Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3107–3111, April 1994
Biochemistry

Isolation of the human peroxisomal acyl-CoA oxidase gene: Organization, promoter analysis, and chromosomal localization

(peroxisomal β-oxidation/lipid metabolism/Zellweger syndrome)

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Communicated by N. Edward Tolbert, December 20, 1993

ABSTRACT **Peroxisomal acyl-CoA oxidase (ACOX; EC 1.3.3.6) is the first enzyme of the fatty acid β-oxidation pathway, which catalyzes the desaturation of acyl-CoAs to 2-*trans*-enoyl-CoAs, and it donates electrons directly to molecular oxygen, thereby producing H_2O_2. The discovery of carcinogenic peroxisome proliferators, which markedly increase the levels of this H_2O_2-producing ACOX in rat and mouse liver, generated interest in peroxisomal β-oxidation system genes. The present study deals with the structural organization of human *ACOX* gene. This gene spans ≈33 kb and consists of 14 exons and 13 introns. Primer-extension analysis revealed three principal cap sites, which were mapped at 50, 52, and 53 nt upstream of the initiator methionine codon. The 5′ flanking region of the *ACOX* gene was sequenced up to 500 bp upstream of the cap sites. This promoter region is G+C-rich and contains three copies of the "GC box" hexanucleotides. Multiple GC boxes are a characteristic feature of the rat ACOX and bifunctional protein genes of the β-oxidation system. A+T-rich TATA-boxlike sequences, TTTATTT and TTATT, have also been identified in this human *ACOX* gene, but typical CCAAT motifs are absent. This *ACOX* gene has been mapped to chromosome 17q25 by *in situ* hybridization, using a biotin-labeled probe.**

Peroxisomes are cellular organelles that are present in virtually all eukaryotic cells. These organelles contain hydrogen peroxide-producing flavin oxidases together with catalase, which decomposes hydrogen peroxide (1). At present >50 enzymes have been found in mammalian peroxisomes, and more than half of these play a role in lipid metabolism (1). Peroxisomes in liver parenchymal cells can be stimulated to proliferate by the administration of a diverse group of chemical compounds known as peroxisome proliferators, which include the plasticizer di(2-ethylhexyl)phthalate, certain herbicides, hypolipidemic drugs, and leukotriene D_4 antagonists (2). These agents induce predictable pleiotropic responses, characterized by hepatomegaly and increase in the number of peroxisomes in hepatic parenchymal cells (2). Concomitantly, there is a rapid induction of the mRNA levels of the enzymes of the peroxisomal fatty acid β-oxidation system, which include the hydrogen peroxide-generating fatty acyl-CoA oxidase (ACOX; EC 1.3.3.6), enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase bifunctional enzyme (PBE), and 3-ketoacyl-CoA thiolase (3, 4). The induction of the β-oxidation enzyme system has been shown to be due to a coordinate increase in the rates of transcription of the corresponding genes for these enzymes (4).

ACOX is the first enzyme of the fatty acid β-oxidation pathway (3). It catalyzes the desaturation of acyl-CoAs longer than C_8 to 2-*trans*-enoyl-CoAs in a rate-limiting step (3). A lethal disorder that is characterized by an isolated deficiency of peroxisomal ACOX is termed pseudoneonatal adrenoleukodystrophy (5). In addition, the marked induction of this hydrogen peroxide-producing enzyme in the livers of rats and mice has been implicated in the oxidative DNA damage and hepatocarcinogenesis resulting from the exposure to peroxisome proliferators (2).

Rat liver peroxisomes contain three acyl-CoA oxidases—namely, palmitoyl-CoA oxidase, pristanoyl-CoA oxidase, and trihydroxycoprostanoyl-CoA oxidase (6). Among the three, only palmitoyl-CoA oxidase is inducible by peroxisome proliferators (6). The gene for this enzyme in the rat spans 25 kb, contains 14 exons/13 introns, and possesses G+C-rich sequences in the promoter region (7). The upstream region also contains peroxisome proliferator response elements (PPREs) having direct repeats of the core motif TGACCT, which is required for steroid hormone-receptor binding (8).

