Identification and Functional Characterization of Zebrafish Solute Carrier Slc16a2 (Mct8) as a Thyroid Hormone Membrane Transporter

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Most components of the thyroid system in bony fish have been described and characterized, with the notable exception of thyroid hormone membrane transporters. We have cloned, sequenced, and expressed the zebrafish solute carrier Slc16a2 (also named monocarboxylate transporter Mct8) cDNA and established its role as a thyroid hormone transport protein. The cloned cDNA shares 56–57% homology with its mammalian orthologs. The 526-amino-acid sequence contains 12 predicted transmembrane domains. An intracellular N-terminal PEST domain, thought to be involved in proteolytic processing of the protein, is present in the zebrafish sequence. Measured at initial rate and at the body/rearing temperature of zebrafish (26 C), T3 uptake by zebrafish Slc16a2 is a saturable process with a calculated Michaelis-Menten constant of 0.8 μM T3. The rate of T3 uptake is temperature dependent and Na+/H+ independent. Interestingly, at 26 C, zebrafish Slc16a2 does not transport T4. This implies that at a normal body temperature in zebrafish, Slc16a2 protein is predominantly involved in T3 uptake. When measured at 37 C, zebrafish Slc16a2 transports T4 in a Na+/H+−independent manner. In adult zebrafish, the Slc16a2 gene is highly expressed in brain, gills, pancreas, liver, pituitary, heart, kidney, and gut. Beginning from the midblastula stage, Slc16a2 is also expressed during zebrafish early development, the highest expression levels occurring 48 h after fertilization. This is the first direct evidence for thyroid hormone membrane transporters in fish. We suggest that Slc16a2 plays a key role in the local availability of T3 in adult tissues as well as during the completion of morphogenesis of primary organ systems. (Endocrinology 152: 5065–5073, 2011)
have shown that passive diffusion represents less than 10% of the total thyroid hormone transport (3, 4), clearly indicating an important role for transporter-mediated pathways. Unfortunately, nothing is known as yet about piscine thyroid hormone transporters.

Several thyroid hormone membrane transporters have been identified in mammals (5, 6), and all of them belong to the solute carrier (SLC) superfamily, which is subdivided into 47 families (7). To date, SLCO1C1/Slco1c1 have shown to transport thyroid hormone in zebrafish (20) and could well contain Slc16a2, which latter substrate is transported by its mammalian counterpart (17).

The zebrafish (Danio rerio) is a member of the cyprinid family and a widely used model species for other vertebrates. Many of the components of the zebrafish thyroid axis have been characterized (18, 19). However, no thyroid hormone membrane transporters have been identified and characterized to date. The Slc16 family is present in zebrafish (20) and could well contain Slc16a2, which would be the piscine ortholog of the mammalian specific thyroid hormone transporter SLC16A2/Slc16a2. The aims of the present study were 1) to identify the zebrafish ortholog of the mammalian SLC16A2/Slc16a2 in the zebrafish genome, 2) to investigate the pattern of Slc16a2 gene expression in zebrafish tissues and during early development, and most importantly, 3) to establish whether zebrafish Slc16a2 protein transports thyroid hormone.

### Materials and Methods

#### Fish and sampling procedures

Adult wild-type zebrafish (1 yr of age) were commercially obtained (De Maanvis, Nijmegen, The Netherlands) and reared in 2-liter tanks at 26 C with recirculating, UV light-treated Nijmegen city tap water. Fish were fed daily with Tetra-min fish feed (Tetra, Melle, Germany) at a ration of 2.5% of the total body weight. Sampling occurred 1 h after feeding; fish were anesthetized in 0.1% (vol/vol) 2-phenoxyethanol (Sigma Chemical Co., St. Louis, MO) and killed by spinal transection. Organs were collected in sterilized 1.5-ml Eppendorf vials, immediately frozen on dry ice, and stored at −80 C until further analyses (tissue distribution and cloning). For the analysis of the temporal pattern of Slc16a2 gene expression during early development, groups of six zebrafish (wild-type) embryos or larvae were collected 3, 6, 12, 24, 48, and 72 h post-fertilization (hpf). The following developmental stages were determined: midblastula (3 hpf), gastrula (6 hpf), 6–10 somite segmentation (12 hpf), pharyngula (24 hpf), late pharyngula (48 hpf), and yolk sac larvae (72 hpf). Animal procedures were performed in accordance with national legislation and were approved by the ethical review committee of Radboud University Nijmegen (The Netherlands).

