RESEARCH ARTICLE

Novel Pathogenic Mechanism Suggested by Ex Vivo Analysis of MCT8 (SLC16A2) Mutations

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Monocarboxylate transporter 8 (MCT8; approved symbol SLC16A2) facilitates cellular uptake and efflux of 3,3′,5-triiodothyronine (T3). Mutations in MCT8 are associated with severe psychomotor retardation, high serum T3 and low 3,3′,5′-triiodothyronine (rT3) levels. Here we report three novel MCT8 mutations. Two subjects with the F501del mutation have mild psychomotor retardation with slightly elevated T3 and normal rT3 levels. T3 uptake was mildly affected in F501del fibroblasts and strongly decreased in fibroblasts from other MCT8 patients, while T3 efflux was always strongly reduced. Moreover, type 3 deiodinase activity was highly elevated in F501del fibroblasts, whereas it was reduced in fibroblasts from other MCT8 patients, probably reflecting parallel variation in cellular T3 content. Additionally, T3-responsive genes were markedly upregulated by T3 treatment in F501del fibroblasts but not in fibroblasts with other MCT8 mutations. In conclusion, mutations in MCT8 result in a decreased T3 uptake in skin fibroblasts. The much milder clinical phenotype of patients with the F501del mutation may be correlated with the relatively small decrease in T3 uptake combined with an even greater decrease in T3 efflux. If fibroblasts are representative of central neurons, abnormal brain development associated with MCT8 mutations may be the consequence of either decreased or increased intracellular T3 concentrations. Hum Mutat 0,1–10, 2008.

KEY WORDS: monocarboxylate transporter 8; MCT8; SLC16A2; mental retardation; novel genotype-phenotype relation; thyroid hormone transport

INTRODUCTION

The thyroid gland synthesizes two iodothyronines, thyroxine (3,3′,5,5′-tetraiodothyronine, T4) and 3,3′,5-triiodothyronine (T3), which together are called thyroid hormone (TH). Most of the T3, the major biologically active TH, is generated from the prohormone T4 by the deiodinating enzymes D1 and D2 [Bianco and Kim, 2006]. The deiodinase D3 inactivates T4 to 3,3′,5′-triiodothyronine (rT3) and T3 to 3,3′-diiodothyronine (T2) [Bianco and Kim, 2006]. The genomic actions of T3 are mediated by nuclear T3 receptors (TRs) [Yen et al., 2006]. As the active centers of the deiodinases and the TRs are located intracellularly, TH metabolism and action require transport of the hormone across the plasma membrane.

Accumulating evidence indicates that uptake of TH into the cell is facilitated by transporter proteins [Hennemann et al., 2001]. Recently, several classes of transporter proteins have been characterized at the molecular level [Visser et al., 2008]. In contrast to most known TH transporters that accept a wide variety of ligands, organic anion transporting polypeptide 1C1 (OATP1C1), monocarboxylate transporter 8 (MCT8; HUGO-approved gene symbol SLC16A2; MIM# 300095), and MCT10 express a high selectivity toward TH [Visser et al., 2008].

TH is critically involved in the development of the CNS during fetal and neonatal life. Minor changes in only one of the factors involved in modulating and mediating TH effects on the brain, may have deleterious neurological effects. Only recently, the first mutations in a TH transporter have been identified [Dumitrescu et al., 2004a; Friesema et al., 2004]. Males with loss-of-function mutations in MCT8 show severe neurological deficits, with axial

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hypotonia, spastic quadriplegia, impaired or absent speech, and muscle hypoplasia. This X-linked syndrome is known as the Allan-Herndon-Dudley syndrome (AHDS; MIM# 300523). Thyroid function tests show low to low-normal serum T4 levels, normal or moderately increased TSH levels, low rT3 levels, and strongly elevated T3 levels. Because MCT8 facilitates cellular T3 uptake and is highly expressed in neurons, it is likely that inactivation of MCT8 results in an impaired supply of T3 to neurons [Friesema et al., 2006a]. Considering the importance of TH for normal brain development, it is understandable that the resulting neuronal T3 deprivation results in neurological damage.

Initially, MCT8 was shown to facilitate T3 and T4 uptake [Friesema et al., 2006b]. However, we recently demonstrated that MCT8 also functions as an efficient T3 and T4 exporter [Friesema et al., in press]. The biological relevance of this function and the possible contribution to the pathogenesis of the MCT8 syndrome is currently unknown.

Here, we present three new Dutch families with mutations in MCT8. We used skin fibroblasts of MCT8 patients as an ex vivo model to elucidate the pathogenic mechanisms resulting in neurological deficits. We provide evidence that MCT8 mutations may differentially affect cellular influx and efflux of T3. This may suggest that, depending on the mutation, decreased as well as increased intracellular T3 concentrations may result in neurological abnormalities in MCT8 patients.

MATERIALS AND METHODS

Materials

[3′-^{125}I]T3 and [3′,5′-^{125}I]T4 were purchased from GE Healthcare (Little Chalfont, UK), nonradioactive iodothyronines were obtained from Henning (Berlin, Germany), and 12-O-tetradecanoylphorbol-13-acetate (TPA) was obtained from Sigma (St. Louis, MO). Real-time PCR primers and probes were purchased from Biosource (Nivelles, Belgium). Oligonucleotides were synthesized by Invitrogen (Paisly, UK). FuGENE6 transfection reagent was obtained from Roche Diagnostics (Almere, The Netherlands).

Serum Analysis

Serum FT4, T3, and TSH were measured by Vitros ECI technology (Ortho-Clinical Diagnostics, Beerse, Belgium) and rT3 was measured by an in-house radioimmunoassay. Neonatal screening data were obtained from the Dutch Health Administration after informed consent of the parents.

Genetic Analysis

The *MCT8* gene (SLC16A2; RefSeq NM_006517.3; genomic reference Z83843.3) was analyzed using standard primers as described previously [Jansen et al., 2007]. We designed additional primers for Patient P6 (Supplementary Table S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). Mutation nomenclature follows the journal guidelines (www.hgvs.org/mutnomen) with coding sequence numbered from the A of the ATG initiation codon.

Cloning and Site-Directed Mutagenesis

The cloning of wild-type (wt) human MCT8 cDNA was described recently [Friesema et al., 2006b]. The mutations of Patients P2, P3, and P4 were introduced in the MCT8 cDNA using the QuickChange Site-Directed Mutagenesis protocol (Stratagene, Amsterdam, The Netherlands). DNA sequencing confirmed the presence of the introduced mutations.

