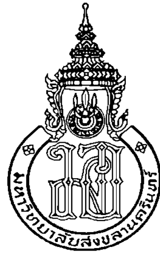

Identification and Characterization of TTR genes of bat and fish

Fitriya Daramae

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biochemistry

Prince of Songkla University

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ชื่อวิทยานิพนธ์	การศึกษาการแยกยีนและคุณลักษณะของยีน TTR ในค้างคาวและปลา
ผู้เขียน	นางสาวฟิตรียา ดาราแม
สาขาวิชา	ชีวเคมี
ปีการศึกษา	2551

บทคัดย่อ

Transthyretin (TTR) เป็นโปรตีนชนิดหนึ่งที่พบในกระแสเลือด มีหน้าที่หลักในการขนส่งฮอร์โมนไทรอยด์ในสัตว์มีกระดูกสันหลังชนิดต่างๆ ในสัตว์เลี้ยงลูกด้วยนมที่มีขนาดใหญ่ TTR ถูกสังเคราะห์ขึ้นที่ตับและ choroid plexus ในสมอง จากการศึกษาการเปลี่ยนแปลงทางโครงสร้างของ TTR ในสายวิวัฒนาการ พบว่า โครงสร้างปฐมภูมิของ TTR มีการอนุรักษ์ไว้ยกเว้นปลาย N-terminal ที่เปลี่ยนแปลงไป นั่นคือ TTR ในสัตว์เลี้ยงลูกด้วยน้ำนม (eutherian) มีปลาย N-terminal ที่สั้นกว่าและมีความชอบน้ำมากกว่า TTR ที่พบในบรรพบุรุษในสายวิวัฒนาการ ปัจจุบันคุณลักษณะของยีน TTR ได้ถูกนำมาใช้และพบว่ามีคุณค่าอย่างมากต่อการศึกษาความเชื่อมโยงทางสายพันธุ์ (phylogenetic tree) ของกลุ่มสัตว์เลี้ยงลูกด้วยน้ำนมชนิดต่างๆ รวมทั้งยังมีประโยชน์ในการอธิบายความสัมพันธ์ระหว่างกลุ่มของสัตว์อีกด้วย อย่างไรก็ตาม ประโยชน์ของยีน TTR ในข้อนี้ขึ้นอยู่กับการมีอยู่ของลำดับนิวคลีโอไทด์ของยีน TTR ในกลุ่มสัตว์ชนิดต่างๆ ในวิทยานิพนธ์เล่มนี้ ได้ทำการศึกษาการจำแนกยีน TTR จากตับของค้างคาวเล็บกุด (*Eonycteris spelaea*) และปลาอีก 3 ชนิด คือ ปลากดเหลือง (*Mystus nemurus*) ปลานิลแดง (*Oreochromis niloticus*) และปลาไหล (*Monopterus albus*) จากการศึกษาชิ้นส่วน TTR cDNA จากปลาทั้ง 3 ชนิด ซึ่งมีความยาว 70% เมื่อเปรียบเทียบกับยีนของสัตว์ที่มีกระดูกสันหลังชนิดต่างๆ และสำหรับ TTR cDNA สายเต็มจากค้างคาวเล็บกุด พบว่าลำดับกรดอะมิโนที่แปลได้ของ TTR จากปลาทั้ง 3 ชนิด มีความสัมพันธ์ใกล้ชิดกับ TTR ที่พบในสัตว์เลี้ยงลูกด้วยน้ำนม มากกว่าที่พบในปลากระดูกแข็ง (teleost) ในขณะที่ลำดับกรดอะมิโนที่แปลได้ของ TTR จากค้างคาวมีความสัมพันธ์ใกล้ชิดที่สุดกับ TTR จากหมู และเมื่อเปรียบเทียบลำดับกรดอะมิโนดังกล่าวจากค้างคาวกับ TTR จากคน พบกรดอะมิโน 2 หน่วยที่ปลาย N-terminal ใน TTR จากค้างคาวเล็บกุดขาดหายไป ซึ่งลักษณะดังกล่าวเหมือนกับที่พบใน TTR ของเม่นแคระพันธุ์ยุโรป (*Erinaceus europaeus*) ซึ่งมีปรากฏในรายงานการวิจัย สำหรับความเชื่อมโยงทางสายพันธุ์ที่ถูกสร้างขึ้นจากการเทียบเคียงเข้าคู่ (pairwise alignment) ลำดับกรดอะมิโนของ TTR จากสัตว์มีกระดูกสันหลังชนิดต่างๆ สนับสนุนความสัมพันธ์ใกล้ชิดระหว่าง TTR จากปลากับ TTR จากสัตว์เลี้ยงลูกด้วยน้ำนม และระหว่าง TTR จากค้างคาวกับ TTR จากหมู นอกจากนี้การศึกษาการแสดงออกของยีน TTR ระหว่างพัฒนาการของลูกปลา

กดเหลือง พบการสังเคราะห์ TTR mRNA ขึ้นในตับ ตั้งแต่ลูกปลากดเหลืองฟักเป็นตัวอ่อนและการสังเคราะห์ลดลงเมื่อลูกปลามีอายุมากขึ้น ชี้ให้เห็นถึงการสังเคราะห์ TTR และการหลั่งสู่กระแสเลือด รวมทั้งความสำคัญของ TTR ที่มีต่อการพัฒนาการของปลากดเหลือง

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ABSTRACT

Transthyretin (TTR) is one of the major thyroid hormone (TH)-binding proteins found in plasma of most vertebrates. In larger mammals, it is mainly synthesized in liver and choroid plexus of brain. The systematic study of the evolutionary change of TTR structure revealed that the primary structure of TTR is highly conserved, except the N-terminal region is shorter and more hydrophilic during evolution of eutherian TTRs from their common ancestors. This coincides with the binding affinity of TTR to THs that increases for T4 but decreases for T3 during the evolution. The gene of TTR is an invaluable source of characters for use in phylogenetic studies of various mammalian groups and also be useful in resolving relationships within an animal group. However, the advantage of TTR gene on evolutionary trees of animals depends predominantly on the available nucleotide sequence of the TTR gene of the animal group. In this thesis, identification of TTR genes from livers of bat *(Eonycteris spelaea)* and 3 species of fish, i.e., green catfish (*Mystus nemurus*), tilapia (*Oreochromis niloticus*) and eel (*Monopterus albus*) was attempted. The partial fragments of TTR cDNA, which each corresponds to ~70% of the whole gene, could be amplified from fish, and the full length cDNA was from bat. The deduced amino acid sequences of TTR from these 3 fish species were more closely related to eutherian than to teleost TTRs, whereas that of bat was mostly related to pig TTR. In comparison to human TTR, two amino acid residues are missing from the N-terminal region of bat TTR, similar to that reported in hedgehog (*Erinaceus europaeus*). The phylogenetic trees, which were constructed from pairwise-alignment of the deduced amino acid sequences of TTRs among vertebrate species, supported the closely relationship between fish and eutherian TTRs, and between bat and pig TTRs. In addition, the expression of TTR gene during

development of the green catfish larvae was studied. The synthesis of TTR mRNA in liver was detected since the larvae emerged from eggs and declined when the animal was older, indicating the presence of TTR in blood and implying to functions of TTR during development of the catfish.

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LIST OF ABBREVIATIONS AND SYMBOLS

^{o}A	=	angstrom
AOX	=	alcohol oxidase
cDNA	=	complementary deoxyribonucleic acid
CSF	=	cerebrospinal fluid
^{o}C	=	degree Celcius
DNA	=	deoxyribonucleic acid
g	=	gram
h	=	hour
kDa	=	kilodalton
kg	=	kilogram
kV	=	kilovolt
l	=	liter
M	=	molar
mg	=	milligram
min	=	minute
ml	=	milliliter
MMLV	=	Mloney murine leukemia virus reverse transcriptase
nm	=	nanometer
OD.	=	optical density
PCR	=	polymerase chain reaction
pmol	=	picomole
RACE	=	Rapid amplification of cDNA ends
RBP	=	retinol binding protein
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcription-polymerase chain reaction
rpm	=	revolutions per minute
s	=	second
T3	=	triiodothyronine
T4	=	tetraiodothyronine

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

TdT	=	terminal deoxynucleotide transferase
TH	=	Thyroid hormone
TTR	=	transthyretin
v/v	=	volume by volume
μg	=	microgram
μl	=	microliter

CHAPTER 1

INTRODUCTION

Introduction

Thyroid hormones (THs), in both forms of L-3,5,3′,5′-tetraiodothyronine (L-thyroxine, T4) and L-3,5,3′-triiodothyronine (L-thyroxine, T3), have a wide range of biological effects on all vertebrate species. They are involved in growth, differentiation, metamorphosis, reproduction, hibernation, and thermogenesis (for review sees Power *et al.*, 2000). The basic molecular structure of TH contains highly hydrophobic phenyl structure, which results to a strong tendency to partition into lipid membranes and lose from bloodstream of the hormones. To ensure distribution throughout the vascular system to target cells, THs bound to specific carrier proteins in blood circulation (Robbins, 1996). In the plasma of larger mammals, three main blood plasma TH-binding proteins have been identified so far. These include thyroxine-binding globulin, transthyretin (prealbumin; TTR), and serum albumin (Larsson *et al.*, 1985).

TTR is a homotetrameric protein that has a molecular mass of ~55 kDa. In human, each subunit of TTR has a molecular mass 14 kDa (Blake *et al.*, 1978) and consists of 127 amino acid residues (Kanda *et al.*, 1974). In eutherians including mammals and marsupials, and birds, liver and choroid plexus are the major sites of synthesis. However, in reptiles, TTR is synthesized only in choroid plexus of the adults (for review sees Power *et al.*, 2000). To date, amino acid or deduced amino acid sequences of TTRs from over 30 vertebrate species have been identified. During evolution of these vertebrates, the primary structure of TTR monomer is highly conserved and has not been altered since more than 400 million years ago (for reviews see Schreiber *et al.*, 2002; Richardson, 2007). The only predominant change occurs at the N-terminal region of TTRs. These N-terminal segments of TTRs in fish, birds, reptiles and amphibians are longer and relatively more hydrophobic than those in mammalian TTRs. These coincide with the binding affinities to THs that increased for T4 but decreased for T3 during evolution of mammalian TTRs from their common

ancestors (Chang *et al*., 1999). In addition, the most characteristic feature of TTR from lower vertebrates such as birds, amphibians, and fish is they have higher affinity for T3 than for T4 (Yamauchi *et al*., 1993, 1999; Chang *et al*., 1999).

To date, several genes including TTR gene has been used in phylogenetic studies of various mammalian groups, such as Carnivora (Flynn and Nedbal, 1998), Bathyergidae and Cavioidea rodents (Rowe and Honeycutt, 2002; Walton *et al*., 2000), Ursidae (Yu *et al*., 2004) and Primates (Yoder and Yang, 2004). They are useful in resolving relationships among and within an animal group. In particular, the gene of TTR showed good concordance with results from mitochondrial gene sequences and no un-resolvable differences with other lines of evidence including comparative morphology or the fossil record (Walton *et al*., 2000) suggested the advantage of the gene in evolutionary filed. However, this usefulness of TTR depends predominantly on the available nucleotide sequence of the TTR gene of the animal group. In this thesis, non-available nucleotide sequences of TTR from particular vertebrates including *E. spelaea*, *M. nemurus*, *O. niloticus* and *M. albus* were identified so that their usage in elucidating the evolutionary interrelationships among or within species or other entities that are believed to have a common ancestor could be attempted.

Review of Literatures

1.1 Structure of TTR

Transthyretin (TTR) was first found in serum and cerebrospinal fluid of human (Kabat *et al*., 1942a, b). It is a homotetrameric protein, which in human, has a molecular mass of ~55 kDa. Each subunit has a molecular mass of 14 kDa (Blake *et al*., 1978) and consists of 127 amino acid residues (Kanda *et al*., 1974). Human TTR was the first TTR whose amino acid sequence was determined from its X-ray crystal structure (Blake *et al*., 1974, 1978; Wojtczak *et al*., 1992; Hamilton *et al*., 1993). The analysis at 2.5 Å (Blake *et al*., 1974) and 1.8 Å resolutions (Blake *et al*., 1978) revealed that it contains high β-sheet content with only a small length of a helix. The amino acid residues of each TTR subunit arranged into two four-stranded β-sheets (denoted as DAGH and CBEF; Figure 1.1), which are anti-parallel to each other and one sheet stacked upon another. A short helix locates at the end of strand E

(see Figure 1.1). The interaction between monomers that forms a stable dimer is hydrogen bonding between the β-strands at the edges of two strands (F and H) of monomer (Blake *et al*., 1978). To form the tetramer, two dimers interact through the hydrophobic contact between residues that situated in AB and GH-loops. Two thyroxine-binding sites, with two sterically equivalent, are situated in the large hydrophobic channel of the tetramer, which formed by two dimers (Figure 1.2) (Wojtcak *et al*., 1996). However, because of a negative co-operativity, only one binding site is occupied by TH under physiological condition (Pages *et al*., 1973; Nillson *et al*., 1975; Naylor and Newcomer, 1999). The unusual high content of β-structure, the interactions between monomers to form dimers and those between dimers to form tetramer, all contribute to make TTR to be one of the most stable globular proteins (Branch *et a*l., 1971 and 1972; Blake *et al*., 1978).

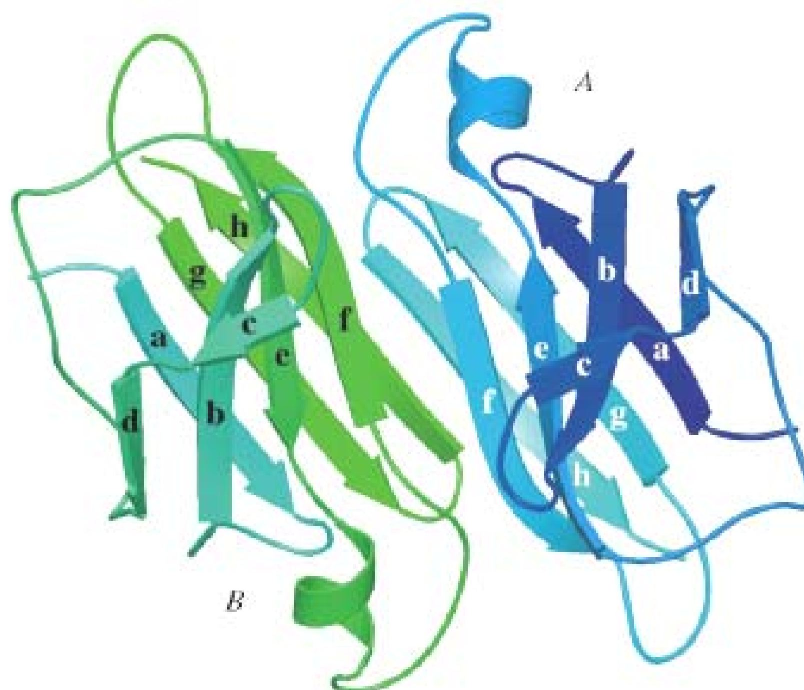


Figure 1.1 Structure of human TTR dimer

Each TTR subunit (A and B) consists two of four- strands (DAGH and CBEF) that are antiparallel to each other. A short helix was found located at the end of strand E. The interaction between monomers that forms a stable dimer with hydrogen bonding between the β-strands at the edges of two monomers strands (F and H) (From: Esnouf, 1997; Merrite and Murphy, 1994; Ghosh *et al*., 2000)