#### RNA isolation and cDNA synthesis

RNA was isolated from zebrafish tissues or individual whole embryos using TRizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Total RNA was precipitated in ethanol, washed, and dissolved in nuclease-free water. RNA concentrations were measured spectrophotometrically, and 1 µg (tissues) or 500 ng (embryos and larvae) of total RNA was used for cDNA synthesis. cDNA synthesis was carried out as previously described (21), and samples were stored at −20 C until further use.

#### Design of gene-specific primers for zebrafish Slc16a2 cloning

TBLASTN searches were performed on the zebrafish whole-genome shotgun database at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the human SLC16A2 as query amino acid sequence (GenBank accession no. NP_006508). Sequences with a high homology with SLC16A2 were selected. Then, contig Zv8_NA2811.6 (GenBank accession no. CAAK05008536) was predicted to encode exon 1, contig Zv8_NA2811.2 (GenBank accession no. CAAK05008532) exon 2, contig Zv8_NA2811.1 (GenBank accession no. CAAK05008530) exon 3, contig Zv8_scaffold1672.31 (GenBank accession no. CAAK05036070) exon 4, contig Zv8_scaffold1672.30 (GenBank accession no. CAAK05036069) exon 5, and contig Zv8_scaffold1672.30 (GenBank accession no. CAAK05036069) exon 6. Based on this analysis, primers were designed and used to amplify the coding sequence (cds) of the zebrafish Slc16a2 gene.

#### Cloning and sequencing of zebrafish Slc16a2 gene coding sequence cds

Amplification was carried out by PCR with Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) according to the manufacturer’s instructions. The cDNA used for the amplification was synthesized from brain total RNA. Thermocycling conditions were programmed as follows: 1 min initial de-
was transformed into competent TOPO expression kit (Invitrogen), and the pcDNA 3.1 construct was cloned using the pcDNA 3.1 directional TOPO expression kit (Invitrogen), and the pcDNA 3.1 construct was transformed into competent Escherichia coli cells. Plasmid DNA was isolated from positive clones using the Quantum Prep miniprep kit (Bio-Rad, Hercules, CA) and stored at −20 C until further use. The cDNA inserted (putative zebrafish Slc16a2 cDNA) into the pcDNA 3.1 vector was sequenced by the Research Bioinformatics (Nijmegen, The Netherlands). The sequence obtained was submitted to the DDBJ/EMBL/GenBank databases under accession number FN687448 (cds) and CBK52149 (protein). Transmembrane domains of zebrafish Slc16a2 protein were predicted using hidden Markov models (TMHMM server version 2.0 at http://www.cbs.dtu.dk/services/TMHMM/) (22). PEST domains [regions rich in proline (P), aspartate (D), glutamate (E), serine (S), and threonine (T) and flanked by positively charged residues, which are thought to target proteins for rapid degradation] were predicted using the epestfind algorithm (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind). Hydrophilic stretches flanked by positively charged amino acids [arginine (R), histidine (H), or lysine (K)] of not fewer than 12 amino acids (or in a medium where Na⁺ would be rapidly depleted in the absence of BSA (25)]. Plates were then incubated at 26 C or 37 C in 1 ml incubation medium to which T₁, or T₂, and 2 × 10⁶ cpm ¹²⁵I-labeled T₁ or T₂, respectively, were added. T₁ and T₂ concentrations as well as incubation times are indicated in the legends to figures. Radiotracer was purified on a 10% (wt/vol) Sephadex LH-20 mini-column shortly before use as described by Mol and Visser (26). After incubation, wells were washed with incubation medium at room temperature and rinsed with 1 ml 0.1 n NaOH to lyse the cells. NaOH fractions were aspirated and counted for ¹²⁵I radioactivity. Cellular thyroid hormone uptake was calculated as femtomoles thyroid hormone contained in the cell lysates. Results were corrected for the amount of ¹²⁵I radioactivity associated with the walls of the culture wells by subtracting the amount of ¹²⁵I radioactivity extracted from the wells incubated without cells (blanks).

To evaluate Na⁺ dependence of thyroid hormone transport by zebrafish Slc16a2, cells were incubated in a medium containing 142.9 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 1.8 mM CaCl₂, 20 mM HEPES, and 0.1% BSA (pH 7.4) or in a medium where Na⁺ was replaced with an equimolar amount of choline⁺. These media were also used during washing steps. Concentrations of thyroid hormone and incubation conditions are indicated in the legends to figures.

