In Mouse α-Methylacyl-CoA Racemase, the Same Gene Product Is Simultaneously Located in Mitochondria and Peroxisomes*

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α-Methylacyl-CoA racemase, an enzyme of the bile acid biosynthesis and branched chain fatty acid degradation pathway, was studied at the protein, cDNA, and genomic levels in mouse liver. Immunoelectron microscopy and subcellular fractionation located racemase to mitochondria and peroxisomes. The enzymes were purified from both organelles with immunoaffinity chromatography. The isolated proteins were of the same size, with identical N-terminal amino acid sequences, and the existence of additional proteins with α-methylacyl-CoA racemase activity was excluded. A racemase gene of about 15 kilobases was isolated. Southern blot analysis and chromosomal localization showed that only one racemase gene is present, on chromosome 15, region 15B1. The putative initial ATG in the racemase gene was preceded by a functional promoter as shown with the luciferase reporter gene assay. The corresponding cDNAs were isolated from rat and mouse liver. The recombinant rat protein was overexpressed in active form in Pichia pastoris. The presented data suggest that the polypeptide encoded by the racemase gene can alternatively be targeted to peroxisomes or mitochondria without modifications. It is concluded that the noncleavable N-terminal sequence of the polypeptide acts as a weak mitochondrial and that the C-terminal sequence acts as a peroxisomal targeting signal.

α-Methylacyl-CoA racemase (Amacr) catalyzes the racemization of a wide spectrum of α-methyl-branched carboxylic acids coenzyme A thioesters (1, 2). The enzyme is thought to have its major physiological role in the biosynthesis of bile acids, because mitochondrial hydroxylation at C-26 of the cholesterol side chain affords specifically the (25S)-isomers (4, 5). Thus, inversion of the configuration at C-25 is required to connect the two pathways. THCA-CoA was shown to be racemized efficiently by Amacr (1). Other physiologic substrates are methyl-branched fatty acids of dietary origin such as pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), which is the α-oxidation product of phytanic acid, a metabolite of the chlorophyll component phytol (for review see Ref. 6), and possibly also endogenously synthesized isoprenoids. Amacr has also attracted the interest of pharmacologists for its participation in the biotransformation of 2-arylpropionic acids (2-methylarylacetic acids) used as nonsteroidal anti-inflammatory and analgesic drugs (Ibuprofen®, from the inactive (2R)- to the pharmacologically active (2S)-enantiomer (7)).

Side chain cleavage of bile acids as well as β-oxidation of methyl-branched fatty acids take place in peroxisomes (8, 9). However, Amacr activity is found to be distributed between peroxisomes and mitochondria in varying proportions, depending on the species. In human tissues, 80–90% of the activity is associated with peroxisomes (2), in line with its presumed function. In mouse and Chinese hamster, a roughly equal distribution between the two organelles is seen (10), whereas in rats, Amacr activity is found almost exclusively in mitochondria (1). The molecular basis of this distribution is not yet known. Only one cDNA sequence for the enzyme (cAMACR) has so far been found in mice, rats (10), and humans (11) alike. To gain insight into possible mechanisms governing this distribution, we isolated the enzymes from highly purified mouse liver mitochondria and peroxisomes, respectively, and compared their properties. At the genomic level, only one gene encoding Amacr (AMACR) was found and characterized. The data are discussed in view of the dual compartmentalization of the Amacr activity in mouse liver.

EXPERIMENTAL PROCEDURES

Materials—Inorganic salts were from Merck; sucrose (molecular biology grade) and Tris were from Applichem (Darmstadt, Germany); Zymolase (Lyticase®, EDTA, and Percoll were from Sigma; and morpholino propane sulfonic acid was from Aldrich. All other chemicals were of analytical grade or of the highest purity available. Ingredients for bacterial and yeast growth media were from Difco Laboratories (Detroit, MI). Restriction enzymes and the corresponding buffers were from MBI Fermentas (Heidelberg, Germany).

Nitrocellulose blotting membranes were from Schleicher & Schuell GmbH (Dassel, Germany). Radionucleic acids were purchased from Amer sham-Buchler (Braunschweig, Germany), from Hartmann (Bra unschweig, Germany), or from NEN Life Science Products.

