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Targeted Disruption of the α Isoform of the Peroxisome Proliferator-Activated Receptor Gene in Mice Results in Abolishment of the Pleiotropic Effects of Peroxisome Proliferators

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To gain insight into the function of peroxisome proliferator-activated receptor (PPAR) isoforms in rodents, we disrupted the ligand-binding domain of the α isoform of mouse PPAR (mPPARα) by homologous recombination. Mice homozygous for the mutation lack expression of mPPARα protein and yet are viable and fertile and exhibit no detectable gross phenotypic defects. Remarkably, these animals do not display the peroxisome proliferator pleiotropic response when challenged with the classical peroxisome proliferators, clofibrate and Wy-14,643. Following exposure to these chemicals, hepatomegaly, peroxisome proliferation, and transcriptional activation of target genes were not observed. These results clearly demonstrate that mPPARα is the major isoform required for mediating the pleiotropic response resulting from the actions of peroxisome proliferators. mPPARα-deficient animals should prove useful to further investigate the role of this receptor in hepatocarcinogenesis, fatty acid metabolism, and cell cycle regulation.

Over the last decade, considerable efforts have been focused on attempting to understand the mechanism of how certain chemicals induce peroxisome proliferation and hepatocarcinogenesis in rodents. These compounds, termed peroxisome proliferators, consist of a broad spectrum of chemicals with little obvious structural similarity except for the presence of an aromatic ring, carboxylic acid, and aliphatic chain. Examples include the widely used hypolipidemic drugs such as clofibrate, prescribed for prevention of coronary heart disease in the United States and in Europe, certain phthalate ester plasticizers, herbicides, and several chlorinated hydrocarbons (57). Administration of peroxisome proliferators to rodents results in a remarkably stereotypical pleiotropic response which is tissue specific (19, 54). It appears to affect most prominently the liver. After short-term treatment with peroxisome proliferators, rat and mouse livers exhibit hepatomegaly due to both cellular hypertrophy and hyperplasia (15, 19). Hepatocytes display a marked proliferation of peroxisomes and, to a lesser extent, of the smooth endoplasmic reticulum. Concurrent with the marked increase in number and size of peroxisomes, there is a transcriptional induction of the enzymes responsible for β oxidation of fatty acids, including acyl coenzyme A (acyl-CoA) oxidase (AXO), bifunctional enzymes (BIEN), and 3-ketoacyl-CoA thiolase (thiolase), located in the peroxisomal matrix, the cytochrome P450 CYP4A enzymes found in the endoplasmic reticulum (39), and the cytosolic liver fatty acid-binding protein (L-FABP) (6, 33, 72). These morphological and biochemical changes are associated with marked alterations in hepatic lipid metabolism (41), as reflected by the lowering of the plasma triglyceride and cholesterol levels in the treated animals (53, 54, 59, 61). Under normal circumstances, peroxisomal β oxidation is only a minor pathway for fatty acid oxidation relative to the mitochondrial system (31). Peroxisomal enzymes become engaged when an animal is under a lipid-overloaded crisis such as that stimulated by peroxisome proliferators, a high-fat diet (48, 50), and metabolic dysregulation (26, 49, 67). The coordinate induction of lipid-metabolizing enzymes and hepatocyte proliferation is an adaptive response that maintains the homeostasis of cellular lipids. All of these data point to a central role for peroxisome proliferation in regulation of fatty acid metabolism. However, the mechanism by which peroxisome proliferators induce the pleiotropic response is still not clear.

Earlier studies suggested that intracellular accumulation of lipid is the major factor for the induction of peroxisome proliferation (5, 31, 41). An increase in circulating fatty acids resulting from either diet (27, 48, 50, 58) or metabolic dysfunction (26, 49, 67) stimulates peroxisomal β oxidation and microsomal ω oxidation of fatty acids in the liver. This ultimately results in peroxisomal proliferation (12). The precise mechanism by which peroxisome proliferators function in this process was never adequately addressed.

A receptor-based mechanism for the pleiotropic response of peroxisome proliferation was suggested by identification of the first peroxisome proliferator-activated receptor (PPAR) cDNA from mice (later referred to as the mPPARα isoform) by Issemann and Green (28). PPARα has a common modular structure which consists of six functional domains (37, 45),

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defined as A and B (transactivation), C (DNA-binding), and D, E, and F (ligand-binding, dimerization, and transactivation). These structural motifs indicated that PPARα is a member of the steroid receptor superfamily (13). Since the cloning of the first PPAR in mice, other isoforms were identified in mice and in other species. To date, three isoforms of PPAR have been identified in *Xenopus laevis* (xPPARα, -β, and -γ [11]), two have been identified in humans (hPPARα [64] and hPPAR/NUC-1, a β homolog [62]), one has been identified in rats (rPPARα [18]), and six have been identified in mice (mPPARα [28], mNUCI [9], mPPARδ [35], mPPARγ [35], mPPARγ1 [75], and mPPARγ2 [68]). It is unknown exactly how many isoforms exist in an animal, but the current isoform classification of mammalian PPARs is relative to that for the *Xenopus* α, β, and γ receptors. The sequence homologies in the domains of PPARs are high enough to distinguish them from other members of the steroid hormone receptor superfamily. Differences in the responsiveness to peroxisome proliferators among isoforms (10, 42) and the levels of expression during development and in various tissues have been reported (10, 11, 35, 69).

Early evidence that PPAR modulates the ligand-induced activation of responsive genes was obtained from experiments using chimeric nuclear hormone receptors containing the putative ligand-binding domain of the PPARα isoform and N-terminal and DNA-binding domains of either estrogen or glucocorticoid receptors in the presence of peroxisome proliferators (11, 18, 28, 62). Although experiments with chimeric receptors were suggestive of a role of PPARα as a transcription factor, direct evidence has become available only recently when a peroxisome proliferation responsive element (PPRE) was identified in the 5′ upstream regions of several target genes. PPRE was identified as an almost perfect direct repeat of the sequence TGA/TCCT separated by one base pair in the genes of the peroxisomal AXO (52, 71), BIEN (2, 42, 73, 74), and thiolase (24), the microsomal CYP4A subfamily (3, 47), and the cytosolic L-FABP (6, 29, 72). Gene activation by PPARα is potentiated by the dimerization partner retinoid X receptor (2, 30, 36). Direct interaction of peroxisome proliferators with PPARα has not been demonstrated, although the structural similarities with other steroid receptor superfamily proteins suggest the existence of a ligand-binding domain. Although mPPARα was shown to be activated by peroxisome proliferators in in vivo cotransfection assays, it is not known if peroxisome proliferation can also be stimulated by other receptor isoforms (mPPARδ, mPPARγ, and mNUCI) or not yet identified isoforms. No direct evidence links any of the individual PPAR isoforms to the peroxisome proliferation response in an intact animal model. It is also unclear whether the retinoid X receptor is required for mPPARα activity in vivo. The generation of a single PPAR isoform gene knockout mouse by homologous recombination should help to answer these questions.

In this report, we describe the production of mPPARα isoform knockout mice by targeted gene disruption the putative ligand-binding domain coding region. Our studies demonstrate that mice lacking expression of PPARα protein do not respond to the prototypical peroxisome proliferators, clofibrate and Wy-14,643, and lack detectable hepatomegaly, proliferation of peroxisomes, or induction of the mRNA encoding the peroxisomal and microsomal lipid-metabolizing enzymes. These results demonstrate that the PPARα isoform is required for mediating the pleiotropic response resulting from the action of peroxisome proliferators in rodents.

