Evolution of structure, ontogeny of gene expression, and function of Xenopus laevis transthyretin

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of the TTR subunits. Also, the binding properties of bullfrog TTR for l-thyroxine (T₄) and 3,5,3'-triiodothyronine (T₃) (66) drastically differ from those of the TTRs in other species (10). In this paper, we report the cloning and sequencing of the cDNA for TTR in Xenopus laevis (xTTR; the African clawed toad) and the analysis of the TTR gene in genomic DNA by Southern blotting. We also analyze the timing and tissue pattern of TTR gene expression in X. laevis. For physicochemical studies of the properties of xTTR, its cDNA was incorporated into two protein expression vectors for the yeast Pichia pastoris. The cleavage recognition site of the α-factor (using the pPIC9 vector) of yeast and the xTTR presegment (using the pPIC3.5 vector) allowed normal processing of the recombinant xTTR in the yeast. The obtained protein product was characterized as TTR by sequencing of the NH₂-terminus, determination of the association of subunits into a tetramer, binding to human retinol-binding protein, and quantification of the binding of T₄ and T₃. The nucleotide sequence of the genomic DNA in the 5' area of the xTTR gene was determined. Comparison of the nucleotide sequence of the genomic DNA with the cDNA sequence derived from mature RNA gave the position of the splice site between exons 1 and 2. Comparison with splice-site positions in other vertebrate TTR mRNAs allowed conclusions about the molecular mechanism of the evolution of the NH₂-terminal region of TTR in vertebrates.

Comparisons of the amino acid sequences of TTRs from different species, with TTR genelike segments in the genomes of Caenorhabditis elegans, Schizosaccharomyces pombe, Salmonella dublin, and Escherichia coli were used to identify the position of xTTR in the evolution of TTR from bacteria to humans. A most parsimonious phylogenetic tree was constructed based on the amino acid sequences of the TTR subunits derived from the nucleotide sequences of TTR cDNAs.

MATERIALS AND METHODS

Materials, sources of animals, cDNA library, cDNA probe, and yeast expression vectors. T₃, T₄, T₅, and 3,5,3'-triiodothyroacetic acid were purchased from Sigma. X. laevis tadpoles were from Hassel Biological (Hamamatsu, Japan). Developmental stages were assessed according to the criteria of Nieuwkoop and Faber (41). A cDNA library, from the middle regions of the bodies of X. laevis tadpoles at stages 53–54, 58–59, 60, 62–63, and 66, deposited in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted by the acid guanidinium isothiocyanate-phenol-chloroform method (11). Total RNA (15 μg/lane) was analyzed by electrophoresis in 1% agarose gel containing 2.6 M formaldehyde. Hybridization with 32P-labeled xTTR cDNA and washing were performed under high-stringency conditions, as described in Ref. 27. Autoradiography was carried out at −80°C for 1 to 7 days. Hybridization signals of elongation factor 1α-mRNA on the same filter were used as loading controls. The amount of radioactive cDNA probe hybridizing with mRNA was quantitated by computer-assisted image analysis (software Photoshop from Adobe). The amount of the TTR mRNA is shown as the ratio of the density for the TTR mRNA bands to that for the elongation factor 1α-mRNA bands.

Northern blot analysis: timing of gene expression during development. Livers were collected from 10 to 20 metamorphosing tadpoles at stages 53–54, 58–59, 60, 62–63, and 66, deposited in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted by the acid guanidinium isothiocyanate-phenol-chloroform method (11). Total RNA (15 μg/lane) was analyzed by electrophoresis in 1% agarose gel containing 2.6 M formaldehyde. Hybridization with 32P-labeled xTTR cDNA and washing were performed under high-stringency conditions, as described in Ref. 27. Autoradiography was carried out at −80°C for 1 to 7 days. Hybridization signals of elongation factor 1α-mRNA on the same filter were used as loading controls. The amount of radioactive cDNA probe hybridizing with mRNA was quantitated by computer-assisted image analysis (software Photoshop from Adobe). The amount of the TTR mRNA is shown as the ratio of the density for the TTR mRNA bands to that for the elongation factor 1α-mRNA bands.

Northern blot analysis: sites of gene expression. Total RNA was extracted from heads, livers, and carcasses (heads and livers removed) of X. laevis tadpoles at stages 57–59. An amount of 10 μg of RNA per lane was analyzed by electrophoresis in 1% agarose gel (37). Hybridization was performed at 65°C using digoxigenin (DIG)-labeled xTTR cRNA or at 60°C using 32P-labeled cane toad (Bufo marinus) chroid plexus lipocalin cDNA (2) as probes. Filters were washed twice for 15 min in 0.3 M NaCl, 0.03 M trisodium citrate (2× SSC), 0.1% SDS at room temperature, once for 15 min in 1× SSC and 0.1% SDS at hybridization temperature and once for 15 min in 0.5× SSC and 0.1% SDS or 0.1× SSC and 0.1% SDS at hybridization temperature. Color detection of the digoxigenin-labeled DIG probe was performed with anti-DIG alkaline phosphatase-conjugated antibody followed by the reaction with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, essentially according to the instruction of Roche Diagnostics.

Construction of expression vectors. BamH I and EcoR I (pPIC3.5 vector) or Xho I and EcoR I (pPIC9 vector) sites were introduced by polymerase chain reaction into xTTR cDNA such that cleavage by BamHI occurred immediately before nucleotide 39 of xTTR cDNA or by Xho I such that...
cleavage occurred immediately before nucleotide 96 of xTTR cDNA or by EcoR I to cleave immediately after the stop codon TAA (498–500) of the xTTR cDNA. The PCR product was ligated with the Pichia expression vectors pPIC3.5 or pPIC9. The inserted vector was linearized by digestion with Sal I and transformed into P. pastoris strain GS115 by electroporation.

Affinity chromatography of recombinant xTTR on human retinol-binding protein. Recombinant xTTR was purified by affinity-column chromatography on human retinol-binding protein coupled to Sepharose 4B, as described by Larsson et al. (34).