Cell Cultures and Transfection

We obtained human skin fibroblasts from Patients P1–P4 by punch biopsy after informed consent by the parents. Fibroblasts from three nonaffected subjects were used as controls (kindly provided by Dr. B. Thio, Erasmus MC). We grew fibroblasts in 75-cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Invitrogen) supplemented with 9% FBS (heat-inactivated; Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 100 nM sodium selenite (Sigma). At confluency, fibroblasts were harvested and seeded at equal densities in six-well dishes for TH transport assays and in 28-cm^2 dishes for metabolism experiments and RNA isolation.

JEG3 cells were cultured in six-well culture dishes with DMEM/F12 medium plus 9% FBS and 100 nM sodium selenite. For TH transport studies, cells were transfected with pcDNA3.hMCT8 (wt or mutant) using empty pcDNA3 as control.

For iodothyronine metabolism experiments, cells were cotransfected with pcDNA3.hMCT8 (wt or mutant) and pCIneo.hD3, as previously described [Jansen et al., 2007].

Immunoblotting and Immunocytochemistry

The MCT8-specific (C-terminal) polyclonal antibody 1306 was used for immunoblotting (IB) and immunocytochemistry (ICC). For ICC, the plasma membrane was stained with the zona occludens 1 (ZO1) antibody (Invitrogen). IB and ICC were performed as reported recently [Friesema et al., 2006b; Jansen et al., 2007].

TH Transport Experiments

At confluency, fibroblasts were washed with incubation medium (Dulbecco's PBS containing 0.1% D-glucose and 0.1% BSA). TH uptake was tested by incubation of the cells for 30 to 60 min at 37°C with 1 nM (2×10^5 cpm) [^{125}I]T3 or [^{125}I]T4 in 1.5 ml of incubation medium. After incubation, cells were washed with the medium, lysed with 0.1 M NaOH, and counted in a gamma counter.

For measurement of TH efflux, cells were loaded for 1 hr with incubation medium containing 1 nM (2×10^5 cpm) [^{125}I]T3 or [^{125}I]T4. After removal of the medium, cells were washed and incubated for 10 to 30 min with incubation medium without ligand. Finally, medium was removed, and cells were washed with incubation medium, lysed with 0.1 M NaOH, and counted in a gamma counter. Values were corrected for protein concentrations (Bradford assay).

This procedure was adapted to JEG3 cells with minor modifications. After 48 hr of transfection, JEG3 cells were incubated for 10 to 30 min with incubation medium containing [^{125}I]T3 or [^{125}I]T4.

TH Metabolism Experiments

To maximize D2 activity, fibroblasts were incubated for 24 hr with DMEM/F12 plus 6% charcoal-treated FBS and 100 nM sodium selenite at confluency. To induce D3 activity, fibroblasts were stimulated for 6 hr with 0.1 μM TPA in DMEM/F12 plus 9% FBS. Subsequently, cells were washed with DMEM/F12 plus 0.1% BSA, and incubated for 4 to 72 hr at 37°C with 1 nM (1×10^6 cpm) [^{125}I]T4 (D2 activity) or for 3 hr at 37°C with 1 nM ($1° 10^6$ cpm) [^{125}I]T3 (D3 activity) in DMEM/F12 plus 0.1% BSA. After incubation, the medium was sampled, processed, and analyzed by HPLC as previously described [Friesema et al., 2006b].

The intact-cell metabolism of T3 in JEG3 cells was investigated as described previously [Jansen et al., 2007].

T3 Effects on Fibroblasts

For the T3 stimulation experiment, culture medium was replaced with DMEM/F12 plus 6% charcoal-treated FBS and 100 nM sodium selenite. After 48 hr, the medium was refreshed with the same medium containing 10 nM T3, and the incubation was continued for 6 hr.

Total RNA was isolated from 1×10^6 fibroblasts using the High Pure RNA isolation kit (Roche). cDNA was synthesized using 0.5 μg RNA and TaqMan RT reagent (Roche). For semiquantitative PCR of MCT8, the sense primer 5′-TGCAGCAGCAGAAAC AAGTACC-3′ and the antisense primer 5′-GCACACAATGGC AAGAAAGG-3′ were used.

SYBR Green I (Eurogentec, Liège, Belgium) was used as the detector dye for quantitative PCR of the T3-responsive genes ZAKI 4α, GLUT1, and MCT4; the primer sequences are presented in Supplementary Table S2. mRNA levels are expressed relative to that of the housekeeping gene cyclophilin A.

Statistical Analysis

All results are the mean of at least duplicate determinations from representative experiments. Values are expressed as mean ± SE. Statistical significance was determined using the Student's *t*-test for unpaired observations.

RESULTS

Clinical Features

Patients P1 and P2 have been reported previously [Friesema et al., 2004, 2006a; Jansen et al., 2007]. Briefly, both patients have severe psychomotor retardation, characterized by truncal hypotonia, quadriplegia, mental retardation, and absence of speech.

Patients P3–P6 are newly identified. Patient P3 is a 4-year-old boy born to nonconsanguineous parents. He presented at the age of 9 months when gross motor milestones were not reached with axial hypotonia, head lag, spastic quadriplegia, microcephaly, and a myopathic face. MRI showed delayed myelination and thinning of the corpus callosum.

Patient P4 is a boy who at the age of 16 months presented with a global developmental delay. He had mild axial hypotonia and mild spastic tetraparesis. He functioned as a 12-month-old infant with respect to communication skills. At the age of 18 months, his head balance was much better than observed in other MCT8 patients; he was able to crawl and he used his hands to grasp toys. A slightly delayed myelination was detected by brain MRI.

Patient P5 is a 38-year-old brother of the mother of Patient P4 (Supplementary Fig. S1). His gross motor milestones were delayed. At a recent examination there was a mild spastic tetraparesis with good head balance. He was able to walk with some support and to communicate by speaking, although his speech was slurred and nonfluent. Furthermore, he was capable of reading by combining separately spelled letters and of writing simple sentences (without verbs) on a computer.

At the age of 4 months, Patient P6 presented with a severe delay in mental and motor development, characterized by a severe axial hypotonia and head lag. He had microcephaly, low muscle mass, and developed spastic quadriplegia. Delayed myelination and thinning of the corpus callosum were demonstrated by MRI.

Table 1 shows the serum TH parameters determined in the patients. In all patients, serum FT4 levels were at or below the lower limit of normal, and serum T3 was increased although only slightly so in Patient P5. Serum rT3 was decreased in Patients P1–P3 and P6, and normal in Patients P4 and P5. Neonatal screening results could be retrieved for Patients P3, P4, and P6, showing decreased T4 levels in Patients P3 and P6, and a normal T4 concentration in Patient P4.

Mutation Analysis

Based on the combination of developmental delay and elevated serum T3, DNA from Patients P1–P6 was tested for mutations in *MCT8*. The results are graphically depicted in Supplementary Figure S2. A deletion of almost 2.4 kb with borders located in exon 3 and intron 4 (c.970_1392+1952del) was found in Patient P1, and a mutation in the acceptor splice site of intron 2 (c.798–1G>C) was identified in Patient P2, as described previously [Friesema et al., 2004, 2006a].

