Studies on the Transcriptional Regulation of Cholesterol 24-Hydroxylase (CYP46A1)

MARKED INSENSITIVITY TOWARD DIFFERENT REGULATORY AXES*

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Mammalian CNS contains a disproportionately large and remarkably stable pool of cholesterol. Despite an efficient recycling there is some requirement for elimination of brain cholesterol. Conversion of cholesterol into 24S-hydroxycholesterol by the cholesterol 24-hydroxylase (CYP46A1) is the quantitatively most important mechanism. Based on the protein expression and plasma levels of 24S-hydroxycholesterol, CYP46A1 activity appears to be highly stable in adults. Here we have made a structural and functional characterization of the promoter of the human CYP46A1 gene. No canonical TATA or CAAT boxes were found in the promoter region. Moreover this region had a high GC content, a feature often found in genes considered to have a largely housekeeping function. A broad spectrum of regulatory axes using a variety of promoter constructs did not result in a significant transcriptional regulation. Oxidative stress caused a significant increase in transcriptional activity. The possibility of a substrate-dependent transcriptional regulation was explored in vivo in a sterol-deficient mouse model (Dhcr24 null) in which almost all cholesterol had been replaced with desmosterol, which is not a substrate for CYP46A1. Compared with heterozygous littermates there was no statistically significant difference in the mRNA levels of Cyp46a1. During the first 2 weeks of life in the wild-type mouse, however, a significant increase of Cyp46a1 mRNA levels was found, in parallel with an increase in 24S-hydroxycholesterol level and a reduction of cholesterol synthesis. The failure to demonstrate a significant transcriptional regulation under most conditions is discussed in relation to the turnover of brain and neuronal cholesterol.

Although the brain is the most cholesterol-rich organ in the body, relatively little is known about the mechanisms by which it maintains steady-state cholesterol levels (1, 2). This is in marked contrast to the situation in virtually every other tissue or organ. One finding that has been consistently confirmed is that, due to the efficiency of the blood-brain barrier, the brain is unable to take up cholesterol from the circulation and relies on de novo synthesis to meet its substantial cholesterol requirements. However, the rate of cholesterol synthesis in the adult brain is very low, and the bulk of brain cholesterol has a half-life that is at least 100 times longer than that of cholesterol in most other organs (3).

One consequence of this “uncoupling” of brain and whole body cholesterol homeostasis has been the evolution of specific mechanisms for maintenance of cerebral cholesterol levels. Two mechanisms for removal of brain cholesterol are currently recognized (1). The first is analogous to classic “reverse cholesterol transport” and is mediated by a flux of cholesterol present in apolipoprotein E containing lipoproteins through cerebrospinal fluid into the circulation (4, 5). In adults, this mechanism is believed to be responsible for elimination of 1–2 mg of cholesterol per 24 h. The details of this particular mechanism for sterol transport are, however, not known. More recently we described a second mechanism for the elimination of brain cholesterol conversion into 24S-hydroxycholesterol (24S-OH), a more polar sterol that can traverse the blood-brain barrier, enter the circulation, and travel to the liver (6, 7). In the liver it may be converted into bile acids or excreted as a sulfated and/or glucuronidated metabolite (8). This efflux of 24S-OH is believed to be the main mechanism by which the brain facilitates the removal of cholesterol, and, in humans, accounts for the removal of 5–7 mg of cholesterol each day (6). The cytochrome P-450 species responsible for 24S-hydroxylation of cholesterol, cholesterol 24-hydroxylase (CYP46A1), has been characterized at the molecular level (9, 10). In normal brain it has been demonstrated to be almost exclusively located in central nervous system neurons (9, 11, 12).

In addition to these mechanisms there is some experimental evidence for a third mechanism for removal of cholesterol from the brain (13). It was recently shown that a disruption of the Cyp46a1 gene in mice leads to a reduction in the net sterol flux from the brain by ~65%, with a parallel decrease in the rate of brain cholesterol synthesis by ~50%, without alteration of the total brain cholesterol levels (13, 14). These mice did not have an obvious phenotype and were biochemically indistinguishable from wild-type littermates.

One possible mechanism for elimination of brain cholesterol involves conversion of cholesterol into 27-hydroxycholesterol by sterol 27-hydroxylase, which is similar to 24S-OH in that it is capable of traversing lipophilic membranes (15). Because we have been unable to demonstrate a net flux of 27-hydroxycholesterol from the human brain into the circulation (7), this mechanism is probably less important from a quantitative point of view. However, because 27-hydroxycholesterol may be metabolized to more polar products in the brain (16), we cannot yet
elaborate the possibility that there may be a net flux of a metabolite of 27-hydroxysterol from the brain.

The human CYP46A1 enzyme has been characterized in some detail with respect to substrate specificity and enzymatic properties (10). At present, however, there are very few data on the regulation of this enzyme. Studies performed when the CYP46A1 was originally cloned demonstrated a significant increase in the expression of the CYP46A1 protein in the brain shortly after birth (9). Regulated spatiotemporal expression was observed in the Cyp46a1 (−/−) mouse, which incorporated a β-galactosidase gene under the control of the endogenous transcriptional regulatory sequences in the Cyp46a1 gene (14). In accordance with these reports, the levels of 24S-hydroxysterol in the circulation are markedly increased during the first months of human life (17). However, at the age of ~1 year in humans (and 2–4 weeks in mouse) expression levels of the enzyme reach a steady state, which is maintained during adulthood (9). In accordance with this, the levels of 24S-hydroxysterol are remarkably stable during human adulthood (7, 18). There is, however, some evidence that the expression of CYP46A1 may be affected in the brain of patients with neurodegenerative conditions such as Alzheimer disease, where the gene displays an ectopic expression pattern. Although predominately confined to neurons in the healthy brain, CYP46A1 immunoreactivity has been detected in astrocytes of brain tissues from Alzheimer disease patients (11, 12). With the exception of some limited studies exploring the liver-X-receptor axis (19), no data have been presented on the regulation of CYP46A1 by nuclear receptors or hormones.

