REVIEW ARTICLE

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis

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Cytosolic and mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthases were first recognized as different chemical entities in 1975, when they were purified and characterized by Lane's group. Since then, the two enzymes have been studied extensively, one as a control site of the cholesterol biosynthetic pathway and the other as an important control site of ketogenesis. This review describes some key developments over the last 25 years that have led to our current understanding of the physiology of mitochondrial HMG-CoA synthase in the HMG-CoA pathway and in ketogenesis in the liver and small intestine of suckling animals. The enzyme is regulated by two systems: succinylation and desuccinylation in the short term, and transcriptional regulation in the long term. Both control mechanisms are influenced by nutritional and hormonal factors, which explains the incidence of ketogenesis in diabetes and starvation, during intense lipolysis, and in the foetal–neonatal and suckling–weaning transitions. The DNA-binding properties of the peroxisome-proliferator-activated receptor and other transcription factors on the nuclear-receptor-responsive element of the mitochondrial HMG-CoA synthase promoter have revealed how ketogenesis can be regulated by fatty acids. Finally, the expression of mitochondrial HMG-CoA synthase in the gonads and the correction of auxotrophy for mevalonate in cells deficient in cytosolic HMG-CoA synthase suggest that the mitochondrial enzyme may play a role in cholesterogenesis in gonadal and other tissues.

Key words: chicken ovalbumin upstream transcription factor, enterocytes, peroxisome-proliferator-activated receptor, succinylation, transcriptional regulator.

INTRODUCTION

The two 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthases

The enzyme HMG-CoA synthase (EC 4.1.3.5) catalyses the condensation of acetoacetyl-CoA and acetyl-CoA to form HMG-CoA plus free CoA. HMG-CoA synthase activity is located in two different compartments: the cytosol and the mitochondria. The HMG-CoA produced by the cytosolic HMG-CoA synthase is transformed into mevalonate by the action of HMG-CoA reductase. This starts the isoprenoid pathway which, in addition to cholesterol as the main end-product, produces several important products, such as ubiquinone, dolichol, isopentenyl adenosine and farnesyl groups, which covalently modify proteins. The HMG-CoA produced inside the mitochondria by the mitochondrial HMG-CoA synthase is transformed into acetoacetate by the action of HMG-CoA lyase. Acetoacetate is transformed into hydroxybutyrate and acetone; all of these are known as ketone bodies.

In 1975 the two HMG-CoA synthase isoforms were characterized by Lane's group [1–3] as different chemical entities, but some uncertainty remained as to whether only one gene produced these two proteins that catalysed the same reaction. In 1986, the group of Goldstein and Brown reported the cloning and sequencing, first of the cDNA [4] and then of the gene [5], of the hamster cytosolic HMG-CoA synthase. The existence of two genes was firmly established in 1990, when we cloned the cDNA [6], and then the gene, for the rat mitochondrial HMG-CoA synthase [7]. Although the percentage identity in amino acid residues between hamster cytosolic and rat mitochondrial HMG-CoA synthases was high (65%), it became clear that they were the products of two different genes, and that they were differently regulated. The cytosolic synthase was repressed by fasting and cholesterol feeding [8]. In contrast, the mitochondrial HMG-CoA synthase was increased by fasting.

Structural and functional comparisons of the promoter regions of the two synthases [5,7] has indeed shown that the two promoters are very different. Cytosolic HMG-CoA synthase contains sterol regulatory elements that modulate transcriptional activity by sterols, mediated by sterol regulatory element binding proteins (SREBP)-1 and -2 [9,10], which have not been observed in the promoter of the mitochondrial HMG-CoA synthase. Conversely, the peroxisome proliferator regulatory element (PPRE) is present in the mitochondrial HMG-CoA synthase promoter, but has not been detected in the promoter of the cytosolic synthase [11]. These lines of evidence emphasize that the promoter of each gene is responsible for the control of one of the two different pathways: the cytosolic HMG-CoA synthase is a control site of the isoprenoid biosynthetic pathway, and the mitochondrial HMG-CoA synthase is an important control site of the ketogenic pathway.

Ketogenesis

Ketogenesis is a mitochondrial process by which acetyl-CoA, mostly derived from the β-oxidation of fatty acids, is converted

Abbreviations used: CAT chloramphenicol acetyltransferase; COUP-TF, chicken ovalbumin upstream transcription factor; CPT, carnitine palmitoyltransferase; CRE, cAMP regulatory element, CREB, CRE binding protein; DR-1, direct repeat-1; FAH, fumarylacetoacetate hydrolase; hGH, human growth hormone; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HNF-4, hepatocyte nuclear factor-4; NRRE, nuclear receptor responsive element; PEPCK, phosphoenolpyruvate carboxykinase; PPAR, peroxisome-proliferator-activated receptor; PPRE, peroxisome proliferator regulatory element; RXR, *cis*-retinoid receptor.

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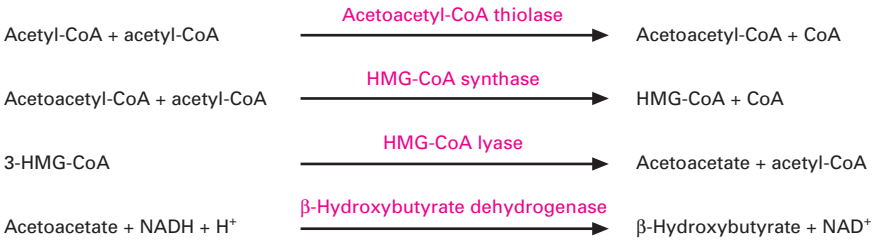

Scheme 1 HMG-CoA pathway

through four reactions, usually known as the HMG-CoA pathway, into acetoacetate, β-hydroxybutyrate and acetone, all of which are commonly called ketone bodies (Scheme 1).

Acetone is formed by the non-enzymic breakdown of acetoacetate and is unlikely to be important in the metabolism of the intact animal. The other two products are used by different tissues as fuels, thus saving glucose. Ketogenesis is mainly hepatic, but it also occurs in the intestines of suckling mammals [12], and to a lesser extent in kidney [13] and in neonatal cortical astrocytes [14,15]. Factors that induce ketogenesis are (in addition to diabetes) fasting and intense lipolysis. In the transition from the fed to the fasted condition, carbohydrate utilization and fatty acid synthesis in the liver cease and are replaced by the oxidation of fatty acids and the induction of ketogenesis.

The influence of fatty acids on ketogenesis depends, in turn, on the metabolic state of the organism. The production of acetoacetate and β-hydroxybutyrate in perfused rat liver incubated with fatty acids shows marked differences depending on whether the hepatocytes are taken from a fed, fasted or diabetic animal [16–20]. The control of ketogenesis by substrate availability in mammals after the entry of fatty acids into mitochondria, regulated by carnitine palmitoyltransferase I (CPT I), has been extensively reviewed [21–23]. A second factor that has aroused vigorous interest as a participant in the control of ketogenesis is the expression of the genes specifically involved in ketogenesis: those for CPT I [24] and mitochondrial HMG-CoA synthase [11].

MITOCHONDRIAL HMG-CoA SYNTHASE AS A CONTROL SITE OF KETOGENESIS

Williamson et al. [25] were the first to propose that mitochondrial HMG-CoA synthase is the rate-limiting enzyme of the ketogenic pathway, in studies of acetoacetate production in sonicated liver particles. Later, the work of Chapman et al. [26] and Clinkenbeard et al. [1] using mitochondrial subfractions supported this view. Dashti and Ontko [27] unequivocally showed that the activity of mitochondrial HMG-CoA synthase was responsible for the control of synthesis of acetoacetate in the HMG-CoA pathway. In aged mitochondria, in which ketogenesis had decreased, only mitochondrial HMG-CoA synthase was decreased in the same proportion, whereas thiolase and HMG-CoA lyase retained 100 % activity.

The question of whether CPT I regulated by malonyl-CoA is the main ketogenic control site, or whether it merely provides the mitochondria with a ketogenic substrate, led to many studies. Grantham and Zammit [28,29] concluded that, although CPT I is an important locus for the control of hepatic fatty acid oxidation, and hence ketogenesis, during the onset of diabetic ketosis [28], it does not appear to play such a role during the acute reversal of ketosis. Moreover, acute depression of the ketogenic capacity of the liver on refeeding is not accompanied during the first few hours of refeeding by any reversal of the changes in CPT I induced by starvation [29]. Analogous results were found during liver regeneration [30,31]. It is now increasingly evident that, during certain transition periods in rats (e.g. foetal–neonatal, suckling–weaning, starved–fed and diabetic–insulin-treated), significant control over ketogenesis may be exerted at another intramitochondrial site distal to CPT I, more specifically HMG-CoA synthase [32–38]. Top-down control analysis in isolated rat liver mitochondria showed that, under conditions of non-regulation of CPT I by malonyl-CoA, the absence of NADH and the inhibition of the Krebs cycle by malonate, 28 % of the control over ketogenesis is invested in the group of enzymes responsible for the production of acetyl-CoA and 72 % resides with the enzymes of the HMG-CoA pathway that convert acetyl-CoA into acetoacetate [39]. Studies on short-term inactivation by succinylation and desuccinylation [40] and on the transcriptional regulation of the HMG-CoA synthase gene by nutritional and hormonal effectors [41] confirmed HMG-CoA synthase as the main control point over ketogenesis, providing that enough substrate is available in mitochondria.

