Crocodylidae transthyretin: structure, function, and evolution

Porntip Prapunpoj, Samantha J. Richardson, and Gerhard Schreiber. Crocodile transthyretin: structure, function, and evolution. Am J Physiol Regul Integr Comp Physiol 283: R885–R896, 2002. First published July 11, 2002; 10.1152/ajpregu.00042.2002.—Structure and function were studied for Crocodylus porosus transthyretin (crocTTR), an important intermediate in TTR evolution. The cDNA for crocTTR mRNA was cloned and sequenced and the amino acid sequence of crocTTR was deduced. In contrast to mammalian TTRs, but similar to avian and lizard TTRs, the subunit of crocTTR had a long and hydrophobic NH2-terminal region. Different from the situation in mammals, triiodothyronine (T3) was bound by crocTTR with higher affinity than thyroxine (T4). Recombinant crocTTR and a chimeric construct, with the NH2-terminal region of crocTTR being replaced by that of Xenopus laevis TTR, were synthesized in the yeast Pichia pastoris. Analysis of the affinity of the chimeric TTRs showed that the NH2-terminal region modulates T3 and T4 binding characteristics of TTR. The structural differences of the NH2-terminal regions of reptilian and amphibian TTRs were caused by a shift in splice sites at the 5’ end of exon 2. The comparison of crocodile and other vertebrate TTRs shows that TTR evolution is an example for positive Darwinian evolution and identifies its molecular mechanism.

thyroid hormone-binding plasma proteins; choroid plexus; Crocodylus porosus; thyroid hormone homeostasis; eye; lizard; Tiliqua rugosa

TRANSTHYRETIN (TTR), thyroxine-binding globulin, and albumin are the major thyroid hormone-binding plasma proteins in larger placental mammals (27, 49). Thyroid hormones have a pronounced tendency to partition into and accumulate in cell membranes because of their higher solubility in lipids than in water (13, 21, 52). In the body, a system of plasma proteins that strongly binds thyroid hormones is necessary to counteract the loss of these hormones from the vascular and interstitial compartments by permeation into cell membranes. It has been convincingly shown, using single-pass perfusion of liver in vitro, that thyroid hormones quickly disappear from the central vein of the liver lobule by permeation into the periportal hepatocytes when no thyroid hormone-binding proteins are present in the perfusion medium (29). The affinities of thyroid hormones for thyroid hormone-binding proteins partially overlap, forming a “buffering” system to maintain appropriate levels of thyroid hormones in the blood (48, 49). This results in 99.98% of total blood thyroxine and 99.8% of triiodothyronine (T3; values for human) bound to protein. Nevertheless, it is the level of free thyroxine (T4) in the blood that is kept constant by regulation of T4 production and release from the thyroid gland, maintaining the euthyroid state of the individual. The individual components of the thyroid hormone-binding protein system are functionally redundant. The loss of one of the proteins due to genetic alteration is compensated by increased binding to remaining plasma proteins, and the euthyroid state is maintained (for T4-binding globulin deficiency in human, see Refs. 5, 6, 30, 33, 37; albumin deficiency in humans, see Ref. 24, in rats, Ref. 28; TTR null mutants of mice, see Refs. 17, 32).

Recently, the evolution of the structure and the expression pattern of the synthesis of TTR were studied for a variety of species from fish to humans (for reviews, see Refs. 34, 47, 49). The analysis of whole genome nucleotide sequence data has shown the presence of TTR gene-like open reading frames in microorganisms, primitive eukaryotes, and nematodes (36). The two main sites for TTR synthesis in the body of mammals and birds are the choroid plexus and the liver: the choroid plexus providing TTR for distribution in the brain, and the liver producing TTR for distribution in the vascular compartment of the body (for review, see Refs. 48, 49). In the liver, the TTR gene was found to be expressed in eutherians (38; for review, see Ref. 48), diprotodont marsupials (15, 38), birds (14, 53), and juvenile fish (44), but not in monotremes (38, 47), Australian polyprotodont marsupials (38), turtles (39), adult amphibians (2), or adult trout, salmon, or carp (38). The choroid plexus synthesized TTR in all these species, except in the amphibians and fish (2, 36, 57). The question arose as to whether and where in the
body TTR might have been synthesized in the common ancestor of birds and mammals, the stem-reptiles. During evolution, the first traces of a neocortex appeared in the reptiles (25). Until now, the tissues of only one reptilian species, the Australian stumpy-tailed lizard *Tiliqua rugosa*, have been analyzed for TTR gene expression. *Tiliqua rugosa* strongly expressed the TTR gene in the choroid plexus in the brain, but not at all in the liver (1). It was desirable to ascertain whether TTR synthesis in choroid plexus, but not in liver, is a general feature of reptilians, suggesting the evolutionary origin of TTR synthesis in the brain at the stage of the stem-reptiles. Therefore, the TTR gene was cloned for *Crocodileus porosus* and sequenced and the tissue pattern of TTR gene expression was characterized.

