Isolation of pig mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene promoter: characterization of a peroxisome proliferator-responsive element

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Low expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase gene during development correlates with an unusually low hepatic ketogenic capacity and lack of hyperketonaemia in piglets. Here we report the isolation and characterization of the 5′ end of the pig mitochondrial HMG-CoA synthase gene. The 581 bp region proximal to the transcription start site permits transcription of a reporter gene, confirming the function of the promoter. The pig mitochondrial HMG-CoA synthase promoter is *trans*-activated by the peroxisomal proliferator-activated receptor (PPAR), and a functional response element for PPAR (PPRE) has been localized in the promoter region. Pig PPRE is constituted by an imperfect direct repeat (DR-1) and a downstream sequence, both of which are needed to confer PPAR-sensitivity to a thymidine kinase promoter and to form complexes with PPAR · retinoid X receptor heterodimers. A role of PPAR *trans*-activation in starvation-associated induction of gene expression is suggested.

Key words: fatty acids, gene expression, ketogenesis.

INTRODUCTION

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase is a potential regulatory site in the pathway converting acetyl-CoA into ketone bodies [1–3]. In rats, hepatic ketogenic capacity increases rapidly during postnatal development [4] or fasting [5], when the liver mRNA, protein, and activity of mitochondrial HMG-CoA synthase increase [6–11]. Factors responsible for the postnatal induction and starvation-associated changes in mitochondrial HMG-CoA synthase gene expression remain to be fully characterized, but probably involve changes in circulating hormones and fatty acids. The rat mitochondrial HMG-CoA synthase gene contains elements that mediate its multihormonal regulation and tissue specificity [12]. Recently, it has been shown that the peroxisomal proliferator-activated receptor (PPAR) is a mediator of the fatty-acid-responsiveness of the gene, and a DNA response element for peroxisomal proliferator-activated receptor (PPRE) has been characterized in the promoter region of the rat mitochondrial HMG-CoA synthase gene [13].

In pigs, ketone-body metabolism is much lower than in other mammals. Thus the suckling pig has a limited capacity for liver ketogenesis [14–18], and liver mRNA levels and the activity [16,19] of mitochondrial HMG-CoA synthase are very low in this period. In addition, while fasting of suckled rat pups does not lead to an increase in mitochondrial HMG-CoA synthase mRNA levels [20], starvation of 2-week-old piglets elicits a large stimulation of liver mRNA and activity [19], raises ketogenesis from long-chain fatty acids *in vitro* [14] and increases circulating ketone bodies [21]. These starvation-associated changes in pig mitochondrial HMG-CoA synthase gene expression correlate with increased plasma non-esterified fatty acid (NEFA) levels, without significant changes of insulin or glucagon levels [19], suggesting that PPAR could also *trans*-activate the pig mitochondrial HMG-CoA synthase gene.

Since the cloning of some peroxisome proliferator-activated receptors (PPARs) (for reviews, see [22–25]) it has been suggested that fatty acids may modulate gene expression through these receptors. In mammals there are at least three PPAR isotypes: α, γ and δ (FAAR, Nuc I, β), which display widely divergent patterns of expression during embryogenesis and in the adult and also have different sensitivities to activation by fatty acids and fibrates [22,26–28]. All PPAR isotypes are able to bind to a PPRE as heterodimers with the retinoid X receptor (RXR) [22,23,29]. PPREs can be mapped, in an orientation-independent fashion, on target genes [22]. They contain imperfect direct repeats of the consensus binding sequence for the nuclear receptor superfamily, TGACCT, with a spacing of a single base pair [DR (direct repeat)-1] [22,30]. In addition, a novel conserved sequence, flanking the second repeat of DR-1, is determinant for RXR · PPAR heterodimer binding to PPREs [30,31].

Here we study the expression of genes related to lipid metabolism in a low-ketogenic mammal. We have cloned the 5′ flanking region of the pig mitochondrial HMG-CoA synthase gene and characterized PPAR *trans*-activation. We demonstrate that there is a functional PPRE in the pig mitochondrial HMG-CoA synthase promoter, since it confers PPAR-sensitivity to a thymidine kinase promoter and forms complexes with PPAR · RXR heterodimers. Therefore, fatty-acid *trans*-activation does not appear to be a species-specific difference between high-ketogenic and low-ketogenic mammals.

EXPERIMENTAL

Reagents

A pig liver genomic library in Lambda EMBL3 SP6/T7 vector was purchased from Clontech (Palo Alto, CA, U.S.A.). pCAT-Basic plasmid and p-GEM-T vector were purchased from Promega (Madison, WI, U.S.A.). ^{32}P-labelled nucleotide was

Abbreviations used: 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; EMSA, electrophoretic-mobility-shift analysis; RACE, **r**apid **a**mplification of **c**DNA **e**nds; NEFA, non-esterified fatty acids; DR, direct repeat.

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obtained from ICN (Irvine, CA, U.S.A.). Acetyl-CoA and *o*-nitrophenyl β-D-galactopyranoside (ONPG) were from Sigma (St. Louis, MO, U.S.A.). D-*threo*-[*dichloroacetyl*-1-^{14}C]Chloramphenicol (57 mCi/mmol) was purchased from Amersham (Amersham, Bucks., U.K.). Oligonucleotides were purchased from Genosys Europe (Cambridge, U.K.).

