0026-895X/03/6306-1296–1303$7.00
MOLECULAR PHARMACOLOGY
Vol. 63, No. 6
2158/1063956
Mol Pharmacol 63:1296–1303, 2003
Printed in U.S.A.

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

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Received September 19, 2002; accepted February 21, 2003

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′-tri-iodothyronine (T_3) receptor (TRβ) as a heterodimer and mediating T_3 responsiveness of *ABCD2* in its promoter context. After a T_3 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 T_3-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 T_3 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 T_3 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 demyelination 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 (Mosser 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

This work was supported by grants from the European Association against Leukodystrophies (ELA; Nancy, France) and the Regional Council of Burgundy. This study was also funded by the Austrian Federal Ministry of Science and Transport (GZ 70.040/2-PR/4/98) and the Austrian National Bank (grant 9043). S.F. was supported by a fellowship from the ELA.

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; T_4, 3,5,3′,5′-tetra-iodo-L-thyronine (thyroxine); T_3, 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 fibrates 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 (T_3) and 3,5,3′,5′-tetra-iodo-L-thyronine (or thyroxine; T_4) 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′-A(G)GGTCA-3′ separated by a 4-base pair spacer (DR+4). Inspection of the *ABCD2* promoter sequence revealed a region conserved in rat, mouse, and human containing a DR+4 motif (in bold) that differs from the consensus TRE by only 1 base pair when reverse orientation is considered (rat, −392/−367, 5′-GCAGT**TGACCT**TATT**CGACCT**CTCCA-3′; mouse, −387/−362, 5′-GCAGC**TGACCT**CATT**CGACCT**CTCCA-3′; and human, −402/−377, 5′-GCAGA**TGGCCT**GATT**CGACCT**CTCCA-3′) (Fourcade et al., 2001).

In the present study, we first demonstrated that the DR+4 motif is a functional TRE that binds TRβ1 and mediates T_3 activation. We then investigated the regulation of *ABCD2* (and *ABCD3*) expression in tissues of rat and cultured murine nervous cells upon T_3 treatment. Finally, we examined whether T_3 treatment could restore β-oxidation and VLCFA levels in fibroblasts from *ABCD1*-deficient mice and patients with X-ALD and whether the restoration was correlated with the up-regulation of *ABCD2*.

Materials and Methods

Animals and Treatments. Male Sprague-Dawley rats weighing 300 g (Janvier, Le Genest St. Isle, France) were injected with T_3 (1 mg/ml i.p., pH 10.4) (Sigma, St. Louis, MO). Thyroidectomized rats were kept for 3 weeks before they were killed. Free T_3 and T_4 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 chow pellets impregnated with 0.15% 6-propyl-2-thiouracil (Harlan, Gannat, France) for 18 days and injected with T_3 (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 starting T_3 treatment (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 rats (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 Mini (QIAGEN, Courtaboeuf, France) were used to prepare total RNA from nervous cells and fibroblasts, respectively. Membranes containing 20 μg/lane of total RNA were hybridized with α-^{32}P–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 P0 (*36B4*). The *36B4* probe was a gift from Dr C. Le Jossic-Corcos (University of Burgundy, Dijon, France).

Semiquantitative PCR. To study *ABCD2* and *ABCD3* expression in cultured nervous cells, conventional PCR was performed as described previously (Fourcade et al., 2001) except that 25 cycles were used for *ABCD2*. Amplification of the control *36B4* was carried out using the forward (F) and reverse (R) primers 5′-AAYGTGGGCTCCAAGCAGATG-3′ and 5′-GAGATGTTCAYCATGTTCAGCAG-3′, respectively, with 17 cycles and 60°C as the annealing temperature. When *ABCD2* expression was studied in fibroblasts, PCR was conducted using the primers 5′-GAAGCCTCGGACTTTCATCATC-3′ (F) and 5′-GTGTAATTATGGGAACATTTTCAC-3′ (R) with 32 cycles and 58°C as the annealing temperature. Gels 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 1959) 5′-CACAGCGTGCACCTCTAC-3′ (F) and (nt 2032) 5′-AGGACATCTTTCCAGTCCA-3′ (R) and the TaqMan fluorescent probe (nt 1986) 5′-HEX-CAAAGAGAAGGAGGATGGGATGC-TAMRA-3′ were used for amplification and detection of ABCD2 mRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA used as the control was analyzed with the primers (nt 525) 5′-AGGTCATCCATGACAACTTT-3′ (F) and (nt 601) 5′-AGTCTTCTGGGTGGCAGT-3′ (R) and the probe (nt 562) 5′-FAM-CATGACCACAGTCCATGCCA-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 20 s and 58°C for 50 s.