The present study was initiated to define the basal characteristics of the human promotor of CYP46A1 and to investigate if different endogenous or exogenous compounds are able to significantly alter the transcriptional activity. Because the activity of this enzyme is likely to be of importance for cerebral cholesterol homeostasis, it was regarded to be of interest to study if there is a change in the expression of Cyp46a1 during the neonatal phase, in parallel with a rapid expansion of the cholesterol pool in the brain and also in a mouse model with markedly reduced levels of brain cholesterol.

### EXPERIMENTAL PROCEDURES

**Materials**—The GeneAmp High Fidelity PCR System is a product of Applied Biosystems (Foster City, CA). A TA Cloning kit was purchased from Invitrogen. TransFast™ Transfection Reagent, Dual-Glo™ Luciferase Assay System, pGL3-Basic vector, and phRL-TK vector were from Promega (Madison, WI). Human Brain RACE-ready cDNA was from Ambion (Cambridge, UK). SH-SY5Y, HepG2, and HEK293 cell lines were from ATCC (Manassas, VA). Oligonucleotides for PCR and sequence analyses were obtained from Cybergene (Stockholm). All other chemicals were of the highest grade commercially available.

**Mouse with a Cholesterol Deficiency**—Mice with an inactive Dicer24 gene were generated and maintained as described in detail before (20). Following the suckling period, the animals were provided with a commercial rodent diet (Harlan Teklad Rat/Mouse Diet) ad libitum.

**Sterol Analysis**—Sterol concentrations were determined in the brain of 3-month-old mice as previously described (20, 21). Briefly, the brain tissue was snap-frozen in liquid nitrogen and pulverized mechanically prior to extraction with chloroform/methanol (2:1, v/v). Butyldihydropolyoxylone (0.5 μg/mg tissue) was added at the time of extraction. To detect both free and esterified sterols a hydrolysis step was included in all cases. Cholesterol and desmosterol were analyzed by isotope dilution mass spectrometry using [2H6]cholesterol as internal standard, whereas the concentration of lathosterol was analyzed by use of [2H4]lathosterol (25). The following ions were monitored: [2H6]cholesterol, m/z 464; cholesterol, m/z 458; and desmosterol, m/z 456. Oestrostereol in brain were determined by isotope dilution-mass spectrometry, essentially as described previously (21).

**Quantification of mRNA Levels by Real-time PCR**—Total RNA was prepared from the brains of the wild-type and knock-out mice with the Quick Prep™ Total RNA Extraction kit (Amersham Biosciences), DNase-treated using an RNase Protect Mini Kit (Qiagen GmbH, Hilden, Germany), and quality-checked on a 2.2 m formaldehyde/ MOPS 1.2% agarose gel. A maximum of 10 μg was reverse transcribed into cDNA with the SuperScript III RTase (Invitrogen).

PCR amplification was performed in triplicate using 5 μl of a 1:10 dilution of each cDNA preparation (25 μl/well) according to the manufacturer’s recommendations. The primers used for the real-time PCR analysis are shown in Table 1. The specificity of the reaction was confirmed by sequencing the PCR product. A final concentration of 250 nM of probes and 900 nM of primers was used for all fluorescence probe assays, whereas in SYBR Green assays a final concentration of 200 nM of primers was used. For all SYBR Green assays a melting curve was obtained to confirm that the signal corresponded to a unique amplification. The relative amount of target mRNA was quantified by singleplex real-time RT-PCR analysis using an ABI PRISM 7000 Sequence Detection System, with hypoxanthine guanine phosphoribosyltransferase as the internal standard in all cases. Relative mRNA levels were calculated according to the comparative threshold cycle (ΔΔCT) method according to the manufacturer’s instructions with all comparisons performed relative to the situation in the adult brain.

**Comparative Genomic Analysis**—Genomic regions of the human, chimpanzee, rhesus macaque, dog, cow, mouse, and rat cholesterol 24-hydroxylase genes were analyzed by orthologous sequence comparison to localize conserved non-coding regions, i.e. potential regulatory regions. The default conservation settings of the MULAN and VISTA software (available online at www.dcode.org and gsd.lbl.gov/vista/index.shtml, respectively) were used to examine the regions of interest (22). Selected sequences were further analyzed for the presence of putative regulatory elements (23) or promoter modules (24) using regulatory VISTA (vVISTA, URL as above) and Frame Worker (www.genomatix.de), respectively.