Determination of the mass of the TTR monomer by mass spectrometry. The mass of the recombinant xTTR subunit was determined by a Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry (Finnigan MAT), using α-cyano-4-hydroxycinnamic acid (10 mg/ml) in 70% acetonitrile with 0.1% trifluoroacetic acid as the matrix. The spectrum was detected with laser power 100 μJ and laser aim 4.

Determination of the mass of the TTR tetramer by gel filtration. The molecular mass of recombinant TTR was estimated by fast-performance liquid chromatography gel filtration on Superose-12 (HR 10/30, Pharmacia) equilibrated in 50 mM potassium phosphate buffer saline (pH 7.4). Column and sample volumes were 24 ml and 50 μl, respectively, the flow rate was 0.5 ml/min, and the standards were bovine serum albumin (68 kDa), ovalbumin (45 kDa), horse heart myosin (16.7 kDa), and horse heart cytochrome c (12 kDa).

Analysis of NH2-terminal amino acid sequence. The NH2-terminal amino acid sequences of the recombinant TTRs were determined on a commercial basis by the Australian Proteome Analysis Facility (Macquarie Univ., Sydney, New South Wales, Australia).

Analysis of T4 and T3 binding to recombinant xTTR. Purified xTTR (100 nM) was incubated with T3 or T4 concentrations from 0 to 1,000 nM in the presence of tracer [125I]T3 or [125I]T4 at 4°C, and free T3 or T4 was separated from the TTR-bound hormones according to the method described by Chang et al. (10). The radioactivity was determined using an LKB 1270 Rackgamma II counter with an efficiency of 70%. Nonspecific binding was estimated by extrapolation of the saturation curve, and other corrections were performed (as described in Ref. 10) before analysis by Scatchard plotting (52).

For binding-competition studies with T3, purified recombinant xTTR (7 μg/ml) was incubated in 250 μl of 20 mM Tris, 97 mM NaCl, 1 mM MgCl2, and CaCl2 (pH 7.4). Column and sample volumes were 24 ml and 50 μl, respectively, the flow rate was 0.5 ml/min, and the standards were bovine serum albumin (68 kDa), ovalbumin (45 kDa), horse heart myosin (16.7 kDa), and horse heart cytochrome c (12 kDa).

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For binding-competition studies with T3, purified recombinant xTTR (7 μg/ml) was incubated in 250 μl of 20 mM Tris, 97 mM NaCl, 1 mM MgCl2, and CaCl2 (pH 7.4), with 0.1 mM [125I]T3 in the presence of various concentrations of unlabeled T3 or analogs of thyroid hormones, at 4°C for 1 h. All processes were carried out at 4°C. At the end of the incubation, 100 μl of 10% AGX-Cl (anion resin AG 1-X8 chloride, a product of BioRad) resin was added to the incubation sample. Free [125I]T3, absorbed to the resin was removed by centrifugation at 1,200 g for 5 min. [125I]T3 bound to xTTR in the supernatant was determined in an Aloka gamma spectrometer. Nonspecific [125I]T3 binding was calculated from the samples with 5 μM unlabeled T3. Specific binding was estimated by subtracting nonspecific binding from total binding.

Amplification of the TTR gene from genomic DNA. Genomic DNA from adult X. laevis liver was prepared by phenol extraction, as described by Sambrook et al. (49). Oligonucleotide primers consisting of xTTR cDNA segments near the 3′ end of exon 1 (5′-ACACCTCTTGAAAAACATG-GGC-3′) and complementary to sequences near the 5′ end of exon 2 (5′-TTCACACTACAGATTTGACGG-3′) were used in polymerase chain reactions to amplify TTR genomic DNA fragments containing the exon 1/intron 1 and intron 1/exon 2 regions, as previously described (3). Polymerase chain reactions were carried out in 0.02 ml reaction mixture for 25 cycles at a denaturation temperature of 94°C for 5 min, an annealing temperature of 55°C for 30 s, and an extension temperature for 1 min. The product from the polymerase chain reaction mixture was then purified and subcloned into pCR2.1 vector.

Phylogenetic analysis of TTR sequences. TTR cDNA sequences were obtained from databases: human (Homo sapiens X59498), pig (Sus scrofa X82258), sheep (Ovis aries X15576), cow (Bos taurus AB009591), rat (Rattus norvegicus X14876) mouse (Mus musculus X03351), shrew (Sorex araneus AJ223149), hedgehog (Erinaceus europaeus AJ277290), wallaby (Macropus eugenii X79113), kangaroo (Macropus giganteus X97688), sugar glider (Petaurus breviceps X80998), dunnart (Sminthopsis macroura X80998), opossum (Monodelphis domestica X80193), chicken (Gallus gallus X60471), lizard (Tiliqua rugosa X75950), bullfrog (Rana catesbeiana AB006134), Xenopus (Xenopus laevis AB026996), sea bream (Sparus aurata AF059193) and imported into an Australian National Genomic Information Service (ANGIS) project. Bases before the first ATG were deleted, and amino acid sequences were derived using the Etranslate program. Derived sequences were checked against published derived sequences, and additional residues in the COOH-terminal region were deleted. For species in which the NH2-terminal amino acid residues had been determined by Edman degradation, residues comprising the presegment were deleted. For species in which the NH2-terminal residue had not been determined (pig, shrew, mouse, sugar glider, X. laevis, sea bream, dunnart, and lizard), it was designated as the residue corresponding to residue 1 in human TTR. These “mature TTR sequences,” together with those from rabbit (Oryctolagus cuniculus P07489), were put into multiple-sequence format using the Pileup program. Bootstrap resampling (500 replicates) was carried out using the Eseqboot program. Eprotpars was used for calculation of maximum parsimony, and Econsense was used to generate the consensus tree. All programs were accessed via the ANGIS. Specific programs used were from the Genetics Computer Group (21).

