0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 8
Printed in U.S.A.

Cloning and Characterization of Type III Iodothyronine Deiodinase from the Fish *Oreochromis niloticus**

JO P. SANDERS†, SERGE VAN DER GEYTEN†, ELLEN KAPTEIN, VEERLE M. DARRAS, EDUARD R. KÜHN, JACK L. LEONARD, AND THEO J. VISSER

Department of Internal Medicine III (J.P.S., S.V.d.G., E.K., T.J.V.), Erasmus University Medical School, 3000 DR Rotterdam, The Netherlands; Laboratory of Comparative Endocrinology (S.V.d.G., V.M.D., E.R.K.), K.U. Leuven, 3000 Leuven, Belgium; Department of Nuclear Medicine (J.L.L.), University of Massachusetts Medical Center, Worcester, Massachusetts 01655

ABSTRACT

Type III iodothyronine deiodinase (D3) catalyzes the inner ring deiodination (IRD) of T_4 and T_3 to the inactive metabolites rT_3 and 3,3′-diiodothyronine (3,3′-T2), respectively. Here we describe the cloning and characterization of complementary DNA (cDNA) coding for D3 in fish (*Oreochromis niloticus*, tilapia). This cDNA contains 1478 nucleotides and codes for a protein of 267 amino acids, including a putative selenocysteine (Sec) residue, encoded by a TGA triplet, at position 131. The deduced amino acid sequence shows 57–67% identity with frog, chicken, and mammalian D3, 33–39% identity with frog, fish (*Fundulus heteroclitus)* and mammalian D2, and 30–35% identity with fish (tilapia), chicken, and mammalian D1. The 3′ UTR contains a putative Sec insertion sequence (SECIS) element. Recombinant tilapia D3 (tD3) expressed in COS-1 cells and native tD3 in tilapia brain microsomes show identical catalytic activities, with a strong preference for IRD of T_3 (K_m ~20 nM). IRD of [3,5-^{125}I]T_3 by native and recombinant tD3 are equally sensitive to inhibition by substrate analogs ($T_3 > T_4 \gg rT_3$) and inhibitors (gold thioglucose ≫ iodoacetate > propylthiouracil). Northern analysis using a tD3 riboprobe shows high expression of a 1.6-kb messenger RNA in gill and brain, although D3 activity is much higher in brain than in gill. The characterization of tD3 cDNA provides new information about the structure-activity relationship of iodothyronine deiodinases and an important tool to study the regulation of thyroid hormone bioactivity in fish. (*Endocrinology* **140:** 3666–3673, 1999)

THE MAJOR secretory product of the thyroid is a prohormone, T_4, which is activated in peripheral tissues by outer ring deiodination (ORD) to T_3. T_4 and T_3 are converted by inner ring deiodination (IRD) to the metabolites rT_3 and 3,3′-diiodothyronine (3,3′-T2), respectively (1–5). Three iodothyronine deiodinases are involved in these processes (1–5). In mammals, the type I deiodinase (D1) is located in liver, kidney, and thyroid. It has both ORD and IRD activities, in particular toward rT_3 and sulfated iodothyronines (1–5). The type II deiodinase (D2) only catalyzes ORD with T_4 as the preferred substrate. In rats, D2 is expressed predominantly in brain, pituitary, and brown adipose tissue, and recent findings suggest additional expression in human thyroid, skeletal muscle and, perhaps, heart (1–7). D3 has only IRD activity with preference for T_3 as the substrate. In mammals, D3 is mainly found in brain, skin, placenta, and fetal tissues (1–5). The three deiodinases have recently been cloned from different species, showing that they are homologous selenoproteins featuring an essential selenocysteine (Sec) residue in their catalytic centers (6, 8–20).

Whereas D3 expression in placenta appears to be independent of thyroid state (21–23), D3 activity in rat brain is increased in hyperthyroidism and decreased in hypothyroidism (24). High D3 activities are expressed in the fetal human liver (25) and the embryonic chicken liver (26–28). Acute down-regulation of hepatic D3 gene expression has been observed after administration of GH or dexamethasone to the chick embryo (29–31). Although T_3 is essential for normal brain development, high D3 expression levels in mammalian placenta and fetal tissues, including brain, are thought to protect the developing fetus against undue levels of maternal thyroid hormone (32, 33).

The three iodothyronine deiodinases have also been identified in *Oreochromis niloticus* (tilapia) and other fish, although their tissue distributions are very different from those of the mammalian enzymes (5, 18, 20, 34–39). In tilapia, D1 activity is much higher in kidney than in any other tissue. By far the highest D2 activity is expressed in liver. D3 activity is high in brain, low in gill, and negligible in all other tissues (5, 36, 37). The catalytic properties of fish D2 and D3 are very similar to those of the mammalian enzymes (5, 34–39). However, at least in tilapia and trout, fish D1 is insensitive to inhibition by 6-propylthiouracil (PTU), in contrast to the potent inhibition of mammalian (and chicken) D1 by this thyrostatic drug (5, 36–38). To investigate the molecular basis for this difference in PTU sensitivity between fish and mammalian D1, we have recently cloned and characterized com-

Received October 6, 1998.

Address all correspondence and requests for reprints to: Theo J. Visser, Ph.D., Department of Internal Medicine III, Erasmus University Medical School, Room Bd 234, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: visser@inw3.azr.nl.

* The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number Y11111. This work was supported by The Netherlands Organization of Scientific Research (Grant 903–40-168), the Belgian FKFO (Project 2.0114.94), the KULeuven Onderzoeksraad (Projects OT/94/11 and OT/97/22), and the FWO Vlaanderen.

† These authors contributed equally to this work.

plementary DNA (cDNA) coding for D1 in tilapia (20). In contrast to our hypothesis, we found that tilapia D1 contains a Sec residue in a position corresponding to the Sec residue in PTU-sensitive D1s, indicating that differences in PTU sensitivity are determined by other structural elements (20).

Simultaneous with the cloning of D1 from tilapia kidney, we also attempted to clone other deiodinases from tilapia liver. This involved RT-PCR of tilapia liver messenger RNA (mRNA) using oligonucleotide primers based on amino acid sequences (NFGSCTSecP, YIEEAH and VVVDTM) highly conserved in the D1 and D3 sequences available at that time (8–13). The RT-PCR products were sequenced and used as probes for cDNA library screening. This resulted in the isolation of TL31, a cDNA clone coding for tilapia D3 (tD3).