### Table 1

| Gene | Forward (5′→3′) | Reverse (5′→3′) | Probe |
|------|----------------|----------------|-------|
| Apolipoprotein E | GCAGGGCAGGCTCTTCCA | CCACTGCGGATGCTGT | SyBR Green |
| SREBP-1c | TTGTACCTGCGATTCAG | GCAGATTGTTATGTTGATTAC | TGGTGGGGCCCTCTGTCACCTTCC |
| CYP46 | CCCGCAACTAAACGATGCTTC | GTCAAGGGATGCGATGCA | TGGTCGTGGCCAGTACCTTCAG |
| HMG-CoA reductase | CTTGTGTCATAGTTCCTCCTGC | TCCAATCCTCTCCCTGGC | TGGTCGTGGCCAGTACCTTCAG |
| HMG-CoA synthase | GTTGAAAGGGCCTCCTCGAGTG | ATAGTCAGGGGATCATAAACAA | TGGTCGTGGCCAGTACCTTCAG |
| HPRT | HPRT | HPRT | HPRT |

* 5′-3′ Blue 6-carboxyfluorescein.
* 3′-4′(−3′)-dimethylaminophenylazo)benzoic acid.  
* Tetramethylrhodamine.  
* Hypoxanthine phosphoribosyltransferase.

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### TABLE 2
Sequences of oligonucleotide primers used in this study

The underlined sequence represents the position of the restriction site used in subsequent cloning.

| Name      | Sequence (5′ to 3′) | Position | Usage        |
|-----------|--------------------|----------|--------------|
| F2        | gcatgcttcacaggtggaaac | −2713    | Cloning      |
| R1        | ggtttgtagcgcgctg    | +100     | Cloning, RACE|
| R15       | cctccaaacatggtgctg  | +661     | RACE         |
| R14       | accgactagcttcggcag   | +278     | RACE         |
| Rev       | gcagcaagttgagcagcag  | +706     | RACE         |
| PA-F2a    | tctcagctcctcagcaagatggaaac | −2711 | pGL3        |
| PA-1      | atggccttgtcttgtcttgtctg | −2102   | pGL3        |
| PA-F11    | ctgcagctgctcatttttcgcag | −988    | pGL3        |
| PA-F12    | tctcagctgctcatttttcgcag | −811   | pGL3        |
| PA-R      | ttcagctgcctgcagcttttcgcag | −65    | pGL3        |
| EXP-F     | tctcagctgcctgcagcttttcgcag | +502   | Reverse transcription-PCR |
| EXP-R     | tctcagctgcctgcagcttttcgcag | +915   | Reverse transcription-PCR |

*Position indicates the distance of the 5′-end of the indicated primer from the translation start site (where +1 refers A of ATG).

The location of the 5′-end of the primer.

Analysis of Transcriptional Start Site—Human brain FirstChoice RACE-ready cDNA (Ambion), which is a library of brain cDNA with complete 5′-ends, was used for amplification of the 5′-end of CYP46 cDNA in two separate RACE experiments. Each experiment used a different combination of primer sets as per the manufacturer’s instructions. One RACE experiment was done with the outer primer supplied by the manufacturer and primer R15 (for primers sequences, see Table 2), with a subsequent nested PCR using the kit inner primer and primer R11. The second experiment was conducted with the outer primer and primer R14, followed by the inner primer and reverse primer. A typical PCR reaction consisted of denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at (94 °C, 15 s), annealing (58 °C, 30 s), and extension (72 °C, 1 min), with a final extension (72 °C, 7 min) using the reaction buffer. The PCR products were resolved by agarose gel electrophoresis and recovered using a GenElute Agarose Spin Column. The DNA fragments were cloned into pCR 2.1 by T/A cloning. Nucleotide sequence of insert DNA was determined using the primer EXP-F and EXP-R. Human fetal and adult brain total RNA were included as a positive control. Reaction conditions were as follows: 52 °C for 30 min, 94 °C for 2 min, followed by 35 cycles of denaturation at (94 °C, 30 s), annealing (53.8 °C, 1 min), and extension (72 °C, 1 min), with a final extension (72 °C, 10 min).

Cell Culture—SH-SYSY (a human neuroblastoma cell line derived from metastatic bone marrow) and HEK293 cells (transformed human embryonic kidney cells) were routinely cultured in minimal essential medium (Sigma) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 × non-essential amino acid mix (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. HepG2 cells (human hepatocellular carcinoma) were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were grown at 37 °C in a humidified atmosphere of 5% CO₂.

Construction of Luciferase Vectors—Four fragments of the 5′-upstream region of the human CYP46 gene were amplified with upstream primers (see Table 2) containing an overhanging XhoI site (PA-R) using the plasmid p10–12 as a template. PCR conditions were essentially as described above. The amplified fragments were subcloned into pCR 2.1, sequence-verified, and recloned into the XhoI and HindIII site of pGL3-basic vector (Promega). These plasmids were named pG-2700 (5′-end, −2711 bp), pG-2100 (−2102 bp), pG-1000 (−988 bp), and pG-500 (−510 bp), according to the positions 5′-end from the translation start site. pG-1300 (−1280 bp) was created by digestion of the pG-2700 with KpnI, followed by self-ligation of the fragment containing vector, because pG-2700 has two KpnI sites at −1280 bp and upstream of the introduced XhoI site in the multiple cloning site of the vector. Similarly, digestion and self-ligation for pG-1000 with Smal produced pG-650 (−659 bp). MuI and Sacl digestion and self-ligation for pG-500 were used to create pG-400 (−410 bp) and pG-250 (−258 bp), respectively. All reporter constructs shared a common 3′-end 65 bp upstream of translation start site. A schematic drawing of the reporter constructs is shown in Fig. 7.

