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REVIEW

Evolution of the Thyroid Hormone-Binding Protein, Transthyretin

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Transthyretin (TTR) belongs to a group of proteins, which includes thyroxine-binding globulin and albumin, that bind to and transport thyroid hormones in the blood. TTR is also indirectly implicated in the carriage of vitamin A through the mediation of retinol-binding protein (RBP). It was first identified in 1942 in human serum and cerebrospinal fluid and was formerly called prealbumin for its ability to migrate faster than serum albumin on electrophoresis of whole plasma. It is a single polypeptide chain of 127 amino acids (14,000 Da) and is present in the plasma as a tetramer of noncovalently bound monomers. The major sites of synthesis of TTR in eutherian mammals, marsupials, and birds are the liver and choroid plexus but in reptiles it is synthesised only in the choroid plexus. The observation that TTR is strongly expressed in the choroid plexus but not in the liver of the stumpy-tailed lizard and the strong conservation of expression in the choroid plexus from reptiles to mammals have been taken as evidence to suggest that extrahepatic synthesis of TTR evolved first. The identification and cloning of TTR from the liver of an amphibian, *Rana catesbeiana,* and a teleost fish, *Sparus aurata,* and its absence from the choroid plexus of both species suggest an alternative model for its evolution. Protein modelling studies are presented that demonstrate differences in the electrostatic characteristics of the molecule in human, rat, chicken, and fish, which may explain why, in contrast to TTR from human and rat, TTR from fish and birds preferentially binds triiodo-L-thyronine.

Key Words: distribution and function; evolution; structure; transthyretin; thyroid hormones.

THYROID GLAND AND HORMONES

Thyroid hormones (TH) have been identified in the plasma of all groups of vertebrates; they are pluripotent and are involved in growth, differentiation, metamorphosis, reproduction, hibernation, and thermogenesis. The basic unit of all vertebrate thyroid glands has been conserved throughout evolution and consists of a follicle, formed by a single layer of epithelial cells enclosing a fluid-filled space or "colloid." The follicle traps inorganic iodide, which is incorporated into thyroid hormone precursors, which are stored in the colloid. The organisation of the gland is characteristic for each vertebrate group; in mammals the thyroid is composed of two lobes connected by an isthmus across the ventral surface of the trachea; birds have two isolated, rounded lobes lying on either side of the trachea; in snakes and turtles there is a single discoid structure located anterior to the heart; the amphibian thyroid is bipartite; and in most bony fish there is no organised gland and the follicles are scattered singly

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or in small groups in the loose connective tissue of the pharynx (Bentley, 1998; Gorbman *et al.*, 1983). Thyroid-like hormones are also apparently synthesised by nonfollicular tissue in the endostyle of protochordates and urochordates (Barrington, 1962; Monaco *et al.*, 1981; Thorndyke, 1978; Dunn, 1980).

TH have a common chemical structure (Fig. 1) consisting of a hydrophobic thyronine nucleus, which accounts for their poor water solubility, a hydrophilic hydroxyl group attached to the phenolic ring and four iodines in positions 3, 5, 3′, and 5′ in thyroxine (T_4) and three similarly at 3, 5, and 3′ in triiodo-l-thyronine (T_3). Inactivation of TH occurs by the mediation of iodothyronine-deiodinase, resulting in deiodination of the inner ring and production of a series of inactive metabolites e.g. reverse T_3, di, and monoiodothyronine, as well as totally unsubstituted thyronine. T_4 is the main secreted product of the vertebrate thyroid gland and deiodination of its outer ring (ORD) produces the more active triiodo-l-thyronine (T_3), which has a much higher affinity for the thyroid hormone receptors (Leonard and Visser, 1986; Darras *et al.*, 1998).

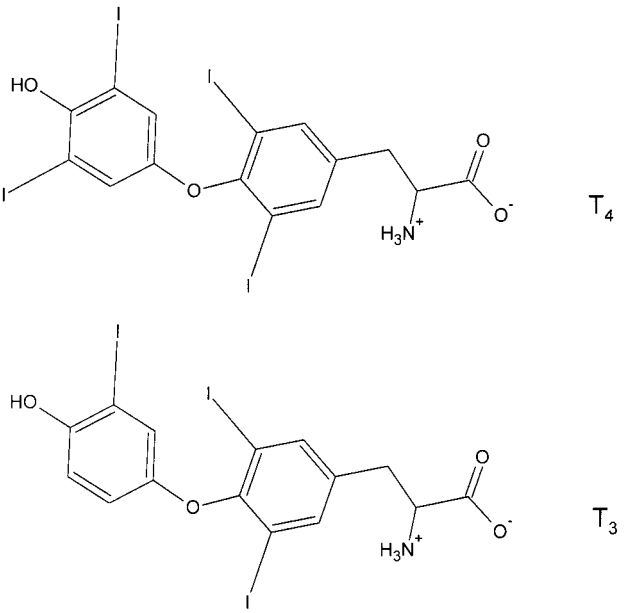


FIG. 1. The structure of the thyroid hormones, T_4 (top) and T_3 (bottom).

THYROID HORMONE-BINDING PROTEINS

The synthesis and secretion of thyroid hormones appear to be similar in all vertebrates studied. Thyroid hormones are stored bound to thyroglobulin in the follicular colloid. Hydrolysis of iodothyroglobulin in the epithelial cell layer liberates principally T_4, which diffuses into the surrounding capillaries and rapidly partitions into lipid membranes (Dickson *et al.*, 1987; Eales, 1985; Hillier, 1970). To counteract this effect and ensure an even distribution of thyroid hormones in perfused tissues (Mendel *et al.*, 1987, Mendel and Weisiger, 1990), T_3 and T_4 are generally transported in the blood bound to serum proteins (Larsson *et al.*, 1985; Robbins and Edelhoch, 1986). In the blood of larger mammals three main blood plasma thyroid hormone-binding proteins have been identified—thyroxine-binding globulin (TBG), transthyretin (prealbumin, TTR), and serum albumin (ALB) (Larsson *et al.*, 1985)—and their binding characteristics have been determined (see Table 1). In humans TBG is present in the circulation at lower concentrations than other binding proteins; nevertheless it carries about 75% of all plasma T_4 as a consequence of its greater affinity for the hormone (100-fold higher; Robbins and Edelhoch, 1986). In rodents TTR is the major carrier of T_4 and in birds both TTR and ALB are carriers. There are relatively few studies of thyroid hormone-binding proteins in lower vertebrates. Moreover the results from such studies appear contradictory (Larsson *et al.*, 1985; Richardson *et al.*, 1994; Tanabe *et al.*, 1969). In one study radiolabelled T_4 was incubated with plasma from adult reptiles, amphibia, and fish and the resulting products were subsequently fractionated by SDS–PAGE followed by autoradiography. Only one protein, ALB, bound the radiolabeled T_4 (Richardson *et al.*, 1994) and it has been proposed that this protein is the principal thyroid hormone-binding protein in the blood of such organisms. The identification in salmon of thyroid hormone-binding proteins with characteristics similar to transthyretin (Larsson *et al.*, 1985) and the recent isolation of TTR and its cDNA from amphibian tadpoles before the metamorphic climax (*Rana catesbeiana*, Yamauchi *et al.*, 1993, 1998) and from the teleost fishes sea bream (Santos and Power, 1996, 1999) and masu salmon (*Oncorhychus masu*) prior to smoltification (Yamauchi *et al.*, 1999) suggest that it may also have a role in transporting TH in these species. Since