Materials and Methods

Materials

Tilapia (*O. niloticus*) were obtained from CERER-University of Liège (Tihange, Belgium) and kept as described before (36, 37). TRIzol reagent was obtained from Life Technologies, Inc. (Breda, The Netherlands); oligo-dT-cellulose was from New England Biolabs, Inc. (Beverly, MA); SuperTaq DNA polymerase was from HT Biotechnology Ltd. (Cambridge, UK); AMV reverse transcriptase and pCI-Neo were from Promega Corp. (Madison, WI); Klenow DNA polymerase was obtained from Roche Molecular Biochemicals (Mannheim, Germany); pCR-II was from Invitrogen (San Diego, CA); synthetic oligonucleotides were from Amersham Pharmacia Biotech (Roosendaal, The Netherlands) or Life Technologies, Inc.; Hybond membranes, [α-^{32}P]dATP and [α-^{32}P]UTP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK); polyethyleneglycol (PEG6000) was from Merck (Hohenbrunn, Germany); DEAE-dextran and Sephadex LH-20 were from Amersham Pharmacia Biotech. Nonradioactive iodothyronines were obtained from Hen-

```
CTAAATTCCAGTTGTCTCGTTTGCCGGCACAGTCTCATCCTATCAGCCGGGGGAGAGATG    60
ATGGACGACTCCGGCGGTGTCCAAATGGCGAAGGCGCTGAAGCATGCAGCCCTCTGCCTG   120
 M  D  D  S  G  G  V  Q  M  A  K  A  L  K  H  A  A  L  C  L    20
ATGCTGCTTCCCCGGTTCCTTCTGGCCGCAGTTATGCTGTGGCTCCTGGATTTCTTGTGC   180
 M  L  L  P  R  F  L  L  A  A  V  M  L  W  L  L  D  F  L  C    40
ATTAGGAAAAAAGTGCTGCTGAAAATGGGAGAGAGGCAGGAGAGCCCGGACGACCCGCCG   240
 I  R  K  K  V  L  L  K  M  G  E  R  Q  E  S  P  D  D  P  P    60
GTGTGCGTCTCTGACTCTAACAAGATGTTCACCTTGGAGTCCCTGAGGGCCGTGTGGCAT   300
 V  C  V  S  D  S  N  K  M  F  T  L  E  S  L  R  A  V  W  H    80
GGTCAGAAATTGGACTTTCTCAAATCTGCGCACCTTGGGCACCCTGCGCCCAACACCGAG   360
 G  Q  K  L  D  F  L  K  S  A  H  L  G  H  P  A  P  N  T  E   100
GTGGTGCTTGTCCAGGAGCGGAAGCAGGTGCGAATCCTGGACTGCGTGAAAGGGAATAGA   420
 V  V  L  V  Q  E  R  K  Q  V  R  I  L  D  C  V  K  G  N  R   120
CCGCTCATTCTTAACTTTGGCAGCTGCTCCTGACCGCCATTCATGACGCGTCTGACGGCG   480
 P  L  I  L  N  F  G  S  C  S  X  P  P  F  M  T  R  L  T  A   140
TTTCAGCGCGTCGTGAGTCAGTACGCAGACATTGCGGACTTTTTAGTTGTATATATCGAG   540
 F  Q  R  V  V  S  Q  Y  A  D  I  A  D  F  L  V  V  Y  I  E   160
GAGGCGCATCCCTCGGACGGCTGGGTGAGCTCGGACGCGCCGTATCAGATCCCCAAGCAT   600
 E  A  H  P  S  D  G  W  V  S  S  D  A  P  Y  Q  I  P  K  H   180
CGCTGTTTGGAAGACAGACTTAGAGCCGCTCAGCTGATGCTCACTGAGGTGCCGGAGACC   660
 R  C  L  E  D  R  L  R  A  A  Q  L  M  L  T  E  V  P  E  T   200
AACGTGGTGGTGGATAATATGGACAACTCGTGTAACGCCGCGTACGGAGCCTACTTTGAG   720
 N  V  V  V  D  N  M  D  N  S  C  N  A  A  Y  G  A  Y  F  E   220
AGACTTTACATCGTGAGGGATGAAAAAGTGGTGTACCAGGGGGGCAGGGGTCCAGAGGGA   780
 R  L  Y  I  V  R  D  E  K  V  V  Y  Q  G  G  R  G  P  E  G   240
TACCGGATTTCCGAGCTTAGAAACTGGCTGGAACAATACAGGAACGATCTGCCGAATTCC   840
 Y  R  I  S  E  L  R  N  W  L  E  Q  Y  R  N  D  L  P  N  S   260
CAAACAGCGGTACTCCATGTGTAGATGCTGAACTGCCCGCTGCTCTGCCCACTCTGCTTA   900
 Q  T  A  V  L  H  V  *                                        267
ATAAGTATCCAACCCACAGTGCAAAATATCCAGATGCTGCTATCAGATGTTCACCCATGG   960
CACATTGTTGTTGTTTTGGTTTTTTTTTTTGCAAAAAAACATGAGGAAAAAAGAAACATT  1020
TTCAGGACTCTTTGTCAAAATAGCCTAAAGTCATGTTGAAGCATAGACCGTAGGCTGTGA  1080
TTTTGTGCTTGTCTCGACTTTCATTGATTGGCCATAGCGATTCTCTTTATTTCTTCTTTT  1140
GTATGAAAAAGTCTCAGTTGGATTCAATTGGTAATCACTCTATTTTTCTACAATACCTCT  1200
CTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTTCGGGCTAATATCGCCTTCTGG  1260
TGTCTCTGTGAAGTTCGGTTTTTAAAAGGGTCATCCAGAAAACCGACACTGATGTTTCCG  1320
ACACTGGTAGCGGGCCATATTAGCGAGACGCCTCACTCGGTGACCGACTGCTAACGGTGT  1380
AACGTTGAAGCAATTGTAACTGAACAACTGTTTTTAATAAATGTCAGATCACACACTGAT  1440
GACGCACTTTTGAGTGTTGAAAAAAAAAAAAAAAAAAA                        1479
```

FIG. 1. Nucleotide and deduced amino acid sequence of cDNA clone TL31. The Sec residue is denoted by X. The putative SECIS element in the 3′ untranslated region is *underlined*.

ning Berlin R&D (Berlin, Germany), [3′, 5′-^{125}I]T_4 (~1200 Ci/mmol) and [3′-^{125}I]T_3 (~2000 Ci/mmol) from Amersham Pharmacia Biotech, and [3,5-^{125}I]T_3 (~35 Ci/mmol) from Mr. R. Thoma (Formula GmbH, Berlin, Germany) courtesy of Dr. G. Decker (Henning, Berlin, Germany). (3′, 5′-^{125}I)rT_3 (~2000 Ci/mmol) and [3,5-^{125}I]T_3S were prepared in our laboratory as described previously (40, 41). 6-*n*-Propyl-2-thiouracil (PTU), iodoacetate (IAc), gold thioglucose (GTG), dithiothreitol (DTT), and chloroquine were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest purity commercially available.

Cloning and sequence analysis

Total RNA was isolated from tilapia liver using TRIzol reagent, and poly(A^+) RNA was isolated on oligo-dT-cellulose. cDNA was obtained by oligo-dT-primed RT using AMV reverse transcriptase. PCR was performed using the primers 5′-AATTTTGGCAGTTGTACCTGACC-3′ and 5′-RTGIGCTTCCTCIATGTA-3′ and SuperTaq DNA polymerase. The PCR products were TA-cloned into pCR-II and sequenced. The tilapia liver cDNA library was constructed in Lambda ZAP-Express (Stratagene, La Jolla, CA). The library was blotted on Hybond-N^+ and screened with the RT-PCR products labeled with [α-^{32}P]dATP by primer extension using Klenow DNA polymerase. The phagemids carried in selected positive bacteriophages were excised, generating cDNA clones in pBK-CMV. The inserts were sequenced manually and by automatic sequencing in both directions using the dideoxy method (42).

