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Peroxisome Proliferator-activated Receptor Mediates Induction of the Mitochondrial 3-Hydroxy-3-methylglutaryl-CoA Synthase Gene by Fatty Acids*

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Fatty acids induce an increase in the transcription of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase gene, which encodes an enzyme that has been proposed as a control site of ketogenesis. We studied whether the peroxisome proliferator-activated receptor (PPAR) is involved in the mechanism of this transcriptional induction. We found that cotransfection of a rat mitochondrial HMG-CoA synthase promoter-chloramphenicol acetyltransferase reporter plasmid and a PPAR expression plasmid in the presence of the peroxisome proliferator clofibrate led to a more than 30-fold increase in chloramphenicol acetyltransferase activity, relative to the activity in the absence of both PPAR and inducer. Linoleic acid, a polyunsaturated fatty acid, increased this activity as potently as does clofibrate and more effectively than does monounsaturated oleic acid. We have identified, by deletional analysis, an element located 104 base pairs upstream of the mitochondrial HMG-CoA synthase gene, which confers PPAR responsiveness to homologous and heterologous promoters. This is the first example of a peroxisome proliferator-responsive element (PPRE) in a gene encoding a mitochondrial protein. This element contains an imperfect direct repeat that is similar to those described in the PPREs of other genes. Furthermore, gel retardation and cotransfection assays revealed that, as for other genes, PPAR heterodimerizes with retinoid X receptor and that both receptors cooperate for binding to the mitochondrial HMG-CoA synthase PPRE and subsequent activation of the gene. In conclusion, our data demonstrate that regulation of mitochondrial HMG-CoA synthase gene expression by fatty acids is mediated by PPAR, supporting the hypothesis that PPAR has an important role at the transcriptional level in the regulation of lipid metabolism.

Hepatic ketogenesis produces fuel for the brain and peripheral tissues during episodes of starvation or sustained exercise (see Ref. 1 for a review). Ketone bodies can also act as fuel for the developing brain and lactating mammary gland. For these reasons complete understanding of how this metabolic process is controlled is of great importance. Although carnitine palmitoyltransferase I, the enzyme responsible for the regulation of the entry of acyl-CoA to mitochondria, might control the rate of ketogenesis, several authors suggest that it is not the only site of control (2–4) and that mitochondrial 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[1] synthase, the enzyme that catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA and CoA in mitochondria, is another important site of control of ketogenesis in different metabolic situations (5–8). Fat feeding increases ketogenesis, and there is evidence that the activity of mitochondrial HMG-CoA synthase is regulated by fatty acids; when these compounds are added to isolated mitochondria, desuccinylation and activation of the enzyme is induced, resulting in a stimulus to ketogenesis (9). A fat diet given to rats increases mitochondrial HMG-CoA synthase activity, mRNA, and protein levels (10–12), and fatty acids added to HepG2 cells increase rat mitochondrial HMG-CoA synthase-CAT chimeric gene transcription (13). The combination of these processes results in a dual control of ketogenesis by increasing both the amount and the activity of the enzyme.

Fatty acids can regulate gene expression mediated by a member of the nuclear hormone receptor superfamily termed the peroxisome proliferator-activated receptor (PPAR) (14, 15). Such receptors are ligand-activated transcription factors. Recent results have demonstrated that PPAR can bind to a specific DNA sequence (peroxisome proliferator-responsive element, PPRE) located upstream of the rat fatty acyl-CoA oxidase gene (16–18) and that this element is also present in the 5′ region of the rat liver fatty acid-binding protein gene (19), the rabbit cytochrome P450 fatty acid ω-hydroxylase gene (20), and the rat bifunctional enzyme gene of the peroxisomal β-oxidation pathway (21–23). The binding of ligand-activated PPAR to these genes produces transcriptional induction. This activation process is dependent on heterodimer formation between PPAR and retinoid X receptor (RXR) (15, 23–27).

Regulation of the expression of genes involved in lipid metabolism by fatty acids is of great physiological and clinical interest. The identification of further PPAR activators and target genes may lead to a better understanding of the complete role of PPARs in the hormonal control of lipid metabolism.

This report reveals that the mitochondrial HMG-CoA synthase gene promoter contains a responsive element to PPAR that has been localized precisely. In HepG2 cells the response to this receptor is increased by the addition of exogenous ligand. In addition, it is shown that activation of mitochondrial HMG-CoA synthase gene expression by fatty acids could be mediated

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[1] The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; CAT, chloramphenicol acetyltransferase; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element; RXR, retinoid X receptor; hRXRα, human 9-*cis*-retinoic acid receptor α; mPPARα, mouse peroxisome proliferator-activated receptor α; bp, base pair(s).

by this receptor. Furthermore, we demonstrate that PPAR binds to this element as a RXR-PPAR heterodimer.