Oligonucleotides were synthesized with an Applied Biosystems model 394 oligonucleotide synthesizer at the Department of Biochemistry (University of Oulu) or were purchased from Carl Roth GmbH &
Immunoelectron Microscopy—Livers were harvested from control and cobalftale-treated Balb/c mice (fed with 0.5% (w/w) cobalte-supplemented chow for 2 weeks) after vascular perfusion fixation (17). Small tissue blocks (~1 mm) were processed for thin sectioning by progressive lowering of temperature (18). Briefly, small tissue blocks were dehydrated in graded series of ethanol concurrent with decreasing temperature from 0 to ~70 °C before infiltration with Microbed resin (Electron Microscopy Sciences, Fort Washington, PA) at ~35 °C; tissues embedded in the same resin were then polymerized under UV light at ~35 °C for 6 h. For immunogold labeling, thin sections (80 nm) on nickel grids were incubated with a blocking medium containing 1% bovine serum albumin and 0.1% fish skin gelatin (Sigma) in phosphate-buffered saline and then exposed to polyclonal anti-Amacr antibody followed by washing in phosphate-buffered saline. Similar grids were incubated with catalase and mitochondrial reductase antibodies as controls for the labeling. Antibody distribution was identified by gold particles (20 nm) conjugated to protein A.

Protein N-terminal Sequence Analysis—The proteins were separated by SDS-PAGE in a 12.5% gel (19) and transferred by electroblotting onto a polyvinylidene difluoride membrane (ProBiot, Perkin-Elmer, Applied Biosystems Division, Foster City, CA) in 10 mM CAPS (pH 11/10) methanol (w/v) with a constant potential of 50 V for 120 min (20). After staining with Coomassie Brilliant Blue, the protein bands of interest were cut off and loaded into the sequencer. Protein N-terminal sequencing was performed with a Procise 494A sequencer (Perkin-Elmer, Applied Biosystems Division, CA).

cDNA Analysis—Rat liver total RNA (30 μg) was prepared from rat liver with the BiozumPUREscript RNA isolation kit (Biozym Diagnostik, Oldendorf, Germany), according to the instructions of the manufacturer. For the first strand synthesis of cAMACR, the Titan^™ One Tube reverse transcription-PCR system from Roche Molecular Biochemicals was used according to the instructions of the manufacturer, with 1 μl of 100 μM sequence-specific primer RRaceI (for oligonucleotide sequences see Table I). The first strands were polyadenylated and amplified by a PCR reaction using the polyT primer and primer HisR. The PCR products were purified, and the second-round PCR was performed with the same polyT primer and MR9 as nested cAMACR-specific primer. The purified PCR products were ligated into pCR2.1 with the Invitrogen original TA cloning kit (Invitrogen, De Schelp, Netherlands) and transformed into competent INV-α cells, following the instructions of the manufacturer. Plasmids from 50 colonies were isolated and treated with EcoRI. The size of the excised inserts was determined by agarose gel electrophoresis. The three largest inserts were sequenced, using plasmid-specific primers (M13fow and M13rev).

Expression of Recombinant Rat Liver Amacr—For amplification of the complete rat liver cAMACR, a PCR was performed, using 1 μl of rat liver Marathon-Ready cDNA (CLONTECH, Palo Alto, CA), primers RS-F and HisR, and Stratagene native Pfu DNA polymerase. The resulting product was purified from the agarose gel, cloned in pCR2.1, and transformed into INV-α. The corresponding plasmids were amplified, purified, and digested with NdeI. The insert was cloned into NdeI-digested and dephosphorylated pET3a (pET Expression System 3, Novagen, Madison, WI) and transformed into INV-α cells. Plasmids containing the insert in the right orientation were transformed into competent BL21(DE3)-LysS. Plasmid C1–9, containing the coding sequence for rat liver racemase, was produced in the same manner, except that oligo R-YN-2-Kozak was used as forward primer and oligo R-YC-wt as reverse primer. The resulting insert was isolated from a 1% agarose gel and ligated into NdeI-digested and dephosphorylated pET3a vector. The plasmid was amplified in INV-α cells, isolated, linearized with NotI, and transformed into Pichia pastoris strain GS115. The expression of recombinant Amacr from 90 MutX transformants was analyzed according the

