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Tissue-specific Expression and Dietary Regulation of Chimeric Mitochondrial 3-Hydroxy-3-methylglutaryl Coenzyme A Synthase/Human Growth Hormone Gene in Transgenic Mice*

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Dolors Serra‡, Cristina Fillat§, Raquel Matas‡, Fatima Bosch§, and Fausto G. Hegardt‡¶

From the ‡Unit of Biochemistry, University of Barcelona, School of Pharmacy, 08028 Barcelona, Spain and §Department of Biochemistry and Molecular Biology, School of Veterinary Medicine, Bellaterra, Spain

We have studied the role of the mitochondrial 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthase gene in regulating ketogenesis. The gene exhibits expression in various tissues and it is regulated in a tissue-specific manner. To investigate the underlying mechanisms of this expression, we linked a 1148-base-pair portion of the mitochondrial HMG-CoA synthase promoter to the human growth hormone (hGH) gene and analyzed the expression of the hGH reporter gene in transgenic mice. mRNA levels for hGH were observed in liver, testis, ovary, stomach, colon, cecum, brown adipose tissue, spleen, adrenal glands, and mammary glands from adult mice, and also in liver and stomach, duodenum, jejunum, brown adipose tissue, and heart of suckling mice. There was no expression either in kidney or in any other nonketogenic tissue. The comparison between these data and those of the endogenous mitochondrial HMG-CoA synthase gene suggests that the 1148 base pairs of the promoter contain the elements necessary for expression in liver and testis, but an enhancer is necessary for full expression in intestine of suckling animals and that a silencer prevents expression in stomach, brown adipose tissue, spleen, adrenal glands, and mammary glands in wild type adult mice. In starvation, transgenic mice showed higher expression in liver than did wild type. Both refeeding and insulin injection reduced the expression. Fat diets, composed in each case of different fatty acids, produced similar expression levels, respectively, to those found in wild type animals, suggesting that long-, medium-, and short-chain fatty acids may exert a positive influence on the transcription rate in this 1148-base-pair portion of the promoter. The ketogenic capacity of liver and the blood ketone body levels were equal in transgenic mice and in nontransgenic mice.

Ketogenesis in mitochondria is mainly controlled by two enzymes: carnitine palmitoyl transferase I and mitochondrial HMG-CoA[1] synthase. The activity of the latter increases with fasting, fat feeding, diabetes, glucagon administration, and with the transition from the fetal to the suckling state (1, 2), and these effects are accompanied by an increase in the mRNA levels for this gene (3). The close correspondence between the increase in mRNA levels, enzyme activity, and ketogenesis has also been observed in the intestine and liver of suckling pups (4, 5), which reinforces the hypothesis that it is the main regulatory point in ketogenesis, both in adult and neonatal rats. Mitochondrial HMG-CoA synthase is also expressed in specialized cells of testis and ovary, although it appears that the function in these gonadal tissues is not concerned with ketogenesis but with the gonadal function in the synthesis of sexual hormones (6).

The long term goal of our research is to understand the molecular mechanisms underlying mitochondrial HMG-CoA synthase gene expression, its tissue-restricted distribution, and its accurate hormonal control. The potential regulatory properties of the 1148 bp of the 5′ sequence have been studied. This promoter is sufficient to direct the tissue-specific expression of a reporter gene *in vitro*, as shown by transient chloramphenicol acetyltransferase expression assays in hepatoma cells (7). In addition a promoter fragment located at −104 bp has very recently been shown to contain a *cis*-responsive element that activates transcription by fatty acids and clofibrate through the peroxisome proliferator activated receptor in transfected Hep G2 cells (8). However, the various cells cultured *in vitro* are imperfect models of the situation *in vivo*, and these models do not reveal the influence of the chromatin structure on gene expression, or the hormonal response. Therefore, the investigation of the promoter elements that regulate the expression of the mitochondrial HMG-CoA synthase gene in transgenic mice is of interest.

Transgenic animal technology is a powerful tool with which to study the contribution of regulatory genes to the control of metabolic pathways. We have studied the expression in transgenic mice of human growth hormone in different tissues under the control of a 1148-bp portion of the proximal promoter of mitochondrial HMG-CoA synthase. Our results show that adult transgenic animals express the reporter gene in liver, testis, ovary, colon, and cecum as in wild type mice, although quantitative differences in mRNA levels were observed. It is also expressed in stomach, brown adipose tissue, spleen, adrenal glands, and mammary glands at variance with the natural promoter, from which it is concluded that a silencer may be located outside the −1148 bp. Suckling transgenic mice also express the reporter gene in different parts of the thin intestine, confirming previous data on expression in wild type mice. In addition, we have also studied, in the transgenic animal, the influence of fasting-refeeding processes, of feeding with a fat diet, and of insulin on the expression. Results confirm previous studies in rat liver.

