Methylation of the regulatory region of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene leads to its transcriptional inactivation

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The mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase gene is expressed in a limited set of tissues in the adult rat. Methylation of the 5' flanking region of the gene *in vitro* leads to its transcriptional inactivation when transfected in hepatoma-derived cell lines. In liver and kidney, expression of the gene correlates inversely with its degree of methylation, indicating that the methylation of the 5' flanking region and the first exon of the gene may be one of the factors responsible for the repression of its transcription. During the fetal/neonatal

transition, a process of selective undermethylation of specific sites takes place in the 5' flanking region of the mitochondrial HMG-CoA synthase gene. Moreover, treatment with the hypomethylating agent 5-azacytidine of a hepatoma-derived cell line that presents barely detectable levels of mitochondrial HMG-CoA synthase mRNA leads to a significant increase in the mRNA levels. These results point to methylation as one of the regulatory mechanisms that operate on the mitochondrial HMG-CoA synthase gene.

INTRODUCTION

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (EC 4.1.3.5) catalyses the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA and CoA. Two isoforms of the enzyme have been reported to be located in different subcellular compartments: cytoplasm and mitochondria. The cytosolic form produces HMG-CoA that is converted into mevalonate through the action of microsomal HMG-CoA reductase, which is the starting point of the isoprenoid biosynthetic pathway [1]. A second pathway, which takes place in the mitochondria, converts HMG-CoA into acetoacetate and then into other related compounds through the ketogenic pathway. Despite its location, mitochondrial HMG-CoA synthase is encoded by a single-copy nuclear gene [2].

Carnitine palmitoyltransferase I and mitochondrial HMG-CoA synthase have been described as rate-limiting steps in ketogenesis [3,4]. Total activity and rate of synthesis of active HMG-CoA synthase protein, as well as mRNA levels, are controlled by a number of hormones, including glucagon acting via cyclic AMP and insulin [5–7].

Transcriptional control of gene expression involves the interaction of a large number of cellular *trans*-acting factors with *cis*-acting DNA sequences. Temporal and tissue-specific regulation of gene expression depends on the activity of various DNA-binding proteins, which may be modulated by a variety of cellular signals, as well as by the structure and local configuration of the gene (for a review see [8]). Modification of DNA structure by DNA methylation may have an important role in controlling the interactions between DNA and the *trans*-acting factors, resulting in either activation or repression of gene expression in animal cells [9].

Here we present evidence that DNA methylation influences mitochondrial HMG-CoA synthase gene expression, and is related to both the tissue-specific expression of the gene and its loss of expression in cultured cell lines.

MATERIALS AND METHODS

Animals

Fed male Sprague–Dawley rats (180–200 g) were subjected to a 12 h light/12 h dark cycle (light periods starting at 08:00 h). The animals were killed by decapitation and the livers were removed and quickly freeze-clamped in liquid nitrogen.

Methylation *in vitro*

The bacterial methyltransferases *HpaII* and *SssI* were used to methylate pSMCAT1 [10], which contains 1149 pb of the 5' flanking region and 28 bp of the first exon of the rat mitochondrial HMG-CoA synthase gene fused to the chloramphenicol acetyltransferase (CAT) coding region of pCAT Basic plasmid (Promega). Plasmid DNA was incubated with 1.0 unit of *HpaII* methyltransferase or *SssI* methyltransferase/ μ g of DNA at 37 °C for 48 h, to ensure complete methylation. To test for complete methylation, the DNA was cut with *HpaII* and *MspI* restriction endonucleases and analysed by agarose-gel electrophoresis. Only samples that were completely methylated were used in transfection experiments.

Cell culture and transfection

HepG2 cells were grown to confluence in minimal essential medium (MEM) with non-essential amino acids and Earle's salts containing 5% (v/v) newborn calf serum, 5% (v/v) fetal calf serum and antibiotics. Transient transfection experiments were carried out by the calcium phosphate method as described [11,12]. It has been reported that methylation does not affect transfection efficiency [13].

β -Galactosidase assay

Cell lysates were obtained by the freeze–thaw method [11], and

Abbreviations used: CAT, chloramphenicol acetyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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Figure 1 Restriction map of the 5' flanking, exon 1 and 5' boundary of intron 1 of the rat mitochondrial HMG-CoA synthase gene

The box denotes exon 1 of the gene. The probe used in Southern-blot experiments and the situation of the 35 CpG dinucleotides in the 2.1 kb *HindIII* genomic fragment sequenced are shown. These are potential sites for CpG methylation. The position of the TATA box and Sp1 and CTF/NF1 recognition sites are also indicated.