MATERIALS AND METHODS

Construction of the targeting vector. Genomic clones corresponding to mPPARα were obtained by screening a library, established by using λEMBL3 and BALB/c mouse DNA, with a mPPARα cDNA (64). Exon mapping of these clones revealed a partial mPPARα gene on a 16-kb *Bam*HI-*Bam*HI fragment spanning from introns 6 to 8. To disrupt the mPPARα gene, we constructed a targeting plasmid containing a deletion of 83 bp between the *Pst*I and *Sph*I sites in exon 8 of the ligand-binding domain (16) which was replaced by the 1.14-kb phosphoribosyltransferase II gene conferring neomycin resistance (Neo; derived from plasmid pMC1NeoPolyA; Stratagene) inserted in the opposite direction of transcription of the genomic clone. The targeting vector contained 3.7 kb of homologous sequence 5′ and 2.6 kb of homologous sequence 3′ of the Neo cassette. A herpes simplex virus thymidine kinase (TK) gene inserted at the 3′ end of the construct allowed the use of a positive-negative selection scheme. To construct the targeting vector, a 6.4-kb *Xba*I-*Xba*I genomic fragment of mPPARα containing intron 6, exons 7 and 8, and intron 8 was subcloned into a pGEM3Z plasmid. This plasmid was then digested with *Sph*I to release the 2.6-kb 3′ genomic fragment or with *Pst*I to release the 3.7-kb 5′ genomic fragment plus pGEM3Z for subsequent subcloning. The 3.7-kb 5′-plus-pGEM3Z fragment was self-religated, and the *Hin*dIII site in the polylinker site of pGEM3Z was converted to a new *Bam*HI site by treatment with Klenow polymerase. This plasmid was then digested with *Bam*HI to release the 3.7-kb 5′ genomic fragment for subsequent subcloning. The Neo cassette, digested with *Bam*HI and *Xho*I, was subcloned into a pGEM3Z plasmid, and this plasmid (NeopGEM3Z) served as the backbone for building the targeting vector. The 2.6-kb *Sph*I-*Sph*I 3′ and the 3.7-kb *Bam*HI-*Bam*HI 5′ genomic fragments were then subcloned into the *Sph*I and *Bam*HI sites, respectively, of plasmid NeopGEM3Z. The 1.9-kb TK cassette (derived from plasmid pMC1TK), digested with *Xho*I and *Hin*dIII, was subcloned into the *Sal*I and *Hin*dIII sites of the targeting construct.

Electroporation and selection conditions. Frozen J1 (40) Sv/129 mouse embryonic stem (ES) cells (passage 12) were thawed at 37°C, diluted with ES medium (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-buffered Dulbecco's modified Eagle medium, 15% fetal bovine serum [HyClone], 55 μM β-mercaptoethanol, 0.1 mM nonessential amino acids, penicillin-streptomycin, 1,000 U of murine leukemia inhibitory factor [LIF] per ml), and pelleted at 1,000 × *g* for 3 min at room temperature (RT). The ES cell pellet was resuspended in 5 ml of ES medium and plated onto a 6-cm-diameter tissue culture dish previously seeded with mitomycin-treated (10 μg/ml of ES medium at 37°C for 3 h) G418-resistant mouse embryonic fibroblasts (NEF) at 37°C in a CO_2 incubator. After 48 h, the ES cells were freshly fed for 3 to 4 h, trypsinized with 0.25% trypsin-EDTA buffered with HEPES, and resuspended at 2.5×10^5/6-cm-diameter plate with electroporation buffer (Hanks' balanced salt solution, 20 mM HEPES buffer, 0.11 mM β-mercaptoethanol; pH adjusted to 7.2 with 1 M NaOH) and used for electroporation.

The targeting construct was purified by banding twice with cesium chloride gradient centrifugation. Supercoiled DNA was removed from the gradient and linearized with *Hin*dIII. The DNA was extracted three times with phenol-chloroform and twice with chloroform, ethanol precipitated, washed with 70% alcohol, and resuspended at a concentration of 2 μg/μl with sterile distilled water. On the day of electroporation, 50 μg of linearized DNA was ethanol precipitated and resuspended in 50 μl of electroporation buffer. The linearized DNA was pipetted into the electroporation cuvette containing 1 ml of ES cells at 2.5×10^5 per plate and electroporated (400 V, 25-μF capacitance, and 0.4-s time constant) with a Bio-Rad Gene Pulser. The ES cells were plated onto 6-cm-diameter dishes containing mitomycin-treated NEF at 3.5×10^5 per dish with ES medium. Twenty-four hours after electroporation, cells were doubly selected with the drugs G418 (300 μg/ml) and ganciclovir (2 μM; gift of Syntex). After 8 days of double selection, the ES clones were picked up under a dissecting microscope, transferred to individual wells of a 96-well plate containing 25 μl of 0.025% diluted HEPES-buffered trypsin-EDTA, and dissociated with multichannel pipettors by pipetting up and down five times. Dissociated clones were transferred to wells of a 24-well plate containing mitomycin-treated NEF with continued double-selection pressure. When clones were expanded and became visible to the naked eye, they were trypsinized individually with 400 μl of 0.25% HEPES-buffered trypsin-EDTA. Twenty-five percent of each clone was expanded in each well of a 24-well plate without a feeder layer for a source for making DNA, and the remaining portion of each clone was stored frozen in 10% dimethyl sulfoxide (DMSO)–20% fetal bovine serum in ES medium in liquid nitrogen.

Generation of chimeric mice. One targeted ES cell clone (PR 86) was injected into C57BL/6N blastocysts (3.5 days), using standard procedures (25). Briefly, blastocysts were flushed from the uteri of superovulated females, and 10 to 15 ES cells were microinjected into the blastocoel of each blastocyst. Groups of 10 to 15 blastocysts were reimplanted in the uteri of pseudopregnant females. Five chimeras (Sv/129 × C57BL/6N) were obtained from 200 injected blastocysts. Three male chimeras were mated to C57BL/6N females, and germ line transmission was scored by the presence of agouti offspring in the F_1 litter. The genotypes of these progenies and the subsequent generations were analyzed by Southern blot analysis of DNA isolated from the tail tip of 4-week-old adult mice.

Southern blot analysis. DNA was isolated from ES cells and mouse tails as described by Laird et al. (38). DNAs were digested overnight to completion with

*Eco*RI or *Hin*cII. The digested DNAs were electrophoresed through 0.6% agarose gels and transferred to GeneScreen Plus nylon membranes (DuPont) in 0.4 N NaOH overnight. Membranes were baked at 80°C for 2 h in an vacuum oven and washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) for 1 h and then prehybridized with hybridization solution containing 50% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 5× Denhardt's solution, 200 μg of salmon sperm DNA per ml, 0.1% SDS, and 10% dextran sulfate for at least 5 h. Hybridization probes were labeled with [^{32}P]dCTP by random priming (Pharmacia). Hybridization was done at 42°C overnight, and washes were performed with 2× SSC–0.5% SDS at 65°C for 20 min twice followed by shaking at RT for 15 min each time. Filters were analyzed by use of a PhosphorImager (Molecular Dynamics) and, in some cases, subjected to autoradiography at −80°C overnight, using Kodak XAR film. The 3′ flanking probe A, an 800-bp *Xba*I-*Sma*I fragment, was used to hybridize with ES cell and mouse tail DNAs that were digested with *Eco*RI or *Hin*cII. Probe A hybridizes to a 9.9-kb *Eco*RI or 6.3-kb *Hin*cII restriction fragment from wild-type genomes (Fig. 1A and B). When one allele of the mPPARα gene is replaced with the targeting vector sequences by homologous recombination, additional hybridizing 5-kb *Eco*RI and 7.5-kb *Hin*cII restriction fragments appear (Fig. 1A and B). Of the 120 G418-gancilovir-selected clones screened, one had undergone the homologous recombination event. An internal Neo probe (1.14-kb *Xho*I-*Hin*dIII fragment of plasmid pMC1NeoPolyA) was used to hybridize with DNA that was digested with *Eco*RI and *Hin*cII to demonstrate single-copy insertion of the targeting vector by a homologous recombination event (Fig. 1B).

