Gene expression of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in a poorly ketogenic mammal: effect of starvation during the neonatal period of the piglet

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The low ketogenic capacity of pigs correlates with a low activity of mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase. To identify the molecular mechanism controlling such activity, we isolated the pig cDNA encoding this enzyme and analysed changes in mRNA levels and mitochondrial specific activity induced during development and starvation. Pig mitochondrial synthase showed a tissue-specific expression pattern. As with rat and human, the gene is expressed in liver and large intestine; however, the pig differs in that mRNA was not detected in testis, kidney or small intestine. During development, pig mitochondrial HMG-CoA synthase gene expression showed interesting differences from that in the rat: (1) there was a 2–3 week lag in the postnatal induction; (2) the mRNA levels remained relatively abundant through the suckling–weaning transition and at maturity, in contrast with the fall observed in rats at similar stages of development; and (3) the gene expression was highly induced by fasting during the suckling, whereas no such change in mitochondrial HMG-CoA synthase mRNA levels has been observed in rat. The enzyme activity of mitochondrial HMG-CoA synthase increased 27-fold during starvation in piglets, but remained one order of magnitude lower than rats. These results indicate that post-transcriptional mechanism(s) and/or intrinsic differences in the encoded enzyme are responsible for the low activity of pig HMG-CoA synthase observed throughout development or after fasting.

INTRODUCTION

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase is a potential regulatory site in the pathway converting acetyl-CoA to 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]. Ketogenesis is also associated with increased substrate availability and changes in the activities of enzymes central to β-oxidation. Perinatal and dietary responses of rat hepatic mitochondrial HMG-CoA synthase coincide with changes in the expression and activity of liver carnitine palmitoyl transferase I (CPT-I) [6,12–15], which suggests common regulatory mechanisms for these genes.

Factors responsible for the postnatal induction and starvation-associated changes in mitochondrial HMG-CoA synthase gene expression remain to be characterized, but probably involve changes in the hormonal and fatty acid milieux. Administration of dexamethasone or dibutyryl-cAMP to adult rats elicits increased liver mRNA, protein and enzymic activity [7,8,16], whereas opposite results are observed with insulin [7]. In addition, the rat mitochondrial HMG-CoA synthase gene contains elements that mediate its multihormonal regulation and tissue specificity [17]. A response element for peroxisomal proliferator-activated receptor (PPAR) has been characterized in the 5′ promoter region of the rat mitochondrial HMG-CoA synthase gene, and is a mediator of the responsiveness of the gene to fatty acid [18].

An unusually low hepatic ketogenic capacity [19–23] and a lack of hyperketonaemia [24,25] in piglets contrast with what is observed in newborns of other species. A major degree of control is probably exerted by mitochondrial HMG-CoA synthase in pigs, because (1) low ketogenesis *in vitro* [19,21–23] is evident despite a postnatal rise in total CPT activity [26], (2) experimental use of $C_{8:0}$ (which bypasses the CPT-I system in liver) as a ketogenic substrate fails to induce ketosis [20], and (3) mitochondrial HMG-CoA synthase specific activity and immunodectable protein in 48 h-old unsuckled pigs are negligible [21]. There is evidence that ketogenesis improves during development in pigs [19,20,27], which might be linked to a change in mitochondrial HMG-CoA synthase activity.

The impact of transcriptional and post-transcriptional modulation of mitochondrial HMG-CoA synthase activity on ketogenesis in rats is not fully understood. Companion studies examining this enzyme in non-ketotic species promise to uncover novel or subtle control mechanisms that might be overlooked in work with rats alone. To this end, we isolated the cDNA of pig mitochondrial HMG-CoA synthase, and examined its mRNA and enzyme activity during development and the suckling–fasting transition. Expression of the gene was detected only in liver and large intestine; increasing mRNA levels were associated with age

Abbreviations used: CPT-I, carnitine palmitoyl transferase I; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; NEFA, non-esterified fatty acids; PCS, pig-specific cytosolic HMG-CoA synthase probe; PEPCK, phosphoenolpyruvate carboxykinase; PMS, pig-specific mitochondrial HMG-CoA synthase probe; PPAR, peroxisome proliferator-activated receptor; RACE, rapid amplification of cDNA ends.

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The cDNA sequence data for pig mitochondrial HMG-CoA synthase will appear in the GenBank Database under the accession number U90884.

and fasting. During fasting, piglet mRNA rose (similar to starved adult rat mRNA), despite low enzyme activity. Our results suggest that the transcriptional control of mitochondrial HMG-CoA synthase is critical during the neonatal period in pigs, and that post-transcriptional mechanism(s) and/or intrinsic differences in the encoded enzyme are also responsible for the low HMG-CoA synthase activity observed in this species.

EXPERIMENTAL

Reagents

The following reagents were purchased from Sigma (St. Louis, MO, U.S.A.): acetyl-CoA, acetoacetyl-CoA, dextran sulphate, EDTA, guanidine thiocyanate, Mops, Tris and Triton X-100. Other chemicals, including agarose, CsCl, dithiothreitol, formaldehyde, formamide, KH_2PO_4, 2,5-diphenyloxazole and sucrose were from Boehringer Mannheim (Mannheim, Germany), Carlo Erba (Milan, Italy) or Merck (Darmstadt, Germany). Radiochemicals ([1-^{14}C]acetyl-CoA and [α]^{32}P]CTP) were from ICN (Irvine, CA, U.S.A.).

Animals

Sprague–Dawley rats (120 g) fed *ad libitum* were used in this study. To induce starvation, food was withdrawn 24 h before the beginning of the experiment. Rats were decapitated and their livers were quickly removed and processed for either isolation of mitochondria or RNA extraction in the same way as pig liver (see below).

Two litters of commercial crossbred pigs (*Sus scrofa*) were used in studies examining developmental changes in mitochondrial HMG-CoA synthase gene expression and enzyme activity. Typical factory production techniques involve a weaning process initiated much earlier (i.e. at 2–3 weeks) and more acutely than observed in nature [28]. To characterize naturally occurring developmental and/or diet-related changes in mitochondrial HMG-CoA synthase, and to avoid possible artifacts introduced by the stress of early weaning, piglets were allowed access to the sow until 8 weeks of age, with adult feed available after the fifth week. Tissues were obtained from one piglet per litter at 24 h after birth and at each week from weeks 1–8, as well as from two mature (60 kg) pigs. Animals seemed to be in the fed state when sampled, but time of last suckling/feeding was not determined. For fasting experiments, tissue or blood was obtained from additional 14-day-old piglets previously starved for 48 h or allowed to suckle. Fasting at the age of 2 weeks was chosen because of previous reports of improved ketogenesis [19,27] with starvation (see the Discussion section).

