Mutations in Thyroid Hormone Transporter MCT8: genotype, function and phenotype

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Mutaties in schildklierhormoontransporter MCT8:
genotype, functie en fenotype

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HET COMPLOT

Wetenschap we lachen er wel om
maar het is natuurlijk
om ten hemel te schreien
nooit klikte het tussen ons
    de dichter
    en jou
afgemeten man plus chemische vrouw
laboratoire tweeling der vooruitgang
samen petrischalen vullen jaja
leg het maar uit
op witte gangen heb ik anders gehoord
wisselen jullie in smetvrije witte jassen
onderling eiwitten uit kan dat
nucleotide baseparen zoiets
dan smiespelen jullie samen
en waarom doen jullie dat en voor wie
zeg op! Wat zijn jullie in godsnaam!
o mijn god met ons van plan

dit heb ik allemaal gehoord
onlangs uit vertrouwde bronnen
maar let op mijn woord
en – lees – lippen – knul
of ook wij begaan ongelukken
via titanendonder en weerlicht
hou je vast aan schalen van richter
hoogachtend hou me vast
    hou me vast
    uw dichter

Ramsey Nasr

THE CONSPIRACY

Science laugh it off
but it's only natural
for crying out loud
it's never clicked between us
    the poet
    and you
measured man plus chemical woman
laboratory twins of progress
filling petri dishes together sure thing
explain that away
down white corridors I've heard otherwise
in immaculate coats exchanging
proteins between yourselves
nucleotide base pairs maybe
then whispering together
and why do you do it and who for
spit it out! What are you in god's name!
oh my god up to

i've heard all this recently
from confident sources
but heed my words
and – read – lips – kiddo
or we too will cause disaster
with sheet lightning and titan's thunder
hold on to those richter scales
sincerely hold on to me
    hold me
    your poet

Ramsey Nasr

translation: David Colmer

CONTENTS

Chapter 1

General introduction

INTRODUCTION

Thyroid hormone is essential for the proper metabolic function of all tissues throughout life, and for the development of many organs, in particular the brain. Thyroid hormone increases energy expenditure, and regulates the production of numerous proteins and enzymes, thus stimulating metabolic rate, growth and differentiation (1). Excess exposure to thyroid hormone, most frequently caused by autoimmune hyperthyroidism (Graves' disease), leads to increased heart rate, weight loss, restlessness, heat intolerance and osteoporosis (2). Hypothyroidism, in adults most often caused by autoimmune thyroiditis (Hashimoto's disease), causes the opposite symptoms: bradycardia, weight gain, cold intolerance, and constipation. It is also associated with reduced cognitive function and depression (3). As thyroid hormone is essential for normal brain development, prenatal and postnatal hypothyroidism (but also hyperthyroidism) can severely impair psychomotor development (see below).

The most important *modus operandi* of thyroid hormone is stimulation or inhibition of gene transcription. This is achieved through binding of its active form T3 (3,3',5-tri-iodothyronine), to the nuclear T3 receptor (TR). The biological activity of thyroid hormone is therefore determined by the intracellular T3 concentration. This depends on a number of factors, including the circulating levels concentrations of T3 and its precursor thyroxine (T4,), the activity of the activating and inactivating iodothyronine deiodinases D1, D2 and D3, and the presence of transporters in the plasma membrane, facilitating the cellular uptake and/or efflux of T4 and T3 (Figure 1.1). The effects of loss of function mutations in thyroid hormone transporter MCT8 are the subject of the work presented in this thesis.

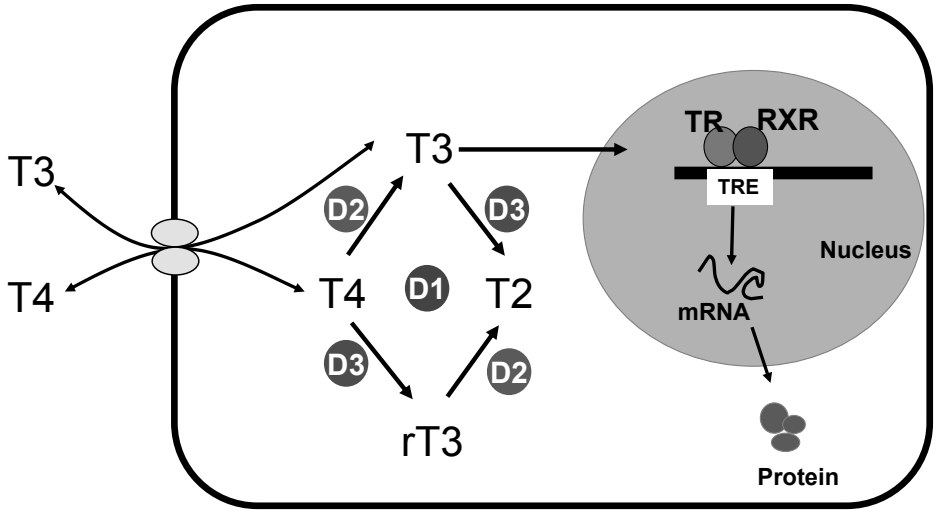

Figure 1.1 Transport, action and metabolism in a thyroid hormone target cell.

THYROID HORMONE SYNTHESIS AND THE HYPOTHALAMUS-PITUITARY-THYROID AXIS.

Thyroid hormone is produced in the thyroid gland, which is located in the neck, ventral to the trachea, just below the thyroid cartilage. Thyrocytes form follicles, in which the hormone is synthesized and stored. Synthesis requires the uptake of the essential trace element iodine, which is incorporated into the tyrosine residues of thyroglobulin to form mono- or diiodotyrosine (MIT and DIT) (4). The uptake of iodide at the basolateral membrane is facilitated by the sodium-iodine symporter (NIS), oxidation of iodide and incorporation into tyrosine residues of thyroglobulin in the follicle involves the enzyme thyroid peroxidase (TPO). T4 is formed from two DIT elements, and thus contains four iodine atoms. T3 contains three iodines (Figure 1.2). The human thyroid mainly produces T4, which is considered to be a biologically inactive prohormone. Only circa 20% of the hormone produced by the thyroid is T3, the active form of thyroid hormone**.** In serum, T3 and T4 are mostly bound to proteins like thyroxine-binding globuline (TBG), transthyretin and albumin. Only a small fraction (~0.02% of T4, and ~0.2% of T3) is unbound, and can be measured as free T4 and free T3 (FT4 and FT3). This free fraction is available for transport into cells, where it can be metabolized by the deiodinases, or bind to the nuclear TR. The large amount of thyroid hormone bound to plasma proteins can be regarded as a quickly recruitable storage pool.

Production of thyroid hormone is tightly regulated by the hypothalamus-pituitary-thyroid axis (HPT) (Figure 1.3). This is a classic example of an endocrine negative feedback loop. Thyroid hormone production is stimulated by thyroid-stimulating hormone (TSH or thy-

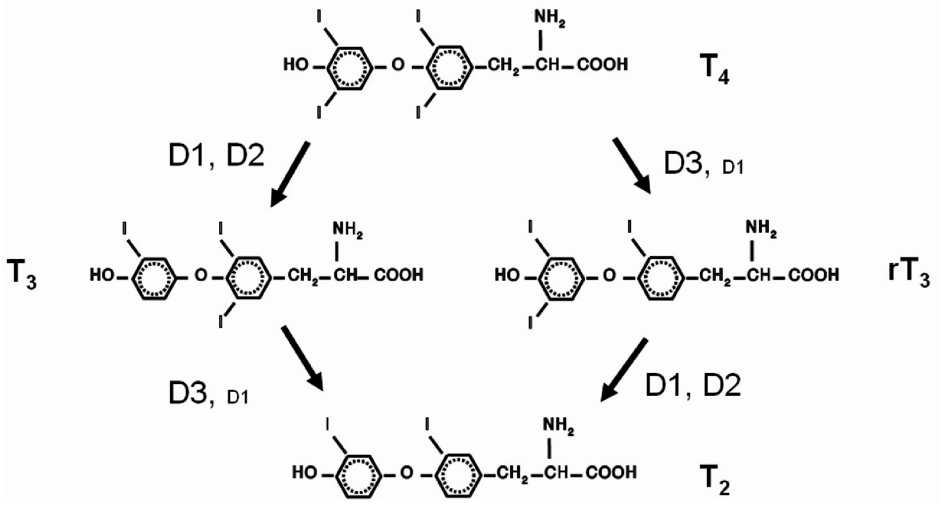

Figure 1.2 Structure of the iodothyronines and their activation and inactivation by type 1, type 2 and type 3 deiodinase (D1, D1 and D3).

rotropin), produced in the anterior pituitary. TSH stimulates a.o. growth of thyrocytes, iodine uptake via NIS, and the secretion of T4 and T3. Production and secretion of TSH is stimulated by thyrotropin-releasing hormone (TRH), which is produced in the paraventricular nuclei of the hypothalamus (5) These nuclei are in close connection with other hypothalamic structures like the arcuate and dorsomedial nuclei, which influence TRH secretion. Through TRH, the basal TSH secretion is modified, enabling adjustment of the

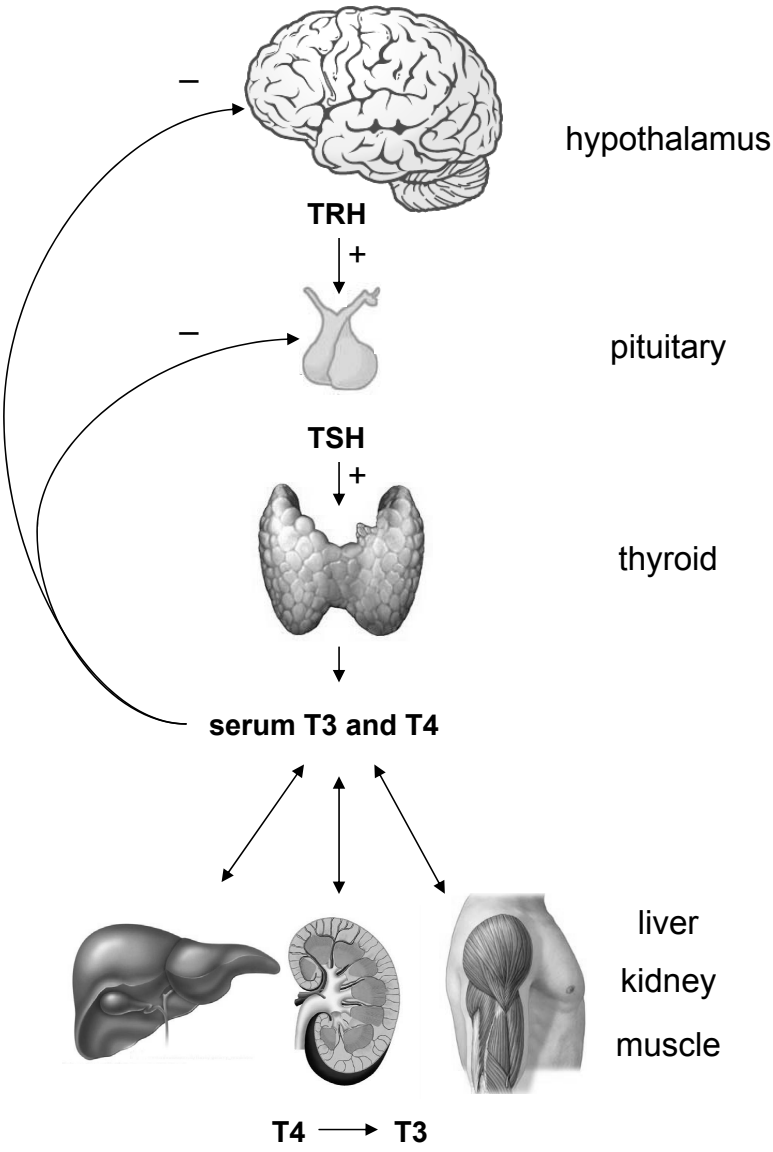

Figure 1.3 The hypothalamus-pituitary- thyroid axis.

thyroid hormone production to adapt to (external) homeostatic challenges, such as cold, stress, starvation and disease (6, 7). Secretion of TSH and TRH is downregulated by thyroid hormone, thus completing the negative feedback loop (8).

THYROID HORMONE ACTION AND METABOLISM

Although the thyroid gland mainly secretes T4, most actions of thyroid hormone are initiated by binding of T3 to the nuclear TR (Figure 1.1). The TR, of which several isoforms exist (TRα1, α2, β1 and β2), forms a heterodimer with the retinoid X receptor (RXR) and binds to so-called T3 response elements (TREs) in the promoter region of thyroid hormone-responsive genes (1). Binding of T3 to its receptor effects a change in gene expression. In the case of positive gene regulation, the T3-TR interaction induces the release of co-repressors and the recruitment of co-activators, resulting in the stimulation of transcription. The mechanism of negative gene regulation by T3, for instance the suppression of thyroid-stimulating hormone (TSH) subunit synthesis in the anterior pituitary, is less well understood

The three deiodinases are homologous selenoproteins (9) which regulate the (local) availability of active thyroid hormone. D1 and D2 convert T4 to T3, thus 'activating' thyroid hormone, whereas D3 catalyzes the degradation of T4 to 3,3',5'-triiodothyronine (rT3) and of T3 to 3,3'-diiodothyronine (3,3'-T2) (Figures 1.1 and 1.2). D1 is located predominantly in liver and kidney. Although it has both outer and inner ring deiodinase activities, its major physiological functions are the generation of plasma T3 and clearance of plasma rT3. D2 is expressed in brain, anterior pituitary, thyroid and skeletal muscle, and has only outer ring deiodinase activity. Although D2 may contribute to circulating T3, it appears especially important for the local production of T3 in these tissues, in particular the brain. D3 is expressed in brain, especially in fetal brain, but also in other fetal tissues, the placenta and the pregnant uterus (9). This suggests an important function of D3 during fetal development, perhaps by preventing growing tissues from exposure to excess T3 that could lead to their premature differentiation.

THYROID HORMONE TRANSPORTERS

Since the thyroid hormone receptor is located in the nucleus, and the active sites of the deiodinases are in the cytoplasm, thyroid hormone action and metabolism are intracellular events. Due to its lipophilic structure, the transmembrane passage of thyroid hormone was first believed to be based on passive diffusion. However, it has become increasingly clear that cellular uptake of thyroid hormone is mediated by transporters, and that the activity

of these transporters determines the access of iodothyronines to the intracellular sites for action and metabolism (10).

In the last three decades, a vast body of experimental evidence has been collected for the involvement of specific transporter mechanisms in cellular thyroid hormone uptake. For example, Mol *et al.* produced a monoclonal antibody directed against a putative thyroid hormone transporter, which was able to inhibit the uptake of T4 and T3 in rat hepatocytes completely. Inhibition of thyroid hormone transport resulted in a reduced production of iodide by the intracellular deiodinases, indicating that transport is rate-limiting for the metabolism of thyroid hormone (11). Furthermore, several studies demonstrated that thyroid hormone transport can be energy dependent. For example, liver perfusion experiments showed that hepatic uptake of T_4 and T_3 is reduced in fasted *versus* normally fed rats. Repletion of energy sources by preperfusing the starved livers with insulin, cortisol and/or glucose, restored T4 and T3 uptake within 30 minutes (12, 13).

In recent years, several transmembrane proteins have functionally been identified as thyroid hormone transporters. Amongst these are organic anion transporters, such as members of the Na^+/taurocholate cotransporting polypeptide (NTCP) and the (Na^+-independent) organic anion transporting polypeptide (OATP) families (14, 15) and several L and T type amino acid transporters. (16-20). In 2001, a T type amino acid transporter (TAT1, also known as MCT10) was cloned and shown to transport Phe, Tyr and Trp, but not the structurally related iodothyronines (21, 22). The relatively high homology of MCT10 with MCT8, another member of the monocarboxylate transporter (MCT) family, lead to the identification of MCT8 as a very active and specific thyroid hormone transporter (23). In addition, it was recently established that in fact MCT10 also is a potent thyroid hormone transporter (see chapter 6). This thesis focuses on the consequences of loss of function mutations in MCT8. For a better understanding of the tissue specific effects of these mutations, as described in chapter 3, the other known thyroid hormone transporters will be introduced briefly.

The NTCP family

NTCP belongs to the family of Na^+-dependent organic anion transporters (SLC10 family), which also comprises the apical sodium-dependent bile acid transporter (ASBT), and the newly discovered sodium-dependent organic anion transporter (SOAT). NTCP is only expressed in hepatocytes, where it is localized to the basolateral cell membrane. It is the major transporter of conjugated bile acids in liver, but it also mediates uptake of unconjugated bile acids and a number of non-bile acid amphipathic compounds such as steroid conjugates (14, 15, 24). Na^+-dependent uptake of the iodothyronines T4, T3, rT3 and 3,3'-T2 as well as the iodothyronine sulfates T4S and T3S and sulfamates T4NS and T3NS has been demonstrated in *Xenopus* oocytes injected with mRNA coding for human or rat

NTCP (25). Iodothyronine preference decreases in the order T4 ~T3 > rT3 ~3,3'-T2, but apparent K_m values have not been determined.

The HAT family

Heterodimeric amino acid transporters (HATs) consist of a heavy chain and a light chain, linked through a disulfide bond (26-28). There are 2 possible heavy chains (4F2hc and rBAT) belonging to the SLC3 gene family, and 7 possible light chains belonging to the SLC7 gene family. 4F2hc (or CD98) is expressed in many tissues, especially on activated lymphocytes and tumor cells. The heavy chains are glycosylated proteins with a single transmembrane domain, whereas the light chains are not glycosylated and have 12 transmembrane domains. The 4F2hc is capable of forming functional heterodimers with 6 light chains (LAT1, LAT2, y^+LAT1, y^+LAT2, Asc1, XCT), whereas rBAT dimerizes only with one light chain ($b^{0,+}$AT).

Transport of the iodothyronines T4, T3, rT3 and 3,3'-T2 by heterodimeric transporters, consisting of human 4F2hc and either human LAT1, mouse LAT2, or human y^+LAT1 or y^+LAT2 was studied in *Xenopus Laevis* oocytes (29). Coexpression of 4F2hc and LAT1 resulted in marked increases in (Na^+-independent) uptake of the different iodothyronines, as well as the 'typical' L type ligands Leu, Phe, Tyr and Trp. Expression of either 4F2hc or LAT1 alone did not induce transport. The affinity of the 4F2hc/LAT1 transporter decreased in the order 3,3'-T2 > T3 ~ rT3 > T4. Apparent K_m values were found to be in the micromolar range, being lowest for T3 (1.5 μM). Both apparent K_m (>10 μM) and V_{max} values were highest for 3,3'-T2 (29). Significant, but smaller, increases in uptake of the different iodothyronines was observed in oocytes coexpressing 4F2hc and LAT2.

It was demonstrated by Ritchie *et al.* that overexpression of the heterodimeric L type transporter in cells results in increased intracellular T3 availability and augmented T3 action (30). Furthermore, they demonstrated T3 uptake via the 4F2hc/LAT1 transporter into the human placental choriocarcinoma cell line BeWo, indicating that this transporter might play an important role in supplying the placenta and developing fetus with thyroid hormone (31). The 4F2hc/LAT1 complex, also referred to as System L, may also play a role in thyroid hormone uptake in adipocytes and across the blood-brain-barrier (32).

The OATP family

The OATP family (now also known as SLCO family) represent a large group of multi-specific proteins responsible for the Na^+-independent transmembrane transport of amphipathic organic compounds, including bile salts, bilirubin and bilirubin glucuronides, estrogen conjugates, thyroid hormone, oligopeptides, numerous drugs, neutral steroids (ouabain and digoxin), and selected lipophilic organic cations (33, 34). To date, approximately 40

OATPs have been identified in humans, rats and mice. OATPs are large proteins of 652-848 amino acids in length and contain 12 transmembrane domains; both N- and C-terminal domains are located inside the cell. There is a particularly large extracellular loop 5 between transmembrane domain 9 and 10, which contains many conserved Cys residues, and *N*-glycosylation sites are present in extracellular loops 2 and 5.

OATPs are expressed in a wide variety of tissues, including liver, kidney, brain (blood-brain barrier, choroid plexus), lung, heart, placenta, testis, eye and small intestine. OATPs facilitate cellular uptake of the organic anions in exchange for intracellular anions such as reduced glutathione and bicarbonate. The multispecific OATPs appear play an important role in the overall body detoxification system. However, some OATPs show more restricted substrate specificity and tissue distribution, and a more highly conserved structure. For example, human OATP1B1 and 1B3 is a liver-specific member of this family. It is expressed on the basolateral membrane of hepatocytes, where it mediates translocation of –amongst other substrates- iodothyronines and bile acids.

Several human OATPs have been shown to transport thyroid hormones. These include members of the OATP1 subfamily (OATP1A2, 1B1 and 1C1) (35-38) and members of the OATP4 subfamily (OATP4A1 and 4C1) (36, 39). Most of these transporters do not show specific preference for particular iodothyronines above others, and some of them have also been shown to transport iodothyronine sulfamates and sulfates efficiently (25, 40). Where this was determined, K_m values for T4 and T3 are usually in the low micromolar range.

Perhaps the most interesting OATP superfamily member in terms of thyroid hormone transport is OATP1C1. Human OATP1C1 was recently characterized by Pizzagalli *et al.*, who demonstrated its widespread distribution in the human brain as well as testis (Leydig cells). In functional studies in *Xenopus* oocytes and CHO cells, OATP1C1 showed high affinity for both T4 and rT3 (K_m ~0.1 μM) but not for T3 (38). The extensive expression of human OATP1C1 in particular in choroid plexus and capillaries throughout the human brain, as well as its high affinity for T4 suggest that this transporter is critical for brain uptake of T4 across the blood-brain barrier. This is a crucial step before conversion of T4 to T3 by D2 in astrocytes and the subsequent action of T3 in neurons.

In agreement with these findings regarding human OATP1C1, the group of Sugiyama has recently reported on the characterization of the orthologous rat and mouse OATP1C1 transporters (41, 42). They describe a similar brain-specific expression, with immunohistochemical staining indicating localization in brain capillary endothelial cells. In functional studies in transfected HEK239 cells, OATP1C1 increased transport of T4 and rT3, amongst other substrates, showing highest affinity and specificity for T4 and rT3. It was demonstrated that transport of T4 is bidirectional, as the efflux of labeled T4 from pre-incubated OATP1C1-transfected cells was largely increased compared to vector-transfected controls.

Sugiyama *et al.* also showed that expression levels of OATP1C1 in isolated brain capillaries is regulated by thyroid hormone concentration. In hypothyroid rats, OATP1C1 mRNA

and protein are upregulated, whereas in hyperthyroid rats the expression is downregulated. Thus, the thyroid state-dependent regulation of OATP1C1 counteracts the effects of alterations in circulating T4 levels on brain T_4 uptake. In addition to parallel changes in D2 expression, they help to maintain the concentration of T3 in the central nervous system.

Whether these observations in rats may be extrapolated to humans remains to be determined. However, there is a high level of homology between rodent and human OATP1C1 regarding amino acid structure, tissue expression and substrate specificity. Based on these studies and the work of Pizzagalli *et al.*, it seems logical to conclude that OATP1C1 is critical for T4 transport across the human blood brain barrier. Sugiyama *et al.* speculate that functional loss of the OATP1C1 gene may lead to thyroid hormone-related neurological disorders. To our knowledge, patients suffering from this form of thyroid hormone resistance have not been identified so far.

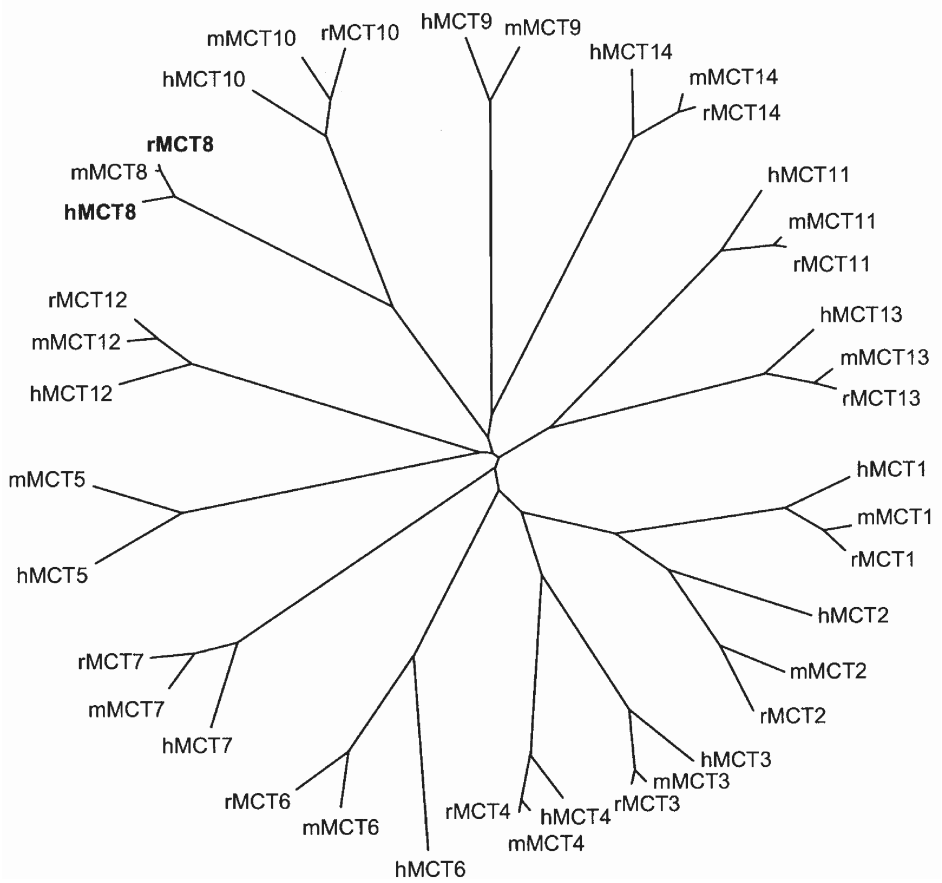

Figure 1.4 Phylogenetic tree of the monocarboxylate transporter (MCT) family, showing relative high homology between MCT8 and MCT10.

The MCT family

The MCT family, now also referred to as SLC16 family, was named after the first four members identified, as they have been characterized as transporters of monocarboxylates such as lactate, pyruvate and ketone bodies (43). These MCTs co-transport monocarboxylates together with a proton, and influx rates therefore increase at lower pH. However, MCTs also facilitate efflux of their ligands. To date, 14 members of the MCT family have been identified in various tissues from different species (Figure 1.4). MCT1 through 4 are expressed in many tissues, including embryonic tissues, muscle, the brain and several tumors (44-47), and expression levels appear to be regulated by substrate demand. For example, the expression of MCT1 and MCT4 in human skeletal muscle is increased by exercise (48). Human MCT10, first identified as a transporter for aromatic amino acids, but now also shown to transport thyroid hormone (see chapter 6), is expressed highest in skeletal muscle and kidney, whereas lower levels are found in heart, placenta and intestine. The tissue distribution of MCT8 will be discussed in detail below.

Five MCT genes are located on human chromosome 17 (MCT11 and MCT13 on 17p; MCT4, MCT6 and MCT7 on 17q), while the other MCTs are dispersed over other autosomal chromosomes, with MCT10 located on chromosome 6q. MCT8 is located on the X chromosome (Xq13.2), which is of major importance in the pathogenesis of MCT8 mutation related psychomotor retardation.

The MCT genes code for proteins of 426 to 565 amino acids with 12 predicted transmembrane domains. The N- and the C-terminal domains of the protein are located inside the cell (Figure 1.5). The largest sequence variation between the different MCTs is observed in the C-terminal end and in the large intracellular loop between transmembrane domains 6 and 7. It is suggested that especially the C-terminal half of the proteins is important for substrate specificity. Conversion of a Phe residue in transmembrane domain 10 to Cys in Chinese hamster MCT1 decreases the ability to transport lactate and pyruvate but increases the transport of mevalonate (49). It is thought that a positively charged group is present in all MCTs, which is necessary for binding of the anionic ligand. An Arg residue in transmembrane domain 8 of many MCTs is a likely candidate, since mutation of this residue greatly reduces the affinity of MCT1 for lactate (50, 51).

For expression at the plasma membrane, MCTs may require ancillary proteins. CD147 (OX-47), a widely distributed cell surface glycoprotein, enables the proper expression and function of MCT1 and MCT4 at the cell surface. Without CD147, the proteins accumulate in the endoplasmic reticulum or Golgi apparatus (51). However, for the thyroid hormone transporters MCT8 and MCT10, no ancillary protein has been identified so far.

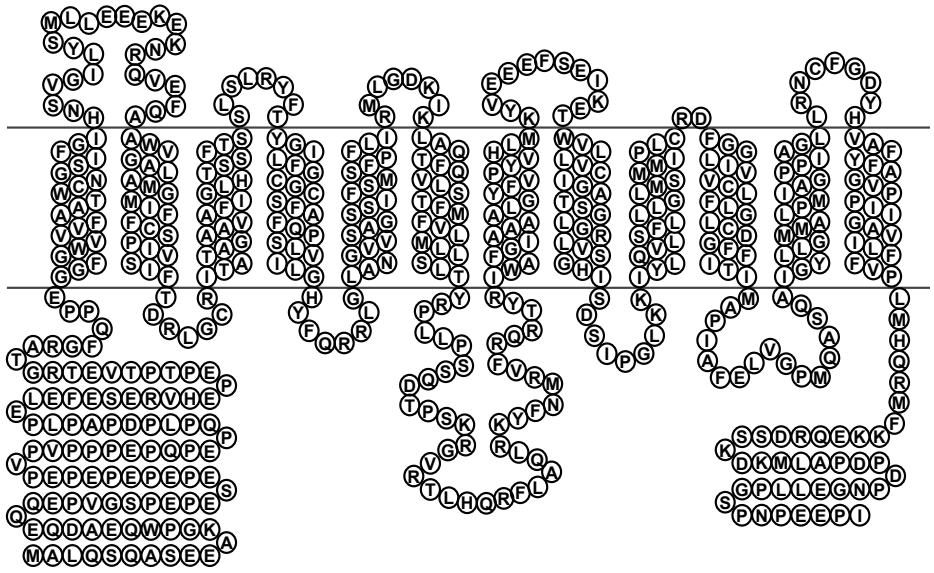

Figure 1.5 Putative structure of MCT8, showing 12 transmembrane domains, intercellular localization of the N- and C-terminal domains and the N-terminal PEST-domains.

MCT8

The MCT8 gene, now known as SLC16A2, was first identified by Lafrenière *et al.* in 1994. They showed that it is located on chromosome Xq13.2, consists of 6 exons, and codes for a protein of ~60 kDa (52). The protein contains 12 predicted transmembrane domains, characteristic of a transporter protein. Near the N-terminal end of the protein so-called PEST domains are present, rich in Pro (P), Glu (E), Ser (S) and Thr (T) residues. PEST-domains serve as proteolytic signals, targeting the protein for rapid degradation. They are found in proteins with a short half-life (53). Lafrenière *et al.* called the gene X-linked PEST containing transporter (XPCT). Expression was demonstrated in human heart, brain, placenta, lung, kidney, skeletal muscle and, most strongly, in human liver. Based on the homology with MCT1, they suggested XPCT to be a hepatic monocarboxylate transporter. Already in this first publication, Lafrenière *et al.* indicated that XPCT, later renamed MCT8, might be a candidate gene for several X-linked hereditary defects mapped to the proximal long arm of the X chromosome.

After the cloning of XPCT (MCT8) in 1994, no reports on the biological function or the transported ligands have been published until Friesema *et al.* identified MCT8 as a specific thyroid hormone transporter (23). Rat MCT8 was cloned and its mRNA injected in *Xenopus* oocytes, which were then tested for transport of several radioactively labeled ligands. These studies demonstrated that expression of MCT8 induced a ~10-fold increase in uptake of T4 and T3 relative to uninjected oocytes. Compared to other thyroid hormone

transporters, such as NTCP and members of the OATP family, the rate of T4 and T3 transport by MCT8 is much higher. Rat MCT8 was shown to transport T4, T3, rT3 and 3,3'-T2, but not iodothyronine sulfates and sulfamates, the amino acids Tyr, Trp, Leu and Phe, nor the monocarboxylates lactate and pyruvate. The specificity of MCT8 for iodothyronines is also supported by the minor inhibitory effects of large concentrations of aromatic amino acids on the transport of T4 and T3. Transport of T4, T3 and rT3 by MCT8 was shown to be saturable, with K_m values of 4.7 μM for T4, 4.0 μM for T3 and 2.2 μM for rT3 in the absence of protein in the medium. T4 transport is modestly inhibited in the absence of Na^+, whereas transport of T3 is Na^+-independent.

There is a high degree of homology in the amino acid sequences of MCT8 between different species, especially between human, mouse and rat MCT8. However, there are significant differences in the 5' end of the coding sequence between human and rodent MCT8. The human gene contains two alternative translation start sites (TLSs), the most upstream of which is lacking in the MCT8 gene of other species. Use of this upstream TLS would result in the generation of a protein which is 74 amino acids longer than use of the downstream (common) TLS. It is unknown to what extent the long or the short isoform of human MCT8 are expressed in different tissues. It is also unknown if there are any functional differences between them. From the experiments presented in this thesis, conducted with the short form of human MCT8, we know that this short isoform is an active thyroid hormone transporter. Within this thesis, the numbering of amino acids starts at the first ATG (and the numbering of nucleotides starts at the A of this start codon). This in contrast to the annotation used in a previous publication, where positions of mutations were related to the second ATG (54).

As has been mentioned by Lafrenière *et al.*, the MCT8 gene is located in a region on the X chromosome that is associated with X-linked diseases. After it was established that MCT8 is an active and specific thyroid hormone transporter, it was hypothesized that mutations in MCT8 could cause an X-linked form of thyroid hormone resistance. This hypothesis was first tested in a 6-year-old boy with severe psychomotor retardation of unknown origin and highly elevated serum T3 levels (54). In this boy, other possible causes of thyroid hormone resistance, such as mutations in the T3 receptors or the deiodinases, had already been excluded. Using PCR, it proved impossible to amplify the first exon of MCT8 in this patient. The borders of the deletion were identified, and it was shown that it comprised of 24.5 kbp. Obviously, the deletion of the first exon is fatal for the expression of MCT8.

To date, mutations in MCT8 have been identified in over 25 families around the world (54-62). All show psychomotor retardation and elevated serum T3 levels. Prominent symptoms are central hypotonia with poor head control, spastic quadriplegia, paroxysmal dyskinesia, and severe cognitive impairment. Most patients are not able to sit, stand or walk without support, and do not develop any speech. However, patients identified in some families show a more advanced psychomotor development, and are able to walk

and/or develop some elementary speech (56). Patients with mutations in MCT8 have remarkable abnormalities in their serum thyroid hormone levels, which can help in the diagnosis. T3 levels are elevated, which appears to be a hallmark of the disease. T4 and FT4 are mostly decreased, and TSH is usually in the normal range. The mothers of most patients have proven to be carriers of the mutations. None of the mothers show psychomotor retardation, and their serum thyroid hormone levels are mostly within the normal range. However, Dumitrescu *et al.* report mothers to have slightly increased serum T3, and slightly decreased serum T4, placing their serum thyroid functions between non-carrier controls and patients (55).

A finding of historic interest is that the clinical phenotype associated with mutations in MCT8 was first described already in 1944 (63). Allan, Herndon and Dudley report on a family with 'sex-linked idiocy', in which male patients have axial hypotonia, spastic quadriplegia and impaired cognition. In this family, they are referred to as 'limber necks'. This report is one of the first on what we now call X-linked mental retardation (XLMR). Recently, in younger members of this family, elevated serum T3 levels and a point mutation in MCT8 were found, leading to an amino acid substitution L568P (56). Named after the original authors, the MCT8 related phenotype is now referred to as Allan-Herndon-Dudley syndrome (AHDS).

THE ROLE OF THYROID HORMONE AND MCT8 IN BRAIN DEVELOPMENT

It has long been established that thyroid hormone is essential for normal human brain development (64). Thyroid hormone influences many important developmental processes, such as neuronal cell migration and differentiation, synaptogenesis and myelination (65-67). Many animal studies, in particular in rats, have provided insight into the mechanisms of thyroid hormone dependent brain development. These show, for example, reduced myelination and reduced dendritic outgrowth of cerebellar Purkinje cells in rats that were made hypothyroid during fetal life (67). Also, it is shown that many genes involved in brain development have thyroid hormone response elements (TREs). These include genes involved in processes like myelination (MBP, myelin basic protein), neuronal cell migration (NGF, BDNF) and the formation of synapses (RC3/neurogranin). mRNA levels of these genes are 2-4 fold reduced in hypothyroid neonatal rat brains (68).

In humans, during the first trimester of pregnancy, the fetal thyroid gland is not yet functional, and transplacental passage of maternal thyroid hormone is essential. MCT8, expressed in placenta, is likely to contribute to this (69, 70). Later, as the fetal HPT axis has developed, the relative importance of maternal hormone is smaller, but supply of iodine via the mother remains crucial. If the thyroid hormone supply to the developing brain is disturbed, the time period in which this occurs determines the clinical consequences (71)

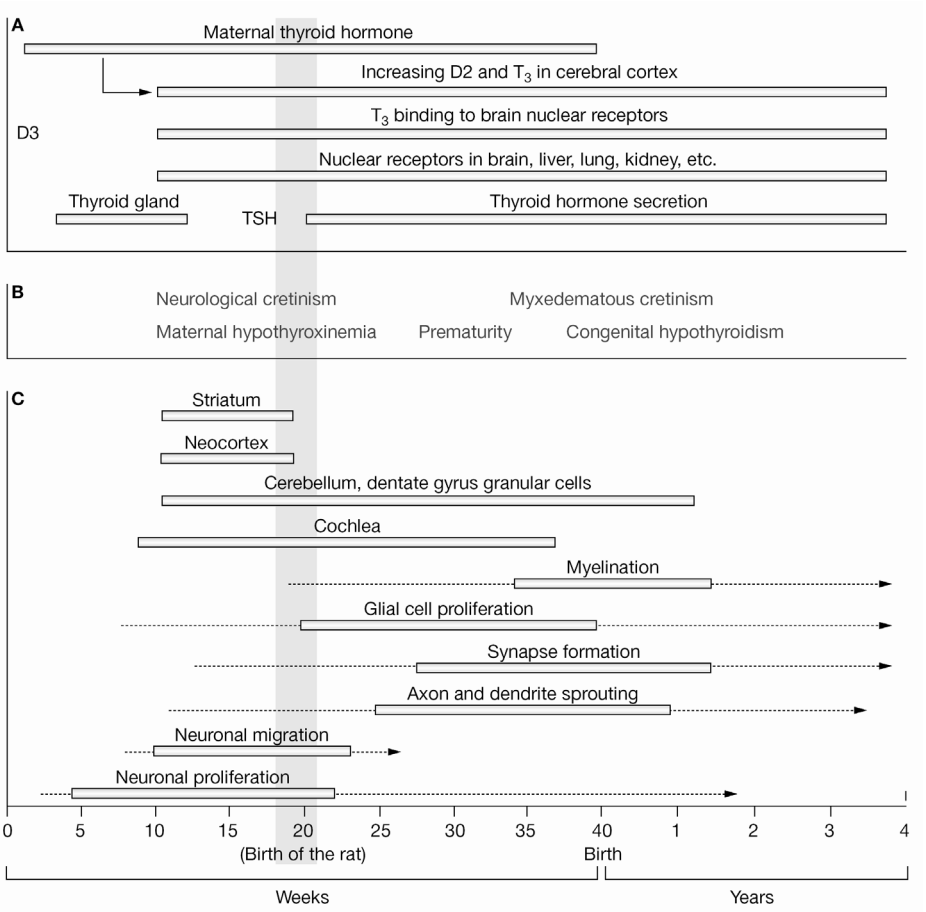

Figure 1.6 Human fetal and neonatal brain development in relation to thyroid hormones. **A.** Time points of thyroid hormone related events. **B.** Approximate timing of disturbances in thyroid hormone supply and their clinical consequences. **C**. Time points of thyroid hormone influenced neurodevelopmental processes. Most data are from studies in rats, which are born with relatively immature brains, equivalent to approximately mid gestation in humans (gray vertical bar). Reproduced from Bernal J, Nat Clin Pract Endocrinol Metab. 2007 Mar;3(3):249-59.

(Figure 1.6). Severe maternal hypothyroidism from early pregnancy, for example due to endemic iodine deficiency, may lead to severe irreversible neurological impairment with spastic quadriplegia, deaf-mutism, and mental deficiency, a syndrome known as neurological cretinism (72). Recently, also more subtle disturbances in maternal thyroid hormone status during pregnancy have been shown to affect the neuropsychological outcome of the child. Pop *et al.* showed that in healthy women with normal TSH, maternal FT4 below the 10th percentile at week 12 was associated with a significantly delay in development at 10 months of age (73). Similar findings by Haddow *et al.* correlate high maternal TSH during pregnancy with delayed development of the child (74).

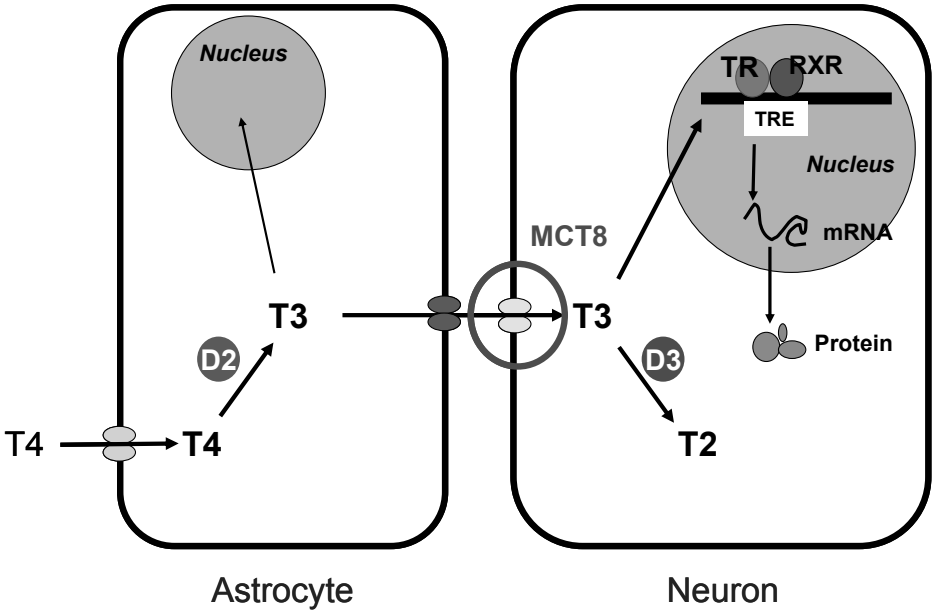

Figure 1.7 Role of MCT8 in the supply of thyroid hormone to neurons in the brain.

Congenital hypothyroidism, due to impaired development of the thyroid gland or inborn errors in thyroid hormone synthesis, may lead to impaired development if not treated promptly after birth (64). However, supply of maternal thyroid hormone during gestation has protective effects. Postnatal treatment with thyroid hormone greatly improves, although may not completely normalize, the neurological outcome in these children (75, 76). This is the basis of the congenital hypothyroidism screening programs that are now effective in many developed countries.

The supply of T3 to neurons in the central nervous system requires interplay between several cell types that regulate uptake, efflux and deiodination of thyroid hormones. MCT8 plays a crucial role in this process (Figure 1.7). T4, taken up into the brain by presumably OATP1C1, is locally deiodinated into T3 by D2 that is expressed in astrocytes (77). T3 must than be exported from the astrocyte to be taken up by neurons and other target cells. Neurons express MCT8 (78-80), which facilitates T3 influx, but the mechanisms involved in the export from astrocytes are still unknown. Neurons also express D3, which is responsible for the inactivation of T3 (78). MCT8 expression in mouse brain is high in the choroids plexus, olfactory bulb, cerebral cortex, hippocampus and amygdala. Moderate expression is observed in the striatum and the cerebellum. To date, little is known about the spatial and temporal expression of MCT8 in the human brain, making this an important field for further research.

HYPOTHESIS AND OUTLINE OF THE THESIS.

The identification of the first patient with a mutation in MCT8 established a major breakthrough in the field of thyroid hormone physiology. It did not only underline the crucial role of thyroid hormone in brain development, but also empowered the idea that functional transporters are essential for thyroid hormone action.

The work presented in this thesis is based on the hypothesis that loss of MCT8 function results in an impaired supply of T3 to the neuron. This has detrimental effects on neuronal migration, differentiation and myelination, causing the psychomotor phenotype that is observed in AHDS patients. Furthermore, mutations in MCT8 are associated with elevated serum T3 levels. The combination of impaired MCT8 function and elevated serum T3 levels might result in a hypothyroid state in cells expressing MCT8, but a hyperthyroid state in cells expressing other thyroid hormone transporters.

The studies presented in **chapter 2** demonstrate that the mutations in MCT8 identified in patients lead to a loss of T3 and T4 transport function in an *in vitro* model system using transfected cells. In **chapter 3**, the tissue specific thyroid hormone status caused by loss of MCT8 function and its associated rise in serum T3 in patients with AHDS is discussed. In **chapter 4**, we correlate the psychomotor development of AHDS patients with MCT8 function *in vitro*, showing that relatively advanced development is associated with residual thyroid hormone transport. In **chapter 5**, we present studies performed with fibroblasts from AHDS patients, from which we deduct that not only impaired influx, but also impaired efflux of thyroid hormone may contribute to the observed psychomotor retardation. **Chapter 6** focuses on thyroid hormone transporter MCT10 and the role of MCT8 in thyroid hormone efflux. In **chapter 7**, we investigate the role of the N-terminal PEST domains and the ubiquitin system in the functional expression of MCT8. **Chapter 8** summarizes the clinical and functional aspects of MCT8 mutations, and introduces the phenotype of the MCT8 knockout mouse. **Chapter 9** provides a general discussion of the work.

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Chapter 2

Functional analysis of MCT8 mutations identified in patients with X-linked psychomotor retardation and elevated serum triiodothyronine

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**) Both authors contributed equally to this study*

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ABSTRACT

Context: T3 action in neurons is essential for brain development. Recent evidence indicates that monocarboxylate transporter 8 (MCT8) is important for neuronal T3 uptake. Hemizygous mutations have been identified in the X-linked *MCT8* gene in boys with severe psychomotor retardation and elevated serum T3 levels.

Objective: The objective of this study was to determine the functional consequences of *MCT8* mutations regarding transport of T3.

Design: MCT8 function was studied in wild-type or mutant MCT8 transfected JEG3 cells by analyzing a) T3 uptake, b) T3 metabolism in cells cotransfected with human type 3 deiodinase, c) Immunoblotting, and d) immunocytochemistry.

Results: The mutations identified in *MCT8* comprise 4 deletions (24.5 kb, 2.4 kb, 14 bp and 3 bp), 3 missense mutations (Ala224Val, Arg271His, Leu471Pro), a nonsense mutation (Arg245stop) and a splice site-mutation (94-amino acid deletion). All tested mutants were inactive in uptake and metabolism assays, except MCT8 Arg271His which showed ~20% activity vs. wild-type MCT8.

Conclusion: These findings support the hypothesis that the severe psychomotor retardation and elevated serum T3 levels in these patients are caused by inactivation of the MCT8 transporter, preventing action and metabolism of T3 in central neurons.

INTRODUCTION

Thyroid hormone is essential for the development of the brain (1, 2). The bioavailability of T3 in the brain is locally regulated through the interplay of two types of cells, astrocytes and neurons. Astrocytes express the type II deiodinase (D2) that converts the prohormone T4 to T3 which is subsequently transported to the neurons, the major target cells for thyroid hormone in the brain. (3, 4). In addition to nuclear T3 receptors, neurons express the type III deiodinase (D3) which catalyzes termination of T3 activity. Multiple transporters are involved in cellular iodothyronine uptake and efflux in different tissues (5, 6). Recent evidence suggests that monocarboxylate transporter 8 (MCT8) is important for T3 uptake into central neurons (7, 8).