MATERIALS AND METHODS

Plasmids—pSMPCAT1 was constructed by subcloning 1148 bp[2] of the 5′-flanking region of the rat mitochondrial HMG-CoA synthase gene and the first 28 bp of exon 1 into the CAT vector pCAT-BASIC reporter gene (Promega) as previously described (13). pSMPCAT2, pSMPCAT3, pSMPCAT4, pSMPCAT5, pSMPCAT6, and pSMPCAT7 contain deleted constructs of the 5′ regulatory region and the same 3′ deletion end point in exon 1 of the gene as previously described (13). pSMPCAT6A was constructed by the application of the polymerase chain reaction, using a pair of oligonucleotide primers, SM1 (5′-AGACGTCGACTGACTTGTTCTGAGACCTTTG, corresponding to coordinates −116 to −96 of the 5′-flanking region of the mitochondrial HMG-CoA synthase gene), and CATOLIGO (5′-CTTTACGATGCCATTGGGATATATCAACGGTGGTATATCC, 2333 to 2372 of pCAT-BASIC vector), and pSMPCAT6 as a template. The *Sal*I-*Xba*I-digested polymerase chain reaction product was cloned into pCAT-BASIC. pSMPCAT6B was constructed in an identical manner to pSMPCAT6A but using SM2 (5′-AGACGTCGACAGACCTTTGGCCCAGTTTTTC, −104 to −84) instead of SM1. The underlined sequences are the restriction sites for *Sal*I. The sequence of the polymerase chain reaction-amplified DNA was confirmed by dideoxy sequencing with a T7 sequencing kit (Pharmacia Biotech Inc.). In order to prepare pSMPCAT6C (−95 to +28), a 199-bp fragment was isolated from the digestion of pSMPCAT6 with *Hin*dIII and was digested with *Sau*96I to yield a 127-bp fragment. It was made blunt-ended, using the Klenow fragment of DNA polymerase I, and cloned into pCAT-BASIC. Heterologous promoter plasmids were constructed in pBLCAT2, which contains the herpesvirus thymidine kinase gene promoter upstream of the CAT reporter gene (28). pTKCATSM116-96 contains a fragment corresponding to coordinates −116 to −96 of the mitochondrial HMG-CoA synthase gene. It was constructed by cloning the oligonucleotide 5′-agctTGACTTGTTCTGAGACCTTTG annealed to 5′-tcgaCAAAGGTCTCAGAACAAGTCA into pBLCAT2 (nucleotides designated in lower case were added to provide cohesive *Hin*dIII-*Sal*I ends at the 5′ and 3′ termini, respectively). pTKCATSM116-69 contains one copy of a fragment corresponding to coordinates −116 to −69. To prepare it, pSMPCAT6A was digested with *Sal*I and *Stu*I to give a 51-bp fragment, which was cloned into pBLCAT2. The cloning of three copies of this fragment, one of them in inverted orientation, yielded pTKCAT(3×)SM116-69. Mutant pSMPCAT1M was generated by replacing the sequence flanked by *Nhe*I and *Stu*I sites of pSMPCAT1 with a *Nhe*I-*Stu*I fragment generated by polymerase chain reaction, using a pair of oligonucleotide primers, SM3 (5′-ATCCCCACTCTGCAGCTTTC, −758 to −739) and SMMStuI (5′-CCTGCGAGGCCTCTGCCTGCCTCAGAAAAACTcaagtggctgcagCAGAACAAGTCAAAG, −119 to −60), and pSMPCAT1 as a template. Mutant pSMPCAT6AM was constructed in an identical manner to pSMPCAT6A but using the oligonucleotide SMMSalI (5′-CAGACGTCGACTGACTTGTTCTGctgcagcca, −116 to −96) instead of SM1 and pSMPCAT1M as a template. Nucleotides designated in lower case correspond to those that have been scrambled from the wild type sequence. A plasmid expressing mouse PPARα was kindly provided by Dr. S. Green (Macclesfield, United Kingdom). cDNA encoding this PPAR was originally cloned in the expression vector pSG5 (29). pSKXR3-1 containing a cDNA for human RXRα cloned into pBluescript SK vector was generously provided by Dr. R. M. Evans (Salk Institute, San Diego). The hRXRα cDNA was digested with *Eco*RI and religated to change the orientation of the insert. The resulting plasmid was digested with *Hin*dIII and *Xba*I to produce a fragment that was cloned into the expression vector pCDM8 (Invitrogen) to yield plasmid pJCXR8. The inserts in all these plasmids, with the exception of pTKCAT(3×)SM116-69, had the same 5′→3′ orientation as found in the natural mitochondrial HMG-CoA synthase gene promoter. The chimeric constructs were verified by DNA sequence analysis using a dideoxy chain termination technique (30). DNA fragments smaller than 100 bp were separated by 4.5% Metaphor agarose (FMC BioProducts) gel electrophoresis and recovered using activated DEAE paper.