Before experimentation, the required ratio FuGENE 6 to DNA (microliters per microgram) was optimized using the vector pEGFP-C1 (BD Biosciences Clontech, Sparks, MD). A 3:1 ratio resulted in the highest transfection efficiency (i.e. 25–30%) and was used during all experiments.

### Temporal pattern of zebrafish Slc16a2 gene expression during early development and tissue distribution in adult tissues

The relative expression of zebrafish Slc16a2 was assessed by real-time quantitative PCR (RT-qPCR) in adult tissues and embryos/larvae. Primers were designed using the Primer Express software (Applied Biosystems, Carlsbad, CA), and they are shown in Table 1. Five microliters of cDNA and 600 (adult tissues) or 200 nM (embryos and larvae) forward and reverse primers were added to 12.5 µl SYBR Green Master Mix (Applied Biosystems). The total volume was adjusted to 25 µl with deionized H₂O. RT-qPCR (10 min at 95 C, 40 cycles of 15 sec at

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<th>Primer</th>
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<tr>
<td>dare Slc16a2 fw</td>
<td>CACCCATGCACCTGGAAGACGGATGA</td>
<td>dare qSlc16a2 fw</td>
<td>ATGGCACTCGGAAAGCGATGA</td>
</tr>
<tr>
<td>dare Slc16a2 rv</td>
<td>TCATATGGTGTTCCTCCATGTC</td>
<td>dare qSlc16a2 rw</td>
<td>CCGTCTGATGGGTCTTTTGC</td>
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<td></td>
<td>GGTTCCAAACAGCTCGAA</td>
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Primer names that include “q” indicate the primers used for RT-qPCR measurements. Note the sequence CACC (in bold) at the 5’ end in primer dare Slc16a2 fw to enable directional cloning in the vector pcDNA 3.1 and to achieve an adequate Kozak consensus sequence for the initiation of translation processes.

Phylogenetic analysis

Multiple sequence alignments were carried out using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/) (23). A phylogenetic tree was constructed based on amino acid difference (p-distance) with the neighbor-joining algorithm (pairwise deletion) using the MEGA software version 4 (24). The reliability of the tree was assessed by bootstrapping using 1000 replications. Only full-length coding sequences were used for analysis.

Thyroid hormone uptake studies

COS-1 cells were seeded in six-well plates at densities of 2 × 10⁵ cells per well. Per plate, two wells served as blanks and were not seeded with cells. Cells were grown in DMEM/F12 medium supplemented with GlutaMAX (1×; Invitrogen) and containing 9% bovine calf serum (HyClone, Logan, UT) at 37 C. Blank wells were rinsed with the same volume of DMEM/F12 medium as used in the wells containing cells. Forty-eight hours after seeding (at 75% confluence), cells were transfected in duplicate with 1 µg empty pcDNA 3.1 vector (control cells) or vector containing zebrafish Slc16a2 cDNA using FuGENE 6 transfection reagent (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Twenty-four hours after transfection, plates were preincubated at 26 C (the rearing temperature of our zebrafish) or 37 C for 20 min and washed with prewarmed incubation medium (DMEM/F12 plus 0.1% BSA). BSA replaced the bovine calf serum during assays. The reasons for this replacement are two: 1) presence of thyroid hormones in the bovine calf serum and 2) BSA provides a buffer of loosely bound ligand in the unstirred water layer surrounding the cell, from which ligand (thyroid hormones) would be rapidly depleted in the absence of BSA (25). Plates were then incubated at 26 C or 37 C in 1 ml incubation medium to which T₁ or T₂ and 2 × 10⁶ cpm ¹²⁵I-labeled T₁ or T₂, respectively, were added. T₁ and T₂ concentrations as well as incubation times are indicated in the legends to figures. Radiotracer was purified on a 10% (wt/vol) Sephadex LH-20 mini-column shortly before use as described by Mol and Visser (26). After incubation, wells were washed with incubation medium at room temperature and rinsed with 1 ml 0.1 n NaOH to lyse the cells. NaOH fractions were aspirated and counted for ¹²⁵I radioactivity. Cellular thyroid hormone uptake was calculated as femtomoles thyroid hormone contained in the cell lysates. Results were corrected for the amount of ¹²⁵I radioactivity associated with the walls of the culture wells by subtracting the amount of ¹²⁵I radioactivity extracted from the wells incubated without cells (blanks).