In Patient P3, a c.1690G>A mutation was found, which results in a Gly to Arg substitution at position 564 (p.G564R). A 3-bp deletion (c.1497_1499delCTT) was identified in Patients P4 and P5, causing a deletion of Phe at position 501 (p.F501del). We were not able to amplify exon 1 in Patient P6. We investigated the extension of the deletion further by PCR using sets of primers aligning to chromosome X from 3.3 Mb upstream to 105 kb downstream of exon 1. Eventually, we identified two most proximal primer sets (P6del1fwd/rev, P6del2fwd/rev), which each yielded PCR products of the expected size (Fig. 1A). Combination of P6del1fwd and P6del2rev resulted in an amplicon of ~0.75 kb in Patient P6 but not in a control. Sequencing of this amplicon refined the borders of the deletion, demonstrating a deletion of 336,997 nucleotides, which includes 252 kb of the upstream region of *MCT8*, exon 1, and 84 kb of intron 1 (Z83843.3:g.1322_MCT8:c.650-15128del; Fig. 1B).

TABLE 1. **Serum Thyroid Hormone Levels in MCT8/SLC16A2 Patients***

	Normal values	Patients					
		P1[a]	P2	P3	P4	P5	P6
Mutation		2.4 kb del[b]	c.798-1G>C; p.S267_S360del	c.1690G>A; p.G564R	c.1497_1499delCTT; p.F501del	c.1497_1499delCTT; p.F501del	337 kb del[c]
Age at testing		5 years	6 years	10 months	18 months	38 years	3.5 years
TSH (mU/L)	0.4–4.3	3.99	2.97	2.8	2.82	1.78	4.24
fT4 (pmol/L)	11–25	11.2	8.8	7.5	11.1	8.9	8.2
T3 (nmol/L)	1.4–2.5	5.09	3.17	4.15	3.78	2.84	4.08
rT3 (nmol/L)	0.14–0.34	0.07	0.08	0.04	0.22	0.25	0.04
Neonatal T4		NA	NA	−1.5 SD	+0.2 SD	NA	−2.4 SD

*Mutation nomenclature follows the journal guidelines (www.hgvs.org/mutnomen) with coding sequence numbered from the A of the ATG initiation codon; RefSeq NM_006517.3; genomic sequence Z83843.3.
[a]Patient P1 was treated with thyroxine (57 μg/day).
[b]Official nomenclature: c.970_1392+1952del.
[c]Official nomenclature: Z83843.3:g.1322_MCT8:c.650-15128del.
NA, not available; SD, standard deviation.

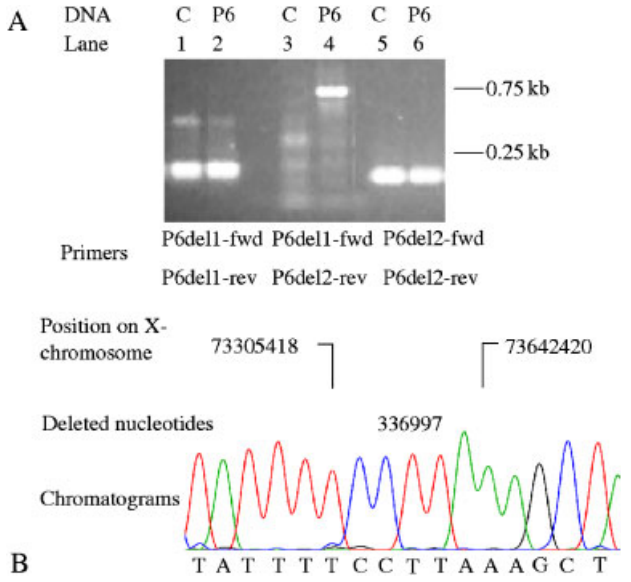

FIGURE 1. **A: PCR analysis of a deletion in the *MCT8* gene in Patient P6 compared to a control. No DNA is amplified in Patient P6 in the large 336-kb region between the two amplicons in the left and right lanes. Combination of P6del1-fwd and P6del2-rev generates an amplicon in DNA of Patient P6, but not in control DNA. B: Partial sequencing profile of the amplicon in lane 4 refines the borders of the deletion in P6 to a loss of 336,997 nucleotides. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

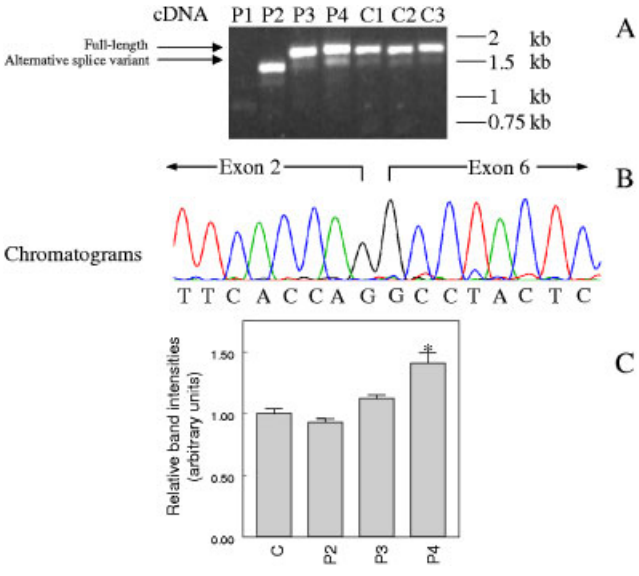

FIGURE 2. **MCT8 mutations at the mRNA level. A: RT-PCR of MCT8 mRNA in skin fibroblasts of Patients P1–P4. The 1.8-kb band indicates full-length MCT8 mRNA and the 1.5-kb band suggests an alternative splicing variant. B: Part of sequencing profile of cDNA derived from mRNA in Patient P1 demonstrating a loss of exons 3, 4, and 5. C: Densitometric analysis of the MCT8 splice variant (lower band) detected in controls and Patients P2–P4. Significance represent values obtained in patient fibroblasts compared to control fibroblasts. $^{*}P < 0.005$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

Delineation of the Mutations at the mRNA Level

To determine the effects of the mutations on mRNA structure, we performed PCR on cDNA prepared from fibroblasts using a forward primer located just before the second putative translation start site and a reverse primer located just after the stop codon of the *MCT8* gene. Figure 2A demonstrates a band of ~1.8 kb for controls and Patients P3 and P4, corresponding to the predicted length of the coding region of *MCT8*. In Patient P1, a vague band appears of ~0.9 kb. Sequence analysis indicated that exons 3, 4, and 5 have been deleted from the mRNA (r.798_1621del; Fig. 2B).