It has been recently reported that human liver peroxisomes contain only palmitoyl-CoA oxidase and an unusual branched-chain acyl-CoA oxidase (9). Immunoblot analysis has demonstrated that human palmitoyl-CoA oxidase cross-reacts with antibodies raised against the rat enzyme and that both have similar subunit compositions (9). Although peroxisome proliferation in human liver is reported to be very low or nonexistent as a result of therapeutic exposure to peroxisome proliferators such as clofibrate (2), knowledge of the possible risk posed by these agents is essential.

Molecular analysis of the human *ACOX* gene is an important step in our attempts to understand the implications of peroxisome proliferator-induced pleiotropic responses to human health. In this report, we describe the structure of the human peroxisomal *ACOX* gene, including the exonic sequences, exon/intron boundaries, and the deduced amino acid sequence.§ We also present the chromosomal localization, some features of the 5′-flanking region including promoter analysis, and the putative transcript size.

MATERIALS AND METHODS

Isolation of Genomic Clones. A human liver genomic library cloned in the *Sac* I site of EMBL3 (Promega) was screened by using rat ACOX cDNA as a probe (10). An 18-kb human ACOX genomic clone was isolated. This clone contained coding sequences corresponding to exons 3–6 of the rat *Acox*

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Abbreviations: ACOX, peroxisomal acyl-CoA oxidase; RT, reverse transcription; PPRE, peroxisome proliferator response element; PPAR, peroxisome proliferator-activated receptor; PBE, enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase bifunctional enzyme.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U03254–U03268).

gene. Oligonucleotide sense primer ACOX 51 (5'-CAGTTGGAAAGACTTCA-3') from exon 5 and antisense primer ACOX 62 (5'-CCTGGGCATACTTCATC-3') from exon 6 of human *ACOX gene* were used to screen a human foreskin fibroblast P1 bacteriophage library (Genome Systems, St. Louis). Three clones, 175, 176, and 177, which contained inserts ranging from 60 to 75 kb in length, were obtained.

Restriction Mapping, Determination of Exon/Intron Boundaries, and DNA Sequencing. The P1 clones were digested with a single restriction enzyme or a combination of restriction enzymes, electrophoresed on 0.8% agarose gels, transferred to nitrocellulose, and analyzed by Southern blotting (11), using the rat ACOX cDNA as a probe (10). Fragments identified as containing the coding regions from Southern analysis were subcloned into the *Eco*RI, *Pst* I, or *Sac* I sites of pBluescriptKS vector (Stratagene). Double-stranded sequencing of denatured plasmid DNA templates was done by using modified T7 DNA polymerase Sequenase and the dideoxynucleotide chain-termination method (12). The putative human exonic sequences were identified by comparing our sequences with those of rat *Acox* gene (7).

Northern Blot Analysis. Total RNA was prepared from human liver by the guanidinium isothiocyanate method, as described by Chirgwin *et al.* (13). A multiple human tissue Northern blot was purchased from Clontech. Prehybridization/hybridization was done as described (14).

Reverse Transcription (RT)-PCR. This procedure was done with total human liver RNA and the sense primer at the 3' end of exon 10 with the sequence 5'-TCAACAGCCCCGAAAGCC-3' and the antisense primer of exon 14 with the sequence 5'-TCACTGCAGTGACTTCAGGTGCTT-3'. The protocol followed for RT of RNA, and PCR amplification of the cDNA was exactly according to the GeneAmp RNA-PCR kit (Perkin–Elmer/Cetus).

Primer Extension. This extension was done according to a described method (14) with total human liver RNA and an antisense primer in the middle of exon 1 with the sequence 5'-GCAGCTCCGGGTTGAAGCTGGCGA-3'. The oligonucleotide was end-labeled by using [γ-^{32}P]dATP (Amersham) and T4 polynucleotide kinase at 37°C for 45 min. Labeled oligonucleotide (5×10^6 counts) was mixed with 40 μg of total RNA and hybridized overnight at 30°C in aqueous buffer. RT was done by using Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) at 42°C for 90 min (14). The reaction was analyzed on an 8% polyacrylamide/urea gel.