TABLE 1
Principal Characteristics of Plasma Proteins Involved in the Transport of Thyroid Hormone in Humans

Binding protein	Serum concentration (mg/liter)	Binding capacity (μg/liter plasma)	Relative affinity for T4 and (/) T3	Molecular weight of binding protein (kDa)	Number of peptide chains in each binding protein molecule
Thyroxine binding globulin (TBG)	15	200	100/9.0[a]	54	1
Transthyretin (TTR)	300	2300	100/9.2[b]	54	4
Albumin (ALB)	45,000	100	100/49.0[c]	66	1

Note. K_A is the affinity constant. Table adapted from de la Paz *et al.* (1992).
[a] Snyder *et al.* (1976): $K_A\ T_4 = 2.5 \times 10^9\ M^{-1}$.
[b] Andrea *et al.* (1980): $K_A\ T_4 = 3.5 \times 10^7\ M^{-1}$.
[c] Sterling (1964): $K_A\ T_4 = 5.0 \times 10^5\ M^{-1}$.

in teleost fish and amphibia, TTR appears to have a higher affinity for T_3 than for T_4 (Yamauchi *et al.*, 1993, 1999), the contradictory observations mentioned earlier may be explained by suggesting that albumin may be the principal T_4-binding protein and TTR the principal T_3-binding protein. The relative importance of these two thyroid hormone-binding proteins at different stages of the life cycle of different organisms remains to be determined, particularly as earlier studies were carried out with plasma samples from adult teleost fish (Richardson *et al.*, 1994) while more recent studies have been carried out with samples from juvenile teleost fish (Santos and Power, 1996, 1999; Yamauchi *et al.*, 1993, 1998, 1999). The identification of TTR in fish other than teleosts and the characterisation of its function will be important in defining its role in TH transport in fish. Throughout this review the term fish indicates teleosts, the only group from which this protein and its gene have been characterized.

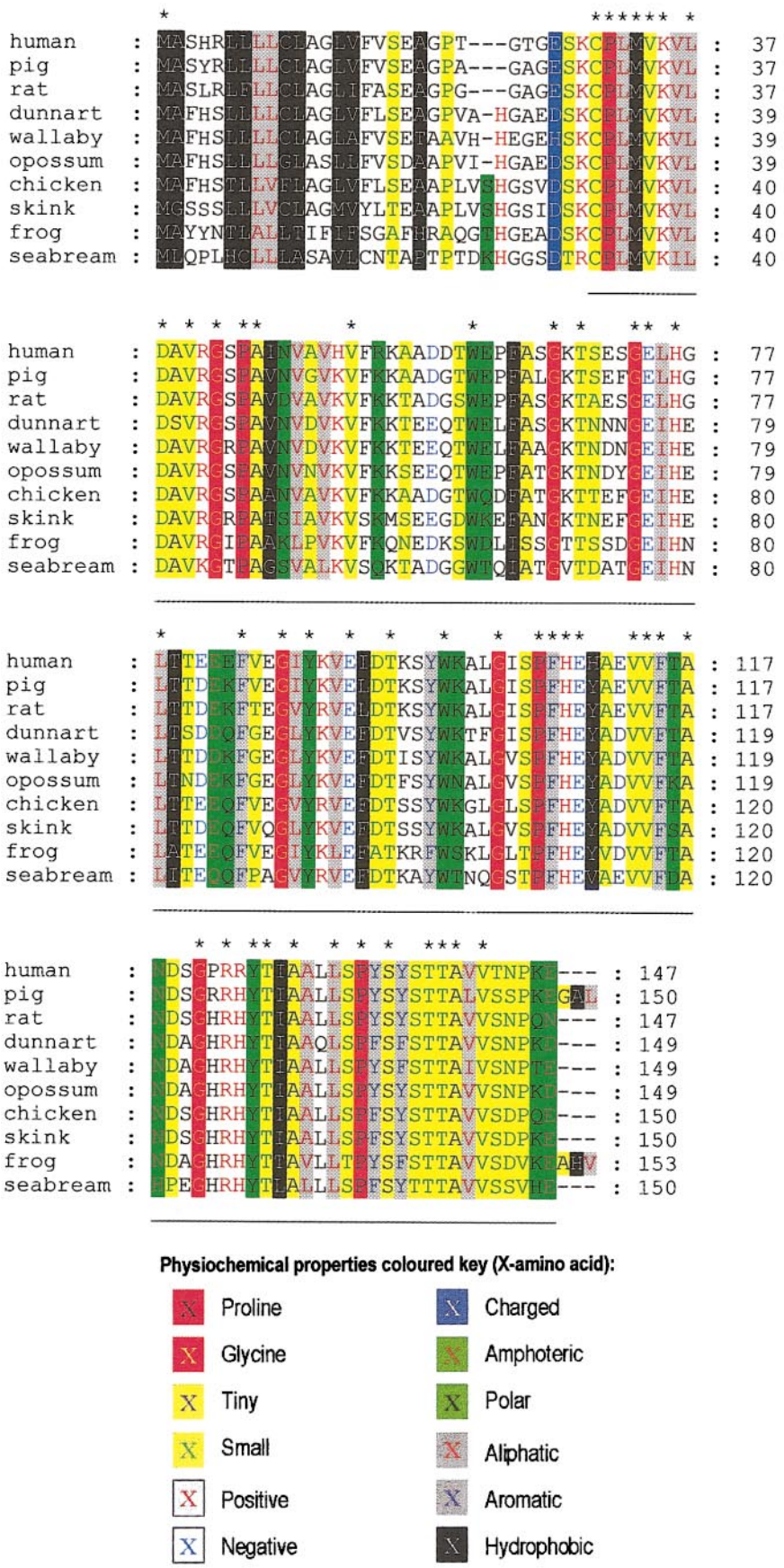
TRANSTHYRETIN—CHEMISTRY AND STRUCTURE

The chemistry and structure of human TTR are well characterised because of its stability and relative abundance, rendering it easy to purify in sufficient quantities for study. Moreover, its importance in a human disease, familial amyloidotic polyneuropathy (FAP), has also provided further incentive for study. There is an abundance of structural studies of this protein in humans and mouse but few in other species.