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¶ To whom correspondence and reprint requests should be addressed: Unidad de Bioquímica, Facultad de Farmacia, Diagonal 643, 08028 Barcelona, Spain. Tel.: 34-3-402-4523; Fax: 34-3-402-1896; E-mail: hegardt@far.ub.es.

[1] The abbreviations used are: HMG, 3-hydroxy-3-methylglutaryl; hGH, human growth hormone; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); PEPCK, P-enolpyruvate carboxykinase.

EXPERIMENTAL PROCEDURES

Construction of the Mitochondrial HMG-CoA Synthase/Human Growth Hormone (mtHMG-CoA synthase/hGH) Chimeric Gene and Generation of Transgenic Mice—The *Hin*dIII-*Bam*HI fragment of plasmid pΦGH (Nichols Institute, San Juan Capistrano, CA), which encompasses the entire hGH structural gene was introduced at the *Hin*dIII-*Bam*HI sites of the Bluescript polylinker. The construction of mtHMG-CoA synthase/hGH fusion gene was begun by inserting a 1.2-kb *Cla*I restriction fragment containing nucleotides −1148 to +28 of the rat mtHMG-CoA synthase gene (7) into *Cla*I digested pBShGH. The proper orientation of the recombinant plasmid produced (pMShGH) was tested by digestion of restriction enzymes.

Production and Maintenance of Transgenic Mice—The purified 3.2-kb *Xho*I-*Eco*RI fragment from pMShGH (Fig. 1*A*) was microinjected into fertilized eggs. The general procedures for microinjection of the mtHMG-CoAS/hGH chimeric gene were as described elsewhere (9). Fertilized mouse eggs were flushed from the oviducts of superovulated C57bl56/SJL mice 6–8 h after ovulation. Male pronuclei from the fertilized eggs were injected with 2 pl of DNA solution (approximately 2 ng/μl), and viable embryos were reimplanted in the oviducts of pseudopregnant mice. Founder mice were mated to C57bl56/SJL animals to establish pedigrees that were heterozygous for the transgene. Animals were maintained under a strictly controlled light-dark cycle of 12 h (lights on at 8:00 a.m.). Other conditions of housing and feeding were standard.

Characterization of the Transgenic Mice—At either 9 days or 3 weeks of age the animals were tested for the transgene by PCR amplification of DNA from tail samples. Small sections of the tail from potentially transgenic mice were added to an extraction buffer containing 100 mg/ml proteinase K, 0.5% SDS, 0.1 M NaCl, 50 mM Tris, pH 7.5, and 1 mM EDTA, and then incubated overnight at 55 °C. RNase T was added to a final concentration of 10 units/ml, and the samples were incubated for 1 h at 37 °C. After RNase treatment, the DNA was extracted with a mixture which contained equal volumes of phenol and chloroform alone, extracted with an equal volume of chloroform, and then ethanol precipitated.

The transgene was identified with primers that yield a fragment of 1095 bp (upstream primer from HMG-CoA synthase gene 5′-CTCACTCAGCGTGTGCTCATCTGCCTGC; downstream primer from hGH gene, 5′-GGGCTACATAGGAAGAACGGGGATTGCAGG). PCRs were typically carried out as follows. A standard 100-μl mixture contained 1 μg of DNA from tail samples, 10 μl of 10 × PCR buffer (Mg^{2+}-free), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1.25 units of *Taq* polymerase (Life Technologies, Inc.), and 50 pmol of each primer. PCR was performed for 30 cycles. Each cycle consisted of denaturation at 94 °C for 1 min, primer annealing at 65 °C for 1 min, and primer extension at 72 °C for 90 s. Ten microliters of the PCR sample were electrophoresed in a 1% agarose gel.

The transgene copy number was subsequently determined by Southern blot analysis of the tail DNA prepared from F_1 mice. 10 μg of DNA were digested with *Pvu*II, electrophoresed in 1% agarose, and transferred to Hybond-C membrane (Amersham Corp.) in 10 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate). The DNA was fixed to the membrane at 80 °C for 2 h. The membrane was prehybridized in buffer containing 0.7 M NaCl, 40 mM NaH_2PO_4, pH 7.6, 0.4 mM EDTA, 0.2% poly(vinylpyrrolidone), 0.2% Ficoll, 0.1% SDS, and 0.2 mg/ml salmon sperm DNA and hybridized in the same solution plus 9% (w/v) dextran sulfate and the ^{32}P-labeled 1.4-kb *Pvu*II fragment from pMShGH (2×10^6 cpm/ml). Washes were performed in 0.2 × SSC, 0.1% SDS at 68 °C. The intensity of the signal generated from genomic DNA was measured by densitometric scanning of the autoradiograms in a Bioprofil (Vilber-Lourmat) photodensitometer and compared with the intensity of signal produced from a known amount of a 1.4-kb *Pvu*II fragment from pMShGH DNA.

