Evidence for the existence of a pristanoyl-CoA oxidase gene in man

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In the rat, 2-methyl branched fatty acids and the bile acid intermediates di- and tri-hydroxycoprostanic acids are desaturated by pristanoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase respectively. In the human, these compounds are oxidized by a single enzyme, branched-chain acyl-CoA oxidase, which according to its amino acid sequence is the human homologue of rat trihydroxycoprostanoyl-CoA oxidase. Pristanoyl-CoA oxidase is apparently absent from human tissues as indicated by immunoblot analysis [Van Veldhoven, Van Rompuy, Fransen, de Béthune and Mannaerts (1994) Eur. J. Biochem. **222**, 795–801] and Northern-blot analysis [Vanhooren, Fransen, de Béthune, Baumgart, Baes, Torrekens, Van Leuven, Mannaerts and Van Veldhoven (1996) Eur. J. Biochem. **239**, 302–309] of human tissues. In this paper we present evidence, however, that at least the gene for pristanoyl-CoA oxidase is present in the human. A human liver cDNA encoding a protein of 700 amino acids, showing 75% amino acid identity with rat pristanoyl-CoA oxidase and harbouring a peroxisomal C-terminal-targeting signal (SKL), was isolated. Bacterial expression of the cDNA resulted in a fusion protein that was cross-reactive with antibodies directed against rat pristanoyl-CoA oxidase and the C-terminal SKL sequence. Screening of a genomic library with the isolated cDNA as a probe resulted in a genomic clone in which four introns were localized. By means of fluorescence *in situ* hybridization the gene for human pristanoyl-CoA oxidase was mapped at chromosome position 4p15.3. We conclude that a gene for pristanoyl-CoA oxidase is present in the human genome. The gene appears to be expressed to such a low extent in liver that its mRNA cannot be detected by routine Northern-blot analysis and that its product remains undetected by standard immunoblotting or by enzyme activity measurements. We speculate that the gene may be expressed under special (e.g. certain developmental stages) conditions or in certain specialized tissues not examined thus far.

INTRODUCTION

Peroxisomes β-oxidize a variety of lipophilic carboxylates including straight-chain fatty acids, 2-methyl branched fatty acids such as pristanic acid and the side chain of the bile acid intermediates di- and tri-hydroxycoprostanic acids [1]. The first reaction of peroxisomal β-oxidation, which consists of the desaturation of an acyl-CoA ester to a 2-*trans* enoyl-CoA, is catalysed by an FAD-dependent acyl-CoA oxidase that donates electrons directly to molecular O_2, yielding H_2O_2 [2].

Over recent years it has become clear that peroxisomes contain multiple acyl-CoA oxidases. Three acyl-CoA oxidases have been purified from rat liver and characterized: (1) palmitoyl-CoA oxidase, which oxidizes the CoA esters of straight-chain fatty acids and prostanoids; (2) pristanoyl-CoA oxidase, which oxidizes the CoA estes of 2-methyl branched fatty acids; (3) trihydroxycoprostanoyl-CoA oxidase, which oxidizes the CoA esters of the 2-methyl branched side chains of the bile acid precursors di- and tri-hydroxycoprostanic acids and which also has some activity towards 2-methyl branched fatty acids [3–7]. Palmitoyl-CoA oxidase and pristanoyl-CoA oxidase are expressed in all rat tissues examined thus far [6,8], but trihydroxycoprostanoyl-CoA oxidase is expressed only in liver [3,9].

From human liver only two acyl-CoA oxidases have been purified and characterized: (1) palmitoyl-CoA oxidase, the substrate spectrum of which resembles that of its rat counterpart; (2) branched-chain acyl-CoA oxidase, which oxidizes the CoA esters of 2-methyl branched fatty acids and those of the bile acid intermediates di- and tri-hydroxycoprostanic acids [10–12]. Both enzymes are expressed in liver as well as in extrahepatic tissues.

Molecular cloning of the rat [8,9,13] and human acyl-CoA oxidases [14–17] revealed that, despite its expression in extrahepatic tissues, branched-chain acyl-CoA oxidase [17] is in fact the human homologue of trihydroxycoprostanoyl-CoA oxidase [9], both enzymes consisting of 681 amino acids and showing 75% amino acid identity. In contrast, the amino acid identity between human branched-chain acyl-CoA oxidase [17] and rat pristanoyl-CoA oxidase [8] is only 20%. Enzyme activity measurements, immunoblot analysis with antibodies raised against rat pristanoyl-CoA oxidase [6] and Northern-blot analysis with rat pristanoyl-CoA oxidase probes [8] all indicated that pristanoyl-CoA oxidase is absent from human tissues. However, to our surprise we found in the EMBL databank human expressed sequence tags (ESTs) that are highly homologous (70–80%) to stretches of bases in the rat pristanoyl-CoA oxidase cDNA sequence. In this paper we present evidence that a pristanoyl-CoA oxidase gene is, unexpectedly, present in the human and we describe the isolation of a cDNA encoding a protein that shares 75% amino acid identity with rat pristanoyl-CoA oxidase.

