0888-8809/06/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 20(11):2761–2772

doi: 10.1210/me.2005-0256

Thyroid Hormone Transport by the Human Monocarboxylate Transporter 8 and Its Rate-Limiting Role in Intracellular Metabolism

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Cellular entry of thyroid hormone is mediated by plasma membrane transporters. We have identified rat monocarboxylate transporter 8 (MCT8) as an active and specific thyroid hormone transporter. The *MCT8* gene is located on the X-chromosome. The physiological relevance of MCT8 has been demonstrated by the identification of hemizygous mutations in this gene in males with severe psychomotor retardation and elevated serum T_3 levels. We have characterized human (h) MCT8 by analysis of iodothyronine uptake and metabolism in cell lines transiently transfected with hMCT8 cDNA alone or together with cDNA coding for iodothyronine deiodinase D1, D2, or D3. MCT8 mRNA was detected by RT-PCR in a number of human cell lines as well as in COS1 cells but was low to undetectable in other cell lines, including JEG3 cells. MCT8 protein was not detected in nontransfected cell lines tested by immunoblotting using a polyclonal C-terminal hMCT8 antibody but was detectable in transfected cells at the expected size (61 kDa). Transfection of COS1 and JEG3 cells with hMCT8 cDNA resulted in 2- to 3-fold increases in uptake of T_3 and T_4 but little or no increase in rT_3 or 3,3′-diiodothyronine (3,3′-T_2) uptake. MCT8 expression produced large increases in T_4 metabolism by cotransfected D2 or D3, T_3 metabolism by D3, rT_3 metabolism by D1 or D2, and 3,3′-T_2 metabolism by D3. Affinity labeling of hMCT8 protein was observed after incubation of intact transfected cells with *N*-bromoacetyl-[^{125}I]T_3. hMCT8 also facilitated affinity labeling of cotransfected D1 by bromoacetyl-T_3. Our findings indicate that hMCT8 mediates plasma membrane transport of iodothyronines, thus increasing their intracellular availability. (*Molecular Endocrinology* 20: 2761–2772, 2006)

ALTHOUGH T_4 IS the major product secreted by the thyroid follicular cells, most actions of thyroid hormone are exerted by binding of T_3 to its nuclear receptors, which are associated with the T_3 response elements in thyroid hormone-sensitive genes (1, 2). This alters the association of these receptors with protein factors (corepressors and coactivators) that either suppress or activate the basal transcription machinery and thus control the expression of the relevant genes. The biological activity of thyroid hormone is therefore determined by the intracellular T_3 concentration, which depends on a number of factors, including the concentrations of circulating T_3 and its precursor T_4, the activity of the iodothyronine deiodinases D1–D3 that either produce T_3 from T_4 (D1, D2) or catalyze the inactivation of both T_4 and T_3 (D3) (3, 4), and the activity of transporters that mediate the influx and/or efflux of T_3 and T_4 (5, 6).

During the last three decades, studies have demonstrated the importance of transporters for the cellular uptake of thyroid hormone (5). However, only recently, such transporters have been identified at the molecular level, including Na/taurocholate-cotransporting polypeptide (NTCP) and multiple members of the Na-independent organic anion-transporting polypeptide (OATP) family (5–9). The most interesting of these is OATP1C1, which shows a high degree of tissue selectivity and ligand specificity, being expressed predominantly in brain and testis and showing high preference for T_4 and rT_3 as the ligand (10–12). In brain, OATP1C1 is localized in particular in capillaries and is thought to be very important for uptake of T_4 across the blood-brain barrier (11).

Previously, two types of amino acid transporters have been implied in the cellular uptake of iodothyronines into different tissues (5, 6). One of these is the heterodimeric L-type amino acid transporter, which facilitates the exchange of neutral branched-chain and aromatic amino acids over the plasma membrane (13, 14). The T-type amino acid transporter shows speci-

First Published Online August 3, 2006

Abbreviations: DTT, Dithiothreitol; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; IRD, inner ring deiodination; MCT8, monocarboxylate transporter 8; NTCP, Na/taurocholate-cotransporting polypeptide; OATP, organic anion-transporting polypeptide; ORD, outer ring deiodination; PM, plasma membrane; 3′-T_1, 3′-iodothyronine; 3,3′-T_2, 3,3′-diiodothyronine; TLS, translation start site.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

ficity for aromatic amino acids, and interaction between cellular transport of Trp and iodothyronines has suggested the involvement of this transporter in thyroid hormone uptake in different tissues (15, 16). One such transporter (TAT1) has recently been cloned and characterized in rats and humans (17, 18). However, although TAT1 showed high transport of the aromatic amino acids Trp, Tyr, and Phe, it was found to be inactive toward T_4 and T_3. TAT1 appears to be a member of the so-called monocarboxylate transporter (MCT) family and is also named MCT10 (19). It shows particularly high amino acid homology with one other member of this family, MCT8, and we have characterized rat (r) MCT8 in *Xenopus laevis* oocytes as an active iodothyronine transporter, whereas it does not transport the aromatic amino acids or the typical monocarboxylate ligands, lactate and pyruvate (20).

The pathophysiological relevance of MCT8 as a thyroid hormone transporter has been demonstrated dramatically in patients with a novel syndrome of severe X-linked mental retardation and strongly elevated serum T_3 levels (21, 22). This severe phenotype is explained by the recent demonstration that MCT8 is localized in different tissues, including the brain, where it is expressed specifically by thyroid hormone-sensitive neuronal populations (23, 24). MCT8 is thought to be important for neuronal uptake of T_3 produced from T_4 in neighboring astrocytes that express D2 (25). Neurons are the primary targets for thyroid hormone action in the (developing) brain, and mutations in MCT8 would deprive neurons of essential T_3 and thus result in psychomotor retardation. Many of these neurons also express D3, which catalyzes termination of T_3 action (25). A defect in MCT8 would also block T_3 access to neuronal D3, thus resulting in a decrease in T_3 clearance and increase in serum T_3 level. This novel syndrome therefore represents another mechanism of thyroid hormone resistance in addition to the well-known resistance syndrome due to mutations in the thyroid hormone β-receptor.

In the present study we set out to characterize human (h) MCT8 as a thyroid hormone transporter. In particular, we investigated the ligand specificity of iodothyronine transport by hMCT8 in transfected cells, and its rate-limiting role in determining intracellular iodothyronine metabolism in cells cotransfected with the different deiodinases. Furthermore, we explored the possible identification of hMCT8 in cell lines by affinity labeling using radiolabeled bromoacetyl-iodothyronine derivatives as well as by immunological methods. The results demonstrate that expression of hMCT8 indeed increases intracellular thyroid hormone availability.

RESULTS

Cloning of hMCT8 cDNA

The putative full-length hMCT8 mRNA is 4.4 kb in size (NM_006517) and contains two alternative translation start sites (TLSs) in the same reading frame, one at nucleotides 167–169 and another one at nucleotides 389–391. Depending on which of these alternative TLSs is used, a protein is produced of 613 or 539 amino acids, respectively. Both TLSs have the general sequence GxxATGG and thus appear to adhere to the Kozak rule, suggesting that they are genuine start codons (26). However, amino acid homology with MCT8 from other species is only apparent downstream from the second TLS. We assumed therefore that this is the true start codon, and we cloned the hMCT8 coding sequence by RT-PCR of RNA isolated from human adult liver. The primers were located immediately upstream of the second ATG and immediately downstream of the TAA stop codon and contained convenient restriction sites for directional cloning in the pcDNA3 expression vector. The cDNA clone obtained by these means was sequenced and found to be identical to the published hMCT8 coding sequence.