Transfection and Luciferase Assay—Plasmids for transfection were prepared using a plasmid purification kit (Qiagen). Luciferase assay was performed using a Dual-Glo luciferase assay system (Promega) with pRL-TK vector as an internal control for normalization of transfection efficiency. Transfection experiments were performed in 96-well culture plates, and luciferase activities were measured using a Victor 1420 Multilabel counter (Wallac). Briefly, 1 day before transfection, recipient cells were seeded into 96-well plates at a density of 2–6 × 10⁴ cells/well. After removal of culture medium, the cells were cotransfected with 100 ng of reporter construct DNA and 20 ng of control plasmid (pRL-TK) per well using TransFast reagent according to the manufacturer’s recommendations. Three hours later, 200 μl of medium (or medium containing a test agent) was added to each well, and the cells were incubated for an additional 48 h. Luciferase activities were measured by the Dual-Glo luciferase assay system according to the manufacturer’s instructions. Firefly luciferase activity was normalized to Renilla luciferase activity. In the experiments testing effects of oxidative stress, the cells were transfected with use of 15 μl of Lipofectin (Invitrogen), 2.5 μg of pGL3 Enhancer plasmid, and 1 μg of pHL-TK vector. The cells were incubated in serum-free media at 37 °C for 3–5 h. The following agents were tested: 22R-hydroxycholesterol (1 and 10 μg/ml), 25-hydroxycholesterol (1 and 10 μg/ml), 24S-hydroxycholesterol (1 and 10 μg/ml), cholesterol (10 and 50 μg/ml), pravastatin (1 μg/ml), testosterone (100 nM), dehydroepiandrosterone (100 nM), estradiol (100 nM), WY-14643 (50 nM), growth hormone (500 ng/ml), cortisol (1.4 μM), triiodothyronine (2.5...
μM), insulin (0.1 IU/ml), rifampicin (12.4 nM), progesterone (0.1 μM), dibutyryl cyclic AMP (0.2 mM), lithocholic acid (25 μM), interleukin-6 (50 pg/ml), chenodeoxycholic acid (25 μM), and tert-butylhydroperoxide (0.05–5 μM). Final concentrations are shown in parentheses after the compound.

RESULTS

Changes in Brain Sterol Synthesis and Elimination during the Early Life of a Mouse—The ontogeny of Cyp46a1 mRNA expression and the corresponding concentration of the enzymatic product, 245-hydroxycholesterol, in mouse brain are shown in Fig. 1. The mRNA levels
increased by a factor of ~2 (Fig. 1A), whereas the levels of 24S-hydroxycholesterol increased by a factor of ~6 (Fig. 1B) during the first 3 weeks of life. The production of 24S-hydroxycholesterol was well correlated to the transcriptional activity of the Cyp46a1 gene (Fig. 1C). As Fig. 1 (D and E) illustrates, the mRNA levels of the Hmg-CoA synthase and Hmg-CoA reductase both decreased markedly in parallel with the increase in Cyp46a1 mRNA levels. As Fig. 1F shows this decrease is matched by a corresponding decrease in the lathosterol/cholesterol ratio, an index of tissue cholesterol biosynthesis, and a reciprocal increase in the 24S-hydroxycholesterol/cholesterol ratio. Moreover, both Hmg-CoA reductase and Hmg-CoA synthase genes were strongly and significantly inversely correlated to both 24S-hydroxycholesterol levels and Cyp46a1 mRNA levels (results not shown).

Apparent Lack of Transcriptional Regulation of Cyp46a1 in Dhcr24 (−/−) Mice—The observed increase in the cerebral expression of Cyp46a1 mRNA during the first 3 weeks of mouse life may be a direct consequence of the increasing accumulation of cholesterol or due to some other factor. To study this, we used Dhcr24 (−/−) mice with a cholesterol deficiency (20). Brain cholesterol, desmosterol, and 24S- and 27-hydroxycholesterol content were determined in the brain of Dhcr24 (−/−) mice and their wild-type littermates (Fig. 2, A and B). The total sterol concentration in the brain of the knock-out mice was reduced by ~25%, whereas the levels of 24S-hydroxycholesterol were reduced by >98%. It should be pointed out that desmosterol cannot be hydroxylated in the 24-position (Fig. 2c). Levels of 27-hydroxycholesterol in the Dhcr24 (−/−) mice were below the detection limit.

In a pilot study we compared expression of mRNA levels of in Dhcr24 (−/−) mice and heterozygous littermates (we have previously shown that heterozygous animals are phenotypically and biochemically indistinguishable from wild-type animals and are suitable as controls in these experiments). We used real-time PCR to measure mRNA levels of Hmg-CoA reductase, Hmg-CoA synthase, Cyp46a1, Srebp-1, Srebp-2, and Apoe in 4 Dhcr24 (−/−) mice and in 6 heterozygous littermates (mean age in each group was 10 weeks). As shown in Fig. 3, Hmg-CoA reductase and Cyp46a1 expression levels were similar in Dhcr24 (−/−) mice and heterozygous littermates. However, both Srebp-1 and Apoe expression levels were significantly higher in the Dhcr24 (−/−) mice (p < 0.001 and p < 0.05, respectively). An increased expression of Srebp-1 mRNA is an expected response to the profound alterations in brain sterols of Dhcr24 (−/−) mice. Surprisingly, there was no significant alteration in Srebp-2 expression.