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95 C and 1 min at 60 C) was carried out using a GeneAmp 7500 sequence detection system (Applied Biosystems).

Statistics
Net T3 uptake kinetic data were analyzed using a non-weighted nonlinear regression computer program (SigmaPlot for Windows version 11.0; Systat Software, Inc., Chicago, IL) in which the Levenberg-Marquardt algorithm for least-squares estimation of parameters is employed. Results from thyroid hormone uptake studies are presented as the mean ± SD of two to three independent experiments performed in duplicate. In experiments where temperature and Na+ dependence was assessed, statistical significance was determined using Student’s unpaired t test and accepted at P < 0.05. The effects of the type of tissue and the time after fertilization on the zebrafish Slc16a2 gene expression in different tissues and during early development, respectively, were analyzed by a one-way ANOVA followed by a post hoc Tukey’s test. The significance level was set at P < 0.05.

Results
Molecular cloning of zebrafish Slc16a2 cds
The full-length zebrafish Slc16a2 cDNA contains 1581 nucleotides, encoding a peptide of 526 amino acids and with an estimated molecular mass of 57 kDa (Supplemental Fig. 1). The amino acid identity between zebrafish Slc16a2 protein and its predicted amino acid sequence obtained by analysis of the whole-genome shotgun database is 99%.

Sequence identity with mammalian SLC16A2/Slc16a2 proteins from human, mouse and rat (the only species where these proteins have been demonstrated to transport thyroid hormones) is 57, 57, and 56%, respectively (Fig. 1). In general, the 12 hydrophobic transmembrane domains (TMD) are particularly conserved between zebrafish Slc16a2 protein and its mammalian counterparts. The loops between TMD are less conserved, especially the large intracellular loop between TMD6 and TMD7. Two amino acids, Arg445 and Asp498, which are supposed to be critical for the molecular interactions between the transporter and the thyroid hormone substrate in humans (27) are present in zebrafish Slc16a2. An intracellular N-terminal PEST domain [a region rich in proline (P), aspartate (D), glutamate (E), serine (S) and threonine (T) and flanked by positively charged residues] is also present in zebrafish Slc16a2. It has a length of 33 amino acids and is flanked by histidine and arginine (amino acids 2 and 36, respectively, in the zebrafish Slc16a2 sequence). In mouse and rat Slc16a2, a second C-terminal PEST motif is present, and the human SLC16A2 sequence even contains three PEST domains (Fig. 1).

Phylogeny of zebrafish Slc16a2 protein
A phylogenetic analysis of zebrafish Slc16a2 protein is presented in Fig. 2. Neighbor-joining analysis grouped the zebrafish sequence within the vertebrate SLC16A2/Slc16a2 clade. All vertebrate SLC16A2/Slc16a2 sequences cluster separately from SLC16A10/Slc16a10 sequences; teleostean, avian, and amphibian sequences form separate clusters again within Slc16a2.

Functional characterization of zebrafish Slc16a2 protein
Figure 3 shows the characterization of T3 uptake by zebrafish Slc16a2. Because fish are ectotherms, we decided...
to perform uptake assays at the rearing/body temperature of zebrafish (26 C). Figure 3A shows the time course of T3 uptake by cells transfected with empty pcDNA 3.1 vector (control cells) and cells transfected with pcDNA 3.1 containing the zebrafish Slc16a2 cds as an insert. The uptake of T3 by cells expressing zebrafish Slc16a2 was higher than that in control cells at all time points analyzed (from 3–300 min). Net T3 uptake (difference between T3 uptake in cells expressing zebrafish Slc16a2 gene and control cells transfected with an empty plasmid) is linear up to 10 min and is adequately described by a first-order exponential equation, confirming the activity of a single transporter (Fig. 3B). The maximum rate, calculated from the slope of the tangent to the progress curve at time point 0, is 0.8 fmol T3/min. The calculated first-order rate constant equals 0.06 min⁻¹. Figure 3C shows T3-dependent saturable uptake of T3 at an initial rate in control cells and cells transfected with zebrafish Slc16a2. Net initial uptake rates are well described by a single-site Michaelis-Menten function (calculated kinetic parameters were Vmax = 582 fmol T3/min, and Km = 0.8 μM T3). Data points and calculated Vmax and Km converge on a linear Eadie-Hofstee transformation, confirming the activity of a single active transporter (Fig. 3D). T3 uptake by COS-1 cells expressing zebrafish Slc16a2 and control cells increased when the incubation temperature during transport assays was increased from 26 C to 37 C (Fig. 3E). This increase in incubation temperature resulted in a 3- and 2-fold increase in net uptake of T3 after 10 and 30 min incubation time, respectively. Replacement of Na⁺ by choline⁺ in the incubation medium did not significantly affect T3 uptake (Fig. 3F).

