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Low Activity of Mitochondrial HMG-CoA Synthase in Liver of Starved Piglets Is Due to Low Levels of Protein Despite High mRNA Levels

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The unusually low hepatic ketogenic capacity of piglets has been correlated with lack of expression of the mitochondrial HMG-CoA synthase gene. However, we have shown that starvation of 2-week-old piglets increased the mRNA levels of mitochondrial HMG-CoA synthase to a level similar to that observed in starved rats (S. H. Adams, C. S. Alho, G. Asins, F. G. Hegardt, and P. F. Marrero, 1997, *Biochem. J.* 324, 65–73). We now report that antibodies against pig mitochondrial HMG-CoA synthase detected the pig enzyme in mitochondria of 2-week-old starved piglets and that the pig mitochondrial HMG-CoA synthase cDNA encodes an active enzyme in the eukaryotic cell line Mev-1, with catalytic behavior similar to that of the rat enzyme when expressed in the same system. We also show that low activity of pig mitochondrial HMG-CoA synthase correlates with low expression of the pig enzyme. The discrepancy in mitochondrial HMG-CoA synthase gene expression between the high levels of mRNA and low levels of enzyme was not associated with differences in transcript maturation, which suggests that an attenuated translation of the pig mRNA is responsible for the diminished ketogenic capacity of pig mitochondria.

Key Words: **mitochondrial HMG-CoA synthase; enzymatic activity; transcript maturation; translation; starvation; postnatal.**

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)[3] synthase has been considered as a potential regulatory site in the pathway converting acetyl-CoA to ketone bodies (1–3). In rats the hepatic capacity to produce ketone bodies increases rapidly during postnatal development (4) or fasting (5), when the liver mRNA, protein, and activity of mitochondrial HMG-CoA synthase increase (6–11). In contrast, an unusually low hepatic ketogenic capacity (12–16) and a lack of hyperketonemia (17, 18) are observed in piglets. A major degree of control is probably exerted by mitochondrial HMG-CoA synthase in pigs, since: (i) low ketogenesis *in vitro* (12, 14–16) is evident despite a postnatal rise in total CPT-I activity (19), (ii) experimental use of C8:0 (which bypasses the CPT-I system in liver) as a ketogenic substrate fails to induce ketosis (13), and (iii) mitochondrial HMG-CoA synthase-specific activity (14, 20) and immunodetectable protein in 48-h-old unsuckled pigs are negligible (14). However, pig mitochondrial HMG-CoA synthase gene is transcriptionally active (21) and starvation of 2-week-old piglets elicits a large stimulation of liver mRNA and activity (20), raises ketogenesis from long-chain fatty acids *in vitro* (14), and increases circulating ketone bodies (22), suggesting a physiological role for the expression of the pig enzyme.

Piglet starvation-associated changes involve stimulation of liver mRNA and activity of mitochondrial HMG-CoA synthase (20). Interestingly, mRNA rose to levels that were similar to those in liver from starved

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[3] Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; CPT I, carnitine palmitoyltransferase I; RT-PCR, polymerase chain reaction associated to reverse transcriptase reaction; HMS, human specific mitochondrial HMG-CoA synthase probe; oligo(dT), oligodeoxithymidine; DTT, dithiothreitol; LB, Luria–Bertani, PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline.

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TABLE I

Oligonucleotides Used in This Study

Oligo.	Position	Sequence	Restriction site
		Pig mitochondrial HMG-CoA synthase	
P6	1151–1132 Reverse	5′GTTTTCTTGTTGAACAAGTC	
PE6F	1189–1210 Forward	5′ATGTACACCTCATCCCTGTATG	
P3E	1588–1569 Reverse	5′TCTCCAAGTcgacTTAGACC	*Sal*I
P5E	146–177 Forward	5′ACCAAAGaaTTcaTAtgGTCCCTGCCGTCCCC	*Eco*RI, *Nde*I
		Rat mitochondrial HMG-CoA synthase	
R6	1152–1133 Reverse	5′GTTTTCTTGTTGAACATGTC	
RE6F	1190–1211 Forward	5′ATGTACACCTCGTCCCTCTACG	
R3E	1589–1570 Reverse	5′TGGATTGGTCgaCTTAGACG	*Sal*I
HR3	101–122 Forward	5′ATGCAGGAAgCTTCGCTCTCAC	*Hin*dIII

Note. Position 1 refers to the transcription star site. Lowercase indicates substitutions of the original sequences to introduce the specific restriction sites.

adult rats, while the increased enzyme activity in pig remained 50 times below that in starved adult or suckling rat (20). This suggested that posttranscriptional mechanisms could also control mitochondrial HMG-CoA synthase expression in pigs.

To assess the molecular mechanisms responsible for low activity of pig mitochondrial enzyme we have expressed recombinant proteins in prokaryotic and eukaryotic cell systems and obtained specific antibodies against pig mitochondrial HMG-CoA synthase. The results indicate that pig mitochondrial gene encodes a catalytically active enzyme and that the low activity found in pig liver mitochondria is due to a low level of enzyme, which suggests that an attenuated translation of the pig mRNA is responsible for the diminished ketogenic capacity of pig mitochondria.

