Thyroid Hormone Induction of the Adrenoleukodystrophy-Related Gene (ABCD2)

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ABSTRACT

X-linked adrenoleukodystrophy (X-ALD) is a demyelinating disorder associated with impaired very-long-chain fatty-acid (VLCFA) β-oxidation caused by mutations in the ABCD1 (ALD) gene that encodes a peroxisomal membrane ABC transporter. ABCD2 (ALDR) displays partial functional redundancy because when overexpressed, it is able to correct the X-ALD biochemical phenotype. The ABCD2 promoter contains a putative thyroid hormone-response element conserved in rodents and humans. In this report, we demonstrate that the element is capable of binding retinoid X receptor and 3,5,3′-triiodothyronine (T3) receptor (TRβ) as a heterodimer and mediating T3 responsiveness of ABCD2 in its promoter context. After a T3 treatment, an induction of the ABCD2 gene was observed in the liver of normal rats but not that of TRβ–/– mice. ABCD2 was not induced in the brain of the T3-treated rats. However, we report for the first time that induction of the ABCD2 redundant gene is feasible in myelin-producing cells (differentiated CG4 oligodendrocytes). The induction was specific for this cell type because it did not occur in astrocytes. Furthermore, we observed T3 induction of ABCD2 in human and mouse ABCD1-deficient fibroblasts, which was correlated with normalization of the VLCFA β-oxidation. Finally, ABCD3 (PMP70), a close homolog of ABCD2, was also induced by T3 in the liver of control rats, but not that of TRβ–/– mice, and in CG4 oligodendrocytes.

X-linked adrenoleukodystrophy (X-ALD; McKusick OMIM 300100) is a peroxisomal disorder with an inflammatory demyelinating of the cerebral white matter and/or with adrenocortical failure (Moser et al., 2001). The gene responsible for X-ALD (ABCD1) belongs to the subfamily D in the ABC transporter family and encodes a peroxisomal membrane protein called ALDP (Moser et al., 1993). The subfamily D includes the three other genes ABCD2, ABCD3, and ABCD4 encoding the peroxisomal half-transporters ALDRP (the closest homolog of ALDP with 63% of identity) (Lombard-Platet et al., 1996), PMP70 (Kamijo et al., 1990), and PMP69 (Holzinger et al., 1997b), respectively. X-ALD is associated with defective peroxisomal β-oxidation of very-long-chain fatty acids (VLCFA), which leads to their accumulation in plasma and tissues. It has been postulated that ALDP, as homodimerized or heterodimerized with one of the three other related proteins, could provide an entry for VLCFA into the peroxisome.

At present, no completely satisfactory therapy for X-ALD is available (Moser et al., 2001). Recently, it has been shown that the drug phenylbutyrate is capable of normalizing VLCFA levels in fibroblasts from X-ALD patients (Kemp et al., 1998). Furthermore, a reduction of the VLCFA excess in plasma and erythrocytes of X-ALD patients treated with lovastatin has been observed (Pai et al., 2000). The studies revealed the induction of the ABCD2 gene expression, providing a possible mechanism through which the drugs may lower VLCFA levels in patients with X-ALD (Kemp et al., 1998; Pai et al., 2000). Induction of ABCD2 expression and normalization of VLCFA β-oxidation have also been observed in livers of ABCD1-deficient mice treated with fenofibrate (Pai et al., 2000). A possible role for ABCD2 in the clinical course of the disease had already been suggested because its expression was increased in the liver of X-ALD-deficient mice treated with lovastatin (Kemp et al., 1998).