RNA secondary structure prediction was done using the MFOLD program provided by Dr. M. Zuker (Institute for Biomedical Computing, Washington University, St. Louis, MO) on the internet (http://www.ibc.wustl.edu/~zuker; Ref. 43). Hydropathicity analysis of the protein was done according to Kyte and Doolittle (44) with a window of 11 using the ProtScale program provided on the website of the Swiss Institute of Bioinformatics (http://expasy.hcuge.ch).

Expression

cDNA was cut out of pBK-CMV with *Sal*I/*Not*I and ligated into *Xho*I/*Not*I digested pCI-Neo and expressed in COS-1 cells grown in DMEM/F12 containing 10% FCS (Life Technologies, Inc.) and 40 nM Na_2SeO_3 (45). One day before transient transfection, COS-1 cells were seeded at 50% confluence in 55 cm^2 cell culture dishes. Expression constructs (7 μg) isolated by alkaline lysis and polyethyleneglycol precipitation (46) were added to serum-free DMEM/F12 medium containing 100 μg/ml DEAE-dextran. After 2 h, the medium was replaced by serum-free DMEM/F12 medium containing 100 μM chloroquine. Again, 2 h later the medium was replaced by DMEM/F12 containing 10% FCS and 40 nM Na_2SO_3. After 3 days, the cells were rinsed with PBS, collected in 0.3 ml 0.1 M phosphate (pH 6.9), 1 mM EDTA and 10 mM DTT, sonicated, snap-frozen on dry-ice/ethanol, and stored at −80 C.

Northern blots

Total tissue RNA (20 μg per lane) was separated on 1% (wt/vol) formylaldehyde-agarose gels and blotted onto Hybond-N^+ membranes by overnight capillary transfer using 20 × SSC. For preparation of a riboprobe, the TL31-pCI-Neo plasmid was double-digested with *Eco*RI/*Xba*I and religated to remove nonspecifically hybridizing repetitive 3′UTR sequences. The 3′UTR-deleted construct was linearized with *Nhe*I, and the riboprobe was generated using the T_3 Ampliscribe kit (Epicentre Technologies, Madison, WI) and [α-^{32}P]UTP. Hybridization of the Northern blot was performed in NorthernMax buffer (Ambion, Inc., Austin, TX) overnight at 67 C. Blots were washed once for 30 min at 50 C with 0.1 × SSC, 0.1% SDS, and twice for 30 min at 70 C with 0.1 × SSC, 0.1% SDS. Autoradiographs were prepared by exposure of the blots at −70 C to Fuji Photo Film Co., Ltd. RX film. Analysis of the ethidium bromide-stained gels indicating 20–30% variation in the amount of applied RNA.

Enzyme assays

Tilapia tissue homogenates and microsomal fractions were prepared as described before (37). Deiodinase activities of native and recombinant enzyme preparations were determined by measuring the radioiodide released from either [3′, 5′-^{125}I]T_4 or [3′, 5′-^{125}I]rT_3 by ORD, or from [3,5-^{125}I]T_3 or [3,5-^{125}I]T_3 sulfate (T_3S) by IRD (40, 41). In short, appropriate amounts of tissue or lysate protein were incubated in triplicate for 30–60 min at 37 C with 10 nM [^{125}I]substrate in 0.2 ml, 0.1 M sodium phosphate buffer (pH 7.2), 2 mM EDTA, and 10 mM DTT. Reactions were stopped and [^{125}I]iodothyronines were precipitated by successive addition of 0.1 ml 5% BSA and 0.5 ml 10% TCA. Radioiodide was further isolated from the supernatant on Sephadex LH-20 minicolumns (40, 41).

For HPLC analysis of the deiodination products, 1 nM [3′, 5′-^{125}I]T_4 or [3′-^{125}I]T_3 was incubated in duplicate for 1 h at 37 C with (1 mg protein/ml) or without cell lysate in 0.2 ml, 0.1 M phosphate (pH 7.2),

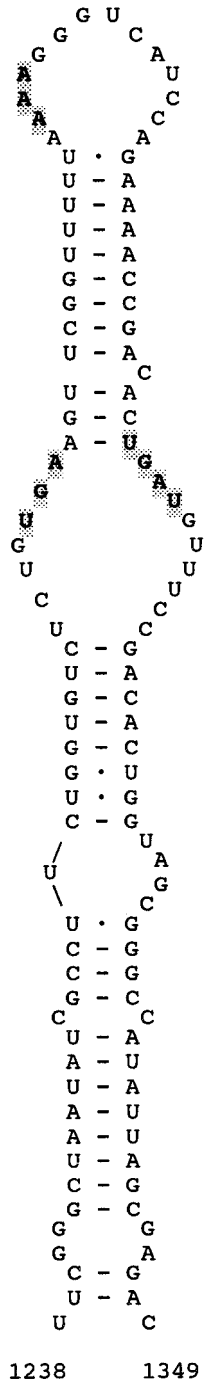

FIG. 2. Predicted stem-loop structure of the SECIS element in the TL31 3′UTR. Consensus nucleotides are indicated in *bold*.

2 mM EDTA and 50 mM DTT. The reactions were stopped by addition of 0.2 ml ice-cold methanol. After centrifugation, 0.2 ml of the supernatant was mixed with 0.2 ml 0.02 M ammonium acetate (pH 4), and 0.1 ml of the mixture was applied to a 250 × 4.6 mm Symmetry C18 column (Waters, Etten-Leur, The Netherlands) connected to an Alliance HPLC system (Waters) and eluted isocratically with a mixture of acetonitrile and 0.02 M ammonium acetate (33:67, vol:vol) at a flow of 1.2 ml/min. Radioactivity in the eluate was monitored on line using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

Results

By RT-PCR of tilapia liver mRNA using oligonucleotide primers corresponding to the conserved amino acid sequences NFGSCTSecP and VVVDTM, a 246-bp cDNA fragment was obtained, the sequence of which showed high homology with the corresponding region in *Xenopus laevis* and *Rana catesbeiana* D3 (12, 13). The labeled PCR product was used as a probe to screen the tilapia liver cDNA library (200,000 independent clones). Seven double-positive clones were identified after plating 500,000 pfu's of the amplified library. Using vector- and PCR product-specific primers, several possibly full-length clones were identified. One cDNA clone (TL31) was found to be 1479 bp long with a reading frame coding for a 267-amino acid protein, assuming that TGA at codon 131 is translated as Sec (Fig. 1). The protein has a calculated molecular weight of 30,356 kDa and an isoelectric point (pI) of 6.2. Analysis of the 3′UTR region of TL31 by RNA secondary structure prediction reveals a stem-loop structure containing consensus SECIS element nucleotides (Fig. 2). SECIS elements are essential for the incorporation of Sec at the in-frame UGA opal stop codon (47–50). Evidence presented below indicates that TL31 represents cDNA coding for tD3.

Figure 3 shows the alignment of the deduced amino acid sequence of tD3 with the D3 sequences of *X. laevis*, *R. catesbeiana*, chicken, human, and rat. The amino acid identity of tD3 amounts to 62–65% with frog D3 (12, 13), 67% with chicken D3 (19), and 57% with mammalian D3 (14, 15). The amino acid sequence of tD3 shows 35% identity with tilapia D1 (20), 33% identity with chicken D1 (19) and 30–33% identity with mammalian D1 (8–11). The amino acid identity of tD3 with fish (*F. heteroclitus*) D2 is 36% (18), with frog D2 33% (16) and with mammalian D2 38–39% (6, 17).