5′-RACE (rapid amplification of cDNA ends) of the pig HMG-CoA synthase cDNA

In the 5′ end amplification [32], a RACE kit from Clontech was used as previously described [19]. The PCR product was cloned taking advantage of an *Eco*RI restriction site introduced in the anchor sequence and AMP5 primers [19]. The insert of the resulting plasmids (pSIM 2, pSIM 3, pSIM6) were sequenced to determine the transcription start site.

Genomic cloning of the pig HMG-CoA synthase exon 1

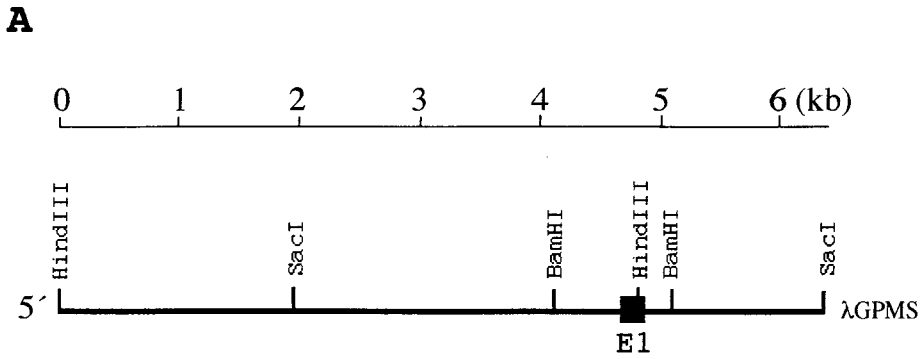
Approx. 600000 recombinants of the pig genomic DNA library were plated (30000 plaque-forming units/150 mm-diameter dish) and transferred to nylon membranes. Recombinants were screened with ^{32}P-labelled double-strand pSIM3 cDNA, which encodes for the 5′ end (417 bp) of pig liver mitochondrial HMG-CoA synthase cDNA [19], and three bacteriophages were isolated. The λGPMS clone (22 kb insert) was the only one that also hybridized with an *Eco*RI/*Hin*dIII 5′ fragment (111 bp probe) of pSIM3 clone, and it was taken through three rounds of plaque purification. A *Bam*HI (954 bp) fragment of λGPMS clone, carrying exon 1 of pig mitochondrial HMG-CoA synthase, was cloned into Bluescript (SK$^+$), generating pGPMS.