MATERIALS AND METHODS

Reagents. Pig liver cDNA library in λgt10 vector was purchased from Clontech (Palo Alto, CA), Radiochemicals ([1-^{14}C]acetyl-CoA and [^{32}P]CTP) were from ICN (Irvine, CA). Acetyl-CoA and acetoacetyl-CoA were from Sigma (St. Louis, MO). Oligonucleotides and the hemocyanin-conjugated peptide (STVPAVPVAKADT) were purchased from Genosys (Cambridge, UK). Other chemicals, including agarose, CsCl, dithiothreitol (DTT), formaldehyde, and formamide, were from Boehringer-Mannheim (Mannheim, Germany), Carlo Erba (Milan, Italy), or Merck (Darmstadt, Germany). Anti-Xpress antibodies and the expression plasmids pRSET and pcDNA3 were purchased from Invitrogen (Groningen, Netherlands). Western blotting detection reagent, ECL kit, was purchased from Amershan (Buckinghamshire, UK). The pET-8c prokaryotic expression vector (23) and the *Escherichia coli* strains for its expression were a gift from Dr. F. W. Studier (Biology Department, Brookhaven National Laboratory, Upton, NY).

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 as described (20). Commercial crossbred pigs were used for fasting experiments, liver was obtained from 14-day-old piglets previously starved for 48 h or allowed to suckle. Tissues were excised from pentobarbitol-anesthetized (20–60 mg/kg, ic) animals and portions of liver were used to obtain mitochondria or RNA preparation as described (20).

Prokaryotic expression constructs. We had previously isolated pig mitochondrial full-length cDNA by RT-PCR-derived methods (20). To express recombinant proteins, we first isolated a λSIM7 clone from a pig liver cDNA library (λgt10, Clontech Inc.) using as a probe the insert generated by *Cla*I–*Xba*I digestion of the plasmid pPMS (20). *Eco*RI digestion of λSIM7 liberated a mitochondrial HMG-CoA synthase cDNA, which was cloned into the BSSK$^+$ *Eco*RI site, generating pSIM7 plasmid. pSIM7 did not contained the 5′ untranslated region previously isolated by RACE (20) or characterized in a pig λGPMS genomic clone (21), but its sequence confirmed the pig mitochondrial HMG-CoA open reading frame sequence previously obtained by RT-PCR (20). To generate a recombinant plasmid encoding histidine-tagged pig mitochondrial HMG-CoA synthase fusion protein (his–pig) we used a unique *Hin*dIII restriction site located at position 59 from the translation start site in the pig cDNA. Thus, we isolated a *Hin*dIII fragment from pSIM7 and cloned it into the *Hin*dIII site of the prokaryotic expression vector pRSET B, generating the plasmid pHisPig. This recombinant plasmid encodes a fusion protein of 532 amino acids in which the amino end of the protein (44 amino acids) is encoded by the expression plasmid sequence while the 488 carboxyl end amino acids correspond to the carboxyl end (amino acids 20 to 508) of the pig mitochondrial HMG-CoA synthase (20). pSIM7 was also used to generate a recombinant plasmid expressing an amino end deletion (37 amino acids) of pig mitochondrial HMG-CoA synthase. For this deletion 100 ng of pSIM7 was used as a template in a PCR reaction (2 min, 95°C; 3 min, 40°C; 2 min, 72°C; six cycles) with P5E and P3E oligonucleotides (see Table I). The PCR product generated was then digested with *Eco*RI and *Sal*I and cloned into BSSK$^+$ *Eco*RI–*Sal*I sites. The new plasmid pΔ37SIM7 was digested with *Nde*I and *Sal*I (filled-in) and the resulting insert was cloned into the *Nde*I and *Bam*HI (filled-in) sites of the prokaryotic expression vector pET3a to generate the construct pΔ37Pig.

To generate a recombinant plasmid encoding a histidine-tagged rat mitochondrial HMG-CoA synthase fusion protein we used 100 ng of the plasmid pJA118B (24) as a target in a PCR reaction (2 min, 95°C; 3 min, 40°C; 2 min, 72°C; 6 cycles) in which the forward primer HR3 introduced a single nucleotide substitution which generated a *Hin*dIII restriction site into the rat cDNA. In this PCR reaction the

T7 (5′TAATACGACTCACTATAGGG3′) oligonucleotide was used as a reverse primer. The PCR product was digested with *Hin*dIII and cloned into pRSET B to generate the plasmid pHisRat, which encodes a fusion protein in which the first 46 amino acids are encoded by the expression plasmid sequence while the 486 carboxyl end amino acids correspond to amino acids 22 to 508 of the rat mitochondrial HMG-CoA synthase cDNA (24).

Expression of the pig and rat mitochondrial HMG-CoA synthase in E. coli. BL21 (DE3) strain was transformed with plasmids pHisPig, pHisRat, and pΔ37SIM7. Cells were grown in LB media to an OD_{600} of 0.6 and then the cultures were induced for 2 h at different temperatures (27, 30, and 37°C) by adding different concentrations of IPTG (from 0.1 to 1 mM). Cultures were centrifuged at 8000 rpm and pellet was resuspended in 4 ml of sonication buffer (20 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and glycerol 10%) per gram of pellet. Sonication was performed in four pulses of 30 s at 16 μm. The resulting extract was centrifuged at 12,000 rpm and the pellet was resuspended in sonication buffer. The protein content of the pellet and supernatant was quantified using the Bradford method and extracts were analyzed in a 10% SDS–polyacrylamide gel electrophoresis.