Figure 4 shows the hydropathicity plot of the tD3 protein using the Kyte and Doolittle algorithm (44), indicating a strongly hydrophobic domain between amino acids 16 and 41 which probably represents a transmembrane domain.

Figure 5 shows the analysis of the enzyme activity expressed in tD3 cDNA-transfected COS-1 cells using different iodothyronine derivatives as substrates. In agreement with the well-known catalytic profile of native D3 from tilapia and other species (1–5), recombinant tD3 catalyzes the IRD of T_3 but much less so of T_3S, whereas it does not catalyze the ORD of T_4 and rT_3. The deiodination products of T_4 and T_3 were also analyzed by HPLC (Fig. 6). The results show that recombinant tD3 specifically catalyzes the IRD of T_4 to rT_3, whereas ORD of T_4 to T_3 is undetectable (Fig. 6A). T_3 undergoes only IRD to 3,3′-T2, whereas release of radioiodide through ORD of [3′-^{125}I]T_3 is undetectable (Fig. 6B). Similar data were obtained using tilapia brain homogenate as source of native tD3 (data not shown).

Figure 7 demonstrates that recombinant tD3 expressed in COS-1 cells has exactly the same substrate specificity and inhibitor sensitivity as the native enzyme in tilapia brain microsomes. IRD of 10 nM [^{125}I]T_3 by both recombinant and native tD3 is inhibited progressively by increasing concen-

```
til    MDDSGGVQMAKALKHAALCLMLLPRFLLAAVMLWLLDFLCIRKKVLLKMGERQ-------      53
xen    mlhca.phtg.lv.qv.a.cl.....tg.......q...rr...tar.es-------      53
ran     mlpaphtccrl.qq.la.cl.....tvll.....p.v.rr.irgak.edpg-----      54
chi                 .a.il.f.....t.............m.tmptaeeaag----      40
rat    mlr.lllhslrlcaqt.s..v.f....gt.f.........hf.rrrhpdhpepevel      60
hum    mlh.lllhslrlcaqt.s..v.f....gt.f.........hf.grrrrgkpepevel      60

til    ------ESPDDPPVCVSDSNKMFTLESLRAVWHGQKLDFLKSAHLGHPAPNTEVVLVQER     107
xen    -----taehe...l.....r.c.v.............yf.....cs.......mleg.     108
ran    -----apere...l....t.r.c.....k...y.....f.....gg.......tlegq     109
chi    --agegpp...........r.......k.........f....v.s....p..iqldgq      98
rat    nsegeemp.....i....d.rlc..a..k.........f.q..e.g....s...rpdgf     120
hum    nsegeevp.....i....d.rlc..a..k.........f.q..e.g....s....pdgf     120

til    KQVRILDCVKGNRPLILNFGSCSXPPFMTRLTAFQRVVSQYADIADFLVVYIEEAHPSDG     167
xen    rlck...fsq.k...vv.....t.....a..q.yr.laa.hvg.....l...........     168
ran    rlc....fs..h...v......t.....a..q.y..laa.rl.f....l........c..     169
chi    .rl....far.k..........t.....a..rs.r.laadfv......l...........     158
rat    qsq....yaq.t...v......t.....a.ms....l.tk.qrdv...ii..........     180
hum    qsqh...yaq.....v......t.....a.ms....l.tk.qrdv...ii..........     180

til    WVSSDAPYQIPKHRCLEDRLRAAQLMLTEVPETNVVVDNMDNSCNAAYGAYFERLYIVRD     227
xen    .l.t..s....q.q..q...a......qga.gcr....t....s.............le     228
ran    .l.t..a....t.q..q..........qga.gcr..a.t.t.as............vil.     229
chi    ......a.s....q..q.........rega.dcpla..t...ass...........viqe     218
rat    ..tt.s..v..q..s....vs..rvlqqga.gcal.l.t.a..sss.........viqs     240
hum    ..tt.s..i..q..s....vs..rvlqqga.gcal.l.t.a..sss.........viqs     240

til    EKVVYQGGRGPEGYRISELRNWLEQYRNDLPNSQTAVLHV                       267
xen    g.............k.....m.....qqg.mgtkgsgqv.iqv                    271
ran    g.............k.g......d..qtratgngal.iq.                       269
chi    ...m..........k.....t..d..ktr.qspgav.iq.                       258
rat    gtim.......d..qv....t...r.deq.hgtrprr.                         278
hum    gtim.......d..qv....t...r.deq.hgarprrv                         278
```

FIG. 3. Alignment of the cDNA-deduced amino acid sequences of tilapia (til), *X. laevis* (xen), *R. catesbeiana* (ran), chicken (chi), human (hum) and rat D3. The Sec residue is denoted by X. Identical amino acids are indicated by *dots*, and gaps are indicated by *hyphens*.

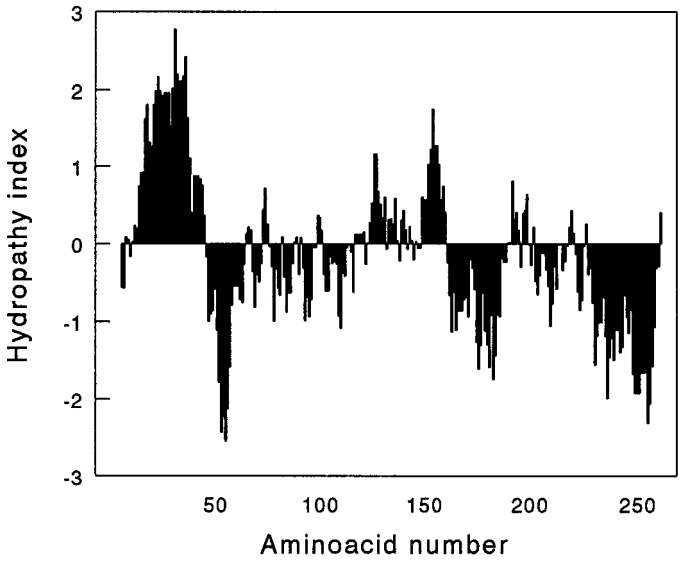

FIG. 4. Kyte and Doolittle hydropathicity plot of the tD3 amino acid sequence. Positive values indicate hydrophobic regions and negative values indicate hydrophilic regions.

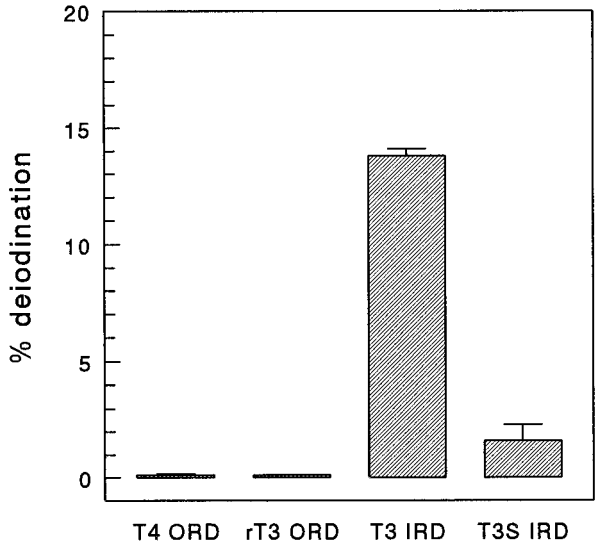

FIG. 5. Catalytic profile of recombinant tD3 expressed in TL31-transfected COS-1 cells. Cell sonicates (1 mg protein/ml) were incubated for 1 h at 37 C with 10 nM substrate and 10 mM DTT. Results are the means ± SD of triplicate incubations in a representative experiment.