The analysis of the amino acid sequence of TTR for a wide variety of species suggested a dichotomy of the evolution of the NH2-terminal section of TTR. A longer and more hydrophobic NH2-terminal region of TTR persisted in avians, and a shorter, more hydrophilic NH2-terminal TTR section evolved in eutherians. The NH2-terminal section of TTR in *Tiliqua rugosa* TTR genes with those of the TTR genes in other species presented here provided insight into the position of reptilians in evolution and into the molecular mechanism of the evolution of TTR.

Mammalian TTRs have a higher affinity for T4 than for T3, whereas avian TTRs have a higher affinity for T3 than T4 (7). This difference may be important for the understanding of the evolution of thyroid hormone distribution in vertebrates, in particular in the brain. Therefore, it was of interest to obtain data for the binding of thyroid hormones by TTR from reptilians. The reptilians were probably the first class of vertebrates to synthesize TTR in the choroid plexus (1). Unfortunately, because TTR is not expressed in the reptilian liver, it was not possible to obtain enough TTR from reptilian blood for TTR purification and physicochemical studies of thyroid hormone binding. A recombinant protein synthesizing system of the yeast *Pichia pastoris*, synthesizing and secreting *Crocodileus porosus* TTR, was established. TTR was purified from the supernatant of incubated recombinant *P. pastoris* cultures and used for the analysis of the binding of T4 and T3 to compare its binding characteristics with those of amphibian, avian, and mammalian TTRs.

The NH2-terminal section of the TTR subunit seems to have evolved much faster than the rest of the molecule, whereas the thyroid hormone binding sites remained unchanged during vertebrate evolution (35, 49). The hypothesis was developed that this NH2-terminal section of the TTR subunit is responsible for the preference in binding of T3 or T4. It is not possible to determine the three-dimensional structure of this region of TTR by X-ray crystallography because the first nine amino acids of human TTR and the first 13 amino acids of chicken TTR do not form ordered structures (4, 54). Here, the synthesis of a recombinant, chimeric crocodile TTR (crocTTR) is reported in which the NH2-terminal section of crocTTR was replaced by the NH2-terminal section of *Xenopus laevis* TTR. The chimeric *Xenopus/crocTTR* synthesized and secreted by *P. pastoris* was purified and the binding of thyroid hormones was analyzed. The obtained results indicate that the NH2-terminal section of TTR influences thyroid hormone binding.

**MATERIALS AND METHODS**

**Tissues and chemicals.** Saltwater crocodiles (*Crocodylus porosus*) were obtained from Hartley’s Creek Crocodile Farm, Queensland, Australia, and were housed in the Department of Zoology, University of Melbourne. Animals were 1–10 yr old and weighed between 995 and 1,359 g. Animals were killed with 3 ml ketamine, and tissues were dissected and then immediately frozen in liquid nitrogen and kept at –70°C until used. Experiments were carried out under permit number 10000206 from the Victorian Department of Natural Resources and Environment.

**Development of the recombinant system.** Australian stumpy-tailed lizards (*Tiliqua rugosa*) were from Bredl’s Reptile Park and Zoo, South Australia. Lizards were anesthetized by intraperitoneal injection of 17 mg/kg body wt pentobarbital sodium and killed by inducing a pneumothorax. Tissues were removed and frozen in liquid nitrogen and kept at –70°C until used. Permit numbers were Y20992 and RP-92–054 from the Victorian Department of Conservation and Environment.

**[125I]T4.** [125I]T4 (1.2 Ci/mg) was from NEN-DuPont, Sep Pak C18 cartridges were from Millipore Waters, thin-layer chromatography plates were from Merck, the enhanced chemiluminescence kit was from Amersham, and X-ray film was from Eastman-Kodak. All reagents were of analytic grade.

**Cloning and sequencing of TTR cDNA from crocodile brain.** Total RNA was extracted from *C. porosus* brain and polyadenylated RNA was purified by affinity chromatography using Oligotex beads (Qiagen). The first and second strands of cDNA were synthesized and an EcoRI-adapted cDNA library was constructed in phage vector λMOSElox, as described by Palazzolo et al. (31), using the cDNA rapid cloning module-AMOSElox (Amersham, RP1716). The library was screened with 32P-labeled lizard TTR cDNA (1). Four positive clones with inserts (with the expected size of 0.6 kb) were selected for sequence determination. The entire sequence of crocTTR cDNA was determined for both strands by the dideoxynucleotide chain termination method (43), using a kit with T7 Sequenase DNA polymerase (Amersham).

