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CpG islands around exon 1 in the succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene are hypomethylated even in human and mouse hepatic tissues where SCOT gene expression is completely suppressed

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Abstract. In ketone body metabolism, hepatocyte-specific silencing of the succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene appears to be physiologically important to avoid a futile cycle in the liver, whereas the SCOT gene is expressed in extrahepatic tissues. It is not possible to explain hepatocyte-specific silencing by cis-elements in the 2.2-kb 5' flanking region. The molecular basis of this gene silencing is unknown thus far. In the present study, the methylation status of CpG islands around exon 1 in the SCOT gene was analyzed by sodium bisulfite treatment and by sequencing of genomic DNA from the HepG2, Chang liver and HeLa human cell lines, and also from mouse liver, heart and kidney cells. Most CpG dinucleotides in the CpG island of the human SCOT promoter region were not methylated in the DNA of HeLa and Chang cells, while HepG2 DNA was hypomethylated in this CpG island. CpG dinucleotides in the mouse SCOT CpG island were almost completely unmethylated in the liver DNA as well as in the heart and kidney DNA. CpG islands around the promoter region of the SCOT gene were hypomethylated in the DNA from both human HepG2 cells and mouse liver. Hence, methylation status does not contribute to hepatocyte-specific SCOT gene silencing.

Introduction

Ketone bodies are important vectors of energy transfer from the liver to extrahepatic tissues, especially when glucose is in short supply (1). Succinyl-CoA:3-ketoacid CoA transferase (SCOT; EC2.8.3.5; locus symbol OXCT) catalyzes the rate-determining step of ketone body utilization (ketolysis) in extrahepatic tissues. SCOT protein is abundant in the heart, brain and kidney, and has been detected in all extrahepatic tissues tested (2). In extrahepatic tissues, SCOT-activated acetoacetyl-CoA is cleaved to acetyl-CoA by mitochondrial acetoacetyl-CoA thiolase (T2). Acetyl-CoA is then converted to fuel via the Krebs cycle.

SCOT mRNA expression is almost completely suppressed in the human liver, the site of most ketone body synthesis (2). This can be viewed as a mechanism to avoid futile cycling. On the other hand, T2 is abundant in the liver, where it is also involved in ketogenesis and isoleucine catabolism. Notably, rat hepatoma cell lines exhibit various degrees of SCOT protein expression, while expression was scarcely detected in rat hepatocytes (3). Presumably, this may allow hepatoma cells to use ketone bodies as an energy source.

Hereditary SCOT deficiency is one cause of ketoacidosis and, typically, elevated serum levels of ketone bodies are present even when a patient is well nourished and not acutely ill. We previously cloned human SCOT cDNA (4) and the human SCOT gene and reported its structural organization (5). We also previously investigated the basis of SCOT deficiency at the molecular level (5-9).

In previous studies, we investigated the control of SCOT gene expression, especially the mechanism of SCOT gene silencing in hepatic tissue. We recently demonstrated that two GC boxes in the SCOT promoter region are essential for promoter activity, but failed to identify cis-elements as responsible for the complete hepatocyte-specific suppression of the SCOT gene in the 2.2-kb 5' flanking region (10).

Genomic analysis also showed high GC contents in the promoter, in which there are many CpG sites, both in human and mouse SCOT genes. It is well known that gene expression is affected by epigenetic control. A DNA region with a high level of CpG methylation in association with a low level of chromatin histone acetylation is inactive for transcription, whereas a DNA region with a low level of CpG methylation in association with a high level of chromatin histone acetylation is active for transcription. In the present study, we investigated the methylation status of CpG islands around exon 1 of the

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SCOT gene in both human cell lines and mouse tissues (including the liver) and found that these SCOT CpG islands were, in general, hypomethylated in both human and mouse hepatic DNA.

Materials and methods

Samples. Genomic DNA was extracted from HeLa, HepG2 and Chang liver cells. Adult mouse genomic DNA was obtained from the heart, kidney and liver. Animal handling and experimentation were carried out in accordance with the guidelines set by the Institutional Animal Care and Use Committee of Gifu University.