Northern (RNA) blot analysis. Total RNA from livers was isolated by the use of guanidine thiocyanate and cesium trifluoroacetate (CsTFA) gradient centrifugation. Ten to twenty micrograms of total RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde and then blotted onto a GeneScreen Plus (DuPont) nylon membrane in 20× SSC overnight. The conditions for baking, washing, prehybridization, hybridization, washing, and labeling hybridization probes were the same as described above for the Southern blot analysis. Seven cDNA probes were used for the analysis. The rat peroxisomal AXO, BIEN, and thiolase cDNAs were obtained by reverse transcription PCR (Invitrogen) from 8 μg of total RNA isolated from clofibrate-treated rat liver. The second-strand cDNAs were amplified by subsequent PCRs with designed primers specific for each gene (see below). The amplified PCR products were subcloned in plasmid pDirect (Clontech). cDNA inserts were then isolated from pDirect by digestion with *Xba*I and *Xho*I for the AXO, *Cla*I and *Sac*II for the BIEN, and *Xba*I and *Xho*I for the thiolase. The digested cDNAs were separated on 1% agarose gel, excised, and purified by electroelution. The cDNA sequences of the genes were confirmed by *Taq* DyeDeoxy-terminator automated sequencing from both DNA strands. The PCR primers selected were based on the published cDNA sequences of the genes. The forward PCR primers for rat AXO (43), BIEN (51), and thiolase (23) were 5′-ATGGATAACGGCTACCTGAAGATG-3′ (776 to 799), 5′-ATGCAGTCGTGAAGTCAGACCCAG-3′ (527 to 550), and 5′-TGAGTGGCATCCCAGAGACC-3′ (345 to 364), respectively. The backward PCR primers for rat AXO, BIEN, and thiolase were 5′-ATGATGCTCCCCT CAAGAAAGTC-3′ (1812 to 1790), 5′-GCGAATTTTCCAACCCACATCT AG-3′ (1620 to 1597), and 5′-TGTACCATCCCTCTCCAGACAC-3′ (1361 to 1340), respectively. The amplified cDNA fragments for the rat AXO, BIEN, and thiolase were 1,037, 1,094, and 1,017 bp, respectively. Rat cytochrome P450 CYP4A1 and CYP4A3 cDNAs were obtained from our laboratory (21, 34). The 450-bp L-FABP cDNA was provided by Sam Sorof (Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pa.). A β-actin probe, which corresponds to a 1,150-bp *Pst*I-*Pst*I fragment containing most of the mouse β-actin cDNA, was used to monitor the loading of the gels. Quantification analysis was performed by scanning the filter, using a phosphor storage and PhosphorImager (Molecular Dynamics) apparatus (32).

Animal feeding studies. Eight male mPPARα$^{-/-}$ mice (F_2 homozygotes; hybrids of Sv/129 × C57BL/6N genetic background; 10 to 11 weeks old; 25 to 35 g) were used for the clofibrate diet study. They were divided into two groups of four. Group 1 was fed with 0.5% (wt/wt) clofibrate (Sigma) rodent chow diet (Bio-Serv), and group 2 was fed with rodent chow diet without chemicals. Equal numbers of male wild-type littermates were used for the study. Ten male mPPARα$^{-/-}$ mice (8 weeks old; 20 to 33 g) were used for the Wy-14,643 diet study, and they were divided into two groups of five. One group was fed with 0.1% (wt/wt) Wy-14,643 (Chemsyn Science Laboratories) rodent chow diet (Bio-Serv). The other group was fed with control rodent chow diet. All animals received water and food ad libitum for a treatment period of 2 weeks. They were housed in groups of two or three in plastic microisolator cages at 25°C with a 12-h light/12-h dark cycle. At the end of the treatment, one mouse from each group was treated for the cytochemical demonstration of peroxisomes by electron microscopy (see below). The remaining mice from each group were asphyxiated with CO_2, and their tissues were quickly removed, weighed, and placed in liquid nitrogen until used.

Electron microscopy. Mice were anesthetized with Metofane, and the livers were fixed in situ by intraventricular perfusion with saline for 5 min followed by a fixative containing 1.5% glutaraldehyde, 0.05% $CaCl_2$, and 4% polyvinylpyrrolidone in 0.1 M sodium cacodylate buffer (pH 7.4) for 15 min (14). The tissues were then excised and stored in the same fixative at 4°C overnight. After they had been washed in 0.1 M sodium cacodylate buffer (pH 7.4) for 5 h, the tissues were cut into 100-μm sections with an Oxford vibratome. The peroxisomes in the livers were stained selectively for catalase with 5 mM 3,3′-diaminobenzidine (DAB) substrate in 0.1 M glycine-NaOH (pH 10.5)–0.15% H_2O_2 at RT for 1 h (1). Subsequently sections were postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at RT for 1 h, dehydrated in graded alcohol-propylene oxide, and embedded in Epon. One-micrometer sections cut with a glass knife were examined unstained, showing only the contrast of the DAB reaction, in a light microscope. The ultrathin sections cut with a diamond knife were counterstained for 1 min with uranyl acetate and lead citrate and examined in a Philips 201 electron microscope.

Generation of mPPARα-specific antisera. The 1.14-kb mPPARα cDNA was digested with *Bam*HI from the original Bluescript plasmid and ligated to the *Bam*HI site located at the 3′ terminus of the glutathione *S*-transferase (GST) gene of the bacterial expression vector pGEX-2T (Pharmacia). The ATG initiation site of the mPPARα cDNA was in the correct orientation and amino acid reading frame to yield GST-mPPARα fusion proteins upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG). The resulting GST-mPPARα expression vector was used to transform two different *Escherichia coli* strains (BL21 DE3/pLysS and HMS174/pLysS) for expression of the fusion protein (~77 kDa). The GST-mPPARα fusion protein was expressed in YT medium containing 25 μg of chloramphenicol per ml, 150 μg of ampicillin per ml, and 0.1 mM IPTG at 37°C for 18 h. The whole bacterial homogenate was pelleted, resuspended in Tris-EDTA, and subjected to preparative SDS-polyacrylamide gel electrophoresis (PAGE) on an 8% polyacrylamide gel. The gel was stained with 0.05% Coomassie brilliant blue in distilled water for 10 min and destained in distilled water for 2 h, and the ~77-kDa (GST-mPPARα) strongly induced band was excised and stored in phosphate-buffered saline. These SDS-PAGE bands were used to raise antibodies in a rabbit (Assay Research). The resulting antisera obtained after three different boosters were administered in about 3 months and were used for Western blot (immunoblot) analysis without purification.

Western blot analysis. Nuclear extracts and cytosols from livers were prepared as described by Gebel et al. (17). Five micrograms of nuclear extract and 50 μg of cytosol were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane by a semidry transfer method. Immunodetection was carried out by the method of Towbin et al. (70), using mPPARα-specific antisera raised in a rabbit as the first antibody (1:200) and alkaline phosphatase-coupled second antibody (1:1,000). The positive immunoreactive bands were visualized by staining with the 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitroblue tetrazolium phosphatase substrate detection system (Kirkegaard & Perry).