Fluorescence *in Situ* Chromosomal Hybridization. Human metaphase cells were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes. Fluorescence *in situ* hybridization was done by using a biotin-labeled ACOX probe (70-kb genomic DNA fragment in P1 clone 177) prepared by nick-translation using Bio-11-dUTP (Enzo Diagnostics) as described (15). Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories), and chromosomes were identified by staining with 4',6-diamidino-2-phenylindole.

Promoter Activity. Human hepatoma HepG2 and rat hepatoma H4IIEC3 cells were cultured as monolayers in Dulbecco's modified Eagle's medium/10% horse serum/5% fetal bovine serum. The construct HAOX-LUC was made by amplifying a 1.7-kb promoter fragment from a 3-kb *Pst* I subclone (see Fig. 1) in pBluescriptKS containing the first and second exons. The primers used for the amplification were the universal T7 primer (Stratagene) and an oligonucleotide primer close to the ATG site with a *Hin*dIII site tagged on that has the sequence 5'-CGGAAGCTTGGCGACGACCAGCTGGC-3' (HAOXP). PCR conditions were similar to those previously stated. The amplified fragment was subcloned in the correct orientation into the *Hin*dIII site of the vector pgL2basic (Promega). Transfections were done by a modification of the calcium phosphate procedure (16) into H4IIEC3 and HepG2 cells at an 80% confluence. After transfection, the cells were washed twice with phosphate-buffered saline. Then, they were lysed in buffer containing 100 mM K_2HPO_4, 1 mM dithiothreitol, and 1% Triton X-100, after which the supernatant was assayed for luciferase activity. This assay was done in 100 μl of cell extract with D-luciferin as substrate in a monolight luminometer. To account for transfection efficiency, luciferase values were normalized to β-galactosidase activity from the cotransfected plasmid CMV-β-gal (16).

RESULTS AND DISCUSSION

Isolation of Human *ACOX* Gene. An 18-kb clone, HA1, was isolated from a human liver genomic library using rat ACOX cDNA as a probe (Fig. 1). Subsequent analysis of HA1 sequence revealed that it contained exons 3-II, 4, 5, and 6, corresponding to the rat *Acox* gene (Fig. 1). Because we were unable to obtain additional longer or overlapping clones, we synthesized primers to human exons 5 and 6 and obtained three clones for human ACOX by screening a P1 human fibroblast genomic library (17). The P1 cloning vector is designed to permit the efficient recovery of insert DNA that has been ligated appropriately to vector sequences and minimizes the rearrangement of DNA during isolation and subsequent amplification steps (17).

All three P1 clones exhibited similar restriction fragment patterns (data not shown). Therefore, we characterized only clone 177, which contained an insert of ≈70 kb. Because information on human ACOX cDNA was not available when these studies were initiated, it was necessary to use different fragments of rat ACOX cDNA (10) for characterization of genomic clone 177.

Structural Organization of Human *ACOX* Gene. As shown in Fig. 2, the gene spans ≈33 kb. All the exons, exon/intron boundaries, 500 nt of the 5'-untranslated region, and up to 300 bp of the 3'-untranslated region were sequenced (Fig. 2). The gene contained 14 exons, which ranged in size from 48 to 207 bp, and 13 introns. All of the exon–intron junctions conformed to the GT/AG rule.