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ABBREVIATIONS: X-ALD, X-linked adrenoleukodystrophy; VLCFA, very-long-chain fatty acids; DR+4, direct repeat with a 4-base pair spacer; RXR, retinoid X receptor; Tα, 3,5,3′,5′-tetra-iodo-L-thyronine (thyroxine); Tβ, 3,5,3′-tri-iodothyronine; TR, thyroid hormone receptor; TRE, thyroid hormone response element; PCR, polymerase chain reaction; F, forward; R, reverse; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; MMLV-TRE, Moloney murine leukemia virus thyroid hormone response element; RT-PCR, reverse transcriptase-polymerase chain reaction; WT, wild type; kb, kilobase.
expression is maximal in the brain and adrenals (Lombard-Platet et al., 1996). At the same time, ABCD2 as well as ABCD3 have been shown to be functionally redundant because their overexpression in X-ALD fibroblasts allows VLCFA β-oxidation to be restored (Braiterman et al., 1998; Kemp et al., 1998; Flavigny et al., 1999; Netik et al., 1999; Albet et al., 2001). That suggests a novel therapeutic strategy for X-ALD because pharmacological induction of ABCD2 is clearly possible (Albet et al., 1997; Kemp et al., 1998; Pai et al., 2000). At present, however, the clinical efficacy (improvement in neurological examinations) of treatment with lovastatin or phenylbutyrate has not been demonstrated (Pai et al., 2000), and fibrate seem to be unable to cross the blood-brain barrier (Berger et al., 1999). Thus, there is a need to identify new molecules capable of inducing ABCD2 and possibly ABCD3. Our strategy is derived from the capability of ligand-modulated transcription factors to activate transcription by binding to DNA response elements. The in silico study of a gene promoter provides putative response elements, which can then be studied in vitro and in vivo to define their function.

The thyroid hormones 3,5,3′-tri-iodothyronine (T₃) and 3,5,3’,5’-tetra-iodothyronine (or thyroxine; T₄) play a major role in lipid metabolism and brain maturation (Bernal and Nunez, 1995). They stimulate peroxisomal fatty-acid β-oxidation (Just and Hartl, 1983) and peroxisome biogenesis (Fringes and Reith, 1982). Thyroid hormones modulate gene expression by interacting with thyroid hormone receptors (TR), which are members of the steroid/thyroid hormone nuclear receptor superfamily. Two distinct genes (TRα and TRβ) encode several isoforms, mainly TRα1 and TRβ1, which have a wide tissue distribution, including liver and brain (Apriletti et al., 1998). TR binds to a thyroid hormone response element (TRE) characteristically as a retinoid X receptor RXR/TR heterodimer. A TRE consists of an imperfect direct repeat of the consensus hexamer 5’-AGGTCC-3’ (DR1) and mediates T 3/5 γ-oxidation (Just and Hartl, 1983) and peroxisome biogenesis.

We then investigated the regulation of ABCD3 expression in tissues of rat and cultured cells, and TRβ expression in cultured fibroblasts from X-ALD-deficient mice and patients.

Materials and Methods

Animals and Treatments. Male Sprague-Dawley rats weighing 300 g (Janvier, Le Genest St. Isle, France) were injected with T₃ (1 mg/ml i.p., pH 10.4) (Sigma, St. Louis, MO). Thyroidectomized rats were kept for 3 weeks before they were killed. Free T₃ and T₄ levels were assessed in the serum of each animal to confirm the treatment efficacy. Female 6-week-old 129/SvPas mice, wild-type (WT) (Charles River Laboratories, L’Arbresle, France) or TRβ-deficient (Ecole Normale Superieure, Lyon, France) were given carboxy cell-impregnated with 0.15% 6-propyl-2-thiouracil (Harlan, Gamay, France) for 18 days and injected with T₃ (20 μg i.p. per animal per day) or with 0.9% NaCl solution (control mice) for the last 3 days.

Cell Culture and VLCFA Analysis. COS-7 cells were grown as described previously (Fourcade et al., 2001). C6 rat glioma cells were cultured in a 1:1 mixture of Ham’s F-10 (Invitrogen, Carlsbad, CA) and Dulbecco’s modified Eagle’s medium supplemented with 7.5% fetal calf serum. CG4 rat glial cells were propagated in B104 neuroblastoma cell-conditioned medium and differentiated to mature oligodendrocytes by culture in the absence of B104 medium for 3 days before treatment with T₃ (Louis et al., 1992). Pure astrocytes were prepared from brain of 18-day-old Sprague-Dawley rat fetuses (Pallud et al., 1999). Primary cultures of mixed glial cells were derived from the brains of newborn rat pups (Besnard et al., 1989). Control and ABCD1-deficient human and mouse fibroblasts were cultured as described previously (Netik et al., 1999). The content and the β-oxidation rate of VLCFA (24:0) in fibroblasts were determined as described previously (Netik et al., 1999).