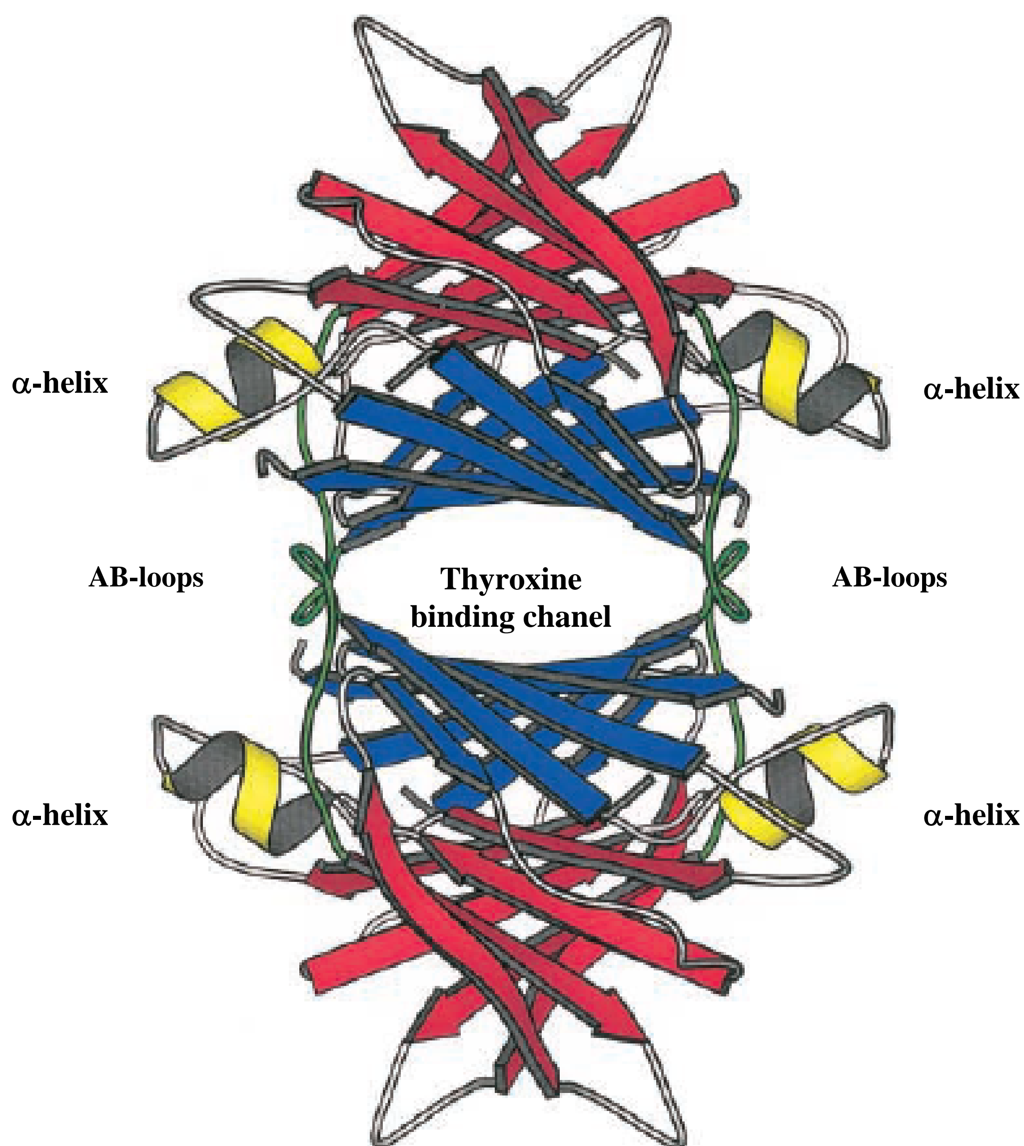


Figure 1.2 Structure of human TTR tetramer

To form the tetramer, two dimers interact together via AB loops. The thyroxine-binding sites are situated in one large hydrophobic channel that is formed between two dimers at the tetramer interface. (modified from Hamilton and Benson, 2001).

1.2 Evolutionary structure of TTR

The comparison of the X-ray structure of TTRs from several vertebrates such as human (Wojtczak *et al*., 2001), rat (Wojtczak, 1997), chicken (Sunde *et al*., 1996) and fish (*Sparus auratus*) (Folli *et al*., 2003) showed that almost structures are similar. Only differ is the absence of helical structure in chicken TTR subunits. This indicated that the overall three-dimensional structure of TTR has not been changed during evolution of the vertebrates (for review sees Richardson 2007). Alignment of the TTR primary structures, either obtained by directly from amino acid sequencing or derived from the nucleotide sequence of TTR cDNA, from over 20 vertebrates including mammals (small eutherians, insectivores, marsupials), birds, reptilians, amphibians and fish shows that residues in all positions in the central channel of TTR including those involved in the binding interaction with THs (Blake and Oatley, 1977; Blake, 1981; Wojtczak et al., 1996) are conserved and have not been altered since primitive fish (Figure 1.3). On the contrary, the predominant change occurred during evolution locates only in the first 10 amino acids (based on the amino acid numbering of human TTR) of the N-terminal region of the TTR subunit. Three to nine (for birds, reptiles, amphibian and fish) and two (for marsupials) additional amino acids (compared with human TTR) are found in this region. A systematic change of the N-terminal region of the TTR subunit from longer and relatively hydrophobic to shorter and more hydrophilic is observed in the evolution of TTRs from eutherians from their common ancestors (Chang *et al*., 1999; Schreiber *et al*., 1998; for reviews see Schreiber and Richardson, 1997; Schreiber *et al*., 1998).

During evolution of vertebrates, the N-terminal region of TTR subunit was changed from longer and more hydrophobic in lower vertebrates, e.g., avian, reptilian, amphibian and fish, to shorter and more hydrophilic in euterians. The systematic analysis and comparison of the nucleotide sequences TTR mRNAs from vertebrate including eutherians, marsupials and a bird revealed shifting in successive steps of the 3′ splice site of the TTR intron 1 in the 3′ direction during evolution of eutherian from their ancestors similar to bird (Aldred et al., 1997). Shifting of the 3′ splice site of the TTR intron 1 in the 3′ direction was postulated to occur in successive steps, which led to a successive shortening of the TTR N-terminal region during

evolution of eutherian TTRs from their ancestral TTRs (Aldred *et al*., 1997; for reviews see Schreiber and Richardson, 1997 and Schreiber *et al*., 1998). The mechanism underlying the 3′ splicing site movement is a series of single base mutations that converted the particular amino acid codons into new splice recognition sites. Recently, isolation of TTR from lamprey, the most ancient living vertebrates, was determined (Manzon *et al*., 2007). The results showed that the N-terninal region of TTRs in *Petromyzon marinus* and *Lampetra appendix* are nine amino acids longer than eutherians. These data strongly supports the changes in the N-terminal region of TTR and the mechanism underlying during vertebrate evolution.

Changes, but with less extent, also occurred at C-terminal region of TTR subunit during evolution of vertebrates. The C-termini of TTRs from pig and bullfrog have additional amino acid residues in comparing to that of TTRs from other vertebrates. In addition, the C-terminal sequences of TTRs from reptilian, amphibian and fish were more hydrophobic than in mammalian TTRs. Since the N- and C-terminal segments locate near the entrance to the central channel that harbors the binding sites for THs, they have been suggested to affect the accessibility of the hormones to the binding sites (Duan *et al.,* 1995a).

The influence of the N-terminal regions on the binding of TTR to THs was demonstrated using chimeric TTRs (Prapunpoj *et al*., 2002, 2006). The heterologous expression system of *Pichia pastoris* was used to produce the recombinant chimeric TTRs in which the N-terminal regions were altered or truncated. The affinities for binding to T4 and T3 of the recombinant proteins were determined. The Kd values for T3 and T4 of the chimeric TTRs significantly differed from the native TTR, leading to the postulation that the N-terminal region has role in determining the binding affinities for T3 and T4 to TTR (Prapunpoj *et al*., 2006). This hypothesis was later experimentally confirmed in fish TTR (Morgado *et al*., 2007). The affinity of T4 for the seabream TTR decreased when the first six amino acid residues of the N-terminal segment were removed, suggesting a possible role of these residues in the binding to T4.

```
                                                       Start of mature protein        β-strand                 β-strand              β-strand
                                                                  ↓                       A                        B                     C
HOMO_SAPIENS              : MASHRLLLLC LAGLVFVSEA GPT - - - - - - - - - GTGESKC PLMVKVLDAV RGSPAINVAV HVFRKAADDT WEPFASGKTS
PAN_TROGLODYTES           : .......... .......... ... - - - - - - - - - ....... .......... .......... ...K....E. ..........
MACACA_FASCICULARIS       : .......... .......... ... - - - - - - - - - .VD.... .......... .....V.... N..K....E. .A........
MUS_MUSCULUS              : ...L..F... .......... ..A - - - - - - - - - .A..... .......... .....VD... K..K.TSEGS .........A
RATTUS_NORVEGICUS         : ...L..F... ....I.A... ..G - - - - - - - - - .A..... .......... .....VD... K..KRT..GS .........A
SUS_SCROFA                : ...Y...... .......... ..A - - - - - - - - - .A..... .......... .....V..G. K..K....G. .....L....
OVIS_ARIES                : ...F...... .......... S.A - - - - - - - - - .A..... .......... .....A..G. K..K....E. ..........
BOS_TAURUS                : ...F..F... .......... .SV - - - - - - - - - .A..P.. .......... .....A..G. K..K....E. ..........
SOREX_ARANEUS             : ...R...... .....L.T.. ... - - - - - - - - - ...Q... .......... Q....V.... R..K....E. ..........
MACROPUS_GIGANTEUS        : ..F.S..... ....A....T AAV - - - - - - - H H ESEH... .......... ..R..V..D. K..K.TEEQ. ..L..A...N
MACROPUS_EUGENII          : ..F.S..... ....A....T AAV - - - - - - - H H EGEH... .......... ..R..V..D. K..K.TEEQ. ..L..A...N
PETAURUS_BREVICEPS        : ..F.S..... ....L..... ..V - - - - - - - A H .GED... .......... ..R..V..D. K..K.TEKQ. ..L......N
SMINTHOPSIS_MACROURA      : ..F.S..... ......L... ..V - - - - - - - A H .AED... ........S. .....V..D. K..K.TEEQ. ..L......N
MONODELPHIS_DOMESTICA     : ..F.S....G ..S.L...D. A.V - - - - - - - I H .AED... .......... .....V..N. K..K.SEEQ. .....T...N
TRACHYDOSAURUS_RUGOSUS    : .G.SS...V. ...M.YLT.. A.L - - - - - - V S H .SID... .......... ..R..TSI.. K.SKMSEEGD .KE..N...N
GALLUS_GALLUS             : ..F.ST..VF ......L... A.L - - - - - - V S H .SVD... .......... .....A.... K..K....G. .QD..T...T
ANAS PLATYRHYNCHOS        : ..F.SA.FVF ......LA.. A.L - - - - - - V S H .SVD... .......... .....A.... K..K.G..GS .QD..T...T
RANA CATESBEIANA          : ..YYNT.A.L TIFIFSGAFH RAQ - - - - - - G T H .EAD... .......... ..I..AKLP. K..KQNE.KS .DLIS..T..
XENOPUS LAEVIS            : ...FKSF..L ALLAIVSE-. A.P - - - - G H A S H .EAD... .......... ..I..A.LL. N...QTESGK ..QIT....T
CROCODYLUS_POROSUS        : ..F.SM..VF ......LT.. A.L - - - - - - V S H .SID... .......... .....A...I K..K.TS.GD .QE..A...T
SPARUS_AURATA             : .LQPLHC..L ASAVLCNTAP T.. - - - - - - D K H .GSDTR. .....I..-. K.T..GS..L K.SQ.T..GG .TQI.T.V.D
CYPRINUS_CARPIO           : ..KAVICV.L VSLFACCRS- A.V - - - - - - G I H .GSDVH. ..T..I.... K.T..G.I.L D.Y.QDQGG. ..KI....VD
PETROMYZON_MARINUS        : .--RF.C..V .VASSSLLCR ADD D H K S H E S H E .GVKDS. .....AI.S. Q.K..AG.KL S.MK.QD.AS .KEV.T.V.G
LAMPETRA_APPENDIX         : .T-RF.C..V .VASSSLLCS ADD D H K S H E S H E .GVKDS. .....AI.S. ..K..AG.KL S.MK.QE.AS .KEV.T.V.G
                           -20       -10          1 3 -ι-θ-η-ζ-ε-δ-γ-β-α 4     10        20         30         40          50
```

```
                                   β-strand           β-strand    α-helix              β-strand             β-strand      β-strand
                                      D                  E                                 F                    G             H
HOMO SAPIENS                  : ESGELHGLTT EEEFVEGIYK VEIDTKSYWK ALGISPFHEH AEVVFTANDS GPRRYTIAAL LSPYSYSTTA VVTNPKE--
PAN TROGLODYTES               : .......... .......... .......... .......... .......... .......... .......... ...I...--
MACACA FASCICULARIS           : .......... .......... .......... S......... .......... ...H...R.. .......... .......--
MUS MUSCULUS                  : .......... D.K....V.R ..L....... T........F .D........ .H.H...... .......... ..S..QN--
RATTUS_NORVEGICUS             : .......... D.K.T..V.R ..L....... .........Y .......... .H.H...... .......... ..S..QN--
SUS_SCROFA                    : .F........ D.K....... ..L....... .........Y .......... .R.H...... .......... L.SS...GAL
OVIS_ARIES                    : D......... .DK....L.. ..L....... S........Y .......... .L.H...... .......... L.SS...--
BOS_TAURUS                    : .......... .DK....L.. ..L....... S........F .......... ...H...... .......... L.SS..A--
SOREX_ARANEUS                 : .F........ D.K.....I. ..L...T... .........Y V....H.... .K........ .......... L.SD...--
MACROPUS_GIGANTEUS            : DN..I.E... DDK.G..L.. ..F..I.... ...V.....Y .D.......A .H.H.....Q .....F.... I.S..T.--
MACROPUS_EUGENII              : DN..I.E... DDK.G..L.. ..F..I.... ...V.....Y .D.......A .H.H...... .....F.... I.S..T.--
PETAURUS_BREVICEPS            : DN..I.E..S DDK.G..L.. ..F..I.... ...V.....Y .D.......A .H.H.....Q .....F.... I.S..T.--
SMINTHOPSIS_MACROURA          : NN..I.E..S DDQ.G..L.. ..F..V.... TF.......Y .D.......A .H.H.....Q ...F.F.... ..S...D--
MONODELPHIS_DOMESTICA         : DY..I.E..N D.K.G..L.. ..F..F...N ...V.....Y .D...K...A .H.H...... .......... ..S...D--
TRACHYDOSAURUS_RUGOSUS        : .F..I.E... D.Q..Q.L.. ..F..S.... ...V.....Y .D...S.... .H.H...... ...F...... ..SD...--
GALLUS_GALLUS                 : .F..I.E... ..Q....V.R ..F..S.... G..L.....Y .D........ .H.H...... ...F...... ..SD.Q.--
ANAS_PLATYRHYNCHOS            : .Y..I.E... ..Q....T.R ..F..S.... G..L.....Y .D........ .H.H...... ...F...... ..SD.Q.--
RANA_CATESBEIANA              : SD..I.N.A. ..Q....... L.FA..RF.S K..LT....Y VD.......A .H.H..T.V. .T...F.... ..SDV..AHV
XENOPUS_LAEVIS                : .L..I.N... D.Q.T..V.. I.FA..AF.G K..L.....Y VD.......A .H.Q....V. .T...F.S.. I.SE.HDDL
CROCODYLUS_POROSUS            : .F..V.E..S D.K......R ..F..S.... ...L.....Y .D........ .H.H...... ...F...... ..SD.Q.--
SPARUS_AURATA                 : AT..I.N.I. .QQ.PA.V.R ..F...A..T NQ.ST....V .....D.HPE .H.H..L.L. ...F..T... ..SSVH.--
CYPRINUS_CARPIO               : MT..V.N.I. .Q..TP.V.R ..F...A... .E.RT...QL .D...E.HAE .H.H..L.L. ...F..T... ..VKAH.--
PETROMYZON MARINUS            : KT..S.H.IS DKD.T..T.. .RFE.QQ..T KT..T....A .....M.HGA .HKH.H.PM. ....FFA.G. I.VDG.GH-
LAMPETRA_APPENDIX             : KT..S.H.IG DKD.T..T.. .RF..QA..T KA..T....A .....M.HGA .HKH.H.PM. ...FF.A.GT I.GDAEGH-
                                          60         70         80         90        100        110        120
```

Figure 1.3 Comparative alignment of the primary structure of vertebrate TTRs

Dots indicate residues that are identical to those of human TTR. The first amino acid of the mature human TTR subunit is indicated with arrow, secondary structural elements of human TTR subunit are present above the alignment, and amino acids that reside in the central channel and are thought to participate in hormone binding are shade. The region where amino acids have been lost at 5′ end of exon 2 during the evolution of the TTR subunit is boxed. Numbering of amino acids that denotes below the alignment is based on the mature human TTR subunit; -α, -β, -γ, -δ, -ε, -ζ, -η, -θ, -ι indicate the positions of residues absent in eutherians. (From: Manzon *et al*., 2007)

1.3 Synthesis sites and secretion of TTR

The major synthesis site of TTR in many vertebrates is liver (for review sees Richardson, 2007). After synthesis, TTR is secreted into bloodstream, similar to other plasma proteins (Dickson *et al.,* 1982; Schreiber, 1987). The synthesis in liver was confirmed by the presence of large amounts of TTR mRNA detected by *in situ* hybridization (Dickson *et al.,* 1985; Jacobsson, 1988). TTR is synthesized as a pre-protein with a signal peptide, which is cleaved during translocation of the protein into an endoplasmic reticulum (ER), and TTR tetramer is formed within the ER of hepatocytes (Bellovino *et al*, 1996; 1998). TTR is distributed throughout the body in equilibrium with the intestinal fluid by penetrating the capillaries. In human plasma, the level of TTR increases successively after birth and reaches 250 μg/ml when becomes adult (Smith and Goodman, 1971). However, the concentration of plasma TTR decreases after the fifth decade of life. In addition, TTR is also synthesized and secreted by the choroid plexus of all vertebrate species, except amphibians and fish (Richardson *et al*, 1994). The TTR synthesized by choroid plexus is secreted into cerebrospinal fluid (CSF), and the majority of T4 in brain binds to this TTR (Hagen and Solberg, 1974; Palha *et al*., 2002). The concentration of TTR in CSF, in comparing to that of albumin and other plasma proteins in CSF, is higher than in the blood. This led to the suggestion to a major role of TTR in the transfer of T4 from the blood to the brain through the blood-choroid-plexus-CSF barrier (Schreiber *et al*, 1990; Schreiber, 2002). Besides liver and choroid plexus, TTR was also found in several other tissues including the endocrine cells of stomach, small intestine and colon (Gray *et al*., 1985; Liddle *et al*., 1985), retinol pigment epithelium of the eye (Martone *et al*., 1988; Herbert *et al*., 1986; Cavallaro *et al*., 1990 a,b; Dwork *et al*., 1990; Mizuno *et al*., 1992; Ong *et al*., 1994; Jaworowski *et al*., 1995), kidney (Katoh *et al*., 1982; Kato *et al*., 1984), visceral extraembryonic endoderm and foregut endoderm (Makover *et al*., 1989), and placental tissue (McKinnon *et al*., 2005), however, only with less amount of TTR.