**Splicing of the precursor TTR mRNA in the salt water crocodile and the stumpy-tailed lizard.** Genomic DNA was prepared from livers of crocodile using pancreatic RNase and proteinase K (Ref. 42, p. 9.16–9.19). PCRs were carried out to amplify TTR genomic DNA fragments containing the exon 1/intron 1 and intron 1/exon 2 regions. Oligonucleotide primers consisting of TTR cDNA segments near the 3’ end of exon 1 (crocodile: 5’–GGCTTTCTACTCTGTTCCTCG–3’; lizard: 5’–TACATCGGGTCTCCTCCTGTTC–3’) and complementary to sequences near the 5’ end of exon 2 (crocodile: 5’–TCCAGAACCTTACCATAGTTG–3’; lizard: 5’–CTTCTCTGAACTGCATCCAGG–3’) were used in the PCRs. PCRs were carried out in 0.1 ml reaction mixtures containing 1,000 ng of genomic DNA, 10 pmol of each primer pair, and 0.5 U of Taq DNA polymerase. Amplification started with denaturation for 5 min at 94°C, followed by 30 cycles of annealing (at 65°C for crocTTR genomic DNA fragments, or at 60°C for lizard TTR genomic DNA fragments) for 30 s, extension at 72°C for 1 min and denaturation at 94°C for 30 s. The final extension was carried out for 1 cycle of 72°C for 5 min. The PCR
mixtures were analyzed in a 1% low melting agarose in 0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0, gel and purified by phenol-chloroform extraction (Ref. 42, p. 6.30–6.31).

Construction of the expression vector for wild-type crocTTR. Because TTR was synthesized in the crocodile only in choroid plexus and the eye, i.e., relatively small organs, in only relatively small total amounts, a recombinant protein expression system was established to obtain sufficient amounts of crocodilian TTR for analysis and characterization. Recombinant TTRs from Xenopus laevis (36) and Sorex araneus (35) had been successful synthesized previously in the yeast Pichia pastoris. Thus, in this paper, P. pastoris was chosen as the gene expression system for production of recombinant TTR from crocodile brain. BamH I and EcoR I sites were introduced by PCR into the TTR cDNA such that cleavage by BamH I occurred immediately before nucleotide 22 and by EcoR I immediately after the stop codon TAA (472–474) of the cDNA. The PCR product was ligated to the bacterial expression vector pPIC9.5 (Invitrogen), which had been digested with BamH I and EcoR I. The inserted vector was linearized by digestion with HindII and used for transformation of Pichia pastoris strain SMD1168 by electroporation. Screening of recombinant colonies and heterologous protein synthesis was performed as described in the user's manual (Invitrogen).

Construction of the expression vector for the production of Xenopus/crocodile chimeric TTR. PCR was used to amplify and generate crocTTR cDNA with the NH2-terminal sequence of Xenopus TTR cDNA, i.e., cDNA that would code for residues Ala1 to Lys9 of Xenopus TTR and residues Cys20 to Gln137 of crocTTR. The cDNA contained compatible restriction sites (XhoI at 5’ end and EcoR I at 3’ end) for ligation into the expression vector pPIC9. The pPIC9 vector containing the alpha factor secretion signal was chosen for expressing the recombinant protein, as this was simpler than creating a chimeric Xenopus/crocodile DNA with a TTR pre-segment. An amount of 50 ng of crocTTR cDNA plasmid was amplified using 40 pmol of the appropriate primers to generate EcoR I and XhoI restriction sites in 100 μl of the reaction mixture. The amplification was started with an initial denaturation step at 94°C for 5 min. This was followed by 25 cycles of 94°C for 30 s, 50°C annealing temperature for 30 s, and extension at 72°C for 1 min. The final extension was carried out for 1 cycle of 72°C for 5 min. The DNA was purified using a PCR purification kit (Qiagen). Purification was followed by double digestion with EcoR I and XhoI for 2 h. Ligation of the DNA to linearized Pichia expression vector was carried out with T4 DNA polymerase kinase at 2 h. Ligation of the DNA to linearized pPIC9 was followed by double digestion with HindII and XhoI and used for transformation of Pichia pastoris strain SMD1168 by electroporation. Screening of recombinant colonies and heterologous protein synthesis was performed as described in the user's manual (Invitrogen).

Purification of recombinant crocTTR from yeast culture supernatant. Recombinant TTR was purified from the Pichia culture supernatant by affinity chromatography using a human retinol-binding protein-Sepharose-4B matrix, as described by Larson et al. (37), or by preparative discontinuous nondenaturing-PAGE using the BioRad Prep Cell.

Determination of the masses of the recombinant TTR tetramers by gel filtration. Native molecular masses of recombinant TTRs were estimated as described previously (36).

Analysis of NH2-terminal amino acid sequences. The NH2-terminal amino acid sequences of the recombinant TTRs were determined in the commercial facility of the Department of Biochemistry, La Trobe University, Melbourne, Australia.