Source of Tissues, RNA Extraction, and Analysis—Transgenic mice and normal littermates were killed at 12 noon by cervical dislocation. The gastrointestinal tract was removed entirely from the esophageal-gastric junction to the rectum and subsequently subdivided into stomach, duodenum, jejunum, ileum, cecum, and colon. The brown adipose tissue was taken from the suprascapular region. The white adipose tissue, liver, testicle, ovary, kidney, heart, lung, brain, muscle, spleen, and adrenal glands were also recovered for RNA hybridization. The mammary glands were taken from 6–8-month-old rats and 1 day after delivery.

All tissues were rapidly frozen in liquid nitrogen, then powdered, and total cellular RNA was extracted with guanidine isothiocyanate and then purified by centrifugation through a CsCl cushion (10). Aliquots of 15 μg were fractionated on 1% agarose gel containing formaldehyde, and subjected to Northern transfer on NY13 nytran filter (Schleicher & Schuell). Filters were fixed at 80 °C at 254 nm for 2 h. After 6 h of prehybridation using high stringency conditions (at 42 °C in 1 M NaCl, 50% formamide, 7.5 × Denhardt's solution, 0.1% SDS, 50 mM NaH_2PO_4, pH 6.3, 10% dextran sulfate, and heat denatured salmon sperm DNA, 0.2 mg/ml), the filters were hybridized overnight using the random-primed ^{32}P-labeled 150-bp partial cDNA probe (see below). The radioactivity was 2×10^6 cpm/ml. Filters were washed briefly at 42 °C in 300 mM NaCl, 30 mM sodium citrate, pH 7.0, and 1% SDS, followed by three 20-min washes at 65 °C in 30 mM NaCl, 3 mM sodium citrate, pH 7, and 0.1% SDS. Filters were autoradiographed at −70 °C in contact with Kodak x-ray film with an intensifying screen.

The levels of mRNA were determined by densitometric scanning of the autoradiograms in a Bioprofil (Vilber-Lourmat) photodensitometer. Densitometry values were corrected by using a fragment from the cDNA clone (pRSA13) for rat serum albumin as a constitutive probe (11). Filters were dehybridized for 30 min at 100 °C with the same washing system and then rehybridized. Statistical analysis was carried out by Student's t test with significance levels chosen as $p < 0.005$ and $p < 0.01$.

Use of cDNA Probes—The following cDNA fragments were used as probes: a 150-bp *Bgl*II-*Pvu*II fragment from exon V of the hGH and a specific 1.5-kb *Kpn*I fragment corresponding to the cDNA for rat mitochondrial HMG-CoA synthase (12).

Determination of Human Growth Hormone in the Serum—A radioimmunoassay was developed to determine the concentration of hGH in the serum of the transgenic mice. Blood samples were collected from a cardiac puncture and hGH levels were determined in 50-μl aliquots of serum using inmunoradiometric assay (BioMérieux, France) according to the manufacturer's protocol.

Determination of the Ketogenic Capacity of Liver and of Serum Ketone Bodies—The blood was collected by capillarity from the eye. The animals were then killed by cervical dislocation, and the liver was removed and cut into small slices for the determination of the rate of liver ketogenesis as in Williamson *et al*. (13) and Bekesi and Williamson (14). Serum ketone bodies were determined following Williamson *et al.* (13).

RESULTS AND DISCUSSION

Generation of the mtHMG-CoA Synthase/hGH Transgenic Mice—The mtHMG-CoA synthase/hGH chimeric gene used to obtain transgenic mice is shown in Fig. 1*A*. Previously to the microinjection in oocytes of mice, this construct was assayed in Hep G2 and Chinese hamster ovary cells in transient transfection experiments (data not shown). Subsequently, this chimeric gene was introduced into the mouse genome to create the transgenic mice. Of 106 mice initially screened for the presence of the transgene, one was positive for integration.

The copy number of the transgene in the positive mouse was determined by Southern blot analysis (see "Materials and Methods"). The founder and the first generation offspring of the founder indicated that the mtHMG-CoA synthase/hGH gene was integrated in one or two copies/haploid genome (Fig. 1*B*). Matings between two heterozygous animals which were offspring of the founder female resulted in the death of the total progeny within a few hours of birth. It was observed that many pups were eaten by their mothers, in a much greater proportion than usual. Neonatal mortality in the transgenics differed from that in the nontransgenic animals, and there were no adults deaths.

Unexpected Phenotype of the mtHMG-CoA Synthase/hGH Transgenic Mice—Pups (whether transgenic or nontransgenic) suckling from transgenic females were observed to be smaller throughout the suckling period than pups of the same age suckling from nontransgenic mothers. At 3 weeks of age (weaning), pups weighed an average of one third as much as control pups of the same age (see Table I). This phenotype was not observed when pups (transgenic or nontransgenic) came from transgenic males, but were suckling from nontransgenic females. 10 days after weaning the small mice doubled their own weight. Moreover, all transgenic and nontransgenic mice equalized their weight at the age of 9 months (data not shown).