Expression of hMCT8 mRNA in Cell Lines

Expression of MCT8 mRNA was investigated by RT-PCR in various human cell lines to study the relationship with hMCT8 protein expression as well as to identify cells devoid of endogenous MCT8, which could then be used for transfection studies (Fig. 1). Among the various cell lines tested, significant hMCT8 mRNA expression was found in HepG2 hepatocarcinoma cells, human embryonic kidney (HEK)293 cells, U87, U373, and CCF-STTG astrocytoma cells, SH-SY5Y neuroblastoma cells, ECC1 and Ishikawa endometrium carcinoma cells, and MCF7 breast carcinoma cells, but not in WRL68 embryonic liver cells, BON pancreatic carcinoid tumor cells, or JEG3, JAR, and BeWo choriocarcinoma cells. MCT8 mRNA was also expressed in COS1 monkey kidney-derived fibroblasts (Fig. 1). For characterization of hMCT8, transfection studies have been carried out largely in COS1 and JEG3 cells, which do or do not express MCT8 endogenously, respectively.

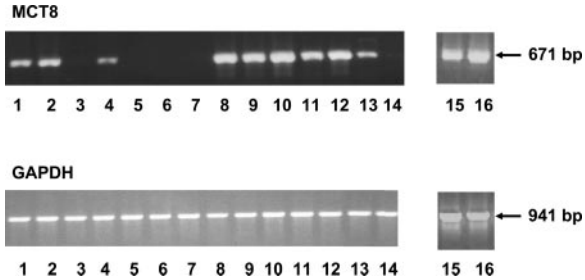

Fig. 1. RT-PCR of MCT8 and GAPDH mRNA in Different Cell Lines

1) ECC1; 2) ECC1 incubated for 4 h with 0.1 μM 12-*O*-tetradecanoylphorbol 13-acetate; 3) WRL68; 4) HepG2; 5) JEG3; 6) JAR; 7) BeWo; 8) U87; 9) U373; 10) CCF-STTG; 11) SH-SY5Y; 12) MCF7; 13) Ishikawa; 14) BON; 15) COS1; 16) HEK293.

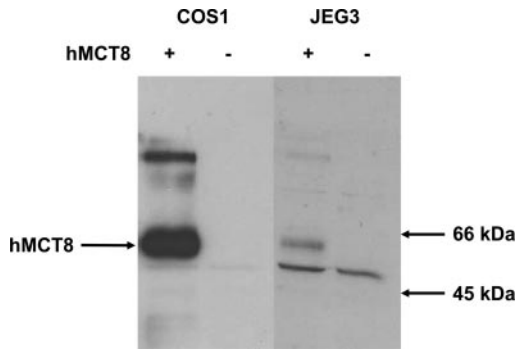

Fig. 2. Western Blot of Homogenates from COS1 and JEG3 Cells Transfected with Empty pcDNA3 or with pcDNA3-hMCT8

The blot was probed with polyclonal antiserum no. 1305 against the C terminus of hMCT8 (1:500).

Immunoblotting of hMCT8 Protein

Immunoblotting of extracts of the various cell lines using our polyclonal C-terminal hMCT8 antibody did not result in the detection of hMCT8 protein, not even in the cells showing significant expression of hMCT8 mRNA, such as COS1 cells. The lack of MCT8 protein detection in COS1 African green monkey kidney-derived fibroblasts is probably due to insufficient sensitivity of the antibody rather than poor cross-reactivity with monkey MCT8, because the MCT8 sequences deduced from the chimpanzee and macaca genomes are identical with the human (h) MCT8 sequence at the site of the epitope. Transfection of COS1 and JEG3 cells with the hMCT8 cDNA resulted in the clear immunostaining of a protein with apparent molecular mass of 61 kDa, in good agreement with the calculated molecular mass of 59.5 kDa (Fig. 2). As the hMCT8 sequence does not contain consensus N-glycosylation sites, the protein will not be glycosylated. A higher band of approximately 260 kDa is also observed in transfected COS1 and JEG3 cells which may represent complex formation with an ancillary protein (Fig. 2).

Affinity Labeling of hMCT8 Protein

N-Bromoacetyl-[^{125}I]iodothyronines such as BrAc[^{125}I]T_3 and BrAc[^{125}I]T_4 are highly effective and specific affinity labels for D1 (27–29). However, it has also been reported that incubation of isolated rat liver cells with unlabeled $BrAcT_3$ results in the inhibition of subsequent uptake of radioactive T_3 by these cells, suggesting covalent modification of T_3 transporter(s) in these cells by $BrAcT_3$ (30). Therefore, we tested in the present study whether native and recombinant hMCT8 in different cell lines may be labeled specifically with BrAc[^{125}I]T_3 and/or BrAc[^{125}I]T_4. This was compared with the affinity labeling of rat (r) D1 in cells (co)transfected with cDNA coding for the deiodinase. Figure 3 shows the results obtained using COS1 cells transfected with hMCT8 cDNA or rD1 cDNA alone or in combination.

Exposure of intact COS1 cells transfected with empty vector to 0.05 nM BrAc[^{125}I]T_3 resulted in the modest labeling of a protein with apparent molecular mass of 61 kDa. Transfection of COS1 cells with hMCT8 cDNA resulted in a large increase in the affinity labeling of the 61-kDa protein. The affinity labeling of this protein was obviously more intense after incubation of cells with BrAc[^{125}I]T_3 than with BrAc[^{125}I]T_4 (Fig. 3). Cells transfected with rD1 cDNA and incubated with BrAc[^{125}I]T_3 showed marked labeling of a 29-kDa protein, corresponding to the calculated molecular mass of rD1. Interestingly, the affinity labeling

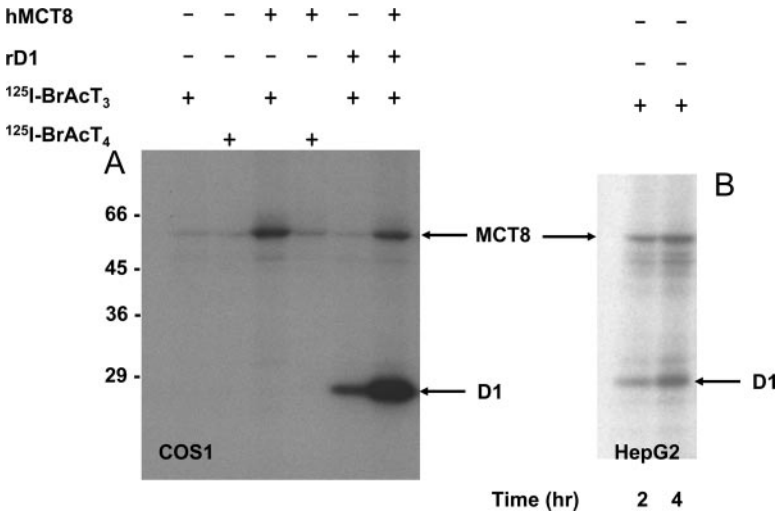

Fig. 3. Affinity Labeling of Recombinant and Native MCT8 and D1

COS1 cells were transfected with pcDNA3-hMCT8 and/or pcDNA3-rD1 and incubated with BrAc[^{125}I]T_3 or BrAc[^{125}I]T_4 (A), and nontransfected HepG2 cells were incubated with BrAc[^{125}I]T_3 (B). Incubations were done for 2–4 h at 37 C as described in *Materials and Methods*.

of rD1 was much greater in cells cotransfected with hMCT8 than in cells without recombinant transporter. Similar results were obtained with transfected HEK293 cells (data not shown). Affinity labeling of endogenous MCT8 with BrAc[^{125}I]T_3 was demonstrated in nontransfected HepG2 cells (Fig. 3) but not in WRL68 and JEG3 cells (data not shown), in keeping with the hMCT8 mRNA expression data. In addition, native D1 was identified by BrAcT_3 affinity labeling in HepG2 cells, in agreement with previous observations of D1 expression in these cells (31).