Purification of radioiodinated thyroid hormones. Commercially available [125I]T3 and [125I]T4 typically contain up to 5% [125I]T3 by the reference date. Therefore, iodinated thyroid hormones were purified and then analyzed as described previously (38). All assays were done using the same batch of [125I]T3 and [125I]T4. Purifications were done in the morning, and measurements of Kd values (see below) were done on the same day.

Analysis of thyroid hormone binding to recombinant TTRs. The binding of thyroid hormones to proteins is difficult to quantitate because of unspecific binding of thyroid hormones to surfaces. One pipetting act can lead to the loss of half of the T4 from a dilute solution. The unspecific binding is a particular problem with equilibrium dialysis. Deionized and MilliQ water often contain an inhibitor of the binding of T4 to TTR. The values given for the Kd of T4 and TTR in the literature vary from 2.8 to 128 nM (see Ref. 7). Recently, a method was developed for the analysis of the binding of thyroid hormones to TTR, in which the contact of T4 in solution with surfaces was minimized. Strictly using only double-distilled (not MilliQ filtered) water, a Kd of 13.6 nM was obtained for human TTR and T4. Calculated from 12 independent analyses, the SE was 0.6 mM and the SD was 2.1 mM (7), despite determinations being made on different days with different preparations of human TTR. This method was used to measure the binding of T3 and T4 to crocTTR and Xenopus/crocTTR. Purified TTR (100 nM) was incubated with T4 or T3 from 0 to 1,000 nM in Irvine buffer in the presence of tracer amounts of [125I]T3 or [125I]T4 at 4°C overnight, as described by Chang et al. (7). Nonspecific binding was extrapolated and other corrections were performed before analysis by Scatchard plot (45). For analyses of Kd values for T3 and T4 and crocTTR, analyses for chicken TTR were always determined simultaneously. For analyses of Xenopus/crocTTR, crocTTR and chicken TTR were analyzed simultaneously.

Northern analysis. Total RNA from crocodile heart, liver, eyes, and brain was extracted with acid guanidinium thiocyanate-phenol-chloroform, as described by Chomczynski and Sacchi (8). Separation of RNA was performed in a 1.5% formaldehyde-agarose gel under denaturing conditions. RNA was transferred by capillary action onto Hybond-N membrane (Amersham), and hybridization was performed at 60°C with 32P-labeled crocTTR cDNA using Rapid-hyb buffer (Amersham). The filter was washed twice for 15 min in 2× SSC (20× SSC: 3 M NaCl, 0.3 M Na2HPO4–2H2O, pH 7.0, 0.1% SDS at room temperature, then once for 15 min in 1× SSC, 0.1% SDS at hybridization temperature, and once for 15 min in 0.5× SSC, 0.1% SDS at hybridization temperature.

SDS-PAGE. Analysis of proteins under denaturing conditions was performed in SDS-polyacrylamide slab gels (15% polyacrylamide, pH 8.6) using a 4% polyacrylamide stacking gel (pH 6.8) and the discontinuous buffer system of Laemmli and Favre (26).

Western analysis. This was performed as described previously (40).

RESULTS
cDNA cloning and sequencing of crocodile brain TTR cDNA. The total number of independent clones in the crocodile brain cDNA library was 1.7×105 and ~94% of these clones were recombinants. The cDNA library was screened with a 32P-labeled lizard TTR cDNA probe. Isolated clones with a DNA insert of ~0.6 kb were selected, and the recombinant plasmids were isolated. The entire nucleotide sequence of crocTTR cDNA was determined for both strands with overlapping fragments. The TTR cDNA consisted of 667 nucleotides (Fig. 1), followed by 30 adenylated residues. The reading frame started with an AUG codon at

AJP-Regul Integr Comp Physiol • VOL 283 • OCTOBER 2002 • www.ajpregu.org

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nucleotide 22 downstream from the 5' end of the cDNA and ended with an in-frame stop codon TAA at nucleotides 472–474. Three additional in-frame stop codons were found in the 3' untranslated region of the cDNA at nucleotides 520–522, 538–540, and 583–585. The consensus polyadenylation segment, AATAAA was located 17 base pairs upstream from the 5' end of the polyadenylation segment. The deduced amino acid sequence of crocTTR was aligned with that of human TTR. By comparison with the amino acid sequence of human TTR, crocTTR had three additional amino acid residues at the NH2-terminal region of the TTR subunit, similar to chicken TTR. These amino acids were designated α, β, and γ. Interestingly, for the close evolutionary relationship between crocodiles and birds, the crocTTR is more similar in amino acid sequence to chicken TTR than to lizard TTR. CrocTTR showed 90% amino acid sequence identity with the sequence of chicken TTR and only 81% with that of lizard TTR. The molecular mass of the TTR subunit from crocodile brain, calculated from the amino acid sequence deduced from the cDNA sequence, is 16,411 Da.
mRNA was detected in 20 μg of total RNA extracted from brain and eyes (Fig. 2). The intensity of the crocodile brain TTR mRNA band was much greater than that of the mRNA band from eyes.