RESULTS

Disruption of the ligand-binding domain of the mPPARα gene in mouse ES cells. The organization of the gene encoding mPPARα is illustrated in Fig. 1AI (16). To generate mice lacking an mPPARα protein, we disrupted exon 8 of the gene, which together with exon 7 encodes the ligand-binding domain of the protein. The targeting vector (Fig. 1AII) contains 6.4 kb of nonisogenic genomic DNA of the BALB/c strain spanning from introns 6 to 8. To ensure disruption of the gene, the positive selectable marker Neo was inserted and replaced 83 bp of the coding region of exon 8 (*Pst*I to *Sph*I). For negative selection against random integration during selection of specific recombinant ES cells, a viral TK gene under the control of the pMCI promoter was included at the 3′ end of the vector.

The targeting vector was linearized at the *Hin*dIII site 3′ of the pMCI-TK sequences and electroporated into the J1 ES cell line derived from a male agouti mouse strain Sv/129 embryo. Cells were subjected to double selection with the drugs G418 and ganciclovir. The correct gene targeting event in doubly resistant individual clones was identified by Southern blot analysis. Of 120 clones screened, one (PR 86) had undergone homologous recombination with the targeting vector (Fig. 1AIII and B). Additional Southern transfer analysis with the Neo probe confirmed that the PR 86 cell line contained the predicted replacement of one of the wild-type mPPAR alleles with sequences from the targeting vector (Fig. 1B). This cell line was used to generate chimeric males that transmitted the mutant allele to their progenies. Three male chimeras were produced and test bred with C57BL/6N females. All chimeras transmitted the targeted mutation to their progenies. Mice heterozygous for the disrupted mPPARα gene were mated, and homozygous mutant offspring were identified by Southern blot analysis (Fig. 1C) and upon breeding exhibited the ex-

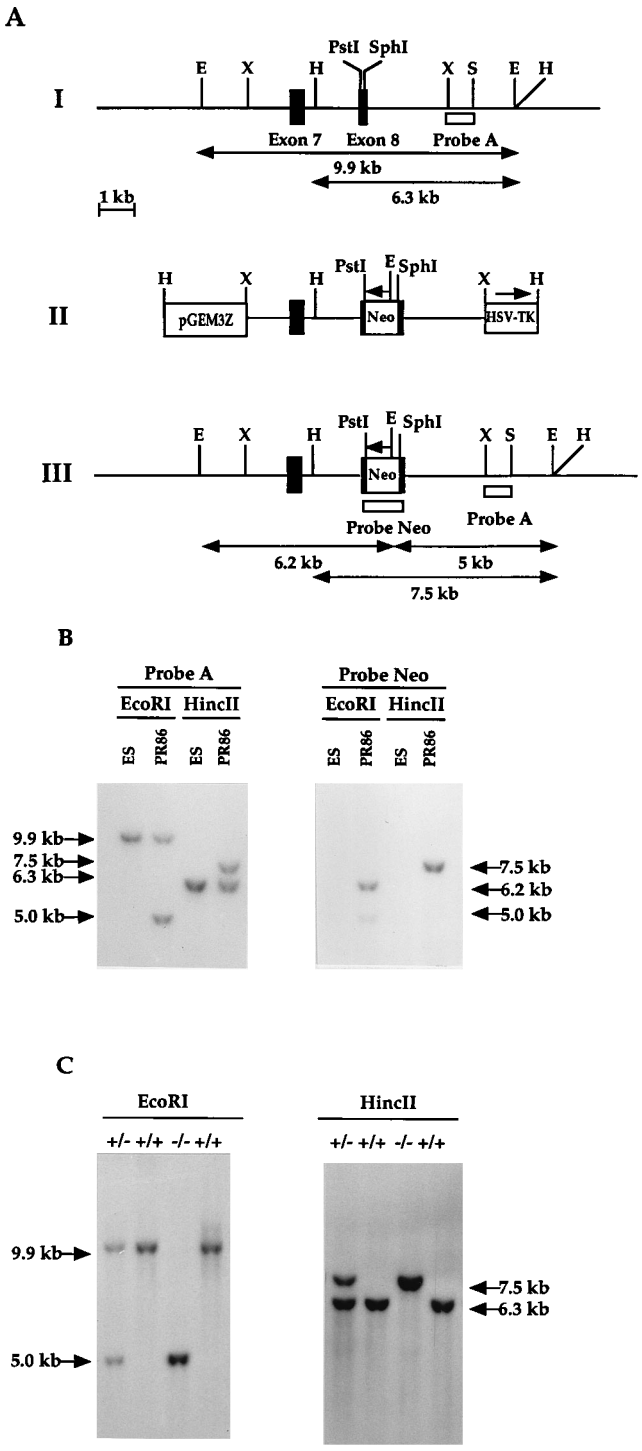

FIG. 1. Targeted disruption of the mouse mPPARα gene. (A) Strategy for the mPPARα knockout. I, partial map of a mouse genomic fragment containing exons 7 and 8 encoding the mPPARα ligand-binding domain. Restriction enzymes: E, *Eco*RI; X, *Xba*I; H, *Hin*cII; S, *Sma*I. The wild-type 9.9-kb *Eco*RI and 6.3-kb *Hin*cII fragments detected by probe A, an 800-bp *Xba*I-*Sma*I fragment from the 3′ end of the mPPARα genomic DNA, are indicated. The targeting vector (II) possesses a total of 6.4 kb of homologous sequence, including 3.7-kb 5′ *Xba*I-*Pst*I and 2.6-kb 3′ *Sph*I-*Xba*I mPPARα genomic fragments. A deletion of 83 bp between the *Pst*I and *Sph*I sites in exon 8 of the ligand-binding domain was replaced by the 1.14-kb Neo gene in reverse orientation relative to the direction of mPPARα transcription. The Neo cassette introduces a novel *Eco*RI restriction site for genotyping by Southern blot analysis. A pMCITK expression cassette (herpes simplex virus TK [HSV-TK]) was added at the 3′ end of the contruct for negative selection. III, the expected homologous recombination event of mPPARα. When one allele of the mPPARα gene is replaced with the targeting vector sequences by homologous recombination, additional hybridizing 5-kb *Eco*RI and 7.5-kb *Hin*cII restriction fragments appear when analyzed with probe A. (B) Genomic Southern blots of ES cell DNA. DNA was extracted from untransfected (ES) as well as targeted (PR 86) ES cell clones and digested with the designated restriction enzymes. DNA was separated in a 0.6% agarose gel, blotted onto a nylon membrane, and hybridized with random-primed ^{32}P-labeled probes shown in panel A. (C) Southern blots of mouse tail DNA. Tail DNA was extracted from the wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) mice and processed as for panel B.

pected Mendelian distribution, with 25% homozygous mutant offspring. Mice homozygous for the mutation were viable, healthy, and fertile and appeared normal. A PPARα-deficient mouse line was derived from these animals.

Mutant mice lack wild-type receptor mRNA and protein. To establish that mPPARα mRNA expression was abolished in mutant mice, Northern blot analysis was carried out with total RNA prepared from the liver, an organ that constitutively expressed PPARα. The cDNA probe of mPPARα detected a 8.5-kb fragment corresponding to the wild-type mRNA in the wild-type (+/+) and heterozygous (+/−) mice but not in the homozygous (−/−) mutants (Fig. 2A). A larger RNA of about 9.5 kb was detected in the +/− and −/− mice but not in the +/+ mice. This RNA in the −/− mice also hybridized to the Neo probe, suggesting that the ~1-kb increase in the mRNA transcript size is due to a read-through transcript initiated at the mPPARα promoter and terminated at the endogenous poly(A) site. The mutant mRNA was very faint compared with the wild-type mRNA, suggesting that it is an unstable truncated transcript. Taken together, the results in Fig. 2A indicate that the mutant gene does yield an abnormal mPPARα mRNA as a result of the presence of stop codons in all frames of the Neo cassette.