Cell Culture and Transfections—HepG2 and HeLa cells were cultured in minimal essential media supplemented with nonessential amino acids and 10% fetal calf serum. Cells were cotransfected typically with 5 μg of the reporter mitochondrial HMG-CoA synthase-CAT gene construct and, when indicated, with 0–100 ng of pSG5-mPPARα and/or pJCXR8 expression plasmids. Effector plasmid dosage was kept constant by the addition of appropriate amounts of the empty expression vector pSG5. 4 μg of plasmid pRSVβGAL (Rous sarcoma virus promoter β-galactosidase) was included as an internal control in cotransfections. Transfection experiments were carried out by the calcium-phosphate method as described (31, 32). After removal of the calcium-phosphate-DNA precipitate, cells were refed with the appropriate medium. Experiments with ligand included either vehicle (dimethyl sulfoxide or ethyl alcohol) or ligand (1 mM clofibrate, 1 μM all-*trans*-retinoic acid, 150 μM linoleic acid, or 250 μM oleic acid). All ligands used were from Sigma. Cells were harvested 48 h after refeeding.

β-Galactosidase and CAT Assays—Extracts of harvested cells were prepared by liquid nitrogen freeze/thaw disruption (three times) after resuspension in 100 μl of 0.25 M Tris-HCl, pH 7.5. β-Galactosidase activity was determined (32) in a 10–20-μl volume of extract to normalize for transfection efficiency. All samples assayed for CAT activity were first incubated at 65 °C for 10 min. CAT assays were performed (31) for either 60 or 180 min for HepG2 and HeLa extracts, respectively. Radioactivity of samples was measured on an LKB-1217 liquid scintillation counter.

In Vitro Transcription/Translation—cDNAs for mPPARα and hRXRα were transcribed and translated (in rabbit reticulocyte lysate) directly from 1 μg of pSG5-mPPARα and 1 μg of pJCXR8, respectively, by using a commercially available kit according to the instructions of the manufacturer (Promega). In order to obtain an unprogrammed lysate as a negative control for electrophoretic mobility shift analysis, a translation reaction was performed with 1 μg of pSG5.

Electrophoretic Mobility Shift Analysis—mPPARα (2.5 μl) and/or hRXRα (2.5 μl) synthesized *in vitro* were preincubated on ice for 10 min in 10 mM Tris-HCl, pH 8.0, 40 mM KCl, 0.05% (v/v) Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, and 1 μg of poly(dI-dC). The total amount of reticulocyte lysate was kept constant in each reaction (5 μl) through the addition of unprogrammed lysate. For competition experiments a 75-fold molar excess of MSPPRE, MMSPPRE, MSA, or MSB double-stranded probes, relative to the labeled probe, was included during preincubation. (MSPPRE is the fragment corresponding to coordinates −116 to −69 of the mitochondrial HMG-CoA synthase gene, which was used to prepare pTKCATSM116-69; MMSPPRE is equivalent to MSPPRE, but the corresponding nucleotides −104 to −92 have been scrambled from the wild type sequence, and MMSPPRE was obtained by digestion of pSMPCAT6M with *Sal*I and *Stu*I; MSA is a synthetic oligonucleotide corresponding to coordinates −116 to −96, which was used to construct pTKCATSM116-96; MSB is an unrelated synthetic oligonucleotide (5′-AGCTAGTCTTATAAAGCCCA) including the TATA box of the mitochondrial HMG-CoA synthase gene.) Then 0.8 ng of MSPPRE, ^{32}P-labeled by fill in with Klenow polymerase, was added, and the incubation was continued for 15 min at room temperature. The final volume for all reactions was 20 μl. Samples were electrophoresed at 4 °C on a 4.5% polyacrylamide gel in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0).