The *MCT8* gene is located on chromosome Xq13.2; depending on which of the 2 possible translation start sites is used, it codes for a protein of 613 or 539 amino acids, containing 12 putative transmembrane domains (TMDs). MCT8 is expressed in numerous human tissues, including brain, heart, placenta, lung, kidney, skeletal muscle and liver. We and others have reported on patients with mutations in the *MCT8* gene (9-13). These patients, all male, show a distinct phenotype of severe psychomotor retardation in combination with elevated serum levels of T3.

In the present study, we provide functional characteristics of 6 *MCT8* mutations. The functional consequences of mutations in MCT8 on cellular uptake and metabolism of T3 were determined in JEG3 cells transfected with wild-type or mutant MCT8 alone or in cells cotransfected with MCT8 and D3.

MATERIALS AND METHODS

Serum and DNA analyses

Serum T4, free T4 (FT4), T3 and TSH were measured by Vitros ECI technology (Immunodiagnostic System, Ortho-Clinical Diagnostics, Beerse, Belgium). The coding sequence of *MCT8* was analyzed in patient DNA using intronic primers flanking the 6 exons.

Cloning and site-directed mutagenesis of human MCT8

Construction of a hMCT8 cDNA-containing pcDNA3 expression vector was described previously (14). The point mutations identified in patients (Table 2.1) were introduced in MCT8 cDNA using the QuickChange Site-Directed Mutagenesis protocol (Stratagene, Amsterdam, The Netherlands) and confirmed by sequencing.

Transfection of JEG3 cells

JEG3 cells were grown at 37 C in DMEM-F12 medium containing 9% FBS and 100 nM sodium selenite and transfected at 70-80% confluency using 0.3 µl FuGENE 6 transfection reagent (Roche Diagnostics, Almere, The Netherlands) per 100 ng DNA. For T3 uptake studies and immunoblotting, cells were grown in 6-well plates and transfected with 500 ng empty pcDNA3 or pcDNA3 containing wild-type or mutated MCT8 cDNA. 50 ng renilla luciferase vector (pRL-SV40, Promega, Leiden, The Netherlands) was cotransfected to enable correction for transfection efficiency. For intact-cell T3 metabolism assays, cells were cultured in 24-well plates and cotransfected with 100 ng pcDNA3 containing wild-type or mutant MCT8 and 100 ng pCI-Neo containing D3 cDNA (14). For analysis of D3 activity in cell lysates, JEG3 were cotransfected for 48 h with 500 ng wild-type or mutant MCT8 and 500 ng hD3 cDNA in 6-well plates. For immunocytochemistry (ICC), cells were cultured on 15 mm coverslips and transfected with 100 ng cDNA.

T3 uptake and metabolism assays, immunoblotting and immunocytochemistry

Two days after transfection, cells were rinsed with DMEM-F12 medium plus 0.1% BSA. For the T3 uptake assay, the cells were incubated for 5 min at 37 C in 1.5 ml DMEM-F12 / 0.1% BSA containing 1 or 100 nM ($2x10^5$ cpm) [^{125}I]T3 (Amersham Biosciences, Roosendaal, The Netherlands). Incubation was stopped by removing the medium and washing once with DMEM-F12 / 0.1% BSA. Cells were lysed with 0.1 M NaOH, and the lysates were counted in a gamma counter. Renilla luciferase activity was measured in parallel wells according to the manufacturer's protocol.

For the intact-cell T3 metabolism assay, MCT8 and D3 (co)transfected cells were incubated for 4 h at 37 C in 0.5 ml DMEM-F12 / 0.1% BSA containing 1 nM ($1x10^6$ cpm) [^{125}I]T3. After incubation, medium was harvested, and analyzed by HPLC as described previously (14). For analysis of D3 activity in cell lysates, cells were harvested in 0.1 ml PED1 buffer (0.1 M phosphate, pH 7.2, 2 mM EDTA and 1mM DTT) and sonicated. Appropriate dilutions of the sonicates were incubated for 1 h at 37 C with 1 nM ($2x10^5$ cpm) [^{125}I]T3 in 0.1 ml PED10 buffer. Incubations were stopped and samples were analyzed by HPLC. Immunoblotting of transfected cell homogenates was performed as described previously (14). For ICC, cells were fixed and permeabilized with 4% paraformaldehyde and 0.2% Triton X100, and stained with MCT8 specific polyclonal antibody 1306. The plasma membrane was stained using zona occludens 1 (ZO-1) antibody (Invitrogen, Breda, The Netherlands). Alexa fluor 488 and 633 (Invitrogen) were used as detection antibodies; analyses were performed on a Zeiss Axiovert 100 confocal microscope using Zeiss LSM software.

RESULTS

We report here on 9 boys with severe psychomotor retardation, 5 of which have been presented previously (Table 2.1). The clinical phenotypes and serum thyroid parameters of the additional 4 patients are similar to those of patients with *MCT8* mutations described by us and others. All our patients have been identified with severe psychomotor retardation, characterized by axial hypotonia, spastic or flaccid quadriplegia, dystonic movements and absence of speech. Mean serum T4 and FT4 are decreased, TSH is mildly increased, and serum T3 is markedly elevated. Patients are between 2.5 and 18 years old and come from various ethnic backgrounds.

All 9 patients were found to have different hemizygous mutations in *MCT8* (Table 2.1). Patient 1 has a 24,527 bp deletion, stretching from 15 kb upstream to 9 kb downstream of exon 1. Patient 2 has a 671C>T missense mutation in exon 2, resulting in an Ala224Val substitution located in the 2nd putative TMD (9, 15). Patient 3 has a 1412T>C mutation in exon 5, resulting in a Leu471Pro substitution located in the 9th TMD (9). Patient 4 has a 2374 bp deletion which comprises a large part of exon 3, entire intron 3 and exon 4, and part of intron 4. Patient 5 has a nonsense 733C>T mutation in exon 2, resulting in premature translation termination (Arg245stop) (9). Patient 6 has a missense 812G>A mutation in exon 3, resulting in an Arg271His substitution located in the second extracellular loop. Patient 7 has a 14-bp deletion (nucleotides 631-644), resulting in a frame shift and truncation of the protein at amino acid residue 235. Patient 8 has a G>C mutation in the acceptor splice site of exon 3, *i.e.* ACCT instead of AGCT. RT-PCR analysis of mRNA isolated from fibroblasts of his affected brother indicated the loss of 282 nucleotides from exon 3 and, thus, of 94 amino acids, including TMDs 4-6. Patient 9 has a 3-bp (TCT) deletion in exon 2, leading to deletion of Phe230.

Figure 2.1 A shows the uptake of T3 by JEG3 cells transfected with wild-type or mutant MCT8 cDNA after 5 min of incubation at 37 C. Significant T3 uptake was observed in empty pcDNA3-transfected cells. JEG3 cells do not show endogenous expression of MCT8 (14). Therefore, this background uptake is likely facilitated by other, as yet unidentified, transporter(s). Transfection with wild-type MCT8 increased T3 uptake 2.8 fold. Transfection of cells with MCT8 mutants Ala224Val, Leu471Pro, Arg245stop, splice site mutant ex3 -1G→C and delPhe230 did not increase T3 uptake over control cells. However, transfection with the MCT8 Arg271His mutant induced a modest, but significant 1.4-fold increase in T3 uptake ($p<0.05$). Uptake experiments using 100 nM T3 produced similar results (data not shown).

JEG3 cells transfected with control plasmid or MCT8 cDNA alone did not show significant metabolism of T3, indicating very low deiodination capacity of these cells (not shown). Figure 2.1 B shows that transfection with D3 cDNA resulted in 11% metabolism of T3 after 4 h. Cotransfection of cells with wild-type MCT8 and D3 greatly increased T3 metabolism to

Table 2.1 Serum thyroid hormone levels in MCT8 patients

	Patient								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Year of birth	1997	1999	2001	1998	2002	1987	1995	1999	2002
Mutation	del ex 1	671C→T	1412T→C	del ex 3,4	733C→T	812G→A	del 631–644	ex3 -1G→C	del 689-691
Protein	absent	A224V	L471P	truncated	R245X	R271H	truncated	del 267–370	del F230
T3 (N 1.40 – 2.55 nmol/l)	6.10	5.44	4.51	5.20	7.28	3.95	3.45	3.17	5.46
T4 (N 54 – 127 nmol/l)	58	57	56	58	35	44	36	42	88
FT4 (N 11 – 25 pmol/l)	9.0	9.3	8.2	11.2	5.7	10.6	9.6	8.8	15.1
TSH (N 0.10 – 4.0 mU/l)	8.79	3.76	1.81	3.99	4.72	1.66	1.73	2.97	0.20
In vitro **function (% of wild-type)**	0*	0	0	0*	0	10-20	0*	0	0

*) synthesis of functional protein impossible.

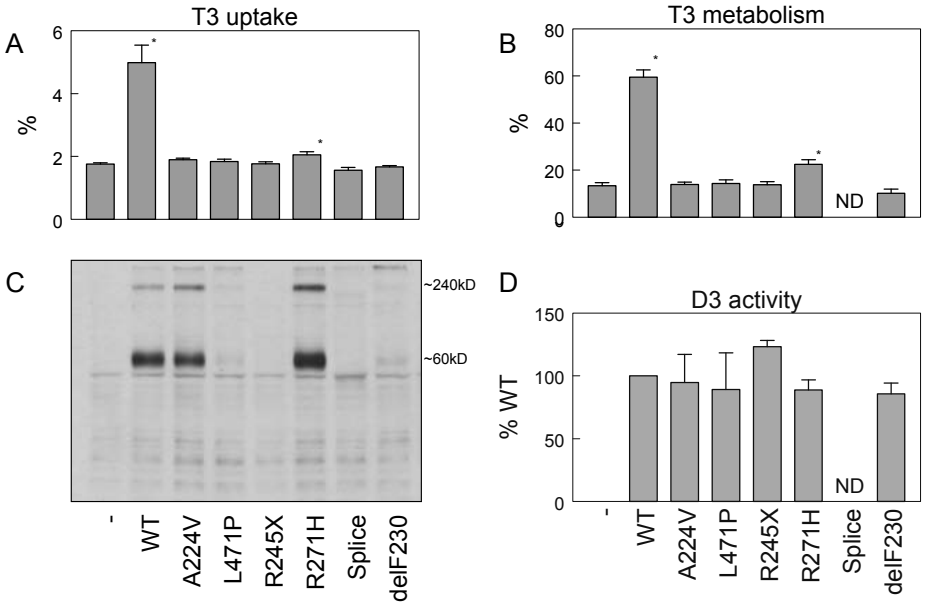

Figure 2.1 A. T3 uptake in wild-type or mutant MCT8-transfected JEG3 cells, shown as percentage of added T3 after 5 min incubation. Transfection of wild-type MCT8 induces uptake ~2.8 fold compared to empty vector transfected controls. Most mutants do not induce uptake, but Arg271His mutant induces a significant 1.4-fold increase. **B.** Metabolism of T3 in intact JEG3 cells co-transfected with wild-type or mutant MCT8 and D3. Metabolism is shown as percentage of metabolites (3,3'-T2 and 3'-T1) in the medium after 4 h incubation. Wild-type MCT8 induces metabolism ~6 fold and Arg271His ~2 fold. The other mutants do not show induction of metabolism. **C.** Western blot of homogenates of JEG3 cells transfected with wild-type or mutant MCT8. Specific bands of ~60kD and ~240 kD, representing monomeric MCT8 and a MCT8-containing protein complex, are detected clearly in wild-type, Ala224Val and Arg271His transfected cells. Less protein is detected for Leu471Pro and delPhe230; no expression is seen of splice site mutant ex3 -1G→C and Arg245stop. **D**. D3 activity in cell lysates, expressed as percentage of activity in cells co-transfected with wild-type MCT8 and D3. No significant differences in D3 activity are observed, indicating that reduced metabolism in intact cells transfected with mutant MCT8 is due to decreased intracellular availability of T3, not to differences in D3 activity. Uptake and metabolism data are presented as mean±SE of 4 experiments, * $p<0.05$ versus control.

~60%. Cotransfection of cells with the MCT8 mutants Ala224Val, Leu471Pro, Arg245stop or delPhe230 and D3 did not increase T3 metabolism compared to D3 transfection alone. However, cotransfection with the MCT8 Arg271His mutant and D3 showed 20% metabolism, again indicating that some T3 transport is preserved in this mutant.

D3 activity in lysates of JEG3 cells cotransfected with the different MCT8 mutants amounted to 85-123% of that in cells cotransfected with wild-type MCT8 (Figure 2.1 D), indicating that the impaired T3 metabolism in intact cells cotransfected with mutant versus wild-type MCT8 is indeed due to inhibited T3 uptake rather than decreased D3 expression. Immunoblotting (Figure 2.1 C) showed marked expression of wild-type MCT8 and of mutants Ala224Val and Arg271His, little expression of Leu471Pro and delPhe230 and

no expression of splice site mutant ex3 -1G→C and Arg245stop. Control cells transfected with pcDNA3 also showed no expression of MCT8. ICC demonstrated marked plasma membrane expression of wild-type MCT8 and mutant Arg271His, whereas Ala224Val was mainly localized in the cytoplasm. Leu471Pro, ex3 -1G→C and delPhe230 showed very little expression of protein (data not shown).

DISCUSSION

We present 9 unrelated young males with severe X-linked psychomotor retardation and elevated serum T3 levels with mutations in the *MCT8* gene. We show that these mutations result in loss of function, demonstrated as reduced uptake and subsequent metabolism of T3 *in vitro*. Several mechanisms may be involved in this loss of function, including reduced protein expression, impaired trafficking to the plasma membrane or reduced substrate affinity. The mutant Ala224Val protein is clearly detectable by IB. However, ICC shows the protein to be mostly distributed in the cytoplasm, suggesting that this mutation inhibits trafficking to the plasma membrane. Much less Leu471Pro protein is detected by IB and ICC, suggesting that loss of function is correlated with reduced expression of the protein. The premature Arg245stop found in patient 5 results in a severely truncated MCT8 protein that cannot be detected with our polyclonal MCT8 antibody, and does not have any functional activity. Mutant ex3 -1 G→C is not detected by IB, and only very limited protein is observed in ICC. This suggests that the expression of this splice variant, although clearly detectable at the RNA level, is very limited at the protein level. The lack of T3 transport by mutant delPhe230 can also be explained by the low expression of this protein. Mutant Arg271His shows significant residual transport capacity. IB shows high expression of the protein, and ICC indicates expression at the plasma membrane. Possibly, the partial loss of function is caused by reduced affinity for T3.

Our findings associate the psychomotor retardation observed in MCT8 patients with loss of T3 transport capacity. This illustrates that MCT8 is crucial for normal thyroid hormone-dependent development of the CNS in humans. Thyroid hormone plays a crucial role in processes such as cell migration, dendritic outgrowth, the formation of synapses and myelination (16). Neurons are the major target cells for thyroid hormone, expressing T3 receptors (17), D3 and MCT8 (8). Loss of function mutations in MCT8 lead to reduced or absent supply of T3 to neurons, resulting in impaired neurological development as well as a reduced clearance of T3 by neuronal D3. The role of MCT8 in neuronal T3 uptake was recently studied in MCT8 knockout mice by Dumitrescu *et al.* (18) and Trajkovic *et al.* (19). They show reduced T3 concentrations, increased D2 activity and reduced D3 activity in brain, reflecting local hypothyroidism, in spite of elevated serum T3. Trajkovic *et al.* also show increased expression of the thyroid hormone-downregulated gene *TRH*, and reduced

expression of the positively regulated gene *RC3* in neurons, supporting hypothyroid state at the cellular level. It must be noted, however, that although MCT8 deficient mice show reduced T3 concentrations in the brain, they do not show an apparent neurological phenotype. This suggests differences in the role of MCT8 in the development of the CNS between the two species.

In conclusion, the experiments presented here support the hypothesis of reduced supply of T3 to neurons in patients with mutations in *MCT8*. The severe psychomotor retardation observed in these patients clearly illustrates the important role of thyroid hormone in human neuronal development.

ACKNOWLEDGEMENTS

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Chapter 3

Tissue-specific thyroid hormone status in patients with mutations in MCT8

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Submitted

ABSTRACT

Objective. For proper thyroid hormone (TH) action, essential for the function of numerous organs and the development of the CNS, transport over the plasma membrane is required. Monocarboxylate transporter 8 (MCT8), an active and specific TH transporter, is expressed in numerous tissues, including the CNS. Mutations in MCT8 cause severe X-linked psychomotor retardation and elevated serum T3 levels, known as Allan-Herndon-Dudley syndrome (AHDS). We hypothesize that, like the brain, MCT8-expressing tissues are hypothyroid, whereas the elevated serum T3 exposes tissues expressing other transporters to excess TH.

Study design. We performed detailed clinical and biochemical studies on AHDS patients from 8 families, examining serum markers for thyroid hormone status in liver, muscle, bone and the hypothalamus-pituitary-thyroid axis.

Results. Patients show severe psychomotor impairment, with axial hypotonia, spastic quadriplegia, dystonic movements and lack of speech. Reduced muscle and fat mass, mild tachycardia and delayed bone age are observed. Serum SHBG and lactic acid are elevated, whereas (HDL) cholesterol, creatine kinase and bone alkaline phosphatase are low or low normal, and Ntx levels are normal.

Conclusions. Mutations in MCT8 and the associated rise in serum T3 lead to hypothyroidism in the brain, thyrotoxicosis of liver and muscle, and eu- or hypothyroidism of bone in AHDS patients. This demonstrates the essential role of transporters in the tissue-specific regulation of TH bioactivity.

INTRODUCTION

Thyroid hormone is essential for the development of the CNS, and for the regulation of metabolism in many tissues (1, 2). Thyroid hormone mainly acts through binding of 3,3',5-tri-iodothyronine (T3) to its nuclear receptors. This leads to increased or decreased transcription of T3-responsive genes, regulating the expression of numerous proteins and enzymes (3).

In recent years it has become increasingly clear that thyroid hormone requires functional transporters to facilitate the cellular influx over the plasma membrane. (4, 5). Several transporter proteins have been identified, amongst which members of the organic anion transporting polypeptide (OATP) family, the sodium taurocholate co-transporting polypeptide (NTCP) family and members of the heterodimeric amino acid transporter (HAT) family (6). MCT8 and MCT10, members of the monocarboxylate transporter family (SLC16), have recently been identified as active and specific thyroid hormone transporters (7, 8). In a number of recent studies, we and others have reported on patients with loss-of-function mutations in the MCT8 gene (SLC16A2), located on chromosome Xq13.2 (9-17). Affected males show severe psychomotor retardation in combination with elevated serum levels of T3. This X-linked condition is known as Allan-Herndon-Dudley syndrome (AHDS, OMIM no. 300523). In AHDS patients, decreased uptake of thyroid hormone in MCT8 expressing neurons appears to impair the thyroid hormone dependent brain development.

MCT8 is not only expressed in neurons, but in numerous human tissues, including liver, skeletal muscle, heart, bone, placenta, lung, and kidney. Like in the brain, loss of MCT8 function might lead to a hypothyroid state in these tissues. However, as serum T3 levels are increased, tissues expressing other transporters might be exposed to excess thyroid hormone, resulting in local thyrotoxicosis. In the present study we present a detailed description of AHDS patients in four families. We provide insight into the effects of MCT8 mutations on the feedback of the HPT axis and examine tissue-specific thyroid hormone status in liver, muscle and bone in a group of 8 patients.

PATIENTS

Family A

The first patient identified in this family (patient A1) was born at term in 1997 as the 5th child of consanguineous parents. Pregnancy and delivery were uncomplicated. At the age of 2 mo, he presented with general hypotonia and developmental delay. There was severe head lag, no eye fixation or pursuit movements, and no smile. Screening for congenital

infections, chromosomal defects and metabolic diseases revealed no abnormalities. MRI at the age of 12 mo showed delayed myelinization but normal brain structures.

Endocrine evaluation at the age of 3 mo showed a serum TSH of 4.9 mU/l (normal [N] 0.1-4.0), FT4 of 9.3 pmol/l (N 11-25) and total T3 of 6.1 nmol/l (N 1.4-2.55). A TRH stimulation test gave a low-normal TSH response. Treatment with L-thyroxine had no effect on serum thyroid hormone levels or clinical symptoms, and was discontinued after several months.

At the age of 7 yr, psychomotor development is largely absent. The patient is unable to sit without support, and cannot stand, walk or talk. Generalized epilepsy with tonic and tonic-clonic seizures, occurring form the age of 2 yr, is treated with valproic acid and lamotrigine. In addition, patient receives mild laxatives for constipation. Physical examination shows frequent dystonic movements of the arms and spontaneous, uncontrolled activity of the facial muscles. Primitive reflexes, such as glabella reflex, snout reflex and asymmetric tonic neck reflex (ATNR), are easily elicited or occur spontaneously. Intentional movement is absent. Patient shows dysmorphic features, including a flat occiput, secondary microcephaly (head circumference 46 cm, << -2.5 SD), a narrow, pointed nose and microphthalmia and cataract of the right eye. He is blind. Low muscle and fat mass are observed, but no other signs of hyper- or hypothyroidism.

An older brother (patient A2), born in 1988, shows similar clinical symptoms, including proximal hypotonia, lack of voluntary movements, secondary microcephaly and severe developmental retardation. No dystonic movements are observed, and vision and eye movements are normal. He makes eye contact, and responds to people by smiling and making sounds. Physical examination shows mild tachycardia, but no other signs of hyper- or hypothyroidism. Pubertal development at age 17 is limited to pubic hair Tanner stage 3, with cryptorchidism and a prepubertal genital. Serum analyses at 16 yr of age show elevated T3 of 5.0 nmol/l (N 1.5-2.5), reduced FT4 of 8.1 (N 11-25) and normal TSH of 2.1 (N 0.1-4.0). A third patient in this family, born in 1984, died at the age of 5 yr. He is reported to have had similar neurological symptoms as his brothers.

PCR Analysis of MCT8 in patients A1 and A2 showed that it was impossible to amplify exon 1 using various standard primer sets, indicating a deletion of at least the coding sequence of this exon. We identified the borders of this deletion containing exon1 by using forward primer F-DELfamA located upstream of MCT8, and reverse primer R-DELfamA located in intron 1. A 321-bp PCR fragment was amplified in the patients and their mother, but not in the father and a female control. Sequence analysis of the PCR product identified the exact size (24,527 bp) and location of the deletion, stretching from 15 kb upstream to 9 kb downstream of exon 1 (Figure 3.1).

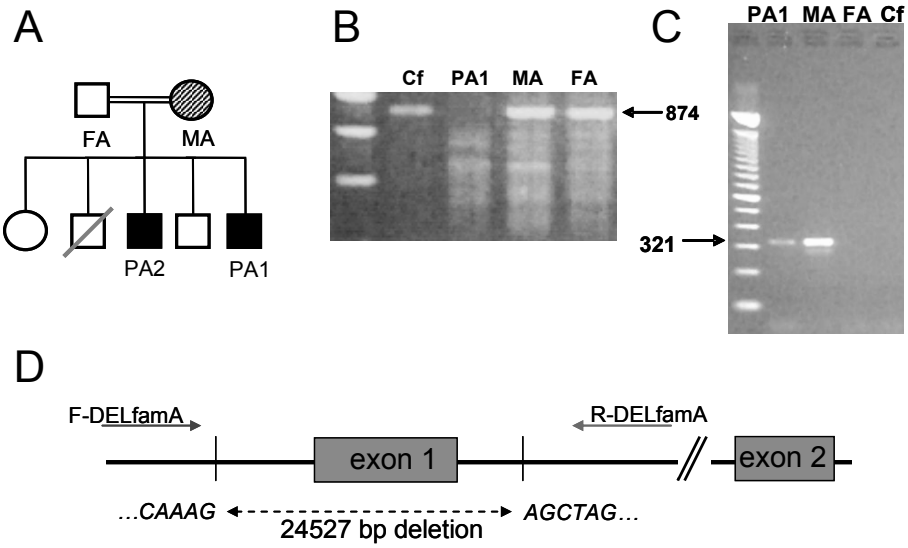

Figure 3.1 A. Pedigree of family A. B. PCR products of exon 1 from a female control (Cf), patient A1 (PA1), his mother (MA) and his father (FA) using standard primers. In patient A1, exon 1 is not amplified. This was later repeated for patient A2 (not shown). **C**. PCR products of patient A1, mother A, father A and a female control using primers F-DELfamA and R-DELfamA. A 321 bp PCR product was formed in patient A1, patient A2 (not shown) and mother A, proving she is carrier of the deletion. **D**. Schematic representation of the 24,527 bp deletion in MCT8 in family A.

Family B

The patient in this family was born in 1998. After an uncomplicated pregnancy, delivery was by caesarean section due to breech position and failure to progress. At 4 mo of age, he presented with axial hypotonia, movement induced hypertonia of arms and legs, and developmental delay. Thyroid evaluation showed serum TSH of 6.2 mU/l (N 0.7-5.7), FT4 of 5.2 pmol/l (N 10-27), and T3 of 5.2 nmol/l (N 1.6-4.1). Treatment with L-thyroxine (57-112 µg/d) suppressed TSH, but did not normalize serum FT4, while serum T3 remained elevated (Figure 3.2). High-dose L-thyroxine treatment caused hypertension, which disappeared when the dose was adjusted. MRI showed normal intracranial structures, but delayed myelination at the age of 5 yr.

At age 7 yr, psychomotor impairment is severe. There is axial hypotonia and spastic tetraplegia. The patient cannot sit, stand, walk, or hold his head, and intentional movement is absent. Speech has not developed, but there is interaction with his surroundings by smiling and making sounds. There is no evidence of sensory involvement. Ophthalmologic evaluation is normal, with unremarkable eye movements, and no signs of nystagmus, strabismus or cataract. Height is at the 5th percentile and weight is at the 10th percentile. Bone age is approximately 5 yr, which is <-2 SD for his chronological age. Head circumference is normal. Patient is thin, but shows no other physical signs indicating hyper- or hypothyroidism.

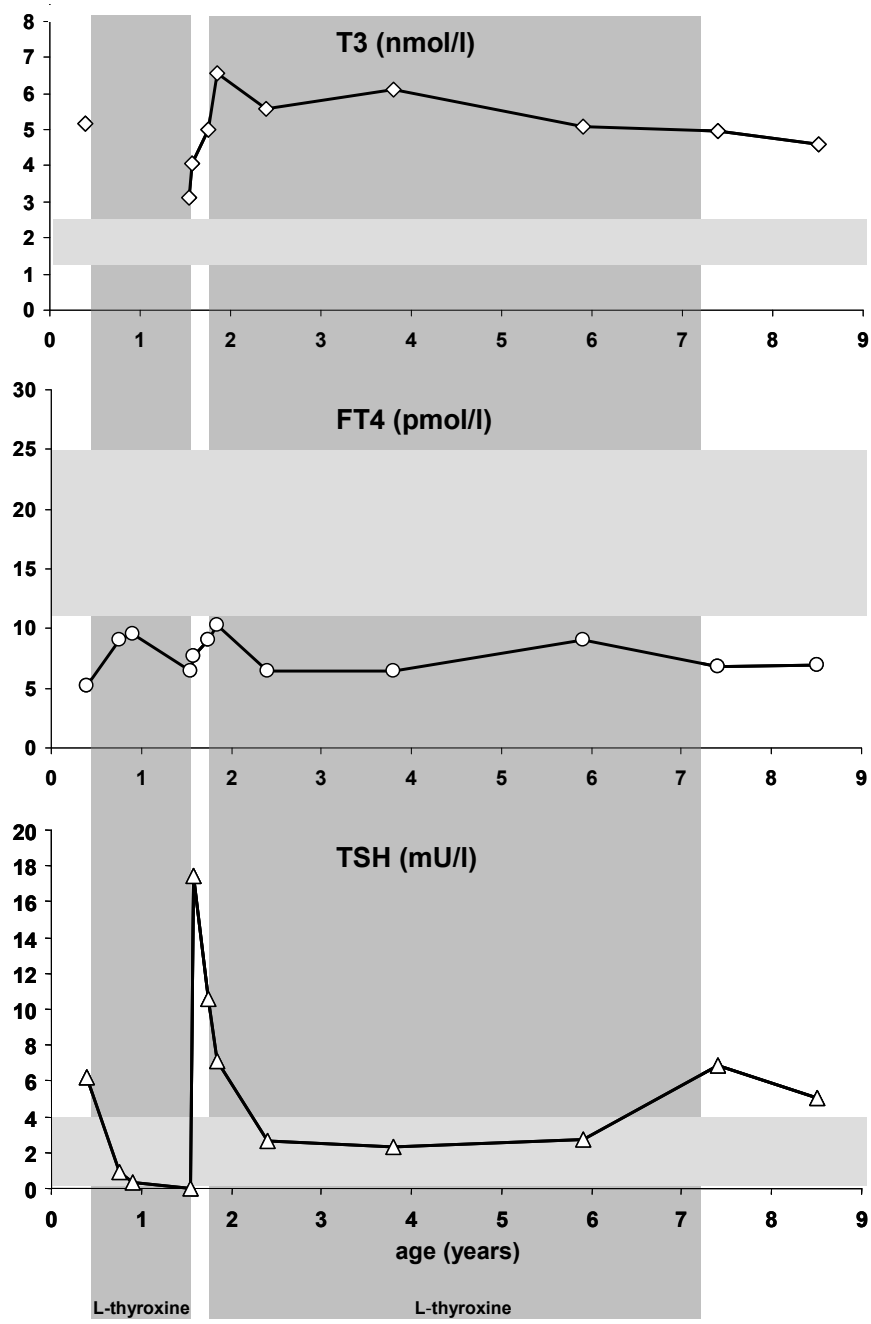

Figure 3.2 T3, FT4 and TSH measurements in patient B. Light gray areas indicate reference ranges, dark gray indicates treatment with L-T4. Between 0.5 and 1.6 years, high dose treatment (50-112 μgr/day) was given, between age 1.8 and 7.2 years 57 μgr/day. Treatment suppresses TSH, but does not normalize T3 and FT4.

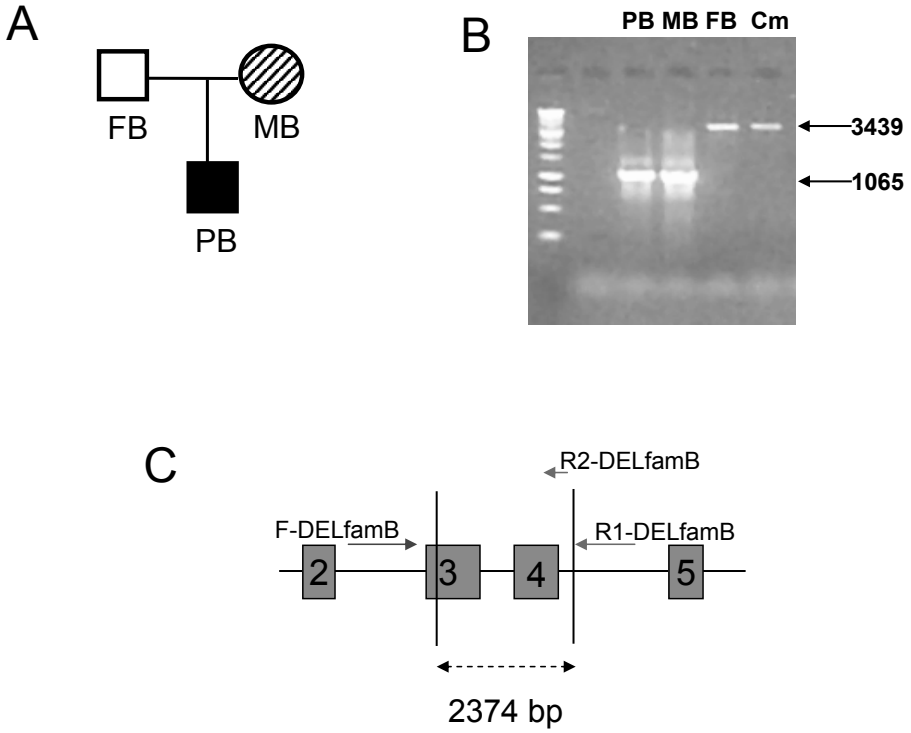

Figure 3.3 A. Pedigree of family B. **B**. PCR products of patient B, mother B, father B and a male control using primers F-DELfamB and R1-DELfamB showing a 2374 bp size difference in mutated vs. non-mutated DNA. In mother B, only the smaller, mutated allele is amplified due to preferential amplification of small fragments. However, using forward primer F-DELfamB with reverse primer R2-DELfamB, located within the deleted area, a DNA fragment of appropriate size was amplified in mother 4, indicating that she is a heterozygous carrier (data not shown). **C**. Schematic representation of the 2374 bp deletion in family B.

PCR analysis of the MCT8 exons showed a deletion encompassing exons 3 and 4. Using forward primer F-DELfamB upstream of exon 3 and reverse primer R-DELfamB downstream of exon 4, a ~1.1 kb fragment was amplified in patient 4 and his mother instead of the ~3.4 kb fragment obtained in his father and healthy controls. Sequence analysis of the short fragment allowed the identification of the exact size (2374 bp) and location of the deletion, which comprises a large part of exon 3, entire intron 3 and exon 4, and part of intron 4 (Figure 3.3).

Family C

The patient identified in this family was born in 1987. He shows severe psychomotor retardation, including hypotonia of the muscles of the neck and back and quadriplegia with

brisk deep tendon reflexes. Speech development is absent. The face shows dysmorphic features, including a high, arched palate and a straight, long mandibula. MRI of the brain showed atrophy of the vermis, and enlarged cerebral ventricles and frontal pericerebral spaces. Also, microphtalmia of both eyes is noted. This patient suffers from severe feeding problems causing malnutrition (BMI 11.4). Fluoxetine is given to increase appetite. Dystonic crises occurred frequently when patient was younger, but now they are rare.

At age 17 yr, there is delayed onset of puberty, with no clinical signs of pubertal development and pre-pubertal testosterone levels. Serum analysis shows an increased serum FT3

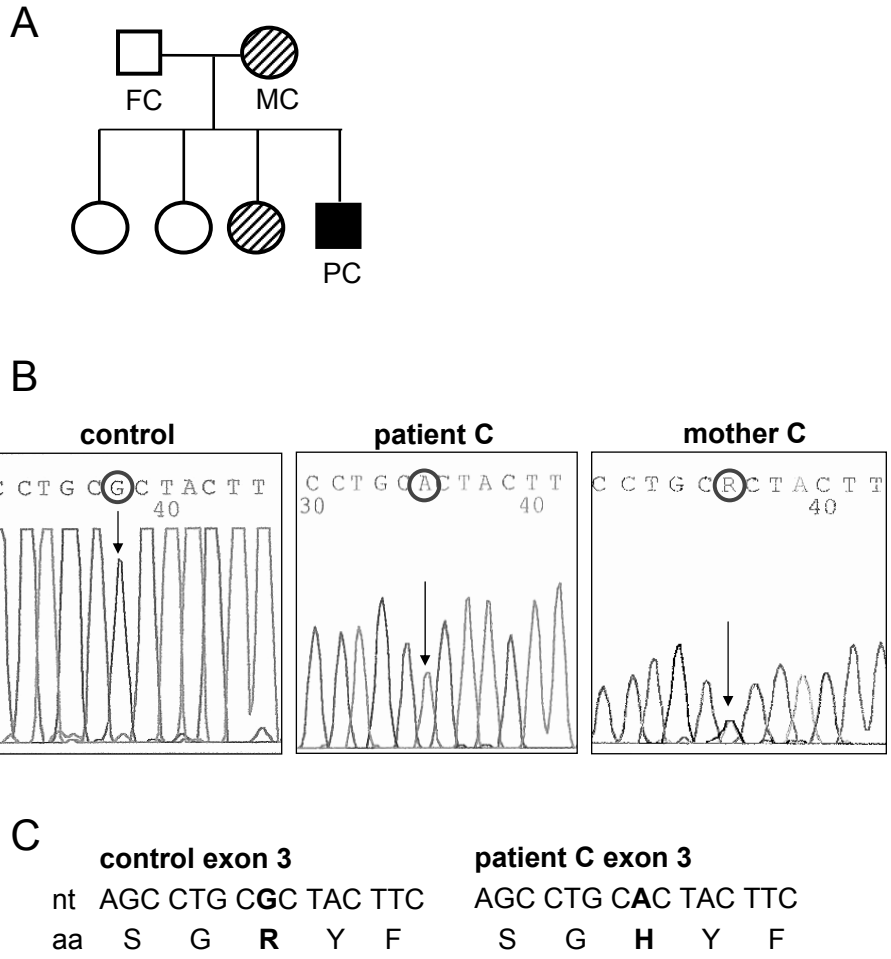

Figure 3.4 A. Pedigree of family C. **B**. Sequence profile of exon 3 in a control, patient C and mother C, showing a G to A mutation. Mother and one sister (not shown) are identified as carrier of the mutation. For color figure see page 193. **C**. Nucleotide and amino acid sequences of exon 3 in a control subject and patient C. The G to A nucleotide mutation and deduced Arg to His amino acid substitution are indicated in bold.

of 10.7 pmol/l (N 3.3-6.1), a normal FT4 of 13.2 pmol/l (N 10.5-25.5) and a normal TSH of 1.5 mU/l (N 0.4-3.6). Physical examination shows profound cachexia, with limited muscle and fat mass, and a relatively high heart rate (110/min).

The coding sequence of MCT8 was amplified and sequenced, demonstrating a missense 812G>A mutation in exon 3, resulting in an Arg271His substitution in the second extracellular loop. The mother and 1 of his 3 sisters were found to be carrier of the mutation (Figure 3.4).

Family D

The first patient identified in this family (patient D1) was born in 1999 after an uneventful pregnancy. Delivery at 43 wk gestation was complicated by progress difficulties, resulting in a mild lesion of the left plexus brachialis. Birth weight was 5070 g. In the first months, he presented with axial hypotonia and dystonic, choreoathetoid movements and hypertonia of the extremities. At age 4 yr, psychomotor development is severely impaired, although this patient is able to crawl and walk with support. He uses his hands to grasp and to make signs to support non-verbal communication. Severe dystonic crises occur, which are treated with intrathecal baclophen infusion. A mild epileptic disorder with onset at 3.5 yr is characterized by short-lasting tonic absences followed by short myoclonic fits. With lamotrigene the patient is seizure-free. MRI at 4 yr shows mild general atrophy, and a moderate signal increase in the white matter.

Because of eating difficulties causing malnutrion, a gastrostomy feeding tube was introduced. However, even with a high nutrient intake, it is difficult to increase or maintain body weight. Thyroid function tests were: TSH 6.6 mU/l (N 0.3-4.7), FT4 8.5 pmol/l (N 12-22), and FT3 12 pmol/l (3.1-6.9). Repeatedly, peripheral hypertension was noted.

Patient D2, born in 2002, showed a similar but more severe clinical phenotype. The neonatal period was characterized by prolonged hyper-bilirubinemia and failure to thrive. At age 2 mo, there was axial hypotonia, head-lag, and no smile nor eye contact. Intermittent, severe hypertonia of the arms and legs was triggered by tactile stimulation, such as (un)dressing. At 1 yr of age, head control had improved, and patient laughed and made good eye contact. ATNR was present in both directions, and deep tendon reflexes were very brisk. Small, irregular nystagmoid eye movements and a small outward squint were observed. Dystonic crises persisted, which were treated with diazepam. Thyroid parameters included serum TSH ranging from 1.1 to 4.6 mU/l (N 0.3-4.7), FT4 8.4 to 16 pmol/l (N 12-22) and FT3 7.7 to 16 pmol/l (N 3.1-6.9). At 2 yr of age, patient was able to hold his head steady, and he used his hands a little. Following febrile episodes, patient suffered two severe dystonic crises with hyperthermia, dehydration, decreased consciousness and rhabdomyolysis, which were treated with rehydration and i.v. midazolam. A month after the second crisis, this patient died at home.

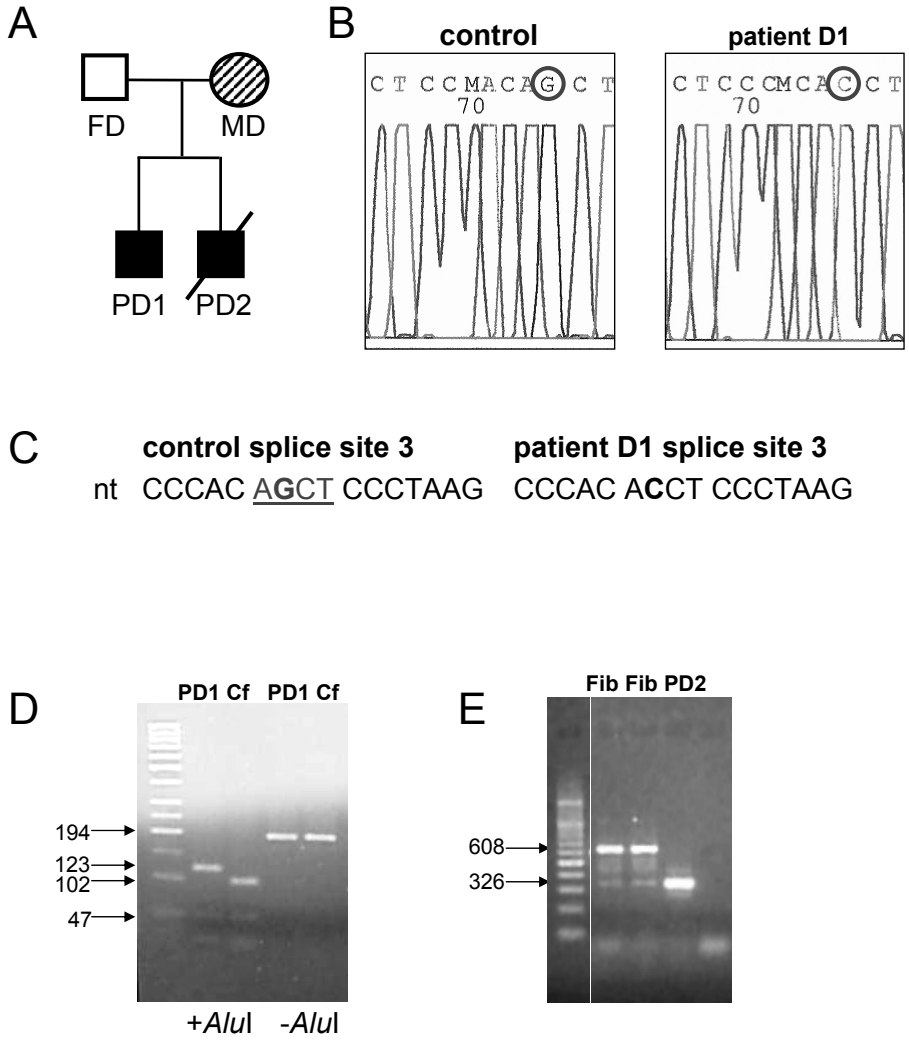

Figure 3.5 A. Pedigree of family D. **B.** and **C.** Sequence profile of the border of intron 2 and exon 3 in patient 8 and a control, identifying a G to C mutation in the splice-site (AGCT). This site is also a A*lu*I restriction site, which is inactivated by the mutation. For color figure see page 193. **D.** RFLP analysis of a 194 bp PCR product in patient D1 and a control obtained using primers F-EX3famD in intron 2 and R-EX3famD in exon 3. This fragment contains 3 A*lu*I restriction sites, one of which is inactivated by the mutation. **E.** RT-PCR products from fibroblast RNA of patient D2 and controls using primers F-FIBfamD, located in exon 1 and R-FIBfamD located in exon 3. A 326 bp fragment was amplified from patient D2, but not the expected 608 bp. The 326bp splice variant RNA is also present in the control fibroblasts.

Sequence analysis of MCT8 in patient D1 revealed a G to C mutation in the acceptor splice site of exon 3, i.e. ACCT instead of AGCT. The consequences of this mutation were investigated by RT-PCR of RNA isolated from preserved fibroblasts of patient D2. Using forward primer F-FIBD2 in the coding sequence of exon 1 and reverse primer R-FIBD2 at

the end of exon 3, we amplified a PCR product of 326 bp, instead of the expected 608 bp. Sequence analysis of this PCR product indicated the use of an alternative splice site (AGCT), located within exon 3, 282 nt downstream from the mutated splice site. Interestingly, this 326 base pair PCR fragment was also found in control fibroblasts, suggesting that this MCT8 mRNA splice variant is also generated in normal subjects. The MCT8 mutation in patient 8 and his brother thus results in the loss of 94 aa, including transmembrane domains 4-6. No DNA was available for analysis from the parents, but clearly the mother is carrier of the mutation (Figure 3.5).

Biochemical evaluation

To investigate the effects of mutations in MCT8 and the high serum T3 levels on the tissue-specific thyroid hormone status we evaluated the HPT axis and TH dependent markers of liver, muscle and bone function. Table 3.1 shows the results obtained in the patients presented above and other AHDS patients we previously identified (18). TH function tests in AHDS patients show high serum T3, low or low-normal serum FT4, normal to elevated TSH levels and a decreased serum rT3 (Table 3.1; Figure 3.6 A). Based on the low or low-normal FT4 levels, several AHDS patients have been treated with L-thyroxine. This treatment has not shown beneficial effects on clinical symptoms, but did increase heart rate, blood pressure and sweating in some patients. Treatment suppressed TSH in patient C and several other patients, without affecting normalizing FT4 and T3. A normal TSH response to TRH administration was observed in patients A1, patient B and several other patients. Abnormal responses have not been observed.

As measure for hepatic thyroid hormone status, serum sex hormone-binding globuline (SHBG), total cholesterol and HDL-cholesterol levels were determined. SHBG levels, measured in patients, carriers and unaffected family members, were increased in most patients, and showed a strong positive correlation with serum T3 (Figure 3.6 B) In patients, we found low or low-normal cholesterol, and reduced HDL-cholesterol levels. Lactic acid was moderately increased; creatine kinase (CK) was in the low-normal range or decreased. Bone specific alkaline phosphatase (BAP), a marker for bone formation, was low-normal in most patients; bone resorption marker collagen type 1 cross-linked N-telopeptide (Ntx) was within the normal range (Table 3.1).

DISCUSSION

MCT8 is an active transporter of thyroid hormones, expressed in the CNS (7, 19). In several recent publications, the X-linked Allan-Herndon-Dudley syndrome (AHDS) has been associated with elevated serum T3 levels and mutations in MCT8. We provided a functional basis

Table 3.1. Serum markers of thyroid hormone action in AHDS patients

	Patient							
	A1	**B**	**C**	**D1**	**e**	**f**	**g**	**h**
Mutation	**del ex 1**	**del ex 3,4**	**R271H**	**ex3 -1G→C**	**R245X**	**del 631–644**	**L471P**	**del F230**
Age at evaluation	**1.7 years**	**5 years**	**17 years**	**6 years**	**1.7 years**	**9.2 years**	**2.2 years**	**2.7 years**
T3 (nmol/l)	**6.10** (1.40-2.55)	**5.20** (1.40-2.55)	**3.95** (1.40-2.55)	**3.17** (1.40-2.55)	**7.28** (1.40-2.55)	**3.45** (1.40-2.55)	**4.51** (1.40-2.55)	**5.46** (1.40-2.55)
T4 (nmol/l)	**58** (54-127)	**58** (54-127)	**44** (54-127)	**42** (54-127)	**35** (54-127)	**36** (54-127)	**56** (54-127)	**88** (54-127)
FT4 (pmol/l)	**9.0** (11- 25)	**11.2** (11- 25)	**10.6** (11- 25)	**8.8** (11- 25)	**5.7** (11- 25)	**9.6** (11- 25)	**8.2** (11- 25)	**15.1** (11- 25)
TSH (mU/l)	**8.79** (0.10-4.0)	**3.99** (0.10-4.0)	**1.66** (0.10-4.0)	**2.97** (0.10-4.0)	**4.72** (0.10-4.0)	**1.73** (0.10-4.0)	**1.81** (0.10-4.0)	**0.20** (0.10-4.0)
SHBG (nmol/l)	-	**318** (34-141)	**303** (20-77)	**73.8** (34-141)	**385** (20-114)	**232** (30-169)	**309** (20-114)	**194.6** (20-114)
total cholesterol (mmol/l)	**1.8*** (2.20-4.21)	**2.9** (2.57-5.46)	**3.0** (2.81-4.89)	**2.1** (2.81-4.89)	**3.1** (2.57-5.46)	**3.4** (2.82-5.27)	**3.2** (2.57-5.46)	**2.7** (2.57-5.46)
HDL-cholesterol (mmol/l)	-	-	**0.42** (0.65-1.81)	**0.21** (0.65-1.92)	**0.21** (0.67-1.65)	**0.56** (0.65-1.92)	**0.17** (0.67-1.65)	**0.39** (0.67-1.65)
Lactic acid (mmol/l)	**2.95*** (0.5-2.2)	**2.4** (0.6-2.4)	**2.52** (0.55-2.4)	-	**3.7** (0.7-2.5)	-	-	-
Creatine Kinase (U/l)	**53*** (24-170)	**78** (30-150)	**22** (33-145)	**20** (30-150)	**31** (30-150)	-	**29** (30-150)	**32** (30-150)
Bone Alk. Phos. (U/l)	-	**-**	**35** (30-89)	**66.3** (63-135)	**78.9** (63-132)	**3.3** (63-135)	**69.7** (62-132)	**60.9** (63-135)
Ntx (nmol/l)	-	**-**	**39.3** (-2SD – 2SD)	**29.7** (-2SD – 2SD)	**-**	**48.6** (-2SD – 2SD)	**-**	**-**

age specific reference ranges:

SHBG (39); cholesterol / HDL-cholesterol (40); lactic acid: local reference ranges;CK (41); BAP (42); Ntx (43).