Database searches for sequences similar to those of TTRs. Database searches for protein sequences and open reading frames (ORF) were performed using the BCM Search Launcher General Protein Sequence/Pattern Searches with BLASTP+BEAUTY postprocessing. The same sequences were suggested by the program as being most closely related to TTR, regardless of which TTR amino acid sequence (from different species) was entered as the query sequence. When the xTTR sequence was entered, the number of matches with the non-TTR sequences was higher than when a reptilian, avian, marsupial, or eutherian TTR sequence was entered. Hydrophobicity profiles were generated by the method of Kyte and Doolittle (33), using a scanning window of seven amino acids.

RESULTS

Molecular cloning and sequencing of xTTR cDNA. In preliminary experiments, TTR cDNA from R. catesbeiana (67) was found to hybridize with RNA extracted from X. laevis tadpole livers but not with RNA from the livers of adult X. laevis frogs. Therefore, a cDNA library from the livers of X. laevis tadpoles in middevelopment (stages 54–57) was screened with a Rana TTR cDNA probe. Six independent cDNA clones with the
lengths of cDNA in the range of 0.4 to 0.65 kb were isolated. The longest was sequenced on both strands (Fig. 1). The alignment of the deduced amino acid sequence with those of other TTRs from various species makes it likely that the coding sequence for xTTR starts with nucleotide 39 and ends with nucleotide 497. The partial matching of the flanking region of the possible start site satisfied Kozak’s rules (31). The deduced amino acid sequence of xTTR was 153 residues long and included a signal peptide. The molecular weight was calculated to be 16,683. A putative polyadenylation signal was present at nucleotide positions 639 to 644.

Partial nucleotide sequences were obtained for the remaining five cDNA clones. Except in one or two positions, these sequences were identical to that of the longest cDNA clone, which is shown in Fig. 1. The difference in one or two nucleotides per strand could be due to a sequencing error or to the existence of more than one TTR gene per haploid genome. The latter possibility was explored further as described in Southern analysis of genomic DNA from X. laevis.

Southern analysis of genomic DNA from X. laevis. The number of chromosomes (61) and the DNA content per nucleus (58) suggest that a duplication of the genome occurred in the ancestor of the X. laevis group. Immunochemical analysis of albumins allowed dating of that duplication event to ~10 million years ago, after the branching of TTR evolution into the lines leading to X. tropicalis and to the X. laevis group ~30 million years ago (8). Furthermore, Southern analysis of the genomic DNA from R. catesbeiana was consistent with the presence of a single copy of the albumin gene per haploid R. catesbeiana genome (4). There is only one TTR gene copy per haploid genome in the rat (18). To clarify the situation for X. laevis, a Southern analysis was carried out for genomic DNA from X. laevis, which had been digested with the restriction enzymes EcoRI, BamHI, PstI, or SacI, and either xTTR exon 1 cDNA or xTTR exon 2 cDNA as probes. The nucleotide se-

Fig. 1. Nucleotide sequence of the cDNA for tadpole [Xenopus transthyretin (xTTR)] and its deduced amino acid sequence. A: sequencing strategy. xTTR cDNA is represented as a small checkered bar. Arrows indicate direction and extent of sequencing. B: nucleotide sequence and derived amino acid sequence of xTTR cDNA. Nucleotide numbers at the right, beginning with the first nucleotide of cDNA insert. Signal peptide amino acids are numbered -19 to -1. Amino acid numbers are indicated underneath the nucleotide sequence. The NH2-terminal residue of the mature transthyretin (TTR), obtained from NH2-terminal amino acid sequencing of recombinant xTTR, is designated as +1. The first in-frame stop codon is represented by 3 asterisks. The putative polyadenylation signal in the 3′ untranslated region of cDNA is underlined.
sequences of exons 1 and 2 do not contain a cleavage site for any of the restriction endonucleases used in the Southern analysis. More than one band was obtained in the analysis for all of the used restriction enzymes (Fig. 2), suggesting that more than one copy of the TTR gene is present per haploid genome of X. laevis.

During the evolution of the TTR gene in vertebrates, the structure of TTR changed slowly over a period of 10 million years. For example, rats and mice had a common ancestor ~8–12 million (fossil data) or 20–35 million (molecular comparisons) years ago (for critical discussion, see Ref. 64). TTRs from mice and rats differ by only six amino acids, and five of these are conservative changes (15, 63). Only one band was observed for TTR in the electrophoresis of serum from X. laevis tadpoles (results not shown). Therefore, it seemed that the TTR whose cDNA is described in Fig. 1 was sufficient representative for X. laevis TTR to justify its further investigation.

**Western analysis of serum of adult X. laevis for TTR.** Western analysis of the serum from adult X. laevis was performed using antiserum against a mixture of TTRs from a human, tammar wallabies, and chickens (see MATERIALS AND METHODS). Human serum was used as a positive control, koala serum was used to demonstrate cross-species recognition of the TTRs by the antiserum, and serum from Tasmanian devil (which does not contain TTR (45)) was used as a negative control. Background nonspecific binding in the globulin region was present for all four species, a strong signal corresponding to the TTR subunit was observed in the lanes containing serum from human and koala but not in the lanes containing serum from Tasmanian devil or adult X. laevis (Fig. 3A, left).

**Levels of TTR mRNA in X. laevis tadpole liver during development.** RNA was extracted from the livers of X. laevis tadpoles from the premetamorphic stage 53 to the end of the metamorphic climax stage 66 and hybridized in Northern analysis to xTTR cDNA (Fig. 3A, right top). For standardization, the filter was simultaneously analyzed with a cDNA probe for elongation factor 1α (Fig. 3A, right middle). Hybridization signals were quantitated by densitometry. The levels of TTR mRNA were found to reach a maximum at prometamorphic stages 58 to 59. Thereafter, they declined and became 1.1% of the maximum level at stage 66 at the end of metamorphic climax (Fig. 3A, right bottom). No signal was obtained for the RNA from the livers of adult X. laevis frogs (data not shown).

