Materials and Methods

Materials

Reagents, enzymes and radioisotopes

A *Pichia* expression kit version E (Invitrogen) with license were kindly gifted from Professor Schreiber, Australia. A PCR and plasmid purification kits were from Gibco BRL (USA). A ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase from Perkin Elmer. T4 DNA ligase was purchased from New England Biolabs. Reaction endonucleases and Taq DNA polymerase were from Promega. Oligonucelotide primers was synthesized by Gibco BRL. L-[^{125}I]-thyroxine (1.25 Ci/mg) and L-3,5,3'-[^{125}I]-triiodothyronine (1.25 Ci/mg) were purchased from Dupont NEN (USA), and stored in the dark at 4°C in lead containers. Sep-Pak C-18 reversed-phase chromatography cartridges were from Water (USA) and a Superose-6 column from Pharmacia LKB Biotechnology (USA). Protein molecular weight markers were products of Pharmacia LKB Biotechnology (USA). All other chemicals used were analytical grade.

Bacterial and yeast strains, and plasmid vectors

E.coli strain DH5α from Promega. *Pichia* SMD 1168 was purchased with license from Invitrogen. *Pichia* expression vectors pPIC 3.5 and pPIC 9 (Invitrogen) were kindly gifted from Professor Schreiber, Australia.

Methods

1. <u>Construction of crocodile TTR expression plasmids and recombinant TTR synthesis</u>

1.1 PCR amplification of TTR fragments and cloning into Pichia pastoris expression vectors

PCR was used to amplify and generate the crocodile TTR cDNAs with compatible restriction ends for ligation into the pPIC 3.5 (*Bam*HI and *Eco*RI ends) or the pPIC 9 (*Xho*I and *Eco*RI ends) expression vector. 50 μg of crocodile TTR cDNA plasmid was amplified using 40 pmol of each appropriate primers (Table 1) into 100 μl of the reaction mixture. The amplification was started with an initial denaturation step at 94 °C for 5 min. This was followed by 25 cycles of 94 °C for 30 s, annealing temperature (see Table 1) for 30 s and 72 °C for 1 min. The final extension was carried out for 1 cycle of 72 °C for 5 min. The DNA was purified using the PCR purification kit. Purification was followed by double digestion with *Eco*RI and *Xho*I or *Eco*RI and *Bam*HI, for 2 h. Ligation of the DNA to linearized *Pichia* expression vector was carried out with T4 DNA polymerase kinase at 16 °C for 16 to 18 h in a 10 μl total reaction volume.

1.2 Preparation of expression vectors for transformation to Pichia patoris

TTR cDNA ligated to a *Pichia* expression vector was introduced into competent *E. coli* DH5α. Transformed *E. coli* cells were plated onto LB agar plates containing 100 μg/ml ampicillin. 10 ampicillin resistant transformants were inoculated into 2 ml LB medium with 100 μg/ml ampicillin and grown overnight at 37 °C. Isolation of plasmid DNA was carried out using the plasmid purification kit. Plasmid DNA was checked for the DNA insert by restriction analysis. Sequencing was performed to confirm the direction of the DNA insert in the plasmid, using *5'AOX1* primer (5'-GACTGGTTCCAATTGACAAGC-3') and *3'AOX1* primer (5'-GCAAATGGCATTCTGACATCC-3'). The DNA plasmid was linearized by digestion with *Sal*I at 37 °C for 2 to 3 h. The linearized DNA was extracted with phenol/chloroform, precipitated with ethanol and dissolved in 10 mM Tris, 1 mM EDTA, pH 8.0.

1.3 Transformation of Pichia pastoris

Plasmid DNA was introduced into *Pichia pastoris* SMD 1168 cells by electroporation. To prepare *Pichia* cells for electroporation, a single colony of *Pichia pastoris* from a fresh yeast extract peptone dextrose (YPD) agar plate was grown in 5 ml of YPD in a McCartney bottle at 30 °C overnight. 100 μl of the overnight culture was inoculated in 250 ml of fresh medium in a 1-liter flask and grown overnight to an OD_{600} of 1 to 1.2. Cells were collected by centrifugation at 1500 x *g* for 5 min at 4 °C and resuspended with 250 ml of ice-cold water. Cells were collected and resuspended once more with 125 ml of water. Then cells were resuspended with

10 ml of ice-cold 1 M sorbitol and finally resuspended in 0.5 ml of ice-cold sorbitol to give a final volume of approximately 0.8 ml.