Electrophoretic Mobility Shift Assay. EMSA was performed as described previously (Fourcade et al., 2001), except for the oligonucleotides 5′-AGCTTCA**GGGTCA**TTTC**AGGTCC**TTGG-3′ (F) and

5′-GATCCCAA**GGACCT**GAAA**TGACCC**TGA-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 RXRα-pSG5 (a gift from Dr. S. Green, Zeneca Pharmaceuticals, Cheschire, 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 RXRα 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 p2206Δ and p748Δ plasmids, deprived of the DR+4 motif, were prepared by deletion of the region −748/−312 and −391/−373, respectively. The p2206Δ plasmid was constructed by replacing the *Bgl*II/*Hin*dIII region with the PCR product used to obtain the p312 construct (Fourcade et al., 2001). The p748Δ plasmid resulted from ligation between *Bgl*II/*Hin*dIII 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 *Eco*RI site. After ligation, the junction was as follows: 5′-CCAGgaatTCTCCAG-3′ (−395/−366), where lowercase letters represent modified nucleotides from the original sequence allowing the creation of an *Eco*RI 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 RXR/TR heterodimer. In the presence of RXRα 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 RXRα/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 T_3, 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 T_3 (Fig. 2). The induction was completely abolished when transfection was performed with the cognate plasmids deleted for the DR+4 motif (p2206Δ and p748Δ). The proximal part of the *ABCD2* promoter in rat, mouse, and human is strikingly conserved (Fourcade et al., 2001), suggesting that the results obtained in rat might be fully applicable to human.

T_3 Induces *ABCD2* Expression in the Liver. To determine whether our in vitro findings have a physiological relevance, we studied the in vivo effects of T_3 on *ABCD2* expression in the liver, a major target of T_3 (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 T_3 (Hartl and Just, 1987). A preliminary study carried out in adrenalectomized and castrated rats revealed that a T_4 treatment [12.5 μg/100 g of b.wt./day] for 3 days was sufficient to induce the expression of the *ABCD2* and *ABCD3*