A 1.5-kb PCR product was obtained in Patient P2. Sequence analysis of this product indicated the use of an alternative splice site downstream in exon 3, resulting in the deletion of 282 nt from the mRNA (r.798_1079del) and a predicted loss of 94 amino acids (aa) from the protein (p.S267_S360del).

In addition to the full-length 1.8-kb band, a 1.5-kb band was also demonstrated in controls and Patients P3 and P4. This splice variant has the same sequence as the MCT8 mRNA in Patient P2. Densitometric analysis demonstrated a 41% increased intensity of the splice variant in Patient P4 compared to controls, suggesting enhanced splicing (Fig. 2C).

IB and ICC

Using IB and ICC, we were not able to detect MCT8 in fibroblasts from controls and patients. Subsequently, JEG3 cells, which do not express endogenous MCT8, were transfected with wt-MCT8 or the splice site (Patient P2), G564R (Patient P3) or F501del (Patients P4 and P5) mutants. On IB, minimal MCT8 expression was seen with the G564R mutant, whereas the F501del mutant showed only slightly diminished protein expression compared to wt-MCT8 (Fig. 3A). A band of ~50 kDa was detected for the splice site variant, in agreement with the 94-aa loss. ICC was carried out to assess the cellular distribution of the F501del mutant. Figure 3B shows a clear membrane distribution of the F501del mutant, similar to wt-MCT8.

TH Uptake and Efflux

Fibroblasts from Patients P1–P4 and controls were incubated for 30 min with [^{125}I]T3. This resulted in a reduction of T3 uptake in fibroblasts from Patients P1–P3 by ~70% and in Patient P4 by ~50% compared to controls (Fig. 4A). Uptake of [^{125}I]T4 in fibroblasts from all patients was ~40% of controls (Fig. 4B).

Subsequently, we measured uptake of ^{125}I-labeled T3 and T4 after incubation for 30 or 60 min. There was no significant difference in T4 uptake between patients and controls. However, although initial T3 uptake rates were higher in controls than in patients, the T3 uptake rate between 30 and 60 min was significantly higher in Patient P4 (0.23%/mg protein/min) than in controls and Patients P1–P3 (0.07–0.08%/mg protein/min), indicating that the equilibrium phase has not been reached in Patient P4 (Fig. 4C and D).

Furthermore, we tested the characteristics of the mutants in transfected JEG3 cells, providing the same cellular background for all mutants. After transfection, cells were incubated for 10 or 30 min with ^{125}I-labeled T3 or T4. No induction of T3 and T4 uptake was seen in the splice site and G564R mutants (Fig. 4E and F). Both T3 and T4 uptake by the F501del mutant increased from ~55% to ~75% of wt-MCT8 after 10 to 30 min of incubation.

Because the above results suggest an impaired T3 efflux from fibroblasts of Patient P4, we directly tested T3 efflux from fibroblasts. Figure 5A shows a higher T3 efflux rate (1.6%/mg protein/min) in controls than in Patients P1–P3 and in Patient P4 (both 0.6%/mg protein/min). Similarly, the T4 efflux rate was higher in controls compared with patient fibroblasts (Fig. 5B).

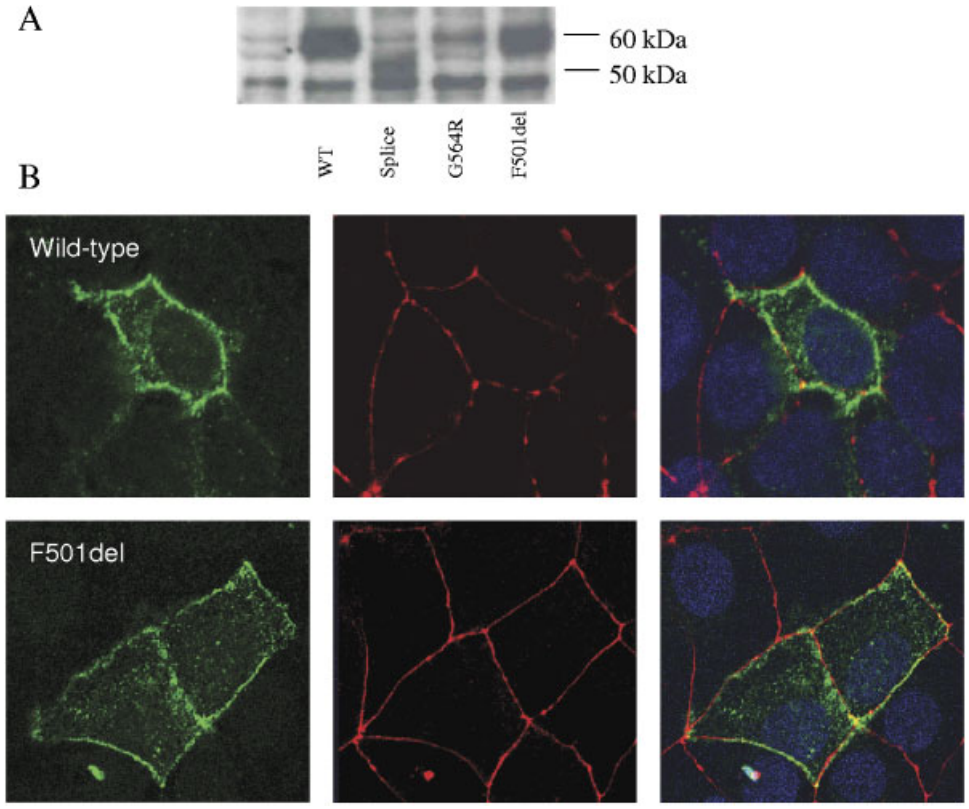

FIGURE 3. **MCT8 mutations at the protein level. A: Immunoblot analysis of JEG3 cell lysates transfected with wild-type or mutant MCT8. B: Immunofluorescent detection of JEG3 cells transfected with wt and the F501del mutation in MCT8. The plasma membrane was stained by a ZO1 antibody.**

Figure 5C and D shows that in transfected JEG3 cells efflux of both T4 and T3 by the F501del mutant is markedly slower than with wt-MCT8.

In addition, we studied the metabolism of T3 in intact JEG3 cells cotransfected with wt or mutated MCT8 and human D3. Cells expressing wt-MCT8 demonstrated 22% metabolism of T3, whereas the splice-site and G564R mutants produced results similar to the empty vector (Fig. 6). We observed 13% T3 metabolism in cells expressing the F501del mutant.