1.4 The biological functions of TTR

1.4.1 as a TH distributor

TTR is a plasma protein that plays important physiological roles in bind to and transports THs, both T4 and T3 forms, in the blood stream (Robbins, 1996). TTR also had a major role in transport T4 in CSF of the brain (Hagen and Solberg, 1974). However, some TTR in the CSF may not bind T4 at all near the choroid plexus and T4 is also likely to permeate through the blood–brain barrier away from the ventricular region (for review sees Schreiber, 2002). In plasma of human, TTR carried T4 on it with about 10% to 15% of other thyroxine binding proteins (Palha, 2002) and it bound to T4 stronger than T3 (Pages *et al*., 1973). The affinity of TTR to T4 and T3 was changed during evolution of vertebrates (for review, see Schreiber and Richardson, 1997; Schreiber *et al*., 1999).

The formation between two dimers to TTR tetramer generates a channel with two binding sites for THs (Blake *et al*. 1978; Wojtcak *et al*., 1996). The interaction of TH to TTR is negative cooperativity (Wojtczak *et al*., 1996), i.e., the binding of one molecule of T4 impedes the binding of the other (for review sees Raghu and Sivakumar, 2004). This is results from the conformational changes that induced by ligand binding to the molecule of TTR (Neumann *et al*., 2001). By comparing diameter of the TTR channel of the two binding sites, it revealed that binding of TH to the first site can change diameter of second.

1.4.2 as a carrier protein for retinol via binding to retinol binding protein (RBP)

TTR also binds retinol-binding protein (RBP), the specific protein carrier of all-*trans*-retinol. The interaction between RBP and TTR was examined by using mass spectrometry and the result showed two RBP molecules per a TTR tetramer (Rostom *et al*., 1998). This consisted to the crystallography experiment that revealed two molecules of RBP bind to one TTR tetramer (Figure 1.4) (Rask *et al*., 1971; Hornberg, 2004). The complex formation of TTR and RBP was found to occur at physiological pH and then falling gradually above and below this pH (van Jaarsveld *et al*., 1973). It also showed that the formation involved with a hydrophobic interaction (Peterson, 1971). The binding of RBP to TTR had no influence on its

binding to T4 (van Jaarsveld *et al*., 1973). Under physiological conditions, the complex between TTR and RBP is believed to play an important physiological role in prevention lose of low molecular weight RBP from glomerular filtration in kidneys (for review sees Hugo 2000). However, this is not likely occurred in some animals such as adult Australian polyprotodonta marsupials, which do not have TTR in bloodstream (Richardson *et al*., 1994). A difference in glomerular filtration size cut-off or other plasma protein than TTR binds to RBP in the marsupials was postulated (for review sees Richardson, 2007).

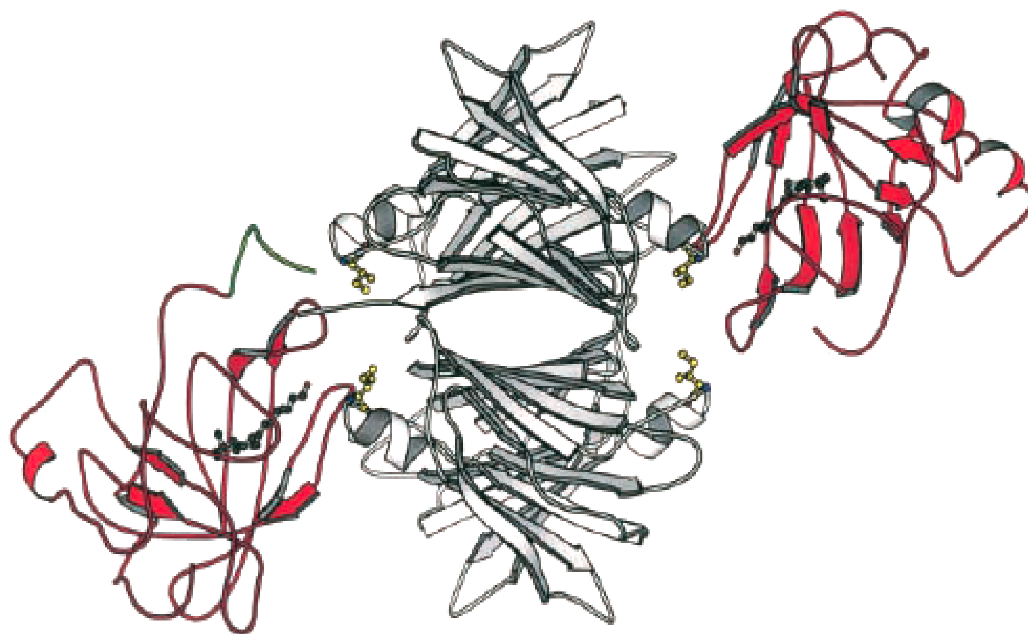


Figure 1.4 Ribbon model of the TTR-RBP complex

Two molecules of RBP (shown in red) bind to the TTR tetramer

(From: Monaco *et al*., 1995)

1.5 Evolution of TTR gene expression

1.5.1 In the liver

The ontogenesis of TTR gene expression in liver was studied in rat (Fung *et al*., 1988), sheep and chicken embryos (Southwell *et al*., 1991). TTR mRNA was appeared in the body of the embryo at the same time as the anlage of the liver (Southwell *et al*., 1991). The expression of TTR gene in the liver is under negative acute phase control (Dickson *et al*., 1982; Bartalena *et al*., 1992; Richardson *et al*., 1998; for reviews see Schreiber *et al*., 1989; Aldred and Schreiber, 1993). The rate of TTR synthesis and the level of TTR mRNA in the liver decreased dramatically during the acute phase response to trauma and inflammation (Birch and Schreiber, 1986; Milland *et al*., 1990; Richardson *et al*., 1998). The consensus sequence CTGGGAA in the 5'-flanking region of TTR gene showed response to interleukin 6 during acute phase and trauma (Fung *et al*., 1988).

TTR was found in the blood plasma of all eutherian and avian species. In the Australian marsupials, TTR was present in the plasma of diprodonts, but absent from the plasma of polyprotodonts (Richardson *et al*., 1994). All of the American marsupials belong to the order Polyprotodonta, however, only some of them contain TTR in their blood (Duan *et al*., 1995a; Richardson *et al*., 1996). Amphibians do not have TTR in their blood as adult. TTR gene is synthesized just before or at the climax of metamorphosis. However, TTR could be detected in the serum of the tadpoles of *Rana catesbeiana* (Miyauchi *et al*., 1977; Yamauchi *et al*., 1993, 1999; Prapunpoj *et al*., 2000b). For adult fish TTR absent in serum but TTR gene was expressed during early development or juvenile of *Sparus aurata* (Santos and Power, 1999), during smoltification in salmonids (Richardson *et al*., 2005; Yamauchi *et al*., 1999) and lifecycle including larval, metamorphic, parasitic feeding, and adult phases in lampreys (Manzon *et al*., 2007). Recently, TTR mRNA was observed in variety of larval tissues, with the highest levels found in the liver of the ancient living fish, lampreys (*Petromyzon marinus* and *Lampetra appendix*) (Manzon *et al*., 2007). In the liver TTR mRNA was found at all phase of the lifecycle. The lampreys TTR cDNA was cloned and characterized. The deduced amino acid sequences for lamprey TTR cDNAs are highest identity to that of *Sparus aurata* (Figure 1.3) (Manzon *et al*., 2007).

1.5.2 In the brain

TTR is also synthesized in choroid plexus of the brain. The concentration of TTR mRNA in choroid plexus is much higher than that in liver (Schreiber *et al*., 1990). Hormone status (Blay *et al*., 1993), trauma, inflammation (Dickson *et al*., 1986; 1987b) and, changes in morphology and multicellular organization of fetal choroids plexus epithelium (Thomas *et al*., 1992) did not affect TTR gene expression. The expression of TTR gene occurs before the formation of the blood-brain barrier and before the period of thyroid hormone dependency (Tu *et al*., 1990; Southwell *et al*., 1991). The kinetics of TTR gene expression in the developing choroid plexus differ among animal species (Thomas *et al*., 1989; Tu *et al*., 1990; Southwell *et al*., 1991). This suggested that TTR gene expression pattern is related to functional maturation of the brain (Tu *et al*., 1990; Southwell *et al*., 1991).

The expression of TTR gene in choroid plexus has been shown for vertebrate species including human (Dickson *et al*., 1986), rats (Dickson *et al*., 1985), sheep, dogs, cattle, pigs, rabbits, guinea pigs, mice (Harm *et al*., 1991), kangkaroos (Schreiber *et al*., 1993), dunnarts (Richardson *et al*., 1993; Duan *et al*., 1995), South American short tailed gray opossums (Duan et al., 1995a), chicken, pigeons, ducks, quails (Duan *et al*., 1991; Harm *et al*., 1991; Southwell *et al*., 1991), crocodiles (Prapunpoj *et al*., 2002), lizards (Achen *et al*., 1993), and turtles (Richardson *et al*., 1997). In crocodiles and lizards, TTR gene was expressed only in the brain (Prapunpoj et al., 2002). In amphibian, no TTR is expressed in choroids plexus of adults nor in tadpoles (Yamauchi *et al*., 1998; Prapunpoj *et al*., 2000b). Recently, TTR mRNA was detected in the brain of lampreys, although the researcher have not confirmed the presence of TTR in the CSF. This latter data are consistent with the finding for the teleost fish, *Sparus aurata*, (Santos and Power, 1999; Funkenstein *et al*., 1999). These findings led to, and confirmed, the hypothesis that the expression of TTR gene in the choroid plexus of the brain first appeared in evolution at the stage of the stem reptiles (Achen *et al*., 1993)

Objectives

1. To isolate and determine nucleotide sequence of TTR cDNAs from bat and fish whose database of the gene are not available.
2. To reveal gene structure of the TTRs.
3. To compare nucleotide and amino acid sequences of bat and fish TTRs to those deposited in GenBank.

CHAPTER 2

MATERIALS AND MATHODS

1. Materials

1.1 Instruments

Instruments	Model	Company
Autoclave	ES-315	Tomy
Balance (4 digits)	AB204-S	Mettler
Balance (2 digits)	PG5002-S	Mettler
Gel Document (Labworks 4.0)	C-80	UVP
Horizontal Electrophoresis	B1	Owl Scientific
Incubator		Memmert
Microcentrifuge	260D	DENVILLE
Micropipett		Gilson, Labnet, Labmate
pH meter	713	Metrohm
Spectrophotometer	8453	Hewlett-Packard
Spectrophotometer	G20	Thermo
Vortex-mixer	VX 100	Labnet
Instruments	Model	Company
Water Bath	WB-710M	Optima

1.2 Chemicals

1.2.1 Analytical grade

Chemical	Company
Absolute ethanol	Normapur
Agar	Merck
Calcium chloride	Merck
Dithiothreitol	Bio-Rad
Ethylene diamine tetraacetic acid (EDTA)	Carlo
Glycerol	Univar
Tryptone	Merck
Yeast extract	Merck

1.2.2 Molecular biology grade

Chemial	Company
Agarose	GenePure
Ampicillin	Calbiochem
100 bp DNA ladder	New England
	Biolabs (NEB)
*Eco*RI	NEB
Ethidium bromide	Promega
pGEM-T Easy vector	Promega
RNase H	Biolabs
Terminal transferase	Biolabs

1.2.3 Reagent kits

Reagent	Company
High-speed plasmid Mini Kit	Geneaid
QIA kit for PCR purification	QIAGEN
Gel/PCR DNA fragments extraction Kit	Geneaid

1.3 Bacterial cell

E. coli DH5α was gifted from Professor Schreiber, University of Melbourne, Australia.

1.4 Animals

Green catfish (*Mystus nemurus*) larvae (0.1-2 g) of 4, 8, 10, 16, 20, 26, 30, 40, 44 and 60 days old were from Songkhla inland Fisheries Station. The animals were killed, immediate frozen in liquid nitrogen and stored at -80°C until RNA was extracted.

Tilapia *(Oreochromis niloticus)*, 8.7 g body weight, and eel (*Monopterus albus*), 44.64 g body weight, were purchased from local market in Hat Yai, Songkhla. Bat *(Eonycteris spelaea)* was caught from natural habitat in Songkhla. The animals were anesthetized and their organs, e.g., liver, heart, spleen, were carefully removed. All organs were immediate frozen in liquid nitrogen and stored at -80°C until used.

2. Methods

2.1 Preparation and amplification of cDNA library by RT-PCR

2.1.1 Synthesis of first strand cDNAs by reverse transcription

The first strand of cDNAs was synthesized from total RNA by Moloney murine leukemia virus reverse transcriptase (MMLV RT) using random primer (Promega) to initiate the synthesis. In the reaction mixture (50 µl), 1 µg of total RNA was incubated in 50 mM Tris-HCl (pH 7.5) containing 20 mM NaCl, 0.22 mM dithiothreitol (DTT), 0.05 mM EDTA, 0.02% Triton X-100, 0.2 mM of dNTPs mixture (0.2 mM each of dTTP, dCTP, dGTP and dATP), 0.5 µg of random primer, and 500 units of MMLV RT. The reaction was performed at 42ºC for 1 h, and subsequently to 85ºC for 5 min to terminate reaction. The reaction mixture was stored at -20ºC for the second strand cDNA synthesis.

2.1.2 Synthesis of the second strand cDNAs and amplification of the cDNAs by PCR

PCR was performed to synthesize the second strands of cDNAs using the first strands as template. In general, the reaction mixture contained Tris-HCl (pH 8.3), KCl, $MgCl_2$, dNTPs and Taq DNA polymerase at appropriate concentrations. In addition, the synthesis and amplification were carried out at the conditions based on primers and type of PCR reaction, i.e., degenerate or specific PCR. In the final step, the reaction mixture was stored at -20°C until used.

2.2 Amplification for cDNA fragment by PCR

2.2.1 by degenerate PCR

The cDNA fragment of a target gene was amplified from the first strand cDNA library by PCR using degenerate primers (Table 2.1), which were designed based on the consensus amino acid sequence of TTRs from several vertebrate species (Manzon *et al.*, 2007). The reaction mixture (40 µl) comprised 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 mM dNTPs, 3.75 mM $MgCl_2$, 0.5 pmoles of each forward and reverse degenerate primers, 0.05 units of Taq DNA polymerase and 2 µl of the single stranded cDNA mixture. The hot-start amplification was

initiated at 94°C for 45s, followed by 35 cycles of annealing at 55°C for 45s, extension at 72°C for 1 min and denaturation at 94°C for 45s. A final extension was performed at 72°C for 10 min. Then, the PCR product was analyzed by electrophoresis on 1.5% agarose gel, and DNA band was visualized by staining gel with ethidium bromide.

2.2.2 by using specific primers

To amplify a specific gene fragment from the mixture of the double stranded cDNAs, PCR was performed using an appropriate specific primer set (Table 2.1). The reaction mixture (40 μl) was set up the same as described in section 2.2.1, except 0.5 pmoles of each appropriate specific primer (Table 2.1) was added to the reaction mixture. The hot-start amplification was initiated at 94°C for 45s (for TTR) or 30s (for β-actin), followed by 35 cycles of annealing at the annealing temperature (Table 2.1) for 45s (for TTR) or 30s (for β-actin), extension at 72°C for 1 min and denaturation at 94°C for 45s (for TTR) or 30s (for β-actin). A final extension was performed at 72°C for 10 min.