For conversion to metric units, divide by the following factors: T_3: 0.0154; (F)T_4: 12.9; SHBG: 0.0288; total and HDL-cholesterol: 0.0259

* determined at age 3 months. Thyroid hormone and TSH values have been published previously (18).

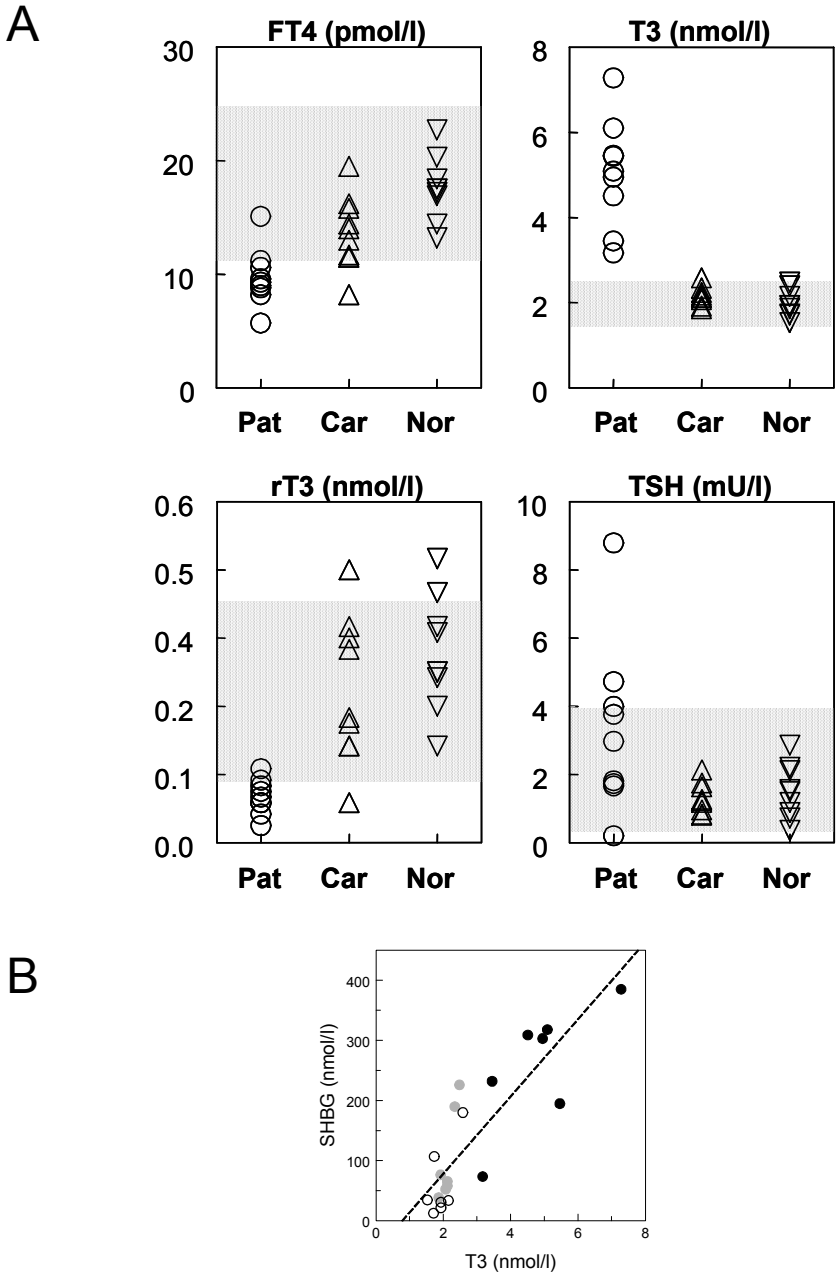

Figure 3.6 A. Serum T4, FT4, T3, rT3 and TSH levels in 9 patients (Pat), carrier females (Car), and unaffected family members (Nor). Included are data from a patient with mutation A224V, described in detail previously (14). Shaded areas represent the normal ranges in healthy individuals. Patient B with was treated with 3.8 µg T4/kg/d; Patient g with 50 µg T4/d; and patient h with 25 µg T4/d. **B.** Correlation between serum levels of T3 and SHBG in patients (black dots), carriers (gray dots) and controls (open dots).

for this association by demonstrating that these mutations lead to loss of T3 transport capacity (18). This results in reduced or absent supply of T3 to central neurons, causing severe psychomotor retardation. The neurological and cognitive impairment of AHDS patients, extensively described in a recent review by Schwartz and Stevenson (20), demonstrates that MCT8 plays a vital role in normal human brain development.

As MCT8 is expressed ubiquitously throughout the human body (21, 22), it is possible that in AHDS patients, not only the brain, but also other tissues are subject to decreased supply of T3. On the other hand, the high levels of serum T3 could be thyrotoxic to tissues that express other transporters, such as OATPs, NTCP, or MCT10 (8, 23). We hypothesize that mutations in MCT8 lead to a tissue-specific hypo-, eu- or hyperthyroid state, depending on the particular repertoire of thyroid hormone transporters that is expressed. We studied in detail the effects of impaired MCT8 function and elevated serum T3 on the hypothalamus-pituitary-thyroid axis, the liver, muscle and bone.

Increased serum T3 levels, a diagnostic hallmark of AHDS, result from reduced T3 clearance and/or increased T3 production. T3 is importantly cleared via deiodination by D3 expressed in neurons. Thus, impaired neuronal T3 uptake might lead to reduced clearance. Furthermore, T3 stimulates hepatic D1 activity, increasing the deiodination of T4 to T3 (24). This will not only further increase serum T3 levels, but also reduce FT4, as is observed in many AHDS patients. Indeed, increased hepatic D1 mRNA and activity are observed in MCT8 knockout mice by Trajkovic *et al.* (25) and Dumitrescu *et al.* (26).

In spite of reduced FT4 levels and increased T3 levels, TSH in AHDS patients is mostly within the normal range, or only slightly elevated. Several factors contribute to this unusual equilibrium. MCT8 is expressed in hypothalamus and pituitary, and is therefore likely to play a role in thyroid hormone feedback (27). Both serum T4, through local D2-mediated conversion to T3, and serum T3 exert negative feedback on TSH secretion. The lack of TSH suppression by the highly elevated T3 levels suggests that mutations in MCT8 lead to impaired T3 sensing of the hypothalamus and pituitary. This is confirmed in TSH suppression experiments in MCT8 knockout mice (25, 26). In hypothyroid (i.e. methimazole or PTU treated) MCT8 knockout mice, 5 to 6-fold higher T3 doses were needed to suppress TSH than in hypothyroid wild-type controls. In AHDS patients, hypothalamic and/or hypophyseal insensitivity to T3 is illustrated also by the fact that treatment with L-thyroxine (or a combination of T4 and T3) does suppress TSH in several patients, whereas T3 alone does not (14). Studies by Trajkovic *et al.* in MCT8 knockout mice indicate that this reduced negative feed-back of T3 is predominantly located at the hypothalamic level, where TRH expression shows little inhibition after T3 administration (25).

We found elevated serum levels of SHBG, strongly positively correlated with T3, and low-normal levels of total cholesterol and low HDL cholesterol in AHDS patients. Similar findings have been reported by others (14, 15). Production of SHBG and metabolism of cholesterol are increased by thyroid hormone, with high SHBG levels (28) and low choles-

terol levels (29, 30) found in patients with primary hyperthyroidism. Our findings indicate hepatic thyrotoxicosis in AHDS patients, despite loss of MCT8 function. This is in keeping with the increased liver D1 activity observed in MCT8 knockout mice (25, 26). In mice and man, elevated serum T3 levels presumably lead to increased hepatic T3 uptake via other transporters.

Most AHDS patients show reduced muscle and fat mass. High circulating T3 levels may affect myocytes and adipocytes, leading to 'wasting' of muscle and fat tissue. Herzovich *et al.* report on increased lactic acid and ammonium concentrations in one patient, indicative of muscle catabolism (15). We found elevated levels of lactic acid in several patients, but normal values have also been reported (14). We observe low or low-normal CK levels. CK levels are elevated in hypothyroidism (31), and low in hyperthyroidism (32). In juvenile hyperthyroidism, thyrotoxic myopathy with muscular weakness, proximal muscle atrophy of the legs and low CK levels has been reported (33). The elevated lactic acid and low CK levels, in combination with low muscle mass, might indicate that in AHDS patients, muscle is in a hyperthyroid state.

Although most AHDS patients appear to have normal heart rate and blood pressure, two patients presented here (A2 and C) show mild tachycardia, even when not treated with thyroid hormones. Other patients (B, D1, (14)) showed tachycardia and/or hypertension when treated with high doses of T4 and/or T3. Although MCT8 is expressed in the human heart, bradycardia has not been observed in AHDS patients. This indicates that thyroid hormone sensitivity of the heart is maintained despite loss of MCT8 function, and that the cardiovascular system might be thyrotoxic in some cases.

We observed delayed bone age in two AHDS patients, whereas advanced bone age (15) and normal bone age (10) have also been reported. This prompted us to evaluate serum markers for bone metabolism. BAP and Ntx mostly showed (low-)normal values, with a clearly reduced BAP in one child with delayed bone age. In children, hypothyroidism leads to delayed bone age, whereas hyperthyroidism advances bone age (34). Hyperthyroidism increases bone turnover with elevated BAP (35) and (urinary) Ntx levels (36), whereas in hypothyroidism bone metabolism markers may be reduced, but can also be normal (37). Data obtained in our patients does not indicate thyrotoxicosis of the bone, but is compatible with a hypothyroid state.

Biochemical evaluation of liver, muscle and bone parameters in AHDS patients suggest thyrotoxicosis of liver and muscle, and possibly hypothyroidism of the bone. It must be noted, however, that the specificity of these markers for the thyroid hormone status of tissues is limited. In AHDS patients, several factors, including feeding problems and immobility, might affect the evaluated characteristics. For example, high SHBG and low cholesterol are seen in malnutrition (38). However, even with adequate nutrient intake via a gastrostomy tube, signs of muscle and fat wasting and elevated SHBG levels persisted in patients C and D1.

The identification of mutations in MCT8 as a cause of severe psychomotor retardation shows that MCT8 is essential for normal development of the human brain. Loss of MCT8 function and the associated rise in serum T3 lead to a tissue-specific hypothyroid, euthyroid or hyperthyroid state, depending on the local repertoire of thyroid hormone transporters and deiodinases that is expressed. Treatment of AHDS patients with T4 and T3 has not shown to improve the clinical condition. However, the apparently thyrotoxic state of liver and muscle might provide a rationale for normalizing serum T3. In conclusion, the intriguing AHDS phenotype clearly underlines the important role of transporters in the tissue-specific regulation of thyroid hormone bioactivity.

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Chapter 4

Genotype-phenotype relationship in patients with mutations in thyroid hormone transporter MCT8

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ABSTRACT

Loss of function mutations in thyroid hormone transporter MCT8 lead to severe X-linked psychomotor retardation and elevated serum T3 levels. Most patients, for example those with mutations Val235Met, Ser448stop, inslle189 or delPhe230, cannot stand, walk or speak. Patients with mutations Leu434Trp, Leu568Pro and Ser194Phe, however, walk independently and/or develop some dysarthric speech. To study the relationship between mutation and phenotype, we transfected JEG3 and COS1 cells with wild-type or mutant MCT8. Expression and function of the transporter were studied by analyzing T3 and T4 uptake, T3 metabolism (by cotransfected type 3 deiodinase), western blotting, affinity-labeling with N-bromoacetyl-T3, immunocytochemistry and quantitative RT-PCR. Wild-type MCT8 increased T3 uptake and metabolism ~5 fold compared to empty vector controls. Mutants Val235Met, Ser448stop, inslle189 and delPhe230 did not significantly increase transport. However, Ser194Phe, Leu568Pro and Leu434Trp showed ~20%, ~23% and ~37% of wild-type activity. RT-PCR did not show significant differences in mRNA expression between wild-type and mutant MCT8. Immunocytochemistry detected the non-functional mutants Val235Met, inslle189 and delPhe230 mostly in the cytoplasm, whereas mutants with residual function were expressed at the plasma membrane. Mutants Ser194Phe and Leu434Trp showed high protein expression, but low affinity for N-bromoacetyl-T3; Leu568Pro was detected in low amounts, but showed relatively high affinity. Mutations in MCT8 cause loss of function through reduced protein expression, impaired trafficking to the plasma membrane or reduced substrate affinity. Mutants Leu434Trp, Leu568Pro and Ser194Phe showed significant residual transport capacity, which may underlie the more advanced psychomotor development observed in patients with these mutations.

INTRODUCTION

Mutations in the MCT8 gene (SLC16A2) impressively illustrate the detrimental effects that mutations in X linked genes can have on psychomotor development. MCT8 is a potent thyroid hormone transporter (1, 2), which is expressed in cells of various tissues, including neurons in the central nervous system (CNS). Loss-of-function mutations in MCT8 are associated with severe psychomotor retardation and elevated serum T3 levels in males (3-12). This indicates that MCT8 plays an essential role in the development of the CNS, most likely by facilitating the supply of thyroid hormone to the neurons.

Reduced thyroid hormone action in the developing brain has long been recognized as an important cause of developmental disorders. Fetal and neonatal hypothyroidism, due to dysgenesis of the thyroid gland or impaired synthesis of thyroid hormone (13, 14) or iodine deficiency (15), severely inhibit neurological development (16). Its most severe clinical presentation is referred to as cretinism, a syndrome comprised of growth retardation, impaired cognitive function, spastic diplegia and deafness (17). However, recently even mild fetal hypothyroidism due to marginally impaired maternal thyroid function in pregnancy has been associated with adverse neurological outcome (18, 19).

Thyroid hormone has to cross numerous membranes before it reaches the nuclear T3 receptor in the neurons, the primary target for thyroid hormone action in the brain. It has become increasingly clear that uptake of thyroid hormone requires transporter proteins, and that simple diffusion through membranes is unlikely if not impossible (20-23). As T3 is produced locally in the brain, T4 has to be transported over the blood-brain-barrier, a process that may include thyroid hormone transporter OATP1C1 (24). T4 is then converted to T3 by type 2 deiodinase (D2) in astrocytes, and transferred to the neurons. As yet it is not known which transporters are involved in the uptake of T4 and the release of T3 from astrocytes. Neurons express MCT8 (25), which is likely to facilitate the uptake of the locally produced T3. Neurons also express type 3 deiodinase (D3); they are considered the main site of thyroid hormone inactivation in the brain (26).

Mutations in MCT8 have been reported in over 25 families around the world (3-12, 27). Affected males show severe psychomotor retardation, hallmarked by hypotonia of the axial muscles, spastic or dystonic quadriplegia, and severe cognitive impairment. Development of speech is usually absent, and the majority of patients are not able to sit, stand or walk without support. Other symptoms include athetoid movement of hands and arms, paroxysmal dyskinesia, muscle hypoplasia, seizures, nystagmus and secondary microcephaly. The first identification of this MCT8 related neurological phenotype was published in 1944 (28). Named after the authors, the syndrome is known as Allan-Herndon-Dudley syndrome (OMIM #300523).

In patients with mutations in MCT8, serum thyroid hormone profiles are abnormal. Total and free T4 levels range between low-normal and clearly decreased, whereas T3 levels are

strongly increased, and reverse T3 (rT3) is decreased. TSH is usually higher than in controls, but mostly falls within the normal range (27). The elevated serum T3 level, especially in combination with decreased serum T4, appears to be a specific biochemical marker for mutations in MCT8.

Although psychomotor impairment is severe in all MCT8 patients, some significant differences in development are observed between families. Most notably, the majority of patients with the Leu568Pro and Leu434Trp mutations reported by Schwartz *et al.* (6) are able to walk without support, although the gait is ataxic. They also develop limited and dysarthric speech. Elementary speech development is also observed in patients with mutation Ser194Phe (6). Independent walking or development of speech was not observed in any of the patients reported by Friesema *et al.* (3), Dumitrescu *et al.* (4), Maranduba *et al.* (7), Holden *et al.* (9), Kakinuma *et al.* (10), Herzovich *et al.* (11) and Jansen *et al.* (12).

It was recently demonstrated that mutations in MCT8 result in reduced uptake and subsequent metabolism of T3 and T4 *in vitro* (12). The aim of the present study was to determine whether differences in psychomotor development observed between families correlate with functional characteristics of these mutants *in vitro*. Possible residual activity of the mutant transporter might underlie the apparent genotype - phenotype relation observed in patients with mutations in MCT8.

MATERIALS AND METHODS

Plasmids and transfections

The cloning of wild-type human (h) MCT8 in the expression vector pcDNA3 (pcNDA3-hMCT8) was described previously (2). Point mutations identified in patients (6, 9) (Table 4.1) were introduced in this plasmid using the QuickChange Site-Directed Mutagenesis pro-

Table 4.1 Mutations and clinical phenotype of MCT8 patients

	Protein	Mutation	No. of patients	Independent walking	Speech	Ref
1	**V235M**	703G→A	5	-	-	6
2	**L434W**	1301T→G	9	ataxia, awkward gait	dysarthric, limited	6
3	**S448X**	1343C→A	4	-	-	6
4	**L568P**	1703T→C	28	ataxia, awkward gait	dysarthric, limited	6
5	**S194F**	581C→T	10	-	dysarthric, limited	6
6	**insI189**	565insATC	1	-	-	9
7	**delF230**	683delTCT	6	-	-	6

- = absent

tocol (Stratagene, Amsterdam, The Netherlands) to produce mutant proteins Val235Met, Leu434Trp, Ser448stop, Leu568Pro, Ser194Phe, inslle189 and delPhe230. Mutagenesis primers were ordered from Invitrogen (Breda, The Netherlands) without additional purification. Introduction of the mutation was confirmed by sequencing. Construction of pCINeo-hD3 and pcDNA3-rD1 (rat type 1 deiodinase) were described previously (2) . A full-length image clone of human mu-crystallin (hCRYM) was obtained from RZPD GmbH (Berlin, Germany). and subcloned into pSG5 (Stratagene) using restriction sites *Eco*R1 and *Bam*H1. All transfections were performed using 3 μl FuGene-6 transfection reagent (Roche Applied Science, Almere, The Netherlands) per 1000 ng plasmid DNA according to the manufacturer's protocol. Transfection of empty pcDNA3 vector was used as control in all experiments. Possible differences in transfection efficiency between MCT8 mutants were studied in lysates of MCT8 and hD3 co-transfected JEG3 cells using D3 activity as control as described previously (12). No significant differences between mutants were observed (data not shown). Uptake and metabolism data are presented as mean ± SE of four experiments without further corrections.

Iodothyronines

Nonradioactive iodothyronines were obtained from Henning (Berlin, Germany) or Sigma (St. Louis, MO). [3'-1^{25}I]T3 and [3',5'-^{125}I]T4 were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Radioactive N-bromoacetyl-T3 (BrAc[^{125}I]T3) was synthesized as described previously (29).

Cell culture

COS1 and JEG3 cells were cultured in 6, 12 or 24-well dishes (Corning, Schiphol, The Netherlands) with DMEM/F12 medium (Invitrogen), containing 9% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 100 nM sodium selenite (Sigma-Aldrich).

Iodothyronine transport experiments

COS1 and JEG3 cells were cultured in 6-well culture dishes, and transfected in duplicate with 500 ng wild-type or mutant pcDNA3-hMCT8 and 500 ng pSG5-hCRYM. hCRYM is a cytosolic thyroid hormone-binding protein. Addition of hCRYM reduces the efflux of T3, greatly increasing the net cellular T3 uptake. After 24 h (COS1) or 48 h (JEG3), cells were washed with assay buffer (Dulbecco's PBS with Ca2+/Mg2+, 0.1% BSA, 0.1% glucose) and incubated for 30 min at 37 C with 1 nM ($2x10^5$ cpm) ^{125}I-labeled T3 or T4 in 1.5 ml assay buffer. After incubation, cells were washed with assay buffer, lyzed with 0.1 M NaOH and counted.

Iodothyronine metabolism experiments.

JEG3 cells were cultured in 24-well culture dishes (2 cm^2), and transfected in duplicate with 100 ng pCIneo-hD3 and 100 ng wild-type or mutant pcDNA3-hMCT8. Two days after transfection, cells were washed with DMEM/F12 plus 0.1% BSA, and incubated for 4 or 24 h at 37 C with 1 nM ($1x10^6$ cpm) [^{125}I]T3 or [^{125}I]T4, respectively, in 0.5 ml DMEM/F12 plus 0.1% BSA. After incubation, the medium was analyzed by HPLC as described previously (2).

Western blotting

Polyclonal antisera were raised in rabbits by Eurogentec SA (Seraing, Belgium) against the keyhole limpet hemocyanin (KLH) conjugate of the synthetic peptide (C)ELLPGSPNPEEPI (hMCT8 C-terminal amino acid residues 527-539). Antiserum (designated 1306) from the final bleed was IgG purified.

JEG3 cells cultured in 6-well plates were transfected with 500 ng wild type or mutant pcDNA3-hMCT8. After 48 h incubation, the cells were rinsed with PBS, collected in 0.1 M phosphate / 2 mM EDTA buffer (pH 7.2) and sonicated on ice. 30 µl homogenate (diluted to 1 µg protein per µl) was mixed with 10 µl 4x loading buffer containing 10 mM DTT, and denatured at 80 C for 5 min. Samples were separated on 10% SDS-PAGE mini gels, blotted to nitrocellulose membranes, and probed with antiserum 1306 (1:1000) as described previously (30).

Affinity-labeling of MCT8 with BrAcT3

COS1 cells in 6-well plates were co-transfected with 1000 ng empty pcDNA3, wild-type or mutant pcDNA3-hMCT8 and 1000 ng pcDNA3-rD1. After 24 h, the cells were washed with serum-free DMEM/F12, incubated 4 h at 37 C with $5x10^5$ cpm BrAc[^{125}I]T3 and processed as described previously (2). 75 µg protein samples were analyzed by SDS-PAGE; gels were blotted and radioactivity on the blots was visualized by phosphor imaging (Typhoon, Amersham Biosciences, Roosendaal, the Netherlands). For statistical analyses, signals of 4 gels were quantified using ImageQuest software (Amersham).

Immunocytochemistry

JEG3 cells were cultured on 15 mm glass coverslips coated with poly-D-lysine (Sigma) and transfected with 50-100 ng wild-type or mutant pcDNA3-hMCT8. After 48 h, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS. Samples were blocked for 30 min in PBS containing 2% BSA, and stained with

rabbit anti-hMCT8 antibody 1306 (1:1000) and monoclonal mouse anti-Zona Occludens 1 (tight junction protein 1) antibody (Invitrogen, 1:250). After secondary staining with goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 633 (Invitrogen), coverslips were mounted with Prolong Gold containing DAPI (Invitrogen). Samples were examined on a Zeiss Axiovert 100 confocal microscope using Zeiss LSM software (Carl Zeiss B.V., Sliedrecht, The Netherlands).

mRNA expression in transfected cells.

JEG3 cells were cultured in 6-well plates and transfected with 500 ng wild-type or mutant pcDNA3-hMCT8. After 48 h, cells were trypsinized, counted and suspended at $5x10^6$/ml. RNA was isolated using the High Pure RNA Isolation kit (Roche); cDNA was generated by reverse transcription of 500 ng RNA using the Taqman RT Reagents kit (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Quantitative PCR was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems) using 2 µl cDNA, 12.5 µl Taqman PCR mastermix (Applied Biosystems) and a hMCT8 specific primer-probe mix containing forward primer 5'-CCATAACTCTGTCGGGATCCTC-3' located in exon 1, reverse primer 5'-ACTCACAATGGGAGAACAGAAGAAG-3' located in exon 2, and probe 5'-FAM-ATACCCATCGGAGGGCTCCGA-TAMRA-3' located just upstream of the reverse primer. MCT8 mRNA levels were expressed relative to GAPDH mRNA levels, which were obtained using pre-developed human GAPDH Taqman Assay reagents (Applied Biosystems).

Statistical analyses.

Comparisons between means were performed using Student's *t* tests in the Statistical Package for the Social Sciences (SPSS) version 12.

RESULTS

Uptake of iodothyronines in MCT8 and CRYM cotransfected cells

Figure 4.1 A shows the uptake of T3 by JEG3 cells cotransfected with wild-type or mutant pcDNA3-hMCT8 and pSG5-hCRYM after 30 min. Control cells were cotransfected with empty pcPNDA3 and pSG5-hCRYM. T3 uptake by control cells was 4.5% after 30 min. Transfection with wild-type hMCT8 increased T3 uptake 5.6-fold ($p<0.001$). T3 uptake in cells transfected with the hMCT8 mutants Val235Met, Ser448stop, insIle189 and del-Phe230 did not significantly differ from control. T3 uptake was increased 2.6-fold ($P<0.03$) by mutant Leu434Trp, 1.8-fold ($P=0.08$, NS) by mutant Leu568Pro and 1.6-fold ($P=0.12$,

NS) by mutant Ser194Phe. Similar results were obtained for T4 uptake in transfected JEG3 cells as well as for T3 and T4 uptake in transfected COS1 cells (data not shown).

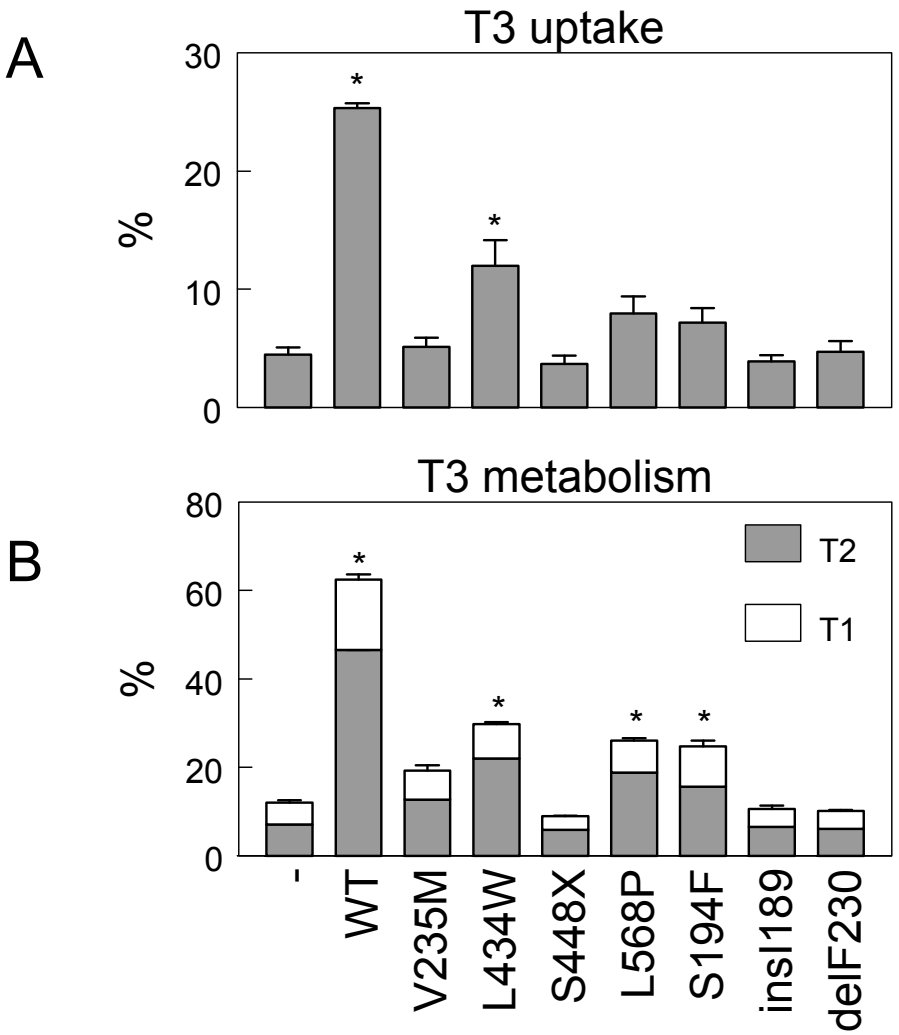

Figure 4.1 A. Uptake of T3 by JEG3 cells cotransfected with wild-type or mutant hMCT8 cDNA and pSG5-hCRYM, shown as percentage of added T3 after 30 min. Empty pcDNA3 + pSG5-hCRYM served as control. **B.** Metabolism of T3 after 4 h in JEG3 cells cotransfected with wild-type or mutant hMCT8 and hD3-pCIneo, shown as percentage of T_2 and T_1 in the medium. Empty pcDNA3 + hD3-pCIneo served as control. Uptake and metabolism data are presented as mean ± SE of four experiments, *$p<0.05$ vs. control.

Metabolism of iodothyronines in MCT8 and D3 co-transfected cells

Figure 4.1 B shows the metabolism of T3 in transfected JEG3 cells. Control cells transfected with hD3 cDNA plus vector without hMCT8 metabolized 13% of T3 after 4 h. Cotransfection of cells with hD3 and wild-type hMCT8 cDNA increased T3 metabolism 4.6-fold to ~60% in 4 h. T3 metabolism in cells cotransfected with hD3 and hMCT8 mutants Val235Met, Ser448stop, insIle189 and delPhe230 did not significantly differ from control. T3 metabolism was increased 2.6-fold ($p<0.01$) by the Leu434Trp mutant, 2.2-fold ($p<0.01$) by the Leu568Pro mutant, and 2.1-fold ($p<0.01$) by the Ser194Phe mutant. Similar results were obtained after 24 h incubation of JEG3 cells with T4 (data not shown).

Western blotting

Immunoblotting of lysates from JEG3 cells transfected with wild-type or mutant hMCT8 is shown in Figure 4.2 A. All lanes were loaded with 30 µg protein. As reported before (2), no band of the expected size of hMCT8 (~60 kDa) could be detected in JEG3 transfected with control plasmid. Transfection with wild-type hMCT8 results in a clear band of the expected size, but also of a higher band of ~240 kDa. After transfection with the Val235Met, Leu-

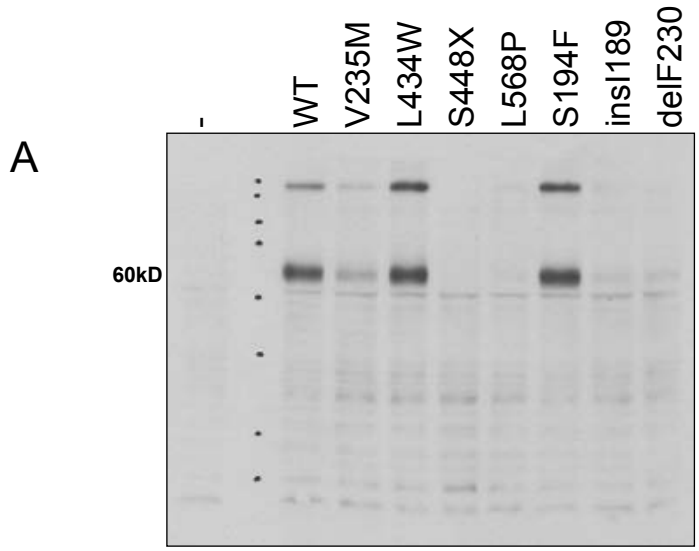

Figure 4.2 A. Immunoblot of JEG3 cells transfected with wild-type or mutant hMCT8. All lanes were loaded with 30 micrograms of protein. Transfection with wild-type hMCT8 results in a band of the expected size (60 kDa), but also of a higher band of ~240 kDa. Mutants Leu434Trp and Ser194Phe are expressed at similar or somewhat higher levels than the wild-type protein. Val235Met is intermediately expressed; faint bands are observed for Leu568Pro, insIle189 and delPhe230. The truncated protein encoded by premature stop mutant Ser448stop is not detectable with the antibody used.

568Pro, insIle189 and delPhe230 mutants, (much) weaker bands of the same sizes were detected, indicating that the expression of the mutant proteins is impaired, but not absent. Mutants Leu434Trp and Ser194Phe are expressed at similar or somewhat higher levels than the wild-type protein. The truncated protein encoded by premature stop mutant Ser448Phe cannot be detected because of the loss of the epitope.

BrAc[^{125}I]T3 affinity-labeling

BrAc[^{125}I]T3 is a specific affinity-label for MCT8, which also transports the label, and for D1 (2). Figure 4.2 B shows the autoradiograph of labeled proteins from COS1 cells co-transfected with pcDNA3, wild-type or mutant pcDNA3-hMCT8 and pcDNA3-rD1 after incubation for 4 h at 37 C with BrAc[^{125}I]T3. Incubation of cells transfected with pcDNA3 and rD1 resulted in labeling of a protein of ~60 kDa, consistent with the size of MCT8, and of a protein of ~30 kDa, the size of D1. Two unknown proteins of ~50 and ~37 kDa are also labeled. Transfection with wild-type hMCT8 increases the intensity of the MCT8 and D1 bands 3.5 fold ($p<0.03$). Transfection with mutant hMCT8 plasmids does not significantly increase the intensity of the MCT8 band compared to the control, except for mutant Leu568Pro. D1 labeling is at control level in cells transfected with Ser194Phe

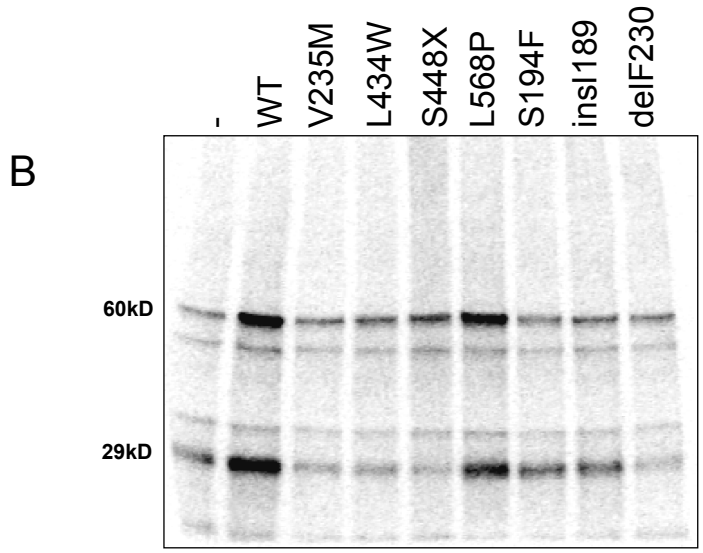

Figure 4.2 B. BrAc[^{125}I]T3 affinity-labeling of COS1 cells co-transfected with hMCT8 and D1 Transfection of wild-type hMCT8 and mutant Leu568Pro increase the labeling of the specific ~60 kDa band by 3.5 and 2.7-fold, respectively, versus cells transfected with empty pcDNA3 ($p<0.05$). Labeling of D1 (~30 kDa) is increased 3.5-fold in cells transfected with wild-type MCT8, and 1.9-fold in Leu568Pro transfected cells, and reduced (50-70%) in cells transfected with Val235Met, Leu434Trp, Ser448stop and delPhe230 (all $p<0.05$).

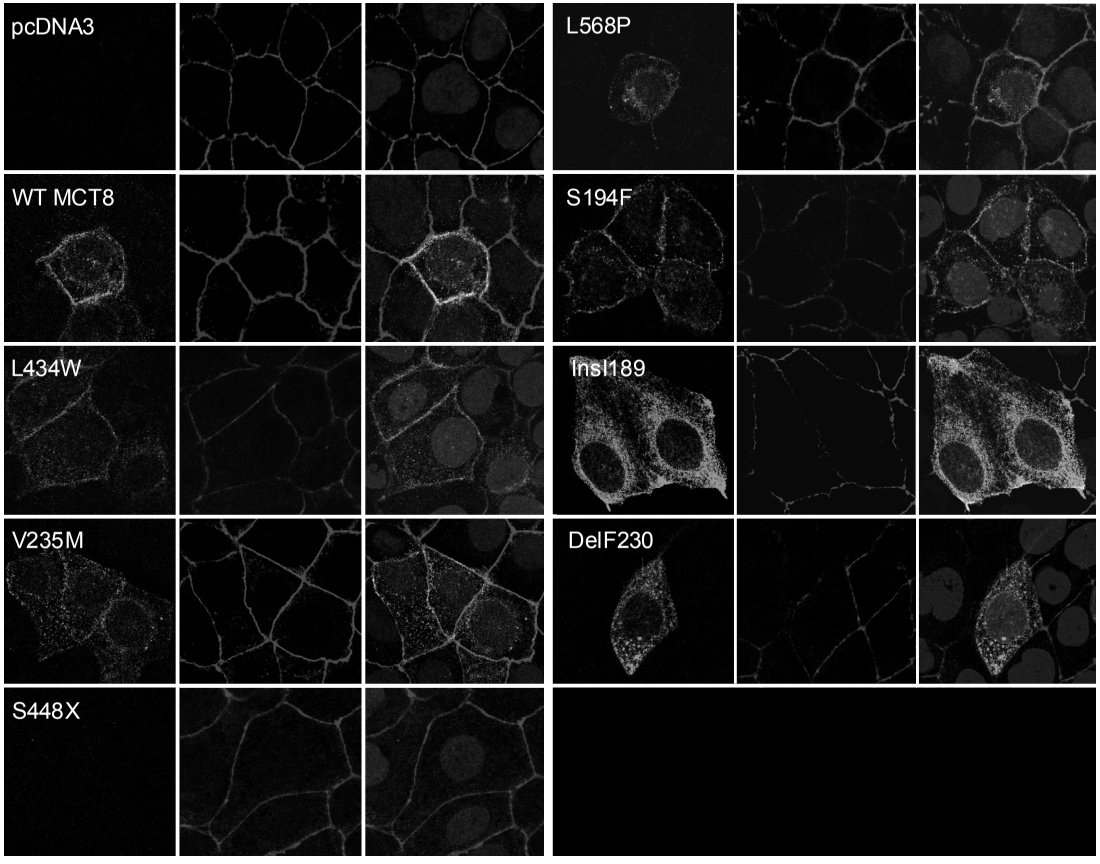

Figure 4.3 Immunocytochemistry of transfected JEG3 cells. hMCT8 specific antibody 1306 is stained green, plasma membrane marker ZO-1 (zona occludens protein 1) is stained red. Nuclear DNA is stained with DAPI (blue). Wild-type hMCT8 and mutants Leu568Pro, Leu434Trp and Ser194Phe co-localize with the plasma membrane marker. Expression of mutants Val235Met, insIle189 and delPhe230 is mostly limited to the cytoplasm. For color figure see page 194.

and insIle189, and significantly reduced in cells transfected with Val235Met, Leu434Trp, Ser448stop and delPhe230 ($p<0.01$). Mutant Leu568Pro shows a 2.7-fold increase of the MCT8 signal, and a 1.9-fold increase of the D1 signal ($p<0.01$), indicating a relatively high affinity for and transport of BrAcT3.

Immunocytochemistry

Following fixation and permeabilization, wild-type or mutant hMCT8 transfected JEG3 were stained with hMCT8 specific antibody 1306. ZO-1 antibody was used as plasma membrane marker. For detection, hMCT8 antibody was stained with Alexa fluor 488 (green) and ZO-1 antibody with Alexa Fluor 633 (red). Cells were mounted using Prolong Gold anti fade reagents containing the DNA marker DAPI (blue). Figure 4.3 shows representative cells for all conditions. In cells transfected with control vector, no specific hMCT8 staining could be observed. In cells transfected with wild-type or mutant MCT8 cDNA, perinuclear MCT8 staining is detected, suggesting protein in the endoplasmatic reticulum or Golgi apparatus. Wild-type hMCT8 also co-localizes clearly with the tight-junction marker ZO-1, indicating expression at the plasma membrane of transfected JEG3 cells. For hMCT8 mutants Val-235Met, insIle189 and delPhe230, expression of the protein appears to be predominantly cytoplasmatic, whereas mutant Ser448stop is not detected by our MCT8 antibody. Mutants Leu568Pro, Leu434Trp and Ser194Phe, which are reported here to have residual transport capacity for T3 and T4, are expressed at the plasma membrane.

Quantitative PCR

To investigate possible effects of the mutations on hMCT8 mRNA stability, we performed quantitative RT-PCR on mRNA isolated from JEG3 cells transfected with wild-type or mutant hMCT8. hMCT8 mRNA expression was corrected for the expression of the housekeeping gene GAPDH (Figure 4.4). As reported previously, JEG3 cells do not endogenously express MCT8 mRNA (2). This is reflected in the absence of MCT8 signal in the cells transfected with control vector. No significant differences in mRNA expression were observed between wild-type MCT8 and the various mutants, indicating that loss of function of the mutants investigated here is not associated with reduced mRNA stability. Mutant delPhe230 mRNA could not be detected with the primer combination used for this RT-PCR, as the reverse primer contains the three deleted nucleotides.

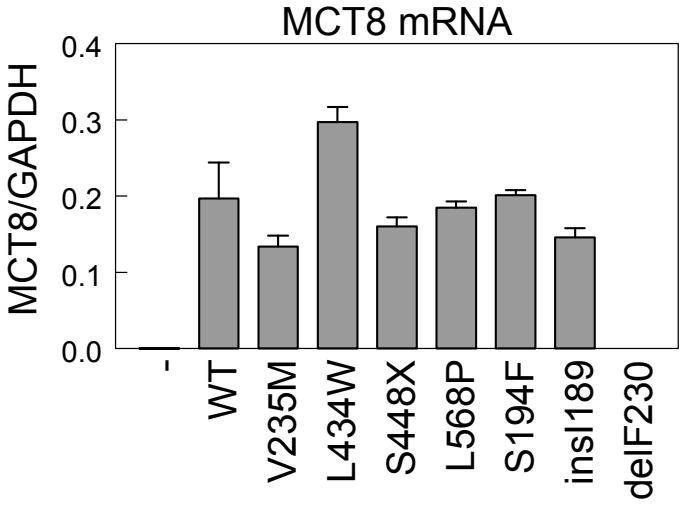

Figure 4.4 Quantitative RT-PCR of wild-type or mutant hMCT8 transfected JEG3 cells. No hMCT8 mRNA is detected in empty vector transfected controls. No significant differences in expression between wild-type hMCT8 and the various mutants are observed. DelPhe230 mRNA could not be detected with the primers used in this assay.

DISCUSSION

The first publication on *MCT8* (SLC16A2) by Lafrenière *et al.* in 1994 noted that the gene is located in a region associated with X-linked diseases (31). The function of MCT8, however, remained elusive until it was demonstrated that it codes for an active and specific thyroid hormone transporter (1). The important physiological role of MCT8 in the development of the CNS became apparent with the identification of patients with mutations in *MCT8*. These patients, all boys, exhibited elevated serum T3 levels and severe psychomotor retardation, confirming that mutations in *MCT8* indeed were a cause of X-linked psychomotor retardation (XLMR) (3, 4). In all initially reported patients, motor impairment is such that they are unable to hold up their head and can not sit, crawl, stand or walk. None of these patients developed any speech beyond making some sounds.

With the identification of MCT8 mutations in patients diagnosed with the Allan-Herndon-Dudley syndrome (6), it has become clear that mutations are associated with a considerable variety in phenotypical manifestations. Most striking is the ability of most patients with mutations Leu568Pro, the original family described by Allan, Herndon and Dudley, and Leu434Trp to walk independently, with an ataxic, awkward gait. Development of walking is delayed, usually after 2 or 3 years of age, and may be lost later in life. Patients with these mutations usually also show some elementary development of speech, although it is described as 'largely unintelligible' (Leu568Pro) (28, 32), and 'dysarthric and difficult to understand' (Leu434Trp) (33). Patients with the Leu568Pro mutation are reported to be

able to obey simple commands (28). In one other family, with the mutation Ser194Phe, most patients also develop some speech, but this is 'limited to some words or phrases' (6). None of the patients in this family ever developed independent walking.

The apparent correlation between genotype and phenotype raised the question whether mutant proteins differ in functional characteristics regarding the uptake of T3 and/or T4. We recently demonstrated for several MCT8 mutations that transport of T3 and T4 is completely absent *in vitro* (12). One mutant protein, Arg271His, showed significant residual transport capacity of 10-20% of wild-type MCT8. The patient with this mutation, however, does not walk or talk nor shows any other signs indicating more advanced psychomotor development.

In the present study, we demonstrate that mutant proteins Leu568Pro, Leu434Trp and Ser194Phe significantly increase uptake and subsequent metabolism of T3 and T4 compared to empty vector transfected controls, whereas for mutants Val235Met, Ser448stop, insILe189 and delPhe230 no significant difference was observed. Residual functionality of the mutants, most clearly demonstrated in the metabolism assays, ranged from 18-27% of wild-type activity for Leu568Pro, 35-40% for Leu434Trp and 15-25% for Ser194Phe (Table 4.2).

Table 4.2 Functional characteristics, protein expression and affinity of WT and mutant MCT8

Protein	T3 uptake (% of WT)	T3 metabolism (% of WT)	Western blot	BrAcT3 affinity-labeling	Cellular localization	Ref
Wild-type MCT8	100%	100%	+++	+++	plasma membrane	1
V235M	NS	NS	++	-	mostly cytoplasm	6
L434W	40%	35%	+++	-	plasma membrane	6
S448X	NS	NS	ND	-	ND	6
L568P	18%	28%	+	++	plasma membrane	6
S194F	15%	25%	+++	-	plasma membrane	6
insI189	NS	NS	-	-	limited expression, mostly cytoplasm	9
delF230	NS	NS	-	-	limited expression, mostly cytoplasm	6
A224V	NS	NS	+++	-	mostly cytoplasm	3, 12
L471P	NS	NS	+	-	limited expression, mostly cytoplasm	3, 12
R245X	NS	NS	ND	-	ND	3, 12
R271H	18%	20%	+++	-	plasma membrane	12
del267-370	NS	NS	-	-	limited expression, mostly cytoplasm	12

NS = not significant compared to control,
ND = not detectable due to lack of epitope

Mutations in MCT8 could lead to a loss of function via several mechanisms, including reduced expression at the RNA or protein level, impaired trafficking to the plasma membrane and reduced affinity for thyroid hormone. As we demonstrate in Figure 4.4, quantitative RT-PCR did not show any significant differences in mRNA expression between wild-type and mutant MCT8. The expression of protein, demonstrated by immunoblotting in Figure 4.2 A, however, shows clear differences between the mutants. Mutant Leu434Trp and Ser194Phe proteins are expressed at levels comparable to wild-type MCT8, and Val-235Met shows intermediate expression, whereas mutant insIle189, delPhe230, Leu568Pro and Ser448stop proteins are expressed at much reduced levels, or are not detected. As mRNA expression does not differ between wild-type MCT8 and mutants, low expression of mutant proteins may result from fast degradation. Affinity-labeling of MCT8 protein with BrAcT3 (Figure 4.2 B) is readily observed in COS1 cells transfected with wild-type MCT8 or with mutant Leu568Pro, but not with other mutants. The significant residual affinity of Leu568Pro suggests that this mutant protein is present in higher concentrations than are detected by immunblotting. Immunocytochemistry (Figure 4.3) localizes wild-type MCT8 and mutants Leu434Trp, Leu568Pro and Ser194Phe at the plasma membrane, whereas the mutants V235M, insI189 and delP230 are mostly located in the cytoplasm.