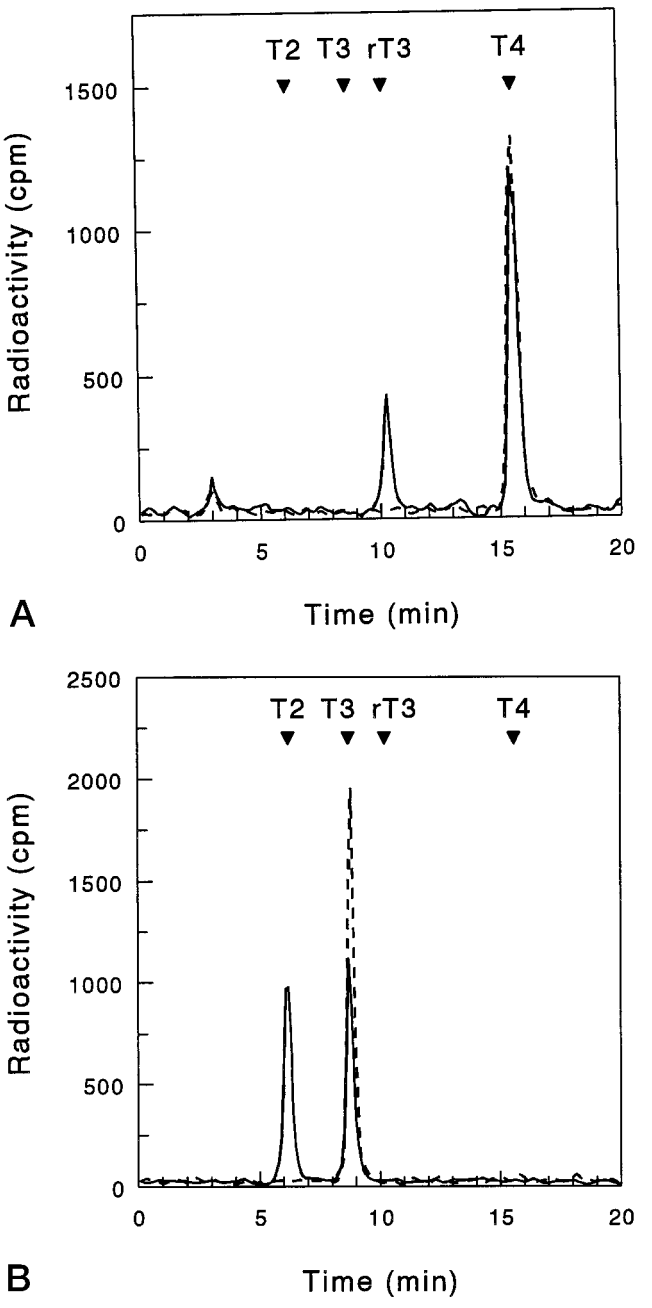

FIG. 6. HPLC analysis of the deiodination of T_4 (A) and T_3 (B) by tD3. Conditions were: 1 nM (3′, 5′-125I)T_4 or (3′-125I)T_3, 50 mM DTT, 1 mg/ml TL31-transfected cell lysate protein, and 1 h incubation at 37 C. Representative results of duplicate incubations with or without enzyme are illustrated with the *continuous* and *interrupted lines*, respectively.

trations of unlabeled T_3, T_4, and rT_3, with IC_{50} values of ~10, ~100 and ≫1000 nM, respectively (Fig. 7, A and B). The apparent K_m value of T_3 for both native and recombinant tD3 amounts to ~20 nM, which is close to the K_m values found for other D3 enzymes (1–5). Native and recombinant tD3 also show equal patterns of inhibition by increasing concentrations of GTG, IAc, and PTU (Fig. 7, C and D). Under the conditions used, IC_{50} values for these inhibitors amount to ~1 μM, ~1 mM and ≫1 mM, respectively.

Figure 8 compares the tissue distributions of D3 activity and mRNA levels in tilapia. Analysis of the IRD of T_3 in tissue homogenates indicates high D3 activity in brain and much lower activities in other tissues (Fig. 8A). On the Northern blots, a prominent 1.6-kb mRNA species is detected in gill and brain by hybridization with the tD3 riboprobe (Fig. 8B). Gill shows extensive hybridization of progressively shorter mRNA species, with a prominent band of ~1.2 kb which is clearly visible after shorter exposure times (not shown). Much weaker bands are present in liver and kidney. Furthermore, smaller mRNA species of ~1 kb are observed in heart and spleen. Gut and muscle show very little tD3 mRNA.