10–50 μ l of the cell lysate was assayed for β -galactosidase activity as described [12].

CAT assay

CAT was assayed as described [11]; the products were separated by t.l.c. and autoradiographed. All the samples assayed for CAT activity were first heated to 65 $^{\circ}$ C for 10 min and then centrifuged (13000 rev./min) to inactivate the acetylases present in crude samples.

RNA analysis

Total RNA from FTO-2B cells was isolated as described [14]. RNA samples were fractionated on 1.2% agarose/formaldehyde gels, transferred and u.v.-cross-linked to Nytran-N membranes (Schleicher and Schuell). Hybridization and washes were carried out as described [15]. To detect the mitochondrial HMG-CoA synthase mRNA, the 0.5 kb *SacI* fragment [2] was used as a probe. Northern blots were normalized after quantification of the RNA loaded by scanning the ethidium-bromide-stained gel. A 1.8 kb *XhoI* fragment of mouse cDNA [16] was used in GATA-1 hybridizations. GATA-1, a transcription factor expressed exclusively in haematopoietic organs such as fetal liver, was used as a control for fetal rat liver RNA.

Southern-blot analysis

Total DNA from Sprague-Dawley rat liver or kidney was purified as described [17]. Digested DNA was transferred to Hybond C extra membranes (Amersham) in $10 \times$ SSC ($1 \times$ SSC = 0.15 M NaCl/0.015 M sodium citrate). The membranes were 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 sulphate. Washes were performed in $0.2 \times$ SSC/0.5% SDS at 68 $^{\circ}$ C.

RESULTS

Methylation of the 5' flanking region of rat mitochondrial HMG-CoA synthase gene *in vitro* leads to its transcriptional inactivation

Our specific aim was to determine whether the degree of methylation of the mitochondrial HMG-CoA synthase gene could be related to its transcriptional inactivation. We chose the first exon of the gene and its 5' flanking region for study, as this region contains most of the transcriptional regulatory mechanisms of the gene [10]. Figure 1 shows the restriction map of a 7.7 kb *EcoRI* genomic fragment which includes 6.5 kb of the rat mitochondrial HMG-CoA synthase gene 5' flanking region, its first exon and 1.0 kb of the first intron [10]. The complete sequence of a 2.1 kb region revealed the existence of 35 CpG dinucleotides, which could be targets for the methylation of the gene (Figure 1).

To study the effect of the methylation on the transcriptional rate of the gene, we performed a complete *in vitro* methylation of a plasmid which includes 1149 bp of the 5' flanking region and the first 28 bp of exon 1 of the mitochondrial HMG-CoA synthase gene fused to the CAT coding sequence [10]. The plasmid construct, pSMCAT1, was methylated with *HpaII* and *SssI* methyltransferases. The DNA was then tested for completeness of methylation by digestion with *HpaII* and *MspI* restriction endonucleases (results not shown). In all cases, only DNA that was completely methylated was used in transfection experiments. Transcription activity in HepG2 cells was reduced by a factor of 2.5 and 10.0 by *HpaII* and *SssI* methyltransferases respectively (Figure 2).

Rat mitochondrial HMG-CoA synthase gene is expressed in tissues in which it is hypomethylated

The rat mitochondrial HMG-CoA synthase gene is tissue-specific, being expressed only in liver, testis and ovary [18]. We chose liver as a prototype of a tissue that expresses this gene and kidney as a negative control, as neither mitochondrial HMG-CoA synthase mRNA nor protein is detectable in this tissue [2,18]. We

Figure 2 Transient transfection of *HpaII*- and *SssI*-methyltransferase-methylated pSMPCAT1 plasmid into HepG2 cells