PROPERTIES OF MITOCHONDRIAL HMG-CoA SYNTHASE

The purification of a homogeneous form of mitochondrial HMG-CoA synthase from avian liver showed that it is a homodimer of 53–57 kDa monomers [2]. Antibodies against mitochondrial HMG-CoA synthase do not inactivate the cytosolic enzyme [3]. Kinetic assays show that acetoacetyl-CoA, one of the substrates of the reaction, is inhibitory and that the K_m for acetyl-CoA depends on the acetoacetyl-CoA concentration in the assay. Mg^{2+} is also inhibitory, and this distinguishes the mitochondrial from the cytosolic HMG-CoA synthase, which is activated by this cation. Palmitoyl-CoA inhibits the enzyme with a K_i of 0.5 μM, and succinyl-CoA at 0.2 mM inhibits 50 % of mitochondrial HMG-CoA synthase activity.

Mechanism of enzyme reaction

Work on yeast [42,43] and avian [44–51] mitochondrial HMG-CoA synthases established that the synthesis of HMG-CoA occurs in three steps. The first, which is rate-limiting, is the acetylation of the enzyme in a cysteinyl thiol group. The partial reactions are:

$$\text{EnzSH} + \text{acetyl-CoA} \rightarrow \text{acetyl–SEnz} + \text{CoASH}$$

$$\text{Acetoacetyl-CoA} + \text{acetyl–SEnz} \rightarrow \text{EnzS–HMG-CoA}$$

$$\text{EnzS–HMG-CoA} + H_2O \rightarrow \text{EnzSH} + \text{HMG-CoA}$$

The formation of HMG-CoA proceeds by a Bi Bi Ping Pong kinetic mechanism [52], in which the enzyme (EnzSH) reacts first

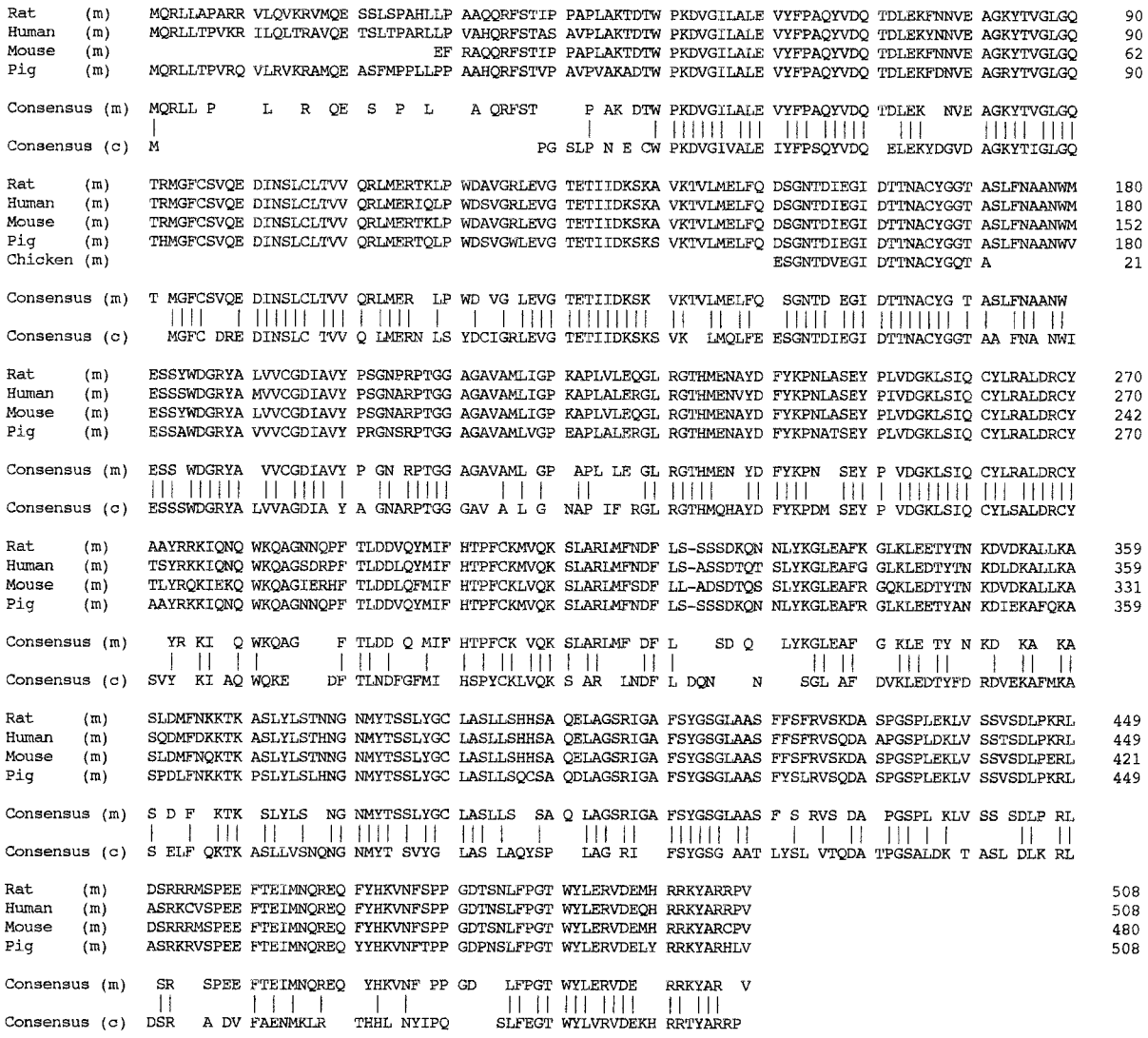

Figure 1 Protein alignment of HMG-CoA synthase sequences

Mitochondrial (m) HMG-CoA synthases from the rat [6], human [67], mouse [68] and pig [69], and 21 amino acids of the sequence of the chicken enzyme [64], are aligned. Position 1 is assigned to the initiation methionine. The consensus sequence for the mitochondrial HMG-CoA synthases is defined below their sequences, and that of the cytosolic HMG-CoA synthases (c) was obtained from the sequences of the Syrian hamster [4], rat [70], human [71] and chicken [72] enzymes. Vertical lines indicate the general vertebrate HMG-CoA synthase consensus sequence.

with acetyl-CoA to form a covalent acetyl–enzyme intermediate (acetyl–SEnz), with the release of CoA. This intermediate then condenses with the second substrate, acetoacetyl-CoA, to form HMG-CoA bound to the enzyme (EnzS–HMG-CoA), which is then liberated by hydrolysis [44–48]. Three cysteine residues at positions 166, 261 and 305 of the mitochondrial HMG-CoA synthase sequence [6] (positions 129, 224 and 268 of the cytosolic enzyme [4]) may be closely juxtaposed within the catalytic domain [49]. Site-directed mutagenesis of all three cysteines showed that Cys-166 (cytosolic Cys-129) plays a crucial role in HMG-CoA synthesis [50]. In addition, His-264 participates in the anchoring of acetoacetyl-CoA, as indicated by the important changes in the V_{max} or the K_m for acetoacetyl-CoA [51] when this amino acid is mutated. The conserved residues His-197 and His-436 do not play a significant role in the kinetics of the enzyme.

As for the yeast and avian enzymes, acetoacetyl-CoA in the concentration range 1–50 μM is a potent inhibitor of ox liver HMG-CoA synthase [53,54], and it is competitive with respect to acetyl-CoA, confirming the enzyme-substitution (Ping Pong) mechanism of catalysis. In the presence of [^{14}C]acetyl-CoA, the enzyme forms a covalently acetylated enzyme that can be hydrolysed by further acetyl-CoA or, to a lesser extent, by succinyl-CoA [55].

Succinylation of mitochondrial HMG-CoA synthase as a mechanism of regulation of its activity

Since succinyl-CoA inhibits mitochondrial HMG-CoA synthase from yeast [42], rat liver [2] and ox liver [54], and glucagon lowers the mitochondrial succinyl-CoA content in perfused livers and in hepatocytes [56], which stimulates ketogenesis, it appears that glucagon must increase the ketogenic flux through the HMG-CoA pathway by decreasing the concentration of succinyl-CoA and also the extent of succinylation of HMG-CoA synthase [57]. [^{14}C]Succinyl-CoA not only inhibits the enzyme, but also succinylates it by a covalent reaction. Mannoheptulose (a com-

pound that raises glucagon levels in blood) produces a decrease in succinyl-CoA that correlates with the increase in HMG-CoA synthase activity [40,58]. 2-Oxoglutarate, which increases the intramitochondrial content of succinyl-CoA, lowers the enzyme activity [59]. Spontaneous desuccinylation is accelerated by acetyl-CoA, and to a lesser extent by acetoacetyl-CoA. Succinyl-CoA is the only CoA thioester that inhibits HMG-CoA by covalent modification

HMG-CoA synthase is substantially succinylated and inactivated in the starved–fed transition [60], in term foetal and in high-carbohydrate/low-fat-weaned rats, and slightly succinylated and active in the livers of foetal and high-fat/carbohydrate-free-weaned rats [61]. There also is a negative correlation between HMG-CoA synthase activity and succinyl-CoA content in mitochondria isolated from full-term foetal, suckling and carbohydrate-weaned rats. The same effect of succinylation of intestinal mitochondrial HMG-CoA synthase and a decrease in enzyme activity is observed in 12-day-old suckling rats fasted for 24 h [62]. A recent report showed that hepatic HMG-CoA synthase in fed humans is also partially succinylated [63].