Electroporation was performed using an Electroporator II model of Invitrogen. 80µl of the *Pichia* cells was mixed with 10 to 20 µg of linearized DNA and transferred to a 0.2 cm electroporation cuvette. Cells were incubated on ice for 5 min and electroporation was carried out at a voltage of 1500 V, capacitance of 50 µF and resistance of 200 Ω, generating pulse lengths of ~10 millisecond with a field strength of ~7500 V/cm. Immediately thereafter, 1 ml of ice-cold 1 M sorbitol was added to the cuvette, the cells were flushed a couple of times and then transferred to a microfuge tube. An aliquot of the cell suspension was plated on a minimal dextrose medium (MD) agar plate and incubated at 30 °C for 2 days.

1.4 Screening for Mut$^+$ transformants

Pichia transformants with phenotype His$^+$ Mut$^+$ were separated from His$^+$ Mut^s by growing the transformant mixture on minimal dextrose medium (MD) and minimal methanol medium (MM) agar plates. A colony of His$^+$ transformant from a MD plate was picked with a sterile toothpick and patched onto MM, then onto MD plate. Screening was performed with 50 to 100 His$^+$ transformants. Patches of GS 115/His$^+$ Mut^s albumin and GS 115/His$^+$ Mut$^+$ β-gal were included as His$^+$ Mut^s and positive controles, respectively. Plates were incubated at 30 °C for 2 days. The His$^+$ Mut$^+$ transformants grow very well in both MD and MM plate, while His$^+$Mut^s transformants grow well in only MM plate.

1.5 Expression of rècombinant Pichia clones

The expression of the TTR gene was first carried out in a small scale. A single colony of His$^+$Mut$^+$ was inoculated into 5 ml of a glycerol or dextrose containing media in a 35 ml McCartney bottle. Cells were grown at 30 °C in an orbital shaking incubator (~200 to 220 rpm) for 18 h (OD_{600} of 2 to 6). Cells were collected by centrifugation at 1500 x *g* for 5 min. Cells were then grown in 5 ml of methanol containing medium (OD_{600} of cell suspension at the start point of growing was 1) for 3 days. 25 µl of 100 % methanol was added every 24 h to maintain the level of methanol in the culture at about 0.5 %. Thereafter, the culture was centrifuged for 5 min and supernatant was analyzed for secreted proteins by Coomassie blue or silver stained SDS-PAGE.

2. <u>Time course of TTR gene expression in yeast</u>

Expression of a selected clone was performed as described in Materials and Methods section 1.4. 500 µl aliquots of culture were removed after 6 h, 24 h, 48 h, and 72 h for

determination of TTR content. Culture samples were centrifuged at 1500 x *g* for 5 min at room temperature. Supernatant was collected and stored at −70 °C before assaying.

3. Media selection

To compare the expression of the recombinant TTR gene in different media, an aliquot of colony of transformed *Pichia* was inoculated into BMG, MMGY BMGY or YPD (see composition of each media in Table 2). After overnight culturing, cells were transferred to BMY, MMMY BMMY or YPM and incubated for another 3 days. Thereafter, supernatant was collected and levels of expressed protein were analyzed by SDS-PAGE and protein bands were detected by silver staining.

4. Purification of recombinant crocodile TTR from yeast culture supernatant

4.1 by affinity chromatography

Yeast cell was separated from culture by centrifugation at 1500 x *g* for 5 min. Culture supernatant was centrifuged once at 12000 x *g* for 20 min before the recombinant TTR was purified from the clear supernatant by affinity column chromatography on human retinol-binding protein Sepharose 4 B, as described by Larsson et. al (1985).

4.2 by preparative gel electrophoresis

TTR was purified from the *Pichia* culture supernatant by preparative discontinuous Native-PAGE using the Prep Cell model 491 of Bio-Rad. The gel concentration (10% resolving gel and 3% stacking gel) and running conditions were optimized and performed according to the instruction manual of the company.

5. DNA sequencing

Nucleotide sequences were determined using a cycle sequencing kit with AmpliTaq DNA polymerase (Perkin Elmer). For each reaction, an appropriate amount (200 to 500 ng in 1.5 to 2.5 µl) of double stranded DNA was mixed with 8 µl of sequencing reagent (including the AmpliTag polymerase) and 5 pmole of primer in a total volume of 20 µl. The amplification was started with an initial denaturation step at 96 °C for 1min, then followed by 25 cycles of 96 °C for 30 s, 45 °C for 15 s and finally 60 °C for 4 min. Thereafter, the DNA was precipitated with 2 µl of 3 M sodium acetate and 50 µl of ethanol. The DNA pellet was washed once with 70 % ethanol, air-dried and dissolved in 3µl of gel loading buffer. DNA separation according to size was performed in 4 % polyacrylamide gel under denaturation using ABI PRISM 373 DNA sequencer (Perkin Elmer).

6. Preparation of competent cells and DNA transformation

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 single colony was picked from colonies freshly grown overnight on Luria-Bertani (LB) agar plates and transferred into 100 ml of LB medium in a 1-litre flask and incubated at 37 °C until the OD_{600} reached 0.5 (2 to 3h). Cells were transferred to polypropylene tubes (50 ml), cooled to 0 °C and collected by centrifugation. All supernatants were removed and tubes were inverted for 1 to 2 min to drain away traces of media. 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/50 ml original culture). The competent cells were stored at –70 °C (as glycerol stocks).