Tissues were excised from pentobarbitol-anaesthetized (20–60 mg/kg, intracutaneously) pigs, and immediately immersed in liquid nitrogen; the animals were then killed by exsanguination. Water-rinsed regions of small intestine corresponding to duodenum, jejunum and ileum, and large intestine samples of proximal (caecum) and distal (colon) regions were obtained. Portions of liver used for isolation of mitochondria (see below) were placed in ice-cold homogenization buffer containing 250 mM sucrose, 0.1 mM EDTA and 5 mM Tris/HCl, pH 7.4. For analysis of plasma hormones and metabolites in fed and fasted piglets, blood was obtained by jugular venipuncture, combined with potassium-EDTA (1.5 mg/ml) and placed on ice until centrifugation at 5000 ***g*** to retrieve plasma. Plasma for glucagon analysis was treated with 500 k-i.u./ml aprotinin (Boehringer-Mannheim). All plasma samples were stored at -80 °C.

Mitochondrial HMG-CoA Synthase Activity

All procedures were performed at 4 °C. Liver tissue was homogenized in 5 vol. of cold homogenization buffer by using three strokes of a motorized loose-fitting apparatus with Teflon pestle. The supernatant from a 350 ***g*** (10 min) centrifugation of homogenate was centrifuged at 15000 ***g*** (15 min) to pellet the mitochondria. After washing and resuspension with homogenizing buffer, the preparation was centrifuged again at 15000 ***g***, then the pellet was washed and resuspended in buffer containing 0.4 mM dithiothreitol, 100 mM Tris/HCl and 1.5 % (v/v) Triton X-100, pH 8, at a ratio of 2:1 (v/w of homogenized tissue). Mitochondria were dialysed overnight in 1000 vol. of buffer containing 20 mM KH_2PO_4 and 12 mM EDTA, pH 7 [29], and samples were stored at -20 °C. Mitochondrial HMG-CoA synthase activity was measured as the incorporation of [1-^{14}C]acetyl-CoA into HMG-CoA at 30 °C for 5 min [29]. The reaction was initiated by adding protein (500–2000 μg of piglet or 20–150 μg of rat) preparation to a reaction mixture (final volume 200 μl) composed of 100 mM Tris/HCl, 1 mM EDTA, 20 μM acetoacetyl-CoA, 200 μM acetyl-CoA (specific radioactivity between 2000 and 4000 c.p.m./nmol). Protein concentrations for activity assays were determined by the biuret method. Radioactivity was counted in a cocktail containing 67 % toluene, 33 % Triton X-100 and 0.56 % 2,5-diphenyloxazole (v/v/w).

Plasma hormones and non-esterified fatty acids (NEFA)

Plasma insulin and glucagon were measured by standard radioimmunoassay procedures employing insulin (Cys Bio International, Gif-Sur-Yvette, France) or pancreatic glucagon [30], human antibodies and standards for human insulin or pig glucagon respectively. Fatty acid concentration was determined enzymically with an automated system (Bio Merieux). Raw insulin and glucagon values (μ-i.u./ml and pg/ml) are reported as molar equivalents by using an insulin conversion factor of 6 (American Diabetes Foundation) and a factor of 3485 pg/pmol for glucagon.

Isolation of DNA fragments encoding part of the catalytic site from mitochondrial and cytosolic pig HMG-CoA synthase

All enzymic manipulations were performed following the manufacturers' (Boehringer-Mannheim, Promega) instructions. The sequence of rat mitochondrial HMG-CoA synthase cDNA between positions 197 and 216 and positions 542 and 561 [31] is highly similar to human mitochondrial HMG-CoA synthase [32], cytosolic HMG-CoA synthase from human [33], rat [34], hamster [35] and chicken [36]; and cockroach HMG-CoA synthase 1 [37] and 2 [38]. PIG1 (5′-CC<u>ATCGAT</u>**GGCCNAARGAYGTNGGNAT**-3′) and PIG2 (5′-GC<u>TCTAGA</u>**GCNGTNCCNCCRTARCANGC**-3′) degenerate primers were thus designed to represent all possible combinations of the different sequences in the similar regions (bold in the PIG1 and PIG2 primer sequences). Pig genomic DNA was obtained, of which 1 μg was used as a template in a PCR amplification (95 °C, 1 min; 55 °C, 45 s; 72 °C, 1 min; 35 cycles) with PIG1 and PIG2 primers. The 380 bp PCR product was cloned into Bluescript (SK$^+$) plasmid using *Cla*I and *Xba*I restriction sites introduced at the 5′ end of PIG1 (*Cla*I) or PIG2 (*Xba*I) primers (underlined in the PIG1 and PIG2 sequences). Seven recombinant plasmids were obtained, of which six were cut by *Nco*I, as in rat and human mitochondrial HMG-CoA synthases, which contain such a site [31,32], and one remained undigested (human [33] and rat

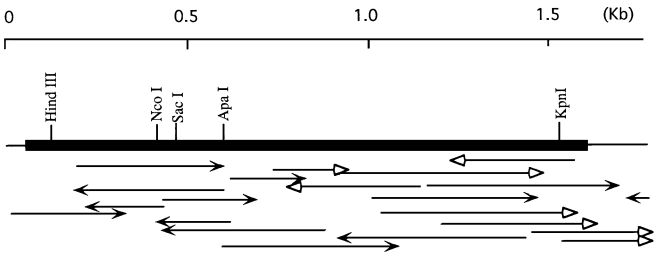

Figure 1 Restriction endonuclease map and sequencing strategy of pig mitochondrial HMG-CoA synthase cDNA

The solid bar indicates the coding sequence. Filled or open arrowheads indicate sequence reaction lengths obtained with specific pig or human primers respectively. The scale at the top is in kb.

[34] cytosolic HMG-CoA synthase lack an *Nco*I restriction site; not shown). All inserts were digested by *Rsa*I and *Sac*I (not shown), which act on sites present in both mitochondrial and cytosolic HMG-CoA synthase cDNA from rat and human [31–34]. A representative clone of each family was sequenced by the dideoxynucleotide method [39] with the use of an automated fluorescence-based system (Applied Biosystems), and plasmids pPMS (region of pig mitochondrial HMG-CoA synthase cDNA, positions 196–560) and pPCS (region of pig cytosolic HMG-CoA synthase cDNA) were characterized (see the Results section).