**Sites of TTR gene expression.** Total RNAs were isolated from livers, whole heads, and carcasses (bodies without livers and heads) of X. laevis tadpoles at prometamorphic stages 57–59. RNAs were separated by electrophoresis and transferred to a Hybond N-nylon membrane. Northern analysis was performed using 32P-labeled xTTR cDNA (Fig. 3B) as a probe. A strong signal for TTR mRNA was detected in RNA isolated from tadpole liver (48-h exposure). In contrast, only an extremely faint TTR mRNA signal was detected in RNA isolated from tadpole heads, and no signal at all from RNA from carcasses without liver and head but including skin.

The major protein synthesized and secreted by the choroid plexus in amphibians is lipocalin (2). Similarly to adult amphibians (2), lipocalin mRNA was found to be present in RNA isolated from livers and in the RNA isolated from whole heads of X. laevis tadpoles (results not shown). The intensity of the lipocalin mRNA band in tadpole liver RNA was comparable with that of TTR mRNA. Crocodile heart (no TTR mRNA) and rat liver RNA (rat TTR mRNA does not cross-hybridize with xTTR cDNA) were used as negative controls. No signals for either TTR mRNA or lipocalin mRNAs were detected in RNAs from rat liver or crocodile heart.

**Synthesis of xTTR in Pichia pastoris.** To obtain sufficient amounts of xTTR for characterization, a recombinant xTTR gene was constructed and cloned using two different signal sequences: the xTTR presegment and the α-factor signal sequence. The two different plasmids constructed for the expression of xTTR are shown in Fig. 4. In plasmid pPIC3.5-xTTR, the xTTR cDNA had been placed under the control of the native AOX1 promoter at BamH I and EcoRI sites, and the xTTR presegment sequence was used in expression. The second plasmid was constructed by ligating the 5’
end of xTTR cDNA to the 3' end of the α-factor signal sequence in the vector pPIC9. (The α-factor signal sequence synthesized in the yeast is removed within the Golgi by the KEX2 protease specific for dibasic amino acid sequences (28, 29). This is known to occur efficiently in *P. pastoris* (48).) The plasmids were linearized with *Sal*I before transformation into the *P. pastoris* strain GS115. Twenty putative *Pichia* recombinants of each expression vector were selected and incubated for protein synthesis for 72 h. The analysis of the culture supernatant by SDS-PAGE and silver staining showed the efficient synthesis and secretion of a protein with a subunit molecular mass of ~16 kDa (data not shown). This value is consistent with the molecular mass of the TTR subunits from other species. The recombinant xTTRs from both plasmid constructs were purified by affinity chromatography on a human retinol-binding protein-Sepharose column. The purified TTR-like proteins were sequenced for the first five amino acids from the NH2-terminus. They had the same sequence, namely alanine, proline, proline, glycine, and histidine. Apparently, the cleavage of the α-factor signal sequence by KEX2 protease occurred at exactly the same site as that of the xTTR presegment by the signal peptidase in *Pichia*. Because the protein was expressed with similar efficiency in all of the 40

Fig. 3. *A*, left: Western analysis for detection of TTR in adult *X. laevis* serum. Aliquots of 6 μl serum from adult *X. laevis* and, for comparison, aliquots of 2 μl serum from Tasmanian devil (*S. harrisii*), koala (*P. cinerius*), and human (*H. sapiens*) were separated in SDS-PAGE, then transferred onto a nitrocellulose membrane. Triple the volume of amphibian serum was analyzed (lower protein concentration compared with mammalian serum). Antiserum (1:5,000) had been generated against a mixture of purified TTRs from human, wallaby, and chicken. The secondary antibody (1:10,000) was horseradish peroxidase-linked anti-rabbit immunoglobulin, raised in sheep. Detection used an enhanced chemiluminescence kit (Amersham) and X-ray film. The positions of TTRs are indicated by a bracket. *A*, right: TTR mRNA expression in *X. laevis* tadpole liver during development. Total RNAs were prepared from tadpole liver at stages 53–54, 58–59, 60, 62–63, and 66 (*lanes* 1–5). Electrophoretically separated RNA (15 μg) was transferred to a nylon membrane and probed with a 32P-labeled 0.65-kb *EcoR*I fragment of xTTR cDNA (*top*) and with a 32P-labeled 0.35-kb fragment of the *X. laevis* elongation factor 1α (EF-1α)-cDNA (*middle*). Hybridization was in 50% and 30% formamide hybridization buffer for TTR mRNA and EF-1α-mRNA, respectively, at 42°C. The amounts of xTTR mRNA relative to those of EF-1α-mRNA are shown below the autoradiograms (*bottom*). Three analyses were carried out for each stage. SE are indicated by vertical bars. MW, molecular weight. *B*: sites of TTR gene expression. Total RNA was prepared from livers (*lane* 1), heads (*lane* 2), and the rest of the body (*lane* 3) of *X. laevis* tadpoles, stage 57 to 59. An amount of 10 μg of RNA per track was subjected to Northern analysis. Digoxigenin (DIG)-labeled cRNAs for xTTR and *X. laevis* EF-1α were synthesized with T3 or T7 RNA polymerase after linearizing the plasmids with appropriate restriction enzymes. Hybridization was in 50% formamide hybridization buffer for TTR and EF-1α messages, respectively, at 65°C.
screened clones, one recombinant clone of each vector was selected for further characterization. Similar to TTRs from other vertebrates, the recombinant xTTR was found to bind specifically to retinol-binding protein (Fig. 5A). This feature was used for the purification of recombinant xTTR from the yeast culture. Approximately 4 mg of the recombinant xTTR were obtained from 1 liter of Pichia culture.

The recombinant xTTR was characterized for its physicochemical properties. SDS-PAGE (15% polyacrylamide) analysis of purified xTTRs expressed from pPIC3.5 and pPIC9 showed the same relative mobility corresponding to the mass of a polypeptide chain of ~16 kDa (Fig. 5B). The electrophoretic migration at pH 8.6 of the recombinant xTTR was found to be faster than that of albumin (data not shown). The mass of the TTR subunit determined by mass spectrometry was 15,013 (Fig. 5C). Fast protein liquid chromatography on a Superose 12 column showed a molecular mass of 66 kDa (Fig. 5D), indicating a tetrameric structure for the xTTR produced by P. pastoris. This is in contradiction to a report by Bellovino et al. (6), who only found the formation of TTR dimers in transfected Cos-1 cells and interpreted the lack of tetramer formation as being due to the absence of liver cell microsomes. The xTTR showed cross-hybridization with rabbit anti-serum against a mixture of human, wallaby, and chicken TTRs (Fig. 5E). The position of the xTTR monomer is indicated by an arrow, at ~18 kDa. The second band of ~36 kDa corresponds to xTTR dimers.