Deiodinase Activity

The dramatic reduction in T3 uptake in fibroblasts from Patients P1–P3 probably results in a decreased intracellular T3 concentration. In contrast, the even larger defect in T3 efflux from fibroblasts of Patient P4 may result in a greater accumulation of intracellular T3. Therefore, we assessed intracellular TH status by measuring D2 and D3 activities. However, we were not able to obtain reliable results for D2 activity in lysates of fibroblasts.

Mean D3 activity in fibroblasts from patients P1–P3 was decreased by 35% compared with controls (Fig. 7). In contrast, a significant 3.8-fold increase in D3 activity was observed in fibroblasts from Patient P4. Subsequently, we aimed to assess T3 metabolism in intact fibroblasts. However, even after 72 hr of incubation with [^{125}I]T3 we could not detect degradation of T3 (data not shown).

Analysis of T3-Responsive Genes in Fibroblasts

We further investigated the intracellular T3 status by studying the expression of T3-responsive genes in the fibroblasts. As a first approach, we measured total RNA after a 6-hr treatment with 10 nM T3. This resulted in a 38% increase in controls, a 27% increase in Patients P1–P3 and a 96% increase in Patient P4 (Fig. 8A). We next examined the effect of 10 nM T3 on transcript levels of specific T3-responsive genes in the fibroblasts. There was a significantly higher induction of ZAKI 4α expression in fibroblasts of Patient P4 than in controls and in other patients (Fig. 8B). Although no significant effects of T3 on MCT4 and GLUT1 mRNA were observed, the expression of these genes tended to increase in cells from Patient P4 (Fig. 8C and D).

DISCUSSION

We describe three new *MCT8* mutations in males with psychomotor retardation and abnormal TH levels. The mutation in Patient P6 is the largest deletion in the *MCT8* region described until now. It is obvious that this deletion is devastating for the function of MCT8. The deletion includes the other genes *ZCCHC13* and *BMPKL2*. The possible contribution of the deletion of these genes, of which the functions are currently unknown, to the clinical phenotype of Patient P6 is not clear. We did not observe additional abnormalities in this patient that have not been detected in other MCT8 patients.

We observed a remarkable difference in phenotype of Patients P4 and P5 vs. Patients P3 and P6 and other previously reported MCT8 patients. Although all patients have psychomotor retardation, the clinical features of Patients P4 and P5 appear milder with less severe hypotonia and much better motor and communication skills. Patient P5 was even capable of reading, writing, and talking. Compared to other patients, the serum TH levels are also less disturbed in Patients P4 and P5. So far, no relationship has been observed between TH levels and the severity of the MCT8

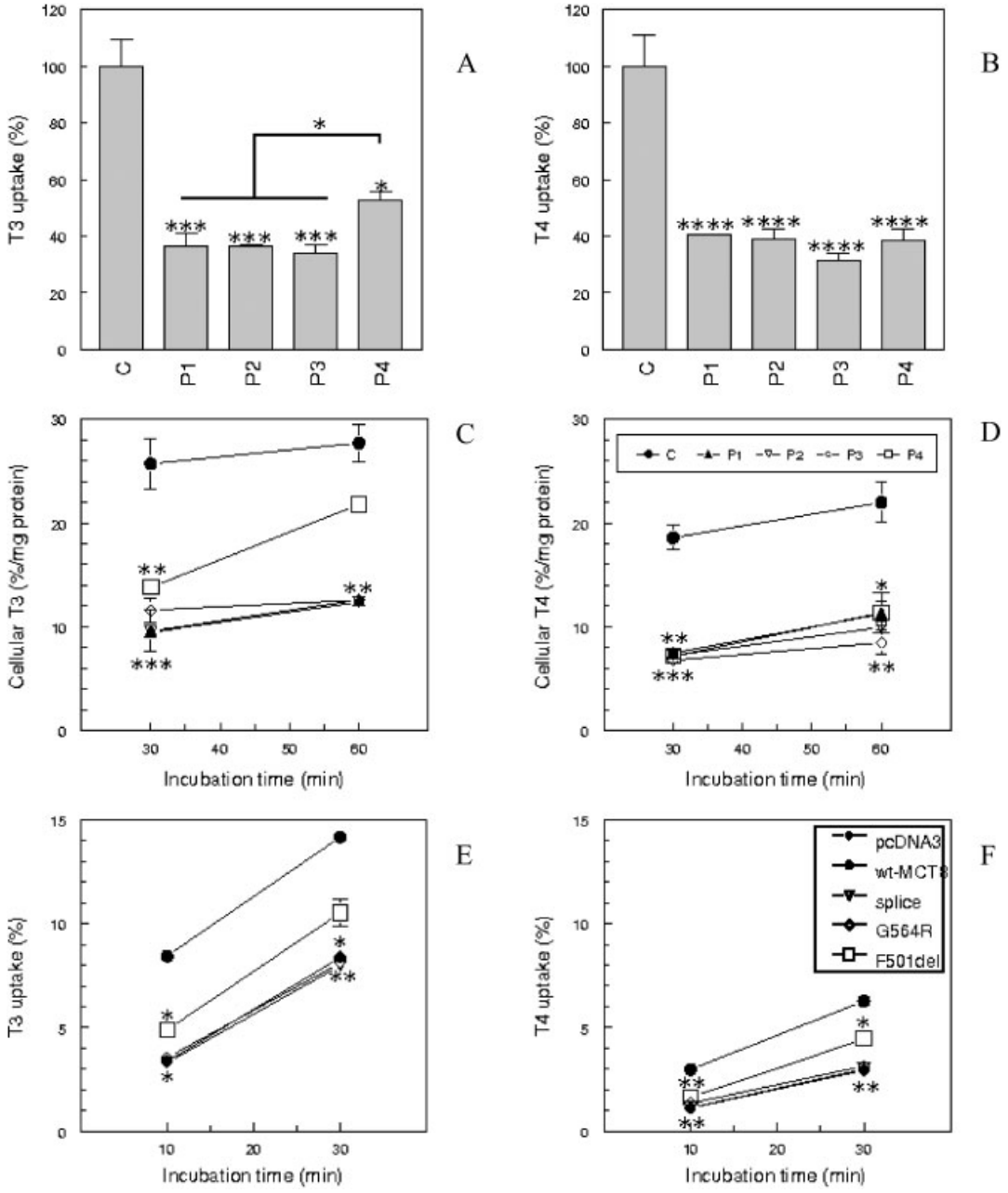

FIGURE 4. **Uptake of ^{125}I-labeled T3 (A) and ^{125}I-labeled T4 (B) after 30 min incubation in fibroblasts of MCT8 patients. T3 and T4 uptake in control fibroblasts is defined as 100%. Uptake of [^{125}I]T3 (C) and [^{125}I]T4 (D) after 30 and 60 min incubation in fibroblasts of MCT8 patients. Results are corrected for protein concentrations. Significances represent values obtained in patient fibroblasts compared to control fibroblasts. Uptake of [^{125}I]T3 (E) and [^{125}I]T4 (F) after 10 and 30 min incubation in wt or mutant MCT8-transfected JEG3 cells are shown as percentage of added ligand. Significances represent values of empty vector or mutant MCT8 compared to wt MCT8. *P < 0.05; **P < 0.01; ***P < 0.005; ****P < 0.001.**

syndrome [Refetoff and Dumitrescu, 2007]. However, the less abnormal TH levels associated with the less severe clinical features in Patients P4 and P5 may indicate that such a relationship exists. Our findings also imply that slightly elevated T3 and normal rT3 concentrations in subjects with psychomotor retardation may be associated with MCT8 mutations. Furthermore, our findings suggest that T4 levels are already lowered at birth in MCT8 patients with a severe phenotype. It should be investigated whether neonatal T4 measurement may be used as a screening tool for earlier identification of this genetic defect.