Table I

| Name     | Sequence                                      |
|----------|-----------------------------------------------|
| ALE17    | 5'-ATG ACC CTA CCA CCG AAT-3'                 |
| ALE18    | 5'-TAA AGA CAT CCA TCG TTA-3'                 |
| ALE22    | 5'-TGA CAT CTA CTA TTT GGC TTT A-3'          |
| ALE28    | 5'-ACG GTG TGG CCG CAG GTG-3'                 |
| HisR     | 5'-CAT ATG CTC TCT CCA GGA CAG A-3'          |
| INT14    | 5'-GGA TGC ATC ACG AAT TCT CTT C-3'          |
| K100     | 5'-CAC ATC TCC AAA TTT CTT CTT C-3'          |
| K50      | 5'-CCC ATC AGT GAT GAA GAA GG-3'             |
| K51      | 5'-GCA AAG AAT GAG CAA GAA T-3'              |
| LO2      | 5'-ATG CCC ACC ATG TGG ATG AT-3'             |
| M13fow   | 5'-GAA CAA CCG CCA GGG AAA G-3'              |
| M13rev   | 5'-CAG GAA ACA GCT AGC AC-3'                 |
| M9R      | 5'-ATG TGA CTT TTG GAT TTT-3'                |
| polyT    | 5'-TTT TTG TTT TTG VV-3'                     |
| PR-3     | 5'-ACG CCT AGG AGA GCG GCC G-3'              |
| PR-4     | 5'-CTT CCT ACT GTT TAG ATC TTG TT-3'         |
| RaseI    | 5'-TTC TGC GGA ATG GCT GTC G-3'              |
| RaseII   | 5'-CAT TCG GTA TCA ATC TTA CCA TCC T-3'      |
| RS4.0    | 5'-ACA GGA AAG AAC TAG ATG C-3'              |
| RS-F     | 5'-CAT ATG ATC TGC GGG GAC TTT C-3'          |
| RU1.0    | 5'-GGA GAA CCC TCA ACC O-3'                  |
| RU3.0    | 5'-GGA CAT GTG CAG CTA CAG CAT-3'            |
| R-YC-1-Mut| 5'-GAA TTC TCA GAG TTT GAG TTT ATC TAG TT-3'|
| RY-C2-wt | 5'-GAA TTC TCA GAG TTT GGT TTT TAG CTT-3'    |
| RY-F-wt  | 5'-GAA TTC GGG TTT TAG GTG TCG CCA TGG-3'    |
| R-YN-2-Kozak| 5'-GAA TTC ATG GTG AAG GGA GTG CAG C-3'     |
| ST10.1   | 5'-ACA ACC TAG AAC AGA CCA GA-3'             |
| SR19.0   | 5'-TGA TTG GGA TAT GTG GTG GAC T-3'          |
| SR19.1   | 5'-TGT GAT GTG CTT GAT ATT T-3'              |

(20)
Isolation of the Amacr Gene (AMACR)—Genomic DNA was extracted from kidney of BALB/c mouse (21) and used as template in PCR. Amplification (Expand™ High Fidelity PCR system; Roche Molecular Biochemicals) with primers ST10.1 and SR19.1, resulted in a 5-kb fragment in the cloned into the vector pUC18 (Cloning kit; Amersham Pharmacia Biotech) and partly sequenced from the 5′ end. A pair of oligonucleotide primers, INT14 and ST10.1, was designed to give in PCR a 600-bp fragment from the exon-intron boundary. This set of primers was sent to Genome Systems Inc. (St. Louis, MO) for screening of the mouse ES-128/SvJ1 germline library. Received bacterial artificial chromosome (BAC) clones were BACM-235K4, BACM-13L1, and BACM-151H13. BAC DNA was isolated with the K-100 Magnum kit (Genome Systems) and digested with restriction enzymes HindIII and EcoRI. Digested DNA was subjected to Southern blotting (21), and the presence of AMACR in BAC clones was detected with three probes made by PCR and random prime labeling to correspond to the 5′ end of the mouse cAMACR with primers Rasel and RS4.0, the middle part with primers RU1.0 and K100, and the 3′ end with primers K51 and SR19.1.

Sequencing of 3′ end of the gene was done on the subcloned 2-kb HindIII fragment of clone BACM-13L1 with oligonucleotide primers SR19.1 and LO2. Upstream sequencing was done directly on the BACM-13L1 clone. Oligonucleotide primers used in sequencing were designed according to sequences obtained from previous reactions. Sequencing was performed on both DNA strands. PCR reactions were used to determine the size of the introns. Intron 1 was amplified with primers ALE28 and ALE18, intron 2 with ALE22 and ALE17, intron 3 with RU3.0 and RS4.0, and intron 4 with primers K50 and ST 10.1. In addition, introns 1 and 2 were sequenced through. DNA sequencing was done with an automated ABI Prism 377 DNA sequencer (Perkin-Elmer).