2.3 Amplification of 5′ and 3′ ends of cDNA by rapid amplification of cDNA ends–polymerase chain reaction (RACE-PCR)

Rapid amplification of cDNA ends (RACE) is the technique to amplify the nucleotide sequence from mRNA template between a defined internal site and unknown sequence at 5′ and 3′ ends of the mRNA. This technique was adopted to identify and amplify the nucleotide sequences at the 5′ and 3′ ends of bat liver TTR gene in which the full sequence could not be obtained by RT-PCR. The procedure was followed as described by the company (Invitrogen) with modifications.

2.3.1 Amplification of the 5′ end (5′ RACE)

To amplify the 5′ end, the first strand cDNA of bat TTR was first synthesized from bat liver mRNAs. In the reaction mixture (28.5 μl), it composed of

0.4 µg of the mRNAs, 2.5 pmoles of the internal specific primer, 5GSP1_BAT (Table 2.1), MMLV buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl), 2.2 mM $MgCl_2$, 0.35 mM dNPTs, 8 mM of DTT and 7 units of MMLV RT. The mixture was incubated at 42ºC for 50 min and, subsequently, at 70ºC for 15 min to terminate the reaction. Then, RNase H (0.2 units) was added prior the mixture was further incubated at 37ºC for 30 min to degrade the RNA template. The reaction mixture was chilled on ice for 5 min, then, the first strand cDNA was purified by extraction with the solution of phenol: chloroform: isoamyl (25:24:1). The DNA was precipitated with ethanol and dissolved in 50 µl of TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.5). In the next step, a homopolymeric tail was added to the 3′ end of the first strand cDNA. The tailing reaction comprised terminal buffer (10 mM Tris-HCl (pH 8.4), 25 mM KCl, 1.5 mM $MgCl_2$), 0.22 mM dCTP, 0.9 units of terminal deoxynucleotide transferase (TdT; Biolabs) and 10 ul of the first strand cDNA. The mixture was incubated at 37ºC for 10 min and, then, at 65ºC for 10 min to terminate the reaction. The dC tailed-cDNA was stored at -20°C until used.

The second strand cDNA was synthesized and amplified by PCR using other specific internal primers. To 5 µl of the dC tailed-cDNA, the primary PCR reaction mixture was set up in a final volume of 50 µl. The reaction comprised of PCR buffer (10 mM Tris-HCl, pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 pmoles of 5GSP3_BAT primer (Table 2.1), 0.4 pmoles of the deoxyinosine-containing anchor primer AAP(5) (Table 2.1), and 0.05 units of Taq DNA polymerase. The PCR was carried out for 35 cycles of denaturation at 94 ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 1 min. In the final step, an extension was performed at 72ºC for 5 min. The DNA product was analyzed by electrophoresis on 1% agarose gel for sufficient amount of the specific product; otherwise it was carried on in the next step by nested PCR.

By nested PCR, the reaction mixture (50 µl) was set up the same as that described for the primary PCR, except 5 µl of the primary PCR product was used as template and the primers incorporated in the reaction were 5GSP3_BAT (0.4 pmoles) and AUAP (0.4 pmoles) (Table 2.1). The PCR was carried out for 35 cycles

of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 1 min. The final extension at 72ºC was accomplished for 5 min. The PCR product was analyzed for the target band by electrophoresis on 1% agarose gel.

2.3.2 Amplification of the 3′ end (3′ RACE)

To amplify the 3′ end of cDNA, the mRNAs purified from bat liver was used as template for the first strand cDNA synthesis. The reaction mixture (21 μl) composed of 50 ng of mRNAs, 0.35 pmoles of AP(3) (Table 2.1), PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 2.4 mM $MgCl_2$, 9.5 mM DTT, 0.95 mM dNTPs, and 9.5 units of MMLV RT. The synthesis was performed at 42^oC for 50 min and the reaction was terminated by incubation at 70^oC for 15 min. To degrade the RNA template, RNase H (0.2 units) was added and the mixture was incubated at 37ºC for 30 min.

To synthesize and amplify the second strand, the PCR reaction (50 μl) that contained 2 μl of the first strand cDNA, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.2 pmoles of 5GSP2_BAT (Table 2.1), 0.2 pmoles of abridged universal amplification primer, AUAP (Table 2.1), to which targets the 3′ end of the AP(3) sequence, and 0.05 units of Taq DNA polymerase was set up. The synthesis and amplification was carried out for 35 cycles of denaturation at 94ºC for 30s, annealing at 65ºC for 30s and extension at 72ºC for 30s. The final extension was accomplished at 72ºC for 5 min. The specific target DNA band was analyzed by electrophoresis on 1% agarose gel.

2.4 Nucleotide sequcncing

Nucleotide sequence of DNA was determined at the Scientific Equipment Center, Prince of Songkla University (PSU) by termination sequencing method (Sanger *et al.*, 1977) using a Dye Terminator Cycle Sequencing kit (Applied Biosystems). Then, the DNA products were separated according to size in a polyacrylamide gel under denaturation. The data processing was performed using ABI Prism 377 Automated DNA sequencer (Applied Biosystems).

2.5 Alignment of nucleic acid and amino acid sequences of TTRs and phylogenetic analysis of the amino acid sequence

The CLUSTAL W was employed to perform phylogenetic analysis of TTR amino acid sequences from vertebrate species using pairwise-alignment and the neighbor-joining method (Saitou and Nei, 1987) option of the Phylip program (Galtier *et al.*, 1996) with 100 bootstrap replicates. The rooted and unrooted phylogenetic trees were constructed from the alignment by DRAWTREE and DRAWGRAM options of the Phylip program, respectively.

2.6 Total RNA extraction

Total RNA was isolated from whole body or organ tissue of animal by acid guanidinium-isothiocyanate-phenol-chloroform extraction method as described by Chomzynski and Sacchi, 1987.

In brief, tissue (100 mg) was homogenized in a solution (1 ml) contained 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol, using a glass-Teflon homogenizer. The homogenate was transferred to a polypropylene tube and reagents were added to the homogenate in sequential: 0.1 ml of 2 M sodium acetate (pH 4.0), 1 ml of phenol-chloroform (5:1) and 4 µl of isoamyl alcohol. The suspension was mixed by inversion after each addition, and then vigorously shook for 10 s prior being cooled on ice for 15 min. The mixture was centrifuged at 10,000xg for 20 min at 4ºC. The aqueous phase was transferred to a fresh tube and 1 ml of isopropanol was added to precipitate RNA. The mixture was kept at -20ºC for at least 1 h. The RNA pellet was collected by centrifugation at 10,000xg for 20 min and dissolved in 0.3 ml of 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1 M β-mercaptoethanol. Re-precipitation of the RNA was performed with 1 volume of isopropanol, and then the RNA pellet was washed once with 70% ethanol. The pellet was collected by centrifugation, vacuum dried and dissolved in RNase-free water. The RNA was stored at -80ºC until used.

2.7 Purification of polyadenylated RNAs

To prepare polydenylated RNA, an Oligotex mRNA kit from Qiagen was chosen. The isolation was carried out as described by the company (Kuribayashi *et al*., 1988). In brief, total RNA sample was mixed with the Oligotex suspension, which contains the oligo dT_{30} primers that are covalently linked to polystyrene latex beads. Polyadenylated RNAs bound to the primers with their polyadenylated tails while other RNA species were removed in washing step. Then, the polyadenylated RNA were eluted out from the beads with water. The solution of mRNAs was stored at -80°C until used.

2.8 Extraction and purification of DNA from agarose gel

The DNA fragment on agarose gel was isolated and purified by using Gel/PCR DNA fragments extraction kit (Geneaid) and followed the method described by the company (Vogelstein and Gillespie, 1979). In brief, a piece of the target DNA fragment was excised with a sterile single-edged razor blade. Then, the gel slice was incubated in the solution containing guanidine thiocyanate (gel: solution was 300 mg : 500 µl) at 55-60°C until the gel slide was completely dissolved (usually took 10-15 min). Then, the DNA solution was applied onto a spin column and DNA was allowed to bind onto the column membrane. Other impurities were washed out with a buffer containing ethanol. In the final step, DNA was eluted out from the column with an appropriate volume (15-50 µl) of 10 mM Tris-HCl, pH 8.5 and stored at -20°C until used.

2.9 Purification of PCR product

The DNA amplified by PCR was purified using a Gel/PCR DNA fragments extraction kit (Geneaid). An aliquot (100 µl) of a PCR reaction was mixed with 500 µl of the buffer containing guanidine thiocyanate. Then, the solution mixture was carried as already described in section 2.8. The DNA that bound to the column was eluted out with 15-50 µl of 10 mM Tris-HCl, pH 8.5 and stored at -20°C.

2.10 Purification of plasmids

The DNA plasmid was isolated from bacterial cell by the alkaline lysis procedure published by Birnboim and Doly (1979) and purified using the high-speed plasmid mini kit (Geneaid). In brief, an overnight culture of *E. coli* was prepared in the presence of an appropriate antibiotic. Cells were collected by centrifugation, and suspended in a buffer solution containing RNase A. Then, the bacterial membrane was disrupted by incubated cells in the solution of 0.1 M NaOH and 0.5 % SDS for 3-5 min at room temperature. Thereafter, the cell lysate was neutralized and adjusted with a chaotropic salt (1.6 M guanidine hydrochloride) for binding to silica material. The precipitated chromosomal DNA and cell debris that occurred were removed out by centrifugation prior the clear supernatant was loaded onto a spin column containing silica filter. DNA was allowed to adsorbed onto the silica matrix, while, all other unbound impurities were washed out with the solution of 20 mM NaCl and 2 mM Tris-HCl, pH 7.5 in 80 % methanol. The DNA plasmid was finally eluted out with TE.

2.11 Ligation and cloning of DNA fragment

The purified DNA of interest was cloned into pGEM-T easy vector (Promega) according to the method provided by the company. The ligation mixture included 25 ng of purified DNA, 50 ng of pGEM-T easy vector and Rapid Ligation buffer. The reaction was initiated by addition of 3 Weiss units of T4 DNA ligase. The ligation was performed at 16°C, overnight. Thereafter, 5 µl of the ligation product was transformed into 80 µl of the *E. coli* DH5α competent cell. The bacterial cells were stored on ice for 10 min, immediate heat-shocked at 42°C for 90s, and cooled down on ice for 90s. An aliquot of SOC medium was added prior the cell mixture was incubated at 37°C for 45-60 min. All transformation mixture was plated onto LB/amplicillin (100 µg/ml)/X-Gal agar, and cells were incubated at 37°C for 14-16 h. White single colonies that generally contain the DNA insert plasmid were selected.

2.12 Determination of RNA and DNA concentration

The RNA and DNA concentrations of sample solution were determined by spectrophotometric absorption at wavelengh 260 mm. One unit of the absorbance corresponds to 40 μg/ml of RNA or 50 μg/ml of double strand DNA according to Sambrook *et al*., 1989. Purities of the RNA and DNA in a preparation were determined from the ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}).

2.13 Preparation of *E. coli* competent cell

Competent cells were prepared from *E. coli* strain DH5α with a transformation efficiency of 10^6 to 10^7, using calcium chloride, as described by Cohen *et al*. (1972). A freshly grown single colony was picked from Luria-Bertani (LB) agar plates and transferred into 5 ml of LB broth in a 50-ml tube. Cells were grown overnight at 37°C in an orbital shaking incubator at 150 rpm. Then, 50 μl of the overnight culture was transferred to 25 ml of LB broth and cells were grown until the OD_{600} reached 0.4 to 0.5 (took 2 to 3 h). Thereafter, cells were cooled to 0°C and prior to collect by centrifugation at 2,500 rpm for 10 min at 4°C. The supernatant was removed and tube was inverted for 1 to 2 min to drain away any trace of medium. Cell pellets were then suspended in ice-cold 0.1 M calcium chloride (10 ml per 50 ml original culture) and cooled to 0°C. After centrifugation and draining, cells were suspended in ice-cold 0.1 M calcium chloride (2 ml per 50 ml original culture). Thereafter, the competent cells were aliquot and 60% glycerol was added to the final concentration of 15.5% (v/v). Immediately, cells were frozen in liquid nitrogen and stored at -80°C as a glycerol stock.

To transform with DNA, 50 μl of the competent cell suspension was mixed with DNA in a polypropylene tube and stored on ice for 30 min. Then, tube was incubated in a water bath at 42°C for exactly 90 s without shaking. Thereafter, cells were incubated at 37°C for 45 min and plated onto LB agar plate with an appropriate antibiotic.

Table 2.1 Oligonucleotide primers for amplification of TTR and β-actin genes in bat and fish

Gene	Method	Primer	Nucleotide sequence (5′→3′)	Direction
Bat TTR/ fish TTR	Degenerate PCR	**FTTR_degen_F**	CGACACCAAGTGCCCTCTGATGGTAARRT	Forward
		FTTTTR_degen_R	GGTGAACACCACCTCGGCGTACTCRTGRAAIGG	Reverse
Bat TTR (5′ end)	5′RACE (1° strand)	**5GSP1_BAT**	AGTTGTGAGCCCGTGGAGCTCTCCAAATTCACTGGTTTTCC	Reverse
	5′RACE (2° strand & amplification)	**5GSP3_BAT**	TGTGGCGAACGGCTCCCAGGTCTCATC	Reverse
		AAP(5)	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIGG	Forward
		AUAP	GGCCACGCGTCGACTAGTAC	Forward
Bat TTR (3′ end)	3′RACE	**5GSP2_BAT**	TGATGAGACCTGGGAGCCGTTCGCCACAG	Forward
		AP(3)	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTT	Reverse
Fish TTR (expression)	Specific PCR	**SPCFTTR2_F**	AGAAAGGCTGCTGATGACACC	Forward
		SPCFTTR2_R	AAGTGCCTTCCAGTAAGATTTGG	Reverse
β-actin	Specific PCR	**β-actin_F**	ATCGTGCGTGACATTAAGGAG	Forward
		β-actin_R	CGTACAGGTCTTTGCGGATG	Reverse

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Isolation, structure and gene expression of fish TTRs

In order to amplify and determine expression of a gene such as TTR by RT-PCR, one or more specific primer sets is required, and the specific primer design is a critical step for the success in the determination by this technique. Normally, the specific primers could be designed and synthesized based on the available nucleotide sequence either full-length or partial fragment of that gene. Unfortunately, neither nucleotide sequence nor amino acid sequence TTRs from green catfish (*M. nemurus*), tilapia (*O. niloticus*) and eel (*M. albus*) is available in database. Even though, nucleotide sequences of TTR have been reported in some fish species (Marchand *et al*., 2001; Yamano *et al*., 1994), those are quite low in similarity. Thus, in this thesis, isolation for full-length or partial fragment of TTR gene from these fish species was attempted, therefore, a specific primer could be generated and determination of the gene expression could be pursued.

3.1.1 cDNA cloning

3.1.1.1 Total RNA preparation

Total RNAs were prepared from liver of catfish, tilapia and eel by acid quanidinium-isothiocyanate phenol-chloroform extraction as described in section 2.6. In general, 348 μg of RNA could be isolated from 1 g of catfish liver, whereas, 443 and 224 μg of RNA could be obtained from 1 g of eel and tilapia livers, respectively. The total RNAs were analyzed on 1.5% formaldehyde agarose gel. Two major discrete bands corresponding to 18S and 28S rRNAs were clearly observed with only little degradation (Figure 3.1). The smear bands that are expected to be mRNAs could be detected. These indicated that the RNA preparations had a good quality to be a material for TTR gene isolation.