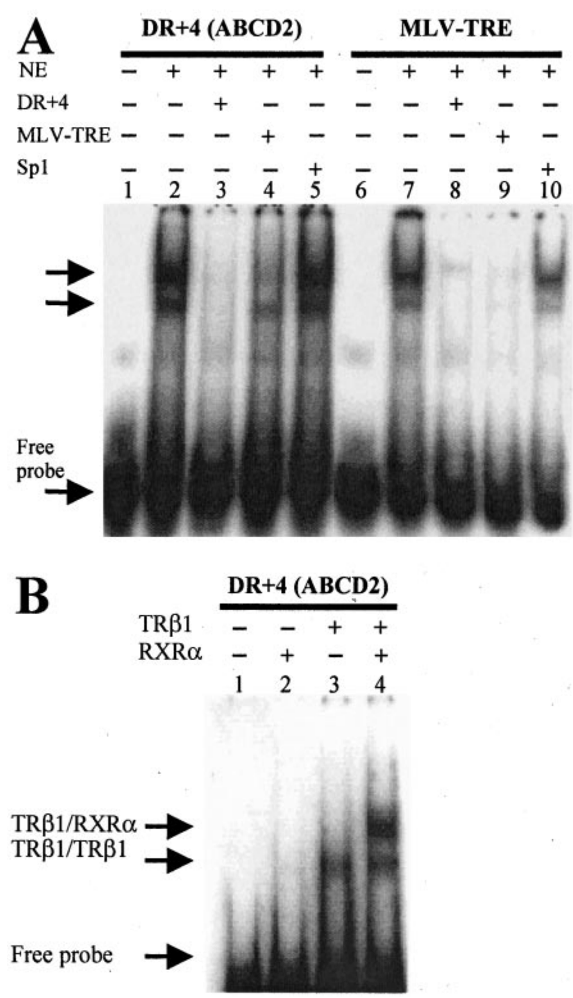

Fig. 1. The DR+4 motif binds RXRα and TRβ1. EMSA with nuclear extracts (NE) from rat liver (A) or in vitro translated proteins (B). A, each reaction contained 20 μg of nuclear extracts and labeled probe DR+4 (lanes 1 to 5) or MMLV-TRE (lanes 6 to 10). Competition experiments were performed with a 50-fold molar excess of unlabeled probe DR+4 (lanes 3 and 8), MMLV-TRE (lanes 4 and 9), or Sp1 (lanes 5 and 10). Specific bands are marked by arrows. B, reticulocyte lysate (lane 1), RXRα (lanes 2 and 4), or TRβ1 (lanes 3 and 4) was incubated with a labeled DR+4 probe.

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 *ABCD2* and *ABCD3* expression in the brain did not seem to be sensitive to T_4, we treated normal rats by substituting T_4 by T_3 (the most metabolically active hormone) and increasing either the duration (7 days) or the dose (100 μg/100 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-μg 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).

T_3 Does Not Induce *ABCD2* and *ABCD3* Expression in *TRβ−/−* Mice. To confirm the involvement of TR in the T_3 induction of *ABCD2* observed in the liver, we treated wild-type and *TRβ−/−* 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 TRβ (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 *TRβ−/−* 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* (×2.0) and *ABCD3* (×2.8) occurred in the liver of wild-type mice as expected but not in the *TRβ−/−* mice. In the animals not injected with T_3, the levels of *ABCD2* and *ABCD3* were higher in the *TRβ−/−* mice than in the wild-type mice (Fig. 4), as a result of the differences in the serum levels of T_3 and T4 through pleiotropic effects of the thyroid hormones.

***ABCD2* and *ABCD3* Are Up-Regulated by T_3 in CG4 Oligodendrocytes but not in Astrocytes.** We observed no

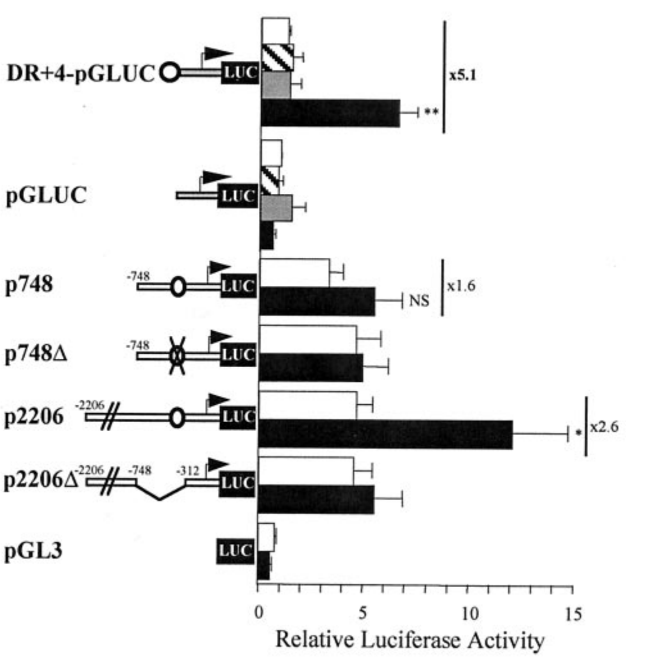

Fig. 2. T_3 responsiveness is mediated by the DR+4 motif. COS-7 cells were transfected with pGLUC or DR+4-pGLUC and with p748, p748Δ, p2206, or p2206Δ, which contain rat *ABCD2* promoter fragments deleted or not for the DR+4 motif. The cells were either treated with 50 nm T_3 for 48 h (▧) or cotransfected 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.