The specificity of the affinity labeling of the hMCT8 and rD1 proteins was tested by adding excess unlabeled (10 μM) T_3 or T_4 to the incubations with intact COS1 cells. The results are shown in Fig. 4, indicating that the affinity labeling of hMCT8 in transfected cells was inhibited to a greater extent in the presence of a high concentration of T_3 than by T_4. Densitometric analysis indicated that MCT8 labeling in the presence of excess T_3 is about one-third of that in the presence of excess T_4. Conversely, affinity labeling of rD1 was inhibited to a greater extent by excess T_4 than by T_3. These results are compatible with a higher affinity of hMCT8 for T_3 than for T_4, whereas the opposite is true for rD1. The affinity labeling of endogenous MCT8 in nontransfected COS1 cells, despite the lack of detection of MCT8 protein on immunoblots, suggests a lower sensitivity of the latter method.

Transport of Iodothyronines by hMCT8

COS1 and JEG3 cells were transfected with pcDNA3 without (control) or with hMCT8 cDNA and after 24 h or 48 h, respectively, incubated for 5, 10, and 30 min at 37 C with 1 nM radioactive T_3 in DMEM-F12 medium with 0.1% BSA (Fig. 5, A and B). COS1 cells showed a somewhat higher uptake of T_3 compared with JEG3 cells, partly due to endogenous MCT8. Transport of T_3 in hMCT8-overexpressing cells measured in several independent experiments showed the same fold increase in both cell lines used. Transfection of COS1 cells with rMCT8 cDNA induced an increase in T_3 uptake that was somewhat smaller than the increase induced by hMCT8 (Fig. 5A).

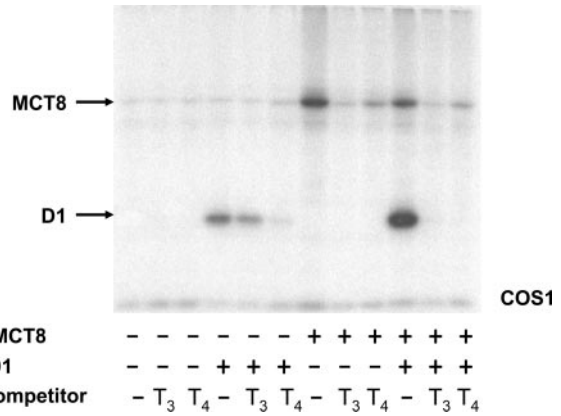

Fig. 4. Effects of unlabeled T_3 and T_4 on the Affinity Labeling of hMCT8 and rD1 by Incubation of Intact COS1 Cells with BrAc[^{125}I]T_3

Cells were transfected with pcDNA3-hMCT8 and/or pcDNA3-rD1 and incubated for 2–4 h at 37 C with the affinity labels in the absence or presence of 10 μM T_3 or T_4 as described in *Materials and Methods*.

COS1 and JEG3 cells were also incubated for 10 min at 37 C with 1 nM of the different iodothyronines, T_4, T_3, rT_3, and 3,3′-T_2 (Fig. 5, C and D). hMCT8 showed a substrate preference of T_3 (~3-fold) > T_4 (~2-fold) > rT_3 ~ 3,3′-T_2. The data in Fig. 5, C and D, suggest that endogenous transporters in COS1 and JEG3 cells show preference for T_3 as iodothyronine ligand, but it should be realized that the free fraction of T_3 in BSA-containing medium is approximately 5-fold higher than that of T_4 (32).

Iodothyronine Metabolism in Cells Cotransfected with hMCT8 and Deiodinases

In this set of experiments the hypothesis was tested that transport of iodothyronines into the cell by hMCT8 facilitates their intracellular metabolism by iodothyronine deiodinases. Because COS1 and JEG3 cells express very little endogenous deiodinase activities, they were transfected with rD1, hD2, or hD3 alone, with hMCT8 alone, or with deiodinase plus hMCT8. The different radioactive iodothyronines were incubated for 4 or 24 h with the transfected cells, and medium radioactivity was analyzed by HPLC (Fig. 6). The results are presented in Figs. 7–10.

T_4 Metabolism

Incubation of T_4 with cells transfected with control (empty) vector did not result in appreciable metabolism even after 24 h (Figs. 7 and 8). Transfection of COS1 or JEG3 cells with hMCT8 alone did not result in a significant increase in T_4 metabolism; neither did transfection of cells with rD1 alone or in combination with hMCT8 (data not shown). However, some conversion of T_4 to T_3 and I^- was observed in cells transfected with hD2 alone, and this was strongly increased in cells cotransfected with hD2 and hMCT8 (Fig. 7). Low but significant conversion of T_4 to rT_3 was observed in cells transfected with hD3 alone, and a dramatic increase in T_4 metabolism was observed in cells cotransfected with hD3 and hMCT8 (Fig. 8). Because native COS1 cells express some D1 activity, a substantial proportion of the initial product rT_3 was further metabolized to 3,3′-T_2 and I^-, and the 3,3′-T_2 was further converted by exogenous hD3 to 3′-iodothyronine (3′-T_1). In JEG3 cells, rT_3 produced by deiodination of T_4 by hD3 was not further metabolized (Fig. 8).

T_3 Metabolism

T_3 was not significantly metabolized in cells transfected with empty vector, but some production of

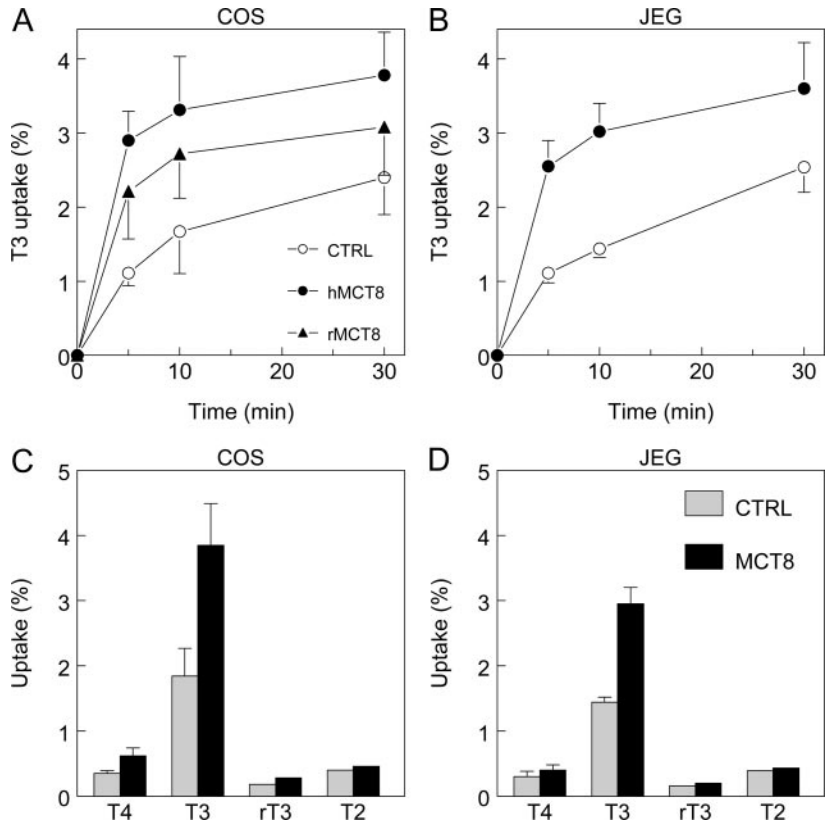

Fig. 5. Transport of 1 nM ^{125}I-labeled T_4, T_3, rT_3, or 3,3′-T_2 in COS1 or JEG3 Cells Transfected with Empty pcDNA3 (CTRL), pcDNA3-hMCT8 (hMCT8), or pCIneo-rMCT8 (rMCT8)

Time-dependent uptake of T_3 is measured in COS1 (A) or JEG3 cells (B) and expressed as percent of added radioactivity. Ligand specificity was determined after incubation of control and hMCT8-expressing COS1 (C) or JEG3 cells (D) for 10 min with the different iodothyronines. Data are expressed as percent uptake of added radioactivity per 10 min. Results are the means ± SEM of two to eight experiments. CTRL, Control.