Northern Blot Analysis. Total RNA was extracted from rat tissues as described previously (Fourcade et al., 2001). The kits GenElute Mammalian Total RNA (Sigma) and RNeasy Multi (Qiagen, Courtabouef, France) were used to prepare total RNA from nervous tissues and fibroblasts, respectively. Membranes containing 20 μg/lane of total RNA were hybridized with α-32P-labeled ABCD2 and ABCD3 cDNA probes as described previously (Albet et al., 2001). Autoradiograms were quantified by digital imaging, and the relative abundance of ABCD2 and ABCD3 mRNA was determined by comparison with the mRNA levels for rat acidic ribosomal phosphoprotein (H11001) and the probe for ABCD3 mRNA as defined by Digital Imaging (Fringes and Reith, 1982), with 17 cycles and 60°C as the annealing temperature. When ABCD2 expression was studied in fibroblasts, PCR was conducted using the primers 5′-GAAGCTTCGACCTCTCATCAGTCCAGCAGTCCACGATTGCCTGAGCT-3′ (F) and 5′-GGGCTCCAAGCAGATG-3′ (R) primes with 32 cycles and 58°C as the annealing temperature. Gels were stained with ethidium bromide and photographed with a gel documentation system. Autoradiograms were quantified by digital imaging, and the relative abundance of ABCD2 and ABCD3 mRNA was determined by comparison with the 36B4 mRNA levels.

Real-Time Quantitative PCR. cDNA generated by reverse transcription from total RNA extracted from fibroblasts was analyzed by quantitative PCR using the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). The primers (nt 525) 5′-AGTCTTCTGGGTGGCAGT-3′ (F) and the TaqMan fluorescent probe (nt 1986) 5′-AGGACATCTTTCACACTTAC-3′ (R) and the TaqMan fluorescent probe (nt 1986) 5′-HEX-CAAAGAGAGGAGATGGGATGAC-TAMRA-3′ were used for amplification and detection of ABCD2 mRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the control. The primers (nt 562) 5′-GACTTCTGCGGTCCACCTACACACTT-3′ (F) and the probe (nt 562) 5′-FAM-CATGACCACAGTCCATAGGCA-TAMRA-3′. Standard curves for quantification were obtained using plasmid containing the mouse ABCD2 or GAPDH cDNA (Berger et al., 1999). For each assay, 1 and 6 ng of reverse-transcribed RNA was used for the PCR analysis of GAPDH and ABCD2 mRNA, respectively. The thermocycler was programmed as follows: 95°C for 10 min, and 50 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The thermocycler was programmed as follows: 95°C for 10 min, and 50 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The thermocycler was programmed as follows: 95°C for 10 min, and 50 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s.
5'-GATCCCGAGGACCTGAATGTGACCTGA-3' (R), which contain the functional TRE in the long terminal repeat of Moloney murine leukemia virus (MMLV-TRE). To carry out EMSA with the receptors RXR and TR, proteins were synthesized in vitro using the transcription-translation-coupled Reticulocyte Lysate System (Promega, Madison, WI) from the rat RXXα-pSG5 (a gift from Dr. S. Green, Zeneca Pharmaceuticals, Cheshire, UK) and human TRβ1-pSG5 (provided by Dr. V. Laudet, ENS, Lyon, France) plasmids. Binding experiments were performed as described above, except that the probe (30,000 cpm) was incubated with 1 to 4 µl of the unlabeled RXXα and/or TRβ1 synthesis mixture (or 2 µl of reticulocyte lysate for the negative control) instead of nuclear extracts.

**Plasmids and Cell Transfection.** The plasmids DR+4-pGLUC, p2206, and p748 were described elsewhere (Fourcade et al., 2001). The p2206A and p748A plasmids, deprived of the DR+4 motif, were prepared by deletion of the region −748/−312 and −391/−373, respectively. The p2206A plasmid was constructed by replacing the BglII/HindIII region with the PCR product used to obtain the p312 construct (Fourcade et al., 2001). The p748A plasmid resulted from ligation between BglII/HindIII double-digested pGL3-Basic and two PCR fragments. The PCR fragments were amplified from the original promoter subclone SH-pKS (Fourcade et al., 2001) using primers that start in the DR+4 motif and contain an EcoRI site. After ligation, the junction was as follows: 5'-CCAGaaATCTCCAGCG-3' (−395/−366), where lowercase letters represent modified nucleotides from the original sequence allowing the creation of an EcoRI site (underscored). The constructs were used in the transient transfection of COS-7 cells as described previously (Fourcade et al., 2001).