From these results it becomes apparent that complete loss of thyroid hormone transport function, as observed for mutants Val235Met, insIle189 and delPhe230, results from decreased protein expression and/or impaired trafficking to the plasma membrane. These mutations are located in highly conserved loci in the first (insI1le89) and second (delPhe230 and Val235Met) transmembrane domain (TMD) and are all likely to effect the helical structures of these domains. For the protein encoded by mutant Ser448stop, expression and localization could not be studied with our antibody. Complete loss of function is, however, expected from the premature stop, truncating the protein in TMD8.

Not surprisingly, all mutants showing significant residual transport capacity are expressed at the plasma membrane, the functional localization of MCT8. From immunoblotting it appears that the Leu434Trp and Ser194Phe mutants are expressed at relatively high protein levels. Labeling with BrAcT3, however, did not show a marked increase of MCT8 signal, suggesting that the mutations lead to reduced substrate affinity. This was also observed for mutant Arg271His (12) (Table 4.2). The Ser194Phe mutation is located in the first extracellular loop, just outside TMD1, whereas mutation Leu434Trp is predicted just inside TMD8. Arg271His is located in the second extracellular loop, between TMDs 3 and 4. Of all mutant proteins studied so far, these three are the only ones showing preserved protein expression but reduced affinity. Although for some members of the MCT family specific domains have been suggested to be involved in substrate recognition (for example TMDs 8 and 10 in MCT1 (34), little is known about which domains are involved in MCT8. From our findings it appears that mutations throughout a large part of the protein can affect the

affinity for (BrAc)T3 without resulting in increased breakdown of the protein. Whether the 'extracellular' localization of these mutations plays a role in this remains to be elucidated.

Protein expression of mutant Leu568Pro, with ~25% residual functionality, appears very limited on immunoblotting. Nonetheless, affinity labeling shows the highest intensity of all mutants tested. The Leu568Pro mutation is located in TMD12; possibly, substitution of Leu with the rigid helix-breaking Pro residue influences protein expression, but has little effect on the affinity for (BrAc)T3. However, L568P is the mutation situated most closely to the epitope of antibody 1306, which might impair detection of the protein by immunoblotting.

The observed phenotypical characteristics of patients with mutations in MCT8 appear to be consistent in patients carrying the same mutation. Of 12 patients examined with mutation Leu568Pro, 11 walked, as did 7 of 8 patients carrying the Leu434Trp mutation. All four individuals with Ser194Phe that were examined developed some speech. It is important to realize, however, that all patients carrying a certain mutation come from one family. Although the residual transport of thyroid hormone we report here possibly contributes to the psychomotor development of these patients, other genetic or environmental factors are likely to contribute to this as well. Comparing patients with the same mutation from different families would provide valuable insight in the effects of residual MCT8 function. Until now, only mutation delPhe230, which we report here to lack transport capacity, has been reported in two unrelated families (6, 12). Patients with this mutation have not been reported to walk independently or develop speech. In general, only patients with mutations showing significant residual transport capacity appear to reach these milestones of advanced psychomotor development.

In conclusion, significant residual uptake and subsequent metabolism of T3 and T4 was demonstrated for mutant MCT8 proteins Leu434Trp, Leu568Pro and Ser194Phe *in vitro. In vivo*, these mutations are associated with more advanced psychomotor development than is observed in most MCT8 patients, especially with regard to walking and, to a lesser extent, talking. The functional characteristics of mutant proteins we describe here may underlie the genotype – phenotype relation observed in patients with mutations in MCT8.

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Chapter 5

Novel pathogenic mechanism suggested by *ex vivo* analysis of MCT8 (SLC16A2) mutations

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ABSTRACT

Monocarboxylate transporter 8 (MCT8; SLC16A2) facilitates cellular uptake and efflux of 3,3',5-triiiodothyronine (T3). Mutations in MCT8 are associated with severe psychomotor retardation and high serum T3 and low 3,3',5'-triiodothyronine (rT3) levels. Here we report 3 novel MCT8 mutations. Two subjects with the delPhe501 mutation have mild psychomotor retardation with slightly elevated T3 and normal rT3 levels. T3 uptake was mildly affected in delPhe501 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 delPhe501 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 delPhe501 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 delPhe501 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.

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 T3, the major biologically active TH, is generated from the prohormone T4 by the deiodinating enzymes D1 and D2 (1). The deiodinase D3 inactivates T4 to 3,3',5'-triiodothyronine (rT3) and T3 to 3,3'-diiodothyronine (T2) (1). The genomic actions of T3 are mediated by nuclear T3 receptors (TRs) (2). 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 (3). Recently, several classes of transporter proteins have been characterized at the molecular level (4). 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 code SLC16A2; MIM#300095) and MCT10 express a high selectivity towards TH (4).

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 (5, 6). Males with loss-of-function mutations in MCT8 show severe neurological deficits, with axial hypotonia, spastic quadriplegia and impaired or absent speech, and muscle hypoplasia. This X-linked syndrome is known as the Allan-Herndon-Dudley syndrome (AHDS, OMIM#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 (7). 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 (8). However, we recently demonstrated that MCT8 also functions as an efficient T3 and T4 exporter (9). The biological relevance of this function and the possible contribution to the pathogenesis of the MCT8 syndrome is currently unknown.

Here, we present 3 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). Non-radioactive iodothyronines were obtained from Henning (Berlin, Germany). 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 reagens 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 (RefSeq, NM_006517.3) was analyzed using standard primers as described previously (10). We designed additional primers for patient P6 (Table 5.1).

Cloning and site-directed mutagenesis

The cloning of wild-type (wt) human MCT8 cDNA was described recently (8). 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.

Table 5.1 Synthetic oligonucleotides for identicifaction of the deletion in patient P6

Name	Primer (5'-3')
P6del1-fwd	GGGAGGTGTGTTGGTCATG
P6del1-rev	AGGTGAAGGGGAAAAAGGTG
P6del2-fwd	GCTCAGGGTTCCTTTTCCTAAC
P6del2-rev	AGGTCTCAGGTCTCCTCCATC

Cell cultures and transfection

We obtained human skin fibroblasts from patients P1-4 by punch biopsy after informed consent by the parents. Fibroblasts from 3 non-affected subjects were used as controls (kindly provided by Dr. B. Thio, Erasmus MC). We grew fibroblasts in 75 cm2 flasks in 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 cm2 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 co-transfected with pcDNA3.hMCT8 (wt or mutant) and pCIneo.hD3, as previously described (10).

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 (8, 10).

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 30-60 min at 37 C with 1 nM ($2x10^5$ cpm) [^{125}I]T3 or [^{125}I]T4 in 1.5 ml incubation medium. After incubation, cells were washed with the medium, lyzed with 0.1 M NaOH and counted in a gamma counter.

For measurement of TH efflux, cells were loaded for 1 h with incubation medium containing 1 nM ($2x10^5$ cpm) [^{125}I]T3 or [^{125}I]T4. After removal of the medium, cells were washed and incubated for 10-30 min with incubation medium without ligand. Finally, medium was removed, and cells were washed with incubation medium, lyzed 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 h transfection, JEG3 cells were incubated for 10-30 min with incubation medium containing [^{125}I]T3 or [^{125}I]T4.

TH metabolism experiments

To maximize D2 activity, fibroblasts were incubated for 24 h with DMEM/F12 plus 6% charcoal-treated FBS and 100 nM sodium selenite at confluency. To induce D3 activity, fibroblasts were stimulated for 6 h 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 4-72 h at 37 C with 1 nM ($1x10^6$ cpm) [^{125}I]T4 (D2 activity) or for 3 h at 37 C with 1 nM ($1x10^6$ cpm) [^{125}I]T3 (D3 activity) in DMEM/F12 plus 0.1% BSA. After incubation, medium was sampled, processed and analyzed by HPLC as previously described (8).

The intact-cell metabolism of T3 in JEG3 cells was investigated as described previously (10).

T3 effects on fibroblast

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

Total RNA was isolated from $1x10^6$ 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'-TGCAGCAGCAGAAACAAGTACC-3' and the antisense primer 5'-GCACACAATGGCAAGAAAGG-3' were used.

SYBR Green I (Eurogentec) was used as detector dye for quantitative PCR of the T3-responsive genes ZAKI 4α, GLUT1 and MCT4. Specific primer sequences are presented in Table 5.2. mRNA levels are expressed relative to that of the house-keeping gene cyclophilin A.

Statistical analysis

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

Table 5.2 Synthetic oligonucleotides of T3-responsive genes

Gene	Sense primer (5'-3')	Antisense primer (5'-3')
ZAKI 4α	TCTTTACCAATCAGGAGGTTAAGGA	ACACTGCAAGGTCGATAAATTCTCAA
GLUT1	ACGGGTCGCCTCATGCT	GTCTGTACCCAGGTGGCG
MCT4	GATCGGCTACAGCGACACAG	GTGTTCAAGAGGTCACGGTAAC

RESULTS

Clinical features

Patients P1 and P2 have been reported previously (5, 7, 10). Briefly, both patients have severe psychomotor retardation, characterized by truncal hypotonia, quadriplegia, mental retardation and absence of speech.

Patients P3-6 are newly identified. Patient P3 is a 4 yr old boy born to non-consanguineous parents. He presented at the age of 9 mo when gross motor milestones were not reached with axial hypotonia, headlag, 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 mo presented with a global developmental delay. He had mild axial hypotonia and mild spastic tetraparesis. He functioned as a 12 mo old infant with respect to communication skills. At the age of 18 mo, 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 yr old brother of the mother of patient P4 (Figure 5.1 A). 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 slurred and influently. Furthermore, he was capable of reading by combining separately spelled letters and of writing simple sentences (without verbs) by computer.

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

Table 5.3 shows the serum thyroid 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 P1-3 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 P1-6 was tested for mutations in MCT8. The results are graphically depicted in Figure 5.1 D. A deletion of almost 2.4 kb with borders located in exon 3 and intron 4

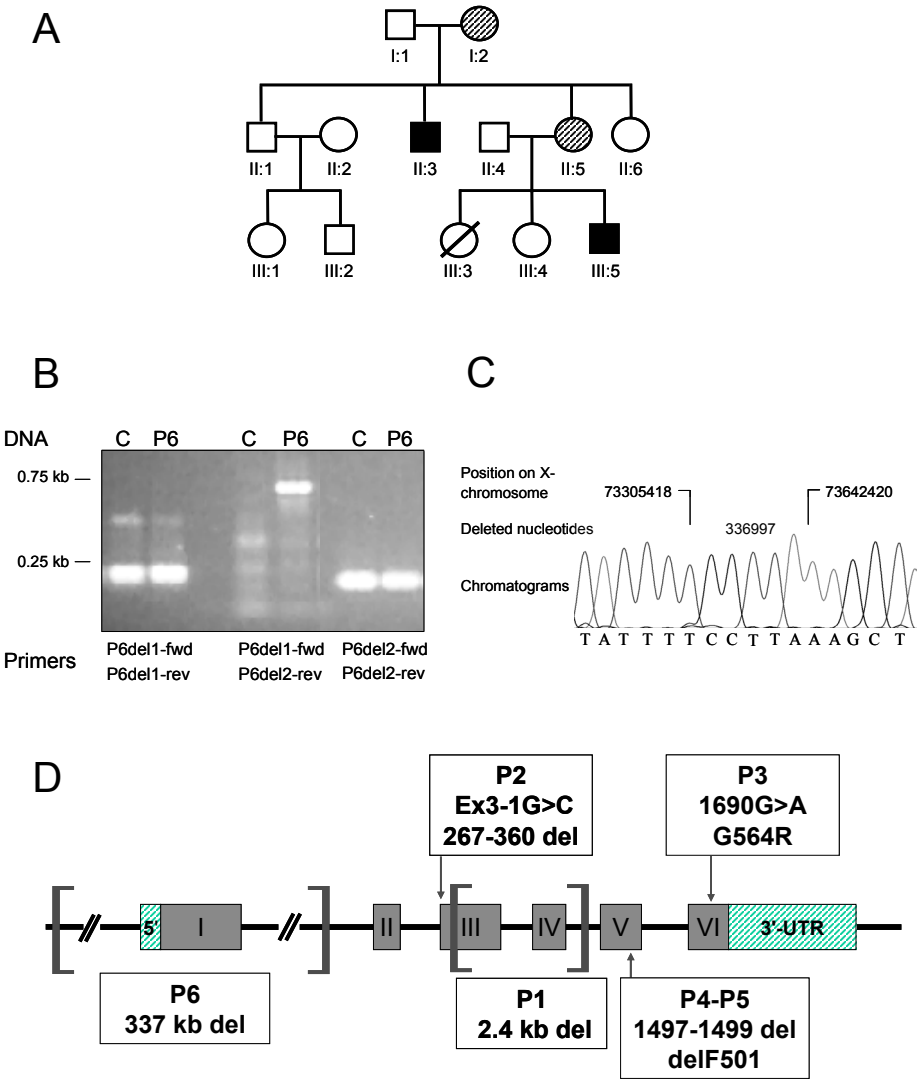

Figure 5.1 A. Pedigree of the family with the delPhe501 mutation. Filled squares indicated affected males. Striped circles represent unaffected carriers. Patient P4 and P5 are denoted as II:5 and III:3, respectively. **B.** PCR analysis of a deletion in the *MCT8* gene in patient 6 (P6) compared to a control. No DNA is amplified in P6 in the 336 kb large region between the two amplicons in the left and right lanes. Combination of P6del1-fwd and P6del2-rev generates an amplicon in DNA of P6, but not in control DNA. **C.** Partial sequencing profile of the amplicon in lane 4 refines the borders of the deletion in P6 to a loss of 336997 nucleotides. **D.** *MCT8* gene structure with the location of the different mutations in patients P1-6. For color figures 5.1 C. and D. see page 195.

Table 5.3 Serum thyroid hormone levels in MCT8 patients

	Normal values	P1*	P2	P3	P4	P5	P6
Mutation		2.4 kb del	Ex3-1G>C 267-360 del (splice site)	1690G>A G564R	1497-1499 delCTT delF501		337 kb del
Age at testing		5 yrs	6 yrs	10 months	18 months	38 yrs	3.5 yrs
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

*Patient P1 was treated with thyroxine (57 mcg/day)

(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 (5, 7).

In patient P3 a c.1690G>A mutation was found, which results in a Gly to Arg substitution at position 564 (p.Gly564Arg). A 3-bp deletion (c.1497_1499delCTT) was identified in patients P4 and P5, causing a deletion of Phe at position 501 (p.delPhe501). We were not able to amplify exon 1 in patient P6. We further investigated the extension of the deletion 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 (Figure 5.1 B). 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:g1322_MCT8:c650-15128del; Figure 5.1 C).

Delineation of the mutations at the mRNA level

To determine the effects of the mutations on mRNA structure, we performed a 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 5.2 A 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; Figure 5.2 B).

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 dele-

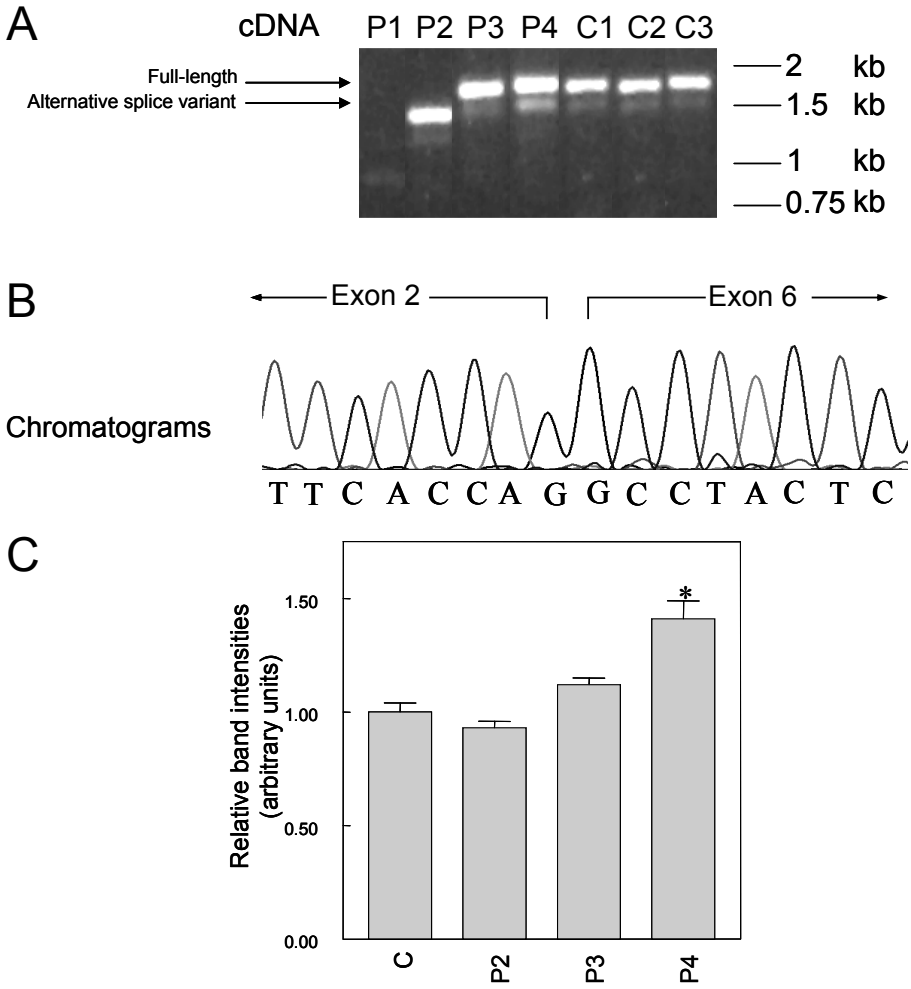

Figure 5.2 MCT8 mutations at the mRNA level. **A.** RT-PCR of MCT8 mRNA in skin fibroblasts of patients 1-4 (P1-4). The 1.8 kb band indicates full-length MCT8 mRNA and the 1.5 band suggests an alternative splicing variant. **B.** Part of sequencing profile of cDNA derived from mRNA in patient P1 demonstrating a loss of exons 3, 4 and 5. For color figure see page 196. **C.** Densitometric analysis of the MCT8 splice variant (lower band) detected in controls and patients P2-4. Significance represent values obtained in patient fibroblasts compared to control fibroblasts. * $P < 0.005$.

tion of 282 nt from the mRNA (r.798_1079del) and a predicted loss of 94 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 intensitiy of the splice variant in patient P4 compared to controls, suggesting enhanced splicing (Figure 5.2 C).

Immunoblotting and immunocytochemistry

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 (P2), Gly564Arg (P3) or delPhe501 (P4/P5) mutants. On IB, minimal MCT8 expression was seen with the Gly564Arg mutant, whereas the delPhe501 mutant showed only slightly diminished protein expression compared to wt-MCT8 (Figure 5.3 A). 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 delPhe501 mutant. Figure 5.3 B shows a clear membrane distribution of the delPhe501 mutant, similar to wt-MCT8.

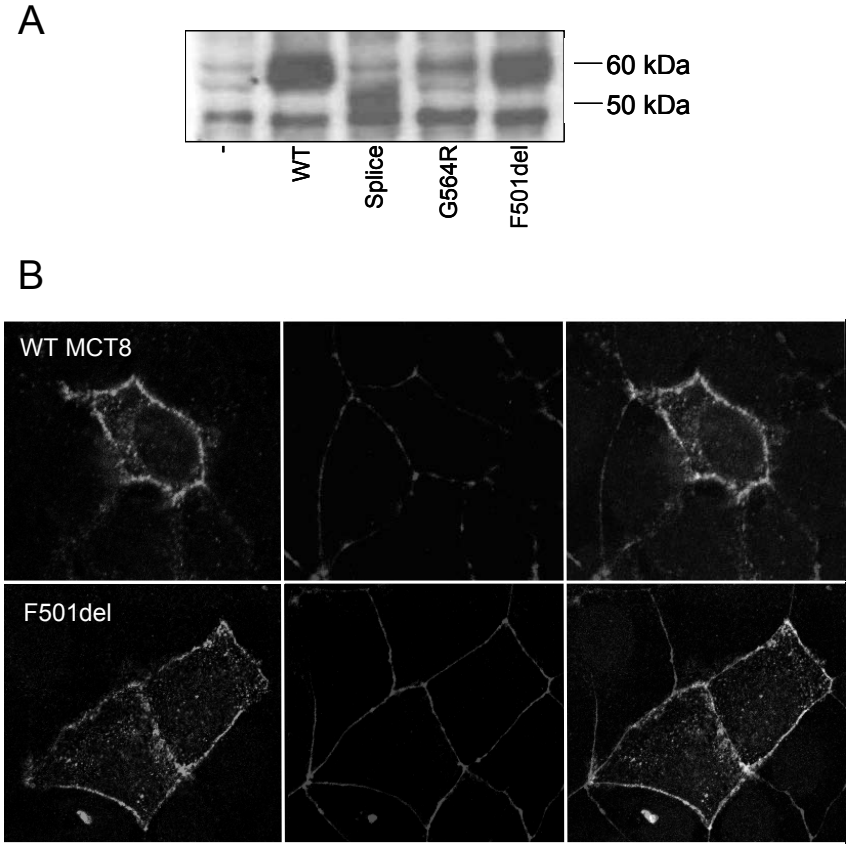

Figure 5.3 MCT8 mutations at the protein level. **A.** Western blot analysis of JEG3 cell lysates transfected with wild-type or mutant MCT8. **B.** Immunofluorescent detection of JEG3 cells transfected with wild-type and the delPhe500 mutation in MCT8. The plasma membrane was stained with a ZO-1 antibody. For color figure see page 196.

TH uptake and efflux

Fibroblasts from patients P1-4 and controls were incubated for 30 min with [^{125}I]T3. This resulted in a reduction of T3 uptake in fibroblasts from patients P1-3 by ~70% and in patient P4 by ~50% compared to controls (Figure 5.4 A). Uptake of [^{125}I]T4 in fibroblasts from all patients was ~40% of controls (Figure 5.4 B).

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 P1-3 (0.07-0.08%/mg protein/min), indicating that the equilibrium phase has not been reached in patient P4 (Figure 5.4 C 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 Gly564Arg mutants (Figure 5.4 E and F). Both T3 and T4 uptake by the delPhe501 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 5.5 A shows a higher T3 efflux rate (1.6%/mg protein/min) in controls than in patients P1-3 and P4 (both 0.6%/mg protein/min). Similarly, T4 efflux rate was higher in controls compared with patients' fibroblasts (Figure 5.5 B).

Figure 5.5 C and D show that in transfected JEG3 cells efflux of both T4 and T3 by the delPhe501 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 Gly564Arg mutants produced results similar to the empty vector (Figure 5.6). We observed 13% T3 metabolism in cells expressing the delPhe501 mutant.

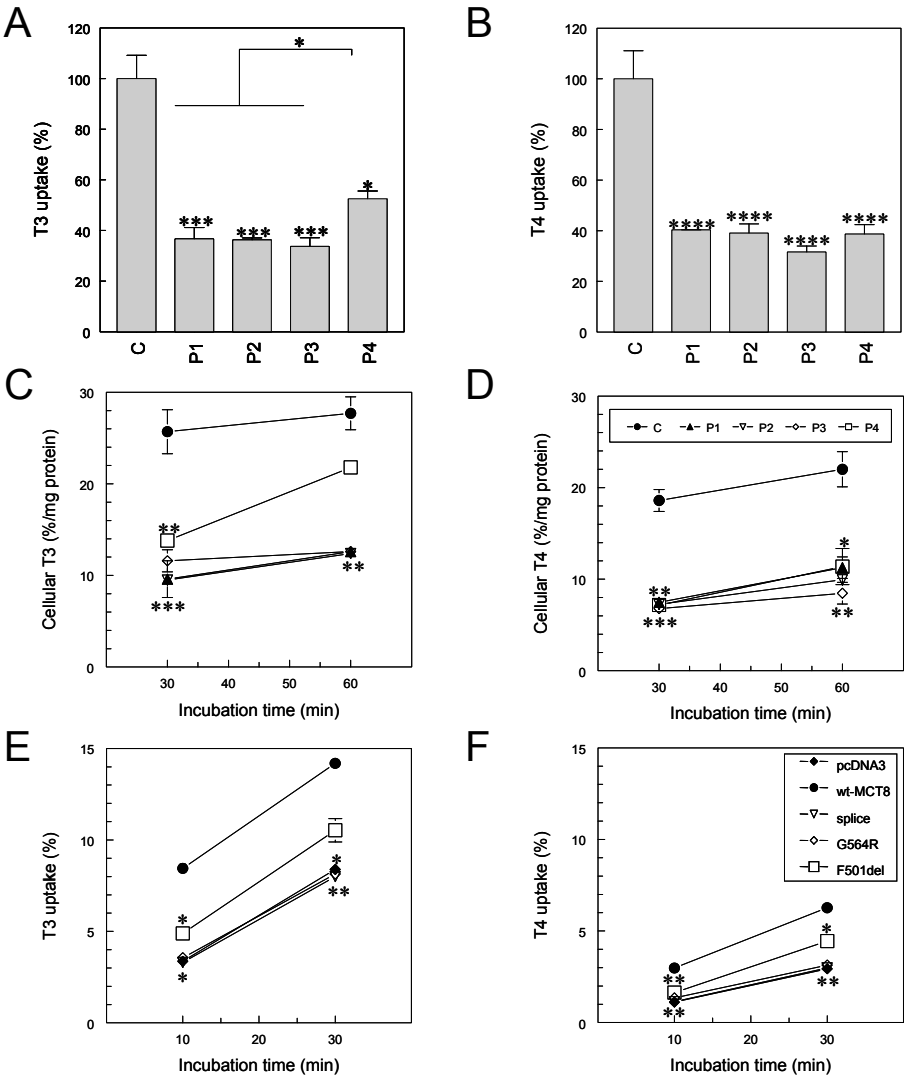

Figure 5.4 A. and **B.** Uptake of ^{125}I-labeled T3 and ^{125}I-labeled T4 in fibroblasts of MCT8 patients after 30 min incubation. T3 and T4 uptake in control fibroblasts is defined as 100%. **C.** and **D.** Uptake of ^{125}I-labeled T3 and ^{125}I-labeled T4 in fibroblasts of MCT8 patients after 30 and 60 min incubation, expressed as percentage of added T3 or T4. Results are corrected for protein concentrations. Significances represent values obtained in patient fibroblasts compared to control fibroblasts. **E.** and **F.** ^{125}I-labeled T3 and ^{125}I-labeled T4 in wild-type or mutant MCT8-transfected JEG3 cells after 10 and 30 min incubation. Uptake is shown as percentage of added T3 and T4, respectively. Significances represent values of empty vector or mutant MCT8 compared to wild-type MCT8. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$.

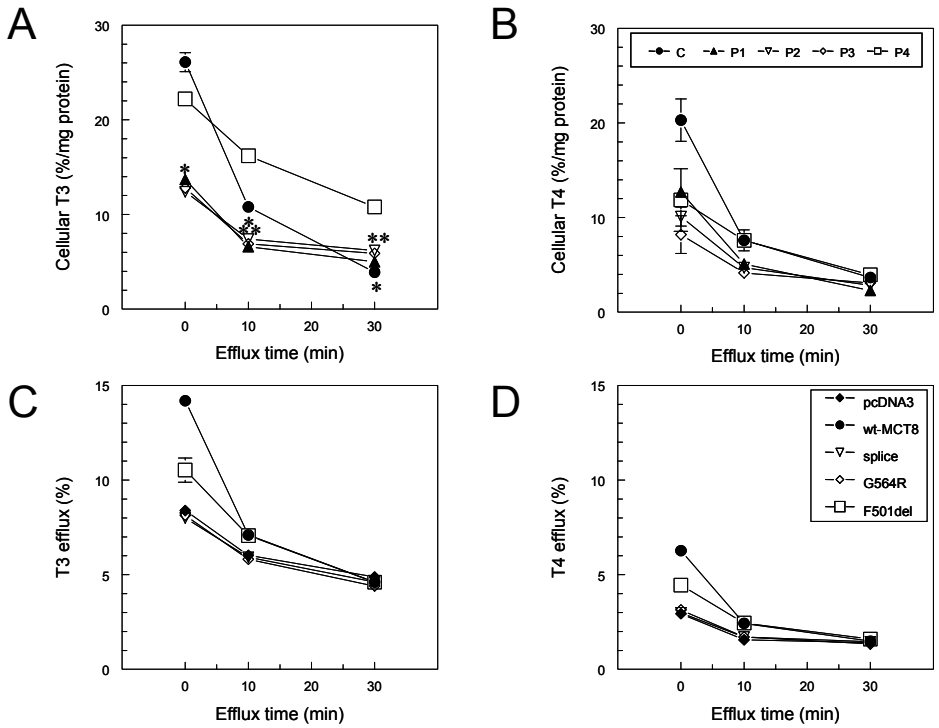

Figure 5.5 A. and **B.** Efflux of cellular T3 and T4 from fibroblasts of MCT8 patients relative to control fibroblasts. Efflux was measured after 10 and 30 min after a 60 min loading with [^{125}I]T3 or [^{125}I]T4. Results are corrected for protein concentrations. Significances represent values obtained in fibroblasts of P4 compared to controls and other patients. **C.** and **D.** Efflux of cellular T3 and T4 from wt or mutant MCT8-transfected JEG3 cells. Efflux was measured in after 10 and 30 min after 30 min loading with [^{125}I]T3 or [^{125}I]T4 and is shown as percentage of added ligand. Significances represent values of the delPhe501 mutant *versus* other mutants and wt MCT8. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

Deiodinase activity

The dramatic reduction in T3 uptake in fibroblasts from patients P1-3 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 P1-3 was decreased by 35% compared with controls (Figure 5.7). 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 h incubation with [^{125}I]T3 we could not detect degradation of T3 (data not shown).

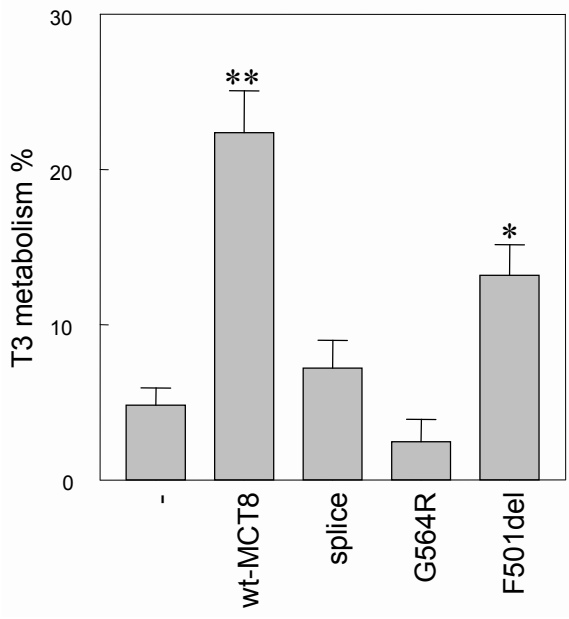

Figure 5.6 Metabolism of T3 in intact JEG3 cells cotransfected with D3 and wt or mutant MCT8. Metabolism is shown as percentage of metabolites in the medium after 4 h incubation. Significances represent values obtained in (wt or mutant) MCT8 *versus* empty vector. * $P < 0.005$; ** $P < 0.001$

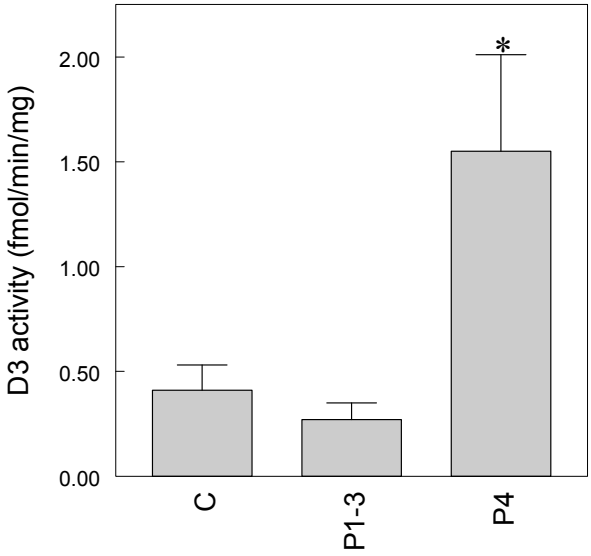

Figure 5.7 Analysis of D3 enzymatic activity in lysates of fibroblasts from MCT8 patients. The means ± SE of triplicate experiments with fibroblasts from patients P1, P2 and P3 measured separately is denoted as P1-3. Significances represent values obtained in patient fibroblasts compared to control fibroblasts. * $P < 0.01$.

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-h treatment with 10 nM T3. This resulted in a 38% increase in controls, a 27% increase in patients P1-3 and a 96% increase in patient P4 (Figure 5.8 A). 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 other patients (Figure 5.8 B). 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 (Figure 5.8 C and D).

DISCUSSION

We describe 3 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, which 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 versus 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 syndrome (11). 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 implicate that also slighty 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 P1-4 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

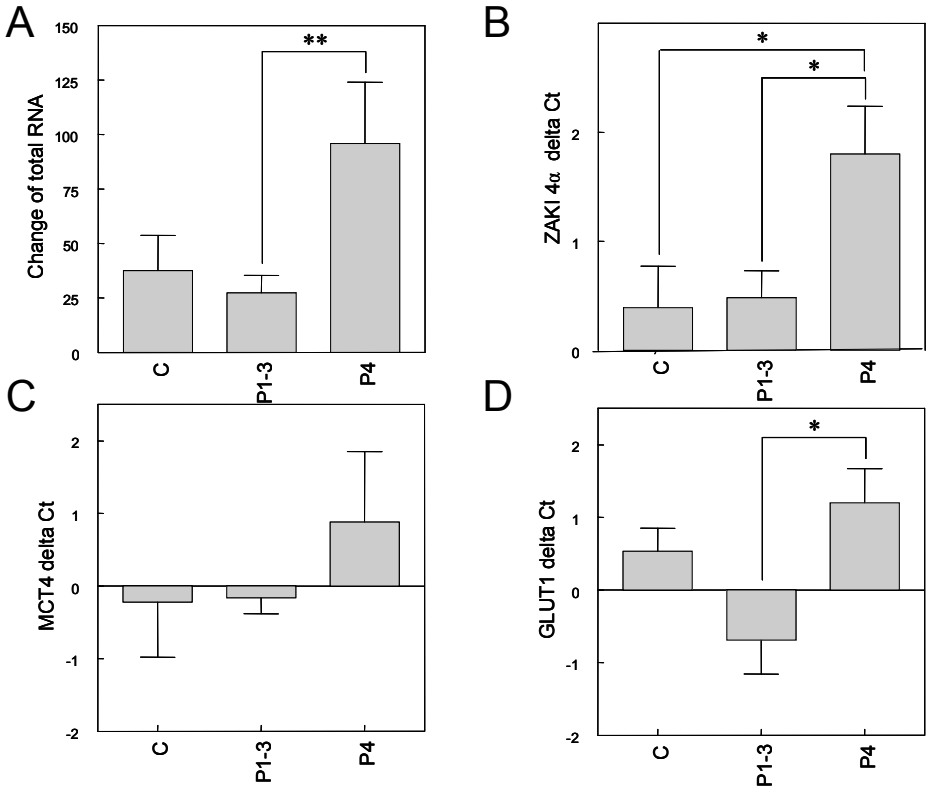

Figure 5.8 A. Effects of incubation for 6 h with 10 nM T3 on total RNA content and mRNA levels of the T3-responsive genes **B.** ZAKI 4α, **C.** MCT4 and **D.** GLUT-1. The changes of mRNA levels after T3 treatment are expressed by the ΔCt method (where ΔCt is the value obtained by subtracting the Ct 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 patient P1, P2 and P3 measured separately is denoted as P1-3. Significances represent values obtained in fibroblasts of P4 compared to controls and other patients. * $P < 0.05$; ** $P < 0.01$.

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 delPhe501 mutation gives rise to an enhanced alternative splicing. This corresponds to the location of the delPhe501 mutation in a predicted exonic splicer enhancer (12). 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 (13).

At the protein level, the splice site and the delPhe501 mutant were clearly detected by IB. ICC showed a plasma membrane localization for the delPhe501 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 (14). 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 was less diminished in patient P4 than in patients P1-3. When T3 uptake by fibroblasts from controls and patients P1-3 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 P4. This was confirmed in efflux experiments, showing that the T3 efflux rate from fibroblasts of patients P4 was similar to that of patients P1-3, 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 delPhe501 mutant facilitated marked T3 metabolism compared to wt-MCT8, whereas both other mutants did not facilitate T3 metabolism.

Considering the different behaviour in cellular T3 transport in patient P4 compared to the other patients, it was highly interesting to study the intracellular TH status in the fibroblasts. Since D2 is negatively and D3 is positively regulated by TH (1), we measured the activity of these deiodinases. MCT8 KO mice are reported to have increased cerebral D2 activities (15, 16). In addition, there is an isolated report that D2 activity is increased in fibroblasts of MCT8 patients (14). 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 P1-3 than in controls, whereas it was increased in patient P4. It was not possible to detect T3 metabolism in intact fibroblasts. This is in keeping with the observation that D3 activity is much easier to detect

in cell lysates than in intact cells (Kester, M.H. and Visser, T.J., unpublished observations). MCT8 KO mice demonstrate diminished D3 activity in brain (16). 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 4 decades ago, Tata described an early acceleration of RNA synthesis after T3 administration in rats (17). 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 3 genes, which are expressed in brain as well as in human skin fibroblasts, which are positively regulated by T3 (18). 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 patients' 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 P1-3. Indeed, we (Visser, W.E. *et al.*, unpublished observations) and others (18, 19) observed an increased expression of these genes when cells were treated with T3 for at least 24 h. 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, they may be explained by assuming a different mechanism for the pathogenesis of the psychomotor retardation in patients P1-3 versus patients P4 and P5. The results in skin fibroblasts of patients P1-3, 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 (20). 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 (7). 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 mimick the abnormal human serum TH levels, T3 clearance is not affected (15, 16). 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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Chapter 6

Effective cellular uptake and efflux of thyroid hormone by human monocarboxylate transporter 10 (MCT10)

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ABSTRACT

Cellular entry of thyroid hormone is mediated by plasma membrane transporters, among others a T-type (aromatic) amino acid transporter. Monocarboxylate transporter 10 (MCT10) has been reported to transport aromatic amino acids but not iodothyronines. Within the MCT family, MCT10 is most homologous to MCT8 which is a very important iodothyronine transporter but does not transport amino acids. In view of this paradox, we decided to re-investigate the possible transport of thyroid hormone by human (h) MCT10 in comparison with hMCT8. Transfection of COS1 cells with hMCT10 cDNA resulted in a) the production of a ~55 kD protein located to the plasma membrane as shown by immunoblotting and confocal microscopy, b) a marked stimulation of cellular T4 and, particularly, T3 uptake, c) a marked increase in the intracellular deiodination of T4 and T3 by different deiodinases, and d) a strong increase in the affinity-labeling of intracellular type I deiodinase by N-bromoacetyl-[^{125}I]T3. Co-transfection studies using the cytosolic thyroid hormone-binding protein μ-crystallin (CRYM) indicated that hMCT10 facilitates both cellular uptake and efflux of T4 and T3. In the absence of CRYM, hMCT10 and hMCT8 increased T3 uptake up to 4.0 and 1.9 fold, and in the presence of CRYM up to 7.3 and 6.5 fold, respectively. hMCT10 was less active towards T4 than hMCT8. These findings establish that hMCT10 is at least as active a thyroid hormone transporter as hMCT8, and that both transporters facilitate iodothyronine uptake as well as efflux.

INTRODUCTION

Thyroid hormone is metabolized and exerts its actions intracellularly, processes which require the transport of extracellular iodothyronines across the plasma membrane (1, 2). Diffusion of iodothyronines across the lipid bilayer of cell membranes is limited, if not impossible, and transmembrane transport of thyroid hormone is predominantly facilitated by transporters (3). Different types of transporters are likely to be involved in the uptake of thyroid hormone in different tissues (3). A number of these has recently been characterized at the molecular level, including the Na/taurochlate-cotransporting polypeptide (NTCP), different members of the (Na-independent) organic anion transporting polypeptide (OATP) family, the heterodimeric L-type amino acid transporter (LAT) and fatty acid translocase (FAT) (4, 5).

Work in particular by the group of Francon and Blondeau has strongly suggested the involvement of a T-type amino acid transporter in the uptake of T4 and T3 in different tissues (6-10). A T-type amino acid transporter facilitates transport of aromatic amino acids, and specific interaction between cellular uptake of T3 and Trp has been documented in red blood cells and other tissues (6-10). Recently, one such T-type amino transporter, termed TAT1, has been characterized in rats and humans (11, 12). Although TAT1 indeed facilitates in- and efflux of Phe, Tyr, Trp and Dopa, it was reported to be inactive toward iodothyronines (11-13). TAT1 is also known as MCT10 (SLC16A10) as it is a member of the monocarboxylate transporter (MCT) family, so called because the first 4 members of this family transport lactate, pyruvate and other monocarboxylates in a H+-dependent manner (14).

Among other members of the MCT family, MCT8 shows by far the highest homology with MCT10, which prompted us to test the possibility that MCT8 is the long-sought T-type amino acid transporter that also facilitates cellular uptake of iodothyronines. Indeed both rat (r) and human (h) MCT8 have been shown to be active and specific iodothyronine transporters, although they do not transport (aromatic) amino acids (15, 16). The pathophysiological relevance of MCT8 has been demonstrated subsequently in male patients with a syndrome, also known as the Allan-Herndon-Dudley syndrome (AHDS) (17) combining severe psychomotor retardation and elevated serum T3 concentrations (18-26). The gene coding for MCT8 is located on the X chromosome, and in all patients with this form of X-linked psychomotor retardation mutations in MCT8 have been identified (18-26).

The hMCT10 gene is located on chromosome 6, and the structure of this gene is very similar to that of the hMCT8 gene (12, 27). Both genes consist of 6 exons and 5 introns, with a particularly large (~100 kb) first intron. The major form of the hMCT8 protein consists of 539 amino acids and the hMCT10 protein of 515 amino acids; both proteins contain 12 putative transmembrane domains (TMDs). Both the N- and C-terminal domains are predicted to be located on the inside of the plasma membrane (4, 28). The N-terminal

regions of both proteins harbor a PEST domain rich in Pro (P), Glu (E), Ser (S) and Thr (T) residues, which may play a role in the regulation of the turnover of these proteins (29).

The lack of iodothyronine transport reported for MCT10 is surprising in view of the documented involvement of a T-type amino acid transporter in tissue uptake of thyroid hormone as well as the homology between MCT10 and MCT8. Therefore, we decided to reinvestigate possible iodothyronine transport by hMCT10 in comparison with hMCT8. These studies were carried out in mammalian cells transfected with cDNA coding for hMCT8 or hMCT10. To study the effects of an increase in the intracellular thyroid hormone-binding capacity, cells were cotransfected with cDNA coding for the cytosolic thyroid hormone-binding protein mu-crystallin (CRYM) (30-32). Our findings demonstrate that both hMCT8 and hMCT10 facilitate bidirectional transport of T4 and, in particular, T3 across the plasma membrane, and that hMCT10 transports T3 even better than hMCT8.

MATERIALS AND METHODS

Materials

Nonradioactive iodothyronines and aromatic amino acids were obtained from Henning (Berlin, Germany) or Sigma (St. Louis, MO). [3'-^{125}I]T3 and [3',5'-^{125}I]T4 (1500-2000 mCi/μmol), [3,5-^{3}H]Tyr (52 Ci/mmol), [5-^{3}H]Trp (30 Ci/mmol) and [2,3,4,5,6-^{3}H]Phe (116 Ci/mmol) were obtained from GE Healthcare UK Limited (Little Chalfont, Buckinghamshire, UK). Radioactive N-bromoacetyl-T3 (BrAc[^{125}I]T3) was synthesized as previously described (33). FuGENE6 transfection reagent was obtained from Roche Diagnostics (Almere, The Netherlands). The pcDNA3 and pcDNA3.1 expression vectors were obtained from Invitrogen (Breda, The Netherlands) and pSG5 from Stratagene (Amsterdam, The Netherlands).

Plasmids

Cloning of hMCT8 cDNA in the pcDNA3 expression vector has been described previously (16). IMAGE clones of full-length hMCT10 cDNA and hCRYM cDNA were obtained from the RZPD German Resource Center for Genome Research (www.rzpd.de). hMCT10 cDNA was subcloned into the expression vector pcDNA3.1 using *Eco*RI and *Xba*I restriction sites, and hCRYM cDNA was subcloned into pSG5 using *Eco*RI and *Bam*HI. Expression vectors containing rat type I deiodinase cDNA (pcDNA3.rD1) or human type III deiodinase cDNA (pCIneo.hD3) were obtained as previously described (16).

Cell culture

COS1 cells were cultured in 6 or 24-well dishes (Corning, Schiphol, The Netherlands) with DMEM/F12 medium (Invitrogen), containing 9% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 100 nM sodium selenite (Sigma). Cells were cultured 24 h after transfection.

Immunoblotting and immunocytochemistry

Polyclonal antisera were raised in rabbits by Eurogentec SA (Seraing, Belgium) against synthetic peptides comprising amino acids 473-487 and 503-515 of hMCT10 conjugated to keyhole limpet hemocyanin. Antiserum (#1758) from the final bleed was used after IgG purification (Eurogentec). IgG purified hMCT8 antibody 1306 was obtained as previously described (16).

hMCT10 and hMCT8 proteins were expressed in COS1 cells cultured in 6-well plates by transfection with 500 ng pcDNA3.1.hMCT10 or pcDNA3.hMCT8 with 500 ng pcDNA3 or 500 ng pSG5.hCRYM using 3 µl FuGENE6 transfection reagent. Empty pcDNA3 or hCRYM alone were used as a control. After 24 h, the cells were rinsed with PBS and collected in 200 µl 0.1 M phosphate buffer (pH 7.2) and 2 mM EDTA (P100E2). The cells were sonicated on ice, aliquoted and stored at -80 C. Homogenates (10-15 µg protein) were separated on 12% SDS-PAGE minigels. Thereafter, the proteins were blotted on nitrocellulose membranes, probed with antiserum 1758 or 1306 (1:1000) and further processed as described previously (46).

COS1 cells were cultured on 15 mm glass coverslips coated with poly-D-lysine. After 24 h, cells were transfected with 400 ng hMCT10 cDNA using 1.2 µl FuGENE6. After 24 h, cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 5 min. Samples were blocked in PBS containing 2% BSA for 30 min and stained with polyclonal rabbit anti-MCT10 antibody 1758 (1:1000) and monoclonal mouse anti- zona occludens 1 (ZO-1) antibody (Invitrogen) (1:250). After secondary staining with goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 633 (Invitrogen) (1:250), coverslips were mounted with Prolong Gold containing DAPI for nuclear staining (Invitrogen). Samples were examined on a Zeiss Axiovert 100 confocal microscope using Zeiss LSM software (Carl Zeiss, Sliedrecht, The Netherlands).

Affinity-labeling of transfected proteins with BrAcT3

COS1 cells grown in 6-well plates were cotransfected in duplicate with either 1000 ng empty pcDNA3 or 500 ng pcDNA3.1.hMCT10 or pcDNA3.hMCT8 plus 500 ng empty pcDNA3, pcDNA3.rD1 or pSG5.hCRYM using 3 µl FuGENE6. After 24 h, the cells were

washed with serum-free DMEM/F12 medium, and incubated for 4 h at 37 C with 400,000 cpm BrAc[^{125}I]T3 in 1.5 ml serum-free DMEM/F12 medium per well. The cells were washed with PBS and duplicate wells were pooled and lysed in 200 µl SDS-PAGE loading buffer containing 10 mM DTT and sonicated on ice. The samples were analyzed by SDS-PAGE (12% gels), followed by autoradiography to BioMax MS film (Kodak, Rochester, NY) at -80 C with intensifying screen (2 to 10 days exposure).