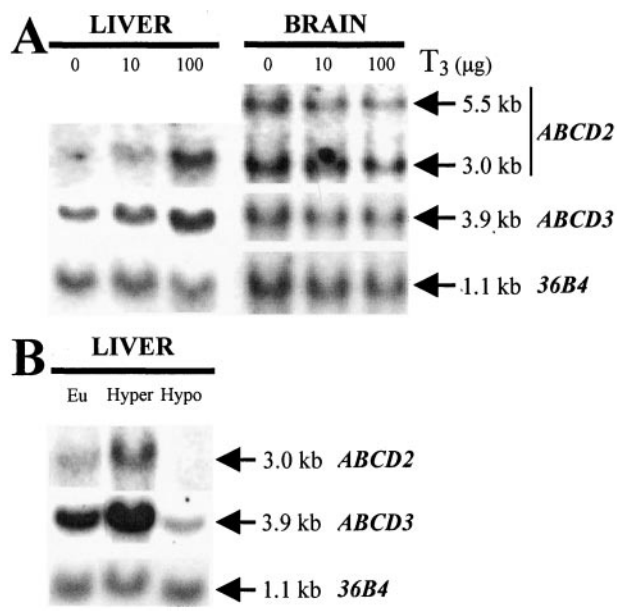

Fig. 3. *ABCD2* and *ABCD3* expression is regulated by T_3 in the liver. A, Northern blot analysis of *ABCD2* and ABCD3 mRNA in the liver and brain of rats untreated (0) or treated with 10 μg of T_3/100 g of b.wt./day for 7 days (10) or 100 μg of T_3/100 g of b.wt./day for 3 days (100). B, thyroidectomized (Hypo) rats were compared with control (Eu) and 10 μg of T_3/100 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.

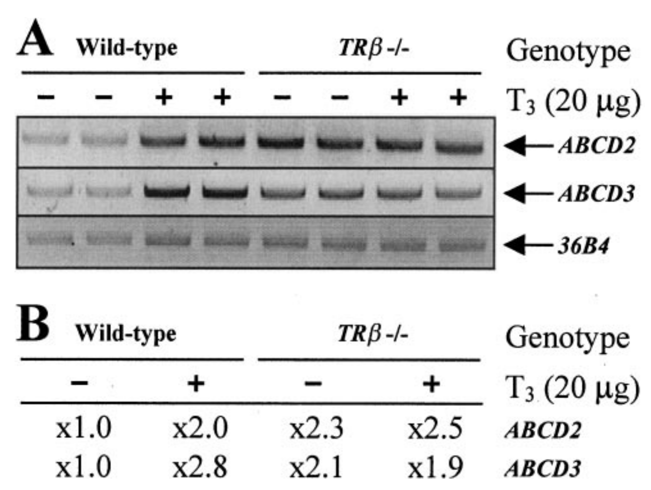

Fig. 4. TRβ is required for T_3 induction of *ABCD2* and *ABCD3* in the liver. Semiquantitative RT-PCR of *ABCD2* and ABCD3 mRNA in the liver of wild-type and *TRβ−/−* mice after a daily injection of 20 μg of T_3 i.p. for 3 days. Total RNA was extracted from three mice for each treatment and pooled before 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 T_3 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 T_3 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 T_3 (Fig. 5A). When CG4 cells were exposed to 100 nm T_3 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 T_3 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 T_3 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 T_3 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.