Sodium bisulfite treatment. The bisulfite conversion of genomic DNA was performed using a previously described protocol with minor modifications (11-13). Approximately 500 ng of genomic DNA was digested overnight with *Hin*dIII, boiled for 1 min, denatured by adding freshly prepared 3 M NaOH for a final concentration of 0.3 M, and incubated at 42˚C for 30 min. A fresh solution of 3.8 M sodium bisulfite was prepared and adjusted to pH 5.0 with NaOH and 20 mM hydroquinone by gentle mixing at 37˚C. Final concentrations of 3.4 M sodium bisulfite and 1 mM hydroquinone were added to the denatured DNA for a final volume of 100 μl. The DNA was gently mixed in this sodium bisulfite/hydroquinone solution, overlaid with mineral oil and incubated at 55˚C for 6 h. After recovering the aqueous phase from under the oil, the unbound bisulfite was removed from the DNA using microspin S-200HR columns (Pharmacia Biotech). The purified DNA sample was subsequently mixed and incubated with freshly prepared NaOH (0.3 M final concentration) at 37˚C for 20 min. The NaOH was removed using microspin S-200HR columns, and the flow-through (<100 ml) contained the converted DNA ready for amplification.

PCR conditions. PCR amplifications were carried out in 50-μl reaction mixtures containing 2-8 μl of bisulfite-treated genomic DNA. PCR amplification was performed under the following general conditions: 94˚C for 5 min, followed by 40 cycles at 94˚C for 1 min, 60˚C for 1 min, 72˚C for 1 min and 1 cycle at 72˚C for 7 min.

For human SCOT CpG islands around exon 1, including the basic promoter region, the following two sets of primers were used: fragment 1, hMETH1 (sense) 5'-GGGTTTTGAATTTTAGGTTAAGATTTATTT-3'; hMETH11 (antisense) 5'-ACTTTACCTTATACCAAATTACCCAAATC-3'; and fragment 2, hMETH2 (sense) 5'-GATTTGGGGTAATTTGGTATAAGGTAAAGT-3'; hMETH22 (antisense) 5'-CCATAACTAACCCAACCTCAATTCTAAAC-3'.

For mouse SCOT CpG islands around exon 1, including the basic promoter region, the following two sets of primers were used: fragment 1, mMETH1 (sense) 5'-TTAGTAAGAGATTTTTTAGGTTTTTGGTAA-3'; mMETH11 (antisense) 5'-CCCTACACCTTCAATTTACCTTATACAAA-3'; and fragment 2, mMETH2 (sense) 5'-TTGAAGGTGTAGGGGGGTAAGAGGAAGGTT-3'; mMETH22 (antisense) 5'-CCTTCCCAAAAAC(G/A)TC(G/A)ACCTAAAACC-3'.

The primer positions are shown in Figs. 1 and 3.

Cloning sequencing and analysis of PCR products. Amplified fragments were separated following electrophoresis on a 1% (w/v) agarose gel and extracted using a Geneclean II kit (Bio 101, Vista, CA). Isolated PCR fragments were ligated into a pGEM-T Easy vector (Promega) for subcloning. Sequencing was carried out using ABI PRISM™ Cycle Sequencing kits (Perkin-Elmer Corp., Foster City, CA) and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). We sequenced ten randomly picked clones after subcloning them into a pGEM-T Easy vector.

Results

Methylation status in CpG islands around exon 1 in the human SCOT gene. As shown in Fig. 1, more than 80 CpG dinucleotides exist within 1,000 bases. The 5' flanking region of the SCOT gene includes two GC boxes and transcription starting sites, and its GC content is 68.3%. Exon 1 consists of 79 nucleotides and its GC content is 63.3%. The 5' region of intron 1 (~500 bp) also has a high GC content of 64.4%. Genomic DNA was extracted from HeLa, Chang liver and HepG2 cells. This region was divided into two fragments, which were analyzed by bisulfite sequencing.

Fig. 2 shows the percentage of CpG methylation at individual CpG dinucleotides (the numbering of CpG dinucleotides is as shown in Fig. 1) in these cell lines. In general, this region was almost entirely composed of non-methylated DNA in the HeLa and Chang liver cells. CpG dinucleotides no. 63-83 were rather more methylated than the other dinucleotides in HepG2 DNA. DNA from HepG2 cells was the most methylated among these three cell lines, and was regarded as hypomethylated in this region.