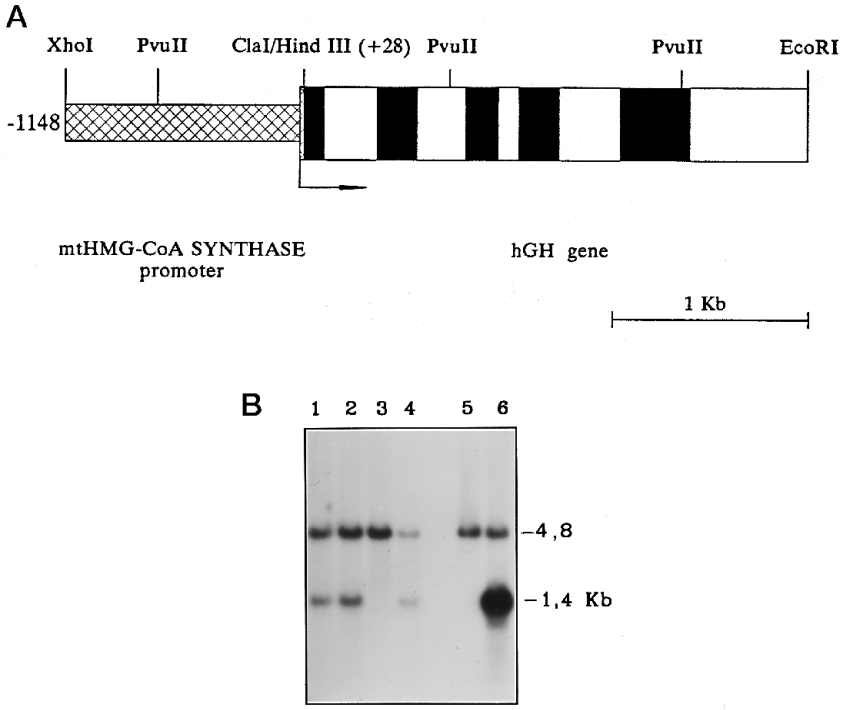

FIG. 1. **Mitochondrial HMG-CoA synthase/human growth hormone (mtHMG-CoAS/hGH) chimeric gene used to create transgenic mice and genomic characterization of transgenic founders.** *A*, structure of the mtHMG-CoAS/hGH fusion construct in which DNA derived from mtHMG-CoA synthase gene is indicated by *hatched areas*, and hGH sequences are indicated by *solid boxes* (exons) and *open boxes* (introns). The transcription initiating site and direction of transcription from the mtHMG-CoA synthase promoter are indicated by the *arrow*. *B*, Southern blot of genomic DNA isolated from each of the transgenic F_1 mice and from a nontransgenic mouse. Genomic DNA (10 μg) was digested with *Pvu*II restriction enzyme. The 1.4-kb fragment was produced by the restriction of *Pvu*II at the site of hGH gene and the site of mtHMG-CoAS promoter inserted in the genome in a head-to-tail manner. Control and transgenic mice showed a 4.8-kb fragment, coming from the *Pvu*II restriction of the endogenous mtHMG-CoAS gene, which hybridized with the 1.4-kb *Pvu*II probe. *Lanes 1*, *2*, *3*, and *4* are F_1 mice. *Lane 5* is a control. *Lane 6* is a control DNA plus 10 copies.

TABLE I

Comparison of the weights of transgenic mice with nontransgenic mice of the same age

Weights of animals (in g) were determined at different ages suckling milk from either transgenic females or nontransgenic females. Pups whether transgenic or nontransgenic suckling from the transgenic mother weighed, on average, the same.

	21 days	31 days	3 months
Transgenic female			
♂ $n = 40$	7.0 ± 1.4	17.7 ± 1.1	20.7 ± 1.0
♀ $n = 48$	6.9 ± 0.5	15.0 ± 0.9	15.7 ± 0.7
Nontransgenic female			
♂ $n = 36$	22.3 ± 0.6	nd	27.3 ± 0.8
♀ $n = 42$	19.7 ± 0.5	nd	21.3 ± 0.4

This phenotype is the opposite of the increased somatic growth generally associated with use of the hGH as a reporter gene. Jetton *et al*. (15) also observed low weight in transgenic mice produced by the chimeric construct glucokinase promoter/human growth hormone, but no explanation was offered.