Many features of the human *ACOX* gene closely resemble those of the rat (7). (*i*) The human *ACOX* gene, like its rat counterpart, consists of 14 exons and 13 introns. (*ii*) Positions

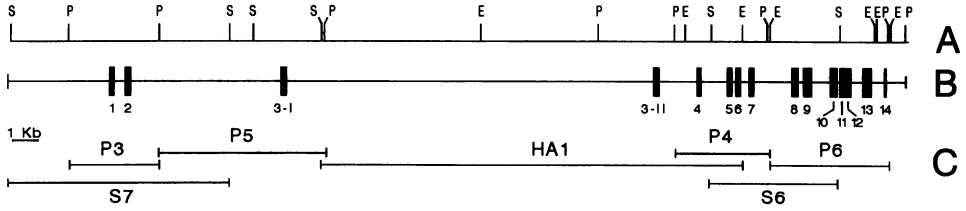

FIG. 1. Structural organization of the human *ACOX* gene. (*A*) Restriction enzymes *Eco*RI (E), *Sac* I (S), and *Pst* I (P) were used to subclone and characterize fragments. (*B*) Intron/exon pattern. Exons are represented by solid boxes, numbered below each box. 3-I and 3-II are two different forms of exon 3, which are possibly used alternatively during splicing as in rat ACOX. (*C*) Overlapping clones that were isolated. Clone HA1 is the 18-kb clone initially isolated from the human liver genomic library by using the rat partial cDNA as probe. HA1 sequence contains exons 3-II, 4, 5, and 6. P3, P4, P5, P6, S6, and S7 are *Pst* I (P) and *Sac* I (S) subclones derived from the 70-kb P1 bacteriophage clone 177.