**Results**

**The DR+4 Motif Binds RXRα and TRβ1.** We first determined whether nuclear proteins could interact with the DR+4 motif present in the rat ABCD2 promoter in EMSA experiments. The incubation of probe DR+4 or MMLV-TRE (a well-known functional TRE) with nuclear extracts from rat liver resulted in the formation of two complexes (Fig. 1A, lanes 2 and 7), which were reduced by an excess of unlabeled oligonucleotide (Fig. 1A, lanes 3 and 8). Cross-competition experiments also showed reduced complexes (Fig. 1A, lanes 4 and 8). The unrelated competitor Sp1 did not alter the complexes (Fig. 1A, lanes 5 and 10). We further investigated whether the DR+4 motif could bind an RXX/TRA heterodimer. In the presence of RXXα and TRβ1, two retarded complexes migrated at the same position as the complexes observed with nuclear extracts (Fig. 1B). The upper complex probably corresponded with an RXXα/TRβ1 heterodimer (Fig. 1B, lane 4) because TRβ1 alone was sufficient to form the lower complex (Fig. 1B, lane 3).

**The DR+4 Motif Is a Functional TRE.** To determine whether the DR+4 motif is a functional TRE, COS-7 cells were transfected with DR+4-pGLUC plasmid (pGLUC contains a β-globin promoter upstream of the reporter gene). When cells were cotransfected with TRβ1-pSG5 and treated with T3, a 5-fold increase in luciferase activity was observed (Fig. 2). As expected, similar results were obtained using a construct containing the human ABCD2 DR+4 motif (data not shown).

To confirm that the DR+4 motif functions in its promoter context, we transfected COS-7 cells with constructs containing fragments of the rat ABCD2 promoter cloned upstream from the promoterless luciferase gene (p2206 and p748). Luciferase activity was induced 2.6-fold by using p2206 and 1.6-fold by using p748 in cells cotransfected with TRβ1-pSG5 and treated with T3 (Fig. 2). The induction was completely abolished when transfection was performed with the cognate plasmids deleted for the DR+4 motif (p2206Δ and p748Δ).

**T3 Induces ABCD2 Expression in the Liver.** To determine whether our in vitro findings have a physiological relevance, we studied the in vivo effects of T3 on ABCD2 expression in the liver, a major target of T3 (Feng et al., 2000) and the only organ in which ABCD2 has so far proved to be inducible (Albet et al., 1997), and in the brain, which is the most important organ for an X-ALD therapy. ABCD3 was also examined because its expression is positively regulated by T3 (Hartl and Just, 1987). A preliminary study carried out in adrenalectomized and castrated rats revealed that a T3 treatment [12.5 µg/100 g of b.wt./day] for 3 days was sufficient to induce the expression of the ABCD2 and ABCD3.
genes in the liver by 2.6- and 2.0-fold, respectively, but we did not detect any induction in the brain (data not shown). Because the expression of ABCD2 and ABCD3 in the brain did not seem to be sensitive to 

\[ T_3 \]

, we treated normal rats by substituting 

\[ T_4 \]

 (the most metabolically active hormone) and increasing either the duration (7 days) or the dose (100 \( \mu g \) of b.wt./day). Again, we observed induction of both genes in the liver, i.e., 1.8- and 3.5-fold increases in the level of ABCD2 mRNA and 1.7- and 2.5-fold increases in the level of ABCD3 mRNA at the 10- and 100- \( \mu g \) of \( T_3 \) doses, respectively (Fig. 3A). No change occurred in the brain (Fig. 3A). Interestingly, thyroidectomized rats exhibited a lowered expression for both genes in the liver (Fig. 3B), indicating that 

\[ T_3 \]

 plays a role in their basal hepatic expression; this lowering did not take place in the brain (data not shown).