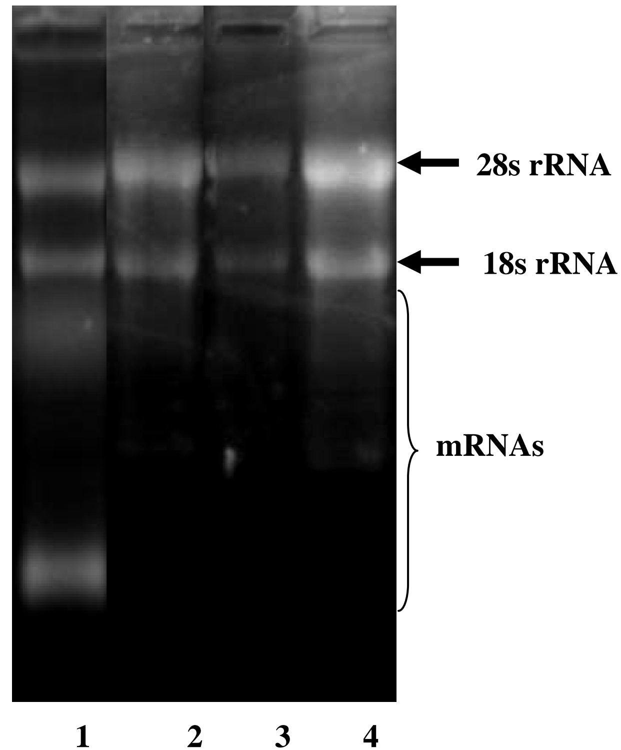


Figure 3.1 Patterns on agarose gel of total RNAs from catfish (1), tilapia (2), eel (3) and bat (4)

Total RNAs (~1.6 µg) isolated from livers of catfish, tilapia, eel and bat by acid guanidinium-isothiocyanate and phenol-chloroform extraction were incubated with a reagent contained 99.5% formamide at 65°C for 15 min prior they were loaded onto 1.5% formaldehyde agarose. The RNA bands were detected by staining gel in a solution of 4.5% ethidium bromide. The 18S, 28S rRNAs and positions of mRNAs were indicated.

3.1.1.2 Construction of cDNA libraries from fish livers by reverse transcription-polymerase chain reaction (RT-PCR) using degenerate PCR

The synthesis of the first strand of cDNA was primed to random primers. One microgram of liver total RNA was used in the synthesis reaction catalyzed by 500 units of Moloney murine leukemia virus (MMLV) RT, the RNA-dependent DNA polymerase that synthesizes the first strand of a cDNA from a single-stranded RNA template to which a primer has been hybridized. The random hexamers used in the reaction are short segments of single-stranded DNA that consist of every possible combination of bases, i.e., 4^6 or 4096 different combinations in the mixture. Because every hexamer was present, these primers can bind to any section of RNA. These double stranded segments are required as primers to start operation of reverse transcriptase. When the reaction was initiated by adding the RT, the enzyme moved and read codons along the mRNA, and synthesized sequences of DNA that complements the mRNA template as called complementary DNA or cDNA. The incubation temperature of 42°C for MMLV RT favored the more efficient reading through of secondary structure and increased the probability of the synthesis of full length cDNA. Success of a cDNA synthesis requires consideration of the characters and activities of both endogenous and contaminating RNase. In comparison to avian myeloblastosis virus (AMV) RT, MMLV has weaker endogenous RNase activity, which degrades the RNA strand of an RNA:DNA hybrid that would allow secondary priming and reduce the yield of full length cDNA. This makes MMLV RT more preferable and successful enzyme for synthesis of long length cDNA. In addition, since MMLV RT has low RNase H activity, pyrophosphate is not necessary to be included in the reaction mixture to inhibit the RNase activity to maximize the yield of full-length cDNA as it does require when AMV RT is used (Myers and Spiegelman, 1978).

The synthesis of the second strand of cDNA was carried out using the mRNA-cDNA hybrid as a substrate and degenerate primers, the mixtures of similar primers. These primers were designed based on the consensus sequence of the deduced nucleotide sequences or the amino acid sequences of TTR from several vertebrates (Manzon et al., 2007). In the primer, some positions of the primer had

several possible bases (Kwok *et al*., 1994) and some had inosine, a purine that occurs naturally in tRNAs. These degenerate primers primed to the first strand cDNA, and in the presence of Taq DNA polymerase, a thermostable DNA polymerase that can go through cycles of denaturation and renaturation of DNA template, the synthesis of the second strand DNA was catalyzed and the complete double-stranded cDNA was generated within the first cycle of PCR. An exponential amplification of the cDNA template was started from second cycle to end of the amplification reaction.

After amplification, the DNA was analyzed on agarose gel, and the conditions were optimized for only most specifics bands. A few discrete most specific bands were observed as shown in Figure 3.2. Thereafter, bands with an expected size were excised from gel and ligated into pGEM Teasy. The ligated plasmid was transformed into *E. coli* DH5α. The *E. coli* clones that obtained the plasmid were isolated prior the plasmid was purified and the nucleotide sequence was determined.

3.1.2 Partial nucleotide sequences of fish TTRs and their structures

Plasmid DNAs that were prepared from the isolated clones were subjected to nucleotide sequencing as described in section 2.10. The nucleotide and the deduced amino acid sequences of the partial TTR cDNAs from catfish, tilapia and eel were shown in Figure 3.3, 3.4 and 3.5, respectively. The TTR cDNA fragments were 270, 271 and 270 nucleotides in length could be isolated from catfish, tilapia and eel, respectively. These all were equivalent to 90 amino acid residues, which was approximately 70% of the entire TTR premature polypeptide strand reported in other vertebrate species (Marchand *et al*., 2001; Essner *et al*., 1997; Yamano *et al*., 1994; Lazar *et al*., 1988). When these deduced amino acid sequences of TTR cDNAs were aligned with that of human TTR, it indicated that the fragments were in most exon 2, whole exon 3 and a few exon 4 (amino acid residue ~10 to ~95 of the full-length human TTR) (Figure 3.3, 3.4, 3.5), which covered β-strand A, B, C, D, E and F, and α-helix domains of the TTR monomer. The identities of nucleotide and amino acid sequences among these partial cDNA fragments from catfish, tilapia and eel TTRs, and with sequences of other teleost fish or other vertebrate TTRs were calculated by the CLUSTAL W multiple sequence alignment (Higgins *et al*., 1992; Thomson *et al*.,

1994) as shown in Table 3.1A and 3.1B, respectively. The results showed there was quite low conservation between the TTR sequences studied in this thesis, i.e., from catfish, tilapia and eel, and those of other telelost fish (Table 3.1). The identities of the amino acid sequences were only 48% to 57%. In contrast, TTRs from catfish, tilapia and eel showed very high identity of the sequences to higher vertebrate TTRs, e.g., 82% to 93% to shrew TTR; 63% to 83% to pig TTR; and 80% to 95% to human TTR (Table 3.1B). In sea bream, the identity of the amino acid sequence of TTR was only 47% to 54% in comparing to those of human, pig and chicken TTRs (Santos and Power, 1999).

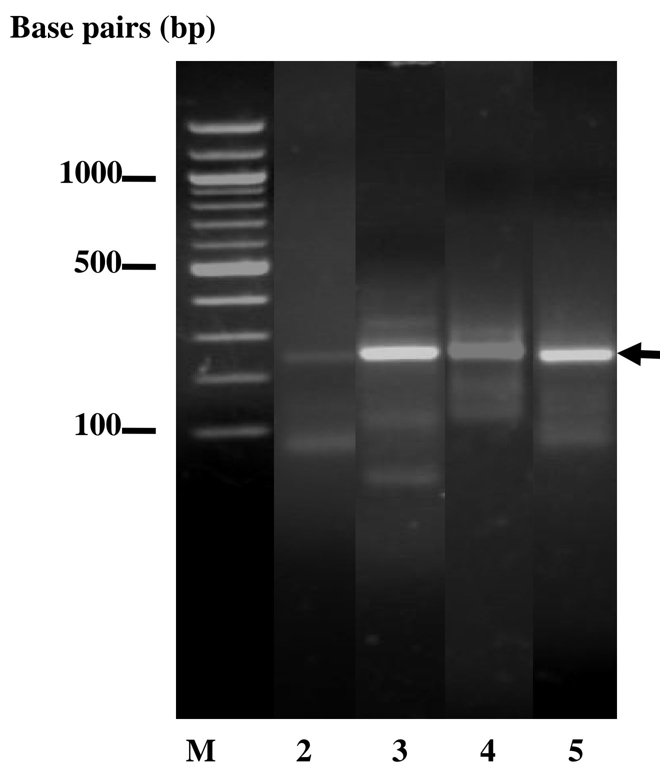


Figure 3.2 Amplified TTR cDNA fragments

TTR cDNA fragments of catfish, tilapia, eel and bat (lane 2, 3, 4 and 5) were synthesized and amplified by PCR using degenerate primers. The PCR products were analyzed by electrophoresis on 1.5% agarose gel. M is standard DNA marker. The expected band is indicated by arrow.

```
GAC ACC AAG TGC CCT CTG ATG GTG AAG ATT CTA GAT GCT GTC CGA GGC      48
Asp Thr Lys Cys Pro Leu Met Val Lys Ile Leu Asp Ala Val Arg Gly
             10                                      20

AGT CCT GCC ATC AAT GTG GCC GTG CAT GTG TTC AGA AAG GCT GCT GAT      96
Ser Pro Ala Ile Asn Val Ala Val His Val Phe Arg Lys Ala Ala Asp
                             30

GAC ACC TGG GAG CCA TTT GCC TCT GGG AAA ACC AGT GAG TCT GGA GAG     144
Asp Thr Trp Glu Pro Phe Ala Ser Gly Lys Thr Ser Glu Ser Gly Glu
     40                                      50

CTG CAT GGG CTC ACA ACT GAG GAG GAA TTT GTA GAA GGG ATA TAC AAA     192
Leu His Gly Leu Thr Thr Glu Glu Glu Phe Val Glu Gly Ile Tyr Lys
                     60                                      70

GTG GAA ATA GAC ACC AAA TCT TAC TGG AAG GCA ATT GGC ATC TCC CCC     240
Val Glu Ile Asp Thr Lys Ser Tyr Trp Lys Ala Ile Gly Ile Ser Pro
                                     80

TTC CAC GAG TAC GCC GAG TGT GTG TTC ACC                             270
Phe His Glu Tyr Ala Glu Cys Val Phe Thr
             90
```

Figure 3.3 Partial nucleotide sequences and the deduced amino acid sequence of TTR from catfish

Nucleotides are shown in bold. The deduced amino acids are indicated underneath the nucleotide sequence using standard triplet-letters abbreviations. Numbers on the right of the sequence refer to nucleotide in the 5′ to 3′ direction of the sequence, beginning with the first nucleotide of the cDNA insert. Numbering of the deduced amino acid residues as indicated underneath the sequence is based on human TTR.

```
                                                               C     1

GAC ACC AAG TGC CCT CTG ATG GTG AAA ATT CTA GAT GCT GTC CGA GGC     49
Asp Thr Lys Cys Pro Leu Met Val Lys Ile Leu Asp Ala Val Arg Gly
             10                                      20

AGT CCT GCC ATC AAT GTG GCC GTG CAT GTG TTC AGA AAG GCT GCT GAT     97
Ser Pro Ala Ile Asn Val Ala Val His Val Phe Arg Lys Ala Ala Asp
                             30

GAC ACC TGG GAG CCA TTT GCC TCT GGG AAA ACC AGT GAG TCT GGA GAG    145
Asp Thr Trp Glu Pro Phe Ala Ser Gly Lys Thr Ser Glu Ser Gly Glu
     40                                      50

CTG CAT GGG CTC ACA ACT GAG GAG GAA TTT GTA GAA GGG ATA TAC AAA    193
Leu His Gly Leu Thr Thr Glu Glu Glu Phe Val Glu Gly Ile Tyr Lys
                     60                                      70

GTG GAA ATA GAC ACC AAA TCT TAC TGG AAG GCA CTT GGC ATC TCC CCC    241
Val Glu Ile Asp Thr Lys Ser Tyr Trp Lys Ala Leu Gly Ile Ser Pro
                                     80

TTT CAT GAG TAC GCC GAG GTG GTG TTC ACC                            271
Phe His Glu Tyr Ala Glu Val Val Phe Thr
             90
```

Figure3.4 Partial nucleotide sequences and the deduced amino acid sequence of TTR from tilapia

Nucleotides are shown in bold. The deduced amino acids are indicated underneath the nucleotide sequence, using standard triplet-letters abbreviations. Numbers on the right of the sequence refer to nucleotide in the 5′ to 3′ direction of the sequence, beginning with the first nucleotide of the cDNA insert. Numbering of the deduced amino acid residues as indicated underneath the sequence is based on human TTR.

```
GAC ACC AAG TGC CCT CTG ATG GTG AAG GTT CTA GAT GCT GTC CGA GGC    48
Asp Thr Lys Cys Pro Leu Met Val Lys Val Leu Asp Ala Val Arg Gly
             10                                      20

AGT CCT GCC ATC AAT GTG GCC GTG CAT GTG TTC AGA AAG GCT GAT GAT    96
Ser Pro Ala Ile Asn Val Ala Val His Val Phe Arg Lys Ala Asp Asp
                             30

GAC ACC TGG GAG CCA TTT GCC TCT GGG AAA ACC AGT GAG TCT GGA GAG   144
Asp Thr Trp Glu Pro Phe Ala Ser Gly Lys Thr Ser Glu Ser Gly Glu
     40                                      50

CTG CAT GGG CTC ACA ACT GAG GAG GAA TTT GTA GAA GGG ATA TAC AAA   192
Leu His Gly Leu Thr Thr Glu Glu Glu Phe Val Glu Gly Ile Tyr Lys
                     60                                      70

GTG GAA ATA GAC ACC AAA TCT TAC TGG AAG GCA CTT GGC ATC TCC CCC   240
Val Glu Ile Asp Thr Lys Ser Tyr Trp Lys Ala Leu Gly Ile Ser Pro
                                     80

TTC CAC GAG TAC GCC GAG GTG GTG TTC ACC                           270
Phe His Glu Tyr Ala Glu Val Val Phe Thr
             90
```

Figure 3.5 Partial nucleotide sequences and the deduced amino acid sequence of TTR from eel

Nucleotides are shown in bold. The deduced amino acids are indicated underneath the nucleotide sequence, using standard triplet-letters abbreviations. Numbers on the right of the sequence refer to nucleotide in the 5′ to 3′ direction of the sequence, beginning with the first nucleotide of the cDNA insert. Numbering of the deduced amino acid residues as indicated underneath the sequence is based on human TTR.

Table 3.1 Comparison of nucleotide and amino acid sequences of catfish, tilapia and eel TTRs with other teleost fish.

Nucleotide and amino acid sequences of TTRs from catfish, tilapia and eel were aligned with other teleost fish. Percentage of identities of the nucleotide (A) and the deduced amino acid sequences (B) was calculated by CLUSTAL W multiple sequence alignment program (Higgins *et al*., 1992; Thomson *et al*., 1994). Sources of the nucleotide sequences: human (NM000371); shrew (AJ223149); pig (NM214212); chicken (NM205335); crocodile (AJ223148); xenopus (BC170446); seabream (AF059193); zebrafish (BC164894); carp (AJ544193);; Am. brook lamprey (DQ855961); and sea lamprey (DQ855960).

(A)

	human	shrew	pig	chicken	crocodile	xenopus	sea bream	zebra fish	carp	Am. lamprey	sea lamprey	catfish	tilapia
shrew	80												
pig	84	79											
chicken	49	60	57										
crocodile	50	57	55	82									
xenopus	52	53	54	59	57								
sea bream	35	38	38	35	34	41							
zebrafish	26	35	37	40	41	42	42						
carp	47	43	47	53	58	46	58	89					
Am. lamprey	20	37	35	27	28	34	34	23	42				
sea lamprey	20	44	32	27	27	37	34	23	45	81			
catfish	93	81	84	74	72	65	61	56	57	57	55		
tilapia	95	82	84	75	73	67	63	56	57	57	54	97	
eel	95	82	84	75	74	67	62	57	58	59	57	97	98

Sources of the amino acid sequences: human (CAA42087); shrew (CAA11130); pig (AAZ94915); chicken (CAA43000); crocodile (CAA11129); xenopus (AAI70446); seabream (AAF212451); zebrafish (CAX14014); carp (CAD66520); Am. brook lamprey (ABI93606); and sea lamprey (ABI93605).

(B)

	human	**shrew**	**pig**	**chicken**	**croco-dile**	**xenopus**	**sea bream**	**zebra fish**	**carp**	**Am. lamprey**	**sea lamprey**	**catfish**	**Tilapia**
shrew	85												
pig	87	87											
chicken	71	74	75										
crocodile	71	71	74	90									
xenopus	53	53	58	61	59								
sea bream	51	51	50	56	53	47							
zebrafish	46	53	43	52	51	45	63						
carp	49	45	46	52	51	46	65	91					
Am. lamprey	42	41	43	46	56	37	47	37	38				
sea lamprey	42	42	43	40	40	37	45	40	36	90			
catfish	80	93	83	74	70	60	56	53	56	48	46		
tilapia	93	80	83	75	70	60	57	54	57	50	47	97	
eel	95	82	63	76	73	63	55	53	56	50	48	95	97

3.2 Isolation, structure of bat TTR

The Chiroptera is the second most diverse order of mammals in which more than 900 species occurring around the world (Nowak, 1999). In comparison to other mammals, thyroid gland activity and concentrations of THs in blood of bats are highly depended on seasons and reproductive stages (Damassa *et al*., 1995; Singh *et al*., 2002). These indicate important of THs on development of the animal. The presence of TTR in the bloodstream of bats has been reported (Müller *et al*., 2007), and one of its functions is to transport a retinol. However, in similar to catfish, tilapia and eel TTRs, nucleotide sequence of bat TTR was not available in database. To reveal partial fragment or full length cDNA is, therefore, necessary for further studies in particular expression of the gene during development. In this thesis, identification of nucleotide sequence of full length TTR cDNA in bat liver was attempted with the powerful techniques of RT-PCR and rapid amplification of cDNA ends (RACE).