Eukaryotic expression constructs. Pig mitochondrial HMG-CoA synthase full-length cDNA was generated from two overlapping plasmids which shared a unique *Hin*dIII restriction site: pSIM6 (encoding the 5′ region of the cDNA (20)) and pSIM7 (encoding the 3′ end of the cDNA, see above). Those plasmids were digested with *Hin*dIII and the purified insert ligated in the presence of $BSSK^+$ *Hin*dIII digested. One of the generated plasmids, pSIM10, carried the full-length cDNA of pig mitochondrial HMG-CoA synthase, which was liberated after *Eco*RI digestion and then cloned into pcDNA3 *Eco*RI site to generate the construct pSMpig. The cDNA of rat mitochondrial HMG-CoA synthase was liberated by pJA118A (24) *Hin*dIII–*Bam*HI digestion and then cloned into pcDNA3 *Hin*dIII–*Bam*HI sites to generate the construct pSMrat.

Transfection experiments and cell culture analysis. Mev-1 a somatic cell mutant of the Chinese hamster ovary (CHO)-K1 cell devoid of detectable cytosolic HMG-CoA synthase and auxotrophic for mevalonate was kindly provided by Dr. Sinensky (25). Mev-1 cells were cultured as described by Ortiz *et al.* (26). DNA transfections with plasmids pSMpig and pSMrat were carried out by the standard calcium phosphate method (27). Forty-eight hours after transfection, the trypsinized cells were cultured for 10 days in HAM's F12, 10% fetal bovine serum supplemented with 0.43 mM mevalonate, and 10 mg/ml of geneticine. Surviving cells, from more than 500 independent clones, were denominated either Mev-$pig^{+/+}$ or Mev-$rat^{+/+}$. Geneticine and mevalonate were then removed from the media of those cell lines. The revertant cells surviving in the absence of mevalonate were called either Mev-$pig^{-/-}$ or Mev-$rat^{-/-}$.

Cells were allowed to reach confluency (10^7 cells per dish) and harvested in cold PBS, and cells from independent dishes were then centrifuged (3000 rpm for 5 min) and processed for Southern, Northern, Western, and activity analysis. For Southern Dot analysis, cells were treated with proteinase K (0.4 mg/ml)/20 mM EDTA/0.4% SDS for 3 h at 37°C. Samples were extracted successively with chloroform, 1:1 mix of phenol/chloroform, and again with chloroform. DNA was ethanol-precipitated, washed in 70% ethanol, and resuspended in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA containing 20 mg/ml of RNAsa A. DNA was analyzed with HMS probe (20), a 365-bp human cDNA fragment (from position +148 to +512, using the translation start codon as +1) that shares the highest identity with pig (91.0%) and rat (89.9%) HMG-CoA synthase cDNA. For Northern Dot analysis cells were resuspended in guanidine isothiocyanate. RNA was obtained by centrifugation in CsCl as indicated in (28) and analyzed with HMS probe. For Western blot analysis, cells were resuspended in 0.2 ml of PBS (PMSF 0.1 mM; leupeptin, 2 μg/ml; aprotinin, 200 units/ml) and lysed by three freeze/thaw (5 min liquid nitrogen/2 min at 37°C) cycles. The supernatant was collected after centrifugation (15,000 rpm for 5 min) and desalted through a Bio-Spin chromatography columns (BioRad). The protein extracts obtained were processed for Western blot or mitochondrial HMG-CoA synthase assay. Mitochondrial HMG-CoA synthase activity was measured as the incorporation of [1-^{14}C]acetyl-CoA into HMG-CoA at 30°C in 5 min (29). The reaction was initiated by adding protein preparation to a reaction mixture (final volume 200 μl) composed of 100 mM Tris–HCl, 1 mM EDTA, acetoacetyl-CoA, and [^{14}C]acetyl-CoA (4000 cpm/nmol). HMG-CoA synthase-specific activity was determined with 200 μM acetoacetyl-CoA and 20 μM of acetyl-CoA. Protein concentrations for activity assays were determined following Bradford. Radioactivity was counted in a cocktail containing 67% toluene, 33% Triton X-100, and 0.56% PPO (v/v/w).

Antibodies and Western blot analysis. Polyclonal antibodies against pig mitochondrial HMG-CoA synthase were elicited against a hemocyanin-conjugated peptide STVPAVPVAKADT (position 37 to 49) corresponding to the N terminus of the pig protein (20), following immunization protocol described in (30). Polyclonal antibodies against rat mitochondrial HMG-CoA synthase were elicited against peptides corresponding to the C terminus of the protein (sequence 469–484, QFHYKVNFSPPGDTSN) as reported previously (31). Immunoblotting was carried out using an ECL kit following manufacture instructions.