Isolation of pig mitochondrial HMG-CoA synthase full-length cDNA

Specific primers for PCR amplification of the 5′ and 3′ ends of pig cDNA were designed on the basis of the sequence of pPMS, thus avoiding the amplification of cytosolic HMG-CoA synthase cDNA. In the 5′ end amplification [40] a rapid amplification of cDNA ends (RACE) kit from Clontech (Palo Alto, CA, U.S.A.) was used following the manufacturer's instructions. Briefly, 2 μg of poly(A)$^+$ mRNA from the liver of a 30 kg pig was the template in the first-strand cDNA synthesis, using RTP5 (5′-TCTCAGTGCCCACTTCCAGC-3′) pig-specific reverse primer. After the RNA degradation, an anchor sequence (3′-GGAGACTTCCAAGGTCTTAGCTATCA**CTTAAG**CAC-3′) was ligated to the 3′ end of the cDNA first strand with T4 RNA ligase. The 5′ end of pig mitochondrial cDNA was subsequently amplified by PCR with an anchor primer (5′-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG-3′) and an AMP5 primer (5′-AAGCTT**GAATTC**ACAGAGTCCCATGGGAGCTG-3′), a pig-specific primer located upstream of RTP5. The PCR product was cloned by taking advantage of an *Eco*RI restriction site introduced in the anchor sequence and AMP5 primers (bold in the respective sequences). The insert of the resulting plasmid (pSIM 3) was sequenced and used as a probe in Northern blot analyses, and in the isolation of the pig mitochondrial HMG-CoA synthase full-length cDNA.

In the 3′ cDNA amplification, a modification of the method reported by Frohman et al. [41] was used. A sample (10^9 plaque-forming units) of a pig cDNA library (λgt10, from Clontech) was boiled and used as a template in a PCR reaction performed with PIP3 (5′-AAGCTTCCTTCATGCCGCCTCTCCTG-3′) pig-specific primer and the left arm of λgt10 reverse primer (5′-TTATGAGTATTTCTTCCAGGG-3′). After the first amplification a new PCR was performed with AMP3 (5′-AAGCTT**GAATTCC**AGCTCCCATGGGACTCTGTG-3′) pig-specific primer and REVT (5′-GACTCGAG**TCGACA**TCGATTTTTTTTTTTTTTTT-3′) primers. This nested PCR yielded a single amplification product of approx. 1.4 kb that hybridized in Southern blot analysis with pPMS (result not shown). *Eco*RI (bold in the AMP3 primer sequence) and *Sal*I (bold in the REVT primer sequence) restriction sites introduced at the 5′ end of the primers allowed ligation of the PCR product into Bluescript, generating the recombinant plasmid pSIM4.

The cloned 5′ (pSIM3) and 3′ (pSIM4) ends were joined by using the single restriction site *Nco*I (underlined in AMP5 and AMP3 pig-specific primers). The insert from pSIM3 was liberated by using *Eco*RI–*Nco*I enzyme digestion and the isolated fragment was cloned into pSIM 4 to generate pSIM5, which carried the full-length cDNA.

Sequencing strategy of pig mitochondrial HMG-CoA synthase

Figure 1 shows the sequence strategy used. pPMS and pSIM3 were sequenced by using T3 and T7 Bluescript-specific primers; pSIM 4 was sequenced by using AMP3 pig-specific primer; and pSIM 5 was sequenced by using AMP5 and PIP3 (5′-CCTTCATGCCGCCTCTCCTG-3′) pig-specific primers. Two new plasmids, pSIM 4.1 and pSIM 4.2, were generated from pSIM 4, by using an *Apa*I single restriction site present in the pig cDNA, and sequenced with T3 and T7 Bluescript-specific primers. Using as a target pSIM4 or pSIM5, we observed that specific human mitochondrial HMG-CoA synthase primers were able to PCR-amplify DNA of the expected size when used with a pig-specific or Bluescript-specific primer (results not shown); therefore we were able to sequence this DNA-product with the human-specific primers. The human reverse primers I5R (5′-CCATTGTGAGTGGAGAGG-3′) and I8R (5′-ATGCTGCTCGTCCACTCG-3′) were used with the pig AMP3 primer; the human forward primers I3 (5′-GGAGCTGTGGCTATGCTG-3′), I4 (5′-CATCCAGTGCTACTTGCG-3′), I5 (5′-ACCCTTCACCCTTGACGA-3′), F4 (5′-GGCCTCTCAGGACATGTTCG-3′) and F5 (5′-TTTCGAGTATCCCAGGATGC-3′) were used with a Bluescript T7 primer. The sequence obtained with this approach was used to design five new pig-specific primers: F1 (5′-TGCTCATGGAGCTCTTCCAGG-3′), F2 (5′-TCACTCTCGACGATTTACAG-3′), F3 (5′-AAGCTGGAAGACACCTACGC-3′), R1 (5′-GCTTGCTTCCACTGCTTCTCG-3′) and R2 (5′-GTGTGAAATTCACCTTGTGGTAG-3′); such primers were used to confirm the pig mitochondrial HMG-CoA synthase cDNA sequence.

Isolation of a pig HMG-CoA lyase cDNA probe

After studying the HMG-CoA lyase cDNA alignments [42] we followed a reverse transcriptase–PCR approach to isolate a pig HMG-CoA lyase probe with human primers HL-F2 (5′-CTGCCTCAGAGCTCTTCACC-3′), HL-F3 (5′-AGATCTCCCTGGGGACACC-3′) and HL-R4 (5′-TGGCCAAGTTTCCTGATGCC-3′), surrounding the catalytic Cys-266. Poly(A)$^+$ RNA of pig intestine was used as a template for the first cDNA synthesis, using oligo(dT) as a primer, then an amplification was performed with HL-F2 and HL-R4 primers (94 °C for 1 min, 94 °C for 1 min, 50 °C for 45 s, 72 °C for 45 s; 35 cycles; 72 °C for 20 min), yielding a smear of the expected size (447 bp). A second semi-nested PCR performed with HL-F3 and HL-R4 yielded a single band of the expected size (237 bp). The isolated pig-specific HMG-CoA lyase fragment was 87.8 % similar to human HMG-CoA lyase cDNA (between positions 616 and 813 [42]) and recognized a constitutive single transcript of approx. 1.5 kb.