**T3 Does Not Induce ABCD2 and ABCD3 Expression in TR\( \beta \)/-/- Mice.** To confirm the involvement of TR in the 

\[ T_3 \]

 induction of ABCD2 observed in the liver, we treated wild-type and TR\( \beta \)/-/- mice with 

\[ T_3 \]

 and examined the gene expression in the liver. All of the mice were pretreated with 6-propyl-2-thiouracil because 

\[ T_3 \]

 and 

\[ T_4 \]

 are markedly increased in mice lacking TR\( \beta \) (Gauthier et al., 1999). After 18 days of pretreatment, the serum levels of free 

\[ T_4 \]

 and 

\[ T_3 \]

 were 24.4 and 7.9 pmol/l in the 

\[ TR\beta/-/- \]

 mice (not injected with 

\[ T_3 \]

), respectively, indicating that the levels of thyroid hormones were normalized. The serum levels of free 

\[ T_4 \]

 (3.8 pmol/l) and 

\[ T_3 \]

 (1.4 pmol/l) in the pretreated wild-type mice (not injected with 

\[ T_3 \]

) were similar to those observed in thyroidectomized animals. Figure 4 shows that 

\[ T_3 \]

 induction of ABCD2 (\( \times 2.0 \)) and ABCD3 (\( \times 2.8 \)) occurred in the liver of wild-type mice as expected but not in the 

\[ TR\beta/-/- \]

 mice. In the animals not injected with 

\[ T_3 \]

, the levels of ABCD2 and ABCD3 were higher in the 

\[ TR\beta/-/- \]

 mice than in the wild-type mice (Fig. 4), as a result of the differences in the serum levels of 

\[ T_3 \]

 and 

\[ T_4 \]

 through pleiotropic effects of the thyroid hormones.

**ABCD2 and ABCD3 Are Up-Regulated by T3 in CG4 Oligodendrocytes but not in Astrocytes.** We observed no

![Fig. 2. T3 responsiveness is mediated by the DR+4 motif. COS-7 cells were transfected with pGLUC or DR+4-pGLUC and with p748, p748Δ, p2206, or p2206Δ, which contain rat ABCD2 promoter fragments deleted or not for the DR+4 motif. The cells were either treated with 50 nm T3, for 48 h [□] or cotransfected with TRβ1-pSG5 [□] or both [□]; the control cells were neither treated nor cotransfected [□]. Luciferase activity was expressed as the x-fold induction in relation to untreated pGLUC-transfected cells. Data represent the mean ± S.E. of three to five independent experiments performed in triplicate wells. Statistically significant differences (Student’s t test) from controls are indicated by *, \( P < 0.01 \); **, \( P < 0.001 \). NS, not significant.

![Fig. 3. ABCD2 and ABCD3 expression is regulated by T3 in the liver. A. Northern blot analysis of ABCD2 and ABCD3 mRNA in the liver and brain of hyperthyroid (Hyper) rats for their mRNA levels in the liver and brain of rats untreated (0) or treated with 10 \( \mu g \) of T3/100 g of b.wt./day for 7 days (10) or 100 \( \mu g \) of T3/100 g of b.wt./day for 3 days (100). B, thyroidectomized (Hypo) rats were compared with control (Eu) and 10 \( \mu g \) of T3/100 g of b.wt./day-treated (Hyper) rats for their mRNA levels in the liver. In each lane, total RNA was pooled from four rats. The 5.5-kb ABCD2 mRNA was not detected in the liver.