Table 1 Potential regulatory *cis* elements in the mitochondrial HMG-CoA synthase gene

Abbreviations: IRE, insulin regulatory element; CTF, CCAAT-binding transcription factor; NF1, nuclear factor 1; C-EBP, CCAAT enhancer binding protein; GRE, glucocorticoid regulatory element.

Cis element	Sequence	Gene position
TATA	TATAAA	−28
Sp1	GGCGGG	−54
NRRE	AGACCTTTGGCCC	−92
IRE	TGATGTTTTC	−130
CTF-NF1	TGGCA	−520, −836
CRE	GTGCGTCA	−546
C-EBP	AGTCAAAAG	−778
GRE	GCTACAGGTTGTGCT	−995
NF1-like	TGGCA	−520, −836, −885, −1140
c-Jun	TGTGTCA	−249
	TGCGTCA	−554
	TGACTCC	−781

PRIMARY STRUCTURE OF MITOCHONDRIAL HMG-CoA SYNTHASE

Isolation of cDNA clones from different vertebrates

Miziorko and Behnke [64] isolated the polypeptide constituting the catalytic site of mitochondrial HMG-CoA synthase by treating the purified chicken enzyme [2] with the abortive inhibitor [^{14}C]propionyl-CoA, which binds to the catalytic cysteine. The isolation of the labelled peptide after trypsin treatment and sequencing revealed for the first time the 21-amino-acid sequence of the catalytic domain (Figure 1).

The strategy used to isolate the cDNA for rat mitochondrial HMG-CoA synthase [6] took advantage of the probable high percentage identity of nucleotides in the 5′ regions and low percentage identity of nucleotides in the 3′ regions of the mRNAs of the two HMG-CoA synthases, since the catalytic sites of the two enzymes (in the 5′ region) are nearly identical [4,64]. In addition, antibodies raised against a C-terminal peptide of the cytosolic protein do not recognize the mitochondrial protein [3]. Two restriction fragments of the cytosolic HMG-CoA synthase cDNA located in the 5′ region and the 3′ region were used differentially as probes in the screening of the cDNA library. The full-length transcript for rat mitochondrial HMG-CoA synthase spans 1994 nucleotides. The transcript contains a 49-nucleotide upstream non-coding sequence preceding the first AUG codon, which starts an open reading frame of 1524 bases [65]. Rat mitochondrial HMG-CoA synthase comprises a protein of 508 amino acids, with a molecular mass of 56.9 kDa. The N-terminal end of the primary translation product contains a sequence of 37 amino acids that is absent from the cytosolic enzyme. The amino acid composition of this sequence is similar to the general composition of leader peptides that translocate cytosolically synthesized proteins into mitochondria [66].

Figure 1 shows the protein sequences derived from all cDNAs of mitochondrial HMG-CoA synthases published so far, i.e. from the rat [6], human [67], mouse [68] and pig [69], as well as the 21-amino-acid peptide from chicken [64]. It also shows the consensus sequences for both the mitochondrial and the cytosolic enzymes. Some conclusions can be derived from comparison of the sequences. (i) The mammalian mitochondrial HMG-CoA synthases all contain 508 amino acid residues. (ii) The catalytic site, defined as the 21-amino-acid peptide by Miziorko and Benke [64], is 100% identical in mammals. (iii) The percentage identity between all proteins is about 80%. (iv) All enzymes contain the 37-amino-acid leader peptide sequence, with a sequence identity of 50%. A total of 15 HMG-CoA synthases have been cloned, of which four are mitochondrial and 11 cytosolic: from rat [70], human [71], Syrian hamster [4] and chicken [72], two from yeast [73,74], two from plants [75,76], two from insects [77,78] and one from *Caenorhabditis elegans* [68]. It is noticeable that the two cytosolic HMG-CoA synthases from the cockroach *Blattella germanica* [77,78] and the one from *C. elegans* [68] end at the same position as the mitochondrial synthases, suggesting that, before that the evolution towards vertebrates took place, the ancestral coding region was the same size as that for the cockroach enzyme. Probably, in the evolution of ketogenesis, the ancestral HMG-CoA synthase acquired a leader peptide to transport the enzyme into mitochondria. The cytosolic HMG-CoA synthase in mammals gained an additional peptide of 49 amino acids at the C-terminus, whose function is unknown.

Isolation of mitochondrial HMG-CoA synthase genes

The rat mitochondrial HMG-CoA synthase gene contains 10 exons, spanning 24 kb [7]. Comparison of the size of these exons with those of the rat cytosolic HMG-CoA synthase gene [5] shows that exons 2–8 of the mitochondrial HMG-CoA synthase gene are identical in size to exons 3–9 of the cytosolic HMG-CoA synthase gene. The human mitochondrial HMG-CoA synthase appears to have identical genomic organization to that in the rat [79].

In addition, 1148 bp of the 5′ flanking region [7] of the rat mitochondrial HMG-CoA synthase gene has also been sequenced. Comparison of this 5′ flanking region with the canonical sequences of several *cis* elements shows that several known sequences appear in this 5′ flanking region (Table 1). Transfection studies in HepG2 cells [7] and in transgenic mice [80] suggest multi-hormonal regulation of gene transcription. The occurrence of a nuclear receptor responsive element (NRRE), which binds peroxisome-proliferator-activated receptor (PPAR), chicken ovalbumin upstream promoter transcription factor (COUP-TF) and hepatocyte nuclear factor-4 (HNF-4), has been demonstrated (see below) [11,81,82], in addition to the cAMP regulatory element (CRE) binding site (A. Eckers, C. Caudevilla, G. Asins, F. G. Hegardt and D. Serra, unpublished work). Some of these sequences present in the rat gene are also observed in the

human [80] and pig [83] mitochondrial HMG-CoA synthase gene 5′ flanking regions.

REGULATION OF EXPRESSION OF MITOCHONDRIAL HMG-CoA SYNTHASE IN ADULT MAMMALS

Adult rat liver

As stated in the Introduction section, ketogenesis is stimulated in a number of physiological situations (fasting, prolonged exercise, high-fat diet and the foetal–suckling transition), and also in diabetes. All these conditions also modify the expression of the gene for mitochondrial HMG-CoA synthase [41]. Starvation (24 h) increases not only the mRNA levels (4-fold) but also the amount of HMG-CoA synthase protein, which increases 2-fold [84]. Upon refeeding of 24 h-starved rats, the levels of HMG-CoA synthase mRNA decrease very rapidly and return to the basal level of fed animals within 2.5 h. After an injection of dibutyryl cAMP, the level of mitochondrial HMG-CoA synthase mRNA increases in a time-dependent manner. Injection of fasted rats with insulin produces a sudden decrease in expression, reaching levels similar to those in control fed rats, not only for mRNA but also for protein [41].

Feeding rats with a diet containing 40 % saturated fat produces a net increase in mitochondrial HMG-CoA synthase mRNA and protein levels by a factor of three [41,84], in parallel with the ketogenic process induced by these nutrients. This activation is mediated by PPAR [11] (see below). Dexamethasone also induces an increase in mRNA and protein levels in fasted rats. In fed rats, however, the effect of dexamethasone is very small [41]. These results are similar to those observed on the dominant effect of insulin over dexamethasone for expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene [85].

Rats in which diabetes has been induced by streptozotocin show increased mitochondrial HMG-CoA synthase mRNA levels compared with the basal values found in normal rats [41]. Protein levels also increase, but not as much as the mRNA [84]. Insulin, which is able to counteract the effects on ketone-body concentrations produced by diabetes, also reduces the expression of the mitochondrial HMG-CoA synthase gene when injected into diabetic rats.

Immunofluorescence microscopy of frozen sections of adult rat liver revealed fluorescence inside all hepatocytes, with no evidence of zonation, indicating that ketogenesis may not be limited to specific regions of the rat liver, but is extended to all hepatocytes (Figure 2A). Immunoelectron microscopy using specific antibodies on cryo-ultrathin hepatic sections confirmed the mitochondrial localization of HMG-CoA synthase in hepatocytes (Figure 2B) [86].

Adult rat and mouse intestine

Ketogenesis in the small intestine of suckling rats (see below) ends at weaning. Simultaneously, the expression of mitochondrial HMG-CoA synthase is arrested. In contrast with the small intestine, the colon and caecum are able to synthesize ketone bodies, mostly from the butyrate produced by the colonic bacteria [87]. In parallel, the colon mucosa expresses mitochondrial HMG-CoA synthase, this expression being dependent on the bacterial status of the tissue. This effect is revealed by the low expression of mitochondrial HMG-CoA synthase in germ-free animals, shown not only by the mRNA levels but also by the immunoprecipitable HMG-CoA synthase protein. Similar patterns of expression were observed in mice: levels of mRNA for mitochondrial HMG-CoA synthase are undetectable in the stomach, duodenum, jejunum and ileum. However, appreciable levels are seen in the colon and caecum in adult mice [80].