3.2.1 Total RNA preparation and partial cDNA fragment of bat liver TTR

Total RNA was prepared from liver of bat with the same method described for catfish, eel and tilapia in section 2.6. Approximate 940 µg of RNA could be isolated from 1 g of bat liver. Analysis on 1.5% formaldehyde agarose gel revealed two major discrete bands corresponding to 18S and 28S rRNAs (Figure 3.1). The A260/A280 was ~ 1.7. This indicated quality of the RNA preparation was good enough for the cDNA amplification.

3.2.2 Amplification and nucleotide sequencing of the partial cDNA fragment of bat liver TTR

The first and second strands of the TTR cDNA were synthesized and amplified by RT-PCR from total RNAs of bat liver using random and degenerate primers as previously described for fish TTRs in section 2.1. Analysis on agarose gel of the PCR products showed a major single DNA band with ~ 250 bp in size (Figure 3.2). The DNA was purified from gel prior it was inserted into pGEM Teasy vector, and

nucleotide sequence of the inserted DNA was determined by sequencing. The nucleotide and the deduced amino acid sequences of the partial TTR cDNA were shown in Figure 3.6. The TTR cDNA fragment with 190 nucleotides in length, which coded for 55 amino acid residues, was obtained. Alignment of this amino acid sequence with that of human TTR revealed the fragment covered presegment, exon 1, 2 and 3 of the mature TTR mRNA.

3.2.2 Full length of TTR cDNA from bat liver

3.2.2.1 Rapid amplification of cDNA ends (RACE)-PCR

Rapid Amplification of cDNA Ends (RACE) is a molecular biological technique to obtain the full length sequence of an mRNA such as TTR, which found within a cell. In the procedure, nucleic acid sequences are amplified from an mRNA template between a defined internal site and unknown sequences at either the 3' or the 5' -end of the mRNA (Frohman *et al*., 1988). Since RACE requires a defined internal site, a short internal sequence must already be known from the mRNA of interest. In general, a cDNA fragment is copied from a region of the mRNA by RT-PCR in the first step of RACE. From this partial cDNA sequence, specific internal primers are designed and extension of the partial cDNA from the unknown ends of the transcript back to the known region is achieved using primers that anneal to the pre-existing poly(A) tail at 3′ end or an appended homopolymer tail at 5′ end (for review see Frohman, 1994).

In this thesis, 5′ and 3′ RACES were performed slightly different according to the protocol described by the company (Invitrogen). The purified mRNAs were used as templates for the first round of cDNA synthesis by MMLV RT using a gene specific primer (GSP) (Table 2.1), an anti-sense oligonucleotide primer that recognizes the partial cDNA sequence of TTR. Thereafter, polyC was added by terminal deoxynucleotidyl transferase (TdT) to the 3′ end of the new synthesized single-stranded cDNA. This homopolymeric tail, i.e., polyC, was used as a string in next round of PCR, which used a second anti-sense TTR gene specific primer (GSP2 or GSP3) (Table 2.1) that also binds to the partial cDNA sequence of TTR, but at different site from that of the GSP. In the presence of a sense universal primer that binds the polyC tail, the cDNA product with the nucleotide sequence covered from the site for gene specific primer to 5′ end of the single-stranded cDNA was amplified. By analysis on

1.5% agarose gel, a single DNA band with ~ 400 bp in size was observed (Figure 3.7). The DNA band was excised from gel, purified, ligated into pGEM Teasy, and transformed into *E. coli* DH5α to be amplified *in vivo*. The plasmid was isolated and nucleotide sequence was determined.

To generate the 3′ end partial cDNA of bat liver TTR, RT-PCR was performed using the natural polyA tail that pre-exists at the 3′ end of all eukaryote mRNAs for priming during the reverse transcription of MMLV RT. In the presence of the oligo dT adaptor primer (AP; Table 2.1), priming of the primer to the polyA tail facilitated the RT to generate a single-stranded cDNA fragment. Then, this synthesized cDNA fragment worked as a template for PCR to generate double stranded 3′ cDNA from a known region by using a sense specific primer (GSP or nested GSP; Table 2.1) and an anti-sense primer, AUAP (Table 2.1), that has sequence complementary to the AP sequence. The 3′ end cDNA of bat TTR showed ~ 420 bp in size by electrophoresis on agarose gel (Figure 3.8). The DNA was purified from gel prior to ligating into pGEM Teasy and in vivo amplified in *E. coli* DH5α. The insert DNA was determined for nucleotide sequence by DNA sequencing as described in section 2.4.

3.2.2.2 Nucleotide sequences and structure of bat TTR cDNA

DNA sequences of 5′ and 3′ ends of the TTR cDNA were determined by dye cycle sequencing method described in section 2.4. Together with the cDNA fragment obtained by degenerate RT-PCR in section 2.2.1 (Figure 3.6), the entire nucleotide sequence of bat liver TTR cDNA was as shown in Figure 3.9B. Summary of the strategy to obtain the full length sequence was shown in Figure 3.9A. The nucleotide sequence together with the deduced amino acid sequence of the TTR was given in Figure 3.9B. The bat liver TTR cDNA was 599 nucleotides in length, followed by a sequence of 19 polyadenylate residues with G and C interrupting at positions 604 and 605, respectively. The start codon ATG was present 26 bases downstream from the 5′ end of the cDNA. The stop codon located at bases 461-463. The whole coding sequence was 435 nucleotides. Three additional in-frame stop codons were found in the 3′ end of untranslated region of the cDNA. When the deduced amino acid sequence of bat TTR cDNA was aligned with that of human TTR, two amino acid residues (glycine

and threonine) were found “missing” from the first 10 amino acid residues at the N-terminus of the mature bat TTR. This observation was similar to that was previously reported in hedgehog TTR (Prapunpoj *et al.*, 2000a). The identities of bat TTR nucleotide sequence to those of TTRs from other vertebrates was summarized in Table 3.2A, while those of the deduced amino acid sequences are in Table 3.2B. The results showed very high conservation between bat liver TTR sequences and those of other vertebrates, ranging from eutherians to fish. Among mammalians, bat TTR was the most closely related to pig TTR. The molecular mass of the bat TTR subunit, calculated from the deduced amino acid sequence, was 14 kDa.

```
                                   CAGAGGCCCACTGATTCTGGGCAGG      25
ATG GCT TCT CGG CGG CTA CTC CTC CTC TGC CTG GCT GGA CTG GTA TTT   73
Met Ala Ser Arg Arg Leu Leu Leu Leu Cys Leu Ala Gly Leu Val Phe
-20                                     -10

GTT TCA GAG GCT GGC CCT GTG GCT GAA CCC AAG TGT CCT CTG ATG GTC  121
Val Ser Glu Ala Gly Pro Val Ala Glu Pro Lys Cys Pro Leu Met Val
            -1  +1       3   6   7   8   9   10

AAA GTC CTG GAT GCT GTG CGG GGC AGT CCT GCT GTC GAC GTG GCT GTG  169
Lys Val Leu Asp Ala Val Arg Gly Ser Pro Ala Val Glu Val Ala Val
                     20                                      30

AAA GTG TTC AAG AAG GCT GCT                                      190
Lys Val Phe Lys Lys ALa ALa
```

Figure 3.6 Partial nucleotide sequences and the deduced amino acid sequence of TTR from bat

Nucleotides are shown in bold. The deduced amino acids are indicated underneath the nucleotide sequence, using standard triplet-letters abbreviations. Numbers on the right of the sequence refer to nucleotide in the 5′ to 3′ direction of the sequence, beginning with the first nucleotide of the cDNA insert. Numbering of the deduced amino acid residues as indicated underneath the sequence is based on human TTR.

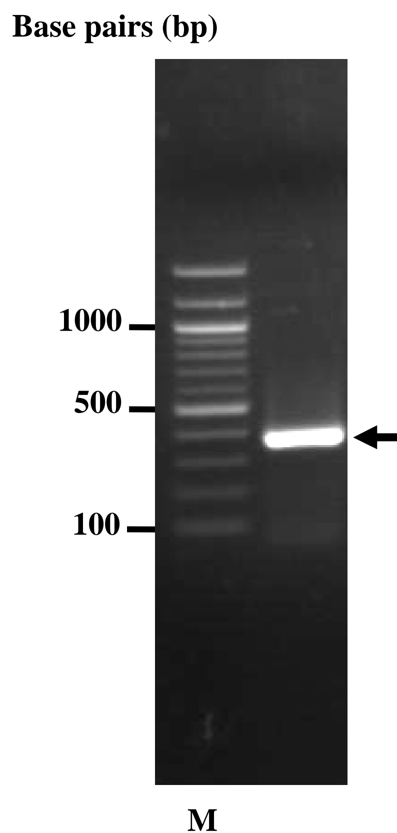


Figure 3.7 Analysis on agarose gel of the 5′ end fragment of bat liver TTR cDNA

The 5′ end of bat TTR cDNA was amplified by RT-PCR and RACE prior the PCR product was separated on 1 % agarose gel. M is standard DNA marker. The expected DNA fragment is indicated by arrow.

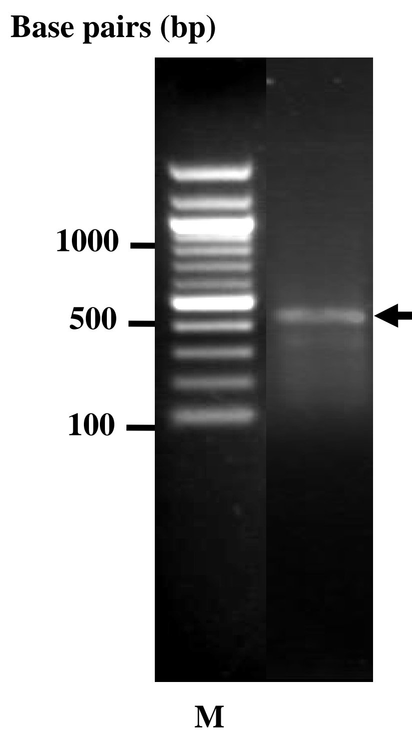
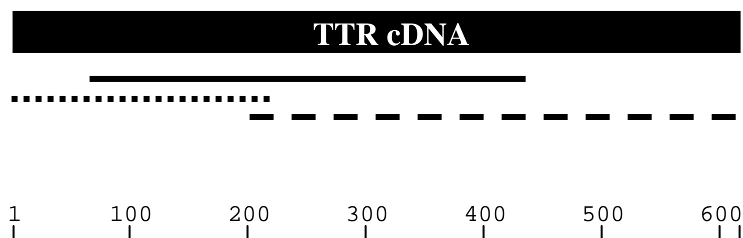


Figure 3.8 Analysis on agarose gel of the 3′ end fragment of bat liver TTR cDNA

The 3′ end of bat TTR cDNA was amplified by RT-PCR and RACE. Thereafter, the PCR product was separated on 1 % agarose gel. M is standard DNA marker. The expected cDNA fragment is indicated by arrow.

(A)



(B)

```
                                       CAGAGGCCCACTGATTCTGGGCAGG    25

PRESEGMENT ──────────→
ATG GCT TCT CGG CGG CTA CTC CTC CTC TGC CTG GCT GGA CTG GTA TTT    73
Met Ala Ser Arg Arg Leu Leu Leu Leu Cys Leu Ala Gly Leu Val Phe
-20
               MATURE PROTEIN ──────────→
GTT TCA GAG GCT GGC CCT GTG GCT GAA CCC AAG TGT CCT CTG ATG GTC   121
Val Ser Glu Ala Gly Pro Val Ala Glu Pro Lys Cys Pro Leu Met Val
             -1  +1                                  10

AAA GTC CTG GAT GCT GTG CGG GGC AGT CCT GCT GTC GAC GTG GCT GTG   169
Lys Val Leu Asp Ala Val Arg Gly Ser Pro Ala Val Asp Val Ala Val
                             20

AAA GTG TTC AAG AAG GCT GCT GAT GAG ACC TGG GAG CCG TTC GCC ACA   217
Lys Val Phe Lys Lys Ala Ala Asp Glu Thr Trp Glu Pro Phe Ala Thr
     30                                      40

GGG AAA CTC AGT GAA TTT GGA GAG CTC CAC GGG CTC ACA ACT ACT GAT   265
Gly Lys Leu Ser Glu Phe Gly Glu Leu His Gly Leu Thr Thr Thr Asp
                     50                                      60

AAA TTT GTA GAA GGA ATA TAC AAA GTG GTG CTA GAC ACC AAG TCC TAC   313
Lys Phe Val Glu Gly Ile Tyr Lys Val Val Leu Asp Thr Lys Ser Tyr
                                     70

TGG AAG GCG CTT GGC ATT TCC CCC TTC CAT GAA TAC GCA GAG GTG GTG   361
Trp Lys Ala Leu Gly Ile Ser Pro Phe His Glu Tyr Ala Glu Val Val
             80                                      90

TTC ACA GCC AAT GAC TCT GGC CAA CGC AAG TAC ACC ATC GCA GCT CTG   409
Phe Thr Ala Asn Asp Ser Gly Gln Arg Lys Tyr Thr Ile Ala Ala Leu
                             100

CTC AGT CCC TAC TCT TAC TCC ACC ACA GCC CTC GTC AGC GCC CCC AAA   457
Leu Ser Pro Tyr Ser Tyr Ser Thr Thr Ala Leu Val Ser Ala Pro Lys
     110                                     120

GAA TGA GCAAGCCCACCTTTGGTCAGTTTGAAGGAGGAGAAACAGGATTTCATGTAACCAT   515
GLU ***
125

CAGTATTCCATTTGTATTAAAGCAGTGTTTTCACTTTATAAGCTATGTTAGAAACTCAGGCAG   578
AGACAATAAAATATTCTTATTAAAAGCAAAAAAAAAAAAA                          618
```

Figure 3.9 Strategy to get full length (A), nucleotide and deduced amino acid sequences (B) of bat liver TTR cDNA

(A) To obtain full length of the TTR cDNA, degenerate RT-PCR, and rapid amplification of 5′ and 3′ ends were adopted. Solid, dot and broken lines indicate the nucleotide fragments generated by degenerate RT-PCR, 5′ RACE and 3′ RACE, respectively. The scale bar underneath the arrows shows length of the cDNA (in base pairs).

(B) Nucleotides are numbered in the 5′ to 3′ direction, as shown at the right of the figure. Nucleotides are presented in bold letters with the amino acid residues deduced from the cDNA sequence indicated below. Numbering of the nucleotides begins with the first nucleotide of the cDNA insert. Numbers for amino acid residues are given underneath the sequence. The amino acid residues of TTR presegment are numbered -20 to -1. The N-terminal residue of the mature protein, as determined from alignment with known mature TTR from other vertebrates, is designed as +1. The first in-frame stop codon, TGA is shown by three asterisks. A polyadenylation signal, AATAAA, near the 3′ end of the cDNA is underlined. Three additional in-frame stop codons (TAA) in the untranslated region at the 3′ end were highlighted in grey.

Table 3.2 Comparison of nucleotide and amino acid sequences of bat liver TTR with other vertebrates

Nucleotide and amino acid sequences of TTR from bat were aligned with those of other vertebrates. Percentage of identities of the nucleotide (A) and the deduced amino acid sequences (B) was calculated by CLUSTAL W multiple sequence alignment program (Higgins *et al*., 1992; Thomson *et al*., 1994). Sources: human (CAA42087); shrew (CAA11130); chicken (CAA43000); xenopus (AAI70446); pig (AAZ94915); gilthead seabream (AAF21245); crocodile (CAA11129); common carp (CAD66520); zebrafish (CAX14014); Am. brook lamprey (ABI93606); sea lamprey (ABI93605).