Dissociation constants of the recombinant TTR and thyroid hormones were determined according to Chang et al. (10). Scatchard analyses and plots indicated $K_d$ values for $T_3$ of 508 ± 34 nM (Fig. 6B) and for $T_3$ of 248 ± 19 nM (Fig. 6A). The $K_d$ for $T_3$ was 0.49. These values were substantially different from those reported for bullfrog TTR, whose $K_d$ for $T_3$ was 241 nM and for $T_3$ was 0.67 nM (66), giving a $K_d$ of 0.003.

The stronger affinity of recombinant xTTR for $T_3$ than for $T_4$ could also be shown in a competition experiment measuring displacement of bound radioactive $T_3$ by $T_3$, $T_3$, $T_4$, or 3,3',5-triiodothyro-l-acetic acid (Fig. 6C).

Evolution of the NH$_2$-terminus by changes of mRNA splicing. Changes in the primary structure of TTR occurred during evolution predominately within the first 10 amino acids from the NH$_2$-terminus (for reviews, see Refs. 54 and 55). The NH$_2$-terminal segment of the TTR subunit is longer and more hydrophobic in avian and reptilian than in eutherian TTRs. X. laevis TTR has an even longer and more hydrophobic NH$_2$-terminal region than avian and reptilian TTRs.

The analysis of the sites of splicing near the 5’ end of the precursor xTTR mRNA was expected to show whether or not a shift in splice sites could be a mechanism for a systematic evolutionary change of the NH$_2$-termini of TTR. Thus the nucleotide sequence of the genomic xTTR DNA was determined in the region coding for the 5’ end of precursor xTTR mRNA. Oligonucleotide primers corresponding to sequences near the exon 1/intron 1 border of xTTR cDNA were designed. They were used in a polymerase chain reaction with adult X. laevis liver genomic DNA leading to the synthesis of DNA segments containing the exon 1/intron 1 and the intron 1/exon 2 regions of precursor xTTR mRNA. The comparison of the genomic DNA nucleotide sequence with the xTTR mRNA derived cDNA sequence gave the location of the splice sites. Splicing at the 5’ end of intron 1 was found to occur at the site corresponding to amino acid position three of the mature protein (Fig. 7A), identical to previously studied vertebrate species (3). Splicing at the 3’ end of intron 1 of the xTTR gene occurred in the position -e at the 5’ end of exon 2 (Fig. 7B). During evolution of the TTR gene of birds from amphibianlike ancestors, the histidine codon in position 5 of TTR was changed into the 3’
splice site recognition sequence CAG. This required only a single base change from U to G.

Evolution of the TTR gene. A most parsimonious phylogenetic tree was constructed from the TTR amino acid sequences available in protein data banks and the derived amino acid sequence of TTR from *X. laevis*. Sea bream TTR was used as the outgroup. The obtained most parsimonious tree is shown in Fig. 8. *X. laevis* and *R. catesbeiana* TTRs are in the same evolutionary branch. The close similarity of the bootstrap numbers for the pairs of rat and mouse and *R. catesbeiana* and *X. laevis* reflect the great similarity of TTR amino acid sequences in each of the pairs. TTRs in *X. tropicalis* and *X. laevis* or in other *X. laevis* species would be still more similar.

The amino acid sequence of xTTR is similar to those of all other known vertebrate TTR sequences, e.g., 62% identical and 97% similar to that of human TTR, despite the distant evolutionary relationship of the two species. Therefore, we searched for a precursor of the xTTR gene in the genomes of some “prevertebrate” species. In this search for ancestral sequences of xTTR, the entire mature xTTR amino acid sequence was used as the query sequence (for details, see MATERIALS AND METHODS). Five ORF “translations” were found to have substantial overall similarities and identities to the sequence of xTTR. These were found in the genomes of *S. pombe* (AL031545), *E. coli* (AE000288), two in *C. elegans* (R09H10.3 in chromosome IV: Z177134 and ZK697.8 in chromosome V: AF039042), and in the *S. dublin* (AF060858) genome (Fig. 9A). Regions of similarity begin at either residue 12 or 16 (numbering for mature human TTR amino sequence) and cease at residue 118. The lengths of the ORFs from *S. pombe*, *E. coli*, *C. elegans* R, *C. elegans* Z, and *S. dublin* are 124, 137, 121, 190, and 172 amino acid residues, respectively. The identities and similarities to xTTR are 32% and 52%, 29% and 53%, 27% and 52%, 27% and 52%, and
...and 28% and 46%, respectively, over the region of residue 12 or 16 to 118 of the xTTR. There are only two regions where gaps had to be introduced into the ORFs: between residues 31 and 40 and 101 and 104. The only ORF in which residues were deleted to align with TTR sequences was that of *S. pombe*: two residues deleted between 42 and 43 and one between 66 and 67. A total of 13 residues is identical in all 16 TTR sequences and 5 ORFs, an additional 19 positions tolerate only two amino acid residues, and a further seven residues are similar in all 21 sequences. Of the 13 identical amino acids, seven are first or last residues of *b*-sheets, eight are in the core of the TTR subunit, and three are in the A-B loop that is responsible for dimer-dimer interaction to form the tetramer of TTR and comprises part of the thyroid hormone binding site. Subgroups of these residues are close to each other in space on *b*-sheets running antiparallel to their own, e.g., residues 12 and 105 and 17 and 111.