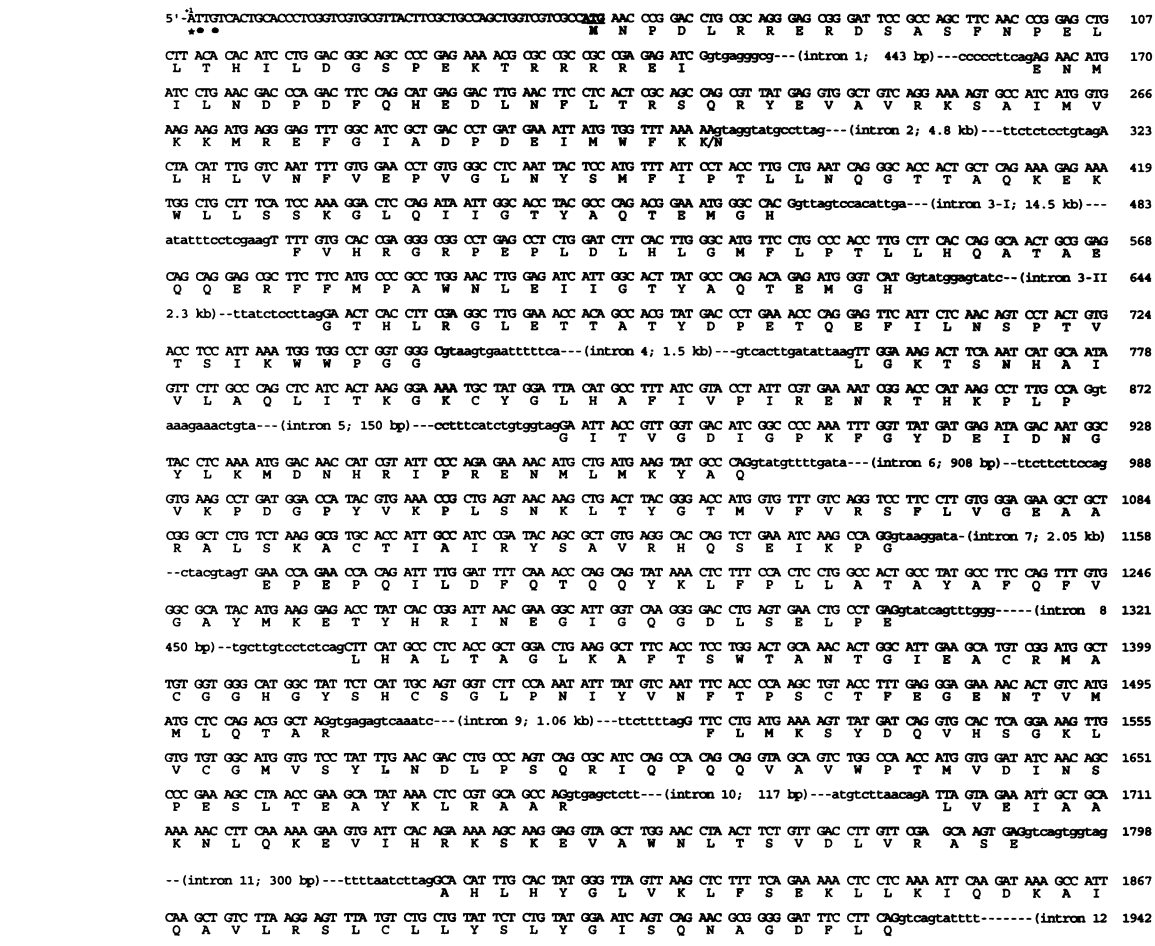

FIG. 2. Nucleotide sequences of the human *ACOX* gene. Uppercase letters indicate the coding sequence and 5′-untranslated regions; lowercase letters indicate the exon/intron junctions and the 3′-untranslated region. The initiation codon ATG (bold underlining) and the stop codon TGA (boldface type) are shown. The three putative transcription start sites are indicated by a star and two filled circles; the first transcription start site (star) is designated as +1. Numbering of nucleotides indicates their position relative to the major transcription start site +1. A single amino acid letter appears below each codon. Intron sizes are indicated in parentheses.

of the introns and length of the exons, except exon 14, are essentially identical. The lengths of the introns, except for introns 2, 3-I, 3-II, 4, 7, and 12, are similar. The deduced amino acid-sequence homologies between rat *Acox* (10) and human *ACOX* are ≈93% (data not shown), implying that the function of the ACOX enzyme has been highly conserved during evolution. Such high sequence similarities have also been reported for the rat and human genes encoding other peroxisomal proteins (18–20). Reports of immunoblot analysis demonstrated that human palmitoyl CoA-oxidase cross-reacts with antisera raised against the rat enzyme, and both these proteins consist of three subunits of 72, 52, and 21 kDa (9).

Two different nucleotide sequences were detected for exon 3 (3-I and 3-II); the homology between them was especially high at the middle and 3′-end portions. Alternative use of these exons during the splicing process produces two species of mRNA in rat (10). Because the nucleotide homology between the human and rat is ≈85%, one would speculate that the same event occurs in human, but this remains to be determined. Sequence analysis of the 3′ noncoding region, up to 300 bp beyond the stop codon, did not reveal a putative polyadenylylation signal, AATAAA. Additional sequence data of the 3′ untranslated region of the human *ACOX* gene are needed to determine the polyadenylylation (AATAAA) and the transcription termination signal (GTGTTTT).

Isolation of a Partial ACOX cDNA Clone. Using the sense primer from exon 10 and the antisense primer from exon 14, we isolated a partial-length cDNA fragment by RT-PCR using the total RNA isolated from human liver. Sequence analysis of this fragment revealed that it exactly matches the corresponding exonic sequences in the *ACOX* gene (data not shown). Isolation of additional ACOX cDNA clones is necessary to obtain full-length cDNA for expression studies.

Transcription Initiation Site(s). The transcription initiation sites for the human *ACOX* gene were determined by primer-extension analysis. Several cap sites were detected, but three different ones located at 50, 52, and 53 nt upstream from the translation start site ATG were the strongest (Fig. 3). This result indicates that the gene is transcriptionally active. The oligonucleotide used was an antisense primer located in the middle of the first exon. The product of the primer-extension reaction was separated on an 8% polyacrylamide/urea gel and detected by autoradiography. The distance of migration was assessed by counting the number of base pairs on the sequencing ladder from the 5′ end of the primer to the position of the bands visible on the film (Fig. 3). Among the three putative transcription start sites, the first one, which is an adenosine, has been designated as +1 (Fig. 2).