3,3′-T_2 was observed in cells transfected with hMCT8 alone (Fig. 9). Significant conversion of T_3 to 3,3′-T_2 and in particular 3′-T_1 was observed in cells transfected with hD3 alone, and this was markedly augmented by cotransfection with hMCT8. The majority of added T_3 was converted within 4 h by COS1 and JEG3 cells cotransfected with hD3 and hMCT8 (Fig. 9). Because T_3 is a poor substrate for D1 and no substrate at all for D2, metabolism of T_3 was not studied in cells transfected with rD1 or hD2.

rT_3 Metabolism

Significant metabolism of rT_3 was observed neither in cells transfected with empty vector nor in cells transfected with hMCT8 alone (Fig. 10). Only at prolonged incubation, significant conversion of rT_3 to 3,3′-T_2 was observed in cells transfected with rD1 alone. However, rT_3 metabolism was strongly augmented in cells cotransfected with rD1 and hMCT8. After 24 h, rT_3 was completely converted in COS1 cells to 3,3′-T_2, 3,3′-T_2 sulfate (T_2S) and I^-. Significant deiodination of rT_3 was also observed in cells transfected with hD2 alone, and this was also strongly increased by cotransfection with hMCT8 (data not shown). Because rT_3 is not a substrate for D3, rT_3 metabolism was not investigated in cells transfected with hD3.

3,3′-T_2 Metabolism

Little metabolism of 3,3′-T_2 was observed in cells transfected with empty vector or with hMCT8 alone (Fig. 9). Conversion of 3,3′-T_2 to 3′-T_1 took place in JEG3 cells transfected with hD3 alone, and this was markedly increased in both COS1 and JEG3 cells by cotransfection with hD3 and hMCT8. 3,3′-T_2 metabolism was not investigated in cells transfected with rD1 or hD2.

Iodothyronine Metabolism in Cell Lysates

The above results indicate that hMCT8 stimulates the cellular metabolism of iodothyronines by facilitating their access to the intracellular deiodinases. An alternative explanation is that transfection with hMCT8 increases the expression of the different deiodinases. To exclude this possibility, we also studied deiodinase activities in lysates of cells transfected with rD1, hD2,

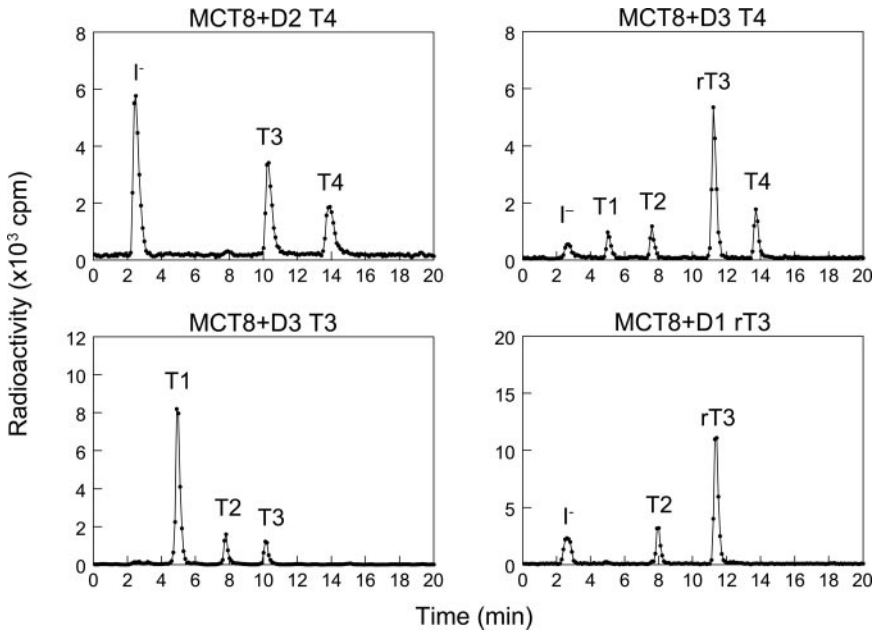

Fig. 6. HPLC Analysis of Medium Radioactivity

HPLC analysis of medium radioactivity after incubation of 1 nM [3′,5′-^{125}I]T_4 for 24 h at 37 C with COS1 cells (A) transfected with pcDNA3-hMCT8 and pcDNA3-hD2; or (B), transfected with pcDNA3-hMCT8 and pCIneo-hD3; C) after incubation of 1 nM [3′-^{125}I]T_3 for 4 h with COS1 cells transfected with pcDNA3-hMCT8 and pCIneo-hD3; or D) after incubation of 1 nM [3′,5′-^{125}I]rT_3 for 4 h with COS1 cells transfected with pcDNA3-hMCT8 and pcDNA3-hD1.

or hD3 in combination with empty plasmid or with hMCT8. Figure 11 shows that cotransfection with hMCT8 did not increase but, if anything, decreased the activities of the different deiodinases. Similarly, cotransfection with D1, D2, or D3 did not change MCT8 protein expression as indicated by immunoblotting (data not shown).

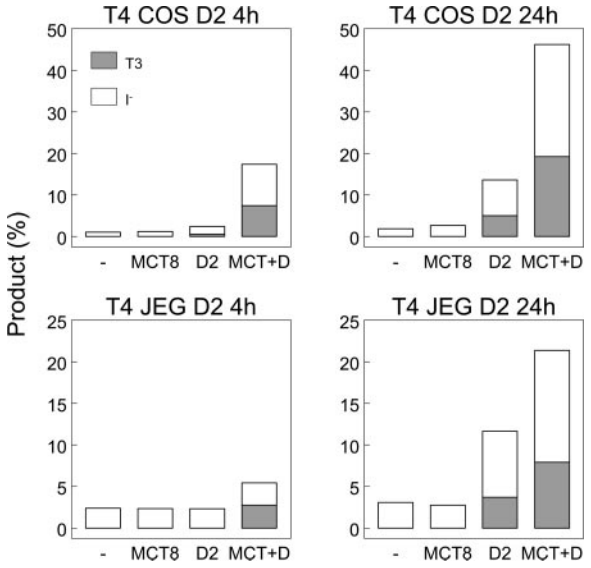

Fig. 7. Metabolism of T_4 in COS1 or JEG3 Cells Transfected with Empty pcDNA3, pcDNA3-hMCT8, or pcDNA3-hD2, or a Combination of the Latter (MCT+D)

Cells were incubated for 4 or 24 h at 37 C with 1 nM [3′,5′-^{125}I]T_4. Results are the means of duplicate determinations from a representative experiment.