The analysis by Kyte and Doolittle (33) of the xTTR amino acid sequence showed a hydrophobic region with a maximum at about residue 20 (Fig. 9B), then a sharp increase in hydrophilicity until about residue 30, a steady increase in hydrophobicity until a maximum around amino acid 80, then an increase in hydrophilicity until about amino acid position 90, and a sharp increase in hydrophobicity until about residue 98, then a final increase in hydrophilicity. A similar profile was obtained for the plot of the *S. pombe* ORF in the region that is similar to residues 12–118 of xTTR. The plot of *C. elegans* Z ORF was similar, except for a region of hydrophilicity around residue 70 before the hydrophobic region at 80, and a less hydrophobic COOH-terminal domain. The profile for the *E. coli* ORF was generally more hydrophilic than those of the others. However, the pattern of increases in hydrophobicity and hydrophilicity were similar: the more hydrophilic NH₂-terminal region was followed by a hydrophilic central region, gradually increasing in hydrophobicity until a maximum around 80, then a sharp increase in hydrophilicity at around 90, and thereafter an increase in hydrophobicity until about residue 98, then an increase in hydrophilicity for the COOH-terminal region. The *E. coli* ORF had the hydrophobic region around residue 70, similar to the *C. elegans* ORF (Fig. 9B).

According to the “normalized alignment score” versus ORF length (amino acid residues) model of Doolittle (16), it is probable that the TTR sequences, the two *C. elegans* ORFs, the *S. pombe* ORF, *E. coli* ORF, and *S. dublin* ORF are homologous; i.e., these sequences came from a single common ancestor. It is interesting to note that there were no splice-site consensus sequences identified in any of the five ORFs, whereas vertebrate TTRs contain three introns.

**DISCUSSION**

**Evolution of TTR function and structure.** The evolutionarily oldest feature essential for life is compartmentation, i.e., the concentration of components and reactions in a compartment separated from the extra-
cellular environment by membranes (43). Through the use of imported energy, the increase in entropy in the cellular compartment can then be kept smaller than that of the environment. With the evolution of multi-cellular organisms, communication across cell membranes became necessary. Lipid-soluble compounds were the obvious choice for the transfer of information across cell membranes. The intracellular aggregation of cellular proteins and the formation of amyloid fibrils are the prominent pathological features in a variety of TTR-related diseases, raising the possibility that TTR constitutes a common morphogenetic element underlying amyloidosis. A common theme in the pathogenesis of TTR amyloidosis is the formation of amyloid fibrils in the absence of the TTR gene product. The TTR gene is the most prevalent genetic factor identified in TTR amyloidosis, and the TTR gene product is the major protein component in the fibrils, suggesting that amyloid fibrils are formed by the interaction of the TTR gene product with other proteins in the cellular environment.
EVOLUTION OF THE XENOPUS LAEVIS TRANSTHYRETIN GENE

**Fig. 8.** Phylogenetic tree resulting from maximum parsimony analysis of mature TTR amino acid sequences from 19 vertebrate species. The fish (*Sparus aurata*) TTR sequence was used as the out group. The consensus tree is shown with bootstrap numbers based on 500 replicates.

of the signal compounds with a receptor protein can then control the interaction of the signal compound with intracellular systems. The observation that the nuclear receptors for all thyroid and steroid hormones form a single large superfamily of proteins suggests that the development of these regulator proteins occurred only once during evolution.

The high lipid solubility of thyroid hormones can cause complications. In a perfused rat liver, thyroid hormone binding proteins had to be added to the perfusion medium to counteract the depletion of hormones from the perfusate by accumulation of the hormones in the surrounding cells (36). Thyroxine-binding globulin, TTR, and albumin are found in the blood of larger mammals (34). Thyroxine-binding globulin and TTR do not occur in the blood of reptilians or of polyprotodonts, the evolutionarily older species of the marsupials (45). The affinity for T4 is higher for eutherian TTRs than for marsupial TTRs. The binding affinity between T4 and marsupial TTRs is again much higher than that for avian TTRs, which resemble reptilian TTRs in structure (10). Thus evolution seems to have favored the development of mechanisms for establishing sufficient extracellular thyroid hormone pools. During the evolution of higher vertebrates, the functional properties of TTRs have distinctly changed.

The comparison of the primary structure of xTTR reported here with those of TTRs in reptiles, birds, marsupials, and eutherians (for reviews, see Refs. 54 and 55) shows that the overall structure of xTTR is similar to that of TTR in other vertebrates. We conclude that a substantial part of the evolution of TTR must have occurred before the stage of the amphibians and fish. The ORFs identified in the genomes of *S. pombe*, *E. coli*, two in *C. elegans*, and in *S. dublin* reflect this part of the early evolution of the ancestral TTR gene.

The Southern analysis of genomic DNA from *X. laevis* with TTR cDNA probes corresponding to regions of the gene lacking recognition sites for the restriction endonucleases used to digest the genomic DNA suggested that the TTR gene was involved in the *X. laevis* genome duplication that occurred after the branching of the evolutionary lineage of *Xenopus* into the branch leading to *X. tropicalis* (no duplication) and into that leading to the *X. laevis* group (duplication). However, because this duplication event occurred relatively recently in evolution, i.e., ~10 million years ago (8), it is unlikely that the structures of the two TTR genes of *X. laevis* differ substantially. The TTRs of rats and mice, which also had a common ancestor ~10–30 million years ago, differ by only one nonconservative change of an amino acid, i.e., a valine for a glycine residue in position 65 from the NH2-terminus, and five conservative amino acid changes in positions 3, 37, 38, 90, and 92 (15, 63). A phylogenetic tree for the evolution of TTR structures, such as that presented in Fig. 8, gives closely similar bootstrap numbers for bullfrog/*X. laevis* (i.e., 90) and rat/mouse (i.e., 92) sequences.

It has been suggested that genome expansion takes place predominantly by sequence amplification involving, in particular, repetitive DNA (35). The results reported in this paper and those of Bisbee et al. (8) for albumin show that the duplication of structural gene DNA sequences can also contribute to increasing genome size.