Northern blot hybridizations

Total RNA was extracted from frozen tissues by the guanidinium thiocyanate method [43] and stored at −80 °C. The concentration of RNA was measured from the A_{260}. Northern blot analysis of total RNA (10 μg) was performed after 1 % (w/v) agarose-gel electrophoresis in 2.2 M formaldehyde, as described [9]. cDNA probes were labelled with [α-^{32}P]dCTP to a specific radioactivity of approx. 9×10^9 c.p.m./μg. Reported absorbance (A) units derived from Northern blot analyses have been corrected for β-actin mRNA abundance.

cDNA probes

In tissue distribution experiments, purified inserts of pPMS or pPCS were used. In developmental and starvation-induction studies the SIM 5 (419 bp 5′ RACE product) was used as a pig mitochondrial HMG-CoA synthase-specific probe. In some experiments a pig-specific HMG-CoA lyase (237 bp reverse transcriptase–PCR product) and human β-actin [9] probes were also used. For the quantification of mRNA, specific 365 bp probes corresponding to the human (human probe), pig (pig probe) or rat (rat probe) mitochondrial HMG-CoA synthase (positions +148 to +512 in each case, taking the translation start codon as +1) were generated individually by PCR, with PIG1 and PIG2 primers and human cDNA [32], pig cDNA (this study) or rat cDNA [31] as templates.

RESULTS

Isolation of mitochondrial HMG-CoA synthase full-length cDNA from pig

The isolation of DNA fragments encoding part of the pig catalytic domain from mitochondrial and cytosolic HMG-CoA synthases was approached by PCR from genomic DNA. PIG1 and PIG2 degenerate primers, which represent sequences of all HMG-CoA synthases isolated so far [31–38], were used in the amplification. Recombinant plasmids derived from the resulting PCR products showed two different DNA inserts identified as mitochondrial or cytosolic HMG-CoA synthase by DNA sequence alignment with published HMG-CoA synthase cDNA species. PMS (380 bp insert; *Nco*I-sensitive) showed greater DNA similarity to rat and human mitochondrial HMG-CoA synthases (89.1 % and 90.8 % respectively) than to rat or human cytosolic HMG-CoA synthases (67.1 % and 67.5 % respectively), and was thus identified as pig mitochondrial HMG-CoA synthase DNA. Conversely, PCS (380 bp insert; *Nco*I-insensitive) showed a greater similarity to rat and human cytosolic HMG-CoA synthases (86.6 % and 91.6 % respectively) than to rat or human mitochondrial HMG-CoA synthases (65.0 % and 65.9 % respectively), and was therefore identified as pig cytosolic HMG-CoA synthase DNA.

To obtain the nucleotide sequence of a complete pig mitochondrial HMG-CoA synthase cDNA, a RACE approach was used. Plasmid pSIM5, carrying the full-length cDNA of pig mitochondrial HMG-CoA synthase, was created by pSIM 3 (5′ end) and pSIM 4 (3′ end) cDNA fusion by using a single *Nco*I restriction site present either in the reverse primer used in the 5′ RACE or in the forward primer used in the 3′ RACE (see the Experimental section for details). Figure 2 shows the nucleotide sequence of this cDNA (SIM 5) encoding pig mitochondrial HMG-CoA synthase, together with the predicted amino acid sequence of the protein. The pig mitochondrial HMG-CoA synthase full-length cDNA spans 1744 nucleotides (see Figure 2) and encodes a protein of 508 amino acid residues with a predicted molecular mass of 56715 Da. Amino acid sequence alignment of pig cDNA with rat and human mitochondrial synthase shows an identity of 82.1 % and 83.2 % respectively. The N-terminal end of the primary translation product shows a sequence of 37 residues that is in agreement with the general composition given for leader peptides that translocate cytosolic synthesized proteins into mitochondria [44]. A putative polyadenylation signal was detected in the 3′ untranslated region, at position +1613 (AATAAA).

Tissue specificity of pig mitochondrial HMG-CoA synthase

A Northern blot experiment with PMS or PCS as probes concurred with the identity of mitochondrial and cytosolic clones previously assigned by homology analysis. The PMS probe hybridized with an approx. 1.8 kb transcript (Figure 3). This transcript size agrees with the full-length pig mitochondrial HMG-CoA synthase cDNA cloned by RACE (see Figure 2) and is similar to the rat and human mitochondrial HMG-CoA synthase transcripts [31,32]. When PCS was used as a probe, Northern blot analysis of pig mRNA showed a constitutive expression of an approx. 3.6 kb transcript (Figure 3), similar in size to the rat cytosolic HMG-CoA synthase [31]. Figure 3 also shows that a significant expression of mitochondrial HMG-CoA synthase was detected only in liver, caecum and colon of pigs. Thus subsequent analyses of developmental or fasting-induced changes in expression focused on these tissues. This tissue specificity of expression did not seem to be related to age because an identical pattern was observed in piglets aged 1, 2 or 3 weeks (results not shown).

mRNA and activity levels of mitochondrial HMG-CoA synthase during development and starvation

Figure 4(A) shows Northern blot analyses of pig liver RNA during development. When RNA samples were processed with SIM3 as a probe, large variations in mitochondrial HMG-CoA synthase mRNA levels were observed. In contrast, when the same samples were analysed with a pig-specific probe for HMG-CoA lyase, the mRNA level was essentially unchanged during suckling. Despite this variability in mitochondrial HMG-CoA synthase transcript it was clear that (1) mRNA levels remained minimal for at least the first week of life, and (2) transcript amounts began to rise by the second and the third weeks and remained high until maturity, despite initiation of weaning. An increasing trend in enzyme specific activity did not become apparent until the fifth week (Figure 4B), after which weekly mean activities (0.03–0.19 m-units/mg of protein) were 3–19-fold that observed in the first month of life (approx. 0.01 m-units/mg of protein). The adult pig liver HMG-CoA synthase activity remained more than one order of magnitude smaller than adult rat liver activity (approx. 6 m-units/mg of protein, measured in the same assay; see also [21]). Expression of mitochondrial HMG-CoA synthase was also investigated in large intestine: in caecum, mRNA was detected throughout the suckling period but decreased in mature animals; in colon, mRNA was present at all stages of development (results not shown).