(a) pSMPCAT1 plasmid was methylated *in vitro* with *HpaII* (lanes H1 and H2) and *SssI* methyltransferases (lanes S1 and S2) and transiently transfected into HepG2 cells. Control experiments (lanes C1, C2, C3 and C4) were carried out by transfecting plasmids treated as in the experimental conditions, but no methyltransferase was added. Blank lanes (B1 and B2) correspond to CAT assay negative controls. Cellular extracts were assayed 48 h after removal of the DNA/calcium phosphate coprecipitate. The pRSV β gal plasmid was co-transfected with pSMPCAT1 (2.5 μ g of each plasmid). Transfection efficiency was normalized by the β -galactosidase activity of the cell extract. (b) CAT activity levels compared with the non-methylated control plasmid. Lane *HpaII* M, mean of the relative CAT activity of experiments performed with pSMPCAT1 methylated with *HpaII* methyltransferase. *SssI*, mean of the relative CAT activity of experiments performed with pSMPCAT1 methylated with *SssI* methyltransferase.

performed Southern-blot analysis of rat liver and kidney genomic DNA which were digested with either *MspI* or *HpaII* plus *EcoRI* and *CfoI* endonucleases. As a probe, we used a 1.2 kb fragment (see Figure 1) which encompasses 1149 bp of the proximal 5' flanking region and 28 bp of the first exon of the mitochondrial HMG-CoA synthase gene. *HpaII*, a methylation-sensitive enzyme, produced similar restriction patterns for liver and kidney DNA, although some larger bands appeared in the kidney sample, indicating the presence of methylated CpGs (Figure 3). The small fraction of larger bands detected could represent highly methylated mitochondrial HMG-CoA synthase sequences derived from non-parenchymatous liver cells, as occurs in the case of phosphoenolpyruvate carboxykinase [19]. A somewhat clearer result was obtained from *CfoI*-digested genomic DNA, in which a more slowly-migrating high-molecular-mass band was detected in the case of kidney DNA, a reflection of hypermethylation of the region.

Hypomethylation of the mitochondrial HMG-CoA synthase promoter and the first exon is linked to the expression of the gene

It is well known that many cell-type-specific functions are lost in stable cell lines as a result of high methylation of the CpG islands associated with the genes [20]. In four hepatoma-derived lines so far tested in our laboratory, the mitochondrial HMG-CoA

Figure 3 Southern-blot analysis of genomic DNA from liver and kidney digested with methylation-sensitive restriction enzymes

Liver (lanes L) and kidney (lanes K) genomic DNA was digested with *MspI* or *HpaII* plus *EcoRI* or *CfoI* and electrophoresed in a 1.2% agarose gel. The molecular sizes (kb) of the main hybridization bands are shown. The probe used in the genomic Southern blot is shown in Figure 1. T and P bands represent total and partial digestion fragments respectively (see Figure 6). E band is the fragment between the *EcoRI* ends of the plasmid shown in Figure 6.

Figure 4 Methylation status of the mitochondrial HMG-CoA synthase gene in FTO-2B cells

FTO-2B rat hepatoma cells were treated with 3 μ M 5-azacytidine for 24 h, after which they were washed and fed with fresh medium. Cells were harvested at 0, 24, 48 and 72 h after exposure to 5-azacytidine and their RNA was purified. Mitochondrial HMG-CoA synthase mRNA levels were estimated by scanning the autoradiograph, and normalized after quantification of the RNA loaded in the Northern blot (approx. 20 μ g) by scanning the ethidium bromide-stained gel.

Figure 5 Expression of the mitochondrial HMG-CoA synthase gene during the fetal/neonatal transition in the rat and Southern-blot analysis of liver genomic *HpaII* sites of its regulatory region during this period

(a) Northern-blot was performed with RNA obtained from fetal liver or liver from newborn rats at the time points indicated. d, days; h, hours; Ad, adult rat (3 months). As a control, the filter was rehybridized with a GATA-1 probe [16]. (b) Liver genomic DNA from fetuses 7 days before birth was digested with *MspI* or *HpaII* plus *EcoRI* and analysed by Southern blot. Lanes L, genomic DNA from adult liver (3 months); lane -7, genomic DNA from fetal liver 7 days before birth. The molecular sizes (kb) of the main hybridization bands are shown. The probe used in the genomic Southern blot is described in Figure 1.

synthase gene is expressed at much lower levels than in rat liver. CHO-K1 and R2C cell lines, derived from ovary and Leydig cells respectively, show a similar level of expression to that of the hepatoma cell lines (results not shown). We therefore indirectly tested the methylation status of the mitochondrial HMG-CoA synthase gene in the hepatoma-derived FTO-2B cell line. Cultured cells are a good system for the study of the effects of 5-azacytidine, which has been shown to promote hypomethylation of DNA [21]. Treatment of FTO-2B cells with 5-azacytidine resulted in a 3.2-fold increase in mitochondrial HMG-CoA synthase mRNA levels 48 h after the pulse (Figure 4a).