DISCUSSION

In a previous study we have demonstrated that rMCT8 facilitates transport of the iodothyronines into *Xenopus laevis* oocytes injected with the mRNA coding for this transporter (20). Although the transporter did not appear to discriminate between the different iodothyronines, its specificity was indicated by the lack of transport of sulfonated iodothyronine derivatives, of typical ligands for T-type (Phe, Tyr, Trp) and L-type (Leu) amino acid transporters, and of monocarboxylates (lactate and pyruvate). Its relatively high activity toward iodothyronines was demonstrated by the much higher rate of uptake of T_4 and T_3 compared with other transporters mediating iodothyronine uptake, including rat Oatp1, rat Ntcp, and human LAT1. Subsequent studies have provided strong evidence that MCT8 also plays an important role in thyroid hormone transport into human tissues, in particular the brain (21–23, 33). However, transport of iodothyronines by hMCT8 has not been directly studied thus far. In this paper, we have determined the properties of hMCT8 after transfection of its cDNA into COS1 and JEG3 cells. Cells transfected with hMCT8 showed higher rates of uptake of T_4 and T_3 than cells transfected with control vector without cDNA insert. In neither cell line did uptake of rT_3 and 3,3′-T_2 appear to be stimulated by transfec-

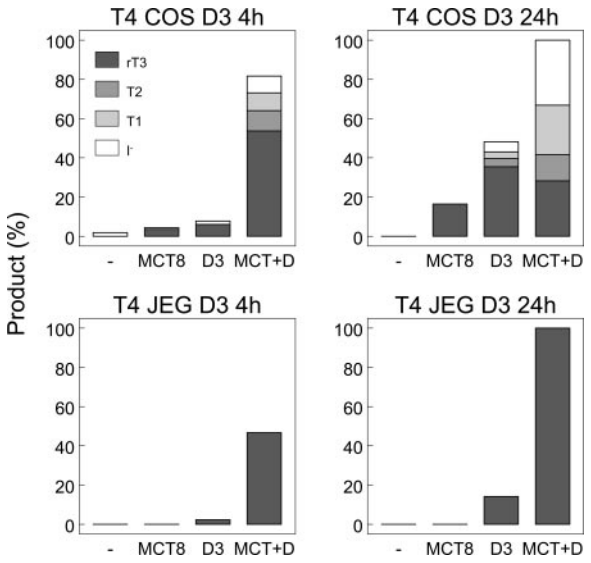

Fig. 8. Metabolism of T_4 in COS1 or JEG3 Cells Transfected with Empty pcDNA3, pcDNA3-hMCT8, or pCIneo-hD3, or a Combination of the Latter (MCT+D)

Cells were incubated for 4 or 24 h at 37 C with 1 nM [3′,5′-^{125}I]T_4. Results are the means of duplicate determinations from a representative experiment.

tion with hMCT8. However, the metabolism of all iodothyronines was markedly stimulated in cells expressing different iodothyronine deiodinases if these cells were also transfected with hMCT8. Another iodothyronine derivative, $BrAcT_3$, was also found to be transported by hMCT8 as indicated by the strong increase in the affinity

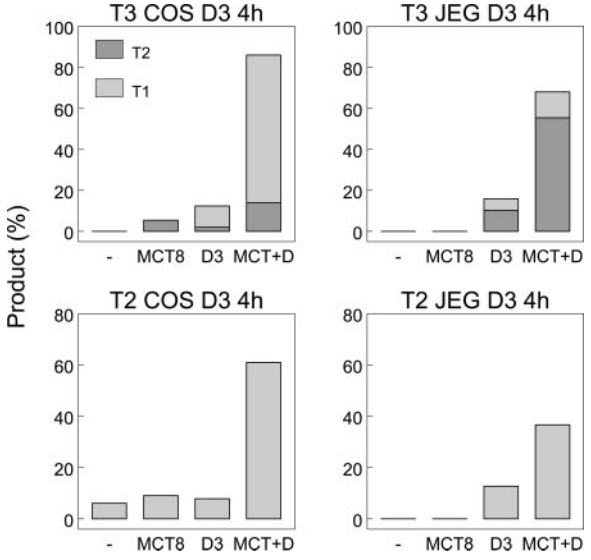

Fig. 9. Metabolism of T_3 or 3,3′-T_2 in COS1 or JEG3 Cells Transfected with Empty pcDNA3, pcDNA3-hMCT8, or pCIneo-hD3, or a Combination of the Latter (MCT+D)

Cells were incubated for 4 h at 37 C with 1 nM [3′-^{125}I]T_3 or [3′-^{125}I]3,3′-T_2 Results are the means of duplicate determinations from a representative experiment.

labeling of rD1 in cells that were transfected with hMCT8 cDNA in addition to rD1 cDNA. At the same time, hMCT8 itself was also found to undergo affinity labeling by $BrAcT_3$.

The relative stimulation of iodothyronine uptake induced by expression of MCT8 in mammalian cells depends on the presence of endogenous transporters in these cells. Both COS1 and JEG3 cells used in the present study also show significant endogenous transport of the different iodothyronines. In COS1 cells this is mediated, at least in part, by MCT8 as indicated by the significant expression of MCT8 mRNA and the identification of MCT8 protein by affinity labeling with $BrAc[^{125}I]T_3$. Very little expression of MCT8 was detected in JEG3 cells as well as in the other JAR and BeWo choriocarcinoma cell lines, although significant expression of MCT8 has been demonstrated in placental trophoblasts (33). The transporter(s) responsible for endogenous transport of iodothyronines in nontransfected JEG3 cells remains unknown.

The function of MCT8 in transfected cells may depend on their expression of ancillary proteins. For several members of the MCT family, including MCT1 and MCT4, it has been demonstrated that their proper targeting to the plasma membrane requires the coexpression of CD147 (also called basigin), a member of the Ig superfamily (19). MCT2 does not associate with CD147 but with the homologous Ig protein embigin (34). The possible requirement of ancillary proteins for MCT8 function needs to be investigated.

The stimulation of iodothyronine uptake in COS1 and JEG3 cells by transfection with hMCT8 is modest, decreasing in the order T_3 (~3-fold) > T_4 (~2-fold) > rT_3 > 3,3′-T_2. When this is compared with the greater degree of induction of iodothyronine uptake in MCT8-expressing oocytes, the limited transfection efficiency in mammalian cells should be taken into account. A similar consideration applies to the comparison of the MCT8-induced increase in iodothyronine uptake with the stimulation of iodothyronine metabolism in cells cotransfected with MCT8 plus deiodinase, because most cells expressing deiodinase will also express MCT8. It seems logical, therefore, that the MCT8-induced increase in iodothyronine metabolism by cotransfected deiodinases is greater than the MCT8-induced increase in T_4 and T_3 uptake. However, the magnitude of this difference is remarkable in particular for rT_3 and 3,3′-T_2, the uptake of which appears to be stimulated very little by MCT8 transfection, in contrast to the large stimulation of their metabolism.