Chromosomal Localization—Chromosomal localization of the gene was detected by Genome Systems, Inc. The fluorescent in situ hybridization was carried out by using the digoxigenin-labeled BACM-13L1 clone as probe. Hybridization was done to normal metaphase chromosomes derived from mouse embryo fibroblasts.

Southern Hybridization—Genomic DNA was extracted from kidney of BALB/c mouse with the SDS-proteinase K method (21), and 33 μg of isolated DNA was digested with EcoRI or HindIII or with both enzymes. Southern blotting and hybridization was done on Hybond-N+ nylon membranes (Amersham Pharmacia Biotech) at 65 °C (21, 22) using 32P-labeled mouse cAMACR as a probe. The filter was washed twice before autoradiographing at −70 °C for 24 h (Kodak X-Omat AR).

Northern Hybridization—For Northern hybridization, a mouse Multiple Tissue Northern™ blot (CLONTECH) nylon membrane was used, on which 2-μg aliquots of poly(A+) RNA from various mouse tissues had been blotted. Hybridization was done according to the manufacturer’s instructions in ExpressHyb™ hybridization solution with 32P-labeled mouse cAMACR as probe. The hybridized fragments were autoradiographed at −70 °C for 72 h.

Peroxide Activity—Primers PR-3 and PR-4 were designed to amplify a 1670-bp-long piece of the 5′ noncoding region of AMACR by PCR (Pfu polymerase; Stratagene, La Jolla, CA). The amplified fragment was cloned into the Smal-digested pG3-LBasic reporter vector (Promega, Madison, WI). Correct orientation of the insert was ascertained by sequencing. HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s minimal essential medium containing penicillin (25 units/ml), streptomycin (25 units/ml), gentamicin (50 μg/ml), 1% (v/v) nonessential amino acids, and 10% (v/v) fetal bovine serum. Cells were transfected by using LipofectAMINE-transfection reagent according to the manufacturer’s instructions (Life Technologies Inc.). Briefly, 4 × 105 cells were plated onto a 60-mm dish 24 h before transfection with a solution containing 4 μg of luciferase reporter plasmid with amplified promoter or the same promoter region but in inverted orientation or 2 μg of luciferase reporter plasmid without promoter or 2 μg of pGL3-control vector (Promega). In addition, in all above-mentioned transfections, 0.6 μg of pSV-β-galactosidase control plasmid (Promega) was used as normalization vector. Cell extracts were prepared using reporter lysis buffer (Promega), and luciferase and β-galactosidase activities were measured according to the manufacturer’s instructions (CLONTECH) with Labsystems Luminoscan RS luminometer (Labsystems, Helsinki, Finland). All luciferase values were normalized with β-galactosidase activity in the same extract.

Sequence Analysis—DNA sequences were analyzed with the DNASIS program (Hitachi Software Engineering Co., Yokohama, Japan). Transcription factor binding sites were searched for by using a MatInspector manual of the manufacturer.

RESULTS

Subcellular Fractionation and Amacr Distribution—Mouse liver homogenate was subjected to subcellular fractionation on a Nycodenz gradient. As shown in Fig. 1 (lower panel), Amacr activity was found in three regions of different density. Two regions were found in the gradient, concomitantly with the marker enzymes for peroxisomes (catalase) as well as for mitochondria (succinate dehydrogenase), and one region was in the fractions floating at the top of the gradient. Furthermore, when subjecting the fractions to Western blot analysis using rabbit antibodies against rat liver Amacr, the intensity of the visualized bands correlated with the distribution of the Amacr activity. The size of the visualized polypeptides was the same in each region (Fig. 1, upper panel).

To study the subcellular distribution of the Amacr activity further, experiments were carried out with CHO fibroblasts. When control CHO fibroblasts were fractionated, a distribution of the Amacr activity similar to that in mouse liver was found (Fig. 2a). When the fractionation was carried out with CHO peroxisome-deficient mutant cells, which were devoid of peroxisomes, Amacr activity was still concomitantly with the mitochondrial fractions and showed the same specific activity as in the mitochondrial fractions of the control CHO fibroblasts (Fig. 2b). Neither particle bound catalase activity was found in these cells, in agreement with earlier findings (13), nor particle bound Amacr activity in the fractions with densities corresponding to the peroxisome-containing fractions of the control cells.