RESULTS

Rat Mitochondrial HMG-CoA Synthase Gene Contains an Element That Is Responsive to Peroxisome Proliferators—Chloramphenicol acetyltransferase expression from pSMPCAT1 containing the promoter and 5′-flanking sequences of the rat mitochondrial HMG-CoA synthase gene (−1148 to +28) greatly exceeded the activity observed for a promotorless CAT construct transfected in human hepatoma HepG2 cells. Cotransfection of a mouse PPARα expression vector (pSG5-mPPARα) led to a more than 30-fold increase in CAT activity in the presence of the peroxisome proliferator clofibrate (1 mM) (Fig. 1).

We examined the effect of varying the amount of this expression vector on reporter plasmid stimulation in HepG2 cells. As seen in Fig. 1, both in the presence and in the absence of clofibrate, increasing amounts of pSG5-mPPARα resulted in greater stimulation of CAT activity. It is interesting to note the substantial activation observed in the absence of clofibrate, amounting to 50% of the activity seen in the presence of clofibrate.

Localization of PPRE—In order to determine the approximate location of the PPRE, progressively larger 5′ deletions of the 5′-flanking region of pSMPCAT1 were used to define the locations of the control elements that mediate the effects of PPAR. These constructs were tested in cotransfection experi-

[2] The coordinates are different from those described in Ref. 12 due to an incorrect extra G in the position −75 of that sequence.

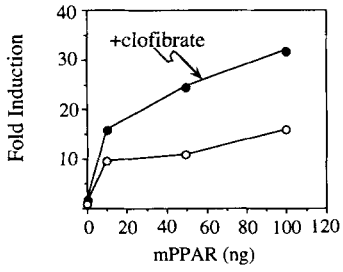

FIG. 1. **PPAR-dependent activation of the mitochondrial HMG-CoA synthase gene promoter by clofibrate.** pSMPCAT1 (−1148 to +28) was cotransfected with increasing amounts of pSG5-mPPARα into HepG2 cells either in the absence (○) or presence (●) of 1 mM clofibrate. Average values of β-galactosidase-normalized CAT activity, from three independent transfections with two plates each, are expressed as -fold induction relative to the activity in the absence of both PPAR and clofibrate.

ments with pSG5-mPPARα using HepG2 cells (Fig. 2).

Deletions of 5′ nucleotides upstream of −982 resulted in increased activation. Furthermore, deletion of sequences between −982 and −333 resulted in almost a 50% decrease in trans-activation. Nucleotides up to position −104 can be deleted without loss of the response to PPAR, which was eliminated by further deletion up to nucleotide −95 (Fig. 2*B*, compare construct *6B* with construct *6C*).

These results identified positions between −104 and −95 as part of the putative mitochondrial HMG-CoA synthase PPRE. Next, several fragments around this position were inserted into pBLCAT2, a plasmid containing the CAT gene under the control of the thymidine kinase gene promoter. As can be seen in Fig. 3, the fragment between −116 and −69, but not that included between −116 and −96, conferred PPAR responsiveness to the otherwise unresponsive thymidine kinase gene promoter. More than one copy of this fragment multiplied induction by PPAR. Together, these data suggest that the PPRE is located between −104 and −69. This region contains an imperfect direct repeat separated by one nucleotide (AGACCTTTGGCCC), corresponding to coordinates −104 to −92, which seems to mediate the response to PPAR. This was confirmed in two ways. First, in transfection experiments the putative PPRE was altered by mutation in the context of both the larger and deletion −116 promoter constructs (pSMPCAT1 and pSMPCAT6A, respectively). As can be seen in Fig. 4, the scrambling of this sequence in both constructs obliterates the response to PPAR. Second, in gel mobility shift assays (see below) MMSPPRE, an oligonucleotide corresponding to coordinates −116 to −69 with nucleotides −104 to −92 scrambled, was not able to compete with the wild type probe for the formation of the complex. This sequence is similar to the imperfect direct repeats of the PPRE that have been described in other genes (see Table I). The results demonstrate that this mitochondrial HMG-CoA synthase element is able to confer PPAR responsiveness both on its natural context and on a normally unresponsive promoter.

