinol-binding protein; kb, kilobase(s); bp, base pair(s); cpm, counts per minute.

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prominent in choroid plexus of mammals (Kato et al., 1986). The FoxA2-binding sequence at −100 bp upstream of the transcription start site was highly conserved in the frog, reptile, bird, and mammal, all vertebrates in which TTR is specifically expressed in the liver. In contrast, the fish species did not have this sequence and, interestingly, in this animal TTR is expressed ubiquitously. Furthermore, the expression of TTR is stage-dependent in the amphibian and reptilian liver. Thus, the amphibian species may be a good model to study about the localized expression of TTR in the liver and the effects of several transcription factors on its mechanisms of tissue specific expression.

Steroid hormones, such as estrogen, progesterone, and testosterone, positively regulate the expression of TTR in the liver and choroid plexus of rodents (Goncalves et al., 2008; Quintela et al., 2008, 2009, 2011). Although the mechanism of TTR expression has been clearly identified in higher vertebrates, it is still unclear in lower vertebrates such as amphibians and fishes. While the expression pattern was different between fishes and amphibians, negative regulation of TTR expression by estrogen was reported in both species (Funkenstein et al., 2000; Urbatzka et al., 2007). All observations suggest that the transcriptional control of TTR is becoming more complex with evolution. Thus, it is important to clarify the genomic structure, including 5′-flanking region that may control the expression of TTR, and the mechanism of transcription of TTR in lower vertebrates such as amphibian, but molecular evidence as to how TTR expression is controlled remains scarce. In this study, we cloned the *Xenopus laevis TTR* (*xTTR*) gene, including regulatory regions, and analyzed the expression pattern, and the role of *X. laevis* FoxA2 (xFoxA2) in controlling the xTTR expression.

2. Materials and methods

2.1. Reagents

All chemicals used in this study were of the highest grade available and were purchased from Wako (Osaka, Japan), Nacalai Tesque (Kyoto, Japan) or Kanto (Tokyo, Japan).

2.2. Animals

X. laevis tadpoles were obtained by injecting adult frogs with human chorionic gonadotropin (ASKA Pharmaceutical, Tokyo, Japan). Tadpoles were reared in dechlorinated tap water under natural lighting conditions and fed Sera Micron commercially available for tropical fishes (Heinsberg, Germany), every other day. The animals were classified according to the developmental stages outlined by Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

2.3. Screening of the genomic DNA library

The *X. laevis* partial genomic libraries constructed with DNA from liver were screened with ^{32}P-labeled *xTTR* cDNA or intron 3 region of the *xTTR* gene. Partial genomic libraries previously digested *Eco*RI or *Pst*I were ligated into the same restriction site of λgt11 and screened by plaque hybridization (Huynh et al., 1985). Recombinant DNA adsorbed on the nylon membranes was hybridized overnight at 42 °C with xTTR cDNA or intron 3 probe (1×10^8 cpm/μg) in a hybridization buffer containing 10× Denhardt's solution, 5× standard sodium citrate (SSC), 50% formamide, 50 mM HEPES (pH 7.0), and 100 μg/ml salmon sperm DNA (Sambrook et al., 1989). The nylon membranes were washed and dried. The dried membranes were autoradiographed on Fuji RX-U film for 1 day at room temperature. The inserts were subcloned into pBluescript II SK (−) and the entire sequence of the cloned genomic DNA was determined for both strands by Sanger sequencing (Sanger et al., 1977).

2.4. Southern blot analysis of X. laevis genomic DNA

Total genomic DNA from liver of adult *X. laevis* was isolated as described in Sambrook et al. (1989). Enzyme-digests of genomic DNA were subjected to electrophoresis on 1% agarose gel, and the DNA was transferred onto a nylon membrane. Southern hybridization was performed with ^{32}P-radiolabeled TTR cDNA fragment or intron 3 fragment (1×10^8 cpm/μg), with prehybridization for 2 h and hybridization for overnight at 42 °C. The filters were washed twice with 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature for 20 min each, followed by washing twice with 1× SSC, 0.1% SDS at 42 °C for 20 min each. The filters were then exposed to Kodak XAR-5 film at −70 °C, for 1–3 days.