Chloramphenicol acetyltransferase (CAT) constructs

Pig promoter CAT constructs were obtained by subcloning the first 43 bp of exon 1 of pig mitochondrial HMG-CoA synthase gene and 581 bp (pGPMSCAT1), 134 bp (pGPMSCAT2), or 74 bp (pGPMSCAT3) of the 5′-flanking region into CAT vector pCAT-Basic reporter gene (Promega). The subcloning used a six-cycle PCR with specific primers in which restriction recognition sequences were introduced at the 5′ end of each primer (underlined in the primer sequence). The PCR product was then enzymically digested and cloned into the pCAT-Basic plasmid. In the construction of pGPMSCAT1, 750 ng of pGPMS was used as target DNA, with T3 and PIP5 primers. PIP5 primer (CTGCAG**TCTAGA**GGAATAGCAGAAAGCCAGC), corresponds to co-ordinates +43 to +22 of exon 1 of pig mitochondrial HMG-CoA synthase. Then the PCR product was digested with *Sal*I (Bluescript polylinker) and *Xba*I (**bold** in PIP5 primer) and ligated into the pCAT-Basic. pGPMSCAT1 was then used as a target DNA with the reverse primer PIP5 and forward primer UPPPRE (GAATTC**GTCGAC**GTGTTGGAGGGGATGTTTCTC, corresponding to co-ordinates −134 to −115 of 5′-flanking region of the pig mitochondrial HMG-CoA synthase gene), and the PCR product was digested with *Xba*I and *Sal*I (**bold** in UPPPRE primer) and ligated into the pCAT-Basic to generate pGPMSCAT2. pGPMSCAT3 was constructed in an identical manner to pGPMSCAT2 but using DOWNPPRE (ACGC**GTCGAC**TAAGCAGGGGCAGGGCTTCC, corresponding to co-ordinates −74 to −54 of 5′-flanking region of the pig mitochondrial HMG-CoA synthase gene) instead of UPPPRE. Heterologous promoter plasmids were constructed in pBLCAT2, which contains the herpes-virus thymidine kinase gene promoter upstream of the CAT reporter gene [33]. pGPMSTKCAT1 contains a fragment corresponding to co-ordinates −116 to −78 of the pig mitochondrial HMG-CoA synthase gene. It was constructed by cloning the oligonucleotide 5′ - agctTCTTTGACTGGGCCAAGCTCTGAGTGAGACCTTTCACATg annealed to 5′-tcgacATGTGAAAGGTCTCACTCAGAGCTTGGCCCAGTCAAAGA into pBLCAT2. pGPMSTKCAT2 contains a fragment corresponding to co-ordinates −100 to −67 of the pig mitochondrial HMG-CoA synthase gene, and it was constructed by cloning the oligonucleotide 5′-agcttGCTCTGAGTGAGACCTTTCACATAGATAAGCAGG annealed to 5′-tcgaCCTGCTTATCTATGTGAAAGGTCTCACTCAGAGCa into pBLCAT2 (nucleotides shown in lower case were added to provide cohesive *Hin*dIII–*Sal*I ends at the 5′ and 3′ termini respectively). For annealing, complementary oligonucleotides were mixed in a total volume of 100 μl of TE buffer (0.1 μg/ml of each oligonucleotide) supplemented with 150 mM NaCl. This solution was incubated for 10 min at 65 °C and then for 20 min at 22 °C. The annealed oligonucleotides were placed on ice and stored at −20 °C, also to be used for electrophoretic-mobility-shift assays (EMSA). Mutant pGPMSCAT1-M was generated from pGPMSCAT1 in which the DR-1 was 'scrambled' by site-directed mutagenesis using an overlap extension PCR [34]. A 100 ng portion of pGPMSCAT1 was used as a target in two independent PCR reactions (95 °C, 30 s; 50 °C, 2 min; and 72 °C, 1 min; 10 cycles). The first PCR used a forward primer, DR1M-F (CCAAGCTCTGAGTG**CACGAATTCCTTA**AGATAAGCAGGGG), which corresponds to co-ordinates −104 to −64, and a reverse primer CATrev (which annealed with the vector downstream arm). The second PCR used a forward primer CATfw (which annealed with the vector upstream arm) and a reverse primer DR1M-R (CCCCTGCTTATCT**TAAGGAATTCGTG**CACTCAGAGCTTGG), which overlapped with DR1M-R. DR1M-F and DR1M-R scrambled the DR-1 within the PPRE (**bold** in DR1M-F and DR1M-R primers). In the subsequent overlap extension reaction, 200 ng of each PCR product was used as a target and CATfw and CATrev were used as primers, in a PCR (94 °C, 1 min; 60 °C, 1 minute; and 72 °C, 1 min; 10 cycles). The PCR product was cloned in the p-GEM-T vector and subsequently subcloned into pCAT-Basic, thus generating pGPMSCAT1-M. Both strand sequences of all CAT plasmids were confirmed by the dideoxynucleotide-chain-termination method [35] with the use of an automated fluorescence-based system (Applied Biosystems). The plasmid pSG5-mPPARα [36], expressing mouse PPARα, was kindly provided by Dr. S. Green [ICI (now Zeneca) Central Toxicology Laboratory, Macclesfield, Cheshire, U.K.]. Construction of pJCXR8 plasmid [13], expressing human RXRα, used pSKXR3-1 plasmid kindly provided by Dr. R. M. Evans (Salk Institute, San Diego, CA, U.S.A.).

Transfections and enzymic assays

HepG2 cells were cultured in minimal essential medium supplemented with non-essential amino acids and 10% (v/v) fetal-calf serum. Cells were co-transfected by the calcium phosphate method [37,38] with 10 μg of the reporter pig mitochondrial HMG-CoA synthase-CAT gene construct (pGPMSCAT1-3, pGPMSTKCAT1-2) or empty plasmids (pCAT-Basic, pBLCAT2) and 500 ng of eukaryotic expression vectors (pSG5-mPPARα or pSG5-hLXRα) or an equal amount of salmon sperm DNA. In all experiments, 3 μg of plasmid pRSVβGAL (Rous-sarcoma-virus promoter β-galactosidase) was included as internal control in transfections. Cell extracts were prepared by



B

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-581                                                    GGATCCCGTGCTTTCACGGAGGCACTTAATTTCATTCCACT

-540   TGGAAATTGTGTAAAGTCATACACATACAGTAAAAATAGAAGAAATATAACAAGTTCCATATCCCGGGAATACCCAGCCCTTTTGCTATG

-450   AGCCAGCATGCAACTACTTACAAAGCTACTACGAAAAGACTTGTTCCTTTGGGTTGTGAAAGATTTTTTTTTTTTCTTGTTGTGTGGAAT

-360   ACATTAAAATCCCAGCAGGTAACTAGACAGCTTTCTGGACCCCCAAGGGAGAGTAATTCAGACAAGAGTCCAAGACAGACCAGGTGGGGT

-270   TACTCGGGATGTGTTAGAAGGAACACCACAGCCTCTTGAGGCAGCTGAGCATACACAGGGCTGCTGCGAGAACTCAGGAAAGCCGGAATC

-180   AAGCCACCTTTTCCTACTCATGCCCAGGAATGGGGCAGCTATGTAGGTGTTGGAGGGATGTTTCTCTTTGACTGGGCCAAGCTCTGAGTG

 -90   AGACCTTTCACATAGATAAGCAGGGGCAGGGCTTCCGGAGGCAGGAGTCTCAGGGCTCTATAAAGCCTGGCCCTGGGCACGACGGGGCAA

   1   CTCTCCCAAAGACTGTGAACTGCTGGCTTTCTGCTATTCCTCTGAAAATGCAGCGCCTGTTGACTCCAGTGAGGCAGGTCTTGCGAGTGA
                                                       MetGlnArgLeuLeuThrProValArgGlnValLeuArgValL