(A)

	human	shrew	hedgehog	pig	bat	Chicken	crocodile	xenopus	sea bream
shrew	80								
hedgehog	79	79							
pig	84	79	85						
bat	79	79	80	85					
chicken	49	60	55	57	69				
crocodile	50	57	70	55	66	82			
xenopus	52	53	45	54	44	59	57		
sea bream	35	38	34	38	35	35	34	41	
carp	47	43	45	47	44	53	58	46	58

(B)

	human	shrew	hedgehog	pig	bat	chicken	crocodile	xenopus	sea bream
shrew	85								
hedgehog	87	90							
pig	51	87	89						
bat	84	85	86	89					
chicken	74	53	76	75	73				
crocodile	71	71	73	74	71	90			
xenopus	53	53	60	58	55	61	59		
sea bream	51	49	51	50	49	56	53	47	
carp	49	45	43	46	71	52	51	46	65

3.3 Phylogeny of fish and bat TTR genes

In molecular phylogenetics or molecular systematics, the structure of molecules in particular DNA, RNA and protein is analyzed and the data is expressed in a phylogenetic tree in order to obtain information on evolutionary relationships of an organism. The most common analysis of the structure is the comparison of gene, either nucleotide or amino acid, sequences using sequence alignment techniques to identify similarity. Degree of similarity in the molecular structure of gene sequences of the closely related organisms is high, while, the molecules of organisms distantly related usually show a pattern of dissimilarity. A relationship tree is built from these similarities so that the probable evolution of organisms could be identified.

In this thesis, the partial amino acid sequences of TTRs from catfish, tilapia and eel were aligned to the amino acid sequences (including presegment) of TTRs from other teleost fish (Figure 3.10), and a phylogenetic tree was drawn by CLUSTAL W algorithm (Thomson *et al*., 1994) as shown in Figure 3.11. The tree revealed that the genetic distance between TTRs from catfish, tilapia and eel and those from seabream, carp and zebrafish was more closely related than to TTRs from lampreys. These data are consistent with the fact that while all fish are teleosts, which belong to Actnopterygii, only lampreys are the primitive non-teleost that belongs to Cephalaspidomorphi, the only extant member of the class Agnatha in which lineage dates back over 530 million years (Sower *et al*., 2006). Interestingly, in comparison to other vertebrates, TTRs from catfish, tilapia and eel were more closely related to larger mammal than to other teleost TTRs (Figure 3.11). This was different from that were previously reported in TTRs from other teleosts (Santos and Power, 1999; Kawakami *et al*., 2006; Manzon *et al*., 2007). The TTR amino acid sequence is highly conserved, in particular the amino acids those are involved in TH binding are virtually 100% conserved across taxa. Most of these amino acids locate in the middle of the sequence, the same region as the partial sequences of catfish, tilapia and eel TTRs. The sequence similarities of these 3 fish TTRs, thus, did not represent for the whole and, therefore, the phylogenetic tree obtained may not provide an absolutely interpretation. To confirm this observation, the full length sequences of catfish, tilapia and eel TTRs are necessary.

The deduced amino acid sequence of bat was aligned with the sequences of TTR from other vertebrates including eutherians, bird, reptile, amphibian and fish (Figure 3.12). The alignment supported the closely relationship between bat and other mammals. Only one of all amino acids those have been reported to line the central channel of the TTR tetramer and to be involved in the binding to THs (for review sees Hamilton and Benson, 2001) was conservative substitution, i.e., Val121 was substituted with Leu, whereas the rest were identical. Comparison of amino acid sequences of TTRs shows that the region coding for the N-terminal of the TTR subunit is the most variable region of the molecule (for reviews see Schreiber *et al*., 2002; Richardson, 2007). A shift in the 3′ direction of the intron 1/exon 2 splice site is the underlying mechanism, which results in the successive loss of amino acids and shorter, more hydrophilic N-termini during evolution of mammalian TTR from its ancestor (Aldred *et al*., 1997). Alignment of the deduced amino acid sequence of the bat TTR subunit with those from other vertebrates showed that bat TTR had two fewer amino acids in its N-terminal region than most of eutherians including human TTR (Figure 3.12). This observation is the same as that reported for hedgehog TTR (Prapunpoj *et al*., 2000a).

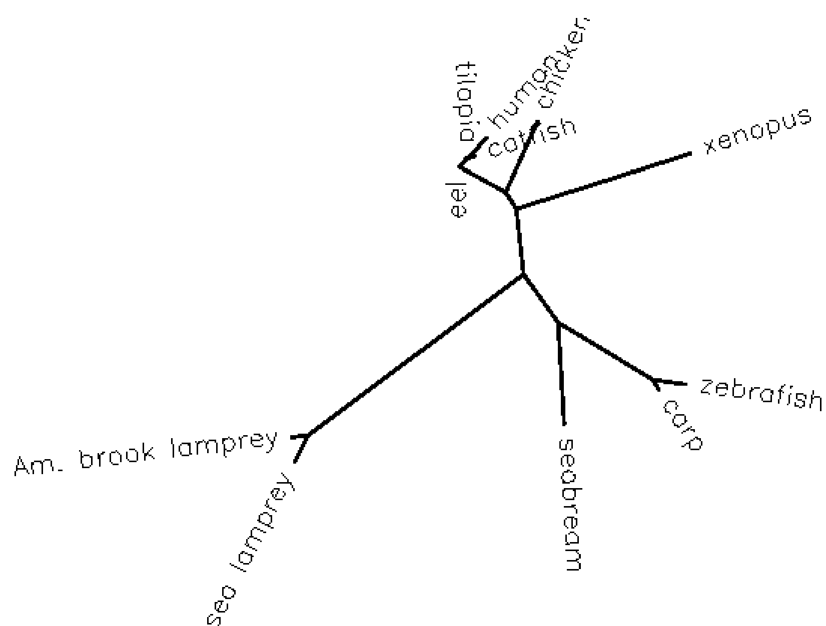
Phylogenetic analysis of the deduced amino acid sequence of bat liver TTR cDNA and TTRs from other vertebrates revealed bat TTR in a group of mammalians (Figure 3.13). This was consistent with phylogenetic trees based on fossil records. Within the mammalian group, bat TTR was most closely related to pig TTR. This might suggest to the evolutionary relationship between these two genera.

```
Carp               -MAKAVICVL  LVSLFACCRS  AP--VGIH--  -GGSDVHCPL  TVKILDAVKG  TPAGNIALDV
Zebrafish          -***E*****  *A****L***  **--*AF*--  -****A****  **********  *********L
Seabream           -*LQPLH*L*  *A*AVLL*NT  **TPTDK*--  -****TR***  M*********  ****SV**K*
Catfish            ----------  ----------  ----------  ----*TK***  M*******R*  S**I*V*VH*
Tilapia            ----------  ----------  ----------  ----*TK***  M*******R*  S**I*V*VH*
Eel                ----------  ----------  ----------  ----*TK***  M**V****R*  S**I*V*VH*
Am. brook lamprey  *TRFLCLL**  VA*SSLL*SA  DDDHKSHESH  E**VKDS***  M**AI*S*R*  K**AGVK*S*
Sea lamprey        -MRFLCLL**  VA*SSLL**A  DDDHKSHESH  E**VKDS***  M**AI*S*Q*  K**AGVK*S*

Carp               YRQDQGGTWE  KIASGKVDMT  GEVHNLITEQ  EFTPGVYRVE  FDTKAYWKAE  GRTPFHQLAD
Zebrafish          F*********  **********  *******A**  **********  ***LT***T*  **********
Seabream           SQKTAD*G**  Q**T*VT*A*  **I*******  Q*PA******  *******TNQ  *S****EV*E
Catfish            F*KAADD***  PF****TSES  **L*G*T**E  **VE*I*K**  I***S****I  *IS***EY*E
Tilapia            F*KAADD***  PF****TSES  **L*G*T**E  **VE*I*K**  I***S****L  *IS***EY*E
Eel                F*KADDD***  PF****TSES  **L*G*T**E  **VE*I*K**  I***S****L  *IS***EY*E
Am. brook lamprey  MKKQEDAS*K  EV*T*VTGK*  **S*H**GDK  D**E*T*K*R  ***QA**TKA  *I****EA*E
Sea lamprey        MKKQDDAS*K  EV*T*VTGK*  **S*H**SDK  D**E*T*K*R  *E*QQ**TKT  *I****EA*E

Carp               VVFEAHAEGH  RHYTLALLLS  PFSYTTTAVV  VKAHE-
Zebrafish          **********  **********  **********  ****D-
Seabream           ***D**P***  **********  **********  SSVR*-
Catfish            C**T------  ----------  ----------  ------
Tilapia            ***TN*----  ----------  ----------  ------
Eel                ***T------  ----------  ----------  ------
Am. brook lamprey  ***M**GA**  K**HIPM***  **F*A*GTI*  GD*EGH
Sea lamprey        ***M**GA**  K**HIPM***  PYFFA*G*I*  VDGKGH
```

Figure 3.10 Comparitive sequences alignment of TTR from some teleost fish

The deduced amino acid sequence of TTRs from catfish, tilapia, eel, seabream *(Sparus aurata)*), zebrafish *(Danio rerio)*, Am. brook lamprey *(Lampetra appendix)* and sea lamprey *(Petromyzon marinus)* were aligned with the amino acid sequence of common carp *(Cyprinus carpio)* TTR. The alignment was performed using the CLUSTAL W multiple sequence alignment program. Asterisks show residues identical to those found in carp TTR.

A



B

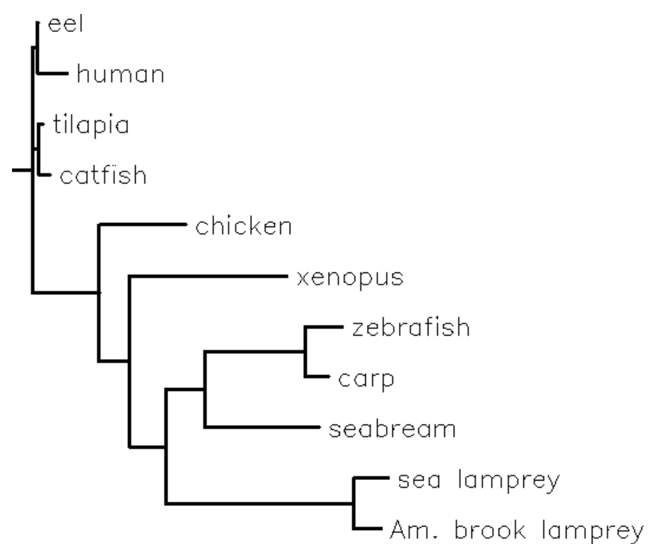


Figure 3.11 Phylogenetic tree of fish and other vertebrate TTRs

The partial amino acid sequences of catfish, tilapia and eel TTRs and the amino acid sequences (including presegment) of TTRs from other teleosts and vertebrates were used for analysis and building phylogenetic trees by the CLUSTAL W program. Unrooted (A) and rooted (B) trees were drawn.

```
Human       MASHRLLLLC  LAGLVFVSEA  GPT           GTGESKC  PLMVKVLDAV  RGSPAINVAV
Hedgehog    ******F***  ******M*E*  ***           --*Q***  **********  *****V****
Shrew       ***R******  *****L*TE*  ***           ***Q***  **********  Q****V****
Pig         ***Y******  **********  **A           *A*****  **********  *****V**G*
Bat         ***R******  **********  **V           --A*P**  **********  *****VD***
Chicken     **F*ST**VF  ******L***  A*L     V S H *SVD***  **********  *****A****
Crocodile   **F*SM**VF  ******LT**  A*L     V S H *SID***  **********  *****A***I
Xenopus     ***FKSF**-  **L*AI****  A*P G H A S H *EAD***  **********  **I**A*LL*
Carp        -MAKAVICVL  *VS*FACCRS  A*V     * I H *GSDVH*  **T**I****  K*T**G*I*L
Seabream    MLQPLHC**L  ASAVLCNTAP  T**     D K H *GSDTR*  *****I****  K*T**GSV*L
           -20         -10        -1 +1  3 -ε -δ -γ -β -α +4   10          20          30

Human       HVFRKAADDT  WEPFASGKTS  ESGELHGLTT  EEEFVEGIYK  VEIDTKSYWK  ALGISPFHEH
Hedgehog    K**K****E*  **********  **********  D*K****V**  **L*******  T********Y
Shrew       R**K****E*  **********  *F********  D*K*****I*  **L***T***  *********Y
Pig         K**K****G*  *****L****  *F********  D*K*******  **L*******  *********Y
Bat         K**K****E*  *****T**L*  *F********  TDK*******  *VL*******  *********Y
Chicken     K**K****G*  **D**T***T  *F**I*E***  **Q****V*R  **F**S****  G**L*****Y
Crocodile   K**K*TS*GD  *QE**A***T  *F**V*E**S  D*K******R  **F**S****  ***L*****Y
Xenopus     N***QTESGK  **QIT****T  *L**I*N***  D*Q*T**V**  I*FA**AF*G  K**L*****Y
Carp        D*Y*QDQGG*  **KI****VD  MT**V*N*I*  *Q**TP*V*R  **F***AYWK  AE*RT***QL
Seabream    K*SQ*T**GG  *TQI*T*VTD  AT**I*N*I*  *QQ*PA*V*R  **F***AYWT  NQ*ST****V
                    40          50          60          70          80          90

Human       AEVVFTANDS  GPRRYTIAAL  LSPYSYSTTA  VVTNPKE
Hedgehog    V*********  *Q********  **********  L*SD***
Shrew       V****H****  *K********  **********  L*SD***
Pig         **********  *R*H******  **********  L*SS***GAL
Bat         **********  *Q*K******  **********  L*SA***
Chicken     *D********  *H*H******  ***F******  **SD*Q*
Crocodile   *D********  *H*H******  ***F******  **SD*Q*E
Xenopus     VD*******A  GH*H****V*  *T***F*S**  I*SE*HDDL
Carp        *D***E*HAE  *H*H**L*L*  ***F**T***  **VKAH*
Seabream    *****D*HPE  *H*H**L*L*  ***F**T***  **SSVR*
                   100         110         120         130
```

Figure 3.12 Comparative sequences alignment of bat liver and other vertebrate TTRs

Amino acid sequence alignments of vertebrate TTRs. Amino acid sequence of TTRs from human (*Homo sapien*), shrew *(Sorex araneus)*, hedgehog (*Erinaceus europaeus*), pig *(Sus scrofa)*, chicken *(Gallus gallus)*, crocodile (*Crocodylus porosus*), xenopus (*Xenopus laevis)*, common carp *(Cyprinus carpio*), and seabream *(Sparus aurata)* were aligned with amino acid sequence of bat liver TTR using the CLUSTAL W multiple sequence alignment program. The alignment and numbering system are based on human TTR. The negative numbers represent the presegment. The amino acid residues that are identical to those found in human TTR are indicated by asterisks. The missing residues are indicated by dashes. The symbols -ε, -δ, -γ, -β and -α were introduced into the numbering of amino acids to indicate the additional residues at 5′ end of exon 2, which are absent from human TTR. The variable N-terminal region is indicated in box.

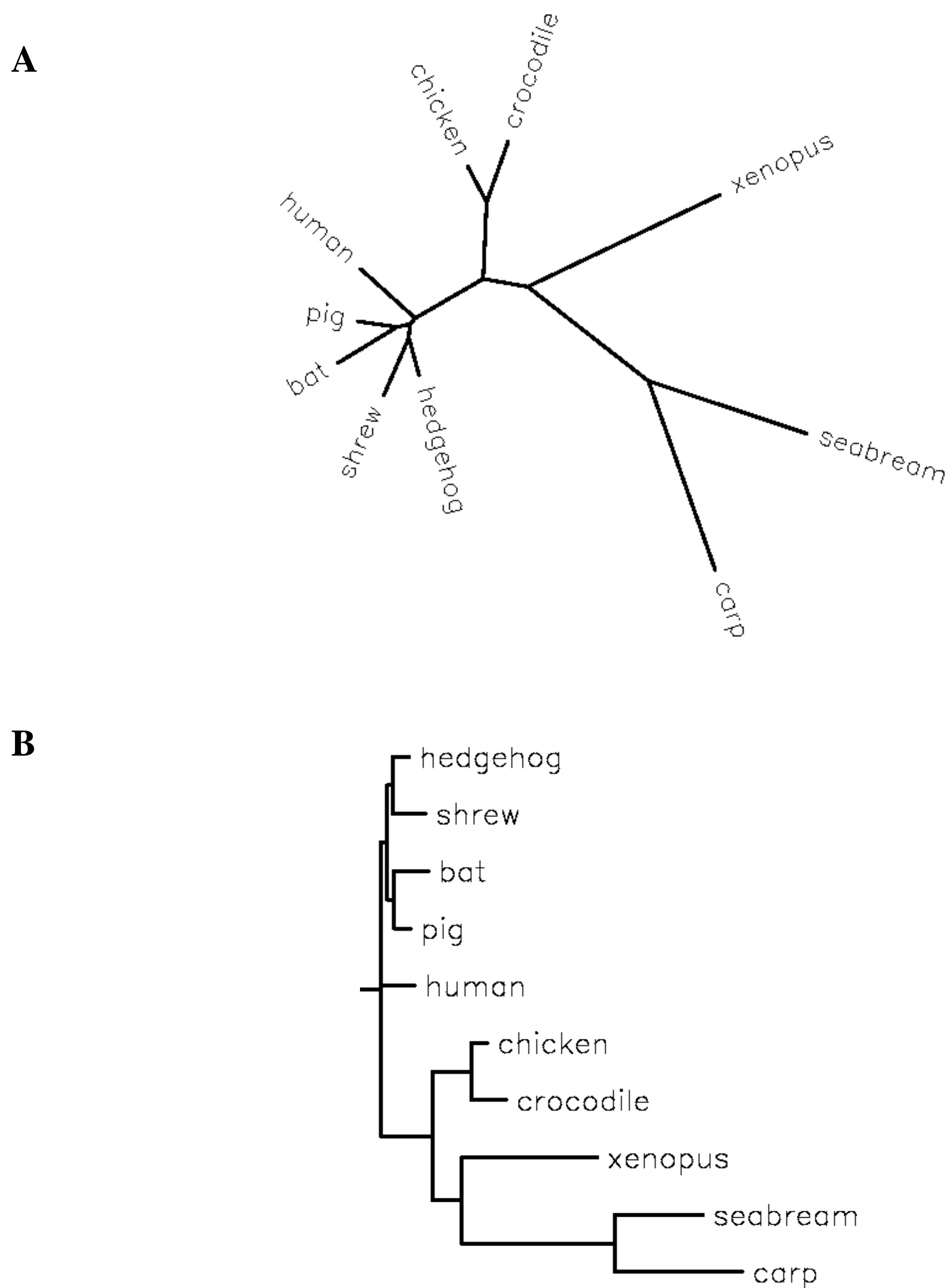


Figure 3.13 Phylogenetic tree of bat and other vertebrate TTRs

The amino acid sequences (including presegment) of bat and other vertebrates were used for analysis and building phylogenetic trees by the CLUSTAL W program. Unrooted (A) and rooted (B) trees were drawn.