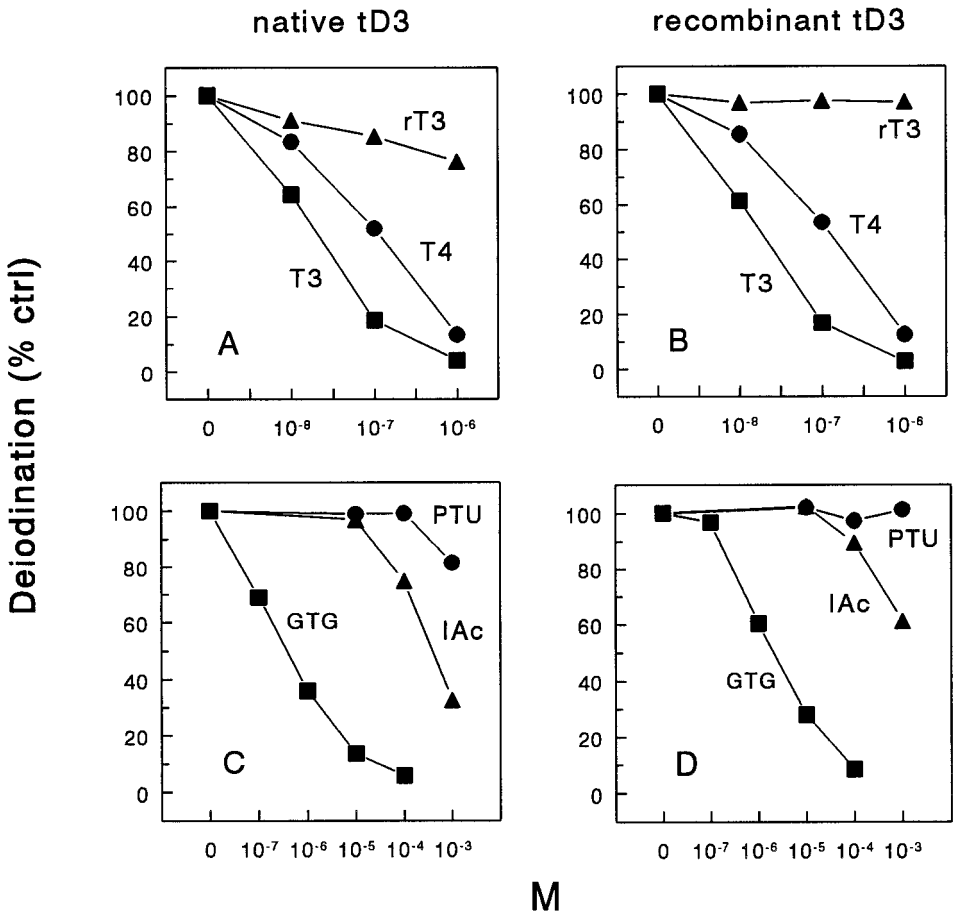

FIG. 7. Inhibition of the IRD of [^{125}I]T_3 by native tD3 in tilapia brain microsomes (A, C) and recombinant tD3 expressed in TL31-transfected COS-1 cell lysates (B, D) by increasing concentrations of unlabeled T_3, T_4 and rT_3 (A, B), or PTU, IAc and GTG (C, D). Conditions were: 10 nM [^{125}I]T_3, 10 mM DTT, 0.1–0.25 mg protein/ml, and 1 h incubation at 37 C. Results are the means of closely agreeing triplicates in a representative experiment.

Discussion

Evidence that the TL31 cDNA cloned and characterized in this study codes for tD3 may be summarized as follows. 1) The nucleotide and deduced amino acid sequences of TL31 show much higher homologies with D3 sequences from various species than with D1 and D2 sequences from fish and other species. 2) The catalytic properties of the enzyme expressed in TL31-transfected cells are characteristic for D3. It efficiently catalyzes the IRD of relatively low concentrations of T_4 and T_3 but is much less effective in the IRD of T_3S. This is in contrast to D1 from tilapia and other species, which show much higher IRD rates with T_3S than with T_3 as substrate (1–5, 20). Furthermore, the TL31-encoded enzyme does not catalyze the ORD of rT_3 and T_4, which are the most prominent reactions catalyzed by D1 and D2, respectively (1–5). 3) IRD of T_3 by TL31-transfected cells and native tD3 expressed in tilapia brain is characterized by identical K_m values and equal sensitivities to inhibition by the substrate analogs T_4 and rT_3 and the deiodinase inhibitors GTG, IAc, and PTU. The higher concentrations of unlabeled T_4 than of T_3 required to inhibit the IRD of labeled T_3 by recombinant and native tD3 are in agreement with the higher apparent K_m values of T_4 than of T_3 for D3 in general (1–5).

GTG, IAc, and PTU are potent inhibitors of D1 from different species, where GTG and IAc are thought to react with the selenolate anion of the native enzyme and PTU is supposed to react with a selenenyl iodide enzyme intermediate (1–5). However, tilapia D1 is much less sensitive to inhibition by GTG and IAc and is virtually insensitive to PTU (20, 36, 37). D2 and D3 from different species are even less sensitive than tilapia D1 to the effects of these inhibitors (1–5). Because Sec is supposed to be the catalytic center in all deiodinases, the reason for their differential sensitivities to these inhibitors remains an enigma (1–20).

The alignment of tD3 with the human, rat, *R. catesbeiana*, *X. laevis*, and chicken D3s reveals regions of high homology. The Kyte and Doolittle hydropathicity plot strongly suggests that the highly conserved N-terminal sequence from Ala^{16} to Ile^{41} in tD3 represents a hydrophobic membrane-spanning domain that anchors the protein in the membrane of the endoplasmic reticulum or in the plasma membrane. Such a transmembrane domain has also been identified near the N terminus of other deiodinases (6, 8–20). Studies of the topography of mammalian D1 suggested that the N terminus is hidden in the lumen of the endoplasmic reticulum with the major part of the protein exposed to the cytoplasm (51). Such an orientation fits with the requirement of thiols as cofactors for the deiodination of iodothyronines which are abundant in the reductive environment of the cytoplasm (52). These studies of D1 topography have also shown that basic amino acids flanking the transmembrane domain, which are located in positions 11 and 42–44 of tD3, are essential for proper insertion in the membrane (51).

The His residues located at positions 163 and 180 of tD3

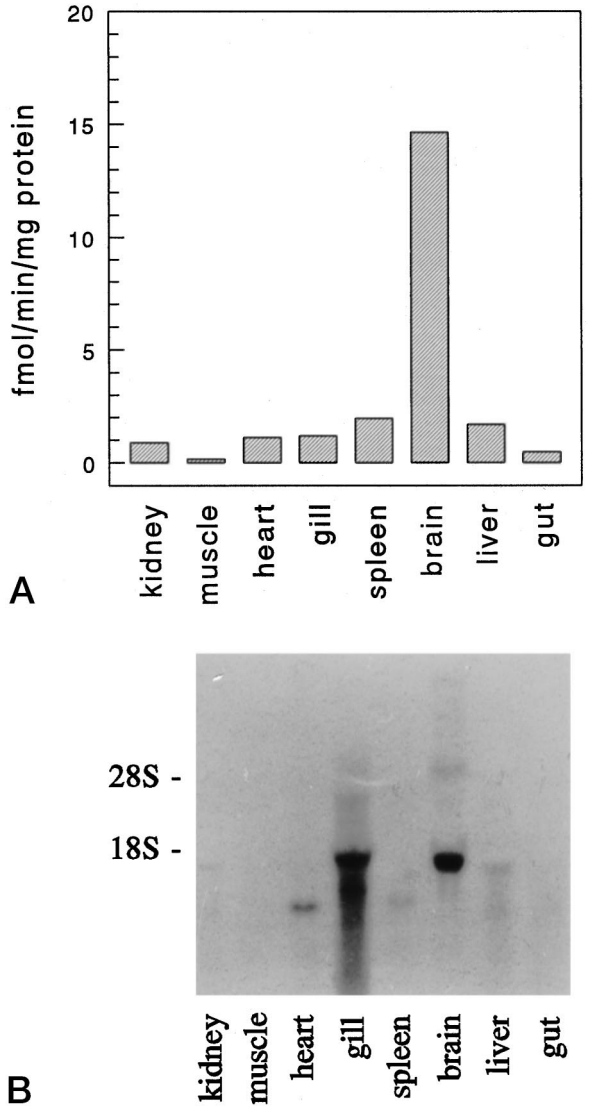

FIG. 8. A, Tissue distribution of D3 activity in tilapia. Homogenates (1 mg protein/ml) were incubated for 1 h at 37 C with 10 nM [^{125}I]T_3 and 50 mM DTT. Results are the means of closely agreeing triplicates in a representative experiment. B, Northern blot of total tissue RNA (20 μg) hybridized with radiolabeled tD3 riboprobe.

are conserved throughout the iodothyronine deiodinase family and have been shown in rat D1 to be essential for enzyme activity (53). One of these may directly participate in the catalytic process by forming a hydrogen bond with the selenol group, further increasing its nucleophilicity (53, 54). Phe^{65} in rat and human D1 (11, 41) has been shown to be involved in binding of rT_3. The absence of Phe in a corresponding position of D3 may contribute to the low affinity of rT_3 for this enzyme.