Molecular Structure

TTR is a 127-residue monomer that has been shown, in human and chicken, to function in the form of a 55-kDa tetramer composed of four identical subunits. The resolution of the crystal structure of human, chicken, and rat TTR has been determined at 1.8 ÅA (Blake *et al.*, 1974, 1978; Wojtczak *et al.*, 1992; Hamilton *et al.*, 1993), 2.9 ÅA (Sunde *et al.*, 1996), and 2.5 ÅA (Wojtczak, 1997), respectively. Moreover, X-ray diffraction indicates that the dominant secondary structure is the β-sheet. Such studies have revealed that the functional tetramer contains two binding sites that are structurally identical and are deeply buried in a narrow cylindrical channel that runs through the centre of the protein molecule. The amino acids that are thought to be in-

FIG. 2. Multiple sequence alignment carried out using Clustal X (Gibson *et al.*, 1994) of transthyretin with the prepeptide from representative species of all vertebrate groups: human (Sasaki *et al.*, 1985), pig (*Sus scrofa,* Duan *et al.*, 1995), rat (*Rattus norvegicus,* Dickson *et al.*, 1985), dunnart (*Sminthopsis macroura,* Duan *et al.*, 1995), wallaby (*Macropus eugenii,* Brack *et al.*, 1995), opossum (*Monodelphis domestica,* Duan *et al.*, 1995), chicken (*Gallus gallus,* Duan *et al.*, 1991), skink (*Tiliqua rugosa,* Achen *et al.*, 1993), frog (*Rana catesbeiana,* Yamauchi *et al.*, 1998), and sea bream (*Sparus aurata,* Santos and Power, 1999). Coloured blocks refer to the physiochemical properties of the amino acids according to the key. The underlined residues correspond to the region modelled. Residues marked with an asterisk are identical in all species analysed.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pig : MASYRLLLLCLAGLVFVSEAGPA---GAGESKCPLMVKVL : 37
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dunnart : MAFHSLLLLCLAGLVFLSEAGPVA-HGAELSKCPLMVKVL : 39
wallaby : MAFHSLLLLCLAGLAFVSETAAVH-HEGEESKCPLMVKVL : 39
opossum : MAFHSLLLLGLASLLFVSDAAPVI-HGAEDSKCPLMVKVL : 39
chicken : MAFHSTLLVFLAGLVFLSEAAPLVSHGSVDSKCPLMVKVL : 40
skink : MGSSSLLLVCLAGMVYLTEAAPLVSHGSIDSKCPLMVKVL : 40
frog : MAYYNTLALLTIFIFSGAFHRAQGTHGEADSKCPLMVKVL : 40
seabream : MLQPLHCLLLASAVICNTAPTPTDKHGGSDTRCPLMVKIL : 40
human : DAVRGSPAINVAVHVFKKAADDTWEPFASGKTSESGELHG : 77
pig : DAVRGSPAVNVGVKVFKKAADGTWEPFALGKTSEFGELHG : 77
rat : DAVRGSPAVDVAVKVFKKTADGSWEPFASGKTAESGELHG : 77
dunnart : DSVRGSPAVNVDVKVFKKTEEQTWELFAAGKTNNNGEIHE : 79
wallaby : DAVRGRPAVNVDVKVFKKTEEQTWELFAAGKTNDNGEIHE : 79
opossum : DAVRGSPAVNVNVKVFKKSEEQTWEPFATGKTNDYGEIHE : 79
chicken : DAVRGSPAANVAVKVFKKAADGTWQDFATGKTTEFGEIHE : 80
skink : DAVRGRPATSIAVKVSKMSEEGDWKEFANGKTNEFGEIHE : 80
frog : DAVRGIPAAKLPVKVFKQNEDKSWDLISSGTTSSDGEIHN : 80
seabream : DAVKGTPAGSVALKVSQKTADGGWTQIATGVTDATGEIHN : 80
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pig : LTTDEKFVEGIYKVELDTKSYWKALGISPFHEYAEVVFTA : 117
rat : LTTDEKFTEGVYRVELDTKSYWKALGISPFHEYAEVVFTA : 117
dunnart : LTSDTQFGEGLYKVEFDTVSYWKTFGISPFHEYADVVFTA : 119
wallaby : LTTDDKFGEGLYKVEFDTISYWKALGVSPFHEYADVVFTA : 119
opossum : LTNDEKFGEGLYKVEFDTFSYWNALGVSPFHEYADVVFKA : 119
chicken : LTTEEQFVEGVYRVEFDTSSYWKGLGLSPFHEYADVVFTA : 120
skink : LTTDEQFVQGLYKVEFDTSSYWKALGVSPFHEYADVVFSA : 120
frog : LATEEQFVEGIYKLEFATKRFWSKLGLTPFHEYVDVVFTA : 120
seabream : LITEEQFPAGVYRVEFDTKAYWTNQGSTPFHEYAEVVFDA : 120
human : NDSGPRRYTIAALLSPYSYSTTAVVTNPKE--- : 147
pig : NDSGRRHYTIAALLSPYSYSTTALVSSPKEGAL : 150
rat : NDSGHRHYTIAALLSPYSYSTTAVVSNPQN--- : 147
dunnart : NDAGHRHYTIAAQLSPFSFSTTAVVSNPKD--- : 149
wallaby : NDAGHRHYTIAALLSPYSFSTTAIVSNPTE--- : 149
opossum : NDAGHRHYTIAALLSPYSYSTTAVVSNPKD--- : 149
chicken : NDSGHRHYTIAALLSPFSYSTTAVVSDPQE--- : 150
skink : NDSGHRHYTIAALLSPFSYSTTAVVSDPKE--- : 150
frog : NDAGHRHYTIAVLLTPYSFSTTAVVSDVKEAHV : 153
seabream : PPEGHRHYTLALLLSPFSYTTTAVVSSVHE--- : 150
Physiochemical properties coloured key (X-amino acid):
X Proline
X Glycine
X Tiny
X Small
X Positive
X Negative
X Charged
X Amphoteric
X Polar
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X Aromatic
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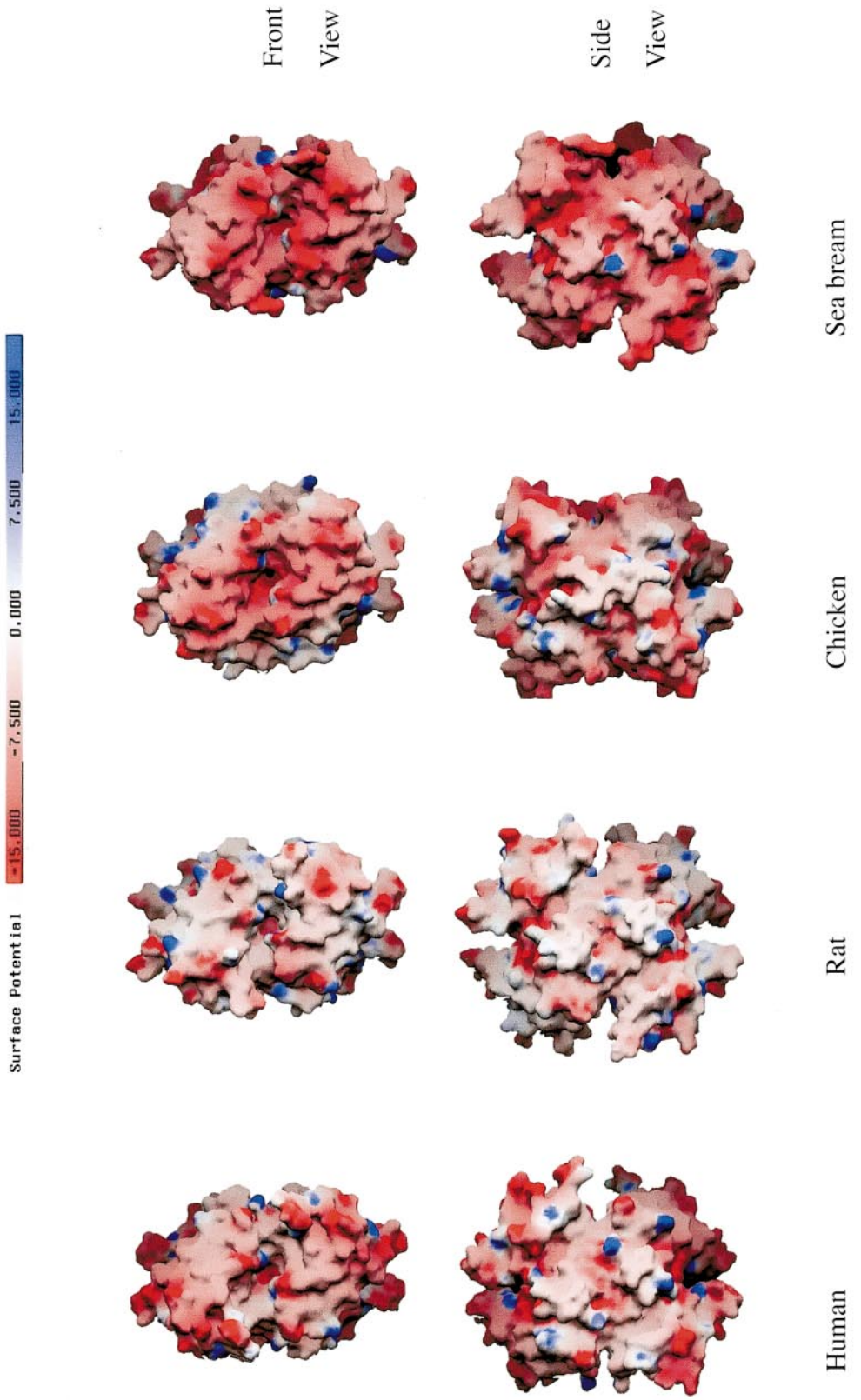