Thyroid hormone uptake and efflux experiments

COS1 cells were cultured in 6-well dishes, and cotransfected in duplicate as described for immunoblotting. After 24 h, cells were washed with DMEM/F12 or Dulbecco's PBS (D-PBS) medium containing 0.1% BSA, and incubated for 5-30 min at 37 C with 1 nM (2x10^5 cpm) [^{125}I]T4 or [^{125}I]T3 in 1.5 ml DMEM/F12 or D-PBS medium plus 0.1% BSA. To study the effects of aromatic amino acids, we incubated (transfected) COS1 cells with [^{125}I]T3 in the absence or presence of 1 mM unlabeled Phe, Trp or Tyr. After incubation, cells were washed with medium with 0.1% BSA, lyzed with 0.1 M NaOH and counted. For efflux studies, COS1 cells were loaded for 10 min with 1 nM (2x10^5 cpm) [^{125}I]T4 or [^{125}I]T3 in D-PBS, briefly washed and subsequently incubated for 2-10 min with 1.5 ml fresh medium with 0.1% BSA without radioactive ligand and with or without excess unlabeled T3 or T4 or Trp. After incubation, medium and cells were collected without further washing.

Amino acid uptake

COS1 cells were cultured in 6-well dishes, and transfected in duplicate with 500 ng empty pcDNA3, pcDNA3.1.hMCT10 or pcDNA3.hMCT8. After 24 h, cells were washed with D-PBS without BSA, and incubated for 0.5-30 min at 37 C with 10 µM [^{3}H]Phe, [^{3}H]Tyr, or [^{3}H]Trp in 1.5 ml D-PBS. After incubation, cells were washed, lyzed with 0.5% SDS and counted.

Iodothyronine metabolism experiments

COS1 cells were cultured in 24-well culture dishes, and transfected in duplicate with 200 ng empty pcDNA3, 100 ng pcDNA3.1.hMCT10 or pcDNA3.hMCT8 plus 100 ng empty pcDNA3 or pCIneo.hD3 using 0.6 µl FuGENE 6. After 24 h, cells were washed with DMEM/F12 plus 0.1% BSA, and incubated for 2-24 h at 37 C with 1 nM (1x10^6 cpm) [^{125}I]T4 or [^{125}I]T3 in 0.5 ml DMEM/F12 plus 0.1% BSA. After incubation, medium was sampled, processed and analyzed by HPLC as previously described (16). Over 80% of added radioactivity was recovered from the medium, and recovery of injected radioactivity over the HPLC was almost 100%.

MCT10 knockdown experiments

HEK293 cells were transfected with 3 different HP GenomeWide siRNAs against hMCT10 (Qiagen), i.e. Hs_SLC16A10_2_HP siRNA with target sequence CACAATAATTGGGAAATA-GAA located at the beginning of the 3'UTR, Hs_SLC16A10_4_HP siRNA with target sequence CACGTTTCTGAATTTGTTTAA located at the end of the 3'UTR, and Hs_SLC16A10_5_HP siRNA with target sequence TACCTTACCTATGGAATCATA located in the coding region. Control transfections were performed with a nonsilencing control siRNA labeled with Alexa Fluor 488 allowing easy monitoring of transfection efficiency. Transfection was performed according to the manufacturer's protocol using HiPerfect Transfection Reagent (Qiagen) and 20 nM of siRNA. After 48 h transfection, HEK cells were incubated for 5 min with [^{125}I] T3 as described above.

RESULTS

Among the 14 members of the MCT family, hMCT10 is most homologous with hMCT8; the amino acid identity of hMCT10 with hMCT8 amounts to 49%, whereas with the other hMCTs it varies between 21% (hMCT12) and 34% (hMCT3). Figure 6.1 shows the amino

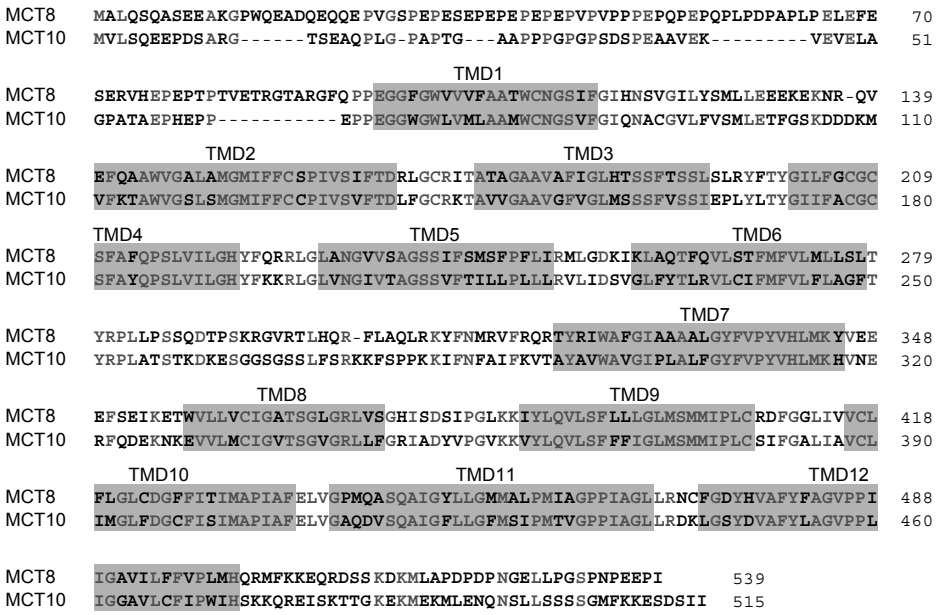

Figure 6.1 Alignment of the amino acid sequences of hMCT8 and hMCT10. Identical amino acids occupying corresponding positions in these proteins are indicated in red. The putative 12 transmembrane domains (TMDs) are indicated by blue shading.For color figure see page 197.

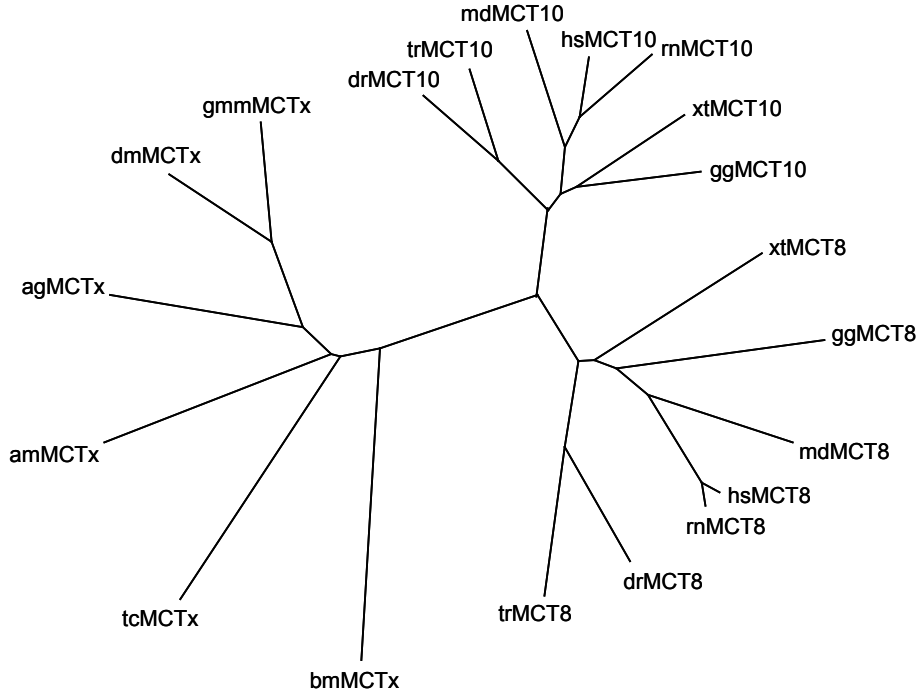

Figure 6.2 Phylogenetic tree of the MCT8/MCT10 protein family. The construction of the tree is based on amino acid sequences from the following species: hs, Homo sapiens (human); rn, Rattus norvegicus (rat); md, Monodelphis domestica (opossum); gg, Gallus gallus (chicken); xt, Xenopus tropicalis (frog); dr, Danio rerio (zebrafish); tr, Takifugu rubripes (pufferfish); gmm, Glossina morsitans morsitans (tsetse fly); dm, Drosophila melanogaster (fruitfly); ag, Anopheles gambiae (mosquito); am, Apis mellifera (honey bee); tc, Tribolium castaneum (beetle); bm, Bombyx mori (silkworm)

acid sequence alignment of hMCT8 and hMCT10. In this figure are also indicated the putative TMDs, reflecting our interpretation of the predictions by various TMD prediction programs which showed considerable variation in the number (11 or 12) and location of the putative TMDs in both proteins. Obviously, the highest degree of amino acid identity between hMCT8 and hMCT10 exists in the putative TMDs and intervening loops and much less so in the N- and C-terminal domains. Considering only this core domain, the amino acid identity between hMCT8 and hMCT10 amounts to 58%.

Figure 6.2 shows a phylogenetic tree of a selection of proteins encoded by genes identified in Genbank because of their homology with the core hMCT8 and hMCT10 sequences using the tBLASTn program. This partial tree includes 2 mammals (human, rat), a marsupial (opossum), an amphibian (Xenopus tropicalis), a bird (chicken) and 2 teleost fish (zebrafish and fugu). The amino acid identity between the core sequences varies from 63 to 96% within the MCT8 subfamily, from 70 to 87% within the MCT10 subfamily, and from 50 to 60% between these subfamilies.

Surprisingly, several genes were identified in insects (flies, mosquitoes, bee, beetle and silkworm) that showed high amino acid identities among each others (40-81%) and with members of the MCT8 (34-41%) and the MCT10 (40-46%) subfamilies. Homologous genes were also identified in 2 prevertebrate marine organisms, the sea squirt (Ciona intestinalis) and the sea urchin (Strongylocentratus purpurata), with core amino acid identities of 29-32% and 38-44%, respectively, with MCT8 and MCT10 proteins.

The amino acid sequences of hMCT8 and hMCT10 do not contain consensus N-glycosylation sites. Previous studies with cells transfected with hMCT8 cDNA have indicated the production of MCT8 protein with a molecular mass of 61 kDa, the expected size for a nonglycosylated protein of 539 amino acids. This is again illustrated in Figure 6.3, which also demonstrates that transfection of COS1 cells with pcDNA3.1.hMCT10 results in the expression of a protein with a molecular mass of 55 kDa, corresponding with the size of a 515-amino acid protein. Transfection efficiency was determined using an YFP-coupled hMCT8 construct and found to be maximally 25% (data not shown). Little or no endogenous monkey MCT10 or MCT8 protein could be detected in nontransfected COS1 cells, although relatively high endogenous amounts of MCT10 and MCT8 mRNA were expressed (data not shown, (16)). The two antibodies also show no cross-reactivity as no hMCT8 protein is detected with antibody 1758 against hMCT10 and no hMCT10 protein is detected with antibody 1306 against hMCT8.

Immunocytochemistry of COS1 cells transiently over-expressing hMCT10 shows the cellular localization of MCT10 at the plasma membrane (Figure 6.4 left panel) as is also shown for the plasma membrane marker ZO-1 (middle panel). The right panel shows the

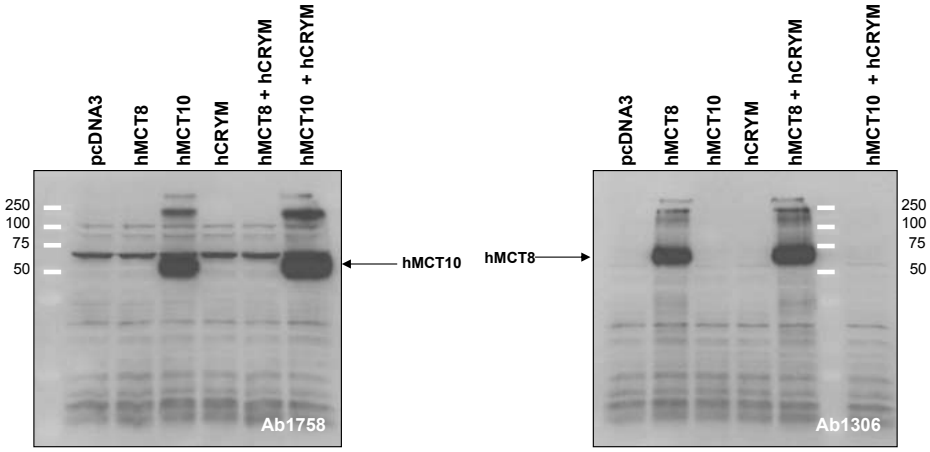

Figure 6.3 Immunoblot of hMCT8 and hMCT10 protein in transfected COS1 cells. Left: staining with polyclonal anti-hMCT10 antibody 1758. Specific bands of ~55 and ~240 kDa are detected in hMCT10 but not in hMCT8 transfected cells, irrespective of cotransfection with hCRYM. Right: staining with polyclonal anti-hMCT8 antibody 1306. Specific bands of ~60 and ~240 kDa are detected in hMCT8 not in hMCT10 transfected cells, irrespective of cotransfection with hCRYM.

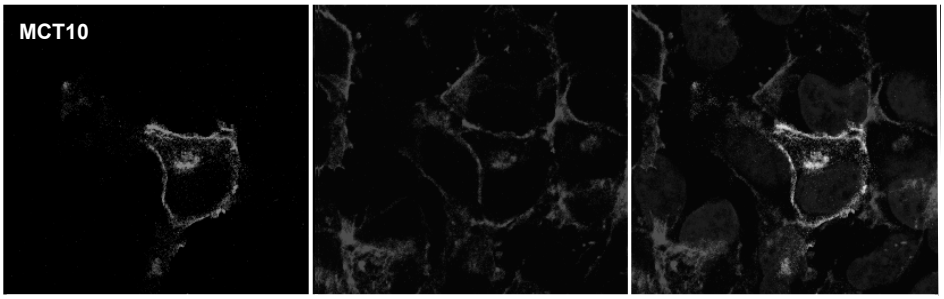

Figure 6.4 Immunocytochemistry of COS1 cells transfected with hMCT10. Left panel: hMCT10 protein detected with polyclonal antibody 1758 and stained with goat anti-rabbit Alexa Fluor 488. Middle panel: plasma membrane staining using antibody against tight junction protein ZO-1 and goat anti-mouse Alexa Fluor 633. Right panel: merged images of hMCT10, plasma membrane marker and nuclear marker (DAPI staining). For color figure see page 197.

merged images of hMCT10 and ZO-1 together with the nuclear staining with DAPI. Similar conclusions were drawn from experiments using JEG3 cells (data not shown).

Native and recombinant hMCT8 has been identified by incubation of intact nontransfected and hMCT8-transfected cells with the affinity-label BrAc[^{125}I]T3 (16). This is also a highly effective and specific affinity-label for the type 1 iodothyronine deiodinase (D1) (33). Therefore, we tested the possible affinity-labeling of exogenous hMCT10 in COS1 cells transfected with hMCT10 cDNA. The results indicate that, in contrast to hMCT8, hMCT10 is not labeled with BrAc[^{125}I]T3 (Figure 6.5). Exposure of cells transfected with rD1 cDNA alone to BrAc[^{125}I]T3 resulted in a minor labeling of the 29 kDa rD1 protein, which was greatly increased by cotransfection with hMCT10 or hMCT8 cDNA. These results indicate that BrAcT3 is transported by hMCT10 resulting in an increased intracellular labeling of rD1, but the transporter itself does not undergo covalent modification by BrAc[^{125}I]T3. Transfection of cells with hCRYM cDNA alone or together with hMCT10 or hMCT8 cDNA did not result in the obvious labeling of a protein with the expected size of hCRYM (36 kDa), indicating that BrAcT3 does not modify this protein.

Incubation of control transfected COS1 cells with [^{125}I]T3 in DMEM/F12 medium containing 0.1% BSA resulted in a time-dependent increase in cell-associated radioactivity amounting from 1.1% at 5 min to 2.3% at 30 min (Figure 6.6 A). In keeping with previous findings, transfection of COS1 cells with hMCT8 cDNA induced a 1.4-1.6 fold increase in cellular T3 uptake. However, transfection of the cells with hMCT10 cDNA produced even larger increases in T3 uptake, changing from 2.9 fold after 5 min to 2.0 fold after 30 min (Figure 6.6 A).

The rapid plateauing of the uptake of T3 by COS1 cells, in particular by cells transfected with hMCT8 or hMCT10 cDNA, suggested that a rapid equilibrium was reached between T3 in- and efflux. To decrease the rate of T3 efflux from the cells, their intracellular thyroid hormone-binding capacity was increased by (co)transfection with hCRYM, a cytosolic

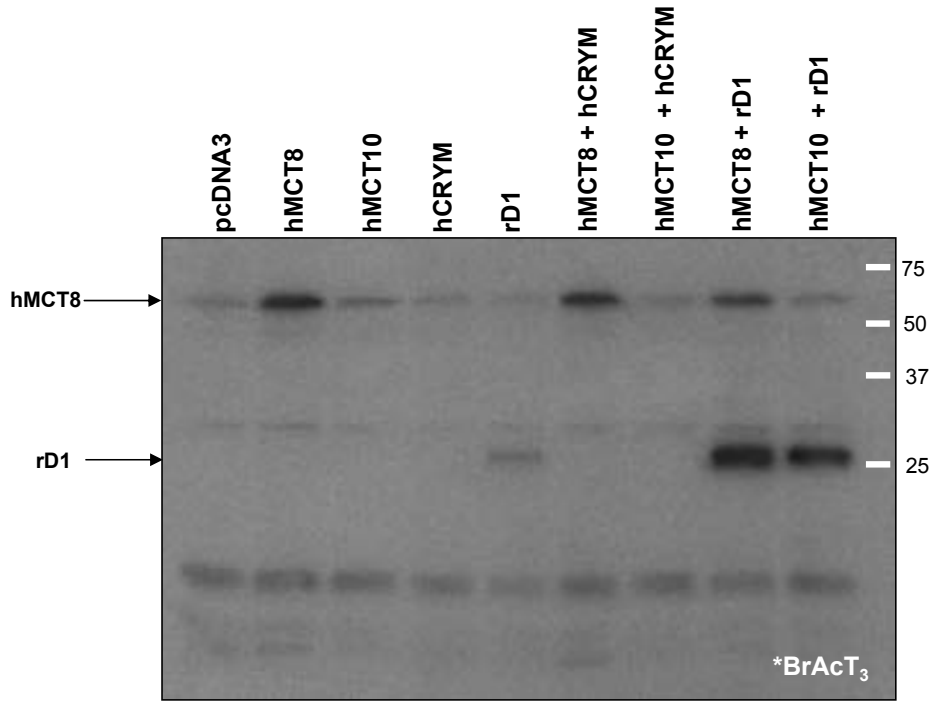

Figure 6.5 Affinity-labeling of hMCT8, hMCT10, hCRYM and/or rD1 (co)transfected COS1 cells with BrAc[125I]T3. A band of ~60 kDa is present in control plasmid transfected cells. This signal is greatly increased in hMCT8 but not in hMCT10 transfected cells, irrespective of cotransfection with hCRYM or rD1. rD1 transfected cells show affinity-labeling of a protein of ~30 kDa, the appropriate size for rD1. Cotransfection of hMCT8 and hMCT10 increases labeling of rD1, indicating transport of BrAcT3 by hMCT8 as well as hMCT10.

protein with high affinity for iodothyronines, preferring T3>T4>rT3 (30-32). Transfection of COS1 cells with hCRYM alone did not affect T3 uptake (not shown), but transfection of COS1 cells with either hMCT8 or hMCT10 in addition to hCRYM resulted in a much larger increase in T3 uptake than induced by hMCT8 or hMCT10 alone (Figure 6.6 C). In the presence of hCRYM, the fold increase in T3 uptake by hMCT8 varied between 6.5 after 5 min and 4.4 after 30 min. Corresponding values for hMCT10 were 6.9 after 5 min and 5.4 after 30 min.

In the absence of hCRYM, transfection of COS1 cells with hMCT8 or hMCT10 alone resulted in a modest increase in T4 uptake over control cDNA-transfected cells (not shown). Although transfection of cells with hCRYM cDNA alone did not affect T4 uptake (not shown), cotransfection with hMCT8 led to marked time-dependent increases in T4 uptake, i.e. 5.8-fold after 5 min and 17.9 fold after 30 min (Figure 6.6 E). The fold increase in T4 uptake after transfection with hCRYM plus hMCT10 compared with hCRYM alone varied between 1.5 after 5 min and 4.5 after 30 min.

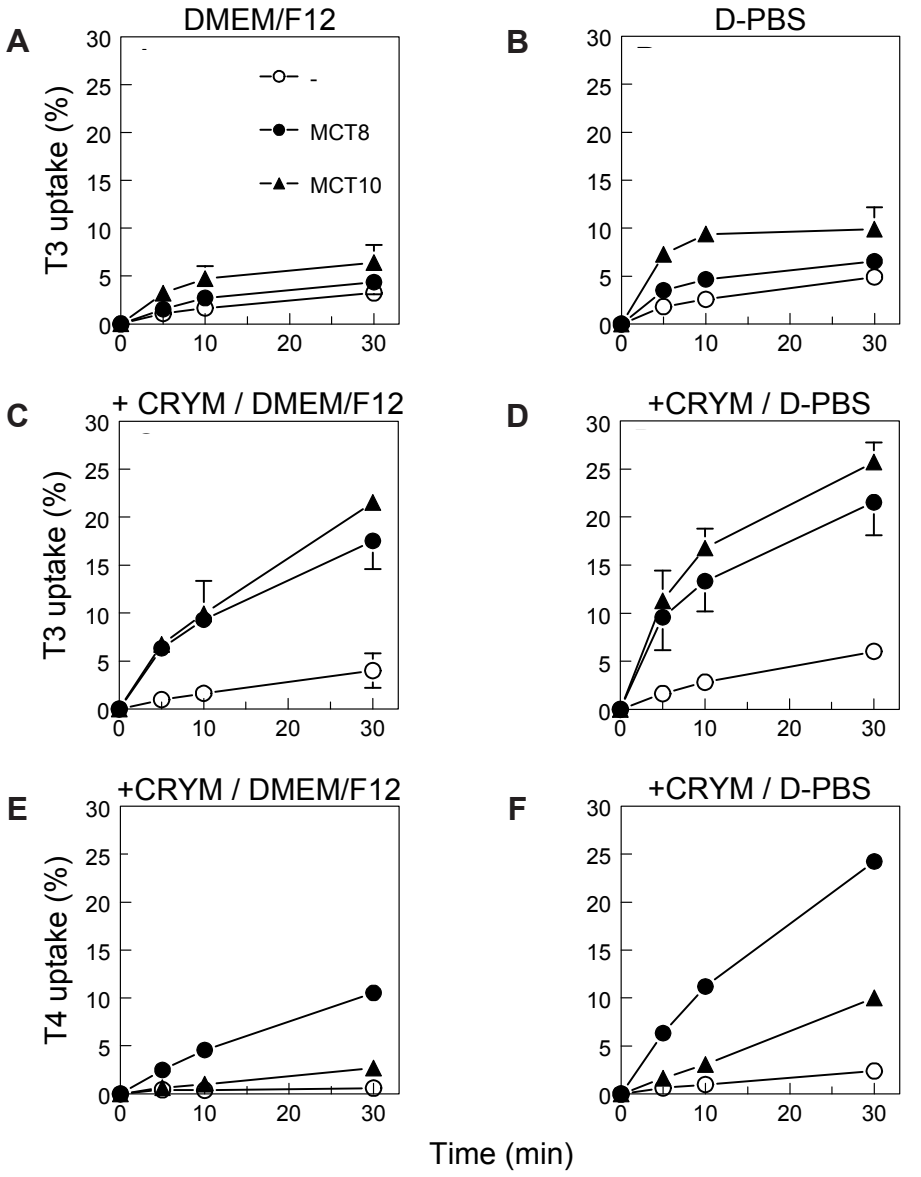

Figure 6.6 Uptake of T3 and T4 by COS1 cells transfected with hMCT8 or hMCT8 cDNA without or with hCRYM cDNA. Cells were transfected with control cDNA (○), hMCT8 cDNA (●) or hMCT10 cDNA (▲) without (A,B) or with (C-F) hCRYM cDNA, and incubated for 5-30 min at 37 C with 1 nM [^{125}I]T3 (A-D) or [^{125}I]T4 (E-F) in DMEM/F12 (A,C,E) or D-PBS medium (B,D,F) containing 0.1% BSA. After incubation, cells were processed and cellular radioactivity was determined as described in Materials and Methods. Results are presented as means ± SD (n=2-4).

We studied the concentration dependence of T3 uptake in COS1 cells transfected with hCRYM cDNA without or with hMCT8 or hMCT10 cDNA. Increasing the T3 concentration

from 1 nM to 10 μM resulted in a progressive decrease in the percentage uptake of [^{125}I] T3 in cells not transfected with transporter as well as in cells transfected with hMCT8 or hMCT10. Irrespective of the expression of hMCT8 or hMCT10, [^{125}I]T3 uptake showed 50% inhibition at approximately 1 μM T3 (not shown). Considering the possible saturation of multiple processes, such as T3 uptake by endogenous and transfected transporters, T3 binding to BSA, and T3 binding to hCRYM, no attempts were made to derive kinetic constants from these data.

The above studies were carried out using DMEM/F12, a culture medium containing high concentrations of compounds, in particular (aromatic) amino acids, which may interfere with the transport of iodothyronines by hMCT8 and hMCT10. Therefore, we compared T3 and T4 transport by hMCT8 and hMCT10 in transfected COS1 cells incubated with ligand in D-PBS medium, a simple buffered salt solution with MgCl2 and CaCl2 plus 1 g/L D-glucose added. Figures 6.6 B and D show T3 uptake by cells transfected without or with hCRYM, respectively, and Figure 6.6 F shows results for T4 uptake in cells transfected with hCRYM.

The findings indicate an increase in T3 and T4 uptake if cells were incubated with ligand in D-PBS medium compared with DMEM/F12 irrespective of transfection with hMCT8 or hMCT10 cDNA or with control vector. This suggests that compounds present in DMEM/F12 but not in D-PBS medium do not specifically interfere with iodothyronine transport by hMCT8 or hMCT10.

We have also investigated the influence of BSA on T3 uptake in COS1 cells. In general, T3 uptake is higher in the absence than in the presence of 0.1% BSA, but the fold increase induced by transfection with hMCT8 or hMCT10 is greater in the presence of BSA (data not shown).

We next investigated if the increased accumulation of T3 and T4 in hCRYM-co-expressing cells transfected with hMCT8 or hMCT10 compared with non-CRYM expressing cells was indeed caused by a diminished iodothyronine efflux. This was done by loading MCT8 or MCT10-transfected cells for 10 min with [^{125}I]T3 or [^{125}I]T4, after which cells were briefly washed and subsequently incubated for 2, 5 or 10 min with fresh medium without ligand. The results are presented in Figures 6.7 and 6.8, showing a rapid release of most cell-associated T3 and T4 in the first 2 min of the efflux incubation. Cellular efflux of T3 and T4 appeared faster in cells expressing hMCT8 than in cells expressing hMCT10 which in turn appeared faster than in control cells. Addition of 10 μM unlabeled T3 (Figure 6.7) or T4 (Figure 6.8) to the efflux medium had little effect on the efflux of [^{125}I]T3 or [^{125}I]T4 in the absence of hCRYM.

This rapid release of cellular T3 and T4 was largely prevented if cells also expressed hCRYM, indicating that the increased accumulation of T3 and T4 in cells expressing hCRYM in addition to hMCT8 or hMCT10 is indeed explained by the increased intracellular binding and, thus, decreased efflux of T3. The addition of excess unlabeled T3 or T4 to the efflux

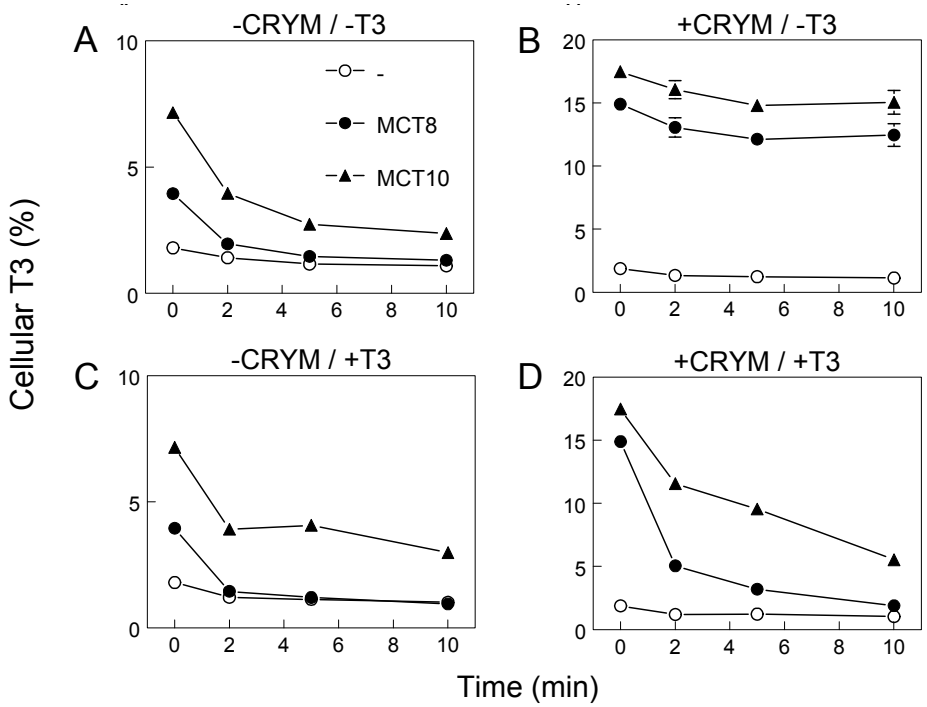

Figure 6.7 Effects of hCRYM on efflux of cellular T3. COS1 cells were transfected with control cDNA (○), hMCT8 cDNA (●) or hMCT10 cDNA (▲) without (A,C) or with (B,D) hCRYM cDNA, and incubated for 10 min at 37 C with 1 nM [^{125}I]T3 (A-D) in D-PBS medium containing 0.1% BSA. After brief washing, cells were incubated with fresh D-PBS medium plus 0.1% BSA without (A,B) or with (C,D) 10 μM unlabeled T3. After incubation for 2-10 min at 37 C, cells were processed and radioactivity was determined in the medium as described in Materials and Methods. Results are presented as means ± SD (n=2-4).

medium greatly increased the rate of [^{125}I]T3 and [^{125}I]T4 efflux from cells co-transfected with hCRYM and hMCT8. Excess medium T3 also stimulated efflux of [^{125}I]T3 from cells co-transfected with hCRYM and hMCT10. Little efflux of [^{125}I]T4 was observed from cells co-transfected with hCRYM and hMCT10 even in the presence of excess unlabeled T4 in the medium. Although displacement of [^{125}I]T3 and [^{125}I]T4 from hCRYM and inhibition of their reuptake by excess unlabeled T3 and T4 are important contributing mechanisms, the results suggests that efflux is better facilitated by hMCT8 than by hMCT10.

Initially, MCT10 has been characterized as a T-type amino acid transporter primarily using Xenopus oocytes as an expression system (11, 12). Using this system, we were unable to detect transport of aromatic amino acids by rMCT8 (15). Therefore, we decided to investigate possible transport of ^{3}H-labeled Phe, Tyr and Trp by hMCT8 or hMCT10 in transfected COS1 cells. Figure 6.9 shows a very rapid uptake of all aromatic amino acids in COS1 cells transfected with empty control vector. Since native COS1 cells express MCT10, this background uptake may be explained by endogenous MCT10 but also alternative

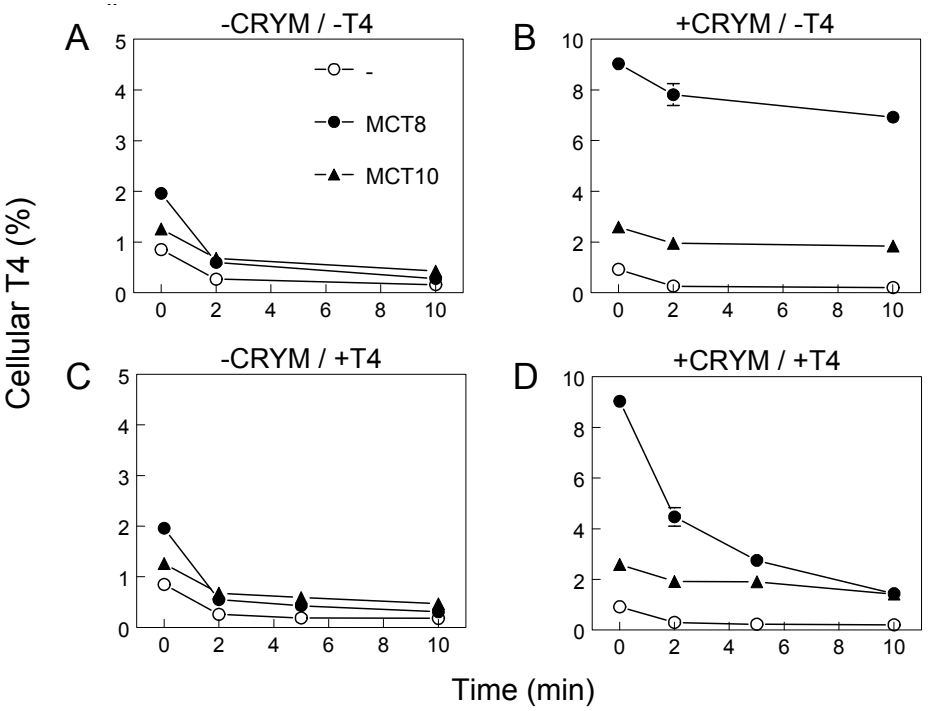

Figure 6.8 Effects of hCRYM on efflux of cellular T4. COS1 cells were transfected with control cDNA (○), hMCT8 cDNA (●) or hMCT10 cDNA (▲) without (A,C) or with (B,D) hCRYM cDNA, and incubated for 10 min at 37 C with 1 nM [^{125}I]T4 (A-D) in D-PBS medium containing 0.1% BSA. After brief washing, cells were incubated with fresh D-PBS medium plus 0.1% BSA without (A,B) or with (C,D) 10 µM unlabeled T4. After incubation for 2-10 min at 37 C, cells were processed and radioactivity was determined in the medium as described in Materials and Methods. Results are presented as means ± SD (n=2-4).

transporters such as the L-type amino acid transporter that is also capable of transporting aromatic amino acids (34).

Transfection of COS1 cells with hMCT8 did not affect uptake of any of the amino acids, which confirms our previous findings with rMCT8 in oocytes indicating that MCT8 is highly specific for iodothyronines. Surprisingly, transfection of COS1 cells with hMCT10 cDNA resulted in a rapid and large decrease in the accumulation of Phe, Tyr and Trp by these cells. This suggests that MCT10 primarily mediates the efflux rather than uptake of the different amino acids, which is in agreement with a recent study carried out in Xenopus oocytes (35).

Figure 6.10 shows the effects of 1 mM unlabeled Phe, Tyr or Trp on the uptake of [^{125}I] T3 by hMCT8 or hMCT10 in COS1 cells co-transfected with hCRYM. Neither Phe nor Tyr significantly affected T3 uptake by hMCT8 or hMCT10. Whereas 1 mM Trp did not inhibit hMCT8, it produced a 33% inhibition of T3 uptake by hMCT10. Addition of 1 mM Trp to the efflux medium had little effect on the efflux of [^{125}I]T3 from cells transfected with hMCT8 or hMCT10 alone or in combination with hCRYM (data not shown).

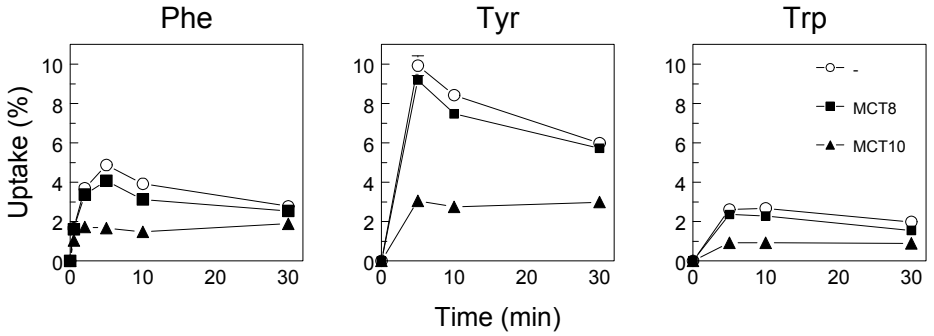

Figure 6.9 Uptake of aromatic amino acids by COS1 cells transfected with hMCT8 or hMCT10 cDNA. Cells were transfected with control cDNA (○), hMCT8 cDNA (●) or hMCT10 cDNA (▲), and incubated for 0.5-30 min at 37 C with 10 µM [^{3}H]Phe, [^{3}H]Tyr or [^{3}H]Trp in D-PBS medium without BSA. After incubation, cells were processed and cellular radioactivity was determined as described in Materials and Methods. Results are the means ± SD from a representative experiment.

We have previously demonstrated that transfection of cells with hMCT8 facilitates intracellular metabolism of different iodothyronines by intracellular D1, D2 and D3 (16). In this study we addressed the question if hMCT10 similarly facilitates intracellular metabolism of T4 and T3. Therefore, we transfected COS1 cells with hD3 alone or in combination with hMCT8 or hMCT10, and subsequently incubated the cells for 2 h with [^{125}I]T3 or for 24 h with [^{125}I]T4. In cells transfected with D3 alone, 9% of T3 was converted to 3,3'-T2 and 3'-T1 and 11% of T4 was converted to rT3 and further metabolites (Figure 6.11).

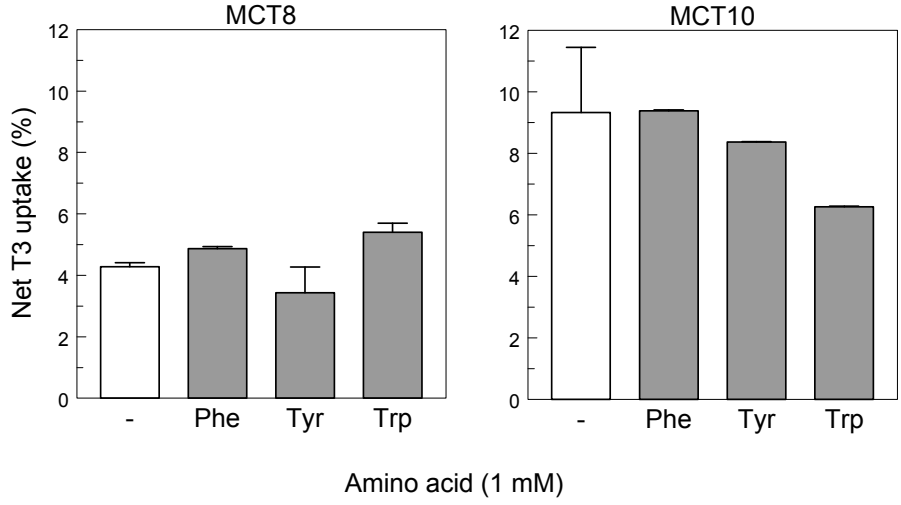

Figure 6.10 Influence of aromatic amino acids on the uptake of T3. COS1 cells were transfected with hMCT8 or hMCT10 cDNA and incubated for 10 min with 1 nM [^{125}I]T3 in D-PBS medium containing 0.1% BSA and with or without 1 mM Phe, Tyr or Trp. Results are presented as uptake minus uptake from control transfected COS1 cells. Results are the means ± SD from a representative experiment.

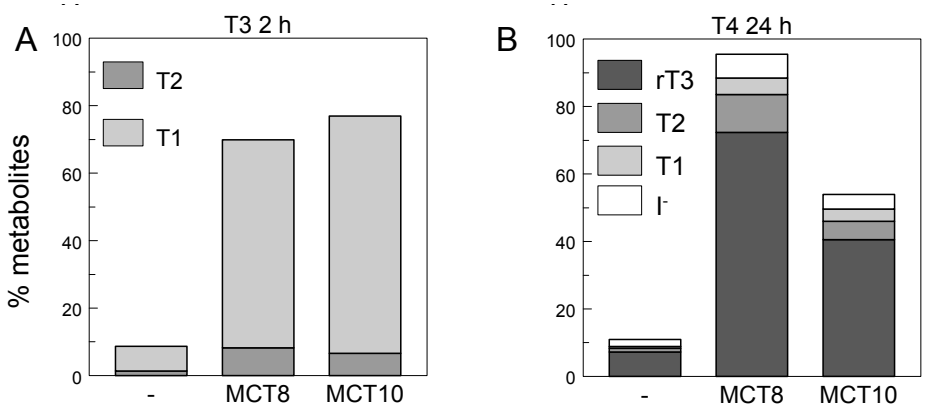

Figure 6.11 Effects of hMCT8 and hMCT10 on intracellular metabolism of T3 and T4 by hD3. COS1 cells were cotransfected with hD3 cDNA and control, hMCT8 or hMCT10 cDNA, and incubated for 2 h or 24 h at 37 C with 1 nM [^{125}I]T3 (A) or [^{125}I]T4 (B), respectively, in DMEM/F12 medium containing 0.1% BSA. Metabolism of T3 and T4 was analyzed by HPLC as described in Materials and Methods. Results are the means of duplicate determinations from a representative experiment.

Co-transfection with hMCT8 or hMCT10 resulted in an 8.1 and 8.9-fold increase in the metabolism of T3 to 70% and 77%, and an 8.8 and 5.0-fold increase in T4 metabolism to 96% and 54%, respectively. Similar facilitation of intracellular T4 metabolism was observed in cells transfected with hD2 plus hMCT8 or hMCT10 compared with cells transfected with hD2 alone (data not shown).

To demonstrate the physiological importance of hMCT10 for T3 transport, we performed siRNA-mediated knockdown studies in HEK293 cells. These cells have relatively high endogenous hMCT8 and hMCT10 mRNA expression levels with CT values of 23-24 determined by Q-PCR (data not shown). HEK293 cells transfected with either one of the three hMCT10-targeted siRNAs showed a significant 20% ($p<0.0001$) reduction of T3 uptake compared with cells transfected with nonspecific siRNA after 48h transfection (data not shown). Transfection efficiency as determined using control siRNA labeled with Alexa Fluor 488 amounted to only 25%.

DISCUSSION

Our studies in transfected COS1 cells demonstrate that hMCT10 is an active thyroid hormone transporter. Compared with the homologous hMCT8 thyroid hormone transporter, hMCT10 is more effective in transport of T3 and less effective in transport of T4. Both hMCT8 and hMCT10 appear to facilitate bidirectional transport of T4 and T3 across the plasma membrane with a net increase in intracellular hormone levels compared with non-transfected cells. This is substantiated by the increase in intracellular iodothyronine

metabolism by hD3 if cells are cotransfected with hMCT8 or hMCT10. The efflux of T3 and T4 from hMCT8 or hMCT10-expressing cells is strongly diminished if their intracellular binding is increased by expression of the cytosolic binding protein hCRYM, providing a convenient system for studying specifically the uptake of thyroid hormone. In contrast to hMCT8, hMCT10 also transports aromatic amino acids, although it is more effective in the release than in the uptake of these ligands. Given the relatively low transfection level of 25%, the level of inhibition in HEK293 cells observed in our siRNA experiments suggests that hMCT10 is responsible for the majority of T3 transport in these cells.

MCT10 has been cloned recently from rats and humans and characterized as a T-type amino acid transporter mediating uptake of aromatic amino acids such as Phe, Tyr, Trp and Dopa (11, 12). However, recent studies indicate that the transporter is much more efficient in facilitating efflux of Phe injected into Xenopus oocytes than in facilitating Phe uptake by oocytes (35). Furthermore, the affinity of MCT10 for Phe in both in- and efflux processes is very low, with apparent Km values exceeding 10 mM (35). In the studies of Endou and coworkers, iodothyronine transport by MCT10 could not be detected in oocytes. Furthermore, addition of T4 and T3 were found to be without effect on the transport of aromatic amino acids by MCT10 (11, 12).

It is difficult to reconcile the negative findings of the group of Endou with our findings of active transport of iodothyronines, in particular T3, by hMCT10 that even exceeds T3 transport by hMCT8. Endou and coworkers used a very high ligand concentration (100 µM) to study T3 and T4 uptake by MCT10, which may be supersaturating the transporter and, thus, masking the specific uptake of the labeled iodothyronines (11, 12). This is supported by our findings that 1 µM T3 is sufficient to produce 50% saturation of hMCT10. However, the same high concentrations (100 µM) of unlabeled T3 and T4 should then be expected to inhibit transport of radioactive amino acids, which was not observed (11, 12). This may be explained if iodothyronines and the aromatic amino acids do not share the same binding sites on the transporter or if amino acid in- and efflux are equally affected by iodothyronines. Vice versa, we observed a significant 33% inhibition of hMCT10-mediated T3 uptake by 1 mM Trp, indicating competition between Trp and T3 transport by hMCT10.

Of special interest is the lack of affinity-labeling of hMCT10 by BrAc[^{125}I]T3 in contrast to the efficient labeling of hMCT8. Although other amino acids may also be modified, the primary targets for protein labeling by bromoacetylated ligands are the Cys residues. In this regard, it is interesting to note that hMCT8 has 10 Cys residues, most of which are located in putative TMDs. Eight of these Cys residue are also present at corresponding positions in hMCT10, but the Cys residues at positions 423 and 472 in hMCT8 correspond to Phe and Lys in hMCT10, respectively.

We are currently investigating if one of these Cys residues is the site of BrAcT3 labeling of hMCT8 and if their absence in hMCT10 explains why this protein is not labeled. The lack of labeling of hMCT10 is not due to a decreased affinity of BrAcT3 for this transporter,

since hMCT10 is at least as efficient as hMCT8 in facilitating the cellular entry of BrAcT3, allowing its labeling of the intracellular rD1 active center. The lack of hMCT10 labeling by BrAcT3 may thus be explained by the absence of a (SH) group susceptible to covalent modification by the bromoacetyl derivative. Within rD1, the selenocysteine residue is thought to be the target for BrAcT3 labeling.

What could be the physiological relevance of MCT10 as a thyroid hormone transporter? Our studies indicate that the transporter facilitates the cellular entry of T4 and, in particular, T3, allowing their access to intracellular processes such as metabolism by deiodinases. It is equally likely that MCT10 also facilitates T3 access to its nuclear receptors. Although this has been clearly demonstrated for the L-type amino acid transporter, which also transports T3, further studies are required to establish such a role for MCT8 and MCT10 in thyroid hormone action (36).

However, since hMCT8 and hMCT10 mediate bidirectional transport of iodothyronines, they may also be important for the release of T3 from cells where the hormone is produced by deiodination of its precursor T4. In D1-expressing tissues such as liver and kidney this T3 is released into the circulation to be transported to its various target tissues (2). In D2-expressing tissues such as the brain T3 is preferentially supplied to local targets (2, 37-39). Such a paracrine regulation of local T3 supply involves the conversion of T4 to T3 in D2-expressing astrocytes and subsequent routing of T3 to its major target cells, the neurons (37-39). MCT8 appears crucial for T3 uptake by neurons, and hemizygous mutations of the MCT8 gene results in severe psychomotor retardation (18-26). In the regulation of local T3 levels in the brain, T3 release from astrocytes is an equally important process as T3 uptake by neurons and T4 uptake by astrocytes. Equally essential is the transport of T4 across the blood-brain barrier. Other than the localization of the T4-specific OATP1C1 transporter in brain capillaries, nothing is known about this important process (40-42).

The exact cellular and subcellular localization of MCT10 should provide clues about its physiological role. MCT10 mRNA expression has been detected in different tissues, including the entire gastrointestinal tract, liver, kidneys and skeletal muscle, with relatively low levels of expression in brain (11, 12, 35). In kidney, MCT10 protein was located in the basolateral membranes of proximal tubular cells, in liver it was localized to the basolateral membrane of centrilobular hepatocytes, and in the intestine a basolateral localization was observed in entrocytes increasing towards the tip of the villi (35). In liver and kidney, MCT10 may be involved in plasma↔tissue transfer of thyroid hormone, and it is tempting to speculate that in the intestine MCT10 may be important for thyroid hormone absorption.