![Fig. 4. TRβ is required for T3 induction of ABCD2 and ABCD3 in the liver. Semiquantitative RT-PCR of ABCD2 and ABCD3 mRNA in the liver of wild-type and TRβ/-/- mice after a daily injection of 20 \( \mu g \) of T3 i.p. for 3 days. Total RNA was extracted from three mice for each treatment and pooled before analysis. A, PCR was performed in duplicate and amplified DNA was analyzed on a 2% agarose gel. B, the levels of gene expression are given as x-fold induction in relation to untreated wild-type animals. Data represent the means of two independent experiments of RT-PCR.
induction of ABCD2 and ABCD3 by T3 in the whole brain. However, such an analysis might not detect induction restricted to only one type of nervous cell. We therefore performed analyses by Northern blotting and semiquantitative PCR on cell lines and primary cultures of rat glial cells. We treated oligodendrocyte-differentiated CG4 cells with 125 or 500 nm T3 for 3 days. The ABCD2 mRNA level was enhanced by 2.3- and 4.8-fold, and the ABCD3 mRNA level by 1.2- and 2.6-fold, after the dose of T3 (Fig. 5A). When CG4 cells were exposed to 100 nm T3 for different times (2 to 10 days), the induction of ABCD2 and ABCD3 was maintained for all the period of treatment (Fig. 5B). On the other hand, we observed no induction for both genes in C6 cells treated with 100 nm T3 for 3 days (Fig. 5A) or shorter times (6, 24, and 48 h) (data not shown). When mixed primary cultures of oligodendrocytes (approximately 40%) and astrocytes were treated with 100 nm T3 for 3 days, the induction of ABCD2 was still visible although low (×1.24 ± 0.04 as mean ± S.E. (n = 11) from three independent cell preparations; several cultures were conducted from each cell preparation and analyzed by RT-PCR in duplicate) (Fig. 5C). Indeed, no change in ABCD2 and ABCD3 expression occurred in primary cultures of pure astrocytes treated with 0.1 or 1 μM T3 for 3 days (Fig. 5C). The results indicate for the first time that the induction of ABCD2 and ABCD3 in cells of the central nervous system is possible. Furthermore, they suggest that the induction can occur in an oligodendrocytic cell type, the target for X-ALD therapy.

T3 Induction of the ABCD2 Gene Is Correlated with Normalization of the X-ALD Biochemical Phenotype. Overexpression of ABCD2 in fibroblasts from ABCD1-deficient mice or from X-ALD patients is known to restore β-oxidation of VLCFA and to reduce their intracellular level (Kemp et al., 1998; Flavigny et al., 1999; Netik et al., 1999; Albet et al., 2001). We thus treated such fibroblasts with T3 to induce ABCD2 gene expression and to examine the effects of this induction on β-oxidation of VLCFA. We first investigated the dependence of the ABCD2 mRNA expression on the T3 dose (10 to 100 nm) and the duration (2 to 10 days) of treatment in ABCD1-deficient mouse fibroblasts using quantitative PCR. Although large differences between individual experiments probably obscured statistically significant differences between treatments, we observed a T3 dose-dependent increase in the amount of ABCD2 mRNA in cells T3-treated for 2 days (Fig. 6A). However, the ABCD2 induction seemed to be transitory, because after treatment with 100 nm T3 for 10 days, the ABCD2 mRNA level was close to the level measured in untreated cells (Fig. 6A). The pattern of ABCD2 expression was similar when semiquantitative RT-PCR was used (data not shown). In ABCD1-deficient mouse fibroblasts exposed to 100 nm T3 for 2 days, the rate of C24:0 β-oxidation increased by 4.3-fold and thus reached a higher level than in untreated WT fibroblasts (Fig. 6B). C24:0 β-oxidation returned to its initial rate after 6 days of T3 treatment. With a delay of a few days, the transitory effect of T3 on VLCFA β-oxidation affected the C26:0 cell level. Indeed, we observed an approximately 45% decrease in the C26:0 content in cells treated with T3 for 4 or 6 days (Fig. 6C). The effect disappeared in fibroblasts treated for 10 days. We obtained similar results with X-ALD human fibroblasts (Fig. 6C). The present data suggest that normalization of the X-ALD biochemical phenotype by T3 results from up-regulation of the ABCD2 gene expression.