2.5. RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the frozen liver, intestine, kidney, brain, eye, and skin of 6 tadpoles (NF52, 54, 56, 58, 60, and 62) or 2 to 4 adult frogs using the LiCl-urea procedure (Auffray and Rougeon, 1980). The amounts of specific RNA transcripts were estimated by quantitative real-time PCR using the Power SYBR Green Master Mix and ABI Prism 7000 (Applied Biosystems, Foster City, CA) after the RNA samples were treated with reverse transcriptase (TaqMan Reverse Transcription Reagents, Applied Biosystems), as previously described (Kudo and Yamauchi, 2005). Each PCR was run in triplicate alongside a control to account for PCR variation. To standardize each experiment, the amount of each gene transcript was divided by the amount of glyceraldehydephosphate dehydrogenase (GAPDH) RNA in the same sample. The primer sequences used to amplify each gene included TTR, forward 5′-GCCCTACTGGCAATTGTCTCA-3′, reverse 5′-GGCTTCT CCATGGGAAGCA-3′; FoxA2, forward 5′-GTTACCTGCGGCGACAGAA-3′, reverse 5′-TGCCTCCCCCTTCCCTAAG-3′; GAPDH forward 5′-CTCATGA CAACAGTCCATGCTTTC-3′, reverse 5′-CTCTGCCATCTCTCCACAGCTT-3′.

2.6. Construction of luciferase reporter plasmid and reporter gene assay

The 5′-flanking region of *xTTR* gene was amplified by PCR. The screened *Eco*RI fragment of *xTTR* gene was used as template. Deletion constructs were generated by deletion of the 5′-flanking region of the *xTTR* with exonuclease III (Takara Bio Inc., Shiga, Japan) and the ΔTATA construct was cloned by PCR. The reporter plasmids PGV-B-*xTTR* promoter were prepared by introducing 5′-flanking region of *xTTR* or each deletion products into the *Sma*I/*Sac*I site of PicaGene Basic Vector, PGV-B (Toyo Ink Mfg. Co. Ltd., Tokyo, Japan). The plasmid pcDNA3-xFoxA2 was prepared by introducing xFoxA2 cDNA amplified by PCR into the *Eco*RI/*Xho*I site of pcDNA3 (Invitrogen, Carlsbad, CA).

Xenopus culture cells used in this study were; XL58 derived from *Xenopus* embryos (Li et al., 1998), XL177 derived from *Xenopus* tadpole epithelium (Miller and Daniel, 1977), XTC-2 derived from *Xenopus* tadpole carcass (Pudney et al., 1973), and KR derived from *Xenopus* adult kidney (Rafferty, 1969). These cells were seeded at a density of 2×10^5/well in 24-well plates and cultured in 70% Leibovitz's L-15 medium containing 10% fetal bovine serum (FBS) (Samuels et al., 1979) for 15 h at 25 °C with air. The following day, the cells were transfected with 350 ng of PGV-B-*xTTR* promoter and 14 ng of pRL-CMV vector with 4 μl of the lipofection reagent DOSPER (Roche, Mannheim, Germany). In some experiments, KR cells were transfected with 35 ng, 3.5 ng, or 0.35 ng of pcDNA3-xFoxA2. After 24 h, the cells were replenished with 70% Leibovitz's L-15 medium containing 10% FBS and were further cultured in 70% Leibovitz's L-15 medium for 24 h. The cells from each well were harvested and assayed for firefly *Photinus pyralis* and sea pansy *Renilla reniformis* luciferase activity, derived from PGV-B-*xTTR* promoter, and pRL-CMV, respectively, using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's directions. Transfection efficiency was