MATERIALS AND METHODS

Materials

Restriction enzymes and Expand® High Fidelity DNA polymerase were from Boehringer-Mannheim (Heidelberg, Germany). *Taq* polymerase (5 units/μl) was obtained from Perkin–Elmer (Foster City, CA, U.S.A.). Human liver marathon 5′-rapid amplification of cDNA ends (5′-RACE) ready cDNA was from Clontech (Palo Alto, CA, U.S.A.). The human liver 5′ stretch λgt11 cDNA library was purchased from Stratagene (La

Abbreviations used: BAC, bacterial artificial chromosome; dNTPs, deoxyribonucleoside triphosphates; EST, expressed sequence tag; FAD, flavin adenine dinucleotide; FISH, fluorescence *in situ* hybridization; 5′-RACE, 5′-rapid amplification of cDNA ends.

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The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number Y11411.

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      -70                                                                            1
5'--CGGATCCTTTCCTGCTTTTGGTTTCCCTGGCAGGGGTTGAACTGTGGAGTGTGTGGGCTCTTATCACGCG   ATG GCA TCC ACT GTG     15
                                                                             Met Ala Ser Thr Val      5

GAA GGA GGC GAC ACA GCT CTG CTC CCA GAA TTC CCC AGG GGG CCC CTC GAT GCC TAC CGA GCA AGA GCG TCC TTC     90
Glu Gly Gly Asp Thr Ala Leu Leu Pro Glu Phe Pro Arg Gly Pro Leu Asp Ala Tyr Arg Ala Arg Ala Ser Phe     30

AGC TGG AAG GAC GTG GCG CTG TTC ACG GAA GGG GAG GGC AAT GTC CGC TTT AAG AAA ACC ATC TTC TCA GCT CTT    165
Ser Trp Lys Asp Val Ala Leu Phe Thr Glu Gly Glu Gly Asn Val Arg Phe Lys Lys Thr Ile Phe Ser Ala Leu     55

GAG AAT GAC CCT CTT TTC GCT CGT TCC CCT GGA GCC GAC CTG TCC TTG GAG AAG TAT CGC GAG CTG AAC TTC CTT    240
Glu Asn Asp Pro Leu Phe Ala Arg Ser Pro Gly Ala Asp Leu Ser Leu Glu Lys Tyr Arg Glu Leu Asn Phe Leu     80

CGA TGC AAG CGG ATC TTC GAG TAT GAC TTC CTC AGT GTC GAA GCA ATG TTC AAG AGC CCT CTG AAG GTC CCC GCC    315
Arg Cys Lys Arg Ile Phe Glu Tyr Asp Phe Leu Ser Val Glu Ala Met Phe Lys Ser Pro Leu Lys Val Pro Ala    105

TTG ATT CAG TGC CTG GGC ATG TAT GAC TCT TCT CTG GCT GCC AAG TAC CTC CTC CAT AGC TTG GTT TTT GGA TCA    390
Leu Ile Glu Cys Leu Gly Met Tyr Asp Ser Ser Leu Ala Ala Lys Tyr Leu Leu His Ser Leu Val Phe Gly Ser    130

GCA GTT TAC AGT TCT GGT TCT GAA AGA CAT CTC ACA TAT ATT CAA AAG ATC TTC AGG ATG GAG ATT TTT GGA TGT    465
Ala Val Tyr Ser Ser Gly Ser Glu Arg His Leu Thr Tyr Ile Glu Lys Ile Phe Arg Met Glu Ile Phe Gly Cys    155

TTT GCT CTG ACC GAA TTA AGC CAC GGC AGT AAT ACC AAG GCC ATT CGC ACA ACT GCC CAC TAC GAT CCT GCC ACT    540
Phe Ala Leu Thr Glu Lys Ser His Gly Ser Asn Thr Lys Ala Ile Arg Thr Thr Ala His Tyr Asp Pro Ala Thr    180

GAG GAA TTC ATC ATA CAT TCC CCT GAT TTC GAA GCT GCC AAG TTT TGG GTT GGC AAC ATG GGC AAG ACA GCC ACT    615
Glu Glu Phe Ile Ile His Ser Pro Asp Phe Glu Ala Ala Lys Phe Trp Val Gly Asn Met Gly Lys Thr Ala Thr    215

CAC GCG GTG GTG TTT GCT AAG CTG TGT GTG CCA GGG GAC CAG TGC CAT GGG CTG CAT CCC TTT ATC GTG CAG ATC    690
His Ala Val Val Phe Ala Lys Leu Cys Val Pro Gly Asn Gln Cys His Gly Leu His Pro Phe Ile Val Gln Ile    240

CGG GAC CCG AAG ACC CTT CTT CCC ATG CCT GGA GTG ATG GTT GGC GAC ATA GGA AAA AAA CTC GGG CAG AAC GGT    765
Arg Asn Pro Lys Thr Leu Leu Pro Met Pro Gly Val Met Val Gly Asp Ile Gly Lys Lys Leu Gly Gln Asn Gly    265

CTA GAT AAT GGT TTC GCC ATG TTC CAC AAG GTC AGA GTT CCT CGC CAG AGC CTT CTG AAC CGG ATG GGA GAC GTC    840
Leu Asp Asn Gly Phe Ala Met Phe His Lys Val Arg Val Pro Arg Gln Ser Leu Leu Asn Arg Met Gly Asp Val    280

ACC CCC GAG GGC ACC TAT GTC AGC CCC TTT AAG GAC GTC AGG CAG CGC TTT GGA GCG TCC CTG GGG AGC CTG TCC    915
Thr Pro Glu Gly Thr Tyr Val Ser Pro Phe Lys Asp Val Arg Gln Arg Phe Gly Ala Ser Leu Gly Ser Leu Ser    305