Discussion

Although ALDRP is apparently unable to compensate for ALDP deficiency at the intrinsic levels of expression in humans with X-ALD and ABCD1-deficient mice, its overexpression partially restores VLCFA β-oxidation (Kemp et al., 1998; Flavigny et al., 1999; Netik et al., 1999; Albet et al., 2001). In the present study, we were interested in identifying novel molecules that could up-regulate ABCD2 expression to provide the basis for pharmacological treatment for patients with X-ALD. By a molecular analysis of the ABCD2 promoter in rat, mouse, and human, we found a conserved DR+4 motif.
fitting the TRE consensus sequence (Fourcade et al., 2001). In the present study, we demonstrated that the rat or human ABCD2 DR+4 was able to mediate T3 up-regulation of the reporter luciferase gene in transient cell transfection experiments. The T3 induction obtained with the plasmids containing the proximal rat ABCD2 promoter was moderate, as already seen for other promoters transfected in COS-7 cells (Simonides et al., 1996). Moreover, it has been reported that the cotransfection of an RXR expression plasmid enhances gene induction by T3 (Simonides et al., 1996). This finding, as well as our demonstration that the ABCD2 DR+4 can bind RXRα/TRβ1 in vitro, suggests that the DR+4 motif might mediate the 9-cis-retinoic acid induction of ABCD2 observed in an embryonal carcinoma human cell line (Troffèr-Charlier et al., 1998). Indeed, the 1.0-kb proximal region of the mouse or human ABCD2 promoter, which is also highly conserved in rat, has been shown to be sufficient to promote the 9-cis-retinoic acid activation of a reporter gene cotransfected with RXRα (Pujol et al., 2000). Thus, retinoic acid would be likely to boost T3 induction of ABCD2.

The effects of T3 on ABCD2 expression were then studied in vivo. We observed an increase in the levels of ABCD2 mRNA in the liver but not in the brain of the hyperthyroid rats. The T3 induction of ABCD2 in the liver requires the presence of TRβ. Moreover, ABCD2 expression was decreased in the liver but not in the brain of the thyroidectomized rats. Thus, thyroid hormone seems to be necessary to maintain the steady-state level of ABCD2 expression, at least in the liver. Thyroid status might be involved in the clinical variability of X-ALD because physiological changes in the regulation of the ABCD2 redundant gene may be beneficial (induction) or not (no induction) for the biochemical status of patients. Our results demonstrate that alteration of the thyroid status could be used to modify the expression of T3-sensitive redundant genes. The doses of T3 used in our experiments may seem unacceptable for humans because of potential side effects. However, the possibility of selectively targeting the TRβ receptor with T3 analogs, such as GC-1, might reduce some deleterious effects of thyroid hormones (Baxter et al., 2001).

Thyroid hormone plays an essential role in the developing brain (Bernal and Nunez, 1995; Rodriguez-Pena, 1999). In the brain of the young rat, postnatal days 8 to 30 correspond to the period of most extensive oligodendrocyte maturation and myelination. T3 concentration in the brain reaches a peak approximately 2 weeks after birth, which correlates with an increase in TRβ expression and in the activity of type II iodothyronine deiodinase, the enzyme responsible for the conversion of T3 to T3 in the brain. Expression of myelin protein-encoding genes in the rodent brain, including the myelin-basic-protein gene in which the presence of a TRE has been demonstrated (Pombo et al., 1999) and of genes encoding the peroxisomal β-oxidation enzymes, reaches its highest point during the postnatal period. ABCD2 expression, which progressively increases after birth and reaches a maximum level at approximately days 15 to 21 in the brain of rat (Albet et al., 2001) and mouse (Berger et al., 1999), seems to match the local T3 bioavailability. Furthermore, the population of microperoxisomes reaches a peak at the period of myelin formation (Adamo et al., 1986). All of these findings, and our observation that ABCD2 is T3-inducible in CG4 oligodendrocytes, suggest that during brain development, ALDRP is involved in the transport of high amounts of a lipid required for myelination through T3 induction of ABCD2 expression. This lipid might be docosahexaenoic acid (C22:6 n-3) or its precursor C24:6 n-3 (Su et al., 2001). Docosahexaenoic acid accumulates specifically in the brain during development (Martinez, 1992). Recently, docosahexaenoic acid has been presented as a ligand for RXR and thus could participate with T3 in ABCD2 induction (de Urquiza et al., 2000).