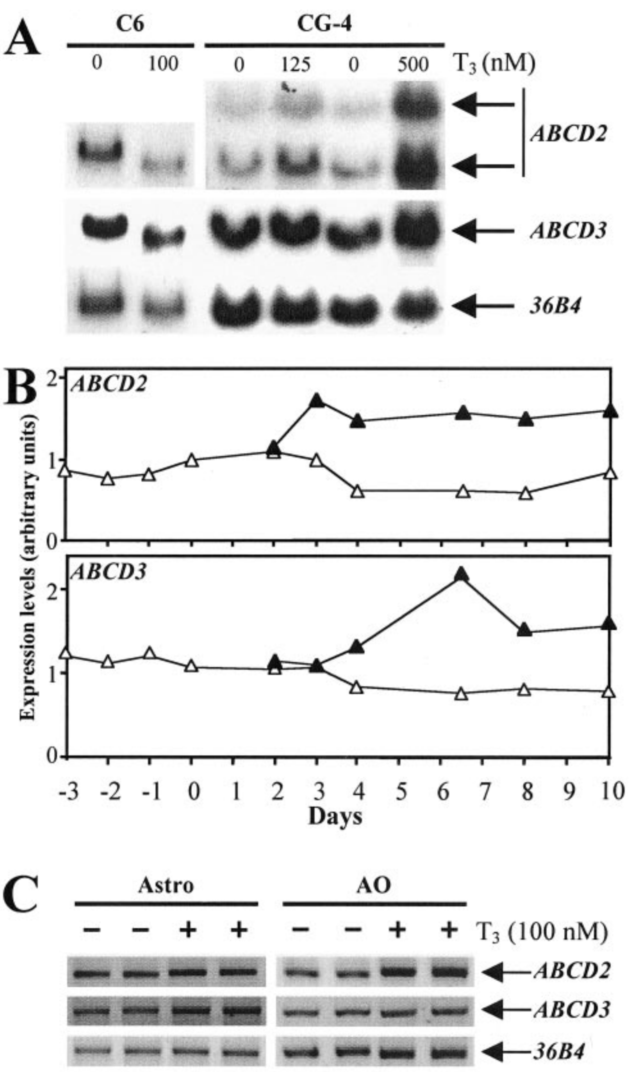

Fig. 5. T_3 induces *ABCD2* and *ABCD3* expression in CG4 oligodendrocytes. A, Northern blot analysis of ABCD2 and ABCD3 mRNA from CG4 or C6 cells after treatment with different doses of T_3 for 3 days. The 5.5-kb ABCD2 mRNA was not detected in C6 cells. B, semiquantitative PCR analysis of ABCD2 and ABCD3 mRNA from CG4 cells treated with 100 nM T_3 for different times (2 to 10 days). Days −3 and 0 correspond to the start of differentiation and T_3 treatment, respectively. The expression level is 1.0 at day 0 for both genes. △, control; ▲, T_3-treated CG4 cells. Assays were conducted in duplicate, and data represent the means of two independent experiments. C, semiquantitative RT-PCR analysis of ABCD2 and ABCD3 mRNA from primary astrocytes (Astro) and from mixed primary cultures of astrocytes and oligodendrocytes (AO) treated with 100 nM T_3 for 3 days.

T_3 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 T_3 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 T_3 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 T_3 dose-dependent increase in the amount of ABCD2 mRNA in cells T_3-treated for 2 days (Fig. 6A). However, the *ABCD2* induction seemed to be transitory, because after treatment with 100 nm T_3 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 T_3 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 T_3 treatment. With a delay of a few days, the transitory effect of T_3 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 T_3 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 T_3 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 T_3 up-regulation of the reporter luciferase gene in transient cell transfection experiments. The T_3 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 T_3 (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 (Troffer-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 T_3 induction of *ABCD2*.

The effects of T_3 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 T_3 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 T_3-sensitive redundant genes. The doses of T_3 used in our experiments may seem unacceptable for humans because of potential side effects. However, the possibility of selectively targeting the TRβ receptor with T_3 analogs, such as GC-1, might reduce some deleterious effects of thyroid hormones (Baxter et al., 2001).