RNase H protection assay. RNA (10 μg) from fed or starved rat or pig was annealed to 500 ng of oligonucleotide (P6 for pig and R6 for rat) either alone or together with 500 ng of $oligo(dT)_{20}$. After denaturation of the nucleic acids at 65°C for 3 min hybridization was performed at 30°C for 30 min in 0.1 M KCl, 10 mM $Mg\,Cl_2$, 0.1 mM EDTA, 20 mM Hepes–KOH, pH 7.4, in a final volume of 10 μl, followed by the addition of 1 unit of RNase H and incubation at 37°C for 1 h. The samples were extracted with phenol/chloroform, precipitated, and electrophoresed through 1.3% agarose/formaldehyde gels and blotted. The filter was hybridized with a specific probe for the rat or pig mitochondrial HMG-CoA synthase 3′ end, which was obtained by PCR (95°C, 1 min; 37°C, 1 min; 72°C, 1 min 30 s; 20 cycles) with oligonucleotides PE6F and P3E using 200 ng of the construct pSIM7 as a template for pig, and oligonucleotides RE6F and RE3 and 200 ng of plasmid pJA118B as a template for rat.

RESULTS AND DISCUSSION

In pigs, ketone body metabolism is much lower than in other mammals (12–16). The lack of expression of mitochondrial HMG-CoA synthase has been correlated with this peculiar phenotype (14). Thus, there is a 2- to 3-week lag in the postnatal induction of the gene (20) and negligible specific activity or immunodetectable protein in 48-h-old unsuckled piglets (14). However, after starvation of 2-week-old piglets a huge induction of the pig liver mRNA levels is accompanied by a 27-fold increase in pig mitochondrial HMG-CoA synthase specific activity (20) and an 8-fold increase in circulating ketone bodies (22). Despite improvements in mRNA expression by fasting, specific activity of the enzyme in mitochondria remained relatively low (50 times lower than that observed in suckled or starved rats (14, 20)), suggesting that posttranscriptional mechanism(s) and/or kinetic differences in the mitochondrial HMG-CoA synthase enzyme also control HMG-CoA synthase gene expression in piglets.

To determine which mechanism best explains the low enzymatic activity observed in pig we studied the expression level of the protein in pig mitochondria and

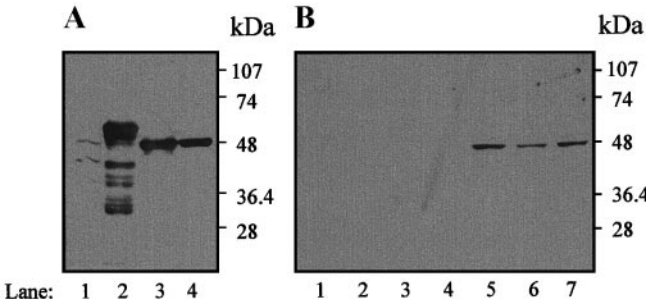

FIG. 1. Western blot analysis of recombinant and liver pig mitochondrial HMG-CoA synthase with antibodies specific against the pig enzyme. (A) 5 μg of *E. coli* control cellular extract (lane 1), *E. coli* cellular extract expressing his-tag fusion protein (his–pig, lane 2), *E. coli* cellular extract expressing mitochondrial HMG-CoA synthase without the first 37 amino acids (p37Pig, lane 3), and 10 μg of Mev-pig$^{-/-}$ cellular extract expressing pig mitochondrial HMG-CoA synthase (lane 4). (B) 50 μg of liver mitochondrial protein from 48-h-old piglets (lanes 1 and 2) 2-week-old piglets (lanes 3 and 4), and 2-week-old piglets starved for 48 h (lanes 5–7). Mobility of the molecular weight markers is indicated in kDa by straight lines to the right of the figures.

measured apparent kinetic parameters of a pig recombinant enzyme.

Low Expression of Mitochondrial HMG-CoA Synthase Protein in Liver of Starved Piglet

To measure the levels of the mitochondrial enzyme in pig liver we generated antibodies against a pig mitochondrial HMG-CoA synthase peptide. Figure 1A shows that this antibodies specifically recognized pig mitochondrial HMG-CoA synthase recombinant proteins. *Escherichia coli*-expressed histidine-tag fusion protein (lane 2) has an apparent molecular mass of ~56 kDa, while a truncated protein (lacking the first 37 amino acids (lane 3) has an apparent molecular mass of ~50 kDa; both protein sizes are consistent with the predicted molecular mass calculated from the pig mitochondrial HMG-CoA synthase cDNA sequence (20). Histidine-tagged protein (lane 2) is also recognized by a commercial antibody (Xpress, Promega) raised against an epitope present in the amino fusion peptide (data not shown). Recombinant protein expressed in Mev-pig$^{-/-}$ cell line (see below) is also recognized by the antibodies (lane 4) with an apparent molecular mass of about 50 kDa, lower than that predicted from the pig mitochondrial HMG-CoA synthase cDNA nucleotide sequence (20) and similar to the molecular mass of the recombinant protein lacking the first 37 amino acids (compare lanes 3 and 4), indicating that the leader peptide was removed in the eukaryotic cell line. Figure 1B shows that, in agreement with previously published data (14), the amount of pig mitochondrial HMG-CoA synthase protein was negligible in liver mitochondria of 48-h-old piglets (lanes 1–2). The expression was also absent in liver of 2-week-old suckling piglets (lanes 3–4). However, in agreement with the induction of gene expression by starvation (20), pig mitochondrial HMG-CoA synthase was present in liver mitochondria from starved 2-week-old piglets (see lanes 5–7).