A 48 h fast in 2-week-old piglets elicited a marked rise in hepatic mitochondrial HMG-CoA synthase mRNA levels without affecting the β-actin mRNA levels (Figure 5). When the same samples were analysed by using the pig cytosolic HMG-CoA synthase-specific probe PCS no changes were detected in the mRNA levels (results not shown). The low mRNA content in the suckled fed pig hinders the quantification of the degree of induction, but it seemed to correlate with a 27-fold rise in liver-specific activity of the mitochondrial HMG-CoA synthase after fasting (0.463 ± 0.102 m-units/mg from fasted animals, com-

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                                                -48   GCTCTCCCAAAGACTGTGAACTGCTGGCTTTCTGCTATTCCTCTGAAA

   1  ATGCAGCGCCTGTTGACTCCAGTGAGGCAGGTCTTGCGAGTGAAGAGAGCGATGCAGGAAGCTTCCTTCATGCCGCCTCTCCTGCCTCCA
      MetGlnArgLeuLeuThrProValArgGlnValLeuArgValLysArgAlaMetGlnGluAlaSerPheMetProProLeuLeuProPro
                                10                                  20                                  30
  91  GCAGCCCACCAAAGGTTTTCTACAGTCCCTGCCGTCCCCGTGGCCAAAGCGGATACTTGGCCAAAGGATGTGGGCATCCTTGCCCTGGAG
      AlaAlaHisGlnArgPheSerThrValProAlaValProValAlaLysAlaAspThrTrpProLysAspValGlyIleLeuAlaLeuGlu
                                40                                  50                                  60
 181  GTCTATTTCCCAGCCCAATATGTGGACCAAACAGACCTGGAGAAGTTTGACAATGTGGAGGCAGGGAGGTACACAGTGGGCTTGGGCCAG
      ValTyrPheProAlaGlnTyrValAspGlnThrAspLeuGluLysPheAspAsnValGluAlaGlyArgTyrThrValGlyLeuGlyGln
                                70                                  80                                  90
 271  ACCCACATGGGCTTCTGCTCCGTCCAGGAGGACATCAACTCCTTGTGCCTGACGGTGGTGCAGCGGCTGATGGAACGCACGCAGCTCCCA
      ThrHisMetGlyPheCysSerValGlnGluAspIleAsnSerLeuCysLeuThrValValGlnArgLeuMetGluArgThrGlnLeuPro
                               100                                 110                                 120
 361  TGGGACTCTGTGGGCTGGCTGGAAGTGGGCACTGAGACCATCATTGACAAGTCCAAGTCTGTCAAAACCGTGCTCATGGAGCTCTTCCAG
      TrpAspSerValGlyTrpLeuGluValGlyThrGluThrIleIleAspLysSerLysSerValLysThrValLeuMetGluLeuPheGln
                               130                                 140                                 150
 451  GACTCAGGCAACACTGACATCGAGGGCATAGATACCACCAATGCCTGCTATGGTGGCACAGCCTCCCTCTTCAACGCTGCCAACTGGGTG
      AspSerGlyAsnThrAspIleGluGlyIleAspThrThrAsnAlaCysTyrGlyGlyThrAlaSerLeuPheAsnAlaAlaAsnTrpVal
                               160                                 170                                 180
 541  GAGTCCAGCGCCTGGGATGGTCGCTATGCGGTAGTGGTCTGTGGAGACATCGCTGTCTACCCCAGGGGAAACTCCCGCCCCACAGGCGGG
      GluSerSerAlaTrpAspGlyArgTyrAlaValValValCysGlyAspIleAlaValTyrProArgGlyAsnSerArgProThrGlyGly
                               190                                 200                                 210
 631  GCTGGAGCCGTGGCCATGCTGGTTGGGCCTGAGGCCCCTCTGGCCTTGGAGAGAGGGCTTAGAGGAACCCACATGGAGAATGCGTATGAC
      AlaGlyAlaValAlaMetLeuValGlyProGluAlaProLeuAlaLeuGluArgGlyLeuArgGlyThrHisMetGluAsnAlaTyrAsp
                               220                                 230                                 240
 721  TTCTACAAGCCAAATGCCACTTCTGAGTACCCACTGGTGGATGGGAAGCTCTCCATCCAGTGCTACCTTCGGGCCCTGGACCGATGCTAC
      PheTyrLysProAsnAlaThrSerGluTyrProLeuValAspGlyLysLeuSerIleGlnCysTyrLeuArgAlaLeuAspArgCysTyr
                               250                                 260                                 270
 811  ACATTATACCGTCAAAAGATCGAGAAGCAGTGGAAGCAAGCTGGCATCGAGCGACATTTCACTCTCGACGATTTACAGTTTATGATCTTC
      ThrLeuTyrArgGlnLysIleGluLysGlnTrpLysGlnAlaGlyIleGluArgHisPheThrLeuAspAspLeuGlnPheMetIlePhe
                               280                                 290                                 300
 901  CACACACCCTTCTGCAAGTTGGTCCAGAAATCCTTGGCTCGCCTGATGTTCAGTGACTTCCTGTTGGCCGACAGCGACACCCAGTCCAGC
      HisThrProPheCysLysLeuValGlnLysSerLeuAlaArgLeuMetPheSerAspPheLeuLeuAlaAspSerAspThrGlnSerSer
                               310                                 320                                 330
 991  CTCTACAAGGGGCTGGAGGCCTTCAGGGGACAAAAGCTGGAAGACACCTACGCAAATAAAGACATAGAAAAGGCATTTCAAAAGGCCTCT
      LeuTyrLysGlyLeuGluAlaPheArgGlyGlnLysLeuGluAspThrTyrAlaAsnLysAspIleGluLysAlaPheGlnLysAlaSer
                               340                                 350                                 360
1081  CTGGACTTGTTCAACAAGAAAACCAAGCCCTCCCTCTACCTCTCCCTGCACAACGGGAAATATGTACACCTCATCCCTGTATGGATGCCTG
      ProAspLeuPheAsnLysLysThrLysProSerLeuTyrLeuSerLeuHisAsnGlyAsnMetTyrThrSerSerLeuTyrGlyCysLeu
                               370                                 380                                 390
1171  GCCTCACTTCTGTCCCAGTGCTCTGCCCAAGACTTGGCTGGTTCCAGGATTGGTGCCTTCTCTTATGGCTCTGGCTTGGCAGCAAGTTTC
      AlaSerLeuLeuSerGlnCysSerAlaGlnAspLeuAlaGlySerArgIleGlyAlaPheSerTyrGlySerGlyLeuAlaAlaSerPhe
                               400                                 410                                 420
1261  TATTCACTTCGAGTGTCCCAGGATGCTTCTCCGGGCTCCCCCCCTGGAGAAGCTGGTATCCAGTGTGTCAGACCTGCCAGAACGTCTCGCC
      TyrSerLeuArgValSerGlnAspAlaSerProGlySerProLeuGluLysLeuValSerSerValSerAspLeuProGluArgLeuAla
                               430                                 440                                 450
1351  TCCCGGAAGCGTGTCTCCCCTGAGGAGTTCACGGAAATAATGAACCAAAGAGAACAATACTACCACAAGGTGAATTTCACACCACCTGGT
      SerArgLysArgValSerProGluGluPheThrGluIleMetAsnGlnArgGluGlnTyrTyrHisLysValAsnPheThrProProGly
                               460                                 470                                 480
1441  GACCCAAACAGCCTTTTCCCAGGCACCTGGTACCTGGAGCGAGTGGATGAGCTGTATCGCCGGAAGTATGCCCGGCATCTGGTCTAAAGA
      AspProAsnSerLeuPheProGlyThrTrpTyrLeuGluArgValAspGluLeuTyrArgArgLysTyrAlaArgHisLeuValEnd
                               490                                 500
1531  TACTTGGAGAGGTTCCTGGGGAACGTGTGCTACACAACTTCTGCCCTCCATCGATCATATACAACCCCCCACTTAACTGGTAAATAAATT