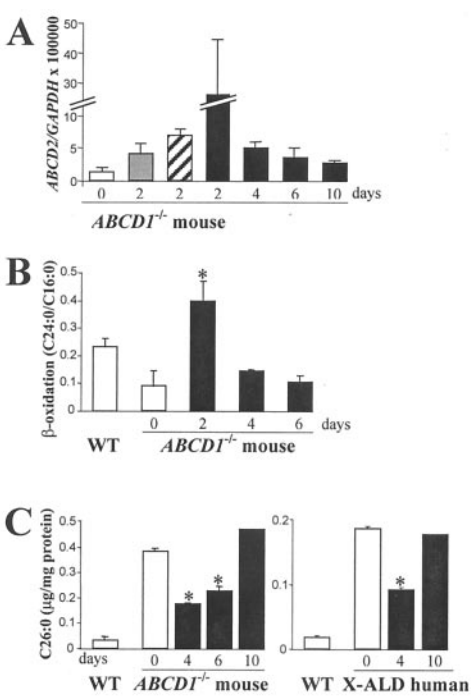

Fig. 6. T_3 affects transitorily *ABCD2* expression and VLCFA β-oxidation in *ABCD1*-deficient fibroblasts. Cells were cultured in the absence (□) or presence of 10 (▤), 50 (▨), and 100 nM T_3 (■) for different times (2 to 10 days). A, the number of ABCD2 mRNA copies was evaluated by quantitative PCR in *ABCD1*-deficient mouse fibroblasts ($n = 3$) and was referred to the number of GAPDH mRNA copies. The level of *ABCD2* expression in untreated fibroblasts remained constant during culture (data not shown). B, the C24:0 β-oxidation/C16:0 β-oxidation ratio was determined in WT ($n = 5$) and *ABCD1*-deficient mouse fibroblasts ($n = 5$ for untreated cells and $n = 2$ for T_3-treated cells). C, the C26:0 levels were determined in WT and *ABCD1*-deficient mouse fibroblasts ($n = 2$ except for the 10-day treatment: $n = 1$) and in WT and X-ALD human fibroblasts ($n = 2$). Assays were conducted in duplicate, and data represent the mean ± S.E. Statistically significant differences (Student's t test) from controls are indicated by *, $P < 0.01$.

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. T_3 concentration in the brain reaches a peak approximately 2 weeks after birth, which correlates with an increase in TRβ1 expression and in the activity of type II iodothyronine deiodinase, the enzyme responsible for the conversion of T_4 to T_3 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 T_3 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 T_3-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 T_3 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 T_3 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 T_3-independent mechanism, which may explain why *ABCD2* expression levels do not vary in the brain of T_3-treated or hypothyroid rats. Indeed, Strait et al. (1997) observed a rapid increase in *myelin-basic-protein* expression upon T_3 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 T_3, indicating that the terminal expression levels were maintained independently of T_3 in differentiated oligodendrocytes. In contrast, we observed no increase in the ABCD2 mRNA levels in the absence of T_3 during the culture of differentiated CG4 oligodendrocytes. Our findings do not support the hypothesis that the high levels of *ABCD2* expression in the adult brain may be independent of T_3. On the other hand, although *ABCD2* is expressed in astrocytes as well as in oligodendrocytes of adult mouse brain (Troffer-Charlier et al., 1998), we observed T_3 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 T_3-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 multipotential stem cells, which are capable of de novo generation of neurons and glia. Interestingly, when exposed to T_3, the multipotential 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 T_3 up-regulation of *ABCD3* expression in rat liver as well as in CG-4 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 (Gärtner et al., 1998). The T_3 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 T_3 regulation levels are known to play an important role in maintaining the intracerebral T_3 content that is relatively constant during changes in thyroid status. Thus, increased T_3 concentrations result in depleted TR and type II iodothyronine deiodinase levels and in enhanced activities of type I and III deiodinases, the two enzymes that inactivate T_3 (Ortiz-Caro et al., 1987; Kohrle, 1999). Similar T_3 regulation may occur in fibroblasts and may explain the transitory effects of T_3 treatment on VLCFA β-oxidation and *ABCD2* expression. Such regulation might be different in oligodendrocytes, because the induction of *ABCD2* was maintained in T_3-treated CG4 cells for 10 days.

In conclusion, we demonstrated that *ABCD2* is a T_3-responsive gene both in rodent and human cells through a classic TRE and that T_3 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 T_3, although *ABCD2* up-regulation was not found in the whole brain, unlike the liver, of the rat upon T_3 treatment. Further studies using *ABCD1*-deficient mice will be required to evaluate the efficacy of T_3 (or T_3 analogs) treatment on ABCD2 mRNA and ALDRP protein levels and VLCFA content in the brain.

Acknowledgments

We thank Martina Krammer for the excellent technical assistance, Hervé Goudonnet for providing thyroidectomized rats, Frederic Flamant for providing *TRβ−/−* mice, and Hugo Moser, Sonja Forss-Petter, and Pamela Talalay for critical reading of the manuscript.

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