An important question is whether ABCD2 is inducible in the adult brain. Once brain development ends, ABCD2 expression remains at a high level in human (Holzinger et al., 1997a) and rodents (Berger et al., 1999; Albet et al., 2001), perhaps through a T3-independent mechanism, which may explain why ABCD2 expression levels do not vary in the brain of T3-treated or hypothyroid rats. Indeed, Strait et al. (1997) observed a rapid increase in myelin-basic-protein expression upon T3 treatment during the first 3 days of differentiation of O-2A oligodendrocytes. However, the levels of myelin-basic-protein mRNA were not different any longer in O-2A cells cultured for 10 days in the presence or absence of T3, indicating that the terminal expression levels were maintained independently of T3 in differentiated oligodendrocytes. In contrast, we observed no increase in the ABCD2 mRNA levels in the absence of T3 during the culture of differentiated CG4 oligodendrocytes. Our findings do not

![Fig. 6](image)
support the hypothesis that the high levels of ABCD2 expression in the adult brain may be independent of T3. On the other hand, although ABCD2 is expressed in astrocytes as well as in oligodendrocytes of adult mouse brain (Troffeer-Charlier et al., 1998), we observed T3 induction of ABCD2 only in CG4 oligodendrocytes and not in astrocytes. This suggests that ABCD2 induction could be restricted to only a single cell type in adult brain, providing an explanation for the absence of detectable variation in ABCD2 expression observed during an analysis of whole-brain mRNA from T3-treated and hypothyroid rats. Induction may also occur in one or a few specific brain regions. The subependymal zone and hippocampus of the adult rodent and human brain are known to contain multipotent stem cells, which are capable of de novo generation of neurons and glia. Interestingly, when exposed to T3, the multipotent stem cells generate clones composed entirely of cells with oligodendrocyte morphology (Johe et al., 1996). A study of the regulation of ABCD2 expression in stem cells expanded from neurogenic regions should be of great interest because the stem cells can rapidly generate myelin-forming cells.

Despite only 38% homology with ALDP, the half-transporter PMP70 can also partially substitute for ALDP because VLCFA β-oxidation is restored in X-ALD fibroblasts transfected with ABCD3 cDNA (Braiterman et al., 1998; Kemp et al., 1998). Thus, ABCD3 could become a target gene in the same way as ABCD2 for pharmacological therapy of X-ALD. We observed T3 up-regulation of ABCD3 expression in rat liver as well as in CG4 cells. Computer analysis of the mouse and human ABCD3 promoters (0.4 and 3.3 kb, respectively) did not reveal the existence of a putative TRE (Gartner et al., 1998). The T3 induction levels observed for ABCD3 in the present study were lower than those for ABCD2. However, the higher content of PMP70 in the peroxisomal membrane in comparison with ALDRP could compensate for a relatively low induction level in the context of partial functional redundancy.

Pharmacological therapy for genetic disease is aimed at up-regulating redundant genes to compensate for a biochemical defect. Even if the assumption that VLCFA excess in brain triggers the inflammatory response associated with progressive demyelination in X-ALD is still in debate, a decrease in the VLCFA content in brain may be beneficial for patients. In ABCD1-deficient fibroblasts, we observed a transitory decrease in C26:0 accumulation correlated with an increase in ABCD2 expression, suggesting that the restoration of the VLCFA β-oxidation resulted from up-regulation of ABCD2. Several T3 regulation levels are known to play an important role in maintaining the intracerebral T3 content that is relatively constant during changes in thyroid status. Thus, increased T3 concentrations result in depleted TR and type II idothyronine deiodinase levels and in enhanced activities of type I and III deiodinases, the two enzymes that inactivate T3 (Ortiz-Caro et al., 1987; Köhrl, 1999). Similar T3 regulation may occur in fibroblasts and may explain the transitory effects of T3 treatment on VLCFA β-oxidation and ABCD2 expression. Such regulation might be different in oligodendrocytes, because the induction of ABCD2 was maintained in T3-treated CG4 cells for 10 days.

In conclusion, we demonstrated that ABCD2 is a T3-responsive gene both in rodent and human cells through a classic T3 and that T3 treatment can induce ABCD2 expression and correct transiently the VLCFA accumulation in X-ALD fibroblasts. Furthermore, we observed that ABCD2 in CG4 oligodendrocytes is responsive to T3, although ABCD2 up-regulation was not found in the whole brain, unlike the liver, of the rat upon T3 treatment. Further studies using ABCD1-deficient mice will be required to evaluate the efficacy of T3 (or T3 analogs) treatment on ABCD2 mRNA and ALDRP protein levels and VLCFA content in the brain.

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