FIG. 4. Surface electrostatic characteristics (Nicholls, 1992) of TTR from different species (the potential values range from −15 to +15 kT/e). The proteins are oriented as follows: (a) front view, with one active site facing; (b) side view (rotated 90° from a).

TABLE 2

Sequence Identity (First Line of Each Row, %) and Similarity (Second Line of Each Row, %) Based on Clustal X (Gibson *et al.,* 1994) Alignments between Transthyretins from Eutherian Mammals—Human (Sasaki *et al.,* 1985), Pig (*Sus scrofa,* Duan *et al.,* 1995), and Rat (*Rattus norvegicus,* Dickson *et al.,* 1985); Marsupial Mammals—Dunnart (*Sminthopsis macroura,* Duan *et al.,* 1995) (Australian Polyprotodonta), Wallaby (*Macropus eugenii,* Brack *et al.,* 1995) (Australian Dyprotodonta), and Opossum (*Monodelphis domestica,* Duan *et al.,* 1995) (American Polyprotodonta); Birds—Chicken (*Gallus gallus,* Duan *et al.,* 1991); Reptiles—Skink (*Tiliqua rugosa,* Achen *et al.,* 1993); Amphibians—Frog (*Rana catesbeiana,* Yamauchi *et al.,* 1998); and Fish—Sea Bream (*Sparus aurata,* Santos and Power, 1999)

	Pig	Rat	Dunnart	Wallaby	Opossum	Chicken	Skink	Frog	Sea bream
Human	85	82	70	69	70	73	66	62	48
	92	92	85	81	83	86	84	67	67
Pig		84	71	71	73	73	68	54	48
		91	84	80	84	85	83	68	66
Rat			72	71	72	74	67	51	52
			87	82	85	85	84	67	70
Dunnart				83	82	74	71	54	48
				91	92	87	86	73	67
Wallaby					83	72	70	54	47
					87	83	84	71	64
Opossum						74	72	54	49
						88	86	72	68
Chicken							79	57	55
							90	73	70
Skink								53	52
								67	68
Frog									47
									64

volved in T_4 binding appear to have been conserved between the human and the chicken TTR (Duan *et al.,* 1991) and two identical binding sites for T4 and T3 are buried deep in the central channel. The chemistry of the channel is characterised by three elements arranged linearly along it: (1) a hydrophilic patch formed by the hydroxyls of Ser 117 and Thr 119, (2) a hydrophobic patch formed by the methyl groups of the Leu 17, Thr 106, Ala 108, Leu 110, Thr 119, and Val 121 pairs; and (3) a group of charged residues including the paired side chains of Lys 15, Glu 54, and His 56 (Blake and Oatley, 1977). The proximity of the paired side chains of Leu 110, Ser 115, and Ser 117 causes a marked local constriction.

A model of the three-dimensional structure of bullfrog (*R. catesbeiana*) TTR has been reported in which the crystal structure coordinates of chicken TTR were used. The resulting model showed that despite differences in the primary amino acid sequence of bullfrog TTR, the three-dimensional structure is highly conserved at the thyroid hormone-binding sites and other important structural regions of the subunits (Yamauchi *et al.,* 1998).