  91   AGAGAGCGATGCAGGAAGCTTCCTTCATGCCGCCTCTCCTGCCTCCAGCAGCCCACCAAAGgtgagtcacgttggtcccaggacacccccc
       ysArgAlaMetGlnGluAlaSerPheMetProProLeuLeuProProAlaAlaHisGlnArg

 181   ccccccccaggtcactctcagaagtggcacacaaccagcaaaagatgagcagagagcctgctctagggagaattccagtcaaaagttggca

 271   aggcttgtgcacagcctcagctgagcaagttaacctctcaaggtctcgcttacctcatctctaaacaagacagcccaggtctgttcacag

 361   aaccgcaggatcc
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Figure 1 Restriction-endonuclease map and 5′ flanking nucleotide sequence of the pig mitochondrial HMG-CoA synthase gene, showing the exon 1/exon 2 boundary

(**A**) Restriction map of a 6.3 kb *Hin*dIII–*Sac*I fragment of λGPMS clone including 5′ flanking region, exon 1, and a flanking part of the intron 1. The black box denotes the exon 1 of the gene. The scale at the top is in kb. (**B**) Nucleotide sequence of a 581 bp 5′ flanking region, exon 1, and 223 bp of 5′ intron-1 boundary of the pig mitochondrial HMG-CoA synthase gene. Nucleotide position +1 is assigned to the C of the transcription start site determined by 5′ RACE of three different clones; negative numbers refer to the 5′ flanking sequence. The whole sequence was determined in both strands of DNA. The PPRE located here is underlined. Putative SP1 [51] and TATA box, 72 and 32 bp respectively upstream of the mRNA start site, are denoted by the double underline. The intron-1 boundary sequence is in lower-case letters.

liquid-nitrogen freeze–thaw disruption (three times) after resuspension of harvested cells in 100 μl of 0.25 M Tris/HCl, pH 7.5. β-Galactosidase activity was determined [38] in 5 μl of extract to normalize for transfection efficiency. CAT assays were performed [37] for 60 min with extracts previously incubated at 65 °C for 5 min. Radioactivity of samples was measured on an LKB-1217 liquid-scintillation counter. The plasmid pSG5-hLXRα, expressing human LXR (a nuclear receptor that defines a distinct retinoid response pathway), was kindly provided by Dr. J. Lehmann (Glaxo Wellcome, Research Triangle Park, NC, U.S.A.).

EMSA

cDNAs for mPPARα and human 9-*cis*-retinoic acid receptor (hRXRα) were transcribed and translated (in a rabbit reticulocyte-lysate system) 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 the EMSA, a translation reaction was performed with 1 μg of pSG5. mPPARα (2 μl) and/or hRXRα (2 μl) synthesized *in vitro* were preincubated on ice for 10 min in 10 mM Tris/HCl (pH 8.0)/40 mM KCl/0.05% Nonidet P40/6% glycerol/1 mM dithiothreitol/poly(dI-dC) (1 μg). The total amount of reticulocyte lysate was kept constant in each reaction (4 μl) through the addition of unprogrammed lysate. For competition experiments a 10–100-fold molar excess of pig-DR-1, pig-PPRE or rat-PPRE double-stranded probes, relative to the labelled probe, was included during preincubation. DR-1-pig is the fragment corresponding to co-ordinates −116 to −78 of the pig mitochondrial HMG-CoA synthase gene, which was used to

prepare pGPMSTKCAT1; pig-PPRE is the fragment corresponding to co-ordinates −100 to −67 of the pig mitochondrial HMG-CoA synthase gene, which was used to prepare pGPMSTKCAT2; and rat-PPRE is the fragment corresponding to co-ordinates −116 to −69 of rat mitochondrial HMG-CoA synthase gene [13]. Then, 1 ng of either pig-PPRE or pig-DR-1, ^{32}P-labelled 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 non-denaturing 4.5%-(w/v)-polyacrylamide gel in 0.5 × TBE buffer (45 mM Tris/45 mM boric acid/1 mM EDTA, pH 8.0).

RESULTS

Isolation of pig HMG-CoA synthase promoter

A 417 bp 5′ fragment corresponding to the 5′ end of the pig HMG-CoA synthase cDNA [19] was used as a probe to isolate λGPMS, a 22 kb mitochondrial HMG-CoA synthase pig genomic clone. Southern-blot analyses of λGPMS, using 417 or