Cloning of the 5′-Upstream Region of the Human CYP46A1 Gene—Using sequence data deposited in GenBank™, a PCR primer set was designed to amplify a region between −2713 and +100 nucleotides relative to the translational start site of CYP46A1 (Table 2). Due to the
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FIGURE 4. Transcriptional start site of human CYP46A1. Rapid amplification of cDNA ends was performed using FirstChoice RACE-ready cDNA and two unique sets of primers (see "Experimental Procedures"). Nucleotides that are candidate transcriptional start sites are shown as white characters and two unique sets of primers (see "Experimental Procedures"). Nucleotides that are candidate transcriptional start sites are shown as white characters in filled boxes. The RACE analysis revealed numerous potential transcriptional start sites (open boxes), with only a small overlap with previous deposits in the EST data base (filled boxes).

extremely high GC content (~80%) of the proximal 300 bp of the 5′-untranslated region, cloning of this region was non-trivial, and it was necessary to empirically determine the amplification conditions. The DNA fragment was subcloned into a T/A-vector (pCR2.1) and five independent clones were sequenced to confirm the integrity of the PCR product. Four of five clones contained two to five nucleotide deviations from the registered sequence (Fig. 4). This clone (p10–12) was, therefore, used for all subsequent analyses, because a difference of the number of Ts would not be expected to be critical for binding of transcription factors.

Attempts to Define the Transcriptional Start Site—Examination of the proximal promoter region of human CYP46A1 did not reveal any canonical TATA or CCAAT boxes, which is similar to the promoters of some other genes coding for cytochrome P450 enzymes, e.g. steroid 27-hydroxylase (CYP27A1) or steroid 12α-hydroxylase (CYP8A1). The GC composition of the proximal 1 kb of the human CYP46A1 gene was 68%, which is a common finding in genes considered to have a largely housekeeping function. Examination of the EST data bases for transcripts corresponding to human CYP46A1 revealed that the 5′ boundaries of the available cDNA clones cluster over ~150 bp region within the CYP46A1 5′-untranslated region (Fig. 4). Such a clustering of EST ends may be indicative of the presence of transcriptional initiation sites in this particular region.

Due to the high GC content in the CYP46A1 upstream region, determination of the actual transcription start site is problematic and canonical primer extension and S1 nuclease mapping methods may produce obscure results. Therefore, we used FirstChoice RACE-ready cDNA (Ambion). This template cDNA is prepared by using the capped endspecific RNA ligation method and is therefore expected to include intact 5′-transcript ends. RACE reactions were performed with two different sets of two gene specific primers for the first and the nested PCR steps, using adult human brain FirstChoice RACE-ready cDNA as a template. Both RACE reactions produced multiple products, and sequencing of these cDNA fragments identified numerous novel potential transcriptional start sites, with only one overlap with the previously reported EST sequences (Fig. 4). Interestingly, the majority (91%) of these sites were clustered within a 60-bp region located 73 bp upstream of the translational start site.

Computational Analysis of CYP46—The 5′-upstream sequence of CYP46A1 was examined using a combination of different computational methods and manual inspection. Initially the genomic sequences from all available orthologous cholesterol 24-hydroxylase genes were examined using the Vista Genome Browser. The overall alignment of genomic DNA from human, rat, and mouse is shown in Fig. 5 (A and B). The proximal regions were highly conserved among the species investigated. Detailed examination of this region was performed using Orthologous Sequence Comparison via the ConSite (mordor.cgb.ki.se/cgi-bin/CONSITE/consite) and rVISTA interfaces. Because CYP46A1 is a gene involved in the maintenance of brain cholesterol homeostasis, attention was primarily focused on the binding sites for transcription factors known to be involved in lipid homeostasis. Several individual candidate sites were identified, including the Krox-24 (also known as NGFI-A, Zif268, Egr-1, Tis8, and Zenk) and GC-box factors SPI1/GC (SPIF). However, as responsive elements for these factors are often abundant in GC-rich regions, it is impossible to evaluate the functional significance of these sites at present. With the exception of possible putative glucocorticoid element and PPARγ sites, no canonical sites for any of the major families of nuclear hormone receptors were identified. The potential transcription factor binding sites present in the human gene are depicted schematically in Fig. 5C. However, as transcription factors often associate into complexes to correctly activate the transcription of a given gene, orthologous sequences were examined for the presence of distance-correlated transcription factor binding sites using Frame-Worker. One such complex composed of three SPI1 binding sites and one glucocorticoid response element was identified between positions −450 and −300 and was conserved across multiple species. A schematic view of the human and mouse sequences is presented in Fig. 5B. The module was rated a Frame Worker score of 1.0, indicating that it is unlikely to have arisen by chance. However subsequent examination of the putative glucocorticoid element present in this module failed to demonstrate its response to glucocorticoids (compare with below).

Basal Activity of the CYP46A1 Promoter—To discover a suitable cell system to investigate the regulation of CYP46A1, several cell systems (both cell lines and primary cultures) were analyzed for the expression of CYP46A1 mRNA and production of 24S-hydroxycholesterol. Of the different cell systems tested, SH-SYSY was found to be most suitable. However, although these cells expressed detectable levels of CYP46A1 (Fig. 6) RNA, there was no significant production of 24S-hydroxycholesterol (<1 ng/10⁶ cells/48 h) in them. In view of these limitations, it was only possible to study transcriptional regulation in cells transfected with CYP46A1 reporter constructs.