We used fibroblasts from Patients P1–P4 as an ex vivo model to investigate the consequences of MCT8 mutations. Studying the effects of mutations on the mRNA level, the size of MCT8 mRNA in Patients P3 and P4 was similar to wt-MCT8, whereas a smaller band was detected in Patient P2, due to the deletion of 282 nt. In the MCT8 mRNA of Patient P1, exons 3–5 are deleted. Apparently, the donor splice site of intron 4, which is deleted in this MCT8 DNA, is needed for normal splicing of exon 5. The resultant MCT8 mRNA encodes a short out-of-frame protein.

Evidence was obtained for the existence of a MCT8 splice variant in Patients P3 and P4 and in controls, which is ~0.3 kb smaller than wt-MCT8 mRNA and identical to the splice site mutant mRNA in Patient P2. Interestingly, the intensity of this band was markedly increased in Patient P4, suggesting that the

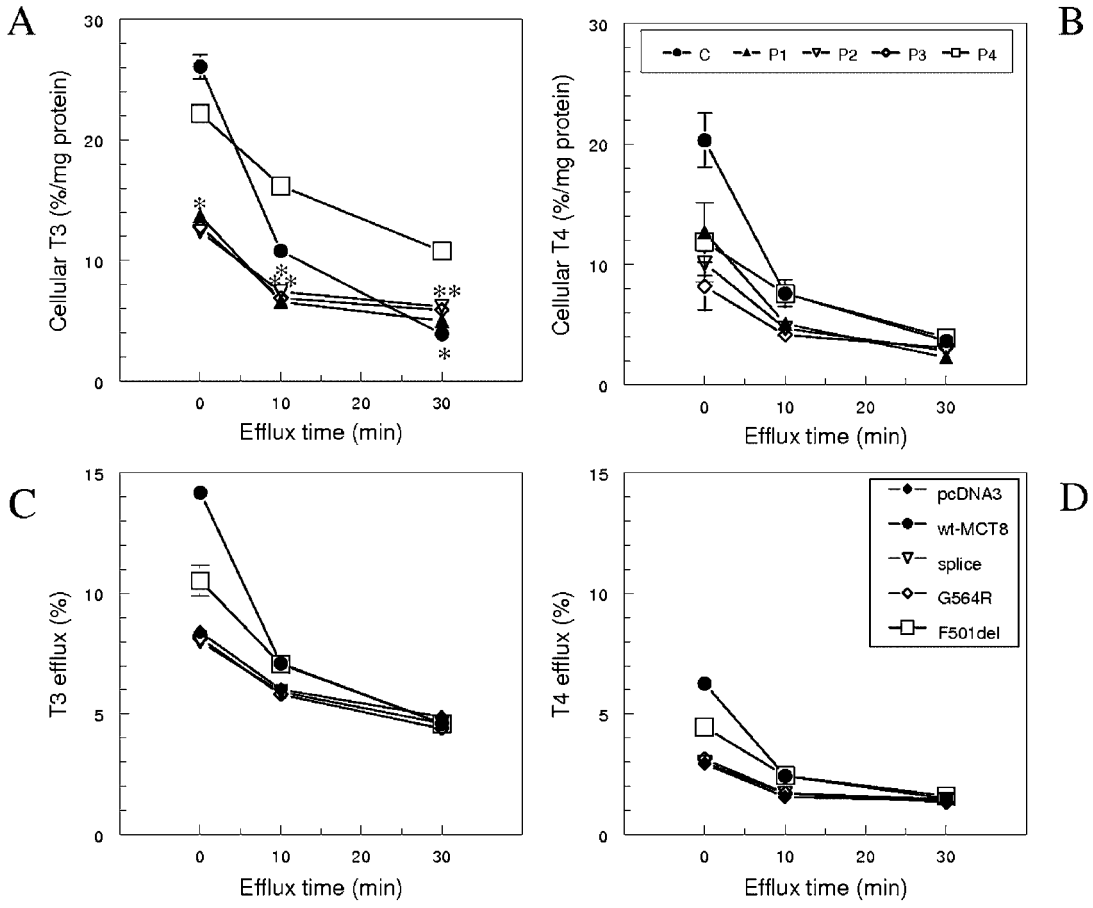

FIGURE 5. **Efflux of cellular T3 and T4 from fibroblasts of MCT8 patients relative to control fibroblasts measured after 10 and 30 min after a 60 min loading with [^{125}I]T3 (A) and [^{125}I]T4 (B). Results are corrected for protein concentrations. Significances represent values obtained in fibroblasts of Patient P4 compared to controls and other patients. Efflux of cellular T3 and T4 measured after 10 and 30 min after 30 min loading with [^{125}I]T3 (C) and [^{125}I]T4 (D) in wt or mutant MCT8-transfected JEG3 cells, shown as percentage of added ligand. Significances represent values of the F501del mutant vs. other mutants and wt MCT8. *P < 0.05; **P < 0.005; ***P < 0.001.**

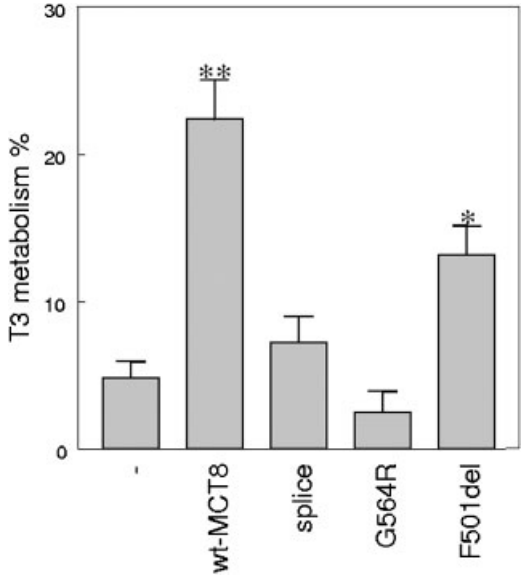

FIGURE 6. **Metabolism of T3 in intact JEG3 cells cotransfected with D3 and wt or mutant MCT8. Metabolism is shown as percentage of metabolites in the medium after 4 hr incubation. Significances represent values obtained in (wt or mutant) MCT8 vs. empty vector. *P < 0.005; **P < 0.001.**

F501del mutation gives rise to an enhanced alternative splicing. This corresponds to the location of the F501del mutation in a predicted exonic splicer enhancer [Fairbrother et al., 2002]. The pathophysiologic relevance of this finding is not clear, but it is known that alternative splicing variants may have (partial) dominant negative effects on the function of transporter proteins [Gamba, 2001].