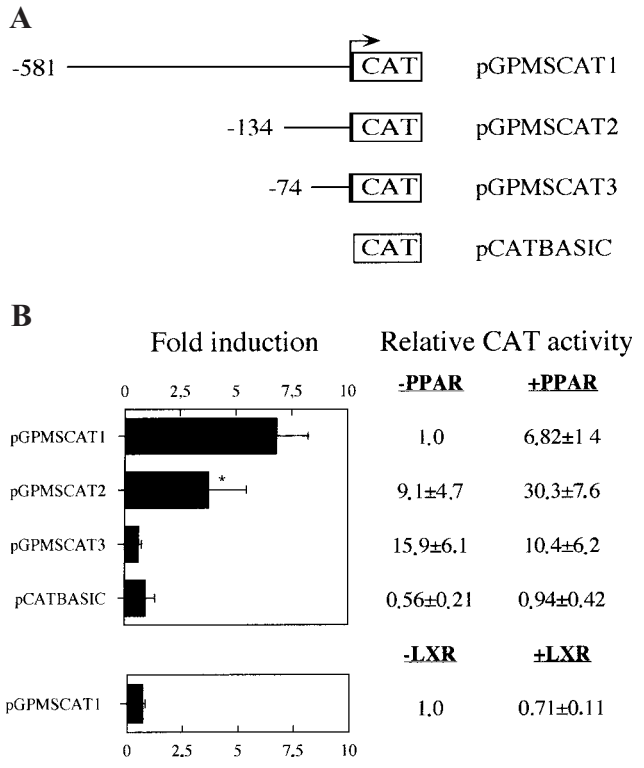

Figure 2 Transcriptional activation of the pig mitochondrial HMG-CoA synthase gene promoter by PPAR: localization of the response element of the gene by promoter deletion analysis

(**A**) Deleted constructs of the pig mitochondrial HMG-CoA synthase promoter. The number at the left denotes the co-ordinate of the 5′ deletion end point of the construct. The small black box indicates the first 28 bp of exon 1 present in all the constructs. (**B**) CAT reporter constructs were co-transfected with 500 ng of salmon sperm DNA, pSG5-mPPARα or pSG5-hLXRα into HepG2 cells. Average values of β-galactosidase-normalized CAT activity (means ± S.D.), from three independent transfections with two plates each, are expressed as 'fold induction' relative to the activity to each construct in the absence of either PPAR (top) or LXR (bottom). Average values of CAT activity (means ± S.D.) in the absence or presence of PPAR or LXR are also indicated, with the activity of pGPMSCAT1, the longest pig mitochondrial HMG-CoA synthase promoter construct, in the absence of either PPAR or LXR defined as 1.0. *Indicates that the value is significantly different at the $P < 0.025$ level from the pGPMSCAT1.

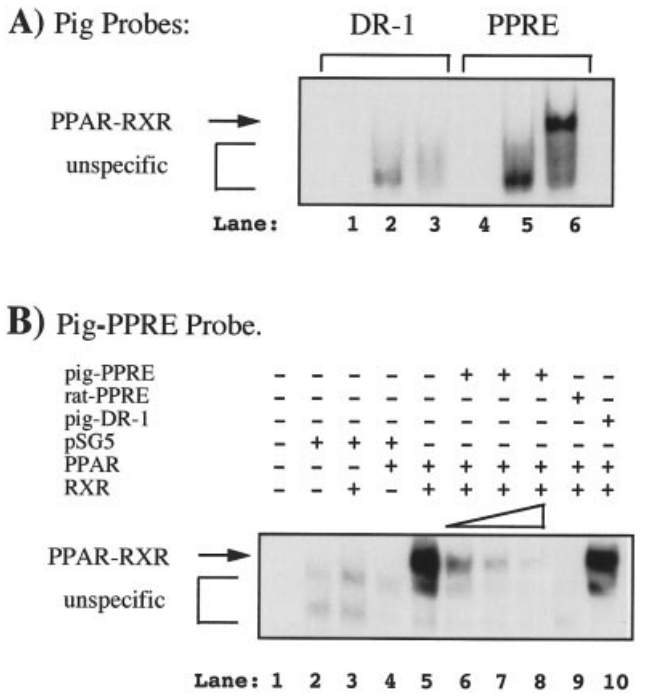

Figure 3 EMSA of the pig mitochondrial HMG-CoA synthase PPRE (DR-1 and 3′ flanking region) with PPAR · RXR heterodimers

(**A**) Pig-DR-1 probe (lanes 1–3) and pig-PPRE probe (lanes 4–6), used in the assay, are the fragments corresponding respectively to co-ordinates −100 to −67 and −116 to −78 of the pig mitochondrial HMG-CoA synthase gene. After incubation with water (lanes 1 and 4), unprogrammed (pSG5) reticulocyte lysate (lanes 2 and 5) or mPPARα and hRXRα translated *in vitro* (lanes 3 and 6), the probes were analysed by EMSA. (**B**) The pig-PPRE probe was incubated with water (lane 1), unprogrammed retyculocyte lysate (lane 2), or *in vitro*-translated hRXRα (lane 3), mPPARα (lane 4), or mPPARα and hRXRα (lanes 5–10). pSG5 (unprogrammed) lysate was used to maintain the amount of reticulocyte lysate in each reaction constant (4 μl). Additions of unlabelled probes (see the Experimental section) are indicated at the top of the Figure and were as follows: lanes 6, 7 and 8 respectively, 10-, 25- and 100-fold excess of unlabelled pig-PPRE probe; lane 9, 100-fold excess of unlabelled rat-PPRE probe; and lane 10, 100-fold excess of unlabelled pig-DR-1 probe (sequence from −116 to −78 of pig mitochondrial HMG-CoA synthase probe). The specific PPAR · RXR · PPRE complex is indicated by an arrow. Shift of pig-PPRE probe by PPAR · RXR heterodimers was observed in four independent experiments.