Basal activity of CYP46A1 promoter was analyzed in these three cell lines by transfection of reporter constructs expressing Firefly luciferase gene under the control of variable length 5′-upstream sequences of the human CYP46A1 gene. These deletion constructs were generated in the pGL3 expression vector and contained between 250 and 2700 bp
upstream to the initiating ATG (Fig. 7). The promoter activity, as estimated by a dual luciferase assay, was studied following transient transfection of HepG2, HEK293, and SH-SY5Y cell. The results are summarized in Fig. 8. In all the three cell lines maximum transcriptional activity was observed with the pGL-650 construct, whereas the highest activity was observed in SH-SY5Y cells, in complete agreement with the observed mRNA expression levels of the endogenous gene. The results indicate that the basal CYP46A1 promoter is most likely located between nucleotides $^{510}$ and $^{659}$, and that certain silencing elements may be present further upstream. Although these results also suggest that the region between $^{659}$ and $^{510}$ may harbor binding sites for neuron specific transcription factors, no such obvious sites were found during the analysis of this region.

Attempts to Uncover Transcriptional Regulatory Factors—A wide variety of different molecules targeting many different regulatory axes were tested with three different constructs: pGL-2700, pGL-1300, and pGL-650. These compounds were selected on the basis of some previously reported regulatory potential, and particular emphasis was placed on compounds known to influence cellular cholesterol homeostasis (cf. sequence comparison. Genes from species other than human and mouse were identified via BLAST and as such represent putative cholesterol 24-hydroxylases. The region immediately proximal to the initiating ATG is shown in A with each species scored by percentage for its similarity to the human sequence, which is not shown. 3 kb of the putative promoter of CYP46A1 was extracted and investigated for the presence of distance-correlated transcription factor binding sites using FrameWorker. For the sake of clarity, only the human and mouse modules are illustrated (B). 1 kb of the 5' -flanking region immediately proximal to the translational start site was examined both individually and in multispecies comparison (C). In similarity with some other cytochrome P450s cholesterol 24-hydroxylase appears to possess a TATA box-free promoter. No canonical sites were detected for factors known to be involved in lipid and cholesterol homeostasis. Numerous putative SP1F binding sites were present in the extremely GC-rich region between $^{150}$ and $^{400}$. The putative Krox-24 (boxed) and Th1-E47 binding sites are also highlighted. Bold type corresponds to a sequence present in human only, whereas underlining indicates those preserved across more than one species. The shaded region indicates the putative transcriptional start site region (see Fig. 4).
Experimental Procedures). All of these interventions have been shown to regulate genes with the appropriate response elements present.

The results of some of these analyses are summarized in Fig. 9a, illustrating that treatments well known to influence the expression of other genes involved in cholesterol homeostasis (e.g., oxysterols or statins) failed to have a significant effect on the activity of the reporter gene under the control of CYP46A1 promoter elements. With one exception (a combination of interleukins and dexamethasone, Fig. 9B), extensive screening efforts with the wide variety of pharmacological compounds spanning a variety of regulatory axes failed to identify a treatment which significantly altered the transcriptional activity of any of the CYP46A1 promoter constructs. The lack of a demonstrable direct effect of either dexamethasone or rosiglitazone indicates that the computer-predicted putative glucocorticoid element and PPARγ binding sites may be non-functional. It should be emphasized that all of the above experiments were carried out in SH-SY5Y, HepG2, and HEK293 cells, with essentially identical results were found in all cases.

In the above experiments the reporter system was exposed to the different treatments for 16 h. To exclude the possibility of transient "acute" effects the experiment shown in Fig. 9A was repeated with a series of shorter exposure times: 1, 2, and 4 h in addition to the 16 h. No time-dependent significant differences were observed (results not shown). However, when using the largest construct (pGL-2700) and 1 h of exposure, the degree of variation was such that a very rapid effect cannot be excluded.

"Experimental Procedures"). All of these interventions have been shown to regulate genes with the appropriate response elements present.

When exposing SH-SY5Y cells to the organic hydroperoxide tert-butylhydroperoxide, the promoter activity was stimulated ~2-fold (Fig. 9C). Additionally, H2O2 stimulated the promoter activity to about the same extent in some cell systems (results not shown). However, when exposing SH-SY5Y cells to the same conditions, they did not survive the treatment.

DISCUSSION

The present studies were undertaken to evaluate the biological importance of transcriptional regulation of CYP46A1. Ideally this type of study would use a cell system, which expressed significant levels of both CYP46A1 mRNA and produced measurable amounts of 24S-hydroxycholesterol. Unfortunately such cell systems are not available. Despite considerable efforts, we found trace amounts only of CYP46 mRNA in untransfected neuroblastoma cells and have not been able to measure any significant secretion of 24S-hydroxycholesterol from them. It should be emphasized that the production of 24S-hydroxycholesterol from cholesterol in vivo in brain microsomes in vitro is extremely low, corresponding to a conversion of only ~0.03% of the substrate per hour (26). In view of this, our studies on transcriptional regulation have been restricted to in vivo experiments in mice and in vitro studies on cells transfected with various promoter constructs.