Particularly interesting is the high expression of MCT10 mRNA in placenta (see expression profile in Genbank http://www.ncbi.nlm.nih.gov/UniGene/ESTProfile Viewer.cgi?uglist=Hs.591327). Placental transfer of maternal thyroid hormone is essential for fetal development, in particular of the brain (43). This is especially important during the first trimester of gestation when the fetal thyroid has not yet developed. Subsequently, there

is an increasing contribution of the fetal thyroid to circulating hormone levels, although in case of defective thyroid development or thyroid hormone synthesis maternal thyroid hormone remains an important source for the fetus throughout gestation (43). Possibly, MCT10 is involved in the placental transfer of maternal thyroid hormone to the fetus. However, fetal brain development depends on the supply of T4 as a substrate for local T3 generation rather than on supply of circulating T3 (2, 38, 43). Placental transport may also be greater for T4 than for T3, which argues against an important role for MCT10 that prefers T3 over T4 as the ligand. However, maternal-fetal transfer of thyroid hormone may well be controlled to a large extent by the high placental expression of D3 which is more effective in the degradation of T3 than of T4 (44, 45). It is important to investigate the involvement of MCT10 in the placental transfer of thyroid hormone.

In conclusion, our studies clearly demonstrate that MCT10 is a thyroid hormone transporter with preference for T3 over T4. It appears an even more effective T3 transporter than MCT8, which is likely to play a crucial role in neuronal T3 supply in the brain. The importance of MCT10 for cellular entry or efflux of T3 in different tissues remains to be fully explored.

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Chapter 7

Regulation of MCT8 expression

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Manuscript in preparation

ABSTRACT

Objective: The MCT8 protein contains several N-terminal PEST domains, enriched with proline (P), glutamic acid (E), aspartic acid (S) and threonine (T). PEST domains occur in many rapidly degraded proteins. Regulated protein degradation importantly takes place via the ubiquitin-proteasome pathway. In the present study we test the hypothesis that the PEST domains target MCT8 for rapid degradation via the ubiquitin-proteasome pathway.

Design: We constructed Flp293-hMCT8Y, a cell line stably expressing a hMCT8-YFP fusion protein. Synthesis of hMCT8-YFP was inhibited with MCT8 siRNA, after which expression was analyzed using FACS. MCT8 expressing JEG293 cells were incubated with proteasome inhibitor MG132, aiming to determine (poly) ubiquitinated MCT8 molecules on immunoblots. Finally, COS1 and JEG 3 cells were transfected with a hMCT8-ΔPEST cDNA, devoid of the PEST domains, and uptake of T3 was analyzed.

Results: Function of YFP containing hMCT8 proved to be similar to wild-type MCT8. No decrease of hMCT8-YFP expression was observed within 24 h after inhibition of synthesis. However, a dose dependent decrease was observed after 48 and 72 h. Immunoblots revealed ubiquitinated MCT8 after inhibition of the proteasome. Transfection with hMCT8-ΔPEST did not increase T3 uptake compared to control cells. hMCT8-ΔPEST protein could not be detected.

Conclusion: MCT8 is a target for the ubiquitin-proteasome pathway, indicating specific and controlled breakdown. However, in spite of the N-terminal PEST domains, no rapid degradation was observed. In contrast, elements within or close to these domains appear essential for normal protein synthesis and expression.

INTRODUCTION

Among the most intriguing structural aspects of the MCT8 protein are the N-terminal PEST domains. These domains were already noted at the identification of the MCT8 gene by Lafrenière *et al.* in 1994 (1). They called the gene, now known as SLC16A2, XPCT, for X-linked PEST containing transporter. PEST domains, enriched with proline (P), glutamic acid (E), aspartic acid (D), serine (S) and threonine (T), occur in many proteins and may be associated with rapid protein degradation. PEST domains are hydrophilic stretches of 12 or more amino acids containing at least one P, E or D and one S or T, flanked by lysine (K), arginine (R) or histidine (H) residues (2). Many PEST containing proteins, such as p53, HSP70 and ornithine decarboxylase (ODC) have very short half-lives of 0.5 to 2 h (3). Involvement of PEST domains in this rapid turnover was demonstrated, for example, by Ghoda *et al.*, who truncated the mouse ODC protein just before the C-terminal PEST domain (4). The shortened protein showed to be at least 10 fold more stable than the native protein. Adding the PEST domain of mouse ODC to the stable Trypanosoma ODC converted it to a rapidly degraded protein (5). the ProtParam protein analysis program (www.expasy.org/tools/protparam.html) regards full length hMCT8 as an unstable protein, but predicts a half-life of over 30 h (in reticulocytes), which does not suggest rapid protein turnover. hMCT8 without PEST domains is predicted to be a stable protein, but with a similar half-life as the full length protein. These intriguing data prompted us to study whether MCT8 is in fact rapidly degraded, and if a hMCT8 variant devoid of PEST domains is more stable.

One of the most important pathways for intracellular protein degradation is the ubiquitin-proteasome pathway. It allows for tight regulation of protein breakdown. Proteins targeted by this system are 'ubiquitinated' (or ubiquitilated), a process in which 8.5 kDa ubiquitin molecules are covalently attached to a lysine residue in the target protein. This highly specific process involves ubiquitin-activating enzyme (E1), ubiquitin-carrier enzymes (E2) and ubiquitin ligases (E3) (6). A polyubiquitin chain is formed by binding an additional ubiquitin to the lysine in position 48 of the previous molecule. This polyubiquitin chain serves as a recognition marker for the proteasome, in which the tagged protein is degraded. Many different E3 enzymes have been identified, recognizing specific motifs on target proteins. These include primary and secondary protein structures, but also post-translational modifications like phosphorylation or binding to ancillary proteins. Various proteins containing PEST domains are degraded via the ubiquitin-proteasome pathway. Examples are the intracellular domain of Notch 1 (7) and NPDC-1 (neural proliferation and differentiation control protein 1) (8).

In the present studies we tested the hypothesis that the N-terminal PEST domains target MCT8 for rapid protein turnover, possibly via the ubiquitin-proteasome pathway. We constructed a stable yellow fluorescent protein (YFP) labeled MCT8 expressing cell line to study MCT8 expression after transfection with MCT8 siRNA using fluorescence assisted cell

sorting (FACS). Furthermore, we incubated MCT8 transfected cells with MG132, a potent inhibitor of the proteasome (9), aiming to detect an increase in (poly)ubiquitinated MCT8 molecules. Co-transfection of cells with MCT8 and His-tagged ubiquitin enabled specific isolation of such molecules. Finally, we constructed a hMCT8 variant without N-terminal PEST domains in order to study effects on protein stability and function. We demonstrate that MCT8 is indeed ubiquitinated. However, we were not able to detect a rapid decrease of MCT8 expression after inhibition of its synthesis, suggesting that in spite of the N-terminal PEST domain, MCT8 is not subject to rapid protein degradation.

MATERIALS AND METHODS

Construction of a stable cell line expressing hMCT8-YFP

The coding sequence of hMCT8 (10) was subcloned into a pEYFP-N1 expression vector (Clontech, BD Biosciences, Breda, The Netherlands) using *Hind*III and *Age*I. To enable this, the hMCT8 stop codon was transformed into an AgeI restriction site by site-directed mutagenesis. The resulting vector pEYFP-N1-hMCT8 codes for a hMCT8-YFP fusion protein, with the YFP linked to the C-terminal end of hMCT8 via a 4- amino acid spacer (Pro-Val-Ala-Thr). Functional characteristics of the fusion protein were compared to unlabeled hMCT8 in transient transfection studies in JEG3 cells. (see results section). To obtain stably hMCT8-YFP transfected Flp-in 293 cells (Invitrogen, Breda, the Netherlands), the chimeric coding sequence was subcloned into pcDNA5/FRT (Invitrogen) using *Hind*III and *Not*I. HEK293 derived Flp-in 293 cells (Invitrogen) were co-transfected with pcDNA5/FRT-hMCT8-YFP and pOG44 (Invitrogen), a Flp-recombinase containing vector, in various ratios using FuGene 6 transfection reagent (Roche Applied Science, Almere, The Netherlands). Transfected cells were selected using Hygromycin (100 µg/ml medium). Four single colonies were cultured separately and compared functionally. One clone was named Flp293-hMCT8Y and selected for further studies.

Construction of a hMCT8 variant without PEST domains

The human MCT8 gene (SLC16A2) codes for a protein containing three N-terminal PEST domains, two of which are included in the short hMCT8 variant that starts at the second putative ATG start site. This variant, which is homologous to the rodent MCT8, is encoded by the pcDNA-hMCT8 vector used in our studies. We constructed a hMCT8 variant without PEST domains by introducing two *Eco*RI restriction sites, one starting at nucleotide position 10 from the second ATG, and one at position 271. Deleting 87 codons by *Eco*R1 digestion

and religation, the vector (pcDNA3-hMCT8-ΔPEST) codes for a protein with only 9 amino acids before the first predicted transmembrane domain.

Cell culture, transfections, T3 uptake studies and fluorescence microscopy

Flp-in 293 cells stably transfected with hMCT8 (Flp239-hMCT8 cells) and stably transfected controls (Flp239-C cells) were kindly provided by Drs. Alex Ianculescu and Tom Scanlan (UCSF, San Francisco, CA). All stable Flp293 cells were cultured in DMEM/F-12 medium supplemented with 9% heat-inactivated FBS, 100 nM sodium selenite, penicillin/streptomycin and hygromycin (100 µg/ml). HEK293, COS1 and JEG3 cells were cultured in DMEM/F12 with FBS and sodium selenite, but without antibiotics. All transient cDNA transfections were performed using 3 µl FuGene-6 transfection reagent per 1000 ng plasmid DNA (11). Uptake of T3 was tested as reported previously (12). Briefly, cells were incubated with 1 nM (2 x 10^5 cpm) [^{125}I]T3 in 1.5 ml incubation medium at 37 C. After incubation, cells were washed, lyzed with 0.1 M NaOH and counted in a gamma counter. Fluorescence microscopy of hMCT8-YFP expressing JEG3 cells was performed as described previously (11).

siRNA transfections and FACS analysis

Flp293-hMCT8Y cells were transfected with 5-10 nM SCL16A2 chimera RNAi (Abnova, Taipei, Taiwan) using HiPerfect siRNA transfection reagent (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Small interfering RNA (siRNA) directed against house keeping gene MAPK1 (Qiagen) was used as negative control. Flp293-C cells transfected with Allstars Alexa FLuor 488 labeled nonspecific siRNA (Qiagen) served to determine transfection efficiency. After 24, 48 and 72 h, cells were trypsinized and fixated in 4% paraformaldehyde. Mean fluorescence of 10^4 cells was determined by FACS analysis on a Beckman-Coulter Flow Cytometer.

Inhibition of the proteasome and ubiquitination of MCT8

Flp293-hMCT8 and Flp293-C cells were cultured in 6 well plates. At confluence, cells were incubated with culture medium containing 1 µM MG132, a potent inhibitor of the proteasome, for 1, 3, 6 and 24 h. After incubation, we tested T3 uptake and analyzed cell sonicates by western blotting using purified hMCT8 polyclonal antibody 1306 as reported previously (11).

HEK293 cells were transiently co-transfected with pcDNA3-hMCT8 and a pcDNA3.1 vector coding for 6xHis-tagged ubiquitin (pcDNA3.1-6HUB) in the ratio 2:1. Controls were transfected with pcDNA3-hMCT8 or pcDNA3.1-6HUB only, complemented with empty

pcDNA3 vector to obtain equal total cDNA concentrations. After 24 h, cells were incubated for 4 h with medium containing 20 µM MG132. Cells were then harvested and diluted to 10^7 cells per 500 µl in 6 M urea in PBS. After sonication, supernatants were incubated for 2 h at 4 C with 10 µl 5% Ni-NTA beads (Qiagen). Beads were washed and eluted according to the manufacturer's protocol. Eluates were diluted to 0.4 mg protein per ml and analyzed by western blotting using hMCT8 antibody 1306.

RESULTS

Function of YFP labeled MCT8

The function of the hMCT8-YFP fusion protein encoded by the pEYFP-N1-hMCT8 vector, was tested by measuring T3 uptake in transiently transfected JEG3 cells (Figure 7.1 A). Compared to empty pcDNA3 transfected controls, 3.3, 3.9 and 3.0 fold increases of intracellular T3 were measured after 2, 5 and 10 min, respectively. Fold increases were slightly higher than in cells transfected with pcDNA3-hMCT8 (3.0, 3.3 and 2.4 after 2, 5 and 10 min). T3 uptake in JEG3 cells transiently transfected with pcDNA5/FRT-hMCT8-YFP, the vector constructed for stable transfection into Flp-in 293 cells, showed similar results (data not shown). Fluorescence microscopy of JEG3 cells transciently transfected with pEYFP-N1-hMCT8 showed clear distribution of the hMCT8-YFP fusion protein at the plasma membrane (Figure 7.1 B). T3 uptake in Flp293-hMCT8Y cells after 5 min was 2.6 fold higher than in Flp293-C controls, showing roughly the same induction of transport as in non-YFP tagged Flp293-hMCT8 cells (2.8 fold) (Figure 7.1 C).

Inhibition of MCT8 expression with siRNA

Flp293-hMCT8Y cells were used to study MCT8 expression after transfection with MCT8 siRNA as the YFP tag enables rapid determination of MCT8 protein levels by FACS. The efficiency of the HiPerfect siRNA transfection protocol was determined at 45, 58 and 42 % after 24, 48 and 72 h, respectively, in Flp293-hMCT8 cells using a Alexa Fluor 488 labeled nonspecific siRNA. Transfection of Flp293-hMCT8Y with 5 or 10 nM MCT8 siRNA did not lead to a significant decrease of fluorescent intensity within 24 h (Figure 7.2). However, a dose dependent decrease was observed after 48 and 72 h. After 72 h, transfection with 10 nM MCT8 siRNA resulted in a decrease of YFP fluorescence of ~50 %. Transfection of Flp293-hMCT8Y with siRNA targeting RNA of housekeeping gene MAP-kinase resulted in an non-significant decrease of YFP signal after 72 h. Transfection of untagged Flp293-hMCT8 cells with MCT8 siRNA showed that the background fluorescence signal of these cells is not affected by MCT8 knockdown. These controls confirm that the observed

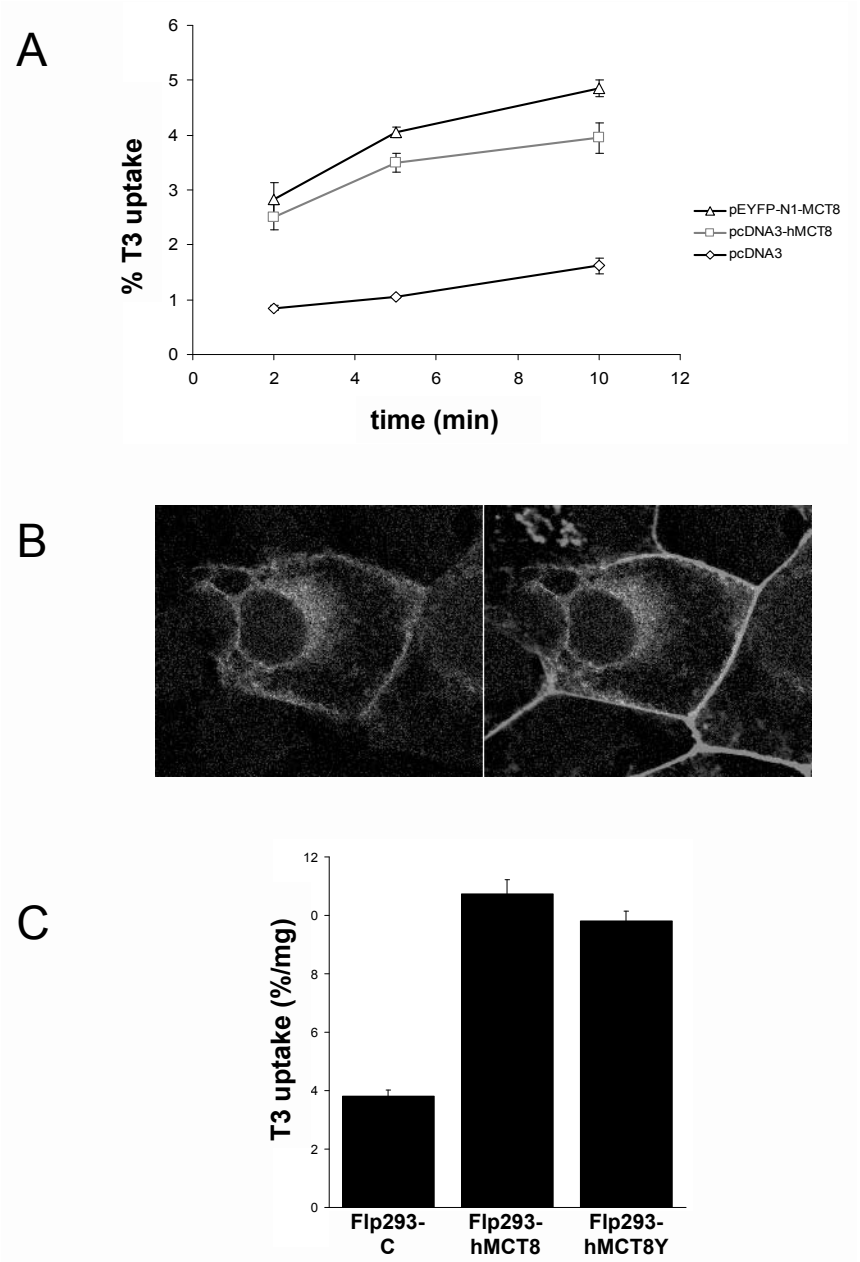

Figure 7.1 A. Uptake of T3 after 2, 5 and 10 min in JEG3 cells transfected with pEYFP-N1-hMCT8, pcDNA3-hMCT8 and empty pcDNA3 controls. **B.** Cellular distribution of the hMCT8-YFP fusion protein (green) in JEG3 cells transfected with pEYFP-N1-hMCT8. Right panel: plasma membrane localization is indicated by co-localization with tight-junction protein ZO-1 (red). For color figure see page 198. **C.** Uptake of T3 after 5 minutes, shown as percentage of total T3 added taken up per mg protein in Flp293-C, Flp293-hMCT8 and Flp293-hMCT8Y cells. No significant differences in T3 uptake between YFP-labeled and non-labeled MCT8 are detected.

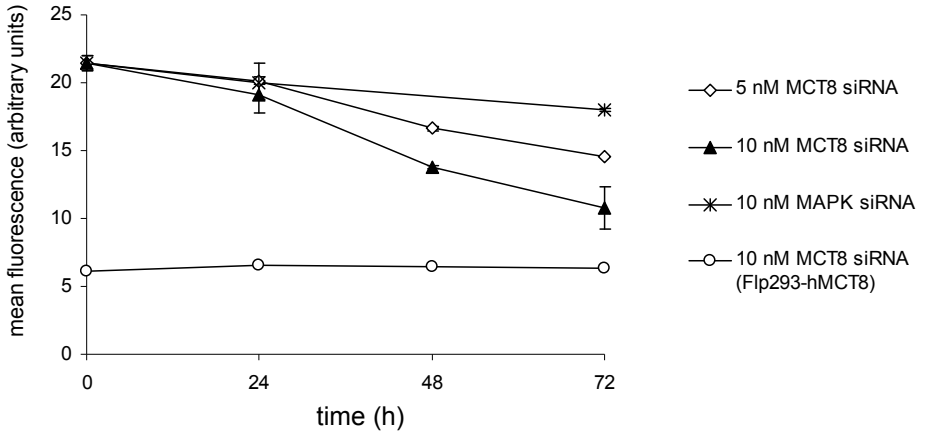

Figure 7.2 Mean fluorescence intensity of Flp293-hMCT8Y cells transfected with SLC16A2 siRNA after 0-72 h. A significant, dose-dependent decrease in intensity is observed after 24 h of transfection.

decreases in fluorescent signal in Flp293-hMCT8Y cells reflect reduced expression of YFP tagged MCT8.

Inhibition of the proteasome and ubiquitination of MCT8

Incubation of Flp293-hMCT8 cells for 1-24 h with 1 µM MG132 resulted in an increase in MCT8 containing protein complexes between 60 and 120 kDa in size, as detected by western blotting (Figure 7.3 A). No clear increase in intensity of these bands was observed in lysates of Flp293-C cells, although HEK293 cells are known to express MCT8 endogenously. Functional analyses of Flp293-hMCT8 and Flp293-C cells showed that MG132 treatment did not significantly change T3 uptake after 5 min incubation (Figure 7.3 B). Sonicates of hMCT8 + 6HUB co-transfected HEK293 cells were analyzed by western blotting using MCT8 specific antibody 1306 before and after incubation with Ni coated beads (Figure 7.3 C).

Pre-incubation samples show bands of ~60 and ~120 kDa, the expected size for MCT8 monomers and dimers, in hMCT8 and hMCT8 + 6HUB co-transfected cells. Bands of ~240 kDa are also observed, suggesting the presence of MCT8 tetramers. Large quantities of MCT8 containing complexes with sizes between ~60 and ~120 kDa and higher are present in the MCT8 + 6HUB co-transfected cells. In smaller amounts these are also observed in cells transfected with hMCT8 only. A ~60 kDa band is also observed in 6HUB only transfected cells, in keeping with endogenous expression of MCT8 monomers in HEK 293 cells.

After incubation with Ni-coated beads, isolating His-tagged ubiquitinated protein complexes, MCT8 is detected in lysates of hMCT8 + 6HUB co-transfected cells. A ~60 kDa band is also seen in hMCT8 only transfected cells, suggesting incomplete removal of

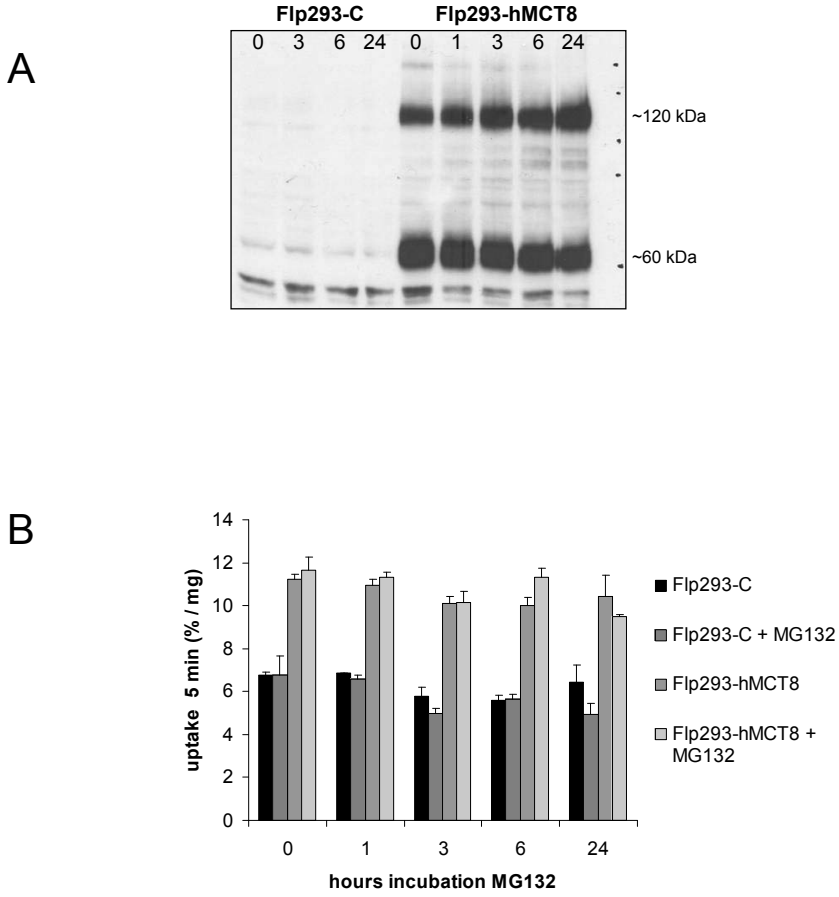

Figure 7.3 A. Western blot of homogenates of Flp293-C and Flp293-hMCT8 cells after 0-24 h incubation with 1 μM MG132, probed with MCT8 specific antibody 1306. Note the increase of MCT8 containing fragments between 60 and 120 kDa, suggesting (poly)ubiquitinated MCT8. **B.** Uptake of T3 after 5 min in Flp293-C and Flp293-hMCT8 cells stimulated with 1 μM MG132 for 0-24 h. No significant differences in uptake are observed after inhibition of the proteasome.

non-His-tagged proteins in this sample, or nonspecific staining. Faint bands of ~60 kDa and higher are also detected in 6HUB only transfected cells.

Function of hMCT8-ΔPEST

The vector pcDNA3-hMCT8-ΔPEST encodes for a MCT8 variant devoid of the N-terminal PEST domains (Figure 7.4 B). However, transfection of JEG3 and COS1 cells with pcDNA3-hMCT8-ΔPEST did not increase T3 uptake compared to empty pcDNA3 transfected controls. Western blotting of sonicates of these cells did not identify MCT8 specific bands (data not

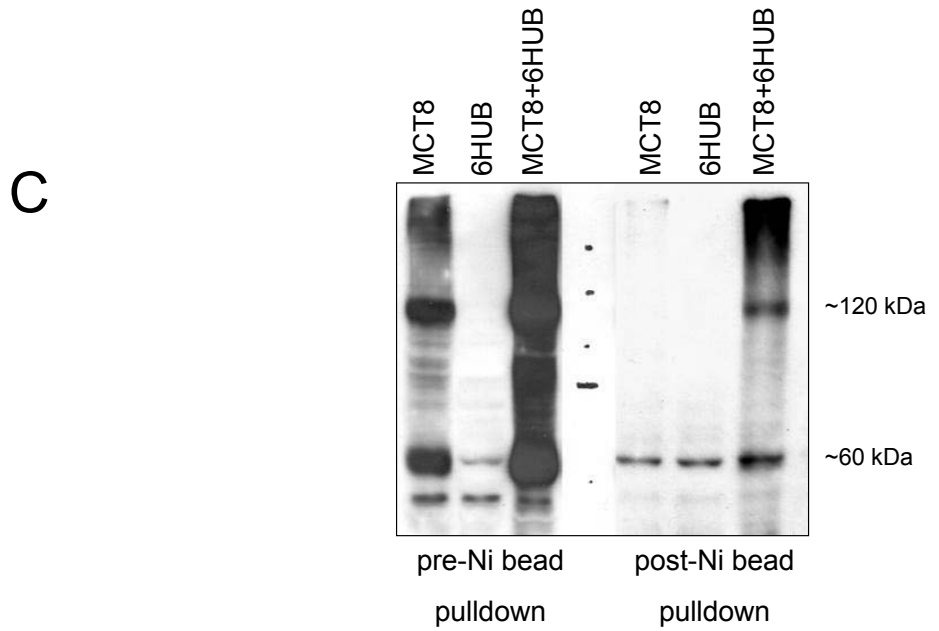

Figure 7.3 C. Western blot of HEK293 homogenates after transfection with hMCT8, 6xHis-tagged ubiquitin (6HUB) and hMCT8 + 6HUB. Left: samples prior to pull-down of ubiquitinated proteins with Ni beads. Ubiquitinated MCT8 is observed in hMCT8 and hMCT8 + 6HUB transfected cells. Right: samples after Ni-bead pull-down, confirming the presence of MCT8 in these ubiquitinated proteins.

shown). From this it was concluded that this hMCT8-ΔPEST variant is not expressed at the protein level.

DISCUSSION

The N-terminal PEST domains contained in hMCT8 suggests the protein is subject to rapid turnover, possibly via the ubiquitin-proteasome pathway. To enable quantitative analyses of MCT8 expression in cells, we constructed a Flp293 cell line stably expressing hMCT8 with a C-terminal YFP tag. Benefits of such a cell line include rapid determination of protein expression by FACS and the possibility to study spatio-temporal aspects of expression in live cells. We demonstrate that YFP labeled MCT8, both in transient and stably transfected cells, functions similar to non-labeled MCT8 regarding the uptake of radiolabeled T3. Not surprisingly, we show that the hMCT8-YFP fusion protein is expressed at the plasma membrane of transfected cells, the functional localization of the transporter. From this it appears that the 26.9 kDa YFP tag does not interfere with the synthesis, trafficking or function of MCT8.

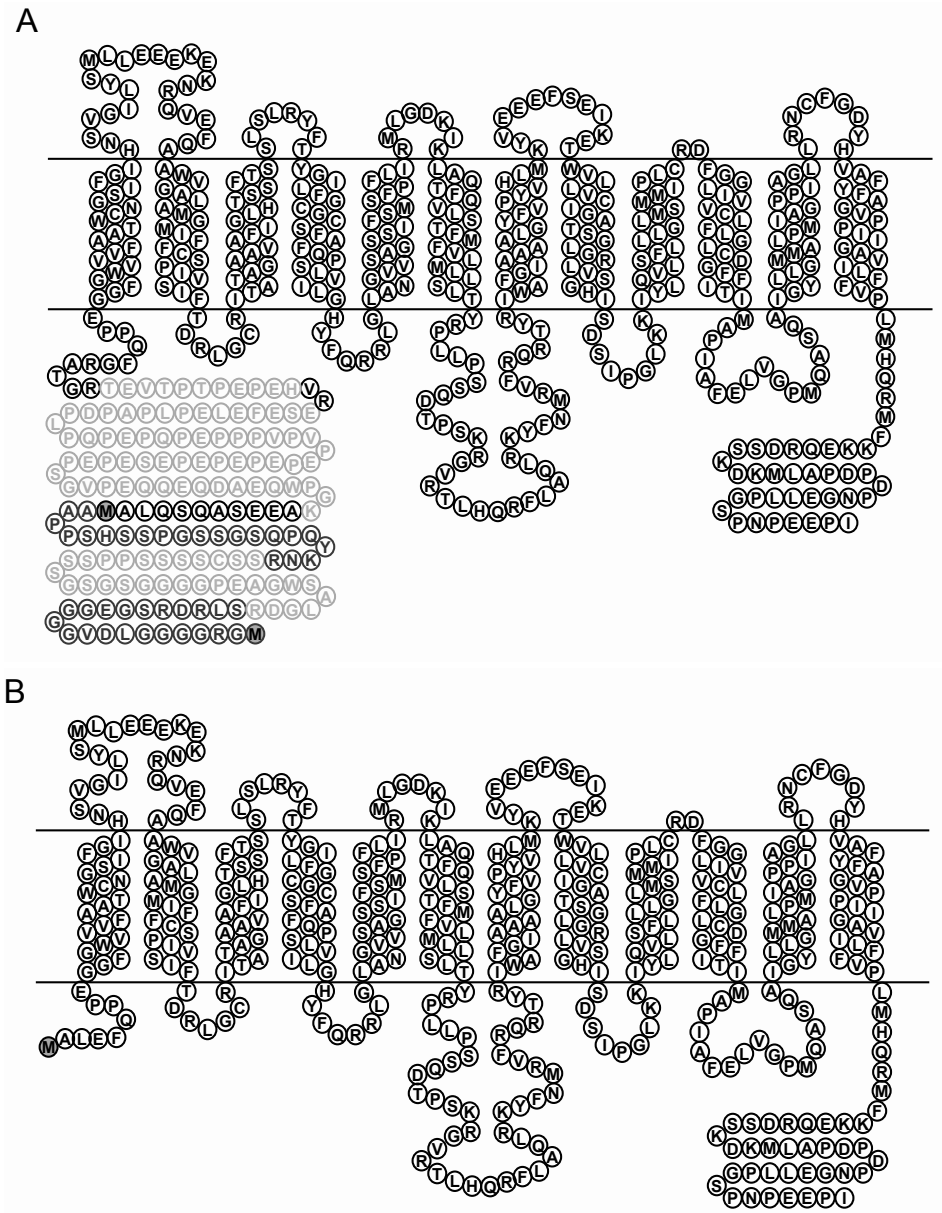

Figure 7.4 A. Putative structure of hMCT8. PEST domains predicted with the PESTfind algorithm at EMBnet Austria (https://emb1.bcc.univie.ac.at) are indicated in grey. **B.** Putative structure of the hMCT8 variant encoded by pcDNA3-hMCT8-ΔPEST.

Inhibition of the proteasome by MG132 results in an intracellular increase of (poly) ubiquitinated proteins. We detected a clear increase of (poly)ubiquitinated MCT8 in Flp293-hMCT8 cells after 1-24 h incubation with 1 μM MG132. Co-transfection of hMCT8 and 6xHis-tagged ubiquitin in HEK293 cells enabled the identification of MCT8 in isolated

ubiquitinated protein cpmplexes, confirming that MCT8, at least in HEK293 cells, is ubiquitinated. Ubiquitinated MCT8 is found in Flp293-hMCT8 and in hMCT8 only transfected HEK293, indicating that a complete ubiquitination machinery, including a specific E3 ubiquitin ligase, is present is these cells. The observed increase of (ubiquitinated) MCT8 after stimulation with MG132 does not lead to an increase of T3 uptake. It is known that ubiquitination plays an important role in internalization of membrane proteins (13), thus inactivating them prior to the actual degradation in the proteasome. It is therefore likely that ubiquitinated MCT8 is not expressed at its functional localization in the plasma membrane. It is however possible that proteins are de-ubiquitinated and relocated to their functional position in the cell, as has been shown, for example, for type 2 deiodinase (14). As yet, we have not studied whether this occurs in our system.

We demonstrate a specific decrease of hMCT8-YFP expression in Flp293-hMCT8Y cells after transfection with a MCT8 specific siRNA. However, we did not observe a rapid decrease, within hours, as may be expected from the presence of the PEST domains. A significant decrease of expression was noted only after 24 h inhibition of MCT8-YFP synthesis. This is in keeping with the predicted protein half-life of over 30 h by the ProtParam program. This might indicate that, despite the N-terminal PEST domains, MCT8 is not a rapid-turnover protein. However, we must consider that although the YFP tag does not interfere with the production, trafficking and function of MCT8, it might effect the degradation. To confirm this, studies of the expression of non-tagged hMCT8 after siRNA transfection need to be performed. Our attempts to study the expression and functional characteristics of a hMCT8 variant without PEST domains were unsuccessful so far. No MCT8 protein was produced by cells transfected with pcDNA3-hMCT8-ΔPEST. It is therefore likely that the N-terminal sequence of MCT8 is vital for normal protein expression or stabilization.

In conclusion, we demonstrate that, in spite of the N-terminal PEST domains, hMCT8 most likely is not subject to rapid protein degradation. In contrast, elements within or close to these domains appear essential for normal protein synthesis and expression. We demonstrate that MCT8 is a target for the ubiquitin-proteasome pathway, indicating specific and controlled breakdown. Our studies so far provide a first look at the regulation of MCT8 expression, but more extensive work needs to be done. This could, for example, address which lysine residue in MCT8 is ubiquitinated, in order to determine if the PEST domains are in fact involved in this process.

ACKNOWLEDGEMENTS

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Chapter 8

Mechanisms of disease: psychomotor retardation and high T3 levels caused by mutations in monocarboxylate transporter 8

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SUMMARY

The actions and the metabolism of thyroid hormone are intracellular events that require the transport of iodothyronines across the plasma membrane. It has become increasingly clear that this does not occur by simple diffusion but is facilitated by transport proteins. Only recently have iodothyronine transporters been identified at the molecular level, of which organic anion transporting polypeptide 1C1 (OATP1C1) and monocarboxylate transporter 8 (MCT8) deserve special mention because of their high activity and specificity towards iodothyronines. OATP1C1 is almost exclusively expressed in brain capillaries, and may be crucial for the transport of the prohormone T4 across the blood–brain barrier. MCT8 is also expressed in the brain—in particular in neurons—as well as in other tissues. MCT8 appears especially important for the uptake of the active hormone T3 into these cells, which is essential for optimal brain development. This T3 is produced from T4 by type 2 deiodinase in neighboring astrocytes. The neurons express type 3 deiodinase, the enzyme that terminates T3 activity. The MCT8 gene is located on chromosome Xq13.2 and has recently been associated with a syndrome combining severe X-linked psychomotor retardation and high serum T3 levels. In over 20 families, where affected males present this syndrome, different mutations in MCT8 have been identified. The mechanism of this disease is thought to involve a defect in the neuronal entry of T3, and thus in the action and metabolism of T3 in these cells, leading to an impaired neurological development as well as a decrease in T3 clearance.

INTRODUCTION

Thyroid hormone is the common name for two compounds,T4 (3,3′,5,5′-tetraiodothyronine) and T3 (3,3′,5-triiodothyronine), produced by the follicular cells of the thyroid gland. T3 is the main bioactive thyroid hormone, whereas T4 has little intrinsic activity. Thyroidal secretion represents the single source of circulating T4 but only contributes a small fraction of plasma T3. Most T3 is generated by deiodination of the prohormone T4 in peripheral tissues (1, 2).

Thyroid hormone is crucial for the development of different organs, in particular the brain, as well as for the regulation of a variety of metabolic processes and thermogenesis throughout life (3-5). Most actions of thyroid hormone are initiated by the binding of T3 to its nuclear receptors, which are the products of the THRA (thyroid hormone receptor α) and THRB (thyroid hormone receptor β) genes (6, 7). Often, binding of T3 to its receptor results in the activation of gene transcription (6, 7); however, T3 also suppresses the expression of certain genes, in particular those coding for both subunits of TSH and TSH-releasing hormone, which is the molecular basis for the negative feedback action of thyroid hormone at the pituitary and hypothalamus level, respectively (8, 9).

Thus, the biological activity of thyroid hormone is largely determined by the intracellular T3 concentration, which depends on the expression of iodothyronine deiodinases. These enzymes convert the prohormone T4 by outer-ring deiodination to receptor-active T3 or catalyze the inner-ring deiodination of T4 and T3 to the receptor-inactive metabolites 3,3′,5′-triiodothyronione (reverse T3 [rT3]) and 3,3′-diiodothyronine (3,3′-T2), respectively (Figure 8.1 A). Three such enzymes, D1–D3, have been characterized as homologous selenoproteins that all contain a selenocysteine residue in their active centers (1, 2).

D1 is expressed in liver, kidney and thyroid, and has both outer- and inner-ring deiodinase activities. Physiologically, it is most important for the production of plasma T3. D2 is expressed particularly in brain, anterior pituitary, (human) skeletal muscle and thyroid. It has outer-ring deiodinase activity only, and is essential for local T3 production in tissues such as brain; however, D2 in skeletal muscle is likely to contribute as a source of circulating T3 (10). In adults, the brain seems to be the main site for D3 expression, but even higher D3 activities are expressed in fetal brain and other fetal tissues, as well as the placenta and the pregnant uterus (1, 2). Local regulation of the thyroid state in brain is largely determined by the coordinated expression of D2 and D3 (11).

Although a different topology has been suggested for D3 (12), all deiodinases have been identified as integral membrane proteins associated with the plasma membrane (D1, D3) or the endoplasmic reticulum (D2) such that the active center is facing the cytoplasm (1, 2). Not only the T3 receptors but also the deiodinase active sites are therefore located intracellularly, implying that both for thyroid hormone action and for metabolism iodothyronine transport across the plasma membrane is required. It has been assumed previously that this

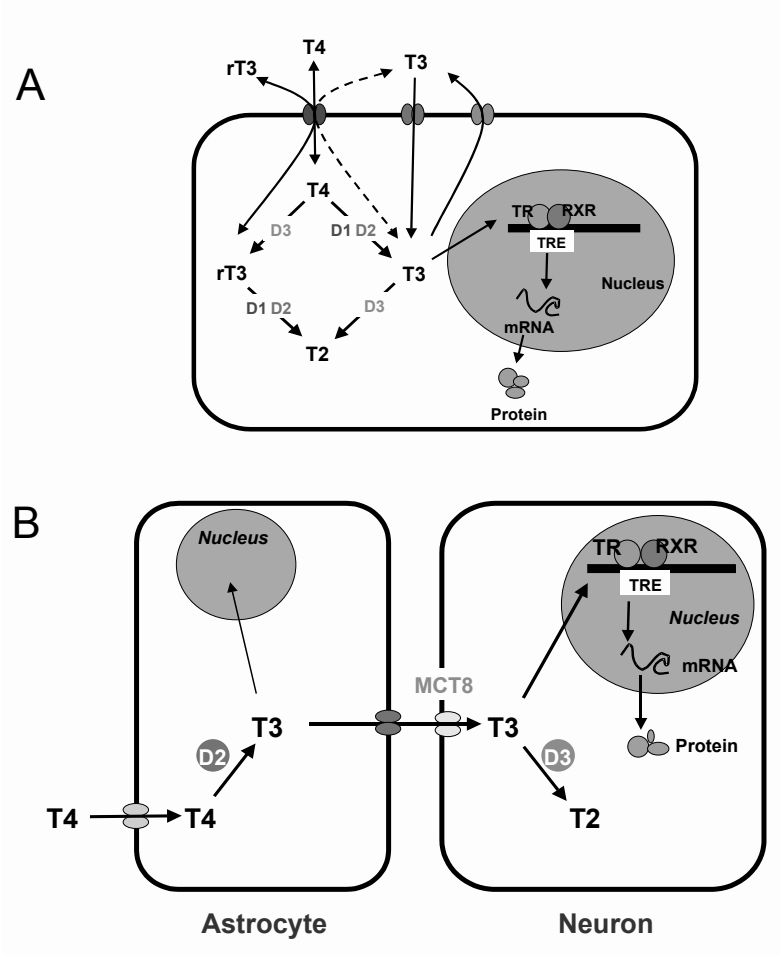

Figure 8.1 A. Model of a T3 target cell, showing the importance of transporters and deiodinases in the regulation of the bioactivity of thyroid hormone. Transporters (shown as paired ovals in the plasma membrane, itself shown as a bold outer line) are required for passage of iodothyronines across the plasma membrane, facilitating uptake, efflux, or both (exchange). They may be specific for T4 and reverse T3 versus T3, or transport all these iodothyronines. The deiodinases catalyze the activation of T4 to T3 (D1, D2) or the inactivation of T4 to reverse T3 and T3 to 3,3′-T2 (D3). Both transporters and deiodinases determine the intracellular T3 concentration available for interaction with its nuclear receptor, which can be expressed as a heterodimer (shown as paired ovals in the nucleus) with retinoid X receptor; this interaction leads to transcription of various genes and consequent generation of proteins. **B.** Local control of T3 bioavailability in the brain. Ultimate delivery of T3 to its primary target, the nuclear receptor in neurons, requires the transfer of T4 across the blood–brain barrier (not shown), its uptake by astrocytes and deiodination by D2 therein, followed by the release of T3 from these cells, and finally the uptake of T3 via monocarboxylate transporter 8 into the neurons. The identity of the other transporters involved in this system is not established. Neurons also express D3 for termination of T3 action. MCT8, monocarboxylate transporter 8; rT3, reverse T3; RXR, retinoid X receptor; TR, T3 receptor; TRE, T3-responsive element. For color figures see page 199.

could take place by the simple diffusion of these lipophilic compounds through the lipid bilayer of the plasma membranes; however, it has been established that cellular influx and efflux of thyroid hormone are carrier-mediated (Figure 8.1 A).

Using cells isolated from different tissues it has been demonstrated that iodothyronine uptake is a saturable process (13). Tissue-specific uptake of thyroid hormone is suggested by the different characteristics reported for the transporters expressed in various tissues: firstly, their specificity for the different iodothyronines; secondly, their dependence on cellular ATP content; thirdly, their requirement for Na+ as a co-ligand; and, fourthly, their interaction with a variety of inhibitors and alternative ligands (13). These results suggested the involvement of different types of transporters in the tissue uptake of thyroid hormone.

Only during the last few years have thyroid hormone transporters been characterized at the molecular level (14-16). These include the Na+-taurocholate cotransporting polypeptide (17, 18), different members of the Na+-dependent organic anion transporting polypeptide (OATP) family (17, 19-23), the heterodimeric L-type amino acid transporters LAT1 and LAT2 (24, 25), and the monocarboxylate transporter 8 (MCT8) (26). Most of these transporters are multispecific, accepting a variety of ligands. Notable exceptions are OATP1C1 (21-23) and MCT8 (26), which show high specificity towards iodothyronines. This review will focus on molecular and clinical aspects of MCT8.

IDENTIFICATION OF MCT8 AS A THYROID HORMONE TRANSPORTER

The MCT family earned its name because the first 4 members (MCT1-4) have been characterized as true monocarboxylate transporters (27). However, MCT10 has been shown to be a T-type (aromatic) amino acid transporter (28, 29), and recently we have demonstrated that rat MCT8 is an active and specific iodothyronine transporter (26).

The human SLC16A2 (formerly MCT8) gene is located on chromosome Xq13.2 and contains 6 exons (Figure 8.2 A) (30). MCT8 contains two in-frame translation start sites, potentially coding for proteins of 613 or 539 amino acids (Figure 8.2 B). In all other nonprimate species studied, MCT8 only contains the second translation start site and is homologous with human MCT8 only downstream from this site (16), suggesting that the short human MCT8 protein represents the functional transporter. Both forms of MCT8 contain 12 putative transmembrane domains (TMDs) and both the N-terminal and the C-terminal domains are located intracellularly. (16, 27). Transient transfection of different cell lines, including COS1 and JEG3 cells, with cDNA coding for human MCT8 results in the marked stimulation of T3 and T4 uptake (31, 32). That MCT8 indeed facilitates the intracellular availability of these iodothyronines has been demonstrated in cotransfection experiments utilizing MCT8 and iodothyronine deiodinases. Incubation of nontransfected

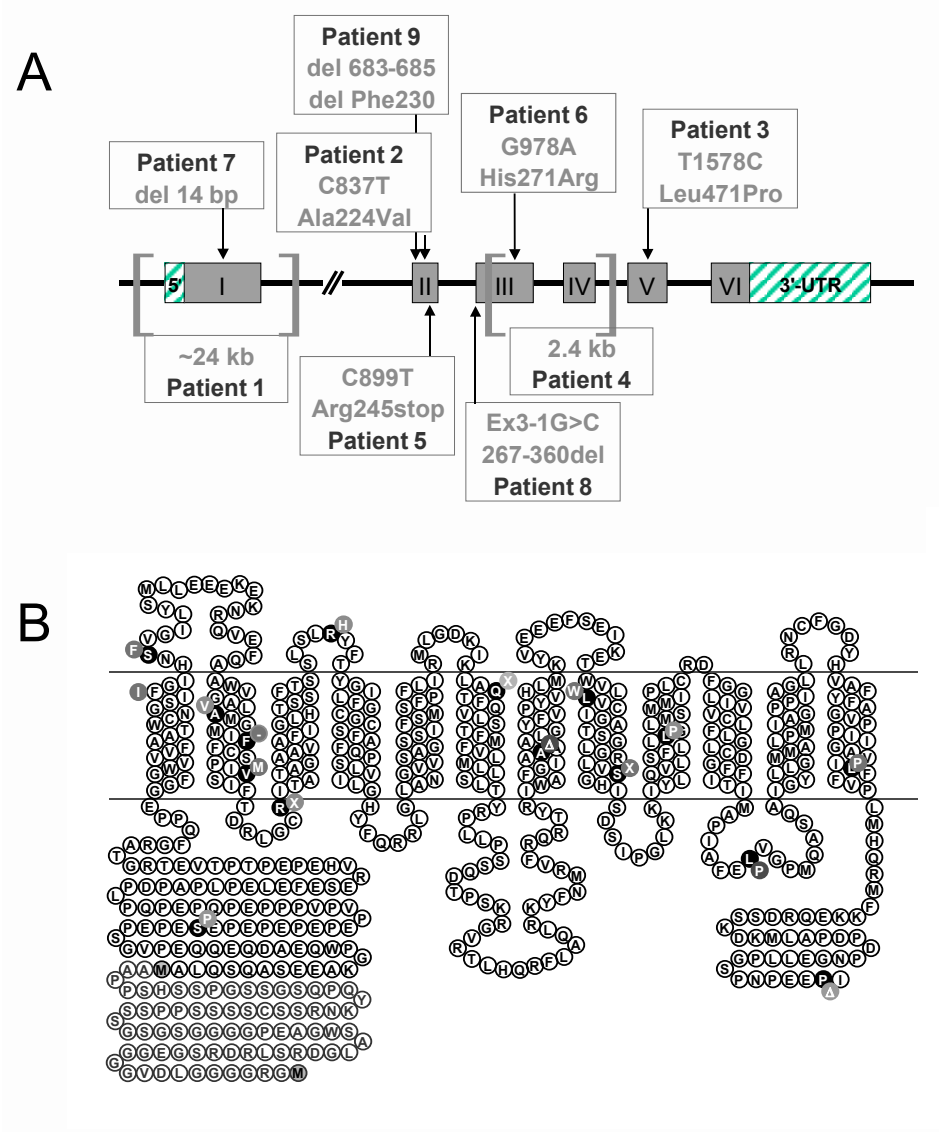

Figure 8.2 A. Structure of the human MCT8 gene and localization of mutations in nine patients. **B.** Predicted topology of human MCT8 in the plasma membrane, showing the twelve putative transmembrane domains, the N-terminal domains that differ by 74 amino acids depending on which translation start codon is used (indicated by the gray-shaded "M" (Met) residues), and the location of both N-terminal and C-terminal domains in the cytoplasm. Indicated are also various mutations identified in our laboratory (32,42) (red), and by the groups of Refetoff (43) (blue), Schwartz (45,46) (green), Passos-Bueno (44) (magenta), and Herzovich (47) (orange). The Ser107Pro alteration in grey represents a single-nucleotide polymorphism, and Δ indicates a single-nucleotide deletion. bp, basepairs; kb, kilobases; UTR, untranslated region. For color figures see page 200.