Heterodimerization of PPAR with RXR—The convergence of the retinoid and PPAR signaling pathways has been shown previously. This led us to examine whether there is a functional interaction of PPAR and RXR in transcriptional activation of the PPRE containing pSMPCAT1 reporter plasmid. Mouse PPARα and human RXRα expression plasmids, either alone or in combination, were cotransfected with pSMPCAT1 reporter plasmid in HepG2 cells, and CAT assays were performed after induction in the presence or absence of 1 μM retinoic acid (Fig. 5). All-*trans*-retinoic acid has been shown to activate RXR, albeit at much higher concentrations than 9-*cis*-retinoic acid (33, 34). Cotransfection of the expression plasmids for both mPPARα and hRXRα in the presence of retinoic acid resulted in a synergistic increase in the activity of the mitochondrial HMG-CoA synthase gene promoter.

We next performed gel mobility shift assays to analyze whether PPAR-RXR heterodimers bind to the PPRE of the mitochondrial HMG-CoA synthase gene. mPPARα or hRXRα alone did not bind significantly to the MSPPRE probe. However, incubation of this PPRE-containing probe with a mixture of mPPARα and hRXRα resulted in a prominent complex. The specificity of this complex was demonstrated by competition with a 75-fold molar excess of unlabeled MSPPRE probe whereas a 75-fold molar excess of three different oligonucleotides (MMSPPRE, which contains a mutated version of PPRE, and MSA and MSB, which do not contain the PPRE) did not lead to the disappearance of the complex (Fig. 6).

Fatty Acids Regulate the Mitochondrial Synthase Gene Expression Mediated by PPAR—There is considerable evidence that fatty acids can activate PPAR as potently as peroxisome proliferators do (14, 15, 19). On the other hand, previous results in our laboratory revealed that fatty acids induce an increase in the mitochondrial HMG-CoA synthase mRNA levels (11) and gene transcription (13). We were therefore interested in determining whether fatty acids could have a role in the regulation of the mitochondrial synthase gene expression mediated by PPAR. HeLa cells were cotransfected with 100 ng of pSG5-mPPARα expression vector and pSMPCAT1 reporter gene, and CAT assays were performed after induction in the presence or absence of fatty acids (150 μM linoleic acid or 250 μM oleic acid). As can be seen in Fig. 7, the highest stimulation of the mitochondrial synthase-CAT reporter plasmid was observed after induction with fatty acids in the presence of PPAR. In agreement with previous results (14, 15), we observed that monounsaturated oleic acid is less potent than linoleic acid in the activation of PPAR.

DISCUSSION

In this paper we show that the 5′ region of the mitochondrial HMG-CoA synthase gene contains an imperfect direct repeat that seems to be important for the response of this gene to the PPAR. To our knowledge, this is the first example of a PPRE in a gene encoding a mitochondrial protein. These studies also reveal that the fatty acid-mediated activation of the mitochondrial HMG-CoA synthase gene is increased in the presence of PPAR. Our data support the hypothesis that PPAR is involved in the regulation of lipid metabolism by activating an alternative pathway for the utilization of fatty acids, such as ketogenesis.

Our first observation was that PPAR is able to activate the mitochondrial HMG-CoA synthase gene expression in a dose-dependent manner. The stimulation is maximal in the presence of the hypolipidemic drug clofibrate, but it is substantial even in the absence of this compound. Similar observations have been reported for other genes (17, 20, 23), and this could be attributed to the presence of an endogenous ligand of PPAR, hypothetically the physiological ligand, the structure of which remains to be demonstrated. Alternatively, it could simply reflect a constitutive activity of this receptor.

We have been able to map the sequence responsible for this response. We have shown by deletion analysis and mutation experiments that the PPRE is located between −104 and −92. This segment contains an imperfect direct repeat AGACCTTTGGCCC similar to the consensus half-site TGACCT for several nuclear hormone receptors (35), which is also present in other PPAR-responsive genes. While the direct repeat is required for induction, it is by itself insufficient for maximal induction (see Fig. 2*A*, *constructs 2–4*). Although the mutation experiment in the context of the larger promoter construct shows that induction is dependent only on this sequence and is not mediated