TCG GGC CGG GTC TCC ATC GTG AGC CTG GCC ATC CTT AAC CTA AAG CTG GCC GTG GCC ATC GCT CTT CGC TTC TCA    990
Ser Gly Arg Val Ser Ile Val Ser Leu Ala Ile Leu Asn Leu Lys Leu Ala Val Ala Ile Ala Leu Arg Phe Ser    330

GCC ACT CGG CGT CAG TTT GGA CCC ACA GAG GAG GAG GAA ATA CCA GTG CTT GAG TAT CCA ATG CAG CAA TGG CGC   1065
Ala Thr Arg Arg Gln Phe Gly Pro Thr Glu Glu Glu Glu Ile Pro Val Leu Glu Tyr Pro Met Gln Gln Trp Arg    355

TTG CTT CCA TAT CTG GCA GCT GTC TAC GGC TTA GAC CAT TTC TCC AAG TCG CTC TTC CTG GAC CTG GTG GAG CTC   1140
Leu Leu Pro Tyr Leu Ala Ala Val Tyr Gly Leu Asp His Phe Ser Lys Ser Leu Phe Leu Asp Leu Val Glu Leu    380

CAG CGA GGA CTT GCA TCG GGA GAC CGC AGC GCC AGA CAG GCA GAG CTT GGA CGT GAG ATC CAC GCC CTG GCA TCG   1215
Gln Arg Gly Leu Ala Ser Gly Asp Arg Ser Ala Arg Gln Ala Glu Leu Gly Arg Glu Ile His Ala Leu Ala Ser    405

GCC AGC AAG CCC CTG GCC TCG TGG ACC ACC CAG CAA GGA ATT CAG GAA TGC CGG GAG GCG TGT GGA GGA CAC GGC   1290
Ala Ser Lys Pro Leu Ala Ser Trp Thr Thr Gln Gln Gly Ile Gln Glu Cys Arg Glu Arg Cys Gly Gly His Gly    430

TAT CTG GCC ATG AAC CGG TTG GGT GTC CTT AGA GAT GAC AAC GAT CCC AAC TGC ACA TAC GAA GGT GAC AAC AAC   1365
Tyr Leu Ala Met Asn Arg Leu Gly Val Leu Arg Asp Asp Asn Asp Pro Asn Cys Thr Tyr Glu Gly Asp Asn Asn    455

ATC CTG CTG CAG CAG ACA AGC AAC TAT TTG CTG GGT CTC CTG GCA CAC CAG GTC CAC GAT GGA GCT TGC TTC CGC   1440
Ile Leu Leu Gln Gln Thr Ser Asn Tyr Leu Leu Gly Leu Leu Ala His Gln Val His Asp Gly Ala Cys Phe Arg    480

AGT CCG CTG AAG TCA GTG GAC TTT CTG GAC GCC TAT CCC GGC ATC CTT GAC CAG AAG TTT GAG GTC TCC AGT GTT   1515
Ser Pro Leu Lys Ser Val Asp Phe Leu Asp Ala Tyr Pro Gly Ile Leu Asp Gln Lys Phe Glu Val Ser Ser Val    505

GCC GAC TGC TTG GAC TCT GCA GTC GCC CTG GCA GCA TAC AAG TGG CTG GTT TGC TAC CTG CTC CGA GAG ACT TAT   1590
Ala Asp Cys Leu Asp Ser Ala Val Ala Leu Ala Ala Tyr Lys Trp Leu Val Cys Tyr Leu Leu Arg Glu Thr Tyr    530

CAA AAA TTA AAC CAA GAG AAA AGA TCA GGA AGC AGT GAC TTT GAA GCA AGG AAC AAA TGC CAG GTG TCC CAC GGC   1665
Gln Lys Leu Asn Gln Glu Lys Arg Ser Gly Ser Ser Asp Phe Glu Ala Arg Asn Lys Cys Gln Val Ser His Gly    555

CGT CCG TTG GCG CTG GCC TTC GTG GAG CTC ACG GTG GTC CAG AGG TTC CAC GAG CAC GTG CAC CAG CCT TCC GTG   1740
Arg Pro Leu Ala Leu Ala Phe Val Glu Leu Thr Val Val Gln Arg Phe His Glu His Val His Gln Pro Ser Val    580

CCG CCC TCG CTG CGG GCC GTG CTG GGG CGG CTC AGT GCT CTG TAC GCC CTG TGG TCC CTG AAG CGC CAC GCG GCC   1815
Pro Pro Ser Leu Arg Ala Val Leu Gly Arg Leu Ser Ala Leu Tyr Ala Leu Trp Ser Leu Lys Arg His Ala Ala    605

CTG CTC TAC CGA GGA GGA TAC TTC TCC GGT GAG CAG GCG GGA GAA GTG TTG GAG AGC GCC GTC CTG GCT TTG TGT   1890
Leu Leu Tyr Arg Gly Gly Tyr Phe Ser Gly Glu Gln Ala Gly Glu Val Leu Glu Ser Ala Val Leu Ala Leu Cys    630