At 26 C, no net uptake of T4 (difference between T4 uptake in cells expressing zebrafish Slc16a2 gene and control cells) by zebrafish Slc16a2 could be measured (Fig. 4A). However, when cells were incubated at 37 C for 30 min, T4 uptake was significantly higher in zebrafish Slc16a2-transfected cells compared with control cells (Fig. 4B). At 37 C, net T4 uptake by zebrafish Slc16a2 was Na⁺ independent (Fig. 4C).

Expression of Slc16a2 transcripts in zebrafish tissues and during early development

Both type of tissue and time after fertilization were sources of variability for zebrafish Slc16a2 gene expression in different tissues and during early development, respectively (one-way ANOVA, P < 0.05 and P < 0.001, respectively). The Slc16a2 gene was ubiquitously expressed in all adult zebrafish tissues tested except in female gonads (Fig. 5). The highest expression levels were found in brain, followed by liver and kidney (brain > liver > kidney > gills, pancreas, pituitary, heart > gut). However, no significant differences were found between Slc16a2 gene expression levels in these tissues. We also detected Slc16a2 mRNA in the following early developmental stages (Fig. 6): midblastula (3 hpf), gastrula (6 hpf), 6–10...
FIG. 3. Characterization of T3 uptake in recombinant COS-1 cells seeded at a nominal density of 2 \times 10^5 cells per well. Results are the mean \pm SD of two to three independent experiments performed in duplicate. A, Time course of T3 uptake in COS-1 cells transfected with an empty pcDNA 3.1 vector (control cells) or a vector containing the cDNA coding for the zebrafish Slc16a2 gene as an insert. B, Progress curve of net T3 uptake in COS-1 cells (difference between T3 uptake in cells expressing the zebrafish Slc16a2 gene and control cells). The progress curve is well described by a first-order exponential equation: \( f(t) = A_\infty \times (1 - e^{-kt}) \). \( A_\infty \) is a limit at \( t = \infty \), \( k \) is the first-order rate constant (minutes\(^{-1}\)), and \( t \) is time (minutes). Calculated kinetic parameters are \( A_\infty = 12 \text{ fmol T3} \); \( k = 0.06 \text{ min}^{-1} \). The tangent to the curve at \( t = 0 \) gives the maximum net T3 uptake rate, which is 0.8 fmol T3/min. A and B, Uptake was measured at the rearing temperature of zebrafish (26 C) with 1 nm T3 plus 2 \times 10^5 cpm 125I-labeled T3 in DMEM/F12 medium plus 0.1% BSA. C, Substrate-dependent uptake of T3 by COS-1 cells transfected with an empty pcDNA 3.1 vector (control cells) or a vector containing cDNA coding for the zebrafish Slc16a2 gene as an insert. D, Net T3 uptake in COS-1 cells, measured at initial rate, as a function of substrate concentration. Data points are described by a single-site Michaelis-Menten function with \( V_{\text{max}} = 582 \text{ fmol T3/min} \) and \( K_m = 0.8 \mu M \) T3. The inset shows an Eadie-Hofstee transformation of the data points and calculated kinetic parameters. C and D, Uptake was measured at initial rate (10 min incubation) and at the rearing temperature of zebrafish (26 C) with 1–2500 nM T3 plus 2 \times 10^5 cpm 125I-labeled T3 in DMEM/F12 medium plus 0.1% BSA. E, Temperature-dependent T3 uptake in COS-1 cells transfected with an empty pcDNA 3.1 vector (control cells) or a vector containing cDNA coding for the zebrafish Slc16a2 gene as an insert. Uptake was measured with 1 nm T3 plus 2 \times 10^5 cpm 125I-labeled T3 in DMEM/F12 medium plus 0.1% BSA. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \). F, Na\(^+\) dependence of the net uptake of 1 nm T3 plus 2 \times 10^5 cpm 125I-labeled T3 in COS-1 cells expressing the zebrafish Slc16a2 gene. Cells were incubated for 10 min at 26 C in a buffer containing NaCl or in a sodium-free medium containing an equimolar amount of choline.
somite segmentation (12 hpf), pharyngula (24 hpf), late pharyngula (48 hpf), and yolk sac larvae (72 hpf). The highest gene expression levels were found 48 hpf at which time zebrafish embryos had not hatched yet. At 48 hpf, Slc16a2 gene expression levels were significantly higher than those at the other time points (Tukey’s test, $P < 0.001$). Pairwise comparisons between Slc16a2 gene expression levels at 3, 6, 12, 24, and 72 hpf were nonsignificant (Tukey’s test, $P > 0.05$). Hatching occurred asynchronously in the 48- to 72-hpf time interval.

