

Identification of Transthyretin in Fish (*Sparus aurata*): cDNA Cloning and Characterisation

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Abstract Transthyretin (TTR) has been proposed to have first evolved in reptiles and is one of the three plasma proteins important in the transport of thyroid hormones in higher vertebrates. A full-length cDNA encoding TTR was isolated from a sea bream (*Sparus aurata*) liver cDNA library using a homologous TTR cDNA probe generated by RT-PCR. Comparison of the deduced amino acid sequence of sea bream TTR with other published sequences, revealed an overall identity of 47-54%, although the amino acids in the active binding site were almost 100% conserved. Distribution of TTR was studied in sea bream adult tissue by RT-PCR and was detected in liver, brain, pituitary, gills, kidney, intestine and testis, although northern blot analysis only revealed TTR in the liver, suggesting that in sea bream, liver is the main source of this protein. TTR was also expressed in larvae from the first day post-hatch (48h post-fertilisation). Analysis of thyroxine (T₄) and triiodo-L-thyronine (T₃) binding to sea bream serum proteins demonstrated that both T₄ and T₃ bind to albumin and TTR. By demonstrating the existence of TTR in teleost fish this study indicates TTR must have evolved in a common fish ancestor of the tetrapod evolutionary line.

The thyroid hormones, thyroxine (T₄) and triiodo-L-thyronine (T₃), are secreted from the thyroid gland and rapidly partition into lipid membranes (1). To counteract this effect and ensure an even distribution of thyroid hormones in perfused tissues (2), T₃ and T₄ are generally transported in the blood bound to serum proteins (3). In humans the main carrier of thyroid hormones is thyroxine binding globulin (TBG); in rodents it is TTR (4) and in birds both TTR and albumin are carriers. In reptiles, amphibians and fish, albumin has been proposed to transport thyroxine in the circulation (5).

The liver and the choroid plexus are the principal sites of TTR synthesis in mammals, birds and diprotodont marsupials. Because in reptiles TTR is only synthesised in the choroid plexus, it was proposed to have first evolved for the transport of thyroxine from the bloodstream to the brain (6, 7, 8).

The results from the present study demonstrate that TTR is present in teleost fish, it is synthesised mainly in the liver and is released into the circulation where it binds thyroid hormones.

Material and Methods

Animals tissues for RNA extraction and blood samples: Juvenile sea bream (mean weight 60g) were obtained from a local fish farm (MARESA, Ayamonte, Spain) and were sacrificed by decapitation. Liver, brain, pituitary, gills, kidney, intestine, ovary and testis were immediately dissected out, frozen in liquid nitrogen and stored at -70°C. Sea bream eggs and larvae were collected at daily intervals until 10 days post-hatch, frozen in liquid nitrogen and stored at -70°C. Blood samples were collected from the caudal vein of sea bream and the wing artery of chicken, left overnight at 4°C. The clot free serum was transferred to a fresh tube

and centrifuged for 30min, 2500g, 4°C. The resulting supernatant was centrifuged for 20min at 1500g, 4°C and the supernatant (blood serum) aliquoted and stored at -70°C.

cDNA library construction and screening: Total RNA was extracted from liver using the guanidium thiocyanate/acid phenol method (9). The poly (A)⁺ fraction was obtained from total RNA by chromatography on oligo-dT cellulose columns (Pharmacia Biotech) and used (5µg) in library production (cDNA synthesis kit, Pharmacia). The cDNA was ligated into Lambda ZAPII/EcoRI/CIAP vector (Stratagene) and packaged into Gigapack[®] II packaging extracts (Stratagene). Two hundred thousand recombinants were screened with a TTR cDNA probe generated by RT-PCR and labelled with [α ³²P-dCTP] (*Rediprime*, random priming labelling kit, Amersham). Plaque lifts were hybridised overnight at 55°C and washed at 65°C for 1h in 0.1xSSC, 0.1% SDS. Four positive clones were identified, automatically excised into pBluescript and two clones were sequenced (Pharmacia automated sequencer) using the universal primers of pBluescript located downstream and upstream of the cloned cDNA.

In vitro translation of cloned TTR cDNA: The purified plasmid containing TTR (1µg) was translated into protein using the TNT/T7 Quick Coupled Transcription/Translation System (Promega). The protein products were separated by SDS-PAGE (12% acrylamide). The gel was fixed in 2-propanol:water:acetic acid (25:65:10) soaked in Amplify (Amersham), dried and exposed to Kodak X-OMAT.

RT-PCR and Southern Blot: Total RNA was extracted from liver, brain, pituitary, gills, kidney, intestine, ovary and testis and from larvae using the guanidium thiocyanate/acid phenol method (9). RNA (5µg) was reverse transcribed in a 30µl reaction containing 0.05M Tris-HCl, pH8.3, 0.075M KCl, 3mM MgCl₂, 0.01M DTT, 1mM dNTP, 15pmol OligodT primer, Rnase inhibitor (3.2U; Pharmacia Biotech)

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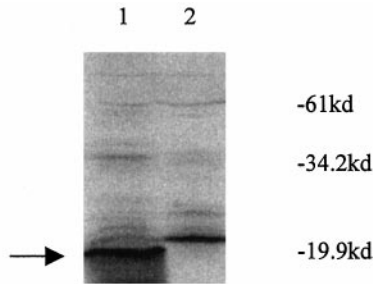


Figure 2. Separation on SDS-PAGE of the *in vitro* transcription/translation product of TTR (arrow). Lane 1- TTR plasmid (1µg) included in reaction, lane 2- no plasmid added to the reaction.