Because it is well established that cholesterol 24-hydroxylase is of importance for elimination of cholesterol from the brain, and because a knock out of this enzyme leads to a reduction of brain cholesterol syn-
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FIGURE 9. Transcriptional regulation of CYP46A1. Treatments known to influence cholesterol homeostasis were not observed to significantly regulate reporter activity (p > 0.05) (A). Although only representative SHSY-5Y results are shown, the results are essentially identical for both HEK293 or HepG2 cells. A combination of dexamethasone (Dex.) and interleukin-6 (IL-6) resulted in a significant activation of CYP46A1-dependent reporter activity (p = 0.02) (B). CYP46A1-dependent reporter activity is increased in SHSY-5Y cells cultured in the presence of increasing concentrations of a radical generator. Treatment with the highest concentration of tert-butylhydroperoxide resulted in a significant increase in reporter activity (p = 0.02) (C).

thesis (13, 14), we first tested the hypothesis that there may be a connection between transcriptional activity of Cyp46a1, Hmg-CoA reductase and Hmg-CoA synthase. Cholesterol synthesis is high in the brain of newborn mammals (1) but is reduced during postnatal development, possibly as a consequence of the accumulation of cholesterol. In principle, the need for CYP46A1 activity would be expected to increase in tandem with increased levels of cholesterol in the central nervous system. On the other hand, the cholesterol accumulation which occurs early in life is presumably mainly in the myelin, while CYP46A1 is present in the neurons, at least in the adult state. It should be mentioned, however, that significant levels of 24S-hydroxycholesterol have been detected in myelin and that these levels increased with age (27) indicating that there is some coupling of 24S-hydroxycholesterol formation to global changes in brain cholesterol (compare with below). Here we show that the decrease in brain cholesterol synthesis during the first 3 weeks of life of a mouse is associated with a marked decrease in the mRNA levels of HMG-CoA synthase and HMG-CoA reductase (Fig. 1, D and E). In parallel with this decrease, there was an increase in the mRNA levels of Cyp46a1. This increase is in accord with a previous report that the activity of the Cyp46a1 enzyme in rat brain microsomes increases by a factor of about two during the early part of life (26). In accordance with a previous report (9) there was a 6-fold increase in the levels of 24S-hydroxycholesterol in the brain during the first 3 weeks of life. After the age of 3 weeks, however, the mRNA levels of the three enzymes, as well as the levels of cerebral sterols were constant, reflecting a balance between cholesterol synthesis and cholesterol elimination under steady-state conditions.

Despite the fact that there is an anatomical separation between the site of cholesterol accumulation (i.e. myelin) and the site of elimination (i.e. neurons) the apparent increase in expression of Cyp46a1 during the first 3 weeks of life may be secondary to the accumulation of cholesterol. To study the possibility that altered cholesterol levels in the brain are able to affect transcriptional activity of Cyp46a1, we measured mRNA levels of Cyp46a1 in the brain of mice with a disruption of the gene coding for the delta-24-reductase, which is responsible for reduction of the Δ24 double bond during cholesterol synthesis. We have previously shown that such a disruption leads to accumulation of desmosterol and an almost complete disappearance of cholesterol with a significant reduction of overall sterol levels (24). Because desmosterol is not a substrate for 24S-hydroxylation by Cyp46a1 (Fig. 2c), the levels of 24S-hydroxycholesterol in the brain of Dhcr24Δ−/− mice were found to be only 1–2% of those of littermate controls. Despite these extremes, the Cyp46a1 mRNA levels were normal. The presence of a general sterol deficiency in the mice was reflected not only by the reduced levels of sterols but also by the (predictable) compensatory increase of Srebp-1 mRNA levels (Fig. 3). The lack of an alteration in both Srebp-2 and Hmgcr mRNA is puzzling and may be a result of a heightened requirement for cerebral fatty acid synthesis. It should be pointed out, however, that desmosterol is also a regulator of Hmgcr and may influence the final mRNA levels (28). However, it has been shown that the capacity to regulate the expression of cholesterol biosynthetic genes may be significantly associated with the cell type (29). Very recently it was reported that treatment of mice with very high doses of a lipophilic statin (simvastatin) causes a significant reduction of cholesterol synthesis in the brain with a compensatory increase in Hmgcr mRNA levels without affecting either Cyp46a1 mRNA levels or 24S-hydroxycholesterol levels (30).

To gain more insight into the mechanism by which CYP46 is regulated, attempts were made to define the basal characteristics of the human CYP46 promoter. The promoter was found to be rich in putative SP1F binding sites, which are potential positive as well as negative regulators of rate of transcription. These are probably responsible for the basal expression of CYP46A1. The presence of multiple SP-1 binding sites in the promoter may allow for transcriptional synergism of SP-1 via direct protein-protein interactions and DNA looping. The high abundance of conserved SP1 sites (Fig. 5c) suggests that this may also occur in the case of CYP46A1. Numerous proteins have been shown to interact with SP1, including NF-kB and Smad, both of which are terminal effectors of inflammatory signaling pathways. Our finding that oxidative stress may influence the expression of CYP46A1 is of interest in light of the fact that SP1 may be induced by oxidative stress, and functions to promote neuronal survival (31). Although our preliminary experiments
revealed a potential for oxidative stress regulation of CYP46A1, further work is needed to evaluate if this is of importance for expression of CYP46A1 under in vivo conditions.

Although it is feasible to suggest that CYP46A1 basal transcriptional activity is governed by SP1 promoter elements, its peak appears to correspond to a region containing a putative Th1/E47 binding site, a transcription factor that has been reported to influence apoE expression in brain and thus the risk of developing late onset Alzheimer disease (32–34). This is of interest in relation to the fact that both apoE and CYP46A1 are important proteins for maintenance of brain cholesterol homeostasis. To date, no polymorphisms have been reported in the promoter of CYP46A1, although several other CYP46A1 polymorphisms are known, some of which have been reported to be related to the risk for development of Alzheimer disease (compare with below).