To establish that mPPARα protein was absent in −/− mice, nuclear and cytosol protein extracts were prepared from livers and subjected to Western blot analysis. Figure 2B shows that the band of about 52 kDa expected for the mPPARα protein was present in the nuclear extract of +/+ (lane 4) and +/− (lane 5) mice but not in −/− mutants (lane 6). No positive immunoreactive band was detected in the cytosol from mice with any of the tested genotypes (lanes 1 to 3). Thus, we conclude that the homozygous targeted mutation resulted in abolishment of PPARα protein expression.

Lack of hepatomegaly in mutant mice fed clofibrate and Wy-14,643. It is well established that administration of peroxisome proliferators such as clofibrate and Wy-14,643 to rodents results in liver enlargement (44, 59). The increase in liver weight was due to hypertrophy and hyperplasia (hepatomegaly) of the hepatocytes (15, 19). As expected, both clofibrate and Wy-14,643 produced a marked increase in liver weights in +/+ mice (1.7-fold with clofibrate and 2.9-fold with Wy-14,643 compared with their corresponding controls) after 2 weeks of treatment with the peroxisome proliferators. The hepatomegaly was most striking in +/+ mice treated with 0.1% (wt/wt) Wy-14,643. In these animals, 2 weeks of Wy-14,643 feeding resulted in massive enlargement of the livers, which accounted for one-sixth to one-fifth of the total body weight, in agreement with results obtained by other investigators (56, 59). In contrast, treatment with clofibrate and Wy-14,643 did not result in significant liver enlargement in the −/− mutants. No significant differences in liver weights between the treated −/− mutant and untreated +/+ wild-type mice were observed. Similar amounts of chow (about 1.5 kg for each group for 2 weeks) were consumed by each group, and no signs of any abnormality were observed in either group during the course of treatment.

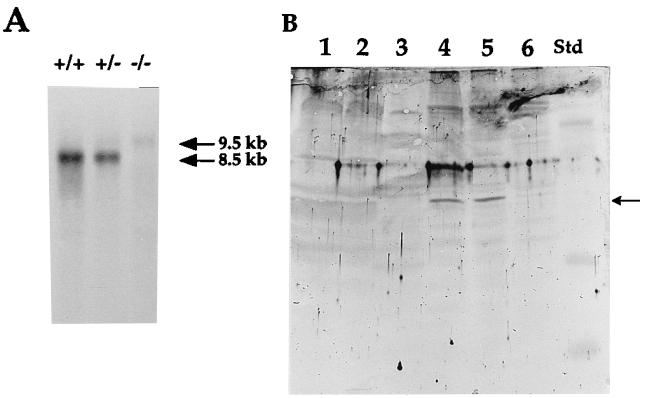

FIG. 2. Expression of mPPARα mRNA and protein in wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice. (A) Northern blot analysis of mPPARα mRNA expression in +/+, +/−, and −/− mice. Total RNA was isolated from the livers of these animals. Ten micrograms of total RNA was separated on a 1% formaldehyde-agarose gel, blotted onto a nylon membrane, and hybridized with a random-primed ^{32}P-labeled 1.4-kb mPPARα cDNA probe. No mPPARα transcript (8.5 kb) was detected in −/− mutant mice. (B) Western blot analysis of mPPARα protein expression in wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice. Cytosol and nuclear extracts were extracted from the livers of these animals. Fifty micrograms of cytosol (lanes 1 [+/+], 2 [+/−], and 3 [−/−]) and 5 μg of nuclear extracts (lanes 4 [+/+], 5 [+/−], and 6 [−/−]) were separated on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. The standard molecular weight markers are represented in lane Std. The blot was probed with antisera raised against GST-mPPARα fusion protein. The arrow indicates the position of the expected 52-kDa mPPARα protein band in both +/+ (lane 4) and +/− (lane 5) mice in the nuclear extract fraction. No mPPARα protein was detected in −/− mutant mice (lane 6).

Thus, the lack of effects of the peroxisome proliferators in the −/− mice strongly implicates a role for the PPARα in controlling the peroxisome proliferator-induced hepatomegaly in rodents.

Lack of proliferation of peroxisomes in mutant mice fed clofibrate and Wy-14,643. It was shown that mouse liver peroxisomes markedly increase in number after the administration of clofibrate and Wy-14,643 to rodents (44, 56). Results from light (Fig. 3) and electron (Fig. 4) microscopy studies indicated that treatment of +/+ mice with clofibrate and Wy-14,643 resulted in increases in the number, size, and staining intensities of peroxisomes in livers of these animals, as revealed by the DAB staining of catalase (14), a marker enzyme for peroxisomes. Swelling of mitochondria was also observed. On the contrary, identical treatment of −/− mice with the peroxisome proliferators did not result in noticeable proliferation of liver peroxisomes (Fig. 3 and 4). In the course of the electron microscopy studies, we also noted abundant accumulation of lipid droplets in livers of −/− mutant mice fed peroxisome proliferators (Fig. 4). This is in marked contrast to that observed in the +/+ mice fed with the same compounds, in which there were no detectable accumulation of lipid droplets in the liver (Fig. 4). Thus, the lack of proliferation of peroxisomes in mPPARα-deficient mice indicates that this isoform mediates the proliferation of peroxisomes in rodent hepatocytes. The observation that mice lacking PPARα expression accumulate lipid droplets in their tissues after feeding on hypolipidemic agents suggests that the α isoform is also essential in maintaining the homeostasis of hepatic lipid metabolism.

Lack of gene activation in mutant mice fed clofibrate and Wy-14,643. In rodents, peroxisome proliferators cause an increase in liver weight and marked proliferation of peroxisomes. Simultaneously, mRNAs encoding the three peroxisomal β-oxidation enzymes (AXO, BIEN, and thiolase) (39), the microsomal ω-oxidation enzymes (CYP4A) (34, 47, 63), and the cytosolic L-FABP (6, 33, 72) are concomitantly induced. The precise mechanism of this induction is largely unknown, though the involvement of the PPAR has been suggested through *trans*-activation transfection studies. In agreement with published results, Northern blot analysis (Fig. 5 and 6) indicated that clofibrate and Wy-14,643 treatment of +/+ mice caused a dramatic increase in the levels of the mRNAs encoding the peroxisomal AXO, BIEN, and thiolase, the microsomal CYP4A1 and CYP4A3, and the cytosolic L-FABP in the livers of the +/+ animals. The levels of induction of the mRNAs of the responsive genes by the peroxisome proliferators in wild-type mice obtained in our study are in general agreement with those observed by other investigators (4, 55, 65). Remarkably, the peroxisome proliferators did not have any significant inducing effects on the expression of the mRNAs of the responsive genes in the livers of the −/− mice lacking mPPARα, except for a slight increase in the thiolase mRNA. This result indicated that mPPARα might not be the only isoform controlling the peroxisome proliferator-induced activation of this gene. The lack of induction of the genes in the −/− mice could not be due to the quantitative differences in the total RNA used for the analysis, as revealed by the uniformity of the expression of β-actin mRNA in the Northern blots (Fig. 5 and 6). Thus, these data strongly implicate the involvement of PPARα as a common regulatory mechanism for controlling the transcriptional activation of the corresponding genes involved in lipid metabolism. These findings, as well as the absence of hepatomegaly and peroxisome proliferation in mice lacking PPARα, further substantiate the contention that PPARα is the major isoform responsible for mediating the pleiotropic response of peroxisome proliferators.