Histological evaluation of the mammary fat pads from 6–8-month-old transgenic animals killed 1 day after delivery revealed moderate epithelial hyperplasias when compared to nontransgenic lactating animals. These hyperplasias resembled in some extend to previously described by Webster *et al.* (16) in transgenic mice expressing a murine mammary tumor virus promoter/activated c-*src* fusion gene. The alveolar lumen and mammary ducts of the transgenic lactating rats presented an accumulation of particulate material and reduced lipid content. According to Bernirschke *et al.* (17) this type of lesion could be considered as preneoplastic in mice, and furthermore most mammary carcinomas are preceded by these lesions. However, our transgenic animals did not develop grossly detectable mammary tumors. It is worthwhile, however, to remark that in one of four transgenic lactating rats examined, we observed a large inflammation area within the mammary fat pad. These lesions were probably produced by the high expression of hGH (see Fig. 2); suckling mice were rejected by their mothers, probably as a results of the pain induced. Indeed, transgenic mothers were seen to eat their pups in a far greater proportion than usual. This interpretation is confirmed by the finding that pups from transgenic males but suckling from a nontransgenic female gained weight as control mice of the same age. Recently Cecim *et al.* (18) reported that mammary tumors are produced in transgenic mice with high serum levels of hGH in which the chimeric genes were either PEPCK/hGH or metallothionein/hGH.

Analysis of Mitochondrial HMG-CoA Synthase/hGH Gene Expression in Different Tissues of Adult and Suckling Transgenic Mice—In order to determine the tissue specificity of expression of mtHMG-CoA synthase/hGH in these animals, the level of RNA in various tissues was analyzed (Fig. 2). The mature RNA transcript of the mtHMG-CoA synthase/hGH gene was 817 bp, whereas the mRNA for the mitochondrial HMG-CoA synthase was 2.0 kb. As indicated in Fig. 2, *Panel A*, the mRNA for hGH is expressed in liver, colon, cecum, testis, and ovary, as expected. There was no expression in tissues such as kidney, small intestine, muscle, white adipose tissue, heart, lung, and brain. It was surprising, however, to find an intense band of expression of the transgene in stomach, brown adipose tissue, spleen, adrenal glands, and mammary glands at variance with what it is commonly observed for mitochondrial HMG-CoA synthase in these tissues in adult animals, *i.e.* the absence of expression.

The results obtained in this study, which is the first to assay the mitochondrial HMG-CoA synthase promoter in transgenic mice, provide evidence that from the comparison between the expression of hGH and of the endogenous mitochondrial HMG-CoA synthase either in transgenic or in nontransgenic animals (data not shown), five different patterns of expression can be seen in adult mice: 1) positive, similar expression, such as in ovary; 2) negative expression, such as in duodenum, jejunum, ileum, white adipose tissue, heart, lung, brain and skeletal muscle; 3) far superior expression in hGH than in the endogenous gene, such as in testis; 4) lower expression but still appreciable levels in hGH, such as in liver, cecum, and colon, in comparison to mitochondrial HMG-CoA synthase; and 5) appreciably high mRNA levels for hGH and no expression at all for the HMG-CoA synthase, such as in stomach, brown adipose tissue, spleen, adrenal glands, and mammary glands.

We conclude from these results that the 1148-bp fragment

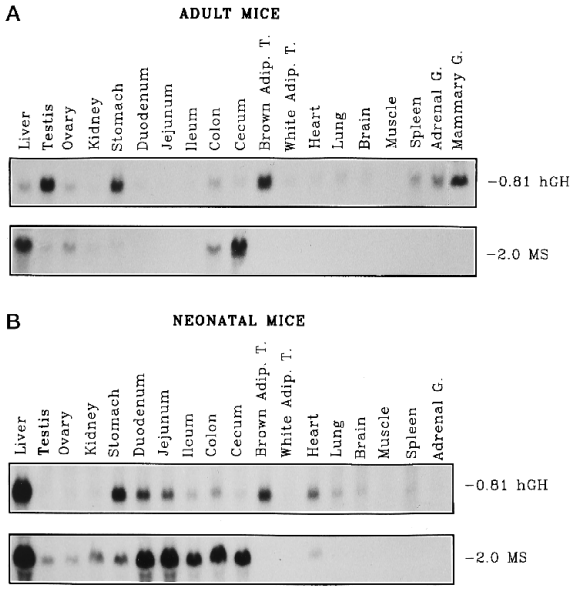

FIG. 2. **Expression of the mthGH-CoAS/hGH chimeric gene.** The expression of the chimeric gene was analyzed by Northern blot from RNA isolated from different tissues indicated under "Materials and Methods." *A*, samples obtained from four adult transgenic mice were pooled, and a representative result is presented. This also applied to the testis and ovary obtained from four males and females, respectively. *B*, samples obtained from four 12-day-old transgenic mice were pooled and analyzed.