Immunoelectron Microscopical Investigations—For further investigation of the subcellular distribution of Amacr, sections of mouse liver were subjected to immunoelectron microscopy with the antisera against rat liver Amacr (2) using the protein A-gold labeling technique (Fig. 3). The gold particles were located in mitochondria and peroxisomes from control and clofibrate-treated mice (10 days) without any observed change in the labeling intensity. For control purposes, the tissue sections were treated with antibodies against rat 120-kDa ∆1-Δ2-dienoyl-CoA reductase (12) and rat catalase for labeling mitochondria and peroxisomes, respectively. In both cases, the antibody recognized the expected compartment as shown in Fig. 3.

Immunopurification of Mitochondrial and Peroxisomal Mouse Liver Amacr—An affinity column was prepared with the rabbit antibodies against Amacr (12). When the soluble extract of ultrasonicated mouse liver homogenate was applied to the column, all Amacr activity was bound to the column, and none could be detected in the flowthrough fractions (data not shown), documenting that the antibodies used recognize all soluble Amacr(s). Subsequently, peroxisomes and mitochondria were isolated from mouse liver on a self-generating Percoll gradient (Fig. 4a), and the soluble extracts were applied separately to the
immunoaffinity column. More than 99% of the Amacr activities were retained on the column.

When the bound proteins from each of the experiments were eluted with acidic buffer and subjected to SDS-PAGE and Western blot analysis, protein staining of the SDS-PAGE gels showed one major protein band in each lane (Fig. 4b), at a position corresponding to the size of the purified Amacr protein (42 kDa). In Western blots, the immunologically cross-reactive bands showed the same size as that from mouse liver homogenate (not shown but see compare with Fig. 1). No Amacr activity could be detected in this eluate. When alkaline buffer instead of acidic buffer was used for elutions, SDS-PAGE and Western blot analysis revealed similar results, and also Amacr activity was detectable, albeit very low, (0.83 and 0.42% of the applied activity for mitochondrial and peroxisomal fractions, respectively). This showed that mitochondrial and peroxisomal Amacrs are equally well bound to and released from the affinity column but are rapidly inactivated under the harsh conditions employed. Similarly, the cytosol (supernatant of the differential centrifugation at 12,000 × g) was applied to and eluted from the affinity column with comparable results.

The immunoisolated proteins of the alkaline elution were, after SDS-PAGE, blotted on a polyvinylidene difluoride membrane, and the Amacr bands were excised and subjected to N-terminal amino acid sequencing. Together with the initial Met and additional 21 new amino acid residues, the open reading frame of the revised cAMACR encodes a polypeptide of 381 amino acid residues with a predicted molecular mass of 41,718 Da.

The Western blot analysis of both rat and mouse Amacr demonstrated that they are of the same size (10). These data, together with the revised mouse cAMACR, suggest that also the earlier published cAMACR for rat liver racemase was not complete either. To investigate this, the reverse transcription-PCR with rat liver total RNA as template was carried out, using RRacaseI for first strand synthesis and then HisR and polyT as primers, followed by nested PCR with M9R and polyT. A cDNA fragment of 570 bp was obtained; nucleotide sequencing of this revealed additional 246 bp, preceding the 5′ end of the hitherto published rat liver cAMACR (10). The extended rat liver cAMACR includes an ATG as putative initial codon, followed by an open reading frame that corresponds to the one for mouse liver (Fig. 5). The identity between the rat and mouse liver Amacrs was 89 and 90.7% at the amino acid- and nucleotide-coding region level, respectively.

The open reading frame of rat liver cAMACR was cloned into the EcoRI sides of the pHL-D2 expression vector, transformed into P. pastoris and overexpressed. The Amacr activity, measured with [2-3H]pristanoyl-CoA as substrate, was 0.5 nmol × min⁻¹ × mg⁻¹ in the soluble supernatant of overexpressing cells but was below the detection limit (10⁻¹⁷ nmol × min⁻¹ × mg⁻¹) in uninduced yeast cells. The immunoblotting analysis of the soluble fraction from overexpressing yeast cells and from rat liver homogenate showed that the detected polypeptide band was of the same size as that found in rat liver.