**Evolution of the TTR gene.** The derived amino acid sequence of crocTTR was aligned with the sequences of TTRs from 26 vertebrate species (Fig. 3). Compared with the amino acid sequence of human TTR, crocTTR is 3 amino acids longer. The comparison of crocTTR with TTRs from other species revealed the following relative identities of amino acid sequences: human, 71%; hedgehog, 73%; shrew, 72%; pig, 74%; sheep, 71%; rabbit, 75%; rat, 75%; mouse, 76%; wallaby, 73%; kangaroo, 73%; sugar glider, 74%; dunnart, 74%; South American gray opossum, 70%; chicken, 90%; lizard, 81%; bullfrog tadpole, 63%; and seabream, 50%. Thus the amino acid sequence of crocTTR was, in this study, placed in a position between those of chicken and lizard TTRs (Fig. 3).

The alignment of amino acid sequences of TTRs from 27 vertebrates, including eutherians, marsupials, avians, reptilians, amphibians, and fish, showed that in the region corresponding to the β-strand A of the human TTR subunit, the amino acid residues are highly conserved throughout evolution. This is also the case for all positions in the central channel (4) (indicated in Fig. 3 by double underlining) and for the residues involved in the binding of thyroid hormones (11) (indicated in Fig. 3 by gray shading).

**Mechanism of evolution of the TTR gene.** In this study, shifts in splice sites of TTR mRNAs from two reptilian species, a lizard and a crocodile, were studied. The nucleotide sequences of the genomic TTR DNAs were determined in the regions coding for the 5’ end of precursor TTR mRNAs. Oligonucleotide primers corresponding to sequences near the exon 1/exon 2 border of the reptilian TTR cDNAs were designed. They were used in a PCR with genomic DNA as the template, leading to the synthesis of DNA segments corresponding to the exon 1/intron 1 and the intron 1/exon 2 regions of reptilian TTR precursor mRNAs. The locations of the splice sites were obtained from the comparison of the genomic TTR DNA nucleotide sequence with the TTR cDNA nucleotide sequences. Splicing at the 5’ end of intron 1 of the TTR precursor mRNAs from both lizard and crocodile was found to occur at the site corresponding to amino acid position 3 of the mature protein based on the amino acid sequence of human TTR (Fig. 4A). The splice site at the 3’ end of intron 1 of the lizard and crocTTR genes occurred in the position γ at the 5’ end of exon 2 (Fig. 4B), similar to that reported for chicken TTR (3). The valine codon in this position in reptilian TTR changed during the evolution of marsupial TTRs from an ancestral TTR gene, similar to that in the reptilian genome, into the 3’-splice-site-recognition sequence CAG.

**Function of crocTTR.** A recombinant crocTTR gene was constructed and cloned into the pPIC3.5 vector using the crocTTR presegment cDNA sequence. The crocTTR cDNA in the plasmid was placed under the control of the native AOX1 promoter at the BamHI and EcoRI sites. The plasmid was linearized with SalI before being transformed into the protease-deficient *P. pastoris* strain SMD 1168 strain. Twenty putative *Pichia* recombinants were selected, inoculated, and incubated for protein synthesis for 72 h. Analysis of the culture supernatants by SDS-PAGE and silver staining showed high-level synthesis and secretion of a protein with an approximate subunit molecular mass of 15 kDa (data not shown), consistent with the molecular mass of the TTR subunits from other vertebrate species. Because the protein was synthesized at a similar rate for each clone, only one recombinant clone was selected for further characterization. Amino acid sequencing of the purified recombinant crocTTR showed that alanine, proline, leucine, valine, serine, histidine, glycine, and serine were the first eight amino acids from the NH₂ terminus. The recombinant crocTTR was found to bind to retinol-binding protein (data not shown), similar to TTRs from other vertebrates. Thus the human retinol-binding protein-Sepharose-4B matrix could be used for the purification of recombinant crocTTR from the yeast culture. With the use of a human retinol-binding protein-Sepharose-4B column, up to 16 mg of recombinant crocTTR was obtained from 1 liter of yeast culture.

The purified recombinant crocTTR was analyzed by SDS-PAGE (15% polyacrylamide). The relative mobility of the subunit corresponded to that of a polypeptide with a mass of 15.5 kDa (Fig. 5A). Fast protein liquid chromatography using a Superose-12 column showed a molecular mass for the native protein of 57.5 kDa (data not shown). Taken together, these results suggest a tetrameric structure for the crocTTR.

The recombinant crocTTR showed cross-hybridization with rabbit anti-serum against a mixture of hu-
TTR dimers in addition to monomers could be detected, as previously reported for TTRs from other species (7, 36).