111 bp probes corresponding to the 5′ end of the pig cDNA [19], localized the 5′ end of the pig gene in a 955 bp *Bam*HI–*Bam*HI fragment (Figure 1A) and indicate that λGPMS does not contain exon 2 of pig mitochondrial HMG-CoA synthase (results not shown). Comparison with three RACE clones indicate that this *Bam*HI–*Bam*HI fragment contains 581 bp of the 5′-flanking region, exon 1 (151 bp) and 222 bp of intron 1 of the pig mitochondrial HMG-CoA synthase gene (Figure 1B).

Localization of pig mitochondrial HMG-CoA synthase PPRE

To characterize the pig 5′-flanking region, a hybrid promoter-CAT plasmid (pGPMSCAT1, see Figure 2A) was transiently transfected into a human hepatoma cell line (HepG2). Figure 2(B) shows that the 5′-flanking region (−581 to +43) of pig mitochondrial HMG-CoA synthase induced, in HepG2 cells, CAT expression above the activity observed for a promoterless pCAT-Basic plasmid. Figure 2(B) also shows that CAT activity of human hepatoma HepG2 cells transfected with hybrid promoter-CAT plasmid pGPMSCAT1 (−581 to +43 bp) was induced more than 5-fold by the co-transfection with a mouse

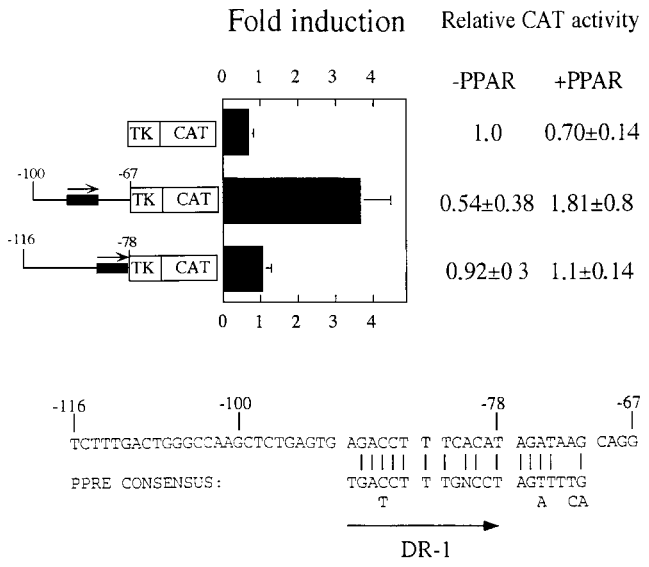

Figure 4 DR-1 of pig mitochondrial HMG-CoA synthase needs 3′ flanking region to constitute a PPRE able to confer PPAR response to the thymidine kinase (TK) gene promoter

HepG2 cells were co-transfected with the expression vector for mPPARα and different reporter plasmids containing the CAT gene under the control of the thymidine kinase gene promoter and two different fragments from the 5′ region of pig mitochondrial HMG-CoA synthase gene as indicated. Average values of β-galactosidase-normalized CAT activity (means $\pm$ S.D.), from three independent transfections with two plates each, are expressed as 'fold induction' relative to the activity of each construct in the absence of the expression vector pSG5-mPPARα. Average values of CAT activity (means $\pm$ S.D.) in the absence or presence of PPAR are also indicated, with the activity of pBLCAT2 in the absence of PPAR defined as 1.0. The black boxes indicate the imperfect direct repeat (DR-1) of the pig mitochondrial HMG-CoA synthase PPRE (-90 to -78). The arrows over the black boxes represent the orientation of the imperfect DR with respect to the natural promoter. The nucleotide sequence between position -116 and position -67 is shown, as well as the sequence of consensus PPRE elements [30]. The arrow at the bottom of the sequence indicates the imperfect repeat and its orientation.

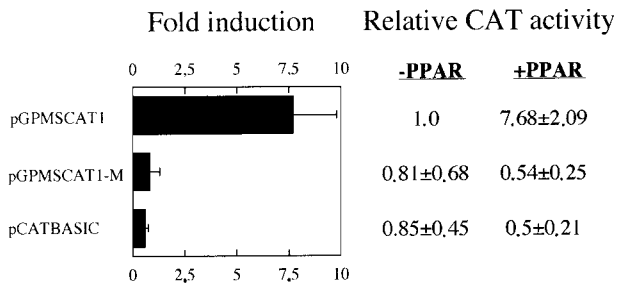

Figure 5 Mutation of the putative pig mitochondrial HMG-CoA synthase PPRE obliterates the response to PPAR

CAT reporter constructs containing the wild-type (pGPMSCAT1) or mutated (by scrambling nucleotides between -90 to -78, pGPMSCAT1-M) 5′-flanking region of the mitochondrial HMG-CoA synthase gene were co-transfected with or without 100 ng of pSG5-PPARα into HepG2 cells. Average values of β-galactosidase-normalized CAT activity (means $\pm$ S.D.), from three independent transfections with two plates each, are expressed as 'fold induction' relative to the activity of each construct in the absence of the expression vector pSG5-mPPARα. Average values of CAT activity (means $\pm$ S.D.) in the absence or presence of PPAR are also indicated, with the activity of pGPMSCAT1 in the absence of PPAR defined as 1.0.

PPARα expression vector (pSG5-mPPARα). PPAR *trans*-activation seems specific, since co-transfection of pGPMSCAT1 (-581 to $+43$ bp) with human LXR expression vector (pSG5-hLXRα) [39] did not increase CAT activity (see Figure 2B).