**Searching for Mouse AMACR**—Screening of the mouse genomic library ES-129/SvJ with the obtained random primed mouse cAMACR (corresponding to nucleotides 2–1503) (10) did not result in correct positive clones. The first exon-intron junction (later identified as exon 4/intron 4 junction) was found by PCR amplification with the primers ST10.1 and SR19.0 using genomic DNA extracted from BALB/c mouse.
kidney as template. After subcloning, the obtained 5-kb fragment was partially sequenced, and it was found that it contained both coding and intronic sequences. The primers INT14, derived from the intronic sequence, and ST10.1 were used for screening and subsequently for the isolation of three positive clones (BACM-235K4, BACM-13L1, and BACM-151H13) from genomic mouse libraries at GenomeSystems, Inc.

Characterization of the Genomic Clones—

The BACM-13L1 clone was digested with HindIII, and the fragments were ligated to pUC18, transformed into *Escherichia coli*, and screened with the mouse cAMACR probe. Among the positive clones, one containing an insert of about 2 kb was selected for further analysis.

**FIG. 4.** Analysis of mitochondrial and peroxisomal Amacr from mouse liver. A postnuclear supernatant of mouse liver homogenate was fractionated on a self-forming Percoll gradient as described under “Experimental Procedures.” The tubes were fractionated starting from the bottom (fraction 1) toward the top (fraction 30). a, marker enzymes catalase (■) and succinate dehydrogenase (●) were assayed in all fractions. The fractions denoted with bars were combined as mitochondrial and peroxisomal fractions, respectively. Amacr protein was isolated from each pool by immunoprecipitation chromatography as described in the text. b, SDS-PAGE of elution fractions 1–4 from immunoprecipitation column with mitochondrial fractions. The horizontal line indicates the position of the Amacr protein.

**FIG. 5.** Complete rat and mouse cAMACRs and their deduced amino acid sequences. Nucleotides are numbered in the 5′ to 3′ direction, beginning with the first nucleotide of the initiation methionine codon as +1. The positions of the introns within the mouse gene are indicated with >.<. Polyadenylation signals are double underlined. The amino acid residues are numbered from the initiation methionine residue as +1. Conservative amino acid replacements are underlined, and white letters on black denote amino acids with different properties in corresponding positions. The mouse sequence upstream of position –26 was not from cDNA but was obtained by sequencing of genomic DNA and is given for comparison with the rat cDNA 5′-UTR. Identical bases are shaded.
was isolated and sequenced and was found to contain the sequence identical to the 5' end of cAMACR up to the poly(A) tail. 766 nucleotides toward the 5' end, the sequence was interrupted by intronic sequences. This intron/exon junction was confirmed by direct sequencing of the BACM-13L1 clone from the 5' direction. Further direct sequencing of the BAC clone toward the 5' end revealed that AMACR contains three additional introns (Fig. 5). All exon-intron boundaries followed the GT-AG rule (Table II). The sizes of the introns 1–4 were 1.3, 1.2, 4, and 5 kb, respectively, as determined with PCR (Table II), and the AMACR gene spans a stretch of 13 kb in the mouse genome (Fig. 6). The characterized gene included with full match the sequence of EST clone AA085247. From the putative initial ATG toward the 5' direction, 1680 bp were sequenced. A GC-rich region was located at the positions –53 to –90 that can serve as an SP1-binding site (Fig. 7). No TATA box was present, but other consensus sequences for transcription factors including sites for GATA1, NF1, AP-1, AP-2, and AP-4 were found.

When the 245 bp preceding the start codon were compared with the 5'-UTR of rat Amacr cDNA, a fairly good similarity (81.4%) was found, assuming a 148-bp deletion in the rat sequence between bases –15 and –16 and four smaller deletions (altogether 43 bp) further upstream in the mouse sequence (Fig. 5).

Promotor Analysis—To test the functionality of the genomic fragment between the base pairs –1670 to –1 as a promotor, it was cloned and ligated to the 5' end of the luciferase gene in the pGL3-Basic reporter vector. Transient transformation of human HepG2 cells with this construct exhibited luciferase values that were 135 S.D.; n = 10) higher than those obtained with the empty reporter construct, indicating that the fragment encompasses sequences that can function as promotor elements in intact cells.