Adult human tissues

The expression of human mitochondrial HMG-CoA synthase mRNA in several adult tissues has been measured. It shows high values in liver [63,67] and colon, low levels in testis, kidney, heart and skeletal muscle, and very low levels in the pancreas [67]. The colon shows 5–10-fold greater expression than the liver, whereas the expression in testis is only 5–10 % of that in the liver. The significance of the very high expression in adult human colon mucosa might be related, as in the rat, to the occurrence of fermentative processes in the large intestine, some of whose end-products, such as butyric acid and propionic acid, could be taken up by the mucosal cells to produce ketone bodies. The expression in testis is also consistent with that in the rat, in which this expression seems to occur only in hormone-producing gonadic cells [88]. Thus, as suggested for the rat (see below), mitochondrial HMG-CoA synthase in human gonads could also be involved in androgen and progesterone synthesis through cholesterol biosynthesis.

DEVELOPMENTAL PATTERN OF THE EXPRESSION OF MITOCHONDRIAL HMG-CoA SYNTHASE IN SUCKLING ANIMALS

Hepatic mitochondrial HMG-CoA synthase in suckling rats

In rats, the oxidation of fatty acids develops soon after birth in the liver, where they are used as precursors for ketone-body synthesis. The concentration of blood ketone bodies increases during the first 24 h following birth in suckling neonates, not only in rats but in many other mammalian species [89–91]. This produces physiological hyperketonaemia, which is maintained throughout the suckling period [92].

The hepatic activity of mitochondrial HMG-CoA synthase shows a specific developmental pattern in the rat. At 1 day before birth, the foetal activity is low, and this can be decreased by one-half by 72 h starvation of the mother. At birth the foetal activity doubles, and starvation of the mother produces an increase of 50 % [93]. The activity continues increasing after birth and is maximal at postnatal day 15, decreasing thereafter [94–97].

The developmental expression pattern for hepatic mitochondrial HMG-CoA synthase shows that mRNA levels for the rat gene are highest on the third day of life and then decrease progressively up to weaning, at which time mRNA levels are half those of the normal fed adult rat [98]. Weaning of the animals on to a high-fat diet maintains mRNA levels higher than in animals given a high-carbohydrate diet [13], suggesting that nutritional factors may influence the delay in the suppression of mitochondrial HMG-CoA synthase seen in normal weaning. This behaviour is probably due to the high glucagon and low insulin levels observed in rats weaned on to a high-fat diet [99]. PCR analysis has revealed [100] a developmental pattern for the pre-mRNA of mitochondrial HMG-CoA synthase that is nearly identical to that for mature mRNA, suggesting that there is transcriptional control in the regulation of this gene, but no detectable control of the splicing to mature mRNA (Figure 3A). The mitochondrial developmental pattern of HMG-CoA synthase protein levels coincides with those of the mRNA and pre-mRNA.

Surprisingly, identical developmental patterns are observed for CPT I and for mitochondrial HMG-CoA synthase in suckling rats (Figure 3B). In contrast, CPT II mRNA levels remain low, without significant changes thoughout the suckling period. The

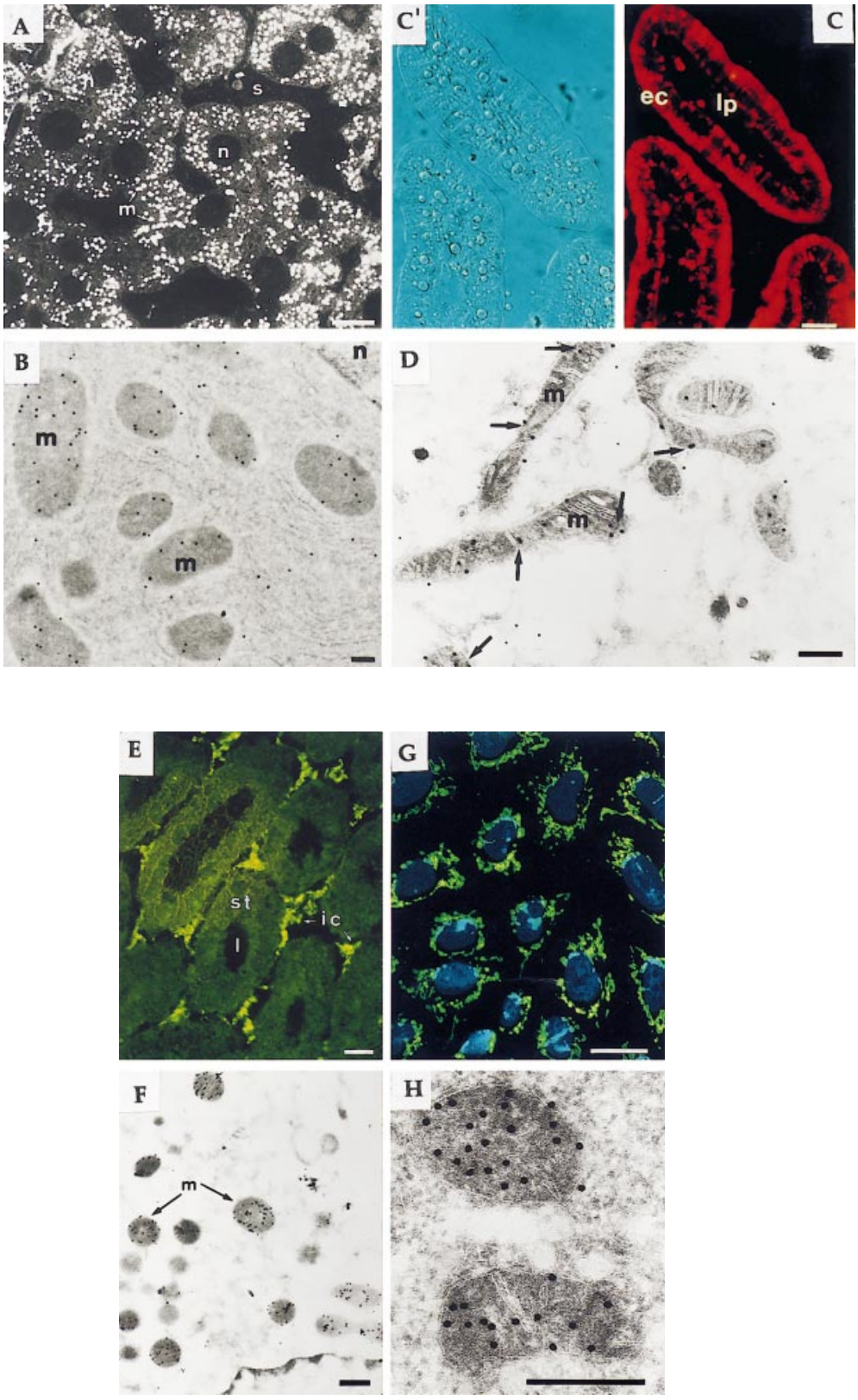

Figure 2 Immunofluorescence localization of mitochondrial HMG-CoA synthase, and immunoelectron localization of mitochondrial HMG-CoA synthase in ultrathin cryosections

(**A**, **C**, **C′**, **E** and **G**) Immunofluorescence localization. (**A**) Thin sections from fasted-rat liver [86]. Bar = 15 μm. Abbreviations: m, mitochondria; n, nucleus; s, sinusoidal space. (**C**) Cryostat section from jejunum of 12-day-old rats using anti-(mitochondrial HMG-CoA synthase) and rhodamine-conjugated goat anti-rabbit [100]. Bar = 25 μm. (**C′**) Phase-contrast micrograph of the same section shown in (**C**). Abbreviations: ec, epithelial cells; lp, lamina propria. (**E**) Immunocytochemical localization of mitochondrial HMG-CoA synthase in frozen sections from rat testis [88]. Bar = 100 nm. Abbreviations: st, seminiferous tubules; ic, interstitial cells. (**G**) Transfected Mev-SM cells [157]. Immunofluorescence was performed using anti-(HMG-CoA synthase)