Three-Dimensional Model of Sea Bream TTR

A detailed model of sea bream TTR structure has been generated using the program Modeller version 4 (Sali and Blundell, 1993), together with the known X-ray structures of TTR from human (Hamilton *et al.,* 1993) (PDB ID: **1tta**), rat (Wojtczak, 1997) (PDB ID: **1gke**), and chicken (Sunde *et al.,* 1996) (PDB ID: **1tfp**) and the sequence alignment (Fig. 2). The first 12 residues of the sea bream structure were not modelled since there were no relevant structural data for this region from crystallised human, rat, or chicken TTR. This approach was possible as a consequence of the high level of identity between the amino acid sequence of TTR from the species used (Table 2). Several models were generated and the best one was selected, on the basis of criteria, such as final PDF value, restraint violations, and conformational analysis performed with PROCHECK (Laskowski *et al.,* 1993). The backbone coordinates were obtained from Modeller and since homology-based methods are not necessarily the best strategy for side chain modelling in high-quality structural models (Burke *et al.,* 1999), the side

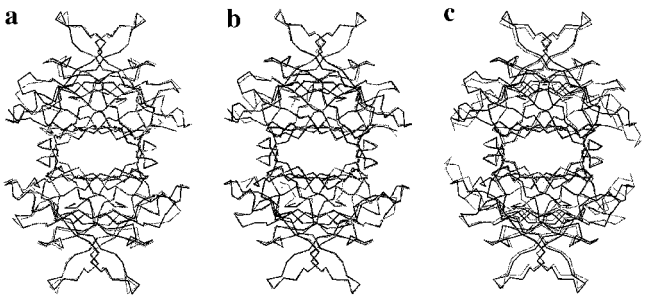

FIG. 3. Comparison between the α-carbon tracing of the TTR structure from sea bream (solid black lines) and the known crystallographic coordinates for human, rat, and chicken (grey lines). The models were produced with Molscript (Kraulis, 1991) and Raster 3D (Merrit and Bacon, 1997). The modelled structures are viewed down the central channel with one thyroid hormone-binding site facing. (a) Comparison between sea bream and human. (b) Comparison between sea bream and rat. (c) Comparison between sea bream and chicken.

chain conformations were modelled using alternative rotamer-based techniques (Mendes *et al.*, 1999a,b).

The structure of sea bream TTR is compared with the corresponding structures from human, rat, and chicken in Fig. 3. The modelled sea bream sequence shows greatest identity with chicken (63.3% versus 55.0% in human) but highest similarity with human (80.0% versus 76.6% in chicken). In common with the predicted three-dimensional structure of bullfrog TTR (Yamauchi *et al.*, 1998) the overall topology of sea bream TTR is conserved and the predicted monomer–monomer and dimer–dimer interfaces and tetrameric structure are similar to those determined by crystallography of human, rat, and chicken TTR. The modelled sea bream TTR structure shows greater similarity with those of human and rat in a defined region, amino acids 74–89, which in chicken is most dissimilar from the mammalian structure. The thyroid hormone-binding site in sea bream is also highly conserved, although Ser 117 (human sequence) is substituted by a Thr residue and given the symmetry of the tetramer, this means that there will be two substitutions per active site. This alteration may have consequences for thyroid hormone binding, as these residues are close to each other in the tetramer.

Despite the high conservation of the structures, analysis of the electrostatic properties of the TTR tetramer models (Fig. 4) shows that the surface potential, most noticeably in the thyroid hormone-binding site, is more negative in the sea bream and chicken structures (sea bream being the most negative) than in the human and rat. Although further studies are required to validate the models it is possible that the differences in electrostatic potential of TTR may be the reasons for reduced T_4 binding by TTR in chicken and fish (Chang *et al.*, 1999; Santos and Power, 1999; Yamauchi *et al.*, 1999), since the OH group of T_4 is predominantly ionised at physiological pH (Nilsson and Peterson, 1971), and hence negative, while in T_3 is predominantly neutral. An increased negative potential in the TTR structures of sea bream and chicken may be an unfavourable factor for T_4 binding.

In humans the association constants of l-thyroxine for the two hormone-binding sites of the functional TTR tetramer differ (Table 1). The difference in affinity between the two sites, despite structural identity, has been explained in terms of a negative cooperative effect, so that the binding of ligand at one site reduces the binding affinity at the second site (de la Paz *et al.*, 1992). This may arise from electrostatic interactions between the two molecules of the hormone when bound.

Ligand-binding studies with TTR have generally been carried out using compounds structurally similar to T_4, such as the fluorescent probes 8-anilinonaphthalene-1-sulphonate (Cheng *et al.*, 1977) and 1-dimethylaminonaphthalene-5-sulphonate (Nilsson *et al.*, 1975). Such binding studies are unlikely to mirror perfectly the binding of T_4 to TTR and *in vivo* the blood concentrations of the hormone are not high enough for the second hormone-binding site to be occupied. T_3 and a number of hormone analogues also bind to both sites in mammalian TTR but with a much lower affinity than T_4.

Transthyretin and Retinol-Binding Protein Interaction

TTR also binds retinol-binding protein (RBP), the specific carrier of all-*trans*-retinol. RBP is a single polypeptide chain of 182 amino acid residues of 21 kDa (Goodman, 1984) that forms a complex with tetrameric TTR under physiological conditions and that prevents glomerular filtration of RBP in the kidney (Kanai *et al.*, 1968; Peterson, 1971). The presence of retinol bound to RBP is essential for the formation of

a stable complex with TTR and crystallographic studies have established the stoichiometry of the complex as a maximum of two RBP molecules per TTR tetramer (Noy *et al.*, 1992; van Jaarsveld *et al.*, 1973; Monaco *et al.*, 1995). The RBP–TTR complex is proposed to be the physiological circulatory retinol transport complex of humans and other terrestrial vertebrates (Vieira *et al.*, 1995). The elimination of the normal RBP–TTR complex, as occurs in the TTR-null mouse model (Episkopou *et al.*, 1993), or by treatment with fenretinide (Berni and Formelli, 1992), results in a significant reduction in plasma RBP and consequently retinol. Despite low plasma levels of retinol (<6% normal) the embryos of TTR-null mice develop normally and have similar viability and fertility to mice carrying the normal TTR gene (Wei *et al.*, 1995). Adult monotremes and Australian polyprotodont marsupials also normally appear to lack plasma TTR, as demonstrated by the absence of T_4 binding (Schreiber and Richardson, 1997), but the consequence of this for the maintenance of normal plasma RBP and retinol levels is unclear. Concentrations of RBP and retinol have not been determined in these species and further studies would provide valuable information concerning the role of the retinol–RBP–TTR complex in retinoid transport.

The possibility of RBP–TTR interaction in fish plasma still requires investigation. RBP has been isolated from several fishes (Shidoji and Muto, 1977; Muto *et al.*, 1982) and in rainbow trout (*Oncorhynchus mykiss*, Berni *et al.*, 1992) it has been shown that despite low sequence conservation (60% identity) with phylogenetically distant vertebrates, RBP has a similar structural organisation. Moreover, rainbow trout, but not *Seriola quinqueradiata*, RBP can interact with mammalian TTR (Berni *et al.*, 1992). As such studies were carried out with heterologous TTR their biological significance is unclear.