Changes in the methylation status of the mitochondrial HMG-CoA synthase gene occur during the fetal/neonatal transition in the rat

To study the expression of the mitochondrial HMG-CoA synthase gene during the fetal/neonatal transition in the rat, we

measured its mRNA levels in liver by Northern-blot experiments (Figure 5). The gene is not expressed until at least 1 day before birth, correlating with the low β -oxidation process and ketogenic capacity of the fetal liver [22,23]. In order to study the methylation pattern of the gene during these changes, we carried out Southern-blot experiments with rat liver genomic DNA from 14-day fetuses and compared the results with data from adult liver (Figure 5b). The *HpaII* plus *EcoRI* digestions indicate that the gene is heavily methylated 7 days before birth, showing slow-migrating bands (E, P4 and P8) with respect to the control adult liver. All these data suggest that the mitochondrial HMG-CoA synthase gene is methylated before birth, correlating with its low transcriptional activity.

DISCUSSION

Methylation of DNA has an important role in the transcriptional inactivation of specific genes. As a first step towards the study of the effect of methylation of mitochondrial HMG-CoA synthase promoter on its transcriptional activity, we have mimicked its methylation status *in vivo* by methylation *in vitro* of a plasmid containing 1149 bp of the 5' flanking region [10]. The aim of this experiment was to study whether the methylation of specific sites on the promoter has any effect on transcriptional activity. When transfected into HepG2 cells, the *HpaII*-methyltransferase-treated plasmid showed a 2.5-fold decrease in CAT activity compared with the unmethylated plasmid (Figure 2). The existence of only two *HpaII* sites in the promoter region tested could explain the residual transcriptional activity observed. To increase the methylation status of the plasmid, we performed similar experiments with *SssI* methyltransferase, which methylates all the cytosine nucleotides of the CpG sequences. This new pattern of methylation may closely mimic the distribution of the methyl groups *in vivo*, as, in mammalian DNA, only the cytosine nucleotides present in the CpG sequences are methylated by DNA methyltransferases [24]. This treatment led to a further fall in the transcriptional activity of pSMPCAT1 in HepG2 cells, to 10% of the non-methylated control (Figure 2). The effect of methylation in the regulatory region of the genes could be a consequence of both a three-dimensional change in the structure of the chromatin and an interference in the binding of critical transcription factors to the promoter region [14,24].

Mitochondrial HMG-CoA synthase is a tissue-specific enzyme, which is expressed only in liver, testis and ovary. As its physiological role in gonads does not appear to be linked to ketogenesis [18], the liver may be the main ketogenic tissue. As a preliminary approach to understanding the features that determine the tissue-specificity of the gene, we tested whether structural modification of the gene, i.e. methylation, could be related to its transcriptional activity. In gonads, the gene is only expressed in steroid-producer cells, which are less abundant [18]. Thus studies on the methylation level of the gene would be difficult to interpret because of the great number of cell types present in the whole testis or ovary. For this reason, we chose liver as a prototype of a tissue that expresses the mitochondrial HMG-CoA synthase gene. As a negative control, we used kidney in which neither detectable mitochondrial HMG-CoA synthase mRNA nor protein is present [2,18]. Southern-blot analysis of rat genomic DNA digested with *EcoRI* and either *MspI* or *HpaII* (in order to obtain DNA fragments that could easily be resolved and analysed by agarose-gel electrophoresis) or separately with *CfoI* showed that the promoter of the gene seems to have a greater degree of methylation in kidney than in liver (Figure 3). The original genomic clone that we studied did not contain the M1 *HpaII* site (Figures 1 and 6). Nevertheless, when an *MspI*

Figure 6 Map of the products of total and partial digestions of the *HpaII* sites in the 7.7 kb *EcoRI* fragment of mitochondrial HMG-CoA synthase gene and scheme of the methylation status of the region

The molecular size of each total (T) or partial (P) digestion fragment is indicated. The box denotes exon 1 of the rat mitochondrial HMG-CoA synthase gene and the arrow indicates the transcriptional initiation site [2]. M1–M4 indicate the *HpaII* (*MspI*) restriction sites and E indicates the *EcoRI* ends of the fragment. The solid circle denotes methylation of an *HpaII* site. The empty circle indicates non-methylated sites and the half-solid circles represent sites of variable degree of methylation.

plus *EcoRI* digestion of rat liver DNA was hybridized with an exon 1-specific probe, only a 1.5 kb fragment was detected (results not shown). We assigned this to the M1–E fragment (Figure 6), demonstrating the existence of the M1 site. These results and the Southern blot shown here consistently demonstrated its existence in the rat genome. We believe that this could be due to a random mutation during cloning and replication of the genomic clones in *Escherichia coli*.