The amount of hMCT8 protein expressed in COS1 cells is higher than that in JEG3 cells, but the increase in T_3 uptake is similar in magnitude if both cell types are transfected with hMCT8 cDNA. Possible explanations for this apparent discrepancy are that 1) a larger proportion of exogenous hMCT8 does not reach the plasma membrane in COS1 cells, or that 2) net uptake of T_3 is not a linear function of the amount of hMCT8 expressed at the plasma membrane. The latter could be the case if MCT8 facilitates not only T_3 uptake but also T_3 efflux. Such

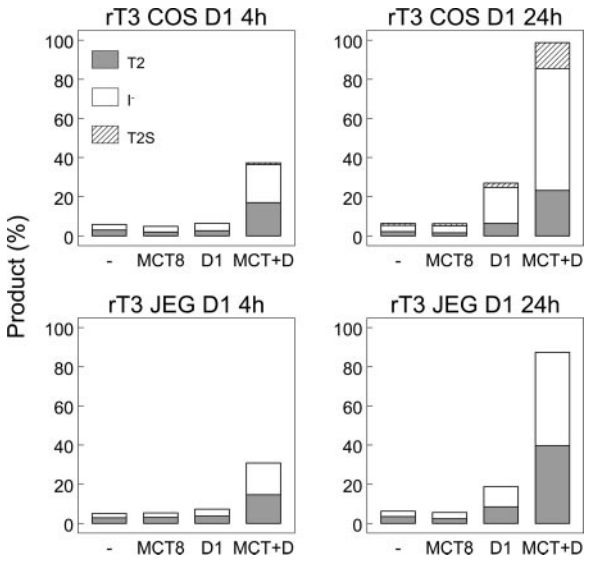

Fig. 10. Metabolism of rT_3 in COS1 or JEG3 Cells Transfected with Empty pcDNA3, pcDNA3-hMCT8, or pcDNA3-rD1, or a Combination of the Latter (MCT+D)

Cells were incubated for 4 or 24 h at 37 C with 1 nM [3′,5′-^{125}I]rT_3. Results are the means of duplicate determinations from a representative experiment.

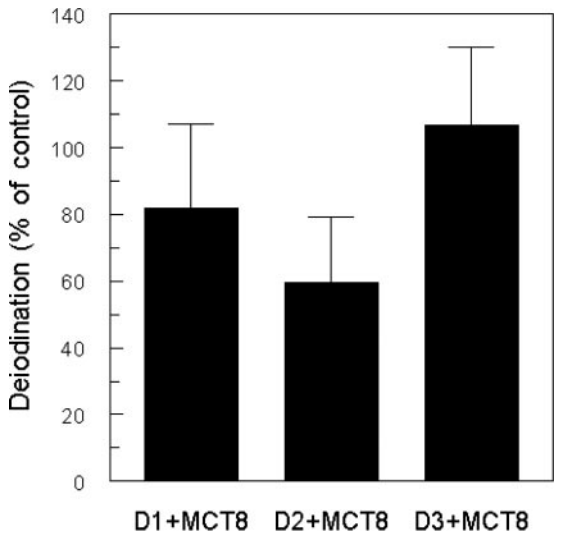

Fig. 11. Deiodinase Activity in Lysates of COS1 Cells Cotransfected with pcDNA3-rD1, pcDNA3-hD2, or pCIneo-hD3 plus pcDNA3-hMCT8, Expressed as Percent of the Deiodinase Activity of Cells Cotransfected with Deiodinase Plasmid plus Empty pcDNA3 (Control)

Results are the means ± SEM of three experiments.

exchange mechanism has been demonstrated for MCT10 and other members of the MCT family (19, 35). Studies are ongoing in our laboratory to further investigate these possibilities.

Very little metabolism was observed in nontransfected COS1 or JEG3 cells incubated for up to 24 h with the different iodothyronines. Some inner ring deiodination (IRD) of T_4 to rT_3 and of T_3 to 3,3′-T_2 was noted in COS1 cells transfected with hMCT8 alone, suggesting the expression of some endogenous D3 activity in these cells. JEG3 cells appeared to be devoid of D3 activity, although they are choriocarcinoma cells, and very high D3 activity is expressed in placenta, in particular in trophoblasts (36). A minor fraction of 3,3′-T_2 was converted to its sulfate by sulfotransferase activity expressed in COS1, but not in JEG3, cells.

Transfection of cells with D3 stimulated the IRD of T_3 to 3,3′-T_2, and in COS1 cells, in particular, this metabolite was rapidly further converted by IRD to 3′-T_1. The latter conversion was also observed in hD3-expressing COS1 and JEG3 cells incubated directly with labeled 3,3′-T_2. Incubation of T_4 with cells transfected with hD3 alone resulted in its IRD to rT_3, which was further converted in COS1 cells by ORD to 3,3′-T_2 and subsequent IRD to 3′-T_1. The nature of this outer ring deiodination (ORD) activity is not clear. Transfection of cells with hD2 alone stimulated the conversion of added T_4 by ORD to T_3 and of rT_3 to 3,3′-T_2, and the latter was also stimulated by transfection of cells with rD1 alone. The stimulation of the conversion of the different iodothyronines by transfection of cells with deiodinases without transfection of MCT8 is explained by the expression of endogenous transporters in these cell lines.

Most remarkable are the results showing that transfection of cells with hMCT8 in addition to the deiodinases facilitates the intracellular metabolism of the different iodothyronines. They represent the most direct evidence that MCT8 indeed increases the intracellular availability of these substrates. It is generally accepted that iodothyronine deiodinases are integral membrane proteins embedded in the plasma membrane (PM) or endoplasmic reticulum (ER) (3, 4). Although previous data suggested that native D1 is localized in the ER of rat liver cells, evidence has been reported for a PM localization of native D1 in rat kidney tubular cells as well as for recombinant D1 in transfected cells (37–40). In cells transfected with D2, this enzyme was found to be associated with the ER (41). The differential localization of D1 in PM and D2 in ER has been regarded in view of the different role of D1 in systemic T_3 production and that of D2 in local T_3 production, assuming that in D2-expressing tissues T_3 acts on the nuclear receptor in the same cell where it is produced from T_4. However, it has become increasingly clear that in the brain, for instance, T_3 is produced in a paracrine rather than an autocrine fashion, *i.e*. T_4 is converted by D2 in astrocytes to T_3, which is then transported to neurons, its primary target particularly during brain development (25). Furthermore, there is evidence indicating that D2 expressed in human skeletal muscle contributes significantly to production of plasma T_3 (42).

Even though D1 and D2 may be localized in different subcellular membrane fractions, it is generally accepted that the active sites of both enzymes are exposed in the cytoplasm. This is logical because the deiodination of iodothyronines catalyzed by these enzymes is a reductive process that requires thiols as cofactor that are only available in the reductive environment of the cytoplasm.

Therefore, iodothyronines incubated with D1- or D2-expressing cells can only be deiodinated after transport across the PM. Our data demonstrate that the deiodination of different iodothyronines by D1 and D2 is strongly facilitated by MCT8, indicating that, indeed, MCT8 markedly increases the intracellular availability of these substrates.

Similar to D1, a PM localization has also been reported for D3 based on studies mostly using transfected cells (37). In contrast to D1 and D2, however, this study also suggested that the active site of D3 is exposed on the external cell surface, which is unexpected regarding the oxidative environment of the extracellular milieu (37). After the deiodination of its substrate, regeneration of native enzyme from an oxidized enzyme intermediate was proposed to involve its internalization and exposure to intracellular thiols. Obviously, our results are not compatible with such a mechanism of action of D3, because transfection of cells with MCT8 also strongly facilitates deiodination of T_4, T_3, and 3,3′-T_2 by D3. These results can be explained only by the intracellular localization of the active site of D3, substrate access to which is facilitated by MCT8.

The above conclusion that MCT8 increases the access of substrates to the intracellular active sites of the deiodinases is supported by the findings obtained using the affinity label $BrAcT_3$. We have previously demonstrated that reaction of liver microsomes with nanomolar concentrations of $BrAcT_3$ results in the complete inactivation of D1, and that reaction with BrAc[^{125}I]T_3 allows the specific affinity labeling of the enzyme (27, 28). The specificity of the affinity labeling of D1 is confirmed in the present study showing little labeling of other proteins if D1-expressing cells are exposed to BrAc[^{125}I]T_3 (28, 43). In intact cells, affinity labeling of D1 is strongly augmented by coexpression of MCT8, indicating that the transporter also increases the intracellular availability of this iodothyronine derivative. However, our results indicate that MCT8 itself also undergoes affinity labeling by BrAc[^{125}I]T_3. Although other amino acids may also be modified, Cys residues are the most likely targets for affinity labels such as $BrAcT_3$. hMCT8 contains 10 Cys residues, eight of which are located in transmembrane domains, one in an intracellular loop, and one in an extracellular loop.