TCC CAG CTG AAA GAC GAT GCA GTT GCC CTG GTA GAC GTG ATC GCT CCT CCT GAC TTT GTT CTG GAC TCA CCG ATT   1965
Ser Gln Leu Lys Asp Asp Ala Val Ala Leu Val Asp Val Ile Ala Pro Pro Asp Phe Val Leu Asp Ser Pro Ile    655

GGC AGA GCC GAC GGC GAG CTC TAC AAA AAC CTC TGG GGC GCT GTC CTG CAG GAA AGC AAG GTG TTG GAG CGG GCA   2040
Gly Arg Ala Asp Gly Glu Leu Tyr Lys Asn Leu Trp Gly Ala Val Leu Gln Glu Ser Lys Val Leu Glu Arg Ala    680

TCC TGG TGG CCA GAG TTT TCT GTG AAC AAA CCT GTC ATA GGA AGT CTG AAA TCG AAG CTC TAG  TGGGACTGGCACAT   2117
Ser Trp Trp Pro Glu Phe Ser Val Asn Lys Pro Val Ile Gly Ser Leu Lys Ser Lys Leu ***                     700

TCAGCCAAGTCTAATGAAACGAAGGGAACTAATCAGACGTGGACCTCAACTTCTGATTCCAGAACACGCCGGAGATTGCTGCTGCTTTCTGAGCCCGCA   2216

CCTGTGCGCCTAAACTGCTGATTGGCCTCAACTTGCCCAGGCGGACGGAGGGAGGCACCCGGCCGGCTGGACCTAATCTGGGATCGCGGTGATTTGCA   2315

CCGTGGAAAAGAAATTGCAGATTGATCATG--3'  2345
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Jolla, CA, U.S.A.). [α-^{32}P]dCTP (300 μCi/mmol) was from ICN (Costa Mesa, CA, U.S.A.). Positively charged Nylon membranes (Hybond N^+) were from Amersham Life Science (Amersham, Bucks, U.K.). Oligonucleotides, deoxyribonucleoside triphosphates (dNTPs) and Ready-to-Go labelling mixtures were obtained from Pharmacia Biotech (Uppsala, Sweden). The PinPoint expression system was from Promega (Madison, WI, U.S.A.).

Isolation of enzymes and preparation of antibodies

Purification of rat liver pristanoyl-CoA oxidase and preparation of antibodies were performed as described previously [6]. Antibodies against the C-terminal SKL peroxisome targeting signal [18], human branched-chain acyl-CoA oxidase [19] and the 52 or 21 kDa subunits of rat palmitoyl-CoA oxidase [6] were obtained as described previously.

Amplification of parts of the human pristanoyl-CoA oxidase gene

PCR on human liver RACE ready cDNA was carried out with a thermocycler (2400 system from Perkin–Elmer) in a reaction volume of 50 μl consisting of 50 ng of cDNA, oligonucleotide hp1 (0.4 μM; for the designation of oligonucleotides, see Figure 1), oligonucleotide hp2 (0.4 μM), 20 mM Tris/HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, dNTPs (200 μM each) and 2.5 units of *Taq* polymerase. Amplification was started with 5 min of incubation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 2 min annealing at 58 °C and 3 min extension at 72 °C. The reaction was completed by 7 min of incubation at 72 °C. PCR fragments were analysed in 1 % (mass/volume) agarose gels.

Cloning of human pristanoyl-CoA oxidase

cDNA clones for human pristanoyl-CoA oxidase were isolated by screening of 1×10^6 independent clones of a human liver λgt11 cDNA library. Plaques were lifted on Hybond N^+ membranes according to the protocol of the manufacturer. The hybridization probe was obtained by random-primed labelling of a *Bam*HI fragment from clone PCox38 (see [8]) with [α-^{32}P]dCTP by using Ready-to-Go labelling mixtures. Hybridization and washing of the membranes were carried out exactly as described previously [8]. Isolated phage, which were positive after three rounds of screening, were kept in SM buffer [0.1 M NaCl, 10 mM $MgSO_4$, 7 H_2O, 50 mM Tris/HCl, pH 7.5, and 0.01 % (mass/volume) gelatin] at 4 °C until further use.

λgt11 phage inserts from positive clones were amplified by PCR. A single plaque was picked from a plate with freshly plated phage by a pipette tip, and transferred to a tube containing 100 μl of SM buffer. A 10 μl portion of this solution was used in a standard PCR mixture of 100 μl containing 2 μM λgt11-specific primers (see Figure 1), 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl, pH 8.3, dNTPs (2 mM each) and 2.5 units of *Taq* polymerase. Inserts were amplified by 5 min of incubation at 94 °C (hot start), 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 54 °C and 2.5 min extension at 72 °C, followed by 7 min at 72 °C. Longer inserts (> 1.8 kB) could only be amplified by transferring the picked plaque directly in the complete PCR mixture and using the Expand High Fidelity DNA polymerase from Boehringer. Amplified inserts were directly sequenced by the dideoxy chain-termination method [20] with vector and rat or human pristanoyl-CoA oxidase-specific FITC-labelled primers (see Figure 1). Sequencing was carried out with the ALF sequencer and the cycle sequencing kit from Pharmacia. Before sequencing, the PCR fragments were purified with the Wizard DNA clean-up system from Promega. The insert of phage clone HPCox11 was subcloned in the pAMP vector from Gibco–BRL, after amplification with uracil-containing λgt11-specific primers, according to the protocol of the supplier (clone pAMPhp11). This clone was sequenced several times in both directions on the ALF with the Autoread sequencing kit (Pharmacia).