**Timing and sites of TTR gene expression.** Adult animals that express the TTR gene in the liver are all endotherm (45, 55). Endotherms appeared relatively recently during evolution. In the choroid plexus of the brain, the TTR gene was expressed well before the endotherms, namely in turtles (46) and lizards (1). Adult toads (2, 23) and bullfrogs (67) lack TTR gene expression in both liver and brain. Apparently, adult amphibians do not possess TTR (2, 23). However, the synthesis of a TTR with an unusual structure of the NH2-terminus was observed during development in the liver of bullfrog tadpoles (66). The brain and, in particular, the choroid plexus of tadpoles expressed the gene for a lipocalin but not the gene for TTR (67). Only traces of TTR mRNA were observed in nonhepatic tissues of lizards (1) and of sea breams (53).

The data reported here show that in *X. laevis* tadpoles, the TTR gene is strongly expressed in the liver during development, similar to the situation in bullfrog tadpoles. A very faint signal was observed in the region of the Northern blot corresponding to xTTR mRNA. This signal could correspond to a very small amount of xTTR mRNA in the retina or, possibly, another region.
of the brain. The in situ hybridization carried out previously for TTR from *R. catesbeiana* did not show silver grains over the area of the choroid plexus but did not clearly exclude the presence of TTR mRNA in the retina (67).

Amphibian brains differ from the brains of more highly developed vertebrates by the absence of a neocortex (30). It seems that the first appearance of TTR gene expression in the brain occurred at the same time as the neocortex, namely at the stage of the stem reptiles. The neocortex is the part of the brain that evolved fastest. The functional selection pressure for the synthesis of TTR in the brain might have been the need for maintaining extracellular thyroid hormone pools in the presence of large lipid volumes. In *X. laevis* tadpoles, TTR gene expression in the liver is found around the same time as high levels of thyroid hormones. One of the functions of thyroid hor-

![Fig. 9. A: comparison of TTR sequences with amino acid sequences (deduced from open reading frames (ORF)) most similar to TTRs. Mature protein sequences from 16 vertebrate species, including representatives from eutherians, marsupials, birds, reptiles, and amphibians, were aligned (sources of the sequences other than xTTR can be found in Refs. 54, 55, and 67). Amino acid sequences (deduced from ORFs) most similar to TTRs were aligned below that of xTTR. Amino acids identical to those in xTTR are indicated by asterisks. Gaps were introduced to aid alignment. The numbering of amino acids is based on that of human TTR. Amino acids present in sequences from marsupials, birds, reptiles, and amphibians that are not present in human TTR are designated -a to -e. Features of secondary structure of human TTR are indicated above its sequence (α-helix and β-sheets), singly underlined residues are in the core of the subunit, and doubly underlined residues are in the thyroid hormone-binding channel (9). Positions that have an identical amino acid in 16 TTRs and the 5 ORFs (*) or in which all amino acids are similar (1) or in which only 2 amino acids are tolerated (ˆ) are indicated. B: comparison of hydrophobicity plots of xTTR with ORFs from *C. elegans*, *E. coli*, and *S. pombe*. Kyte-Doolittle hydrophobicity plots for the region of xTTR with similarity to the ORFs in A (a), *S. pombe* ORF in A (b), *C. elegans* Z ORF in A (c), and *E. coli* ORF in A (d). A scanning window of 7 amino acid residues was used.](http://ajpregu.physiology.org/lookup/doi/10.1152/ajpregu.00221.2017)
mammals is the regulation of growth and differentiation during metamorphosis. Whereas thyroidectomized tadpoles did not metamorphose into frogs, administration of thyroxine to their water resulted in development into frogs (22).

**xTTR synthesis in Pichia pastoris and xTTR properties.** Concerted evolution of TTR structure and deiodinase gene expression? The high yield of xTTR production by *Pichia pastoris* allowed a detailed analysis of the properties of xTTR. The recombinant xTTR was shown to have the size and tetrameric structure expected of TTRs. It bound to human retinol-binding protein (a typical feature of TTRs). The amino acid sequence in its NH2-terminal region was identical to that derived from the xTTR cDNA (Fig. 1).

The binding of T4 and T3 by xTTR could be quantitated by Scatchard analysis. In line with the observations for avian species (10), xTTR bound T3 with higher affinity than T4. The ratio of the affinity to T4 over that to T3 for xTTR was found to be about one-tenth of that in mammalian TTRs. The affinity of xTTR for T3 is much lower than that of mammalian TTRs (see Ref. 10). Regard et al. (44) found lower total T3 levels in tadpole serum than in human serum, whereas total T3 levels in tadpole serum were in the same range as in mammals. The question arises: is the evolution of a precursor-product system for the distribution of thyroid hormones integrated with that of the regulation of 5’-deiodinases? Different strengths of binding of T4 and T3 by TTR will influence the levels of T4 and T3 in the blood and in tissues. The main form of thyroid hormone synthesized in the thyroid gland is T4. T4 can be converted into T3 by deiodination. In the human, about one-third of T3 stems from the thyroid gland. Two-thirds of T3 are produced by extrathyroidal deiodination of T4. The generation of T3 from T4 is catalyzed by 5’-deiodinases. There are several 5’-deiodinases (different proteins) in different organs with tissue-specific regulation. For example, under hypothroid conditions, the 5’-deiodinase in the brain is upregulated, whereas the 5’-deiodinases in muscles, liver, and kidney are downregulated (see Ref. 57). This allows organ- or tissue-specific modulation of the regulation of metabolic functions by thyroid hormones. T3 binds more tightly to nuclear receptors than T4 (50) and is more effective than T4 in the regulation of gene transcription. The comparative analysis of primary TTR structures suggests that the evolution of most of the structure of TTR occurred before the stage of amphibians and fish. The comparison of the binding of T4 and T3 by xTTR (this paper) with that of TTRs in other species (10) suggests ongoing evolution of this feature after the stage of the amphibians. An advantage of preferential T3 binding by TTR would be an increase in the extracellular levels of T3, i.e., the potential substrate for the generation of T3. It is interesting that 5’-deiodinase activity was demonstrated in gut, skin, and tails of metamorphosing *R. catesbeiana* tadpoles but not in liver, kidney, or brain (19, 20). The ratio of the concentration of total T3 over that of T4 was higher in amphibians (peak levels during metamorphosis) than in mammals (44). During metamorphosis of *R. catesbeiana* tadpoles, intracellular activities of 5’-deiodinase and 5-deiodinase were found to peak in different tissues at the same time as metamorphic activity in these tissues (5). The tissue-specific pattern of the expression of deiodinase genes was interpreted as suggesting that the conversion of T4 into T3 could be related to the timing of metamorphic events (5). The generation of T3 from T4 would not be necessary in a tissue synthesizing and containing a preferentially T3-binding TTR, such as tadpole liver. We do not know whether these features observed with *R. catesbeiana* are a general property of amphibians. However, the concept of a concerted evolution of thyroid hormone homeostasis involving evolutionary changes of both TTR structure-function and regulation of 5’-deiodinases is intriguing.