Comparison of the model of sea bream TTR with the structure in mammals and bird indicates, as noted, that in evolution there has been an overall decrease in negative electrostatic potential (Fig. 4) of the tetramer. However, an effect of the electrostatic potential differences observed in TTR from different vertebrate groups on the binding of RBP is not obvious, since analysis of the contact zone for RBP in mammals, birds, and fish suggests neutral electrostatic characteristics. Moreover, there has been conservation of the shape of this domain between TTR of fish, mammals, and birds. The conservation of these characteristics may explain why trout RBP can interact with mammalian TTR (Berni *et al.*, 1992) and suggests a strong selection pressure to retain the structure of this domain. The biological and evolutionary significance of this conservation remains to be determined.

GENE STRUCTURE OF TRANSTHYRETIN IN MAMMALS, MARSUPIALS, BIRDS, AND LIZARDS

Genomic Structure

The genomic structures of TTR have been described in human (Sasaki *et al.*, 1985, 1989), rat (Fung *et al.*, 1988), and mouse (Costa *et al.*, 1986). Human TTR is a single-copy gene, which has been mapped to chromosome 18q11.2–q12.1 (Sparkes *et al.*, 1987), spans 6.9 kb with four exons, three introns, a TATA box-like sequence at nt −24–30 and CAAT box-like sequence at −95–102 (Sasaki *et al.*, 1985; Tsuzuki *et al.*, 1985). The first exon encodes the 5′ untranslated region and contains an 18-amino-acid signal peptide as well as the first 3 amino acid residues of the mature protein, whilst exon 2 codes for residues 4–47, exon 3 for residues 47–92, and exon 4 for residues 93–127.

The rat and mouse genes are also composed of four exons, contain a TATA-box and CAAT-box sequence, and have a gene organisation similar to that found in the human. The 5′ flanking region of the gene contains a short DNA segment, the -50 to 190 region, which is extremely well conserved (93%) between human and rodents (Costa *et al.*, 1986; Fung *et al.*, 1988). It has been proposed that this region is important for the cell-specific regulation of the gene and binding sites for HNF-1, C/EBP, HNF-3, HNF-4, and HNF-6 (Costa *et al.*, 1989; Samadani and Costa, 1996). In addition to these, further regulatory elements, at least 2000 bp upstream of the gene, may be involved in the regulation of gene expression in human and mouse liver via both *cis*-acting and *trans*-acting factors. Studies with transgenic mice have identified two regions, a 100-nucleotide enhancer located -2 kb upstream and a proximal −150 to 90-bp promoter region, sufficient for

directing TTR expression in the liver (Costa *et al.,* 1989; Yan *et al.,* 1990). Abundant TTR gene expression also occurs in the choroid plexus of mammals, birds, and reptiles, but little is known about the regulatory mechanisms. The genomic organisations for TTR of amphibians and fish have not yet been determined but gene expression is restricted mainly to the liver. Comparative studies between the TTR gene from fish and amphibians and that from eutherians, birds, and lizards may provide a means of identifying elements in the 5′ region that regulate TTR expression in the choroid plexus.

cDNA Structure

The cDNA for transthyretin has now been cloned from over 10 different species (Schreiber and Richardson, 1997). A single mRNA transcript has been described in all species and transcript size varies from 0.65 kb in the Australian diprotodont marsupial, sugar glider (*Petaurus breviceps,* Duan *et al.,* 1995), to 0.7 kb in rat (Dickson *et al.,* 1985). The cDNA generally consists of a 5′ untranslated region (14–30 nucleotides), a coding region that corresponds to 127–130 amino acids, and a 3′ untranslated region (115–181 nucleotides) preceding the poly(A) tail. The size of the mature protein is 130 amino acids in the pig (Duan *et al.,* 1995); 127 amino acids in the rat (Dickson *et al.,* 1985), human (Mita *et al.,* 1984; Soprano *et al.,* 1985; Wallace *et al.,* 1986), mouse (Wakasugi *et al.,* 1985), and wallaby (*Macropus eugenii,* Brack *et al.,* 1995); 129 amino acids in short-tailed grey opposum (*Monodelphis domestica*), stripe-faced dunnart (*Sminthopsis macroura*), and sugar glider (Duan *et al.,* 1993); and 130 amino acids in the chicken (Duan *et al.,* 1991), lizard (Achen *et al.,* 1993), bullfrog (Yamauchi *et al.,* 1998), and teleost fish (Santos and Power, 1999). Comparison of the amino acid sequence of TTR from mammals and marsupials with TTR from birds, amphibians, and fish demonstrates the presence in the latter species of 3 additional amino acids at the N-terminus, Val-Ser-His in chicken and lizard; Gly-Thr-His in frog, and Asp-Lys-His in fish. Studies of the 5′ organisation of the gene in eutherians and birds suggest that this is the region that has changed most distinctly during evolution. Changes in the splice sites of intron 1 have led to the production of TTR with a shorter more hydrophilic N-terminal amino acid sequence (Aldred *et al.,* 1997); the effect of this alteration on function remains to be determined. However, TH-binding studies with plasma from birds and mammals show that TTR in birds preferentially binds T_3 while in mammals it preferentially binds T_4, and it has been suggested that the change in the N-terminal amino acid sequence of TTR may be responsible for the change in hormone affinity (Chang *et al.,* 1999). The longer, more hydrophobic N-termini correlate with preferential binding to T_3 and the shorter, more hydrophilic N-termini preferential binding to T_4 (Chang *et al.,* 1999, Yamauchi *et al.,* 1999). The relative importance of the molecular electrostatic potential or the nature of the N-terminus on the binding characteristics of TTR is unclear and to date is an unresolved question.

The TTR mRNA encodes a pro-TTR monomer. The N-terminal region is a hydrophobic signal peptide that, depending on the species, consists of up to 20 amino acids (Soprano *et al.,* 1985). Pro-TTR undergoes a cleavage process during its migration through the endoplasmic reticulum membrane to yield the native TTR monomer (Soprano *et al.,* 1985). Multiple alignment of the sequence from representative species reveals the very high conservation that has occurred in this protein (Fig. 2) between eutherians, marsupials, birds, and lizards (85–65%). The sequence conservation between TTR from the latter species and amphibian and fish is much lower (47–48% identity, Table 2). However, if the protein sequences from various species are compared considering conservative amino acid substitutions, there is a substantial increase in similarity between sequences, suggesting that the overall chemical properties of the protein have been conserved (see Fig. 2) during its evolution.