Dissociation constants for the recombinant TTRs and thyroid hormones were determined according to Chang et al. (7). Purified TTR from chicken blood was included as a control. The $K_d$ values of chicken TTR derived from the Scatchard analysis were 7.67 $\pm$ 1.09 nM for T3 and 35.98 $\pm$ 2.22 nM for T4, with a $K_d$ T3/$K_d$ T4 ratio of 0.21. The values obtained were slightly different from those reported by Chang et al. (7). However, the data confirmed that chicken TTR binds T3 with higher affinity than T4. Recombinant crocTTR showed higher affinity for T3 (7.56 $\pm$ 0.84 nM, Fig. 5B) than for T4 (36.73 $\pm$ 2.38 nM, Fig. 5C), similar to reports for avian (7), amphibian (36), and fish TTRs (44). The $K_d$ T3/$K_d$ T4 ratio obtained for crocTTR was 0.21, the same as for chicken TTR.

Fig. 5. Comparison of the structure of crocTTR with TTRs from other vertebrate species. Amino acid sequence derived from the cDNA sequence of crocTTR is aligned with the amino acid sequences of human TTR and those derived from cDNA sequences for 26 other vertebrate species. Single-letter amino acid abbreviations are used. X indicates that an amino acid could not be unambiguously identified by Edman degradation. *Residues identical to those in human TTR. Gaps were introduced to aid alignment. Features of secondary structure of human TTR are indicated above the sequences. Numbering of residues is based on that for human TTR. Negative numbers indicate the residues in the presegment; positive numbers represent residues of the mature protein. Negative Greek letters $\alpha$, $\beta$, $\gamma$, $\delta$, and $\varepsilon$ were introduced to indicate positions of residues in noneutherian species. Bold letters indicate the first amino acid of mature TTRs. Double underlining indicates amino acid residues located in the central channel (4). Gray shading indicates amino acid residues, in the central channel, that are involved in binding thyroid hormones (11). For origins of sequences, see Refs. 35, 36.
Role of the NH₂-terminal section of crocTTR in thyroid hormone binding. To obtain crocTTR with Xenopus TTR NH₂ termini, a recombinant chimeric TTR gene was constructed using PCR and specific primers to introduce the desired restriction sites. The recombinant chimeric TTR gene was cloned into plasmid pPIC9 at the XhoI and EcoRI sites of the plasmid. The /H9251-factor signal sequence in the vector was used for the synthesis and secretion of the chimeric TTR. The yeast /H9251-factor protein that was synthesized NH₂ terminally to the recombinant chimeric TTR was expected to have been efficiently removed within the Golgi of /H9251 (41) by the KEX2 protease (22, 23). This was checked by Edman degradation (see below).

Fig. 4. Comparison of the nucleotide and derived amino acid sequences of crocodile and lizard TTR precursor mRNAs at the exon 1/intron 1 and intron 1/exon 2 borders with those from other vertebrate species. Nucleotide sequences of the exon 1/intron 1 (A) and intron 1/exon 2 (B) border regions of crocodile and lizard TTR genomic DNAs were determined. Splice sites are indicated by two-ended arrows. The 5' and 3' splice sites of intron 1 of crocodile and lizard TTR precursor mRNAs were aligned with those from other vertebrate species. Consensus recognition sequences for splicing are indicated in bold above the positions of the splice sites of human TTR precursor mRNA. Nucleotides identical to those in the consensus sequences for the 3' splice site are underlined. Nucleotides in exons are in bold uppercase letters, whereas those in introns are in lowercase letters. Deduced amino acid sequences are given below the nucleotide sequences. Asterisks in intron 1 indicate the same base as in human TTR precursor mRNA. Negative numbers indicate amino acids of the human TTR presegment, and positive numbers indicate amino acids of the mature protein. Negative Greek letters /H9251, /H9252, /H9253, /H9254, and /H9280 were introduced into the numbering of amino acid residues to indicate the additional residues at the 5' end of exon 2 observed in marsupial, avian, reptilian, and Xenopus TTRs (36) that were not found in human TTR. NH₂-terminal amino acids, determined by Edman degradation of the mature proteins or of the recombinant TTRs, are indicated by a box open at right. For origins of sequences, see Refs. 35, 36.
From the sequencing data of the purified chimeric TTR, the first eight amino acids from the NH$_2$ terminus were alanine, proline, proline, glycine, histidine, alanine, serine, and histidine. This revealed that the cleavage of the α-factor signal sequence by KEX2 protease occurred at the same site as that of the *Xenopus* TTR presegment by the signal peptidase in *Pichia* (36).