Methylation status in CpG islands around exon 1 in the mouse SCOT gene. As shown in Fig. 3, more than 80 CpG dinucleotides exist within 960 bases around exon 1 as well as the human SCOT gene. Genomic DNA was extracted from mouse kidney, heart and liver tissues. This region was divided into two fragments, which were analyzed by bisulfite sequencing. Unexpectedly, the DNA from mouse liver was completely unmethylated in these 81 CpG dinucleotides, as was the DNA from mouse kidney and heart.

Discussion

Liver-specific SCOT gene silencing appears to be physiologically important. This silencing is observed in humans and mice. Previously, we studied the molecular basis of this liver-specific SCOT gene silencing. In the present study, we compared the methylation status of the CpG islands around exon 1 of the human SCOT gene in two hepatoma cell lines (HepG2 and Chang liver cells) and in the HeLa cervical cancer cell line. We showed that SCOT mRNA and protein were detectable in Chang liver cells and the HeLa cervical cancer cell line, but not in the HepG2 cell line, indicating that the latter maintains the characteristics of liver cells in ketone body metabolism (10). In general, the CpG islands around exon 1 were non-methylated in HeLa and Chang liver cells and were hypomethylated (up to 60%) in the HepG2 cell line. Since SCOT gene silencing was almost complete, the difference among these cell lines

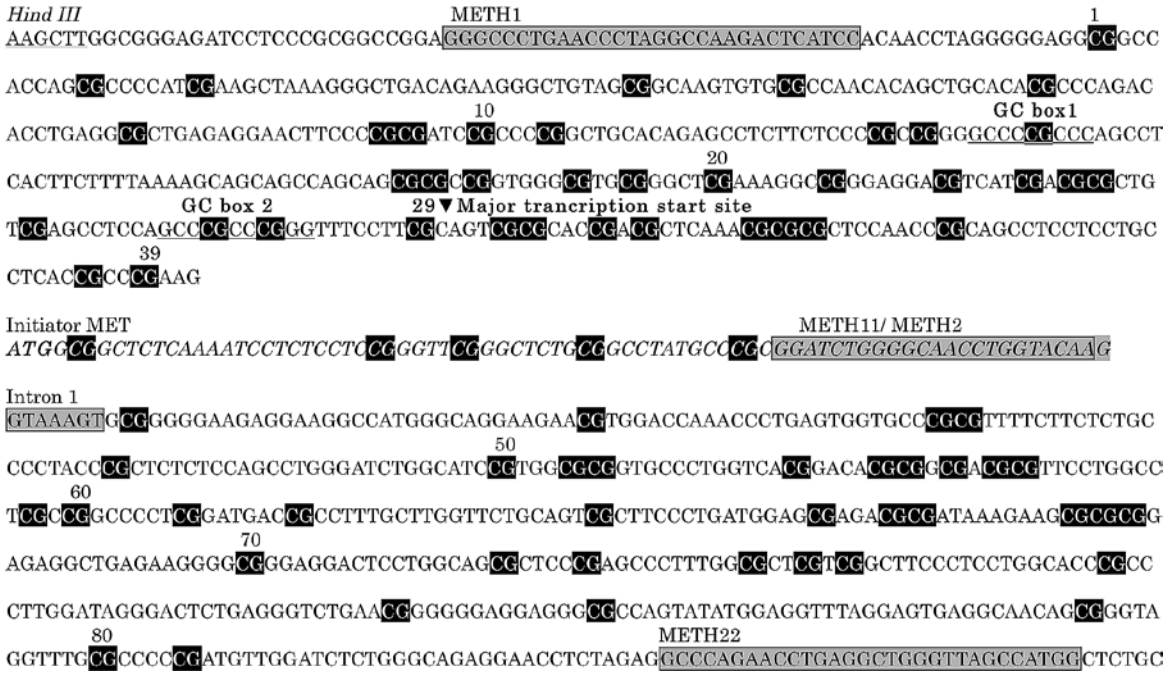

Figure 1. CpG islands around exon 1 of the human SCOT gene. CpGs are indicated by a black background. CpG dinucleotides are numbered from a 5' to 3' orientation. Two GC boxes and major transcription starting points are indicated. The nucleotides in exon 1 are indicated by italicized characters. The positions of the primers used in the PCR after bisulfite treatment are indicated by a grey background.