At the protein level, the splice site and the F501del mutant were clearly detected by IB. ICC showed a plasma membrane localization for the F501del mutant similar to wt-MCT8, indicating that the mutation in Patients P4 and P5 does not hamper an adequate protein expression.

We noticed a severely diminished initial uptake of T3 and T4 in fibroblasts from all patients as compared to controls. This is in accordance with a previous report showing a markedly decreased T3 and T4 uptake in fibroblasts from two MCT8 patients [Dumitrescu et al., 2004b]. These results indicate that MCT8 plays a major role in TH uptake in skin fibroblasts. Therefore, skin fibroblasts may be representative for cells which express MCT8 as the predominant TH transporter and may, thus, be a suitable tool for elucidating the cellular mechanisms in MCT8-expressing neurons involved in the pathogenesis of the MCT8 syndrome.

Cellular TH uptake increased with time in the fibroblasts of all patients, indicating the contribution of other TH transporters. Although T4 uptake was equally affected in all patients, T3 uptake

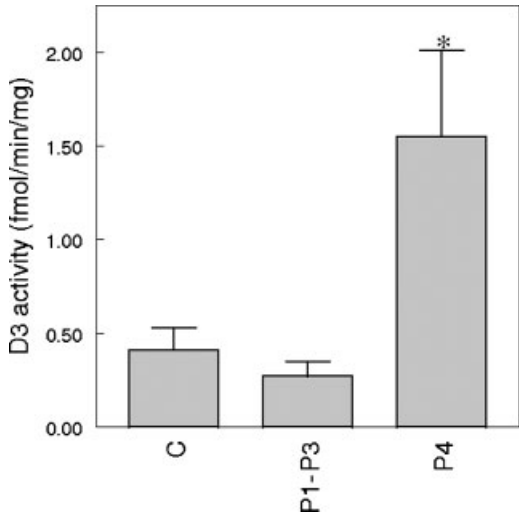

FIGURE 7. **Analysis of D3 enzymatic activity in lysates of fibroblasts from MCT8 patients. The means ± SE of triplicate experiments with fibroblasts from Patients P1, P2, and P3 measured separately are denoted as P1–P3. Significances represent values obtained in patient fibroblasts compared to control fibroblasts. *P < 0.01.**

was less diminished in Patient P4 than in Patients P1–P3. When T3 uptake by fibroblasts from controls and Patients P1–P3 reached a plateau phase, it continued to increase in Patient P4. As the equilibrium is the net result of influx and efflux, these results strongly suggested that T3 export is severely affected in Patient P4. This was confirmed in efflux experiments, showing that the T3 efflux rate from fibroblasts of Patient P4 was similar to that of Patients P1–P3, indicating the involvement of an endogenous export protein other than MCT8.

T3 and T4 transport by the MCT8 mutants was further tested in transfected JEG3 cells, providing the same cellular environment to all mutants. Differences in TH transport, thus, solely represent the specific characteristics of the transfected MCT8 mutants compared to wt-MCT8. The findings in the patients' fibroblasts were replicated in the transfected JEG3 cells, thereby substantiating that the affected T3 efflux in Patient P4 results from the mutated MCT8 and not from an altered expression of other TH transporters in this patient. In addition, in transiently transfected cells, the F501del mutant afforded marked T3 metabolism compared to wt-MCT8, whereas both other mutants did not facilitate T3 metabolism.

Considering the different behavior in cellular T3 transport in Patient P4 compared to the other patients, it was highly interesting