Chromosomal Localization of the Mouse Amacr—To determine the chromosomal localization of the mouse AMACR, the BACM-13L1 clone was labeled and hybridized on metaphase chromosomes derived from mouse embryo fibroblasts. The identification of the mouse chromosomes was based on their G banding pattern. In addition, mouse chromosome 15 was identified with a probe specific for the telomeric region of that chromosome. Only double spot signals were considered as specific hybridization signals. Of 80 metaphase spreads, 73 showed the mouse racemase gene location on chromosome 15, region 15B1 (Fig. 8).

Southern and Northern Blotting—When genomic mouse liver DNA was digested with EcoRI or HindIII or both enzymes and the products were analyzed by Southern blot hybridization with the mouse cAMACR as probe, two fragments were detected in the case of EcoRI digestion with sizes of approximately 13 and 5 kb. Digestion with HindIII revealed five bands of approximate sizes of 6.2, 5.8, 4.0, 2.3, and 1.7 kb. The double digestion yielded five fragments with approximate sizes of 6.0, 4.0, 2.1, 1.7, and 0.9 kb (Fig. 9). This restriction fragment pattern and the restriction map constructed by the DNASIS program (Fig. 6) suggest that the size of AMACR is approximately 15 kb.

For Northern blotting, 2-μg aliquots of mRNA, isolated from various mouse tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis), were hybridized with the mouse cAMACR as probe, as described under "Experimental Procedures." A signal corresponding to a size of 1.9 kb was detected in the samples from liver and kidney tissues (Fig. 10A). Northern hybridization with a β-actin probe showed that there were two signals of β-actin in heart and skeletal muscle, namely a 2-kb one and slightly smaller one of 1.6–1.8 kb (25, 26), as well as a weaker one in testis. The other tissues have one mRNA of approximately 2 kb (Fig. 10B).

**DISCUSSION**

The present work demonstrates that the mouse genome contains only one AMACR, encoding a polypeptide that can be targeted alternatively into mitochondria or peroxisomes, the subcellular compartments containing active Amacr. This statement is based on the combination of observations at the genomic and cDNA level and on the exclusion of other proteins than Amacr with Amacr activity in mouse liver.

Fluorescence in situ hybridization experiments performed on mouse metaphase chromosomes visualized only one region for AMACR (chromosome 15, region 15B1). The analysis of EcoRI/HindIII digests of genomic DNA with Southern blotting gave an estimated gene size of 15 kb. This size is sufficient to accommodate the complete AMACR, whose size was about 13.5 kb, when estimated by direct sequencing and PCR analysis of the mouse genomic BACM-13L1 clone. These data indicate that the haploid mouse genome contains only one copy of AMACR.

By screening of mouse EST libraries and PCR analysis or screening of mouse liver cDNA libraries (10), one cAMACR could be identified, and no other sequences with significant similarities were found. Furthermore, Northern blot analysis of mouse liver and kidney RNA visualized only one signal of about 1.9 kb, in good agreement with the full size of the mouse cAMACR. The cAMACR identified in this study can be assumed to be complete because: (i) the genomic fragment preceding the open reading frame (−1670 to −1) is a functional promotor, as shown by the luciferase reporter gene assay, (ii) when the 5'-flanking nucleotide sequence was translated in the frame, 22 stop-codons were encountered, and (iii) the identified putative initial ATG was embedded in a Kozak consensus sequence.

In accordance with the finding of the existence of one AMACR and Amacr mRNA, only one gene product was found. As shown by Western blot analysis of tissue homogenate, cytosol, and purified mitochondria and peroxisomes, in all cases only one protein band was visualized, corresponding to the same molecular mass. The N-terminal amino acid sequencing of the Amacr proteins, immunoisolated from cytosol and purified mitochondria and peroxisomes, resulted in NAmacr, which matched fully with the amino acid sequence predicted by the
cAMACR minus the leading methionine. This experiment, together with the genomic and cDNA analysis, showed that the Amacr polypeptides recognized by the anti-Amacr antibody in different subcellular compartments were the same. When applying mouse tissue extracts to the affinity column prepared with the anti-Amacr-antibody, the Amacr activity was completely removed from the flowthrough. This excludes the possibility of the existence of Amacr activities not detectable by the antibody.