DISCUSSION

We used a homologous recombination strategy with ES cells to disrupt the ligand-binding domain of the mPPARα gene. The target frequency was one correct homologous recombination event in 120 G418- and ganciclovir-selected colonies. The disrupted mPPARα allele was successfully transmitted in the germ line of mice. The targeted mutation resulted in a mutant mPPARα allele that does not produce a functional mPPARα protein. Crossing of heterozygous mice resulted in the expected Mendelian ratio of 25%. The homozygous mice were viable, fertile, and healthy and lacked any observable gross defects, thus implying that mPPARα is not essential in embryonic development.

PPARα is the major isoform mediating the pleiotropic actions of peroxisome proliferators. We developed a line of mPPARα-deficient mice as a model to test the hypothesis that PPAR mediates the pleiotropic response of peroxisome proliferators. Evidence that PPAR mediates the peroxisome proliferator-induced pleiotropic response was based on the *trans*-activation cotransfection in vivo assays (18, 28, 64) and the recent identification of the PPREs in several of their target genes, including the peroxisomal AXO (52, 71), BIEN (2, 42, 73, 74), and thiolase (24) genes as well as the CYP4A6 (3, 47) and L-FABP (6, 29, 72) genes. If PPARα is in fact the major isoform controlling the pleiotropic response of peroxisome proliferators, then administration of the prototypical peroxisome proliferators, such as clofibrate and Wy-14,643, to the mPPARα mutant mice should not produce any pleiotropic effects characteristic of peroxisome proliferators. Indeed, mice lacking mPPARα expression did not exhibit any noticeable sign of hepatomegaly, proliferation of peroxisomes, or tran-

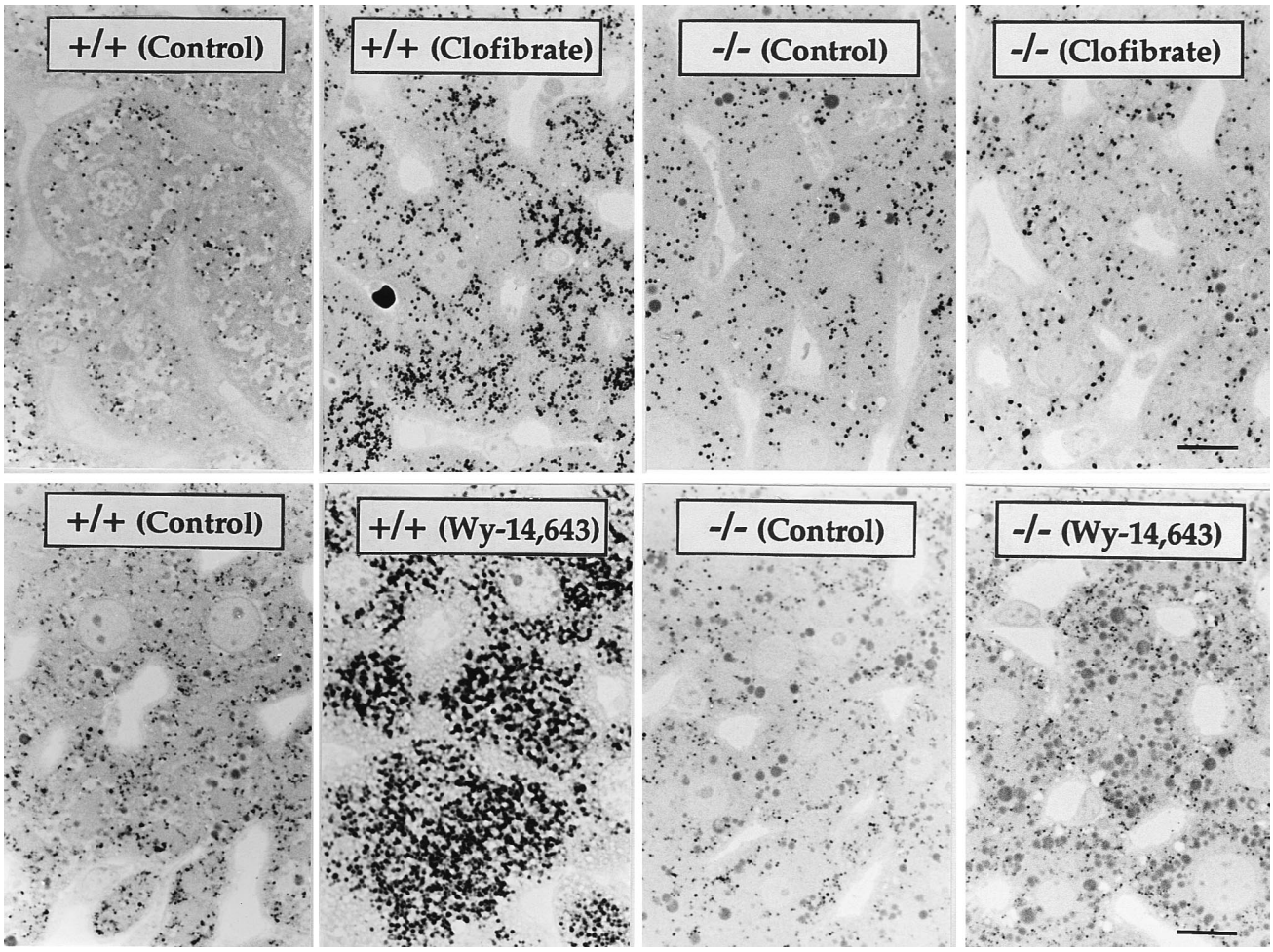

FIG. 3. Light micrographs of DAB-stained peroxisomes in hepatocytes of wild-type (+/+) and homozygous mPPARα mutant (−/−) mice fed control, 0.5% (wt/wt) clofibrate, or 0.1% (wt/wt) Wy-14,643 rodent chow diet for 2 weeks. Note the marked proliferation of peroxisomes in +/+ mice fed clofibrate or Wy-14,643 and the lack of noticeable peroxisome proliferation in −/− mice fed the same compounds. Bar scale = 3.3 μm.

scriptional activation of the responsive genes, including the peroxisomal AXO and BIEN and the cytosolic L-FABP genes. Although the CYP4A genes in mice (the mouse counterparts of the CYP4A6 genes in rabbits) have not been examined for the presence of PPREs, results from our studies clearly demonstrated that expression of the genes encoding mouse CYP4A P450s is under the control of mPPARα. Incomplete abolishment of the transcriptional activation of the thiolase gene in mice lacking the mPPARα suggests the involvement of factors in addition to the mPPARα.

Results obtained from the studies of mPPARα mutant mice strongly support the contention that PPARα is required for the peroxisome proliferator-induced pleiotropic response observed in the wild-type animals. An increase in the number and size of peroxisomes and proliferation of endoplasmic reticulum and resulting liver enlargement were not observed in mice lacking PPARα. However, it should be noted that mice lacking PPARα expression did possess peroxisomes, suggesting that constitutive expression of genes encoding peroxisomal enzymes is not controlled by this receptor. The presence of constitutive expression of the microsomal CYP4A proteins and the cytosolic L-FABP in the mPPARα-deficient mice was comparable to that in the wild-type mice, suggesting that constitutive expression of these genes was also not under the control of PPARα. The findings obtained from our animal model demonstrate that PPARα is the major isoform mediating the pleiotropic response of peroxisome proliferators.

Conclusions and future perspectives. In addition to a lack of peroxisome proliferation and hepatomegaly with a nonfunctional mPPARα protein, several genes encoding the enzymes of peroxisomal β oxidation and microsomal ω oxidation as well as L-FABP were not inducible upon administration of peroxisome proliferators. These findings demonstrate that mPPARα is involved in regulating a battery of lipid-metabolizing enzymes for balancing perturbations in cellular lipid metabolism. Mice lacking mPPARα had an accumulation of numerous fat droplets in the liver upon peroxisome proliferator treatment, suggesting that mPPARα is also essential in maintaining the homeostasis of hepatic lipid metabolism.