COS1 or JEG3 cells with radioactive T3 does not result in significant metabolism of the hormone. Transfection of the cells with MCT8 or D3 alone induces only a modest increase in the rate of T3 metabolism, but cotransfection of cells with MCT8 plus D3 produces a dramatic increase in T3 metabolism. Similarly, MCT8 facilitates the intracellular deiodination of T4 by D2 or D3 and of rT3 by D1 or D2 (31).

TISSUE DISTRIBUTION OF MCT8

In all species studied, MCT8 expression shows a wide tissue distribution (33). Localization of MCT8 mRNA in mice by in situ hybridization (ISH) demonstrates major expression in liver, kidney, pituitary and thyroid (Figure 8.3). In the mouse pituitary, MCT8 does not appear to be localized in hormone-producing cells but rather in folliculo-stellate cells. This has also been demonstrated in the human anterior pituitary (34). Folliculo-stellate cells may be involved in short-loop feedback regulation of TSH secretion as they have been shown to express the TSH receptor (35).

ISH studies have also indicated distinct MCT8 expression patterns in the mouse brain with highest transcript levels in the choroid plexus of the third, fourth and lateral ventricles. In addition, strong MCT8 labeling is found in the olfactory bulb, the cerebral cortex, the hippocampal formation, the amygdala and throughout the striatum (Figure 8.3). In all these regions, MCT8 appeared to be localized in neurons and not in glial cells (36). Marked expression of MCT8 has also been observed in the human hypothalamus, in particular in neurons in paraventricular, supraoptic and infundibular nuclei, and in ependymal cells lining the third ventricle (37). These sites play an important role in the negative feedback regulation of hypothalamic TSH-releasing hormone expression by thyroid hormone.

ROLE OF MCT8 IN NEURONAL SUPPLY OF T3

Most likely, control of local T3 levels in brain takes place in functional units of astrocytes and neurons (Figure 8.1 B). This involves a number of steps: firstly, uptake of T4 in astrocytes by an unidentified transporter; secondly, conversion of T4 to T3 by D2 in these cells; thirdly, release of T3 from the astrocytes by another unidentified transporter; fourthly, uptake of T3 in neurons by MCT8 and possibly also other transporters not identified yet; fifthly, transport of T3 to its nuclear receptor in the neurons; and finally, degradation of T3 by D3 also expressed in the neurons.

This view is supported by ISH studies that localize D2 in astrocytes and MCT8 and D3 in adjacent neurons, which is especially clear in the hippocampal area (Figure 8.3). The T4-specific OATP1C1 transporter is expressed almost exclusively in brain capillaries, prob-

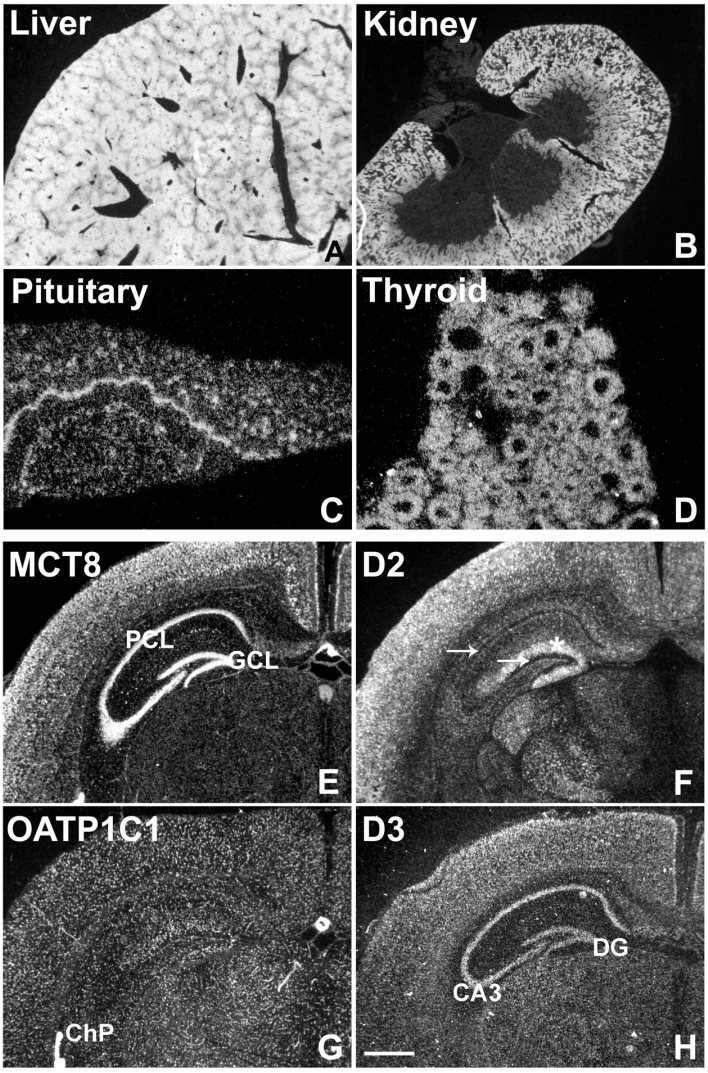

Figure 8.3 A. to **D.** MCT8 expression in peripheral mouse tissues. Dark-field photomicrographs illustrate the localization of monocarboxylate transporter 8 transcripts in liver (A), kidney (B), pituitary (C) and thyroid (D) after hybridization with a 35S-labeled antisense riboprobe. Scale bar (in A): 1.2 mm (A, B); 150 µm (C, D). **E.** to **H.** Comparison of monocarboxylate transporter 8, organic anion transporting polypeptide 1C1, D2 and D3 expression pattern in the mouse brain. As revealed by in situ hybridization, monocarboxylate transporter 8 is highly expressed in the cerebral cortex and in the hippocampus where strongest hybridization signals were observed in the pyramidal cell layer (PCL) as well as in the granule cell layer (GCL) of the dentate gyrus (E). These neurons are devoid of any specific labeling for D2 (F) but do express D3 (H). Organic anion transporting polypeptide 1C1 expression was detected in capillary endothelial cells as well as in the choroid plexus (G). Arrows in F mark the pyramidal and granule cell layer that are devoid of specific labeling for D2. Asterix in F, molecular layer of the dentate gyrus Scale bar (in H): 500 µm. CA3, field CA3 of Ammon´s horn; ChP, choroids plexus; DG, dentate gyrus; MCT8, monocarboxylate transporter 8; OATP1C1, organic anion transporting polypeptide 1C1.

ably playing an important role in T4 transport across the blood–brain barrier (Figure 8.3). Although some OATP1C1 expression is seen in astrocytes, the major route for T4 uptake in these cells is unknown. Many transporters that facilitate cellular uptake of iodothyronines may also mediate efflux of the hormone; furthermore, thyroid hormone may be released from cells by multidrug resistance-related proteins (38, 39). The important role of MCT8 in the uptake of T3 in neurons is strongly supported by clinical studies reviewed in the next section.

ROLE OF MCT8 IN X-LINKED MENTAL RETARDATION

In 2001, it was realized that two patients with apparently identical features were investigated by our group and that of Grüters in Berlin. Both were young boys of Turkish descent, then aged 2 and 4 years, who suffered from severe psychomotor retardation, truncal hypotonia, spastic quadriplegia, and who were unable to sit or stand independently. They showed severe mental retardation, poor communication skills and no development of speech. In addition to this severe neurological phenotype, both also showed abnormal serum thyroid parameters, in particular highly elevated serum T3 levels and decreased total T4 and free T4 (FT4) concentrations.

In view of the important role of thyroid hormone in CNS development (4, 5), the combination of the neurological phenotype and the elevated serum T3 suggested that some form of thyroid hormone resistance was the underlying pathogenesis of this novel syndrome. Until then, heterozygous mutations in THRB had been the only genetic defect identified in patients with thyroid hormone resistance, who usually show increased serum T4, T3 and TSH concentrations and a mild clinical phenotype that may include a retarded mental development (40, 41). Although both clinical and biochemical phenotypes of our patients did not fit with the latter picture, both THRA and THRB genes were screened in one patient, and no pathogenic mutation could be identified. Also, mutations in the different deiodinases were considered and shown to be absent.

It was subsequently hypothesized that the apparent thyroid hormone resistance in our patients was caused by a defect in the cellular uptake of thyroid hormone. By the end of 2002, we had established that rat MCT8 is an active and specific iodothyronine transporter with important expression in the brain, which prompted us to screen the MCT8 gene for possible mutations. Indeed, hemizygous MCT8 mutations were found in both patients—a 24.5-kb deletion in one and a missense mutation in the other. Subsequently, we have been able to study twelve more unrelated boys having the same syndrome of severe psychomotor retardation in combination with high serum T3 concentrations. All were found to have mutations in MCT8.

Table 8.1 shows a detailed summary of the clinical, biochemical and genetic characteristics of our first nine patients (families 1-9) (32, 42), two unrelated patients reported by Dumitrescu *et al.* (families 11,12) (43), one family (10) with affected males reported by Maranduba *et al.* (44), seven families (13–19) reported by Schwartz and colleagues (45, 46), and a male infant reported by Herzovich *et al.* (family 20) (47). Several mutations in MCT8 have been identified by Lenzner *et al.* (48) by screening a large cohort of patients with X-linked mental retardation, but no details are available. Recently, we identified three novel mutations (see chapter 5).

CLINICAL CHARACTERISTICS OF PATIENTS

Our patients vary in age between 3 and 18 years, and show a rather uniform phenotype of severe psychomotor retardation (Table 8.1) (42). There is remarkable hypotonia of the axial muscles, and spastic or dystonic quadriplegia. Motor impairment is severe, making it impossible to hold up the head for a prolonged period of time, to sit upright, crawl, stand or walk. When lying down, the patients are not able to roll over. Intentional, targeted movement is absent, or is poorly coordinated. Several patients show involuntary, athetoid and dystonic movements of the hands and arms, especially when triggered by stimuli such as (un)dressing. They show hyperreflexia with brisk deep tendon reflexes, and persistence of primitive reflexes. Cognitive impairment is severe in all patients, and none of them developed speech; however, they appear to be aware of their surroundings and communicate by smiling, laughing, crying or making sounds. Hearing appears to be normal in all patients by clinical and neurophysiological evaluation. Vision also appears normal in all patients except patient 1, who is blind; however, microphthalmia, nystagmus, saccadic eye movements and/or squint are observed in several patients (Table 8.1) (42). Epilepsy is present in the majority of our patients, varying from absences to tonic-clonic seizures, which respond to treatment with anticonvulsants. No major dysmorphic features are observed in our patients. Secondary microcephaly is present in several patients, with a head circumference below the third percentile. Many patients show markedly reduced body length and an even greater reduction in body weight. This is at least in part explained by the feeding difficulties encountered with many patients; however, even patients who have been fed adequately through a gastrostomy tube show little increase in body weight. Although an asthenic built is often associated with spastic quadriplegia, many patients appear to have muscle hypoplasia (Table 8.1) (42).

The patients reported by the group of Refetoff (43) show a very similar phenotype, although they were said to have impaired "gaze" and hearing. Their studies also suggest that paroxysmal movement disorders are an important feature of the clinical phenotype (49). The patient reported by Herzovich *et al.* (47) also has very similar character-

istics. This phenotype resembles that previously described in patients with the so-called Allan–Herndon–Dudley syndrome, which has recently been shown in eight families to have the same pathogenesis (45, 46, 50-53).

MUTATIONS IN MCT8

The affected males in all 20 recently characterized families have been shown to have mutations in MCT8 (Table 8.1). For many of these mutations it is obvious that they result in a complete loss of MCT8 transporter function. A ~24-kb deletion has been characterized in family 1 that stretches from 9.2 kb upstream to 14.5 kb downstream of exon 1, making it impossible to produce any form of MCT8. A smaller deletion of 2.4 kb has been identified in family 4 that runs from exon 3 into intron 4. In family 7, a 14-bp deletion has been recognized in exon 1, obviously resulting in a frame-shift, and thus a scrambled and truncated protein. This is also true for the single-nucleotide deletion in exon 3 detected in family 12. Equally deleterious are the nonsense mutations identified in families 5, 16 and 20, that result in premature stops at codons 245, 448 and 335, respectively, and thus in truncated and inactive proteins. The splice-site mutation in family 8 should also be devastating to MCT8 function, as it has been shown to result in the deletion of 282 nucleotides from exon 3 in the mRNA and, thus, in the deletion of 94 amino acids, including three TMDs, from the protein.

For the remainder of the mutations detected in the several families it is less predictable to what extent they affect MCT8 function (Figure 8.2). These include the missense mutations resulting in a Ser194Phe substitution in the first extracellular loop, Ala224Val and Val235Met substitutions in the second TMD, an Arg271His substitution in the second extracellular loop, a Leu434Trp substitution in the eighth TMD, a Leu471Pro substitution in the ninth TMD, a Leu512Pro substitution in the fifth intracellular loop, and a Leu568Pro mutation in the twelfth TMD. Other mutations with unpredictable consequences include the three-nucleotide insertion resulting in the insertion of Ile189 in the first TMD, and the three-nucleotide deletion resulting in the deletion of Phe230 in the second TMD. A particularly intriguing mutation was identified in family 10, namely a single-nucleotide deletion in the second-last codon, resulting in a frameshift and a by-passing of the natural stop codon until another stop is encountered 195 nucleotides downstream. This leads to the extension of the MCT8 protein by 65 amino acids, that probably contains a thirteenth TMD (44).

These mutations have been introduced in MCT8 cDNA, and the mutants have been analyzed functionally after transfection of JEG3 cells, which show very little endogenous MCT8 expression (31, 32). T3 transport was tested in cells transiently transfected with wild-type or mutant MCT8 cDNA alone, and T3 metabolism was tested in cells co-transfected with

Table 8.1 Clinical, biochemical and molecular characteristics of patients with *MCT8* mutations

Family	MCT8 mutation		Age (years)	Head circumference<P3	Height<P3	Weight<P3	Truncal hypotonia	Muscle hypoplasia
	Gene	Protein						
1	del ex 1	Absent	8	1/1	1/1	1/1	1/1	1/1
2	671C→T	A224V	6	NR	0/1	1/1	1/1	1/1
3	1412T→C	L471P	4	1/1	0/1	0/1	1/1	1/1
4	Del ex 3,4	Truncated	7	NR	P10	P10	1/1	1/1
5	733C→T	R245X	3.5	1/1	0/1	1/1	1/1	1/1
6	812G→A	R271H	18	NR	1/1	1/1	1/1	1/1
7	Del 631–644	Truncated	10	0/1	1/1	NR	1/1	1/1
8	Ex3 -1G→C	del 267–370	6.5	NR	NR	NR	1/1	1/1
9	**del 683-685**	**del F230**	2.5	1/1	1/1	1/1	1/1	1/1
10	Del 1835T	PI612,613QS+65	24–62	0/6	NR	NR	6/6	6/6
11	1535T→C	L512P	8	0/1	0/1	0/1	1/1	1/1
12	Del 1212T	Truncated	3	NR	NR	NR	1/1	1/1
13	1703T→C	L568P	25–71	0/12	0/6	6/11	12/12	9/12
14	1301T→G	L434W	30–76	0/8	0/8	3/8	8/8	7/8
15	703G→A	V235M	1–39	0/4	2/4	4/4	2/2	4/4
16	1343C→A	S448X	2–16	1/2	2/2	2/2	1/1	2/2
17	581C→T	S194F	2–40	2/4	0/4	4/4	4/4	4/4
18	**del 683–685**	**del F230**	13–25	NR	NR	NR	2/2	2/2
19	Ins ATC565	ins I189	3–9	1/2	0/2	2/2	2/2	2/2
20	1003C→T	Q335X	1	0/1	0/1	0/1	NR	NR

Bold is used for genes and proteins that show the same mutation in different families. NR, not reported; P3, 3rd percentile; P10, 10th percentile.

[a]presumed inactive.

D3 cDNA as described above. In both systems, most mutations were found to result in a complete inactivation of the transporter (Table 8.1) (32, 54). Notable exceptions were the Arg271His mutant in family 6 with 17–20% activity versus wild-type MCT8 (32), the extended MCT8 mutant in family 10 with 18% activity (44), the Leu568Pro mutant in family 13 with 15–28% activity, the Leu434Trp mutant in family 14 with 33–35% activity, and the Ser194Phe mutant in family 17 with 23–25% activity (54) (Table 8.1).

Hyperreflexia/clonus	Paroxysmal dyskinesia	Seizures	Narrow long face	Absent speech	Never walked	Ocular involvement	Serum T_3 (%control)	T_3 uptake (%control)	T_3 metabolism (%control)	References
1/1	1/1	1/1	1/1	1/1	1/1	1/1	309	0[a]	0[a]	32,42
0/1	1/1	NR	0/1	1/1	1/1	1/1	275	5.0	0.0	32,42
0/1	0/1	0/1	NR	1/1	1/1	0/1	228	–0.1	3.1	32,42
1/1	0/1	0/1	NR	1/1	1/1	0/1	258	0[a]	0[a]	32,42
1/1	1/1	1/1	0/1	1/1	1/1	1/1	368	–0.2	0.1	32,42
1/1	1/1	1/1	1/1	1/1	1/1	1/1	251	19.7	17.0	32
1/1	0/1	0/1	1/1	1/1	1/1	0/1	175	0[a]	0[a]	32
1/1	1/1	1/1	NR	1/1	1/1	0/1	160	0[a]	0[a]	32
1/1	1/1	0/1	0/1	1/1	1/1	1/1	276	2.4	–3.5	32
6/6	6/6	NR	6/6	6/6	6/6	0/6	195	NR	18.0	44
0/1	1/1	0/1	NR	1/1	1/1	1/1	186	–4.8	–3.8	43
1/1	1/1	NR	NR	1/1	1/1	1/1	191	0[a]	0[a]	43
12/12	10/12	1/11	6/12	0/12	1/12	0/10		15.3	28.0	45
7/8	0/8	2/7	8/8	2/8	1/8	0/4	266	32.6	35.2	45
3/4	0/4	1/1	4/4	4/4	4/4	0/4	237	8.6	14.3	45
2/2	1/2	1/2	2/2	2/2	2/2	0/2	100	–10.6	–5.9	45
4/4	4/4	0/4	2/4	0/4	4/4	0/4	249	22.8	25.2	45
2/2	0/2	0/2	2/2	2/2	2/2	0/2	NR	2.4	–3.5	45
2/2	2/2	1/2	1/2	2/2	2/2	0/2	NR	3.1	–2.7	46
NR	NR	NR	NR	NR	NR	NR	NR	0[a]	0[a]	47

THYROID STATE

In all patients where thyroid function tests were performed, mean serum T4 and FT4 levels are diminished to about 60% of mean values in healthy controls (42-47). On average, the mean serum T3 level is increased to 230% of mean control (Table 8.1), whereas the mean serum rT3 is decreased to 36% of control. Compared with healthy controls, the mean serum TSH concentration is roughly doubled in the patients. Figure 8.4 shows the data for the patients, the heterozygous carriers, including all mothers, and the unaffected members in the nine families we investigated. Although serum TSH is not different between the unaffected relatives and the carriers, T3 tends to be higher and FT4 and rT3 tend to be lower in the carriers compared with the unaffected members. In none of the families with MCT8 mutations are neurological abnormalities observed in the heterozygous females.

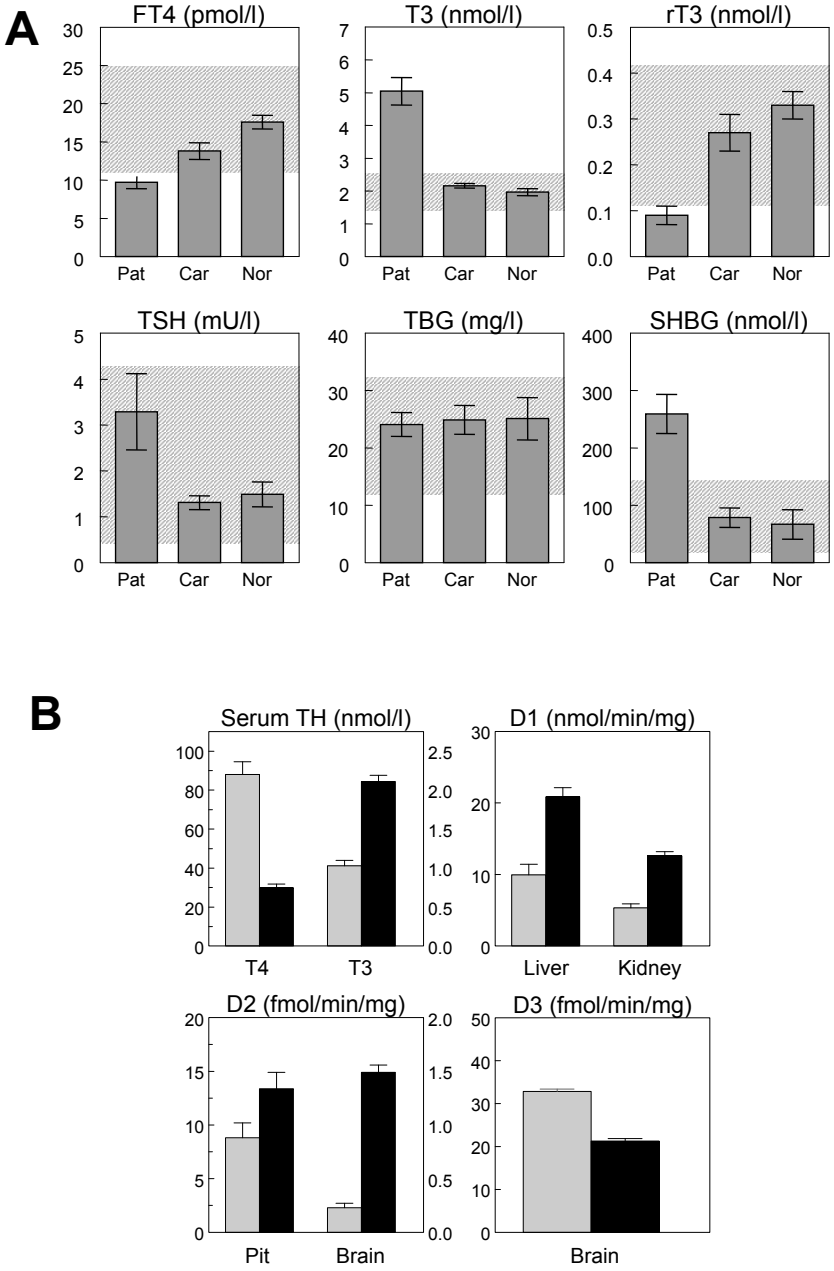

Figure 8.4 A. Serum thyroid parameters (mean ± SEM) in patients, heterozygous females, and unaffected members of families 1–9 (see Table 1). **B.** Serum T4 and T3 levels, liver and kidney D1 activities, pituitary and brain D2 activities, and brain D3 activities (mean ± SEM) in 3-week-old MCT8+ (gray bars) and MCT–black bars) male mice. All analyses were done as previously described (60); *) P<0.05; **) P<0.001 versus wild-type. Car, heterozygous female carriers; FT4, free T4; Nor, unaffected family-members; Pat, patients; Pit, pituitary; SHBG, sex-hormone-binding globulin; TBG, T4-bining globulin.

Despite the highly elevated serum T3 concentration in the various patients, serum TSH on average is rather increased than suppressed, suggesting that the increase in serum T3 does not completely compensate for the decrease in serum T4 regarding the negative feedback regulation of TSH secretion in these patients. Probably, this reflects a defect in the action of serum T3 at the level of the pituitary and/or hypothalamus.

In keeping with the postulated role of MCT8 in neuronal T3 uptake (Figure 8.1 B), hemizygous mutations in MCT8 obviously result in an impaired supply of T3 to its intracellular target in these cells, namely the nuclear T3 receptor. In view of the crucial role of thyroid hormone in brain development, it is fully understandable that the impaired access of T3 to its neuronal target leads to such a severe defect in neurological development as observed in our patients. This defect is at least as severe as that observed in cretinism due to iodine deficiency during fetal and neonatal development as well as the neurological abnormalities resulting from untreated congenital hypothyroidism (4).

This proposed mechanism of disease in our patients is based on the assumption that MCT8 is a specific thyroid hormone transporter, but it has not been excluded completely that MCT8 is capable of transporting other ligands; furthermore, it is unknown if all T3-responsive neurons depend on MCT8 for their T3 supply. If subsets of neurons express other T3 transporters, they may well have toxic intracellular T3 levels as they are exposed to a large T3 excess. Such an imbalance between neurons with deficient and those with excessive T3 supply may be more detrimental than a general decrease in T3 supply to all target cells.

The changes in serum thyroid hormone levels observed in our patients are explained by assuming that mutations in MCT8 will also block the supply of T3 to D3 expressed in these neurons. As an initial event, this could result in a decrease in T3 clearance and, hence, the accumulation of circulating T3. As a secondary event, hepatic and renal D1 expression will be stimulated by the increased serum T3 with a further stimulation of the T4 to T3 conversion as well as other deiodinations catalyzed by this enzyme (2, 55). An increased D1 activity may thus contribute to the low serum T4 and rT3 concentrations. This implies that tissues such as the liver respond to the increased serum T3 despite the mutations in MCT8 that they also express. This may well be the case owing to the expression of other thyroid hormone transporters in the liver, including Na+-taurocholate cotransporting polypeptide and different members of the OATP family (15, 16).

That the liver is actually thyrotoxic in our patients is supported by findings of strongly increased serum levels of sex-hormone-binding globulin ([SHBG]; Figure 8.4). The production of this protein in the liver is strongly stimulated by T3 (56); however, serum SHBG is also increased by malnutrition, which is often the case in patients with MCT8 mutation because of feeding problems (57). Moreover, serum T4-binding globulin (TBG) levels are not different between patients and unaffected family members (Figure 8.4), although the production of TBG in liver is under negative control by thyroid hormones (58). Nevertheless,

the strong positive correlation between serum T3 and SHBG levels suggests an increased T3 stimulation of liver SHBG producing cells in our patients. It is also speculated that the very low body weight relative to body length reflects the increased exposure of adipose tissue and skeletal muscle to T3, resulting in the "wasting" of these tissues (32, 44). A corollary of the above discussion is that the effects of MCT8 mutations in different tissues depend on the importance of MCT8 for the supply of T3 to these tissues. The heart is one of the most sensitive tissues to thyroid hormone (59, 60) and shows marked expression of MCT8; however, except for the patient in family 6 who was reported with tachycardia, most patients with MCT8 mutations have normal heart function. This suggests that mutations in MCT8 results in a partial inhibition of cardiac T3 supply because of the contributions of other transporters.

MCT8-KNOCKOUT MICE

Many of the above considerations concerning the thyroid state of patients with mutations in MCT8 are actually borne out by recent findings in mice with a targeted inactivation of the MCT8 gene (61, 62). Surprisingly, this was not associated with an apparent neurological phenotype, although this needs to be investigated in more detail; however, hemizygous MCT8– male mice do have markedly lower serum T4 and higher serum T3 concentrations than the wild-type MCT8+ mice, with a roughly 7-fold increase in the serum T3:T4 ratio (Figure 8.4). D2 activities in pituitary and much more so in brain are strongly increased in the MCT8- mice compared with the wild-type animals. This is in agreement with the marked decrease in plasma T4, since D2 inactivation by the ubiquitin–proteasome system is enhanced by substrate (1, 55).

Conversely, D3 activity in brain is significantly lower in MCT8– versus MCT8+ mice. A similar decrease in brain D3 expression has been observed in Pax8–/– mice, which completely lack thyroid hormone due to the absent development of the thyroid gland (63). This supports the hypothesis that central neurons in mice and patients with hemizygous mutations in MCT8 are indeed deprived of T3. Finally, MCT8– mice show marked increases in D1 activity in both liver and kidney compared with MCT8+ mice, reflecting increased intracellular T3 contents in these tissues (1, 2). These data therefore support the hypothesis that hepatic T3 uptake in our patients is relatively unimpeded despite the mutations in MCT8.

CONCLUSIONS

The severe phenotype of patients with mutations in MCT8 dramatically underscores the physiological importance of transporters for the metabolism and action of thyroid hormones. In addition to mutations in the T3 receptor, mutations in a thyroid hormone transporter represent a novel mechanism of thyroid hormone resistance. Undoubtedly, other syndromes will be recognized that result from mutations in other thyroid hormone transporters, and OATP1C1 is an important candidate in this respect.

The damage caused by a defect in thyroid hormone action during early fetal brain development is most probably irreversible (4). Treatment with thyroid hormone of infants with MCT8 mutations is therefore unlikely to have a beneficial effect. Only in patients carrying mutations that do not completely inactivate MCT8 may very early postnatal treatment with thyroid hormone alleviate some of the symptoms, especially those related to the (later) development of the cerebellum. Different patients with MCT8 mutations have been treated with T4 and/or T3, but substitution doses have little effect and high doses produce symptoms of hyperthyroidism such as tachycardia. The importance of the recognition of MCT8 mutations as a cause of X-linked psychomotor retardation therefore lies more in carrier identification and prenatal diagnosis than in therapeutic intervention. The highly elevated serum T3 is one of the hallmarks of this syndrome and may be used as a screening method to identify patients with X-linked mental retardation due to mutations in MCT8.

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Chapter 9

Summary and general discussion

SUMMARY

The studies presented in this thesis demonstrate that MCT8 is a transmembrane protein that facilitates both in- and efflux of thyroid hormone. MCT8 function is crucial for normal neurological development, as loss-of-function mutations are associated with severe psychomotor retardation. In **chapter 1**, the crucial role of transporters in thyroid hormone physiology is introduced, as are several families of thyroid hormone transporting proteins. The importance of sufficient supply of thyroid hormone for normal brain development and the role of MCT8, are discussed, leading to the general hypothesis on which the work was based.

Chapter 2 reports on mutations in MCT8 in 9 patients with severe psychomotor retardation and elevated serum T3 levels. It describes results from *in vitro* studies with transfected JEG3 cells, which show that these mutations lead to a loss of T3 and T4 transport. Whereas transfection with normal (or 'wild-type') MCT8 greatly induces uptake and subsequent metabolism compared to controls, mutant MCT8 does not.

Chapter 3 discusses the MCT8-related phenotype, known as Allan-Herndon-Dudley syndrome, in detail, based on case reports of four patients. It also introduces the concept of tissue specific thyroid hormone status. Impaired MCT8 function leads to hypothyroidism in the brain, but the associated rise in serum T3 might expose tissues and organs expressing other transporters to thyrotoxic conditions. Indeed, clinical and biochemical evaluation indicates hyperthyroidism in liver, muscle and adipose tissue, and possibly hypothyroidism in bone.

Chapter 4 describes functional and expressional studies of MCT8 mutations identified in seven families by Schwartz *et al.* These include the original family described by Allan, Herndon and Dudley in 1944. Interestingly, patients in three of the families show more advanced neurological development compared to the MCT8 patients we initially described, in particular with regard to independent ambulation and communicative skills. We demonstrate that this more advanced psychomotor development is correlated with residual T3 transport capacity of the mutants *in vitro*. Furthermore, we investigated the mechanisms leading to loss of function in MCT8 mutants. Immunocytochemistry shows that mutants with residual transport, like wild-type MCT8, are expressed at the plasma membrane. Labeling experiments with BrAcT3 indicate that the mutations reduce the affinity of the transporter. Complete loss of function, however, is associated with low or absent protein expression, or a mainly cytoplasmic distribution indicating impaired trafficking of the mutant.

In **chapter 5** we used primary cultures of skin fibroblasts from MCT8 patients as an *ex-vivo* model to study uptake and efflux of thyroid hormones and the intracellular thyroid hormone status. This revealed that one mutation, delPhe501, impairs in particular the efflux of T3. Increased activity of type 3 deiodinase and increased expression of mRNA of

thyroid hormone-regulated genes in these cells indicate a high rather than low intracellular T3. This leads to a possible second pathophysiological model, in which impaired efflux of T3 leads to thyrotoxicosis in the brain, resulting also in neurological impairment. The two patients identified with this delPhe501 mutation, a 18 months old boy and his 38 year old uncle, show rather advanced psychomotor development. The older patient can walk with support, speaks coherently and is able to spell words. Although formal testing is to be done, general cognitive functioning appears to be at the level of a 6-8 year old.

Chapter 6 focuses on transport of iodothyronines by MCT10, the MCT family member with highest homology to MCT8, and on the characteristics of MCT8 as a thyroid hormone exporter. Interestingly, studies on MCT8 as a thyroid hormone transporter were initiated after it was reported that MCT10 does transport aromatic amino acids, but not the structurally related iodothyronines (1, 2). As it appears now, the conditions used in the original report were not suited to test thyroid hormone transport (saturating amounts of non-labeled thyroid hormone probably blocked entry of the radioactive ligand). It can only be speculated if and when MCT8 function and its associated phenotype would have been identified, if MCT10 would initially have been reported as the excellent thyroid hormone transporter it in fact is.

In **Chapter 7**, some characteristics of MCT8 expression are explored. Two major items influencing this are the N-terminal PEST domains in the protein, and the ubiquitin-proteasome pathway. We demonstrate that MCT8 is ubiquitinated, and thus reversibly targeted for proteosomal breakdown. Although the PEST domains suggests rapid turnover of the protein, expression is stable for over 24 h after the inhibition of MCT8 production by a specific MCT8 siRNA.

Chapter 8 summarizes the clinical and functional aspects of MCT8 mutations, and introduces the MCT8 knockout mouse. This model represents the human MCT8 mutants with respect to the serum thyroid hormone levels, and, to some extent, the tissue specific thyroid hormone status. However, these mice show no clear signs of neurological impairment.

DISCUSSION

On the physiological role of MCT8

For long it was believed that thyroid hormone crosses the lipid bilayer plasma membrane based on simple diffusion. Although experimental evidence accumulated proving that specific transporter proteins were in fact responsible, the full recognition of this fact came only after the identification of mutations in MCT8 in humans. The plausible and, through the work presented here, substantiated hypothesis that the severe psychomotor impair-

ment is caused by lack of thyroid hormone entry (or efflux) in the developing brain leaves little room for passive diffusion as a significant mechanism of uptake. Many of the studies presented here show that mutations in MCT8 lead to a complete or partial loss of thyroid hormone uptake.

Based on the localization of MCT8 in neurons, and the distribution of the activating type 2 deiodinase in astrocytes and of the inactivating D3 in neurons, this led to a model of MCT8 being a T3 importer in neurons. This fits with the important role of thyroid hormone in the differentiation of neurons, a cornerstone of neurological development. However, MCT8 might also have other functions than uptake of T3. From the studies presented in chapters 5 and 6, it has become clear that, at least *in vitro*, MCT8 is also an excellent thyroid hormone exporter. It is likely that MCT8 also has this function *in vivo*. In the brain, neurons are considered to be important target cells for T3, and play a role in its inactivation through D3. However, it is conceivable that T3 is also transported from one neuron to another, in which efflux via MCT8 might play a role. Also, efflux might be a mechanism safeguarding cells form excess intracellular T3, with MCT8 functioning as a 'pressure valve'. This would be in keeping with the observations made in fibroblasts of the patient with mutation delPhe501, in which import, but also, to a larger extent, efflux is impaired. These cells show increased D3 activity, and increased expression of thyroid hormone regulated genes, suggesting high intracellular T3. A physiologically relevant role for MCT8 as a thyroid hormone exporter might also be found in the many extracranial tissues expressing the protein, such as the liver. Loss of MCT8 export function in AHDS patients might, next to the elevated serum T3, contribute to hepatic thyrotoxicosis by decreasing T3 efflux.

Another likely role for MCT8 is in the uptake of thyroid hormone over the blood-brain and the blood-cerebrospinal fluid barriers (3). In mice, MCT8 is highly expressed in the choroid plexus, and also in endothelial cells of larger brain capillaries. In the same structures OATP1C1 is expressed, which has high affinity for T4 and rT3 (4). One might speculate that co-expression of these two specific transporters is based on substrate specificity, with MCT8 responsible for T3 uptake. However, MCT8 is also a T4 transporter. Therefore, co-expression might also indicate that one transporter functions as basolateral T4 importer, and the other as apical exporter. Detailed studies of the (sub)cellular localization of the transporters are needed to test this hypothesis.

Finally, MCT8 might also transport other substrates. After illustrating that MCT8 does not transport aromatic amino acids and iodothyronine sulfates (5), we recently investigated a newly discovered family of thyroid hormone derivatives, the iodothyronamines (6). 3-Iodothyronamine (T1AM) is a naturally occurring substrate that structurally resembles neurotransmitters such as catecholamine and serotonin. Iodothyronamines appear to be responsible for fast, non-genomic actions of thyroid hormone via a G-protein coupled plasma membrane receptor called TAR1 (trace amine receptor 1). In mouse models, T1AM induces profound bradycardia and hypothermia within minutes after intraperitoneal ad-

mission. It might also be responsible for the non-genomic actions of thyroid hormone derivatives that contribute to the psychiatric effects of hyper- and hypothyroidism (7). The TAR1 locus is associated with susceptibility to schizophrenia and bipolar disorder, further indicating that iodothyronamine system may play a role in neurological development and function (8).

We demonstrated that T1AM strongly inhibits T3 uptake by MCT8 transfected COS1 cells *in vitro*, and might be a good substrate for transport itself (9). As intraperitoneal injection in mice rapidly resulted in hypothermia, T1AM must be transported over the blood brain barrier rather efficiently. It is conceivable that MCT8 facilitates this transport. It is currently investigated whether mutations in MCT8 affect possible T1AM transport, which might contribute to the neurological phenotype in AHDS patients.

Clinical considerations: on diagnosis and treatment

The disease-identifying feature of elevated serum T3 levels in AHDS is shown to affect thyroid hormone status is tissues like liver, muscle and fat (chapter 3). Although impaired motor input from the CNS will also affect muscle mass, this local thyrotoxicosis will have major impact on the cachexic state observed in many patients, as it leads to 'wasting' of muscle and fat. Treatment with high-energy diets and gastrostomy feeding tubes does not adequately affect the catabolic state. This prompted Wemeau *et al.* to investigate the effects of a PTU/L-thyroxine regime in the now 18 years old patient identified with mutation R271H (10). This treatment resulted in normalization of FT3 levels, reduction of tachycardia and a weight gain of 3 kg in one year. In the previous year, the severely cachexic patient (BMI 12) gained only 200 grams, despite optimal feeding. Treatment has no effects on the neurological or cognitive functions of the patient, but is nonetheless of obvious value to the general condition. Treatment aimed at the improvement of the psychomotor retardation is not promising. Many patients have been treated with L-thyroxine, sometimes in large dosages, in an attempt to overcome the peripheral resistance. This has not shown to be of any benefit, and likely worsens the thyrotoxicosis in other tissues, as is illustrated by increased heart rate and sweating. Even if it would be possible to increase thyroid hormone levels in the brain, the impaired development during fetal life is not likely to be reversed. One must consider the fundamentally different situation in MCT8 patients compared to patients with congenital hypothyroidism. In the latter, maternal thyroid hormone will have protective effects during pregnancy, whereas neurons in MCT8 patients will have been deprived of thyroid hormone since conception. For this reason, also treatment with thyroid hormone analogs that might penetrate better into the brain does not appear very promising. Nonetheless, diagnosis of MCT8 mutations can be extremely valuable for the families involved. In many cases of severe psychomotor retardation, the etiological diagnosis cannot be made. This leaves many questions, for example regarding the chance that other

children might also be affected. We are now not only able to provide a diagnosis, but also to screen female family members for carriership, and offer early prenatal diagnosis to pregnant carriers.

One might hope that the recent attention for the role of MCT8 in neurological development will help the diagnostic process in patients with unexplained psychomotor retardation. Although the clinical symptoms, especially in the first months of life, appear to be non-specific (general hypotonia, head lag, lack of fixation and smiling), elevated serum T3 values are present in all patients with inactivating mutations in MCT8. The mean total serum T3 of AHDS patients identified in our lab so far is 4.6 nmol/l (normal range 1.4 – 2.5), measured by Vitros ECI technology (Immunodiagnostic System, Ortho-Clinical Diagnostics). Only one patient, the 38 year old uncle with mutation delPhe501 described in chapter 5, had a total serum T3 below 3,0 nmol/l. Of 29 other young males with severe psychomotor retardation of unknown origin, who tested negative for mutations in the coding sequence, only 4 had T3 above this limit. This indicates that, although specificity is not optimal, high serum T3 is a sensitive marker for mutations in MCT8. The low rT3 levels, and in particular, the highly increased T3 over rT3 ratio (T3/rT3) are also sensitive markers for AHDS, but determination might not be available widely. It is still difficult to indicate how high serum T3 should be to warrant investigation of the MCT8 gene. As the number of AHDS patients identified so far is relatively limited, experience is still growing. In our opinion, MCT8 should be screened in any male patient with unexplained psychomotor retardation and serum T3 above the upper limit of normal, and in those who present with the 'classical' combination of symptoms axial hypotonia, spastic quadriplegia and severe cognitive impairment.

It is important to realize that normal results of the congenital hypothyroidism screening program do not allow thyroid hormone abnormalities to be discarded as cause of psychomotor retardation. Screening programs are usually based on determination of serum TSH and/or T4 in the first week of life (11). In AHDS patients, TSH is usually within the normal range, and T4 is low or low-normal. It is therefore unlikely that TSH based screening programs, as are used in most countries in Europe, will identify MCT8 patients. When a T4 based screening is used, patient's serum value has to be 'sufficiently' low to be detected. We analyzed the results of the T4 based screening of the Dutch patients with mutations Gly564Arg, delPhe501, and delEx1 described in chapter 5. The patients with Gly564Arg and delEx1 were identified with low T4, whereas no abnormalities were detected in the serum of the patient with delPhe501. This reflects the situation in later life. Low neonatal T4 is also reported in one patient by Dumitrescu *et al.*, whereas they report normal TSH in another patient (12).

Taken together, these results show that screening of T4, but not TSH, could identify MCT8 patients in the neonatal period. Theoretically, determination of neonatal T3 could contribute greatly to early diagnosis. This is however not measured in any of the screen-

ing programs around the world. The low expected number of patients and the limited therapeutic options will probably prevent inclusion in the population wide screening, but measurement might be of great value in selected cases. It is however currently not known whether serum T3 in AHDS patients is elevated in the neonatal period. Possibly, the high expression of D3 in fetal tissues and placenta prevents this (13).

As yet, the incidence of MCT8 mutations remains unclear, although at least two studies have been performed to investigate this. Lenzner *et al.* examined DNA of a large cohort of 16 families and 180 unrelated males with severe psychomotor retardation of unknown origin (14). These include a 1-bp insertion in exon 1, leading to a frameshift with a premature stop, and two missense mutations (Met1Leu and Gly558Asp). Unfortunately, clinical details of these patients, including serum T3, are not available. Testing the functional characteristics of mutant Gly558Asp in our transfected cell system, we showed that it leads to complete loss of function. We analyzed the coding sequence of MCT8 in a smaller group of 82 boys with unexplained (X-linked) psychomotor impairment without knowing clinical details or serum T3. We identified a C to G mutation in exon 5 of one patient, leading to a Met402Ile substitution in the 9th transmembrane domain. This variant was not found in over 100 alleles tested by RFLP. Testing this mutant *in vitro* demonstrated that it has wild-type characteristics for uptake and subsequent metabolism of T3 and T4. In other words, no loss of function was observed. When clinical data of the patient was revealed, it appeared that there was severe psychomotor retardation, but no prominent axial hypotonia and no elevation of serum T3. Unfortunately, the patient had deceased two years earlier at the age of 3. From the clinical records and the treating pediatric neurologist it was understood that, although no definite diagnosis was made, the patient most likely suffered from a mitochondrial DNA defect. It remains puzzling, however, to identify a rare, apparently normally functioning MCT8 variant in a young boy with psychomotor retardation. As new functions of MCT8 are identified, including the transport of novel ligands, this Met402Ile variant will remain under investigation.

MCT8 mutations in female carriers

It remains controversial if mutations in MCT8 affect female carriers in any way. No clear neurological impairment has been observed in any of the mothers. Thyroid function tests are usually all within the normal range, although Dumitrescu *et al.* describe a mild serum thyroid hormone phenotype, with low or low-normal T4 and rT3, normal TSH and normal or high-normal T3 (12). In the group of female carriers we identified, we found the mean FT4 to be right in between that of patients and of non-carrier controls. As carriers are heterozygous for the mutation, one unaffected copy of the X chromosome is present in every cell in the body. However, it is known that cells only transcribe genes only from one of the X chromosomes, and the other is inactive. This so-called X-inactivation occurs early

in embryonic cells and is persistent is all daughter cells (15). Random X-inactivation leads to a 50:50 ratio of cells expressing either the maternal or the paternal chromosome.

However, various epigenetic phenomena can lead to 'skewing' of the inactivation, with preferential expression of one particular X chromosome in more than 80% of cells (16). This may result in signs and symptoms in female carriers of X-linked diseases, even though an unaffected copy of the gene involved is present. For example, most female carriers of the α-thalassemia X-linked psychomotor retardation syndrome (ATR-X syndrome, OMIM 301040), in males leading to a combination of mental retardation and mild α-thalassemia, are completely normal. Some carriers, however, show signs of thalassemia, without any signs of cognitive impairment. This is the result of unfavorable skewing to the affected chromosome in the hematopoietic cells. (15). Similarly, mutations in the MCT8 gene could have clinical consequences in female carriers. In the mothers studied by Dumitrescu *et al.* (12), no skewed X-inactivation was found in DNA isolated from peripheral blood monocytes. However, inactivation is a tissue or cell line-specific process, and peripheral blood monocytes may not represent skewing in other cell types. The absence of a neurological phenotype might be explained by selective survival of neurons expressing the non-mutated chromosome, enabling normal psychomotor development. Cells, for example in liver and kidney, that express the affected chromosome, but to which the function of the mutated MCT8 is not essential for cell survival, might persist and cause the mild thyroid hormone phenotype.

Future directions: on other causes of peripheral resistance to thyroid hormone

It is clear that impaired action of thyroid hormone on the developing brain, whether caused by iodine deficiency, congenital hypothyroidism or mutations in MCT8, severely hampers psychomotor development (chapter 1). As thyroid hormone action is mostly mediated by binding of T3 to the nuclear T3 receptor (TR), the intracellular concentration of T3 is a fundamental determinant of the biological activity of thyroid hormone. It is the result of the production of hormone in the thyroid gland, the levels of T3 and T4 in the serum, their transport into cells, their conversion by the deiodinases and, finally, their binding to receptors. Mutations in the proteins involved in any of these steps could lead to altered thyroid hormone action. This can be specific for tissues expressing the affected protein, but can also have systemic consequences. As illustrated in this thesis, mutations in MCT8 have both effects.

Another well-described example is thyroid hormone resistance due to mutations in the TRβ gene. Some 1000 patients have been described with mutations in the T3 binding domain of the receptor, leading to reduced affinity (17). These patients are identified by high serum FT4 and non-suppressed TSH. Clinical symptoms vary considerably, but goitre,

hyperactivity, learning disabilities, developmental delay, delayed bone age and tachycardia are common. Some fit with reduced thyroid hormone action, suggesting that the rise in serum FT4 is not able to compensate completely for the reduced affinity of the receptor. Other symptoms, like tachycardia, indicate hyperthyroidism. This is explained by exposure of TRα-expressing tissues, like the heart, to elevated levels of serum thyroid hormone.