1621  GGATTGGACACAGC(A)n
```

Figure 2 Nucleotide and deduced amino acid sequences of pig mitochondrial HMG-CoA synthase

Position +1 of each sequence corresponds to the putative start codon (ATG). The underlined sequence signifies the mitochondrial synthase-specific region, PMS, initially obtained by PCR (see the Experimental section). A putative polyadenylation sequence is shown in bold.

pared with 0.017 ± 0.001 m-units/mg from suckling animals). The activity detected in the starved piglets (approx. 0.5 m-units/mg) was higher than in adult pigs (see Figure 4B) but still one order of magnitude lower than activity detected in fed (approx. 6 m-units/mg) or starved (approx. 25 m-units/mg; see also [11,45]) rats. Fasting resulted in marked differences in transcript levels between the proximal and distal regions of the large intestine. Mitochondrial HMG-CoA synthase mRNA (Figure 5) increased 4.8-fold in the caecum ($P = 0.07$) but did not change in the colon ($P < 0.1$).

In suckling rats no fasting-induced changes in mRNA levels have been observed [46]; thus the significant fasting-induced rise in mitochondrial HMG-CoA synthase mRNA in the liver of piglets (Figure 5) resembled that observed in fasted adult rats

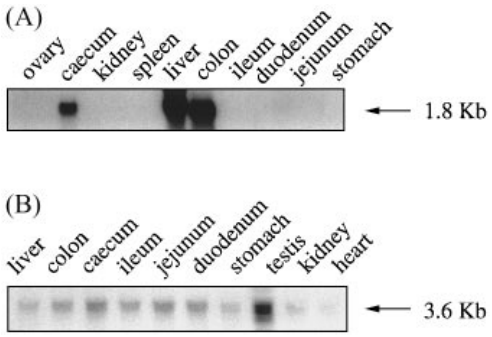

Figure 3 Tissue-specificity of pig mitochondrial HMG-CoA synthase gene expression

Total RNA samples (obtained from a fed 30 kg pig) were analysed for mitochondrial HMG-CoA synthase (**A**) or cytosolic HMG-CoA synthase (**B**) mRNA abundance. mRNA (approx. 1.8 kb) hybridized with mitochondrial synthase specific-probe, PMS, in the liver and large intestine, whereas the cytosolic synthase specific-probe, PCS, recognized an approx. 3.6 kb mRNA species that seemed to be constitutively expressed.

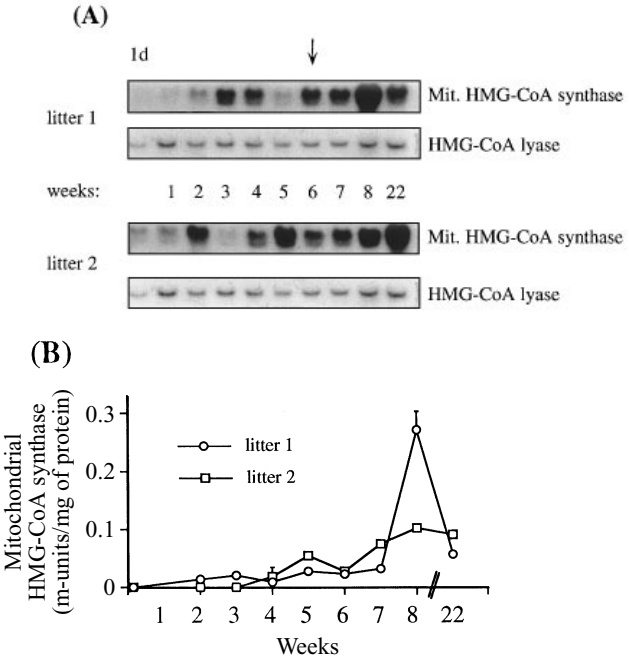

Figure 4 Developmental changes in pig liver mitochondrial HMG-CoA synthase mRNA and activity

(**A**) Abundance of liver mitochondrial HMG-CoA synthase mRNA in two litters of fed pigs aged 1 day (1 d) through to maturity. The arrow denotes the introduction of adult feed during weeks 6–8 while piglets continued to have access to the sow. For comparison, the abundance of pig liver HMG-CoA lyase mRNA (approx. 1.5 kb) was measured in the same RNA samples used for synthase analysis. (**B**) Age-related changes in the specific activity of mitochondrial HMG-CoA synthase in liver mitochondrial preparations from animals used for Northern blot analysis in (**A**).

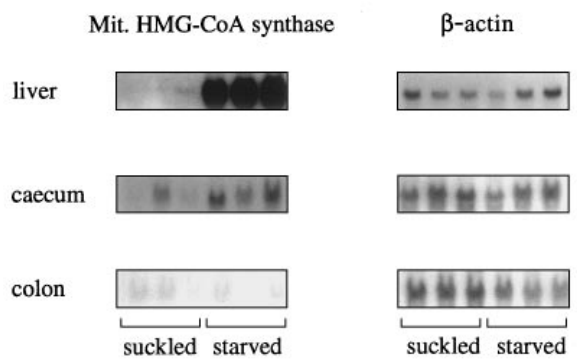

Figure 5 Changes in piglet liver mitochondrial HMG-CoA synthase in the suckling–fasting transition

Northern blot analyses showing a large induction of synthase gene expression in the liver and caecum of fasted piglets (exposure time 1 day and 6 days respectively). The colon does not show any induction. Tissues were collected from 2-week-old fasted (fasted for 48 h; $n = 3$) or suckled ($n = 3$) piglets to determine differences in the abundance and activity of mitochondrial HMG-CoA synthase mRNA.

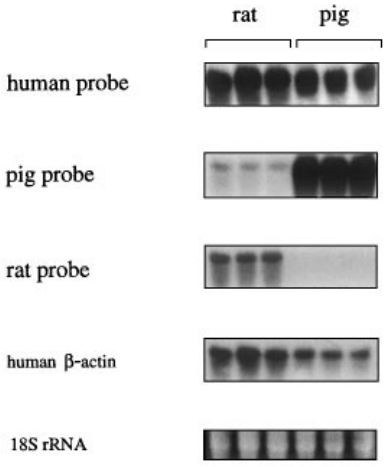

Figure 6 Species comparison of liver mitochondrial HMG-CoA synthase mRNA levels in fasted animals

At the top, liver mRNA levels are compared across the species [starved mature rats ($n = 3$) compared with starved 2-week-old piglet ($n = 3$)] by using a human mitochondrial HMG-CoA synthase probe hybridized at 55 °C (human probe, see below.) Blots were stripped and sequentially probed at 65 °C with pig (pig probe)- or rat (rat probe)-specific 365 bp cDNA fragment. All probes used expand the same region of human, pig or rat mitochondrial HMG-CoA synthase cDNA (from position +148 to +512, taking the translation start codon as +1) in which 51% of mismatches were at the same nucleotide position, and were generated by PCR, with PIG1 and PIG2 primers and human cDNA [32], pig cDNA (this study) or rat cDNA [31] as templates. Two independent experiments showed mRNA induction by starvation in the piglets (Figure 5) or rats (results not shown).

[7,8]. In an attempt to compare transcript amount in the two models, RNA from liver of fasted animals was probed with pig, rat and human mitochondrial HMG-CoA synthase probe (human probe; PCR-produced with PIG1 and PIG2 primers by using the human cDNA clone of mitochondrial HMG-CoA synthase as a target [32]; this 365 bp human probe was 89.9% or 91.0% similar to rat or pig cDNA). The use of homologous pig or rat probes shows, as expected, that the detected level of mitochondrial HMG-CoA synthase mRNA in starved piglets or rats depends on the probe used (see Figure 6). Despite the differences in enzyme activity discussed previously (approx. 50-fold), mRNA levels quantified with the heterologous human probe were only slightly (35%) higher in fasted rat than in fasted pig (Figure 6, top panel; 0.393 ± 0.0204 and 0.291 ± 0.0126 absorbance units respectively; $P < 0.05$).

Circulating hormones and fatty acids could affect the expression of mitochondrial HMG-CoA synthase [7,8,17,18]. To gain an insight into their roles in the fasting-induced rise in mitochondrial HMG-CoA expression (Figure 5), insulin, glucagon and NEFA concentrations were determined in suckled ($n = 7$) and fasted ($n = 5$) piglets. Fasting did not significantly affect plasma insulin (38 ± 3 pM compared with 48 ± 7 pM) or glucagon (131 ± 13 pM compared with 136 ± 19 pM) levels. However, fasting plasma NEFA levels were almost double those observed in suckling animals (602 ± 75 μM compared with 358 ± 17 μM; see also [27,47]).