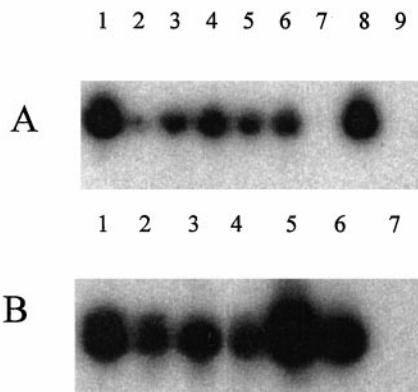


Figure 3. Southern blot of RT-PCR products (292bp) for TTR. Similar amounts of cDNA were used in PCR reactions. (A) - Sea bream tissue distribution; (B) - TTR during ontogeny. (A) 1- liver, 2- gills, 3- brain, 4- intestine, 5- kidney, 6- testis, 7- ovary, 8- pituitary, 9- negative control. (B) 1- 1 day post hatch (dph), 2- 2 dph, 3- 3 dph, 4- 6 dph, 5- 8 dph, 6- 10 dph, 7- negative control.

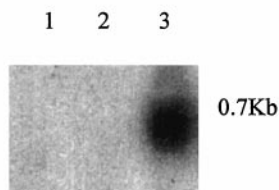


Figure 4. Northern blot analysis of sea bream TTR in 1 -kidney, 2- brain, 3- liver.

Discussion

In the present study we report the isolation of cDNA for TTR from a teleost fish, the sea bream. We have used RT-PCR to demonstrate that it has a widespread distribution in sea bream tissue, although results from northern blots indicate that liver

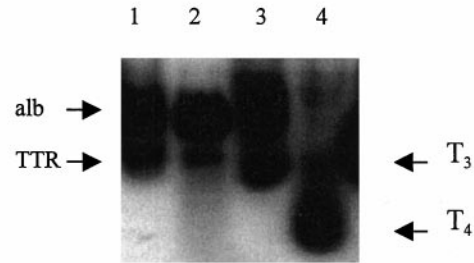


Figure 5. Analysis of serum proteins binding to T_3/T_4 . Autoradiography (1h exposure at room temperature) following non-denaturing polyacrylamide gel electrophoresis of fish and chicken serum incubated with $[^{125}I]T_3$ and $[^{125}I]T_4$. Lane 1-chicken serum/ $[^{125}I]T_3$, lane 2- chicken serum/ $[^{125}I]T_4$, lane 3-sea bream serum/ $[^{125}I]T_3$, lane 4- sea bream serum/ $[^{125}I]T_4$. The migration positions of free T_3 and T_4 , albumin (alb) and TTR are indicated by arrows.

is the main source of TTR in fish.

Multiple sequence alignments show that the overall homology of sea bream TTR with other species is low, ranging from 47% (human and pig) to 54% (chicken), although the regions corresponding to the functional domains of the protein are highly conserved. For example, the organisation of the amino acids involved in the formation of the central channel of TTR to which T_3 and T_4 bind have 90% homology to human TTR (10). The hydrophobic patch formed by the methyl groups of Leu 17, Thr 106, Ala 108, Leu 110, Thr 119, Val 121 and the group of charged residues, which includes the paired side-chains of Lys 15, Glu 54, and His 56 are present. Moreover, the amino acids involved in binding to retinol-binding-protein (RBP) (11), 20, 21, 83-85, 99, 100 and 114 are also highly conserved in sea bream TTR. The conservation of the binding sites for T_3 , T_4 and RBP and the detection of T_3 and T_4 binding to sea bream TTR, suggest that TTR in fish has a similar function to that in higher vertebrates, namely the transport of thyroid hormones and RBP.

In humans, mice and rats where the genomic sequence of TTR is known, the loss of valine (α) and leucine (3) results from a stepwise shift of the splice site between exon 1 and exon 2 in the 5' to 3' direction, causing decrease in length and hydrophobicity of the N-terminus (12). The N-terminus of sea bream TTR, in common with the avian and reptilian molecule, has three additional amino acids when compared to the eutherian proteins. The lengthening of the N-terminus of TTR in the sea bream by the amino acids DKH probably leads to a decrease in hydrophobicity. Since the N-terminus of TTR subunits are located near the entrance to the central channel of the tetrameric binding complex, alterations in this region may alter the affinity between the two thyroid hormones and TTR (12).

The presence of TTR in fish has been subject of some controversy. Larsson et al (3) have shown the presence of a

protein with the same characteristics as TTR in *Salmo salar* but Richardson et al (5) failed to find it in *Salmo trutta*, *Oncorhynchus mykiss*, *Oncorhynchus tshawytscha* and *Cyprinus carpio*. Failure to detect piscine TTR could be attributed to the use of T₄ as ligand, since our study shows that T₃, which is generally more abundant in fish plasma (13), binds to TTR better than T₄. The amphibian, *Rana catesbeiana*, also showed higher affinity for T₃ than T₄ (14).

On the basis of comparative studies of the amino acid sequence of TTR (either isolated and purified or cloned cDNAs) and mapping of gene expression, it has been proposed that TTR first evolved in the choroid plexus of the stem reptiles about 300 million years ago (5, 15). Liver TTR gene expression is suggested to have evolved much later and independently in the lineage leading to birds, Australian diprotodont marsupials, some American polyprotodont marsupials and eutherians (7, 15, 16). The results from the present study indicate it evolved much earlier than has been proposed suggesting that in reptiles and protherians there was a functional specialisation, namely that of a T₄ delivery system to the brain, while avian and eutherian species retained TTR expression in the liver.

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