Tissue Distribution of Human *ACOX* Gene Transcripts. The tissue distribution of *ACOX* gene transcripts was examined by using the 500-bp partial-length human liver ACOX cDNA

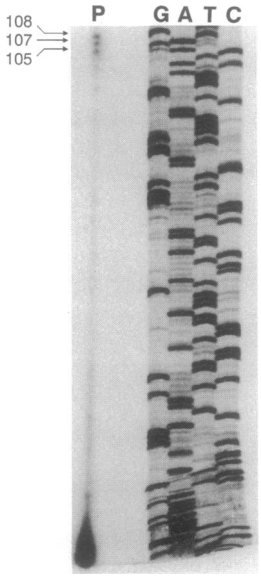

FIG. 3. Determination of the transcription initiation site(s) for the human *ACOX* gene. The three putative sites upstream of the initiator methionine codon of human *ACOX*, corresponding to G (50 nt upstream), T (52 nt upstream), and A (53 nt upstream) were determined by comparing them with the reference sequencing ladder run as a size marker adjacent to the primer-extension reaction product (lane P). Three major bands (arrows) appear at 105, 107, and 108 nt from the 5′ end of the primer.

isolated above as a probe. The results (Fig. 4) indicate that it cross-hybridized with poly(A)$^+$ RNAs of various discrete sizes from liver, skeletal muscle, and kidney with strong signals of 9, 8, and 6 kb in all three lanes. However, only the liver exhibited an additional band, ≈3.6 kb in size, which appears to be the correct size for the ACOX transcript in human. High-molecular-weight RNAs that hybridize to cDNA sequences are known as "precursors" and may reflect variations in 3′- or 5′-untranslated regions (21). The multiple gene transcripts seen in the Northern analyses need to be explored further.

Analysis of a Putative *ACOX* Gene 5′ Sequence. The initiation codon ATG, putative cap sites, and upstream sequences are shown in Fig. 5*A*. A highly G+C-rich region, ≈65%, is found upstream of the cap sites. This area contains three copies of the GC box, GGGCGG or CCCGCC from −84 to −89, −93 to −98, and −308 to −313 upstream of +1. Also, a 24-bp G+C-rich repeat that encompasses the first two GC boxes is located from −76 to −99. More than one cap site is commonly found in such genes. Multiple GC boxes are frequently found in eukaryotic promoters and are a characteristic feature of the mammalian peroxisomal genes, such as those encoding rat ACOX (7), PBE (22), thiolase A (19), as well as human catalase (18) and human thiolase (23). The GC box probably represents a common transcription signal for the genes of the β-oxidation enzymes including human ACOX.

Also, TATA box-like A+T-rich sequences, TTTATTT and TTATT, are present from −289 to −295 and −500 to −504, respectively. These A+T-rich motifs are also present

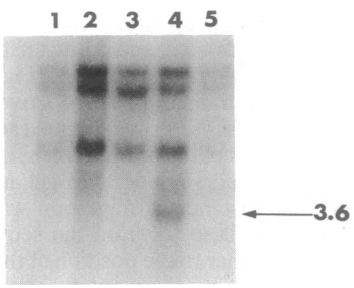

FIG. 4. RNA blot analysis. Multiple human tissue blot of poly(A)$^+$ RNAs probed with ^{32}P-labeled partial-length human ACOX cDNA. The membrane was purchased from Clontech. One band corresponding to 3.6 kb (arrow) is prominent in liver (lane 4). Lanes: 1, skeletal pancreas; 2, kidney; 3, skeletal muscle; and 5, lung.