Although incorporation of Sec into tD3 has not been demonstrated directly, our findings strongly suggest that this enzyme features a Sec residue in a position corresponding to the Sec residue in other deiodinases. Sec is encoded by the UGA opal stop codon if the termination of translation normally signalled by this codon is suppressed in the presence of a SECIS element in the 3′UTR of the mRNA (47–50). The stem-loop structure predicted in the 3′ UTR of the tD3 cDNA contains most but not all of the consensus nucleotides observed in other SECIS elements (47–50). The putative tD3 SECIS element contains the sequence GUGA (nucleotides 1268–1271) instead of AUGA in other SECIS elements (47–50). The function of this first adenosine is not clear, since it is not involved in the nonWatson/Crick base-pairing proposed by Walczak *et al.* (50). A similar deviation from the consensus SECIS element was found in the second putative SECIS element in tilapia D1 cDNA (20). The consequences of this substitution for the efficiency of Sec incorporation are unknown. However, the tD3 SECIS element appears to function effectively in COS-1 cells not only in the context of wild-type tD3 cDNA but also in a chimeric construct combining the coding sequence of human D2 and the 3′UTR of tD3.[1]

The TL31 cDNA clone represents most of the tD3 mRNA because the size of the largest and most prominent band observed on Northern blots is only slightly bigger than TL31. Smaller mRNA species are observed in heart and spleen. The significance of these multiple mRNA species is unknown, but they may represent mRNA processing intermediates. However, the smaller D3 mRNA species in heart and spleen (~1 kb) are not expected to translate into functional protein because they are too short to contain both the initiator codon and the SECIS element. The high-stringency conditions used in the Northern analysis preclude hybridization of the tD3 riboprobe with D1 and D2 mRNA. This is supported by the barely detectable hybridization with RNA from liver and kidney which show abundant expression of D2 and D1, respectively (5, 20, 36, 37). Furthermore, hybridization with D1 (20) and D2 (55) riboprobes shows different hybridization signals.

The translational efficiency of the tD3 mRNA apparently shows substantial differences between tissues. Brain contains high levels of both D3 activity (37) and D3 mRNA. Even higher D3 mRNA levels are found in gill, although this tissue contains only limited D3 activity (37). The tailing observed with D3 mRNA from gill suggests high mRNA degradation. It is also remarkable that the Northern blots showed very little expression of D3 mRNA in tilapia liver, although D3 cDNA fragments were produced by RT-PCR of liver mRNA, and the tilapia liver cDNA library contained several independent TL31-like clones.

In conclusion, we have cloned and characterized D3 cDNA from tilapia. Together with the human, rat, chicken, and frog D3 sequences, the elucidation of a fish D3 sequence helps to define the conserved regions of these proteins which are essential for IRD activity.

Acknowledgments

We thank Hans van Toor for expert assistance with the HPLC analyses.

References

1. **Larsen PR, Berry MJ** 1995 Nutritional and hormonal regulation of thyroid hormone deiodinases. Annu Rev Nutr 15:323–352
2. **Leonard JL, Köhrle J** 1996 Intracellular pathways of iodothyronine metabo-

[1] J. P. Sanders, E. Kaptein, and T. J. Visser, unpublished results.