	5'													
CpG number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
HepG2	10	0	10	10	30	0	10	0	10	0	0	10	10	10
Chang	10	20	0	0	0	0	0	0	0	0	0	0	0	0
HeLa	0	0	10	0	0	0	0	0	10	0	0	10	0	0

CpG number	15	16	17	18	19	20	21	22	23	24	25	26	27	28
HepG2	30	10	30	10	10	10	10	10	10	0	0	10	10	10
Chang	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HeLa	10	0	0	0	0	0	0	0	0	0	0	0	0	10

CpG number	29	30	31	32	33	34	35	36	37	38	39	40	41	42
HepG2	60	0	10	40	30	30	10	30	30	60	10	40	0	0
Chang	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HeLa	0	0	0	0	0	0	0	0	0	0	0	0	0	0

CpG number	43	44	45	46	47	48	49	50	51	52	53	54	55	56
HepG2	0	0	0	0	0	0	0	40	10	0	0	30	10	20
Chang	0	0	0	0	10	10	0	0	0	0	0	0	0	0
HeLa	0	0	0	0	0	0	0	0	10	0	0	0	0	0

CpG number	57	58	59	60	61	62	63	64	65	66	67	68	69	70
HepG2	0	20	10	10	0	30	30	40	40	30	40	40	40	50
Chang	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HeLa	0	0	0	0	0	0	0	0	0	0	0	0	0	0

CpG number	71	72	73	74	75	76	77	78	79	80	81
HepG2	40	40	30	20	40	30	40	30	40	40	40
Chang	0	0	0	0	0	0	0	0	0	0	0
HeLa	0	0	0	0	0	0	0	0	0	0	0

Figure 2. Methylation status in the CpG island around exon 1 of the human SCOT gene. Ten clones of each PCR fragment after bisulfite treatment were sequenced. Percentages of methylated CpG dinucleotides are shown. The number of CpG dinuceltides is shown in Fig. 1.

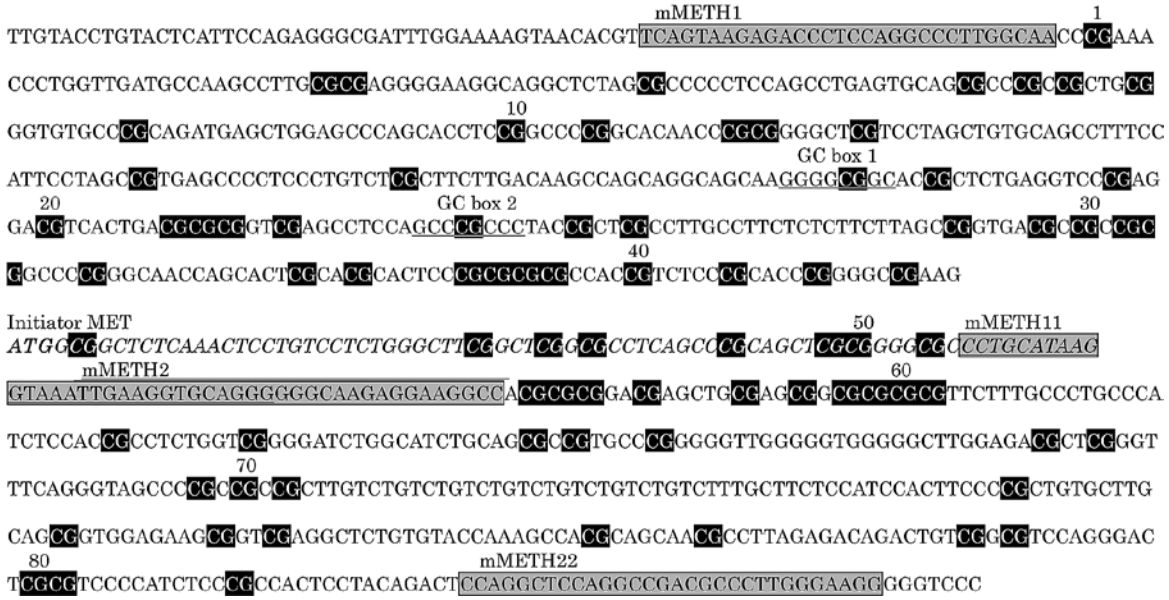

Figure 3. CpG islands around exon 1 of the mouse SCOT gene. CpGs are indicated by a black background. CpG dinucleotides are numbered from a 5' to 3' orientation. Two GC boxes and major transcription starting points are indicated. The nucleotides in exon 1 are indicated by italicized characters. The positions of the primers used in PCR after bisulfite treatment are indicated by a grey background.

