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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 (T_4) and triiodo-L-thyronine (T_3) binding to sea bream serum proteins demonstrated that both T_4 and T_3 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 (T_4) and triiodo-L-thyronine (T_3), 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), T_3 and T_4 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, iot 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^{32}P-dCTP] (*Redi*prime, 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 $MgCl_2$, 0.01M DTT, 1mM dNTP, 15pmol OligodT primer, Rnase inhibitor (3.2U; Pharmacia Biotech)

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and M-MLV reverse transcriptase (20U; GibcoBRL), for two hours at 37°C. RT-PCR was carried out with this cDNA (5µl) in a 50µl reaction containing 10mM Tris-HCl pH 9.0, 50mMKCl, 0.1% Triton® X-100, 1.5mM $MgCl_2$, 1mM dNTP), 50 pmol of each primer and Taq DNA polymerase (1.25U; Promega). Primers were specific for sea bream TTR (forward primer: 5'CTCCAGCCGGATCAGTGGCA; reverse primer: 5'GCGGTGGTGGTGTAGGAGAACG). PCR products were separated on a TBE/Agarose gel (1.5%) and transferred to a nitrocellulose membrane (Hybond C, Amersham). The membrane was baked for 2h at 80°C and hybridized using full-length TTR as probe and the same procedure described for cDNA library screening. The blot was exposed for 5min at room temperature.

Northern Blot analysis: Total RNA (30µg) from liver, kidney and brain was fractionated on a 2.2% formaldehyde / 1.1% agarose gel, transferred to a nylon membrane and fixed by UV crosslinking. This membrane was hybridised at 55°C, overnight with a full length TTR cDNA probe labelled with [α^{32}P-dCTP] . The filter was washed at 60°C in 1xSSC, 0.1% SDS and exposed to Kodak X-OMAT with intensifying screens at -70°C.

Analysis of T_3/T_4 binding to serum proteins: Fish and chicken serum (5µl) were incubated in duplicate with 13.7pmol (1.5µCi) [^{125}I]T_4 or 16pmol (1.5µCi) [^{125}I]T_3 for 1h at room temperature, separated by electrophoresis on a 10% non-denaturing polyacrylamide gel in 0.05M Tris-HCl, pH8.6. The resulting gel was autoradiographed or stained with Coomassie Blue R-250.

Results

cDNA cloning and sequencing: A full length sea bream cDNA sharing high homology with previously isolated TTR cDNAs was isolated from a fish liver cDNA library. The sequence obtained (Figure 1) spans 633bp and contains an open reading frame encoding a protein of 130 amino acids preceded by a leader peptide of 20 amino acids. The 5'-untranslated region comprises 24 nucleotides and the 3'untranslated region 156 nucleotides starting at position 478, corresponding to the first in-frame stop codon. The consensus ATTAAA polyadenylation signal was located 120 bases downstream from the first in-frame stop codon and preceded a polyadenylated tail of 15 nucleotides.

In vitro translation of cloned cDNA: The molecular weight of the protein produced by *in vitro* translation of the TTR containing plasmid was 15kda (Figure 2). The molecular weight of the product is in agreement with the predicted molecular weight of TTR deduced from the nucleotide sequence and is similar to that observed in other species.

TTR expression in adult tissue and in larvae: Southern blot analysis of the PCR products (292bp) from several adult tissue and larval samples of sea bream (Figure 3 A and B),

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-20                                       M   L   Q   P   L   H      -15
  1  AGT CGG TCT ACC CAT CAG CCA GCC ATG CTT CAA CCA CTG CAC        42
-14   C   L   L   L   A   S   A   V   L   C   N   T   A   P       -1
 43  TGT CTG CTT CTA GCC TCC GCC GTG CTG TGC AAC ACC GCC CCC        84
  1   T   P   T   D   K   H   G   G   S   D   T   R   C   P       14
 85  ACC CCC ACG GAC AAA CAT GGA GGC TCA GAC ACC AGG TGT CCT       126
 15   L   M   V   K   I   L   D   A   V   K   G   T   P   A       28
127  CTG ATG GTA AAA ATC CTG GAC GCA GTG AAA GGG ACT CCA GCC       168
 29   G   S   V   A   L   K   V   S   Q   K   T   A   D   G       42
169  GGA TCA GTG GCA CTC AAA GTG TCT CAG AAG ACC GCT GAT GGA       210
 43   G   W   T   Q   I   A   T   G   V   T   D   A   T   G       56
211  GGG TGG ACA CAG ATT GCG ACC GGA GTG ACA GAT GCC ACT GGG       252
 57   E   I   H   N   L   I   T   E   Q   Q   F   P   A   G       70
253  GAG ATC CAC AAT CTG ATC ACA GAG CAG CAG TTC CCA GCA GGA       294
 71   V   Y   R   V   E   F   D   T   K   A   Y   W   T   N       84
295  GTG TAT CGT GTT GAG TTT GAC ACC AAA GCT TAC TGG ACG AAT       336
 85   Q   G   S   T   P   F   H   E   V   A   E   V   V   F       98
337  CAG GGC AGC ACG CCG TTC CAT GAA GTA GCT GAG GTG GTG TTT       378
 99   D   A   H   P   E   G   H   G   H   Y   T   L   A   L      112
379  GAC GCC CAT CCT GAA GGC CAT GGT CAC TAC ACC TTG GCT CTG       420
113   L   L   S   P   F   S   Y   T   T   T   A   V   V   S      126
421  CTG CTC AGT CCG TTC TCC TAC ACC ACC ACC GCC GTG GTC TCC       462
127   S   V   H   E   *                                          131
463  AGC GTA CAC GAG TGA CCC GCG TCA CAC TGA GAT CTT GTT TCT       504
505  CTC ATG TTC TGA TCC AAA CCA ACA TAG TAT TTT TTT TTC CAT       546
547  GCA TCT TCT TTT CCT CTG TGC CAT AAA GCT CCA TCC AAA CAA       588
589  AGA AAA ATG ATT AAA ATA CAT TGA TGA GCC AAA AAA AAA AAA       630
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Figure 1. Complete nucleotide sequence of sea bream TTR cDNA and deduced aminoacid sequence. The signal peptide is numbered –20 to –1. The N-terminal residue 1 of mature sea bream TTR was deduced from multiple sequence alignments. The first in-frame stop codon is marked with an asterisk. The consensus ATTAAA is underlined.