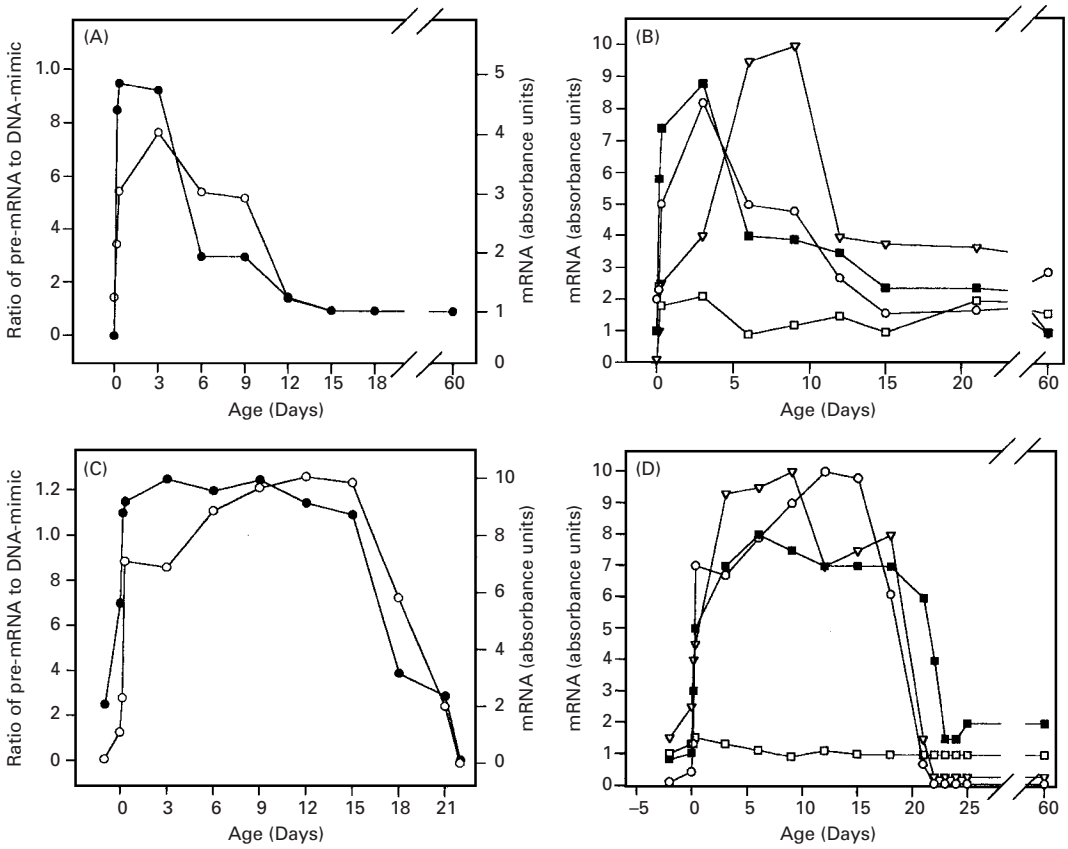

Figure 3 Developmental pattern of gene expression of rat mitochondrial HMG-CoA synthase, CPT I, CPT II and PEPCK

Levels of pre-mRNA (●) and mRNA (○) for mitochondrial HMG-CoA synthase are shown in liver (**A**) and intestine (**C**) from newborn, suckling, weaned and adult rats. Also shown are the mRNA levels in liver (**B**) and intestine (**D**) for PEPCK (▽), CPT I (■) and CPT II (□) from the same animals (○, mitochondrial HMG-CoA synthase). From [98,102].

CPT I protein levels also follow an analogous pattern [101], as do mRNA levels for PEPCK (Figure 3B). The curves of the expression of these three genes also correlate with their mRNA levels [102]. Thus common transcription factors seem to operate in unison in the regulation of gluconeogenesis and ketogenesis in the livers of suckling animals.

Intestinal mitochondrial HMG-CoA synthase in suckling rats

In 1986, Hahn [103] observed that gluconeogenesis occurs in the intestinal mucosa of suckling rats. This suggested that, in infancy, the mucosa behaves to some extent like the liver. Since the same author had observed that, after birth, hepatic ketogenesis rose about 50-fold, he hypothesized that small-intestinal mucosa could also carry out ketogenesis. He found that suckling-rat intestinal mucosa had a high rate of ketogenesis *in vitro*, comparable with that of liver, and that this capacity was not present after weaning [12]. Békési and Williamson [104] observed that mucosa from the small intestine had high mitochondrial HMG-CoA synthase activity, and that the developmental pattern of ketogenesis *in vitro* was similar to that of mitochondrial HMG-CoA synthase activity. Most of the activity is found in the proximal small intestine rather than in the distal region.

Levels of HMG-CoA synthase mRNA in the small intestine of newborn rats are low, but when suckling begins, there is a net increase in gene expression up to day 12. Levels then plateau until day 15, and then decrease progressively throughout the suckling period, becoming zero after weaning (day 21) and remaining so in the adult rat [13,98]. The suckling–weaning transition produces a sharp fall in mitochondrial HMG-CoA synthase expression. The strong correlation between HMG-CoA synthase expression and ketogenesis activity suggests that mitochondrial HMG-CoA synthase is also a major control point of ketogenesis in the intestine of suckling rats, as it is in liver. As observed in the livers of suckling rats, the bell-shaped curve of mRNA levels for mitochondrial HMG-CoA synthase matches the curve for pre-mRNA, suggesting that this gene is

Figure 2 (contd.)

immunoglobulins and fluorescein-isothiocyanate-conjugated pig anti-rabbit immunoglobulins (green stain). Nuclei were stained with bis-benzimide (blue stain). Bar = 20 μm. (**B**, **D**, **F** and **H**) Immunoelectron localization. (**B**) Fasted-rat liver [86]. Bar = 500 nm. (**D**) Rat enterocytes [100]. Colloidal gold particles (arrows) are in mitochondria. Bar = 250 nm. (**F**) Rat Leydig cells [88]; (**H**) SM cells [157] (bar = 500 nm). The methodology involves incubation with anti-(HMG-CoA synthase) antibodies in sections of tissues, followed by Protein A–gold (16 nm) incubation. Abbreviations: m, mitochondria, n, nucleus. Figures taken from the following references, with permission: (**A**, **B**) Royo, Pedragosa, Ayté, Gil-Gomez, Vilaró and Hegardt, J. Cell. Physiol., copyright ©1995, F. Hegardt [86]; (**C**, **C′**, **D**) Serra et al. [100]; (**E**, **F**) Royo et al. [88]; (**G**, **H**) Ortiz et al. [157] copyright © 1994 American Society for Biochemistry and Molecular Biology.

transcriptionally regulated, without significant regulation of the splicing mechanism (Figure 3C) [100]. Equally, there is developmental regulation of mitochondrial HMG-CoA synthase protein in these neonatal rats. Immunocytochemical studies using antibodies against mitochondrial HMG-CoA synthase revealed very intense staining located exclusively in the epithelial cells that line the villi, which correspond to enterocytes (Figure 2C). No difference is seen between the tip and the base of the villi. The immunolabelled cells present a dotted staining pattern that suggests an intramitochondrial localization. Immunoelectron microscopy of cryo-ultrathin sections confirmed that mitochondrial HMG-CoA synthase was located in the mitochondrial matrix of enterocytes (Figure 2D).

The levels of intestinal CPT I [105] and PEPCK [102,106] mRNAs show a pattern that is identical to that for mitochondrial HMG-CoA synthase [105] (Figure 3D). This suggests that these three genes may act in concert at this stage of rat life, that they may have similar DNA regulatory elements in their promoters and that identical transcription factors may regulate gluconeogenesis and ketogenesis co-ordinately in the small intestine of suckling rats.

Kidney mitochondrial HMG-CoA synthase in suckling rats

The developmental pattern of expression of mitochondrial HMG-CoA synthase mRNA in kidney appears to be similar to that in the small intestine, which suggests that this organ may play a role in ketogenesis in the suckling period [13,14]. As in the liver and small intestine, when rats are weaned on to a high-fat diet, expression is increased compared with animals weaned on to a high-carbohydrate diet, suggesting that nutritional factors may modulate the expression of mitochondrial HMG-CoA synthase in the kidneys of suckling rats.

Effects of fasting/refeeding and hormone treatment on the expression of mitochondrial HMG-CoA synthase in suckling rats

Fasting of 12-day-old rats for 24 h leads to decreases in ketogenesis and in mitochondrial HMG-CoA synthase mRNA levels and enzyme activity in the small intestine. Since fasting does not modify the high glucagon/low insulin ratios, this effect is probably due to the absence of fatty acids, which are present in maternal milk [62]. Fasting of 12-day-old rats produces small changes in ketogenesis and in mitochondrial HMG-CoA synthase mRNA levels and activity in the liver [62]. This correlates with unchanged ketogenesis in hepatocytes from fasted rats [107], which is explained [108] by the fact that levels of hepatic cAMP are maximal in suckling animals and are unaffected by fasting. These results are also consistent with the inhibition of esterification in fasted suckling rats [109].

Treatment of suckling rats with insulin produces a decrease in mitochondrial HMG-CoA synthase mRNA levels and activity in the small intestine for 16 h [110]. In the liver, the decreases in mRNA levels and activity observed in the first 2 h of insulin treatment recover thereafter.

Corticosterone also seems to play a role in ketogenesis in suckling rats, as it increases markedly after day 15, and at day 21 and thereafter its levels are maximal. Dexamethasone produces a continuous decrease in mitochondrial HMG-CoA synthase mRNA levels in the small intestine after 4 h [111], which mirrors the decrease in ketogenesis [112]. This may be explained by the concomitant maturation of the intestinal brush border induced by dexamethasone [113–117].