**Mechanism of evolution of TTR.** The analysis of the splice site between intron 1 and exon 2 of precursor xTTR mRNA shows a further shift toward the 5’ end of TTR mRNA compared with avian precursor TTR mRNA (3). The observations reported here for the xTTR gene make it likely that successive shifts in splice sites could be an evolutionary mechanism of general importance. Such shifts could produce stepwise changes in the properties of proteins, such as the affinity for a ligand. A stepwise “improvement” of protein function could be the point of operation of selection pressure. This would be an example for a molecular mechanism of positive neo-Darwinian evolution.

In conclusion, among the physicochemical properties of thyroid hormones there are two outstanding features important for the position of thyroid hormones in the regulation of metabolism. The first is the excellent solubility of thyroid hormones in lipid phases. The second is the presence of three (in T3) or four (in T4) iodine atoms in the thyroid hormone molecule. The iodine atom is probably the largest of the atoms occurring in the molecules of living matter. These large and strongly electronegative iodine atoms help the precise, specific interaction with proteins required for hormone action via receptor proteins. The first feature, the high solubility of thyroid hormones in membranes, means that thyroid hormones can act as hormones without the need for complex signal-transduction systems, such as those involved in mediating the action of the water soluble hormones insulin and adrenalin.

At equilibrium, thyroid hormones can accumulate in lipids and membranes to concentrations that are several thousandfold higher than those in the aqueous environment (13, 24). The increases during evolution in the relative sizes of internal organs [e.g., brain, liver, kidney (25)] in the intensity of metabolism associated with membranes in endotherms compared with ectotherms and in the sizes of intestinal lipid pools in diprotodont (i.e., herbivorous), compared with polyprotodont (i.e., carnivorous or omnivorous) marsupials (26), favored the permeation of thyroid hormones from extracellular aqueous environments into lipid compartments. However, the presence of thyroid hormone-binding proteins in the extracellular compartment can counteract the loss of thyroid hormones from the aque-
ous phase. The most interesting of these proteins in blood plasma is TTR. The data communicated here suggest that the changes in vertebrate species of the tissue pattern of gene expression, the timing of gene expression during development, and the evolution of the structural properties of TTR can all be interpreted as parts of the evolution of a mechanism to ensure the appropriate distribution of thyroid hormones at different times of development (the young amphibian during metamorphosis and the overall body of adult eutheri- ians), in different compartments (the extracellular space of the brain in reptiles, birds, and mammals and the intestinal tissue of herbivorous marsupials), and different metabolic systems (endothermic vs. ectothermic species). However, TTR is only one factor in a larger multicomponent system for maintaining thyroid hormone homeostasis. This system includes other thyroid hormone-binding extracellular proteins, the two thyroid hormones T₃ and T₄, various deiodinas converting T₄ into T₃, and widely different fluid dynamics in extracellular compartments (rapid mixing of the blood stream in the body vs. slower, pipelinelike flow of the cerebrospinal fluid through the ventricle system and subarachnoid space in the brain).

The data reported here for the binding of T₃ and T₄ to xTTR, together with data recently reported for other species (10, 66), suggest that the thyroid hormone distribution system in the form found in larger mammals has evolved only relatively recently. The development of a precursor (dominant transport form)-product system for thyroid hormones allows the independent tissue-specific regulation of local T₃ levels, introducing additional flexibility for regulation by thyroid hormones.

Finally, the observations reported here demonstrate for a part of the TTR molecule, the NH₂-terminal region, that stepwise neo-Darwinian evolution could be the result of a stepwise shift of RNA splice sites. Until now, molecular examples for stepwise, positive neo-Darwinian evolution are rare.

Perspectives

The reported investigations provide important impe-
tus for future work. Characterization of the structure of the xTTR gene showed extensive similarity with TTR genes in reptiles, birds, and mammals. It follows that a major period of TTR evolution must have occurred in prevertebrate organisms. The observation of TTR DNA-like ORFs in bacteria, yeast, Salmonella, and C. elegans supports this conclusion. Future work is necessary to characterize the “precursor” TTR genes and the function of the proteins they encode in prevertebrates.

The type of functional pressure postulated for posi-
tive selection in a Darwinian mechanism of evolution should be investigated. Presumably, it would involve proteins coded for by the ORFs. Efficient synthesis of recombinant TTR in the yeast P. pastoris suggests possibilities for searching for TTR-like precursor pro-
teins in prevertebrate organisms and for studying syn-
thesis and properties of such proteins coded for by TTR genelike ORFs. Whether such proteins would form tetramers, bind thyroid hormones, and, if so, bind T₃ more strongly than T₄ (similar to xTTR but different than mammalian TTRs) is an interesting question.

Finally, the data reported here for the tissue pattern of TTR gene expression in Xenopus support the hypothesis that the need for a special thyroid hormone-bind-

ing protein system in the brain evolved at the stage of the reptiles and did not yet exist in amphibians. The identification of the features in the “postamphibian” vertebrate brain requiring such a thyroid hormone-binding protein system will be an exciting topic for future work. It is likely to provide an in-depth understand-
ing of TTR function.

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