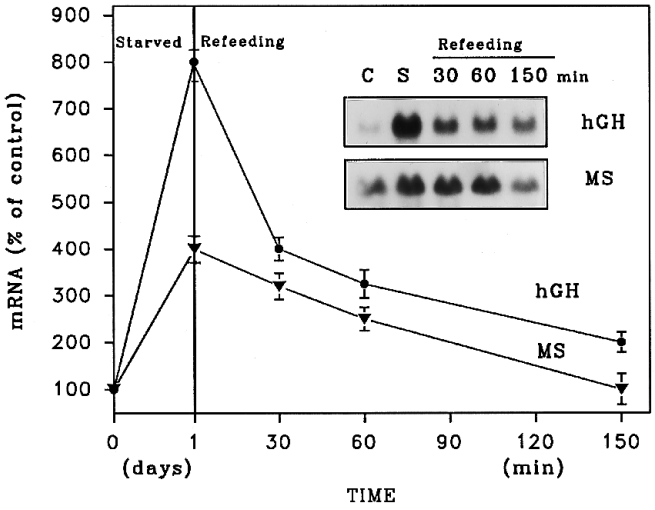

FIG. 3. **Regulation of the expression of mtHMG-CoAS/hGH in fasting/feeding conditions**. Mice were starved for 24 h and then refed for up to 150 min. At the indicated time mice were decapitated, and their livers quickly removed and frozen in liquid nitrogen. Total RNA was isolated and subjected to electrophoresis and to Northern transfer. Data are expressed as a percentage of the mRNA signal of control animals fed a standard diet (means and S.E. of four mice in each group). A representative Northern blot is shown in the *inset*.

contains the *cis*-acting sequences which are required for specific expression in liver. This suggests that an enhancer outside the −1148-bp sequence stimulates the transcription rate in this tissue, since in its absence the mRNA levels are decreased. Studies of several other mammalian genes in transgenic mice have revealed that regulatory elements in the proximal promoter are sufficient to confer liver-specific expression, although maximal expression requires a distal DNA sequence that enhances the expression. For example, this arrangement is found for the gene that encodes transthyretin, where 300 bp of the 5′-flanking region sequence alone results in relatively low levels of expression in the liver, and high levels of expression require the presence of an enhancer found 2 kb upstream (19). The same occurs in albumin and α-fetoprotein genes, where both promoters contain proximal elements that direct low levels of expression in the liver, but high expression levels in the liver are controlled by distinct liver-specific enhancer elements that are located farther upstream from each gene (20, 21). The distal liver-specific enhancer for the albumin gene is located 10 kb upstream of its promoter. For the α-fetoprotein gene, three enhancer elements located between 1 and 7 kb upstream of the transcription start site are required for proper levels of tissue expression in transgenic mice.

The expression pattern seen in testis suggests that outside of the −1148 bp of mitochondrial HMG-CoA synthase promoter there must be negative sequences that partially overcome the expression in this tissue. A similar higher expression in testis in comparison to control mice was also observed by Short *et al.* (22) with the transgene PEPCK/hGH. We did not find any different expression between hGH and HMG-CoA synthase in ovary.

The results in cecum and colon suggest that an enhancer, outside the −1148-bp sequence, stimulates the transcription rate in these tissues, since in its absence the mRNA levels are decreased. Studies of fatty acid binding protein expression in colon and cecum of transgenic mice whose reporter gene was also human growth hormone show that the tissue specific *cis*-elements that promote the expression in these tissues are localized to small DNA sequences, between −1600 and +21 bp. The absence of these sequences strongly modifies the mRNA pattern (23). Analogous results were also observed with intestinal fatty acid binding protein in the sequences comprised between −1178 and +28 (24).

An unexpected result was found: there was an intense hGH expression in brown adipose tissue, stomach, and mammary gland of adult mice, but not so intense in adrenal glands and spleen, at variance with what happens in the adult endogenous gene. The *cis*-acting sequences responsible for the regulation of mitochondrial HMG-CoA synthase expression in stomach, brown adipose tissue, and mammary gland appear to be present in the proximal 1148 bp of the promoter of mitochondrial HMG-CoA synthase. A similar expression in stomach in transgenic but not in control mice was observed by Short *et al.* (22) with the transgene PEPCK/hGH. It appears that some elements, which may repress the transcription, are not present in the 1148 bp of the promoter. On the other hand, if the absence of expression in other tissues, such as muscle heart, brain, lung, kidney, white adipose tissue, and the different parts of the gut is produced under the same mechanism, *i.e.* the occurrence of regulatory sequences, we could affirm that the *cis*-element of other nonexpressed tissues is probably different from those responsible for transcription in brown adipose tissue, stomach, adrenal glands, spleen, and mammary gland.

Analogous experiments of expression were carried out in neonatal mice. As seen in Fig. 2, *Panel B*, in addition to the results seen in adult mice, we can observe bands corresponding to duodenum, jejunum, ileum, and kidney tissues, as expected (4). It was noteworthy, however, that the intensity of the mRNA bands for hGH in small intestine were much lower than that of the endogenous mitochondrial HMG-CoA synthase, suggesting that a possible enhancer or *cis*-acting element for intestinal expression is not present in this promoter fragment. We have also observed that the mRNA levels for hGH in liver are the same than in the mitochondrial HMG-CoA synthase endogenous gene, at variance with what happens in adult transgenic mice. This could be interpreted as a partial repression of the gene, produced in transgenic animals after weaning. Brown adipose tissue was also only expressed in transgenic

mice as happens in adult.