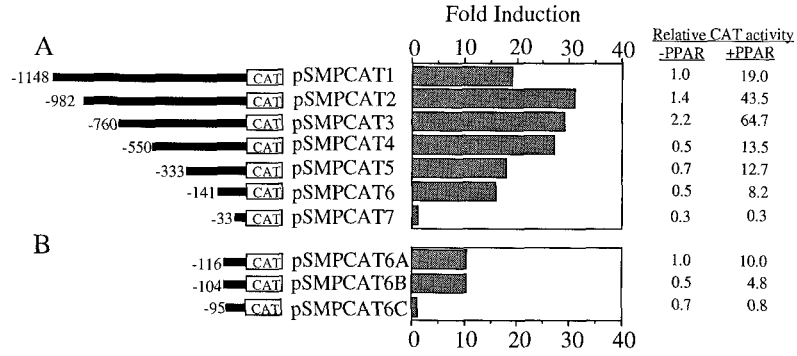

FIG. 2. **Localization of the response element of the mitochondrial HMG-CoA synthase gene by progressive deletion analysis.** CAT reporter constructs containing the indicated position of the 5′-flanking region of the mitochondrial HMG-CoA synthase gene were cotransfected with or without 100 ng of pSG5-mPPARα into HepG2 cells. Average values of β-galactosidase-normalized CAT activity, from at least three independent transfections with two plates each, are expressed as -fold induction relative to the activity to each construct in the absence of PPAR. Relative CAT activity in the absence or presence of PPAR is indicated, with the activity of pSMPCAT1 (*section A*) and that of pSMPCAT6A (*section B*) both in the absence of PPAR defined as 1.0.

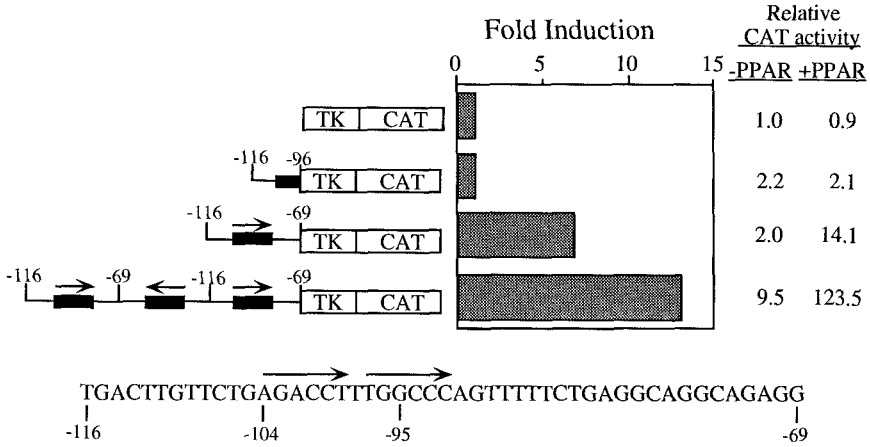

FIG. 3. **The mitochondrial HMG-CoA synthase PPRE confers PPAR responsiveness to thymidine kinase gene promoter.** HepG2 cells were cotransfected with the expression vector for mPPARα and different reporter plasmids containing the CAT gene under the control of the thymidine kinase gene promoter and different fragments from the 5′ region of mitochondrial HMG-CoA synthase gene as indicated. Average values of β-galactosidase-normalized CAT activity, from at least three independent transfections with two plates each, are expressed as -fold induction relative to the activity in the absence of expression vector pSG5-mPPARα. Relative CAT activity in the absence or presence of PPAR is indicated, with the activity of pBLCAT2 in the absence of PPAR defined as 1.0. *Black boxes* indicate the imperfect direct repeat of the mitochondrial HMG-CoA synthase PPRE (−104 to −92). *Arrows* over the *black boxes* represent the orientation of the imperfect direct repeat with respect to the natural promoter. The nucleotide sequence between −116 and −69 is shown. *Arrows* over the sequence indicate the motifs and their orientation.

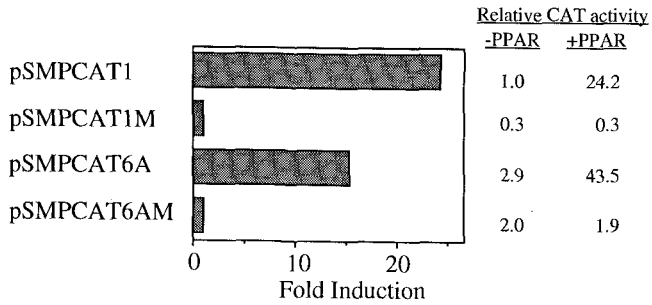

FIG. 4. **Mutation of the putative mitochondrial HMG-CoA synthase PPRE obliterates the response to PPAR.** CAT reporter constructs containing the wild type (pSMPCAT1 and pSMPCAT6A) or mutated (by scrambling nucleotides between −104 to −92, pSMPCAT1M and pSMPCAT6AM) 5′-flanking region of the mitochondrial HMG-CoA synthase gene were cotransfected with or without 100 ng of pSG5-mPPARα into HepG2 cells. Average values of β-galactosidase-normalized CAT activity, from at least two independent transfections with two plates each, are expressed as -fold induction relative to the activity to each construct in the absence of PPAR. Relative CAT activity in the absence or presence of PPAR is indicated, with the activity of pSMPCAT1 in the absence of PPAR defined as 1.0.