To define, within the pig HMG-CoA synthase promoter, the control element that mediates the effects of PPAR, two 5′ deletions of the 5′-flanking region of the pig gene were used. The mPPARα co-transfection induced overexpression of CAT gene within the promoter-CAT plasmids pGPMSCAT2 (-134 bp), while the CAT gene within the shorter promoter-CAT plasmid, pGPMSCAT3 (-74 bp), was not activated by mPPARα co-transfection (Figures 2A and 2B). Although PPAR *trans*-activation of pGPMSCAT1 was significantly higher than observed for pGPMSCAT2 (see the legend to Figure 2), these results indicate that the DNA sequence, about co-ordinates -134 and -74, on the pig gene could be responsible for the interaction with PPAR. Analysis of this sequence shows, between position -90 to -71, a coincidence of 15 out 20 bp with a consensus sequence for functional PPREs [31], indicating the presence of a putative element (PPRE) which could mediate the PPAR response of pig mitochondrial HMG-CoA synthase gene.

Figure 3 shows that a pig promoter sequence (pig-PPRE) between position -100 to -67 formed a prominent complex in an EMSA with a mixture of mPPARα and hRXRα produced *in vitro* (see Figure 3A, lane 6, and Figure 3B, lane 5). The specificity of this complex was demonstrated by competition with different amounts (10–100-fold molar excess) of unlabelled pig PPRE probe (see Figure 3B, lanes 6–8) or 50-fold molar excess of rat HMG-CoA synthase PPRE probe (see Figure 3B, lane 9). Figure 3 also shows that a pig promoter sequence (pig-DR-1) between position -116 and -78, which contains the DR-1 without the 3′ flanking region, was unable to form a complex with a mixture of mPPARα and hRXRα produced *in vitro* (Figure 3A, lane 3), and it was also unable to cause the disappearance of the complex formed between the sequence from position -100 to -67 (pig-PPRE) and mPPARα·hRXRα heterodimers (see Figure 3B, lane 10). Thus pig PPRE behaves like other PPREs, which require a DR-1 motif and the sequence immediately downstream of the second repeat of the DR-1, to form a complex with PPAR·RXR heterodimers in EMSA ([31]).

Furthermore, when the pig-PPRE sequence (-100 to -67) was inserted into pBLCAT2, a plasmid containing the CAT gene under the control of the thymidine kinase gene promoter, it conferred PPAR-responsiveness to the otherwise unresponsive thymidine kinase gene promoter (see Figure 4). Moreover, when the pig-DR-1 sequence (-116 to -78) was inserted in a pBLCAT2 plasmid, which provided a heterologous 3′ flanking region to the DR-1, it did not confer mPPARα-sensitivity to a thymidine kinase promoter (see Figure 4).

As expected, mutation of pig PPRE, by scrambling the DR-1 (-90 to -78) in the context of the largest promoter assayed (-581 to $+43$), obliterated the response to PPAR (see Figure 5). The results demonstrated that this pig mitochondrial HMG-CoA synthase element is able to confer PPAR- responsiveness both on its natural context and on a normally unresponsive promoter.

DISCUSSION

Isolation of pig mitochondrial HMG-CoA synthase promoter

During the neonatal period, emerging liver fatty acid metabolism involves the rise of mRNA levels for at least two main controlling genes (i.e., carnitine palmitoyltransferase I and mitochondrial HMG-CoA synthase) [9,10,40,41]. Such changes of gene expression have been correlated with nutritional and hormonal

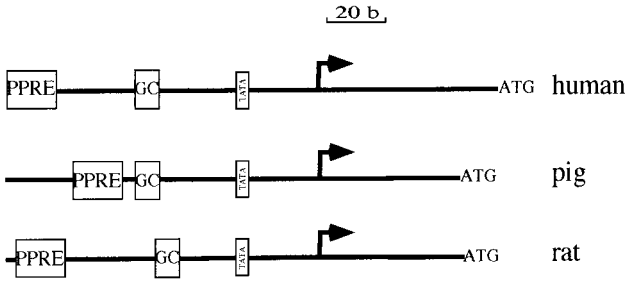

Figure 6 Upstream-element organization of human, pig and rat mitochondrial HMG-CoA synthase genes

The transcription start site, indicated by arrows, was determined by 5-RACE for the human [44] and pig (the present study) genes, and by primer extension and S1 experiments for the rat [52] gene. The functionality of PPRE was shown experimentally for the rat [13] and pig gene (the present study). Human PPRE showed 17/19 identity with rat PPRE [44]. The scale at the top is in nucleotides.

changes in which, respectively, milk fatty acids and low insulin/glucagon ratios may play a pivotal role (for a review see [4]). In contrast with what is observed in the neonates of other species, suckling piglets show low hepatic β-oxidation, low ketogenesis [14–18] and a lack of hyperketonaemia [42,43]. We have recently shown that pig mitochondrial HMG-CoA synthase gene expression differs from what has been observed until now in ketogenic species, since in pigs the mRNA levels remain very low during suckling [19]. The pig mitochondrial HMG-CoA synthase gene is therefore an interesting model in which to study the transcriptional control of fatty-acid-controlled genes.