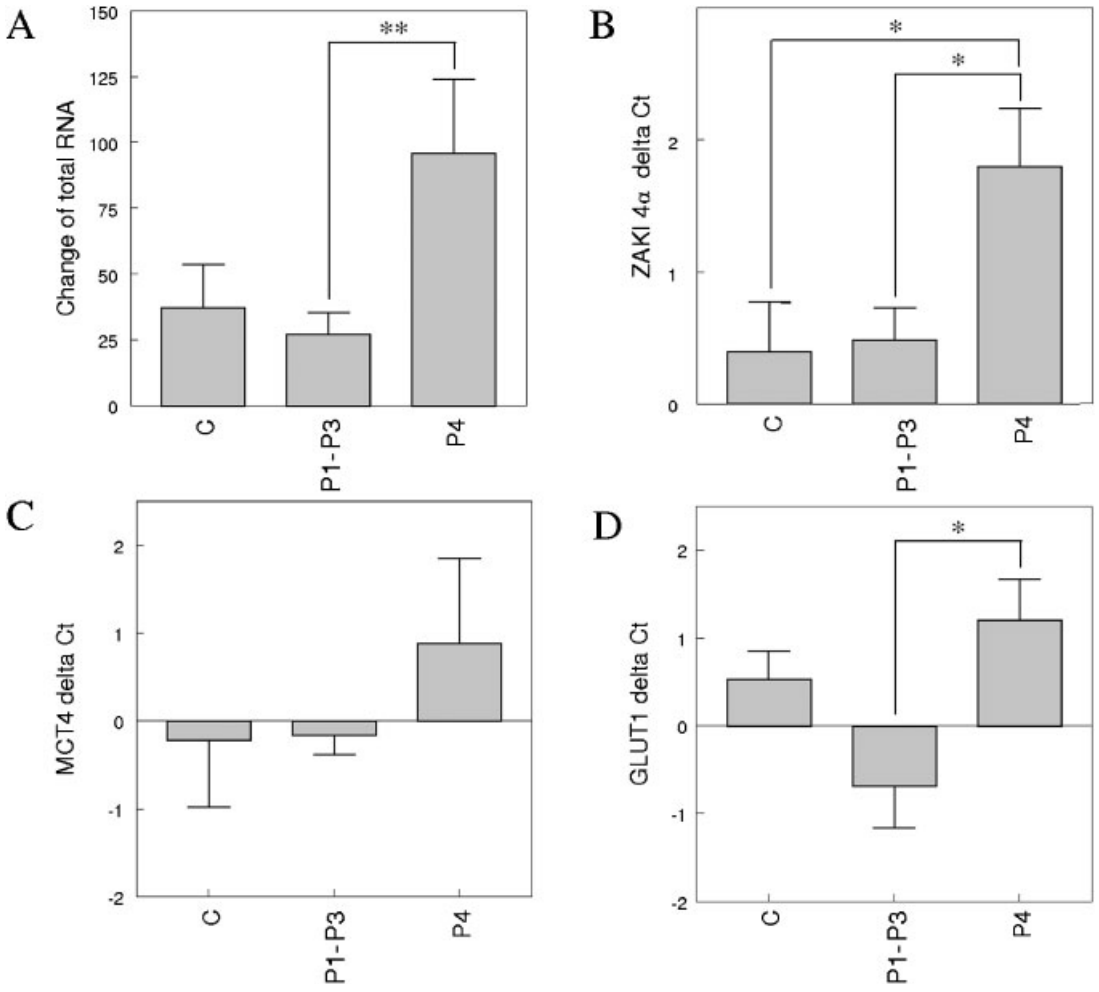

FIGURE 8. **Effects of incubation for 6 hr with 10 nM T3 on total RNA content (A) and mRNA levels of the T3-responsive genes ZAKI 4α (B), MCT4 (C), and GLUT-1 (D). The changes of mRNA levels after T3 treatment are expressed by the δCt method (where δCt is the value obtained by subtracting the Ct value of the target mRNA from the Ct value of the house-keeping gene Cyclophilin A). The means ± SE of triplicate experiments in fibroblasts from Patients P1, P2, and P3 measured separately are denoted as P1–P3. Significances represent values obtained in fibroblasts of Patient P4 compared to controls and other patients. *P < 0.05; **P < 0.01.**

to study the intracellular TH status in the fibroblasts. Since D2 is negatively and D3 is positively regulated by TH [Bianco and Kim, 2006], we measured the activity of these deiodinases. MCT8 KO mice are reported to have increased cerebral D2 activities [Dumitrescu et al., 2006; Trajkovic et al., 2007]. In addition, there is an isolated report that D2 activity is increased in fibroblasts of MCT8 patients [Dumitrescu et al., 2004b]. However, we were not able to obtain reliable measurements of D2 activity, which may be due to methodological differences.

In lysates of fibroblasts, mean D3 activity was lower in Patients P1–P3 than in controls, whereas it was even increased in Patient P4. It was not possible to detect T3 metabolism in intact skin fibroblasts, which is supported by the observation that D3 activity is much easier to detect in cell lysates than in intact cells (M.H. Kester and T.J. Visser, personal observations). MCT8 KO mice demonstrate diminished D3 activity in brain [Trajkovic et al., 2007]. Decreased D3 activity is thought to compensate for a decrease in cellular T3 supply. In contrast, the increased D3 activity in fibroblasts from Patient P4 may represent an increased intracellular T3 availability due to more prominent decrease in the efflux than in the uptake of T3.

It is known that T3 influences transcriptional activity. Already more than four decades ago, Tata [1964] described an early acceleration of RNA synthesis after T3 administration in rats. Indeed, T3 treatment resulted in an induction of total RNA in fibroblasts. However, compared to the modest increase in controls, RNA was almost doubled in fibroblasts from Patient P4, supporting the notion of a higher availability of intracellular T3. It is likely that abnormal gene expression profiles in brain resulting from altered intracellular T3 concentrations play an important role in the pathogenesis of the neurological abnormalities seen in MCT8 patients. We studied three genes, expressed in brain as well as in human skin fibroblasts, which are positively regulated by T3 [Moeller et al., 2005]. Although ZAKI 4α expression was upregulated by T3 in all fibroblasts, the increase was significantly higher in Patient P4 than in controls. Except for Patient P4, we did not notice an increase in GLUT1 and MCT4 expression in control and patient fibroblasts in response to T3 treatment.

The relatively short T3 incubation time, which was optimal for demonstrating differential gene regulation in Patient P4, may be the explanation that GLUT1 and MCT4 expression did not increase in fibroblasts from controls and Patients P1–P3. Indeed, we (Visser et al., unpublished observations) and others [Moeller et al., 2005; Romero et al., 2000] observed an increased expression of these genes when cells were treated with T3 for at least 24 hr. The T3-mediated effects on the expression of these genes in normal brain development are currently unknown, but it is conceivable that aberrations in their regulation may have adverse effects on neurological development.

If our findings in skin fibroblasts can be extrapolated to MCT8-expressing neurons in MCT8 patients, our findings may be explained by assuming different mechanisms for the pathogenesis of the psychomotor retardation in Patients P1–P3 vs. Patients P4 and P5. The results in skin fibroblasts of Patients P1–P3, who fit the "classical" MCT8 phenotype, are in line with the assumed function of MCT8 in neuronal T3 uptake. It is fully understandable that MCT8 mutations result in a diminished intracellular T3 concentration. Considering the crucial role of TH in normal brain development, it is conceivable that neurological defects will be the consequence of this neuronal T3 deprivation. The disturbed balance between T3 uptake and export, leads to increased intracellular T3 levels in Patients P4 and P5. It is known that not only diminished, but also raised TH concentrations have harmful effects on brain development [Kopp et al., 1995]. It is likely that the increased D3 activity in fibroblasts of Patient P4 is an adaptive response to lower toxic levels of T3.

It is thought that the initial event in the disturbed TH levels in MCT8 patients is the result of neuronal T3 deprivation [Friesema et al., 2006a]. The decreased T3 supply to neuronally expressed D3 will result in a decreased T3 clearance. This gives rise to increased serum T3, which will stimulate renal and hepatic D1 activity and, thus, increase T3 production. This increased D1 activity may contribute to the lower T4 and rT3 serum levels. However, the serum T3 levels are also (slightly) increased in Patients P4 and P5. In these patients, the elevated intracellular T3 concentrations and increased D3 activity would result in an increased T3 clearance. Therefore, the current hypothesis concerning the disturbed serum TH levels in the MCT8 syndrome may not be correct. This is underscored by the observation that in MCT8 KO mice, which perfectly mimic the abnormal human serum TH levels, T3 clearance is not affected [Dumitrescu et al., 2006; Trajkovic et al., 2007]. Further research is required to elucidate the initial events resulting in the disturbed TH levels in the MCT8 syndrome.

In conclusion, the data presented in this report suggest a novel mechanism involved in the pathophysiology of the neurological damage associated with human MCT8 mutations. We speculate that abnormal brain development in patients with MCT8 mutations may be the consequence of either decreased or increased intracellular T3 concentrations. Further research is required to expand insights in the pathophysiological mechanisms underlying this dramatic disease.

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