was likely due to the mechanism of gene silencing. Since hepatocyte-specific silencing is observed not only in humans, but also in mice, we analyzed the methylation status around exon 1 of the mouse SCOT gene. Most CpG dinucleotides in the CpG islands around exon 1 of the mouse SCOT gene were not methylated in the hepatic DNA or in the DNA from the heart and kidney. Hence, the methylation status around exon 1 of the human and mouse SCOT genes does not contribute to hepatocyte-specific SCOT gene silencing.

In normal hepatocytes, SCOT gene expression is almost completely suppressed, whereas all extrahepatic tissues tested exhibited SCOT expression to various degrees, as follows: myocardium > brain kidney adrenal glands > other tissues (2). The activation of acetoacetate to acetoacetyl-CoA by SCOT is essential for the use of ketone bodies as an energy source in extrahepatic tissues (1). The absence of SCOT in hepatocytes is an important element in energy metabolism, suppressing ketolysis in the liver that might otherwise create a futile cycle and interfere with the efficiency of ketogenesis. Another important aspect of SCOT gene regulation is that some hepatoma cell lines have detectable SCOT expression (3). Such dysregulation benefits tumor cells since there is a relationship between SCOT expression and their growth rate. Hence, this hepatocyte-specific suppression should be programmed and conserved in mammalians.

We recently demonstrated that two GC boxes in the SCOT promotor region are essential for promotor activity, but failed to identify the specific cis-elements responsible for the complete silencing of the SCOT gene in hepatic tissues in the 2.2-kb 5' flanking region (10). One possibility is that liver-specific elements, such as strong suppressors or silencers in the SCOT gene, may lie outside of the 2.2-kb 5' flanking region. Alternatively, other mechanisms of gene silencing, such as methylation and siRNA, may be involved in hepatocyte-specific SCOT gene silencing.

DNA methylation is an evolutionally conserved mechanism for the regulation of gene expression in mammals. Tissue- and disease-specific *de novo* methylation events are observed during somatic cell development/differentiation. Once established, DNA methylation patterns are thought to be stable. Generally, cytosine residues in CpG are methylated in the genome, especially within non-coding DNA, introns and repetitive sequences. Most CpG clusters, called CpG islands, which are frequently found in the proximal promoter regions of many genes, are unmethylated during normal cell development. However, there are exceptions, such as imprinted genes, genes on the inactive X chromosome and tissue-specific differentially methylated genes. DNA methylation plays a role in the regulation of tissue-specific gene expression (14,15). Comparative analysis between mice and humans suggests that some, but not all, tissue-specific differentially methylated regions are conserved (16).

At least 5% of 15,500 CpG islands in the mouse are differentially methylated (17), and 50 tissue-specific differentially methylated regions have been identified. The majority of the tissue-specific differentially methylated regions are associated with 5' promoter CpG islands, and may play important roles in establishing or maintaining gene silencing during or after tissue differentiation.

Here, we investigated whether the methylation status of CpG islands around exon 1, including the SCOT gene promoter in the liver, is different from other tissues which express the SCOT gene, such as heart and kidney tissue. We clearly demonstrated that most CpG dinucleotides in CpG islands around exon 1 of the SCOT gene were hypomethylated or non-methylated in DNA from human and mouse hepatic tissues, as well as DNA from SCOT-expressed cells and tissues. The tissue-specific-expressed human β-globin (18) and α2(1) collagen (19) genes were found to have CpG islands that remain unmethylated in tested tissues regardless of

expression. Since many CpG islands are located at genes that have a tissue-restricted expression pattern, it follows that CpG islands remain methylation-free, even when their associated gene is silent (20). In other words, a cluster of hypomethylated CpG dinucleotides may be a common characteristic of CpG islands, while some tissue-specific differentially methylated CpG islands have also been identified. Hence, the SCOT gene has a typical CpG island that remains un- or hypomethylated. Further analysis is needed to understand liver-specific silencing of the SCOT gene.

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