Similar synthesis rates were obtained for the chimeric TTR and the wild-type crocTTR. The chimeric TTR possessed physicochemical properties similar to the wild-type TTR. This included subunit mass of 15.5 kDa (Fig. 5A), greater electrophoretic migration than albumin at pH 8.6 (data not shown), and cross-hybridization with anti-serum against a mixture of human, wallaby, and chicken TTRs (Fig. 5A). The chimeric TTR had a molecular mass of 50.1 kDa (data not shown), as determined from relative mobility on a Superose-12 column, which was slightly less than that of the wild-type crocTTR.

Compared with wild-type crocTTR, the *Xenopus* crocodile chimeric TTR showed different binding affinities for both T$_3$ and T$_4$ (Fig. 6, A and B). The chimeric TTR bound thyroid hormones with lower affinity than the wild-type crocTTR, but still bound T$_3$ with higher affinity than T$_4$ ($K_d$ for T$_3$ was $33.03 \pm 0.42$ nM, $K_d$ for T$_4$ was $55.69 \pm 7.69$ nM, $K_d$ T$_3$/$K_d$ T$_4$ ratio was 0.60). In contrast, the chimeric TTR had higher affinities to T$_3$ and T$_4$ compared with those of *Xenopus* TTR ($K_d$ for T$_3$ was $247.8 \pm 19.3$ nM, $K_d$ for T$_4$ was $508 \pm 33.8$ nM; Ref. 36, using the method described in Ref. 7). However, the $K_d$ T$_3$/$K_d$ T$_4$ ratio of *Xenopus/crocodile chimeric TTR was closer to that for *Xenopus* TTR (0.49; Ref. 36, using the method described in Ref. 7).

**DISCUSSION**  

**Tissue pattern of the expression of the TTR gene in C. porosus.** The presence of TTR mRNA in brain (1, 39) and eyes (1) in the lizard *Tiliqua rugosa* has been previously reported, but only with very low levels of gene expression. TTR mRNA in total RNA from lizard eye could only be detected by a very sensitive RT-PCR, but not by Northern analysis. Similar to the observation in lizard, no TTR mRNA was detected (this study) in RNA extracted from crocodile heart or liver. TTR is synthesized by the choroid plexus in all studied species of mammals, birds, and reptiles (see Ref. 49). Similar to the situation for *C. porosus* and *T. rugosa*, the TTR gene is not expressed in liver, but is expressed very strongly in choroid plexus of Australian (15) and some American (40) polyprotodont marsupials and monotremes (20). We propose that the persistence of TTR synthesis in choroid plexus and its absence from the liver during the evolution of marsupial-like species...
from reptilelike ancestors, is due to the selection pressure for providing and maintaining a particular function of TTR in the brain. The reptiles are the first species possessing traces of a cortex (25). During further evolution, the brain, and in particular the neocortex, is the organ growing fastest in relative size (56). It seems to be justified to suspect a special function of TTR synthesis by the choroid plexus in reptiles, birds, and mammals. The choroid plexus secretes cerebrospinal fluid, which has open access to the interstitial space in the brain (10). However, the flow of cerebrospinal fluid through the brain is very slow compared with the rapid distribution and mixing in the bloodstream. It takes ~200 min in most mammals for the secretion of an amount of new fluid equivalent to that of the total volume of cerebrospinal fluid (9). The choroid plexus would appear to be a very efficient site for the synthesis of a substance involved in maintaining homeostasis in the brain for compounds permeating from the interstitial into the intracellular compartment.

Evolution of the TTR gene. During the last 15 years, the amino acid sequences for TTR or the nucleotide sequences for TTR cDNA have become available for a large number of species (for review, see Ref. 48). They allow the construction of a phylogenetic tree for the evolution of the TTR gene (35, 36). In such a tree, the C. porosus TTR gene would be located halfway between prokaryotic organisms and mammals. The higher percentage of the amino acid sequence identity of crocTTR to chicken TTR than to lizard TTR supports the evolution of Archosauria concept by Gauthier et al. (18, 19, for review, see Ref. 50), which considers crocodilians and birds as the same monophyletic group. The sequence of the amino acids forming the binding site for thyroid hormones, as identified by X-ray crystallography (4, for review, see Ref. 49), are 100% conserved during evolution. This suggests that the binding of thyroid hormones is an important function of TTR.

Mutations of the amino acid sequence of TTR in humans are of clinical interest. A large number of such mutations have been described (for reviews, see Refs. 16, 48, 49). They appear to be distributed randomly along the polypeptide chain. Most of these mutations lead to the synthesis of TTRs with the tendency to form insoluble protein aggregates, so-called amyloid, accumulating as intracellular deposits in various organs. In humans, the mutation Ile68→Leu leads to amyloidosis. CrocTTR has Leu as the naturally occurring amino acid in position 68, yet there was no evidence of aggregation of the TTR synthesized by Pichia to suggest amyloid formation of crocTTR. Sheep, cow, and many marsupial and lizard TTRs also have Leu in position 68. We have purified TTRs from sheep (47), bovine (unpublished), wallaby (Macropus eugenii, 38), kangaroo (Macropus giganteus, 38), sugar glider (Petaurus breviceps, 38), and opossum (Monodelphis domestica, 15) serum and found no evidence of TTR amyloid formation during analyses or after long-term storage of these purified TTRs. This suggests that a point mutation in human TTR that leads to amyloid formation must be influenced by the other amino acids in its immediate vicinity.