MITOCHONDRIAL HMG-CoA SYNTHASE IN THE PIG AS A MODEL OF A NON-KETOGENIC MAMMAL

Ketogenesis in pigs is different from that in other mammalian species. While in most species the blood ketone bodies increase during the first day after birth, levels of blood ketone bodies remain very low in suckling pigs, despite high concentrations of non-esterified fatty acids in the plasma. This seems to be due to a limited capacity for hepatic fatty acid oxidation, which diverts the acyl-CoA into esterified fats. A major degree of control is probably exerted by mitochondrial HMG-CoA synthase in pigs, because (1) ketogenesis *in vitro* is low despite a postnatal rise in total CPT activity [118–122], (2) an intraperitoneal dose of octanoate administered to fasted piglets (which bypasses the CPT I system in liver) as a ketogenic substrate fails to induce ketosis [123], and (3) levels of mitochondrial HMG-CoA synthase specific activity and immunodetectable protein in 48 h-old unsuckled pigs are negligible [119].

Adult pigs

The low ketogenic capacity of pigs is well known, and this correlates with a low activity of mitochondrial HMG-CoA synthase. The tissue specificity of mitochondrial HMG-CoA synthase in pigs is similar to, but not identical with, that in other mammalian species [69]. While this gene is expressed in the large intestine and liver of adult pigs, it is not expressed in the small intestine, stomach, kidney, ovary or testis. Regulation of expression also seems to differ across tissues, as fasting induces a significant rise in transcript levels in the liver and caecum, but not in the colon.

Suckling pigs

The rate of oleate oxidation is very low in isolated hepatocytes from 48 h-old starved pigs (5–20 % of that found in hepatocytes from starved adult rats) [124]. This does not appear to be due to a limited entry of oleate into the mitochondria, since CPT I and CPT II activities are high. In contrast, the activity of HMG-CoA synthase in the newborn piglet is only 5 % of that in rat mitochondria [125], which is corroborated by an extremely low amount of HMG-CoA synthase protein, strongly suggesing that mitochondrial HMG-CoA synthase controls the ketogenic rate.

Another important difference with respect to rats is that there is very low expression of mitochondrial HMG-CoA synthase in the liver throughout the suckling period, whereas in the rat expression is high. Substantial mRNA levels are observed only after a postnatal lag of 1–2 weeks, a pattern that is very different from the rapid postnatal rise reported for suckling rats [13,98]. On the other hand, 2-week-old piglets show increased mitochondrial HMG-CoA synthase mRNA levels after 24 h starvation, along with increased ketogenesis and increased levels of circulating ketone bodies, an effect that is not seen in suckling rats [62].

A possible control mechanism for mitochondrial HMG-CoA synthase expression in fasted piglets might be trans-activation by fatty acids of the promoter of this gene. It has been shown that plasma levels of non-esterified fatty acids nearly double after 48 h of fasting [126,127], which may induce trans-activation mediated by PPAR in the pig HMG-CoA synthase promoter. A PPRE has been observed in the 5′ upstream region of the gene [83]. The kinetic parameters of pig mitochondrial HMG-CoA synthase are very similar to those of the rat enzyme (C. Sampaio, F. G. Hegardt and P. F. Marrero, unpublished work). Therefore the low activity of HMG-CoA synthase in pigs compared with

rats is probably due neither to the low mRNA abundance nor to intrinsic kinetic properties, but rather to differences in post-transcriptional regulation.

STUDIES OF MITOCHONDRIAL HMG-CoA SYNTHASE IN TRANSGENIC ANIMALS

Two studies have been reported on the properties of mitochondrial HMG-CoA synthase in transgenic mice. One aimed to correlate increased ketogenesis with overexpression of mitochondrial HMG-CoA synthase [128], and the other investigated the characteristics of the promoter of this gene [80]. The PEPCK promoter seems to behave like the HMG-CoA synthase promoter in response to different stimuli, and could direct the expression of mitochondrial HMG-CoA synthase, in a physiologically regulated manner, in the same tissues as the endogenous gene. This conclusion is reached due to the following evidence: (1) the transcription rates of the PEPCK [129,130] and mitochondrial HMG-CoA synthase genes are markedly increased by starvation and diabetes; (2) the transcription of both genes is inactive during foetal development, but greatly enhanced at birth; (3) the two genes show nearly identical, developmentally regulated expression in liver and intestine; and (4) tissue-specific expression is similar. According to this rationale, transgenic mice over-expressing mitochondrial HMG-CoA synthase under the control of the PEPCK promoter [131] were produced [128]. When the expression of the transgene was induced in the liver by starvation, a 3-fold increase in mRNA levels for mitochondrial HMG-CoA synthase was achieved compared with controls, which was correlated with a 3-fold increase in enzyme activity. Serum analysis indicated that levels of ketone bodies were also increased 3-fold. Control and transgenic animals have similar concentrations of serum glucose and triacylglycerols, and both groups have diminished levels of non-esterified fatty acids. Dibutyryl-cAMP and dexamethasone increase the levels of mitochondrial HMG-CoA synthase mRNA in cultured hepatocytes from transgenic mice, which is followed by an increase in ketogenesis. It appears that, in the livers of the transgenic mice, the expression of the transgene (HMG-CoA synthase) controls ketone-body synthesis.

The second study using transgenic animals aimed to examine the expression of the promoter of the mitochondrial HMG-CoA synthase in different tissues. Transgenic mice were produced with a chimaeric gene composed of a 1.15kb portion of the mitochondrial HMG-CoA synthase promoter fused to the human growth hormone (hGH) gene as a reporter gene [80]. In addition to the transgene being expressed in the same tissues as endogenous mitochondrial HMG-CoA synthase, the expression of the hGH gene in these animals is also high in stomach, brown adipose tissue, spleen, adrenal glands and mammary gland. In suckling animals, increased expression of hGH is also seen in brown adipose tissue and heart, while expression of the endogenous gene is absent in these tissues. This is interpreted as showing that the 1.15 kb portion of the mitochondrial HMG-CoA synthase promoter contains the information required for expression of the gene in most tissues, but that a silencer upstream from the 1148 bp portion probably prevents expression in stomach, brown adipose tissue, spleen, adrenal glands and mammary glands in wild-type adult mice. Starvation, refeeding, insulin injection or feeding with different fatty acids in transgenic animals confirmed that the 1.15 kb portion of the promoter contains the elements necessary to modulate the expression of mitochondrial HMG-CoA synthase by cAMP, insulin and fatty acids, as well as developmental expression in the small intestine.

TRANSCRIPTIONAL REGULATION OF THE MITOCHONDRIAL HMG-CoA SYNTHASE GENE

Trans-activation of the promoter in transiently transfected cells

Transfection of two different cell lines, NIH 3T3 and HepG2, with a plasmid containing 1148 bp of the mitochondrial HMG-CoA synthase 5′ flanking region fused to a chloramphenicol acetyltransferase (CAT) reporter gene [7] allowed studies on transcriptional regulation. Deletion of the fragment between positions −1148 and −760 produces an increase in CAT activity. Further deletions up to −142 bp do not affect CAT activity. Deletion of the promoter to −33 bp leads to a large decrease in the rate of transcription of the chimaeric gene, suggesting that critical elements of the promoter are present in the sequence −142 to −33. The decrease in the transcriptional rate may be due to the deletion of *cis* elements, such as Sp1 located at position −49, and the loss of putative binding sites for liver transcription factors, such as PPAR (see below). An increase in CAT activity is observed when HepG2 cells transiently transfected with the chimaeric plasmid are incubated with dibutyryl cAMP plus theophyllin (Figure 4). Dexamethasone and oleate also increase CAT activity. In contrast, 40 nM insulin consistently leads to repression of CAT activity.

The cAMP-induced transcription of the mitochondrial HMG-CoA synthase gene is mediated by the CRE-binding protein (CREB) through both Sp1 (A. Nadal, J. A. Ortiz, C. Mascaró, P. Marrero, F. G. Hegardt and D. Haro, unpublished work) and CREB (A. Eggers, C. Caudevilla, G. Asins, F. G. Hegardt and D. Serra, unpublished work) binding sites in the promoter of the gene (Figure 5). Both sequences are able to confer responsiveness to protein kinase A and CREB to the otherwise unresponsive thymidine kinase promoter.

Effect of methylation status on the transcription rate of mitochondrial HMG-CoA synthase

Another important effector in the transcriptional regulation of the promoter of the mitochondrial HMG-CoA synthase gene is the methylation of specific regions of the promoter [132]. *In vitro* methylation of a plasmid, which includes 1148 bp of the 5′ flanking region of the mitochondrial HMG-CoA synthase gene

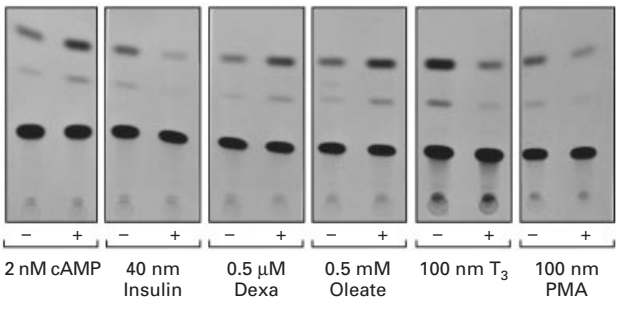

Figure 4 Multi-hormonal regulation of the transfected plasmid pSMPCAT1 in Hep G2 cells

The plasmid pSMPCAT1 contained 1148 bp of the 5′ flanking region of the mitochondrial HMG-CoA synthase gene and the first 28 bp of exon 1 fused to the CAT gene. HepG2 cells were co-transfected with the plasmid and treated with 2 mM dibutyryl cAMP plus 1 mM theophylline (cAMP), 40 nM insulin, 0.5 μM dexamethasone (Dexa), 0.5 mM oleate, 10 nM tri-iodothyronine (T_3) or 100 nM PMA for 48 h after the removal of calcium phosphate/DNA precipitate. Control experiments are also shown (−). From [7], with permission.