The affinity labeling of D1 and MCT8 in transfected cells is reminiscent of previous findings regarding the effects of pretreatment of isolated rat liver cells with $BrAcT_3$ on subsequent uptake and metabolism of T_3 (30). These findings demonstrated that $BrAcT_3$ inactivated the transporter involved in T_3 uptake in hepatocytes as well as the D1 expressed endogenously by these cells. Although MCT8 is expressed in various tissues, including liver, it remains to be investigated whether MCT8 is a major hepatic transporter for T_3 or T_4. There is no evidence for a hypothyroid state of the liver in male patients with hemizygous mutations in MCT8 (21), suggesting that thyroid hormone is taken up by liver cells predominantly via other transporters. Various organic anion transporters, such as NTCP and different OATPs, which also transport iodothyronines, are expressed in liver and may be more important for hepatic thyroid hormone uptake. The finding that $BrAcT_3$ specifically labels MCT8, and perhaps also other hepatic thyroid hormone transporter(s), suggests that this affinity label is a useful tool for the identification of thyroid hormone transporters in different tissues.

The present findings are consistent with our hypothesis that both the severe psychomotor retardation and markedly elevated T_3 levels in male patients with hemizygous mutations in MCT8 are due to defective cellular T_3 uptake in central neurons. The resultant deprivation of these neurons of T_3 during critical periods of differentiation, migration, and arborization results in irreversible defects in central nervous system development (25). It also blocks access of T_3 to D3 expressed in neurons and possibly also in other cells, resulting in a decreased T_3 clearance and, thus, an increased accumulation of circulating T_3. Secondarily, hepatic D1 may be stimulated by the elevated serum T_3 resulting also in an increased peripheral T_3 production. It is logical to assume that MCT8 is also important for allowing T_3 access to its nuclear receptor. Such a role has been demonstrated for the LAT1 transporter (44), but further studies are required to demonstrate that this is also the case for MCT8.

MATERIALS AND METHODS

Materials

Nonradioactive iodothyronines were obtained from Henning (Berlin, Germany) or Sigma Chemical Co. (St. Louis, MO). 12-*O*-tetradecanoylphorbol 13-acetate was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). [3′-^{125}I]T_3 and [3′,5′-^{125}I]T_4 (1500–2000 mCi/μmol) and carrier free Na^{125}I were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK), and [3′,5′-^{125}I]rT_3 was obtained from PerkinElmer (Boston, MA). 3,[3′-^{125}I]T_2 was prepared as previously described (45). Also radioactive *N*-bromoacetyl-T_3 (BrAc[^{125}I]T_3) and BrAc[^{125}I]T_4 were synthesized as previously described (27), with some minor changes. In short, ^{125}I-labeled T_3 or T_4 was dissolved in 5 ml dry ethylacetate. After addition of 20 μl bromoacetyl chloride (Fluka, Buchs, Switzerland), the mixture was incubated overnight at 40 C. The ethylacetate was evaporated under stream of N_2, and the residue was dissolved in 0.5 ml 20% ethanol in 0.1 M NaOH. After acidification with 0.5 ml 1 M HCl, the mixture was purified on a Sephadex LH-20 column (Amersham Biosciences).

Cloning of hMCT8

Total RNA was isolated from human adult female liver using the High Pure Tissue RNA isolation kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's guidelines. cDNA was synthesized using 1 μg RNA and Taqman RT reagents (Roche Diagnostics) in a total volume of 50 μl, 4 μl of which was used for PCR. The sense primer 5′-CAAGCTTTGCAGCAGCAGAAACAAGTACC-3′ (*Hin*dIII site *underlined*) located upstream of the second ATG start codon and the reverse primer 5′-GCTCTAGAGCACACAATGGCAAGAAAGG-3′ (*Xba*I site *underlined*) located just downstream of the TAA stop codon, were used to amplify the coding sequence (1714 bp). PCR was carried out using Taq DNA polymerase and Q-solution of QIAGEN (Venlo, The Nether-

lands) for 40 cycles of 1 min at 94 C, 1 min at 61 C, and 2 min at 72 C. The amplified MCT8 cDNA was cloned in pGEM-T (Promega Corp., Leiden, The Netherlands), and the nucleotide sequence was confirmed on an automated ABI 3100 capillary sequencer, using the Big Dye Terminator Cycle Sequencing method (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Subsequently, the cDNA was cut with *Hin*dIII and *Xba*I and cloned into the corresponding sites of the mammalian expression vector pcDNA3 (Invitrogen, Breda, The Netherlands).

pCIneo-rMCT8 plasmid was kindly provided by Dr. A. P. Halestrap (University of Bristol, Bristol, UK)

Deiodinase Expression Plasmids

The pCDM8-hD3 plasmid was kindly provided by Dr. P. R. Larsen (Harvard Medical School, Boston, MA) (46). The 1.9-kb hD3 insert was excised and subcloned into the *Xho*I/*Not*I sites of the pCIneo mammalian expression vector (Promega). The hD2 expression vector (pcDNA3-hD2-rD1-SECIS) was constructed as previously described (40). It is a chimeric construct containing the hD2 coding sequence and 0.7 kb of the rat D1 3′-UTR containing the SECIS element (40). The rD1 expression plasmid (pcDNA3-rD1) was obtained by subcloning the G21 full-length rat D1 cDNA obtained from Dr. P. R. Larsen (47) into pcDNA3 (Invitrogen).

Cell Culture

Different human cell lines were tested for MCT8 mRNA or protein expression, to identify cell lines that could be used for transfection studies. ECC1 endometrium carcinoma cells were kindly provided by Dr. B. van der Burg (Hubrecht Laboratory, Utrecht, The Netherlands); BON pancreatic carcinoid tumor cells by Dr. M. Rutgers (Netherlands Cancer Institute, Amsterdam, The Netherlands); Ishikawa IK-3H12 endometrium carcinoma cells by Dr. M. Nishida (University of Tsukuba, Tsukuba, Japan); and JAR choriocarcinoma cells by Dr. C. Ris-Stalpers (Amsterdam Medical Center, Amsterdam, The Netherlands); MCF7 mammary carcinoma cells by Dr. J.A. Foekens (Erasmus MC, Rotterdam, The Netherlands); and HepG2 hepatocarcinoma cells by Dr. B. B. Knowles (Wistar Institute, Philadelphia, PA). SH-SY5Y neuroblastoma cells, U87, U373, and CCF-STTG astrocytoma cells, WRL68 embryonic liver cells, and JEG3 and BeWo choriocarcinoma cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). All cells were grown in flasks (75 cm^2) or multiwell dishes of Corning (Schiphol, The Netherlands) with DMEM/Ham's F12 medium (Invitrogen), containing 9% heat-inactivated fetal bovine serum (Invitrogen) and 100 nM sodium selenite (Sigma-Aldrich).