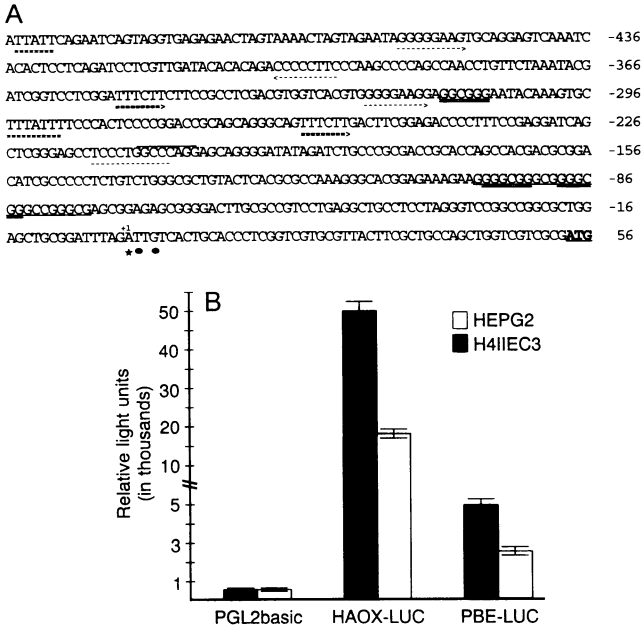

FIG. 5. The human *ACOX* gene 5′ region. (*A*) Sequence of the 5′-untranslated and promoter region from the initiation methionine (boldface underlining). The three transcription initiation start sites are indicated by a star and two solid ellipses. The nucleotide indicated by a star (A) is designated as +1. The core GC box motifs are double underlined. A 24-bp G+C-rich sequence that encompasses the first two GC boxes is underlined. A putative AP-2 motif is overlined. TATA box-like A+T-rich sequences are indicated by double-dashed lines. An imperfect motif, TCCCTGGCCC, is indicated by a single dashed line and appears to be present and conserved in all three rat enzymes of the peroxisomal β-oxidation pathway. Direct and inverted repeats are marked with dashed arrows. (*B*) A 1.7-kb promoter fragment was subcloned into a luciferase expression vector pGL2 and transiently transfected into HepG2 (white bar) and H4IIEC3 (black bar) cells. The levels of luciferase expression, as measured in relative light units, are shown for the promoterless vector alone (pGL2 basic), the human *ACOX* promoter construct (HAOX-LUC), and the rat bifunctional enzyme construct (PBE–LUC). pGL2basic is the negative control, and PBE–LUC serves as a positive control for the experiment. Values are the means of two experiments normalized to a cytomegalovirus β-galactosidase transfection control.

in the upstream region of the rat thiolase B gene, which lacks GC boxes, but are absent in the promoter region of the rat *Acox* gene, which is highly G+C-rich, although both enzymes can be induced by peroxisome proliferators (7, 19).

A putative AP-2 motif is located from −203 to −210 nt upstream of +1. Also, an imperfect motif of sequence TCCCTGGCCC is present from −206 to −215 nt upstream from +1. This sequence appears similar to TCCTGGTCCC, which is present and conserved in all the three inducible rat genes of the β-oxidation pathway. However, CCAAT boxes, which are a common feature of mammalian peroxisomal gene promoters, are absent from the 5′ regions of human (Fig. 5*A*) and rat ACOX-encoding genes (7). Therefore, the upstream region of human *ACOX* contains both GC boxes and A+T-rich motifs, instead of one or the other, as has been observed thus far with the β-oxidation enzymes, suggesting that this gene might be regulated differently.

Promoter Analysis. The identity of the region reported as promoter (Figs. 2 and 5*A*) for the human *ACOX* gene was confirmed by amplifying 1.7 kb upstream of ATG using the primer HAOXP and the universal primer T7. The amplification product was subcloned in the correct orientation into the *Hin*dIII site of the luciferase expression vector pGL2basic. Transfections were done in two cell lines—rat hepatoma H4IIEC3 and human hepatoma HepG2. The promoterless

vector pGL2basic was used as a negative control, whereas the rat bifunctional enzyme–luciferase construct, PBE-LUC, served as a positive control (16). Luciferase activity of HAOX-LUC in H4IIEC3 cells was 125-fold greater than that of pGL2basic alone, whereas in HepG2 cells, the activity of HAOX-LUC was ≈85-fold (Fig. 5*B*).