To compare the levels of the detected protein in pig or rat mitochondria, histidine-tagged fusion proteins were used to normalize the dilution of antibodies used in the Western blot analysis. Figure 2A shows the ability of antibodies against pig or rat HMG-CoA synthase (31) to recognize similar amounts of histidine-tagged pig or rat fusion proteins. Figure 2B shows that in liver mitochondria from starved pigs (lanes 1 and 2) there is less HMG-CoA synthase protein than in liver mitochondria from fed (lanes 3 and 4) or starved rats (lanes 5 and 6, note that different amounts of protein were loaded into the gel), indicating that despite the high levels of mRNA detected (see Fig. 5 and Ref. (20)) pig expressed low levels of mitochondrial HMG-CoA synthase enzyme. Figure 2B also shows that the immunodetected pig (lanes 1–2) or rat (lanes 3–4) mitochondrial HMG-CoA synthase has a molecular mass of about 50 kDa, consistent with the presence of processed proteins inside the liver mitochondria.

Pig Mitochondrial HMG-CoA Synthase Recombinant Protein Is a Catalytically Active Enzyme

To study whether the low expression of pig mitochondrial HMG-CoA synthase was the only reason for the low activity of the enzyme in pig mitochondria (14, 20), we attempted to express recombinant proteins in *E. coli*. A mature protein, in which the first 37 amino acids were removed to produce a protein of similar size

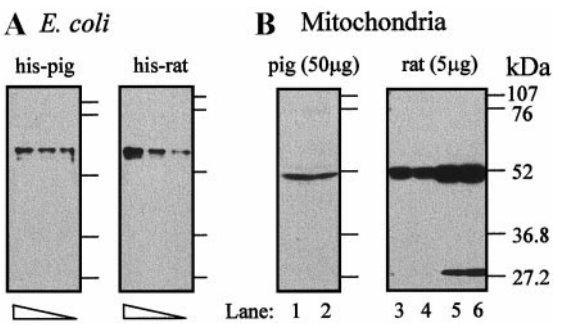

FIG. 2. Comparative analysis of HMG-CoA synthase enzyme levels from pig and rat mitochondria. (A) Different amounts (300–100 ng) of pig (his–pig) or rat (his–rat) recombinant proteins were analyzed using a 1:600 dilution of antibodies specific against pig (left) or a 1:1600 dilution of antibodies specific against rat (right). (B) Using the same dilution of antibodies liver of mitochondrial protein from 2-week-starved piglets (50 μg, lanes 1–2) or fed (5 μg, lanes 3–4) and starved rats (5 μg, lanes 4–5) were analyzed in the same Western Blot. Mobility of the molecular weight markers are indicated in kDa by straight lines to the right of the figures.

to that expected in the liver mitochondria, was expressed as an insoluble protein in the *E. coli* extracts (data not shown). Similarly, a fusion protein in which the first 19 amino acids were replaced by 44 amino acids, carrying a six-histidine tag and specific epitope, was also expressed as an insoluble protein (data not shown). The same result was obtained when a rat mitochondrial HMG-CoA synthase fusion protein was expressed in *E. coli* (data not shown). All the proteins were partially active (~0.5 mU) in the fresh extracts and they were recognized by specific (see Figs. 1A and 2A) or anti-epitope (data not shown) antibodies.

In order to express soluble proteins we exploited the capacity of rat mitochondrial HMG-CoA synthase to correct the mevalonate auxotrophy of Mev-1 cells when introduced ectopically into this cell line (26). Thus, we generated stable cell lines of Mev-1 containing pig (Mev-pig$^{+/+}$) or rat (Mev-rat$^{+/+}$) cDNA of mitochondrial HMG-CoA synthase. In these cells the insertion of the recombinant plasmid into the cell genome (see Fig. 3) allows the cells to grow in the presence of mevalonate and geneticine. When geneticine and mevalonate were removed from the media of both cell lines, a similar amount of vigorously expressing mitochondrial HMG-CoA synthase cells survived and proliferated generating, respectively, two new cell lines Mev-pig$^{-/-}$ and Mev-rat$^{-/-}$. Figure 3A shows that the four stable cell lines (Mev-pig$^{+/+}$, Mev-rat$^{+/+}$, Mev-pig$^{-/-}$, and Mev-rat$^{-/-}$) contained a similar number of cDNA copies in the genome. Figure 3B shows that, as expected, the cell lines Mev-pig$^{-/-}$ and Mev-rat$^{-/-}$, which proliferate without mevalonate, expressed respectively pig or rat mitochondrial HMG-CoA synthase mRNA. The detected transcript was recombinant due to the fusion of the 3′ untranslated region of human growth hormone (data not shown). Figure 3B also indicates that the expression of mitochondrial HMG-CoA synthase mRNA needed for the establishment of the Mev-pig$^{-/-}$ or Mev-rat$^{-/-}$ cell lines was similar, since we use as a probe a DNA fragment from human cDNA (32) which in Southern Dot analysis recognizes pig or rat cDNA (data not shown). Figure 3C shows that, as expected, the cell lines Mev-pig$^{-/-}$ and Mev-rat$^{-/-}$ expressed, respectively, pig and rat recombinant mitochondrial HMG-CoA synthase enzymes while the cell lines incubated in the presence of geneticine and mevalonate (Mev-pig$^{+/+}$ or Mev-rat$^{+/+}$) did not express either of the recombinant enzymes. This result is in agreement with the mitochondrial HMG-CoA synthase specific activities of those cell lines (1.27 mU for Mev-pig$^{-/-}$, 0.029 mU for Mev-pig$^{+/+}$, 2.13 mU for Mev-rat$^{-/-}$, and 0.029 mU for Mev-rat$^{+/+}$). The mechanism by which rat mitochondrial HMG-CoA synthase supports the mevalonate pathway in a cell line defective for cytosolic HMG-CoA synthase (25) is not understood. Nevertheless, the experiment indicates that pig mitochondrial synthase supports the mevalonate pathway in the cell system, as does the rat enzyme, showing therefore that the pig gene encodes a catalytically active enzyme.