showed that TTR is expressed in hatching larvae and in liver, brain, pituitary, gills, kidney, intestine and testis. Only the ovary did not appear to contain TTR. However, the liver was the only tissue in which TTR was detected by northern blot where it was present as a single transcript of 0.7 Kb (Figure 4). No message could be detected in kidney or brain despite the use of similar amounts of RNA.

Analysis of T_3/T_4 binding to serum proteins: The electrophoretogram (Figure 5) obtained for chicken serum incubated with T_4 was similar to previous reports by Richardson et al (5), showing that albumin and TTR, which runs ahead of albumin, bound T_4. Our results show that chicken albumin and TTR also bind T_3. A parallel assay of sea bream serum also gave a much stronger signal of T_3 binding TTR and albumin than T_4. In chicken, as in sea bream, albumin was the main thyroid hormone binding protein.

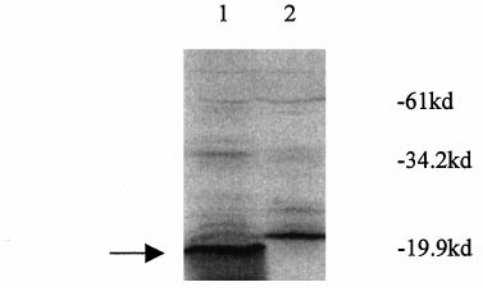

Figure 2. Separation on SDS-PAGE of the *in vitro* transcription/translation product of TTR (arrow). Lane 1- TTR plasmid (1µg) included in reaction, lane 2- no plasmid added to the reaction.

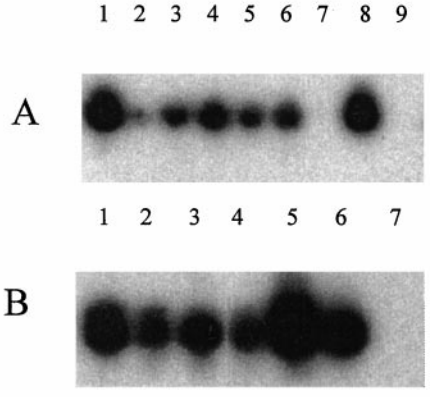

Figure 3. Southern blot of RT-PCR products (292bp) for TTR. Similar amounts of cDNA were used in PCR reactions. (A) - Sea bream tissue distribution; (B) - TTR during ontogeny. (A) 1- liver, 2- gills, 3- brain, 4- intestine, 5- kidney, 6- testis, 7- ovary, 8- pituitary, 9- negative control. (B) 1- 1 day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

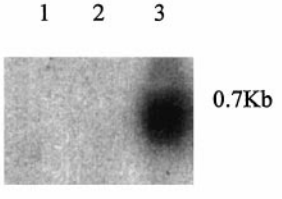

Figure 4. Northern blot analysis of sea bream TTR in 1 -kidney, 2- brain, 3- liver.

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

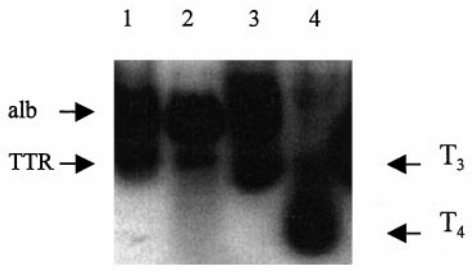

Figure 5. Analysis of serum proteins binding to T_3/T_4. Autoradiography (1h exposure at room temperature) following non-denaturing polyacrylamide gel electrophoresis of fish and chicken serum incubated with [^{125}I]T_4 and [^{125}I]T_3. Lane 1-chicken serum/ [^{125}I]T_3, lane 2- chicken serum/ [^{125}I]T_4, 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 T_4 as ligand, since our study shows that T_3, which is generally more abundant in fish plasma (13), binds to TTR better than T_4. The amphibian, *Rana catesbeiana,* also showed higher affinity for T_3 than T_4 (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 T_4 delivery system to the brain, while avian and eutherian species retained TTR expression in the liver.

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