RT-PCR of MCT8 in Different Human Cell Lines

At confluence, cultured cells were split and seeded in six-well dishes (9.6 cm^2). After 24 h, cells were trypsinized and washed with PBS (pH 7.2). Total RNA was isolated from 10^6 cells using the High Pure RNA isolation kit (Roche Diagnostics) according to the manufacturer's guidelines. RNA concentrations were determined using the RiboGreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). cDNA was synthesized using 0.5 μg RNA and TaqMan RT reagents (Roche Diagnostics) in a total volume of 50 μl, 2 μl of which was used for PCR. Table 1 shows the synthetic oligonucleotides and conditions used for PCR of MCT8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Band intensities derived from PCR of MCT8 were compared with those obtained from PCR of GAPDH during the exponential phase of the reaction.

hMCT8 Antibodies and Western Blotting

Polyclonal antisera were raised in rabbits by Eurogentec SA (Seraing, Belgium) against the keyhole limpet hemocyanin conjugate of the synthetic peptide (C)ELLPGSPNPEEPI (hMCT8 C-terminal amino acid residues 527–539). Antiserum (designated 1305) from the final bleed was used without further purification.

The hMCT8 protein was expressed in COS1 or JEG3 cells after FuGENE mediated plasmid DNA transfection. After 24–36 h incubation, the cells were rinsed with PBS and collected in 0.2 ml 10 mM phosphate buffer (pH 7.2) and 2 mM EDTA. The extract was sonicated on ice, aliquoted, and stored at −80 C. Homogenates (10–15 μg protein) were separated on 10% SDS-PAGE minigels. Thereafter, the proteins were blotted to nitrocellulose membranes and probed with antiserum 1305 (1:500) as described previously (48).

Affinity Labeling of hMCT8 Protein with $BrAcT_3$ or $BrAcT_4$

COS1 and HEK293 cells (six-well plates) were transfected with pcDNA3-hMCT8 and/or pcDNA3-rD1 expression vectors as described below. Parallel experiments were also done using nontransfected HepG2, WRL68, and JEG3 cells. The cells were washed with serum-free DMEM-F12 medium and were preincubated at 37 C with 2 ml serum-free DMEM-F12 medium per well. BrAc[^{125}I]T_3 or BrAc[^{125}I]T_4 (2×10^5 cpm/well; specific activity 1500 mCi/μmol) was added, and the cells were incubated for 2–4 h at 37 C. In some experiments the cells were incubated for 5 min with nonradioactive T_4 or T_3 (10 μM) before addition of radioactive $BrAcT_4$ or $BrAcT_3$. The cells were washed with PBS and lysed in 0.25 ml SDS-PAGE loading buffer containing 10 mM dithiothreitol (DTT). The samples were analyzed by SDS-PAGE (10% gels), followed by autoradiography to BioMax MS film (Kodak, Rochester, NY) at −70 C with intensifying screen (2–4 d exposure).

Iodothyronine Transport by hMCT8

COS1 and JEG3 cells were cultured in six-well culture dishes and transfected in duplicate with 1 μg of empty pcDNA3 or pcDNA3-hMCT8 cDNA using FuGENE6 transfection reagent according to the manufacturer's guidelines. In some experiments cells were also transfected with pCIneo-rMCT8. After 24 h (COS1) or 48 h (JEG3) culturing, cells were washed with DMEM/F12 with 0.1% BSA and incubated for 5–30 min at 37 C with 1 nM (2×10^5 cpm) ^{125}I-labeled T_4, T_3, rT_3, or T_2 in 1.5 ml DMEM/F12 with 0.1% BSA. After incubation, cells were washed with DMEM/F12 with 0.1% BSA, lyzed with 0.1 M NaOH, and counted.

TABLE 1. Oligonucleotide Primers and PCR Conditions Used for PCR.

Gene	Sense Primer (5′–3′)	Antisense Primer (5′–3′)	Annealing Temperature (C)	No. of Cycles
MCT8	TGCAGCAGCAGAAACAAGTACC	GTGAAGTAGCGCAGGCTTAGG	60	35
GAPDH	AAGGTGAAGGTCGGAGTCAAC	TTGTCATACCAGGAAATGAGC	50	27

Iodothyronine Metabolism in Intact Cells Transfected with hMCT8 and/or Deiodinases

COS1 and JEG3 cells were cultured in 24-well culture dishes (2 cm^2) and transfected in duplicate with 1) 0.2 μg of empty pcDNA3; 2) 0.1 μg empty pcDNA3 plus 0.1 μg pcDNA3-rD1, pcDNA3-hD2, or pCIneo-hD3; 3) 0.1 μg empty pcDNA3 plus 0.1 μg pcDNA3-hMCT8; or 4) 0.1 μg deiodinase plasmid plus 0.1 μg pcDNA3-hMCT8 using FuGENE 6 transfection reagent according to the manufacturer's guidelines. Twenty four hours (COS1) or 48 h (JEG3) after transfection, cells were washed with DMEM/F12 plus 0.1% BSA and incubated for 4–24 h at 37 C with 1 nM (1×10^6 cpm) ^{125}I-labeled T_4, T_3, rT_3, or 3,3′-T_2 in 0.5 ml DMEM/F12 plus 0.1% BSA. After incubation, 0.1 ml medium was added to 0.1 ml ice-cold methanol. After centrifugation, 0.1 ml supernatant was mixed with 0.1 ml 0.02 M ammonium acetate (pH 4.0); 0.1 ml of the mixture was applied to a 4.6 × 250 mm Symmetry C18 column connected to an Alliance HPLC system (Waters, Etten-Leur, The Netherlands), and eluted with a gradient of acetonitrile in 0.02 M ammonium acetate (pH 4.0) at a flow of 1.2 ml/min. The proportion of acetonitrile was increased linearly from 28–42% in 15 min. The radioactivity in the eluate was monitored on line using a Radiomatic A-500 flow scintillation detector (Packard Instruments, Meriden, CT).

Iodothyronine Metabolism in Cell Lysates

COS1 cells were transfected as described for the incubations with intact cells. Twenty four or 48 h after transfection, cells were washed with PBS and harvested by scraping the content of each well into 100 mM phosphate buffer (pH 7.2) containing 2 mM EDTA and 1 mM DTT, and disrupted by sonication. The cell sonicates were stored at −80 C until further analysis. Protein content was determined using the method of Bradford (49), with BSA as standard.

D1 activities were determined in cell lysates by incubation of 0.1 μM (1×10^5 cpm) [3′,5′-^{125}I]rT_3 for 60–180 min at 37 C with varying concentrations of cell sonicate in 0.1 ml 100 mM phosphate (pH 7.2), 2 mM EDTA, and 10 mM DTT (PED10). Reactions were stopped by the addition of 0.1 ml ice-cold 5% BSA. Protein-bound [^{125}I]iodothyronines were precipitated by addition of 0.5 ml 10% trichloroacetic acid on ice. After centrifugation, the supernatants were analyzed for $^{125}I^-$ production on Sephadex LH-20 minicolumns (bed volume, 0.25 ml), which were equilibrated and eluted with 0.1 M HCl.

D2 activities were determined by incubation of 1 nM (1×10^5 cpm) [3′,5′-^{125}I]T_4 for 60–180 min at 37 C with varying concentrations of cell homogenate in 0.1 ml PED10. Release of $^{125}I^-$ was determined as described for D1 activity.

D3 activities were determined by incubation of 1 nM (2×10^5 cpm) [3′-^{125}I]T_3 for 60–180 min at 37 C with varying concentrations of cell sonicate in 0.1 ml PED10. Reactions were stopped by the addition of 0.1 ml ice-cold methanol. After centrifugation, supernatants were mixed with an equal volume of ammonium acetate (pH 4.0), and the mixtures were analyzed by HPLC as described above.

Acknowledgments

We thank Wim Klootwijk for technical assistance.

Received June 29, 2005. Accepted July 24, 2006.

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This work was supported by the Netherlands Organisation for Scientific Research (NWO grants 916.36.139 and 916.56.186) (to E.C.H.F. and M.H.A.K.) and by the Sophia Foundation for Medical Research (project no. 438) (to J.J.).

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