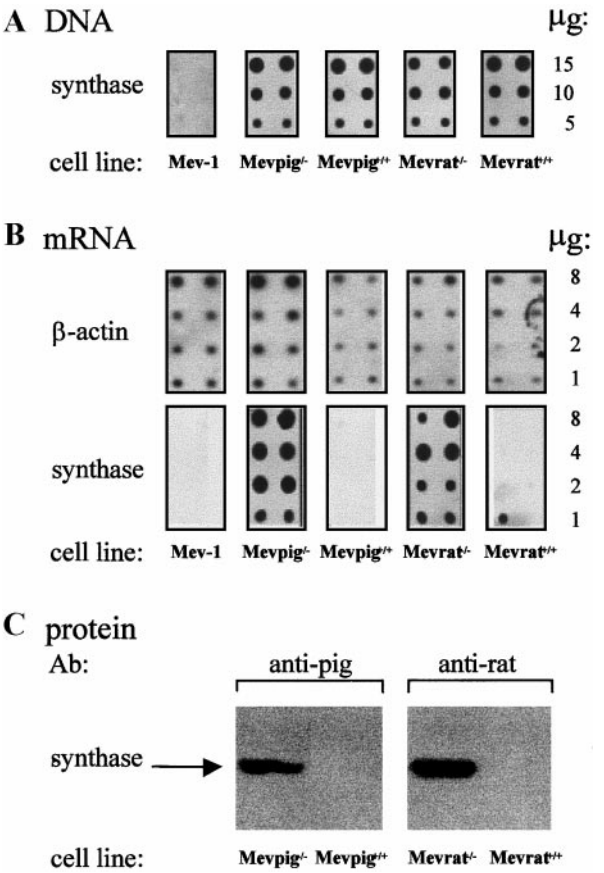

FIG. 3. Comparative analysis of DNA copy number and expression of pig and rat mitochondrial HMG-CoA synthase in different cell lines. Different amounts of genomic DNA (A), total RNA (B), or 50 μg of total protein (C) of cell lines growing in the presence (Mev-pig$^{+/+}$ and Mev-rat$^{+/+}$) or absence (Mev-pig$^{-/-}$ and Mev-rat$^{-/-}$) of geneticine and mevalonate were analyzed. Dot Blots (A and B) were carried out with a 365-bp human cDNA probe 89.9 or 91.0% homologous to rat or pig cDNA (20). This human probe recognized similarly the pig or rat expression vectors indistinctly in dot blot analysis (not shown). Nylons were also hybridized with human β-actin probe to ensure that samples were equally loaded (not shown in A and B). The antibodies used in the analysis of Mev-pig$^{-/-}$ and Mev-pig$^{+/+}$ or Mev-rat$^{-/-}$ and Mev-rat$^{+/+}$ by Western blot were specific for pig or rat, respectively.

The high expression of mitochondrial HMG-CoA synthase in the stable cell lines allowed the kinetic analysis of the pig and rat enzyme. Figure 4 shows double-reciprocal plots, obtained from Mev-pig$^{-/-}$ and Mev-rat$^{-/-}$ cell lines, of initial velocities versus different concentration of one substrate, while the concentration of the other substrate remains constant. Figure 4A shows that, at the concentration tested, any increase in the concentration of acetoacetyl-CoA (between 10 to 400 μM) resulted in either no change or a decrease in the enzyme activity for both pig and rat enzymes. This

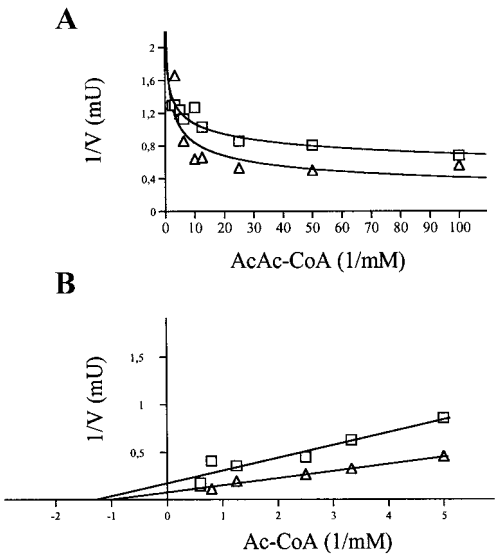

FIG. 4. Double-reciprocal plots of initial velocities versus concentration. (A) Varying the acetoacetyl-CoA concentration (10, 20, 40, 80, 100, 160, 200, 300, and 400 μM) at fixed acetyl-CoA concentrations (200 μM). (B) Varying the acetyl-CoA concentration (0.2, 0.3, 0.4, 0.8, 1,25, and 1,660 mM) at fixed acetoacetyl-CoA concentrations (20 μM). The mitochondrial HMG-CoA synthase activity was measured in cellular extracts obtained from Mev-pig$^{-/-}$ (□) or Mev-rat$^{-/-}$ (△) cell lines.