Bacterial expression of human pristanoyl-CoA oxidase

In order to express human pristanoyl-CoA oxidase, a cDNA fragment containing the complete open reading frame of clone HPCox11 was subcloned in the Xa2 expression vector from Promega (PinPoint expression system). The fragment was generated by PCR with a forward primer (hphind, see Figure 1) containing an artificial *Hin*dIII restriction site just one base in front of the ATG start codon. PCR was carried out in a mixture consisting of 100 ng of pAMPhp11 DNA, 1 μM forward primer hphind (containing a *Hin*dIII restriction site), 1 μM M13 reverse primer (pAMP-specific primer), 2.5 mM $MgCl_2$, 10 mM Tris/HCl, pH 8.3, 50 mM KCl, dNTPs (2 mM each) and 2.5 units of Expand High Fidelity DNA polymerase. Amplification was started by 5 min of incubation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C, 1.5 min extension at 72 °C, and ended by 7 min at 72 °C. After clean up of the PCR fragment with the Wizard DNA clean up system, the fragment was digested with *Hin*dIII and *Kpn*I and ligated to the *Hin*dIII-*Kpn*)-digested Xa2 expression vector. Transformants were checked by restriction enzyme analysis and DNA sequencing. Expression and analysis of the biotinylated fusion protein were performed according to the manufacturer's instructions.

SDS/PAGE and Western blotting

Before PAGE, proteins were solubilized in a buffer containing 0.5 % (mass/volume) SDS. Solubilized proteins were then electrophoresed in a normal (Pharmacia Biotech) or mini (Bio-Rad, Richmond, CA, U.S.A.) vertical gel system as described by Laemmli [21]. After electrophoresis the proteins were semi-dry transferred to nitrocellulose (0.45 μm) [22] and briefly visualized by Ponceau S staining [23].

For immunostaining the membranes were completely destained in 2 mM NaOH and blocked for 1 h with 5 % (mass/volume)

Figure 1 cDNA and deduced amino acid sequence of human pristanoyl-CoA oxidase

Nucleotides are numbered in the 5′ to 3′ direction, starting at the start codon. The 5′ untranslated region is indicated by negative numbers. The stop codon is marked by ***. The part of the sequence that overlaps with the different ESTs (AC: AA207238, Z44451, Z45527, T78298, F12169, T65260) is underlined. These ESTs were obtained after screening of the EMBL databank by running the FastA option [25] from the Genetics Computer Group (GCG) package at the EMBL node in Brussels, with the rat pristanoyl-CoA cDNA as query sequence. Primers used for subcloning experiments, PCR and sequencing were: 5′-gt11, f-TGGCGACGACTCCTGGAGCCCG; 3′-gt11, f-CTGGTAATGGTAGCGACCGGCGC; hphind, CCGGCCGAAGCTTGATGGCATCCACTGTGGAAGGAGG (*Hin*dIII site underlined); 3′-gt11-pAMP, CXACXACXACXAGACCAACTGGTAATGGTAGC (x = uracil); 5′-gt11-pAMP, CAXCAXCAXCAXATATCGACGGTTTCCATATG; oligonucleotide hp1, 1355–1375 (sense); oligonucleotide hp2, 1937–1954 (antisense); oligonucleotide hp3, 1083–1108 (antisense); oligonucleotide hp4, 1123–1143 (antisense); oligonucleotide hp5, 200–224 (antisense); oligonucleotide hp6, 1903–1927 (antisense); oligonucleotide hp7, 1701–1725 (sense); oligonucleotide hp8, 505–528 (antisense); oligonucleotide hp9, 548–572 (antisense): oligonucleotide hp10, 970–991 (sense); oligonucleotide hp11, 1355–1375 (antisense); oligonucleotide hp12, 1687–1709 (antisense); oligonucleotide hp13, 72–93 (sense); oligonucleotide hp14, 2220–2245 (antisense); oligonucleotide hp15, 661–683 (antisense): oligonucleotide hp16, 1456–1479 (antisense). The FITC label is indicated by f.

milk powder in TBST [150 mM NaCl, 65 mM KCl, 25 mM Tris/HCl, pH 7 and 0.05 % (v/v) Tween 20]. Then the membrane was incubated with the primary rabbit antibody, diluted in 1 % (mass/volume) milk powder in TBST, for another hour. After extensive washing with TBST, the blot was incubated with an anti-rabbit antibody coupled to alkaline phosphatase diluted in 1 % (mass/volume) milk powder in TBST. Finally, the immune complexes were visualized by staining with Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Screening of a bacterial artificial chromosome (BAC) library and fluorescent *in situ* hybridization (FISH)

For the genomic assignment of the human pristanoyl-CoA oxidase gene by FISH, a genomic clone (BAC) was isolated which was subsequently used as a probe. High-density filters with a human BAC library (Research Genetics, Huntsville, AL, U.S.A.) were hybridized with a radioactively labelled *Hin*dIII–*Kpn*I fragment of cDNA clone HPCox11 (see above) according to standard hybridization methods. One BAC clone, 267H3, was isolated and its identity confirmed by direct cycle sequencing (Thermosequenase Cycle Sequencing kit from Amersham) of the BAC DNA.