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

7. Analysis of N-terminal amino and sequence of TTR

The N-terminal amino acid sequence of recombinant crocodile TTRs were determined at La Trobe University, Australia, using an automatic Edman degradation.

8. Western Analysis

Protein samples were separated by electrophoresis in 15 % acrylamide SDS-PAGE, pH 8.9, resolving gel and 4 % acrylamide stacking gel, pH 6.8, (Laemmli and Favre, 1973) in duplicate. Then, proteins were transferred onto nitrocellulose membrane using transfer buffer (25 mM Tris, 192 mM glycine and 20 % v/v methanol, pH 8.3) overnight at 4 °C. Protein bands were visualized by staining with Coomassie Brilliant Blue or Amido Black. Specific proteins were detected, immunochemically (Kuno and Kihara, 1967), using enhanced chemiluminescence detection. The membranes were first blocked by incubation with blocking agent (5 % low fat dried milk in 25 mM Tris, 0.15 M NaCl, pH 7.4, and 0.1 % Tween 20) for 1 h at room temperature in an orbital shaker. Thereafter, membranes were incubated with primary antibody (polyclonal antibody against TTRs), diluted in blocking agent, for 1 h at room temperature. Then, the membranes were washed three times with 25 mM Tris, 0.15 M NaCl, pH 7.4, and 0.1 % Tween 20 for 10 min at room temperature. Incubation of the membranes with second antibody (horseradish peroxidase-linked anti-Rabbit immunoglobulins) was

performed for 1 h. Again, membranes were washed (3 x 10 min followed by 2 x 5 min) with 25 mM Tris, 0.15 M NaCl, pH 7.4. This was followed by ECL detection using Kodak Biomax AR film. After various exposure times films were developed immediately.

9. Determination of the mass of TTR tetramer

The molecular mass of recombinant TTRs was estimated by FPLC/gel-permeation chromatography using a Superose-12 column (HR 10/30, Pharmacia), equilibrated in 50 mM potassium phosphate buffer saline pH 7.4. The column volume was 24 ml. A 50 μl sample of purified TTR (~2 mg/ml) was chromatographed at a flow rate of 0.5 ml/min. The absorbance was measured at 280 nm . The column was calibrated with bovine serum albumin (68 kDa), ovalbumin (45 kDa), horse heart myosin (16.7 kDa) and horse heart cytochrome *c* (12 kDa).

10. Purification of [^{125} I]-L-thyroxine and [^{125}I]-L-3,5,3′-triiodothyromine

Commercially available ^{125}I-thyroxine and ^{125}I-3,5,3′-triiodothyronine preparations contain free iodine. This free iodine was removed prior to experiments by chromatography using SepPak C18 prepacked columns, as described by Mendel *et al.* (1989). Briefly, 5 μCi of iodinated thyroid hormone was diluted with 2 ml of methanol, water and phosphoric acid solution (methanol : water 1:1 with 0.3 ml of 85 % phosphoric acid per liter) loaded onto a column, previously washed with 2 ml of methanol and 5 ml water. Then, the column was washed with methanol, water and phosphoric acid solution (5 ml for ^{125}I-3,5,3′-triiodothyronine purification or 10 ml for ^{125}I-thyroxine purification) to remove free iodine. ^{125}I-thyroxine or ^{125}I-3,5,3′-triiodothyronine was eluted with 100 % methanol. The purity of thyroid hormones was checked by thin layer chromatography (Pardridge and Mietus, 1980).

11. Measurement of the thyroid hormone binding by TTR

Thyroid hormone binding to TTR is quantitatively described by the dissociation constant, K_d. Purified TTR (100 nM) was incubated with varied concentration of thyroid hormone, L-thyroxine or L-3,5,3′-triiodothyronine, from 0 to 1000 nM in the presence of trace amounts of ^{125}I-thyroxine or ^{125}I-3,5,3′-triiodothyronine at 4 °C, overnight, as described by Chang *et al.* (1999). A volume of 0.4 ml of the incubation mixture was transferred to a vial for total radioactivity determination. Free thyroxine or 3,5,3′-triiodothyronine from the same volume of incubation mixture was separated from the thyroid hormones bound by TTR by adsorption to a layer of methyl cellulose coated charcoal on a glass microfilter under constant suction pressure. The charcoal and filter were washed once with 0.4 ml of incubation buffer.

The radioactivity was determined using an LKB 1270 Rackgamma II counter with an efficiency of 70 %. Non-specific binding was extrapolated and other corrections were done as described by Chang *et al.* (1999).