DISCUSSION

Isolation of pig mitochondrial HMG-CoA synthase cDNA

The strategy for isolating pig mitochondrial HMG-CoA synthase was based on the assumption that the evolutionary distance between the different HMG-CoA synthases would not be high. We previously observed that the different cytosolic and mitochondrial HMG-CoA synthases contained extensively conserved sequences (results not shown) that would presumably be conserved in the pig. Accordingly we chose fully conserved amino acid sequences from published HMG-CoA synthases (between positions 197 and 216 and positions 542 and 561 of rat mitochondrial HMG-CoA synthase cDNA [31]) and the corresponding degenerate oligonucleotides were used in PCR experiments. This approach was successful in isolating two DNA products (365 bp each) encompassing two different fragments of pig HMG-CoA synthase. By sequence comparison between these DNAs and those of the rat and human, we deduced that the amplified fragments corresponded to pig mitochondrial and cytosolic HMG-CoA synthase. The sequence of the putative cDNA for mitochondrial HMG-CoA synthase was used to design specific primers that allowed the isolation of a full-length cDNA (Figure 2) by RACE.

The identification of the cDNA as mitochondrial HMG-CoA synthase was based on several criteria: (1) the amino acid sequence predicted from this cDNA shares extensive conservation with mitochondrial HMG-CoA synthases from rat (82.1 %) and human (83.2 %); (2) the predicted amino acid sequence from the cDNA contains a region with a high level of identity to that of the active site of other HMG-CoA synthases, whether cytosolic or mitochondrial; (3) the N-terminus contains a putative leader peptide to target the protein into mitochondria [44]; and (4) in the avian enzyme the cysteine residue involved in the formation of the acyl-S-enzyme intermediate has been identified [48]; this is mapped as Cys-166 in the cDNA sequence of mitochondrial HMG-CoA synthase from rat [31] and human [32], and it is conserved in the pig cDNA sequence (Figure 2).

Tissue specificity of pig mitochondrial HMG-CoA synthase

As in rats [9,10,31,49] and humans [32], mitochondrial HMG-CoA synthase expression is tissue-specific in pigs. All three species show mitochondrial HMG-CoA synthase mRNA expression in the large intestine and liver of adult animals (see Figure 3A); but pig differs from human and rat in that the kidney, ovary and testis lack detectable expression of the gene (Figure 3A, and results not shown). During suckling the expression pattern was also different in rats and pigs: no expression was found in small intestine of piglets (results not shown). Regulation of expression also seemed to differ across tissues because fasting induced a significant rise in transcript in liver and caecum but not in colon (Figure 5).

The relevance of intestinal mitochondrial HMG-CoA synthase expression/activity for whole-animal ketone body metabolism in newborns is not well defined. Rat neonatal small intestine possesses the capacity for ketogenesis, as judged by the presence of ketogenic enzymes and active ketogenesis *in vitro* [9,50,51]. Therefore the lack of expression in small intestine and the abundance of mRNA in large intestine of piglets might affect metabolism. The relative mass (percentage of body mass) of the small intestine (4 %) is similar to that in the liver (3 %) in suckling pigs, whereas the large intestine (approx. 1 %) is smaller (S. H. Adams, P. F. Marrero and F. G. Hegardt, unpublished work). Nevertheless the physiological effect of intestinal expression of the gene remains unknown.

mRNA and activity levels of mitochondrial HMG-CoA synthase during development

Hepatic rates of ketogenesis and β-oxidation rise within hours of the onset of suckling in rats [6,52–54] but remain low in newborn pigs [19,21–23,27]. The increased ketogenesis in newborn rats is due to transcriptional and post-transcriptional factors, including the induction of the expression of mitochondrial HMG-CoA synthase and CPT-I gene [9,10,14,15] and the depressed inhibition of these enzymes by succinyl-CoA and malonyl-CoA respectively [4].

Studies reporting insignificant ketogenesis [19,21–23], a relatively low rate of β-oxidation [21] and significant acetogenesis [22,23] in newborn pig liver have made it clear that perinatal changes in lipid metabolism in pigs differ markedly from those described in other species. Although ketogenesis is minimal in newborn pigs, studies performed *in vitro* [19] and *in vivo* [20,27] have shown a significant developmental increase in hepatic ketogenic capacity. Changes in activity of mitochondrial HMG-CoA synthase might be associated with development of ketogenic capacity in pigs, as suggested previously [20,21]. This study is the first to address the molecular phenomena regulating mitochondrial HMG-CoA synthase activity in pigs throughout development or after fasting.

The abundance of liver mRNA encoding mitochondrial HMG-CoA synthase rose with age (Figure 4A), indicating that the synthase gene is developmentally controlled. Substantial mRNA was observed only after a postnatal lag of 1–2 weeks (Figure 4A), a pattern markedly different from the rapid postnatal rise reported for suckling rats [9,10]. A major species difference was also noted during the suckling–weaning transition and maturity: in rat the gene expression and activity of mitochondrial HMG-CoA synthase fall steadily with consumption of adult food [6,9,10,55] but mRNA levels for the enzyme were persistently abundant in the liver of older pigs, even after the introduction of adult food during the final 3 weeks of suckling (Figure 4A). These results illustrate that major differences in regulation of the mitochondrial HMG-CoA synthase gene can occur across species during postnatal development.