Polymorphism at the M2 site may allow the detection of T1 and P1 as two bands of equivalent intensity in samples from heterozygous animals. In Southern blots of *EcoRI* plus *MspI*-digested DNA of FTO-2B cells, only the P1 band is detected (results not shown). This could reflect the monoclonal origin of the FTO-2B hepatoma cells and may confirm the polymorphism of the M2 site.

The *CfoI* analysis of liver and kidney DNA clearly shows the larger molecular size of the hybridization band in the kidney sample, presumably a result of a higher degree of methylation than in the liver. Nevertheless, of the four *HpaII* sites used for the analysis, only M1 is located within the promoter/mRNA leader region, the methylation state of which is known to be critical [25]. Therefore *HpaII*/*MspI* analysis of this region may not be sufficient to uncover the relevant methylation status of the regulatory region of the mitochondrial HMG-CoA synthase gene.

Methylation of HTF (*HpaII* tiny fragments) islands has an important role in the inactivation and consequent loss of expression of some tissue-specific genes when a cell line is derived from the original tissue that expressed the committed gene [20]. We performed Northern-blot analysis on several hepatoma, ovary- and testis-derived cell lines in order to find a line that expressed the mitochondrial HMG-CoA synthase gene, but none

of them expressed levels comparable with those of the rat liver (results not shown).

The methylation status of specific genes changes substantially during the fetal/neonatal transition. The adaptation of the newborn to extra-uterine conditions requires great metabolic changes that lead to changes in the expression of specific genes. Many of these genes have been shown to be switched on and off by methylation [26]. Analysis of the developmental expression pattern of the mitochondrial HMG-CoA synthase in the rat showed that, before birth, its mRNA is not detectable in the fetal liver, correlating with data concerning the very low capacity for long-chain fatty acid oxidation and ketone body production in fetal liver [22,23]. Approximately 1 day before birth, the levels of mitochondrial HMG-CoA synthase mRNA rise (Figure 5a). These results correlate with data from mitochondrial HMG-CoA synthase protein amounts and activity found by Quant et al. [27]. Southern-blot experiments on genomic DNA obtained from fetuses 7 days before birth showed slow-migrating bands (E, P2 and P4 bands) when digested with *EcoRI* and *HpaII* and compared with an adult liver sample (Figure 5b). These bands can be explained by *HpaII* partially digested fragments of the *EcoRI* genomic fragment (Figure 6). These results suggest that methylation of the promoter and 5' regulatory region is one of the factors that lead to developmental regulation, although hypomethylation of the gene may not be the only requirement for the transcription of the mitochondrial HMG-CoA synthase gene. Methylation of the rat mitochondrial HMG-CoA synthase gene seems to be one of the factors responsible for its transcriptional activation and inactivation, as an adaptive response to the new metabolic conditions in the liver.

In order to support a cause-and-effect relationship between methylation and expression, we treated FTO-2B cells with 5-

azacytidine, a compound that is incorporated into newly synthesized DNA, inhibiting DNA methyltransferase and resulting in hypomethylation of DNA and consequent activation of some committed genes [21]. Treatment of FTO-2B cells with 3 μ M 5-azacytidine for 24 h led to a 3.2-fold increase in the levels of mitochondrial HMG-CoA synthase mRNA 48 h later (Figure 4).

All these experiments point to methylation as a regulatory mechanism of the transcription levels of the mitochondrial HMG-CoA synthase gene, being one of the factors that contribute to its tissue specificity and developmental regulation.

We thank Dr. Diego Haro, Dr. Mariela Gómez-García and Dr. Ying-Peng Chen for helpful discussions. We also thank Mr. Robin Rycroft for correcting the English manuscript. This work was supported by Grant PB89-267 from the Dirección General de Investigación Científica y Técnica, Spain. G.G.-G. is recipient of a predoctoral fellowship from the Generalitat de Catalunya.

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