The mPPARα mutant mice should provide an indispensable animal model with which to study other aspects of metabolic regulation associated with the PPAR. mPPARα-deficient mice will be of value for investigating whether PPARα is involved in mediating peroxisome proliferator-induced hepatocarcinogenesis in rodents. Since the hypolipidemic drugs and phthalate ester plasticizers have an important role in our society today, an understanding of their effects on biological systems and the underlying mechanisms is imperative. Also, the mPPARα

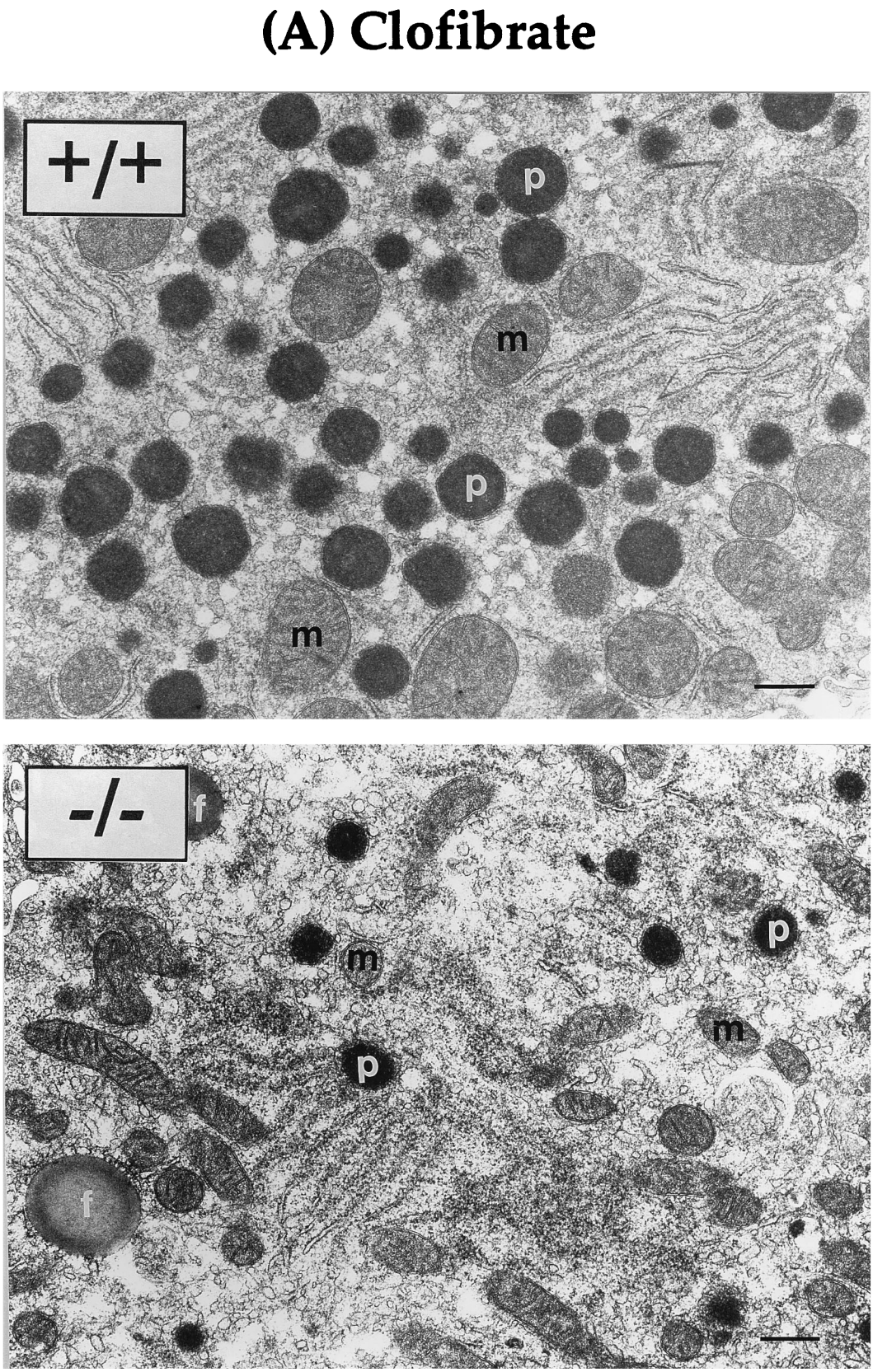

FIG. 4. Electron micrographs of DAB-stained peroxisomes in hepatocytes of wild-type (+/+) and homozygous mPPARα mutant (−/−) mice fed 0.5% (wt/wt) clofibrate (A) or 0.1% (wt/wt) Wy-14,643 (B) rodent chow diet for 2 weeks. Note the marked proliferation of peroxisomes in +/+ mice fed clofibrate or Wy-14,643 and the lack of noticeable peroxisome proliferation in −/− mice fed the same compounds. Tremendous fat droplet accumulation was seen in −/− mice fed Wy-14,643. Abbreviations: p, peroxisomes; m, mitochondria; f, fat droplets. Bar scale = 0.6 μm.