kinetic behavior had previously been showed for pure mitochondrial HMG-CoA synthase from ox (33, 34) and avian (35) liver. No differences are evident between the pig and rat recombinant enzymes with respect to the acetoacetyl-CoA inhibition observed at a constant concentration of acetyl-CoA (200 μM). This plot (Fig. 4B) was used to calculate apparent kinetic constants for the pig (Mev-pig$^{-/-}$) and rat (Mev-rat$^{-/-}$) recombinant enzymes. The apparent K_m was similar for pig (695 μM) and rat (811 μM) enzymes, while V_{max} of pig enzyme (7.7 mU) was lower than for rat (13.7 mU), although the levels of mRNA expressed are similar (see Fig. 3B). Since the kinetic parameters are only apparent we cannot conclude differences in the catalytic capacity of pig and rat enzymes. However, these differences in the V_{max} appear to be related to levels of expression of the recombinant enzymes in the cell lines generated from Mev-1 cells (Fig. 3C) and do not explain the 50-fold differences observed in isolated mitochondria (20).

Transcript Maturation Analysis of Pig and Rat Mitochondrial HMG-CoA Synthase

Pig liver mitochondria express low levels of a catalytically active enzyme despite the high levels of mRNA observed in starved 2-week-old piglets (20), suggesting that an attenuated translation could be responsible for the low activity. Although translation can be regulated by different mechanisms, in a great number of cases the control is exerted by the length of the poly(A) tail, which influences both mRNA stability and translation. Two mayor alternative polyadenylation signals have been reported in the rat 3′ noncoding region and a putative regulatory role of the 3′ untranslated region has been suggested in controlling rat mitochondrial HMG-CoA synthase expression (36). To assess whether polyadenylation site usage, or the length of the poly(A) tail, were involved in the expression of pig liver mitochondria HMG-CoA synthase, RNase H protection assays were performed with pig and rat liver RNA (see Fig. 5). Northern blot analysis of the undigested (lanes 1 of Figs. 5A–5D) RNA, shows two transcripts for pig HMG-CoA synthase (indicated by arrows in Figs. 5A and 5B) and a main transcript for rat HMG-CoA synthase (Figs. 5C and 5D). The transcript sizes are different but in concordance with the cloned cDNAs (20, 24). The RNase H-digested samples (lanes 2 of Figs. 5A to 5D) indicates that a main polyadenyl-

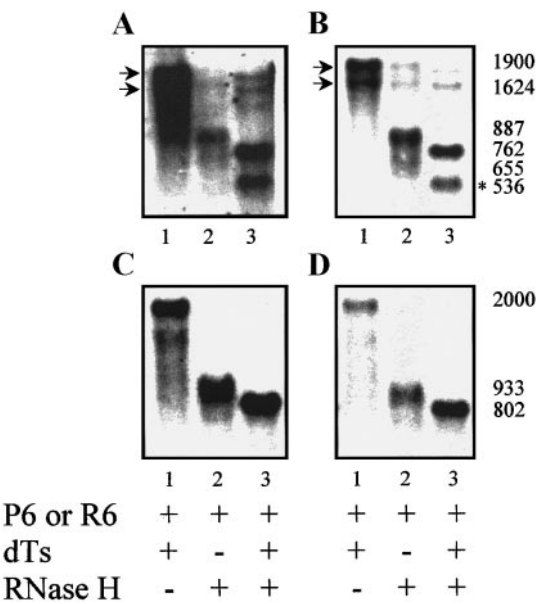

FIG. 5. RNase H protection analysis of the 3′UTR and poly(A) tail lengths of mitochondrial HMG-CoA synthase liver mRNA of fed or starved pigs and rats. Total (10 μg) RNA from the liver of 2-week-old fed pig (A), 2-week-old starved pig (B), adult fed rat (C), and adult starved rat (D) was annealed with a 20-nt single-stranded DNA (oligonucleotide P6 for pig mRNA and R6 for rat mRNA) complementary to a sequence located approximately 800 nucleotides 5′ of HMG-CoA synthase polyadenylation site, in the absence (lines 2) or presence (lines 3) of oligo(dT). The hybrids, except the controls (lanes 1), were treated with RNase H, and the RNA fragments were electrophoresed and transferred. The blot was hybridized to a ^{32}P DNA probe specific for pig or rat synthase, respectively, and complementary to the 3′-terminal 400 oligonucleotides of the pig or rat mitochondrial HMG-CoA synthase mRNA. Due to the different mitochondrial HMG-CoA synthase transcript abundance in each species and metabolic situation, membranes were exposed to the films for 2 h in B and D, 6 h in C, and 2 days in A.