Transthyretin Sequence Heterogeneity

TTR is encoded by a single gene that gives rise to a single protein product. However, in human TTR there is considerable sequence heterogeneity as a consequence of mutations resulting in substitution of a single residue at various positions of the normal amino acid sequence. These mutations may be nonpathogenic or they may cause familial amyloidotic polyneuropathy, an hereditary disease characterised by extracellular deposition of transthyretin-derived amyloid

in various tissues (peripheral nervous system, heart, vitreous body of the eye); see reviews by Sipe (1992); Benson and Uemichi (1996); Ingenbleek and Young (1994), and Schreiber and Richardson (1997). Several mutation "hotspots" have been postulated within the coding sequence of TTR (Gly6Ser, Val30Met, Arg104Cys, Ala109Thr, Thr119Met, Val122Ile, residue position in the mature protein after removal of the 20-residue prepeptide), although most mutations identified so far are not associated with these hotspots and are evenly distributed along the molecule.

Comparison of the primary sequence of TTR from different vertebrate species with that of human variants demonstrates that some of the mutations that cause disease in human are a normal feature of the protein in other species. For example, the mutations identified in human TTR that lead to the substitution of Val30Leu and Ile84Ser are normally present in sea bream TTR and Ile68Leu occurs in skink TTR (Fig. 2). Comparison of human TTR variants associated with pathology with TTR from other species demonstrates that 15 of the 36 substituted amino acids in human TTR have been 100% conserved in TTR of all other species, suggesting that there has been strong evolutionary pressure to conserve these residues probably because they are of structural or functional importance. The remaining 21 amino acids known to be altered in human TTR variants show little conservation between species. It will be of interest to determine why these TTR variants cause disease in humans but similar amino acid substitutions have no obvious effect in other species where they appear to have arisen normally as a consequence of evolution.

The consequence of amino acid substitutions in TTR is clearly complex and analysis of the primary structure alone may be insufficient to understand the consequences of a certain residue change, particularly since other residues in its structural vicinity may have been substituted simultaneously, compensating for the instability introduced by one mutation alone. The overall effects of particular amino acid substitutions may vary depending upon several factors, including ionisation state of amino acid functional groups and pH of the immediate environment within the molecule as well as the metabolic and physiological differences between species.

TRANSTHYRETIN GENE EXPRESSION

TTR has a fairly restricted gene expression, being demonstrated principally in the liver and choroid plexus of rat (Dickson *et al.*, 1985; Fung *et al.*, 1988; Schreiber *et al.*, 1990), human (Dickson and Schreiber, 1986), sheep (Schreiber *et al.*, 1990), pig (Duan *et al.*, 1995), and chicken (Southwell *et al.*, 1991) and in the liver of marsupials (Richardson *et al.*, 1994). In two reptiles, the turtle (*Trachemys scripta*) and lizard (*Tiliqua rugosa*), TTR is present in the brain but little or no expression has been detected in the liver (Achen *et al.*, 1993; Richardson *et al.*, 1997). The protein synthesised in the liver is secreted into the blood, whilst from the choroid plexus epithelium it is secreted into the cerebrospinal fluid, presumably to carry essential TH to the brain (Dickson *et al.*, 1987). TTR expression has been detected in the eye of cattle and sheep (Cavallaro *et al.*, 1990; Dwork *et al.*, 1990; Martone, 1988) and *in vitro* cultures of pigment epithelium of the retina from the rat show synthesis and secretion of TTR into the interphotoreceptor space of the retina (Ong *et al.*, 1994). Low levels of TTR mRNA are also expressed in the rat and human pancreas (Jacobsson *et al.*, 1990). TTR is also reported to be present in the visceral yolk sac during fetal rat development (Soprano *et al.*, 1986), in the developing rat eye (Mizuno *et al.*, 1992), and in developing chicken heart (Barron *et al.*, 1998) although its function during development remains to be determined.

In premetamorphic tadpoles of frog, TTR is expressed principally in the liver and has not been detected in choroid plexus where lipocalcin is the main protein synthesised (Achen *et al.*, 1992). In juvenile sea bream TTR is expressed principally in the liver and appears to be absent from the choroid plexus (Santos and Power, 1999). Northern blot studies with a range of adult sea bream tissues have demonstrated that, in addition to abundant expression in the liver, it was possible to detect significant TTR expression in the intestine and heart (Fig. 5), raising questions about its physiological function at these sites. The different locations of TTR gene expression suggest evolutionary variations and adaptations in TTR function.

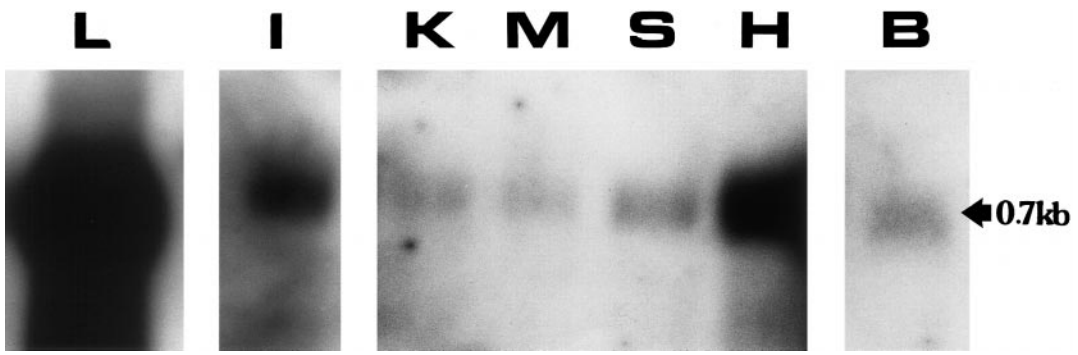

FIG. 5. Northern blot analysis of the expression of TTR in adult sea bream tissues. L, liver; I, intestine; K, kidney; M, muscle; S, skin; H, heart; B, brain. 10 μg of RNA poly(A)$^+$ from each tissue was loaded on a 2.2% formaldehyde/1.1% agarose gel, transferred to a nylon membrane, and fixed by UV crosslinking. This membrane was hybridised at 55°C overnight with a full-length sea bream TTR cDNA probe labelled with [α-^{32}P]dCTP. The filter was washed using high-stringency conditions and exposed to Kodak X-OMAT with intensifying screens at −70°C for 12 h (liver) and 48 h (intestine, kidney, muscle, skin, heart, and brain). A single transcript, 0.7 kb, of varying intensity was detected in all tissues analysed. Liver had the highest expression of TTR, but in the sea bream a strong signal was also detected in intestine and heart.