mRNA and activity levels of mitochondrial HMG-CoA synthase during starvation

During suckling a number of physiological conditions mimic the fasted state of adults. In rats, for example, circulating glucagon and NEFA levels are raised, the insulin level is low and gluconeogenesis coupled to β-oxidation/ketogenesis operates concomitantly with adaptational changes in enzyme activity and transcription [i.e. high for cytosolic phosphoenolpyruvate carboxykinase (PEPCK), mitochondrial HMG-CoA synthase and CPT-I] [4]. Thus the fasting of previously suckled rat pups

does not alter the insulin-to-glucagon ratio [56] or lead to increased levels of mitochondrial HMG-CoA synthase mRNA [46]. In contrast, starvation in piglets aged 2 weeks elicited a large stimulation of expression of the liver mitochondrial HMG-CoA synthase gene (Figure 5), increased ketogenesis from long-chain fatty acids *in vitro* [19] and increased circulating ketone bodies [27]. The latter event was not observed in fasted 3-day-old piglet [27], suggesting that fasting-induced ketogenesis (perhaps coupled with enhanced hepatic mitochondrial HMG-CoA synthase activity) occurs only later in development. These results clearly emphasize that multiple levels of transcriptional control impact the pig synthase gene, including developmental and fasting-induced regulators of expression.

The mechanism(s) by which mitochondrial HMG-CoA synthase expression is induced during the suckling–fasting transition in piglet is not clear. The promoter of the rat mitochondrial HMG-CoA synthase gene is activated *in vitro* by the presence of long-chain fatty acids [17,18], an event mediated by PPAR [18]. Plasma NEFA nearly doubled with 48 h of fasting [27,47]. It is plausible that increased blood fatty acids have a role in the fasting-induced stimulation of expression observed in piglets (Figure 5), and the hypothesis that NEFA/PPAR trans-activate the pig gene is the subject of current investigation in this laboratory.

The induction of mitochondrial HMG-CoA synthase mRNA by starvation (Figure 5) was reflected in a 27-fold increase in specific activity. Despite the large amount of transcript, the increased enzyme activity in pig remained one order of magnitude lower than that in adult or suckling rat. This suggests that post-transcriptional mechanisms and/or kinetic differences in the mitochondrial HMG-CoA synthase enzyme also control activity in pigs. As a first attempt at assessing this hypothesis we performed Northern blot experiments of mRNA from rat or pig liver, using as a probe a fragment of the cDNA from human mitochondrial HMG-CoA synthase. This probe, whose sequence shared similar global homology with the cDNA from rat or pig (90 %), detected a large increase in transcript after fasting in both species, consistent with results from the use of homologous probes. The slight species differences in fasted mRNA levels detected with the human probe do not seem to explain the 50-fold difference in enzyme activity in the two models (see the Results section). If the heterologous human probe hybridizes to HMG-CoA synthase mRNA with similar affinity for rat or pig samples, then the results shown in Figure 6 might also indicate that the very low activity of the enzyme in pig is not due to low mRNA abundance but rather to intrinsic differences in enzyme kinetics and/or differences in post-translational modifications. The expression of recombinant proteins in *Escherichia coli* might elucidate which mechanism best explains the low enzymic activity.

The unusual rise in insulin level in the first 1–2 days of suckling [25,47,57] and the fasting-induced increase in ketogenesis [27] by 2 weeks of age raised the possibility that changes in mitochondrial HMG-CoA synthase expression in fasted piglets might be due to a decrease in the insulin-to-glucagon ratio. However, the results illustrate that stimulation of expression of the mitochondrial HMG-CoA synthase gene in fasted 2-week-old piglets does not seem to be related to changes in circulating insulin or glucagon concentrations. No significant changes in plasma concentrations of hormones were observed, and the liver content of cytosolic PEPCK mRNA as determined by Northern blot hybridization (results not shown) was not different (50 ± 15 compared with 52 ± 7 absorbance units for fed and fasting animals respectively). Abundance of cytosolic PEPCK mRNA is a sensitive marker of the prevailing insulin and glucagon activities in a number of systems [4,58].

In summary, the patterns of expression and activity of mitochondrial HMG-CoA synthase in piglets lend support to the idea that this enzyme could modulate the ketone body profile of suckling and fasting, and the age-related increase of ketogenic capacity described previously [19,20,27]. Despite increases in expression by fasting or suckling, the specific activity of the enzyme in mitochondria remained relatively low, suggesting that post-transcriptional mechanism(s) and/or kinetic differences in the mitochondrial HMG-CoA synthase enzyme also control the expression of the HMG-CoA synthase gene in pigs.

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