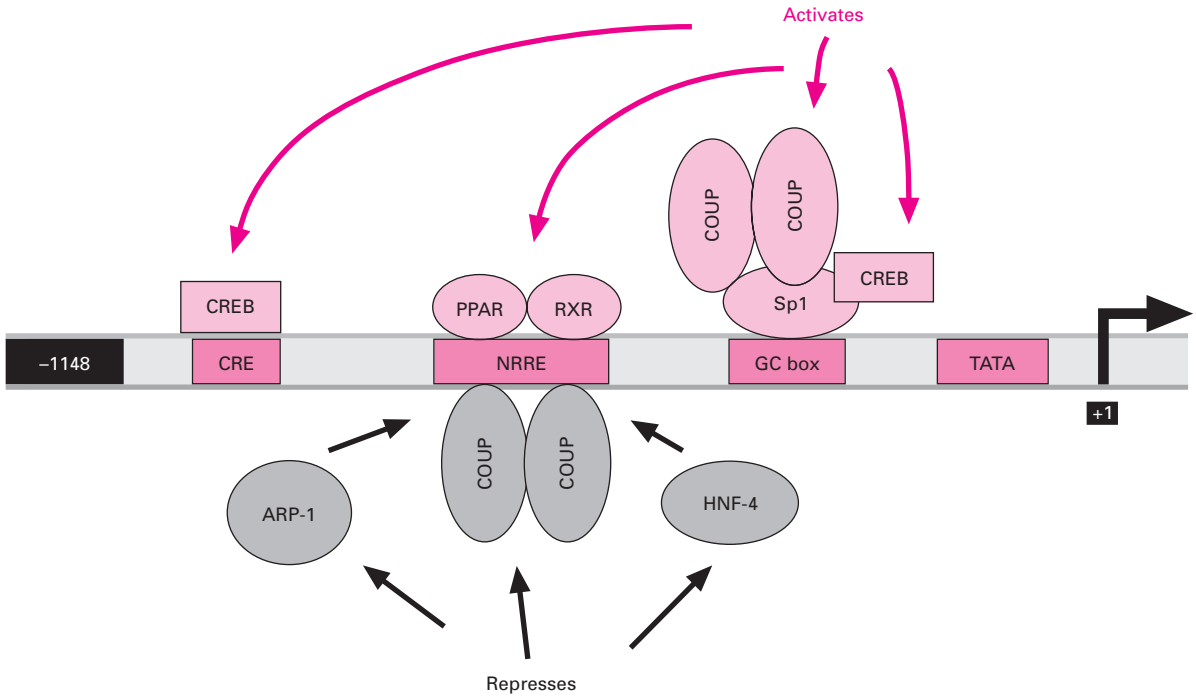

Figure 5 ***Cis*** **elements of the mitochondrial HMG-CoA synthase promoter**

Transcription factors PPAR, RXR, Sp1 and CREB bind to the mitochondrial HMG-CoA synthase promoter at different sites, activating transcription. Other transcription factors, such as HNF-4 and ARP-1, repress transcription by competing with the heterodimer PPAR–RXR at the NRRE site. COUP-TF can either activate or repress transcription, depending on the binding site and the tissue. Abbreviation: ARP-1, apo A1 regulatory protein-1.

fused to the CAT coding sequence, leads to a decrease in CAT activity to a variable degree that depends on the number of CG dinucleotides methylated. Moreover, high expression of the mitochondrial HMG-CoA synthase gene is seen only in tissues in which the gene is hypomethylated. Experiments using genomic DNA from liver (a tissue with high expression) and kidney (a tissue in which the expression is absent in adults) digested with endonucleases sensitive to methylated DNA indicate the presence of methylated CGs in kidney, and their absence in liver.

The methylation status of specific genes changes substantially during the foetal–neonatal transition [133]. Changes in methylation status have also been observed in the neonatal mitochondrial HMG-CoA synthase gene. The gene is heavily methylated 7 days before birth compared with that in control adult liver. Four sites seem to be targets for methylation. One located at −0.8 kb is fully methylated in kidney. The other three are variably methylated in adult liver and foetal liver, depending on the metabolic and developmental conditions. Experiments *in vitro* involving the induction of hypomethylation of DNA in the FTO-2B cell line by azacytidine showed a 3-fold increase in mitochondrial HMG-CoA synthase mRNA levels 48 h after the pulse, confirming the *in vivo* data [132].

Transcriptional regulation by PPAR

Fatty acids induce the transcription of the gene for mitochondrial HMG-CoA synthase both *in vivo* and *in vitro* [7,41]. Transcriptional activation by fatty acids seems to be mediated by a member of the nuclear hormone receptor superfamily, termed PPAR, which is a ligand-activated transcription factor. It is commonly accepted that PPAR can bind to a specific DNA sequence, called the PPRE. Elements of this kind have been located upstream of several genes [134–136]. This activation process is dependent on heterodimer formation between PPAR and the *cis*-retinoid receptor (RXR).

The mitochondrial HMG-CoA synthase gene contains an element that is responsive to PPAR, which mediates the activation by fatty acids (Figure 5) [11]. Co-transfection of constructs formed by progressively longer 5′ deletions of the 5′ flanking region of the synthase promoter fused to the CAT gene together with the PPARα expression factor shows that the nucleotide sequence between positions −104 and −95 comprises a direct repeat (DR)-1 sequence. A short sequence of seven nucleotides located close downstream is necessary for binding of PPAR. Confirmation that the whole sequence is a NRRE comes from three lines of evidence. (1) It confers PPAR responsiveness to the otherwise unresponsive thymidine kinase gene promoter. (2) Mutation by scrambling of the DR-1 sequence obliterates the response to PPAR. (3) Gel mobility shift assays show that, while PPRE-containing DNA results in the formation of the complex with the PPARα protein, which competes with an excess of unlabelled probe, a shift is absent with a scrambled sequence probe. Linoleic acid produces transcriptional activation in the mitochondrial HMG-CoA synthase promoter in the presence of PPAR with the same efficiency as clofibrate. A later study [137] examined 16 NRREs from different genes related to lipid metabolism that are activated by peroxisome proliferators, and reached the interesting conclusion that the strongest NRRE in the binding to the different PPARs (α, β and γ), and also to the different RXR α, β and γ subtypes participating in the active heterodimers, is that corresponding to rat mitochondrial HMG-CoA synthase.

COUP-TF as a potential repressor or activator of the mitochondrial HMG-CoA synthase promoter

COUP-TF is another member of the nuclear hormone receptor superfamily. COUP-TF can stimulate the expression of several genes [138–141], but it usually works as a negative regulator. It inhibits ligand-induced trans-activation by other nuclear receptors (retinoic acid receptor, thyroid hormone receptor, vitamin D receptor, RXR and PPAR). COUP-TF has a dual role in the mitochondrial HMG-CoA synthase promoter in HepG2 cells. First, it activates the transcription of this gene, irrespective of the NRRE locus, by binding to a fragment comprising nucleotides -62 to $+28$ [81] through an interaction with the transcription factor Sp-1, which binds to a GC box present in the promoter (J. C. Rodriguez, F. G. Hegardt and D. Haro, unpublished work). In addition, COUP-TF competes with PPAR for binding to the same NRRE sequence (positions -104 to -92), and this competition leads to inhibition of the transcriptional activation produced by the heterodimer PPAR–RXR (Figure 5). The same results are seen with an artificial construct composed of the thymidine kinase promoter and three copies of the mitochondrial HMG-CoA synthase NRRE. The inhibitory effect of COUP-TF is also seen in rat Leydig tumour R2C cells. It is noteworthy that the activation of COUP-TF seen in HepG2 cells is not seen in Leydig tumour R2C cells, which reveals the specificity of the organ in determining whether either activation or inactivation of transcription of the mitochondrial HMG-CoA synthase occurs. This may be related to the different function of this gene in liver compared with testis (see below).

HNF-4 represses the mitochondrial HMG-CoA synthase gene

HNF-4 is another member of the superfamily of zinc-finger nuclear receptor transcription factors. This factor, in combination with other liver-specific transcription factors, regulates liver-specific gene expression [142–145]: genes of carbohydrate metabolism [146–148], lipid metabolism [149–151], urea biosynthesis [152], blood coagulation and development. HNF-4 represses the mitochondrial HMG-CoA synthase gene in transiently transfected HepG2 cells [82]. This repression seems to be caused by competition between HNF-4 and PPAR in the promoter of mitochondrial HMG-CoA synthase by binding to the same locus that binds PPAR and COUP-TF (Figure 5). This mutual displacement has also been observed in the promoters of other genes [153,154]. PPAR and its antagonists COUP-TF and HNF-4 work as lipostat sensors, which mediate the transcriptional rate of the enzymes related to fatty acid catabolism.