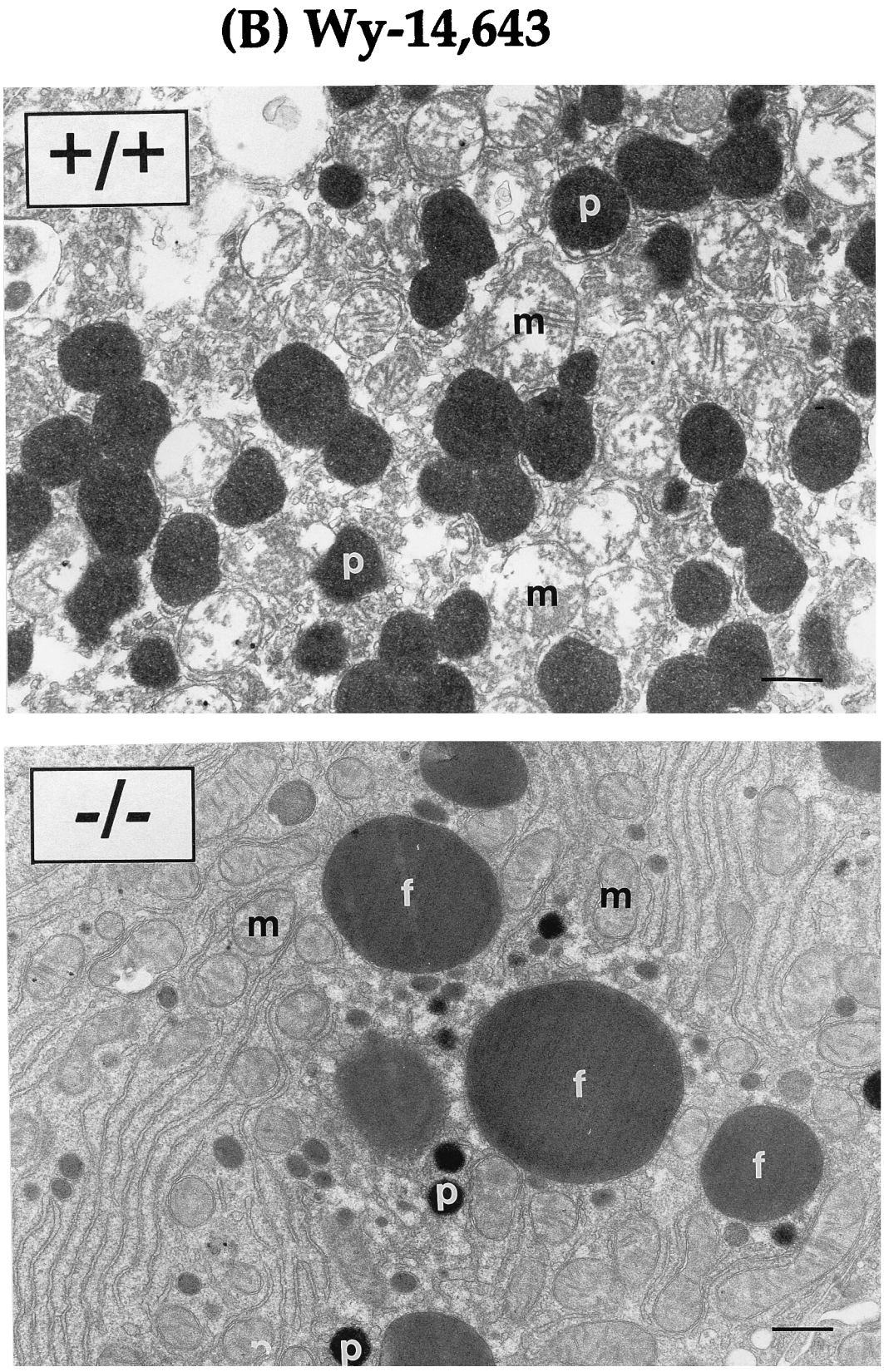

FIG. 4—*Continued.*

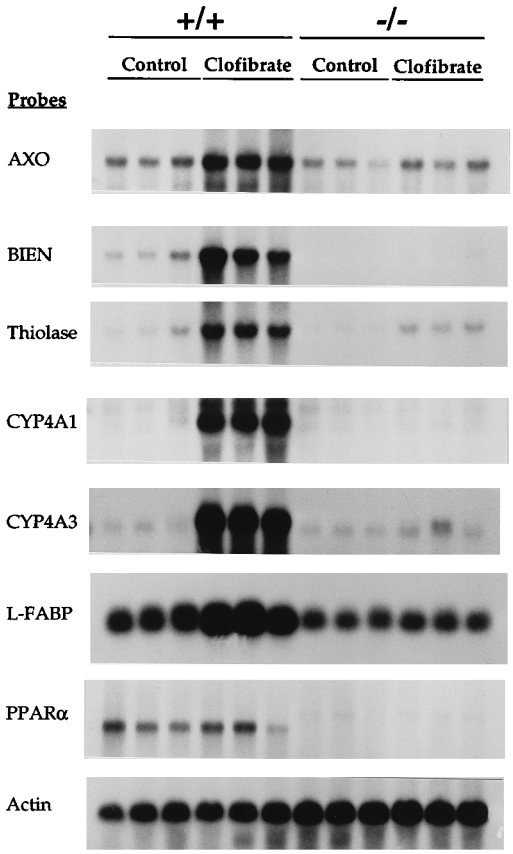

FIG. 5. Northern blot analysis of wild-type (+/+) and mPPARα homozygous mutant (−/−) mice from the clofibrate diet study. Total RNA was extracted from three livers of each of the four experimental groups: +/+ (control diet), +/+ (clofibrate diet), −/− (control diet), and −/− (clofibrate diet). Twenty micrograms of total RNA was electrophoresed on a 1% formaldehyde-agarose gel, blotted onto a nylon membrane, and probed with eight different random-primed ^{32}P-labeled cDNA probes as shown. cDNA probes AXO, BIEN, thiolase, CYP4A1, CYP4A3, and L-FABP are from a rat source, while PPARα and actin are from a mouse source. The exposure times were 24 to 48 h for all probes except actin, which was exposed for 4 h only.

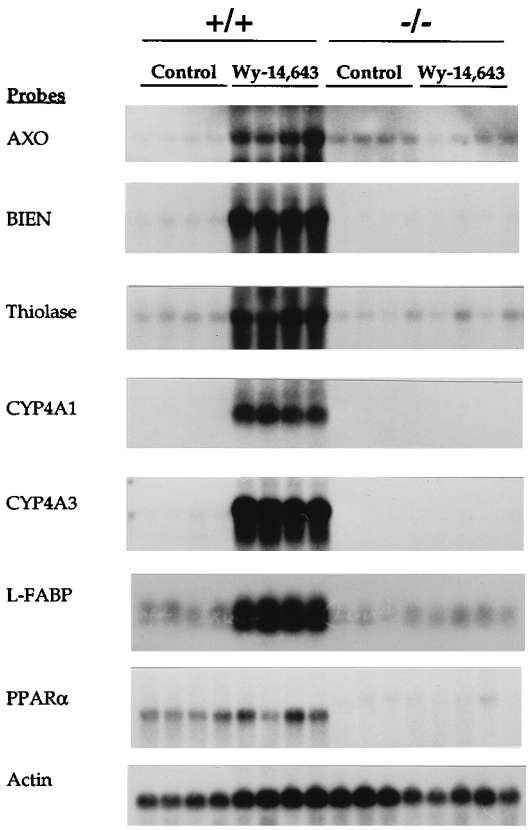

FIG. 6. Northern blot analysis of wild-type (+/+) and mPPARα homozygous mutant (−/−) mice from the Wy-14,643 diet study. Total RNA was extracted from four livers of each of the four experimental groups: +/+ (control diet), +/+ (Wy-14,643 diet), −/− (control diet), and −/− (Wy-14,643 diet). Ten micrograms of total RNA was electrophoresed on a 1% formaldehyde-agarose gel, blotted onto a nylon membrane, and probed with eight different random-primed ^{32}P-labeled cDNA probes as shown. cDNA probes AXO, BIEN, thiolase, CYP4A1, CYP4A3, and L-FABP are from a rat source, while PPARα and actin are from a mouse source. The exposure times were 24 to 48 h for all probes except actin, which was exposed for 4 h only.

mutant animal model should provide insights for studies concerning the PPAR-associated transcriptional activation of malic enzyme, a cytosolic enzyme that catalyzes the oxidative decarboxylation of malate to pyruvate, producing NADPH required for fatty acid biosynthesis (7, 22), mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase, a mitochondrial enzyme important in hepatic ketogenesis during starvation and high-fat feeding (60), and medium-chain acyl-CoA dehydrogenase, a pivotal enzyme that catalyzes the initial reaction in the mitochondrial fatty acid β-oxidation cycle (20). All of these enzymes are involved in lipid metabolism, and their transcriptional activations were also linked to the action of PPARα. Further evidence that PPARα is not limited to peroxisomal and microsomal enzymes has come from a recent study reporting that expression levels of two steroid-regulated genes, encoding transthyretin and metallothionein, were markedly decreased after the administration of peroxisome proliferators (46). Use of the mPPARα-deficient mice might help us to understand the mechanism for the down-regulation of these two genes involved in hormonal homeostasis since variations in transthyretin can influence thyroid function.

Our studies suggest that mPPAR isoforms other than PPARα may not be important in the liver. This is not totally surprising in view of their low levels of expression in this tissue compared with PPARα and their lack of *trans* activation by Wy-14,643 (35). However, they may have important functions associated with nonhepatic tissues and cell types such as adipocytes in which only low levels of mPPARα expression were found but measurable levels of mPPARγ were observed (8, 68, 69), thus suggesting that this isoform may be responsible for signal transduction in fat cells. Finally, it will be of interest to produce mice that lack both PPARα and its dimerization partner, the retinoid X receptor (66), as a means to generate additional phenotypes.

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