Regulation of the Expression of mtHMG-CoA Synthase/hGH in Fasting/Feeding Conditions—Marked alterations in the level of expression of the chimeric mtHMGS/hGH gene in these animals were caused by dietary changes. The dietary conditions chosen led to changes in plasma ketone body concentrations in rat (3). Some transgenic and nontransgenic animals were fasted for 24 h and then placed on a chow diet. mRNA levels for hGH increased 8-fold after the 24 h of fasting (Fig. 3). The fact that mitochondrial HMG-CoA synthase mRNA levels in control animals increased less than 4-fold, suggests that the influence of glucagon (or cAMP) in this promoter may be mediated by a repressor which is not present within the 1148 bp. Other possibilities such as the influence of the site where the transgene has been inserted in the mouse genome cannot be ignored. Upon refeeding of fasted animals, the levels of hGH mRNA levels decreased very rapidly, so that mRNA values nearly reached the control values of fed animals in 2.5 h. The increase observed in mRNA levels of mouse mitochondrial HMG-CoA synthase followed a similar pattern to that observed in rat (3).

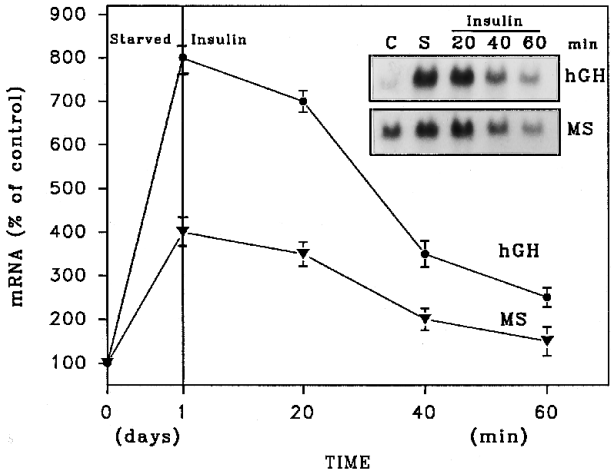

FIG. 4. **Regulation of the expression of mtHMG-CoAS/hGH by insulin.** Starved mice (24 h) were injected intraperitoneally with insulin (40 units/kg), and the specific mRNA levels corresponding to different times of action of hormone were determined. A representative Northern blot is shown in the *inset*.

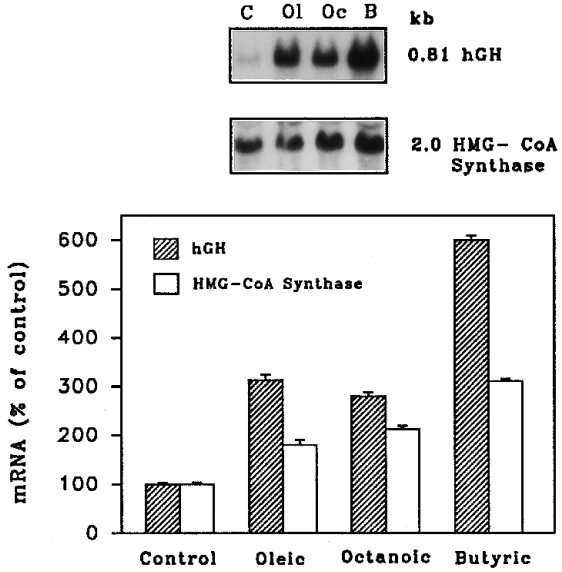

FIG. 5. **Effect of fatty acids in the expression of mtHMG-CoAS/hGH.** Three mice were fed with different fatty acid diet for 5 days. A representative Northern blot is shown. *C*, control animals; *O*, oleate (5% w/w); *Oc*, octanoate (8% w/w); *B*, butyrate (5% w/w).

Regulation of the Transgene Expression by Insulin—We then aimed to test whether insulin could also control the expression of the chimeric gene. Given that the level of expression of the mitochondrial HMG-CoA synthase gene is low in control fed animals, we performed this series of experiments with starved transgenic and nontransgenic mice. Rapid acting insulin was injected intraperitoneally into 24-h starved mice and the levels of specific mRNA hGH were measured at the times indicated. As shown in Fig. 4 insulin caused a time-dependent decrease in mRNA levels; this effect was already observed 20 min after the injection of insulin, and after 1 h the mRNA for hGH decreased nearly to basal levels. Insulin was shown to act in transgenic mice in a similar fashion to the wild type animals. In addition, the mitochondrial HMG-CoA synthase mRNA levels in control mice followed a similar pattern, as previously seen in rats (3).