3.4 TTR gene expression during development of catfish (*Mystus nemurus*)

In vertebrates, TTR plays an important role as a transporter in plasma for THs (for reviews see Richardson *et al*., 1994; Cyr and Eales, 1996), which have essential for metabolism and growth of vertebrates including mammalians (for reviews see Bernal and Nunez, 1995; Bettendorf, 2002), reptilians (Chiu and Lam, 1994), amphibians (for reviews see Tata, 1999; Shi *et al.,* 2001), and fish (Miwa *et al*., 1988). In lower vertebrates in particular fish, THs have predominant effects on development and metamorphosis. Roles of THs have been demonstrated in teleosts including carp (Lam and Shama, 1985), tilapia (Lam, 1980; Nacario, 1983), and zebrafish (Brown, 1997). However, information of TTR in fish is still few.

TTR is synthesized in liver only during development in amphibians, reptiles and polyprotodont marsupials, whereas it is synthesized by the liver during development and in adult life in eutherians, diprotodont marsupials and birds (Richardson *et al*., 2005). In amphibians, TTR is synthesized in the liver on day 7, and just prior to the climax of metamorphosis (Yamauchi *et al*., 1993, 1998; Zorn and Mason, 2001; Richardson, 2002), whereas, TTR is synthesized by the liver of juveniles, and during development of fish (Santos and Power., 1999; Funkenstein *et al*., 1999; Yamauchi *et al*., 1999; Richardson, 2002). In both fish and amphibians, early development, development and metamorphosis, are characterized by an increase in THs in the blood, and this increase in TH concentration would require additional distribution capacity. This was suggested be the selection pressure for the developmentally regulated hepatic synthesis of TTR during development only.

In previous study, effects of THs on catfish larvae were demonstrated (Leelawatwattana, 2003). Significant increase of survival was detected in the larvae at particular ages, i.e., at 15, 30, and 45 days after hatched. The question arises how it is correlated to the present of TH distributor such as TTR. Thus, in this thesis, the expression of TTR gene during development of catfish larvae was attempted to reveal by semi-quantitative RT-PCR.

Total RNA was isolated from the whole bodies of the larvae at 4, 8, 10, 16, 20, 26, 30, 40, 44 and 60 days after hatched by acid guanidinium-isothiocyanate and

phenol-chloroform extraction methods as described in section 2.6. Analysis of the RNA preparations on 1.5% formaldehyde agarose gel as shown in Figure 3.14 demonstrated sufficient quality to determine for amount of TTR mRNA in the next step by RT-PCR. The TTR cDNA fragment was synthesized and amplified by RT-PCR using the specific forward and reverse primers, which were designed based on nucleotide sequence of the partial TTR cDNA of catfish. To quantitative, expression of β-actin gene, a house keeping gene, was also determined as internal control. Thereafter, the PCR products were separated on 1.5% agarose gel. Single DNA bands of TTR and β-actin gene fragments with an expected size of ~ 150 bp and ~ 250 bp were obtained, respectively (Figure 3.15). Intensity of the DNA band was evaluated by gel document. The relative expression of the TTR gene was calculated based on that of beta-actin gene, and plotted against ages of catfish (Figure 3.16).

The expression of the fish TTR gene gradually increased from day 4 and reached maximum at day 16. From day 16 to day 40, the expression was significantly steady. However, slightly, but not significant, down and up of the gene expression could be observed during day 16 to day 26, which reached minimum at day 20. After day 40 until day 60, TTR gene gradually decreased. This result indicated that there was change in the expression pattern of TTR gene during development of the green catfish. Because TTR mRNA could be detected since the fish was 4 days of age, it implied for the presence of TTR since after hatched and requirement of TTR functions during development of the green catfish. In addition, the presence of TTR also detected in liver of 60 days larvae (Figure 3.17). These results absolute confirmed the synthesis by liver and secretion of TTR into blood during development of this fish specie. This observation well agrees with the previous demonstration of TTR in blood of amphibians and other teleosts at pre-metamorphosis or larvae stage (for review sees Richardson, 2007). In addition, the TTR expression post hatching in development of the fish could imply that it plays an important role in transporting THs.

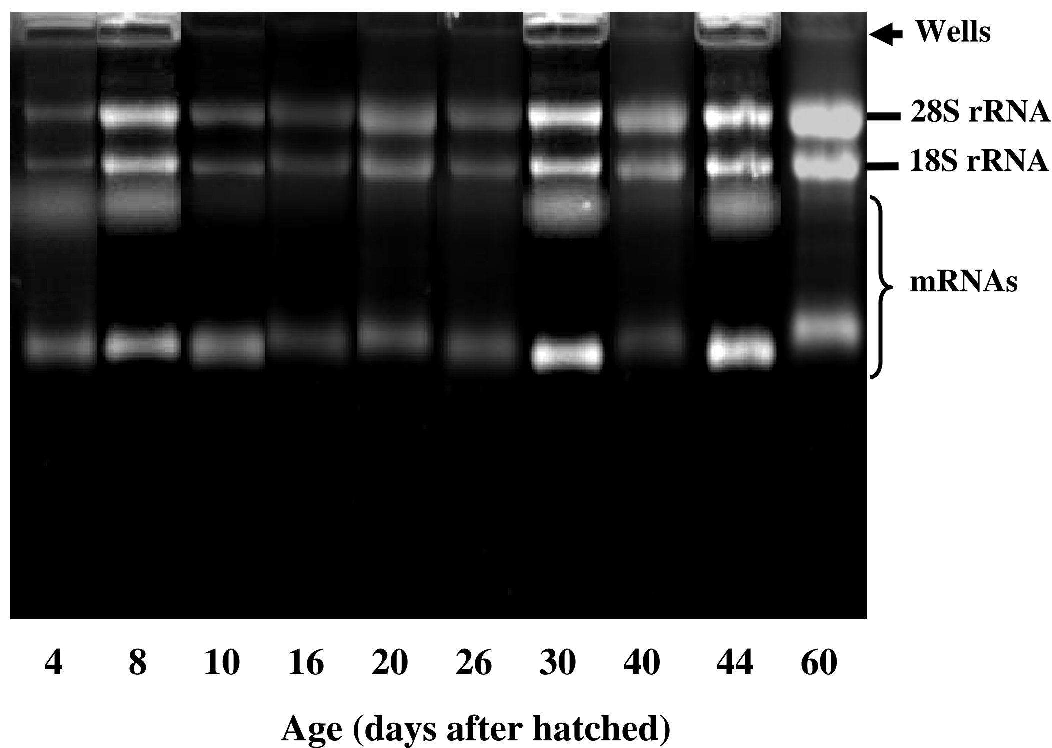


Figure 3.14 Analysis on agarose gel of the total RNA from catfish larvae

Total RNAs, extracted from whole bodies of catfish larvae at 4, 8, 10, 16, 20, 26, 30, 40, 44 and 60 days after hatched by acid guanidinium-isothiocyanate-phenol-chloroform, were separated on 1.5% formaldehyde agarose gel and the RNA bands were visualized by staining gel in ethidium bromide. The positions of 18S and 28S ribosomal RNAs are indicated on the right.

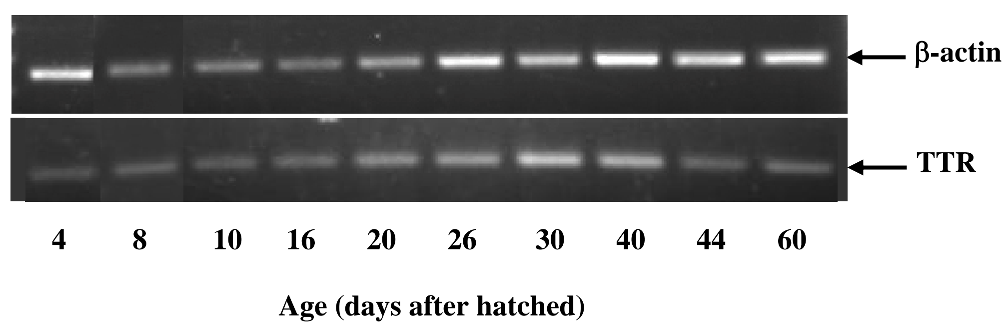


Figure 3.15 Analysis of TTR and β-actin gene fragments on agarose gel

Total RNAs of catfish larvae at defferent ages were determined by RT-PCR using specific primers. The PCR products were analyzed on 1.5% agarose gel. The expected sizes of TTR and and β-actin cDNA fragments are ~150 and ~250 bp, respectively. β-actin was used to normalize the RNA amount of each unknown sample for the analysis by semi-quantitative RT-PCR.

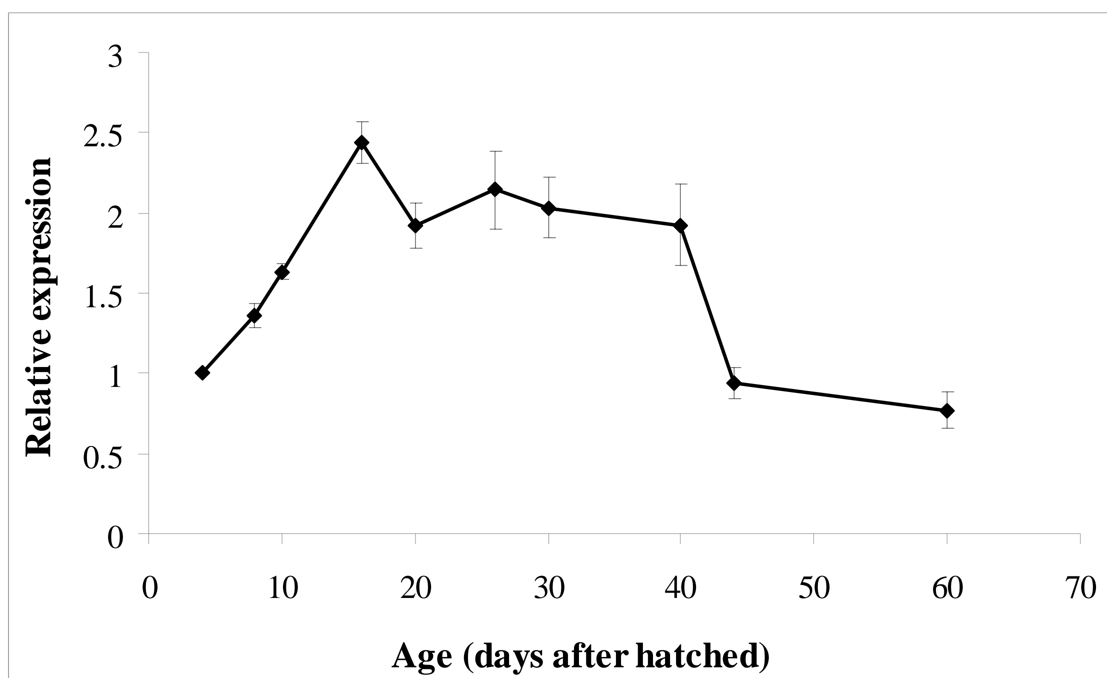


Figure 3.16 Intensity of the TTR cDNA fragment band on agarose gel was determined by the Gel document and analyzed by using the Lab Work 4.0 software (UVP). The relative expression levels of the TTR gene was determined by relatively comparing to the amount of the beta-actin gene. The error bars were from duplicate assays.

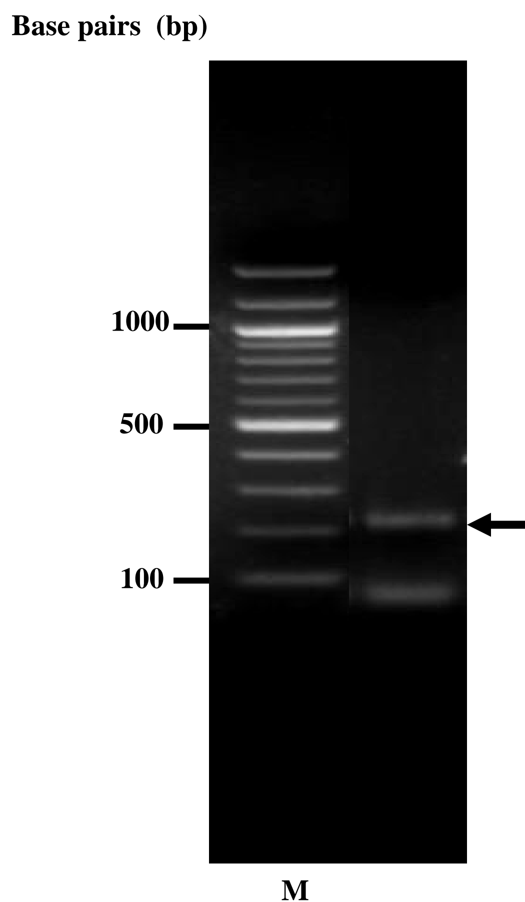


Figure 3.17 Analysis of TTR gene fragment amplified from liver of catfish

Total RNA from 60 days old catfish larvae was determined by RT-PCR using specific primers prior the PCR product was analyzed on 1.5% agarose gel. The expected size of the TTR fragment is indicated by arrow (~150 bp). M is standard DNA marker.

CHAPTER 4

CONCLUSION

1. The partial nucleotide fragments of TTR cDNAs from liver of 3 fish species, i.e., catfish (*M. nemurus*), tilapia (*O. niloticus*) and eel (*M. albus*), were successfully identified by RT-PCR and sequenced. The TTR cDNA fragments of 270, 271 and 270 bp, which each corresponds to 90 amino acid residues, could be isolated from catfish, tilapia and eel, respectively. In comparison, these sequences are approximate 70% of the full length TTR cDNAs reported in other vertebrates.
2. Analysis of the deduced amino acid sequences of these fish TTR cDNA fragment showed higher identity of the sequences to those of larger mammals than to those of teleost fish. This was consistent with the phylogenetic tree, which revealed that TTRs from catfish, tilapia and eel were more closely related to TTRs from larger mammals, e.g., human and shrew, than to those from other teleost fish.
3. The full length TTR cDNA could be identified and amplified from liver of bat *(Eonycteris spelaea)* by RT-PCR and RACE. The cDNA contains 599 nucleotide residues, which covered the 5′-untranslated region (5′UTR), the presegment sequence, the sequence code for mature protein, and 3′-untranslated region (3′UTR).
4. The deduced amino acid sequence of bat liver TTR was 125 residues in length, and it was shown closely related to mammalians in particular pig TTR, with 89% identity of the sequence. This was consistent with the phylogenetic analysis.
5. The alignment to amino acid sequence of human TTR revealed that two amino acid residues (glycine and threonine) were “missing” from the first 10 amino acid residues at the N-terminus of the mature bat TTR. This observation is the same as previously reported in hedgehog TTR.
6. The expressions of TTR gene during development of the green catfish larvae was detected since 4 days after hatched, reached maximum when the larvae was 16 days old, and declined when the larvae were older than 40 days. These results

indicated the presence of TTR in blood and implied to the functions of TTR during development of the catfish.

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