A systematic change seems to have occurred during the evolution of the NH2-terminal section of the polypeptide chain of the TTR subunit. This NH2-terminal section is longer in marsupial than in eutherian TTRs and again longer in reptilian and avian TTRs. Avian and reptilian TTRs are more closely related in structure than the avian and mammalian TTRs. The amino acids at the NH2-terminal end of reptilian and avian TTRs are more hydrophobic than those at the NH2-terminal end of mammalian TTRs.

**Mechanism of the evolution of the TTR gene.** Evolutionary adaptation to changes in environment by inheritable (genetic) changes has been postulated to occur in small steps. During the evolution of the structure of TTR, the predominant region where mutations occurred was within the first 10–13 amino acids from the NH2 terminus, leading to changes in the length and hydropathy of the NH2-terminal regions (for reviews, see Refs. 48, 49). The NH2-terminal segment of the TTR subunit from eutherians is shorter and more hydrophilic than those from marsupial, avian, reptilian, and amphibian TTRs. Stepwise shifts in splice sites at the intron 1/exon 2 border have been
proposed to be the evolutionary mechanism for the shortening of the NH2-terminal region (3). A positive, directional evolution is observed for the NH2-terminal section of the subunit of C. porosus TTR and T. rugosa TTR. Thus here we demonstrate that our hypothesis for the mechanism of evolution of the structure of the NH2 termini of TTR holds true for the Reptilia, representing the first case of the shortening of the NH2 termini compared with that of their common ancestors. This mechanism was subsequently used by avian, metatherian, and eutherian TTRs.

Evolution of the function of C. porosus TTR. Recently, it was suggested that the affinities of TTRs for thyroid hormones evolved from values giving lower ratios for binding of T4 over binding of T3 in bird/marsupial-like ancestors to values giving a higher ratio of T4 over T3 in eutherian species (7). This evolution may be correlated with the evolution of specific 5’-deiodinases, generating T3 from T4 in a tissue-specific manner.

Recently, it was demonstrated that juvenile saltwater crocodiles have negligible activity levels of deiodinases performing deiodination of T4 to T3 in their brains (51). CrocTTR has higher affinity for T3 than for T4 (this study) and probably transports T3 into the brain rather than T4. This is in sharp contrast to the situation for mammals: mammalian TTRs have higher affinity for T4 than for T3 (7), and TTR synthesized by mammalian choroid plexus transports T4 but not T3 into the brain (13). Mammalian brains have type II deiodinases converting T4 to T3. In some areas of the brain, 65% of T3 is derived from local deiodination of T4 (55).

Some recombinant protein synthesizing systems allow the synthesis of relatively large amounts of proteins that are normally made in only small amounts in nature. Here, recombinant Pichia pastoris yeast could be constructed that synthesized, processed, and secreted sufficient amounts of C. porosus TTR for physiological studies of its properties. The obtained results showed that the thyroid hormone-binding properties of C. porosus TTR are more similar to those of avian TTRs than to those of mammalian TTRs.

Role of the NH2-terminal section of C. porosus TTR in thyroid hormone binding. The identity of the amino acid sequences of the thyroid hormone-binding sites in the TTRs of all vertebrate species (comparable to the extent of conservation of the amino acid sequences of histones) suggests that the binding of thyroid hormones is a functionally most important, and therefore conserved, property of TTR. However, the NH2-terminal regions of TTR seem to show changes typical for a positive neo-Darwinian evolution of TTRs from the TTR in reptilelike ancestors to TTRs in eutherians. Furthermore, the NH2-terminal regions are far away from the binding sites for thyroid hormones in TTR. The NH2-terminal regions are located at the entrances of the central tunnel of the TTR tetramer, which contains the thyroid hormone-binding sites. Direct experimental evidence is obviously required to relate the structures of NH2-terminal regions and the thyroid hormone binding properties of TTRs. The P. pastoris system allowed the synthesis and secretion of a chimeric TTR in which the NH2-terminal section of the C. porosus TTR subunit had been replaced by the NH2-terminal region of Xenopus laevis TTR subunits. The analysis of the isolated and purified chimeric Xenopus/crocTTR showed that the NH2-terminal regions strongly influenced thyroid hormone-binding properties of TTR. Very likely, the NH2-terminal sections of the TTR molecule influence access and exit of the thyroid hormone molecules to and from the central channel of TTR. This would also help to explain the different ratios of affinities for T4 and T3 found in TTRs with identical thyroid hormone-binding sites.

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