5′-RACE of liver mRNA with pig mitochondrial HMG-CoA synthase-specific primers [19] generated a cDNA 5′ end, which was used to isolate the 5′ flanking region of the gene (Figure 1A). The 5′ extremity was determined for three RACE clones. Each terminates at the cytidine residue situated 28 nt downstream of the TATA box element, which, in the pig mitochondrial HMG-CoA synthase gene, is located 47 bases upstream of the initiation Met codon. Similarly, the transcription start site of the rat [12] and human [44] genes is situated 28 nucleotides downstream of the TATA box, which is respectively located 49 and 60 bases upstream of the initiation Met codon (see Figure 6). As in rat [12] and human [44], exon 1 of pig mitochondrial HMG-CoA synthase encodes also the leader peptide involved in protein targeting to the mitochondrial matrix (104 nucleotides). The 581 bp 5′ flanking region of exon 1 of the pig HMG-CoA synthase gene permits the transcription of a reporter gene (CAT) in transient expression experiments performed in human hepatoma cell line HepG2 (Figure 2). These results suggest that the function of the 5′ flanking region is to act as the promoter region of the gene.

Deletions of DNA sequences between -581 and -134 caused an increase (9.1 ± 4.7) in the level of basal expression of the chimaeric gene in HepG2 cells (see Figure 2), which suggests an inhibitory sequence between -581 and -134. Similarly, for the rat mitochondrial HMG-CoA synthase promoter, a deletion of DNA sequence between -1148 and -116 caused a threefold increase in the expression of the chimaeric gene [12,13]. The fact that 74 bp of the 5′ region of the promoter still allowed the expression of the reporter gene in HepG2 cells indicates *cis* elements critical for the expression of the gene in the proximal region of the promoter. Besides the PPRE characterized here, the potential regulatory *cis* elements found in the 74 bp upstream of transcription start site are (1) a putative Sp1 box, from position -72 to -62, and (2) a putative TATA box, from position -32 to -27 (see Figure 1B). These elements are also present in similar positions of the rat [12] and human [44] mitochondrial HMG-CoA synthase promoter (see Figure 6).

Localization of pig mitochondrial HMG-CoA synthase PPRE

Starvation of 2-week-old piglets elicits a large stimulation of liver mRNA and activity of mitochondrial HMG-CoA synthase [19], raises ketogenesis from long-chain fatty acids *in vitro* [14] and increases circulating ketone bodies [21]. The mechanism by which mitochondrial HMG-CoA synthase expression is induced during the suckling-fasting transition in piglets is not clear, but it correlates with an increase in plasma NEFA [19,21,45] without any significant changes in insulin or glucagon levels [19]. Because the rat counterpart gene is activated *in vitro* by long-chain fatty acids [12,13], an event mediated by PPAR [13], we assayed in HepG2 cell line the effect of mPPARα co-transfection on CAT activity driven by different fragments of the pig HMG-CoA synthase promoter and localized a putative PPRE between positions -134 and -74 (see Figure 2). Using EMSA (Figure 3), PPAR-unresponsive thymidine kinase promoter (Figure 4) and site-directed mutagenesis (Figure 5) we showed that the pig mitochondrial HMG-CoA synthase contains a PPRE sequence in which the imperfect direct repeat (DR-1) AGACCTTTC-ACAT is necessary, but not sufficient, to confer mPPARα response. Thus the sequence immediately downstream of the second half-site of the DR-1 is essential to confer mPPARα-sensitivity to a thymidine kinase promoter and to form a complex with mPPAR$\alpha \cdot$hRXRα heterodimers.

In starved mammals, a number of physiological conditions mimic the neonatal state of pups (circulating glucagon and NEFA are raised, insulin is low), and therefore we could expect similar changes in the expression of genes to lead to a similar adaptation of liver fatty acid metabolism. Nevertheless, we previously reported that whereas expression of the pig HMG-CoA synthase gene (measured as mRNA levels or enzymic activity) was strongly induced by fasting, there was a lack of neonatal induction [19]. Now, after the isolation of the pig HMG-CoA synthase gene promoter, we report that PPAR is able to *trans*-activate the pig gene through a PPRE, suggesting that PPRE *trans*-activation could be involved in starvation-associated changes. In this regard, Northern-blot experiments with a heterologous probe detected a starvation-associated induction of PPARα mRNA levels in 2-week-old animals (S. H. Adams, F. G. Hegardt and P. F. Marrero, unpublished work).

Recently it has been shown that multiple members of the steroid/nuclear-hormone-receptor superfamily can bind PPRE [46–49] and that a nuclear-receptor-responsive element 1 ('NRRE-1') is required for cardiac developmental-stage-specific transcription of the medium-chain-acyl-CoA dehydrogenase–CAT gene chimaera [50]. Without ruling out the possibility that PPRE has a limited role in the neonatal induction of mitochondrial HMG-CoA synthase, *trans*-activation of pig HMG-CoA synthase by PPAR is similar to that previously reported for the rat gene [13], although the two species show a different neonatal induction pattern of mRNA levels [9,10,19]. Therefore the results presented here support the previous hypothesis [19] that species-specific post-transcriptional mechanisms could be responsible for the low activity of pig HMG-CoA synthase.

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