Therefore, the region identified as promoter is shown to have activity as confirmed by the luciferase assay. The higher activity of HAOX-LUC in H4IIEC3 vs. HepG2 cells can be attributed to the fact that the rat hepatoma cell line is the one known to be inducible by ciprofibrate (16). It has been demonstrated that mouse and human pexoxisome proliferator-activated receptors (PPARs) are capable of transactivating PPREs upstream of the rat *Acox* gene (24). To fully comprehend the effects of peroxisome proliferators or their lack thereof on human systems, it is essential to determine whether the human *ACOX* gene can be induced and if it has PPREs similar to those present in rat. Future experiments to fully characterize the promoter will involve identifying putative PPREs present in the promoter region of human *ACOX* and possible interaction(s) of human PPAR with the PPREs.

Chromosomal Localization. To localize the *ACOX* gene, we performed fluorescence *in situ* hybridization of a biotin-labeled ACOX probe (177) to normal human metaphase chromosomes (Fig. 6). Hybridization of the ACOX probe resulted in specific labeling only of chromosome 17. Specific labeling of 17q24–25 was observed on four (20 cells), three (4 cells), or two (1 cell) chromatids of the chromosome 17 homologues in 25 cells examined. Of 95 signals observed (95 of 100 17q chromatids from 25 metaphase cells were labeled), 72 (76%) were located at 17q25, 10 (11%) signals were located at the junction of q24–q25, and 13 (14%) signals were located at q24. Forty of 72 signals were located at 17q25.1, and 1 (2.5%) was located at q25.3. The remaining 32 signals were localized to q25 with further sublocalization. Specific labeling of 17q25 was obtained in an additional hybridization experiment using this probe. These results indicate that the *ACOX* gene is localized to chromosome 17, band q25.1.

The enzymes of the peroxisomal β-oxidation system of rat liver are markedly induced by peroxisome proliferators by transcriptional activation of the respective genes (4). The identification of a family of PPARs in mouse, rat, human, and *Xenopus* (24–28) and the evidence that these PPARs associate with heat shock protein 72 and form heterodimers with retinoid X receptor α suggest that peroxisome proliferator signaling mechanisms are indeed complex. The binding of various PPARs with PPREs in 5′ upstream regions of rat *Acox*, and PBE-encoding gene suggests that characterization of promoter regions of human peroxisomal β-oxidation genes is essential for a clear understanding of the regulation of these genes by peroxisome proliferators and in extrapolating their risk to humans.

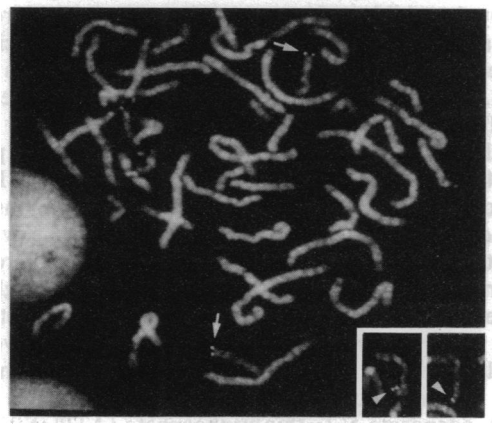

FIG. 6. Chromosomal assignment of the human *ACOX* gene. *In situ* hybridization was performed by using a biotin-labeled ACOX probe to human metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes. The chromosome 17 homologues are identified with arrows in this partial metaphase cell; specific labeling was observed at 17q25. (*Inset*) Partial karyotypes of two chromosome 17 homologues, illustrating specific labeling at 17q25 (arrowheads). Images were obtained by using a Zeiss Axiophot microscope coupled to a cooled charge-coupled device camera. Separate images of 4′,6-diamidino-2-phenylindole-stained chromosomes and the hybridization signal were merged by using image analysis software (IMAGE 1.47 and GENEJOIN).

This work was supported by National Institutes of Health Grant GM23750.

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