EVOLUTION OF TRANSTHYRETIN

Existing models of TTR evolution are largely based on results of binding studies of radiolabelled T_4 with blood plasma from adult representatives of most vertebrate groups, of SDS–PAGE of the secretory products from the choroid plexus, or of Northern blotting of various tissues (Harms *et al.*, 1991; Richardson *et al.*, 1994; Schreiber *et al.*, 1993). Such studies demonstrated T_4 binding to TTR in the plasma of all birds, eutherian mammals, and adult diprotodont marsupials (which includes kangeroos and opossums) and the absence of such binding in monotremes, polyprotodont marsupials, reptiles, amphibians, and fish (Chang *et al.*, 1999; Richardson *et al.*, 1994, 1997; Schreiber and Richardson, 1997). In common with other plasma proteins TTR is expressed and secreted by the liver (Schreiber, 1987) and its expression at this site in homeotherms is proposed to have occurred independently in birds, eutherians, and diprotodont marsupials (Richardson *et al.*, 1994; Schreiber *et al.*, 1993, 1995). The other main site of TTR gene expression in reptiles, birds, eutherians is the choroid plexus (Achen *et al.*, 1993) where it is the only thyroid hormone-binding protein synthesised (Schreiber, 1987), and TTR is presumed to facilitate the transport of TH into the brain.

Strong expression of TTR in the choroid plexus but not in the liver of the stumpy-tailed lizard (Achen *et al.*, 1993), together with the strong conservation of TTR in the choroid plexus from reptiles to mammals (Harms *et al.*, 1991), and the apparent absence from amphibians and fish, on the basis of ligand-binding studies with plasma from adults, led to the hypothesis that the expression of the TTR gene first arose in the brain of reptiles (Achen *et al.*, 1993); it subsequently and independently arose in the liver of birds, eutherians, and diprotodont marsupials. However, the cloning and characterisation of TTR from the liver of premetamorphic amphibians and juvenile fish (Santos and Power, 1996, 1999; Yamauchi, 1998) indicate that the gene for TTR probably arose in or prior to the ancient fishes. The presence in amphibians and fish of gene expression for TTR in the liver and its absence from the choroid plexus suggest that indeed TTR expression in the brain first arose in the reptiles, but that this evolved subsequent to its expression in the brain first arose in the reptiles, but that this evolved subsequent to its expression in the liver. The selection pressure for "turning on" TTR gene expression in the brain may be the rapid evolution of the brain and its concomitant increase in size and complexity, with the first traces of a neocortex appearing in the stem reptiles (Duan *et al.*, 1995, Richardson *et al.*, 1997). TTR, it is proposed, would function to ensure appropriate extracellular and intracellular thyroid hormone distribution in the brain (Schreiber and Richardson, 1997).

In addition to evolution of differential tissue expression of TTR, there appears to have been a change in function from a T_3 transporter to a T_4 transporter (Chang *et al.*, 1999; Yamauchi *et al.*, 1999). The phylo-

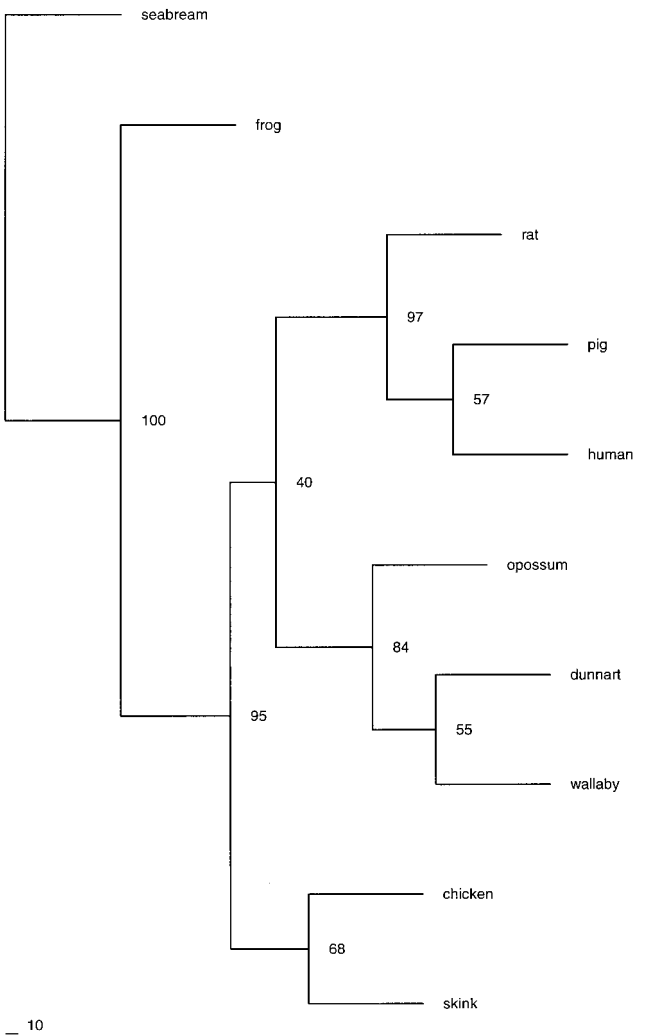

FIG. 6. Phylogram generated by Clustal X (Gibson *et al.*, 1994) of representative species from eutherians mammals—human (Sasaki *et al.*, 1985), pig (*Sus scorfa*, Duan *et al.*, 1995), and rat (*Rattus norvegicus*, Dickson *et al.*, 1985); marsupial mammals—dunnart (*Sminthopsis macroura*, Duan *et al.*, 1995) (Australian Polyprotodonta), wallaby (*Macropus eugenii*, Brack *et al.*, 1995) (Australian Dyprotodonta), and opossum (*Monodelphis domestica*, Duan *et al.*, 1995) (American Polyprotodonta); birds—chicken (*Gallus gallus*, Duan *et al.*, 1991); reptile—skink (*Tiliqua rugosa*, Achen *et al.*, 1993); amphibian—frog (*Rana catesbeiana*, Yamauchi *et al.*, 1998); and fish—sea bream (*Sparus aurata*, Santos and Power, 1999).

gram of TTR (Fig. 6) from representatives of each of the classes from which it has been isolated and sequenced corresponds approximately to the phylogenetic tree for the evolution of vertebrates. Interestingly the TTR from eutherian and marsupial mammals that have the highest affinity for T_4 are clustered into a group separate from TTR from birds, reptiles, amphibians, and fish. The functional significance of the change in affinity of TTR for TH is unclear but may be related to differences in thyroid hormone balance and metabolism; there are reports of higher blood levels of T_3 than T_4 in murine fish (Bjornsson *et al.*, 1998; Eales and Shostak, 1987; Pavlidis *et al.*, 1997), in contrast to mammals, in which T_4 predominates (McNabb, 1992). This is an area of thyroid hormone physiology that clearly merits further attention.

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