POSSIBLE CHOLESTEROGENIC ROLE OF MITOCHONDRIAL HMG-CoA SYNTHASE IN TESTIS AND OVARY

Expression of mitochondrial HMG-CoA synthase in testis and ovary

Testis and ovary contain high levels of mRNAs for mitochondrial HMG-CoA synthase, similar to those in liver. By using specific antibodies, mitochondrial HMG-CoA synthase protein is also detected in gonadal tissues [88]. Immunocytochemical studies reveal that expression is located in Leydig cells (Figures 2D and 2F), but is absent from Sertoli cells and cells that correspond to the different stages of spermatocyte maturation. In the ovary, the protein is located in theca interna cells of the ovarian follicle, in some corpus luteum cells and the ruptured ovarian follicle, and in the epithelial cells lining the proximal portion of the oviduct. In contrast, label is absent from granulosa cells of the maturing follicle and from mature oocytes.

The high level of expression of the mitochondrial HMG-CoA synthase gene is not related to ketogenesis in these gonadal cells, since when rats are fasted, no increase in mRNAs is observed in gonadal cells, despite the fact that a 4-fold increase is observed in liver. In addition, no ketone-body synthesis is observed in testis or ovary. Hypophysectomized rats show no change in the expression of hepatic mitochondrial HMG-CoA synthase, in contrast with the large decreases observed in testis and ovary. Immunolocalization of expression in hypophysectomized rats shows an marked regression of Leydig cells, with expression being absent. In the ovary, the presence of the HMG-CoA synthase protein is clearly decreased in corpus luteum cells and in epithelial cells lining the proximal part of the oviduct.

The common characteristic of Leydig cells and theca interna cells of the pre-ovulatory follicle is their capacity to synthesize androgens or progesterone respectively from cholesterol. However, in cells in which the later steps of the pathways yield oestrogens from androgens (granulosa cells of the pre-ovulatory follicles and Sertoli cells), the mitochondrial HMG-CoA synthase gene is not expressed. Mitochondrial HMG-CoA synthase could be an important mechanism regulating mitochondrial cholesterogenesis [88], which would be consistent with the observation that amounts of digitonin-precipitable labelled sterols synthesized from [1-^{14}C]acetyl-CoA are strongly correlated with levels of HMG-CoA synthase in Leydig cells [155]. The minimal HMG-CoA lyase activity and the occurrence of HMG-CoA reductase in Leydig cell mitochondria challenge the physiological role attributed to mitochondrial HMG-CoA synthase, which may promote cholesterol biosynthesis.

Mitochondrial HMG-CoA synthase corrects auxotrophy for mevalonate in Mev-1 cells deficient in cytosolic HMG-CoA synthase

Mev-1 cells are mutant Chinese hamster ovary (CHO) cells that are deficient in cytosolic HMG-CoA synthase [156]. Consequently, these cells are autotrophs for mevalonate and cannot survive in the usual media unless 0.4 mM mevalonate is present. The introduction of a construct comprising the cDNA for mitochondrial HMG-CoA synthase subcloned into a eukaryotic expression vector in Mev-1 cells produces (as a result of stable transfection) colonies able to grow in the absence of mevalonate [157]. This suggests that the expression of the mitochondrial HMG-CoA synthase gene confers on the cells the capacity to synthesize mitochondrial HMG-CoA, which can be transformed into cholesterol and also into the mevalonate-derived isoprenoids necessary for cell growth and division. The transfected cells contain the intramitochondrial protein (Figures 2G and 2H) and have the capacity to produce [^{14}C]cholesterol from [^{14}C]acetyl-CoA. The mechanism by which intramitochondrial HMG-CoA is converted into cholesterol is unknown at present. One possibility could be that mitochondria utilize endogenously produced farnesyl pyrophosphate for isoprenoid biosynthesis, and that the biosynthetic steps in mitochondria are regulated independently from those occurring in other subcellular compartments [158]. Another possibility is that a metabolite of HMG-CoA, possibly acetoacetate, crosses the mitochondrial membrane and, after the action of acetoacetyl-CoA synthetase and cytosolic acetoacetyl-CoA thiolase, the resulting acetyl-CoA is incorporated into the cytosolic isoprenoid pathway. It is also possible that the HMG-CoA is converted into 3-hydroxy-3-methylglutaric acid by an HMG-CoA hydrolase; this could pass into the cytosol and, due to the activity of a succinyl-CoA transferase, would again be converted into HMG-CoA that is able to follow the isoprenoid pathway.

INHERITED DISEASES RELATED TO MITOCHONDRIAL HMG-CoA SYNTHASE

Two patients aged 16 months and 6 years have been described in preliminary communications [159,160] with fatty liver and hypoketotic hypoglycaemia and dicarboxylic aciduria in which mitochondrial HMG-CoA synthase activity was low. Patients did not show a rise in ketone bodies after fasting, or following a long-chain-fat load. There was a small rise in ketone bodies after a load of leucine. The absence of immunoreactive material on Western blots of liver homogenates using specific antibodies for mitochondrial HMG-CoA synthase confirmed the disease. No molecular study has been attempted to describe the mutations responsible for these cases.

Another mutation that indirectly affects mitochondrial HMG-CoA synthase is that responsible for the lethal albino mouse phenotype. Lethality occurs 12 h after birth and is due to hypoglycaemia and hypoketonaemia, which results from a failure to activate hormone-dependent genes in liver and kidney encoding enzymes that are important for gluconeogenesis (such as glucose-6-phosphatase, tyrosine aminotransferase and PEPCK) and ketogenesis (such as mitochondrial HMG-CoA synthase) [161,162]. Decreased transcription has been shown to be responsible for these and other enzyme deficiencies. The hypoglycaemic effects cannot be corrected by ketone-body synthesis and utilization, because mitochondrial HMG-CoA synthase is not expressed in these mice. The mutation in the albino mice is a large deletion of 3800 kb in chromosome 7. Inside this, a 310 kb region was discovered which contains the gene for fumarylacetoacetate hydrolase (FAH), which is responsible for this phenotype. Accumulation of fumarylacetoacetate, which cannot be hydrolysed, together with its precursor maleylacetoacetate, is toxic for cells and blocks the expression of transcription factors necessary for the activity of the mentioned genes, among them mitochondrial HMG-CoA synthase. Knockout mice for the FAH gene reproduce the phenotype of the albino mice, and animals die 12 h after birth [163]. Overexpression of the FAH gene in transgenic albino mice rescues the phenotype, most of genes responsible for the phenotype are expressed, and mice survive [164]. This albino mouse is an animal model for human hereditary tyrosinaemia type 1, which is a liver disease of varying severity and clinical prognosis, depending on the degree of FAH deficiency. The mouse and human phenotypes differ in that lethal albino mice die within hours after birth, whereas severe FAH deficiency in humans is tolerated for several months, albeit with severe liver failure, renal tubular disfunction, cardiomyopathy and severe neurological crisis.

EPILOGUE

For more than 25 years, many scientists have worked on the elucidation of the regulation of ketogenesis at the level of mitochondrial HMG-CoA synthase. Thanks to the tools of molecular biology, it has been possible to reveal the primary structure of the enzyme, the composition of the gene and its promoter, and the *cis* elements that might regulate its transcriptional rate by the effect of nutrients (particularly fatty acids) and pancreatic hormones. While these advances have given new insights into the control of ketogenesis, some questions constitute major challenges for the future. These include the following. What are the molecular mechanisms that switch the gene off in the gut of suckling rats at weaning, while the hepatic gene remains open to metabolic signals? What are the signals in the promoter that maintain the gene fully activated in the colon and caecum in most adult mammals? Does cAMP trans-activate the mitochondrial HMG-CoA synthase promoter at different sites depending on the tissue? Why does the pig (a non-ketogenic animal) regulate ketogenesis by post-transcriptional control, while in the rat the mechanism is mainly transcriptional? What is the exact involvement of mitochondrial HMG-CoA synthase in cholesterogenesis in gonadal cells? Does the promoter of the gene contain elements that modulate the tissue-specific cholesterogenic response? How common is the hereditary disease of mitochondrial HMG-CoA synthase deficiency and what is its phenotype? Thus the next few years promise to be at least as exciting (and rewarding) in the pursuit of these questions as those of the pioneers were.

Note added in proof (received 5 February 1999)

After submission of this Review, an article was published by L. M. Meertens et al. [165] in which it was shown that human mitochondrial HMG-CoA synthase protein physically interacts with PPARα *in vitro*. Experiments *in vivo* also show that mitochondrial HMG-CoA synthase protein is translocated into the nucleus and potentiates PPARα-dependent transcriptional activation of the mitochondrial HMG-CoA synthase gene via the PPAR regulatory element. Binding to PPARα, stimulation of PPARα activity and nuclear translocation require the integrity of the sequence LXXLL in the protein. These findings suggest a novel mechanism of gene regulation by which the product of the mitochondrial (but not the cytosolic) HMG-CoA synthase gene directly interacts with the PPRE to autoregulate its own nuclear transcription.

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