The enzyme activities of acyl-CoA degradative pathways generally have multiple subcellular localization as exemplified by the mitochondrial and peroxisomal β-oxidative enzymes. The proteins of these different pathways are encoded by separate genes and are often presented as paralogs, even in the same compartment. The Amacr is exceptional not only among the acyl-CoA metabolizing gene products but also in general, because unlike other enzymes, which are modified when present in different compartments, the same Amacr can be targeted to two subcellular compartments. In most cases, proteins with different compartmentation were found to acquire different localization signals by alternative transcription or splicing or, in some cases, different translation start points or proteolytic modifications (for review see ref. 27). No indication for any of these processes was found for the Amacr. Another known dual locating mammalian protein metabolizing CoA thio esters in mitochondria and peroxisomes is the D3,5-D2,4-dienoyl-CoA isomerase. In this case however, an N-terminal amino acid sequence is cleaved off upon mitochondrial targeting, whereas the peroxisomal targeting occurs via the C-terminally located peroxisomal targeting signal type 1 (PTS1). As a consequence, the mitochondrial polypeptide is some 4 kDa smaller than the peroxisomal isoform (17).
An intriguing question is which factor determines the alternative targeting of Amacr either to mitochondria or to peroxisomes. Amacr contains the polypeptide -KANL at the C terminus, which has previously been shown to act as functional PTS1 in catalase (28). The helical wheel of the N terminus of Amacr shows the typical features of a mitochondrial targeting signal (MTS), but it contains a negatively charged residue (glutamate) at the position 11. Although uncommon in MTSs, a negative charge is sometimes accepted; the \( \beta \) subunit of the human pyruvate dehydrogenase has even two glutamates in positions 10 and 14 (29). Conceivably, the Amacr MTS is only poorly recognized by the mitochondrial import machinery, allowing the folding of the protein in the cytoplasm. In this case, the C-terminal PTS1 will, after completion of synthesis, be recognized by the PTS1 receptor Pex5p, and the enzyme will be imported into the peroxisome. The quality of the MTS and the frequency of its recognition by the mitochondrial import system would then determine the relative distribution of the Amacr protein between the mitochondrial and peroxisomal compartments.

This model would also explain why in cells from patients with Zellweger syndrome, which are devoid of functional peroxisomes, Amacr activity is reduced to the 10–20% normally found in mitochondria (2). Any Amacr protein not sequestered by the mitochondrial import system early in its synthesis could not be imported into mitochondria later but would remain in the cytosol to be degraded rapidly.

During the past few years, data have been emerging that provide an alternative route to the direct targeting of enzymes from the cytoplasm to their final destination. Evidence was presented that some proteins are imported into mitochondria and then re-exported and transferred to other compartments (for review see Ref. 30). It cannot be excluded that the Amacr protein follows a similar route. Because its MTS appears to be one of the few that are not cleaved inside the mitochondria, the sizes of the differently located proteins do not yield any information on the possible pathways taken. The transgenic mouse...
model being developed in future should also provide a possibility to study the influence of the different localization signals, after transfection with native and mutated CAMACRs.

The physiological role of Amacr in mammalian peroxisomes can be easily inferred. As indicated in the introduction section, it is required in the bile acid synthesis from THCA-CoA and the degradation of pristanoyl-CoA, processes occurring in peroxisomes. In contrast, the physiological significance of the mitochondrial Amacr activity is still unclear. There is evidence that \( \beta \)-oxidation of branched chain fatty acids in peroxisomes does not go to completion but stops at 4,8-dimethylnonanoic acid, which, after conjugation with carnitine, is imported into mitochondria (31). Because phytanic and pristanic acids have the \((R)\)-configuration at the inner methyl branch points (32) and the \( \alpha \)-methylacyl-CoA dehydrogenases in mitochondria, like the peroxisomal oxidases, appear to be specific for the \((2S)\)-enantiomers (14), the racemase would be required in mitochondria, too.

Additional physiological functions of Amacr in the metabolism of other, as yet unknown, endogenous substrates must also be considered. Patients with inborn defects of the \( \beta \)-oxidation pathway of branched chain fatty acids, e.g., of the peroxisomal multifunctional enzyme type 2, have, in addition to the expected hepatic dysfunction, severe neurological symptoms from birth (33, 34). Obviously, there must be branched chain substrates of endogenous origin for this pathway that occur in all tissues. Conceivably, the racemase may be required for the degradation of at least some of them, in peroxisomes but possibly also in mitochondria. It is hoped that the transgenic mouse model, which is currently being developed, will provide some answers to these questions, too.

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