Regulation of the Transgene Expression by Fatty Acids of Different Length—A group of transgenic and control mice were fed with diets enriched respectively in either oleic acid, octanoic acid or butyric acid (8% w/w) and animals were killed after 5 days. As shown in Fig. 5 the increase in expression produced in the promoter of mitochondrial HMG-CoA synthase by fatty acids, such as oleic, octanoic and butyric acid was similar to that found in rats (3). Although butyric acid promotes the expression of the chimeric gene more strongly than the long- and medium-chain fatty acid, a clear expression can be observed in the three treatments, suggesting that a similar *cis*-element present in the 1148 bp promoter is used for transcriptional activation of either long-, medium-, or short-chain fatty acids. We have shown the occurrence, at −104 bp of the promoter of mitochondrial HMG-CoA synthase, of a *cis*-element (peroxisome proliferator responsive element), which interacts with the peroxisome proliferator activator receptor, which in turn mediates the activation of long-chain fatty acids (8).

Ketone Body Production of Transgenic Mice by Starvation, Refeeding, and Fat Feeding—Groups of four animals under different nutritional conditions were also analyzed for their ketogenic rate in liver and also by their serum content of acetoacetate. Results are seen in Table II. It can be concluded that the transgene did not modify the changes in liver ketogenesis with respect to control nontransgenic mice. In a similar manner, the serum levels of total ketone bodies were not mod-

TABLE II

Hepatic rate of ketogenesis of transgenic and control mice treated under different conditions

Data are presented as the mean ± SE (n = 4 for each group).

Treatment	Control		Transgenic	
	Acetoacetate	3-Hydroxybutyrate	Acetoacetate	3-Hydroxybutyrate
		μmol/h/g		
Control	0.49 ± 0.05	8.28 ± 0.82	0.41 ± 0.04	8.51 ± 0.91
Fasted (24 h)	1.21 ± 0.30	15.62 ± 1.79	1.11 ± 0.32	15.32 ± 2.13
Fasted-refed	0.48 ± 0.06	9.21 ± 0.76	0.52 ± 0.07	10.12 ± 0.36
Fasted-insulin	0.81 ± 0.07	13.80 ± 1.51	0.78 ± 0.08	11.90 ± 0.08
Oleate	0.82 ± 0.08	11.91 ± 1.53	0.80 ± 0.05	10.81 ± 1.21

TABLE III
Analysis of total ketone bodies in serum of mice treated under different conditions

Data are presented as the mean ± SE (n = 4 for each group).

Treatment	Serum ketone bodies	
	Control	Transgenic
	μmol/ml	
Control	0.13 ± 0.08	0.12 ± 0.09
Fasted (24 h)	1.08 ± 0.06	0.98 ± 0.08
Fasted-refed	0.16 ± 0.04	0.17 ± 0.05
Fasted-insulin	0.46 ± 0.09	0.52 ± 0.05
Oleate	0.49 ± 0.10	0.51 ± 0.11

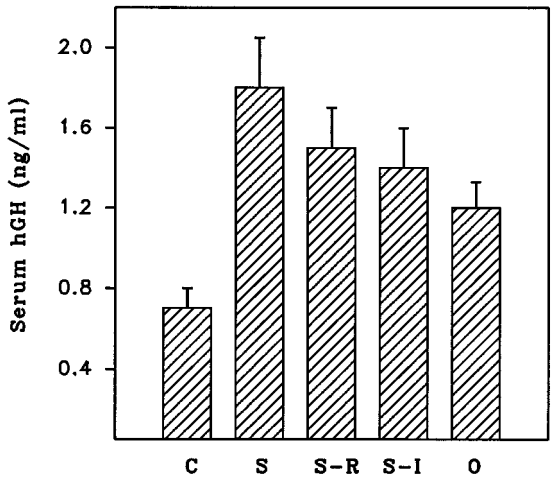

FIG. 6. **Serum hGH concentrations under different treatments.** Serum hGH concentrations were determined as indicated under "Material and Methods." *C*, control; *S*, starved; *S-R*, starved and refed; *S-I*, starved and insulin-treated; *O*, oleic acid-treated. Results are means plus S.E. of four animals in each group.

ified by the occurrence of the transgene (Table III).

Serum Levels of Human Growth Hormone in Transgenic Mice—The hGH levels were determined in transgenic mice under different nutritional conditions. As seen in Fig. 6 there was an appreciable increase in hGH levels upon 24 h of starvation (2.5-fold), which decreased slightly on refeeding or after insulin injection, because the hGH was sufficiently stable after 1 h of either treatment. Oleate also increased the hGH levels. These results agree with the half lives of hGH mRNA levels, which is 2.5 h (25).

In conclusion, the use of transgenic mice has allowed us to extend our knowledge of an important regulatory site in ketogenesis. These results opened the possibility to elucidate the functional characteristics of the mitochondrial HMG-CoA synthase promoter outside 1148 bp and how this gene may modulate ketogenesis in liver and other tissues.

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