Discussion

Our studies demonstrate that the zebrafish Slc16a2 protein, an ortholog to the mammalian SLC16A2/Slc16a2 transporter, facilitates cellular thyroid hormone uptake in a temperature-dependent and Na$^+$-independent manner. This is the first characterization of a thyroid hormone membrane transporter in fish.

Zebrafish Slc16a2 shares not only a high sequence homology with human SLC16A2 and mouse and rat Slc16a2 but also 12 transmembrane $\alpha$-helices with a high degree of conservation and an identical gene structure that consists of six exons. The only difference between the zebrafish and mammalian transporters is the presence of a single N-terminal PEST domain in zebrafish Slc16a2, whereas human, mouse, and rat sequences contain two to three PEST domains in the N and C termini. The PEST domain is thought to act as proteolytic signal, targeting the protein for rapid degradation (28). The low conservation of the zebrafish PEST sequence and the absence of a C-
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proximately 1.5 nM (29), two to three orders of magnitude lower than the calculated K_m value for T_3 uptake in this study. It appears that zebrafish Slc16a2 in vivo is operating well below its limiting rate, and small elevations in plasma T_3 concentrations will result in a linear increase in uptake rate.

Thyroid hormone uptake by zebrafish Slc16a2 is temperature dependent, as it is for mammalian Slc16a2 (11). Zebrafish Slc16a2 transport activities are increased at the physiologically relevant temperature for mammals, 37 C, a temperature that fish do not tolerate. When measured at 37 C, zebrafish Slc16a2, as does human SLC16A2 (12), transports both T_3 and T_4, but only T_3 when the incubation temperature is that of the body temperature (i.e. 26 C). An intriguing but highly speculative notion would be that the acquired capacity for T_4 by the mammalian/endothermic Slc16a2 protein is an example of exaptation, i.e. a necessary byproduct of endothermy (30).

The Slc16a2 gene is preferentially expressed in the adult zebrafish brain, gills, pancreas, liver, pituitary, heart, kidney, and gut. The tissue distribution is similar to that in rat (11), and it indicates these organs as important targets for thyroid hormone action. In the zebrafish brain, most likely, Slc16a2 regulates T_3 availability in a manner similar to that in mammals where SLC16A2 facilitates the transmembrane uptake of T_3 in neurons. This is a key process for neuronal activity because mammalian neurons do not possess a T_4-to-T_3 conversion capacity (2). In addition, zebrafish Slc16a2 is also expressed during early development, where its gene expression reaches a maximum in the late pharyngula stage, at 48 hpf. In this stage, which is immediately before hatching, the morphogenesis of the main primary organ systems such as the thyroid gland (31) and the nervous, muscular, and circulatory systems (32) is completed. The temporal expression pattern indicates that Slc16a2 is involved in these major developmental changes, probably by facilitating an increase in intracellular T_3 availability, which regulates the expression of key morphogenetic target genes.

In conclusion, we have cloned the zebrafish Slc16a2 gene (cds) and functionally characterized the zebrafish Slc16a2 protein as a thyroid hormone membrane transporter. Our findings are the first direct evidence for thyroid hormone membrane transporters in a piscine species. Zebrafish Slc16a2 is likely to play a key role in the local availability of T_3 during early development and in adult tissues.

Acknowledgments

We thank Marnix Gorissen, M.Sc., for technical assistance with zebrafish dissection, and Moniek van’t Root and Iris Peters Rit for their help during procedures with embryos and larvae.
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F.J.A. is a recipient of a postdoctoral grant from the Ramón Areces Foundation (Madrid, Spain).


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