ation site was used in transcript maturation of pig (Fig. 5A) and rat (Fig. 5C) mRNA, generating respectively, fragments of approximately 887 and 933 nucleotides. No change by starvation was observed in the use of this polyadenylation site in pig (compare lanes 2 of Figs. 5A and 5B) or rat (compare lanes 2 of Figs. 5C and 5D) mRNA. The introduction of oligo(dT) in the RNase H reaction (lanes 3 of Figs. 5A to 5D) allows the quantification of the poly(A) tail length from pig (125 nt, compare lanes 2 and 3 of Fig. 5A) and rat mRNA (131 nt, compare lanes 2 and 3 of Fig. 5C). Starvation did not induce any change in the poly(A) tail length either in pig (compare lanes 3 of Figs. 5A and 5B) or in rat (compare lanes 3 of Figs. 5C and 5D) mRNA. In the presence of oligo(dT) the RNase H reaction detected a second polyadenylation site in the mature pig transcript, which generates a fragment of approximately 536 nucleotides (indicated by an asterisk in lanes 3 of Figs. 5A and 5B). The usage of this polyadenylation site, located 5′ to that detected previously, could explain the two transcripts observed for pig mitochondrial HMG-CoA synthase in the undigested or partially digested samples, and will generate a transcript of the size expected from the previously cloned cDNA (20). This transcript was barely detected after RNase H digestion in the absence of oligo(dT) (see lane 2 of Fig. 5A) probably due to the lower abundance and/or to poly(A) tail length heterogeneity. The use of this second site of polyadenylation did not change in the transcript maturation of liver from starved piglets. These data show that changes in the poly(A) tail and changes during 3′ end transcript maturation are not involved in the low amount of pig mitochondrial HMG-CoA synthase enzyme detected in pig liver mitochondria. However, from data presented in Fig. 5, it is also clear that both pig mRNAs have shorter 3′ UTRs than rat mRNA. Therefore, the pig 3′ UTRs could play a role in low translation efficiency.

Our data point out that an attenuated translation could be responsible for the low expression of pig mitochondrial HMG-CoA synthase gene. The mechanism of such putative attenuation is unknown. If we assume the same catalytic capacity for the pig and rat enzyme, then the data showed here may indicate that translation attenuation of pig mRNA versus rat mRNA is only slightly reproduced in the stable cell lines in terms of V_{max} (1.27 mU for Mev-pig$^{-/-}$ and 2.13 mU for Mev-rat$^{-/-}$), suggesting that either the attenuated translation is a *trans*-specific event or that the recombinant transcript generated here had lost its specific translation peculiarities, or both.

Postnatal maturation of liver mitochondria is profoundly controlled at the level of translation of the transcripts encoded by nuclear genes. The translational repression of the β-F1-ATPase mRNA, which accumulates previously to birth and remains masked until the differentiation of mitochondria starts (37), is performed by RNA-binding proteins that interact with an AU-rich sequence located in the 3′UTR of the mRNA. This sequence is highly conserved within the 3′ end of the UTR of many mammalian nucleus-encoded mitochondrial genes including pig and rat mitochondrial HMG-CoA synthase, suggesting that the synthase could be regulated in the same way and that the switch to the translational active state would not be properly acquired in the pig. If this was the case, many other nucleus-encoded mitochondrial genes would remain off, postnatally leading to a global phenomenon of mitochondrial immaturity. However, previously published data (14) suggest that pig mitochondria is mature enough to support certain metabolic pathways.

The pig mitochondrial HMG-CoA synthase mRNA presents a 5′TOP sequence composed by a C residue at the cap site followed by six pyrimidines. These 5′TOP sequences, which are commonly found in genes coding for the translational machinery (38), mediate the translational silencing of these genes during the cell arrest and activate translation during growing. Nevertheless, the presence of the 5′TOP sequence is necessary but not sufficient to confer translational repression. What is more, it seems difficult to understand why the mitochondrial HMG-CoA synthase would be regulated parallel to genes coding for proteins of the translational machinery.

The 5′-untranslated region is highly conserved between the rat, pig, and human synthases. The analysis of the secondary structure of these sequences (data not shown) has revealed the presence of a hairpin preceding the translation initiation codon. Differences in the hairpin stability or the presence of specific *trans*-acting proteins that interact with this region could modulate the rate of translation of these mRNAs. The fact that there are not apparent differences in the translation of the pig and rat mRNAs expressed in Mev-1 lines, suggests the existence of a specific *trans*-acting factor that mediates translational repression in pig liver.

Ketone bodies have traditionally been considered as an alternative metabolic fuel in low-glucose conditions (e.g., suckling, starvation). From this point of view the low expression of mitochondrial HMG-CoA synthase in pig liver reported here explains why pigs do not rely on in circulating ketone bodies as a source of energy in such conditions (17, 18, 39). However, mitochondrial HMG-CoA synthase is expressed in tissues like brain, kidney, testis, or ovary (31, 40), in which an anabolic role of the ketone body production has been suggested (26). In some of these tissues the expression levels of the mitochondrial HMG-CoA synthase gene are so low that it is observed only by RNase protection assays (40). The fact that the pig mitochondrial HMG-CoA synthase mRNA levels are increased by starvation under the control of a typical PPAR responsive promoter

(21) and that pig mitochondrial HMG-CoA synthase is expressed as a catalytically active protein suggests a physiological role for such low activity *in vivo*. These findings support the hypothesis that the low levels of expression could be involved in the anabolic role of ketone bodies in pigs.

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