lism. In: Braverman LE, Utiger R (eds) The Thyroid. Lippincott-Raven, Philadelphia, pp 125–161
3. **Visser TJ** 1996 Pathways of thyroid hormone metabolism. Acta Med Austriaca 23:10–16
4. **St. Germain DL, Galton VA** 1997 The deiodinase family of selenoproteins. Thyroid 7:655–688
5. **Mol KA, Van der Geyten S, Burel C, Kühn ER, Boujard T, Darras VM** 1998 Comparative study of iodothyronine outer ring and inner ring deiodinase activities in five teleostean fishes. Fish Physiol Biochem 18:253–266
6. **Salvatore D, Bartha T, Harney JW, Larsen PR** 1996 Molecular biological and biochemical characterization of the human type 2 selenodeiodinase. Endocrinology 137:3308–3315
7. **Salvatore D, Tu H, Harney JW, Larsen PR** 1996 Type 2 iodothyronine deiodinase is highly expressed in human thyroid. J Clin Invest 98:962–968
8. **Berry MJ, Banu L, Larsen PR** 1991 Type I iodothyronine deiodinase is a selenocysteine-containing enzyme. Nature 349:438–440
9. **Mandel SJ, Berry MJ, Kieffer JD, Harney JW, Warne RL, Larsen PR** 1992 Cloning and in vitro expression of the human selenoprotein, type I iodothyronine deiodinase. J Clin Endocrinol Metab 75:1133–1139
10. **Maia AL, Berry MJ, Sabbag R, Harney JW, Larsen PR** 1995 Structural and functional differences in the dio1 gene in mice with inherited type 1 deiodinase deficiency. Mol Endocrinol 9:969–980
11. **Toyoda N, Harney JW, Berry MJ, Larsen PR** 1995 Identification of critical amino acids for 3,3′,5′-triiodothyronine deiodination by human type I deiodinase based on comparative functional-structural analyses of the human, dog and rat enzymes. J Biol Chem 269:20329–20334
12. **St.Germain DL, Schwartzman RA, Croteau W, Kanamori A, Wang Z, Brown DD, Galton VA** 1994 A thyroid hormone-regulated gene in Xenopus laevis encodes a type III iodothyronine 5-deiodinase. Proc Natl Acad Sci USA 91: 7767–7771 (Erratum, p 11282)
13. **Becker KB, Schneider MJ, Davey JC, Galton VA** 1995 The type III 5-deiodinase in Rana catesbeiana tadpoles is encoded by a thyroid hormone-responsive gene. Endocrinology 136:4424–4431
14. **Croteau W, Whittemore SL, Schneider MJ, St. Germain DL** 1995 Cloning and expression of a cDNA for a mammalian type III iodothyronine deiodinase. J Biol Chem 270:16569–16575
15. **Salvatore D, Low SC, Berry M, Maia AL, Harney JW, Croteau W, St. Germain DL, Larsen PR** 1995 Type 3 iodothyronine deiodinase: cloning, *in vitro* expression, and functional analysis of the placental selenoenzyme. J Clin Invest 96:2421–2430
16. **Davey JC, Becker KB, Schneider MJ, St. Germain DL, Galton VA** 1995 Cloning of a cDNA for the type II iodothyronine deiodinase. J Biol Chem 270:26786–26789
17. **Croteau W, Davey JC, Galton VA, St. Germain DL** 1996 Cloning of the mammalian type II iodothyronine deiodinase. J Clin Invest 98:405–417
18. **Valverde-RC, Croteau W, Lafleur GJ, Orozco A, St. Germain DL** 1997 Cloning and expression of a 5′-iodothyronine deiodinase from the liver of Fundulus heteroclitus. Endocrinology 138:642–648
19. **Van der Geyten S, Sanders JP, Kaptein E, Darras VM, Kühn ER, Leonard JL, Visser TJ** 1997 Expression of chicken hepatic type I and type III iodothyronine deiodinase during embryonic development. Endocrinology 138:5144–5152
20. **Sanders JP, Van der Geyten S, Kaptein E, Darras VM, Kühn ER, Leonard JL, Visser TJ** 1997 Characterization of a propylthiouracil-insensitive type I iodothyronine deiodinase. Endocrinology 138:5153–5160
21. **Suzuki M, Yoshida K, Sakurada T, Kaise N, Kaise K, Fukazawa H, Nomura T, Itagaki Y, Yonemitsu K, Yamamoto M, Saito S, Yoshinaga K** 1986 Effect of changes in thyroid state on metabolism of thyroxine in rat placenta. Endocrinol Jpn 33:37–42
22. **Emerson CH, Bambini G, Alex S, Castro MI, Roti E, Braverman LE** 1988 The effect of thyroid dysfunction and fasting on placenta inner ring deiodinase activity in the rat. Endocrinology 122:809–816
23. **Koopdonk-Kool JM, De Vijlder JJM, Veenboer GJM, Ris-Stalpers C, Kok JH, Vulsma T, Boer K, Visser TJ** 1996 Type II and type III deiodinase activity in human placenta as function of gestational age. J Clin Endocrinol Metab 81:2154–2158
24. **Kaplan MM, Visser TJ, Yaskoski KA, Leonard JL** 1983 Characteristics of iodothyronine tyrosyl ring deiodination by rat cerebral cortical microsomes. Endocrinology 112:35–42
25. **Richard K, Hume R, Kaptein E, Sanders JP, van Toor H, de Herder WW, den Hollander JC, Krenning EP, Visser TJ** 1998 Ontogeny of iodothyronine deiodinases in human liver. J Clin Endocrinol Metab 83:2868–2874
26. **Borges M, LaBourene J, Ingbar SH** 1980 Changes in hepatic iodothyronine metabolism during ontogeny of the chick embryo. Endocrinology 107:1751–1765
27. **Galton VA, Hiebert A** 1987 The ontogeny of the enzyme systems for the 5′- and 5-deiodination of thyroid hormones in chick embryo liver. Endocrinology 120:2604–2610
28. **Darras VM, Visser TJ, Berghman LR, Kühn ER** 1992 Ontogeny of type I and type III deiodinase activities in embryonic and posthatch chicks: relationship with changes in plasma triiodothyronine and growth hormone levels. Comp Biochem Physiol A 103:131–136
29. **Darras VM, Berghman LR, Vanderpooten A, Kühn ER** 1992 Growth hormone acutely decreases type III iodothyronine deiodinase in chicken liver. FEBS Lett 310:5–8
30. **Darras VM, Kotanen SP, Geris KL, Berghman LR, Kühn ER** 1996 Plasma thyroid hormone levels and iodothyronine deiodinase activity following an acute glucocorticoid challenge in embryonic compared with posthatch chickens. Gen Comp Endocrinol 104: 203–212
31. **Van der Geyten S, Buys N, Sanders JP, Decuypere E, Visser TJ, Kühn ER, Darras VM** 1998 Acute pretranslational regulation of hepatic type III iodothyronine deiodinase by GH and dexamethasone in embryonic chicken. J Endocrinol Invest [Suppl] 21:10
32. **Visser TJ, Schoenmakers** 1992 Characteristics of type III iodothyronine deiodinase. Acta Med Austriaca 19:18–21
33. **Burrow GN, Fisher DA, Larsen PR** 1994 Maternal and fetal thyroid function. N Engl J Med 331:1072–1078
34. **Cyr DG, Eales JG** 1996 Interrelationships between thyroidal and reproductive endocrine systems in fish. Rev Fish Biol Fisheries 6:165–200
35. **Frith SD, Eales JG** 1996 Thyroid hormone deiodination pathways in brain and liver of rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol 101: 323–332
36. **Mol K, Kaptein E, Darras VM, de Greef WJ, Kühn ER, Visser TJ** 1993 Different thyroid hormone-deiodinating enzymes in tilapia (*Oreochromis niloticus*) liver and kidney. FEBS Lett 321:140–144
37. **Mol KA, Van der Geyten S, Darras VM, Visser TJ, Kühn ER** 1997 Characterization of iodothyronine outer ring and inner ring deiodinase activities in the blue tilapia, *Oreochromis aureus*. Endocrinology 138:1787–1793
38. **Orozco A, Silva JE, Valverde-R C** 1997 Rainbow trout expresses two iodothyronine phenolic ring deiodinase pathways with the characteristics of mammalian type I and II 5′-deiodinases. Endocrinology 138:254–258
39. **Fenton B, Orozco A, Valverde-R C** 1997 Kinetic characterisation of skin inner-ring deiodinative pathway and its correlation with circulating levels of reverse tri-iodothyronine in developing rainbow trout. J Endocrinol 154:547–554
40. **Moreno M, Berry MJ, Horst C, Thoma R, Goglia F, Harney JW, Larsen PR Visser TJ** 1994 Activation and inactivation of thyroid hormone by type I iodothyronine deiodinase. FEBS Lett 344:143–146
41. **Toyoda N, Kaptein E, Berry MJ, Harney JW, Larsen PR, Visser TJ** 1997 Structure-activity relationships for thyroid hormone deiodination by mammalian type I iodothyronine deiodinases. Endocrinology 138:213–219
42. **Sanger F, Nicklen S, Coulson AR** 1977 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
43. **Zuker M, Stiegler P** 1981 Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res 9:133–148
44. **Kyte J, Doolittle RF** 1982 A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132
45. **Oertel M, Gross M, Rokos H, Köhrle J** 1993 Selenium-dependent regulation of type I 5′-deiodinase expression. Am J Clin Nutr 57:313S–314S
46. **Goldberg GS, Lau AF** 1993 Transfection of mammalian cells with PEG-purified plasmid DNA. Biotechniques 14:548–550
47. **Berry MJ, Banu L, Chen Y, Mandel SJ, Kieffer JD, Harney JW, Larsen PR** 1991 Recognition of UGA as a selenocysteine codon in type I deiodinase requires sequences in the 3′ untranslated region. Nature 353:273–276
48. **Berry MJ, Banu L, Harney JW, Larsen PR** 1993 Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons. EMBO J 12:3315–3322
49. **Shen Q, Leonard JL, Newburger PE** 1995 Structure and function of the selenium translation element in the 3′-untranslated region of human cellular glutathione peroxidase mRNA. RNA 1:519–525
50. **Walczak R, Westhof E, Carbon P, Krol A** 1996 A novel RNA structural motif in the selenocysteine insertion element of eukaryotic selenoprotein mRNAs. RNA 2:367–379
51. **Toyoda N, Berry MJ, Harney JW, Larsen PR** 1995 Topological analysis of the integral membrane protein, type 1 iodothyronine deiodinase (D1). J Biol Chem 270:12310–12318
52. **Berry MJ** 1992 Identification of essential histidine residues in rat type I iodothyronine deiodinase. J Biol Chem 267:18055–18059
53. **Mol JA, Docter R, Hennemann G, Visser TJ** 1984 Modification of rat liver iodothyronine 5′-deiodinase activity with diethylpyrocarbonate and rose bengal; evidence for an active site histidine residue. Biochem Biophys Res Commun 120:28–36
54. **Visser TJ** 1990 The role of glutathione in the enzymatic deiodination of thyroid hormone. In: Viña J (ed) Glutathione: Metabolism and Physiological Functions. CRC Press, Boca Raton, FL, pp 317–333
55. **Sanders JP, Van der Geyten, Kühn ER, Darras VM** 1998 Expression of type II and type III iodothyronine deiodinase in tissues of Oreochromis niloticus. J Endocrinol Invest [Suppl] 21:78