TABLE I

Comparison of the imperfect direct repeats of the described PPRE in different genes and proposed consensus

AOx, rat fatty acyl-CoA oxidase gene; CYP4A6, rabbit cytochrome P450 fatty acid ω-hydroxylase gene; HD, rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene; L-FABP, rat liver fatty acid-binding protein gene; MTHMGS, rat mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene.

Gene	Position	Direct repeat (5′ to 3′)	Ref.
AOx	−570 to −558 (sense)	TGACCT T TGTCCT	17
CYP4A6	−650 to −662 (antisense)	TCAACT T TGCCCT	19
HD	−2939 to −2927 (sense)	TGAACT A TTACCT	37
L-FABP	−68 to −56 (sense)	TGACCT A TGGCCT	18
MTHMGS	−104 to −92 (sense)	AGACCT T TGGCCC	This paper
		AC A A G C	
	Consensus	⇕⇕A⇕CT ⇕ T⇕NCC⇕	
		TG C T T T	

independently by other PPAR-responsive upstream sequences, it seems that maximal transcriptional induction depends on cooperation with other proteins bound to distal sites.

The convergence of retinoid and PPAR signaling pathways has been analyzed by several authors, and it has been shown that both PPAR and RXR stimulate the acyl-CoA oxidase gene through PPAR-RXR heterodimers that bind to PPREs (15, 24–27). This heterodimerization has also been shown to be necessary for the activation of the rat enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene (23, 27). Evidence from several sources led us to conclude that this is also true for the

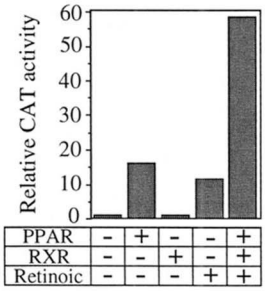

FIG. 5. **Transcriptional activation of the mitochondrial HMG-CoA synthase gene promoter by PPAR and RXR.** HepG2 cells were cotransfected with the expression vectors for mPPARα and hRXRα and reporter plasmid pSMPCAT1 (−1148 to +28) and in the presence or absence of 1 μM retinoic acid as indicated at the *bottom* of the figure. Average values of β-galactosidase-normalized CAT activity, from at least three independent experiments with two plates each, are expressed as relative CAT activity, with the activity in the absence of the expression vectors and in the absence of retinoic acid defined as 1.

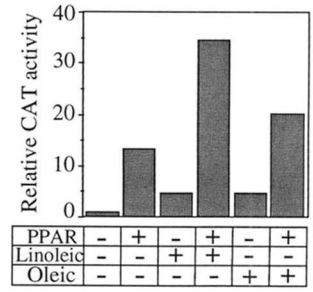

FIG. 7. **Transcriptional activation of the mitochondrial HMG-CoA synthase gene promoter by fatty acids.** HeLa cells were cotransfected with 100 ng of the expression vector for mPPARα and reporter plasmid pSMPCAT1 (−1148 to +28) in the presence or absence of 150 μM linoleic acid or 250 μM oleic acid as indicated at the *bottom* of the figure. Average values of β-galactosidase-normalized CAT activity, from two independent experiments with two plates each, are expressed as relative CAT activity, with the activity in the absence of both PPAR and fatty acids defined as 1.