Another recently identified cause of resistance to thyroid hormone are mutations in selenocysteine insertion sequence-binding protein 2 (SPB2), located on chromosome 9q22 (18). SBP2 is involved in the synthesis of selenoproteins, amongst which the deiodinases. Two families with mutations have been identified, with clinical symptoms including short stature, delayed bone age and delayed puberty. Psychomotor development is unaffected. Serum FT4 is elevated, T3 is and rT3 are low, and TSH is normal or slightly elevated. These findings indicate an impaired deiodination of T4. Indeed, reduced activity of D2 is observed in patients' fibroblasts.

Mutations in MCT8, TRβ and SBP2 currently are the only identified causes of resistance to thyroid hormone. It is likely, however, that mutations in other transporters, TRα, the deiodinases or essential associated proteins would also affect the thyroid hormone status in tissues, possibly with clinical consequences. Of particular interest in this context is OAT-P1C1, involved in the uptake of thyroid hormone over the blood-brain barrier. Analogous to MCT8, loss of OATP1C1 function might impair neurological development. Mutations in MCT10 could reduce thyroid hormone uptake in many tissues. Observations in TRα knockout mice suggest that loss of TRα function in humans might lead to psychomotor retardation, anxiety, depression or bipolar disorders (19). Furthermore, as is observed in patients with mutations in SBP2, mutations in D1 and D2 could affect the deiodination of T4, leading to reduced availability of active thyroid hormone. Analogous to findings in D3 knockout mice, mutations in D3 could lead to central hypothyroidism (20).

As yet, none of these possible mutations mentioned above have been identified. Detection might be hampered by a number of factors. First, all genes involved are autosomal. Possibly, heterozygous carriers have no symptoms, or display only a mild phenotype, whereas homozygosity might not be compatible with life. This is known, for example, from the TRα knockout mouse models, where homozygous knockouts are stillborn (21). Second, If mutations lead to reduced thyroid hormone action in tissues, but this is not reflected in serum, it will be very hard to identify new patients. For example, AHDS patients mostly have a low or low-normal, but not severely decreased, FT4, whereas TSH is usually in the normal range. It is possible that in the workup of patients this only 'mildly' abnormal profile is considered a coincidental finding that cannot explain the severe phenotype. We now know that determination of T3 will provide essential information, but in many clinical settings this is not done routinely. Likewise, in TRα knockout mice TSH is significantly increased, but total and free T4 are normal (21). In humans with mutations in TRα, this combination might be attributed to subclinical hypothyroidism, a prodromal stage of (auto-immune)

hypothyroidism. Again, determination of T3 is essential, as this is significantly increased in knockout mice.

It may be clear that identification of novel phenotypes due to mutations in peripheral thyroid hormone pathway genes is rather challenging. Nonetheless, the results obtained with MCT8 have prompted our group to pursue the identification of novel thyroid hormone related neurological phenotypes. Two main strategies are followed. In the Thyroid Origin of Psychomotor Retardation (TOP-R) study, a large heterogenic cohort of patients with mild to severe psychomotor retardation of unknown origin is collected, in which extensive serum thyroid hormone measurements are performed. Based on the results of this screening, likely candidate genes (TRβ, D2 and D3, MCT8 and MCT10, OATP1C1 and others) will be sequenced to identify mutations or polymorphisms. The exons coding for the T3-binding domain of TRα will be sequenced regardless of serum thyroid hormone levels.

A second research line will focus completely on the genetic variation in TRα and possible consequences for brain function. This study will investigate TRα function in several cohorts. These include a group of patients with bipolar disorder who do not respond to lithium treatment alone, but do respond when high doses of T4 are added. These patients tolerate high dose T4 without signs of tachycardia and osteoporosis, indicating that TRα responsiveness to thyroid hormone is reduced. Another investigated group consists of patients with major depression, in which T3 enhances the effects of antidepressant drugs.

These new studies investigate not only possible causes of impaired thyroid hormone action in the developing brain, but also examine its role in adult human brain function. This might provide new pathophysiological models for developmental disorders, and explain why a subset of patients with psychiatric complaints benefits from co-medication with thyroid hormone. Identification of these patients might be of great clinical importance, as treatment with T3 both prevents delayed postnatal cerebellar development and relieves extreme anxiety in TRα knockout mice.

In conclusion, the identification of mutations in MCT8 as a cause of severe psychomotor retardation was a significant event in many ways. It provided the clinical proof of principle that specific transporter proteins are essential for thyroid hormone action. Identifying the cause of Allan-Herndon-Dudley syndrome, it enables genetic counseling to families with this severely disabling disease. Finally, it stresses that mutations in peripheral pathway genes can lead to a tissue-specific deficiency or excess of thyroid hormone with dramatic consequences, sparking off important and exciting new research.

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Samenvatting

SAMENVATTING

Schildklierhormoon is essentieel voor het metabolisme in de lichaamscellen, en voor de normale ontwikkeling van organen, met name die van het brein. Schildklierhormoon reguleert de productie van veel eiwitten en enzymen, en stimuleert zo het energieverbruik, de groei en de uitrijping van cellen en weefsels. Te veel schildklierhormoon (hyperthyreoïdie) leidt onder andere tot versnelde hartslag, gewichtsverlies, rusteloosheid, warmte-intolerantie en botontkalking (osteoporose). Te weinig schildklierhormoon (hypothyreoïdie) leidt tot een trage hartslag, gewichtstoename, koude-intolerantie en obstipatie. Daarnaast komen geheugen- en concentratiestoornissen en depressie voor. Aangezien schildklierhormoon essentieel is voor de normale ontwikkeling van de hersenen, kan een tekort tijdens de zwangerschap of in eerste jaren na de geboorte, leiden tot ernstige verstoring van de verstandelijke en motorische (oftewel psychomotore) ontwikkeling.

Schildklierhormoon wordt geproduceerd in de schildklier, een vlindervormig orgaan in de hals. Dit gebeurt onder invloed van TSH, het schildklierstimulerend hormoon, dat afkomstig is uit de hypofyse, een klier in de hersenen. De schildklier produceert met name thyroxine (T4), een inactieve vorm van schildklierhormoon. Uit T4 kan door omzetting T3 ontstaat, het actieve schildklierhormoon. Deze omzetting vindt plaats onder invloed van de twee verschillende enzymen, die we type 1 dejodase (D1) en type 2 dejodase (D2) noemen. D1 in de lever en de nieren is verantwoordelijk voor het T3 in het bloed, terwijl D2, onder andere in de hersenen, zorgt voor lokale omzetting naar T3. Type 3 dejodase (D3), dat met name voorkomt in de hersenen en in de placenta, zorgt voor de inactivatie van T3 en T4.

Het belangrijkste werkingsmechanisme van schildklierhormoon is de regulatie van de transcriptie (het aflezen) van schildklierhormoon-gevoelige genen. Deze regulatie, die zowel stimulerend als remmend kan zijn, verloopt via binding van T3 aan de T3-receptor (TR). Deze receptor bevindt zich in de celkern. De biologische activiteit van schildklierhormoon wordt dan ook mede bepaald door de T3-concentratie in de cel. Deze is afhankelijk van verschillende factoren, waaronder de hoeveelheid T3 en T4 in het bloed, de werking van de (in)activerende enzymen D1, D2 en D3 en de functie van transporteiwitten in de celwand. Deze "transporters" faciliteren de opname en afgifte van schildklierhormoon door de cel.

Het onderzoek dat is beschreven in dit proefschrift richt zich op schildklierhormoontransporter MCT8. MCT8 is essentieel voor de opname van schildklierhormoon in zenuwcellen in de hersenen. Mutaties in het MCT8-gen (de bouwtekening voor de transporter) verstoren de aanmaak van normaal MCT8. Hierdoor kan de cel geen of onvoldoende schildklierhormoon opnemen. Omdat het MCT8-gen op het X-chromosoom ligt, hebben mutaties in MCT8 met name consequenties voor mannen. Vrouwen hebben vanwege het tweede X-chromosoom in hun cellen in principe altijd een ongeschonden MCT8-gen beschikbaar. Bij mannen met een mutatie in MCT8 leidt het tekort aan schildklierhormoon in de hersencellen tot ernstige stoornissen in de neurologische ontwikkeling.

In **hoofdstuk 1** beschrijven we de cruciale rol van transporteiwitten in de werking van schildklierhormoon. Aangezien zowel de schildklierhormoonreceptor (TR) als de (in) activerende enzymen D1, D2 en D3 binnen in de cel gelokaliseerd zijn, is opname van het hormoon in de cel essentieel. Aanvankelijk werd gedacht dat dit kon plaatsvinden door passieve diffusie, waarbij stoffen vanzelf door de plasmamembraan (de celwand) heengaan, gedreven door een verschil in concentratie binnen en buiten de cel. In de afgelopen 30 jaar is echter duidelijk geworden dat hiervoor specifieke transporteiwitten nodig zijn, die de doorgang door de plasmamembraan mogelijk maken. Naast MCT8 zijn dit onder andere transporteiwitten uit de familie van "organische anion-transporterende polypeptiden" (OATP), de "heterodimere aminozuur transporters" (HAT) en het "natrium-taurocholaat co-transporterende polypeptide" (NTCP). Deze transporters hebben allemaal hun eigen gevoeligheid voor de verschillende vormen van schildklierhormoon en komen specifiek in verschillende weefsels in het lichaam voor. We beschrijven verder het belang van schildklierhormoon voor de ontwikkeling van de menselijke hersenen, en introduceren de belangrijke rol die MCT8 hierin speelt. Patiënten met een mutatie in MCT8 hebben een gestoorde hersenontwikkeling, als gevolg waarvan ze meestal niet zelfstandig kunnen zitten, staan of lopen, en meestal geen spraak ontwikkelen. Opvallend is dat ze abnormale hoeveelheden schildklierhormoon in hun bloed hebben. Zo is er met name een verhoogde bloedspiegel van het werkzame schildklierhormoon T3. Interessant is dat dit fenotype (de klinische verschijnselen van mutaties in MCT8) al in 1944 beschreven blijkt te zijn door de een aantal Amerikaanse artsen. De specifieke genetische oorzaak ervan was echter jarenlang onbekend. Het blijkt nu dat dit syndroom, dat naar de ontdekkers Allan-Herndon-Dudley syndroom genoemd, wordt veroorzaakt door mutaties in MCT8.

In **hoofdstuk 2** bestuderen we mutaties in MCT8, gevonden in 9 patienten met ernstige psychomotore retardatie en verhoogde T3 spiegels in het bloed (Allan-Herndon-Dudley syndroom). Met het doel de effecten van deze mutaties te onderzoeken in het laboratorium, zijn deze aangebracht in MCT8-cDNA, kleine stukjes DNA met de bouwtekening voor het MCT8. Vervolgens werd normaal MCT8-cDNA of gemuteerd MCT8-cDNA toegevoegd aan gekweekte cellen. Dit heet "transfectie" van cellen. Door cellen te transfecteren met de bouwtekening van een eiwit, kan je ze het eiwit waarin je geïnteresseerd bent, laten maken. In ons geval gaat het dus om het normale of het gemuteerde MCT8. Vervolgens hebben we gekeken hoe goed de getransfecteerde cellen schildklierhormoon konden opnemen. Hierbij bleek dat cellen die werden getransfecteerd met normaal MCT8 veel beter schildklierhormoon opnamen dan cellen getransfecteerd met gemuteerd MCT8. Hiermee werd aangetoond dat mutaties in MCT8 leiden tot verlies aan transportfunctie.

In **hoofdstuk 3** geven we een gedetailleerde beschrijving van een aantal patiënten met het Allan-Herndon-Dudley syndroom, waarbij we laten zien dat mutaties in MCT8 niet in elk weefseltype hetzelfde effect hebben. Terwijl de hersenen waarschijnlijk een tekort hebben aan schildklierhormoon, blijken weefsels die andere schildklierhormoontransporters

gebruiken juist bloot te staan aan de grote hoeveelheid T3 in het bloed. Lichamelijk onderzoek van de patiënten en laboratoriumonderzoek van hun bloed wijst erop dat met name de lever, de spieren en het vetweefsel een overschot aan schildklierhormoon hebben. Dit verklaart (ten dele) waarom patiënten met het Allan-Herndon-Dudley syndroom zo extreem mager zijn: schildklierhormoon bevordert de stofwisseling in hun vet- en spierweefsel.

In **hoofdstuk 4** onderzoeken we de functie van MCT8-mutaties, die door andere onderzoekers zijn gevonden in patiënten uit zeven verschillende families. Onder hen bevindt zich de familie die in 1944 werd beschreven door Allan, Herndon en Dudley. Interessant is dat patiënten uit 3 van de families een betere neurologische ontwikkeling lijken te hebben dan de MCT8-patiënten die wij aanvankelijk ontdekten, met name wat betreft zelfstandig lopen en/of de spraak-taalontwikkeling. Mutaties gevonden in deze drie families laten een relatief betere schildklierhormoon opname zien in getransfecteerde cellen. We tonen hiermee aan dat er een relatie bestaat tussen de mutatie in het MCT8-gen (het genotype), de functie van de transporter, en de klinische verschijnselen van de patiënt (het fenotype).

Vervolgens beschrijven we verschillende manieren waarop mutaties in MCT8 kunnen leiden tot verlies aan transportfunctie. Door middel van microscopisch onderzoek laten we zien dat mutanten die relatief goed functioneren mooi in de celmembraan zitten, net als het normale MCT8. Bij sommige mutanten die helemaal geen functie hebben blijkt het MCT8 wel gemaakt te worden, maar niet in de celmembraan terecht te komen. Dit verklaart direct waarom de opname van schildklierhormoon gestoord is. Bij sommige andere mutanten wordt zelfs (bijna) helemaal geen MCT8 gevormd.

In **hoofdstuk 5** onderzoeken we de opname en afgifte van schildklierhormoon in fibroblasten (huidcellen) van MCT8 patiënten. Deze huidcellen zijn na afname in kweek genomen, en maken onderzoek mogelijk aan materiaal dat direct van de patiënt afkomstig is. Dit onderzoek toont aan dat bij één mutatie (delPhe501) met name de afgifte van schildklierhormoon gestoord is. We laten zien dat er in fibroblasten van deze patiënt een hoge activiteit is van het schildklierhormoon afbrekende enzym D3, en dat verschillende schildklierhormoon gevoelige genen in hoge mate worden afgeschreven. Dit wijst op een *verhoogde*, in plaats van een *verlaagde* hoeveelheid T3 in de cel. Dit suggereert mogelijk een alternatieve verklaring voor de psychomotore retardatie die wordt veroorzaakt door mutaties in MCT8: ook een teveel aan schildklierhormoon tijdens de hersenontwikkeling kan leiden tot neurologische schade. Interessant is dat de twee patiënten bij wie deze mutatie is gevonden, - een 18 maanden oud jongentje en zijn 38 jaar oude oom - een relatief goede neurologische ontwikkeling hebben in vergelijking met andere patiënten met het Allan-Herndon-Dudley syndroom. De oudste patiënt kan zelfstandig lopen, spreekt coherent en kan woorden spellen. Zijn cognitieve ontwikkeling lijkt te liggen op het niveau van een 6- tot 8-jarige.

Hoofdstuk 6 beschrijft het transport van verschillende vormen van schildklierhormoon door MCT10, het lid van de MCT familie dat het meest lijkt op MCT8. Daarnaast beschrij-

ven we hoe MCT8 en MCT10 naast de opname van schildklierhormoon ook de afgifte ervan uit de cel mogelijk maken.

In **hoofdstuk 7** hebben we de afbraak van MCT8 bestudeerd. MCT8 bevat een zogenaamd PEST-domein, een specifiek stukje van het eiwit dat een rol kan spelen bij de eiwitafbraak. Eiwitten met een PEST-domein hebben vaak een korte halfwaardetijd, wat betekent dat zij snel worden afgebroken. We hebben gekeken hoe lang MCT8 aantoonbaar blijft in cellen nadat de aanmaak ervan is stopgezet. De aanmaak van een bepaald eiwit kan specifiek worden gestopt door cellen te transfecteren met een zogenaamd siRNA. We laten zien dat de expressie van MCT8 stabiel blijft voor meer dan 24 uur na transfectie met MCT8 siRNA. Dit wijst erop dat, ondanks de aanwezigheid van het PEST domein, MCT8 niet snel wordt afgebroken. Verder tonen we in dit hoofdstuk aan dat MCT8 een doelwit is van het ubiquitine-proteasoom systeem. Dit systeem zorgt voor een gereguleerde afbraak van eiwitten in de cel.

Hoofdstuk 8 geeft een samenvatting van de klinische en functionele aspecten van mutaties in MCT8. Daarnaast beschrijven we kort de resultaten van onderzoek MCT8 knockout muizen, muizen waarvan het MCT8 gen is uitgeschakeld. Het blijkt dat de schildklierhormoonwaarden in het bloed van deze muizen sterk lijken op die van menselijke MCT8 patiënten. Tegen de verwachting in blijkt echter de neurologische ontwikkeling van deze muizen ongestoord. Dit suggereert dat de hersenontwikkeling bij de muis, anders dan bij de mens, niet afhankelijk is van goed functionerend MCT8.

Concluderend onderstreept het in dit proefschrift beschreven onderzoek dat transporters essentieel zijn voor de goede werking van schildklierhormoon. MCT8 is de eerste schildklierhormoontransporter waarin mutaties zijn beschreven. Deze leiden tot een weefselspecifiek tekort of overschot aan schildklierhormoon, zich uitend in ernstige psychomotore retardatie en een verhoogd metabolisme in lever-, spier- en vetweefsel. Hierbij is de ernst van de neurologische schade gecorreleerd aan de mate waarin de mutatie leidt tot verlies aan transportfunctie. Doordat we met mutaties in MCT8 de oorzaak van het Allan-Herndon-Dudley syndroom hebben gevonden, zijn er meer mogelijkheden gekomen voor genetische diagnostiek en advies. Dit kan van grote waarde zijn voor families met jongens met ernstige psychomotore retardatie. Het behandelen van de gestoorde hersenontwikkeling bij patiënten met het Allan-Herndon-Dudley syndroom is (nog) niet mogelijk. Uit recent onderzoek blijkt echter dat het normaliseren van de schildklierwaarden in het bloed mogelijk positieve effecten heeft op de spieren en het vetweefsel.

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Nu de promotiedag nadert, wil ik ook graag de leden mijn promotiecommissie hartelijk danken. Professor Arts, beste Willem Frans, dank voor de prettige samenwerking de afgelopen jaren. Ik herinner me nog goed dat we samen met Maarten Reeser de eerste MCT8 patiënten onderzochten. Terwijl iemand foto's nam merkte je op dat die ongetwijfeld getoond zouden worden bij mijn promotie. Ik ben benieuwd of ze inderdaad opduiken! Dank voor je kritische blik op onze manuscripten en je enorme kinderneurologische kennis. Professor van der Lelij, beste Aart Jan, dank dat je zitting hebt willen nemen in de leescommissie. Professor Grüters, dear Annette, thank you very much for the collaboration over the last few years. I'm very honoured you are member of my committee, and look forward to visiting you in Berlin one day. Professor Halestrap, thank you very much, it's wonderful to have such an expert on the MCTs in the committee. Professor Krenning, beste Eric, ik vind het heel bijzonder dat één van de schildklierhormoontransporter-mensen van het eerste uur zitting heeft willen nemen in de commissie. Hartelijk dank daarvoor! Professor Wiersinga, beste Wilmar, wat een eer dat je lid van mijn commissie wil zijn. Professor Fetter, beste Willem, er zijn vele redenen om jou te bedanken. Natuurlijk voor je voor je zitting in mijn commissie, maar meer nog voor de kans die je me hebt gegeven in de VU. Jouw steun in de laatste maanden van mijn promotie en je deelname aan mijn promotie zorgen voor een prachtige overgang van lab naar kliniek. Mijn grote dank daarvoor.

Dit dankwoord zou niet compleet zijn zonder Willy Visser en Sabine de Muinck Keizer te noemen. Willy en Sabine, via jullie ben ik het schildklieronderzoek in gerold, en daar ben ik jullie erg dankbaar voor! Onze Graves-studie bleek meer voeten in de aarde te hebben dan we van tevoren dachten, maar Ik ben ervan overtuigd dat er mooie dingen uit gaan komen. Dank ook aan alle andere betrokkenen bij deze studie!

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Hoewel de verdeling in de tijd misschien anders doet vermoeden, zijn vrienden, familie, thuis nog veel belangrijker dan al dat leuke en uitdagende werk. Michiel, Tom, Jochum, onze vriendschap beslaat al (bijna) meer dan een half leven! Michiel, wat ben ik blij dat jij mijn vriend bent. Ik weet nog goed hoe we een paar maanden geleden herinneringen ophaalden aan de tijd dat we net in Amsterdam woonden. Jij met grootse plannen om filmmaker te worden (cineast!), en ik voorzichtig dromend van een baan als specialist. Wat ben ik trots op je! Trots op jouw doorzettingsvermogen, je creativiteit, en je gevoel voor het mooie verhaal. Het is je gelukt! Natuurlijk wil ik ook Swaan hier noemen. Lieve Swaan, je bent een schat, ik ben superblij dat we zo dicht bij jullie staan. Tom, jij supersympathiek multitalent! Je bent een gemeen goede gitarist, een uitstekende kok, en stiekem ook nog eens een grafisch vormgever van formaat. Heel erg bedankt voor het ontwerpen van de prachtige omslag van dit boekje. Het wordt tijd dat we weer eens een hele goede fles opentrekken! Jochum, jij bent ook al zo’n man die van alles kan. Ik ben onder de indruk van hoe jij je plek verovert op je werk, maar ben bovenal blij dat je sinds kort weer toekomt aan jouw werkelijk unieke talent. Er zijn immers nog lang niet genoeg mooie liedjes! Maarten, terwijl ik dit zit te schrijven komt één van onze opnames langs op mijn Ipod, en opnieuw ben ik diep geroerd door jouw spel. Thanks voor alle mooie muziek die je in de rondte strooit, ’t was de soundtrack voor het schrijven van dit proefschrift. Jason, Bart, Tanco en alle andere bandgenoten van de afgelopen jaren: super bedankt. Nu dit proefschrift (bijna) af is hoop ik dat we snel weer de gitaren om zullen hangen. Zoals Lou Reed al zei: You can’t beat guitars, bass, drums.

Geen logischer plek voor Jet, Job en Roel dan hier tussen vrienden en familie in. Wat een geluk om jullie, en natuurlijk ook Edward, Suzanne en Willemijn, cadeau te krijgen. Dank voor alle gezelligheid, alle etentjes, alle steun. Jullie zijn broeders, zusters en vrienden in één. Ide en Pauline, bedankt voor jullie nooit aflatende meeleven. Dat betekent veel voor me!

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ik blij dat wij samen zijn! Al jaren weet ik hoe lief, betrokken en slim je bent. Pas sinds kort weet ik dat je ook de moed hebt om aan de bel te trekken als dat nodig is, en hoe hard je kan knokken voor wat je waardevol vindt. Ik ben je daar ongelooflijk dankbaar voor. Hé lieve Jan, het is ons gelukt! Morgen maak ik koffie voor je! Vier voeten in de modder!

Curriculum Vitae

The author of this thesis was born on November 13th 1973 in Arnhem. After graduating in 1992, he spent a year working as a teaching assistant at the Revalidatie Scholengemeenschap Arnhem, a school for disabled teenagers. In 1993, he moved to Amsterdam to study medicine at the Vrije Universiteit. During his studies he had his first taste of lab work, determining glucocorticoid sensitivity of cells at the department of cell biology and immunology under the supervision of Dr Timo van den Berg. In spite of infected cell cultures, malfunctioning assays and the occasional late night pipetting session, something clicked. During his clinical rotations, Jurgen spent some time with Dr. Julian Shield at the department of pediatric endocrinology of Children's Hospital in Bristol, UK. Again, something clicked. After obtaining his medical degree in October 2000, Jurgen worked as a resident in pediatrics in 'het Westfries Gasthuis' in Hoorn, and in 't Lange Land Ziekenhuis' in Zoetermeer. Although he enjoyed clinical work tremendously, in September 2002 the challenge of doing scientific research brought him to the Erasmus University Medical Center in Rotterdam. Under the supervision of Dr. Willy Visser he set up a nationwide study investigating the effects of maternal Graves' disease on the thyroid function of the neonate. This profound introduction in thyroidology put him in touch with one of the gurus of thyroid research, Prof. Theo Visser. With financial support from the Sophia Foundation for Scientific Research, Jurgen started his PhD research in march 2004. The results of three and a half wonderful years at Theo's lab are presented in the current thesis. For his work on the thyroid hormone transporter MCT8, Jurgen was awarded the European Thyroid Association's Young Investigator's Prize 2007. In November 2007, he started his training residencies in pediatrics at the Free University Medical Center (VUmc) in Amsterdam under the supervision of Prof. Willem Fetter. Jurgen and his girlfriend Janneke live in Amsterdam.

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Color figures

CHAPTER 3

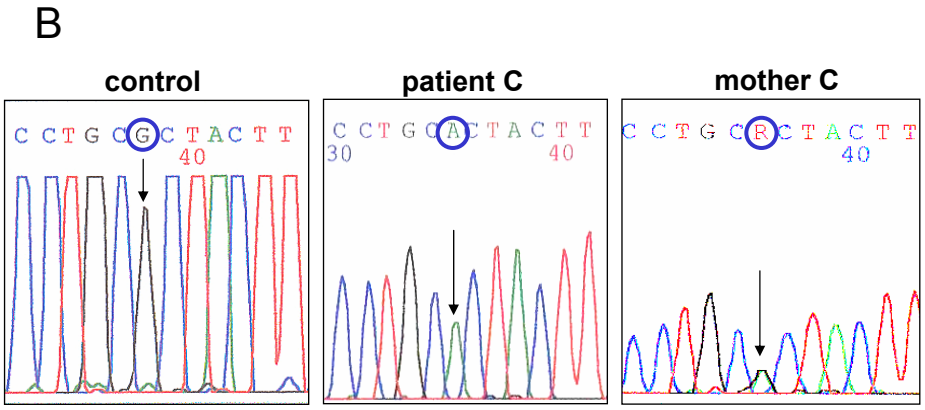

Figure 3.4 B. Sequence profile of exon 3 in a control, patient C and mother C, showing a G to A mutation. Mother and one sister (not shown) are identified as carrier of the mutation.

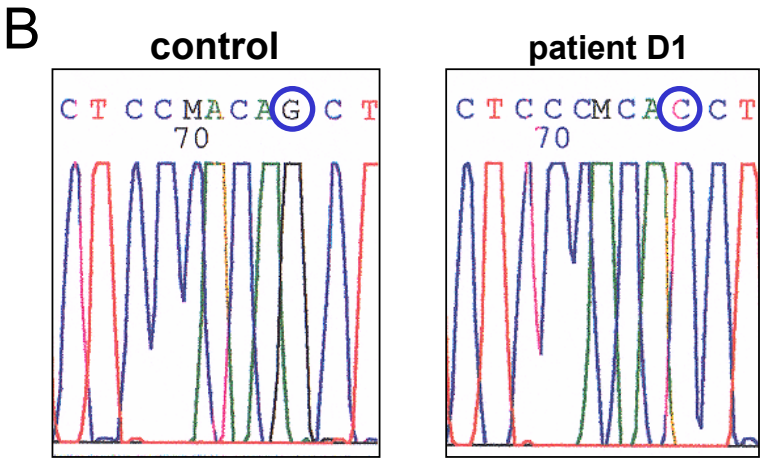

Figure 3.5 B. Sequence profile of the border of intron 2 and exon 3 in patient 8 and a control, identifying a G to C mutation in the splice-site (AGCT). This site is also a A*lu*I restriction site, which is inactivated by the mutation.

CHAPTER 4

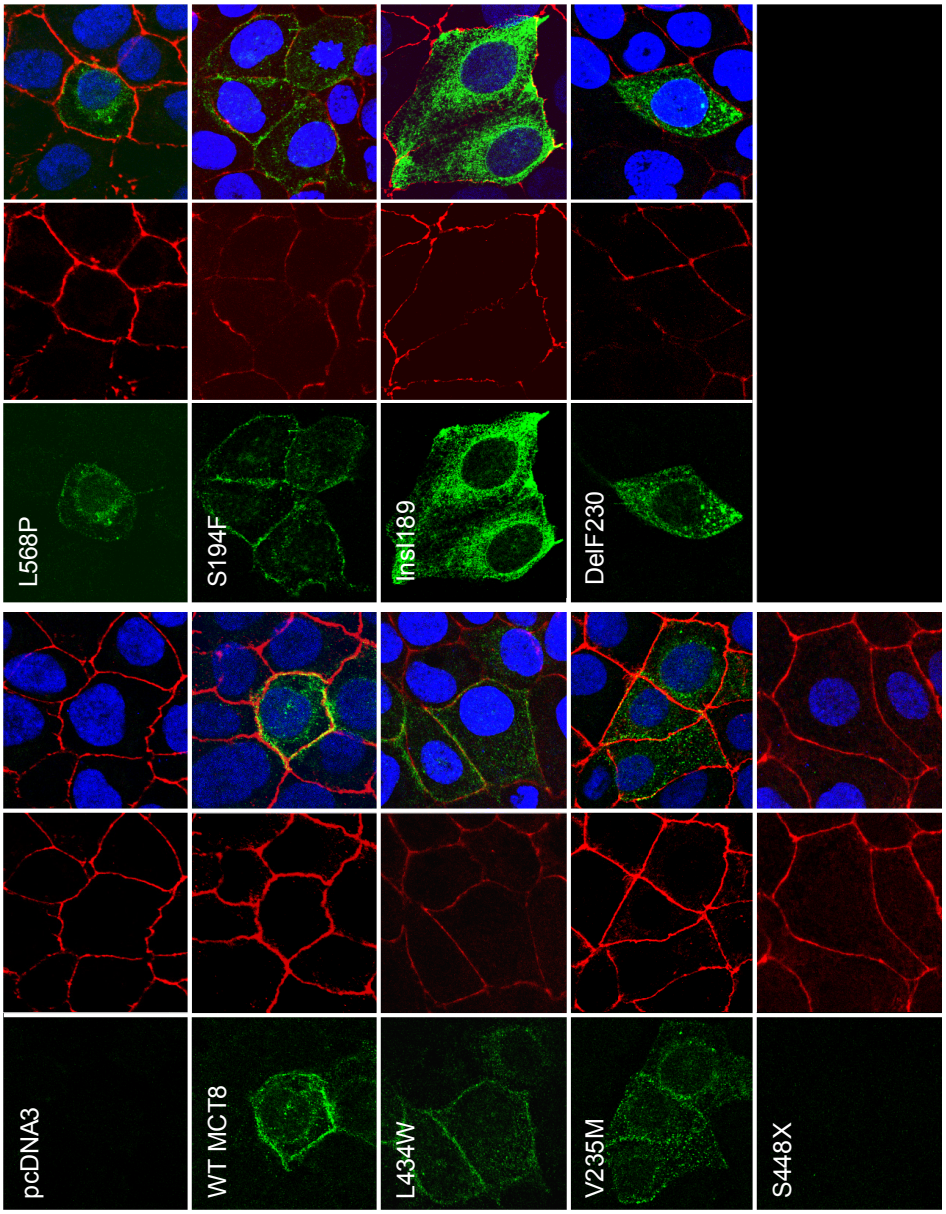

Figure 4.3 Immunocytochemistry of transfected JEG3 cells. hMCT8 specific antibody 1306 is stained green, plasma membrane marker ZO-1 (zona occludens protein 1) is stained red. Nuclear DNA is stained with DAPI (blue). Wild-type hMCT8 and mutants Leu568Pro, Leu434Trp and Ser194Phe co-localize with the plasma membrane marker. Expression of mutants Val235Met, insIle189 and delPhe230 is mostly limited to the cytoplasm.

CHAPTER 5

C

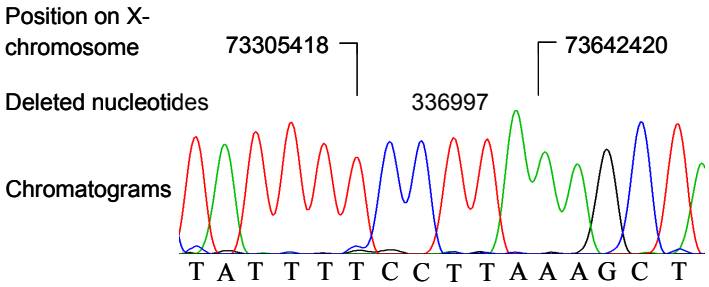

Figure 5.1 C. Partial sequencing profile of the amplicon in lane 4 of figure 5.1 B. refines the borders of the deletion in P6 to a loss of 336997 nucleotides.

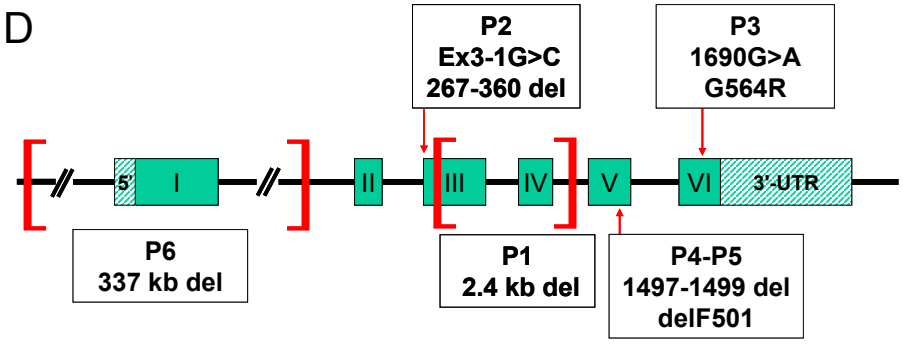

Figure 5.1 D. *MCT8* gene structure with the location of the different mutations in patients P1-6.

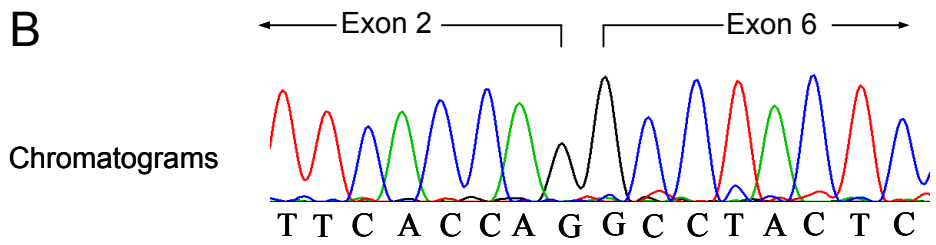

Figure 5.2 B. Part of sequencing profile of cDNA derived from mRNA in patient P1 demonstrating a loss of exons 3, 4 and 5.

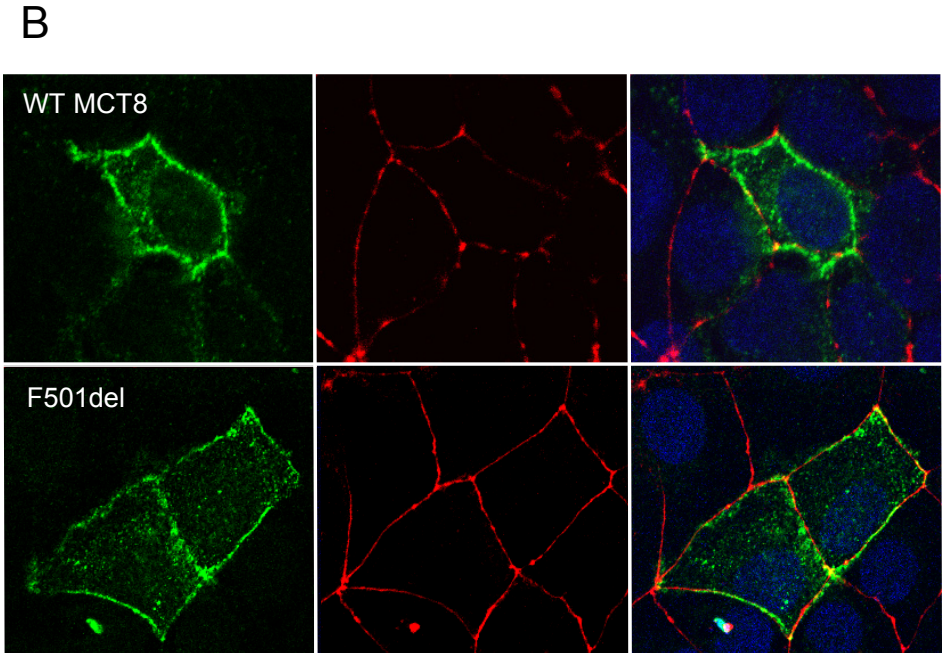

Figure 5.3 B. Immunofluorescent detection of JEG3 cells transfected with wild-type and the delPhe500 mutation in MCT8. The plasma membrane was stained with a ZO-1 antibody.

CHAPTER 6

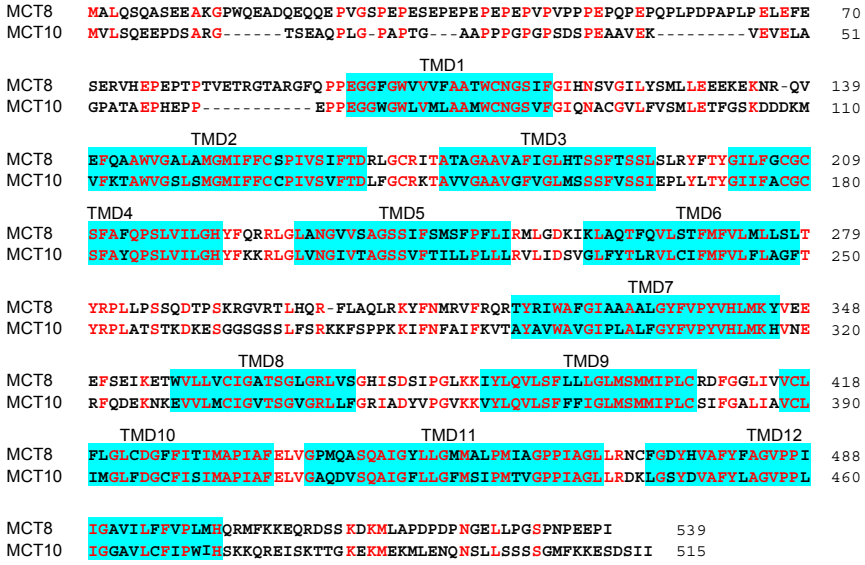

Figure 6.1 Alignment of the amino acid sequences of hMCT8 and hMCT10. Identical amino acids occupying corresponding positions in these proteins are indicated in red. The putative 12 transmembrane domains (TMDs) are indicated by blue shading.

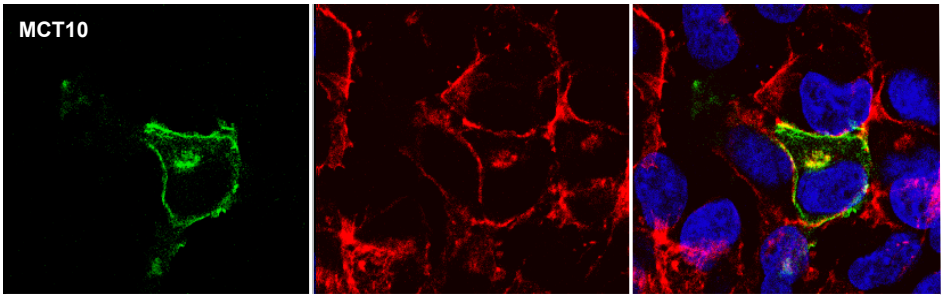

Figure 6.4 Immunocytochemistry of COS1 cells transfected with hMCT10. Left panel: hMCT10 protein detected with polyclonal antibody 1758 and stained with goat anti-rabbit Alexa Fluor 488. Middle panel: plasma membrane staining using antibody against tight junction protein ZO-1 and goat anti-mouse Alexa Fluor 633. Right panel: merged images of hMCT10, plasma membrane marker and nuclear marker (DAPI staining).

CHAPTER 7

B

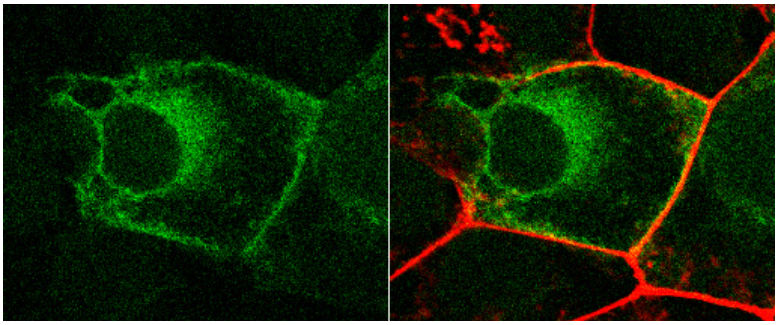

Figure 7.1 B. Cellular distribution of the hMCT8-YFP fusion protein (green) in JEG3 cells transfected with pEYFP-N1-hMCT8. Right panel: plasma membrane localization is indicated by co-localization with tight-junction protein ZO-1 (red).

CHAPTER 8

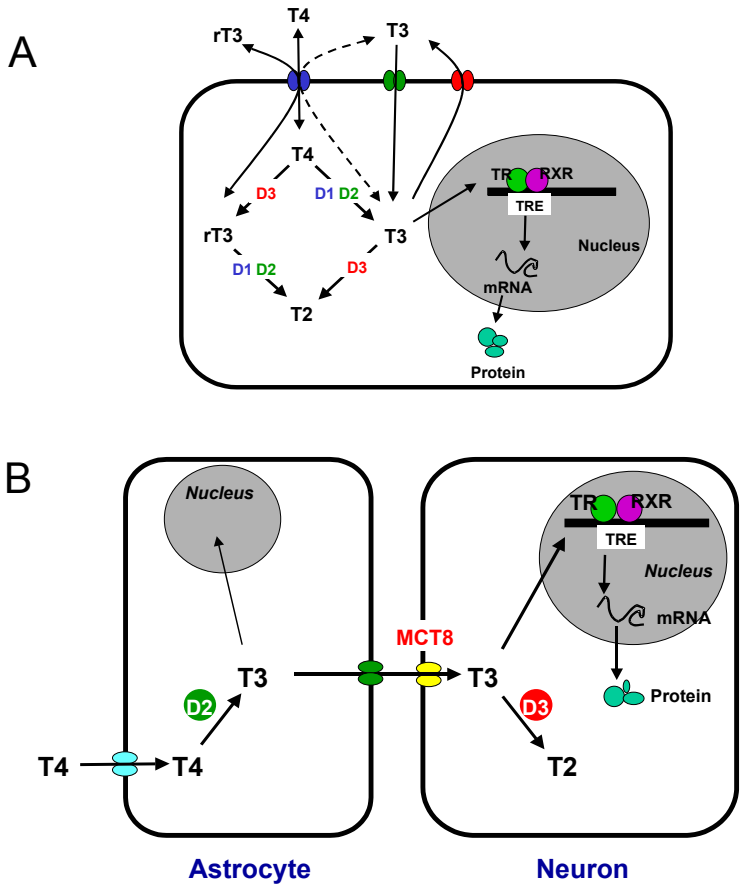

Figure 8.1 A. Model of a T3 target cell, showing the importance of transporters and deiodinases in the regulation of the bioactivity of thyroid hormone. Transporters (shown as paired ovals in the plasma membrane, itself shown as a bold outer line) are required for passage of iodothyronines across the plasma membrane, facilitating uptake, efflux, or both (exchange). They may be specific for T4 and reverse T3 versus T3, or transport all these iodothyronines. The deiodinases catalyze the activation of T4 to T3 (D1, D2) or the inactivation of T4 to reverse T3 and T3 to 3,3′-T2 (D3). Both transporters and deiodinases determine the intracellular T3 concentration available for interaction with its nuclear receptor, which can be expressed as a heterodimer (shown as paired ovals in the nucleus) with retinoid X receptor; this interaction leads to transcription of various genes and consequent generation of proteins. **B.** Local control of T3 bioavailability in the brain. Ultimate delivery of T3 to its primary target, the nuclear receptor in neurons, requires the transfer of T4 across the blood–brain barrier (not shown), its uptake by astrocytes and deiodination by D2 therein, followed by the release of T3 from these cells, and finally the uptake of T3 via monocarboxylate transporter 8 into the neurons. The identity of the other transporters involved in this system is not established. Neurons also express D3 for termination of T3 action. MCT8, monocarboxylate transporter 8; rT3, reverse T3; RXR, retinoid X receptor; TR, T3 receptor; TRE, T3-responsive element.

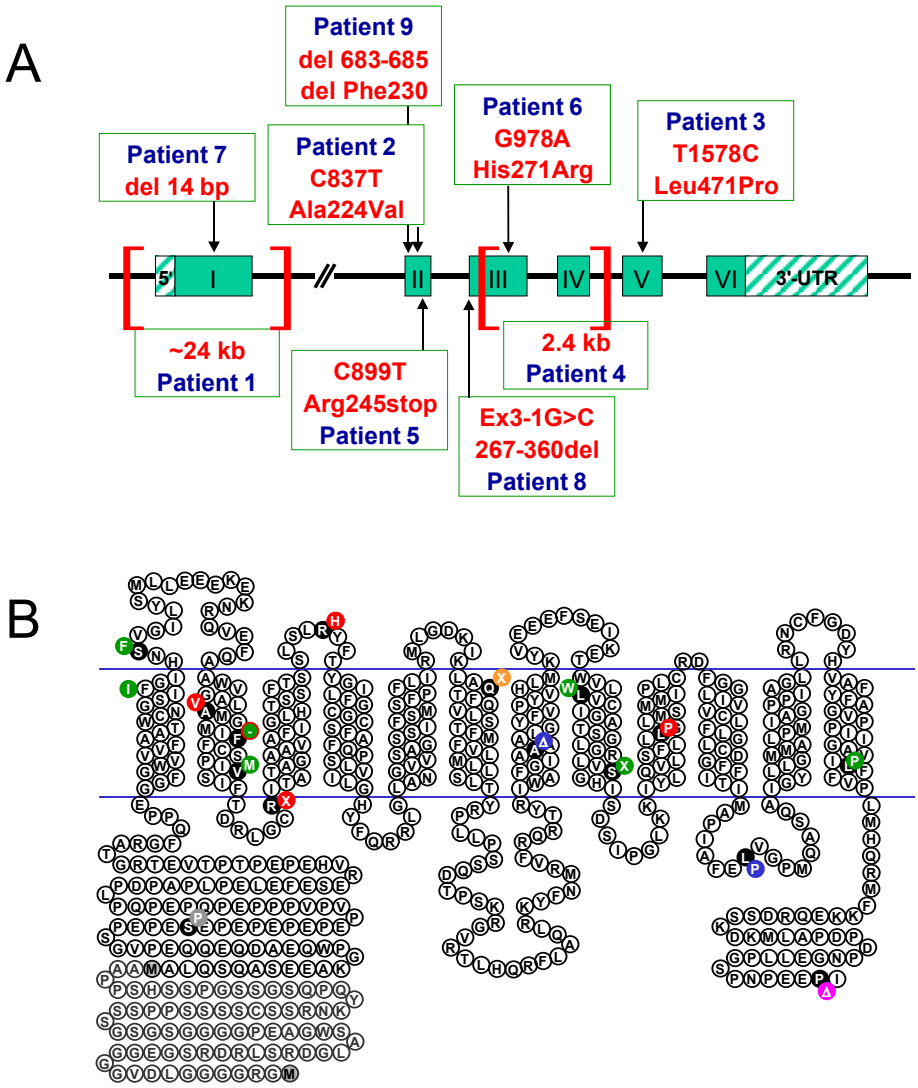

Figure 8.2 A. Structure of the human MCT8 gene and localization of mutations in nine patients. **B.** Predicted topology of human MCT8 in the plasma membrane, showing the twelve putative transmembrane domains, the N-terminal domains that differ by 74 amino acids depending on which translation start codon is used (indicated by the gray-shaded "M" (Met) residues), and the location of both N-terminal and C-terminal domains in the cytoplasm. Indicated are also various mutations identified in our laboratory (32,42) (red), and by the groups of Refetoff (43) (blue), Schwartz (45,46) (green), Passos-Bueno (44) (magenta), and Herzovich (47) (orange). The Ser107Pro alteration in grey represents a single-nucleotide polymorphism, and Δ indicates a single-nucleotide deletion. bp, basepairs; kb, kilobases; UTR, untranslated region.