After labelling of the BAC probe with FITC-labelled dUTP, FISH was performed on metaphase and prometaphase spreads of normal white blood cells as described previously [24].

RESULTS AND DISCUSSION

To discover whether human ESTs were available that showed significant homology to the cDNA for rat pristanoyl-CoA oxidase, the EMBL databank was screened with the FastA option [25] from the GCG package at the EMBL node in Brussels. Several highly homologous (around 75 %) cDNA sequences were obtained, which partly overlapped the sequence in question (Figure 1). The existence of these ESTs was an indication that, despite the fact that pristanoyl-CoA oxidase cannot be detected in human tissues, a gene for pristanoyl-CoA oxidase might be present in the human.

In order to obtain the complete cDNA derived from a putative gene for human pristanoyl-CoA oxidase, we initially performed PCR on human liver RACE ready cDNA with EST-specific primers. With oligonucleotides hp1 and hp2 (see Figure 1) we were able to amplify a fragment of 500 bp that showed 100 % identity with the EST between these two primers (results not shown). RACE ready cDNA was used since it also allows amplification of regions of unknown sequence in both 5′ and 3′ direction. PCR with primer hp2 in combination with the 5′ anchor primer and primer hp1 in combination with the 3′ anchor primer, however, did not result in any fragments. The 5′ and 3′ parts are probably too long to be amplified efficiently. We therefore decided to screen a human liver λgt11 cDNA library with ^{32}P-labelled cDNA from rat pristanoyl-CoA oxidase.

Of the initially 1×10^6 clones, ten appeared to be specific after three rounds of screening. cDNA inserts from positive phage clones were amplified by PCR with vector-specific primers. Sequencing of the PCR products, either directly or after subcloning in an appropriate vector, showed that the isolated clones were highly homologous to the rat pristanoyl-CoA oxidase cDNA. One of the clones (HPCox11) contained a complete open reading frame of 2100 bases encoding a protein of 700 amino acids (Figure 1). Comparison of its amino acid sequence with that of rat pristanoyl-CoA oxidase (Figure 2) showed 75 % amino acid identity, indicating that the enzyme encoded by the isolated cDNA presumably represents the human homologue of rat pristanoyl-CoA oxidase. We therefore, decided to call the protein human pristanoyl-CoA oxidase.

Amino acid identities of human pristanoyl-CoA oxidase with the other human acyl-CoA oxidases, palmitoyl-CoA oxidase and branched-chain acyl-CoA oxidase, are 20 and 22 % respectively. Human pristanoly-CoA oxidase ends with the well-known peroxisomal SKL-terminal targeting sequence, indicating that the enzyme is indeed a peroxisomal protein and that its apparent absence from human tissues cannot be explained by a defective import into the peroxisome. It should be noted that several peroxisomal matrix proteins, including the β-oxidation enzymes, are rapidly degraded in the cytosol when they are not imported into the peroxisome [26].

The cDNA of human pristanoyl-CoA oxidase did not reveal premature stop codons or large deletions that could result in a truncated (and possibly inactive) enzyme. In order to confirm that the isolated cDNA codes for the whole protein, we expressed the cDNA in bacteria. By means of PCR, with appropriate primers, we generated a DNA fragment containing the complete open reading frame of human pristanoyl-CoA oxidase. After subcloning of this fragment in the PinPoint expression vector (Xa2), the protein was expressed in *Escherichia coli*. The bacterially expressed fusion protein had the expected size of 80 kDa (70 kDa plus the 13 kDa fusion part; Figure 3a, lane 2) and cross-reacted with the antibody against rat pristanoyl-CoA oxidase (Figure 3b, lane 2) but not with those against human branched-chain acyl-CoA oxidase or the 21 and 52 kDa subunits of palmitoyl-CoA oxidase (results not shown). In addition, the fusion protein reacted with an antibody directed against the C-terminal SKL peroxisomal targeting sequence (Figure 3c, lane 2) confirming that the protein did not end prematurely. We also investigated whether the fusion protein was enzymically active. The protein did not show any enzymic activity towards 2-methylpalmitoyl-CoA (results not shown), probably because of incorrect oligomerization [6].

By means of genomic analysis we obtained further evidence that a gene for pristanoyl-CoA oxidase is present in the human. A genomic clone (BAC 267H3) was isolated by screening of a BAC library with the complete cDNA of human pristanoyl-CoA oxidase as a probe. Sequencing of this clone with several human pristanoyl-CoA oxidase-specific primers revealed the position of four introns (see Figures 1 and 4). Intron I, situated between cDNA positions 144 and 145, was partially sequenced from both directions (397 bp). The three other introns (II–IV) were localized between cDNA positions 543 and 544, 1300 and 1301 and 1983 and 1984 respectively (see Figures 1 and 4). They were sequenced only partially in the 5′ direction. All intron/exon junctions conformed to the general splice-site rules. PCR on rat genomic DNA with two rat pristanoyl-CoA oxidase-specific primers (rp13 and rp14, see ref. [8]) showed that, in the rat pristanoyl-CoA oxidase gene, an intron is situated at the same position as intron I in the human gene (results not shown). The length of the intron is 300 bp. Obviously, the intron/exon organization of the rat and human pristanoyl-CoA oxidase genes is almost identical. as is also seen for the genes for rat and human palmitoyl-CoA oxidase [14–16,27]. FISH with BAC 267H3 showed clear signals on both chromosome 4pter-p15 regions in 80 % of the metaphases (Figure 5). Analysis of prometaphase chromosomes refined the assignment to 4p15.3 (Figure 5, inset).