In accordance with our findings in vivo in the cholesterol-deficient mouse, we did not detect any significant change in the transcriptional activity of the CYP46A1 promoter after exposing the reporter system to factors known to modulate cholesterol homeostasis, i.e. statins, cholesterol, or oxysterols. With one exception (a combination of dexamethasone and Interleukin-6), we also failed to detect any transcriptional regulation of CYP46A1 by steroid hormones, insulin, growth hormone, thyroid hormone, cAMP, or bile acids. It should be emphasized that three different cell systems were used in these studies, which all gave similar results. Although apparent changes of transcriptional activity up to 50% were noted in some individual experiments, these were not consistent and could not be reproduced in other sets of experiments.

The apparent resistance to regulation of CYP46 at a transcriptional level by changes in cholesterol homeostasis is in marked contrast to the regulation of the rate-limiting enzyme for elimination of cholesterol from the liver, cholesterol 7α-hydroxylase (CYP7A1). The latter is subject to a highly sophisticated transcriptional regulation by the flux of bile acids and cholesterol through the liver, and by a great number of dietary and hormonal factors (for a review, see Ref. 35). The low need for a transcriptional control of elimination of cholesterol from the brain may be a consequence of the effective blood–brain barrier, which blocks entrance of extracerebral cholesterol into the system. At the present state of knowledge, the most important role of the CYP46A1 system in the adult mammalian brain appears to be a compensation for the very low rate of de novo synthesis of cholesterol occurring in this organ. The possibility cannot be excluded, however, that the CYP46A1 product 24S-hydroxycholesterol may have some specific physiological function in the brain in addition to being a mediator of cholesterol transport (compare with below).

Studies performed in the Cyp46a1 (−/−) mouse revealed that the steady-state levels of brain cholesterol were maintained by a reduction in cholesterol synthesis. Because astrocytes appear to be responsible for the majority of cholesterol synthesis in the adult brain (14, 36), this regulation is presumably at the level of the astrocytes. The reduction in cholesterol synthesis observed in Cyp46a1 (−/−) mouse may be an attempt to preserve steady-state neuronal cholesterol levels by reducing the supply of cholesterol to the neurons. However, despite this adaptive response, it has been reported in preliminary form that Cyp46a1 (−/−) mice have severe learning disabilities (37). This may be due to a specific need of 24S-hydroxycholesterol for a normal function of neuronal membranes or synapse formation, or alternatively due to a need for elimination of cholesterol from at least some neurons by the cholesterol 24-hydroxylation mechanism.

It should be emphasized that the half-life of cholesterol is orders of magnitude longer in the adult brain than elsewhere in the body and that the majority of cholesterol present in the brain is in a highly stable pool (i.e. myelin), with a half-life of ~1 year in mice (38). The stability of brain cholesterol is further highlighted by the fact that overall CNS cholesterol concentration is unchanged in animal models where genes known to be involved in cholesterol transport are inactivated. Inactivation of Abca1, Srb1, Ldlr, Apoe, or Apoa1 thus did not cause a significant change in whole CNS cholesterol concentrations (39). Alterations in the flux of 24S-hydroxycholesterol from the brain into the circulation under pathological conditions (40) are thus more likely to reflect changes in the number of neuronal cells in the brain rather than changes in the expression or enzymatic activity of CYP46A1 in individual neurons.

The stability of CYP46A1 expression and, as a result, 24S-OH production, may have direct consequences for oxysterol-mediated activation of liver-X-receptor target genes in the brain, which has been associated with modulation of production of β-amyloid (41). The relatively constant level of 24S-OH in different brain areas (21, 42) implies that oxysterol-mediated regulation of liver-X-receptor activity within the brain may be largely dependent on the availability of ligands for its heterodimeric partner, the retinoid X receptor. This is particularly intriguing, because polyunsaturated fatty acids have been reported to activate retinoid X receptor (43, 44) and have been associated with protection versus dementia, particularly Alzheimer disease (45).

The connection between Alzheimer disease and cholesterol homeostasis has been the focus of renewed interest over the past decade, following the recognition that cholesterol may influence the generation of β-amyloid. Because CYP46A1 is critical for the brain cholesterol levels several groups have investigated the possible relationship between polymorphisms in the CYP46A1 gene and the risk of Alzheimer disease (46–54). These studies have presented conflicting results with some showing an association between an intronic polymorphism and the risk of Alzheimer disease, whereas others have not. Importantly, several of these studies reported an interaction between CYP46A1 polymorphisms and the apoE genotype. The mechanism by which these polymorphisms influence the risk of Alzheimer disease is unclear at present. Intronic polymorphisms would not be expected to alter the protein structure, and there appears to be no generation or destruction of consensus response elements for transcription factors.

To summarize, our results are consistent with the contention that regulation of cholesterol synthesis is more important for maintenance of cholesterol homeostasis in the brain than is the corresponding regulation of cholesterol removal. Under normal conditions substrate availability may be the most critical factor for production of 24S-hydroxycholesterol. Nevertheless, the availability of cholesterol substrate does not seem critical in the maintenance of certain transcription levels of CYP46A1 in adulthood. In addition to the changes in CYP46A1 expression occurring during the neonatal stage, the only factor that influenced transcriptional levels of the enzyme was oxidative stress. This finding is consistent with the observed ectopic expression of CYP46A1 in astrocytes of patients with Alzheimer disease (11, 12). Whether or not the latter induction is mediated by increased gene transcription is not known. Attempts to define the factors responsible for the up-regulation of CYP46A1 during the neonatal period and in Alzheimer disease are ongoing.

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