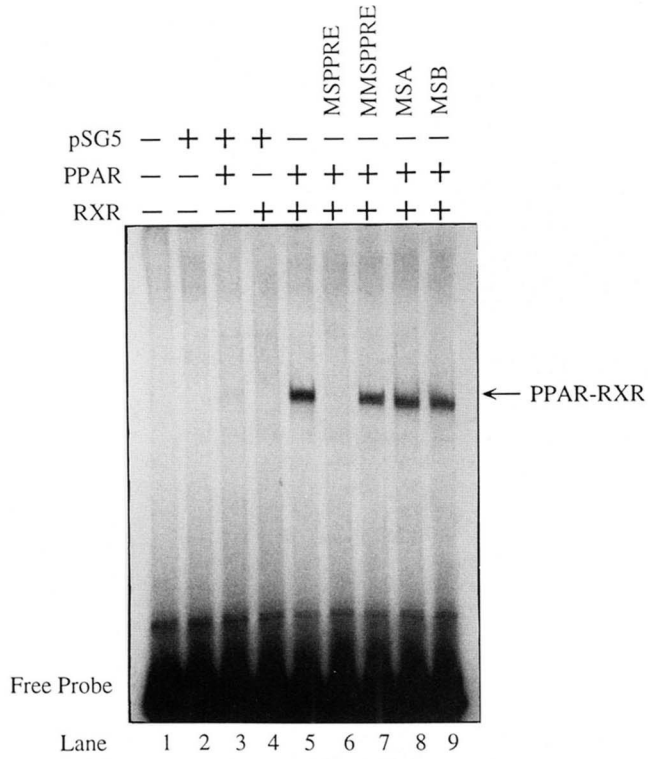

FIG. 6. **Electrophoretic mobility shift assay of the mitochondrial HMG-CoA synthase PPRE with PPAR-RXR heterodimers.** mPPARα and hRXRα were translated *in vitro*, incubated with labeled MSPPRE probe, and analyzed by electrophoretic mobility shift analysis. Additions were as indicated at the *top* of the figure. The total amount of reticulocyte lysate was maintained constant in each reaction (5 μl) through the addition of unprogrammed lysate (pSG5, see "Materials and Methods"). *Lane 5* contained a mixture of equal volumes of mPPARα and hRXRα translation reactions. The specific PPAR-RXR-PPRE complex is indicated by an *arrow*. *Lanes 6–9* contained a competition of the complex with a 75-fold molar excess of different unlabeled mitochondrial synthase gene promoter oligonucleotides: *MSPPRE*, containing the proposed PPRE; *MMSPPRE*, identical probe but with the proposed PPRE mutated by scrambling; *MSA*, containing the region between −116 and −96; *MSB*, containing an unrelated fragment of the mitochondrial HMG-CoA synthase gene promoter.

mitochondrial HMG-CoA synthase PPRE. First, cells transfected with pSMPCAT1, the construct containing the longest mitochondrial synthase 5′-flanking region, have 3.6-fold higher CAT activity (calculated from the data in Fig. 5) when they are cotransfected with both PPAR and RXR in the presence of retinoic acid than when transfected with PPAR alone. It has been reported (33, 34) that all-*trans*-retinoic acid supplemented cells convert this precursor to its stereoisomer 9-*cis*-retinoic acid, the physiological ligand of RXR. We attribute the induction seen in the presence of retinoic acid alone to endogenous RXR in HepG2 cells. Furthermore, added RXR without additional retinoic acid is inactive and does not produce transactivation. Second, in order to interact with the mitochondrial HMG-CoA synthase PPRE-containing probe to produce a bandshift in a gel retardation assay, the presence of both PPAR and RXR proteins is necessary. Neither PPAR nor RXR alone was able to produce a homodimeric interaction with this responsive element. An interesting point is that oligonucleotide MSA containing region between −116 and −96 was not able to compete. Moreover, even though it contained an imperfect direct repeat of the half-site TGACCT between −111 and −99 (TGTTCTGAGACCT), it was not able to confer PPAR responsiveness to a heterologous promoter (see Fig. 3, *construct-containing region between −116 and −96*). This sequence may be the target for the interaction with other transcription factors such as the chicken ovalbumin upstream promoter transcription factor family, which compete with PPAR and RXR, as is the case of the rat enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene (36), adding more complexity to the system and increasing the possibilities of regulation. Experiments are in progress to address this question.

We have previously reported that administration of a fatty diet to rats increases mitochondrial HMG-CoA synthase mRNA levels, and this effect can be explained at the transcriptional level since fatty acids increase mitochondrial HMG-CoA synthase-CAT chimeric gene transcription in tissue culture (11, 13). Here we have shown that in HeLa cells the activation of the mitochondrial HMG-CoA synthase gene promoter by fatty acids is maximal in the presence of PPAR.

PPARs are transcription factors that are activated by fatty acids and regulate the expression of enzymes involved in the metabolism of these nutrients. It has been shown that PPARs activate genes of peroxisomal β-oxidation and microsomal cytochrome P450-mediated ω-hydroxylation, and the cytosolic fatty acid transport-related liver fatty acid-binding protein gene. This study provides evidence supporting the influence of PPAR in the regulation of another lipid metabolism-related gene, mitochondrial HMG-CoA synthase. This enzyme has been proposed by several authors as an important control site of the ketogenesis pathway, which produces ketone bodies that can be used as metabolic fuel in catabolic states such as starvation and diabetes (37). Therefore, these data provide further

evidence that PPAR has an important role in the regulation of lipid metabolism.

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