The fact that the transcription and translation product of the gene has not been detected in the human remains a puzzle [6,8,12]. We repeated several of our earlier immunoblotting experiments [6], but pristanoyl-CoA oxidase was not detectable in either human liver homogenates or purified human liver peroxisomes (results not shown). Although this is not final proof

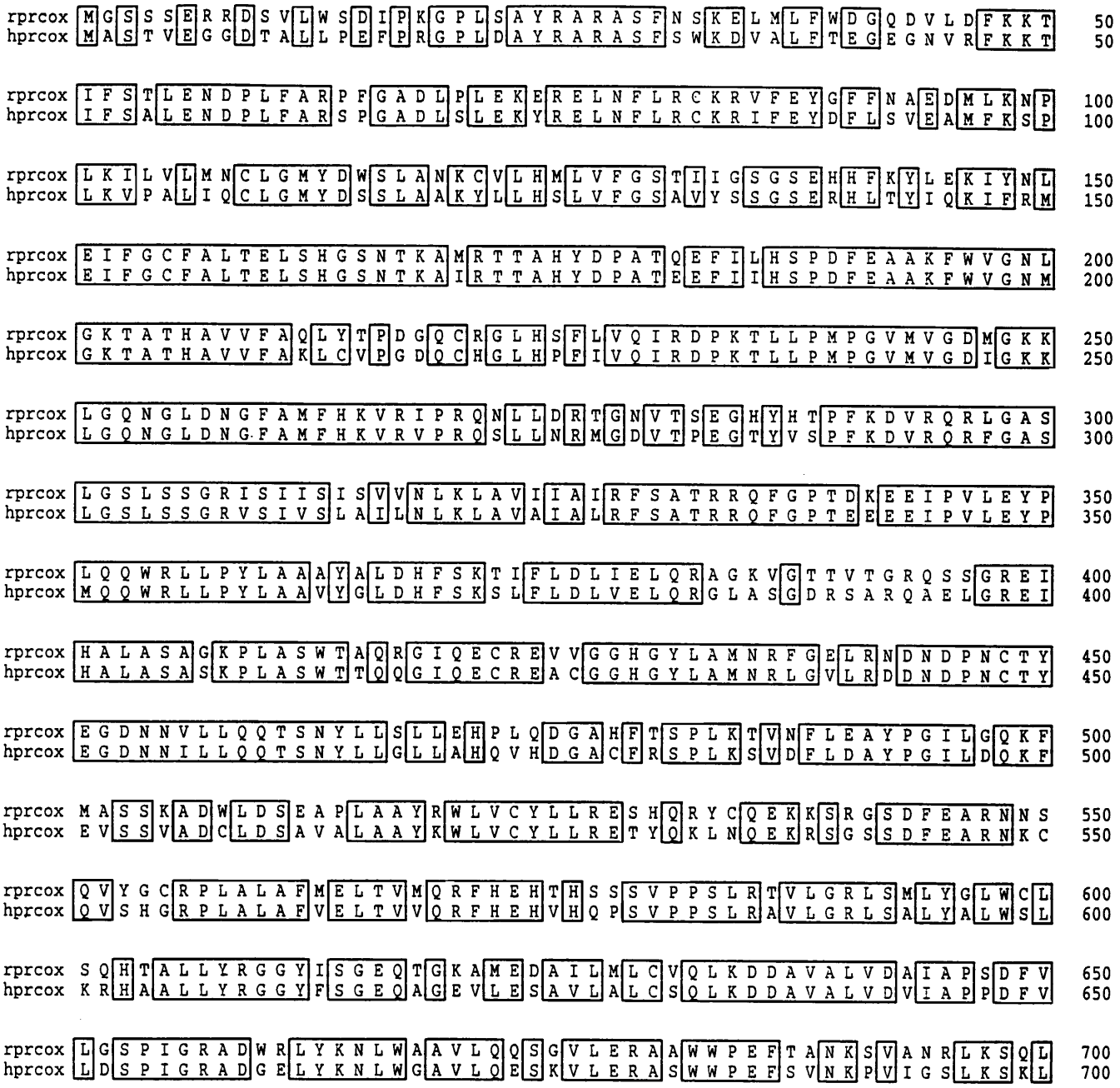

Figure 2 Amino acid comparison of rat and human pristanoyl-CoA oxidase

Rat and human pristanoyl-CoA oxidase (rprcox and hprcox respectively) were aligned by the PileUp option of the Genetics Computer Group (GCG) package at the EMBL node in Brussels. Identical and conserved amino acids are boxed. The numbers on the right represent amino acid positions.

of the absence of the protein from the human, the data suggest that, if the protein is present, it must be in very small amounts. We also repeated some of the column separations starting from partially purified human acyl-CoA oxidases [12]. Again, the protein remained undetectable (results not shown).

We conclude that a gene for pristanoyl-CoA oxidase is present in the human genome. The gene appears to be expressed to such a low extent, at least in liver, that its mRNA cannot be detected by routine Northern-blot analysis, and that its product remains undetected by standard immunoblotting or by enzyme activity measurements. The human pristanoyl-CoA oxidase cDNA does not display large deletions, frame shifts or premature stop codons that could result in a truncated and possibly inactive enzyme. In addition, the cDNA codes for a functional c-terminal peroxisomal targeting signal so defective import into the peroxisome is highly unlikely as an explanation for the apparent absence of the protein. One must keep in mind, however, that the human branched-chain acyl-CoA oxidase has taken over the function of pristanoyl-CoA oxidase in human tissues. It is therefore questionable whether there is a physiological role for human pristanoyl-CoA oxidase, at least under normal conditions. The possibility remains that the enzyme is expressed only under

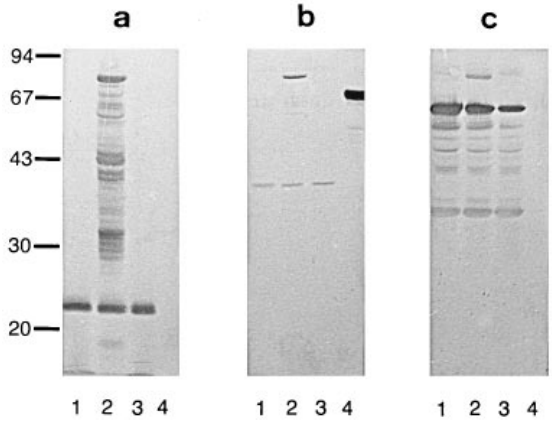

Figure 3 Analysis of bacterially expressed human pristanoyl-CoA oxidase

The cDNA encoding human pristanoyl-CoA oxidase was subcloned in the PinPoint expression system (Promega) as described in the Materials and methods section. All three expression vectors (Xa1, Xa2 and Xa3) were used. In vector Xa2 the cDNA was subcloned in the correct reading frame, and in the other two vectors the reading frame was shifted by one base. In lanes 1, 2 and 3 proteins obtained from cells transfected with the Xa1, Xa2 and Xa3 vector respectively were electrophoresed. In lane 4, 100 ng of purified rat pristanoyl-CoA oxidase was loaded. After blotting, immunostaining was performed with a streptavidin–alkaline phosphatase conjugate (**a**), affinity-purified rat pristanoyl-CoA oxidase antibody (**b**) and an antibody directed against the SKL tripeptide (**c**). In lane 4 of (**c**) one can see that the latter antibody is specific for SKL. Purified rat pristanoyl-CoA oxidase, which has the tripeptide SQL at its C-terminus, showed no cross-reactivity. Numbers on the left indicate molecular mass (in kDa) of the marker proteins.

special circumstances, e.g. at certain developmental stages, or in specialized tissues not examined thus far. This would be supported by the ESTs, which were derived from brain and fetal liver/spleen tissues.

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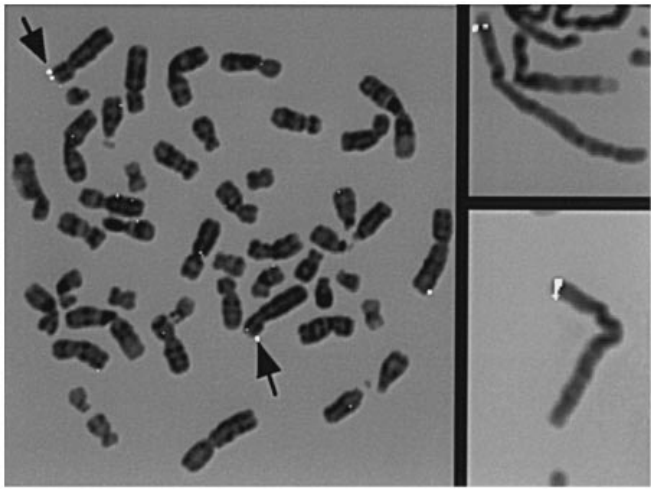

Figure 5 Regional assignment of human pristanoyl-CoA oxidase by FISH

BAC 267H3, carrying the human pristanoyl-CoA oxidase gene, was labelled with FITC and hybridized to metaphase and prometaphase spreads on normal white blood cells. The arrows indicate the signal on distal 4p. The insets show prometaphase chromosomes, with fluorescent signals on 4p15.3.

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                  144                                          145
I      5′-CGC TTT AAG GTGAGGCGT--397 bp--CATTTCCAG AAA ACC ATC-3′
          Arg Phe Lys                                  Lys Thr Ile

                                                       544
II                                        5′-CTTGTGCAG GAA TTC ATC-3′
                                                       Glu Phe Ile

                                                        1301
III                                       5′-TTGTTGCAG  TG AAC CGG-3′
                                                         Asn Arg

                                                       1984
IV                                        5′-TGTTTGCAG CTC TAC AAA-3′
                                                       Leu Tyr Lys
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Figure 4 Partial structure of the human pristanoyl-CoA oxidase gene

A genomic clone, isolated as described in the Materials and methods section, for human pristanoyl-CoA oxidase was sequenced with several fluorescently labelled primers (see legend to Figure 1). With primer hp11 (sense) and hp5 (anti-sense) an intron was localized which was partially sequenced (I). Sequencing of the genomic clone with primers hp15, hp11 and hp14 revealed three other intron/exon junctions (II–IV respectively). All intron/exon junctions were flanked by conserved splice-site bases, which are double underlined. The intron sequence is single underlined. The numbers above the sequences indicate the last or first base of the corresponding exon (numbering according to Figure 1).

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